The Use of Artificial Crabs for Testing Predatory Behavior and Health in the Octopus

Piero Amodio [1§], Paul Andrews [2§], Marinella Salemme [1§], Giovanna Ponte [1,3] and Graziano Fiorito [1,3]

[1]Associazione Cephalopod Research, CephRes-ONLUS, Napoli, Italy; [2]St. George University of London, London, UK; [3]Stazione Zoologica Anton Dohrn, Napoli, Italy

Summary

The willingness of the cephalopod mollusc Octopus vulgaris to attack a live crab is traditionally used as a method to assess the overall health and welfare of octopuses in the laboratory. This method requires placing a crab in the home tank of an animal, measuring the time (latency) taken for the octopus to initiate an attack and withdrawing the crab immediately prior to capture. The same crab is commonly used to assess multiple octopuses as part of daily welfare assessment. Growing concern for the welfare of crustaceans and a review of all laboratory practices for the care and welfare of cephalopods following the inclusion of this taxon in Directive 2010/63/EU prompted a study of the utility of an artificial crab to replace a live crab in the assessment of octopus health. On consecutive days O. vulgaris (N=21) were presented with a live, a dead or an artificial crab, and the latency to attack measured. Despite differences in the predatory performance towards the three different crab alternatives, octopuses readily attacked the artificial (and the dead) crab, showing that they can generalize and respond appropriately towards artificial prey. Researchers should consider using an artificial crab to replace the use of a live crab as part of the routine health assessment of O. vulgaris.

Keywords: octopus vulgaris, Directive 2010/63/EU, animal testing alternative, replacement, crab

1 Introduction

Cephalopods (nautiloids, squid, cuttlefish and octopus) have been utilized for more than one hundred years to explore aspects of their biology, physiology and mostly in neuroscience. These studies provided important information on basic cellular and behavioral processes, including learning and memory, that appears shared among many taxa (Hochner et al., 2006; Borrelli and Fiorito, 2008; Hochner, 2012; Brown and Piscopo, 2013; Huffard, 2013). The inclusion of cephalopods in the legislation of the European Union on the protection of animals for scientific purposes (Directive 2010/63/EU; see: Andrews et al., 2013; Smith et al., 2013) requires the development of guidelines for all aspects of their care and welfare (Fiorito et al., 2014; see also: http://www.cost.eu/domains_actions/fa/Actions/FA1301).

Due to the current limits of breeding cephalopods in captivity (Iglesias et al., 2014), experimental studies should rely on wild born animals. Animals taken from the wild should be allowed to acclimatize to captivity before any experiment starts.

Since the pioneering studies initiated at the end of the 1940s, acclimatization in cephalopods is assessed by measuring the latency to attack a live prey, usually crab or prawn (Boycott, 1954; Messenger, 1968; Duval et al., 1984; review in: Borrelli and Fiorito, 2008; Boal, 2011). In Octopus vulgaris, a rapid predatory response triggered by the prey is used as proxy of achieved acclimatization and for assessment of good health status of the individual (Borrelli, 2007). In brief, each animal is presented daily with a live crab (Carcinus maenas, Crustacea, Decapoda) attached to a cotton thread (tethered crab). The prey lands on the bottom of the tank in less than five seconds, and it generally walks spontaneously; if the crab stays motionless (freezing behavior) the thread is gently pulled (after about 10 s) to solicit movement. Although motion is not considered a necessary prerequisite for the octopus to respond to a stimulus (Wodinsky, 1971), a moving object (more than one staying still) attracts an octopus' attention, and promotes the attack response (Maldonado, 1963; Packard, 1963). The acquisition of experience in captivity reduces the requirements for a moving object, thus revealing contextual learning and plasticity of behavior (Hochner et al., 2006; Borrelli and Fiorito, 2008; Huffard, 2013).

Once the octopus moves towards the crab, this is promptly removed before capture. The same crab is normally used to

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§ These authors mutually contributed to this work

measure willingness to attack in multiple octopuses in a single session and may even be used on consecutive days.

Over the last five years there has been growing concern regarding the ability of decapod crustacea to experience pain and suffering (Gherardi, 2009; Elwood, 2011; Horvath et al., 2013; Magee and Elwood, 2013). Although the welfare impact on the crab of repeated exposure to a predator has not been assessed, it is likely that this is stressful for the animal. This is supported by the observation that when a species (*Liocarcinus sp*.), other than *C. maenas*, is utilized repeatedly during acclimatization of the octopus, the crab dies (M. Salemme, pers. obs.).

In compliance with the 3Rs principles, here we investigated the use of an artificial crab as a replacement, i.e., a dummy or stimulus model (*sensu* Immelmann and Beer, 1989), for a live crab to assess the willingness of an octopus to attack, and hence the potential for this method to be utilized during acclimatization and daily health monitoring of *O. vulgaris*.

2 Materials and methods

Ethical statement

Experiments complied with national legislation for the care and use of animals for research purposes, and the precautionary principles adopted for cephalopods (Andrews et al., 2013; Smith et al., 2013; Fiorito et al., 2014).

Experimental animals

O. vulgaris Cuvier 1797 (N=21) of both sexes (250-600 g body weight) caught in the Bay of Naples (Italy) were individually identified and housed in an experimental tank (60 x 100 x 50 cm) with running seawater (temperature 19.0°C).

Experimental design and procedures

Octopuses were randomly assigned to two different conditions based on the duration of acclimatization: i.) short (N=9, 5 males and 4 females) lasting five days, and ii.) long (N=12, six for each sex) lasting at least 30 days. During this time, animals were exposed daily to live crab (*C. maenas*) and fed with live crabs every other day, see Tab. S1 at http://dx.doi.org/10.14573/altex.1401282s.

To assess the potential for replacing the use of a live crab, we compared the latency of *O. vulgaris* over five consecutive days to attack a live crab or, as alternatives, a freshly dead *C. maenas* or an artificial peeler crab (Berkley Gulp Peeler Crab), see Fig. S1, S2 at http://dx.doi.org/10.14573/altex.1401282s. Experiments were designed to compare the time required (latency to attack, s) by an octopus to attack a live crab or its alternative by using a partial modification of the neophobia test (Greenberg, 1983) designed for *O. vulgaris* by Borrelli (2007). Every day two blocks of two trials each were carried out. Within each block, the animal was presented with a tethered crab first: this provides a measure of the octopus' attack performance in "normal" conditions, enhances the animal's attention, and "prepares" it for the actual task, i.e., reference latency (Borrelli, 2007). During the second trial of the block, the tester presented to the octopus either the dead crab (dead) or the artificial one (dummy). Following Borrelli (2007), the two alternative preys were attached to a cotton thread and always presented in front of the animal, similar to the live crab. Each trial lasted a maximum of five minutes (ceiling latency: 301 s) and a failure to attack within this period was classified as "no attack". The two blocks were spaced apart by approximately four hours (morning and afternoon blocks); inter trial interval was fixed at five minutes. For more detailed information, see supplementary data at http://dx.doi.org/10.14573/altex.1401282s.

To further validate the possible use of a stimulus model as a replacement of a live crab to monitor octopus well-being, at the end of the five days of the experiment we exposed nine *O. vulgaris* (short acclimatization) to an extended acclimatization procedure (i.e., daily presentation of a crab) for 15 successive days. Octopuses were randomly assigned to two groups and presented every day with either the live or the dummy crab.

During the experiments the investigator was hidden behind curtains located on each tank to minimize interaction with the animals and instructed to present the stimulus at approximate similar distance during trials and days and to all animals (Borrelli, 2007).

Data analysis

Data were not normally distributed and non-parametric statistics were used to analyze the performance of animals (Siegel, 1956; Zar, 1999). Data are presented by median and ranges, and using box and whisker plots. Between-group comparisons were assessed with a Mann-Whitney U test; Wilcoxon matched pairs signed-ranks tests were utilized to test related sample comparisons. All tests were two-tailed and the alpha was set at 0.05; SPSS (rel. 14.0, SPSS Inc. – Chicago, 2005) was utilized for statistical analysis.

3 Results

All octopuses met acclimatization criteria (i.e., well adapted to captivity) at the beginning of the experimental phase. However, short and long acclimatization induced a significant difference in behavioral performance of octopus towards a live crab (Mann-Whitney U test: Z=4.39, N_1=9, N_2=12, P<0.001) with the latency being longer and more variable after short acclimatization (Tab. 1; Fig. 1). Despite these differences, the attack performance of *O. vulgaris* towards live *C. maenas* during the five days remained stable and homogeneous (morning vs. afternoon block, pair-wise comparison always not significantly different; data not shown, see Table 1).

Since each octopus was presented with four consecutive tethered crabs (or their alternatives) without being able to capture and feed on any of them, a Wilcoxon matched-pairs signed-ranks test was first performed to measure the potential effect of the repetition of crab presentation on the predatory performance of the animals. Successive crab presentations did not affect octopuses' predatory behavior (live crab morning vs. afternoon: not significant after Wilcoxon signed-ranks test), indicating that the animals continued to readily attack the prey even in the absence of a reward.

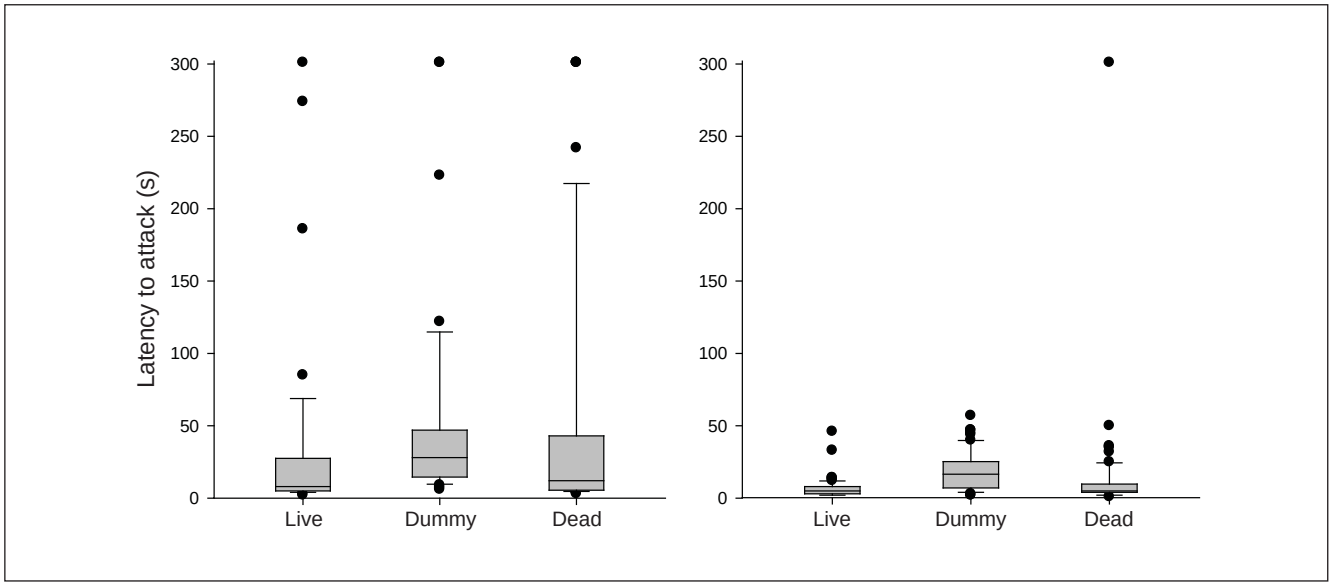

Fig. 1: Box plots showing the latency to attack the crab and their alternatives (dummy and dead) during the experiment by *O. vulgaris* after short (left) or long acclimatization (right)

Boxes represent the interquartile range (25th-75th percentiles), bars within boxes the median values, whiskers the 10th and 90th percentiles, and circles the outliers.

Tab. 1: A summary in tabular form of the performance of *O. vulgaris* (short and long acclimatization) when presented with live, dummy or dead crabs

Behavioral performances are presented as median and ranges (min-max value) of the latencies to attack (in s) over the five days (day 1 - day 5) of the experiment and as overall performance. Values are presented for each pair with values for "live" always in the first row. A value of 301 s correspond to a no-attack. The results of Wilcoxon signed-ranks test (Z and corresponding P) are also provided.

	Short acclimatization								**Long acclimatization**							
	Live vs Dummy				**Live vs Dead**				**Live vs Dummy**				**Live vs Dead**			
	N	median	Z	P	N	median	Z	P	N	median	Z	P	N	median	Z	P
Day 1	9	*22.0 (4-106) 27.0 (7-122)*	1.60	0.133	9	*20.0 (3-301) 7.5 (4-301)*	0.12	0.931	12	*6.0 (3-11) 18.5 (11-45)*	3.06	>0.001	12	*6.0 (2-13) 8.5 (2-50)*	2.12	0.032
Day 2	9	*14.5 (5-301) 20.5 (9-301)*	0.18	0.909	9	*30.5 (6-227) 19.5 (5-301)*	0.70	0.528	12	*6.0 (3-18) 20.5 (5-57)*	2.47	0.011	12	*7.0 (2-33) 7.0 (3-301)*	0.77	0.487
Day 3	9	*12.0 (4-301) 41.0 (10-301)*	1.60	0.128	9	*16.0 (5-274) 25.5 (6-301)*	2.44	0.014	12	*7.0 (3-14) 18.0 (3-47)*	2.40	0.012	12	*6.5 (4-23) 5.0 (3-19)*	0.24	0.835
Day 4	9	*10.0 (4-205) 35.0 (10-301)*	1.60	0.124	9	*9.0 (4-301) 9.0 (3-95)*	0.54	0.646	12	*3.5 (2-16) 8.0 (2-31)*	2.45	0.012	12	*4.0 (2-12) 4.5 (2-36)*	0.28	0.829
Day 5	9	*7.0 (2-26) 29.5 (6-301)*	2.31	0.017	9	*4.0 (2-18) 14.0 (5-301)*	2.03	0.046	12	*4.0 (2-46) 15.5 (2-47)*	2.58	0.006	12	*4.0 (2-18) 4.0 (1-15)*	0.49	0.654
Overall	9	*8.0 (2-301) 28.8 (6-301)*	2.94	0.003	9	*8.0 (2-301) 12.0 (3-301)*	1.53	0.118	12	*5.0 (2-46) 16.5 (2-57)*	5.90	>0.001	12	*5.0 (2-46) 5.0 (1-301)*	1.96	0.046

The presentation of either dummy or dead crabs induced a more variable response in the octopuses, with animals' performance after a short acclimatization markedly variable (Fig. 1). In addition, when alternatives to the live crab were presented, some animals occasionally failed to attack (short acclimatization: in 2 and 3 instances out of 45 trials, dead vs dummy, respectively; long acclimatization: 1 out of 45 trials, dead).

As shown in Table 1, octopuses with a short exposure to captivity did not show a significant difference when attacking the live or the dead crab, but recognized the artificial crab as a potential different prey item on all five days. Comparison between the predatory performance of more experienced (long acclimatization) *O. vulgaris* towards the three different prey alternatives showed highly significant differences (Tab. 1; Fig. 1). The performance over five days (Tab. 1) of the animals under the two conditions revealed that short-acclimatized octopuses performed almost constantly towards one of the two prey alternatives; however, towards the end of the experiment, octopuses started to treat the dummy crab as not a "valid" alternative and attacked it with significantly longer latencies.

In contrast, *O. vulgaris* with longer acclimatization times distinguished between dummy and live crabs over all the five days of the study (Tab. 1; Fig. 1).

Finally, at the end of the experiment, the repeated presentation over the successive 15 days of the artificial crab to octopuses resulted in an improvement in their performance (as indicated by a decrease in latency to attack), but animals never reached a level similar to that when presented with a real crab (median, range (s)=8.8, 5.9 and 12.0; 21.5, 11.0 and 40.9; live vs dummy, respectively).

4 Discussion

The primary aim of this study was to assess whether it was possible to replace the use of a live crab with an artificial one in the test of predatory behavior widely used as part of daily health and welfare assessment of octopuses in captivity. Our results show that whilst *O. vulgaris* would attack the artificial crab, the animal appears to be able to differentiate between the live, artificial and dead crabs. Additionally, the latency and incidence of attack is affected by the period of acclimatization (5 or 30 days, i.e., short or long), an observation that has implications for guidelines for the care and welfare of cephalopods (Fiorito et al., 2014). In particular, while the predatory performance towards the live prey resembled the one established as a criterion for acclimatization, the presentation of a dead and, more evidently the artificial, crab resulted in a more variable response, with latencies that were relatively higher.

Although a live crab has been used to assess willingness to attack in *O. vulgaris* (even for aversive training paradigms) for over 60 years, the method itself has been subjected to little formal study (Boycott, 1954; Maldonado, 1965; Borrelli and Fiorito, 2008; Cartron et al., 2013). Consistent with Borrelli (2007; Borrelli and Fiorito, 2008), here we demonstrate that under the same acclimatization conditions (see below), the latency to attack a live crab is relatively consistent over days and from morning to afternoon. Although the octopus is not "rewarded," the predatory response is not extinguished. These results support the validity of the use of predatory behavior as part of the daily assessment of health in octopus. These data with a live crab provided a suitable stable baseline against which the response to an artificial or a dead crab could be assessed objectively. Although octopuses would attack both artificial and dead crabs, the response was more variable than for the live crab and occasionally animals failed to attack (see above). Overall, the latency to attack a live crab is shorter than for an artificial or a dead crab (only for short acclimatized animals) and this may reflect the fact that movement is known to promote predatory behavior in *O. vulgaris* (Packard, 1963; but see Wodinsky, 1971). Monitoring the attack response to either live or artificial crabs over 15 days revealed that whilst both were attacked, the latency to attack the live crabs remained relatively stable, while that for the artificial crab decreased although it never matched the performance seen with the live crab.

The differences in latency between attacking a live and an artificial crab do not invalidate the use of an artificial crab provided that appropriate values are selected for assessment of "normal attack" as is also the case when a live crab is used.

Our study used two different acclimatization times and this revealed that it has an effect on attack latency that has not previously been reported (but see Borrelli and Fiorito, 2008). With short periods (5 days) of acclimatization the latency to attack all three "crabs" was longer and more variable than after 30 days of acclimatization. It has been reported that recently caught octopuses are reluctant to attack and explore novel objects placed in the tank (Hochner et al., 2006). However, in the short acclimatization group, whilst the latency to attack the live and dead crabs was similar, both were shorter than for the artificial crab. Consideration should be given for the time taken for the artificial crab to reach the tank floor (see Fig. S3 at http://dx.doi.org/10.14573/altex.1401282s) which is longer than for either the live or dead crab and could contribute to the longer latency to attack, although *O. vulgaris* attack crabs in mid-water as well as on the tank floor (Hochner et al., 2006; Borrelli and Fiorito, 2008). However, the artificial crab is lighter than either the live or the dead crabs and may not have the same hydrodynamic properties as a real crab in its descent to the tank floor. This observation may suggest that the type of movement displayed by a potential prey item in the water column may also be important rather than movement *per se* and requires further study. In the short acclimatization group there was also some indication that the octopuses could also distinguish between live and dead crabs (Tab. 1: days 3 and 5). In the long acclimatization group the latency to attack the artificial crab was significantly longer on all five testing days than for the live crab, but on one day (day 1) animals distinguished live from dead crabs. These preliminary indications that octopuses have some capacity to distinguish live from dead crabs are intriguing and require further study.

The finding that duration of acclimatization affects predatory behavior shows the importance of stating acclimatization protocols in the methods sections of publications and particu-

larly those investigating any aspect of cephalopod behavior, as in the ARRIVE guidelines (Kilkenny et al., 2010). As most cephalopods in research are obtained from the wild (Smith et al., 2013; Fiorito et al., 2014) adequate acclimation to the laboratory environment is pivotal to study design and interpretation.

This study shows that an artificial crab could replace the use of a live crab to assess the willingness to attack as an index of overall health and well-being in the octopus. The use of an artificial crab avoids exposing a live crab repeatedly to a potentially stressful situation. In addition, the predatory behavior test can be more readily standardized with an artificial crab as they can be used all year round, which may not be possible with live *C. maenas* necessitating a change of species and/or of the size of the individual crab utilized for different octopuses due to seasonal differences. Additionally, using the artificial crab removes the possibility that an octopus being tested prior to transport, drug administration by gavage or about to undergo a scientific procedure where food deprivation is required, will capture the crab and ingest it, thus confounding the study.

Each laboratory would need to set criteria for "normal values," taking into account the period of acclimatization utilized. Unless there is a compelling scientific justification to use a live crab (especially in repeated testing), we recommend that those responsible for daily care and welfare of cephalopods assess the use of an artificial crab in their own experimental setting. As most laboratories feed their *O. vulgaris* using a live crab, measurement of attack latency can readily be compared with an artificial (or dead) crab. Although we have focused on the use of an artificial crab to measure predatory behavior in *O. vulgaris*, we believe this method has applicability to cuttlefish, possibly using an artificial crab or prawn. Finally, as for any other investigation based on the study of stimulus models, a better designed artificial crab may improve the "quality" of the presentation to the octopuses (and other cephalopod species).

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Correspondence to
Marinella Salemme, PhD
Associazione Cephalopod Research, CephRes
Via dei Fiorentini 21
80133 Napoli, Italy
Phone: +39 339 1316246
e-mail: cephres@cephalopodresearch.org

Addendum

Addendum to Evidence for the Detection of Non-Endotoxin Pyrogens by the Whole Blood Monocyte Activation Test

Nina Hasiwa [1,2], Mardas Daneshian [1], Peter Bruegger [4], Stefan Fennrich [5], Astrid Hochadel [2], Sebastian Hoffmann [6], Felix E. Rivera-Mariani [3], Christoph Rockel [7], Stefanie Schindler [8], Ingo Spreitzer [9], Sandra Stoppelkamp [5], Kranthi Vysyaraju [3], and Thomas Hartung [1,3]

[1]CAAT-Europe, University of Konstanz, Konstanz, Germany; [2]AtaX-Advice, Konstanz, Germany; [3]Johns Hopkins University, Bloomberg School of Public Health, CAAT, Baltimore, USA; [4]Novartis, Basel, Switzerland; [5]Department of Thoracic, Cardiac and Vascular Surgery, Tübingen University Hospital, Tübingen, Germany; [6]seh consulting + services, Paderborn, Germany; [7]Enzler Hygiene AG, Center for Hygiene, Pratteln, Switzerland; [8]Animalfree Research, Zürich, Switzerland; [9]Paul-Ehrlich Institut (PEI), Langen, Germany

Regarding this t4 report, which appeared in ALTEX (2013), 30(2), as co-author Thomas Hartung would like to acknowledge that the respective patents referred to, which he holds as the co-inventor of the whole blood pyrogen test, were at the time of the article licensed to Biotest Pharmaceuticals, Germany, and he received royalties from sales of this test. The license and associated royalty payment obligations were in the meantime transferred to Merck-Millipore, France.

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Review

The Challenge of Ecophysiological Biodiversity for Biotechnological Applications of Marine Microalgae

Lucia Barra †, Raghu Chandrasekaran †, Federico Corato and Christophe Brunet *

Stazione Zoologica Anton Dohrn, Villa Comunale, Naples 80121, Italy;
E-Mails: luciana.barra@szn.it (L.B.); raghu.chandrasekaran@szn.it (R.C.);
federico.corato@szn.it (F.C.)

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: christophe.brunet@szn.it;
Tel.:+39-81-583-3243; Fax: +39-81-764-1355.

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Abstract: In this review, we aim to explore the potential of microalgal biodiversity and ecology for biotechnological use. A deeper exploration of the biodiversity richness and ecophysiological properties of microalgae is crucial for enhancing their use for applicative purposes. After describing the actual biotechnological use of microalgae, we consider the multiple faces of taxonomical, morphological, functional and ecophysiological biodiversity of these organisms, and investigate how these properties could better serve the biotechnological field. Lastly, we propose new approaches to enhancing microalgal growth, photosynthesis, and synthesis of valuable products used in biotechnological fields, mainly focusing on culture conditions, especially light manipulations and genetic modifications.

Keywords: biotechnology; biomass; diatoms; phytoplankton; ecophysiology; biodiversity; functional diversity; cultures; light

1. Introduction

It is now well known that marine biotechnology (the so-called "blue biotechnology") can make a large contribution to the key societal challenges, in relation to the huge biological diversity populating marine ecosystems. As an alternative to higher plants, microbial organisms such as microalgae are

relevant resources for applied societal purposes, due to their rich biodiversity, growth rate, and multiple application potentials.

Microalgae, either prokaryotes or eukaryotes, are oxygenic autotrophs that populate all aquatic ecosystems ranging from freshwater and brackish waters to oligotrophic marine waters. The microalgal world represents rich biodiversity, characterized by different biological, ecological and functional traits. The species number ranges from 30,000 described species to one million, and some estimates report more than 200,000 species only for the *Bacillariophyceae* [1,2]. This group is the most recent and diversified group [3], and until now, more than 8000–10,000 diatom species have been described [4], while only a few species have been employed for biotechnological applications [5].

For Chlorophyceae, a group which comprises of almost 2000 species, only 7–8 species are biotechnologically active, followed by Cyanophyceae, where the number is even smaller [6,7]. Use of microalgae for biotechnological applications has been increasing in recent years, mainly for bioremediation, nutraceutical and pharmaceutical purposes, as well as for bioenergy production [8].

We can distinguish three kinds of algal applications (Table 1). The first application corresponds to the property of microalgae in interacting with the environment in which they grow, such as the use of biomass for O_2 production, reduction of CO_2 [9], bioremediation and bioremoval of some organic and inorganic compounds [10]. Microalgal species or strains are selected for their efficient growth or for their ability to harvest specific toxic compounds from the environment. The second application corresponds to the production of primary metabolites, as for instance carotenoids [11,12], proteins [13], and lipids such as polyunsaturated fatty acids, PUFA [14]. Some of these molecules have antioxidant activities [15–17]. For this purpose, microalgae with high physiological plasticity such as diatoms should be mainly used, since they are able to efficiently adapt to environmental variations [18]. The third application is related to secondary metabolites which are generally produced in low quantities, mainly for pharmaceutical applications [19].

Up to now, biotechnology uses around 20 species of microalgae from marine, estuarine and freshwater (lakes, ponds) ecosystems [6], representing a miniscule percentage of the myriad of known species. The open challenges for achieving the "green revolution" with "blue biotechnology" concern: (i) the cultivation of new species; (ii) identification of new molecules that can contribute to the development of industrial applications from marine ecosystems, and (iii) upscaling microalgal biomass production, while keeping production costs as low as possible. To overcome the aforementioned challenges, an extensive exploration of microalgal biodiversity (taxonomical and functional), chiefly by focusing on morphological, ecological and physiological traits, is mandatory. These traits, resulting from the adaptive evolution of a species to its ecological niche, determine its growth capacity in the marine environment. Many experimental results have shown that algal growth, physiology and biochemistry are strongly controlled by environmental variables, such as hydrology (temperature, salinity [20,21]), chemistry (pH, macronutrients and micronutrients [22]) and light [23]. Improving culture conditions will allow cells to be more efficient in terms of growth, photosynthesis and primary metabolite synthesis, since growth is the result of the energy dynamics balance between production and loss [24,25].

In this review we mainly focus on pelagic photosynthetic micro-organisms. The non-planktonic microalgae, while ecologically relevant, have different growth properties that we are not going to discuss in this paper (see the review by Lebeau and Robert [26]). This review aims to:

(i) draw a general picture on the actual use of microalgae in the biotechnological field; (ii) disseminate the knowledge on biodiversity and richness of the microalgal world, and (iii) propose ways to improve microalgal production in the context of biotechnological applications. For the latter, we focus especially on the improvement of culturing conditions, in addition to the development of genetic engineering technologies [8].

2. Biotechnological Applications of Microalgae and Cultivation

2.1. Biotechnological Applications of Microalgae

Commercial culturing of algae has a history of 440 years, starting from the cultivation of *Porphyra* in the 1640s [7]. According to Hallmann [27], about 10^7 tons of algae are harvested each year by algal biotechnology industries for different purposes, and almost 60 commercial companies are selling algae or algal products worldwide. In this section, we will go through their use as bio-markers/remediation, bio-fuel sources, and primary metabolite producers. Microalgae have gained the attention of the scientific world, as is shown in many studies, for their biochemical and physiological capacity to respond to organic pollutants (POPs) [28–31], polycyclic aromatic hydrocarbons (PHAs) [32–34], polychlorinated biphenyls (PCBs) [35,36] and pesticides [37–41]. These studies have demonstrated that macroalgae and microalgae are important tools in monitoring and controlling the presence of heavy metals in ecosystems, due to several biochemical strategies employed to reduce toxicity of non-essential trace metals [42–48]. Among the species resistant to heavy metal exposure, we can cite *Chlamydomonas reinhardtii* [49] and some macroalgae, such as *Fucus serratus*, alongside the aquatic plant *Lemna minor* [50].

Most organic chemicals can be naturally degraded within the aquatic environment as a result of complex processes mediated both by auto- and heterotrophic organisms [51]. However, when the wild-type xenobiotic detoxification systems, mainly based on metallothionein proteins [52,53], are not sufficient to cope with pollutant biodegradation, a possible alternative route is the creation of consortia made by microalgae and/or cyanobacteria [47,54]. A complete review of the mechanisms and solutions for pollutant management is provided by Torres and collaborators [28].

Many reports have described microalgae as a potential oxygen producer and CO_2 depository [55,56], and as a green energy source for bio-fuels and biogases [57–62], providing feedstock for renewable fuels such as biodiesel, methane, hydrogen and ethanol. Moreover, microalgae grow faster and reach a higher productivity compared to conventional agricultural plants [59], and are able to grow almost anywhere, requiring only sunlight and nutrients [63–65]. This is discussed in greater detail in the fourth section of this review.

The wide amount of bioactive primary metabolite production is one of the key microalgal features to be exploited in many applicative fields, such as nutraceutics [66], animal feeding products and cosmetics [67]. Microalgal species, such as *Spirulina* for diet, *Dunaliella salina* and *Haematococcus pluvialis* for carotenoid production, and several other species for aquaculture, are used in mass culturing [68–70].

The high protein content, amino acid pools, carbohydrate digestibility, pigments, ω3 and ω6 family lipids and the presence of nearly all essential vitamins (e.g., A, B1, B2, B6, B12, C, E, nicotinate,

biotin, folic acid and pantothenic acid) make microalgae an unconventional source for improving the food nutritional state and hence the health of humans and animals [13,66,71,72]. Furthermore, microalgae are considered to be of great interest in the biotechnological field because they are a precious source of lipophilic pigments such as chlorophyll (0.5% to 1% of dry weight), carotenoids (0.1% to 0.2% of dry weight on average and up to 14% of dry weight for β-carotene in some species, including *Dunaliella* sp.), xanthophylls (lutein, zeaxanthin and astaxanthin) and hydrophilic pigments, such as phycobiliproteins.

Recently, commercial forms of microalgal products are incorporated into pasta, snacks, gum, and beverages [73,74], as sources of natural food colorants, or as nutritional supplements [69,72,75]. The market is actually dominated by some species of chlorophytes and cyanophytes, such as *Arthrospira* spp., *Chlorella* spp., *Dunaliella salina*, *Heamatococcus pluvialis* and *Aphanizomenon flos-aquae*. More than 70 companies sell *Chlorella* as a source of β-1,3-glucan, which has properties of an immunosystem stimulator, as a free radical scavenger, and as a reducer of bad blood lipids [76]. *D. salina* is exploited for its β-carotene content that can reach 14% of its dry weight [77]. The chlorophyte *Muriellopsis* sp. is being exploited for the production of the xanthophyll lutein, due to its high content produced under peculiar culture conditions [78], while *Heamatococcus pluvialis* is used for its massive accumulation of astaxanthin, highly synthetized under stressful conditions [79,80]. Due to its high protein and amino acid content, *Arthrospira* is extensively used for human nutrition production in China and India, under the name of *Spirulina pacifica* [81,82]. According to several studies, the cyanophyte *A. flos-aquae* can also benefit health [83,84]. However, the diversity of usable species for aquaculture remains higher than the species that are used for human diets [85,86], since species such as *Phaeodactylum*, *Chaetoceros*, *Skeletonema* and *Thalassiosira*, along with *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova* and *Nannochloropsis*, are easily ingestible and digestible by cultivable organisms, and are used in aquaculture [87,88]. In the field of cosmetics, microalgal extracts are used for face, skin and hair care products, as well as in sunscreen products. Genera such as *Arthrospira* and *Chlorella* are employed in the skin care market [89]. For more information on companies selling microalgae and methods for their cultivation, see the review by Spolaore and collaborators [67].

Last, but not least, primary metabolites from microalgae are also used in diagnostics and application fields. In particular, phycobiliproteins, coloured proteins commonly present in cyanophytes and cryptophytes, are extensively commercialised in clinical and immunological analysis as colorants, fluorescent labelling molecules, and as pharmaceutical agents [90]. Until now, in the research and diagnostics development field, phycobiliproteins have been the object of more than 200 patents [90].

2.2. Algal Cultivation Techniques

The cultivation of microalgae can be performed indoor or outdoor, using enclosed or open systems [69]. These two systems strongly differ in shape, aeration, illumination systems, building material and volume capacity. In the past, natural waters (lakes, lagoons, *etc.*) or artificial ponds were used to grow microalgae. These outdoor open systems that use just natural light for illumination, are inexpensive to install and maintain and present uncertainties in the success of production operation. One of the main concerns is the uncontrolled environmental parameters, such as light, temperature, and air humidity that can induce large variability in cell physiology, altering the growth efficiency (see

following sections). Excessive light or ultraviolet radiation in outdoor open systems might induce photoinhibition or can alter the healthy state of cells. Outdoor culturing for biomass production, with the exception of tropical areas, is strongly season dependent [91–97]. The transition periods of the year (e.g., end of spring and autumn) also show rapid climatological changes; a relevant temperature excursion between night and day might also affect physiological acclimation of microalgal biomass [98]. Other drawbacks, linked to the fact that cultures are open and not axenic, are correlated to the proliferation of bacteria, viruses, other microalgal species and predators that may decimate microalgal cultures [70]. Closed photo-bioreactors that can be set indoor or outdoor (either naturally or artificially illuminated), have been employed to axenically grow microalgae, such as cyanophytes [70,99,100]. For some diatoms, both outdoor and indoor cultivation systems have been established together with a generalised set of conditions [5]. The conditions and growth parameters can be set more accurately in indoor culture batches than in outdoor ones. Indoor culturing techniques prevent many environmental and biological causes of death or reduction in growth rate, and are more efficient for maintaining mass cultivation at high production rate. Section 4 of this review focuses on the possible ways to improve growth quantum yield of microalgal cultivation in indoor systems.

3. Exploring the Richness of Microalgal Biodiversity

Biodiversity is characterized by taxonomical metrics with distinct morphological traits, as well as other variables related to functional diversity such as ecology, physiology, and biochemistry of the species [101]. The cell functional traits regulate the ecophysiological requirements of the species, thereby shaping cell performances under specific conditions or along environmental gradients, as in nature. In this section, we aim to present the richness of microalgal biodiversity, which has been underexploited thus far in the biotechnological field.

3.1. Taxonomical Diversity

Biodiversity is huge in the microalgal realm, with almost 64 classes (phyla) and more than 70,000 species that could represent the basin to draw for a deep investigation of the microalgal world within a blue biotechnology aim [1]. The most recent and successful taxon group from an evolutionary and biogeochemical point of view is the diatoms' group [3,102] that has been estimated to have roughly 10^5 species [103]. Many diatoms comprise cryptic or hidden species not easily recognizable with classical microscopic tools [104,105]. For this reason in recent years, taxonomy has been using molecular tools, in addition to morphological identification to better decipher the richness of the microalgal realm [106] and the evolutionary trends of microalgae (e.g., [107–110]). Molecular tools allow for the investigation of small cell-sized species and uncultivable ones [111], as well as cryptic species [104,105,112]. As mentioned previously, for some taxa of phytoplankton, the known biodiversity does not take into account the hidden and cryptic species present in the groups [113]. Indeed, with the use of ribosomal or organelle markers as LSU, SSU, ITS, 5.8 S, RbcL and COX1 associated with the ultrastructure of a diatom cell wall, the silica frustule helps resolve cryptic species belonging to the following genera: *Chaetoceros*, *Skeletonema* and *Pseudo-nitzschia* [102,104,114] thereby enlarging the possibility of choosing the most appropriate species to the *ad hoc* cultivation

system. For more information on taxonomical diversity and evolution of phytoplankton, we suggest the recent review papers [109,110].

3.2. Functional Diversity: Morphology and Size

Morphological variations amongst species lead cells to be more or less cultivable; these variations mostly imply changes in the surface: volume ratio, cell floatability and sinking rate. A cell's shape can vary from round to elongated or triangular, affecting interactions between cells and the surrounding environment and having consequences also on the assimilation of resources [115].

The main cellular morphology includes the presence of flagella (Table 2), such as in the dinophytes, cryptophytes and some haptophytes, in addition to the presence of spines, referred to as setae and found mostly in diatoms. The setae can have several functions, such as cell protection against predators, sedimentation by trapping air bubbles and generation of photosynthetic oxygen. Indeed, setae from some *Chaetoceros* species contain chloroplasts that are able to move up and down, probably in relation to the light they are exposed to [116].

Another morphological trait is represented by the possibility of forming a colony. Colony-forming species could be considered evolutionarily favoured due to the colony-formation implication in defence mechanism (Table 2), as well as in growth regulation and competition [117]. One example is represented by some species of *Phaeocystis* (haptophyte) that can be present in both colonies and single cells [118].

Chain-forming species (Table 2) are also widespread in an aquatic environment, and mostly belong to the diatom genus [119]. Chain formation confers resistance to predation [120,121], enhances nutrient fluxes [122] and can increase sexual reproduction if chains become entangled [102].

The morpho-functional traits listed above are strongly ecologically relevant, though they do not add any *gain* for biotechnological purposes in relation to the current cultivation techniques that use high nutrient content and shallowness of culture tanks.

Instead, cell size can be considered as a relevant functional trait (Table 2), which has many implications for the ecophysiological requirement of cells and on the ecology of phytoplankton [101,123]. Cells range from 0.3 μm to several millimetres [123]. The smallest photosynthetic organisms, belonging to picophytoplankton class (size <3 μm) are strongly adapted to grow and dominate in oligotrophic waters [124,125]. The tiny cell size confers important ecological and biological properties on picophytoplankton, such as the large surface area per unit of volume, minimal diffusion boundary layer (*i.e.*, higher nutrient consumption rates), low sinking rate, low package effect and efficient light utilization [25,126,127]. Indeed, significant relationships between photosynthetic and growth rates were obtained in different picophytoplankton species, suggesting a direct and functional link between absorbed light [25,98] and metabolic processes. This could be related to a high physiological plasticity and intraspecific variability, as has been revealed in picophytoplankton [24,25,128–130]. These features might be related to a potentially higher rate of speciation in these small organisms than in larger sized ones [128].

Table 1. Discrimination of the three groups of algal applications.

Application	Function	Requirements	Algae
Biomass production	Fluxes of matter and energy	Optimization of culture conditions for growth and photosynthesis maximization	Large-sized coastal species, species with a high constitutive growth and photosynthesis
Primary metabolites	Production of interesting molecules such as carotenoids, phycobiliproteins, proteins, lipids, polysaccharides and antioxidants	Optimization of culture conditions for maximizing interesting molecules production and high growth rates (photosynthetic biotechnology through light manipulation and metabolic engineering)	Physiologically plastic species, such as small diatoms and coastal species
Secondary metabolites	Production of toxin or drugs	Optimal or stressful conditions to produce this kind of molecules	Selected or genetically modified species

Table 2. Functional traits in microalgae: Ecological or physiological relevancies and interests in a biotechnological field. * DMS: dimethylsulfide; ** DMSP: dimethylsulphoniopropionate; DCM: Deep-Chlorophyll Maximum.

Functional trait or adaptive feature	Ecological or physiological relevance	Group of species	Interests and/or problems in biotechnology
Multicellular life forms (chains and colony; [117])	Influence of sinking rate, reduced predation	Diatoms, haptophytes	Little impact in shallow and oxygenated/mixed tanks
Flagellates [131]	Migration and motility	Dinophytes, haptophytes and cryptophytes	Little impact in shallow and oxygenated/mixed tanks
Small cell size [127]	Low nutrient requirements, high growth capacity, low sinking rate	Picoeukaryotes	High growth and production capacity and acclimation
Benthic species [26,132]	Growth on solid support (sediments, leaves), highly resistant species	Some diatoms, cyanophytes	Difficult to cultivate
Toxic species [133]	Defence mechanisms, highly competitive	Cyanophytes, diatoms, dinophytes	Discovery and selection of new molecules
Sexual reproduction [134]	Genetic recombination	Some diatoms	New strains selection with better fitness
Diazotrophy [135]	Atmospheric N_2 fixation	Cyanophytes	Low growth capacity

Table 2. *Cont.*

Mixotrophy [136–138]	Growth under nutrients depletion and darkness	Some dinophytes, diatoms, chrysophytes and cryptophytes	Low growth capacity, interest for bioremediation
Presence of large vacuoles [102]	Internal storage of nutrient	Diatoms	Long-term maintenance, decrease of dilution frequency
Low light adapted [139]	Growth under low light, photoinhibited under high light	Deep chlorophyll maximum (DCM) species	High growth rate under low light, high capacity of photoprotection
Variable light adapted [24]	Growth under low and high light, physiological plasticity	Coastal species (some diatoms and haptophytes)	High capacity of xanthophyll and antioxidant production, high growth rate
Low iron requirement species [140]	Growth in pelagic/oceanic ecosystems, photobiological and physiological adaptation	Some pennate diatoms	Little effect, Fe is provided in high quantity
Oceanic Temperature zones [141,142]	Low temperature growth	Polar species (diatoms, haptophytes)	Cost for low temperature maintenance of the cultures Temperature defence mechanisms, peculiar molecules for allowing photosynthesis and production at low temperature
Calcareous microalgae [143]	Species producing calcified scales around the cell	Coccolithophorids	Calcite production
DMSP, DMS producer species [15]	Antioxidant production (* DMS and ** DMSP) under environmental stresses	Prymnesiophytes, Diatoms, Dinophytes	Highly effective antioxidant systems, well-growing species
Halophilic species [144]	High salinity level, osmotic stress regulation	Chlorophytes and cyanophytes	Low growth capacity, costly culture management Molecules of interest

Although there is not much knowledge of picophytoplankton despite its high biodiversity, in addition to the features discussed earlier, it is implied that exploring picophytoplankton for biotechnological purposes is challenging. The low consumption together with the high growth rate and low loss of energy between light harvesting and division processes might outweigh the scant biomass content per cell.

3.3. Functional Diversity: Uptake of Nutritional Resources

The diversity of culture media available for growth of marine and coastal microalgae (Table 3) reflects the physiological diversity of microalgal groups. The major natural seawater-enriched media are: the f/2 medium, widely used for coastal and diatom species [145]; the K medium for oceanic species; the Pro99 medium for cyanophytes and picoeukaryotes; and the MNK medium mainly used for coccolithophorids [146].

Table 3. The most commonly used culture mediums for growth of marine microalgae (see also [147]).

Name	Microalgae	Specificities
f/2 medium [148,149]	Coastal microalgae, diatoms	Enriched medium
K medium [146,150]	Oceanic microalgae	Trace metals
Pro99 [151]	*Prochlorococcus* spp. and some picoeukaryotes	High ammonia concentrations, No vitamin requirement
MNK medium [152]	Oceanic coccolithophores	Enriched medium

The macronutrients required by microalgae to perform photosynthesis and growth are nitrogen (nitrate, NO_3, nitrite, NO_2 or ammonium, NH_4), phosphorus (phosphate, PO_4) and silicate (SiO_2), the latter being required only for diatoms and silicoflagellates. As reported in many studies [101,153–155], the variability in uptake and the efficiency in using nutrients are high among phytoplankton groups, depending on evolutionary and ecological functional traits [156–161]. The most striking group corresponds to the diatoms, with the presence of a large central vacuole that can store nutrients and carbohydrates [102], which allows cells to maintain their growth during nutrient-depleted periods. This feature provides this group a competitive advantage over other groups.

Nitrogen can be provided as nitrate, nitrite or ammonium, which is a key variable for microalgal growth. It has been observed that different sources of nitrogen are differently metabolized, influencing the growth capacity and protein content [162], as observed in two *Chaetoceros* species [163]. Evidence from Meseck and collaborators [22] show that ammonium uptake efficiency in *Tetraselmis chui* PLY 429 significantly increased when carbon dioxide was added to the culture, while it decreased with a nitrate source. Experimental results obtained with the diatom *Skeletonema marinoi* grown in f/2 medium under different light conditions, revealed that after three or four days, ammonium concentration strongly decreased, while nitrite and nitrate concentrations remained high [164]. This result confirms that cells primarily use NH_4, instead of NO_3. Allen *et al.* [165] reported a decrease in growth rate of *Phaedactylum tricornutum* grown on nitrate as the only nitrogen source. In fact, the use of nitrate is more costly for cells than ammonium, because of the enzymes involved in its reduction into usable form [166,167]. However, high concentrations of ammonium have been shown to inhibit

growth of some phytoplankton species, although the response is variable among the different groups [168]. Eppley *et al.* [169] did not show any differences on the half-saturation constants for nitrate and ammonium uptake among several coastal and oceanic species. Recently, the possible use of the intracellular NO_3 by the benthic diatom *Amphora coffeaeformis* as a possible survival mechanism in darkness and anoxia has been shown [170]. The same mechanism has also been reported in the pelagic *Thalassiosira weissflogii* [171].

Macronutrient limitation affects growth capacity of microalgae, as well as their physiological state. Some of the physiological variations induced by a reduction in nutrients might be used for biotechnological applications. Nitrogen starvation in many microalgal species is commonly linked to an enhancement of triglyceride accumulation [61,172]. By contrast, lipid content tends to decrease with phosphorus limitation [173]; the latter reduces the formation of phospholipids and triggers the production of triglycerides and other neutral lipids [174]. N-limitation in addition to P-limitation induces an increase in non-photosynthetic carotenoids, as for instance astaxanthin in *Haematococcus pluvialis* [175], or β-carotene in *Dunaliella bardawil* upon nitrogen limitation [176]. Generally, nutrient limitation negatively affects photosynthesis and growth rates of microalgae [177–182]. P-limitation seems to repress the carbon-concentrating mechanism (CCM) activity and leads to lower photosynthesis [183], while N-limitation may have a stimulatory effect on the activity of the CCM, as shown in *Chlorella emersonii* [183].

Micronutrients, such as iron, manganese, zinc, cobalt, copper, molybdenum and the metalloid selenium, are also essential for microalgal growth. They are present in insoluble forms and can be limiting in oligotrophic waters [184,185]. To cope with this feature, offshore species have evolved lower iron requirements to survive in iron-poor oceanic waters compared to coastal species [186]. Iron is involved in controlling microalgal growth, as shown in the diatom *Cyclotella meneghiniana*, while its requirement is nitrogen source dependent [187,188]. Manganese is needed in large amounts for cells growing under low light conditions, as it is involved in photosynthesis [189]. Zinc is present in proteins involved in DNA transcription and in alkaline phosphatase; it is also used as a co-factor for carbonic anhydrase, a critical enzyme that transports and fixes CO_2 [190]. Copper is also needed for photosynthesis, being required in cytochrome oxidase and plastocyanin, even though it can be substituted in some species by iron when cytochrome c_6 is present [191]. Nickel is a co-factor of urease, mainly required when urea is present as nitrogen source [192]. Other micronutrients, such as selenium, cadmium and mercury, can be added to the medium, albeit carefully because of their toxic effects. Moreover, three vitamins—vitamin B_{12} (cyanocobalamin), thiamine, and biotin—are also essential for microalgal growth [193].

Peculiar functional groups of microalgae concerning resource uptake are diazotrophs and mixotrophs (Table 2). Diazotrophs, primarily cyanophytes like the species *Trichodesmium*, fix atmospheric nitrogen and are able to grow without external sources of nitrogen. Beside a potential advantage of such strategy for biotechnological mass cultivation, the low metabolic rate efficiency of these species limits their applications in biotechnology [135]. Mixotrophs instead are able to perform both photosynthesis and heterotrophic grazing on particles or assimilate dissolved organic carbon (osmotrophy) [136–138]. Cells can therefore grow under inorganic nutrient depletion and this feature might favour the allocation of intracellular carbon into proteins [194]. This functional group can account for up to 20% of primary production, explaining plankton blooms under extreme conditions [195].

However, growth rate is generally low [196] and is strongly dependent on temperature, together with the efficiency of grazing activity. It has been recently shown that heterotrophic algae, such as *Scenedesmus* spp., can be used for fatty acid production [197]. Mixotroph species might be potentially used in the framework of the first kind of microalgae application, for pollutants bioremediation (Table 1).

3.4. Functional Diversity: Functional Traits versus Environmental Variables

Microalgae can also be characterized by their adaptive traits to optical, physical or hydrological variables. Since production is strictly related to the amount of energy supplied to the photosynthetic apparatus, light is the main variable driving photosynthetic capacity, and therefore growth. In aquatic ecosystems, light intensity is the most variable parameter, changing over different time and spatial scales and ranging from limiting to excessive. Light limitation influences nutrient uptake [198–203] and degree saturation of lipid [204,205]. Excessive light, which is damaging for cells, results in photoinhibition [206], in reduction of maximum quantum yield, and CO_2 uptake [207–209]. Microalgae have developed protective mechanisms, to prevent or limit irreversible photoinhibition, in order to cope with the formation of reactive oxygen species (ROS) by synthesizing certain carotenoids or other molecules with antioxidant activities [66]. The first response to high light is the activation of photoprotective xanthophyll cycle [23], leading to the synthesis of zeaxanthin (in green algae) or diatoxanthin (in many chlorophyll *c*-containing algae). The role of these photoprotective xanthophylls, in countering peroxidative damage, has been reported in diatoms [210]. In *Haematococcus pluvialis* high light conditions increase the astaxanthin content three-fold as compared to low light [211,212].

Two functional groups, discriminated by light responses, have been proposed: high light and shade-acclimated species [139]. The high light group is characterized by low photosynthetic pigment content relative to the shade-acclimated species. More recently, Dimier *et al.* [24] discriminated three functional groups based on their photoregulation capacity: high light adapted, low light adapted and variable light adapted. The latter group, composed by coastal microalgae, is characterized by efficient and fast development of photoprotective processes such as xanthophyll cycle and non-photochemical quenching [24].

Recently, it has been shown that the growth capacity of the toxic *Ostreopsis ovata* (dinophyte) is strongly dependent on the effects of both temperature and light [21]. Temperature influences the growth of photoautotrophs through its control of enzymatic kinetics [213,214] and changes in cellular membrane composition [215]. Generally in microalgae, an increase in temperature leads to an enhancement in growth [216–219], as well as in carbon content [218–222]. Indeed, it has also been shown by Toseland and collaborators [142] that temperature strongly affects phytoplankton metabolism in the field as nutrients and light do. Microalgal responses to temperature also vary between groups (Table 2) [88,216,223,224]. The optimal temperature for growth capacity is usually below 10 °C for polar species [225,226], around 10–25 °C for temperate ones [227,228], close and beyond 25 °C for tropical species [88] and between 25 and 35 °C for desert-inhabiting microalgae [229].

Another relevant variable is salinity (Table 2). Osmoregulation is achieved by uptake of ions [230,231], the synthesis of osmotic active compounds or the expulsion of water [76,230,232–235]. Biochemical changes occur in cells exposed to high salinity, including an increase in ash content [236,237] and

protein synthesis inhibition [238]. Estuarine species are tolerant to a wide range of salinities, though fewer species are able to grow in high salinity conditions. The success of these halophytic species is related to enhanced synthesis of osmotic stress regulation molecules such as the zeaxanthin in cyanophytes [239] or glycerol in the green alga *Dunaliella salina* [240]. The latter also accumulates β-carotene under high salinity stress [176,241,242] while *Haematococcus pluvialis* accumulates astaxanthin [175]. These species are already used in biotechnology to produce stress-response molecules, useful in cosmetics or nutrition [144].

In a culture batch, it is also important to monitor pH since it can vary in relation to photosynthesis. Moreover, pH variations can induce: (i) the differential availability of inorganic carbon sources ($CO_2/HCO_3^-/CO_3^{2-}$) [243–245]; (ii) the co-precipitation of phosphate with calcium, magnesium and carbonate at high carbonate concentrations; (iii) the low solubility and availability of trace metals at high pH [246]; (iv) intracellular pH regulation; and (v) changes in the uptake of essential nutrients, such as nitrate and phosphate [22,247,248]. The diatom *Phaeodactylum tricornutum* presents high plasticity to pH variation being able to grow at pH > 10, making it one of the most common contaminants in poorly buffered cultures [249,250]. This property could be advantageous in outdoor mass cultivation.

4. Enhancing Production and Growth: "Photosynthetic Regulation Biotechnology" and Genetic Modifications

One of the main challenges for increasing biotechnological applications of microalgae is the enhancement of their growth and yield. The growth rate of a microalgal population is the result of the balance between actively dividing and death cells. This "performance index" mainly depends on both cells' acclimation to the culture conditions and the energetic cost of internal processes regulation. The latter depends on how ecophysiological requirements match the environmental conditions and on physiological plasticity of the cultivated strains. Two of the most promising routes to progress, in enhancing biomass or interesting molecules production, are the "Photosynthetic Regulation Biotechnology" (Figure 1) and the genetic engineering of strains.

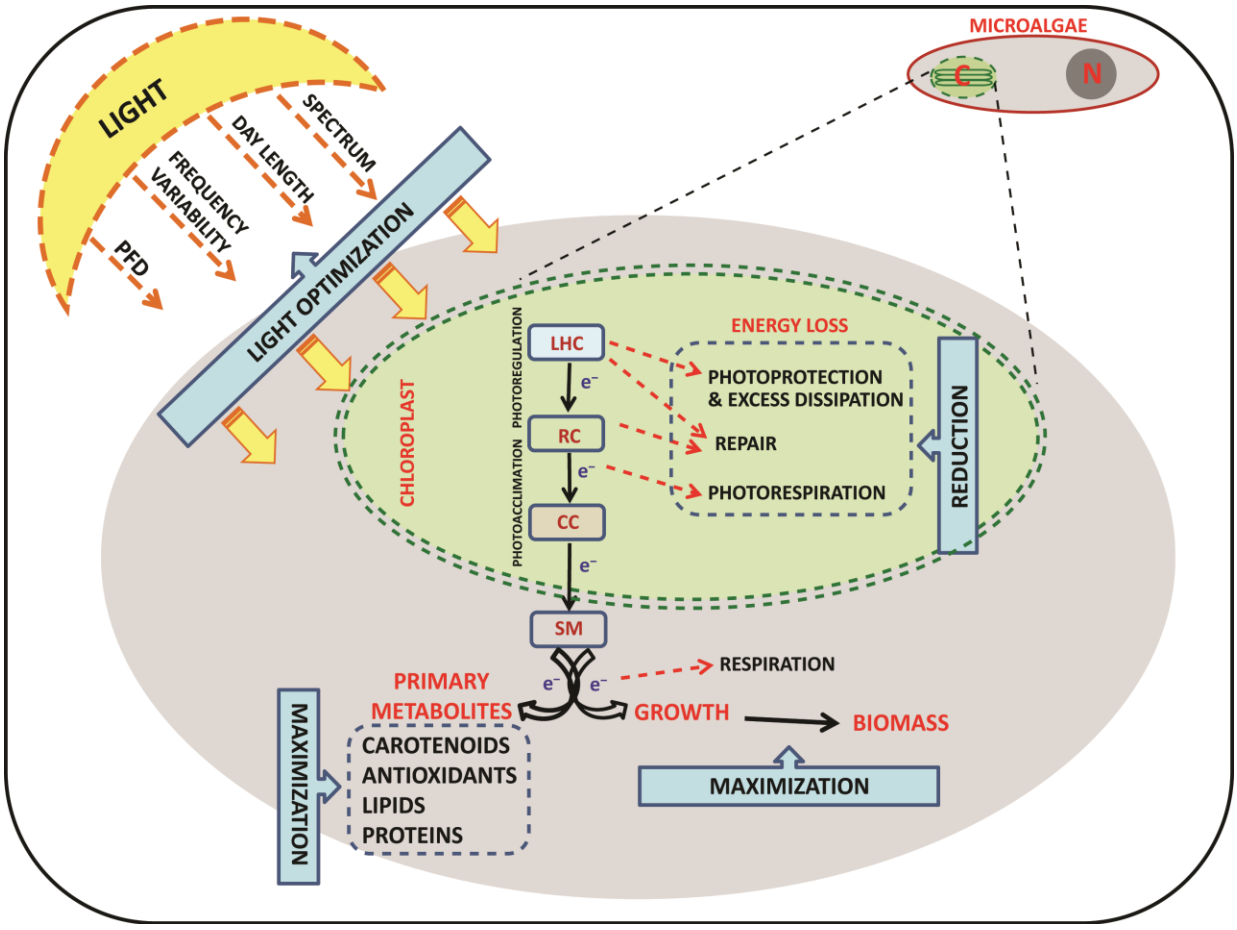
4.1. Light Control and the "Photosynthetic Regulation Biotechnology"

Light is the most relevant trigger for photosynthesis even though it is the most variable parameter at sea over different temporal and spatial scales (see previous section). Many recent studies, using physiological, biochemical or molecular approaches, have shown how light intensity, variability and spectral composition affect the physiological state and growth of cells [24,25,251–253]. By consequence, light manipulation might be a way for modifying microalgal productive performance [254,255], and therefore a relevant issue for biotechnological purposes (see the review by Murchie and collaborators [256]), in the field of "photosynthetic regulation biotechnology".

Photosynthesis is a unique process that converts light energy into biochemical energy, through light and dark reactions. In response to light intensity variations, cells modulate two stages of the photosynthetic process by modifying the photosynthetic apparatus structure and pigments, as well as the enzymes involved in carbon fixation [257]. Recent studies have shown that these two steps do not respond in the same way to light changes, showing an uncoupling of their regulative processes

that depend on the ecophysiological properties of cells, and this also affects the growth rate maintenance [25]. Photosynthetic properties and growth can be poorly coupled, as observed in a diatom and a prasinophyte subjected to fluctuating irradiance [258], reinforcing the observations on the variability of the relationship between photosynthetic organic production and cell growth [259]. This variability can be attributed to many factors, such as variations in respiration, changes in dissolved organic losses and metabolite storage, as for instance polysaccharides [258]. The discrepancy between growth and production is less severe in small cells with smaller intracellular energy storage [25,126,251].

Figure 1. "Photosynthetic Regulation Biotechnology": light manipulation to maximize photosynthesis and growth. The optimization of light in terms of photon flux density, spectral radiations, photoperiod, and frequency variability is investigated in order to reduce energy losses during the photosynthetic process and maximize biomass and primary metabolite production. PFD-Photon Flux Density; e^--electron; LHC-Light Harvesting Complex; RC-Reaction Center; CC-Calvin Cycle; SM-Storage Molecules; C-Chloroplast and N-Nucleus.



The term "light" includes different key variables (Figure 1): instantaneous photon flux density (PFD), daily light dose intensity, photoperiod, light distribution, frequency of PFD variability and spectral characteristics [20,260]. The integrated daily light dose depends on the photoperiod and light distribution. The daily light dose experienced by cells under sinusoidal light distribution compared to

quadratic shape is around 1.9 times less, when provided using the same 12:12 h light–dark photoperiod and maximal light value [164]. Light is generally provided following a quadratic distribution, *i.e.*, with an "on/off" switch system. Recent studies have shown that applying a sinusoidal light regime allows cells to activate a gradual and efficient photoregulation on the contrary of a quadratic distribution [24,251]. It has been observed that the slow increase of light at dawn allows cells to efficiently perform photoregulation and prepares the photosynthetic apparatus to cope with the high light midday peak [24,251]. By contrast, a constant excessive light induces considerable damage, increasing the biochemical costs associated with defence, protection and recovery, and decreasing the energy fuelled towards growth. Reciprocally, when constant light is limiting for optimal photosynthesis, cell performance will be reduced.

The photoperiod, *i.e.*, the succession of illuminated and dark periods during the day, influences microalgal growth and photosynthetic rate [20,261]. It is known that biomass synthesis, in terms of produced carbon, may be higher under continuous light than alternating light–dark phases [203,262]. However, cells do not appear healthy under continuous light [263]; the light–dark succession allows cells to recover and uncouple many biological processes, such as photosynthesis, from cell division [20,264]. Moreover, photosystem repairing and relaxing occur during the dark period; it is also known that during dark phytoplankton cells uptake and assimilate NH_4 [265,266].

The very fast frequency of light variability (scale of milliseconds) positively influences photosynthetic efficiency, due to the re-oxidation of the electron transporters of the photosynthetic apparatus during dark phases [261]. Again, the frequency of the light variability, on an hour scale, is highly relevant. The shade acclimation state of cells is strengthened by the enhancement of the light fluctuations experienced, driving production of different carotenoids and antioxidants [251]. Light fluctuation dynamics also strongly affect the growth capacity of cells [251,258,259,267].

The spectral radiation of light does influence growth, photoacclimation state, and cell biochemistry [268,269]. Blue light affects many physiological processes in algae, such as photo-morphogenesis [270], chloroplast movement [271], cell division and photosynthetic acclimation in diatoms [252,253,270,272]. The spectral composition of light plays a key role in the ability of diatoms to finely balance light harvesting and photoprotective capacities [273]. Indeed, red radiation mixed to blue is necessary for activating the photoprotective pathway, such as the synthesis of xanthophyll pigments with an antioxidant role [274,275]. The spectral properties of microalgal absorption are the base for designing new models of photobioreactors and improving microalgal growth [276].

4.2. Genetic Transformations

Until now, biotechnological production from microalgae, such as food additives, cosmetics, animal feed additives, pigments, polysaccharides and fatty acids, is done in a non-transgenic way. However, genetic engineering is already being applied to microalgal research field, and selectable marker genes, promoters, reporter genes, transformation techniques, together with other genetic tools are available for various species. At present, about 20 species are accessible to genetic transformation [27], and large-scale sequencing projects are in progress for several microalgae species [277]. Sequences are available at the NCBI organelle database [278] and at the Organelle Genome Database [279].

The complete genome for about 30 cyanobacteria have already been sequenced; genome sequences of the cyanophyte *Synechococcus* [280], the green alga *Micromonas* [281] and *Ostreococcus* [128] are also available. For a more exhaustive list, please refer to the website from the Roscoff Culture Collection [282,283]. The following species' genomes (nuclear, mitochondrial and plastidial): *Alexandrium tamarense, Amphidinium operculatum, Aureococcus anophagefferens*, *Chlorella vulgaris*, *Cyanophora paradoxa,* and *Dunaliella salina* are being sequenced, and 37 microalgae transcriptomic projects are currently taking place [8,284].

In the last few years, about 20 new microalgal species have been genetically modified with success: nine green microalgae, five diatoms, three cyanobacteria, two dinophytes, two red microalgae and one euglenoid have been successfully transformed; most of these were achieved by nuclear and stable transformation [27]. Stable transformation of four diatom species (*Phaeodactylum tricornutum, Navicula saprophila, Cylindrotecha fusiformis* and *Cyclotella cryptica*) has also been reported, and 62 promoters have already been tested for microalgae transformations [8]. The use of engineered nucleases to genetically reprogram diatoms, with the aim of producing bio-fuels, has been successfully demonstrated [285]. By targeting specific sequences within diatoms' genomes, nucleases can be used to accurately insert, correct, or inactivate specific genes. With the whole genome sequencing of several diatom species, such as *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, a new era of post-genomics research has begun, with opportunities to improve fundamental understanding of the diatoms biology, and to build a molecular foundation for new industrial applications. Stable mutants of some species of microalgae have been obtained in the last years such as *Phaeodactylum tricornutum*, expressing a heterologous functional glucose transporter, *Dunaliella salina* (*zea1*) overproducing zeaxanthin, and cyanobacteria-synthetizing mosquito larvacides [7,27,80,286–289]. *Euglena gracilis* has been transformed at the chloroplast level [290], while *Thalassiosira weissflogii* has been only transiently transformed [287]. Some groups are more difficult to be transformed by electroporation or conjugation [291], as for instance diatoms with their silica wall, compared to species from other groups such as *Spirulina*, *Anabaena*, or *Synechocystis*. Recently *Synechocystis* sp PCC6803 was deeply analysed, through several growth-coupled knockouts, to identify its main metabolic properties in the aim of biotechnological applications [292].

One of the main goals in biotechnological genetic engineering is their use as "bioreactors" in producing vaccines [293–295], specific proteins [296], or bioenergetic molecules [297]. Some therapeutic proteins have been successfully produced using microalgae, mainly the Chlorophyta *Chlamydomonas reinhardtii*, for which suitable transgenic tools and genomic data are available (for all three genomes: nuclear, chloroplastic and mitochondrial). The chloroplast of *C. reinhardtii* has been used to produce a range of recombinant proteins, including reporters such as glucuronidase (GUS), luciferase (LUC), green fluorescent protein (GFP), industrial enzymes, vaccines and therapeutic enzymes [298]. To date [8], 18 biopharmaceutical proteins have been expressed in *C. reinhardtii* and one in *Chlorella ellipsoidea* [298,299]. Diatoms have not been employed for expression of any biopharmaceutical proteins, but Hempel and collaborators [300] have recently reported the first stable expression of a full-length human antibody and the respective antigen in *P. tricornutum*.

In the bio-fuel production field, the Heterokonta *Nannochloropsis* represents a new model with potential for further development into an integrated photons-to-fuel production platform [301]. Several isolates of *Nannochloropsis* spp. produce large quantities of triacylglycerols, related to over-representation

of genes involved in lipid biosynthesis together with rapid growth capacity, and industrial-scale cultivation [301]. The genome of the high PUFA-content species *Nannochloropsis oceanica* has been recently sequenced by Pan and collaborators [302]. Sequence similarity-based investigation identified new elongase- and desaturase-encoding genes involved in the biosynthesis of PUFAs, which provide a genetic basis for its rich eicosapentaenoic acid (EPA) content, making this species suitable for the genetic engineering of a triacylglycerols pathway.

To conclude, we can assert that genetic transformation could be a valid tool to "revolutionize" blue biotechnology. However, cautious measures have to be taken for ecosystem bio-safety and to monitor transgenic microorganisms in nature.

5. Conclusions

The functional biodiversity of microalgae has to be explored thoroughly, and mass culturing conditions presently used have to be revisited in order to optimize the fitness of cultivated species and decrease production costs. Indoor culturing systems would be preferable to outdoor systems for the reasons already discussed on the controlled *vs.* uncontrolled environmental parameters. We also suggest use of local microalgal species and seawater from which the species have been isolated, *i.e*., cultivation next to aquatic ecosystems, in the frame of what we can call the "Km-0 mass cultivation" strategy. This strategy might reduce the cost of cultivation, allow for the use of freshly isolated species and strains, and potentially also provide high flexibility in species choice that share common ecophysiological requirements. This could also solve the potential loss of growth efficiency due to long-term cultivation maintenance.

Furthermore, we recommend the use of ammonium, as nitrogen source, to increase biomass production, since it is assimilated faster than nitrate, and this allows an increase in the production efficiency (see above). We suggest providing light with an intra-diel light–dark cycle, with a sinusoidal shape instead of quadratic distribution, for the reasons discussed in the previous sections. Turbulence, reproducing coastal ecosystems, can be applied to coastal species cultivation, through a light variation frequency program. We propose the use of illumination systems that allow regulation of the photon flux density and the spectral composition to better manage photosynthetic productivity. For many microalgal groups (e.g., diatoms or chlorophytes), green radiation may be excluded from the light spectrum since it is not harvested, in order to increase the growth yield and reduce production costs [276]. Since the effect of light variations on physiology and growth of autotrophs is relevant, we suggest that genetic engineering should mainly target the photophysiological response system (e.g., [252,253,303]), entering the "photosynthetic regulation biotechnology." The photophysiological pathway transformation of microalgae could be useful but with a high probability of obtaining a productive strain with a slow growth.

Diatoms that include many coastal species might be the ideal model to cultivate with biotechnological aims, for many reasons, such as the huge biodiversity of this group and many biological peculiarities (see previous). Many diatom species grow on benthic substrates, at least during one step of their life cycle; therefore, their cultivation for biotechnological purposes needs a deeper communication between ecophysiological and process engineering researchers. Moreover, they are able to reproduce sexually [134], allowing this group frequent gene recombination processes and

present high physiological flexibility [274]. Due to this relevant feature, we could easily manipulate their photophysiological responses and biochemical pathways to increase the production of targeted molecules by varying the culture conditions.

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Author Contributions

Lucia Barra, Raghu Chandrasekaran contributed equally in writing the manuscript. Christophe Brunet, Federico Corato conceived and designed the format of the manuscript. All the authors contributed in critical reading and discussion on the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Spectral Radiation Dependent Photoprotective Mechanism in the Diatom *Pseudo-nitzschia multistriata*

Christophe Brunet[1]*, Raghu Chandrasekaran[1], Lucia Barra[1], Vasco Giovagnetti[1], Federico Corato[1], Alexander V. Ruban[2]

1 Laboratory of Ecology and Evolution of Plankton, Stazione Zoologica Anton Dohrn, Villa Comunale, Napoli, Italy, **2** School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London, United Kingdom

Abstract

Phytoplankton, such as diatoms, experience great variations of photon flux density (PFD) and light spectrum along the marine water column. Diatoms have developed some rapidly-regulated photoprotective mechanisms, such as the xanthophyll cycle activation (XC) and the non-photochemical chlorophyll fluorescence quenching (NPQ), to protect themselves from photooxidative damages caused by excess PFD. In this study, we investigate the role of blue fluence rate in combination with red radiation in shaping photoacclimative and protective responses in the coastal diatom *Pseudo-nitzschia multistriata*. This diatom was acclimated to four spectral light conditions (blue, red, blue-red, blue-red-green), each of them provided with low and high PFD. Our results reveal that the increase in the XC pool size and the amplitude of NPQ is determined by the blue fluence rate experienced by cells, while cells require sensing red radiation to allow the development of these processes. Variations in the light spectrum and in the blue versus red radiation modulate either the photoprotective capacity, such as the activation of the diadinoxanthin-diatoxanthin xanthophyll cycle, the diadinoxanthin de-epoxidation rate and the capacity of non-photochemical quenching, or the pigment composition of this diatom. We propose that spectral composition of light has a key role on the ability of diatoms to finely balance light harvesting and photoprotective capacity.

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* E-mail: christophe.brunet@szn.it

Introduction

Originating some 2.32−2.45 Gyr ago, oxygenic photosynthesis spread across the Earth, allowing the great diversification of life and globally altering the community structure and ecological function of terrestrial and aquatic habitats [1,2]. Phytoplankton, small floating photosynthetic microorganisms that populate the aquatic realms, thrive in a light environment naturally variable over spatial and temporal extremes [3,4]. While penetrating through the water column, light intensity (photon flux density; PFD) exponentially decreases (Fig. 1A) due to absorption and scattering by dissolved substances and suspended particles [5]. The unpredictable passing of clouds, motion of waves and turbulent mixing, superimposed to long-term diel and seasonal periodicity, create very complex patterns of short-term fluctuations in the instantaneously available light that controls phytoplankton photosynthesis [6]. In response to such a heterogeneous light environment, phytoplankton have evolved protective mechanisms to harvest light in conditions of excess detrimental PFD, and minimize photo-oxidative damage caused by the formation of reactive oxygen species in the photosystems [7−9]. The xanthophyll cycle (XC) and non-photochemical quenching (NPQ) are crucial photoprotective processes that are rapidly activated (seconds to minutes) to dissipate excess absorbed light energy and ensure efficient light harvesting in the photosynthetic membrane [10−12].

Together with the PFD variations along the water column, light spectrum also changes greatly, with different attenuation of red, green and blue lights (Fig. 1B), with a steep decrease of the red : blue ratio over the surface layer (Fig. 1C). Interest concerning how does phytoplankton vary its physiological properties with spectral radiation (e.g., [13−17]) is still open and, recently, moves towards the intriguing question on how do photosynthetic cells acclimate to and sense the marine light radiation [18−23]. Some ecologically-relevant processes, such as aggregation in the dinophyte *Gonyaulax* [24], enhancement of sinking rate [25], and cell motility in diatoms [26] are shown to be triggered by red light, while little information is still available on red light photoreceptors [20]. By contrast, many key physiological processes in algae, such as photomorphogenesis [23], chloroplast movements [27] and cell division in diatoms [23,28] are triggered by blue light. Blue light photoreceptors, belonging to the aureochrome family, identified in the xanthophyte *Vaucheria frigida* [18] have been shown to be only present in photosynthetic stramenophiles [29]. Very recently, by acclimating *Phaeodactylum tricornutum* to different spectral radiation, Schellenberger Costa et al. showed that blue light perception by aureochrome governs the acclimation and protection processes [21,22].

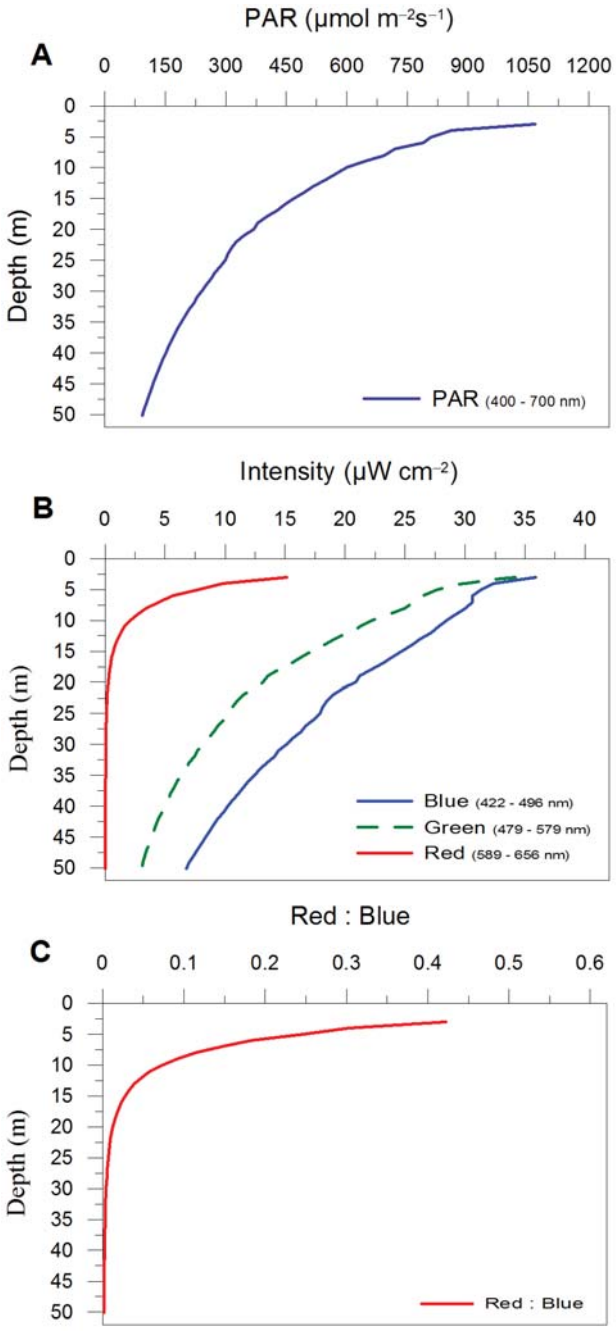

Figure 1. *In situ* light profile *vs* depth. (A) PAR (µmol photons m^{-2} s^{-1}) distribution along the water column, (B) blue, red and green radiation distribution along the water column (µW cm^{-2}; blue –422 to 496 nm, green –479 to 579 nm and red –589 to 656 nm), and (C) distribution of red : blue ratio along the water column. Data are mean of profiles done on 5 stations in the Mediterranean Sea in June–July 2008 (C. Brunet, unpublished data).
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The general objective of our study is to decipher the role of spectral radiation on the photophysiological acclimation properties of coastal diatoms, well-known for their high photosynthetic flexibility and regulative capacity [7,9,10]. We choose the toxic *Pseudo-nitzschia multistriata* species, since the strain has been recently isolated from the Gulf of Naples (coastal area of the Mediterranean sea, Stazione Zoologica Anton Dohrn, Italy, strain number SY717) and its ecological properties are known (e.g., [30]).

We investigate if and how spectral radiation does affect the photoprotective capacity of this diatom, focussing on the regulation of pigment content, and on the rapidly activated protective responses, as the XC and NPQ. Since the different distribution over water column of the red and blue radiations, and their essential eco-physiological roles (as introduced before), we aim to test the hypothesis that photoprotective capacity in diatoms differs between different mixtures of blue and red radiations, compared to the same radiations when provided separately. We also address the question on the biological effect of the red : blue ratio of the light experienced by cells, as trigger for the photoprotective response ([22]).

The results suggest that spectral composition of light has a key role, together with PFD, on the ability of diatoms to finely balance light harvesting and photoprotective capacity. To our knowledge this is the first report demonstrating the dependence of the XC and NPQ to both the blue and red radiation together.

Materials and Methods

Ethics Statement

No specific permits or permissions were required for the field studies, as the cruise for measuring the vertical light profiles was carried out in international waters and the isolation of *Pseudo-nitzschia multistriata* strain SY717 has been done during the long term research Mare-Chiara program in the coastal area of the Gulf of Naples where no specific permits or permissions were required. This work did not involve endangered or protected species.

Experimental Strategy and Sampling

Four spectral light conditions – blue, red and two mixed light conditions, namely blue-red-green and blue-red were applied (Table 1). The two mixed light conditions were characterized by (i) the same photon flux density (PFD) and relative proportion of red radiation provided (18–20%), and (ii) two different red : blue ratios: 0.43 (blue-red-green) and 0.25 (blue-red), determined by the presence or absence of green light (Table 1). These two values of red : blue ratio characterize the high light environment, 2 m (~0.43) and 6 m (~0.25) depths, of the water column during summer in the Mediterranean Sea (Fig. 1).

For each condition, the daily light dose was kept constant, in order to be comparable for the provided photon flux density. Two daily light doses, 6.1 mol m^{-2} d^{-1} and 11 mol m^{-2} d^{-1} (sinusoidal light distribution, peaking at 250 and 450 µmol photons m^{-2} s^{-1}, respectively; Table 1), have been tested, with a 12:12 hours light:dark photoperiod. Light intensity was measured inside each flask by using a laboratory PAR 4 π sensor (QSL 2101, Biospherical Instruments Inc., San Diego, CA, USA), while spectral composition (PAR(λ)) were measured at light peak by using a radiometer (Hyper OCR I, Satlantic, Halifax, CA).

Light was provided by a custom-built illumination system, which allows to monitor and regulate the light intensity and quality. The system is composed by blue, green and red light emitting diodes (peaking at 460, 530 and 626 nm, respectively; Fig. 2). Experiments were conducted on *Pseudo-nitzschia multistriata* strain SY717 isolated in the Gulf of Naples (40° 48′ N, 14° 15′ E, Mediterranean Sea). Cells were cultivated at 20°C in 75 cm^2 polystyrene canted neck flasks (Corning® flask, Corning Inc., NY, USA), containing natural sterile seawater amended with f/2 nutrients. All the experiments, lasting three days, were performed in triplicate during the exponential growth phase (Fig. 3), on

Table 1. Light condition characteristics, and photosynthetic and biochemical properties in *Pseudo-nitzschia multistriata* cells.

	Blue		Blue-red		Blue-red-green		Red
	Low	High	Low	High	Low	High	Low
	(B-L)	(B-H)	(BR-L)	(BR-H)	(BRG-L)	(BRG-H)	(R-L)
PFD	250	450	250	450	250	450	250
Blue	250	450	200	360	105	189	0
Green	0	0	0	0	100	180	0
Red	0	0	50	90	45	81	250
Red : Blue	0	0	0.25	0.25	0.43	0.43	0
a^*	2.51 (0.09)	1.46 (0.07)	2.69 (0.22)	2.89 (0.10)	1.97 (0.63)	1.22 (0.08)	4.44 (0.05)
PUR	2.47 (0.09)	2.4 (0.06)	2.27 (0.23)	4.39 (0.16)	1.49 (0.51)	1.95 (0.12)	1.11 (0.23)
$_{rel}ETR_{max}$	2.72 (0.13)	1.66 (0.11)	3.61 (0.41)	3.45 (0.17)	2.13 (0.57)	1.07 (0.09)	2.63 (0.47)
α	6.7 (1.1)	3.8 (0.27)	7.3 (0.36)	7.1 (0.44)	4.9 (0.96)	3.0 (0.54)	13 (2.2)
Ek	408 (66)	431 (21)	497 (32)	491 (39)	472 (37)	363 (84)	200 (5)
POC	131 (37)	62 (8)	80 (5)	80 (5)	100 (8)	89 (5)	154 (28)
POC/PON	4.9 (0.52)	4.3 (0.63)	4.8 (0.27)	4.7 (0.37)	6.2 (0.76)	4.7 (0.05)	2.9 (0.19)
Chl *a*/POC	8.2 (4.8)	10.3 (2.9)	13.8 (2.6)	10.7 (1.5)	9.8 (2.2)	10.8 (0.9)	6.2 (2.5)

Blue, green and red fluence rates (µmol photon m^{-2} s^{-1}) measured at light peak and red : blue ratio values for the different light conditions. $a^* \times 10^{-11}$, absorption coefficient (m^2 $cell^{-1}$); PUR$\times 10^{-6}$, photosynthetically usable radiation (µW $cell^{-1}$); $_{rel}ETR_{max} \times 10^{-6}$, (maximal relative rate of linear electron transport, nmol e^{-1} s^{-1} $cell^{-1}$), $\alpha \times 10^{-9}$ (maximum light use efficiency, nmol e^{-1} s^{-1} $cell^{-1}$(µmol photon m^{-2} s^{-1}) $^{-1}$), and *Ek* (light intensity for reaching $_{rel}ETR_{max}$, µmol photon m^{-2} s^{-1}); POC, particulate organic carbon (pg $cell^{-1}$); POC/PON, particulate organic carbon (POC) to particulate organic nitrogen (PON) ratio (pg/pg); Chl *a*/POC$\times 10^{-3}$, Chlorophyll *a* to POC ratio (pg/pg). Data represent mean and standard deviation. For a^* and PUR, $n = 3$; For $_{rel}ETR_{max}$, α and *Ek*, $n = 6$ (mean of the two days light peak measurements); For POC, PON, POC/PON and Chl *a*/POC, $n = 21$.

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cultures pre-acclimated to each experimental light condition for two weeks before the experiments. Under red light, at high PFD (450 µmol photons m^{-2} s^{-1}) cells did not grow, preventing any experimental result.

Samples for pigments, variable fluorescence and electron transport rate, and elemental composition analysis were taken at dawn (time 0), midday (time 6 hours) and in the afternoon (time 9 hours), during the first two days of the experiment, and once (time 0) during the third day. Cell counts were performed daily at time 0, while the absorption spectrum were analysed once per experimental condition (time 6 hours on the second day).

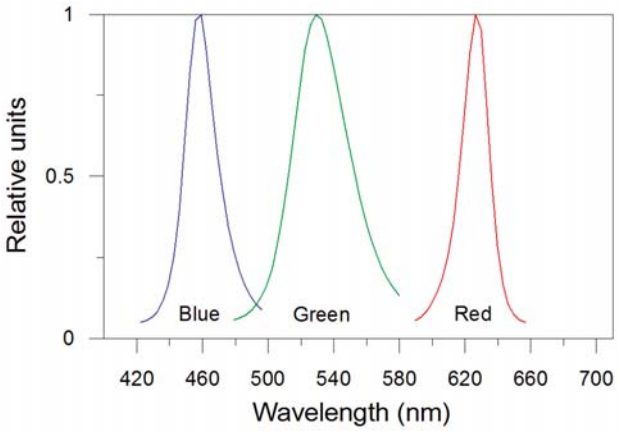

Figure 2. Spectral properties of the LEDs. Blue (422−496 nm), green (480−580 nm) and red light (590−656 nm).

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Cell Concentration

Cell concentration was estimated on triplicate sub-samples. An aliquot of 1 mL was used to fill a Sedgewick Rafter counting cell chamber, and cell counts were performed using a Zeiss Axioskop 2 Plus microscope.

Photochemical Efficiency and Photosynthetic Parameters

Photochemical efficiency of photosystem (PS) II was estimated by a Phyto-PAM fluorometer (Heinz Walz, Effeltrich, Germany). The variable fluorescence analysis was performed on 15-minutes dark-acclimated samples, to measure the maximum photochemical efficiency (Fv/Fm, [31]). Fm was measured after a saturating pulse of red light (2400 µmol m^{-2} s^{-1}, lasting 450 ms), causing a complete reduction of the PSII acceptor pool.

Electron transport rate (ETR) *versus* irradiance curves were determined applying 13 increasing red actinic lights (655 nm) from 1 to 853 µmol photons m^{-2} s^{-1} lasting 1 minute each. The relative electron transport rate ($_{rel}ETR$, expressed in µmol e^{-1} s^{-1} $cell^{-1}$) was calculated as follows: $_{rel}ETR = (Fv'/Fm') \cdot I \cdot 0.5 \cdot a^*$.

where, I is the incident irradiance (expressed in µmol photons m^{-2} s^{-1}), Fv′ and Fm′ are the variable PS II fluorescence yield and maximal PS II fluorescence yield, respectively, for illuminated cells (measured at the end of the 1 min lasting actinic light), a^* is the cell-specific absorption coefficient, expressed in m^2 $cell^{-1}$ (for the determination of a^* see below). A factor of 0.5 was applied to correct for the partitioning of photons between PSI and PSII, assuming that excitation energy is evenly distributed between the two photosystems.

ETR-I curves were fitted with the equation of Eilers and Peeters to estimate the photosynthetic parameters [32], $_{rel}ETR_{max}$ (maximal relative rate of linear electron transport), α (maximum light use efficiency which is the slope of the beginning of the light curve), and *Ek* (light intensity for reaching $_{rel}ETR_{max}$).

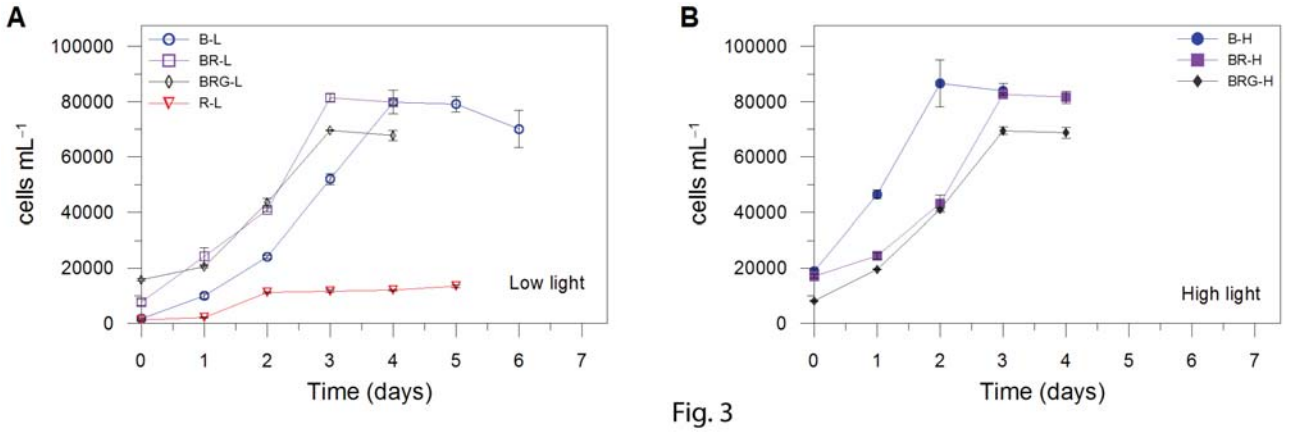

Figure 3. Growth curve of *Pseudo-nitzschia multistriata*. Growth under (A) low and (B) high light. B-L, BR-L, BRG-L, R-L are blue, blue-red, blue-red-green, and red low light conditions, respectively; B-H, BR-H, and BRG-H are blue, blue-red, blue-red-green high light conditions, respectively. Red high light prevented cell growth. Experiments were performed during the exponential phase on days 3 to 5 (B-L), 1 to 3 (R-L, BR-L, BRG-L, B-H) and 2 to 4 (BR-H, BRG-H). Data represent mean ± SD ($n = 3$).
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For the non-photochemical quenching (NPQ) estimation, dark-adapted cells were illuminated with an actinic light setup at 480 µmol photons m^{-2} s^{-1} during 10 minutes, and the maximum fluorescence yield was estimated every min. NPQ was quantified by the Stern-Volmer expression: NPQ = (Fm/Fm′)–1.

Pigments

Pigment measurement was conducted by High Performance Liquid Chromatography (HPLC). An aliquot of algal culture (10 mL) was taken with a pipette, immediately filtered (under low light condition) on 25 mm GF/F glass-fiber filter (Whatman, Maidstone, UK) and stored in liquid nitrogen until further analysis. Pigments were extracted by mechanical grounding during 3 minutes in 2 mL of a 100% methanol solution. Successively, the homogenate was filtered onto Whatman 25 mm GF/F glass-fiber filters and the volume of the extract was accurately measured. Prior to injection into the HPLC, 250 µL of an Ion Pairing Agent (ammonium acetate 1 mol L^{-1}, final concentration 0.33 mol L^{-1}) were added to 0.5 mL of the pigment extract and incubated for 5 minutes in darkness at 4°C. This extract was then injected in the 50 µL loop of the Hewlett Packard series 1100 HPLC (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (2.6 µm diameter C8 Kinetex column; 50 mm×4.6 mm; Phenomenex®, USA). The temperature of the column was steadily maintained at 20°C, and the flow rate of the mobile phase was set up at 1.7 mL min^{-1}. The mobile phase was composed of two solvents mixture: A, methanol/aqueous ammonium acetate (70/30, v/v) and B, methanol. During the 12-minutes elution, the gradient between the solvents was programmed: 75% A (0 min), 50% A (1 min), 0% A (8 min), 0% A (11 min), 75% A (12 min). Pigments were detected spectrophotometrically at 440 nm using a Hewlett Packard photodiode array detector, model DAD series 1100. Fluorescent pigments were detected using a Hewlett Packard standard FLD cell series 1100 with excitation and emission wavelengths set at 407 nm and 665 nm, respectively. Determination and quantification of pigments were carried out using pigment standards from the D.H.I. Water & Environment (Horsholm, Denmark).

Particulate Organic Carbon and Nitrogen

Ten mL aliquots for the determination of particulate organic carbon (POC) and particulate organic nitrogen (PON) were filtered on pre-combusted (450°C, 5 hours) glass-fiber filters (Whatman, Maidstone, UK), conserved in cell culture plates (Corning®, Corning Inc., NY, USA), and immediately stored at –20°C. The analyses were performed with a Thermo Scientific Flash EA 1112 automatic elemental analyzer (Thermo Fisher Scientific, MA, USA), following the procedure previously described by Hedges and Stern, [33]. Filters were thawed just prior to analysis and allowed to dry at 60°C through a desiccator. Then filters were loaded in small tin cups that were crimped closed and transferred to the CHN analyzer. A set of empty filters was processed as ordinary samples to accomplish the blank determination. Cyclohexanone 2,4-dinitrophenylhydrazone (C% 51.79, N% 20.14, H% 5.07) was used as standard.

Absorption Spectrum

The spectral absorption measurements were performed using a spectrophotometer Hewlett Packard HP-8453E equipped with an inverted Labsphere integrating sphere (RSA-HP-53 Reflectance Spectroscopy Accessory). Ten mL aliquot was used to measure the spectral values of absorption coefficient (m^{-1}) by intact cells [34]. Filtered cultures were used as references and the measurements were done in cuvette with 5 cm light path. The $a(\lambda)$ values were measured between 250 nm to 800 nm, and integrated between 400 and 700 nm. This integrated value was divided by cell concentration for the estimation of the cell-specific absorption coefficient, a^*, expressed in m^2 cell^{-1}.

The photosynthetically usuable radiation (PUR) was calculated using the following equation (Morel et al. [34]):

$$PUR = \int_{400}^{700} PAR(\lambda)a(\lambda)\mathrm{d}\lambda$$

Statistical Analysis

Student's *t*-test and Spearman's rank correlation was performed using Systat 7 software.

Results and Discussion

Spectral Radiations and Photoprotective Responses

The synthesis of xanthophyll cycle (XC) pigments, diadinoxanthin (Dd) and diatoxanthin (Dt), is higher under high light than low light (Fig. 4A, $p<0.01$, $n=21$), with the exception of blue high light condition (B-H), in which cells did not increase the XC pigment pool. The low synthesis of both Dd and Dt under B-H (Fig. 4B,C) is not related to a variation in light absorption, since the absorption coefficient (a^*, Table 1) and photosynthetically usable radiation (PUR, Table 1) in B-H were similar to the values found in BRG-H (blue-red-green high; $p>0.05$, $n=3$), in which Dd and Dt were significantly produced.

Therefore, to explain the absence of Dt and Dd synthesis in B-H, compared to BR-H (blue-red high) and BRG-H, we propose that the XC pigment synthesis in diatoms might require sensing of red light to be triggered, as well as the activation of blue-photoreceptors by high blue fluence rate to be activated [21]. Red light might act as a signal for cells to initiate the high light regulatory pathway, the intensity of the photoprotective response being thus determined by the blue fluence rate perceived by cells. This hypothesis fits with the results obtained under low light. Indeed, red radiation alone prevented Dt synthesis, and blue radiation alone was not able to enhance the Dt synthesis (Fig. 4A,D,E). Furthermore, significantly higher XC pigment content (Dd and Dt; Fig. 4A,D,E) was found in the BR-L (blue-red low) condition compared to B-L (blue low) and BRG-L (blue-red-green low; $p<0.01$, $n=21$). The absence of such XC activation under BRG-L is related to the low blue fluence rate experienced by cells (105 µmol photons m^{-2} s^{-1}, Table 1), compared to BR-L (200 µmol photons m^{-2} s^{-1}) or to high light conditions ($\geq$190 µmol photons m^{-2} s^{-1}, Table 1). Behind this interpretation, we know that green light induces much less effect on photoregulative processes than blue light in diatoms, which is in agreement with the lower PUR values measured in BRG (blue-red-green) compared to B (blue) and BR (blue-red) conditions (Table 1). This assumption is also supported by the absence of green-absorbing rhodopsin genes in coastal diatoms [35], as well as the by higher photosynthetic pigments content measured under green compared to blue light (data not shown), and by the fact that the pigments absorbing in blue-green region, Fuco and β-Car, were similar under BRG-L and BRG-H (see discussion below) and their distribution perfectly followed the Chl *a* content.

The de-epoxidation state (DES), i.e. the Dd de-epoxidation into Dt, instead seems to be mainly up regulated by blue fluence rate as indicated by the higher DES under high than low light (Fig. 5A,B). This is found even when the synthesis of Dd and Dt is low, i.e. when red light is absent as in B-H. This feature reveals that Dd de-epoxidation does not depend on XC activation by the presence of both red and blue lights together; being enhanced by high blue fluence rate, as also observed by Schellenberger Costa *et al.* [21]. It would mean that the high light dependent-transthylakoidal ΔpH build-up [12], which activates the Dd de-epoxidase enzyme for transforming Dd into Dt, is not under control of the red perception signal. By contrast, the requirement of red light for enhancing both the Dd and Dt pigments would indicate that at least one of the enzymes involved into the XC photoprotective pathway [19] is under control of the red perception signal. By consequence, the Dd de-epoxidation rate being up regulated by the XC pigment content (Fig. 4A–E, 5A,B), is therefore dependent on the combination of red and blue radiations.

The NPQ capacity was enhanced only in BR-H (Fig. 5C,D), where the strongest NPQ was measured (0.66 ± 0.13 at light peak, $n=6$). Intriguingly, the highest blue fluence rate (B-H) prevented NPQ increase, suggesting that NPQ development, as XC, required red light concomitantly with high blue fluence rate. The higher NPQ in BR-H than in BRG-H (0.25 ± 0.03 at light peak, $n=6$; Fig. 5D) is due to the higher blue fluence rate experienced by cells (360 vs 189 µmol photons m^{-2} s^{-1}, Table 1). In BRG-H, NPQ capacity was as low as the values obtained in B-H (no red radiation) and under low light ($p>0.05$, $n=21$; Fig. 5C,D), despite the highest XC pool size and DES (Fig. 4A, 5B). Therefore, the NPQ development (Fig. 5C,D) is uncoupled with both the XC pool size (Fig. 4A) and DES (Fig. 5A,B), as also reported by Schellenberger Costa *et al.* [21] on *Phaeodactylum tricornutum* grown under different spectral light conditions. This uncoupling between XC and NPQ in BRG-H can be related to a weak functional activation of Dt molecules [10,36] and to the heterogeneous spatial localization of Dt cellular pools [37−39]. Furthermore, the BRG-H condition, and the high red : blue ratio (0.43), might be a potential source of peroxidative damages in cells, that photoprotective xanthophylls can counter, as already observed in diatoms [8,39].

Spectral Radiations and Photosynthetic Pigment Content

Among the photosynthetic pigments, chlorophyll *a* (Chl *a*), fucoxanthin (Fuco) and β-Carotene (β-Car) followed the same trend over light conditions (Fig. 6A–C), with a stable ratio between those pigments (β-Car/Chl *a*, Fig. 6C; Fuco/Chl *a* : $\approx$ 0.70, data not shown). The contents of these three pigment decreased in B-H and BR-H compared to B-L and BR-L (Fig. 6A–C) as expected in a highlight photoacclimation state. The absence of such feature in BRG-H, where cellular pigment content was similar to BRG-L (Fig. 6A–C) can be relied to the lower blue fluence rate (189 µmol photons m^{-2} s^{-1} at the light peak) than in B-H and BR-H ($\geq$360 µmol photons m^{-2} s^{-1}, Table 1). This statement reinforces the strongest role of blue light on photosynthetic regulation in this diatom compared to green light (see discussion above) and coincides with the lower PUR values measured in BRG compared to B and BR conditions ($p<0.05$, $n=3$; Table 1).

The co-variation of Fuco and β-Car with Chl *a* indicates a decreasing number of PSII units under high light, reveals an n-type photoacclimation strategy operated by the coastal diatom *P. multistriata*. This strategy allows to co-regulate the number of antennae and photosystem core complexes to finely tune the amount of absorbed light energy with the biochemical capacity of the cell. This is in agreement with the statement of Six et al. [40] and Lepetit et al. [9], who reported similar photoacclimation strategy by species growing in the upper mixed layer where light is variable.

Furthermore, our results reveal that, the high light-induced pigment variations do not require red radiation to be operated, on the contrary to XC activation or NPQ. This uncoupling between pigment variation occurring in the light harvesting complexes and the photoprotective XC activation, also fits with the absence of a significant relationship between Dd+Dt and Chl *a* or Fuco ($p>0.05$, $n=147$). The reason might be linked to the different Dd content that the fucoxanthin chlorophyll a/c-binding protein (FCP) complexes might bind [41,42]. Indeed, these two studies on two different diatoms revealed that two types of FCPs are present in diatoms, with different content of Dd, and that high light FCPs accommodate more Dd compared to low light FCPs.

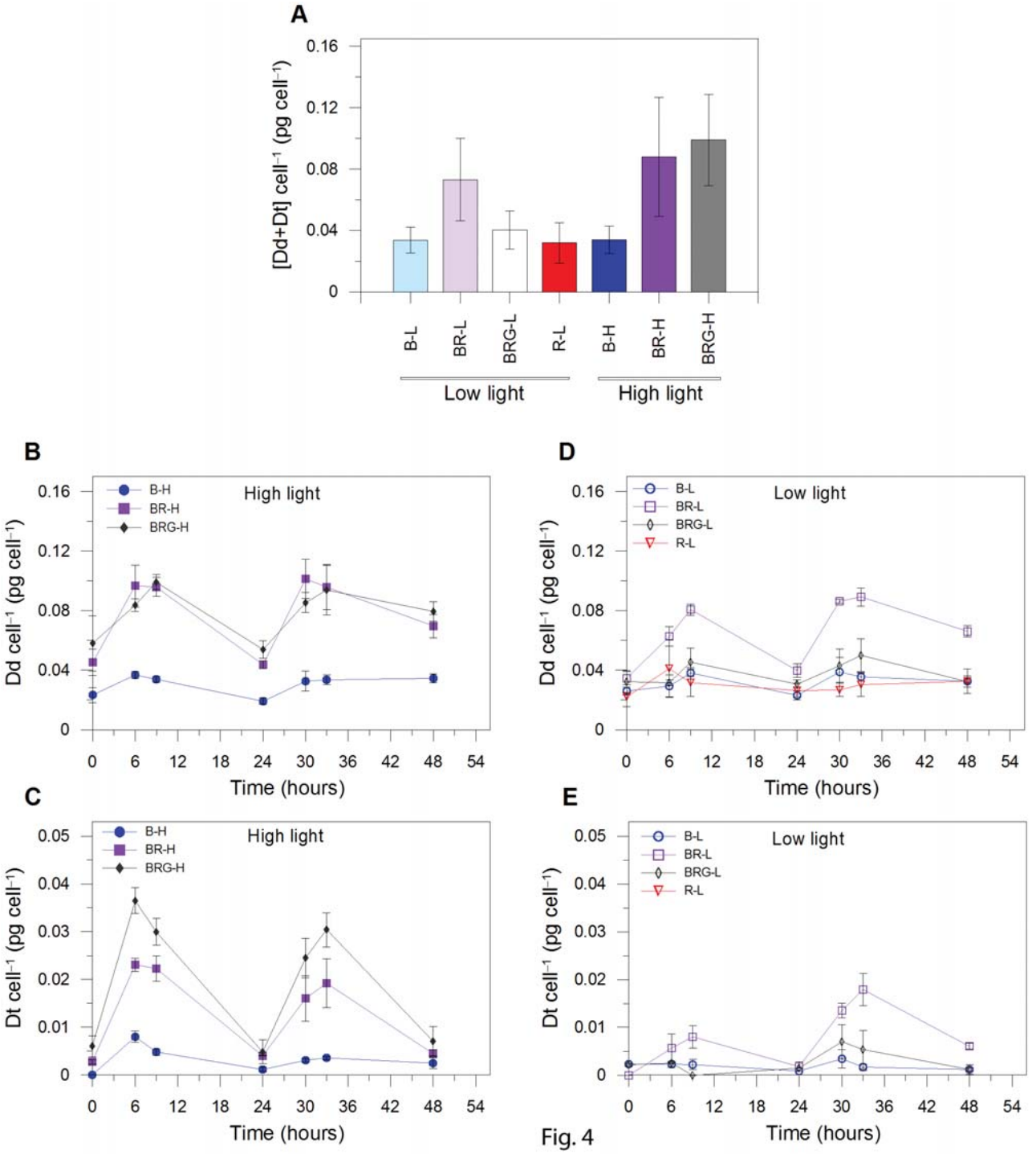

Fig. 4

Figure 4. Variations of xanthophyll cycle pigment content. (A) sum of diadinoxanthin and diatoxanthin ([Dd+Dt]; pg cell^{-1}); diadinoxanthin time distribution (Dd; pg cell^{-1}) in high (B) and low light (D); diatoxanthin time distribution (Dt; pg cell^{-1}) in high (C) and low light (E). B-L, BR-L, BRG-L, R-L are blue, blue-red, blue-red-green, and red low light conditions, respectively; B-H, BR-H, and BRG-H are blue, blue-red, blue-red-green high light conditions, respectively. Dt content was below detectable level in R-L. Time is in hours after the start of the experiment. Data represent (A) mean ± SD ($n = 21$) and (B–E) mean ± SD ($n = 3$).
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Intriguingly, the two other accessory photosynthetic pigments, Chl c_2 and c_3 (Fig. 6D,E), showed unrelated variations with Fuco, β-Car and Chl *a* (Fig. 6A–C), varying both with PFD (decreasing under high light) and spectral conditions. Chl c_2/Chl *a* ratio decreased under only red or low red : blue ratio conditions, as revealed by the significant lower value of this ratio in BR-L and R-L (red low) among low light conditions ($p<0.05$, $n = 21$) and in BR-H among high light conditions ($p<0.05$, $n = 21$). A concomitant

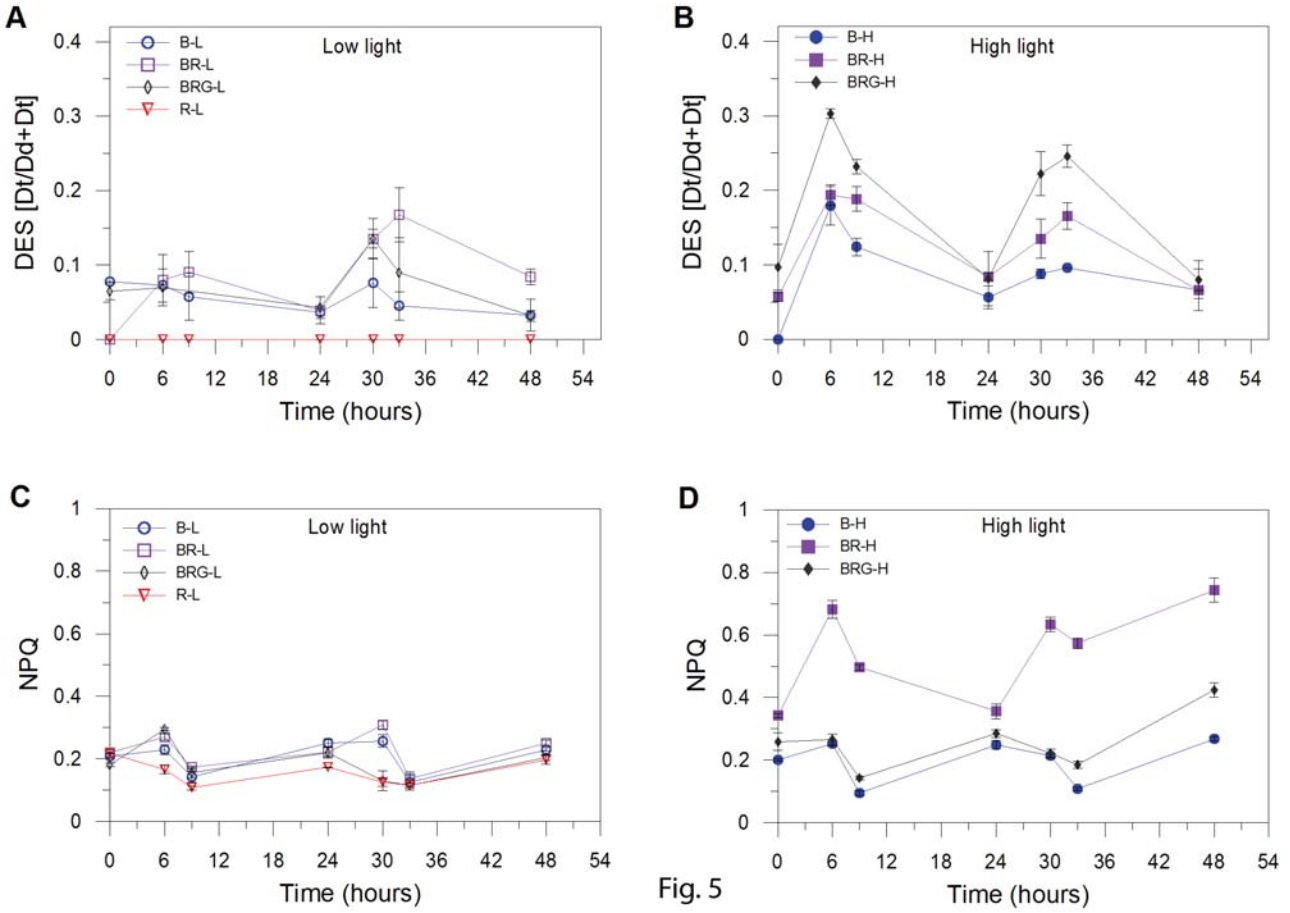

Figure 5. De-epoxidation state (DES = Dt/(Dd+Dt)) and non-photochemical quenching (NPQ). Time distribution of DES in (A) low and (B) high light. Time distribution of NPQ in low (C) and high light (D). B-L, BR-L, BRG-L, R-L are blue, blue-red, blue-red-green, and red low light conditions, respectively; B-H, BR-H, and BRG-H are blue, blue-red, blue-red-green high light conditions, respectively. Time is in hours after the start of the experiment. Data represent mean ± SD ($n = 3$).
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effect of the low red : blue ratio on the decrease of Chl c_2/Chl *a* and NPQ enhancement is observed, as also indicated by the significant correlation between Chl c_2/Chl *a* ratio and NPQ ($p<0.01$, $n = 147$, data not shown).

Therefore, Chl c_2 content in the FCP complexes can be preferentially modulated by light instead of Chl *a* and Fuco content. This result agrees with studies showing independent changes between Chl c_2 and Fuco content (e.g., in *Pelagomonas calceolata*, Dimier et al. [43] and in *Phaeodactylum tricornutum*, see Fig. 6 in Nymark et al. [44]). Indeed recently Gundermann et al. [45] showed that FCPs mainly exist as trimers in *P. tricornutum* and sub-fractioning of FCP complexes from low and high light, yielded different populations of trimeric complexes. Under low light, the trimers mainly containing Lhcf5 proteins were characterised by low Fuco : Chl *c* ratio while under high light Lhcf5 was significantly reduced and trimers containing Lhcf4 proteins were characterised by high Fuco : Chl *c* ratio. From both the Gundermann et al. [45] study and our results, we can hypothesize that the high light regulation of Lhcf5 *vs* Lhcf4 proteins content requires red radiation and high blue fluence rate (i.e., under low red : blue ratio).

Even though the absorption properties of Chl c_3 and Chl c_2 are almost similar, Chl c_3/Chl *a* varied irrespectively to the red : blue ratio value (Fig. 6E). The monospectral light conditions (blue or red), presented higher and similar Chl c_3/Chl *a* ratio (Fig. 6E), while this ratio similarly decreased in the two mixed light conditions (BR and BRG) under low and high light ($p<0.05$, $n = 21$). *Pseudo-nitszchia multistriata* is one of the few diatoms presenting Chl c_3 pigment [46]. The spectral radiation modulation of Chl c_3 content, decreasing when both red and blue radiations are present together, fits with the increase of Chl c_3 observed in the deep, layer below 50 metres depth in the Mediterranean Sea, where only blue light is present and red light is absent [47]. In contrast to Chl c_2, little information is available on the Chl c_3 pigment mainly because this pigment is rarely found in diatoms, but mostly in haptophytes [48] and pelagophytes [43].

Spectral Radiations and Growth and Cell Properties

When provided with low PFD, red light induces a significant decrease of growth rate compared to the other conditions (Fig. 3A,B). Recently, Schellenberger Costa et al. [21] showed on *Phaedoactylum tricornutum* that usage of light energy was less efficient when cells were grown under red light as to blue or white light. The low growth capacity in red condition is related to an undergoing physiological stress, which also could explain the growth inhibition under high red light (data not shown), as revealed by the significantly higher POC content ($p<0.05$, $n = 21$; Table 1) together with the lower particulate organic carbon (POC)

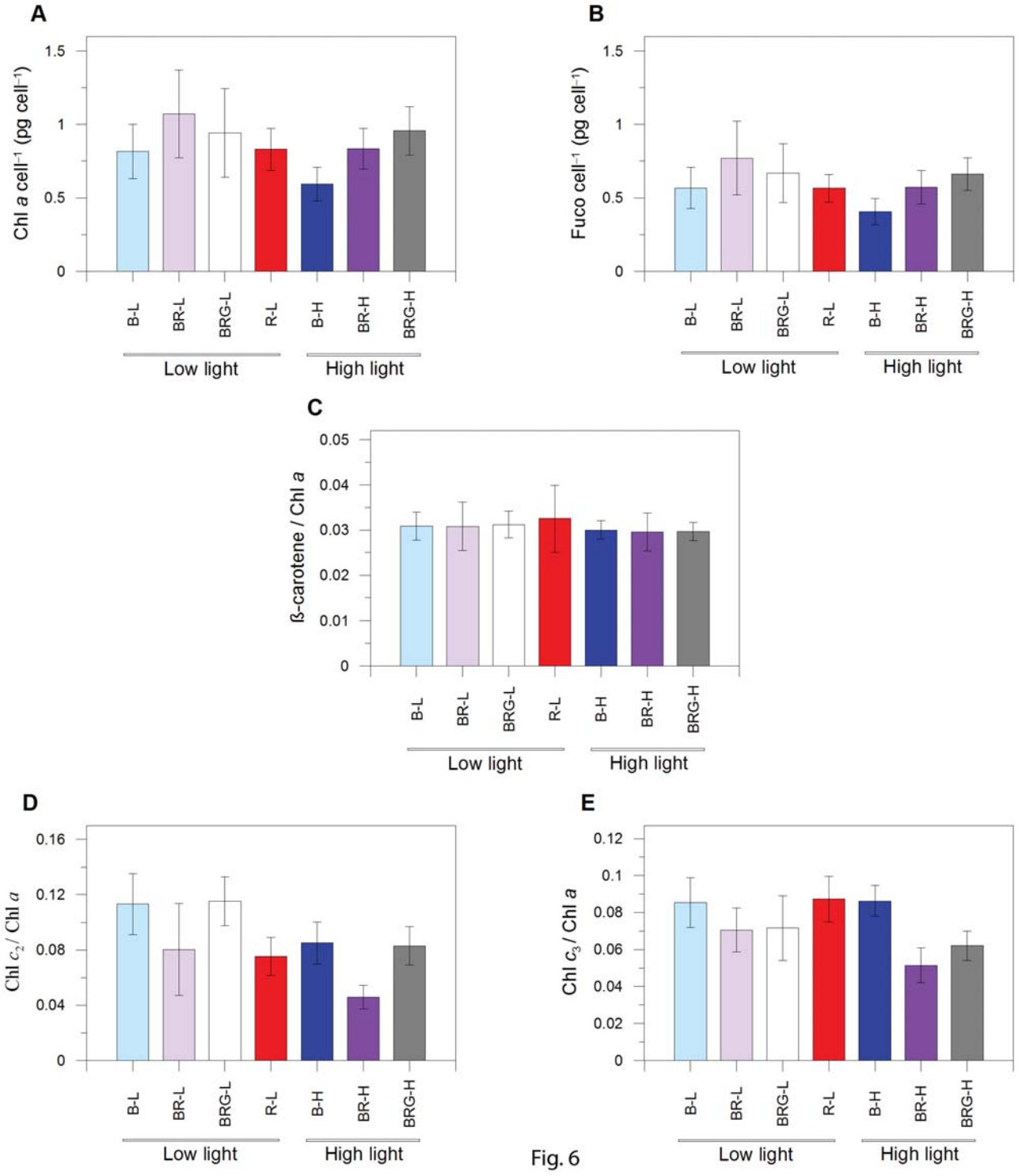

Figure 6. Variations of photosynthetic pigments content. (A) Chlorophyll *a* (Chl *a*; pg cell^{-1}), (B) Fucoxanthin (Fuco; pg cell^{-1}), (C) β-carotene : Chl *a* ratio, (D) Chlorophyll c_2 : Chl *a* ratio and (E) Chlorophyll c_3 : Chl *a* ratio. B-L, BR-L, BRG-L, R-L are blue, blue-red, blue-red-green, and red low light conditions, respectively; B-H, BR-H, and BRG-H are blue, blue-red, blue-red-green high light conditions, respectively. Data represent mean ± SD ($n = 21$).
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: particulate organic nitrogen (PON) ratio ($p<0.01$, $n = 21$; Table 1) and chlorophyll *a* : POC ratio ($p<0.05$, $n = 21$; Table 1). Interestingly, in all conditions with blue light, cells achieve almost similar growth (Fig. 3A,B), revealing an efficient development of acclimative features to the different conditions, by varying their

energy allocation strategies or the arrangement of biochemical pathways [49].

The fastest cell number increase during the exponential growth phase recorded under B-H (Fig. 3B) is paralleled by a low POC/PON ratio (Table 1), in agreement with the findings of Halsey et al. [49]. The lack of red-dependent activation of the xanthophyll cycle and non-photochemical quenching in B-H, limiting the energetic cost of the photoprotective response [43], allows therefore a highest energetic investment for growth. In turn, cells being unable to balance light harvesting and photoprotective capacity, are not able to cope with high light damages, leading cells earlier into stationary phase under this condition (B-H) compared to other high light conditions.

The low red : blue ratio (0.25, BR-H and BR-L) appears to be peculiar, when compared to the other light conditions, since growth capacity (Fig. 3), absorption coefficient (a^*), as well as photosynthetic properties and POC and PON content (Table 1) are similar between low and high PFD. At the exception of R-L, the highest a^* is found in BR (Table 1, $p<0.001$, $n=3$) and is paralleled by the enhancement of the maximal relative rate of linear electron transport ($_{rel}ETR_{max}$), light intensity for reaching the $_{rel}ETR_{max}$ (Ek) and the maximum light use efficiency under low light (α), thus revealing relevant changes of the photosynthetic properties under this condition (Table 1), irrespective of PFD experienced. Moreover, as consequence of the highest PUR in BR-H, which results in an excess energy absorption, cells undergo an efficient photoprotection, by increasing Dt content (Fig. 4) and by developing high NPQ (Fig. 5D). The causes of the high a^* in BR-H, similar to BR-L (Table 1), and therefore the highest PUR in BR-H, are unclear, not being explained by significant variations in pigment content (Fig. 6A–C). The uncoupling between pigment variation and a^* in BR-H might concern variations in pigment package effect. The latter can be induced by structural changes in thylakoid membranes [50,51], occurring in BR-H as revealed by the variations in Chl *c* content, and by the changes in LHCs properties (see discussion above, Gundermann et al. [45]).

Conclusion

Our study leads to consider the spectral composition of light as an essential trigger for photophysiological acclimation of diatoms. Our results suggest that the fast photoprotective processes such as XC and NPQ require red light to be initiated and a high blue fluence rate to be activated. These results logically fit with the optical properties of the water column, since red radiation is only present in the upper layer of the water column, i.e. associated to high PFD (Fig. 1; [5]). Hypothetically, red radiation sensed by cells in the surface layer, act as a relevant environmental cue [20] for signalling high light environment, while blue fluence rate experienced by cells, narrowly correlated to the depth at which cells are upwelled, determines the strength of the photoprotective XC activation, NPQ development and pigment content variations. Furthermore, the red : blue ratio is also a crucial parameter for shaping photophysiological properties of the cells, mainly linked to pigment content related to light-harvesting complex structures.

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Author Contributions

Conceived and designed the experiments: CB FC. Performed the experiments: RC FC CB LB VG. Analyzed the data: RC FC CB LB VG AVR. Wrote the paper: CB RC LB VG AVR.

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Light modulation of biomass and macromolecular composition of the diatom *Skeletonema marinoi*

Raghu Chandrasekaran[a,1], Lucia Barra[a,b,1], Sara Carillo[c], Tonino Caruso[d], Maria Michela Corsaro[c], Fabrizio Dal Piaz[d], Giulia Graziani[e], Federico Corato[a], Debora Pepe[a], Alessandro Manfredonia[a], Ida Orefice[a], Alexander V. Ruban[f], Christophe Brunet[a,*]

[a] *Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy*
[b] *CNR-IBBR, Via Università 133, 80055 Portici (NA), Italy*
[c] *University of Naples "Federico II", Dipartimento di Scienze Chimiche, Complesso Universitario M.S. Angelo, Via Cintia 4, 80126 Napoli, Italy*
[d] *University of Salerno, Dipartimento di Chimica e Biologia, Via Giovanni Paolo II 132, 84084 Fisciano (SA), Italy*
[e] *University of Naples "Federico II", Department of Agricultural and Food Science, Parco Gussone, 80055 Portici (NA), Italy*
[f] *School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK*

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ABSTRACT

The biochemical profile and growth of the coastal diatom *Skeletonema marinoi* was investigated under four different daily blue light doses (sinusoidal light peaking at 88, 130, 250 and 450 μmol photons m^{-2} s^{-1}, respectively). Ability of cells to regulate the light energy input caused alterations in growth and different biosynthetic pathways. The light saturation index for photosynthesis (E_k), which governs the photoacclimative processes, ranged between 250 and 300 μmol photons m^{-2} s^{-1}. Cells that were adapted to low light ($<E_k$) enhanced their carotenoid, lipid and protein contents and lowered carbohydrate content, and vice versa under high light ($\geq E_k$). Variations in fatty acid, pigment and amino acid compositions were a result of light adaptation. Our data show that light is a potent factor for manipulating biomass synthesis in microalgae, such as diatoms for microalgal biotechnology.

1. Introduction

Use of microalgae for biotechnological applications covers many aspects, such as food additives in nutraceutics and animal feeding, cosmetics, pharmaceutics and energetics (Barra et al., 2014; Cadoret et al., 2012; Chisti, 2013; Levitan et al., 2014; Munir et al., 2013). In an economic context, microalgae present many advantages compared to other photosynthetic organisms, such as their high growth rate and the requirement of little space for biomass production. Indeed, Cadoret et al. (2012) reported a productivity of 10 g m^{-2} day^{-1} for microalgae compared to 1–2 g m^{-2} day^{-1} for higher plants. Despite their great potential for the biotechnological applications (Cadoret et al., 2012; Fu et al., 2013), the extensive use of photosynthetic protists has to be enforced. The main challenge is to enhance biomass and bioactive molecules synthesis, and, in parallel, to avoid the increase in production costs (Blanken et al., 2013). To cope with these biotechnological expectations, there is a need to (i) increase the diversity of algal species used, (ii) undertake the genetic engineering of the most adequate strains (Cadoret et al., 2012) and (iii) to deeply investigate alternative ways for enhancing biomass synthesis, such as the "photosynthetic regulation biotechnology", light manipulation being a powerful tool for increasing microalgal productivity (Barra et al., 2014; Perrine et al., 2012). Light intensity and its frequency variability, as well as its spectral composition, strongly affect the growth and photosynthesis of microalgae (Dimier et al., 2009; Fu et al., 2013; Schellenberger Costa et al., 2013a,b).

Among microalgae, diatoms, the most recent and evolutionary diversified group (Kooistra et al., 2007), is still underexploited in the field of biotechnology. However, the siliceous cell wall of diatoms is of huge interest in the field of nanotechnology for designing and producing specific frustules (Lebeau and Robert, 2003; Wang et al., 2013). Furthermore, they can be highly suitable for large-scale cultivation, since most of the diatom species present fast adaptive physiological plasticity, and the presence of large central vacuole

* Corresponding author. Tel.: +39 0815833243.
E-mail address: christophe.brunet@szn.it (C. Brunet).
[1] These authors contributed equally to this work.

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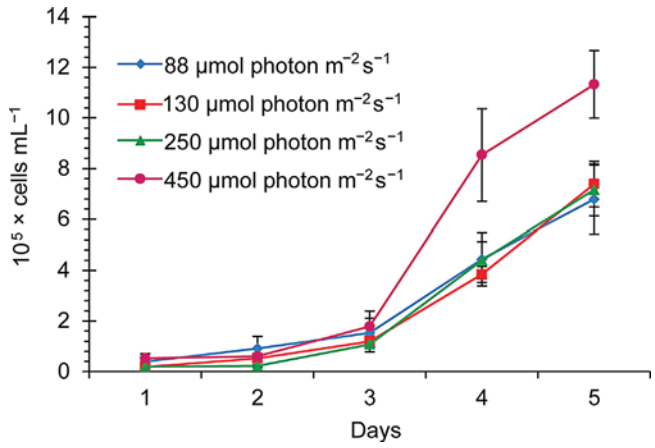

Fig. 1. Growth curve of *S. marinoi* under the four blue fluence rates; experiments were performed during the exponential phase on days 4 and 5 (data represent mean ± SD; $n = 3$).

allows them to be hugely competitive in variable environments (Kooistra et al., 2007). Among the diatom-specific carotenoids, fucoxanthin together with the most common β-carotene is of huge interest in the field of biotechnology (Peng et al., 2011). The two other xanthophylls, diadinoxanthin and diatoxanthin, are not extensively studied but their potential role as antioxidants have been recently reported (Gallina et al., 2014). The lipid profile, both in terms of quantity and quality, makes diatoms suitable candidates for aquaculture (Lebeau and Robert, 2003). Several studies have shown that the diatoms polyunsaturated fatty acids (PUFAs) have positive effect on human health (Hallahan and Garland, 2005), while the monounsaturated fatty acids (MUFAs) are considered to be the potential feedstock for biodiesel production (Levitan et al., 2014).

In this study, we explore the role of light and the potential of the "photosynthetic regulation biotechnology" to better exploit diatoms in the field of biotechnology. Only blue light condition was selected, being the most energetic and fully absorbed wavelengths by microalgae for the photosynthetic activity. Furthermore, only blue light, i.e. without red radiation, strongly limits photoprotective processes in the cells (Brunet et al., 2014), potentially increasing the biochemical energy available for growth.

The centric diatom *Skeletonema marinoi* was submitted to four different blue fluence rates, characterized by daily light dose of 2.2, 3.2, 6.1 and 11 mol m^{-2} day^{-1}, respectively. Our results, both on physiological property and biochemical profiles of the cells, confirm that variation of blue fluence rate does strongly influence the biochemical characteristics of the cells, reinstating that light manipulation is a concrete way to modulate the biochemical energy allocation in microalgae, and thus adequate for biotechnological purposes.

2. Materials and methods

2.1. Experimental strategy and sampling

Experiments were conducted on the coastal centric diatom *S. marinoi* (CCMP 2092), by cultivating at 20 °C in 4.5-L glass flask with air bubbling, containing natural sterile seawater amended with *f*/2 nutrients (Guillard and Ryther, 1962). All the experiments were performed in triplicate, lasting 2 days during the exponential growth phase (Fig. 1), on cultures pre-acclimated to each experimental light condition for 2 weeks before the experiments. Four different sinusoidal blue light distributions, peaking at 88, 130, 250 and 450 µmol photons m^{-2} s^{-1} (daily light dose: 2.2, 3.2, 6.1 and 11 mol m^{-2} day^{-1}, respectively), have been applied, with a 12:12 h light:dark photoperiod. Blue light ($\lambda = 460 \pm 30$ nm) was provided by a custom-built illumination system (Brunet et al., 2014). Light intensity was measured inside each flask by using a laboratory PAR 4π sensor (QSL 2101, Biospherical Instruments Inc., San Diego, CA, USA).

Samples for cell counts, variable fluorescence and electron transport rate measurements, pigments, particulate organic carbon and nitrogen, proteins and RNA were taken three times per day: dawn (time 0), midday (time 6 h) and in the afternoon (time 9 h). Samples for absorption spectrum analysis, lipids, carbohydrates and fibers determination were taken once during the experiment (midday sampling on second day).

2.2. Cell concentration

Cell concentration was estimated on triplicate sub-samples. An aliquot of 1 mL was used to fill a Sedgewick Rafter counting cell chamber, and cell counts were performed using a Zeiss Axioskop 2 Plus microscope.

2.3. Photochemical efficiency and photosynthetic parameters

Photochemical efficiency of photosystem (PS) II was estimated by a Phyto-PAM fluorometer (Heinz Walz, Effeltrich, Germany). The variable fluorescence analysis was performed on 15-min dark-acclimated samples, to measure the maximum photochemical efficiency (Brunet et al., 2014).

Electron transport rate (ETR) versus irradiance curves were determined by applying 13 increasing red actinic lights (655 nm) from 1 to 853 µmol photons m^{-2} s^{-1} lasting 1 min each. The relative electron transport rate ($_{rel}$ETR, expressed in µmol e^{-1} s^{-1} $cell^{-1}$) was calculated as follows:

$$_{rel}\mathrm{ETR} = \left(\frac{Fv'}{Fm'}\right) \cdot I \cdot 0.5 \cdot a^*$$

where I is the incident irradiance (expressed in µmol photons m^{-2} s^{-1}), Fv' and Fm' are the variable PS II fluorescence yield and maximal PS II fluorescence yield, respectively, for illuminated cells (measured at the end of the 1 min lasting actinic light), a^* is the cell-specific absorption coefficient, expressed in m^2 $cell^{-1}$ (for the determination of a^*, see Section 2.6). A factor of 0.5 was applied to correct for the partitioning of photons between PSI and PSII, assuming that excitation energy is evenly distributed between the two photosystems.

ETR-I curves were fitted with the equation of Eilers and Peeters to estimate $_{rel}ETR_{max}$ (maximal relative rate of linear electron transport), α (maximum light use efficiency), and E_k (light saturation index for photosynthesis).

The non-photochemical quenching (NPQ) was estimated on 15-min dark-acclimated cells by illuminating the sample with an actinic light setup at 480 µmol photons m^{-2} s^{-1} during 10 min, and the maximum fluorescence yield was estimated every minute. NPQ was quantified by the Stern–Volmer expression:

$$\mathrm{NPQ} = \frac{Fm}{Fm'} - 1$$

2.4. Pigments

Pigment measurement was conducted by high-performance liquid chromatography (HPLC), following the procedure described in Brunet et al. (2014). Ten mL aliquot of algal culture was filtered (under low light) on 25 mm GF/F glass-fiber filter (Whatman, Maidstone, UK) and stored in liquid nitrogen until further analysis. Pigments were extracted by mechanical grounding during 3 min in

2 mL of a 100% methanol solution. Pigments were separated in a Hewlett Packard series 1100 HPLC (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (2.6 μm diameter C8 Kinetex column; 50 mm × 4.6 mm; Phenomenex®, USA). Pigments were detected spectrophotometrically at 440 nm using a Hewlett Packard photodiode array detector, model DAD series 1100. Determination and quantification of pigments were carried out using pigment standards from the D.H.I. Water & Environment (Horsholm, Denmark).

2.5. Particulate organic carbon and nitrogen

Ten mL aliquot for the determination of particulate organic carbon (POC) and particulate organic nitrogen (PON) was filtered on pre-combusted (450 °C, 5 h) glass-fiber filters (Whatman, Maidstone, UK), conserved in cell culture plates (Corning®, Corning Inc., NY, USA), and immediately stored at –20 °C. The analyses were performed with a Thermo Scientific Flash EA 1112 automatic elemental analyzer (Thermo Fisher Scientific, MA, USA), following the procedure previously described by Brunet et al. (2014). Filters were thawed just prior to analysis and allowed to dry at 60 °C through a desiccator. Then filters were loaded in small tin cups that were crimped closed and transferred to the CHN analyzer. A set of empty filters was processed as ordinary samples to accomplish the blank determination. Cyclohexanone 2,4-dinitrophenylhydrazone (C, N, H, 51.79, 20.14, 5.07 wt%, respectively) was used as standard.

2.6. Absorption spectrum

The spectral absorption measurements were performed using a spectrophotometer Hewlett Packard HP-8453E equipped with an inverted Labsphere integrating sphere (RSA-HP-53 Reflectance Spectroscopy Accessory) following the procedure described in Dimier et al. (2009). Ten mL aliquot of algal culture was filtered onto Whatman GF/F filters and immediately frozen. The absorption ($a(\lambda)$) was measured between 250 and 800 nm, and thus integrated between 400 and 700 nm. This integrated value was divided by cell concentration for the estimation of the cell-specific absorption coefficient, a^*, expressed in m^2 $cell^{-1}$.

The photosynthetically usable radiation (PUR) was calculated as described in Brunet et al. (2014).

2.7. Nutrients

Samples for determining nutrient concentrations were collected in 20 mL polyethylene vials, and quickly frozen and stored at −20 °C. Ammonium, nitrate, nitrite, silicic acid and phosphate concentrations were determined using a Technicon AutoAnalyzer following classical methods (Grasshoff et al., 2009).

Nutrient concentration analyzed from the morning sampling on the first and second day of the experiment was therefore used to estimate the uptake of nutrients reported by cell number increase during the 1st and 2nd day of the experiments:

$$\mathrm{Nu} = \frac{N_2 - N_1}{C_2 - C_1},$$

where Nu is the nutrient uptake (nmol $cell^{-1}$ day^{-1}), C_n is the cell concentration at day n and N_n is the nutrient concentration at day n.

2.8. Cells pellet preparation

The volume sampled (50 mL for RNA and protein, 800 mL for lipids, carbohydrates and fibers) from each triplicate was centrifuged at 4000 rpm (3399 × g) for 20 min at 4 °C (DR15P centrifuge, B. Braun Biotech International, Melsungen, Germany); the supernatant was discarded. For proteins and RNA quantification, the pellet has been transferred in a 2 mL Eppendorf tubes and centrifuged at 14,000 rpm (20,817 × g) for 15 min at 4 °C (5417R centrifuge, Eppendorf, Hamburg, Germany). For lipids and carbohydrates, the pellets obtained after the centrifugation (16 tubes of 50 mL) was pooled together and centrifuged again as previously mentioned and weighed.

2.9. RNA analysis

The total RNA has been extracted from the pellet following the procedure described in Barra et al. (2013). Concentration of RNA was measured by Nanodrop (Agilent Technologies, Santa Clara, CA, USA).

2.10. Protein and amino acids analysis

The total proteins from the pellet was extracted by sonicating the cell pellets for 2.4 min in 600 μL of sterile water and centrifuged at 13,000 rpm (17,949 × g) for 20 min at 4 °C. Then the supernatant was collected and the pellet was re-extracted with 500 μL of 0.1 N NaOH and 0.5% β-mercaptoethanol (v/v). The mixture was kept at RT for 1 h (with occasional shaking) and centrifuged at 13,000 rpm (17,949 × g) for 20 min at 21 °C. The supernatant was mixed and the pellet was discarded. Proteins were then purified with trichloroacetic acid (TCA) before acid hydrolysis for amino acids analysis, according to Barbarino and Lourenço (2005) and the crude extract has been quantified with Folin & Ciocalteu reagent (Sigma).

Samples containing 50 μg of protein were acid hydrolyzed with 1 mL of 6 N HCl in vacuum-sealed hydrolysis vials at 110 °C for 22 h. Norleucine was added to the HCl as an internal standard. Although tryptophan was completely lost with acid hydrolysis and methionine and cysteine + cystine could be destroyed to varying degrees by this procedure, the hydrolysates were suitable for analysis of allotheramino acids. The tubes were cooled after hydrolysis, opened and placed in a dessicator containing NaOH pellets under vacuum until dry (5–6 days). The residue was then dissolved in a suitable volume of dilution Na–S R buffer (pH 2.2; Beckman Instr.), filtered through a Millipore membrane (0.22 μm pore size) and analyzed for amino acids by ion-exchange chromatography in a Beckman, model 7300 instrument equipped with an automatic integrator.

2.11. Lipid and fatty acids analysis

Each wet pellet was sonicated for 15 min at 25 °C in 10 mL of acetic acid/chloroform (1/9), or acetone/methanol (9/1) or pure methanol in order to get respectively triglyceride, glycolipid or phospholipid fraction. The extracted solution was passed through cartridges Supelclean™ ENVI-Florisil® SPE Tubes (by Aldrich), preconditioned with 30 mL of chloroform (Popovich et al., 2012). The samples have been subsequently concentrated under N_2 flux to reach final volume of 1 mL and esterified with KOH 2 M in methanol according to (Graziani et al., 2013). One μL was injected directly in a Thermo Finnigan TRACE gas chromatograph equipped with a fused silica capillary column (FAMEWAX Restek, 30 m × 0.25 mm i.d., 0.25 μm film thickness) and an FID detector. The calibration has been performed using a standard PUFA's (by Supelco) as internal standard.

The degree of unsaturation (DU) was calculated using the following formula as reported in Ramos et al. (2009):

$$\mathrm{DU} = (\text{monounsaturated Cn} : 1, \text{wt\%}) + 2 \times (\text{polyunsaturated Cn} : 2, 3, \text{wt\%})$$

Table 1
Photosynthetic properties of *S. marinoi* under the four blue fluence rates.

	88 μmol photons m^{-2} s^{-1}	130 μmol photons m^{-2} s^{-1}	250 μmol photons m^{-2} s^{-1}	450 μmol photons m^{-2} s^{-1}
PAR dose	2.2	3.2	6.1	11
a^*	1.66 ± 0.59	0.90	0.94 ± 0.29	2.26 ± 0.64
PUR	0.51 ± 0.18	0.37	0.87 ± 0.34	4.1 ± 1.2
μ	0.40 ± 0.27	1.24 ± 0.23	0.49 ± 0.13	0.31 ± 0.11
$_{rel}ETR_{max}$	3.16 ± 1.15	2.21 ± 0.08	2.90 ± 1.02	
E_k	252 ± 36	241 ± 7	306 ± 13	
α	0.012 ± 0.003	0.009 ± 0.0006	0.0095 ± 0.003	
NPQ	0.64 ± 0.05	0.87 ± 0.03	0.61 ± 0.02	
Total RNA	0.48 ± 0.18	0.30 ± 0.06	0.69 ± 0.22	0.38 ± 0.14

Average PAR dose experienced by the cells under different blue light conditions (mol m^{-2} day^{-1}). $a^* \times 10^{-11}$, absorption coefficient (m^2 $cell^{-1}$); PUR × 10^{-6}, photosynthetically usable radiation (μW $cell^{-1}$); μ, growth rate (day^{-1}); $_{rel}ETR_{max} \times 10^{-6}$ (maximal relative rate of linear electron transport, pmol e^{-1} h^{-1} $cell^{-1}$); α (maximum light use efficiency, pmol e^{-1} h^{-1} $cell^{-1}$(μmol photon m^{-2} s^{-1}) $^{-1}$); E_k (light saturation index for photosynthesis, μmol photons m^{-2} s^{-1}) and total RNA content (pg $cell^{-1}$). Data represent mean and SD ($n = 3$ except for total RNA, $n = 15$).
The lack of data for the 450 μmol photons m^{-2} s^{-1} condition is due to the breakdown of the PAM fluorometer during the experiment.

2.12. Carbohydrate analysis

The pellets obtained for carbohydrate analysis were sonicated for 5 min in 5 mL of *f*/2 culture medium and centrifuged. The supernatant and the cellular debris pellet were stored separately for the analysis. Total carbohydrates content, calculated on both supernatant and pellet of each sample, was determined by microphenol assay as reported in Kobata (1972).

2.13. Fiber analysis

Total fiber content of *S. marinoi*, composed by soluble and insoluble fraction, was determined by the AOAC 985.29 gravimetric method (Graziani et al., 2013).

2.14. Statistical analysis

Student's *t*-test and Spearman's rank correlation were performed using Systat 7 software.

3. Results and discussion

3.1. Light modulation of photosynthetic property and growth

Cells grown under 88, 130 and 250 μmol photon m^{-2} s^{-1} had similar growth capacity (Fig. 1) in agreement with a low range of variability of the linear electron transport rate ($_{rel}ETR_{max}$, from 2.2 to 3.1 × 10^{-6} pmol e^{-1} h^{-1} $cell^{-1}$, Table 1). The similar photosynthetic and growth rates measured under the different illumination conditions are probably related to biochemical and physiological adjustments within the cells. Indeed, the light saturation index for photosynthesis (E_k, Table 1), ≈250–300 μmol photons m^{-2} s^{-1}, revealed that cells grown in 88 and 130 μmol photon m^{-2} s^{-1} conditions were potentially light limited, while under the 250 and 450 μmol photon m^{-2} s^{-1} cells were light saturated or oversaturated (450 μmol photon m^{-2} s^{-1}). The pronounced low light acclimation state of the cells under 88 μmol photon m^{-2} s^{-1} was confirmed by the highest value of the maximum light use efficiency parameter (α, Table 1), and by the increase in cell-specific absorption coefficient (a^*, Table 1). On the opposite, under the highest light condition, 450 μmol photon m^{-2} s^{-1}, cells grew faster reaching a higher maximum cell number (11 × 10^5 cells mL^{-1}) compared to the three other conditions (~7 × 10^5 cells mL^{-1}; Fig. 1). Indeed, the light enhancement was only little compensated by adjustments in light absorption, as shown by the highest photosynthetic usable radiation (PUR, Table 1).

None of the nutrients was limiting during the 2 days of experiment (Table 2), while the increase in cell concentration was sustained by the uptake of NO_3^-, PO_4^{3-} and SiO_3^{2-} (Table 2, Fig. 2). By contrast, NO_2^- and NH_4^+ increased between the 2 days of experiment, probably in relation with the rapid and continuous recycling of these nutrients in the medium (Barra et al.,

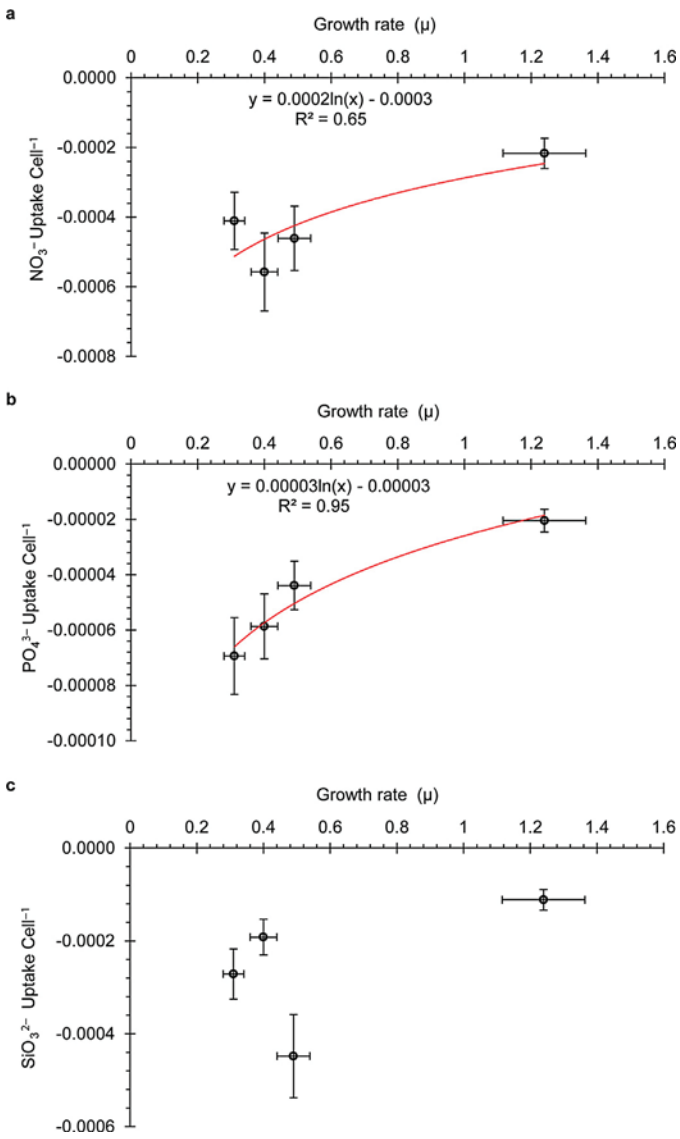

Fig. 2. Relationship between growth rate (μ; day^{-1}) and nutrient uptake per cell (nmol $cell^{-1}$ day^{-1}): (a) NO_3^-, (b) PO_4^{3-} and (c) SiO_3^{2-} (data represent mean ± SD; $n = 3$).

Table 2
Growth rate and nutrient concentrations under the four blue fluence rates during the two days of experiments.

	88 μmol photons m^{-2} s^{-1}	130 μmol photons m^{-2} s^{-1}	250 μmol photons m^{-2} s^{-1}	450 μmol photons m^{-2} s^{-1}
Cell con.				
1st day/2nd day	443,989 ± 205,449	214,214 ± 31,979	439,509 ± 73,147	854,291 ± 282,535
	678,394 ± 336,142	738,348 ± 99,598	715,593 ± 122,325	1,072,880 ± 160,228
Cell con. increase	234,405 ± 225,457	524,134 ± 112,078	276,083 ± 145,257	218,588 ± 176,819
NH4+				
1st day/2nd day	0.60 ± 0.37	0.92 ± 0.21	0.84 ± 0.78	1.81 ± 0.80
	0.93 ± 0.32	1.17 ± 0.70	0.97 ± 1.08	1.05 ± 0.08
NO_2^-				
1st day/2nd day	4.89 ± 1.13	4.21 ± 0.49	4.93 ± 1.06	6.68 ± 0.05
	5.94 ± 0.56	7.12 ± 0.67	8.20 ± 1.29	15.31 ± 1.23
NO_3^-				
1st day/2nd day	369.16 ± 9.5	858.93 ± 46.35	694.16 ± 142.58	594.70 ± 44.01
	300.54 ± 28	756.56 ± 33.23	629.32 ± 60	515.60 ± 42.56
PO_4^{3-}				
1st day/2nd day	10.13 ± 0.91	24.13 ± 0.29	19.54 ± 2.38	14.25 ± 0.72
	2.08 ± 0.04	13.73 ± 2.97	11.10 ± 4.34	1.74 ± 0.45
$[SiO_3]^{2-}$				
1st day/2nd day	68.03 ± 19.89	124.29 ± 17.43	148.69 ± 58.31	64.08 ± 0.76
	38.75 ± 23.79	63.86 ± 27.86	56.58 ± 40	5.38 ± 5.50

Cell concentration (Cell con.; cells mL^{-1}) and nutrient concentration (nmol mL^{-1} day^{-1}).

2014). Growth rate was significantly correlated to the uptake of NO_3^- and PO_4^{3-},($p < 0.05$ for NO_3^- and $p < 0.01$ for PO_4^{3-}; Fig. 2), revealing their relevant role in driving biomass synthesis and that light intensity did not affect directly the uptake of these nutrients. By contrast, the higher SiO_3^{2-} uptake was measured under high light compared to the low light conditions and it was not related to the growth rate ($p > 0.05$, Fig. 2). This agrees with a potential increase in photorespiration rate induced by high light (Brunet et al., 2011; Schnitzler Parker et al., 2004), being this process a way for cells to dissipate the excess biochemical energy, and to the fact that silicate metabolism is more linked to respiration and cell cycle than photosynthesis (Norici et al., 2011).

The RNA content per cell did not show significant variation among the light conditions ($p > 0.05$, $n = 15$), ranging from 0.30 to 0.70 pg $cell^{-1}$ (Table 1). The RNA content per cell displayed circadian oscillation in the cells (data not shown), that might be related to variations in the cellular content of proteins, lipids, carbohydrates or pigments (Fábregas et al., 2002). In all conditions, the RNA content per cell increased at midday compared to the morning and afternoon samples, while some studies (e.g. Berdalet et al., 1992) showed enhanced RNA content at the beginning of the light period. This discrepancy is due to the light distribution, provided in a sinusoidal way in our study and in a quadratic way for the other studies, as recently demonstrated (Orefice, personal communication).

3.2. Light modulation of carotenoids and chlorophylls

The main photosynthetic pigments, chlorophyll *a* (Chl *a*) and fucoxanthin (Fuco), were significantly correlated ($p < 0.001$, $n = 36$) presenting a significant decrease under the high light conditions ($p < 0.05$, $n = 9$; Fig. 3a and b). Under low light ($E < E_k$, 88 and 130 μmol photon m^{-2} s^{-1}), the total carotenoid content and Chl *a* per cell was similar (Fig. 3a, Table 3), despite the difference in daily light dose (2.2 and 3.2 mol m^{-2} day^{-1}). Fuco/Chl *a* was similar among the different light conditions ($p > 0.05$, $n = 9$), except for 450 μmol photon m^{-2} s^{-1} where it significantly decreased ($p < 0.01$, $n = 9$). Thus, *S. marinoi* tends to modify the antenna structure by decreasing Fuco compared to the Chl *a* molecules in the reaction center under the latter condition (Dimier et al., 2009), while under low light condition, *S. marinoi* tends to modify all the reaction center and antenna structure to maintain similar the pigment ratio.

On the opposite of Fuco and Chl *a*, chlorophyll *c* (Chl *c*) content per cell exponentially decreased from the lowest to the highest light dose (Fig. 3a), in agreement with the results obtained on *Pseudo-nitzschia multistriata* (Brunet et al., 2014). The highest Chl *c* content under 88 μmol photon m^{-2} s^{-1} (two times more compared to the other light conditions) enhances the ability of cells to cope with extreme low light, since this pigment is known to efficiently transfer energy to Chl *a* (Di Valentin et al., 2013).

The β-carotene (β-car) content did not show any significant trend over the light gradient (Fig. 3c), while a significantly higher

Table 3
Biochemical properties of *S. marinoi* under the four blue fluence rates.

	88 μmol photons m^{-2} s^{-1}	130 μmol photons m^{-2} s^{-1}	250 μmol photons m^{-2} s^{-1}	450 μmol photons m^{-2} s^{-1}
Total carotenoids	0.015 ± 0.006	0.019 ± 0.003	0.009 ± 0.003	0.005 ± 0.001
Total carbohydrates	0.62 ± 0.12	0.79 ± 0.24	2.60 ± 1.52	2.52 ± 0.06
Total lipids	1.25 ± 0.16	2.60 ± 0.25	0.66 ± 0.06	0.68 ± 0.14
Neutral lipids	0.54 ± 0.13	0.90 ± 0.10	0.21 ± 0.08	0.42 ± 0.08
Phospholipids	0.03 ± 0.02	0.96 ± 0.16	0.29 ± 0.07	0.18 ± 0.15
Glycolipids	0.68 ± 0.04	0.74 ± 0.01	0.16 ± 0.04	0.08 ± 0.04
DU	28	92	5	34
Total proteins	26.73 ± 3.45	45.03 ± 7.88	23.87 ± 0.73	15.89 ± 0.79
Total amino acids	0.00199	0.00356	0.00231	0.00038
IDF (wt%, insoluble fibers)	11.85	12.45	16.80	12.28
SDF (wt%, soluble fibers)	2.15	1.89	nd	nd
POC	14.16 ± 1.87	10.99 ± 0.13	19.37 ± 2.78	15.18 ± 0.83
PON	2.62 ± 0.89	3.86 ± 0.60	3.78 ± 1.31	2.11 ± 0.63

DU, degree of unsaturation [(MUFA, wt%) + 2 × (PUFA, wt%)]; POC, particulate organic carbon (pg $cell^{-1}$); PON, particulate organic nitrogen (pg $cell^{-1}$). Data represent mean and standard deviation (for POC, PON, $n = 15$; carotenoids and proteins, $n = 9$; and for lipids, fibers, and carbohydrates, $n = 3$).

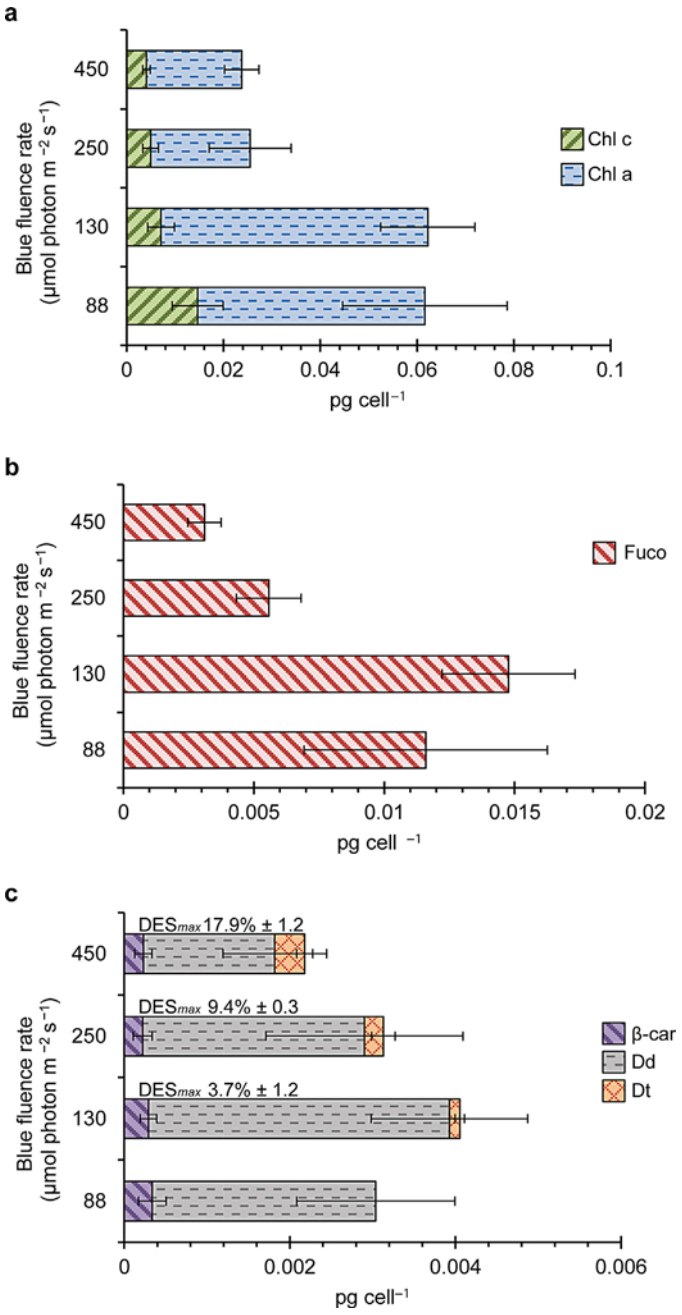

Fig. 3. Variations of photosynthetic and photoprotective pigments content. (a) Chlorophyll *a* and Chlorophyll *c* content per cell (Chl *a* and Chl *c*; pg cell^{-1}); (b) Fucoxanthin content per cell (Fuco; pg cell^{-1}); (c) β-carotene, diadinoxanthin and diatoxanthin content per cell (β-car, Dd and Dt; pg cell^{-1}); maximum de-epoxidation state [DES_{max} = Dt/(Dd + Dt)] (data represent mean ± SD; n = 9 except for DES_{max}, n = 3).

β-car/Chl *a* was found under the two high light conditions ($p < 0.05$, $n = 9$) compared to the low light conditions (data not shown). This feature is related to the Chl *a* decrease and to the involvement of β-car in the biosynthesis of the xanthophyll cycle (XC) pigments (Dambek et al., 2012). Diadinoxanthin over chlorophyll *a* ratio (Dd/Chl *a*) followed the same trend as β-car/Chl *a* and was significantly higher under high light than low light ($p < 0.05$, $n = 9$). As Fuco, Dd concentration decreased from the 130 µmol photon m^{-2} s^{-1} to the highest light intensity (Fig. 3c). The 88 µmol photon m^{-2} s^{-1} condition was out of this trend, revealing a peculiar physiological response of the cells grown under this extremely light limiting condition. Diatoxanthin (Dt; photoprotective pigment) showed an opposite trend to Dd, being significantly higher under the highest intensity compared to the other conditions ($p < 0.05$, $n = 9$; Fig. 3c). Dt concentration was very low under all the light conditions, reinforcing that cells are not able to develop photoprotective process under blue light (Brunet et al., 2014). Indeed, the absence of red spectrum strongly limits Dt synthesis and therefore the development of non-photochemical quenching (NPQ, excess-energy dissipative process, Table 2).

3.3. Light modulation of macromolecular composition

Although the protein and lipid content per cell were significantly correlated to the growth rate ($p < 0.05$, $n = 4$), the biochemical property depends on the light experienced by cells. Similar to carotenoid content, cells increased lipid and protein content under low light compared to high light (Table 3, $p < 0.01$). The highest concentration was found under 130 µmol photon m^{-2} s^{-1}. The high lipid content under the low light conditions may serve as an effective energy and carbon storage (Fábregas et al., 2002) providing a larger sink of the available energy. It also decreases the membrane permeability by forming thick membrane layers and thus reducing the energy loss by passive diffusion of organic matter (Cherrier et al., 2014). The classes of glycolipids and neutral lipids followed the same trend as total lipids, being enhanced under low light, with a maximal concentration at 130 µmol photon m^{-2} s^{-1} (Table 3). The third class, the phospholipids, did not follow any trend over the light gradient (Table 3), while its concentration was strongly enhanced under 130 µmol photon m^{-2} s^{-1} (Table 3). The low phospholipid content under high light suggests that the membrane permeability was higher under this condition compared to the other light conditions. This feature could allow cells to remove excess energy by exudation of organic matter when the photosynthesis outpaces cell growth (Cherrier et al., 2014). Furthermore, the increase in the neutral lipid contribution under the highest light condition (≈62 wt% vs <40 wt%) might be attributed to their role in preventing photo-oxidative damages (Solovchenko, 2012).

Under all the conditions, except for the 130 µmol photon m^{-2} s^{-1} light, the saturated FAs hugely dominated over the others (77–97 wt%; Fig. 4), while the unsaturated FAs (sum of monounsaturated FAs and polyunsaturated FAs, MUFAs + PUFAs) which contributed to 59 wt% of the total lipids under the latter condition. The unsaturated FAs contribution decreased to 23, 29 and 3 wt% of the total lipids under 88, 450 and 250 µmol photon m^{-2} s^{-1}, respectively (Fig. 4).

Among the saturated FAs, cells synthesized predominantly C8:0 (ranged between 82 and 33 wt%; Table S1), except under the 130 µmol photon m^{-2} s^{-1}, in which it was replaced by C20:0 (25 wt%; Table S1). Under 130 µmol photon m^{-2} s^{-1}, cells accumulated C18 monounsaturated FAs (15 wt%) and C20 polyunsaturated FAs (17 wt%); C18:1ω9t dominated the MUFAs, while C20:2ω6 dominated the PUFAs (Table S1). Under the two extreme light conditions, 88 and 450 µmol photon m^{-2} s^{-1}, cells presented high contribution of C16:1 monounsaturated FA (≈17 and 10 wt%, respectively; Table S1), while the contribution of C17:1 monounsaturated FA was high only in the highest light (≈9 wt%; Table S1).

The 130 µmol photon m^{-2} s^{-1} of blue light offers the best condition for enhancing lipid production and for obtaining homogenous distribution of the saturated, polyunsaturated and monounsaturated fatty acid (FA) classes (Fig. 4). The degree of unsaturation (DU, ratio between saturated and unsaturated FA) was higher under 130 µmol photon m^{-2} s^{-1} (92) compared to all the other light conditions (ranged between 5 and 35). While at an economic point of view, the production of algal biodiesel cannot rely on artificial light (Chisti, 2013), the results of our study might be of interest in the field of bio-energy. Indeed, high amount of saturated FAs provides a superior oxidative stability, while polyunsaturated fatty

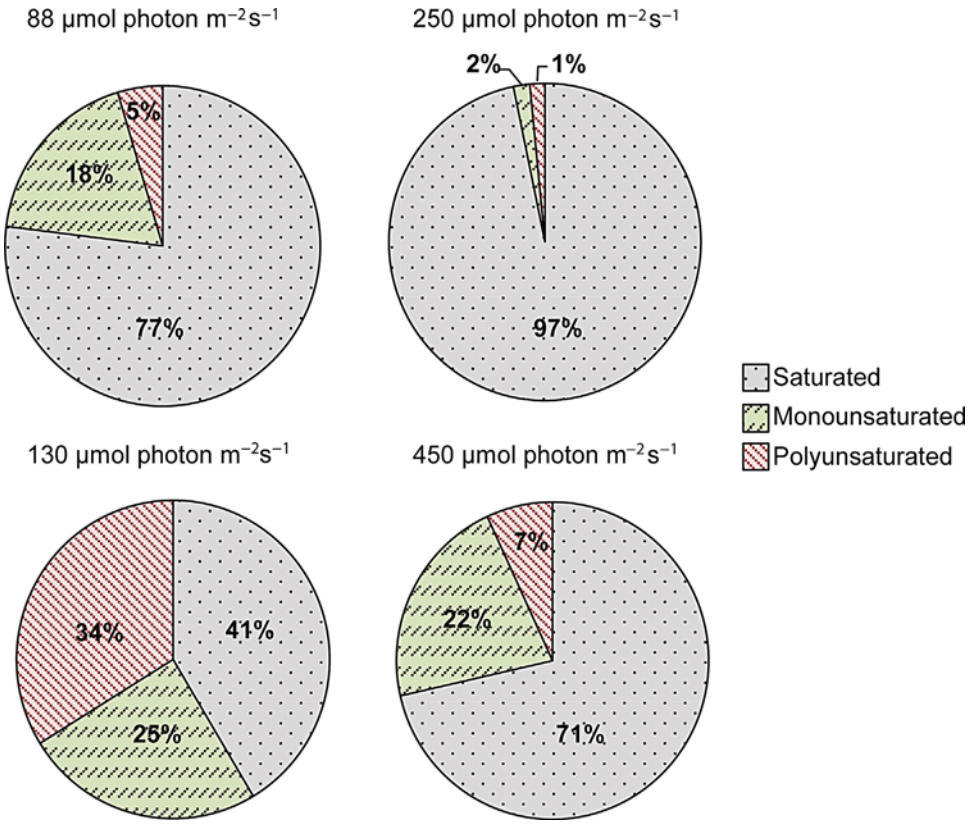

Fig. 4. Relative abundance (wt%) of saturated, mono- and polyunsaturated fatty acids in *S. marinoi* under the four blue fluence rates (data represents mean, $n = 3$).

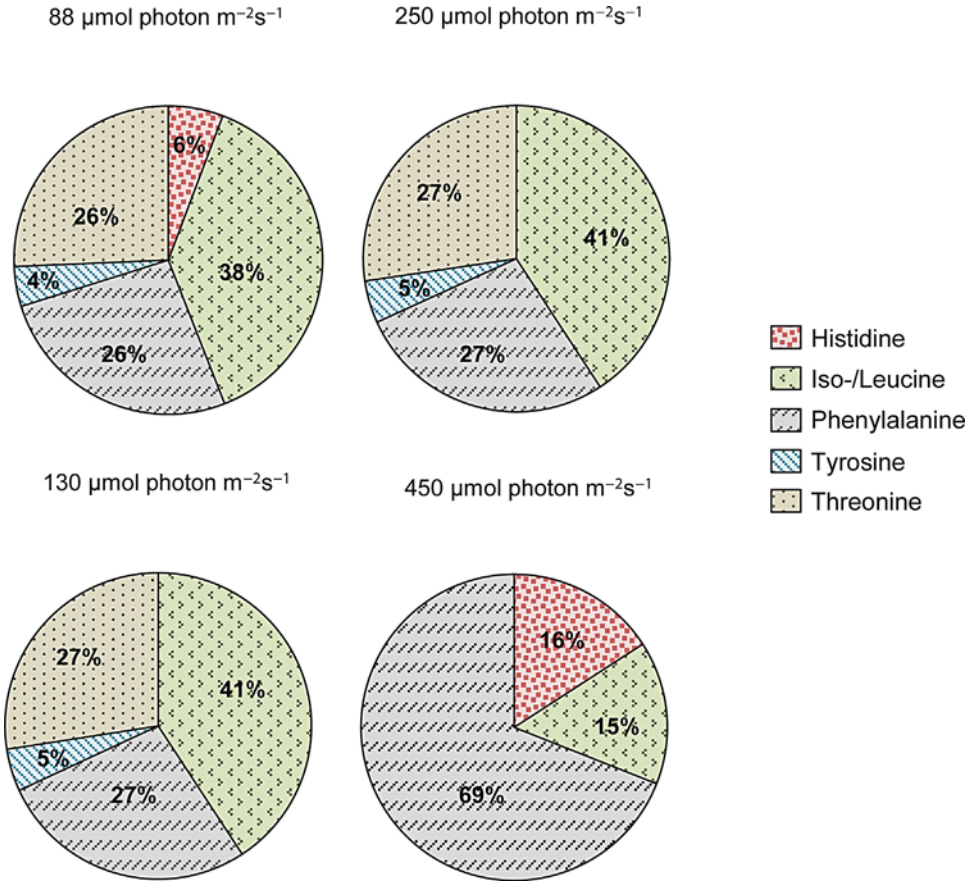

Fig. 5. Relative abundance (wt%) of the essential amino acids present in *S. marinoi* under the four blue fluence rates (data represents mean $n = 3$).

acids provide better cold-flow properties but at the cost of oxidative stability (Ramos et al., 2009), and DU is linearly correlated to the cetane number (CN), a fuel quality parameter which is related to ignition delay and combustion quality of the fuels (Ramos et al., 2009).

Proteins followed the same trend as lipids, i.e. they are enhanced under low light compared to high light, with the highest value under 130 μmol photon m^{-2} s^{-1}, and the lowest found under 450 μmol photon m^{-2} s^{-1} (Table 3). The essential amino acids profiles were similar between the 88, 130 and 250 μmol photon m^{-2} s^{-1} (Fig. 5). Iso-/Leucine's relative abundance was highest (38–41 wt%), followed by phenylalanine (26–27 wt%) and threonine (26–27 wt%). The tyrosine's relative abundance was very low (4–5 wt%), while histidine was present only under 88 μmol photon m^{-2} s^{-1} (Fig. 5). By contrast, under the highest light condition, 450 μmol photon m^{-2} s^{-1}, the essential amino acids profile significantly changed (Fig. 5). Phenylalanine was more abundant (69 wt%, Fig. 5), while tyrosine and threonine were not detected under this condition (Fig. 5). Interestingly, histidine accounted for 16 wt% under the latter condition, while this amino acid was only detected under the two extreme light conditions.

Under high light, the lipid and protein contents per cell were significantly low, whereas the carbohydrate content per cell was significantly higher ($p < 0.01$, Table 3) compared to low light. The high carbohydrate content (~threefold) under high light conditions should serve as an immediate energy source that can be required for repair and maintenance of the damages induced by high light. It could be an efficient strategy for cells to cope up with the high light damages when the XC functioning is limited. The decrease in protein content and increase in the carbohydrate content has been already observed by Terry et al. (1983) in the diatom *Phaeodactylum tricornutum*.

On the contrary to the previously described biochemical variations, the particulate organic carbon (POC) and particulate organic nitrogen (PON) concentration did not vary a lot among the different light conditions (Table 3). POC was ranged between 11 and 19 pg $cell^{-1}$, while PON ranged between 2.1 and 3.8 pg $cell^{-1}$. The POC and PON contents per cell were significantly correlated under all the conditions ($p < 0.001$, $n = 9$), except under 88 μmol photon m^{-2} s^{-1}, highlighting again that extreme low light condition induces great biochemical variations in the cells that might be less suitable for biomass production.

The fiber content, expressed as wt% on dried biomass, was constant under all conditions (Table 3), with values ranged between 11 and 16 wt% for insoluble dietary fibers (IDF) and between 0 and 2 wt% for soluble dietary fibers (SDF). This stability, i.e. its independency from light, was confirmed by the similar values of IDF (14.10 wt%) and SDF (2.20 wt%) under the white light condition (100 μmol photon m^{-2} s^{-1} provided by neon lamps). The values of IDF and SDF were significantly less compared to the reported values in the literature from other microalgae (Graziani et al., 2013).

3.4. Conclusions

"Photosynthetic regulation biotechnology", defined as the manipulation of energy partitioning in photosynthetic organisms by light for optimizing their biotechnological use, allows a deep modulation of the biochemical profile in the diatom *S. marinoi*. Our results highlight the fundamental importance of the "resonance" between light condition and the targeted species for driving macromolecular composition of the cells and enhancing production. Firstly, applying a sinusoidal light distribution allow cells to optimize their biochemical and physiological state during the period of illumination, in agreement with previous studies (Dimier et al., 2009; Giovagnetti et al., 2014). Secondly, blue light is one of the shortest wavelength (together with violet) with high energy that is predominant in aquatic ecosystems, both in terms of fluence rate and penetrating capacity compared to other wavelengths, and is totally harvested by carotenoids (absorbing between 430 and 490 nm). All these features make blue light the most efficient one for providing energy to microalgae (e.g. Schellenberger Costa et al., 2013a,b; Brunet et al., 2014) for biomass production. Moreover, the absence of red wavelength limits the development of photoprotective mechanisms, such as XC and NPQ (Brunet et al., 2014), potentially increasing the energy flow toward the photosynthetic machinery. We show that the sinusoidal distribution of blue light with a midday peak (130 μmol photon m^{-2} s^{-1}) < E_k is optimal for growth and primarily metabolites as lipids, carotenoids and proteins synthesis in the coastal diatom *S. marinoi*.

Furthermore, our study, in agreement with Lebeau and Robert (2003) and Barra et al. (2014), highlights diatoms' great potential for biotechnological applications, mainly concerning the production of primary metabolites. Enhancement of fucoxanthin production, together with the xanthophyll pigments such as diadinoxanthin or diatoxanthin has various applied issues (Peng et al., 2011), while the enhancement of protein production is suitable for aquaculture (Lebeau and Robert, 2003), and the lipid production and composition modulation might be suitable in the fields of bio-energy (Levitan et al., 2014) and nutraceuticals (Hallahan and Garland, 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.10.016.

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Light modulation of biomass and macromolecular composition of the diatom *Skeletonema marinoi*

Raghu Chandrasekaran[a,1], Lucia Barra[a,b,1], Sara Carillo[c], Tonino Caruso[d], Maria Michela Corsaro[c], Fabrizio Dal Piaz[d], Giulia Graziani[e], Federico Corato[a], Debora Pepe[a], Alessandro Manfredonia[a], Ida Orefice[a], Alexander V. Ruban[f], Christophe Brunet[a,*]

[a] *Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy*
[b] *CNR-IBBR, Via Università 133, 80055 Portici (NA), Italy*
[c] *University of Naples "Federico II", Dipartimento di Scienze Chimiche, Complesso Universitario M.S. Angelo, Via Cintia 4, 80126 Napoli, Italy*
[d] *University of Salerno, Dipartimento di Chimica e Biologia, Via Giovanni Paolo II 132, 84084 Fisciano (SA), Italy*
[e] *University of Naples "Federico II", Department of Agricultural and Food Science, Parco Gussone, 80055 Portici (NA), Italy*
[f] *School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK*

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ABSTRACT

The biochemical profile and growth of the coastal diatom *Skeletonema marinoi* was investigated under four different daily blue light doses (sinusoidal light peaking at 88, 130, 250 and 450 μmol photons m^{-2} s^{-1}, respectively). Ability of cells to regulate the light energy input caused alterations in growth and different biosynthetic pathways. The light saturation index for photosynthesis (E_k), which governs the photoacclimative processes, ranged between 250 and 300 μmol photons m^{-2} s^{-1}. Cells that were adapted to low light ($<E_k$) enhanced their carotenoid, lipid and protein contents and lowered carbohydrate content, and vice versa under high light ($\geq E_k$). Variations in fatty acid, pigment and amino acid compositions were a result of light adaptation. Our data show that light is a potent factor for manipulating biomass synthesis in microalgae, such as diatoms for microalgal biotechnology.

1. Introduction

Use of microalgae for biotechnological applications covers many aspects, such as food additives in nutraceutics and animal feeding, cosmetics, pharmaceutics and energetics (Barra et al., 2014; Cadoret et al., 2012; Chisti, 2013; Levitan et al., 2014; Munir et al., 2013). In an economic context, microalgae present many advantages compared to other photosynthetic organisms, such as their high growth rate and the requirement of little space for biomass production. Indeed, Cadoret et al. (2012) reported a productivity of 10 g m^{-2} day^{-1} for microalgae compared to 1–2 g m^{-2} day^{-1} for higher plants. Despite their great potential for the biotechnological applications (Cadoret et al., 2012; Fu et al., 2013), the extensive use of photosynthetic protists has to be enforced. The main challenge is to enhance biomass and bioactive molecules synthesis, and, in parallel, to avoid the increase in production costs (Blanken et al., 2013). To cope with these biotechnological expectations, there is a need to (i) increase the diversity of algal species used, (ii) undertake the genetic engineering of the most adequate strains (Cadoret et al., 2012) and (iii) to deeply investigate alternative ways for enhancing biomass synthesis, such as the "photosynthetic regulation biotechnology", light manipulation being a powerful tool for increasing microalgal productivity (Barra et al., 2014; Perrine et al., 2012). Light intensity and its frequency variability, as well as its spectral composition, strongly affect the growth and photosynthesis of microalgae (Dimier et al., 2009; Fu et al., 2013; Schellenberger Costa et al., 2013a,b).

Among microalgae, diatoms, the most recent and evolutionary diversified group (Kooistra et al., 2007), is still underexploited in the field of biotechnology. However, the siliceous cell wall of diatoms is of huge interest in the field of nanotechnology for designing and producing specific frustules (Lebeau and Robert, 2003; Wang et al., 2013). Furthermore, they can be highly suitable for large-scale cultivation, since most of the diatom species present fast adaptive physiological plasticity, and the presence of large central vacuole

* Corresponding author. Tel.: +39 0815833243.
E-mail address: christophe.brunet@szn.it (C. Brunet).
[1] These authors contributed equally to this work.

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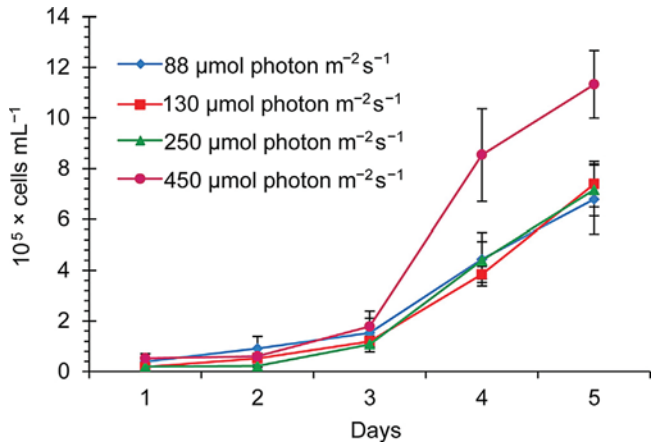

Fig. 1. Growth curve of *S. marinoi* under the four blue fluence rates; experiments were performed during the exponential phase on days 4 and 5 (data represent mean ± SD; $n = 3$).

allows them to be hugely competitive in variable environments (Kooistra et al., 2007). Among the diatom-specific carotenoids, fucoxanthin together with the most common β-carotene is of huge interest in the field of biotechnology (Peng et al., 2011). The two other xanthophylls, diadinoxanthin and diatoxanthin, are not extensively studied but their potential role as antioxidants have been recently reported (Gallina et al., 2014). The lipid profile, both in terms of quantity and quality, makes diatoms suitable candidates for aquaculture (Lebeau and Robert, 2003). Several studies have shown that the diatoms polyunsaturated fatty acids (PUFAs) have positive effect on human health (Hallahan and Garland, 2005), while the monounsaturated fatty acids (MUFAs) are considered to be the potential feedstock for biodiesel production (Levitan et al., 2014).

In this study, we explore the role of light and the potential of the "photosynthetic regulation biotechnology" to better exploit diatoms in the field of biotechnology. Only blue light condition was selected, being the most energetic and fully absorbed wavelengths by microalgae for the photosynthetic activity. Furthermore, only blue light, i.e. without red radiation, strongly limits photoprotective processes in the cells (Brunet et al., 2014), potentially increasing the biochemical energy available for growth.

The centric diatom *Skeletonema marinoi* was submitted to four different blue fluence rates, characterized by daily light dose of 2.2, 3.2, 6.1 and 11 mol m^{-2} day^{-1}, respectively. Our results, both on physiological property and biochemical profiles of the cells, confirm that variation of blue fluence rate does strongly influence the biochemical characteristics of the cells, reinstating that light manipulation is a concrete way to modulate the biochemical energy allocation in microalgae, and thus adequate for biotechnological purposes.

2. Materials and methods

2.1. Experimental strategy and sampling

Experiments were conducted on the coastal centric diatom *S. marinoi* (CCMP 2092), by cultivating at 20 °C in 4.5-L glass flask with air bubbling, containing natural sterile seawater amended with *f*/2 nutrients (Guillard and Ryther, 1962). All the experiments were performed in triplicate, lasting 2 days during the exponential growth phase (Fig. 1), on cultures pre-acclimated to each experimental light condition for 2 weeks before the experiments. Four different sinusoidal blue light distributions, peaking at 88, 130, 250 and 450 µmol photons m^{-2} s^{-1} (daily light dose: 2.2, 3.2, 6.1 and 11 mol m^{-2} day^{-1}, respectively), have been applied, with a 12:12 h light:dark photoperiod. Blue light ($\lambda = 460 \pm 30$ nm) was provided by a custom-built illumination system (Brunet et al., 2014). Light intensity was measured inside each flask by using a laboratory PAR 4π sensor (QSL 2101, Biospherical Instruments Inc., San Diego, CA, USA).

Samples for cell counts, variable fluorescence and electron transport rate measurements, pigments, particulate organic carbon and nitrogen, proteins and RNA were taken three times per day: dawn (time 0), midday (time 6 h) and in the afternoon (time 9 h). Samples for absorption spectrum analysis, lipids, carbohydrates and fibers determination were taken once during the experiment (midday sampling on second day).

2.2. Cell concentration

Cell concentration was estimated on triplicate sub-samples. An aliquot of 1 mL was used to fill a Sedgewick Rafter counting cell chamber, and cell counts were performed using a Zeiss Axioskop 2 Plus microscope.

2.3. Photochemical efficiency and photosynthetic parameters

Photochemical efficiency of photosystem (PS) II was estimated by a Phyto-PAM fluorometer (Heinz Walz, Effeltrich, Germany). The variable fluorescence analysis was performed on 15-min dark-acclimated samples, to measure the maximum photochemical efficiency (Brunet et al., 2014).

Electron transport rate (ETR) versus irradiance curves were determined by applying 13 increasing red actinic lights (655 nm) from 1 to 853 µmol photons m^{-2} s^{-1} lasting 1 min each. The relative electron transport rate ($_{rel}$ETR, expressed in µmol e^{-1} s^{-1} $cell^{-1}$) was calculated as follows:

$$_{rel}\mathrm{ETR} = \left(\frac{Fv'}{Fm'}\right) \cdot I \cdot 0.5 \cdot a^*$$

where I is the incident irradiance (expressed in µmol photons m^{-2} s^{-1}), Fv' and Fm' are the variable PS II fluorescence yield and maximal PS II fluorescence yield, respectively, for illuminated cells (measured at the end of the 1 min lasting actinic light), a^* is the cell-specific absorption coefficient, expressed in m^2 $cell^{-1}$ (for the determination of a^*, see Section 2.6). A factor of 0.5 was applied to correct for the partitioning of photons between PSI and PSII, assuming that excitation energy is evenly distributed between the two photosystems.

ETR-I curves were fitted with the equation of Eilers and Peeters to estimate $_{rel}ETR_{max}$ (maximal relative rate of linear electron transport), α (maximum light use efficiency), and E_k (light saturation index for photosynthesis).

The non-photochemical quenching (NPQ) was estimated on 15-min dark-acclimated cells by illuminating the sample with an actinic light setup at 480 µmol photons m^{-2} s^{-1} during 10 min, and the maximum fluorescence yield was estimated every minute. NPQ was quantified by the Stern–Volmer expression:

$$\mathrm{NPQ} = \frac{Fm}{Fm'} - 1$$

2.4. Pigments

Pigment measurement was conducted by high-performance liquid chromatography (HPLC), following the procedure described in Brunet et al. (2014). Ten mL aliquot of algal culture was filtered (under low light) on 25 mm GF/F glass-fiber filter (Whatman, Maidstone, UK) and stored in liquid nitrogen until further analysis. Pigments were extracted by mechanical grounding during 3 min in

2 mL of a 100% methanol solution. Pigments were separated in a Hewlett Packard series 1100 HPLC (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (2.6 μm diameter C8 Kinetex column; 50 mm × 4.6 mm; Phenomenex®, USA). Pigments were detected spectrophotometrically at 440 nm using a Hewlett Packard photodiode array detector, model DAD series 1100. Determination and quantification of pigments were carried out using pigment standards from the D.H.I. Water & Environment (Horsholm, Denmark).

2.5. Particulate organic carbon and nitrogen

Ten mL aliquot for the determination of particulate organic carbon (POC) and particulate organic nitrogen (PON) was filtered on pre-combusted (450 °C, 5 h) glass-fiber filters (Whatman, Maidstone, UK), conserved in cell culture plates (Corning®, Corning Inc., NY, USA), and immediately stored at –20 °C. The analyses were performed with a Thermo Scientific Flash EA 1112 automatic elemental analyzer (Thermo Fisher Scientific, MA, USA), following the procedure previously described by Brunet et al. (2014). Filters were thawed just prior to analysis and allowed to dry at 60 °C through a desiccator. Then filters were loaded in small tin cups that were crimped closed and transferred to the CHN analyzer. A set of empty filters was processed as ordinary samples to accomplish the blank determination. Cyclohexanone 2,4-dinitrophenylhydrazone (C, N, H, 51.79, 20.14, 5.07 wt%, respectively) was used as standard.

2.6. Absorption spectrum

The spectral absorption measurements were performed using a spectrophotometer Hewlett Packard HP-8453E equipped with an inverted Labsphere integrating sphere (RSA-HP-53 Reflectance Spectroscopy Accessory) following the procedure described in Dimier et al. (2009). Ten mL aliquot of algal culture was filtered onto Whatman GF/F filters and immediately frozen. The absorption ($a(\lambda)$) was measured between 250 and 800 nm, and thus integrated between 400 and 700 nm. This integrated value was divided by cell concentration for the estimation of the cell-specific absorption coefficient, a^*, expressed in m^2 $cell^{-1}$.

The photosynthetically usable radiation (PUR) was calculated as described in Brunet et al. (2014).

2.7. Nutrients

Samples for determining nutrient concentrations were collected in 20 mL polyethylene vials, and quickly frozen and stored at −20 °C. Ammonium, nitrate, nitrite, silicic acid and phosphate concentrations were determined using a Technicon AutoAnalyzer following classical methods (Grasshoff et al., 2009).

Nutrient concentration analyzed from the morning sampling on the first and second day of the experiment was therefore used to estimate the uptake of nutrients reported by cell number increase during the 1st and 2nd day of the experiments:

$$\mathrm{Nu} = \frac{N_2 - N_1}{C_2 - C_1},$$

where Nu is the nutrient uptake (nmol $cell^{-1}$ day^{-1}), C_n is the cell concentration at day n and N_n is the nutrient concentration at day n.

2.8. Cells pellet preparation

The volume sampled (50 mL for RNA and protein, 800 mL for lipids, carbohydrates and fibers) from each triplicate was centrifuged at 4000 rpm (3399 × g) for 20 min at 4 °C (DR15P centrifuge, B. Braun Biotech International, Melsungen, Germany); the supernatant was discarded. For proteins and RNA quantification, the pellet has been transferred in a 2 mL Eppendorf tubes and centrifuged at 14,000 rpm (20,817 × g) for 15 min at 4 °C (5417R centrifuge, Eppendorf, Hamburg, Germany). For lipids and carbohydrates, the pellets obtained after the centrifugation (16 tubes of 50 mL) was pooled together and centrifuged again as previously mentioned and weighed.

2.9. RNA analysis

The total RNA has been extracted from the pellet following the procedure described in Barra et al. (2013). Concentration of RNA was measured by Nanodrop (Agilent Technologies, Santa Clara, CA, USA).

2.10. Protein and amino acids analysis

The total proteins from the pellet was extracted by sonicating the cell pellets for 2.4 min in 600 μL of sterile water and centrifuged at 13,000 rpm (17,949 × g) for 20 min at 4 °C. Then the supernatant was collected and the pellet was re-extracted with 500 μL of 0.1 N NaOH and 0.5% β-mercaptoethanol (v/v). The mixture was kept at RT for 1 h (with occasional shaking) and centrifuged at 13,000 rpm (17,949 × g) for 20 min at 21 °C. The supernatant was mixed and the pellet was discarded. Proteins were then purified with trichloroacetic acid (TCA) before acid hydrolysis for amino acids analysis, according to Barbarino and Lourenço (2005) and the crude extract has been quantified with Folin & Ciocalteu reagent (Sigma).

Samples containing 50 μg of protein were acid hydrolyzed with 1 mL of 6 N HCl in vacuum-sealed hydrolysis vials at 110 °C for 22 h. Norleucine was added to the HCl as an internal standard. Although tryptophan was completely lost with acid hydrolysis and methionine and cysteine + cystine could be destroyed to varying degrees by this procedure, the hydrolysates were suitable for analysis of allotheramino acids. The tubes were cooled after hydrolysis, opened and placed in a dessicator containing NaOH pellets under vacuum until dry (5–6 days). The residue was then dissolved in a suitable volume of dilution Na–S R buffer (pH 2.2; Beckman Instr.), filtered through a Millipore membrane (0.22 μm pore size) and analyzed for amino acids by ion-exchange chromatography in a Beckman, model 7300 instrument equipped with an automatic integrator.

2.11. Lipid and fatty acids analysis

Each wet pellet was sonicated for 15 min at 25 °C in 10 mL of acetic acid/chloroform (1/9), or acetone/methanol (9/1) or pure methanol in order to get respectively triglyceride, glycolipid or phospholipid fraction. The extracted solution was passed through cartridges Supelclean™ ENVI-Florisil® SPE Tubes (by Aldrich), preconditioned with 30 mL of chloroform (Popovich et al., 2012). The samples have been subsequently concentrated under N_2 flux to reach final volume of 1 mL and esterified with KOH 2 M in methanol according to (Graziani et al., 2013). One μL was injected directly in a Thermo Finnigan TRACE gas chromatograph equipped with a fused silica capillary column (FAMEWAX Restek, 30 m × 0.25 mm i.d., 0.25 μm film thickness) and an FID detector. The calibration has been performed using a standard PUFA's (by Supelco) as internal standard.

The degree of unsaturation (DU) was calculated using the following formula as reported in Ramos et al. (2009):

$$\mathrm{DU} = (\text{monounsaturated Cn} : 1, \text{wt\%}) + 2 \times (\text{polyunsaturated Cn} : 2, 3, \text{wt\%})$$

Table 1
Photosynthetic properties of *S. marinoi* under the four blue fluence rates.

	88 μmol photons m^{-2} s^{-1}	130 μmol photons m^{-2} s^{-1}	250 μmol photons m^{-2} s^{-1}	450 μmol photons m^{-2} s^{-1}
PAR dose	2.2	3.2	6.1	11
a^*	1.66 ± 0.59	0.90	0.94 ± 0.29	2.26 ± 0.64
PUR	0.51 ± 0.18	0.37	0.87 ± 0.34	4.1 ± 1.2
μ	0.40 ± 0.27	1.24 ± 0.23	0.49 ± 0.13	0.31 ± 0.11
$_{rel}ETR_{max}$	3.16 ± 1.15	2.21 ± 0.08	2.90 ± 1.02	
E_k	252 ± 36	241 ± 7	306 ± 13	
α	0.012 ± 0.003	0.009 ± 0.0006	0.0095 ± 0.003	
NPQ	0.64 ± 0.05	0.87 ± 0.03	0.61 ± 0.02	
Total RNA	0.48 ± 0.18	0.30 ± 0.06	0.69 ± 0.22	0.38 ± 0.14

Average PAR dose experienced by the cells under different blue light conditions (mol m^{-2} day^{-1}). $a^* \times 10^{-11}$, absorption coefficient (m^2 $cell^{-1}$); PUR × 10^{-6}, photosynthetically usable radiation (μW $cell^{-1}$); μ, growth rate (day^{-1}); $_{rel}ETR_{max} \times 10^{-6}$ (maximal relative rate of linear electron transport, pmol e^{-1} h^{-1} $cell^{-1}$); α (maximum light use efficiency, pmol e^{-1} h^{-1} $cell^{-1}$(μmol photon m^{-2} s^{-1}) $^{-1}$); E_k (light saturation index for photosynthesis, μmol photons m^{-2} s^{-1}) and total RNA content (pg $cell^{-1}$). Data represent mean and SD ($n = 3$ except for total RNA, $n = 15$).
The lack of data for the 450 μmol photons m^{-2} s^{-1} condition is due to the breakdown of the PAM fluorometer during the experiment.

2.12. Carbohydrate analysis

The pellets obtained for carbohydrate analysis were sonicated for 5 min in 5 mL of *f*/2 culture medium and centrifuged. The supernatant and the cellular debris pellet were stored separately for the analysis. Total carbohydrates content, calculated on both supernatant and pellet of each sample, was determined by microphenol assay as reported in Kobata (1972).

2.13. Fiber analysis

Total fiber content of *S. marinoi*, composed by soluble and insoluble fraction, was determined by the AOAC 985.29 gravimetric method (Graziani et al., 2013).

2.14. Statistical analysis

Student's *t*-test and Spearman's rank correlation were performed using Systat 7 software.

3. Results and discussion

3.1. Light modulation of photosynthetic property and growth

Cells grown under 88, 130 and 250 μmol photon m^{-2} s^{-1} had similar growth capacity (Fig. 1) in agreement with a low range of variability of the linear electron transport rate ($_{rel}ETR_{max}$, from 2.2 to 3.1 × 10^{-6} pmol e^{-1} h^{-1} $cell^{-1}$, Table 1). The similar photosynthetic and growth rates measured under the different illumination conditions are probably related to biochemical and physiological adjustments within the cells. Indeed, the light saturation index for photosynthesis (E_k, Table 1), ≈250–300 μmol photons m^{-2} s^{-1}, revealed that cells grown in 88 and 130 μmol photon m^{-2} s^{-1} conditions were potentially light limited, while under the 250 and 450 μmol photon m^{-2} s^{-1} cells were light saturated or oversaturated (450 μmol photon m^{-2} s^{-1}). The pronounced low light acclimation state of the cells under 88 μmol photon m^{-2} s^{-1} was confirmed by the highest value of the maximum light use efficiency parameter (α, Table 1), and by the increase in cell-specific absorption coefficient (a^*, Table 1). On the opposite, under the highest light condition, 450 μmol photon m^{-2} s^{-1}, cells grew faster reaching a higher maximum cell number (11 × 10^5 cells mL^{-1}) compared to the three other conditions (~7 × 10^5 cells mL^{-1}; Fig. 1). Indeed, the light enhancement was only little compensated by adjustments in light absorption, as shown by the highest photosynthetic usable radiation (PUR, Table 1).

None of the nutrients was limiting during the 2 days of experiment (Table 2), while the increase in cell concentration was sustained by the uptake of NO_3^-, PO_4^{3-} and SiO_3^{2-} (Table 2, Fig. 2). By contrast, NO_2^- and NH_4^+ increased between the 2 days of experiment, probably in relation with the rapid and continuous recycling of these nutrients in the medium (Barra et al.,

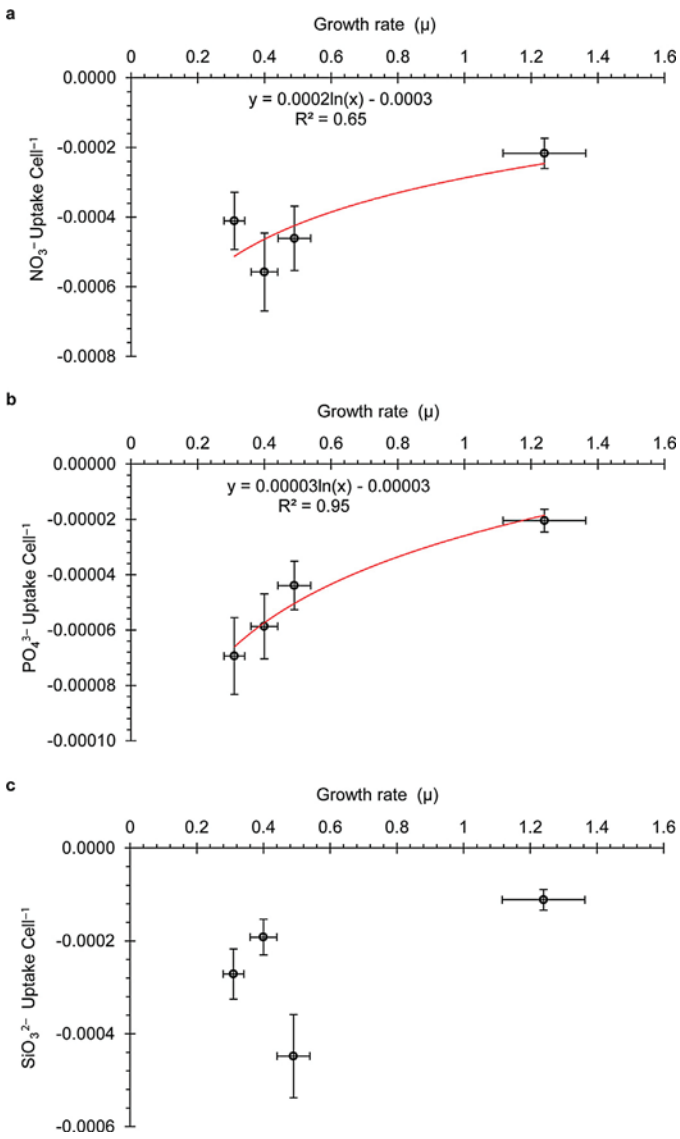

Fig. 2. Relationship between growth rate (μ; day^{-1}) and nutrient uptake per cell (nmol $cell^{-1}$ day^{-1}): (a) NO_3^-, (b) PO_4^{3-} and (c) SiO_3^{2-} (data represent mean ± SD; $n = 3$).

Table 2
Growth rate and nutrient concentrations under the four blue fluence rates during the two days of experiments.

	88 μmol photons m^{-2} s^{-1}	130 μmol photons m^{-2} s^{-1}	250 μmol photons m^{-2} s^{-1}	450 μmol photons m^{-2} s^{-1}
Cell con.				
1st day/2nd day	443,989 ± 205,449	214,214 ± 31,979	439,509 ± 73,147	854,291 ± 282,535
	678,394 ± 336,142	738,348 ± 99,598	715,593 ± 122,325	1,072,880 ± 160,228
Cell con. increase	234,405 ± 225,457	524,134 ± 112,078	276,083 ± 145,257	218,588 ± 176,819
NH4+				
1st day/2nd day	0.60 ± 0.37	0.92 ± 0.21	0.84 ± 0.78	1.81 ± 0.80
	0.93 ± 0.32	1.17 ± 0.70	0.97 ± 1.08	1.05 ± 0.08
NO_2^-				
1st day/2nd day	4.89 ± 1.13	4.21 ± 0.49	4.93 ± 1.06	6.68 ± 0.05
	5.94 ± 0.56	7.12 ± 0.67	8.20 ± 1.29	15.31 ± 1.23
NO_3^-				
1st day/2nd day	369.16 ± 9.5	858.93 ± 46.35	694.16 ± 142.58	594.70 ± 44.01
	300.54 ± 28	756.56 ± 33.23	629.32 ± 60	515.60 ± 42.56
PO_4^{3-}				
1st day/2nd day	10.13 ± 0.91	24.13 ± 0.29	19.54 ± 2.38	14.25 ± 0.72
	2.08 ± 0.04	13.73 ± 2.97	11.10 ± 4.34	1.74 ± 0.45
$[SiO_3]^{2-}$				
1st day/2nd day	68.03 ± 19.89	124.29 ± 17.43	148.69 ± 58.31	64.08 ± 0.76
	38.75 ± 23.79	63.86 ± 27.86	56.58 ± 40	5.38 ± 5.50

Cell concentration (Cell con.; cells mL^{-1}) and nutrient concentration (nmol mL^{-1} day^{-1}).

2014). Growth rate was significantly correlated to the uptake of NO_3^- and PO_4^{3-},($p < 0.05$ for NO_3^- and $p < 0.01$ for PO_4^{3-}; Fig. 2), revealing their relevant role in driving biomass synthesis and that light intensity did not affect directly the uptake of these nutrients. By contrast, the higher SiO_3^{2-} uptake was measured under high light compared to the low light conditions and it was not related to the growth rate ($p > 0.05$, Fig. 2). This agrees with a potential increase in photorespiration rate induced by high light (Brunet et al., 2011; Schnitzler Parker et al., 2004), being this process a way for cells to dissipate the excess biochemical energy, and to the fact that silicate metabolism is more linked to respiration and cell cycle than photosynthesis (Norici et al., 2011).

The RNA content per cell did not show significant variation among the light conditions ($p > 0.05$, $n = 15$), ranging from 0.30 to 0.70 pg $cell^{-1}$ (Table 1). The RNA content per cell displayed circadian oscillation in the cells (data not shown), that might be related to variations in the cellular content of proteins, lipids, carbohydrates or pigments (Fábregas et al., 2002). In all conditions, the RNA content per cell increased at midday compared to the morning and afternoon samples, while some studies (e.g. Berdalet et al., 1992) showed enhanced RNA content at the beginning of the light period. This discrepancy is due to the light distribution, provided in a sinusoidal way in our study and in a quadratic way for the other studies, as recently demonstrated (Orefice, personal communication).

3.2. Light modulation of carotenoids and chlorophylls

The main photosynthetic pigments, chlorophyll *a* (Chl *a*) and fucoxanthin (Fuco), were significantly correlated ($p < 0.001$, $n = 36$) presenting a significant decrease under the high light conditions ($p < 0.05$, $n = 9$; Fig. 3a and b). Under low light ($E < E_k$, 88 and 130 μmol photon m^{-2} s^{-1}), the total carotenoid content and Chl *a* per cell was similar (Fig. 3a, Table 3), despite the difference in daily light dose (2.2 and 3.2 mol m^{-2} day^{-1}). Fuco/Chl *a* was similar among the different light conditions ($p > 0.05$, $n = 9$), except for 450 μmol photon m^{-2} s^{-1} where it significantly decreased ($p < 0.01$, $n = 9$). Thus, *S. marinoi* tends to modify the antenna structure by decreasing Fuco compared to the Chl *a* molecules in the reaction center under the latter condition (Dimier et al., 2009), while under low light condition, *S. marinoi* tends to modify all the reaction center and antenna structure to maintain similar the pigment ratio.

On the opposite of Fuco and Chl *a*, chlorophyll *c* (Chl *c*) content per cell exponentially decreased from the lowest to the highest light dose (Fig. 3a), in agreement with the results obtained on *Pseudo-nitzschia multistriata* (Brunet et al., 2014). The highest Chl *c* content under 88 μmol photon m^{-2} s^{-1} (two times more compared to the other light conditions) enhances the ability of cells to cope with extreme low light, since this pigment is known to efficiently transfer energy to Chl *a* (Di Valentin et al., 2013).

The β-carotene (β-car) content did not show any significant trend over the light gradient (Fig. 3c), while a significantly higher

Table 3
Biochemical properties of *S. marinoi* under the four blue fluence rates.

	88 μmol photons m^{-2} s^{-1}	130 μmol photons m^{-2} s^{-1}	250 μmol photons m^{-2} s^{-1}	450 μmol photons m^{-2} s^{-1}
Total carotenoids	0.015 ± 0.006	0.019 ± 0.003	0.009 ± 0.003	0.005 ± 0.001
Total carbohydrates	0.62 ± 0.12	0.79 ± 0.24	2.60 ± 1.52	2.52 ± 0.06
Total lipids	1.25 ± 0.16	2.60 ± 0.25	0.66 ± 0.06	0.68 ± 0.14
Neutral lipids	0.54 ± 0.13	0.90 ± 0.10	0.21 ± 0.08	0.42 ± 0.08
Phospholipids	0.03 ± 0.02	0.96 ± 0.16	0.29 ± 0.07	0.18 ± 0.15
Glycolipids	0.68 ± 0.04	0.74 ± 0.01	0.16 ± 0.04	0.08 ± 0.04
DU	28	92	5	34
Total proteins	26.73 ± 3.45	45.03 ± 7.88	23.87 ± 0.73	15.89 ± 0.79
Total amino acids	0.00199	0.00356	0.00231	0.00038
IDF (wt%, insoluble fibers)	11.85	12.45	16.80	12.28
SDF (wt%, soluble fibers)	2.15	1.89	nd	nd
POC	14.16 ± 1.87	10.99 ± 0.13	19.37 ± 2.78	15.18 ± 0.83
PON	2.62 ± 0.89	3.86 ± 0.60	3.78 ± 1.31	2.11 ± 0.63

DU, degree of unsaturation [(MUFA, wt%) + 2 × (PUFA, wt%)]; POC, particulate organic carbon (pg $cell^{-1}$); PON, particulate organic nitrogen (pg $cell^{-1}$). Data represent mean and standard deviation (for POC, PON, $n = 15$; carotenoids and proteins, $n = 9$; and for lipids, fibers, and carbohydrates, $n = 3$).

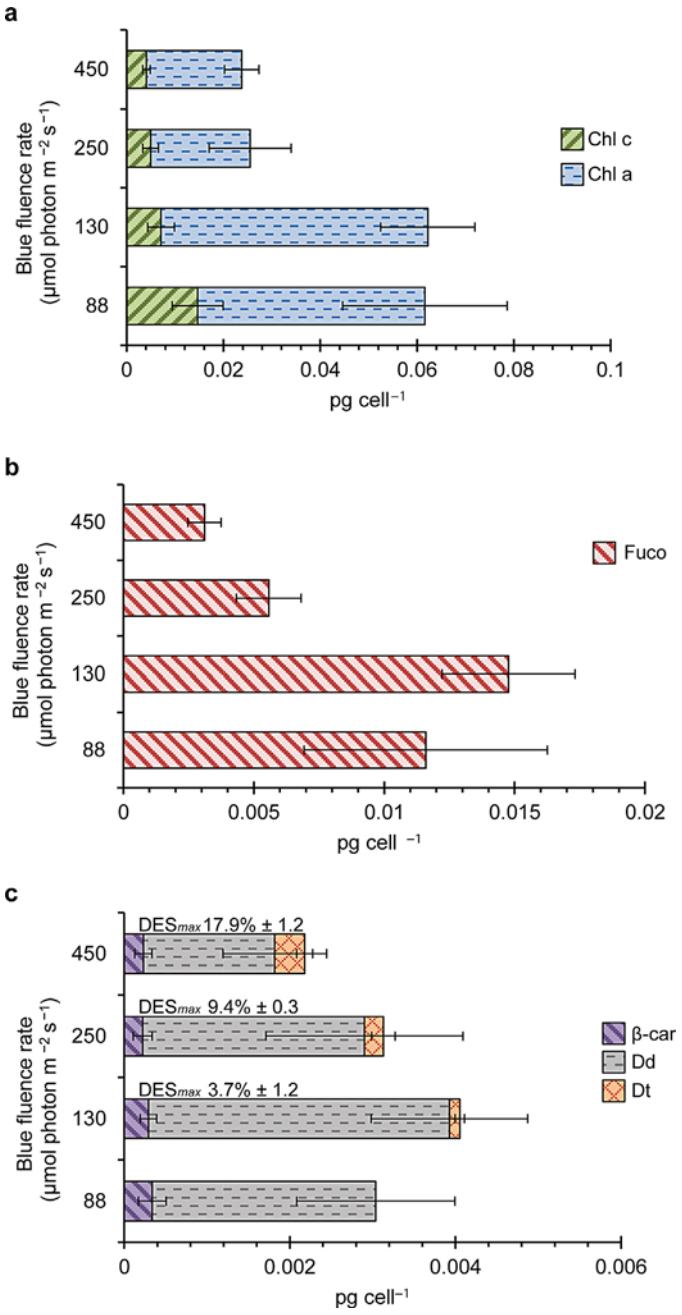

Fig. 3. Variations of photosynthetic and photoprotective pigments content. (a) Chlorophyll *a* and Chlorophyll *c* content per cell (Chl *a* and Chl *c*; pg cell^{-1}); (b) Fucoxanthin content per cell (Fuco; pg cell^{-1}); (c) β-carotene, diadinoxanthin and diatoxanthin content per cell (β-car, Dd and Dt; pg cell^{-1}); maximum de-epoxidation state [DES_{max} = Dt/(Dd + Dt)] (data represent mean ± SD; n = 9 except for DES_{max}, n = 3).

β-car/Chl *a* was found under the two high light conditions ($p < 0.05$, $n = 9$) compared to the low light conditions (data not shown). This feature is related to the Chl *a* decrease and to the involvement of β-car in the biosynthesis of the xanthophyll cycle (XC) pigments (Dambek et al., 2012). Diadinoxanthin over chlorophyll *a* ratio (Dd/Chl *a*) followed the same trend as β-car/Chl *a* and was significantly higher under high light than low light ($p < 0.05$, $n = 9$). As Fuco, Dd concentration decreased from the 130 µmol photon m^{-2} s^{-1} to the highest light intensity (Fig. 3c). The 88 µmol photon m^{-2} s^{-1} condition was out of this trend, revealing a peculiar physiological response of the cells grown under this extremely light limiting condition. Diatoxanthin (Dt; photoprotective pigment) showed an opposite trend to Dd, being significantly higher under the highest intensity compared to the other conditions ($p < 0.05$, $n = 9$; Fig. 3c). Dt concentration was very low under all the light conditions, reinforcing that cells are not able to develop photoprotective process under blue light (Brunet et al., 2014). Indeed, the absence of red spectrum strongly limits Dt synthesis and therefore the development of non-photochemical quenching (NPQ, excess-energy dissipative process, Table 2).

3.3. Light modulation of macromolecular composition

Although the protein and lipid content per cell were significantly correlated to the growth rate ($p < 0.05$, $n = 4$), the biochemical property depends on the light experienced by cells. Similar to carotenoid content, cells increased lipid and protein content under low light compared to high light (Table 3, $p < 0.01$). The highest concentration was found under 130 µmol photon m^{-2} s^{-1}. The high lipid content under the low light conditions may serve as an effective energy and carbon storage (Fábregas et al., 2002) providing a larger sink of the available energy. It also decreases the membrane permeability by forming thick membrane layers and thus reducing the energy loss by passive diffusion of organic matter (Cherrier et al., 2014). The classes of glycolipids and neutral lipids followed the same trend as total lipids, being enhanced under low light, with a maximal concentration at 130 µmol photon m^{-2} s^{-1} (Table 3). The third class, the phospholipids, did not follow any trend over the light gradient (Table 3), while its concentration was strongly enhanced under 130 µmol photon m^{-2} s^{-1} (Table 3). The low phospholipid content under high light suggests that the membrane permeability was higher under this condition compared to the other light conditions. This feature could allow cells to remove excess energy by exudation of organic matter when the photosynthesis outpaces cell growth (Cherrier et al., 2014). Furthermore, the increase in the neutral lipid contribution under the highest light condition (≈62 wt% vs <40 wt%) might be attributed to their role in preventing photo-oxidative damages (Solovchenko, 2012).

Under all the conditions, except for the 130 µmol photon m^{-2} s^{-1} light, the saturated FAs hugely dominated over the others (77–97 wt%; Fig. 4), while the unsaturated FAs (sum of monounsaturated FAs and polyunsaturated FAs, MUFAs + PUFAs) which contributed to 59 wt% of the total lipids under the latter condition. The unsaturated FAs contribution decreased to 23, 29 and 3 wt% of the total lipids under 88, 450 and 250 µmol photon m^{-2} s^{-1}, respectively (Fig. 4).

Among the saturated FAs, cells synthesized predominantly C8:0 (ranged between 82 and 33 wt%; Table S1), except under the 130 µmol photon m^{-2} s^{-1}, in which it was replaced by C20:0 (25 wt%; Table S1). Under 130 µmol photon m^{-2} s^{-1}, cells accumulated C18 monounsaturated FAs (15 wt%) and C20 polyunsaturated FAs (17 wt%); C18:1ω9t dominated the MUFAs, while C20:2ω6 dominated the PUFAs (Table S1). Under the two extreme light conditions, 88 and 450 µmol photon m^{-2} s^{-1}, cells presented high contribution of C16:1 monounsaturated FA (≈17 and 10 wt%, respectively; Table S1), while the contribution of C17:1 monounsaturated FA was high only in the highest light (≈9 wt%; Table S1).

The 130 µmol photon m^{-2} s^{-1} of blue light offers the best condition for enhancing lipid production and for obtaining homogenous distribution of the saturated, polyunsaturated and monounsaturated fatty acid (FA) classes (Fig. 4). The degree of unsaturation (DU, ratio between saturated and unsaturated FA) was higher under 130 µmol photon m^{-2} s^{-1} (92) compared to all the other light conditions (ranged between 5 and 35). While at an economic point of view, the production of algal biodiesel cannot rely on artificial light (Chisti, 2013), the results of our study might be of interest in the field of bio-energy. Indeed, high amount of saturated FAs provides a superior oxidative stability, while polyunsaturated fatty

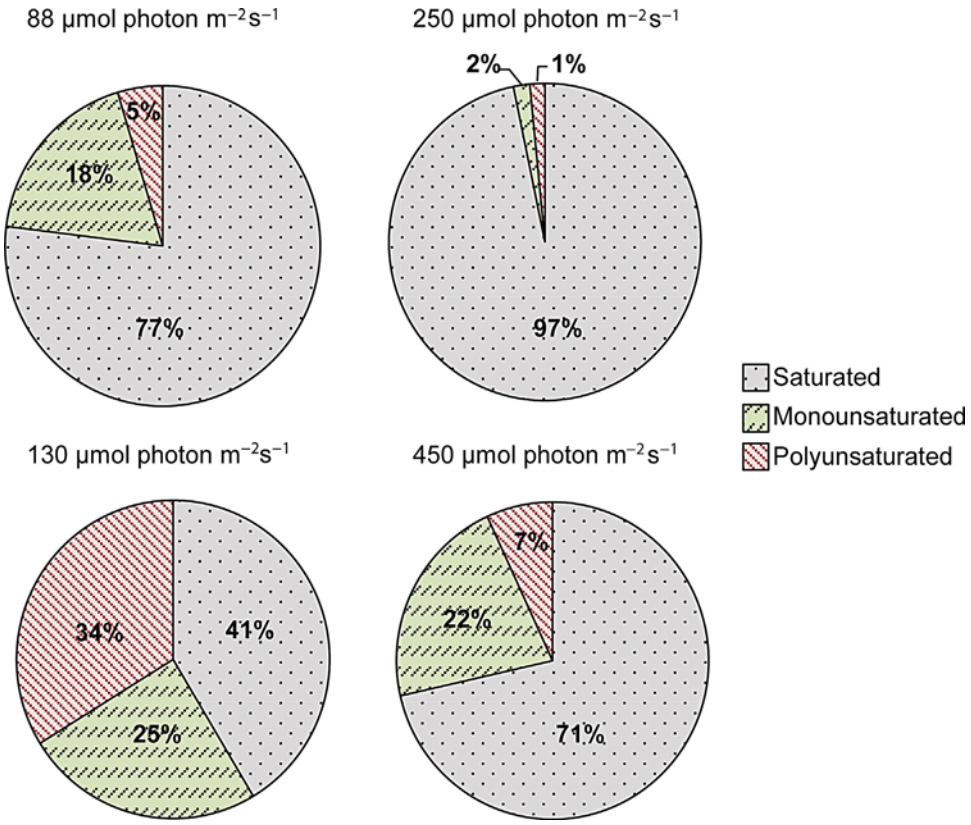

Fig. 4. Relative abundance (wt%) of saturated, mono- and polyunsaturated fatty acids in *S. marinoi* under the four blue fluence rates (data represents mean, $n = 3$).

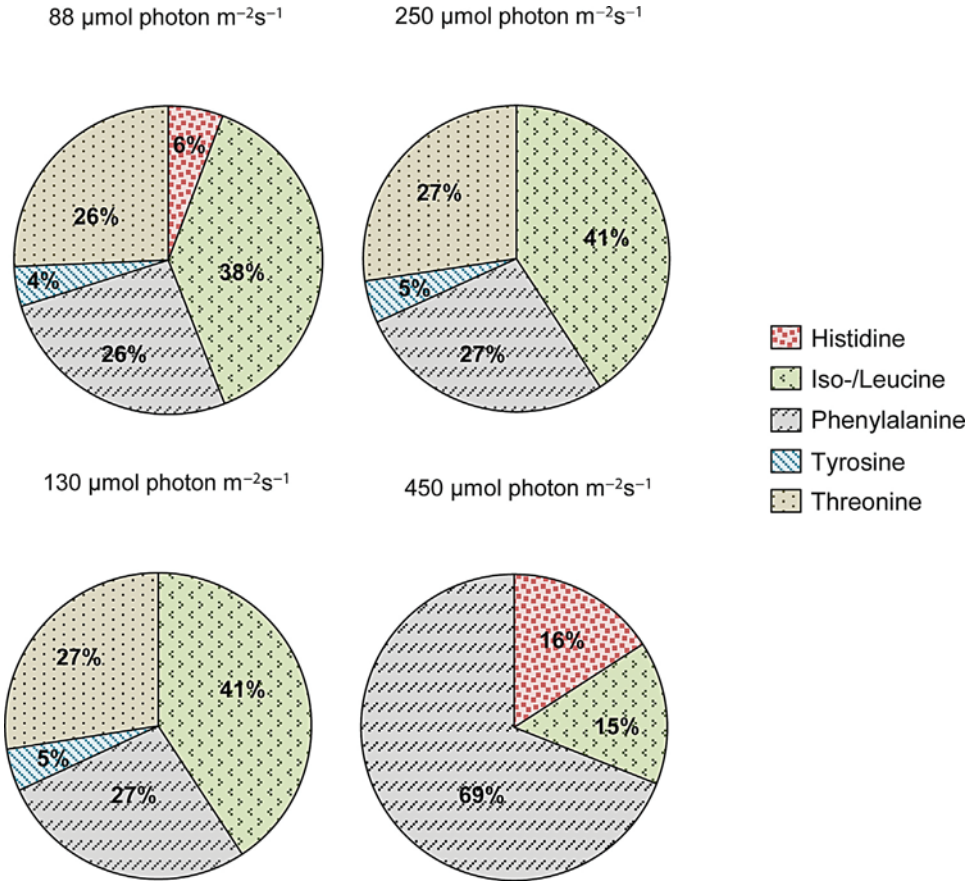

Fig. 5. Relative abundance (wt%) of the essential amino acids present in *S. marinoi* under the four blue fluence rates (data represents mean $n = 3$).

acids provide better cold-flow properties but at the cost of oxidative stability (Ramos et al., 2009), and DU is linearly correlated to the cetane number (CN), a fuel quality parameter which is related to ignition delay and combustion quality of the fuels (Ramos et al., 2009).

Proteins followed the same trend as lipids, i.e. they are enhanced under low light compared to high light, with the highest value under 130 μmol photon m^{-2} s^{-1}, and the lowest found under 450 μmol photon m^{-2} s^{-1} (Table 3). The essential amino acids profiles were similar between the 88, 130 and 250 μmol photon m^{-2} s^{-1} (Fig. 5). Iso-/Leucine's relative abundance was highest (38–41 wt%), followed by phenylalanine (26–27 wt%) and threonine (26–27 wt%). The tyrosine's relative abundance was very low (4–5 wt%), while histidine was present only under 88 μmol photon m^{-2} s^{-1} (Fig. 5). By contrast, under the highest light condition, 450 μmol photon m^{-2} s^{-1}, the essential amino acids profile significantly changed (Fig. 5). Phenylalanine was more abundant (69 wt%, Fig. 5), while tyrosine and threonine were not detected under this condition (Fig. 5). Interestingly, histidine accounted for 16 wt% under the latter condition, while this amino acid was only detected under the two extreme light conditions.

Under high light, the lipid and protein contents per cell were significantly low, whereas the carbohydrate content per cell was significantly higher ($p < 0.01$, Table 3) compared to low light. The high carbohydrate content (~threefold) under high light conditions should serve as an immediate energy source that can be required for repair and maintenance of the damages induced by high light. It could be an efficient strategy for cells to cope up with the high light damages when the XC functioning is limited. The decrease in protein content and increase in the carbohydrate content has been already observed by Terry et al. (1983) in the diatom *Phaeodactylum tricornutum*.

On the contrary to the previously described biochemical variations, the particulate organic carbon (POC) and particulate organic nitrogen (PON) concentration did not vary a lot among the different light conditions (Table 3). POC was ranged between 11 and 19 pg $cell^{-1}$, while PON ranged between 2.1 and 3.8 pg $cell^{-1}$. The POC and PON contents per cell were significantly correlated under all the conditions ($p < 0.001$, $n = 9$), except under 88 μmol photon m^{-2} s^{-1}, highlighting again that extreme low light condition induces great biochemical variations in the cells that might be less suitable for biomass production.

The fiber content, expressed as wt% on dried biomass, was constant under all conditions (Table 3), with values ranged between 11 and 16 wt% for insoluble dietary fibers (IDF) and between 0 and 2 wt% for soluble dietary fibers (SDF). This stability, i.e. its independency from light, was confirmed by the similar values of IDF (14.10 wt%) and SDF (2.20 wt%) under the white light condition (100 μmol photon m^{-2} s^{-1} provided by neon lamps). The values of IDF and SDF were significantly less compared to the reported values in the literature from other microalgae (Graziani et al., 2013).

3.4. Conclusions

"Photosynthetic regulation biotechnology", defined as the manipulation of energy partitioning in photosynthetic organisms by light for optimizing their biotechnological use, allows a deep modulation of the biochemical profile in the diatom *S. marinoi*. Our results highlight the fundamental importance of the "resonance" between light condition and the targeted species for driving macromolecular composition of the cells and enhancing production. Firstly, applying a sinusoidal light distribution allow cells to optimize their biochemical and physiological state during the period of illumination, in agreement with previous studies (Dimier et al., 2009; Giovagnetti et al., 2014). Secondly, blue light is one of the shortest wavelength (together with violet) with high energy that is predominant in aquatic ecosystems, both in terms of fluence rate and penetrating capacity compared to other wavelengths, and is totally harvested by carotenoids (absorbing between 430 and 490 nm). All these features make blue light the most efficient one for providing energy to microalgae (e.g. Schellenberger Costa et al., 2013a,b; Brunet et al., 2014) for biomass production. Moreover, the absence of red wavelength limits the development of photoprotective mechanisms, such as XC and NPQ (Brunet et al., 2014), potentially increasing the energy flow toward the photosynthetic machinery. We show that the sinusoidal distribution of blue light with a midday peak (130 μmol photon m^{-2} s^{-1}) < E_k is optimal for growth and primarily metabolites as lipids, carotenoids and proteins synthesis in the coastal diatom *S. marinoi*.

Furthermore, our study, in agreement with Lebeau and Robert (2003) and Barra et al. (2014), highlights diatoms' great potential for biotechnological applications, mainly concerning the production of primary metabolites. Enhancement of fucoxanthin production, together with the xanthophyll pigments such as diadinoxanthin or diatoxanthin has various applied issues (Peng et al., 2011), while the enhancement of protein production is suitable for aquaculture (Lebeau and Robert, 2003), and the lipid production and composition modulation might be suitable in the fields of bio-energy (Levitan et al., 2014) and nutraceuticals (Hallahan and Garland, 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.10.016.

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ORIGINAL ARTICLE

The green–blue swing: plasticity of plankton food-webs in response to coastal oceanographic dynamics

Domenico D'Alelio, Maria Grazia Mazzocchi, Marina Montresor, Diana Sarno, Adriana Zingone, Iole Di Capua, Gayantonia Franzè*, Francesca Margiotta, Vincenzo Saggiomo & Maurizio Ribera d'Alcalà

Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

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Correspondence
Domenico D'Alelio, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy.
E-mails: dalelio@szn.it, dom.dalelio@gmail.com

*Present address: Department of Biology, University of Akron, Akron, OH 44325-3908, USA.

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Abstract

The internal organization of plankton communities plays a key role in biogeochemical cycles and in the functioning of aquatic ecosystems. In this study, the structure of a marine plankton community (including both unicellular and multicellular organisms) was inferred by applying an ecological network approach to species abundances observed weekly at the long-term ecological research station MareChiara (LTER-MC) in the Gulf of Naples (Tyrrhenian Sea, Mediterranean Sea) in the summers of 2002–2009. Two distinct conditions, characterized by different combination of salinity and chlorophyll values, alternated at the site: one influenced by coastal waters, herein named 'green', and the other reflecting more offshore conditions, named 'blue'. The green and blue 'phases' showed different keystone biological elements: namely, large diatoms and small-sized flagellates, respectively. Several correlations amongst species belonging to different trophic groups were found in both phases (connectance ~0.30). In the green phase, several links between phytoplankton and mesozooplankton and within the latter were detected, suggesting matter flow from microbes up to carnivorous zooplankton. A microbial-loop-like subweb, including mixo- and heterotrophic dinoflagellates and ciliates, was present in the green phase, but it was relatively more important in the blue phase. The latter observation suggests a more intense cycling of matter at the microbial trophic level in the blue phase. These results show that different modes of ecological organization can emerge from relatively small changes in the composition of aquatic communities coping with environmental variability. This highlights a significant plasticity in the internal structure of plankton webs, which should be taken into account in predictions of the potential effects of climatic oscillations on aquatic ecosystems and biogeochemical cycles therein.

Introduction

Understanding the structure and functioning of plankton communities is a crucial step in tracking biogeochemical cycles and predicting future responses of aquatic ecosystems to environmental changes at different times and spatial scales (de Senerpont Domis *et al.* 2013; Behrenfeld & Boss 2014).

The flux of matter, energy and information in the oceans largely depends on the structure of plankton communities, which are, in turn, characterized by the species present, their abundance and functional roles, and their possible biological inter-connections (Sommer *et al.* 2012). The clear-cut, paradigmatic formalization of a planktonic trophic chain ruled by phytoplankton production and zooplankton grazing – dating back to G. A. Riley's work (Anderson & Gentleman 2012) – and applied to any planktonic system, has been progressively questioned by the increasing levels of awareness of the fine-tuned mechanisms at the base of plankton ecology (Tett & Wilson 2000;

Strom 2008). Plankton communities show multiple traits able to influence biogeochemical cycles with cascade effects. Many microbes can shift their metabolism between autotrophy and heterotrophy and effectively feed on other microbes (Sherr & Sherr 2007; Jeong *et al.* 2010; Schmoker *et al.* 2013), planktonic animals can graze selectively and show niche partitioning (Katechakis 2004; Katechakis & Stibor 2004; Olson *et al.* 2006) and unexpectedly complex trophic cascades can emerge as a consequence of changes in community structure (Stibor *et al.* 2004; Caron & Hutchins 2012).

Depicting the structure of communities and the links amongst their components encompasses two complementary analytical steps: (i) the inference of biological links from empirical data (by describing, comparing and correlating the abundances of species or functional groups) and (ii) the construction and analysis of ecological networks based on pairwise co-variations (Blüthgen *et al.* 2008; Vermaat *et al.* 2009). In this context, ecological networks are structured graphs consisting of species as nodes and biological links as edges; they represent a useful conceptual tool to schematize community structure, *e.g.* in terms of food-web relationships or the presence and relative importance of keystone species (Beckerman *et al.* 2006; Jordán 2009). Such an approach has been applied successfully to marine food-webs, revealing ecologically consistent structures in different systems (Dunne *et al.* 2004; de Santana *et al.* 2013). As for plankton, network approaches have revealed non-random community organization and suggested crucial biological mechanisms, such as symbiosis, parasitism, competition and predation amongst unicellular organisms (Steele *et al.* 2011).

The present study aimed to (i) infer the structure of marine plankton communities, including both unicellular and multicellular organisms, in terms of statistical and presumably trophic links based on field data of species composition and abundance and (ii) relate short-term temporal changes in community structure to differences in the hosting environment. We applied an ecological network approach to plankton data collected weekly at the long-term ecological research station MareChiara (LTER-MC) in the inner Gulf of Naples (GoN, Tyrrhenian Sea, western Mediterranean). This well-studied system shows strong seasonality and resilience in the succession of plankton communities (Zingone *et al.* 1995, 2009; Modigh 2001; Modigh & Castaldo 2002; Mazzocchi *et al.* 2011, 2012) with strong regularity in the species' life histories (D'Alelio *et al.* 2010). Herein, we describe the characteristics of two alternative modes of organization in a plankton community (including phyto-, microzoo- and mesozooplankton) in this coastal area. We call these modes the 'green' and 'blue' phases, occurring in lower and higher salinity water-masses, respectively, in the surface water-layer at LTER-MC during summer. Based on the observation of community properties during the green and blue phases, we present two scenarios of food-web structure that switch through time in the summer plankton of the GoN.

Material and Methods

Study site

The sampling station LTER-MC (40°48.5′ N, 14°15′ E) is located in the GoN, two nautical miles off the coastline over the 75-m isobath. Sampling at LTER-MC has been conducted since 1984, except for a major interruption from 1991 to 1994. The sampling frequency was fortnightly until 1990 and has been weekly since 1995.

The GoN is a relatively deep and wide embayment (average depth = 170 m, area = ~870 km^2) that is relatively open to the offshore Tyrrhenian Sea waters. Land runoff from a very densely populated region influences the water typologies in the GoN; yet, in contrast to other coastal sites, riverine inputs are limited and intermittent, and salinity rarely goes below 37.5 (Ribera d'Alcalà *et al.* 2004; Iermano *et al.* 2012, 2013). The close proximity of oligotrophic offshore waters to the coastline results in the co-existence of two subsystems: a relatively eutrophic coastal zone and an oligotrophic area similar to the offshore Tyrrhenian waters. The position and width of the boundary between the two subsystems are variable over the seasons and the exchange between the subsystems at times can be enhanced by local circulation. Noticeably, in the coastal GoN, summer is not a period of low phytoplankton biomass because of nutrients coming from land. By contrast with the predictions from Margalef's Mandala (Wyatt 2012), the community is dominated by diatoms despite stratification (Zingone *et al.* 1990).

Data collection

Conductivity, temperature, and fluorescence profiles were obtained with a SBE911 mounted on a Rosette sampler equipped with Niskin bottles (12 l). Chlorophyll *a* (chl *a*) concentrations was determined at 0.5, 2-, 5-, 10-, 20-, 40- and 60-m depths, whereas salinity and nutrient concentrations were determined at 0.5, 2-, 5-, 10-, 20-, 30-, 40-, 50-, 60- and 70-m depths. Ammonium (NH_4), nitrate (NO_3), nitrite ($N0_2$), phosphate (PO_4) and silicate (SiO_4) concentrations were determined with a TECHNICON II autoanalyzer up to 2005 and, starting from 2006, using a FlowSys Systea Autoanalyzer, according to Hansen & Grasshoff (1983), modified as described in Ribera d'Alcalà *et al.* (2004). Chl *a* s was determined with a spectrofluorometer (Holm-Hansen *et al.* 1965;

Neveux & Panouse 1987). Phytoplankton and microzooplankton samples were collected from the 0.5-m Niskin bottle and fixed with neutralized formaldehyde (0.8–1.6% final concentration) and acid Lugol's iodine (2% final concentration), respectively. Mesozooplankton samples were collected from 50-m depth to the surface using a Nansen net (113-cm mouth diameter, 200-μm mesh size) and fixed with formaldehyde (2–4% final concentration). Phytoplankton, micro- and mesozooplankton were counted according to standard procedures as reported by Ribera d'Alcalà *et al.* (2004).

Data analysis

We analysed the physical and ecological variability at LTER-MC at a weekly time scale from the end of June to the end of August for 8 years (2002–2009). Salinity and chl *a* values in the 0–2-m layer were used to characterize the superficial water-masses as coastal 'green' or offshore 'blue'. Green and blue phases were identified in each summer season and the plankton community associated with each phase was characterized. Within each of the very large phyto-, microzoo- and mesozooplankton data sets, taxa were kept as such or assembled in homogeneous groups according to different criteria (Table 1). The most abundant species were kept separated. Less abundant taxa were aggregated based on taxonomy (*e.g.* congeneric species). Less common species were aggregated according to size (*e.g.* dinoflagellates and ciliates smaller or larger than 15 μm) or trophic level (*e.g.* carnivorous mesozooplankton, mixotrophic ciliates), according to current knowledge. This aggregation allowed zero values to be limited in the time-series data sets. After grouping, the whole plankton community was represented by 32 elements (Table 1).

Cluster, correlation and principal component analyses were carried out with the open-source software PAST (http://palaeo-electronica.org/2001_1/past/issue1_01.htm). Hierarchical clustering for environmental and planktonic community data were conducted using the unweighted pair group method with arithmetic mean (UPMGA) algorithm and according to Euclidean distance and Bray–Curtis similarity metrics, respectively (Legendre & Legendre 2012). Spearman correlations amongst plankton community elements (Table 1) were carried out for green and blue phases (see Results) including a minimum number of five samples, *i.e.* across the longer time periods that had a coherent match between the environmental and planktonic community data clustering.

All positive and negative correlations with $r > 0.7$ or < -0.7 were considered. The largest fraction of P-values (relating to almost 400 correlations) was within the 0.05 threshold, a lower number (almost 200) was within the 0.10 threshold, a limited fraction (almost 60) exceeded 0.10. A histogram showing the frequency of P-values from all correlations considered in the present study is shown in Supporting Information Fig. S1. A non-stringent statistical limit was imposed upon our analyses because the high P-values of some correlations with high coefficients may have arisen because of the limited number of samples analysed (number of samples in continuous time-series ranging from five to nine). In order to visualize in a single elaboration all of the possible links between community elements, correlations detected in green (light and dark green) and blue (light and dark blue) periods in the different years were compiled into distinct matrices, namely: green positive, green negative, blue positive and blue negative. Correlation networks were built and analysed with the open-source software yED 3.11.1 (yWorks GmbH, http://www.yworks.com). Network connectance was estimated as the number of links/node^2 (Beckerman *et al.* 2006). The relative centrality of network nodes was estimated in the frame of the yED software.

To corroborate patterns detected with these first exploratory analyses, further networks were built including only those links detected in at least two summer seasons and having ecological significance. The structure of plankton communities during the blue and green phases was reconstructed based on correlation networks amongst taxa. The ecological significance of correlations amongst taxa was assessed based on available knowledge from previous studies, as described in the Results and Discussion section. Special emphasis was given to potential trophic links at different levels of the trophic-web, such as amongst protists, between the latter and zooplankton, and between carnivores and non-carnivorous mesozooplankton.

Results and Discussion

The main hindrance to the various approaches to community studies of marine plankton is how to cope with highly diverse microbial communities floating in unstable and non-conservative environments. Like other coastal embayments, the GoN lies at the boundary between offshore and coastal waters with different levels of productivity. To reduce the degrees of freedom, we focused our analysis on the summer season, when the upper layer (0–2 m) is permanently decoupled from the subsurface layer because of thermal stratification. This allowed us to focus on phytoplankton assemblages confined to the surface layer, and to consider the 0–50 m integrated samples for more mobile organisms, *e.g.* mesozooplankton. The physical–ecological oscillations at LTER-MC during summer –

Table 1. Codes and descriptions for plankton taxa (species or supra-specific groups) considered in the present study.

taxon code	trophic role	plankton categories	taxa, genera, species or life stages included
1	A, M, H	Small flagellates	(cell size <10 μm) mainly UIT plus *Ollicola* sp., *Pyramimonas* spp.
2	A	*Chaetoceros*	*Chaetoceros socialis*, *Chaetoceros simplex*, *Chaetoceros throndsenii*, *Chaetoceros tenuissimus*, *Chaetoceros* spp.
3	A	*Leptocylindrus*	*Leptocylindrus danicus, Leptocylindrus aporus*, *Leptocylindrus* spp.
4	A	*Skeletonema*	*Skeletonema pseudocostatum*, *Skeletonema menzelii*
5	A	Small diatoms	(cell size <10 μm) *Bacteriastrum* sp., *Cyclotella* spp., *Minidiscu* ssp., *Minutocellus* sp., *Thalassiosira* spp., UIT
6	A	Pennate diatoms	(cell size >10 μm) *Thalassionema* spp., UIT
7	A	*Pseudo-nitzschia*	*Pseudo-nitzschia delicatissima*, *Pseudo-nitzschia galaxiae*, *Pseudo-nitzschia pseudodelicatissima*, *Pseudo-nitzschia* spp.
8	H	Large dinoflagellates	(cell size >15 μm) mainly UIT (both thecate and naked dinoflagellates) plus *Dinophysis* sp., *Gymnodinium* spp., *Gyrodinium* spp., *Lessardia* sp., *Oxyphysis* sp., *Oxytoxum* spp., *Palaeophalacroma* sp., *Pronoctiluca* sp., *Pyrocystis* sp., *Protoperidinium* spp., *Torodinium* sp.
9	M, H	Small dinoflagellates	(cell size <15 μm) UIT (both thecate and naked dinoflagellates)
10	A	Centric diatoms	(cell size >10 μm) *Cerataulina* sp., *Dactyliosolen* spp., *Eucampia* sp., *Guinardia* sp., *Hemiaulus* sp., *Lauderia* sp., *Lioloma* spp., *Lithodesmium* sp., *Odontella* sp., *Proboscia* sp., *Rhizosolenia* spp., UIT
11	A	Coccolithophores	Mainly UIT plus *Acanthoica* sp., *Calciopappus* sp., *Calciosolenia* sp., *Calyptrosphaera* spp., *Ceratolithus* sp., *Helicosphaera* sp., *Holococcolithophora* sp., *Homozygosphaera* sp., *Emiliania* sp.
12	A, M	Rare flagellates and dinoflagellates	(cell size >15 μm) UIT plus rare autotrophic flagellates and dinoflagellates (*e.g. Alexandrium* spp., *Ceratium* spp., *Gonyaulax* spp., *Karenia* spp., *Prorocentrum* spp., *Scrippsiella* spp.
13	H	Nanociliates	(cell size <25 μm) UIT
14	H	Heterotrophic ciliates	(cell size >25 μm) *Strombidium* spp., *Strobilidium* sp. (Ciliata)
15	M	Mixotrophic ciliates	*Laboea strobila*, *Tontonia* sp. (Ciliata)
16	H	Prostomatids	UIT (Ciliata Prostomatida)
17	A	*Mesodinium rubrum*	*Mesodinium rubrum* (Ciliata)
18	H	Tintinnids	*Eutintinnus* spp., *Helicostomella* sp., *Nolaclusila* sp., *Proplectella* sp., *Salpingella* spp., *Tintinnopsis* spp., *Undella* spp., UIT (Ciliata Tintinnida)
19	H, S	*Penilia avirostris*	*Penilia avirostris* (Cladocera)
20	H, S	Calanoid juveniles	Mainly juvenile stages of *Clausocalanus* spp. and *Paracalanus parvus*
21	H, S	Cladocerans	*Evadne* spp., *Pseudevadne tergestina* (Cladocera)
22	H, S	*Paracalanus parvus*	Adult stages of *Paracalanus parvus* (Copepoda Calanoida)
23	H. S	Appendicularians	UIT (Tunicata Appendicularia)
24	H, S	*Acartia clausi*	Adult stages of *Acartia clausi* (Copepoda Calanoida)
25	H, S	*Temora stylifera*	Adult stages of *Temora stylifera* (Copepoda Calanoida)
26	H, S	*Centropages typicus*	Adult stages of *Centropages typicus* (Copepoda Calanoida)
27	H, O	*Oithona* spp.	Different life stages of *Oithona atlantica*, *O. decipiens*, *O. longispina*, *O. nana*, *O. setigera*, *O. similis* (Copepoda Cyclopoida)
28	H, O	Meroplankton	Larval stages of Anellida Polychaeta, Crustacea Maxillopoda, Echinodermata, Mollusca
29	H, S	Thaliaceans	Salps and doliolids (Tunicata Thaliacea)
30	H, S	Other calanoids	Different life stages of rare calanoids (Copepoda Calanoida) (*e.g. Acartia* spp., *Calocalanus* spp., *Centropages* spp., *Clausocalanus* spp., *Ctenocalanus vanus*, *Paracalanus* spp.)
31	H, S, D	Detritivores	*Corycaeus* spp., *Farranula rostrata* and Oncaeidae (Copepoda Cyclopoida) plus *Euterpina acutifrons* (Copepoda Harpacticoida)
32	H, C	Carnivores	UIT of Chaetognata, Mollusca Pteropoda, Cnidaria Siphonophora plus *Candacia* spp. and *Pleuromamma* spp. (Copepoda Calanoida)

A = autotrophic; C = carnivorous; D = detritivorous (feeding on detritus, *e.g.* faecal pellets and aggregates); H = heterotrophic; M = mixotrophic; O = omnivorous (feeding on both microbes and animals); S = suspension feeders (feeding on microbes, both autotrophic and heterotrophic); UIT = unidentified taxa.
From 1 to 18 are protists, from 19 to 32 are metazoans.

herein, the end of June–end of August – were studied for 8 years (2002–2009) at a weekly time scale. Community composition during the green and blue phases, defined as described above, were tagged and characterized.

Environmental variability

In the summer seasons under investigation, vertical profiles of salinity and chl *a* showed the largest variations

within the upper 10-m layer and rather similar values below 10 m, with a steep halocline often present between 2 and 10 m, and peak chl *a* values in the 0.5–2-m layer (not shown). Salinity and chl *a* also underwent the largest oscillations over time in the upper 2-m layer and showed a significant negative correlation ($r = -0.63$; $n = 87$; $P < 0.001$). The increment of chl *a* values at lower salinity levels was indicative of the presence of waters directly affected by coastal runoff and, therefore, richer in plankton. Thus, the relative shifts of surface salinity and chl *a* represented a good proxy for waters showing the influence of more coastal-green, or more offshore-blue, conditions at LTER-MC. A more detailed discrimination of water types based on chl *a* and salinity values was performed through a cluster analysis. Two main groups were detected, both including two subgroups. The four groups, or phases, namely, light and dark green and light and dark blue phases, showed different average values and combinations of chl *a* and salinity, as shown in Fig. 1a (see also Fig. S2).

Under typical summer conditions, when stable high pressure fields prevail in the Mediterranean Sea, breezes alternating in direction on a diurnal scale are the dominant local forcing in the GoN (Uttieri *et al.* 2011). The surface current field makes a complete clockwise (anticyclonic) rotation over 24 h, with relatively strong coastward-orientated currents under the action of sea breeze, in contrast to relatively weaker offshore-moving waters in the presence of a land breeze. Under these dynamic conditions, the exchange between the coastal area and the open Tyrrhenian Sea is hampered (Uttieri *et al.* 2011). Accordingly, the surface waters in the GoN during summer are characterized by oscillating dynamics, with (i) alternation between phases reflecting the coastal influence to a different extent and (ii) green phases lasting between 2 and 7 weeks.

Ecological variability

Plankton taxa occurring in the summer communities of the years 2002–2009 and their abundance are listed in Table 1 and Supporting Information Table S1, respectively. A cluster analysis based on Bray–Curtis similarity amongst all plankton samples identified two main clusters (Fig. 1b). Further subgroups were present, especially in the left-hand cluster (Fig. 1b). A large number of plankton samples in the latter group corresponded to the green (both light and dark green) clusters based on chl *a* and salinity, whereas the majority of samples in the right-hand group corresponded to the blue chl *a* and salinity clusters (Fig. 1a and b). A high number of plankton samples corresponding to light blue conditions were scattered across the plankton clusters. Nonetheless, green and blue plankton samples were mutually segregated. Such congruence between the environmental and planktonic data was evident in six out of 8 years, but not in 2003 and 2008 (Figs 1c–h, S3 and S4).

The close match between environmental and planktonic clustering and the trophic regime was also reflected by significant differences in the abundance and relative percentage of the dominant taxa between the green and blue phases (Table 2, Fig. 2a–e). The most evident result was the relative shift between small-sized phytoplankton, dominating the blue phase, and relatively large diatoms, prevailing in the green phase (Fig. 2a). From the blue to the green phase, the average density of small flagellates (code 1 in Table 2) only showed a threefold increase, whereas the most abundant diatom genera *Chaetoceros* and *Leptocylindrus* (codes 2 and 3) underwent 15- and eightfold increases in cell density, respectively, and *Skeletonema* and *Pseudo-nitzschia* (codes 4 and 7) increased sixfold (Table 2). As for ciliates, the obligate heterotrophic prostomatids and tintinnids (codes 16 and 18) underwent eight- and sixfold increases in the green phase, respectively (Table 2). Against these significant shifts in protist communities, meso zooplankton showed less marked variations between the green and the blue phases (Table 2, Fig. 2d,e).

Mesozooplankton resilience in the GoN has previously been highlighted at different spatial (Ianora *et al.* 1985) and temporal scales (Mazzocchi *et al.* 2012) against clear gradients and strong spatial heterogeneity for phytoplankton (Zingone *et al.* 1990) and wide variability in environmental parameters and autotrophic biomass across seasons and decades (Mazzocchi *et al.* 2012). This resilience probably emerges from the combined effects of longer life cycles of zooplanktonic organisms and their adaptive behavioural responses, including the capacity to graze on a wide array of food items. Nonetheless, slight changes were observed in the zooplankton community in the present study. The abundance of the copepod *Acartia clausi* (code 24) was three times higher in green than in blue phases, whereas the abundance of the copepod *Centropages typicus* (code 26), the cladocerans (*Evadne* and *Pseudevadne*, code 21) and thaliaceans (salps and doliolids, code 29) underwent a twofold increase (Table 2). Overall, the same protist and mesozooplankton species and groups were present in the green and blue phases' communities, but their abundance and relative contribution significantly changed (Fig. 2), suggesting plankton community re-shaping in the swing between the two phases.

The small flagellates–diatoms alternation is typical of those Mediterranean regions characterized by intermittent nutrient enrichment and mesoscale oceanographic structures, such as fronts (Siokou-Frangou *et al.* 2010). In these systems, remarkable biological heterogeneity is also

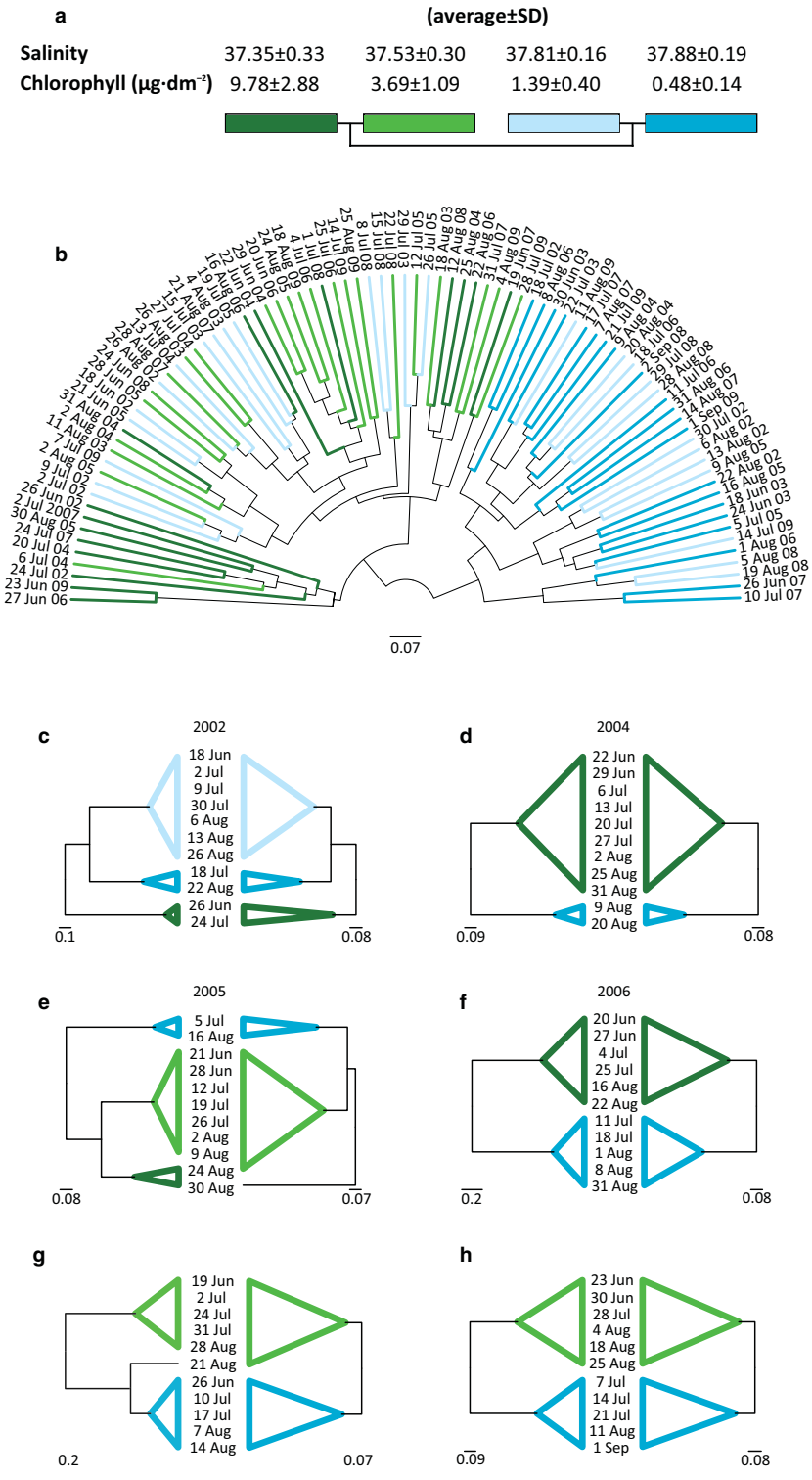

Fig. 1. (a): Schematic dendrogram of combinations of salinity and chlorophyll *a* values measured at st. LTER-MC on different dates in the period under study (summers 2002–2009), based on a UPMGA analysis (Euclidean distance). The complete dendrogram is presented in Supporting Information (Fig. S2). Salinity (average values over the 0–2 m layer) and chl *a* (averages of integrated values over the 0–2 m layer) are indicated for each of the four clusters. Clusters colors used here and in the following figures indicate different domains (phases) alternating over the season. (b): Cluster analysis of planktonic communities at st. LTER-MC. Dendrogram (UPMGA, Bray–Curtis similarity) based on abundance values of species and supra-specific groups (Table 1) on different dates in the period under study. The sampling dates are reported at the tip of each branch. Branch colors are based on the scale of Fig. 1a, each color representing the corresponding range of salinity and chl *a* values on each date. (c–h): Coupled environmental and planktonic community clustering for single years. Left-hand dendrograms: cluster analyses for physical-chemical parameters (salinity and chl *a*) based on UPMGA and Euclidean distance. Right-hand tree: cluster analyses for taxa abundance based on UPMGA and Bray-Curtis similarity. Each color corresponds to the coupled salinity-chlorophyll conditions indicated in Fig. 1a. Results for 2003 and 2008, which did not show a clear correspondence between the environmental and planktonic community clustering, are shown in Figs S3 and S4.

reported, owing to the small-scale fragmentation of the phytoplankton community over time and space. Due to its geomorphology and environmental features, the GoN probably represents an ecotone, *i.e.* a narrow transitional area between different communities, and in this respect it is analogous to other <100-km-wide transition zones in coastal oceans. In these areas, diatom blooms may be inoculated by persistent opportunistic species (Wyatt

Table 2. Average abundances of taxa in the green and blue phases.

taxon code	average abundance: green phase	average abundance: blue phase	unit of measurement	green/blue ratio
1	12,853	4436	10^3 cells·l^{-1}	2.9
2	8636	553	10^3 cells·l^{-1}	15.6
3	3913	489	10^3 cells·l^{-1}	8.0
4	2555	394	10^3 cells·l^{-1}	6.5
5	1797	284	10^3 cells·l^{-1}	6.3
6	575	77	10^3 cells·l^{-1}	7.4
7	1236	182	10^3 cells·l^{-1}	6.8
8	49	10	10^3 cells·l^{-1}	4.9
9	252	90	10^3 cells·l^{-1}	2.8
10	230	68	10^3 cells·l^{-1}	3.4
11	240	96	10^3 cells·l^{-11}	2.5
12	299	115	10^3 cells·l^{-1}	2.6
13	6266	2352	1 cell·l^{-1}	2.7
14	2367	1001	1 cell·l^{-1}	2.4
15	2063	712	1 cell·l^{-1}	2.9
16	1600	191	1 cell·l^{-1}	8.4
17	2955	1062	1 cell·l^{-1}	2.8
18	1866	311	1 cell·l^{-1}	6.0
19	1207	1151	10^{-3} ind.·l^{-1}	1.0
20	457	314	10^{-3} ind.·l^{-1}	1.5
21	773	397	10^{-3} ind.·l^{-1}	1.9
22	289	274	10^{-3} ind.· l^{-1}	1.1
23	265	241	10^{-3} ind.· l^{-1}	1.1
24	155	53	10^{-3} ind.· l^{-1}	2.9
25	73	77	10^{-3} ind.· l^{-1}	0.9
26	76	37	10^{-3} ind.· l^{-1}	2.0
27	63	68	10^{-3} ind.· l^{-1}	0.9
28	57	42	10^{-3} ind.· l^{-1}	1.3
29	40	21	10^{-3} ind.· l^{-1}	1.9
30	76	86	10^{-3} ind.· l^{-1}	0.9
31	48	68	10^{-3} ind.· l^{-1}	0.7
32	31	29	10^{-3} ind.· l^{-1}	1.1

ind. = individuals.

2012) and/or sustained by the germination of resting spores from coastal sediments (Montresor *et al.* 2013). The higher proportion of diatoms *versus* small flagellates under high nutrient conditions reflects the significantly higher growth potential of these opportunistic, r-strategist organisms (Zingone *et al.* 1990; Wyatt 2012).

In the GoN, in the green and blue phases, respectively, dissolved inorganic nitrogen (DIN, *i.e.* NH_4, NO_3 and NO_2) was 0.97 ± 0.38 *versus* 0.75 ± 0.34 μmol·dm^{-2}, phosphate (PO_4) was 0.17 ± 0.07 *versus* 0.10 ± 0.06 μmol·dm^{-2} and silicate (SiO_4) was 1.41 ± 0.75 *versus* 1.76 ± 0.39 μmol·dm^{-2} (for each parameter, median values ± median absolute deviation of the integrated 0–2-m values). However, the frequency of DIN values exceeding 1 μmol·dm^{-2} in the green and blue waters were 90% and 50%, respectively. The percentage of ammonium (NH_4) within DIN was comparable in the two phases (*i.e.* 61% and 59%, in green and blue phases, respectively). The slight differences in nutrient concentrations between the green and blue phases suggests that the two water-masses described herein represent two different types of coastal waters and they cannot be associated with either eutrophic or oligotrophic waters in absolute terms. Therefore, in the GoN, salinity and chlorophyll values represent a more powerful indicator of the green–blue state than nutrient concentrations. Nonetheless, we speculate that the relatively higher amount of inorganic nitrogen and phosphate in green waters may have enabled diatoms to overgrow small flagellates and that the corresponding lower amount of silicate in the green phase as compared with the blue one may account for the higher activity of (and consequently uptake of this nutrient by) these algae.

The changes in micro- and mesozooplankton abundances detected between the green and the blue phases are discussed in the following sections in relation to the potentially different trophic processes associated with the two phases identified above.

Ecological networks

The highest number of positive links based on Spearman correlation coefficients was found amongst mesozooplankton taxa in the green phase and amongst unicellular taxa in the blue phase; negative Spearman correlation coefficients were instead common between mesozooplancton and unicellular taxa both phases (Fig. 3a). Positive links between two species could be suggestive of several distinct mechanisms, such as: (i) functional homogeneity, in the case of species that are not inter-related but that are taking advantage of similar environmental conditions under non-limiting resource availability; (ii) 'mutualistic 'relationships, *i.e.* for species reciprocally benefiting from each other, or (iii) 'balanced' trophic relationships, in which the predator/grazer increases because of an increase of the prey, but does not eliminate the prey. By contrast, negative links could be suggestive of (iv) functional heterogeneity; (v) 'allelopathic' relationships or competition, or (vi) predator–prey relationships – in which the former increases at the expense of the latter. Based on the link distributions shown in Fig. 3a, mechanisms of types i–iii are likely to be acting within the mesozooplankton in the green phase and within unicellular organisms in the blue one. This suggests a differential extension of the 'classical' food-web and 'microbial loop' in the green and blue phases. By contrast, mechanism vi is likely to be acting between mesozooplankton and protists, suggesting a high impact of the grazers on microbes in both the green and blue phases. Further interpretations are presented in the next section, where specific and ecologically relevant

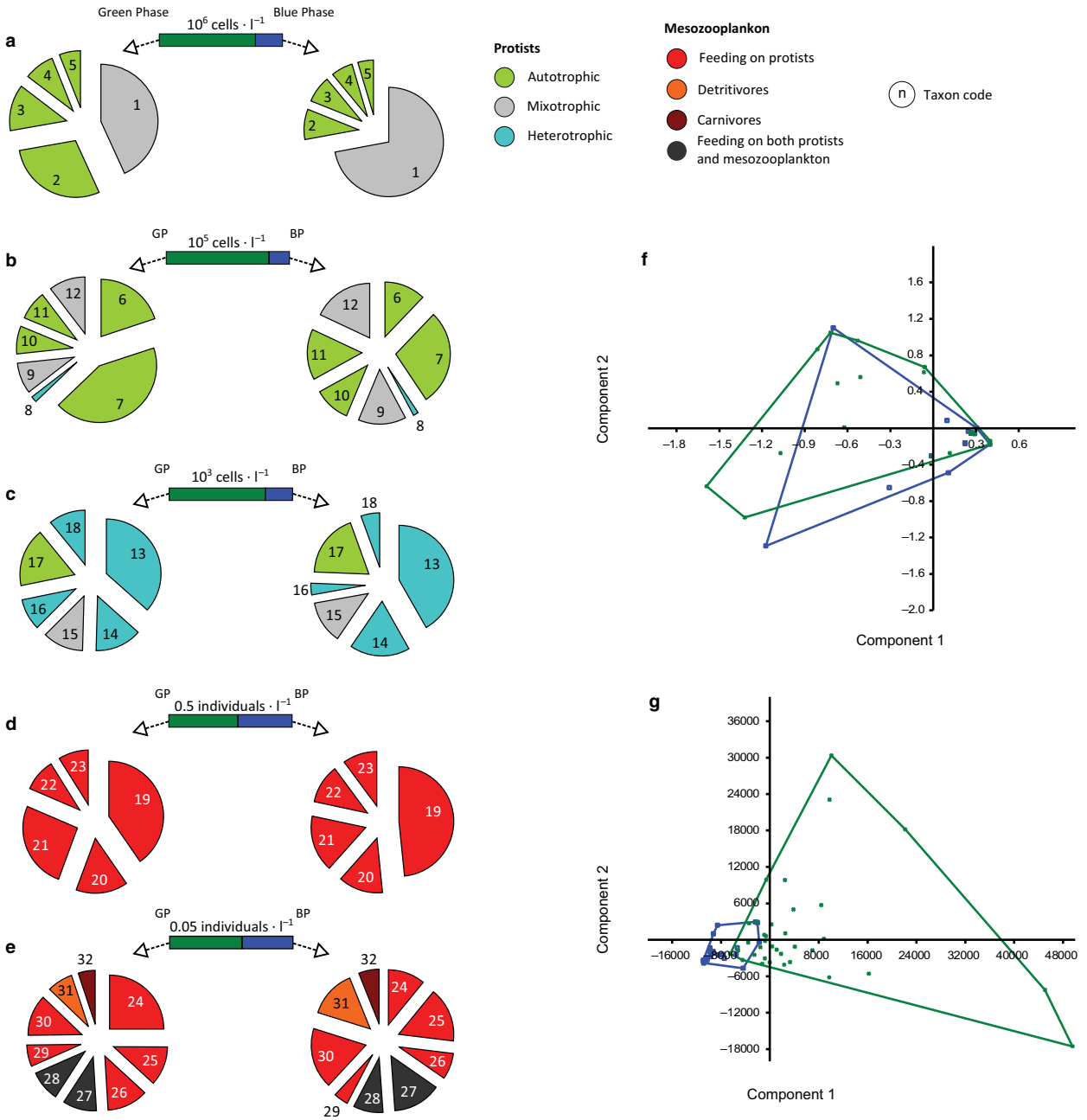

Fig. 2. (a–e): Relative quantitative contribution of plankton categories (codes as in Table 1) to communities detected in green and blue phases (GP and BP, respectively). Couple of diagrams represent each a set of species with comparable mean abundance values, the order of magnitude of which is reported above the colored bars. Green-blue bars at the top of each couple of diagrams indicate the proportional abundance (Ab) of that set of species in the green phase – calculated as $[Ab_{GP}/(Ab_{GP} + Ab_{BP})]$ – and in the blue phase – calculated as $[(Ab_{BP}/(Ab_{GP} + Ab_{BP})]$. The pie charts represent changes in abundance for (a): the five most abundant phytoplankton categories; (b): other phytoplankton taxa; (c): microzooplankton groups; (d): the five most abundant mesozooplankton species; and (e): other mesozooplankton categories. (f–g): Plots from PCA (Principal Component Analysis) of planktonic community samples in green and blue phases. (f): PCA based on taxa presence/absence. (g): PCA based on taxa abundances.

links, based on current scientific knowledge on the organisms involved, are used to build trophic-webs in the green and blue phases.

Regardless of their ecological significance, all significant links were first used to build networks in order to analyse community organization in the green and blue phases

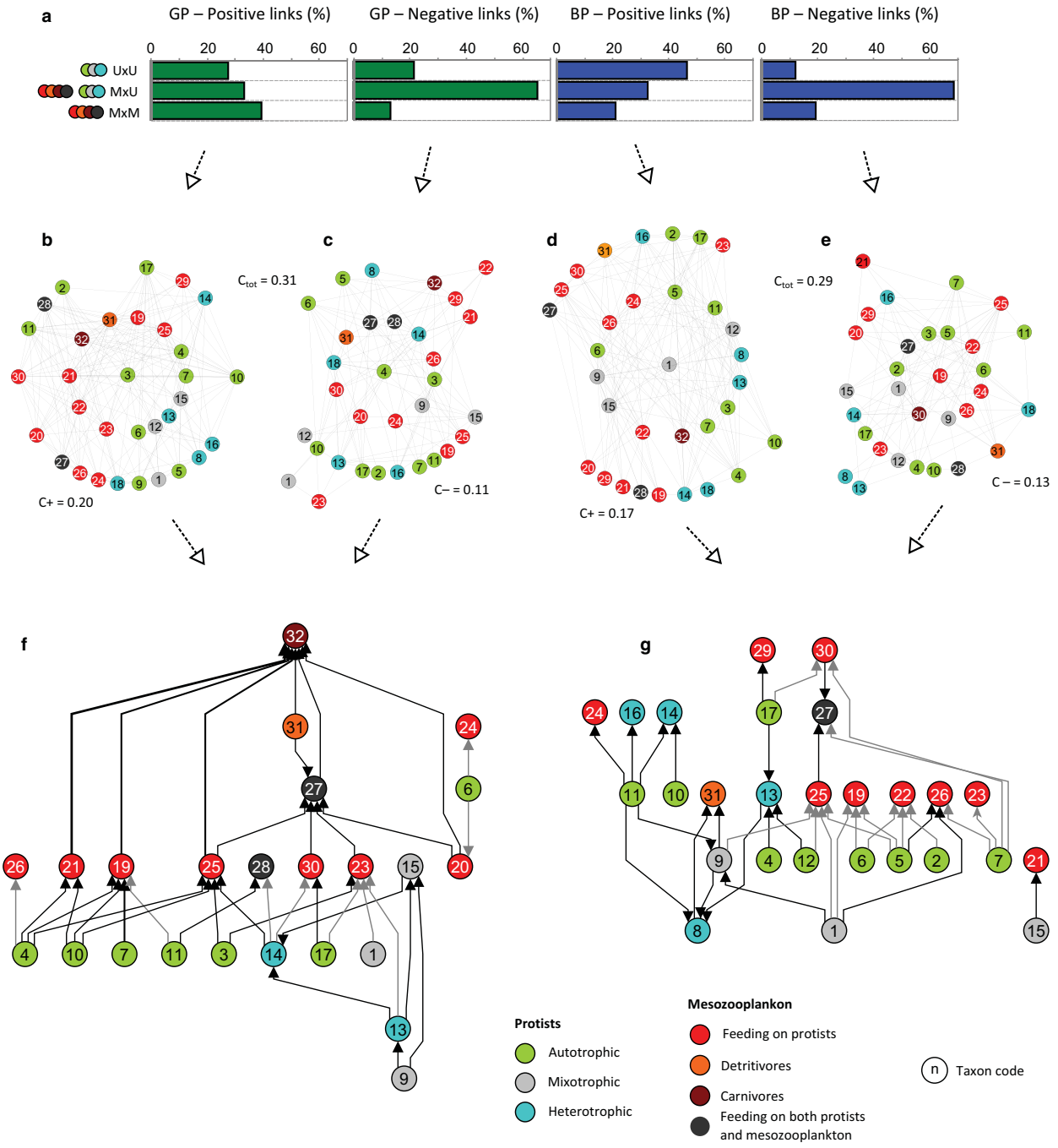

Fig. 3. (a): From the top to the bottom, percentage of links among unicellular organisms (U), among mesozooplankters (M), and between unicellular organisms and mesozooplankton (U × M). From the left to the right, histograms for positive and negative links are shown for green and blue waters. (b–e): Radial networks built from the matrices of positive and negative correlations (links). Nodes and edges in the networks are taxa (see Table 1) and links, respectively. Node color refers to trophic level (see legend) and numbers within nodes are the taxon codes. C stands for connectivity (number of links/node^2): C_{tot} is the overall value; C+ and C− is the value calculated on either positive or negative links' networks, respectively. The radial layout highlights the centrality of some nodes (*i.e.* their participation to interconnected paths). (f–g): Simplified networks including only ecological significant links that occurred at least twice, in different years. Node colors and number as in Fig. 3b–e. Black and grey edges signify positive and negative links, respectively. Edge weight is related to the strength of the link, while the arrowhead indicates the direction of the flux of matter based on biological plausibility. The networks are drawn according to a hierarchical layout, from the bottom to the top. The nodes participating to the most interconnected paths are at the bottom of the web, the others at the top. GP and BP stand for green and blue phases, respectively.

based on topological properties. To reduce complexity, positive and negative links were reported for each phase in distinct networks (Fig. 3b–e). Amongst network properties, we focused on stability and centrality.

Stability is related to network complexity, which is represented by the parameter named 'connectance', calculated as the number of links over the total possible number of links (Beckerman *et al.* 2006). In this study, connectance was very high in both green and blue phases (*i.e.* 0.31 and 0.29, respectively), challenging the view that aquatic food-webs are less stable than terrestrial ones. Connectance values spanning 0.03–0.32 were reported for different communities of multicellular organisms, the lowest values being found in aquatic food-webs in stream ecosystems, which are probably the most unstable ones (Dunne *et al.* 2004). Very low connectance values were also reported for aquatic food-webs in the Arctic (0.05) and Antarctic (0.01) ecosystems (de Santana *et al.* 2013), probably because of stronger environmental oscillations, mainly in the light regime. However, the microbial and photosynthetic components were not included in the analyses by de Santana *et al.* (2013). Hence, the overall stability may have been underestimated and should be further investigated to fully assess the vulnerability of polar systems to climate variations. Interestingly, in the green phase in the GoN, connectance in the positive subnetwork (Fig. 3b) was about double that in the negative one (Fig. 3c). This fact suggests that positive (synergistic) processes may be more effective than negative (competitive) ones in stabilizing the green phase community. By contrast, connectance values of positive and negative subnetworks in the blue phases were not very different from each other. Taken together, these observations suggest that, despite the assumed large variability at small scales of coastal environments, robust biological interactions do take place in communities living therein. We can thus speculate that co-evolution processes and life strategies favouring persistence may have been strong drivers in these environments.

Another relevant piece of information provided by networks is the quantitative assessment of centrality, *i.e.* how links are distributed across the system and whether and to what extent the organization of the latter can rely on a single or a few species. For instance, centrality assessment can be used to identify potentially keystone species in food-webs/ecosystems (Jordán 2009). Based on the radial layout used in Fig. 3b–e, the node present in the most inter-connected paths in the diagram was chosen as the central node, according to an algorithm that measures weighted centrality. Furthermore, nodes were assigned to circles in such a way that edges either connected nodes on subsequent circles or nodes placed on the same circle. In our elaborations, each network was characterized by distinct central nodes and two crown-levels of nodes with a lower centrality (Fig. 3b–e). In positive-links networks (Fig. 3b and d), the central nodes were the diatom *Leptocylindrus* (code 3) in the green phase and a heterogeneous group composed of small (<10 μm) flagellates (code 1) in the blue phase. In negative-links networks (Fig. 3c and e), the diatom genus *Skeletonema* (code 4) and the cladoceran *Penilia avirostris* (code 19) were central in the green and blue phases, respectively. Besides differences in central nodes, distinct motifs characterized the four radial networks. For instance, considering positive links, in the green-phase network the first-crown level showed many links between *Leptocylindrus* in the central node and mesozooplankton. By contrast, in the blue-phase network, a comparable number of links was detected between flagellates in the central node and mesozooplankton and mixo- and heterotrophic protists in the first-crown level.

Correlation networks have at times been used as an exploratory approach to characterize plankton communities in terms of links amongst their components and to sort for possible biological mechanisms at the base of their organization (Steele *et al.* 2011). Yet, connectance and centrality have never been estimated for planktonic communities and no specific comparison with our data was possible. Our analyses showed that the overall community organizations according to the possible links detected amongst the same taxa were significantly different between the green and blue phases (Fig. 3b–e). These differences were only partially explained in terms of the changes in the abundances of taxa in the green and blue phases. In fact, a large fraction of links involved community elements showing comparable densities in the two phases.

The green plankton food-web

The ecological networks shown in Fig. 3b–e report all the links, *i.e.* co-variations, detected amongst the different nodes in the plankton community. These links were statistically relevant – the majority of them significantly so (Fig. S1) – but not necessarily ecologically relevant. The latter property was explored by producing sub-networks including only the links occurring at least twice and having trophic significance. Links between species or species-groups belonging to the same trophic/functional level were not considered because of the still scarce knowledge on synergistic or competitive interactions amongst plankton available to date. Two hypothetical trophic-webs were reconstructed based on Spearman correlations for the green and blue phases, respectively (Fig. 3f and g). The networks included only potential trophic links, both positive and negative, that were detected at least in two of the 8 years under study. Positive and negative links were

interpreted as mechanisms of types 'iii' and 'vi', namely, 'balanced' trophic and predator–prey relationships, respectively, as indicated in the previous section. Networks were arranged according to a hierarchical criterion, with the nodes participating in the most inter-connected paths at the bottom.

In the green trophic-network, positive links largely exceeded negative ones and the elements at the higher trophic levels were mainly generalists. For instance, the suspension-feeding copepod *Temora stylifera* (code 25, Table 1, Fig. 3f) was one of the most linked zooplankters, being positively correlated with three categories of bloom-forming diatoms (namely, codes 3, 4, 10) as well as with large-celled heterotrophic ciliates (code 14). In the GoN, *T. stylifera* was concentrated within the upper 30-m layer during summer (Di Capua & Mazzocchi 2004). Interestingly, the feeding rate of *T. stylifera* has been reported to accelerate in the presence of polyunsaturated aldehydes released by diatoms (Kâ *et al.* 2013). Moreover, this copepod can perform small-scale diel vertical migration and increase nocturnal feeding activity in the chlorophyll-rich surface waters in the Rhone river plume area (Northwestern Mediterranean; Pagano *et al.* 1993). A rapid response of *T. stylifera* to food availability through vertical migrations is likely to occur in the GoN population and could determine the trophic links with diatoms observed in green waters. Thanks to this behavioural plasticity, *T. stylifera* may compete effectively with other suspension feeders typical of the summer in the GoN, such as cladocerans, despite a much lower abundance than the latter.

Unlike copepods, cladocerans reproduce by parthenogenesis and can rapidly increase their population size over a short time (Atienza *et al.* 2008). Thus, high reproduction rates rather than migration are most likely to be the cause of short-term changes in population size in this case. Amongst cladocerans, *Penilia avirostris* (code 19) showed multiple links with phytoplankton, *i.e.* three diatom taxa (*Skeletonema, Pseudo-nitzschia* and centric diatoms >10 μm, codes 4, 7, 10; positive correlations) and coccolithophores (code 11, negative correlation), in the green phase (Fig. 3f). Under experimental conditions, the suspension-feeding *P. avirostris* showed relatively lower grazing pressure on colonial diatoms than on single-celled diatoms, dinoflagellates and ciliates (Atienza *et al.* 2006). As diatoms have shown a limited tendency to form colonies during summer in the GoN (Zingone *et al.* 1990), they might indeed represent suitable food items for *P. avirostris*. Interestingly, a relatively stronger positive link, shown in three out of 5 years, was detected between *P. avirostris* and *Pseudo-nitzschia*, a genus including several potentially toxic species in the GoN (Zingone *et al.* 2006). The rapid increase in abundance of cladocerans through parthenogenesis requires a contemporary increase in grazing rate by females and juveniles. *Pseudo-nitzschia* species, reported to be either selected against or not-preferred by copepods in a multi-diet experiment (Olson *et al.* 2006), could be an available prey to cladocerans, with no need to compete with copepods. Accordingly, the robust and exclusive link of *Pseudo-nitzschia* spp. to *P. avirostris* in our study corroborates the idea that food selectivity in mesozooplankton could be a way to pursue niche partitioning and reduce competition amongst suspension feeders (Katechakis 2004).

At the same trophic level, the relatively abundant and effective filter-feeders appendicularians (code 23) were related positively with the bloom-forming diatom *Leptocylindrus* (code 3, Fig. 3f) and negatively with small unicellular organisms, such as <10-μm-flagellates (code 1), and ciliates (codes 13 and 17). Evidence of multifaceted interactions between these tunicates and ciliates has been obtained previously (Lombard *et al.* 2010). Our observations suggest an active role of appendicularians in the microbial loop as a main mortality factor for slow-growing or non-photosynthetic microbes. Amongst microbial-loop-like interactions, a pathway involving four categories of mixo-and heterotrophic unicellular organisms (codes 9, 13, 14 and 15) was worthy of notice. Small dinoflagellates <15 μm (code 9) were directly linked to larger (≤25 μm) mixo- and heterotrophic ciliates (codes 13 and 15), whereas the latter were linked to even larger (above 25 μm) heterotrophic ciliates (code 14). This result is in line with earlier empirical evidence of intra-guild predation amongst microzooplankton in the GoN at LTER-MC (Franzè & Modigh 2013). Yet, microbial-loop-like links in green waters were far less common than those associated with a classical trophic-web, whereas no relevant links were detected between phytoplankton and microzooplankton.

At a higher trophic level in the green food-web, the ambush-feeding copepods *Oithona* spp. (code 27) were positively correlated with four mesozooplankters– namely, three categories of copepods and appendicularians (codes 20, 23, 25 and 30). *Oithona* spp. are omnivorous cyclopoid copepods feeding on both living (microbes and other animals) and inert (detritus and faecal pellets) organic matter (Gonzalez & Smetacek 1994) by means of raptorial behaviour towards remotely detected preys (Svensen & Kiørboe 2000). In the summertime green phase in GoN, the increasing in abundance of *Oithona* spp. could be caused by the increased fluxes of some of their food, e.g. both faecal pellets of *T. stylifera* and detritus from discharged appendicularian houses. Finally, at the top of the web, carnivorous zooplankters (code 32) showed six positive links with herbivorous or omnivorous zooplankton. In particular, a very strong link, present in

four out of the 5 years, was detected amongst carnivores and the cladocerans, including the genera *Evadne* and *Pseudevadne* (code 21). Nonetheless cladocerans were still abundant over the summer, as they probably are able to counteract the effect of predatory pressures by means of parthenogenetic reproduction.

The blue plankton food-web

In the blue trophic network, a large amount of negative links potentially associated with trophic relationships was detected (Fig. 3g), suggesting a higher impact of animal grazing on microbial populations in the blue than in green phases. This result was predictable to some extent, based on limited resources in the blue phase. Small flagellates and diatoms (codes 1 and 5) in particular appeared to be highly impacted by many grazers. Noticeably, the generalist mesozooplankters *Penilia avirostris* and *Temora stylifera* (codes 19 and 25) kept that trophic status in blue waters, establishing only negative links with unicellular organisms. By contrast, carnivores did not play a significant role in the blue-phase food-web, indicating a relatively low transfer of organic matter to higher trophic levels. Carnivore zooplankton in this study included mainly chaetognaths, which occurred with low abundance, having their annual peak later in the autumn (Mazzocchi *et al.* 2011). These secondary consumers, which prey mainly on copepods, generally have low feeding rates (*e.g.* Feigenbaum 1991; Durò & Saiz 2000), which may have been even negligible during low-biomass conditions at the sampling site, making them disappear from the food-web in the blue phases. The different strategies in the primary and secondary consumers may account for the stability in mesozooplankton composition across the green–blue swing.

Indeed, even though the composition and relative abundance of grazers were similar between the green and blue communities, different links among grazers and their potential food were detected in the two phases. Interestingly, two categories of grazers were only present in one of the two food-webs: juvenile copepods (code 20, copepodites) in the green, and thaliacean salps and doliolids (code 29) in the blue phases. The more relevant feeding activity of juvenile copepods may be related to the larger autotrophic biomass in coastal waters during the green phase, whereas the presence of thaliaceans in the blue food-web may hint at the predominance of a microbial trophic-web sustained by small-sized species. Gelatinous filter-feeders such as salps and doliolids represent important contributors to biogeochemical cycles, with the former more effective than the latter in poorly productive waters (Deibel & Paffenhofer 2009). In the putative blue food-web in the GoN these filter-feeders were linked only to the photosynthetic ciliate *Mesodinium rubrum* (code 17), but it cannot be excluded that they may feed on other ciliates and microbes in general. Thaliaceans feed over a size-window larger than that of copepods, from bacteria to other metazoans (Bone 1998). They are reported to produce large and somewhat unpredictable outbreaks along Italian coasts (Boero *et al.* 2013) and can potentially compete with mesozooplankton and impact food-webs (Atkinson *et al.* 2004). Moreover, outbreaks of the salp *Thalia democratica* have been linked to positive anomalies in temperature in the Bay of Villefranche-sur-mer (Northwestern Mediterranean; Licandro *et al.* 2006). The stable presence of thaliaceans in the GoN during the summer blue phase, emerging also at the level of trophic links, calls for further investigations on the ecological role of these organisms in this area. Changes in the oceanographic regime related to climatic oscillations might perturbate the green–blue swing described herein, *e.g.* causing the predominance of the blue over the green phases and inducing positive effects on the trophic impact of filter-feeding gelatinous plankton.

The extent of inter-connected pathways potentially associated with microbial grazing was higher in blue than in green waters. Interestingly, several potentially trophic links between mixo-heterotrophic ciliates (codes 13, 14 and 16) and photoautotrophic unicellular organisms were detected, whereas larger (code 8) and smaller (code 9) dinoflagellates were well integrated in the (mainly microbial) putative trophic-web (Fig. 3g). Many authors have already suggested that microzooplankton are major consumers of diatoms and ciliates and that dinoflagellates can represent non-univocal trophic levels, being both predator and prey within the same food-web (Calbet & Landry 2004; Sherr & Sherr 2007; Modigh & Franzè 2009; Jeong *et al.* 2010; Franzè & Modigh 2013; Schmoker *et al.* 2013). Our observations of apparently convoluted trophic cascades amongst microzooplankters corroborate the suggestion that a major part of microbial production may be consumed by microbes themselves under conditions of relatively low primary production, as already suggested for the GoN (Modigh & Franzè 2009; Franzè & Modigh 2013).

Despite the lack of observation of links between copepods and ciliates, we cannot exclude that such trophic relationships do exist in the GoN, especially in the blue phase. In fact, microzooplankters are presumably an important element in copepod diet in oligotrophic systems, where autotrophic production is low and carried out by small cells, which are rarely consumed by copepods (Paffenhöfer *et al.* 2006; Saiz & Calbet 2010). Amongst copepods, the species *Paracalanus parvus* (code 22), which is apparently adapted to low-biomass food environments with a relatively low phytoplankton/microzooplankton ratio (Paffenhöfer & Stearns 1988; Sautour & Castel 1993, 1999), was active only in the blue phase in the GoN,

giving further strength to our observations and leading to the hypothesis of a role played by this species in the community's adjustment following the green–blue swing.

Synthesis

The dissection of the internal mechanisms driving the structure of plankton communities is the key to the understanding and prediction of its influence on biogeochemical cycles over a wide range of time scales (Tett & Wilson 2000; Strom 2008; de Senerpont Domis *et al.* 2013; Behrenfeld & Boss 2014). Limited knowledge on marine plankton biology and ecology means that this task is not yet in reach. However, we believe that a robust characterization of structural patterns going beyond the paradigmatic divide between the 'classical food-web' and the 'microbial loop' is an important step in that direction. Two approaches seem particularly suited to that aim: perturbation experiments (Smetacek & Naqvi 2008) and analysis of time series (Ducklow *et al.* 2009). In this study we explored the latter using a coastal time series for which crucial biological information, *i.e.* taxonomic characterization of the community, is available. In this approach perturbations are driven by natural processes and responses are recorded while processes are occurring.

During summer, changes in the planktonic system in the GoN were clearly coupled with horizontal circulation, which may act as a perturbing force. Indeed fluxes at the interfaces of the system resulting from lateral mixing and/or external inputs (terrestrial inputs, exchange with offshore waters) alter the environmental conditions and result in two prevalent patterns, characterizing two environmental and trophic domains, the green and the blue one. This very schematic representation captures an important feature of the planktonic system at our site: in summer it appears to swing between the green and blue phases, which have different organization patterns. An important feature emerging from our analysis is that the two community phases are characterized by the same species/group composition, although the relative weights of individual taxa, and hence the community structure, markedly vary between the domains. This is not unexpected and may be attributed to the plasticity of different players in response to variations in energy and matter fluxes. It also suggests that the internal homogenization, probably resulting from lateral mixing, depends upon the exchange between the coastal and offshore Tyrrhenian waters, which host a totally different community (*e.g.* Siokou-Frangou *et al.* 2010). What is worth noting is that these changes in community structure are associated with changes in the functional links amongst the players, obviously within the assumptions of the network approach. This re-design of the functional links suggests a biological plasticity at a higher level, *i.e.* at the level pertaining to the community.

The overall picture emerging from our study highlights that in a coastal ecosystem, in spite of rather stable and predictable conditions such as those typical of summer in the GoN, different modes of organization of plankton communities can emerge even from relatively low levels of environmental variability. As stated above, the time scale of these readjustments is difficult to assess with our data. However, considering the time scales of lateral mixing of the fluxes at the boundaries of the area and the biological response to this, the community can possibly re-adjust on a time scale in the order of days, which is shorter than our sampling resolution. At the inter-annual scale, the persistence and spatial extension of a green or a blue phase may vary in relation to the wet or dry climatic regime during summer, and the same variability probably affects many other coastal Mediterranean sites. We did not focus on large perturbations and therefore cannot provide insight on the impact of extreme climatic events, such as 'heat waves', on these communities. In fact, even though physiological plasticity can help single species to overcome temperature perturbations, the plasticity of the overall community could lead to substantial changes in its structure and function.

In conclusion, our study demonstrates that long-term time series can provide useful information on the functional response of plankton communities to variable environmental conditions, provided that data are available for a sufficient number of ecologically relevant taxa, and calls for further analyses based on the community approach. This approach would enable the decryption of the ensemble of responses of marine ecosystems to environmental changes at the interannual scale. In addition, evidence of switching responses by planktonic organisms should strongly encourage the formulation of models, both conceptual and numerical, which should include such responses in order to improve the representation of the plankton webs in terms of convolution, stability and relative robustness to environmental oscillations (Wyatt *et al.* 2001).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Frequency distribution for P-values of correlations with $r > 0.7$ or < -0.7 considered in the present study.

Fig. S2. Circular dendrogram of average salinity and integrated chlorophyll *a* values in the 0–2 m layer at st. LTER-MC during summers 2002-2009. Numbers represent samples collected on the dates listed on the right. Hierarchical clustering based on a UPGMA algorithm for a Euclidean distance matrix.

Fig. S3. Coupled hierarchical clustering of salinity and chlorophyll *a* (left) and plankton data (right) based for a UPGMA algorithm for, respectively, Euclidean distance and Bray–Curtis similarity matrices.

Fig. S4. Coupled hierarchical clustering of salinity and chlorophyll a (left) and plankton data (right) based on a UPGMA algorithm for, respectively, Euclidean distance and Bray–Curtis similarity matrices.

Table. S1. Taxa abundance data.

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Inter-specific plastidial recombination in the diatom genus *Pseudo-nitzschia*[1]

Domenico D'Alelio[2]

Integrative Marine Ecology Department, Stazione Zoologica Anton Dohrn

Villa Comunale, I-80121 Naples (Italy)

Maria Valeria Ruggiero

Integrative Marine Ecology Department, Stazione Zoologica Anton Dohrn

Villa Comunale, I-80121 Naples (Italy)

[1] Submitted 3 August 2015

[2] Corresponding author. Domenico D'Alelio, Integrative Marine Ecology Department,

Stazione Zoologica Anton Dohrn - Villa Comunale, I-80121 Naples (Italy)

Emails: dalelio@szn.it, dom.dalelio@gmail.com. Tel: +39 081 5833212. Fax: +39 081

7641355

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ABSTRACT

Plastids are usually uni-parentally inherited and genetic recombination between these organelles is seldom observed. The genus *Pseudo-nitzschia*, a globally relevant marine diatom, features bi-parental plastid inheritance in the course of sexual reproduction. This observation inspired the recombination detection we pursued in this paper over a ~1,400-nucleotide-long region of the plastidial *rbc*L, a marker used in both molecular taxonomy and phylogenetic studies in diatoms. Among all the *rbc*L-sequences available in web-databases for *Pseudo-nitzschia*, forty-two haplotypes were identified and grouped in five clusters by Bayesian phylogeny. Signs of hybridization were evident in four out of five clusters, at both intra- and inter-specific levels, suggesting that, in diatoms, i) plastidial recombination is not absent and ii) hybridization can play a role in speciation of *Pseudo-nitzschia* spp.

Key index words: diatoms, plastids, *Pseudo-nitzschia*, *rbc*L, recombination

Pseudo-nitzschia H. Peragallo is a globally distributed genus of planktonic, potentially toxigenic diatoms (Lelong et al. 2012, Lundholm et al. 2012, Trainer et al. 2012). In order to detect signs of genetic recombination in plastids of *Pseudo-nitzschia* spp. we i) analyzed all available sequences for the single-copy locus *rbc*L (RuBisCO large subunit) of this genus, ii) identified unique haplotypes (Table S1 in the Supporting Information); iii) built a Bayesian phylogeny (Fig. 1a), iv) identified sub-sets of closely related sequences and v) performed recombination detection on each sub-set. Among plastidial genes, *rbc*L was chosen since the largest dataset of sequences was available in the GenBank database. Six out of forty-two sequences analyzed in the present study were derived herein according to Amato et al. (2007), while the other thirty-sex were downloaded from the GenBank database and, among

the latter sequences, twenty were not associated to any paper (Table S1). All sequences were accurate and no-stop codon was detected in protein-translations for all of them. Detection of chimeric sequences was carried out on distinct phylogenetic clades composed of more than three haplotypes and using SplitsTree v.4 (Huson 1998) (http://www.splitstree.org/), Pairwise Homoplasy Index test (or PHI-test; Bruen et al. 2006) and RDP (Recombination Detection Program) v.4 (Martin et al. 2010; http://web.cbio.uct.ac.za/~darren/rdp.html). The latter software included seven different algorithms for recombination detection (see details and specific references in Appendix S1 in the Supporting Information). Such a multiple approach was used since previous methodological studies suggested that definitive conclusions about the presence of recombination should not be taken on the basis of a single method (e.g., Posada 2002, Martin et al. 2011).

Within the eighteen *Pseudo-nitzschia* species analyzed, forty-two *rbc*L haplotypes were detected, distributed in five main clades supported by Bayesian phylogeny (Fig. 1a), which matched the delineation of species and species-groups in the genus *Pseudo-nitzschia* (Lelong et al. 2012, Lundholm et al. 2012, Trainer et al. 2012). Split-decomposition analysis produced Parsimony-supported networks in four out of five phylogenetic clades (differently colored in Fig. 1a). The presence of non-univocal paths connecting *rbc*L-haplotypes suggested the presence of either homoplasy or recombination between them. The Phi-test showed that recombination was statistically supported in the green and pink networks (Fig. 1, b and d), suggesting a complex and reticulate evolutionary history of the *rbc*L gene at both intra- and inter-specific level.

Statistically significant individual recombination events (i.e., chimeric sequences) were found in the blue, pink and red groups (Fig. 1, c – e; Table S2 in the Supporting Information). Among the latter, recombination signals were particularly strong in two events, one in the pink and one in the red clades, which were detected by three and seven out of seven distinct

recombination-detection algorithms implemented in RDP, respectively (Fig. 2, a, pink clade and b, red clade; Table S2). In both events, complementary phylogenies were significantly incongruent, i.e., different regions in the same recombinant sequence descended from distinct and relatively genetically distant parents. Mismatching topologies of trees produced with outer and inner alignment-windows (i.e., respectively, outside and within the crossing-over points) strongly supported the occurrence of past recombination events in the evolution of *rbc*L-haplotypes belonging to *P. fraudulenta* (Cleve) Hasle (haplotypes # 14, 15, 16) and *P. multistriata* (Takano) Takano (haplotypes #32, 33, 34), harbored in the pink and the red clades respectively (Figs. 1 and 2).

The evidence of recombination in *rbc*L, stemming from different analytical tests, convinced us that the pattern detected herein resulted from the occurrence of past crossing-over events rather than from convergent evolution or retro-mutations. On the other hand, chimeric nucleotide sequences can be produced in PCR reactions when two or more haplotypes are contemporarily present in the analyzed DNA (Pääbo et al. 1990, Bradley and Hillis 1997). Nonetheless, we are confident that recombinant haplotypes detected in the present work derived from natural crossing-over events for three reasons: i) recombination signals were wide-spread over the whole *Pseudo-nitzschia* genus; ii) one individual crossing-over event involved parental and recombinant sequences derived in independent works, by different authors, in different labs and times (i.e., in the hybrid origin of *P. multistriata*, Fig. 2b); iii) more than one haplotype derived from one and a single recombination event (same parental sequences and breaking points) - i.e., haplotypes 14-16 (*P. fraudulenta*) and 32-34 (*P. multistriata*; Fig. 2, a and b, respectively). In the latter case, the recombinant haplotypes differed from each other by few single-nucleotide polymorphic sites; it is highly probable that these haplotypes derived from mutations subsequent to the recombination events detected. Recombination between plastids is rare but well documented, in both plants and algae (Sager

and Ramanis 1967, Medgyesy et al. 1985, Lemieux and Leet 1987, Cerutti and Jagendorf 1993, Blanc-Mathieu et al. 2013, Wolfe and Randle 2013). Plastids derive from prokaryotic cells anciently 'captured and domesticated' by proto-eukaryotic cells (Delwiche 1999) but they still maintain autonomous biological functions, including cell-to-cell migrations (Thyssen et al. 2012) and capability of transferring genetic material, as notably observed for RuBisCO genes (Delwiche and Palmer 1996). Moreover, evidence of both recombination between separate chromosomal elements (Zhang et al. 2002) and recombination-mediated Lateral Gene Transfer (Rice and Palmer 2006) have been reported for microalgal plastids. Thus, the interspecific recombination signals that we found in the diatom genus *Pseudo-nitzschia* are neither peculiar nor potentially unique, and they could be found in other diatoms and microalgae, given the presence of bi-parental plastid inheritance.

Crossing-over of plastidial DNA could occur if at least two conditions verify, namely i) heterologous plastids must fuse for the time needed for recombination to occur and ii) recombinant plastids must be inherited, i.e., transmitted to the generations following the crossing-over event. Both conditions seem plausible in the life-cycle of the diatom genus *Pseudo-nitzschia*.

Firstly, in the species *P. multistriata*, time-lapse analyses delineated complex dynamics of chloroplast migration during the sexual phase (Eleonora Scalco & Marina Montresor personal communication), which is resumed as follows. *Pseudo-nitzschia* cells have two relatively large chloroplasts (say, *a* and *b*), which divide at the beginning of the sexual phase. After gametogenesis, each of the two gametes includes both *a* and *b* plastids, derived from the first division. *P. multistriata* is a heterothallic diatom so that conjugation occurs between two heterologous gametes, belonging to distinct clonal lineages. Thus, after the fusion, the zygote includes four heterologous plastids which are tightly packed, at that stage. In such a condition, lasting several hours, recombination between plastids could be likely promoted.

Concerning the second condition, after zygote development, the initial cell of *Pseudo-nitszchia* undergoes degradation of two of the four plastids inherited by each of the two parental cells, but degradation is apparently unselective and follows a Mendelian rule (Levialdi Ghiron et al. 2008). Thus, putatively recombinant plastids would be 'recruited' with the same probability of non-recombinant ones in the new F1 generation. Since some levels of inter-specific hybridization could be hypothesized in the genus *Pseudo-nitzschia* (Amato and Orsini 2015), then the described mechanism could lead to the recombination of heterologous plastid genes.

Recombination between plastids is thought to guarantee these organelles' functional stability, e.g., by repairing genetic injuries (Maréchal and Brisson 2010).. The recombining region in the *rbc*L that we detected did not show any signs of positive selection (data not shown) and we do not know if any evolutionary advantage was gained by the recombinant sequence compared to the parental ones. We analyzed only *rbc*L, as it is, nowadays, the only plastidial locus for which a wide and somewhat complete dataset in terms of sequence-coverage is available for *Pseudo-nitzschia* species. Yet, recombination could have involved also a wider genomic region in the plastid of putatively recombinant *Pseudo-nitzschia* spp. Our observations call for further analyses over the whole plastid genome of *P. multistriata* and *P. fraudulenta* and their own parents, in order to better investigate the biological significance of plastidial recombination.

To date, genetic hybridization in diatoms has been reported only under the species level (e.g., D'Alelio et al. 2009, Tesson et al. 2014). However, the recombinant origin of *P. multistriata* and *P. fraudulenta rbc*L is a proof that hybridization can occur also between distinct *Pseudo-nitzschia* species. Moreover, we cannot exclude that the above-mentioned species derived from hybrid speciation, although the pattern we detected could be likely explained by genetic introgression. Hybrid speciation is a common evolutionary option for several lineages (Mallet

2007, Abbott et al. 2013), including marine and more spatially 'dispersed' organisms (Arnold and Fogarty 2009) and protists, e.g., yeast (Greig et al. 2002) but, to the best of our knowledge, this evolutionary route has never been reported in microalgae. Our study suggests *Pseudo-nitzschia* as a good model genus to investigate hybrid speciation in microalgae, e.g., by using in-vitro breeding and by sequencing other plastidial and nuclear molecular markers.

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Figure legends

Figure 1. Network analysis of the *rbc*L dataset. (a) Bayesian phylogeny of *Pseudo-nitzschia*

*rbc*L gene. Sequences were aligned with standard analytical tools (Bioedit v.7.2.5, Hall

1999). Phylogeny was produced using the substitution model General Time Reversible + Rate Heterogeneity (G) + Proportion of Invariant Sites (I) (GTR + G + I, G = 0.694 and I = 0.742), according to model-tests, by running the MrBayes algorithm (1,000,000 permutations) in Topali v.2.5 (Milne et al. 2009). (b-e) Genetic networks for the four main *Pseudo-nitzschia* clades. Genetic networks produced by SplitsTree v.4 (Huson 1998) were validated with a bootstrap procedure (n = 1,000) and only consensus Parsimony split networks, including only paths showing 95% or higher parsimony, are shown. The result of PHI-test (Bruen et al. 2006) is shown for each network.

Figure 2. Phylogenies of individual recombination events. (a) and (b) refer to complementary phylogenies for the chimeric sequences of *P. fraudulenta* and *P. multistriata*, respectively. (a) corresponds to the pink clade in Figure 1; (b) corresponds to the red clade in Figure 1. For both events (a-b) independent phylogenies were produced for i) the 'outer' alignment window, including the region between the 5' end of the alignment and the first breaking point plus the region between the second breaking point and the 3' end of the alignment (black tree); and ii) the 'inner' alignment window, including the region between the breaking points (gray tree). (a) Bayesian trees (MrBayes algorithm with 1,000,000 permutations, in Topali v.2.5 (Milne et al. 2009) for the outer and inner regions were built using the Hasegawa, Kishino and Yano substitution model (HKY + G, G = 0.010; Hasegawa et al. 1985) and the Symmetrical Model (SYM + G, G = 0.011) (Milne et al. 2009), respectively, according to model tests. (b) Bayesian trees (same procedure as above) for the outer and inner regions were built using the substitution models F81 + G (G = 0.011) (Felsenstein 1981) and the HKY + G (G = 0.017) (Hasegawa et al. 1985), respectively, according to model tests.

Figure 1

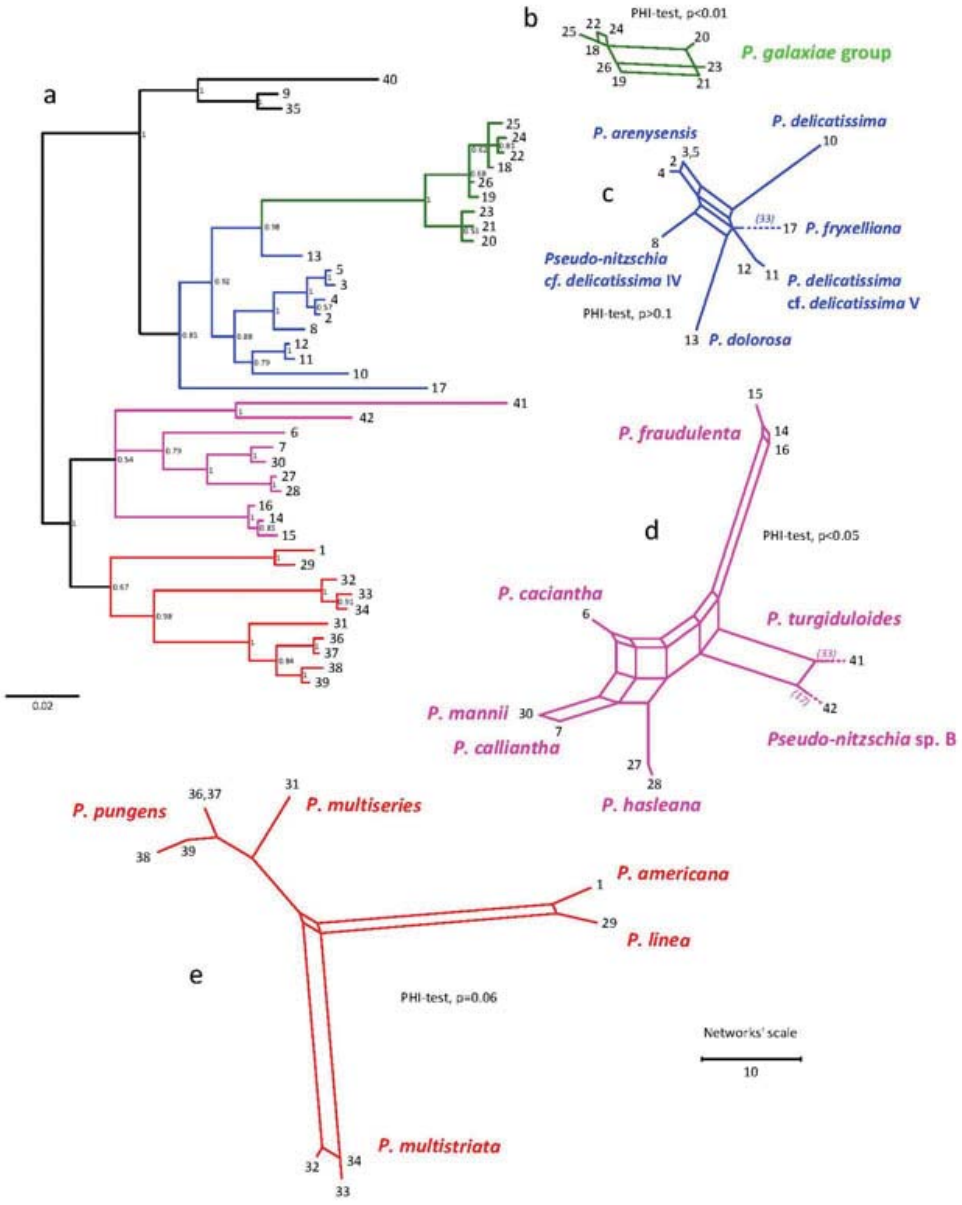
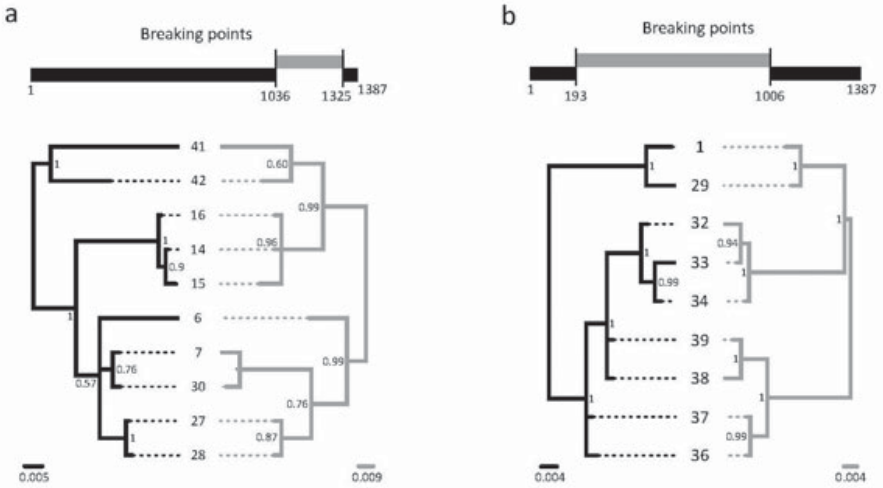


Figure 2



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Acclimation to different depths by the marine angiosperm *Posidonia oceanica*: transcriptomic and proteomic profiles

Emanuela Dattolo[1], Jenny Gu[2], Philipp E. Bayer[2], Silvia Mazzuca[3]*, Ilia A. Serra[3], Antonia Spadafora[3], Letizia Bernardo[3], Lucia Natali[4], Andrea Cavallini[4] and Gabriele Procaccini[1]

[1] *Functional and Evolutionary Ecology Lab, Stazione Zoologica Anton Dohrn, Napoli, Italy*
[2] *Evolutionary Bioinformatics Group, Institute for Evolution and Biodiversity, University of Münster, Münster, Germany*
[3] *Laboratorio di Proteomica, Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria, Arcavacata di Rende (CS), Italy*
[4] *Dipartimento di Scienze Agrarie, Alimentari ed Agro-ambientali, Università di Pisa, Pisa, Italy*

Edited by:
Mats Bjork, Stockholm University, Sweden

Reviewed by:
Ján A. Miernyk, University of Missouri, USA
Ning Li, The Hong Kong University of Science and Technology, China

***Correspondence:**
Silvia Mazzuca, Associate Professor in Plant Biology, Laboratorio di Proteomica, Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria, Ponte Bucci, 12 A, 87036 Arcavacata di Rende (CS), Italy
e-mail: silvia.mazzuca@unical.it

For seagrasses, seasonal and daily variations in light and temperature represent the mains factors driving their distribution along the bathymetric cline. Changes in these environmental factors, due to climatic and anthropogenic effects, can compromise their survival. In a framework of conservation and restoration, it becomes crucial to improve our knowledge about the physiological plasticity of seagrass species along environmental gradients. Here, we aimed to identify differences in transcriptomic and proteomic profiles, involved in the acclimation along the depth gradient in the seagrass *Posidonia oceanica*, and to improve the available molecular resources in this species, which is an important requisite for the application of eco-genomic approaches. To do that, from plant growing in shallow (−5 m) and deep (−25 m) portions of a single meadow, (i) we generated two reciprocal Expressed Sequences Tags (EST) libraries using a Suppressive Subtractive Hybridization (SSH) approach, to obtain depth/specific transcriptional profiles, and (ii) we identified proteins differentially expressed, using the highly innovative USIS mass spectrometry methodology, coupled with 1D-SDS electrophoresis and labeling free approach. Mass spectra were searched in the open source Global Proteome Machine (GPM) engine against plant databases and with the X!Tandem algorithm against a local database. Transcriptional analysis showed both quantitative and qualitative differences between depths. EST libraries had only the 3% of transcripts in common. A total of 315 peptides belonging to 64 proteins were identified by mass spectrometry. ATP synthase subunits were among the most abundant proteins in both conditions. Both approaches identified genes and proteins in pathways related to energy metabolism, transport and genetic information processing, that appear to be the most involved in depth acclimation in *P. oceanica*. Their putative rules in acclimation to depth were discussed.

Keywords: ***Posidonia oceanica*, acclimation, ESTs, proteomic, eco-genomic**

INTRODUCTION

The littoral coastal zone is characterized by severe environmental gradients, which mold distribution of populations and species of marine organisms. In a framework of conservation and restoration of biodiversity and in order to predict responses to environmental changes and to develop *ad hoc* conservation strategies, it is crucial to improve our knowledge about the limits of physiological acclimation, physiological plasticity, and intraspecific traits variation, of species living along environmental gradient (Thomas et al., 2004; Schmidt et al., 2008; Thomas, 2010; Hill et al., 2010).

Along the coastline all over the world, excluding polar areas (Green and Short, 2003), seagrasses form among the most productive and neglected marine ecosystems, providing an high number of ecosystem's services, also in comparison to terrestrial habitats (Costanza, 1997; McArthur and Boland, 2006).

Seagrass meadows are very sensitive to disturbance and are being lost rapidly in both developed and developing parts of the world (Short and Wyllie-Echeverria, 1996; Waycott et al., 2009), with only occasional efforts for mitigation and restoration. Seagrass loss has been attributed to a broad spectrum of anthropogenic and natural causes that largely diminish their habitat, affecting their distribution and diversity (Orth et al., 2006; Waycott et al., 2009). For marine plants, seasonal and daily variations in light availability and temperature represent the mains factors driving their distributions along the bathymetric cline. Changes in these environmental factors, due to climatic and anthropogenic effects, can compromise the survival of these key ecosystem-engineering species (Doney et al., 2002).

In Mediterranean Sea, the endemic seagrass *Posidonia oceanica* (L.) Delile can grow as deep as 50 m, depending on light penetration and water clarity (Pasqualini et al., 1998), being extremely sensitive to changes in light availability (Lee et al., 2007). The increase of water turbidity, widely observed as result of human activities along the coastline, affects particularly the deep distribution of the meadows (Ardizzone et al., 2006). *P. oceanica*

grows according to a phalanx strategy, with sporadic sexual reproduction and slow-growing clonal lineages, which can persist *in situ* for hundreds of years (Ruggiero et al., 2002; Migliaccio et al., 2005; Arnaud-Haond et al., 2012). Plasticity of *P. oceanica* long-living clones must play an important role on the persistence of the species, being able to survive changes of environmental conditions, as the ones experienced by the unstable highly-impacted Mediterranean coastline.

During the last decades, the application of *-omics* technologies at ecological studies provided powerful tools for following the physiological acclimation in response to environmental variations (Feder and Walser, 2005; Foret et al., 2007; Gracey, 2007; Karr, 2008), and helped researchers to correlate the differences of gene's expression profiles to changes in the main ecological cues in many different organisms (Chevalier et al., 2004; Edge et al., 2008; Kassahn et al., 2009; Larsen et al., 2012; Richards et al., 2012).

Despite their high ecological value, seagrasses are poorly understood for what concerns the genetic basis behind their physiological adaptation and plasticity (Procaccini et al., 2007). It's only recently that transcriptomic approaches were implemented for few species, to correlate seagrasses gene expression with ecological factors. In particular, transcriptomic response to temperature changes and thermal stress was studies in the two congeneric species, *Zostera marina* and *Zostera noltii* (Maathuis et al., 2003; Reusch et al., 2008; Massa et al., 2011; Winters et al., 2011), while transcriptional (Bruno et al., 2010; Serra et al., 2012b) and proteomic approaches (Mazzuca et al., 2009) were applied to study light response in natural conditions in *Posidonia oceanica*. In *P. oceanica*, studies were hampered by the fact that available genomic and transcriptomic resources only consisted in a single Expressed Sequences Tags (EST) library, obtained from shoots collected along a depth range (from −5 to −30 m) in a single site (Wissler et al., 2009), and available in Dr.Zompo, a specific seagrasses database containing both *P. oceanica* and *Z. marina* EST sequences http://drzompo.uni-muenster.de/ (Wissler et al., 2009).

Several approaches can be utilized for genomic studies in species for which the whole genome is not available (e.g., Hofmann et al., 2005; Stapley et al., 2010), most of them requiring high computational power and advanced bioinformatics resources (Morozova and Marra, 2008; Pop and Salzberg, 2008; Metzker, 2010). Among the others, Suppressive Subtractive Hybridization (SSH)–EST library (Diatchenko et al., 1996) approach resulted especially powerful to identify differentially expressed genes in the presence of clear differences in physiological status (Jones et al., 2006; Puthoff and Smigocki, 2007) and it was applied to study flowering (Matsumoto, 2006), senescence (Liu et al., 2008a,b), or salt-stress (Zouari et al., 2007) in terrestrial plants.

The aim of this work was to identify differences in transcriptional and proteomic profiles in *P. oceanica*, correlated with its bathymetric distribution, with the ultimate goal to identify the metabolic pathways involved in acclimation. We also aimed to increase genomic resources in *P. oceanica* and to present a powerful approach for studying physiological response at a molecular level in organisms for which genomic resources are limited.

In order to do that, we built a SSH-library between plants growing at two different depths in the same meadow, and we obtained their protein content using the innovative USIS mass spectrometry methodology coupled with 1D-SDS electrophoresis. Proteins identifications were performed using the Global Proteome Machine (GPM) open-source system for analyzing, storing, and validating proteomics information derived from tandem mass spectrometry (Craig et al., 2004; Fenyö et al., 2010) and X!Tandem software (Craig and Beavis, 2003; Craig et al., 2005) against a local database derived by Dr.Zompo and UniProtKB databases.

MATERIALS AND METHODS

SHOOTS SAMPLING

Posidonia oceanica shoots were collected by SCUBA diving in the Lacco Ameno meadow, Island of Ischia (Gulf of Naples, 40°45′52″ N; 13°53′29″ E) at two sampling stations located above and below the summer thermocline (−5 and −25 m depths).

Leaf tissue from 20 shoots for each stand was cleaned from epiphytes and shock frozen in dry ice on the research vessel soon after collection. Tissue was stored at −80°C before RNA and proteins extraction.

Temperature, salinity and Photosynthetic Active Radiation (PAR) were measured at the surface and at six different depths along the bathymetric distribution of the meadow (**Table 1**). Values were obtained right before shoot sampling, using a Seabird Seacat Probe operated from the boat and connected to a wired computer onboard.

RNA EXTRACTION

Total RNA was isolated from leaf tissue of ten shoots for each condition, using hexadecyltrimertihyl ammonium bromide (CTAB) method (Chang et al., 1993) with some modifications. About 4 g of each shoot were weighted and grind to a fine powder in liquid nitrogen in a pre-cooled mortal. The powder was transferred to an Eppendorf tube and 1 ml of pre-warmed extraction buffer was added to the samples (2% CTAB, 0.2% β-mercaptoethanol, 1.4 M NaCl, 20 mM EDTA, 200 mM Tris-HCl pH 7.5). After incubation at 65°C for 10 min, 800 μl chloroform-isoamyl alcohol (49:1 v/v) were added. After centrifugation, at 6500 rpm for 10 min, the RNA was selectively precipitated from the upper phase through the addition of 1/4 volume 10 M LiCl and precipitated for 2–4 h at −20°C. RNA was recovered by centrifugation (Beckman JA-20

Table 1 | Environmental variables.

Depth (m)	Temperature (°C)	Salinity (PSU)	*PAR* (μM/m²/sec)
0	27.55	37.79	960
−5	**26.84**	**37.78**	**703**
−10	24.03	37.75	491
−15	22.24	37.74	355
−20	20.02	37.78	230
−25	**18.99**	**37.78**	**100**
−30	18.12	37.79	50

Values of temperature, T (C°); salinity (PSU); and Photosynthetically active radiation, PAR (μM/m²/sec) collected during the sampling (July, 2010 h ∼14:00) with a Seabird Seacat Probe at the surface and at six depths along the bathymetric distribution of the meadow (depth). Sampling stations are indicated in bold.

rotor) at 11,000 rpm at 4°C for 30 min. Supernatant containing genomic DNA was removed and pellets were washed once with 1 ml 100% EtOH and two times with 1 ml 75% EtOH. Precipitations were followed by centrifuging at 10,000 rpm for 5 min to remove the EtOH and pellets were dried at room temperature for few minutes. RNA was suspended in 50 μl H_2O RNase free. RNA quality and quantity was evaluated by gel electrophoresis and by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies) monitoring the absorbance at 260 nm. Purity was determined by 260/280 nm and 260/230 nm ratios using the same instrument. All samples resulted free from protein and organic solvents used during RNA extraction. RNA was stored at −80°C.

CONSTRUCTION OF SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION (SSH)-LIBRARIES

For each depths considered in the experiment, the same quantity of total RNA extracted from individual shoot was pooled. About 280 μg of each RNA pools were purified using Dynabeads mRNA Purification kit (DYNAL BIOTECH), following the manufactures instructions, in order to select polyA$^+$ mRNA.

The construction of the forward and reverse SSH libraries was performed using the PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA, USA), following the manufacturers instruction. Shallow library (FORWARD subtraction, S) was carried out with shallow mRNA as tester pool and deep mRNA as driver pool. Reversely, in the deep library (REVERSE subtraction, D), deep mRNA was used as tester pool and shallow mRNA as driver pool.

The two resulting subtractive libraries were cloned individually in pCR2.1-TOPO vector (Invitrogen), and transferred into TOP F' cells (Invitrogen) with vector: insert ratio 1:10, following manufacturer's instructions. Colonies were grown overnight in Petri dishes with LB medium and Ampicillin (μg/ml). Afterwards, single colonies were picked and transferred into 96-well plates containing LB and Ampicillin (LB/Amp) to grow overnight. About twenty 96-well plates for each library (S and D) were screened in PCR to identified positive recombinant colonies. Every single colony has been amplified using specific primers of the TOPO vector: T7 forward and M13 reverse. PCR products have been analyzed on 1.5% Agarose gel stained with Ethidium Bromide in 1× TAE buffer. For each library, about 1000 colonies having an insert longer than 500 bp were selected for sequencing (data not shown).

Finally, replicates of selected colonies were stored in LB/Amp-15% glycerol (at −80°C) and shipped to the Biologisch-Technische Produkte Service of the Max Planck Institute for Molecular Genetics (Molgen, Berlin, DE) for ESTs sequencing using ABI 3730xl automated DNA sequencers (Applied Biosystems, USA).

DATA ANALYSES AND BIOINFORMATICS

Bioinformatics analysis of EST data sequences was carried out by the Evolutionary Bioinformatics Group at the Westfälische Wilhelms University Institute for Evolution and Biodiversity (Münster, DE).

Raw sequences of each library were trimmed removing the low quality regions, the vector, the adapter and the poly-A/T regions, using PREGAP4 (Staden, 1996). Only the EST raw sequences longer than 100 nucleotides entered the assembly step. Successfully trimmed EST reads were assembled into tentative unigenes (TUGs) using CAP3 (Huang, 1999). After trimming and deletion of short sequences (94 in total), only sequences of good quality were finally assembled into 486 TUG, which include 2290 ESTs. Considering other 286 single reads (Singletons), a total of 772 SSH-Unigenes were identified. To infer functions of SSH-Unigenes, an homology search, using BLASTN algorithm, was made against public multiple databases: non-redundant NCBI Gene Ontology (GO), KEGG (Kyoto Encyclopedia of Genes and Genomes), SWISSPROT, and NR-NCBI (using BLASTX algorithm with an Expect-value threshold of = 0.001) and Dr.Zompo. Identified Unigenes were stored in the database Dr.Zompo as "Pooc_B" library. Divergence in gene expression patterns at the two different depths, was assessed mapping Unigenes into functional categories using Mapman (Thimm et al., 2004).

PROTEIN EXTRACTION AND ELECTROPHORESIS

Only adult leaves were used for this purpose according to Spadafora et al. (2008). Plant material was grounded to a fine powder in liquid N_2 using mortar and pestle and transferred to a centrifuge tube, where cold 10% trichloroacetic acid in acetone with 0.1 M β-mercaptoethanol, was added. Samples were kept at −20°C for at least 2 h, and then centrifuged at 12,000 g for 15 min at 4°C. The resulting pellet was washed 3 times by suspending in acetone containing 0.1 M DTT and centrifuged as above between each wash. The pellet was air-dried and used for protein extraction. Tissue powder from ten different plants from shallow (A) and deep (B) conditions, respectively, was pooled and used for phenol-based protein extraction (Spadafora et al., 2008). Tissue powder was re-suspended in extraction buffer containing 0.1 M Tris-HCl, pH 8.8, 2% SDS and 0.1 M β-mercaptoethanol. Supernatant was mixed with equal volume of buffered phenol (pH 8.0, Sigma). Phases were separated by centrifugation at 15,000 g for 5 min. The phenol phase was precipitated with 5 volumes of cold methanol containing 0.1 M ammonium acetate overnight (−20°C). Protein phase was recovered by centrifugation and washed twice with cold acetone. The phenol extraction step has been repeated twice for each set of samples and thereafter processed for mass spectrometry analyses.

Protein samples from Sets A and B were processed on 1D SDS-PAGE; the Laemmli buffer system was used to cast a 6% stacking gel and 12.5% resolving gel. After denaturation at 100°C for 3 min, proteins were resolved at constant voltage (200 V) in a Bio-Rad mini Protean II apparatus. CBB stained gels were scanned on a densitometer (GS800, Biorad) and peptide bands were quantified using QuantityOne software (Bio-Rad). 1D gel lines from Sets A and B samples were cut in 24 slices each (**Figure 1**) and digested enzymatically with trypsin. The tryptic fragments were analyzed by LC-ESI MS/MS coupled with the ionization source for mass spectrometers named Universal Soft Ionization Source (USIS) (Cristoni S, patent no. PCT/EP2007/004094). For the experiments, a Bruker HTC Ultra spectrometer, equipped with a Dionex Ultimate 3000 HPLC system, was used. Chromatography separations were conducted on a Thermo Biobasic C18 column (1 mm i.d. _ 100 mm length and 5 μm particle size), using a linear

gradient from 5 to 90% acetonitrile (ACN), containing 0.1% formic acid with a flow of 100 μL/min, including the regeneration step; one run lasted 70 min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of m/z 250–2000 followed by full MS/MS scan for the most intense ion from the MS scan).

PROTEIN IDENTIFICATION

Spectra acquired by LC-MS/MS were used to identify peptide sequences using the open-source system GPM software against the GPM plant database (http://plant.thegpm.org/tandem/thegpmtandem.html). Since the GPM plant database considers only a few species belonging to *Liliopsida*, excluding seagrasses, this procedure can lead to a loss of peptide identification by mass spectrometry. Thus, spectra acquired by LC-MS/MS were also used to identify peptide sequences using X!Tandem software (http://www.thegpm.org/tandem/index.html) against a local database. X!Tandem is a search engine for identifying proteins by searching sequence collections, reducing the time required to match protein sequences with tandem mass spectra (Craig and Beavis, 2003). It scores the match between an observed tandem mass spectrum and a peptide sequence, by calculating a score that is based on the intensities of the fragment ions and the number of matching b- and y-ions. This score is converted to an expectation value using the distribution of scores of randomly matching peptides (Fenyö et al., 2010).

In the local database, sequences from seagrasses and other species belonging to *Liliopsida* available in the UniprotKB database and the amino acid sequences of *P. oceanica* and *Z. marina* deduced from five ESTs libraries (Pooc_A, Pooc_B, Zoma_A, Zoma_B and Zoma_C) collected in the Dr.Zompo database (Wissler et al., 2009, http://drzompo.uni-muenster.de/) were included. In the last case, it has been necessary first to create a protein database from the nucleotide sequences. For this, the most straightforward procedure is listing all possible ORFs from the six reading frames; the resulting list contains a large majority of protein sequences that are unlikely to be real, but MS/MS data allow to discriminate between real and false polypeptide sequences (Armengaud, 2009). The use of all possible reading frames has allowed to optimize the peptide identifications. ESTs are relatively error prone (Alba et al., 2004) and an ORF can be split and displayed over 2 or 3 frames when a frame-shift error exists on the cDNA sequence. Consequently, the deduced protein sequence can be incorrect (Serra et al., 2012a). The translation of each nucleotide sequence was performed using a translation tool available at http://www.ebi.ac.uk/Tools/st/emboss_transeq/5.

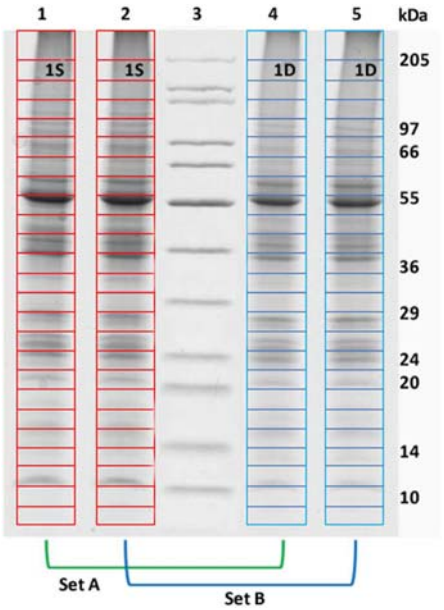

FIGURE 1 | One-dimensional-SDS-PAGE. One-dimensional-SDS-PAGE of two independent biological replicates of leaf proteins purified from shallow (lanes 1 and 2) and deep (lanes 4 and 5) *P. oceanica* plants. Each gel lane was cut in 24 slices, then each couple (1S;1D...24S;24D) from swallow and deep lanes was comparatively analyzed by mass spectrometry as Sets A and B datasets.

RESULTS

SSH-LIBRARY

After assembly process and trimming, ESTs sequences clustered to 772 TUGs, 286 of which were Singletons and 486 were Contigs, consisting of two or more reads. Among the TUGs identified, the 45% (349/772) had a GO annotation, while the 55% (423/772) were not classified in GO. Protein annotation against SwissProt database, gave in total 278 Unigenes classified into putative known functions or unclassified proteins. Based on Dr.Zompo database, only the 39% of the total number of SSH-Contigs (189/486) had homologies with known *P. oceanica* ESTs sequences, while 61% (297/486) were new. The main statistic features of the SSH–EST library are reported in **Table 2**, other data are reported in **Table S1**.

Annotation and other features of SSH-TUGs are listed in **Tables S2a,b**. TUGs were included in the database Dr.Zompo (http://drzompo.uni-muenster.de/) in the *P. oceanica* "Pooc_B" library. The 2576 single ESTs obtained were submitted to the dbEST within GeneBank (LIB EST_Pooc SSH, Genbank Accession Numbers: JZ354020–JZ356595).

COMPARISON OF TENTATIVE UNIGENES FREQUENCIES BETWEEN SHALLOW AND DEEP CONDITIONS

Among the 486 Contigs identified, only 28 (3% of the total) were present in both libraries, while 314 Contigs have been found

Table 2 | EST library features.

	N°
ESTs in shallow library	1330
ESTs in deep library	1246
Contigs in shallow library	200
Contigs in deep library	314
Contigs in common (shallow + deep)	28
Singletons only in shallow library	139
Singletons only in deep library	147

Comparison of main statistical features between shallow and deep Posidonia oceanica EST libraries.

only in the deep-library (D-library) and 200 Contigs only in the shallow-library (S-library). For Singletons, 147 and 139 were present only in the D-library and in the S-library, respectively (**Table 2**).

TUGs more abundant in the S-library include (i) proteins involved in protein turnover, as Proteasome subunit alpha, E3 ubiquitin (F-box protein) and ATP-dependent Clp protease proteolytic subunit and (ii) proteins involved in stress defense, as Heat shock cognate 70 kDa protein, Ketol-acid reductoisomerase, Acyl-CoA-binding protein and Cytochromes c/b subunits (**Table S2a**). TUGs more abundant in the D-library include (i) proteins involved in the photosynthetic pathways as Chlorophyll a-b-binding proteins, Photosystem I/II, Oxygen-evolving enhancer protein and (ii) proteins involved in basal metabolism and in stress response, as Universal stress protein, Zinc-finger protein, Metallothionein-like protein, Cytochrome P450, Caffeoyl-CoA O-methyltransferase, Aquaporin PIP2 and S-norcoclaurine synthase (**Table S2b**).

Among Contigs, only six showed significant differences in frequency ($p \leq 0.05$) between libraries. Five Contigs were up-regulated in S-library and only one was up-regulated in D-library (**Table 3**). The differential expression of two of these Contigs, Pooc_B_c42, encoding for a N(2),N(2)-dimethylguanosine tRNA methyltransferase, and Pooc_B_c217, whose function is unknown, has been tested in RT-qPCR and showed the expected expression profiles (**Figure S1**, also see Serra et al., 2012b).

Since SSH technique can also generate background clones which are not representing differentially expressed sequences but can be false positives, we will consider the remaining transcripts identified here as "putative differentially expressed" until each one will be experimentally validated in future studies.

Differences between libraries were both quantitative, i.e., relative expression of particular Unigenes, assessed as number of reads, and qualitative, i.e., comparing proteins for the same functional categories or the same metabolic pathways. The comparative abundance of each functional category is shown in **Figure 2**. Genes belonging to light related processes (e.g., photosynthesis and energetic metabolism), genetic information processing (e.g., transcription and translation), transport, folding, sorting, and degradation of proteins were abundant in both conditions. Nevertheless, looking at different pathways, differences were observed in their protein composition. For the photosynthetic pathway, in the D-library there are 26 different TUGs encoding for Chlorophyll a-b-binding proteins, whereas only 15 different TUGs were present in the shallow one (**Tables S2a,b**). The opposite trend was observed for proteins related to electrons carrier transport (**Tables S2a,b**). TUGs assigned to PSI and PSII were more abundant in low light (D-library) rather than in high light (S-library), and this difference was particularly strong for PSI (PSI: 2/19, PSII 19/24 reads in S- and D-library, respectively, **Figure 2**). Other striking qualitative differences were observed among stress response proteins. Universal stress proteins, as Zinc-finger, Metallothionein-like, Cytochrome P450, Caffeoyl-CoA O-methyltransferase, Aquaporin PIP2 and S-norcoclaurine synthase were more abundant in the D-library (**Table S2b**), while other proteins involved in stress defense, Heat shock cognate 70 kDa, Ketol-acid reductoisomerase, and Acyl-CoA-binding protein were more abundant in the S-library. TUGs belonging to protein turnover, such as proteasome subunit alpha, E3 ubiquitin (F-box protein), ATP-dependent Clp protease proteolytic subunit (**Table S2a**) were more abundant in the S-library.

Peptide sequences from shallow and deep samples, their relative positive match against the different databases and their functional annotations are reported as in **Tables S3a** (shallow) and **3b** (deep). Database search methods using the GPM and X!Tandem software combined with classical BLASTN searching method to identify peptide sequences, have allowed to assign the large portion of the identified peptides at proteins with known function, enhancing significantly our previous knowledge on the *P. oceanica* proteome. After eliminating redundancies (i.e., proteins common to the two sets of data), the total net protein discovery amounts exactly to 64 proteins, which were principally involved in photosynthesis and energy metabolism, with both structural and regulative functions (**Figure 3**). Mitochondrial and chloroplastic ATP synthase subunits were the most abundant. The chloroplast isoforms of ATP synthase, which take part to the Calvin cycle, were highly expressed in both light conditions, while the mitochondrial isoforms, which take part to respiration, appeared down regulated in low light. Proteins involved in photosynthetic metabolic pathways, such as oxygen-evolving

Table 3 | List of Contigs differentially expressed.

Contig	Annotation	Best hit	*E*-value	Shallow library (−5 m)	Deep library (−25 m)	Regulation	RT-qPCR regulation
Pooc_B_c42	N(2),N(2)-dimethylguanosine tRNA methyltransferase	Q34941	3.0e-14	15	0	*UP-5 m*	*UP-5 m*
Pooc_B_c444	F-box protein At5g67140	Q9FH99	2.0e-28	16	0	*UP-5 m*	
Pooc_B_c209	no hit	–	–	15	0	*UP-5 m*	
Pooc_B_c205	no hit	–	–	341	19	*UP-5 m*	
Pooc_B_c18	no hit	–	–	38	0	*UP-5 m*	
Pooc_B_c217	cellular_component	GO:0005575	–	0	17	*UP-25 m*	*UP-25 m*

*List of Contigs showing significant EST frequency difference between libraries ($p \leq 0.05$). For each Contig, name, annotation, best hits E-value, number of EST reads in each library, and regulation signals are indicated. Differential expression between the two depths reported in this work (shallow −5 m, deep −25 m) was tested in RT-qPCR experiment by Serra et al. (2012b). RT-qPCR results are also reported in **Figure S1** for Contigs Pooc_B_c42 and Pooc_B_c217 (in bold).*

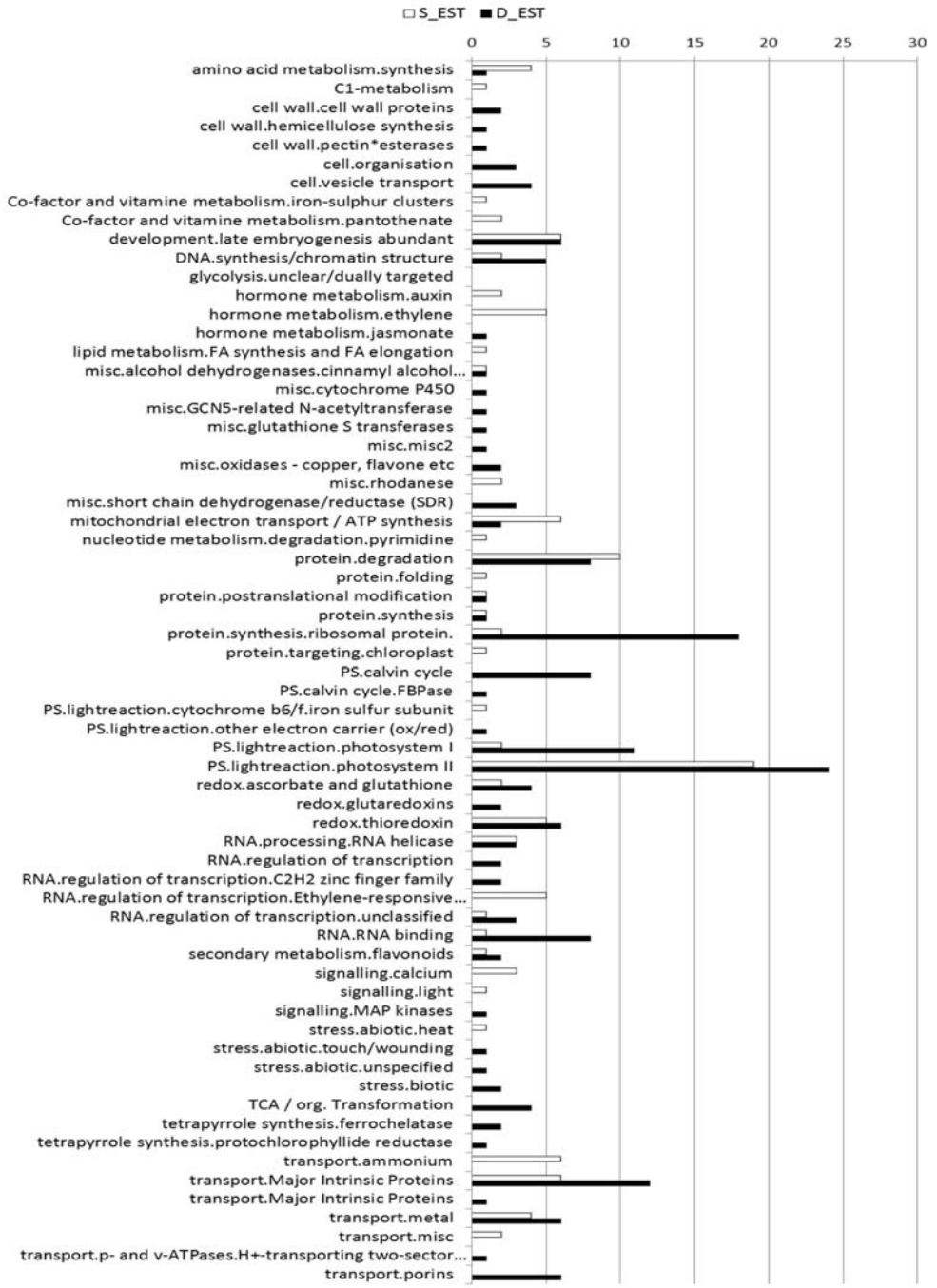

FIGURE 2 | Expression Level of TUGs. Level Expression (number of EST) of TUGs associated to the different metabolic categories in shallow (white column) and deep (black column) conditions.

enhancer proteins, were almost equally represented in the two conditions. Though most of the identified proteins showed little differences in number of peptides between the two conditions, 17 unique peptides were found only in shallow samples corresponding to as many proteins (**Table 4A**); meanwhile, in deep samples, 23 unique peptides that were not found in shallow ones have been assigned to 18 proteins (**Table 4B**). Summarizing the results, among the 64 newly identified proteins, 17 are exclusive of shallow samples and 18 of the deep ones as shown by the Venn diagrams (**Figure 4**). RuBisCO subunits, Chlorophyll a-b-binding proteins and Ferredoxin-NADP reductase (leaf isozyme) were more represented in the deep samples, while Glyceraldehyde-3-phosphate dehydrogenase was more represented in the shallow ones. Moreover, proteins with regulative activity, as the Ribulose

FIGURE 3 | Expression Level of peptides. Level of expression (number of peptides) associated to the different proteins in shallow, (white column) and deep (black column) conditions.

bisphosphate carboxylase/oxygenase activase A and a 14-3-3-like protein, were also recognized as more expressed in shallow in respect to the deep samples.

DISCUSSION

The aim of this work was to investigate physiological acclimation in *Posidonia oceanica* plants along a bathymetric gradient, combining transcriptomic, and proteomic analyses. Plants were collected after the stabilization of the summer thermocline, when light and temperature regimes were well-differentiated between the two selected sampling stations (−5 and −25 m).

A not perfect match between transcriptome and proteome profiles was found, since some targets were identified only in one of the two datasets (e.g., F-box protein only among ESTs; RuBisCO only among peptides). Despite the correlation between transcriptomic and proteomic profiles is usually high (e.g., Guo et al., 2008), the effective level of observed accordance among these data varies in dependence of the system studied (Pascal et al., 2008; Vogel and Marcotte, 2012).

Furthermore, as also reminded from other authors (Feder and Walser, 2005; Diz et al., 2012) in interpreting differences in transcripts and peptides abundance, it should be taken in to account that many different regulative steps during transcription and translation processes influence the expression levels of mRNAs and their corresponding protein.

Besides the problem of reading correctly differences in genes and proteins expression profiles, a main question in combining transcriptional and proteomic data analyses is also in how to asses the interaction between them (Rogers et al., 2008; Huang et al., 2013). In the present work, data obtained from both analyses were discussed jointly, in order to asses the putative role and function of each target recognized in *P. oceanica* acclimation to depth.

Overall, our results suggested that a large portion of genes and proteins which were identified as *putatively* differentially expressed, could be assigned to three principal metabolic pathways: Photosynthesis, Cellular energetic metabolism and Protein turnover. Furthermore, pathways related to Signaling and Stress response, though similar in their overall expression between the two depths, showed different protein compositions.

PHOTOSYNTHETIC PROCESSES

Light availability, both intensity and quality, influences directly and indirectly chloroplast metabolism (Jiao et al., 2007). The modulation of photosynthetic machinery is critical in the short term (day by day) and long-term (season, years) adaptation to environmental light. In photosynthetic organisms, the adaptation to different light conditions happens through adjustments of cellular homeostasis to maintain a balance between energy supply (light harvesting and electron transport) and consumption (cellular metabolism). The regulation of these mechanisms involves changes in the expression levels of both mRNA and mature proteins. During the sampling, the irradiance at the deep stand was about 1/10 of the irradiance present at the shallow stand, with values that are very close to the theoretical minimum light requirement estimated for *P. oceanica* (∼9–16% of surface irradiance, Lee et al., 2007). Hence, many genes and proteins belonging to the photosynthetic machinery resulted differentially regulated

Table 4 | List of unique peptides.

Sample slice	Protein attribution	Log(e) peptide	Peptide sequences	TBLASTN (Drzompo)	*E*-value (Drzompo)	Functional annotation	Mr (kDa)
(A)							
4S	gi|38154488| gb|AY368906|-1 gpmDB [20/28] protein	3.3	MAEAETFAFQAEINQLLSLIINTFYSNK	Zoma_C_c66842	2.00e-08	Heat shock protein 81-3	80.1
4S	gi|38154492| gb|AY368907|-1 gpmDB [21/29] homo (3/3) protein	−6.7	DLVLLLFETALLTSGFSLEEPNTFGNR	Pooc_Contig353	1.00e-10	Heat shock cognate protein 80	80.1
7S	At3g23810.1 gpmDB [83/146] homo (10/10) protein	−4.2	WVFPDTNSGIIVLAEGR	Zoma_C_c68954	0.001	Adenosylhomocysteinase 1	53.1
7S	gi|119350|sp|P25696.1	−2.8	SGETEDTFIADLAVGLSTGQIK	Zoma_C_c67987	3	Enolase 1	47.9
7S	At2g28000.1 gpmDB [61/93] homo (5/5) protein	−10	GGYPILIIAEDIEQEALATLVVNK	Zoma_C_c58171	2	RuBisCO large subunit-binding protein subunit beta, chloroplastic	62.9
8S	ATCG00490.1 gpmDB [74/111] homo (0/33)protein	−56.9	TFQGPPHGIQVER	Pooc_B_rp6_D3_R	2.7	Auxin response factor 24	50.5
10S	gi|48752579| gb|CO083098|-3 gpmDB [10/11] homo (1/1) protein	−3.1	TLLVSAPGLGDYISGAILFEETLYQSTIDGK	Pooc_PC035C04	3.00e-00	Fructose-bisphosphate aldolase, chloroplastic	32.7
10S	gi|156725011| gb|EV229122|-1 gpmDB [1/7] homo (2/93) protein	−30.2	FGIVEGLMTTVHSITATQK	Pooc_Contig14	6e-05	Glyceraldehyde-3-phosphate dehydrogenase	54.7
10S	gi|82621107| gb|DQ284454|-3 gpmDB [8/10] homo (0/3) protein	−8.6	NDLEFAKKLASLADLYVNDAFGTAHR	Pooc_PC021E08	2.00e+00	Phosphoglycerate kinase, cytosolic	52.1
10S	gi|150162092| gb|EE553762|-1 gpmDB [0/1] protein	−2.4	DALFKHANIKPIITSTVWK	Pooc_PC044A11	0.22	Plant protein 1589 of unknown function	16.8
12S	gi|73880486| gb|DT483224|3 gpmDB [0/1] homo (5/5) protein	−1.9	NPLNYTQVSVLADDILK	Zoma_C_c45955	0.004	ATP synthase subunit gamma, mitochondrial	32.7
15S	Pooc_B_rp7_C5_R_6	−12.7	NSPNSFDPLGLAEDPEAFAELK	Pooc_B_rp7_C5_R	2.00e-08	Chlorophyll a-b-binding protein 40, chloroplastic	31.6
16S	gi|52390306| gb|CV233595|3 gpmDB [0/1] homo (12/12)	−2.7	LTGTDVGYPGGLWFDPLGWGSGSPEK	Pooc_B_c320	0.28	Chlorophyll a-b-binding protein CP24 10A, chloroplastic	26.6
16S	gi|48389884| gb|CN917384|-3 gpmDB [9/10] protein	−2.9	NEVPVISPEQLAEADGIIFGFPTR	Zoma_C_c56810	2	Flavoprotein wrbA	22.7
17S	gi|38605705|sp|P05642.2|	−3.1	IVTGVPEAIPVIGSPLVELLR	Zoma_C_c33944	9	Cytochrome b6	33.4
17S	gi|73875282| gb|DT478020|-3 gpmDB [0/1] homo (2/2) protein	−6	IVIGLFGDDVPQTAENFR	Pooc_PC015E05	8	Peptidyl-prolyl cis-trans isomerase CYP20-1	31.3

(Continued)

Table 4 | Continued

Sample slice	Protein attribution	Log(e) peptide	Peptide sequences	TBLASTN (Drzompo)	*E*-value (Drzompo)	Functional annotation	Mr (kDa)
(B)							
4D	sp|Q6L509|Q6L509_ORYSA	−11.2	IINEPTAAAIAYGLDK	Zoma_C_c66491	0.005	Heat shock cognate 70 kDa protein 2	70
6D	gi|116010686| gb|AK241321|-2	−1.8	GGECVGGGGGGGGGGAEAR	Pooc_Contig164	0.0000001	60S ribosomal protein L9	88
6D	gi|194694909|gb|BT036534|-3	−1.8	GDVADGVFLGHADWPR	Pooc_B_c93	9.6	Probable eukaryotic translation initiation factor 5-2	51.6
7D	At2g28000.1 gpmDB [72/113] homo (6/19) protein	−9.3	APLLIIAEDVTGEALATLVVNK	Zoma_C_c57597	0.00003	RuBisCO large subunit-binding protein subunit alpha	52.3
10D	gi|187950292| gb|AY103880|-3 gpmDB [0/1] protein	−4.8	LVDTNGAGDAFVGGFLSQLVLGK	Pooc_PC010D04	0.87	Fructokinase-2	55.5
12D	gi|34959481| gb|CA106174|-2 gpmDB [0/1] protein	−1.7	AARPPPAGTPPPR	Pooc_PC019C02	0.26	Putative low molecular weight protein-tyrosine-phosphatase slr0328	28.5
13D	At5g65430.1gpmDB [53/81]homo (38/38) protein	−3.7	QAFEEAIAELDTLGEESYK	Pooc_PC039D11	0.00001	14-3-3-like protein C	28
14D	At4g10340.1gpmDB [40/73]homo (12/12) protein	−4.1	TGALLLDGNTLNYFGK	Pooc_Contig159	0.00005	Chlorophyll a-b-binding protein CP26, chloroplastic	30.1
14D	gi|110373880|gb|EC938302|3 gpmDB [0/3] homo (0/7) protein	−14	QEDIDGFLVGGASLK	Zoma_C_c59451	0.028	Triosephosphate isomerase, chloroplastic	34.5
15D	gi|45990591| gb|CN149099|-3gpmDB [0/5]homo (9/51) protein	−12	TDEFPGDYGWDTAGLSADPETFAK	Pooc_B_c360	1.00e-09	Chlorophyll a-b-binding protein of LHCII type I	33
15D	gi|73873524| gb|DT476262|2 gpmDB [0/6] homo (0/14) protein	−10.8	SEIPEYLTGEVPGDYGYDPFGLSK		0.00004		
15D	Zoma_C_c15686_5	−10	ELEVIHTRWAMLGTLGCVFPELLSR		0.0006		
15D	gi|157980300| gb|EX528572|-3 gpmDB [0/12] homo (0/15) protein	−29.5	YLGSFSGEAPSYLTGEFPGDYGWD TAGLSADPETFAK		1.00e-15		
15D	Zoma_C_c34383_5	−10.7	EPNSIFGVGGITMRRNTVK	Pooc_B_c132	0.005	Chlorophyll a-b-binding protein 21, chloroplastic	28.2
16D	Pooc_Contig333_3	−21.8	SKVEDGIFGTSGGIGFTK	Pooc_Contig333	0.00003	Photosystem II 22 kDa protein, chloroplastic	29.3

(Continued)

Table 4 | Continued

Sample slice	Protein attribution	Log(e) peptide	Peptide sequences	TBLASTN (Drzompo)	*E*-value (Drzompo)	Functional annotation	Mr (kDa)
16D		−10.3	VAMLGFAASIFGEAITGK		0.00003		
18D	Pooc_PC011B10	−18	TEIEGDGGVGTTTK	Pooc_PC011B10	0.001	14 kDa proline-rich protein DC2.15	14.3
18D	Pooc_Contig48_2	−11.5	VWDFCGSSQLMQLLPK	Pooc_Contig48	0.000002	S-norcoclaurine synthase	23.3
		−10.2	QIEGGHLDLGFLSSHSR		0.000007		
	Pooc_Contig132_3	−18	VWDFCASCQLMQLLPK	Pooc_Contig132	7.00e-07		
21D	At5g38430.1 gpmDB [31/51] homo (6/6) protein	−3	EHGNTPGYYDGR	Pooc_Contig3	0.001	Ribulose bisphosphate carboxylase small chain SSU5B, chloroplastic	18.9
22D	LOC_Os01g61920.1 homo (267/267)	−2.5	TVTAMDVVYALK	Pooc_B_rp10_E10_R	0.018	Histone H4	11.4
23D	gi|27548338| gb|CA766549|	−3.3	FDSLEQLDEFSR	Zoma_C_c60643	1.6	Cytochrome b559 subunit alpha	9.3

Log (e): the base-10 log of the expectation that any particular peptide assignment was made at random.

List of unique peptides identified within the 23 slices of 1DE gel of proteins from (A) shallow samples and (B) deep samples. In the table are shown: the protein attribution obtained by GPM and X!TANDEM softwares with the corresponding log(e) values, the peptide sequence, the annotation obtained with TBLASTN search against Dr.Zompo database, with the corresponding E-values and putative functions, the molecular mass of the protein sequence, in kilodaltons.

FIGURE 4 | Venn diagrams. Venn diagrams comparing the total protein discovery with those found only in shallow or in deep samples. Among the 64 newly identified proteins, 17 are exclusive of shallow samples and 18 of the deep ones.

between stands, in order to perform photosynthesis under such different light conditions.

Transcriptional and proteomic profiles showed high differentiation on Chlorophyll a-b-binding (*Cab*) proteins between the two depths. An increase of Chlorophyll concentration under low-light was reported for other seagrasses (Dennison, 1990; Sharon et al., 2011). In *P. oceanica* chlorophyll rate was reported to vary not only along the depth gradient, but also during different seasons (Pirc, 1986). In addition, differences among *Cab* proteins identified between depths, suggest that in *P. oceanica* different *Cab* proteins are utilized for the assembly of the antenna complex, in response to specific photo-acclimation processes. It seems that, to prevent photo-damage due to high-light, plants evolved different strategies, such as the shrinking of PSII antenna size (Escoubas et al., 1995) and thermal dissipation (Elrad et al., 2002). Changes in antenna pigments compositions in low- light were also suggested for *P. oceanica* and for other seagrasses by Casazza and Mazzella (2002).

The relative quantity of transcripts and proteins recognized in this study also suggests an increase in PSII and PSI transcripts in deep plants in respect to the shallow ones (especially as regards as PSI). Photosynthetic-organisms balance electron flow between the two photosystems by modulating both antenna size and photosystem stoichiometry (Chitnis, 2001), in response to light intensity and quality. The redox status of the whole cell and of the chloroplast and the ratio between ATP and NADPH could also contribute in modulating PSI/II relative abundance (Chitnis, 2001). PSI/II ratio was found modified across depth also in the seagrass *Halophila stipulacea* (Sharon et al., 2011), in macroalgae (Fujita, 1997; Yamazaki et al., 2005) and cyanobacteria (Levitan et al., 2010) as to indicate that this could be a general photo-acclimatory mechanism. At the present, we are not able to explain the regulative mechanisms underlying this differential modulation between shallow and deep plants, but similar patterns of PSI/II ratio were already observed in shallow *P. oceanica* meadows growing under different light conditions (Mazzuca et al., 2009). Authors reported a reorganization of the thylakoid architecture under low-light conditions, that is consistent with the rearrangement between the two photosystems, since approximately 85% of PSII is located in the apprised domains of the grana and 64% of PSI is located in the stroma lamellae.

Another interesting hint suggested from our data for the *P. oceanica* photosynthetic acclimation involves the enzyme RuBisCo. The expression pattern of this enzyme between the two light conditions was different from the expectation: we measured, in fact, a similar content of this protein between shallow and deep stations, with a slightly higher abundance in low-light, especially for what concern the large subunit. This is in contrast with previous results, where Mazzuca et al. (2009) showed a clear decrease of the same protein in low-light condition in *P. oceanica*. The activity of RuBisCo responds to different environmental signals including light, changes in source-sin balance, temperature and circadian rhythms [reviewed in Portis (2003)]. However, regulation of RuBisCo is mediated, among others, by the activity of the chaperone Ribulose bisphosphate carboxylase/oxygenase activase A (RCA). This protein was identified in our collection as over-expressed, even if at low levels, in low-light condition. RCA is thought to have a key role in the regulation of photosynthesis under different environmental stress conditions (Portis, 2003) and during the daily cycle (Yin et al., 2010). In a recently study of Yamori et al. (2012) it was reported that in low-light condition, high expression of RCA contributes to maintain RuBisCo in high active state, helping in assuring high levels of CO_2 assimilation also under shade conditions. These observations open the question regarding the real regulation mechanism of RuBisCo in *P. oceanica* in response to light, especially for what regards limiting light conditions.

CELLULAR ENERGETIC METABOLISM

For what concerns respiration, an overall increase of related transcripts and proteins was recorded in shallow plants, probably related to the higher temperature present in respect to the deeper portion of the meadow plants [overview in Touchette and Burkholder (2000)]. Nevertheless, considering separately the regulation of each of the three main stages of the respiratory process, we see that glycolysis and electron transport chain steps were strongly enhanced in high light, while the tricarboxylic acid (TCA) cycle was higher in low light.

The understanding of the regulations of these pathways in plants is further complicated by the interactions between them and many other key elements (Fernie et al., 2004). Among the putative regulatory enzymes of mitochondrial activity (Bunney et al., 2001), a protein like 14-3-3 was recognized in our peptide collections. Collectively, plant 14-3-3s isoforms, which bind to phosphorylated client proteins to modulate their function, are implicated in an expanding catalogue of physiological functions and are affected by the extracellular and intracellular environment of the plant. They play a central role in the response to the plant extracellular environment, particularly environmental stress, pathogens, and light conditions (Denison et al., 2011).

STRESS RESPONSE

Several transcripts encoding for proteins associated with stress response and plant defense were detected in low-light. Amongst these, metallothionein-like protein, which are implicated in metal tolerance in plants (Cobbett, 2000), Catalase and Oxygen-evolving enhancer proteins, which respond to reactive oxygen species (ROS) stress and are responsible for the breakdown of hydrogen peroxide to oxygen and water (Blokhina, 2003) and also the Cytochromes P450 family, which is implicated in detoxification. It is known that *P. oceanica* may accumulate metals from the sediment in its organs and tissues (Warnau et al., 1996; Schlacher-Hoenlinger and Schlacher, 1998) and the study by Giordani et al. (2000) have demonstrated that treatments with Mercury, Copper and Cadmium may induce the production of Metallothionein proteins in this species. Moreover, in the deep plants several transcripts encoding for Zinc finger domain stress-associated proteins and the 2-caffeic-acido-methyl transferase, were also found. The same proteins were also previously recognized (Mazzuca et al., 2009) in *P. oceanica* in similar environmental condition and associated to biotic and abiotic stress response (Cozza et al., 2006).

All these elements suggest that plants living in the deep stands are more sensitive to oxidative stress than plants growing in shallow stands, due to the higher investment by the former in maintaining basal metabolism and the consequent lower resources available for cell defense and repair. In addition, deep plants could also respond to exogenous oxidative stress due to the local distribution of stressing factors, which seem to be more important in the area of the bay where the deep stand is growing.

PROTEIN TURNOVER

Many clones with sequence homology on components of the (Ub)Ubiquitin-26S proteasome pathway were identified in both ESTs collections. This degradation pathway is involved in the removal of abnormal polypeptides throughout normal protein turnover, and provides the degradation of enzymes and key regulatory factors of signal transduction, making it one of the most elaborate regulatory mechanisms in plants, allowing cells to respond rapidly to signal molecules and changes in environmental conditions (Gagne et al., 2004; Moon et al., 2004). Higher expression level of ubiquitin/26S proteins was already found in *P. oceanica* as consequence of plants acclimation to low-light conditions (Mazzuca et al., 2009). Three components of this complex witch appeared to be more expressed in high-light condition in comparison with low-light are involved in "protein-targeting": the E3 ubiquitin-protein ligase, a U-box and RING-box protein and the SCF-E3, F-Box protein (Moon et al., 2004). The participation of SCFs in plant development is extensive, affecting processes such as hormone response, photo-morphogenesis, circadian rhythms, floral development, and senescence (Du et al., 2009). Moreover, several studies support that F-box proteins such as SCF E3, are also involved in phyA-mediated light signaling and in the regulation of circadian clock, making it possible that SCF proteins degrade a repressor of light response in preparation for light signals at dawn (Harmon and Kay, 2003).

Furthermore, plants growing at the different depths appear to respond not only to different environmental signals, but also to different endogenous signaling, such as hormones. In shallow plants, several component of the Ethylene signaling pathway were detected, such as the above mentioned F-box proteins. At present, information exists on the functions of a relatively small number of F-box proteins and most of these are involved in regulation of the hormone signaling pathway.

The role of the SCF is to degrade repressors of hormone response (auxin, GA, and JA), whereas in response to ethylene, the SCF degrades positive regulators in the absence of the hormone. The existing data strongly suggest that the principal control point of Ethylene signaling regulation is protein degradation via the ubiquitin/26S proteasome pathway (Potuschak et al., 2003; Kendrick and Chang, 2009). Ethylene is an important gaseous hormone's regulator in several plants processes, as (i) the regulation of endogenous rhythms, e.g., seed germination, plant growth, leaf expansion, root hair formation, fruit ripening, and timing of vegetative senescence and (ii) the transduction of environmental signaling, e.g., responses to abiotic stresses and pathogen attack (Potuschak et al., 2003; Raab et al., 2009). According to these indications, the different activity of ubiquitin-mediated proteolysis recognized between shallow and deep growing plants of *P. oceanica* could depend from the different seasonal timing at which they respond. Buia and Mazzella (1991) previously observed in the seagrasses *P. oceanica*, *Cymodocea nodosa* and *Zostera noltii*, a clear shift in life cycle between plants growing in shallow and deep stands in the Mediterranean Sea, with the effect that shallow plants (−5 m) complete their annual cycle in early summer, turning into senescence, while at the same time plants of the deep stands (−25 m) are fully growing. Our data about the photo-acclimation response of *P. oceanica* along the bathymetric gradient probably also reflect the different adjustments in life cycle during the year of plants growing at different depths. This allows plants to growth, optimizing the harvesting and the utilization of the available light in the different seasonal conditions and to minimize the negative effects due to photo-damage.

In conclusion, this study allowed to identify several regulatory networks and metabolic pathways involved in environmental signals response along the depth distribution of *P. oceanica*, and allowed to improve the available molecular resources, which is an important requisite for the application of eco-genomic approaches in this species.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/PlantProteomics/10.3389/fpls.2013.00195/abstract

Figure S1 | RT-qPCR SSH library. Expression levels of Pooc_B_c42 and Pooc_B_c 217 (y-axis, Mean ± SD) in *Posidonia oceanica* shoots collected at −25 m (deep-library). Shoots collected at −5 m (shallo-library) were used as control and the expression level of GOI in the control is represented in the figure by the x-axis. RT-qPCR data were normalized **(A)** with the best RGs in this experimental condition (EF1A, L23, NTUBC, Serra et al., 2012b), **(B)** using "universal" RGs (EF1A, NTUBC, 18S, and UBI) (*$p < 0.05$, ***$p < 0.001$). Additional information on primers sequences and RT-qPCR conditions in Serra et al. (2012b).

Table S1 | Additional statistics features of SSH–EST library. Additional statistics features of SSH–ESTs *Posidonia oceanica* library.

Table S2a | List of Unigenes belonging to the shallow (high-light) library. List of Unigenes belonging to the shallow (high-light) library. Unigenes name, their functional annotation with the *E*-value, number of ESTs identified (S_EST) and sequences lengths (Length) are indicated. For each sequence, the presence of putative ORF (open reading frame), SSRs (simple sequences repeats) and SNPs (single-nucleotide polymorphisms) are also showed.

Table S2b | List of Unigenes belonging to the deep (low-light) library. List of Unigenes belonging to the deep (low-light) library. Unigenes name, their functional annotation with the *E*-value, number of ESTs identified (S_EST) and sequences lengths (Length) are indicated. For each sequence, the presence of putative ORF (open reading frame), SSRs (simple sequences repeats) and SNPs (single-nucleotide polymorphisms) are also showed.

Table S3a | List of peptides identified in the 1DE gel of proteins from shallow samples (S). List of peptides identified in the slices of 1DE gel of proteins from shallow samples (S), the protein attribution obtained with GPM and X!TANDEM sotfwares with the corresponding log(e) value, functional annotation obtained with TBLASTN search against Dr.Zompo database and corresponding *E*-value are shown.

Table S3b | List of peptides identified 1DE gel of proteins from deep samples (D). List of peptides identified in the slices of 1DE gel of proteins from deep samples (D), the protein attribution obtained with GPM and X!TANDEM softwares with the corresponding log(e) value, functional annotation obtained with TBLASTN search against Dr.Zompo database and corresponding *E*-value are shown.

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Response of the seagrass *Posidonia oceanica* to different light environments: Insights from a combined molecular and photo-physiological study

E. Dattolo [a, *, 2], M. Ruocco [a, 1, 2], C. Brunet [a], M. Lorenti [a], C. Lauritano [a], D. D'Esposito [a], P. De Luca [a], R. Sanges [a], S. Mazzuca [b], G. Procaccini [a]

[a] *Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy*
[b] *Laboratorio di Proteomica, Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria, Arcavacata di Rende 87036, Italy*

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ABSTRACT

Here we investigated mechanisms underlying the acclimation to light in the marine angiosperm *Posidonia oceanica*, along its bathymetric distribution (at −5 m and −25 m), combining molecular and photo-physiological approaches. Analyses were performed during two seasons, summer and autumn, in a meadow located in the Island of Ischia (Gulf of Naples, Italy), where a genetic distinction between plants growing above and below the summer thermocline was previously revealed. At molecular level, analyses carried out using cDNA-microarray and RT-qPCR, revealed the *up*-regulation of genes involved in photoacclimation (RuBisCO, ferredoxin, chlorophyll binding proteins), and photoprotection (antioxidant enzymes, xanthophyll-cycle related genes, tocopherol biosynthesis) in the upper stand of the meadow, indicating that shallow plants are under stressful light conditions. However, the lack of photodamage, indicates the successful activation of defense mechanisms. This conclusion is also supported by several responses at physiological level as the lower antenna size, the higher number of reaction centers and the higher xanthophyll cycle pigment pool, which are common plant responses to high-light adaptation/acclimation.

Deep plants, despite the lower available light, seem to be not light-limited, thanks to some shade-adaptation strategies (e.g. higher antenna size, lower E_k values). Furthermore, also at the molecular level there were no signs of stress response, indicating that, although the lower energy available, low-light environments are more favorable for *P. oceanica* growth.

Globally, results of whole transcriptome analysis displayed two distinct gene expression signatures related to depth distribution, reflecting the different light-adaptation strategies adopted by *P. oceanica* along the depth gradient. This observation, also taking into account the genetic disjunction of clones along the bathymetry, might have important implications for micro-evolutionary processes happening at meadow scale. Further investigations in controlled conditions must be performed to respond to these questions.

1. Introduction

Marine angiosperms (*seagrasses*) are able to grow from the surface to ~50 m depth (Duarte, 1991), although records exist for their distribution down to 90 m (see Dalla Via et al., 1998 and references therein). The deeper limit of seagrass distribution depends on specific features of single species and correlates with the local light attenuation coefficients, influenced by both natural and human driven processes (Duarte, 1991; Ralph et al., 2007). Irradiance decreases exponentially along the water column, and also the light spectral quality is rapidly altered, shifting from the sunlight spectrum into a narrow band of blue-green light, due to water absorption and scattering processes (Kirk, 2011). In the clearest oceanic waters, blue light reaches the greatest depth, while in the coastal shallow water, with high scattering, light attenuates first and green light penetrates deeper (Kirk, 2011). Moreover, light is further attenuated due to self-shading inside the

* Corresponding author. Tel.: +39 0815833236.
E-mail addresses: dattolo@szn.it, emanudat@gmail.com (E. Dattolo).
[1] Present address: CCMAR – Centre of Marine Sciences, 8005-139 Faro, Portugal.
[2] These authors made equal contribution to this work.

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seagrass canopy and to epiphytic colonization of leaves (Dalla Via et al., 1998).

In pelagic algae, the single individual, often a single cell, can experience variations of light over a broad range of time and space (Rmiki et al., 1996), and can briskly pass from high to low light environments. Single individuals of benthic photosynthetic organisms, instead, develop the whole life cycle at a stable depth, mainly experiencing light variability related to diurnal and seasonal cycles. Even within a single population, individuals growing at different depths may develop different photo-physiological adaptive traits in order to cope with the different light properties of the specific environment (i.e. ecotypes; Carroll et al., 2007).

Acclimation and adaptation to light gradients occur in seagrasses through a multilevel strategy, which includes changes in (i) meadow structure (Collier et al., 2007, 2009; Olesen et al., 2002; Peralta et al., 2002), (ii) shoot morphology (Dalla Via et al., 1998; Longstaff and Dennison, 1999; Olivé et al., 2013), (iii) leaf-surface features (Enriquez et al., 1992; Durako, 2007), (iv) pigment and protein content (Casazza and Mazzella, 2002; Collier et al., 2008; Mazzuca et al., 2009; Pirc, 1986; Sharon et al., 2009; Silva et al., 2013); (v) ratio between photosystem I and II units (Dattolo et al., 2013; Sharon et al., 2011) and (vi) photosynthetic parameters, such as photosynthetic efficiency (α), maximum electron transport rate (ETR_{max}), and saturating irradiance (E_k) (Campbell et al., 2003; Collier et al., 2009; Larkum et al., 2006; Silva and Santos, 2003). In addition to these responses, seagrasses can alter resource allocation patterns to optimize carbon balance (Alcoverro et al., 2001), and can adapt their reproductive cycle along the bathymetric cline, as observed in Mediterranean species (Buia and Mazzella, 1991).

In seagrasses, the comprehension of the molecular and biochemical mechanisms for regulating light harvesting, carbon utilization and photosynthetic processes is still limited. Besides, there are no studies which relate photo-physiological features and genetic make-up of seagrasses populations, even if the phenotypic plasticity in photosynthetic acclimation is undoubtedly a fundamental trait to take into consideration, for assessing resistance and resilience at population level (Crosbie and Pearce, 1982; Flood et al., 2011). The recent development of molecular resources for different seagrass species (e.g. Franssen et al., 2011; Wissler et al., 2009) has greatly facilitated the investigation of differentially expressed genes in response to environmental variations (Bergmann et al., 2010; Bruno et al., 2010; Gu et al., 2012; Massa et al., 2011; Reusch et al., 2008; Serra et al., 2012; Winters et al., 2011), and first attempts have been already carried out in *Posidonia oceanica*, to couple genomic and eco-physiological approaches (Dattolo et al., 2013; Mazzuca et al., 2013; Procaccini et al., 2012).

The seagrass *Posidonia oceanica* (L.) Delile is endemic of the Mediterranean sea, where it forms extensive monospecific meadows, extending from ~1.0 to 40–50 m depth (Duarte, 1991; Pasqualini et al., 1998). This species supports a highly diverse associate community and is generally known to supply highly valuable ecosystem services as the protection of the coastline from erosion. However, its distribution is undergoing a regression, estimated at 10% over the last 100 years (Pergent et al., 2010), due to the several threats which disturb the coastal ecosystems (Marbà and Duarte, 2010; Waycott et al., 2009).

P. oceanica is hermaphroditic, with sexual reproduction considered overall sporadic (Diaz-Almela et al., 2006). It grows according to a phalanx strategy (Migliaccio et al., 2005) and long living clonal lineages can persist for hundreds of years (Arnaud-Haond et al., 2012; Migliaccio et al., 2005; Procaccini et al., 2007). These features make *P. oceanica* an ideal target for studying plasticity of genotypes in response to changes in environmental conditions (Buia and Mazzella, 1991; Lorenti et al., 2006; Mazzuca et al., 2009; Olesen et al., 2002; Reusch, 2014; Serrano et al., 2011). Moreover, the clear genetic structure existing in *P. oceanica* populations along the depth gradient accounts for inter-genotype competition for colonizing environments characterized by sharp differences in environmental parameters, such as light (D'Esposito et al., 2012; Migliaccio et al., 2005).

This study aims to compare the photoacclimation properties of *P. oceanica* growing in two ecological niches (i.e. in high and low-light environments, −5 and −25 m depth), characterized by differences in light quantity and quality, in order to assess the divergent acclimation mechanisms acting along the depth gradient within a single population. Regulation of metabolic pathways and photo-dependent processes were investigated, through the study of photo-physiological and molecular plant features.

Data were collected in two seasons (summer and autumn) of consecutive years (2010–2011) to investigate plant response in different combination of light and temperature regimes: in summer, when maximum differences in light and temperature exist between the two sampled depths, and in autumn, when only light was different between the two depths, temperature being almost homogeneous along the water column. Together with gene expression analysis, we compared variations in photosynthetic parameters and pigment composition. The molecular information consisted in a large-scale gene expression screening of *P. oceanica* clones growing in the shallow and deep stand of the meadow using cDNA-microarray, in parallel with the assessment of the expression profile of thirteen selected target genes, to specifically investigate the regulation of the molecular machinery of the photosynthesis and photoprotection systems.

2. Materials and methods

2.1. Study area

The study has been performed in the *Posidonia oceanica* meadow located in the Lacco Ameno Bay (Island of Ischia, Gulf of Naples, Italy; 40°45′5″ N, 13°53′4″ E), which extends from 1 to about 33 m depth (Buia et al., 1992; Zupo et al., 2006). The site is characterized by a thermal stratification of the water column present from April to early October, with a summer maximal difference of up to 7 °C between −5 and −25 m (Buia et al., 1992). In this meadow, the canopy growing at 5 m depth receives approximately 50% of surface photosynthetically active radiation (PAR), while plants growing at 25 m depth experience about 10% of surface PAR (Buia et al., 1992). The daily period of photosynthesis-saturating irradiance (H_{sat}) at −25 m is about one half the duration of that experienced by plants at −5 m (Lorenti et al., 1995). For both 5 and 25 m depth, light transmission inside the canopy is approximately 10% (Buia et al., 1992).

Samplings have been conducted in summer 2010 (July, 03th, ~12:00), and in early autumn 2011 (September, 29th, ~11:30). Samples were collected from the shallow (−5 m) and the deep portion of the meadow (−25 m).

During the first sampling (2010), temperature and photosynthetic photon flux density (PPFD) were measured using a multiparameter instrument (SBE 16, Sea-Bird Electronics, Bellevue, WA, equipped with a quantum sensor LI-COR mod. LI-193SA) operated from the boat. During the second sampling (2011), PPFD was estimated using a portable quantameter (Biospherical, San Diego, CA, mod. QSI-140B) and temperature was measured by mean of HOBO loggers (Onset Computer Corp., USA), deployed at both depths.

2.2. Photosynthesis measurements and sampling strategy

In order to assess variations in photosynthetic performance of *P. oceanica* at different depths and between different seasons,

photosynthetic parameters were obtained by pulse amplitude modulated (PAM)-fluorometry using a Diving-PAM instrument (Walz, Effeltrich, Germany), operated by SCUBA divers. Measurements of chlorophyll fluorescence were taken *in situ* on the mid portion of the youngest fully mature leaf in the shoot (usually the second-rank leaf). Its photosynthetic performance is relatively high and stable (Modigh et al., 1998) and the production pattern is representative of the whole shoot (Zupo et al., 1997).

Rapid light curves (RLCs) of irradiance *vs.* ETR (electron transport rate past PSII) were obtained by exposing selected leaf spots to a range of irradiances between ~ 10 and 800 $\mu mol/m^{-2}/s^{-1}$, produced by the Diving-PAM lamp and lasting 10 s each.

Photosynthetic parameters (relative maximum electron transport rate, $rETR_{max}$, initial slope of the curve, α and the saturating irradiance, E_k) were calculated by fitting empirical data to an exponential function (Ralph and Gademann, 2005).

In 2010, light-adapted RLCs were performed on *P. oceanica* leaves ($n = 8$ for each depth) without subjecting them to a dark adaptation period. In 2011, the RLCs were performed for leaves after a 10 min-dark-adaptation period ($n = 5$ for each depth). Thanks to this procedure, in autumn, we also estimated the F_v/F_m ratio (maximum photosynthetic efficiency of PSII), and the maximum capacity of non-photochemical quenching (NPQ_{max}) developed along the RLC (Dimier et al., 2009). NPQ corresponds to the thermal dissipation of excess of energy capacity and was quantified by the Stern-Volmer expression: NPQ = (Fm−Fm')/Fm' (Bilger and Björkman, 1994), where Fm and Fm' are the maximal PSII fluorescence yield, for dark adapted and illuminated leaves, respectively. Average values estimated at the two depths were compared for each sampling season using the Student's *t* test. Significance was determined at $p < 0.05$.

In both seasons (July 2010 and September 2011), the youngest fully mature leaves of individual shoots were collected for molecular and biochemical assays among those utilized for photosynthetic measurements. This allowed a better correlation between photosynthetic performance and biochemical/molecular features.

All samples were collected around midday (between 11:30 and 12:00 h) and placed in black containers to avoid overexposure to full sunlight. On the research vessel, leaf material was rapidly scraped free of epiphytes, towel-dried and shock-frozen in liquid nitrogen (within 15–20 min after collection), prior to storage at −80 °C in the laboratory. This relatively short fixing-time and the comparable time of the day among the different seasons and depths support a direct comparability of the obtained data. The list of all performed analyses, divided per season, is reported in Table 1S.

2.3. cDNA-microarray

As first, we used a cDNA microarray analysis to characterize the whole transcriptome expression of *P. oceanica*, and select pathways more affected by light variation along the depth cline.

2.3.1. Microarray design

The microarray was built using clone's sequences taken from the two ESTs *P. oceanica* libraries presently available and stored in the database Dr. Zompo (http://drzompo.uni-muenster.de/). The whole set of unigenes (495) belonging to the SSH-EST library ("Pooc_B" in Dr. Zompo), which are differentially expressed along the bathymetric gradient (Dattolo et al., 2013), and another set of unigenes (501), selected from a *P. oceanica* non-subtracted EST-library ("Pooc_A" in Dr. Zompo), were included in the microarray. For each unigene, a single EST was used as probe (selected from *P. oceanica* ESTs, Genbank Accession Numbers: GO34959 to GO349047; JZ354020 to JZ356595).

2.3.2. Target preparation

Total RNA was extracted from youngest fully mature leaves ($n = 3$ for each depth), collected in 2010, using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Chang et al., 1993), with some modifications (see Dattolo et al., 2013).

RNA quantity was assessed by Nano-Drop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies), monitoring the absorbance at 260 nm; purity was determined by monitoring the 260/280 nm and 260/230 nm ratios, integrity was tested measuring the RIN number with Agilent RNA 6000 Nano Kit (Agilent 2100 Bioanalyzer) and with 1% denaturing gel electrophoresis. All samples resulted free from proteins and organic solvents used during RNA extraction. Complementary DNA (cDNA) synthesis incorporating dUTP-Cy3 or dUTP-Cy5 was carried out employing the CyScribe first-strand cDNA labeling kit (GE Healthcare, Amersham Biosciences) following the manufacturer's instructions. Equal quantities of labeled cDNA were hybridized using the hybridization solution.

2.3.3. Probe preparation

cDNA clones, stored in phage libraries, were amplified with universal primers T7 and M13 reverse (for primers sequences: pCR2.1-TOPO vector manufacture's protocols. Invitrogen). PCR products were purified and spotted in triplicates on slides. Glass spotting was carried out with the Microarray Spotarray24 (Perkin Elmer) using Micro Spotting Pins (Telechem Corporation, Sunnyvale, CA, United States) on GAPS II Coated Slides. Probe's spotted sequences and their relative *P. oceanica* unigenes are reported in Table 2S (Supplementary material).

2.3.4. Hybridization

Prior to hybridization, microarrays were incubated at 42 °C for 60 min in a pre-hybridization buffer (50% Formamide, 5X SSC, 0.1% SDS and 0.1 mg/ml BSA). Hybridization was performed for 16 h in hybridization buffer (50% Formamide, 5X SSC, 0.1% SDS and 0.1 mg/ml Herring Sperm) at 42 °C, using equal quantities of labeled cDNA. Pre-hybridization and hybridization buffers were removed by several washing with Wash solution 1 (2X SSC + 0.1% SDS in MilliQ H_2O) and Wash solution 2 (0.1X SSC + 0.1% SDS in MilliQ H_2O) and with a last wash in MilliQ H_2O. For both shallow (i.e. high-light condition = S) and deep stand (i.e. low-light condition = D), microarray has been hybridized with three biological replicates (S1-S3; D1–D3), each of them replicated at least four times (i.e. technical replicates), in order to assess the technical variability of each microarray hybridization output (Lee et al., 2000).

2.3.5. Scanning and data analysis

Acquisition and quantification of array images were performed with a Perkin Elmer Scanarray Express confocal microarray scanner. The software package Imagene (Biodiscovery) was used to screen spots and quantify spot signals as fluorescence intensities for each dye channel. According to the software Imagene (Biodiscovery), spots with poor quality and artefacts were excluded from further analysis and only high quality signals were selected to compare the expression profiles among replicates. After discarding low quality replicates, the number of independent technical replicates used for the analysis was the following: S1 = 4; S2 = 5; S3 = 3; D1 = 4; D2 = 5; D3 = 4. To detect differentially regulated genes among shallow and deep samples, differences in the number of replicates were taken into account and significance was assessed using an Unpaired *t* test. A Benjamini and Hochberg false discovery rate (FDR) correction for multiple testing method was applied and only genes with *p*-values < 0.05 were considered significant. In order to illustrate the degree of similarity between depths and among biological replicates, a Principal Component

Analysis (PCA) was performed and data were plotted in a tri-dimensional graph.

A gene ontology (GO) term enrichment analysis was performed to identify biological processes, cellular components and molecular functions involved in acclimation to the different conditions. For each GO class, we compared the proportion of associated genes in the group of significantly differently expressed genes with the same proportion relative to the entire set of genes spotted on the microarray. Proportions were compared using the Fisher's exact test and the *p*-values corrected using the Benjamini and Hochberg method. We considered as significant all the classes showing a significantly higher proportion of genes in the differentially expressed genes group with a corrected *p*-values smaller or equal to 0.1.

2.4. RT-qPCR

A reverse transcription – quantitative Polymerase Chain Reaction (RT-qPCR) analysis has been performed in order to further investigate differences in gene expression of genes belonging to differentially expressed pathways, as resulting from the microarray analysis.

For the summer sampling, RNA from three biological replicates, independent from the ones used for microarrays, was pooled. For the autumn sampling, experiments were conducted on three independent biological replicates per depth, collected from the same shoot replicates on which fluorometric measures were conducted.

2.4.1. RNA extraction and cDNA synthesis

Total RNA was extracted as described in Mazzuca et al. (2013). RNA quantity and purity was assessed by Nanodrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies), RNA quality was evaluated by 1% agarose gel electrophoresis. Total RNA (500 ng) was reverse-transcribed in complementary DNA (cDNA) with the iScript™ cDNA Synthesis Kit (Bio-Rad), following the manufacturer's instructions, using the GeneAmp PCR System 9700 (Perkin Elmer).

2.4.2. Selection of target genes and primer design

For RT-qPCR analysis, we selected thirteen genes encoding for the photosynthetic and the photoprotection molecular machineries (Table 1): (i) four genes from the *Lhc* gene family: Chlorophyll *a-b* binding protein 6A (*CAB-6A*), Chlorophyll *a-b* binding protein 151 (*CAB-151*), Chlorophyll *a-b* binding protein 4 (*LHCA4*) and Chlorophyll *a-b* binding protein CP29.2 (*LHCB4.2*); (ii) three genes belonging to Photosystem II: PSII 22 kDa protein (*PSBS*), PSII protein D1 (*psbA*) and PSII protein D2 (*psbD*); (iii) two genes belonging to Photosystem I: PSI reaction center subunit IX (*psaJ*) and PSI reaction center subunit V (*PSAG*); (iv) the ferredoxin (*SEND33*), and the small subunit of RuBisCO (*SSU5B*) and (v) two genes involved in the photoprotective cycles (due to xanthophyll and tocopherols respectively): Zeaxanthin epoxidase (*ZEP*) and a Homogentisate phytyltransferase 1 (*HPT1*). Twelve genes were already described in Mazzuca et al. (2013).

For two genes (*psbA* and *psbD*), primers were designed on conserved regions of the seagrass *Zostera marina* and other related species. For the other eleven genes, primers were designed on *P. oceanica* ESTs sequences present in Genbank (Table 1); Swiss-Prot and TAIR (The Arabidopsis Information Resource) best scoring hits obtained through BLASTX searches are reported in Table 3S. For all target genes, primers were designed using the software Primer3 v. 0.4.0 (http://frodo.wi.mit.edu/primer3/). Gene Runner v. 3.05 (Hasting Software) was used to predict primer melting temperature (T*m*) and secondary structures. All cDNA amplicons ranged from 100 to 200 bp in size in order to ensure similar PCR efficiency.

PCR conditions were optimized on a GeneAmp PCR System 9700 (Perkin Elmer). For a detailed description, see Serra et al. (2012). Amplification efficiency (*E*) for all primer pairs has been calculated from the slopes of standard curves of the threshold cycle (Ct) *vs.* cDNA concentration, with the equation $E = 10^{-1/\text{slope}} - 1$. All $E > 92\%$ and $R^2 > 0.96$.

Table 1
List of target genes selected for RT-qPCR experiment. Gene and protein names, biological process, GenBank Accession Number, primer sequences, amplicon size (S, base pair), percent efficiency (*E*), and correlation coefficient (R^2) are shown.

Gene	Protein	Biological process	GenBank	Primer sequence 5′→3′	S	*E*	R^2
psbA	Photosystem II protein D1	Photosynthesis	KC954695	F:GACTGCAATTTTAGAGAGACGC R:CAGAAGTTGCAGTCAATAAGGTAG	137	92%	0.99
psbD	Photosystem II protein D2	Photosynthesis	KC954696	F:CCGCTTTTGGTCACAAATCT R:CGGATTTCCTGCGAAACGAA	162	100%	0.98
PSBS	Photosystem II 22 kDa protein, chloroplastic	Photosynthesis	GO346095.1	F:CCGCTCCTGTTGTTCTTCAT R:GGACCTCCTTCCTTGAGACC	158	100%	0.99
psaJ	Photosystem I reaction center subunit IX	Photosynthesis	GO346974.1	F:GGTTTGGGTCTTTAGCAGGTC R:GAATGGGTGGGAGGAGAAAT	160	98%	0.99
PSAG[a]	Photosystem I reaction center subunit V, chloroplastic	Photosynthesis	GO348645.1	F:CTATGTGCTTGCTACGTCCAG R:TCAAACAAACCACCAGCATC	187	100%	0.99
LHCB4.2	Chlorophyll *a-b* binding protein CP29.2, chloroplastic	Photosynthesis	GO346860.1	F:TCGAACACTTGACGGTGGTA R:ACGCTTCAGTTGGCTGAGAT	195	100%	0.98
CAB-151[a]	Chlorophyll *a-b* binding protein 151, chloroplastic	Photosynthesis	GO347467.1	F:AAGCCCATTAGCACAACCTG R:GGGCAATGCTTGGTACTCTC	199	93%	0.99
CAB-6A[a]	Chlorophyll *a-b* binding protein 6A, chloroplastic	Photosynthesis	GO346691.1	F:CGACCGTTCTTGATCTCCTT R:AGTTCATCACCATCGCCTTC	154	96%	0.99
LHCA4[a]	Chlorophyll *a-b* binding protein 4, chloroplastic	Photosynthesis	GO347781.1	F:GGTCCAACACAACGTGACAG R:GACCTCCCTTGGAACCTTTC	200	100%	0.98
SEND33[a]	Ferredoxin, chloroplastic	Electron transport	GO348399.1	F:TCAGACTGGGGGTAAGCAAC R:TCTACATCCTCGACCACTGC	187	100%	0.98
SSU5B	RuBisCO small subunit	Carbon dioxide fixation	GO346679.1	F:AGCATGGTAGCACCCTTCAC R:GGGGGAGGTATGAGAAGGTC	169	100%	0.99
ZEP	Zeaxanthin epoxidase, chloroplastic	Xanthophyll cycle	GO348250.1	F:TGCTCCAGAGAAAGCCAGTT R:TGGCATCCCCAAATGTTATA	197	100%	0.96
HPT1	Putative Homogentisate phytyltransferase 1	Lipid metabolic process	JZ356529	F:CCACTAGCTTTGTCGCCTTC R:ATGGTGTCTGGGGGAGGTAT	185	100%	0.99

[a] EST reverse/complement. Gene names according to Swiss-prot best hit.

2.4.3. RT-qPCR

RT-qPCR was performed as described in Mazzuca et al. (2013). All reactions were conducted in triplicate to capture intra-assay variability and each assay included three no-template negative controls (NTC) for each primer's pair.

Relative expression of each target gene was calculated using the Relative Expression Software Tool REST 2009, version 2.0.13 (Pfaffl et al., 2002), which uses the hypothesis test *P(H1)* to determine significant differences between controls and targets. This software provides proper error propagation and robust statistical analysis by using a random reallocation algorithm with 10,000 iterations. Relative gene expression in plants collected in the shallow stand was analyzed using samples collected in the deep stand as control. L23 (GenBank: GO347779), EF1A (GenBank: GO346663) and NTUBC2 (GenBank: GO347619) were used as reference genes (RGs) for the relative quantification because they have been previously identified as the most stable RGs in *P. oceanica* in the same experimental conditions (Serra et al., 2012).

2.5. Pigment composition and short-term incubation in high light

In order to characterize the pigment pool of plants under different light regimes, during the second sampling campaign (September, 2011) four replicate shoots were collected at each depth, selected among those used also for photosynthetic measurements.

Additionally, a short high-light incubation experiment was conducted on three leaves from each batch of shallow and deep shoots collected for the other analysis. Leaf fragments were incubated on board in seawater to full sunlight ($\approx$1200 μmol photons m^{-2} sec^{-1}) for 20 min before freezing, in order to investigate the photoprotective xanthophyll cycle activation and compare the fast pigment reactions to high-light stress (pigments were extracted after pooling the three leaf replicates from each depth).

For both field and high-light exposure, photosynthetic pigment identification and quantification were accomplished using high-performance liquid chromatography (HPLC). Samples were stored in complete darkness at −80 °C until the chromatographic analysis. Pigments from frozen leaf pieces of approximately 2.5–4 cm were extracted with a mini potter in 3 ml 100% methanol in dim light. 300 μl of ammonium acetate (1M) was added to 1 ml of filtered extract. After 5 min at 4 °C, the extract was injected in a Hewlett Packard Series 1100 HPLC. A 3-μm C_8 BDS column (100 × 4.6 mm) was used and the mobile phase was composed of two solvent mixtures: A (methanol: aqueous ammonium acetate, 70:30) and B (methanol). The gradient between the solvents was the same as in Vidussi et al. (1996). Pigments were detected at 440 nm using an HP photodiode array detector Model DAD Series 1100, which gives the 400–700 nm spectrum for each detected pigment. Single pigments were identified and quantified using chlorophyll and carotenoid standards obtained from the VKI (Water Quality Institute, International Agency for ^{14}C Determination, Denmark). Average values estimated at the two depths were compared using the Student's *t* test. Significance was determined at $p < 0.05$. Statistical analysis was not possible for pooled samples (high-light experiment).

3. Results

3.1. Environmental conditions

Temperature at 5 m depth in the Lacco Ameno *P. oceanica* meadow reached 26.8 °C in July 2010, with a difference in respect to 25 m depth of about 8 °C (18.9 °C). In September 2011, temperatures averaged 23.6 and 22.6 °C at the shallow and at the deep station, respectively.

In July, photosynthetically active radiation (PAR) above the leaf canopies was 703 and 100 μmol/m^{-2}/s^{-1} at 5 and 25 m depth, respectively. In September, PAR was 370 and 150 μmol/m^{-2}/s^{-1} at 5 and 25 m depth, respectively. Note that the higher value of PAR in September compared to July at the deep station, is reflecting the higher clarity of the water column, that generally occurs in early autumn in the center of Lacco Ameno bay, while the relatively low value at −5 m is seemingly due to a near shore turbidity event at the moment of the sampling.

3.2. Photosynthetic properties

In July 2010, the relative maximal electron transport rate value ($rETR_{max}$), as well as the photosynthetic efficiency at sub-saturating irradiance (α), was similar between the two sampled depths, while the light saturation of photosynthesis (E_k) was significantly higher in the shallow stand (24 ± 5 *vs.* 17 ± 4, $p < 0.01$, $n = 8$; Fig. 1 and Table 2). In September 2011, no significant differences in photosynthetic parameters ($rETR_{max}$, α and E_k) were recorded among *P. oceanica* collected at 5 m and 25 m depth.

The non-photochemical quenching response (NPQ_{max}), measured on autumn samples (September, 2011), was significantly higher in the deeper meadow than in the shallow one (0.34 ± 0.056 *vs.* 0.19 ± 0.072, $n = 5$, $p < 0.01$; Table 2), indicating a different capacity of thermal dissipation of excess energy between plants living in shallow and in deep conditions. Values of F_v/F_m in September for −5 m and −25 m plants (0.77 ± 0.008 *vs.* 0.76 ± 0.005; Table 2) were typical for the season (Lorenti et al., 2006) and indicate a similar level of efficiency in the utilization of absorbed radiation between the two depths.

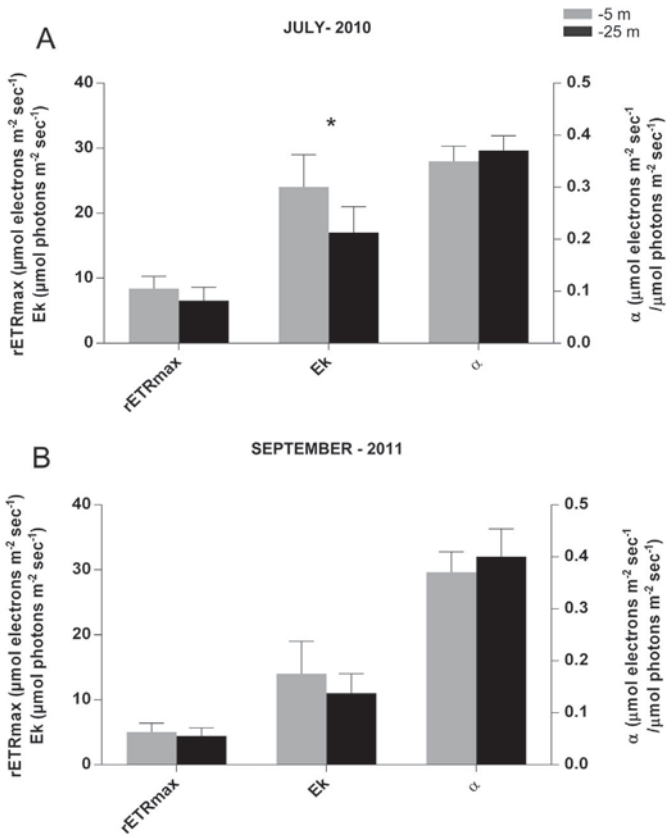

Fig. 1. Photosynthetic parameters. Photosynthetic parameters ($rETR_{max}$, E_k, and α) measured at −5 m (gray bars) and −25 m (black bars). Panels (A) and (B) refer to sampling of July 2010 (summer) and September 2011 (autumn), respectively. *$p < 0.05$.

Table 2
Rapid Light Curve parameters obtained for the two samplings performed in 2010 (July, light-adapted RLCs) and 2011 (September, dark-adapted RLCs). $rETR_{max}$ (relative maximum electron transport rate, μmol electrons m^{-2} sec^{-1}), α (initial slope of the ETR-I curve μmol electrons m^{-2} sec^{-1}/μmol photons m^{-2} sec^{-1}), E_k (saturating irradiance, μmol photons m^{-2} sec^{-1}), F_v/F_m and NPQ_{max} (non-photochemical quenching) values are shown for each depth. Values are averages ±SD ($n = 8$ for 2010, and $n = 5$ for 2011).

	Depth	$rETR_{max}$	α	E_k	F_v/F_m	NPQ_{max}
2010	−5 m	8.4 ± 1.9	0.35 ± 0.029	24 ± 5	–	–
	−25 m	6.5 ± 2.1	0.37 ± 0.029	17 ± 4	–	–
2011	−5 m	5.0 ± 1.4	0.37 ± 0.040	14 ± 5	0.77 ± 0.008	0.19 ± 0.072
	−25 m	4.4 ± 1.3	0.40 ± 0.054	11 ± 3	0.76 ± 0.005	0.34 ± 0.056

3.3. Gene expression pattern by microarray

To determine inter- and intra-site variations in gene expression profiles of plants collected at the two depths, a Principal Component Analysis (PCA) was performed on the whole set of genes spotted on the microarray. Gene expression patterns of shallow (blue dots) and deep plants (red dots) appeared quite distinct (Fig. 2). Plants collected in high light showed a higher degree of homogeneity in comparison to samples collected in low light. In the Heat map of Fig. 1S, shallow and deep samples cluster separately, although one biological replicate within each condition (D3 in the Deep samples and S3 in the Shallow samples), is quite different from the other replicates of the same condition.

About the 50% (497 over 996) of all contigs spotted on the microarray showed significant differences in expression level between depths, and using shallow as tester condition in relation to deep, used as control condition, we found that 379 contigs (38%) were *up*- and 118 contigs (12%) were *down*-regulated in shallow samples (Fig. 3). A full list of the differentially regulated contigs can be retrieved from the Supplementary material (Table 4S).

Most of the differentially regulated contigs (67%) derived from the *P. oceanica* SSH-EST library (Dattolo et al., 2013). Among

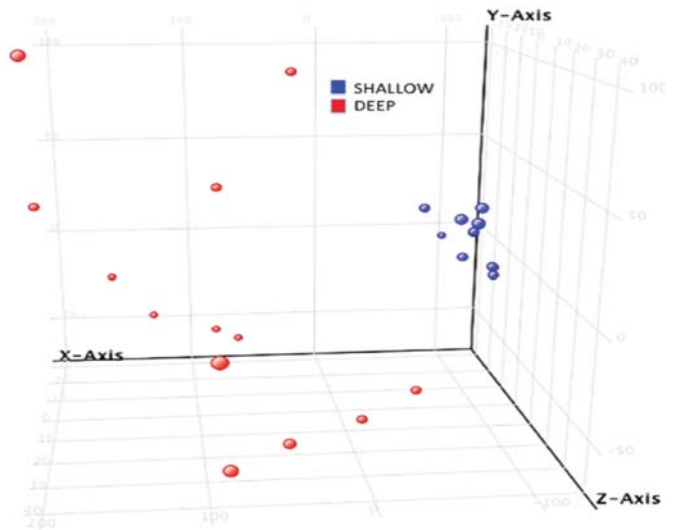

Fig. 2. PCA. PCA plot of microarray gene expression data. The three axis *x*, *y* and *z* explain the 58%, 25% and 11% of the variance, respectively. The analysis highlights a very similar expression profiles of samples collected in high light (i.e. shallow: blue dots) and large differences in expression profiles of samples collected in low light (i.e. deep: red dots). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

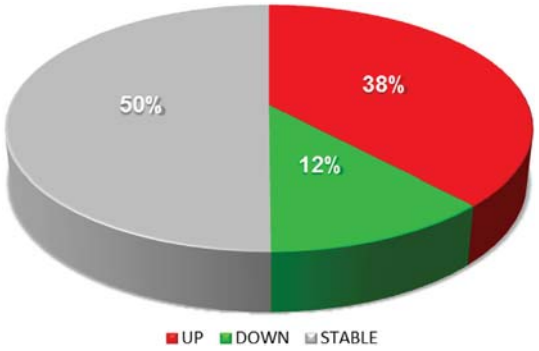

Fig. 3. Pie-Chart. Representation of percent presence of all contigs spotted on the microarray, grouped according to their regulation, using shallow as tester and deep as control condition. The 50% (497) of them did not show significant differences in expression levels (stable; gray slice), while the 38% (379) and the 12% (118) were *up*- (red slice) and *down*-regulated (green slice), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transcripts *up*-regulated in high light, there were genes involved in stress response (Pooc_B_c449, a lipid metabolic process component, homogentisate phytiltransferase – HTP1, involved in tocopherol biosynthesis; Pooc_B_c356 and Pooc_B_c332, annotated for heat shock proteins), in the homeostasis mediated by light-signals (such as Pooc_B_c229, a light regulated protein Lir1), protein kinases (Pooc_B_c170) and ABC transporter member proteins (Pooc_A_PC025C02), chloroplast thylakoid membrane (Pooc_B_c165, Pooc_B_c151) and cellular components (e.g. Pooc_B_c424, Pooc_B_c158, Pooc_B_c275) (Table 4S). Several transcripts associated to the photosynthetic process were differentially regulated. Among the *up*-regulated ones, there were photosystem subunits (e.g. Pooc_B_c240, Pooc_Contig158) and chlorophyll *a/b* binding proteins (Pooc_B_c132, Pooc_B_c293, Pooc_B_c386), whereas other photosynthesis-related transcripts displayed a *down*-regulation in high light (Pooc_Contig192; Pooc_Contig327; Pooc_B_c212 annotated for photosystems subunits, and Pooc_B_c64; Pooc_B_c47; Pooc_Contig79 encoding for chlorophyll binding proteins) (Table 4S). Other genes which appeared among the *down*-regulated in high light were associated to translation (e.g. Pooc_PC014D11, splicing factor; Pooc_PC011G05, translation machinery-associated protein 22; and Pooc_B_c93, eukaryotic translation initiation factor 5-2) and ribosomal components (Pooc_Contig245, ribosomal L32p protein family; Pooc_PC010E04,40S ribosomal protein S11) (Table 4S). Several other contigs which displayed a different regulation between plants growing at the two depths remain unknown. Among genes which were not differentially expressed in our analysis there were enzymes involved in general metabolism, such as glycolysis (data not shown).

Gene Ontology (GO) enrichment results showed that a significant number of transcripts associated with the photosynthetic (GO:0015979, $p = 0.0032$) and oxidation-reduction (GO:0055114, $p = 0.0039$) processes were among those *up*-regulated in high light (Table 3), while there was no significant enrichment for the *down*-regulated processes.

3.4. RT-qPCR

In order to better understand the photosynthetic response of *P. oceanica* at different depths, we selected thirteen genes associated to photosynthesis and photoprotection and we tested their expression via RT-qPCR. In autumn samples (2011), all but one genes showed a higher average expression level in the shallow

Table 3
Gene Ontology enrichment analysis performed on microarray data. Transcripts associated to the GO enriched classes in high light are shown. Differentially expressed contigs are significantly enhanced to be associated with Photosynthesis ($p = 0.0032$) and Oxidation–reduction processes ($p = 0.0039$).

Contig name	Description
GO: 0015979 Photosynthesis (*p* = *0.0032*)	
Pooc_B_c124	Protochlorophyllide reductase, chloroplastic
Pooc_B_c132	Chlorophyll a-b binding protein 21, chloroplastic
Pooc_B_c180	Protochlorophyllide reductase, chloroplastic
Pooc_B_c182	Protochlorophyllide reductase, chloroplastic
Pooc_B_c212	Photosystem II 22 kDa protein, chloroplastic
Pooc_B_c272	Chlorophyll a-b binding protein CP29.1, chloroplastic
Pooc_B_c284	Photosystem II 10 kDa polypeptide, chloroplastic
Pooc_B_c293	Chlorophyll a-b binding protein 1, chloroplastic
Pooc_B_c310	Chlorophyll a-b binding protein 13, chloroplastic
Pooc_B_c320	Chlorophyll a-b binding protein CP24 10A, chloroplastic
Pooc_B_c343	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, chloroplastic
Pooc_B_c360	Chlorophyll a-b binding protein of LHCII type I, chloroplastic
Pooc_B_c386	Chlorophyll a-b binding protein 151, chloroplastic
Pooc_B_c445	Chlorophyll a-b binding protein CP29.2, chloroplastic
Pooc_B_c466	Photosystem I reaction center subunit V, chloroplastic
Pooc_B_c47	Chlorophyll a-b binding protein M9, chloroplastic
Pooc_B_c81	Oxygen-evolving enhancer protein 3-2, chloroplastic
Pooc_B_c83	Oxygen-evolving enhancer protein 2, chloroplastic
Pooc_B_c94	Photosystem I reaction center subunit VI-1, chloroplastic
Pooc_B_c408	Chlorophyll a-b binding protein 1B, chloroplastic
GO:0055114 Oxidation–reduction process (*p* = *0.0039*)	
Pooc_Contig5	Cytochrome b-c1 complex subunit 9
Pooc_B_c139	Peroxidase 42
Pooc_B_c152	Peroxiredoxin Q, chloroplastic
Pooc_B_c180	Protochlorophyllide reductase, chloroplastic
Pooc_B_c182	Protochlorophyllide reductase, chloroplastic
Pooc_B_c193	5'-adenylylsulfate reductase 3, chloroplastic
Pooc_B_c225	Isocitrate dehydrogenase [NADP], chloroplastic
Pooc_B_c226	NADH dehydrogenase [ubiquinone] iron–sulfur protein 8, mitochondrial
Pooc_B_c241	Glutaredoxin
Pooc_B_c30	Cytochrome P450 86A1
Pooc_B_c305	Cytochrome *c* oxidase subunit 2
Pooc_B_c343	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, chloroplastic
Pooc_B_c370	Cytochrome P450 81D1
Pooc_B_c486	Cytochrome *b*
Pooc_B_c98	Naringenin,2-oxoglutarate 3-dioxygenase
Pooc_B_c100	Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a

stand compared to the deep one; four being significant according to REST 2009 analysis (Fig. 4 and Table 4): Photosystem I reaction center subunit V (*PSAG*, *P(H1)* = 0.035), Photosystem II type II chlorophyll *a*/*b*-binding protein (*CAB-151*, *P(H1)* = 0.000), the chloroplast electron carrier ferredoxin (*SEND33*, *P(H1)* = 0.032), and the small subunit of RuBisCO (*SSU5B*, *P(H1)* = 0.035). The only exception was the transcript for the homogentisate phytiltransferase (*HPT1*), which showed no change among shallow and deep replicates.

Interestingly, *HPT1* was *up*-regulated in summer (2010), in accordance with the microarray data, obtained in the same season (Table 4S and Fig. 2S). Comparing RT-qPCR results between the two seasons, all genes were regulated in the same direction, with the exception of *psbD*, which was slightly *down*-regulated in summer shallow samples. Summer samples also showed a higher degree of variation between depths, according to the wider differences encountered in environmental cues (i.e. PAR and T). Of the thirteen genes utilized in RT-qPCR, nine share annotations with genes present among the 497 differentially expressed in the microarray and the 77% of them was expressed in the same direction in summer, when microarray was built, supporting the validity of the data obtained (Fig. 2S).

3.5. Pigment content and short-term incubation in high light

Shallow plants contained significantly more chlorophylls (both *a* and *b*) than deep plants ($p < 0.05$; Table 5S and Fig. 5A), whereas carotenoids (i.e. lutein, neoxanthin and β-carotene) were similar at both sampled depths (Table 5S and Fig. 5A). Chlorophyll *b*/*a* ratio was in average higher in deep plants (although differences were not significant; Table 5S and Fig. 5A). The concentration of the two carotenoids anteraxanthin (A) and zeaxanthin (Z), belonging to the photoprotective xanthophyll cycle (XC), was similar at 5 and 25 m depth, while their precursor, violaxanthin (V), was significantly higher in the shallow stand, as well as the sum of the three xanthophyll cycle pigments (VAZ) ($p < 0.05$; Table 5S and Fig. 5B).

During the short high-light incubation experiment, chlorophylls (both *a* and *b*) and photosynthetic carotenoids (i.e. neoxanthin, lutein and β-carotene) sharply decreased in shallow samples (~60%), in contrast with the slight reduction observed in deep ones (Fig. 5C); the chlorophyll *b*/*a* ratio slightly increased in shallow samples (Fig. 5C). The sum of the three xanthophyll cycle pigments (VAZ) decreased at −5 m while it remained quite similar at −25 m. Violaxanthin decreased in both cases, while anteraxanthin and zeaxanthin increased mainly in the deep samples (Fig. 5D).

4. Discussion

In our study we observed that *Posidonia oceanica* plants living at different depths (i.e. −5 m and −25 m) exhibit different acclimation processes acting at molecular and physiological levels, in order to cope with the specific light conditions in which they grow. The species shows characteristics of shade-adapted plants, as supported by the different responses here observed along the bathymetric gradient. The distinct pattern of responses, therefore, seems to reflect different strategies or needs to be able to live along such intense light gradient. These differences are mainly associated to photoacclimative, photoprotective and reparative processes that shallow plants, in contrast to deep ones, must activate to deal with high light. Plants from deep stands seem to be more photorelaxed and showed a successful photoacclimation to low light.

In plants growing under high-light conditions (−5 m), we observed in the microarray data a significant enrichment of *up*-regulated transcripts in the categories associated to light-dependent reactions of photosynthesis (e.g. light-harvesting proteins, photosystem subunits and electron carriers) and oxidative–reduction processes. We also found a higher abundance of xanthophyll cycle pigments (XCP) compared to those acclimated to low-light conditions. Altogether, these responses suggest adaptations of shallow *P. oceanica* plants to high-light conditions, typical of sun plants (Boardman, 1977; Demmig-Adams and Adams, 1996; Thayer and Björkman, 1990). The *up*-regulation of the photosynthetic machinery reflects an attempt to maintain an efficient photosynthetic performance that enable the species to process the high amount of harvested energy under high-light conditions and to reduce the susceptibility of the photosynthetic apparatus to chronic photo-damage and photoinhibition (Walters, 2005).

In accordance with this, the most *up*-regulated genes in RT-qPCR, in shallow samples, were the transcripts encoding for the RuBisCO small subunit (*SSU5B)* and the ferredoxin (*SEND33*). The *up*-regulation of RuBisCO in high light is in agreement with results obtained on proteins in the same species (Mazzuca et al., 2009), where authors observed a 30% *down*-expression of the RuBisCo large subunit in chronic low-light acclimated leaves. The high light *up*-regulation of RuBisCO, together with the significant *up*-regulation of ferredoxin (*SEND33*), suggests a general increase in the

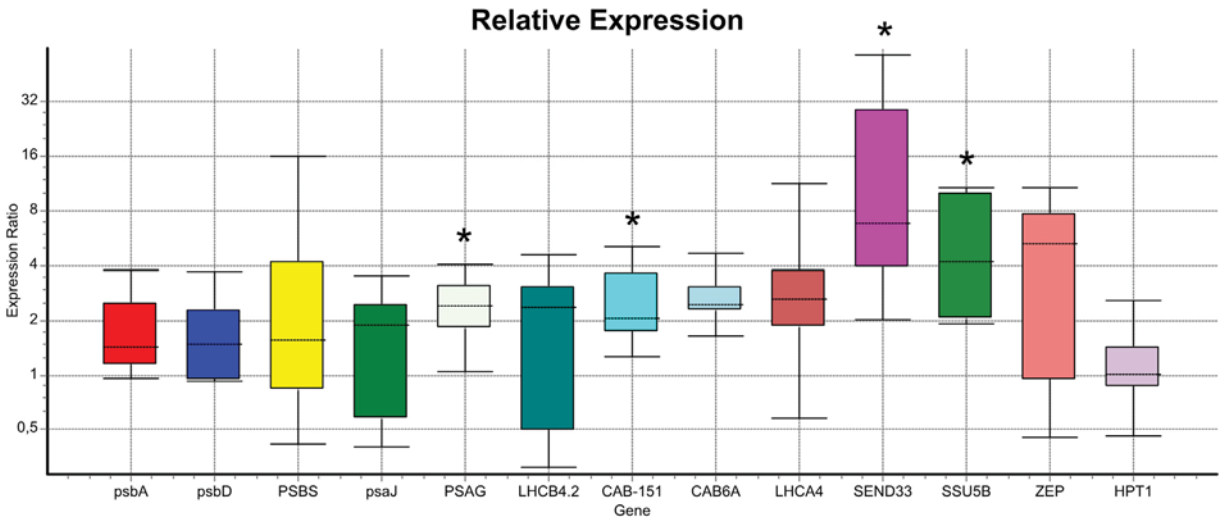

Fig. 4. RT-qPCR. Relative quantification using RT-qPCR of mRNA expression of the 13 genes of interest (*psbA, psbD, PSBS, psaJ, PSAG, LHCB4.2, CAB-151, CAB-6A, LHCA4, SEND33, SSU5B, ZEP* and *HPT1*), in *Posidonia oceanica* shoots collected at −5 m, compared with samples collected at −25 m. The mRNA levels were normalized using *L23*, *EF1A* and *NTUBC2* as reference genes, according to Serra et al., 2012. Relative quantification was obtained using REST 2009 (Pfaffl et al., 2002). The boxes represent interquartile range, or middle 50% of observations. The dotted lines represent median gene expression. The whiskers represent minimum and maximum observations. (*) significant values according to REST 2009 analysis (*P(H1)*, see Table 4).

activity of the electron transport chain, in the generation of ATP and in CO_2 fixation in shallow plants.

Relative-ETR was similar between the two depths, although the real capacity to move electrons along the electronic chain (i.e. absolute-ETR) is expected to be higher in shallow plants, due to the slightly lower leaf absorptance that characterizes deep plants (Olesen et al., 2002; Ralph et al., 2010; Sandoval-Gil et al., 2014). Supporting this assumption, our pigment results have shown that the chlorophyll *b*/*a* ratio (i.e. antenna size) was lower in shallow plants, whereas the content in chlorophylls was significantly higher, which points to an increase in the number of reaction centers and therefore in the capacity to move electrons along the electron transport chain (Falkowski and Raven, 1997; Frost-Christensen and Sand-Jensen, 1992). This is a common photoacclimative response to high light, where the outer-LHCII complex is involved in the acclimative modulation of the PSII antenna size (e.g. Ruban, 2009).

No significant differences in the expression of genes encoding for PSII and PSI, as assessed by RT-qPCR, were found in autumn, with the exception of PSAG which has been proposed as an important regulator of PSI activity (Rosgaard et al., 2005). Differences were higher in summer, as confirmed also by microarray results, where several subunits of PSII and PSI were differentially regulated at the two depths. Previous results obtained in the same light conditions as in the present study also suggest a different regulation of PSI and PSII among different depths (Dattolo et al., 2013). Relative changes in gene regulation, stoichiometry, and antenna size between PSII and PSI, do have a role in the adaptation to different light regimes (Ruban, 2009), and these mechanisms are known to increase the efficiency in the utilization of absorbed light, improving the overall efficiency of photosynthesis (Walters and Horton, 1995). In the seagrass *Halophila stipulacea*, the PSI:PSII ratio was found to increase in the deep-growing plants in relation to a higher PsaC concentration (Sharon et al., 2011). Changes in the protein composition of PSI and PSII were also observed in two marine green algae (*Bryopsis maxima* and *Ulva pertusa*, Yamazaki et al., 2005) and in other seagrass species (Major and Dunton, 2002; Sharon et al., 2011). Further studies are needed in *P. oceanica* to clarify regulation of PSI/PSII stoichiometry in response to changes in light intensity and spectral quality.

Concerning photoprotective responses, plants of the lower stand have shown an *up*-regulation of genes related to oxidative-stress defense, antioxidant metabolisms and general heat-stress response, in particular during the summer season. These molecular responses clearly indicate that shallow plants, in contrast to deep ones that appeared to be photorelaxed, are exposed to light levels that are somewhat stressful and can produce a photodamage. To avoid photoinhibition, which leads to damage of photosynthetic reaction centers, with a reduction in the photosynthetic efficiency and productivity, plants have developed a suite of photoprotective strategies to cope with the excess in excitation energy at which antenna systems are exposed (Ruban, 2009). Specifically, PsbS subunit of PSII and XCP are mainly involved in the regulation of the photoprotective process of thermal energy dissipation (Frenkel et al., 2009; Niyogi et al., 2005; Zia et al., 2011). In our study, the genes *PSBS* and *ZEP* (encoding the Zeaxanthin de-epoxidase enzyme, which catalyze the O_2-NAPDH dependent epoxidation of zeaxanthin to violaxanthin) showed an higher expression level in shallow plants (strongly enhanced in summer, when light is higher).

Table 4
Relative expression report for RT-qPCR experiments obtained with REST 2009 (Pfaffl et al., 2002). Expression values (with Std. Error and 95% C.I.) and *P(H1)* (Probability of alternate hypothesis that difference between sample and control groups is due only to chance) are reported for each gene.

Gene	Expression	Std. Error	95% C.I.	P(H1)	Result
psbA	1.650	1.059–2.898	0.966–3.661	0.105	
psbD	1.482	0.935–2.336	0.931–3.432	0.509	
PSBS	2.073	0.720–6.225	0.472–14.173	0.393	
psaJ	1.389	0.492–2.777	0.413–3.422	0.502	
PSAG	2.297	1.643–3.488	1.160–3.989	0.035	UP
LHCB4.2	1.508	0.405–3.403	0.324–4.394	0.527	
CAB-151	2.377	1.567–4.250	1.315–4.997	0.000	UP
CAB6A	2.633	1.876–3.943	1.663–4.599	0.073	
LHCA4	2.591	1.104–6.667	0.632–10.673	0.204	
SEND33	9.276	3.274–40.089	2.220–54.936	0.032	UP
SSU5B	4.434	2.008–10.573	1.932–10.744	0.035	UP
ZEP	3.178	0.759–8.600	0.504–10.425	0.298	
HPT1	1.069	0.631–1.705	0.481–2.444	0.797	

Significantly differentially regulated genes are underlined ($p < 0.05$).

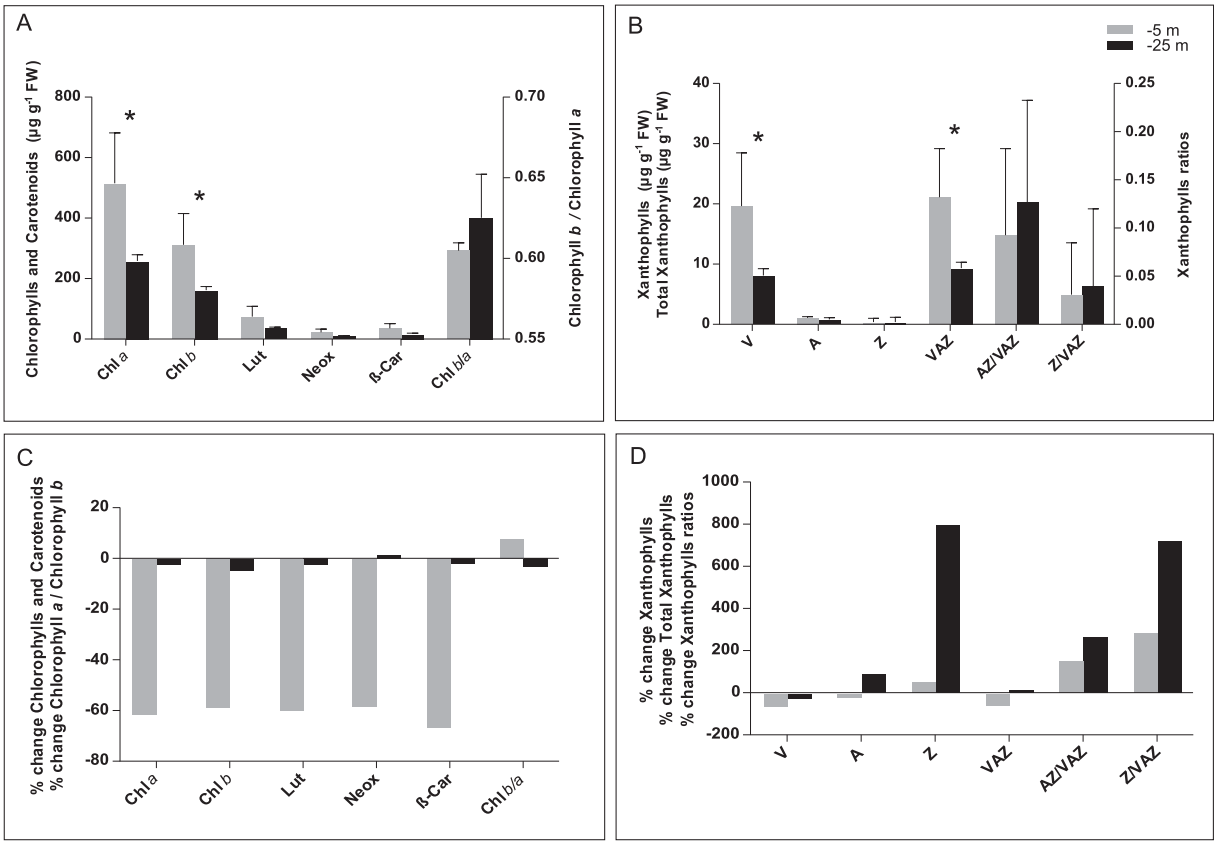

Fig. 5. Pigment content. Pigment content in the September 2011 *P. oceanica* samples, collected at −5 m (gray) and −25 m (black). Panels (A) and (B) refer to plants in natural conditions ($n = 4$), while panels (C) and (D) refer to plants from the short high-light exposure experiment ($n = 3$, pooled).(A) mean chlorophylls and carotenoids concentrations (Chl *a*, Chl *b*, Lut, Neox, β-Car) and chlorophyll *b*/*a* ratio, (B) mean xanthophylls concentration (V, A, Z), total xanthophylls concentration and ratios (VAZ, AZ/VAZ, Z/VAZ), (C) % change of chlorophylls and carotenoids (Chl *a*, Chl *b*, Lut, Neox, β-Car) concentrations and chlorophyll *b*/*a* ratio, (D) % change of xanthophylls (V, A, Z), total xanthophylls concentration and ratios (VAZ, AZ/VAZ, Z/VAZ); $^*p < 0.05$.

Unexpectedly, NPQ, determined under autumn field conditions, was higher in deep plants. Similarly, leaves from the deeper stand also showed a greater capacity for thermal energy dissipation in the short high-light incubation experiment, in accordance with a higher de-epoxidation state of the xanthophyll cycle pigment pool. Although further investigation will be performed to address this issue, the overall observed responses seem to indicate that plants from deep and shallow stands operate different photoprotective strategies when submitted to highly stressing light levels. Shallow samples showed less XC activation and a more drastic response, implying modifications in the structure of photosystems, as indicated by the decrease of the photosynthetic pigment concentrations (e.g. chlorophyll *a*). This photoprotective mechanism was already reported for *P. oceanica* in response to high-light stress in controlled conditions (Marín-Guirao et al., 2013).

Our analyses also revealed the tocopherol biosynthesis activation in shallow *P. oceanica* plants naturally exposed to high light in the summer season, which is another defense mechanism to avoid photoinhibition and photooxidation. The Homogentisate Phytiltransferase (*HTP1*), a key enzyme of tocopherol biosynthesis (Collakova and Della Penna, 2003), showed a clear activation in the microarray and RT-qPCR data in summer samples, but not in the RT-qPCR on autumn samples, when light was lower. Indeed, tocopherols (i.e. Vitamine E) play a role as antioxidant and radical quenching and in response to various stressors, including high light (Havaux et al., 2000; Munné-Bosch and Alegre, 2002), and temperature (Leipner et al., 1999; Streb et al., 1998). The different regulation of this transcript between summer and autumn, might suggest that the increase of tocopherols biosynthesis in *P. oceanica*, acts as protective mechanism principally in response to the prolonged high light and temperature conditions, as occurring during the summer season, like observed for *Arabidopsis* (Havaux et al., 2005).

Due to these photoacclimative and photoprotective responses, shallow plants do not show signs of accumulated photo-damage, as indicate by the high Fv/Fm (i.e. maximum photochemical efficiency of PSII) values. Nevertheless, the over-expression of "general" cell metabolism (e.g. cellular macro-molecules metabolism) and structural membrane components in plants from the high-light condition, supports the notion that these plants require a higher turnover of membrane proteins, most probably due to the constant need of repair.

Deep *P. oceanica* plants do not seem to be light-limited and do not show signs of cellular stress, which suggests a successful adaptation to the existing environmental conditions. Indeed, these plants have shown lower light saturation values for photosynthesis (E_k) and higher antenna sizes than shallow plants, which are common characteristics of shade-plants (Lobban and Harrison, 1994; Falkowski and Raven, 1997; Kirk, 2011).

Results of whole gene expression analysis show that two distinct gene expression signatures were displayed by shallow and deep *P. oceanica* plants. Genetic response in shallow plants seems to be generally more uniform in comparison with what shown by deep samples. Plants in high light have to activate specific defense mechanisms to avoid photo-damage and maintain high photosynthetic performance, which also requires a large energy reinvestment (e.g. in protein-repair process). On the other hand, the

higher degree of variation in gene expression pattern present among deep samples, which also do not show a preferential activation of any metabolic process, might reflect the absence of a single environmental cue driving plant response. Shade tolerance, indeed, is inversely associated with the tolerance to other stressful factors (Valladares and Niinemets, 2008), implying a global rearrangement of plant's homeostasis, that might be achieved by a more plastic response among individuals.

In conclusion, we found that *Posidonia oceanica* is able to adopt different strategies in order to cope with highly different light regimes. The *up*-regulation of many components of the photosynthetic machinery in high light (shallow plants) allows for an efficient photosynthesis, while reducing the susceptibility of the photosynthetic apparatus to environmental stress (Walters, 2005). The *up*-regulation is stronger in summer, when light is higher. Higher xanthophyll cycle pigment content characterizes the shallow sun-growing plants by contrast to the deep plants (shade-plants).

The two stands growing above and below the summer thermocline are genetically distinct (Migliaccio et al., 2005) and show different patterns of gene expression, which could account for their further separation. Two different scenarios can be hypothesized: i) that the species has enough phenotypic plasticity to adapt to the light gradient imposed by its depth distribution, or ii) that selective pressures acting along the bathymetry, have shaped the genetic composition of the meadow, favoring genotypes better adapted to existing conditions (e.g. light and temperature) in each stand. It is well known that patterns of gene-expression regulation can be highly inherited across generations (e.g. Gilad et al., 2006; Whitehead and Crawford, 2006), and this is an important component of adaptive population divergence (e.g. Jeukens et al., 2009; Oleksiak et al., 2005) and ecological speciation (Pavey et al., 2010). Here we identified potential targets of selection in genes involved in the different mechanisms that plants growing at different depths adopt, but further investigation are needed to dig on the adaptive strategies and on the capability of genotypes to switch in their gene expression patterns if environmental conditions change. This has important implications also for forecasting the future of the resource in the framework of climatic changes and for planning adequate restoration strategies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2014.07.010.

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An integration of historical records and genetic data to the assessment of global distribution and population structure in *Octopus vulgaris*

Daniele De Luca*, Gaetano Catanese, Gabriele Procaccini and Graziano Fiorito

Stazione Zoologica Anton Dohrn, Naples, Italy

Edited by:
Melanie April Murphy, University of Wyoming, USA

Reviewed by:
Miguel Arenas, Consejo Superior de Investigaciones Científicas, Spain
Athanasios Exadactylos, University of Thessaly, Greece
Octavio Salgueiro Paulo, Universidade de Lisboa, Portugal

****Correspondence:***
Daniele De Luca, Stazione Zoologica Anton Dohrn, Villa Comunale, Naples 80121, Italy
e-mail: daniele.deluca@szn.it

The common octopus (*Octopus vulgaris* Cuvier, 1797) is one of the most widely distributed species belonging to the genus *Octopus* as well as an important commercially harvested species and a model organism for behavioral biology of invertebrates. It has been described for the first time in the Mediterranean Sea but it is considered a cosmopolitan species inhabiting the temperate and tropical seas of the northern and southern hemispheres. In the last few years, several species previously considered as *O. vulgaris* have been recognized as new species, limiting the distributional range of *"vulgaris"* and reinforcing the thesis of a species complex. Where it is an important fishery resource, numerous studies have been conducted in order to define its genetic structure with the purpose of managing different stocks. However, many locations are still poorly investigated from this point of view and others are under taxonomic revision to exclude or confirm its occurrence. Here we provide a summary of the current status of knowledge on distribution and genetic structure in this species in the different oceanic regions.

Keywords: *Octopus vulgaris*, Cephalopoda, genetic structure, species complex, phylogenetics

FROM POPULATIONS TO SPECIES AND SPECIES COMPLEXES

In its simplest form, a population can be defined as "a group of interbreeding individuals that exist together in time and space" (Hedrick, 2000). Several factors, called evolutionary processes, affect the genetic structure of a population leading to phenomena such as genetic divergence, local adaptation or extinction. In presence of high gene flow, populations lack of clear boundaries and form a continuous population, a condition known as panmixy. On the contrary, over a long time, isolated populations tend to diverge genetically up to not being able to interbreed: a new species is raised (Mayr, 1942). When the time of separation between two species is recent or when hybridization occurs among them, they tend to be well differentiated morphologically but not genetically (Shaffer and Thomson, 2007). Conversely, species can be well differentiated genetically, but not morphologically: this is when "cryptic species complexes" can arise (Bickford et al., 2007; Barley et al., 2013).

Within cephalopods, several "cryptic species complexes" are known (Anderson et al., 2011), especially among octopuses (Norman and Finn, 2001; Amor et al., 2014). One of the most investigated is exactly the *O. vulgaris* species complex. To date, more than 10 species were recognized in this complex (Norman, 2000), and only a few have been validated with molecular markers (Söller et al., 2000; Pérez-Losada et al., 2002). However, Voss et al. (1998) highlight that numerous "forms" or subspecies of *O. vulgaris* exist worldwide, although most of them lack of a description or a reference. Despite several authors consider the common octopus as a cosmopolitan species (**Figure 1**), Norman (2000) suggests that several populations, such as the ones from the Caribbean Sea, Japan and South Africa, are likely to be separated species because of the strong isolation and the different environment in which they live. Warnke et al. (2004) rejected this hypothesis and confirmed the presence of *O. vulgaris* in Japan using mitochondrial genes. More recently, Guerra et al. (2010) showed that the Japanese specimens cluster separately from the others. However, these conclusions deriving from mitochondrial data are not ultimate and need to be integrated with nuclear data too because speciation is not a clockwise process and sometimes recent speciation events have not reached monophyly yet. As outlined by Allcock et al. (2014), more analysis including more specimens and multiple genes should be performed.

CURRENT KNOWLEDGE ON DISTRIBUTION AND POPULATION STRUCTURE

MEDITERRANEAN AND BLACK SEA

Together with the Eastern Atlantic Ocean, the Mediterranean region is considered to be one of the areas in the world where more information exist on cephalopods (Mangold, 1998). Here the common octopus is well known by the time of Aristotle, which provided its earliest written observations in the eastern Mediterranean (Mangold, 1983) and it has been intensively studied from the end of the eighteenth century to date. Despite the descriptions of Cuvier (1797) and Lamarck (1798), the holotype is missing and, as far as we know, a neotype has been designated in 1998 from the Catalonian Sea off Banyuls-sur-Mer and the species is being redescribed (Mangold and Hochberg, 1991). It is found in the entire basin, where it finds suitable environmental

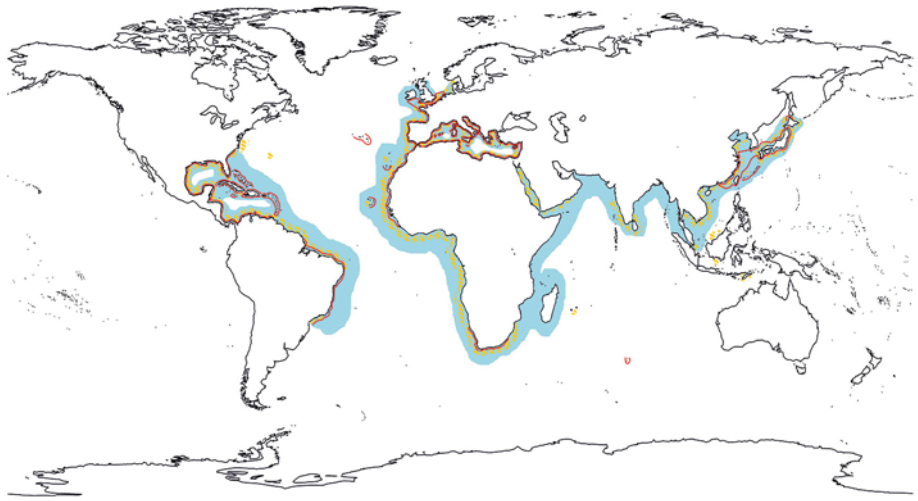

FIGURE 1 | Distribution of *O. vulgaris* after Mangold (1983), Roper et al. (1984) and Norman et al. (2013), in orange, light blue and red respectively.

and ecological conditions, but it is absent in the Marmara and Black Sea, as any other cephalopod species, due to low salinity in the upper waters and reduced gas exchange in the deeper ones (Torchio, 1968; Mangold and Boletzky, 1988).

The first investigation on the genetic structure of *Octopus vulgaris* in the Mediterranean basin has been conducted by Maltagliati et al. (2002) and Casu et al. (2002) using allozymes and a single microsatellite locus respectively (**Table 1**). Both studies focused mainly on the Western and central Mediterranean with just one sample in the Eastern and one in the Atlantic (Casu et al., 2002) and found no isolation-by-distance among populations. Furthermore, the allozyme analysis highlighted a breaking point between western and eastern Mediterranean populations which is not found with microsatellites, probably due to the different resolution of the two markers utilized and to the small representativeness of a single microsatellite locus.

A significant genetic structure has been found in several populations from the central Mediterranean Sea (Strait of Sicily) using mitochondrial markers (Fadhlaoui-Zid et al., 2012; **Table 1**). The authors also mention a significant genetic divergence between western and eastern samples, which could be interpreted as a breaking point between Western and Eastern Mediterranean basin.

The records of *O. vulgaris* in the Levantine Basin (east of 23°E) are less common in the literature compared to the ones from the Western and Central Basin and generally come from Turkish or Israeli waters (Adam, 1967; Ruby and Knudsen, 1972). A recent work by Keskin and Atar (2011) investigated the genetic structure of the common octopus along the Turkish coasts using mitochondrial markers (**Table 1**) and found two clusters compatible with geographical distance, one in the eastern side and the other one in the southern side of the country.

In summary, although it is evident that the use of different molecular markers with different resolution power leads to different scenarios about population structure, the topographic fragmentation of the Mediterranean Basin and the different ecological conditions which occur in the western, central and eastern part should account for a certain degree of population structure (Mona et al., 2014).

NORTHEAST ATLANTIC OCEAN

The Northeast Atlantic region stretches from the coast of Greenland eastward to the North Sea, and from the North Pole southward to the Straits of Gibraltar, including open ocean islands such as the Azores. In this region, *O. vulgaris* reaches its northern distributional limit, being very common (e.g., along the Iberian Peninsula), rare (English Channel) or even absent (North Sea) in different regions. Interestingly, Hoyle (1886) during the "Challenger Expedition" reports this species from the Scandinavian Region and not from the Lusitanian region. On the contrary, Rees (1950) considers it as "a Lusitanian member of our fauna" and reports its occurrence in the English Channel both on British and French coasts up to the German coasts. He also discusses about its abundance during the 1899–1900 years due to a warmer climate in the previous years and hypothesizes that the octopus is probably not able to maintain a breeding population on the English side of the Channel, and so its occurrence is due to an immigrant population from the south. Several records are also reported from Helgoland, in the German part of the North Sea (Hertling, 1936; Rees and Lumby, 1954 both in Jaeckel, 1957) but they are not corroborated by more recent data and might constitute sporadic individuals carried beyond their normal range.

Quite different is, however, the situation in the Iberian Peninsula. Here the occurrence of *O. vulgaris* is unquestioned and information about the population structure is available. Analyzing six populations around the Iberian Peninsula and Canary Islands, Cabranes et al. (2008) found high levels of microsatellite genetic variability and a fine spatial substructure in the Atlantic, which is function of geographical distance (**Table 1**). Furthermore, genetic divergence was also observed between Atlantic and nearby Mediterranean populations, stressing the role of the Gibraltar strait as a genetic break in octopus, as

Table 1 | Resume of the main genetic studies on population structure, phylogeography and phylogenetic relationships in *O. vulgaris* on a global scale.

Region	Area	Molecular markers	Main results	Degree of differentiation	Investigators
Global	Worldwide	16S, COIII	Attribution of the specimens from Taiwan, Japan and Venezuela to *O. vulgaris*	Sequence divergence 0–3.92%	Warnke et al., 2004
Mediterranean and Black Sea	Mediterranean Sea	20 allozymes	No isolation-by-distance between populations and breaking point between western and eastern Mediterranean populations	$F_{ST} = 0.256$	Maltagliati et al., 2002
	Mediterranean Sea	1 microsatellite	High levels of genetic divergence among the populations of the basin, no isolation-by-distance	$F_{ST} = 0.243$	Casu et al., 2002
	Turkey	COI	Two clusters compatible with the geographical distance	n. a.	Keskin and Atar, 2011
	Central Mediterranean Sea	COIII	Genetic structure in the central Mediterranean Sea	$\Phi_{ST} = 0.046$	Fadhlaoui-Zid et al., 2012
Northeast Atlantic Ocean	Iberian Peninsula	5 microsatellites	Fine spatial substructure in the Atlantic which is function of geographical distance	$F_{ST} = 0.014$–0.054*	Cabranes et al., 2008
Eastern central and Southeast Atlantic Ocean	Eastern Africa	3 microsatellites	Genetic differences between the two main African banks and significant structuring within populations	$F_{ST} = 0.0003$–0.0286*	Murphy et al., 2002
	South Africa	COIII	No genetic structure between samples from east and west coasts	n. a.	Oosthuizen et al., 2004
	South Africa	16S, COIII	Two genetically different lineages which reject the findings of Oosthuizen et al. (2004)	Sequence divergence 0.4–1.3%	Teske et al., 2007
Western and Eastern Indian Ocean	Amsterdam and St. Paul Islands	COI, COIII	The specimens from the Southern Indian Ocean belongs to *O. vulgaris*; the Japanese form clusters separately	n. a.	Guerra et al., 2010
Northwest Pacific Ocean	Japan	12S, 16S, COI	Phylogenetic relationships among Japanese coleoid cephalopods	n. a.	Takumiya et al., 2005
	Japan, China	COI, COIII	The Japanese and Mediterranean populations seem to be conspecific due to the low value of sequence divergence between them	Sequence divergence 2%	Kaneko et al., 2011
	China	16S, COI	Phylogenetic relationships among Octopodidae in Chinese waters	n. a.	Lü et al., 2013
Southwest Atlantic and Southeast Pacific Ocean	Brazil	6 microsatellites	Genetic differentiation across the southern coasts of Brazil	$\Phi_{ST} = 0.107$	Moreira et al., 2011
	Brazil	16S, COI	Distinctiveness of *O. vulgaris* from *O. insularis*	Sequence divergence 9.5–11.2%	Sales et al., 2013

*Western central Pacific and Western central Atlantic have been omitted because data are missing or included in the main results of other regions. Degree of differentiation: n.a., not available; F_{ST} and Φ_{ST}, fixation indices; *, only pairwise F_{ST} values were available in the original paper.*

previously showed for many marine taxa (Patarnello et al., 2007). The analysis conducted by Casu et al. (2002) mentioned earlier did not record such a break, probably as a consequence of the use of a single microsatellite locus.

Several reports confirm the presence of the species in the oceanic islands of Azores (Joubin, 1920; Schmidt, 1939), but no genetic studies exist to assess the connectivity between islands and coastal populations.

EASTERN CENTRAL AND SOUTHEAST ATLANTIC OCEAN

Ranging from the Strait of Gibraltar to the South African coasts, this region sustains one of the most productive *O. vulgaris* fishery stock, the Sahara Bank, and studies performed here provided substantial contributions to our knowledge of the biology of the species (Hatanaka, 1979; Mangold, 1983). Its occurrence along the coasts of this region appears in several expeditions' report (Hoyle, 1886; Adam, 1952, 1962; Voss, 1962) and is confirmed in some recent studies which allowed to define the genetic structure in this area. In north-western Africa, two fishery banks occur and they are genetically distinct (Murphy et al., 2002). Furthermore, the authors also hypothesize the existence of a fine spatial structure in this area because samples collected from a research cruise in the same region did not cluster with any of the two banks.

In South Africa the situation is more complex. A first study by Oosthuizen et al. (2004) showed no distinction between the samples collected on the eastern and western coasts using the COIII region. On the other hand, reanalyzing these samples with different molecular markers (16S and COI), Teske et al. (2007) found two different lineages: one containing all the analyzed populations from South Africa and another one characterized by samples from Durban (see **Table 1**). This divergent lineage is interpreted by the authors either as a recent introduction by ships' ballast water or as a long-established lineage disappearing in most of its southern African distribution, but only a larger sampling plan can resolve this controversy.

WESTERN AND EASTERN INDIAN OCEAN

Our knowledge about the occurrence of *O. vulgaris* in this region is limited to the Red Sea and the St. Paul and Amsterdam Islands, because the specimens from the Andaman's and Sri Lanka analyzed by Goodrich (1896) actually belongs to *O. cyanea* according to Adam (1939). Anyway, also in the Red Sea the situation is not controversy free. Despite numerous expeditions and reports, *O. vulgaris* is specifically reported in the area only by Hoyle (1886); other authors such as Wülker (1920) and Adam (1942) just list it based on previous reports, and it was not found in following expeditions (Adam, 1955, 1960). Torchio (1968) considers the species absent in the Red Sea and questions about its occurrence in the Indo-Pacific region. The most recent record from the Red Sea refers to the comparative study between specimens from the Mediterranean (Alexandria) and the Red Sea (Suez) based on the assessment of morphological characters (Riad and Gabr, 2007). In general, due to the scarcity of records, it is possible to assume that the species is rare in the Red Sea, where it could have migrated from the Mediterranean Sea (i.e., anti-Lessepsian migrant).

Different is the situation for the specimens from the St. Paul and Amsterdam Islands in the southern Indian Ocean (Guerra et al., 2010; **Table 1**). According to morphological and genetic analysis, these animals match *O. vulgaris sensu stricto* (from the Mediterranean), even if molecular data rely only on two mitochondrial genes. Anyway, up to now and to new findings, it can be considered the only effective evidence for this region.

WESTERN CENTRAL PACIFIC OCEAN

In this region, which extends from the south of Vietnam up to the northern coasts of Australia including the Malay Archipelago, just historical data of the "Challenger Expedition" exist (Hoyle, 1886). The author reports *O. vulgaris* specimens from what he calls "the Indo-Malayan region" but since such region was intended to extend from the Red Sea eastward up to the Malay Archipelago, it is possible that the specimens where collected in the present western and eastern Indian Ocean region (see paragraph above). If so, the occurrence of the species in this Western central Pacific Ocean region is questioned.

NORTHWEST PACIFIC OCEAN

In this area, the common octopus is reported from the Chinese waters northwards up to Tsugaru Strait, even if it is more common in central and southern Japan (Nesis, 1987). In respect to the populations from China and Korea, only the Japanese ones have been studied for a long time under several aspects of their biology (Sasaki, 1929; Tanaka, 1958), probably because of their commercial value. Despite Norman (2000) argues about this Japanese form as the most likely to be a valid species due to its geographical isolation with the Atlantic and South African ones, Kaneko et al. (2011) consider it conspecific with the Mediterranean populations on the basis of the low value of sequence divergence of mitochondrial markers. Other studies in this area focus on the phylogenetic relationships between coleoid cephalopods (Takumiya et al., 2005) or within the Octopodidae (Lü et al., 2013) but just at a local scale, providing no information about the degree of connectivity between different populations (**Table 1**). The development of a new set of microsatellite loci by Zuo et al. (2012) from samples in Chinese waters might be a starting point for this kind of investigations.

WESTERN CENTRAL ATLANTIC OCEAN

The western-central Atlantic Ocean region embraces the Atlantic Ocean section from Cape Hatteras to the regions of South America within the Northern Hemisphere, including the Caribbean Sea and the oceanic islands. Despite the numerous contributions of some of the major cephalopod workers such as d'Orbigny and Verrill in the nineteenth century and Adam, Pickford and Voss in the twentieth century systematic problems remain. Here this species (or similar species) is distributed along the coasts of United States (Vecchione et al., 1989; Whitaker et al., 1991) and Bermuda (Voss, 1960), in the Gulf of Mexico and Caribbean Sea (Pickford, 1945; Voss, 1955; Judkins, 2009), in Central America (Hochberg and Camacho-García, 2009) and in Venezuelan waters (Arocha and Urosa, 1982). In some regions of Central and northern South America it is known just from few specimens (Pickford, 1945). One of the most evident problems in this geographic region is the abundance of synonymous and uncertain species due to the resemblance of many specimens collected there with the Atlantic-Mediterranean "form" or to the lack of a holotype to be used as reference. Consequently, the western Atlantic "form" of *O. vulgaris* is referred to as *O. americanus* despite no holotype exists for this entity, as *Octopus* cf. *vulgaris*, or just as *O. vulgaris*. Pickford (1945) raised the issue if the American octopus is conspecific with *O. vulgaris* "Lam." and, after a morphological examination, she concluded that "even in respect to the hectocotylus, the American *vulgaris* is identical with its European counterpart." She also reported geographical variations in specimens from Bermuda and coastal waters of United States

and little concrete differences with museum specimens labeled as *Octopus rugosus*.

Up to date, no genetic studies have been conducted in this area to clarify the relationships among the different forms of *O. vulgaris*. Moreover no genetic structure studies exist. The development of microsatellite loci in *O. maya* (Juárez et al., 2013), one of the most harvested octopus species in the Gulf of Mexico, and the following analysis of population structure could stimulate similar analysis also in the common octopus.

SOUTHWEST ATLANTIC AND SOUTHEAST PACIFIC OCEAN

The knowledge of *O. vulgaris* in the southwest Atlantic is limited to Brazil, where it constitutes the most important fishery resource. After the description of a new species (*O. insularis*) from the northeastern coasts of Brazil by morphological and genetic characters (Leite et al., 2008), new genetic data limit the distributional range of *O. vulgaris* to southern Brazil (downstream of Rio de Janeiro) and several localities in the northern and western part (Sales et al., 2013). In southern Brazil, Moreira et al. (2011), using microsatellite loci, highlighted the occurrence of four genetic populations with no significant evidence for isolation by distance, although several bordering populations were the less divergent (**Table 1**).

No records exist about the occurrence of *O. vulgaris* in Argentina and the southeast Pacific Ocean, where it is probably replaced by *O. mimus*, but a deeper investigation in countries such as Peru, Ecuador and Colombia is still needed.

THE PROBLEM OF *O. RUGOSUS*

An important step for the definition of the distributional range in *O. vulgaris* is the assessment of the taxonomic status of *O. rugosus* Bosc (1792). Robson (1929) considers it as a distinct species based on the rough, finely granular skin and shorter arms and hectocotylus compared with *O. vulgaris* but Pickford (1945) and Adam (1952) refer to it as synonymous of *O. vulgaris*. Anyway, its occurrence is recorded from the Red Sea (Adam, 1942), the western and eastern Indian region (Goodrich, 1896; Adam, 1939, 1942), the Caribbean island of Bonaire (Adam, 1937) and along the African, Japanese, Australian and Atlantic coasts (Adam, 1942). If subsequent analysis will prove that this species is actually a synonymous of *O. vulgaris*, all the localities in which it has been reported might be included in the distributional range of the common octopus.

CONCLUSIONS

This review aimed to provide a general picture of the distribution and genetic structure in *Octopus vulgaris* on a global scale, highlighting pitfalls and clues, which could represent the basis for following investigations. The amount of data available in literature is huge and often incomplete, so here we just selected the main and most useful information. In general, few data support the occurrence of *O. vulgaris* in several regions and they are quite doubtful and controversial, making the range hypothesized by Mangold closer to the reality in respect to the one by Roper et al. (**Figure 1**). Regarding the genetic structure, some regions have been investigated more than others, but almost all analysis are concordant in finding genetic structure among populations (**Table 1**), which could derive from low dispersal and enhanced homing of adults, although the potential dispersal of larvae remains to be addressed. Hence, several questions are at the moment unsolved: (i) is *O. vulgaris* a real cosmopolitan species or the hypothesis of species complex is correct? (ii) is there a fine population structure as consequence of the limited adult dispersal or do paralarval meso-scale migrations connect nearby populations? (iii) are these migrations affected by water mass circulation? The answers to all of these questions will contribute to a major comprehension of the ecology of this species and of its biogeographical patterns, with strong impact in fishery and biodiversity management. The FAO statistics reveal that there are real problems in the identification of the cephalopod species caught by the fisheries, with *O. vulgaris* being the only octopus identified to species level (Boyle and Rodhouse, 2005). We know that this can be not always correct. In this context, genetic approaches will constitute a useful tool to investigate biodiversity, assign the catches to the species level and define the stocks in order to prevent their overexploitation.

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MICROSATELLITE LETTERS

A new set of pure microsatellite loci in the common octopus *Octopus vulgaris* Cuvier, 1797 for multiplex PCR assay and their cross-amplification in *O. maya* Voss & Solís Ramírez, 1966

Daniele De Luca · Gaetano Catanese · Graziano Fiorito · Gabriele Procaccini

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Abstract Assessing the genetic structure of cosmopolitan species is essential for conservation purposes, and it assumes particular importance in the case of commercially exploited species and model organisms. Among cephalopods, the common octopus *Octopus vulgaris* Cuvier, 1797 is a good example of cosmopolitan harvested species whose population structure and connectivity is still unknown in many areas of its distribution. Ten new pure microsatellite loci were isolated in Mediterranean specimens of this species and assembled in multiplex for a quickest and cheapest amplification. The number of alleles ranged from 2 to 16, and the expected heterozygosity from 0.097 to 0.900. These loci have been tested in specimens from the Mediterranean Sea, the Atlantic and Pacific Ocean, and in the congeneric species *O. maya*. These markers will be added to the existing ones and used for the determination of genetic structure, connectivity among distant populations and stock management.

Keywords *Octopus vulgaris* · Cosmopolitan species · Microsatellite · Multiplex-PCR · *Octopus maya*

Despite being perhaps the most studied and commercially exploited cephalopod in the world, several aspects of the biology of the common octopus *Octopus vulgaris* Cuvier, 1797 remain unsolved. On a global scale, it is not clear if it is a real cosmopolitan species or constitutes a species complex; in the Mediterranean Sea, supposed to be its type locality, and also in many other areas, the population structure is mostly unknown (De Luca et al. 2014). Twenty-three mostly imperfect microsatellite loci are available for *O. vulgaris* (Greatorex et al. 2000; Zuo et al. 2012). Here we report the development of a new set of pure microsatellites, tested in Mediterranean, Atlantic and Pacific specimens, and in the congeneric *O. maya*.

Total genomic DNA was extracted from 25 mg of fresh arm tip from a Mediterranean specimen (Gulf of Naples, Italy) using the NucleoSpin® Tissue kit (Macherey–Nagel) and then digested overnight with the restriction enzymes *Hae*III and *Rsa*I. Digested DNA was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) and then linked to adapters AP11 (F: 5′-CTCTTGCTTAGATC TGGACTA-3′) and AP12 (F: 5′-TAGTCCAGATCTAAG CAAGAGCACA-3′). A genomic library enriched for microsatellites by the biotin-streptavidin capture method was built (Di Maio and De Castro 2013, *modif.*) After a first enrichment with AP11 primer, the hybridization was performed with 3′-biotinylated probes $(CA)_{13}$ and $(ATT)_9$ and the enriched fragments were captured using Vectrex® Avidin D (Vector Laboratories). After a second enrichment, fragments were purified and cloned using the TOPO®TA Cloning® kit for sequencing (Invitrogen). A total of 116 clones was sequenced on ABI 3730 DNA Sequence Analyzer (Applied Biosystems) using M13 and T7 primers. Out of the 90 positive clones obtained, only 41 contained pure microsatellite repeats and primer design was possible for 15 of them. The 15 loci were first screened on a 2 % agarose gel to test for successful amplification and band patterns. PCR reactions were carried out separately for each locus in a final volume of 25 μL containing 50 ng of DNA, 10X PCR buffer containing 1.5 mM Mg^{2+}, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1.25 U of Taq DNA polymerase (Roche Diagnostics) and were performed at the following conditions: 95°C for 5 min; 35 cycles of 30 s at 95°C, 20 s at the optimum T_a of each primer (Table 1), and 45 s at 72°C; and a

D. De Luca (✉) · G. Catanese · G. Fiorito · G. Procaccini
Stazione Zoologica Anton Dohrn, Villa Comunale,
80121 Naples, Italy
e-mail: daniele.deluca088@gmail.com

Table 1 Microsatellite loci isolated in the common octopus *Octopus vulgaris*

Locus	Primer sequence 5′–3′	Repeat motif	Size range (bp)	T_a (°C)	Plex	GenBank	Atlantic (Faro, Portugal)					Mediterranean (Gulf of Naples, Italy)					Pacific (Keelung,Taiwan)				
							n	N_a	H_o	H_e	HWE	n	N_a	H_o	H_e	HWE	n	N_a	H_o	H_e	HWE
Vulg04	F: NED-TATATTCATACGCACACGATG	$(GT)_{13}$	157–175	55	B	LC003026	20	3	0.200	0.232	ns	20	5	0.250	0.236	ns	5	6	0.800	0.889	ns
	R: GTCCACACATTCGATCACT																				
Vulg06	F: VIC-GTGAACACGTAAACAATAGCTC	$(CA)_{13}$	183–201	55	A	LC003027	20	9	0.800	0.854	ns	20	10	0.700	0.809	ns	5	5	1.000	0.756	ns
	R: TCTTCAACAATCACGTAAAGGC																				
Vulg07	F: FAM-ACTTCAGAGAACGTTTGTGCC	$(CA)_{27}$	174–232	59	B	LC003028	20	12	0.700	0.900	0.019*	20	16	0.750	0.913	ns	5	4	0.400	0.800	ns
	R: GCGTGTATGCATGGATGGAGG																				
Vulg09	F: NED-AAAAGTTCGCTTCTCAAACCACAC	$(TG)_{10}$	208–216	55	A	LC003029	20	2	0.000	0.097	0.026*	20	1	–	–	–	5	3	1.000	0.689	ns
	R: TAGGGTCATAAACAAGCCAACACT																				
Vulg10	F: VIC-ATACAGTCCCTCACACACCCGTAT	$(CA)_{15}$	209–219	55	B	LC003030	20	3	0.300	0.591	0.000***	20	5	0.250	0.277	ns	5	5	1.000	0.800	ns
	R: AGAGCAAGAAGAAGGGAATGAGAA																				
Vulg11	F: PET-TGGACTAACGATTAATTTTTGC	$(CA)_{18}$	227–271	55	B	LC003031	20	7	0.650	0.618	ns	20	9	0.750	0.819	ns	5	5	0.800	0.844	ns
	R: ATTTCCGATCATATAAACCACT																				
Vulg12	F: FAM-CCTGCCAAAATTCTGTGCAAT	$(CA)_{18}$	242–262	59	A	LC003032	20	4	0.050	0.191	0.000***	20	3	0.300	0.349	ns	5	3	0.800	0.600	ns
	R: AAGAAGCTCGTTTTGAAACCAC																				
Vulg13	F: VIC-TGCCTATTTCACAAATGTAGC	$(TG)_{11}$	313–331	55	A	LC003033	20	6	0.250	0.659	0.000***	20	3	0.450	0.497	ns	5	na	–	–	–
	R: TTTATACAGAGAAAAGGCAAG																				
Vulg14	F: NED-TAATATGTAACAGTCACGAGGGTA	$(CAT)_6$	394–406	57	B	LC003034	20	4	0.250	0.345	0.021*	20	4	0.400	0.387	ns	5	3	0.200	0.511	ns
	R: TGCTGTTGACATTTAGCCCAA																				
Vulg15	F: PET-AAAGCTACAGCTCAGTGAGGGAGA	$(GA)_{17}$	195–227	58	A	LC003035	20	9	0.650	0.709	0.028*	20	7	0.650	0.717	ns	5	1	–	–	–
	R: AGATGGCTCTTCTGACTGTCACTTCC																				

A and B indicate the multiplex

n, n° of specimens; N_A, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; HWE, deviations from Hardy–Weinberg equilibrium; ns, not significant; na, not amplified

* ($P < 0.05$), *** ($P < 0.001$)

final extension of 7 min at 72°C. After this first screening, primers were combined in multiplex PCR and polymorphism was tested using fluorescence-labelled oligos (Life Technologies, Table 1). A total of 10 loci met the criteria of selection, i.e. pure repeat, polymorphism, and amplificability by multiplex PCR, and were assembled in two multiplex (A and B). Multiplex PCR was performed using the Type-it® Microsatellite PCR kit (Qiagen) following the manufacturer's protocol and at a T_a of 59°C for both multiplex. Polymorphism was tested in 45 individuals (Table 1). Loci Vulg09 and Vulg15 were monomorphic in Mediterranean and Pacific specimens respectively, while Vulg13 did not show amplification products in Pacific samples. Number of alleles (N_a), expected (H_e) and observed heterozygosity (H_o) and deviations from Hardy–Weinberg equilibrium were assessed using Arlequin v. 3.5.1.3 (Excoffier and Lischer 2010), while linkage disequilibrium (LD) was tested using GENEPOP v. 4.2 (Rousset 2008). The number of alleles ranged from 2 to 16 and the expected and observed heterozygosity from 0.097 to 0.900 and from 0.000 to 1.000, respectively. Significant deviations from HWE were only detected in seven loci in individuals from the Atlantic locality (Table 1). Putative null alleles were detected using Micro-Checker v. 2.2.3 (Van Oosterhout et al. 2004) and found in the Atlantic specimens in loci Vulg07, Vulg09, Vulg10, Vulg12, and Vulg13, and in one locus (Vulg07) in the Mediterranean population. LD among loci was not detected. 8 microsatellites also worked in *O. maya* (except for Vulg07 and Vulg13) but their polymorphism was not assessed due to the low number of available specimens.

These new microsatellite loci can be used together with the others available in literature for the characterization of Mediterranean and Atlantic populations of *O. vulgaris*. Furthermore, they can also be useful for the study of Asian specimens (in contrast to previously developed microsatellites, as reported by Zuo et al. 2012), allowing a better definition of population genetic diversity and stock boundaries at global scale.

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Settlement pattern of *Posidonia oceanica* epibionts along a gradient of ocean acidification: an approach with mimics

L. DONNARUMMA[1,3], C. LOMBARDI[2], S. COCITO[2] and M.C. GAMBI[1]

[1] Stazione Zoologica Anton Dohrn, Laboratorio di Ecologia Funzionale ed Evolutiva, Napoli, Italy
[2] ENEA Centro Ricerche Ambiente Marino, La Spezia, Italy
[3] present address Dipartimento di Scienze e Tecnologie, Università di Napoli "Parthenope", Napoli, Italy

Corresponding author: gambimc@szn.it

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Abstract

Effects of ocean acidification (OA on the colonization/settlement pattern of the epibiont community of the leaves and rhizomes of the Mediterranean seagrass, *Posidonia oceanica*, have been studied at volcanic CO_2 vents off Ischia (Italy), using "mimics" as artificial substrates. The experiments were conducted in shallow *Posidonia* stands (2-3 m depth), in three stations on the north and three on the south sides of the study area, distributed along a pH gradient. At each station, 4 rhizome mimics and 6 artificial leaves were collected every three months (Sept 2009-Sept 2010). The epibionts on both leaf and rhizome mimics showed clear changes along the pH gradient; coralline algae and calcareous invertebrates (bryozoans, serpulid polychaetes and barnacles) were dominant at control stations but progressively disappeared at the most acidified stations. In these extremely low pH sites the assemblage was dominated by filamentous algae and non calcareous taxa such as hydroids and tunicates. Settlement pattern on the artificial leaves and rhizome mimics over time showed a consistent distribution pattern along the pH gradient and highlighted the peak of recruitment of the various organisms in different periods according to their life history. *Posidonia* mimics at the acidified station showed a poor and very simplified assemblage where calcifying epibionts seemed less competitive for space. This profound difference in epiphyte communities in low pH conditions suggests cascading effects on the food web of the meadow and, consequently, on the functioning of the system.

Keywords: Seagrass, epiphytes, mimics, CO_2 vents, colonization pattern, recruitment, biodiversity, Mediterranean Sea.

Introduction

The amount of CO_2 in the atmosphere has steadily increased since the industrial period. According to estimates, if greenhouse gas emissions continue to rise at current rates, the atmospheric CO_2 concentration levels will be 500 ppm by 2050 and 800 ppm by 2100 (IPCC, 2007). The surface ocean pH level may therefore fall to 7.7 or 7.8 with an increase in acidity of 150 % compared to pre-industrial values (Hardt & Safina, 2010). This decrease in the ocean pH level, as a result of atmospheric CO_2 dissolution in the surface waters of the oceans, is termed "ocean acidification" (OA) (Caldeira & Wickett, 2003) or "the other CO_2 problem" (Doney *et al.,* 2009). In particular, OA is the result of carbonic acid formation (H_2CO_3), which dissociates to bicarbonate (HCO_3^-), carbonate ions (CO_3^{2-}) and protons (H^+).

Acidification affects mainly the process of calcification of organisms such as corals, molluscs, and many other organisms with skeletons and shells composed of calcium carbonate (Royal Society, 2005; Doney *et al.,* 2009; Lombardi *et al.,* 2011a, b). In addition, when the seawater is combined with other kinds of natural and/or anthropogenic stress factors (e.g. warming, pollution and overfishing), it may cause sensible changes in the benthic community structure (Kroeker *et al.,* 2010; Rodolfo-Metalpa *et al.,* 2011), impacting most fundamental biological and geochemical processes (Kleypas *et al.,* 2006; Fabry *et al.,* 2008). Many laboratory studies have shown that the early life stages of several organisms are also negatively influenced by acidified seawater (Kroeker *et al.,* 2010) and this has been observed also in *in situ* experiments at CO_2 vents (Cigliano *et al.,* 2010; Ricevuto *et al.,* 2012). However, the response of multispecies assemblages to OA at naturally acidified water is still poorly documented, especially regarding highly complex systems built by structuring, habitat-forming species, such as seagrass meadows (Martin *et al.,* 2008).

Posidonia oceanica (L.) Delile is the endemic and dominant seagrass in the Mediterranean Sea. It forms extensive meadows from the surface down to a maximum of about 40 m depth (Procaccini *et al.,* 2003). Studies on the fauna associated to *P. oceanica* revealed high diversity of species settled both on leaves and rhizomes (Maz-

zella *et al.,* 1992; Cocito *et al.,* 2012) and belonging to different taxonomic groups, which includes many calcifying taxa such as coralline algae, crustaceans, molluscs, serpulid polychaetes, bryozoans (Mazzella *et al.,* 1992; Buia *et al.,* 2000; Borg *et al.,* 2006; Balata *et al.,* 2007; Nesti *et al.,* 2009).

Within the *Posidonia* complex system, the epiphytes settled on the leaves and the rhizomes play an important role in the food web and functioning of the meadows (Mazzella *et al.,* 1992). The epiphytic algal component represents the main item for energy transfer within the "grazing chain" of the seagrass food-web, from primary producers to higher trophic levels through the consumption due to small mesoherbivores, a guild of grazers that not only fed on the system but also found shelter and permanent habitat in the meadow (Brawley, 1992). The animal component of the epiphytes is also consumed by other specialized invertebrates (Gambi & Morri, 2008) and shows interesting examples of adaptation to the special local conditions of the leaf canopy, with several characteristic and/or exclusive species (e.g. the bryozoan *Electra posidoniae*).

In addition, the epiphytes represent sensitive indicators of natural or anthropogenic impacts (e.g. eutrophication) (Morri, 1991), and are able to record the environmental alterations resulting from climate change (e.g. seawater acidification) (Martin *et al.,* 2008). In fact, the epiphytic community of *P. oceanica*, and especially that found on the leaves, mainly consists of short-lived species which show a highly seasonal development, following the cycle of leaf growth (Mazzella *et al.,* 1989), as well as a high degree of small scale spatial variability (Nesti *et al.,* 2009 and literature herein).

Up to date the only study dealing with seagrass epiphytes in relation to OA is that carried out by Martin *et al.* (2008) along an OA gradient at natural CO_2 vents off Ischia. These authors highlighted big differences in the composition and structure of the calcareous epiphytic community of *P. oceanica* leaves in acidified seawaters, where coralline algae and other calcifiers were strongly reduced or absent. However, Martin *et al.* (2008) limited their study to a single spatial transect (on the south side of the study area) and on a single observation period (April), and did not consider the community settled on the rhizomes.

Similar results, showing a reduced colonization of calcareous macroalgae and calcifying benthic invertebrates colonizing the hard rocky reefs, adjacent to the *Posidonia* stands in the same study area, were shown by other authors (Hall-Spencer *et al.,* 2008; Kroeker *et al.,* 2011; Porzio *et al.,* 2011). Some of the patterns observed in the adult populations of various species of the rocky substrate were due to selective pressure on the larval and juvenile stages (Cigliano *et al.,* 2010; Ricevuto *et al.,* 2012).

The overall aim of this study is to highlight the effects of ocean acidification on the colonization and settlement pattern of the *Posidonia* epibiont community of the leaves and rhizomes along a gradient of pH reduction, and increased pCO_2, using a new experimental approach with "mimics" (see Methods) (Gambi *et al.,* 2011; Cocito *et al.,* 2012). The plant mimics were used to reduce the impact of experimental studies on the natural *Posidonia* meadow within the study area. Moreover, mimics are essential for having an un-colonized substrate to examine the seasonal cycles of epiphyte re-colonization at a given time interval and to compare possible quantitative and qualitative differences in species between artificial and natural substrates (i.e. *P. oceanica* tissue). In detail, our goals are i) to characterize the *Posidonia* stands where *Posidonia* mimics were placed; ii) to report colonization pattern on the first three months of exposure along the gradients for both the leaves and rhizome mimics; iii) to study the settlement pattern through time of the main epibionts (limited to the south side only).

Materials and Methods

Study site

The study area is adjacent to Castello Aragonese, a volcanic dome and islet located at the north-eastern side of Ischia island (Gulf of Naples, Italy) (40°43.84'N 13°57.08'E) (Rittmann & Gottini, 1981). Previous and recent gas analyses (Tedesco, 1996; Hall-Spencer *et al.*, 2008) showed that the seawater is acidified by gas comprising 90.1-95.3 % CO_2, 3.2-6.6 % N_2, 0.6-0.8 % O_2, 0.08-0.1 % Ar and 0.2-0.8 % CH_4 (no sulphur present, while both water temperature and salinity do not change respect to normal conditions) (Hall-Spencer *et al.,* 2008; Kerrison *et al.,* 2011; Kroeker *et al.,* 2011). The seawater pH varies from the normal value of 8.17 to as low as 6.57 along a continuous gradient occurring both at the north and south-western sides of the Castello (Kroeker *et al.,* 2011; Lombardi *et al.,* 2011b). On both sides of the Castello islet a shallow *Posidonia oceanica* meadow subjected to CO_2 emission is present (Buia *et al.,* 2003; Hall-Spencer *et al.,* 2008). On the south side, the most intense venting activity include also *Posidonia* meadow, here in some restricted areas *Posidonia* can reach very shallow depth (0.5-1 m), and forms a sort of reef. This side of the Castello is also more sheltered to water movement respect to the north side which is exposed to the dominant north-western winds. At the north side, *Posidonia* meadow is very close to the active venting area, but direct gas bubbling is very limited or absent inside the meadow. In addition, in this area, anecdotal observations of one of us (MCG) as well as other researchers, testify that venting was absent in the early '80 (Russo G.F., Boudouresque C-F., Cinelli F., Ott J., Pronzato R., personal communication), so that water acidification is a relatively recent phenomenon on the north side of the Castello. The experiments are conducted in shallow *Posidonia* stands

at 2.5-3.5 m depth, in three stations located on the north side (N1, N2, N3) and three on the south side (SC, S2, S3) distributed along a gradient of pH. The stations have been selected based on previous studies in relation to proximity to CO_2 emissions and mean pH values recorded (see Cigliano *et al.,* 2010 for a map of the area, Lombardi *et al.,* 2011b). The S3 and N3 stations, acidified sites with very low pH, are located in an area with high bubbling and dense emissions (<10 bubbles emissions to m^2). Mean pH values are approximately 7.2 at the northern and 6.6 at the southern sites, near the rocky reef (Kroeker *et al.,* 2011). In these stations, the *P. oceanica* meadow is very dense (over 900 shoots/m^2) (Buia *et al.,* 2003) with the short leaves due to the frequent grazing by the herbivorous *Sarpa salpa*, which is the most abundant fish species in the area (Guidetti & Bussotti, 1998; Bussotti & Guidetti, 1999). The S2 and N2 stations, low-intermediate pH conditions, are located approximately 60 m far from S3 and N3. S2 and N2 have mean pH values around 7.7-7.8 (Hall-Spencer *et al.,* 2008; Lombardi *et al.,* 2011b), but it has also a considerable variability in time, also at daily scale (Kerrison *et al.,* 2011; Kroeker *et al.,* 2011), and the bubbles emissions are reduced compared to the acidified area (>5 bubbles emissions to m^2). The SC and N1 are control stations, located approximately 80 m from the S2-N2, where the CO_2 emissions are almost absent and the pH values are those of normal sea waters (8.1). The south side station (SC) has been used as a control site for previous transplant experiments (Lombardi *et al.,* 2010, 2011a,b; Rodolfo-Metalpa *et al.,* 2010), and it does not coincide with the south control station of other studies (e.g., Kroeker *et al.,* 2011, 2013a).

Sampling methods and data analysis

Artificial structures mimicking the plant morphology, both rhizome and leaves have been developed *ad hoc* for this study. This methodology, implying the use of artificial substrates, is analogous to the use of panels and volcanic tiles to study the fouling, community re-colonization and succession pattern in hard bottom environments (Relini & Faimali, 2004; Kroeker *et al.,* 2013a). Moreover, artificial structures mimicking the physical structure and morphology of a seagrass have already been used by other authors (Pinkney & Micheli, 1998), especially to study the re-colonization of associated flora and fauna (Bologna & Heck, 1999; Lee *et al.,* 2001; Cocito *et al.,* 2012) and to detect the specificity of the epiphytes for the seagrass substrate (Mazzella *et al.,* 1981). Although seagrass mimics are not exact surrogates of the plant (Pinkney & Micheli, 1998), if appropriately designed, they can simulate plant architecture and structure. So they have the merit to reduce variability of plant features, respect to the natural context. Mimics also allow to test the simple influence of plant architecture and physical structure on the epibiont community colonization *vs* the biological and chemical effect. In addition, in the specific context of the natural CO_2 vents, the use of mimics reduces the impact on the *Posidonia* system caused by shoot collection in the relatively limited extension of the *Posidonia* meadows here available. Mimics (Fig. 1) of *Posidonia* rhizomes consist of hollow cylinders made of earthenware with rough and finely grooved surface to reproduce the roughness of the natural rhizomes as best as possible. The length of the cylinders, set according to the rhizome mean height from literature data, is 13 cm, with a diameter of approximately 3 cm (Gambi *et al.,* 2011; Cocito *et al.,* 2012). Mimics of *Posidonia* leaves are made with non toxic, dark green flexible PVC (IDROEVA ®, Pati s.p.a.); the material is cut in strips 1 cm wide and 36 cm long. Four PVC strips (artificial leaves) are attached together with plastic straps to a hooked iron stake (Fig. 1). The stakes with artificial leaves are then inserted in the hollow of the cylinder and hammered on the bottom among the *Posidonia* natural rhizomes, producing a minimal impact on the meadow. At each of the 6 stations, *Posidonia* mimics were inserted into the meadow, in an area of approximately 3 x 6 m, where the condition of CO_2 emissions and the pH value variability could be considered relatively homogeneous in time according to previous measurements on the same spots (Cigliano *et al.,* 2010; Lombardi *et al.,* 2011b). At each station, 16 mimics were placed in September 2009. Every three months (December 2009, March, June and September 2010), 4 mimics of the rhizome and 6 artificial leaves were randomly sampled. After the sampling, mimics were transported in the laboratory inside of small plastic bags and within a cool box. Wet structures were firstly photographed (Camera: Nikon Coolpix 5700, resolution: 5 megapixel) then fixed in 4 % formol and transferred after 48 hours to 70 % ethanol for species preservation. On the leaves, we randomly selected spots of 1 x 2 cm on the external side of the artificial leaf (generally the most colonized) as to have at least 6-7 spots per artificial leaf available. All the rhizome mimics were photographed. In order to study the pattern of settlement of

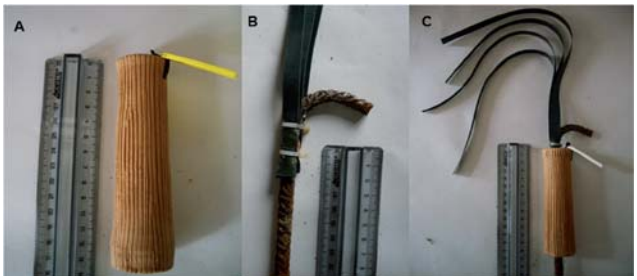

Fig. 1: Artificial structures mimicking the *P. oceanica* rhizome and leaves used to study colonization and settlement patterns of the epibiont populations. A) rhizome mimic; B) the metal hooked stick with artificial leaves tied; C) a whole view of a mimic *Posidonia* shoot.

epibionts over time in the south side stations (SC, S2, S3), 3 additional rhizome mimics and 6 artificial leaves were taken, while 3 new mimics and 6 artificial leaves were reinserted at three month time intervals from September 2009 (December 2009, March, June and September 2010). The south side was selected to reduce the sampling effort, since here the pH gradient is stronger than to the north side (Kroeker et al., 2011). At each station, *Posidonia* shoot density was measured in September 2010, and samplings of *Posidonia* natural shoots (6-10 shoots) for the morphometric analysis were done in five periods through the year in order to characterize the features of the *Posidonia* shoots in the plot used for mimic's deployment (Buia *et al.,* 2004). On rhizome mimics and artificial leaves, the epibiont identification at the lowest taxonomic level possible was performed using a stereo microscope. The percentage of epibiont coverage and the abundance of the main epibiont algal and animal taxa were calculated using the image-analyzing program Vidana 1.1. Data related to cover, as well as those on shoot morphometric measurements were subjected to statistical analysis using ANOVA (one and two ways) to test differences among stations (pH factor), side and sampling periods. Tukey HSD post-hoc analysis was performed to further highlight differences among stations. Data was checked for homogeneity of variance using a Cochran C test ($p > 0.05$). Where data was found to be heterogeneous, data was $\sqrt{(X + 1)}$ transformed (Underwood, 1997). The analyses were performed with the program STATISTICA 7. Only for the community settled on the rhizome mimics, a matrix was produced (taxa/station) for a multivariate analysis. The distance matrix was calculated using the Bray-Curtis algorithm to obtain the ordination model (MDS) and the ANOSIM test was applied to verify the significance of pHs (stations) and side (north *vs* south). These analyses were performed with the program PRIMER+PERMANOVA v.6 (Warwick & Clarke, 1991).

Results

Shoot density and morphometric analysis of Posidonia along the pH gradient

Posidonia oceanica shoot density showed, both at the north and south sides, a significant decrease from the most acidic stations (N3 and S3) to the control ones (N1 and SC) (two-way ANOVA, $F = 14.67$, $p = 0.000$, d.f. 5), although the depth did not vary much between the stations (2-3.5 m). Indeed, the shoot density values were the highest at 2-2.5 m depth at the acidified stations (N3 = mean 858 ± 85 (s.d.) shoot m^2; S3 = 1014 ± 73; N2 = 708 ± 98; S2 = 726 ± 123; N1 = 438 ± 88; SC = 494 ± 125). The mean leaf length and mean shoot surface (pooling the 5 sampling periods) were higher on the south side respect to the north (two-way ANOVA, leaf length $F = 5.25$; $p = 0.02$, d.f. 1; leaf surface $F = 7.01$; $p = 0.013$, d.f. 1). The mean leaf length was also significantly different among stations, with values in N2, N3 and S3 lower than all the others (two-way ANOVA, $F = 2.87$; $p = 0.03$, d.f. 5, Tukey post-hoc comparisons). Leaf surface varied significantly only in S2 which showed much higher values (two-way ANOVA, $F = 3.97$; $p = 0.009$, d.f. 5, Tukey post hoc comparisons) (Fig. 2A, B). The analysis of the apex erosion coefficient (Fig. 2C) reveals that biological erosion, due to various grazers, is the main source of leaf damaging. The biological erosion was significantly higher in N3 and

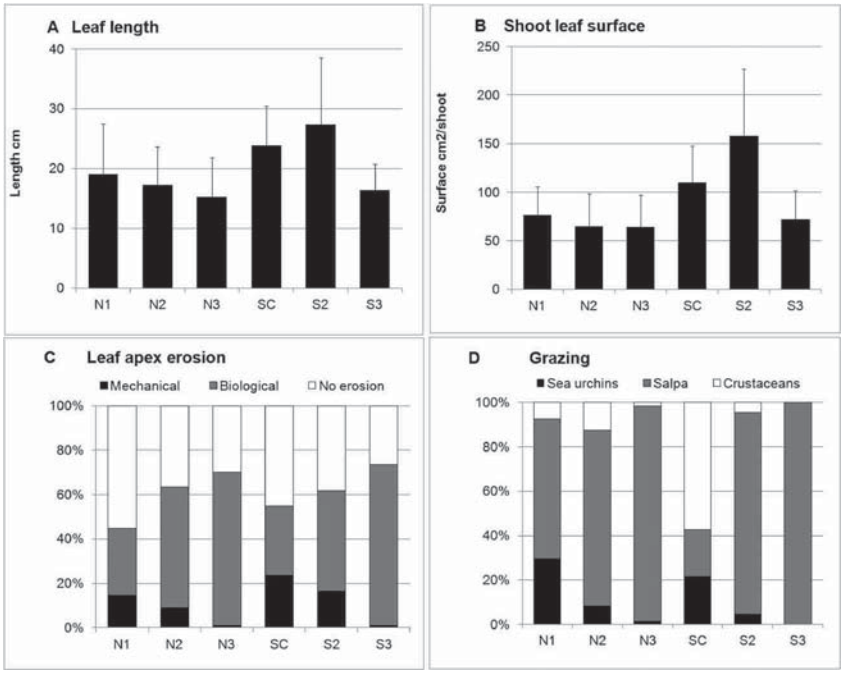

Fig. 2: Trends of *Posidonia oceanica* morphological features measured in the mimic's plots along the pH gradient (each station is the mean of 5 sampling periods between Sept 2009 - Sept 2010). Bars represent standard deviations. A) mean leaf length; B) mean surface of the shoot (cm^2); C) percentage of types of leaf apex erosion; D) percentage of types of biological apex erosion.

S3 stations (two-way ANOVA, F = 5.52; p = 0.001, d.f. 5). Among the type of biological damaging, *Sarpa salpa* grazing (Fig. 2D) resulted the most common and the dominant type in N3 and S3 stations (two-way ANOVA, F = 16.55; p = 0.000, d.f. 5). Grazing by crustaceans (manly isopods) resulted significantly higher in SC (two-way ANOVA, F = 3.69; p = 0.012, d.f. 5, Tukey post-hoc comparisons). So overall, the acidified *Posidonia* stands are characterized by extremely high shoots density, short leaf length and consequently low leaf surfaces, mainly due to intense grazing by *Sarpa salpa* fish.

Colonization of mimics along the pH gradient

For the first three months of exposure (Sept-Dec 2009) (Fig. 3), the algal cover on the artificial leaves at both south and north sides showed a large presence of coralline algae (genera *Hydrolithon/Pneophyllum* and *Titanoderma*), found under control or intermediate pH conditions (N1, N2, SC) and significantly reduced or absent at the acidified stations (N3, S3) (Fig. 3) (one-way ANOVA, Table 1). The genus *Titanoderma* was present only at the stations on the north side. On the contrary, the filamentous macroalgae, belonging to Chlorophyceae and Phaeophyceae (Chloro/Phaeo in Fig. 3), were abundant at the most acidified stations (ANOVA, Table 1). The sessile fauna was represented only by Hydrozoa and only at the control station N1. Grazing traces, due to radular marks of large gastropods (e.g. *Gibbula* spp.) on epiphytic biofilm and slime on artificial leaves, were visible at the acidified stations (N3, S3) and in S2, where Chloro/Phaeo algal cover dominated. Rhizome mimics (Fig. 4) showed a large presence of encrusting coralline algae at the control and intermediate stations of the north side (N1, N2) and at the control station of the south side (SC). These coralline algae were absent at the acidified station S3, while they were reduced at N3 and S2 (one-way ANOVA, Table 2). On the contrary, at the acidified stations green and brown filamentous algae (Chloro/Phaeo) increased, and non-calcifying red algae (other Rhodophyceae) were also present (Fig. 4A). The abundance of calcifying sessile fauna, including Cirripedia (*Balanus* spp.), Bryozoa, and Serpulidae (which include Serpulinae and Spirorbinae) polychaetes, showed a significant decrease from control stations to the acidified ones where all these groups were absent (Fig. 4B). Spirorbinae polychaetes, characterized by spiral calcareous tubes, showed high abundances at control stations and a trend consistent with the other calcifying groups:

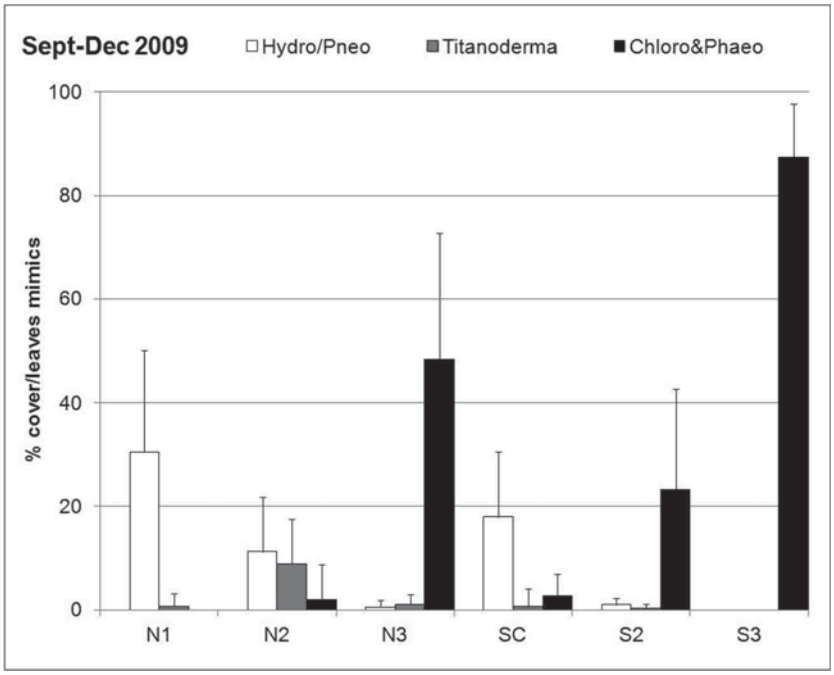

Fig. 3: Trend of cover (%) of the main macroalgal taxa in the artificial leaves of *P. oceanica* after the first 3 months of exposure (Sept. - Dec. 2009) at the north and south sides of Castello Aragonese. Bars represent standard deviations.

Table 1A. One-way ANOVA of the main macroalgal forms, on the artificial leaves after the first 3 months of exposure (Sept - Dec 09), among stations (SC - N3) and between sides (North vs. South) of Castello Aragonese.
df: degree of freedom, p: probability with ns (p>0.05), * (p<0.05), ** (p<0.01); *** $P < 0.001$.

	Stations	SC - N3		Side	S - N	
	***F* values**	**df**	**p**	***F* values**	**df**	**p**
Hydro/Pneo	55.46	5	***	15.16	1	***
Titanoderma	30.06	5	***	27.58	1	***
Chloro/Phaeo	275.82	5	***	26.22	1	***

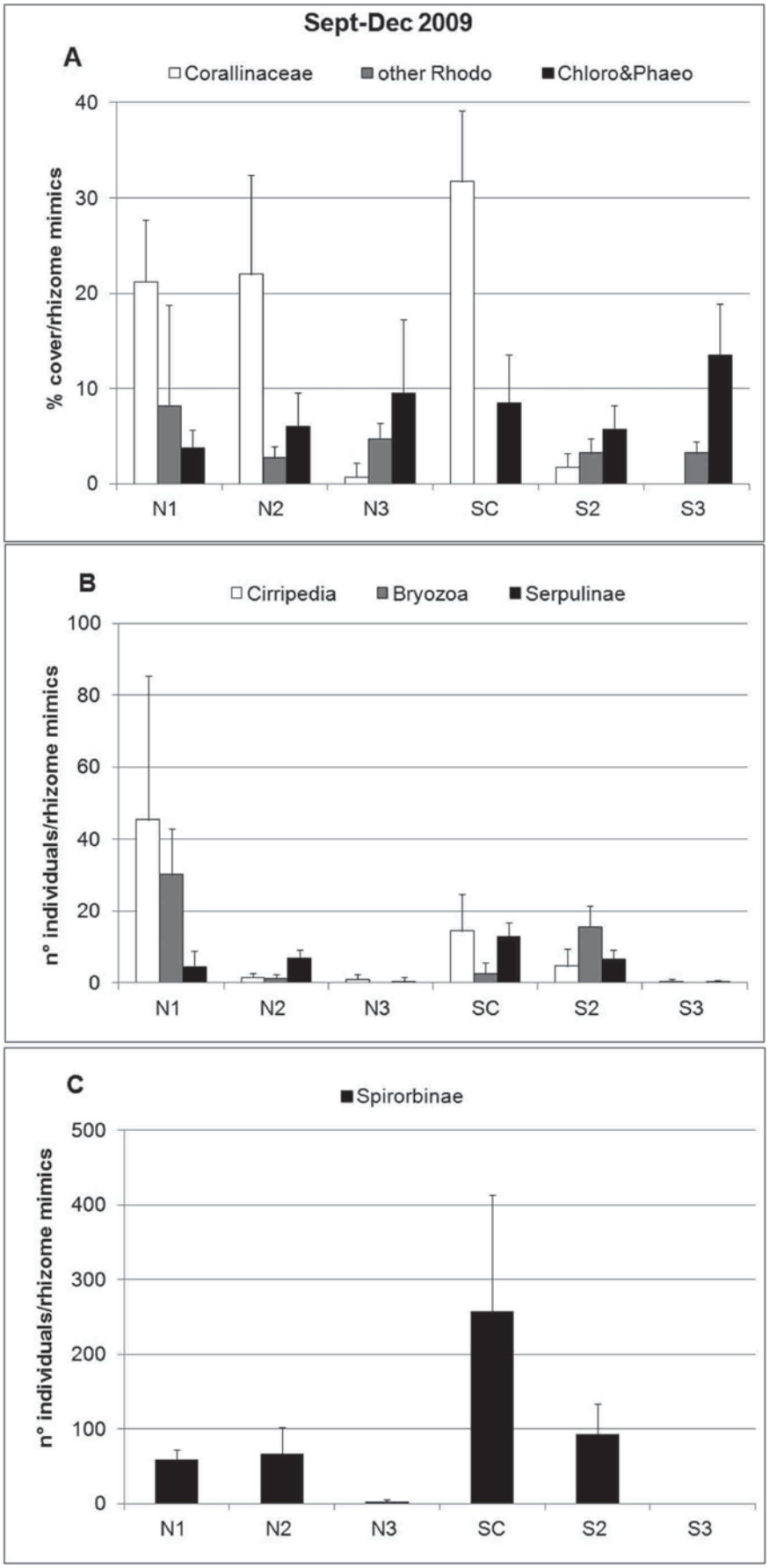

Fig. 4: Trend of cover (%) of the main macroalgal taxa and abundance of the main sessile invertebrates on the rhizome mimics of *P. oceanica* after the first 3 months of exposure (Sept. - Dec. 2009), at the north and south sides of Castello Aragonese. Bars represent standard deviations.

Table 1B. One-way ANOVA of epibiont on the rhizome mimics after the first 3 months of exposure (Sept - Dec 09), among stations (SC - N3) and between sides (North vs. South) of Castello Aragonese.
df: degree of freedom, p: probability with ns (p>0.05), * (p<0.05), ** (p<0.01); *** $P < 0.001$.

	Stations SC - N3			Side S - N		
	***F* values**	**df**	**p**	***F* values**	**df**	**p**
Corallinaceae	22.09	5	***	0.47	1	n.s.
Rhodophyceae	1.46	5	n.s.	3.23	1	n.s.
Chloro/Phaeophyceae	9.04	5	***	4.45	1	**
Cirripedia	4.29	5	**	1.08	1	n.s.
Bryozoa	17.46	5	***	0.72	1	n.s.
Serpulinae	6.85	5	***	0.66	1	n.s.
Spirorbinae	8.03	5	***	3.17	1	n.s.

absent at the acidified stations (N3, S3) (Fig. 4C). The multivariate analysis (nMDS model, graph not shown) showed a clear separation between mimics at the acidified S3 and N3, and all other stations, with mimics of the control station SC forming a relatively compact subgroup. The ANOSIM test highlighted a global R = 0.71, P 0.1 % for the station factor (pH), while the side factor (north *vs* south) was not significant.

Settlement pattern through time on mimics

The study of the settlement pattern over time was conducted only on the south side (replacement of 3 mimics and 6 artificial leaves every three months). The analysis of succession on artificial leaves (Fig. 5) showed that coralline algae were absent in S3 in all periods of exposure, and always low in S2. Settlement at control station occurred mainly in June-Sept 2010 with mean cover significantly higher than in the other periods (ANOVA, Table 2A). The filamentous macroalgae (Chloro/Phaeo) were present in all stations, but with a greater abundance at the acidified station S3 with a peak in Sept-Dec 2009, and in S2 in Jun-Sept 2010 (ANOVA, Table 3). The sessile fauna showed a scarce occurrence (not shown) of a few taxa represented by both calcifying (Spirorbinae, Bryozoa) and non-calcifying organisms (Hydrozoa, Ascidiacea), without any trend according to the pH or time.

Rhizome mimics (Fig. 6) showed absence of coralline algae in S3 in all periods and a strong reduction in S2, consistently with pattern observed on the artificial leaves (ANOVA, Table 2B). At the control station coralline settlement peaked in Sept-Dec 2009, although they are present throughout the year. On the contrary, the green and brown filamentous macroalgae (Chloro/Phaeo) prevailed in S3 and S2, although scarcer in Dec-Mar 2010, and increased in Mar-June 2010 especially at the acidified station S3 (ANOVA, Table 2B). Also the sessile fauna showed different settlement pattern along the pH gradient and in time (Fig. 7). The Cirripedia (represented by two *Balanus* species), always scarce at the acidified station and absent in June-Sept 2010, settled on the other

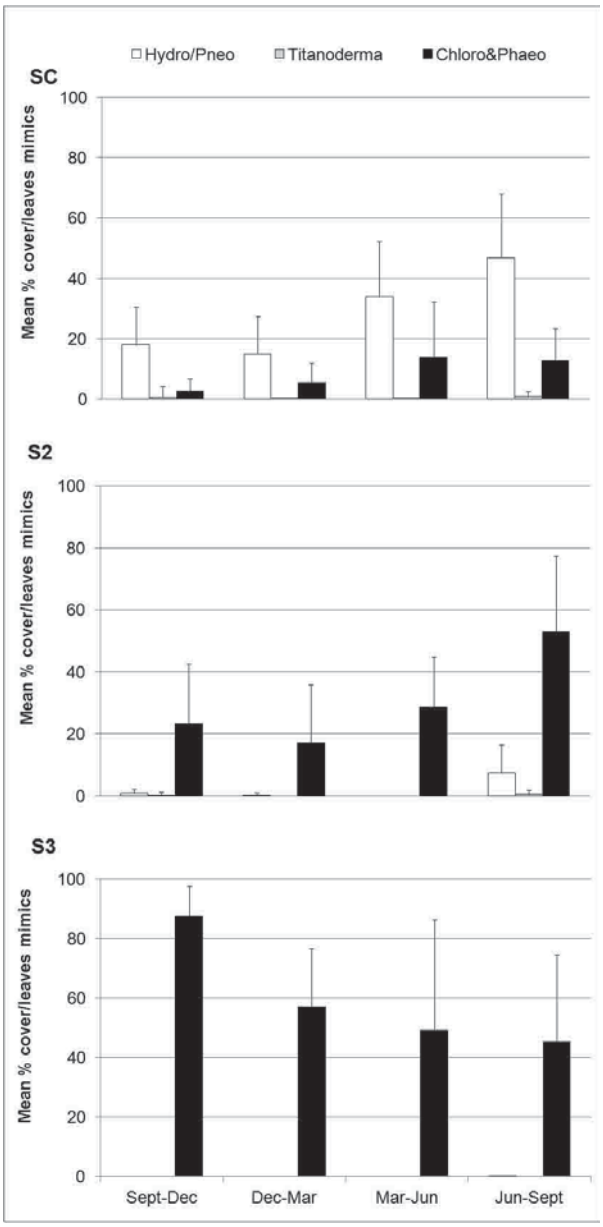

Fig. 5: Trend of settlement (algal cover) of the main macroalgal taxa in the artificial leaves of *P. oceanica* in the period Sept. 2009 - Sept. 2010 along the gradient, in the south side of Castello Aragonese. Bars represent standard deviations.

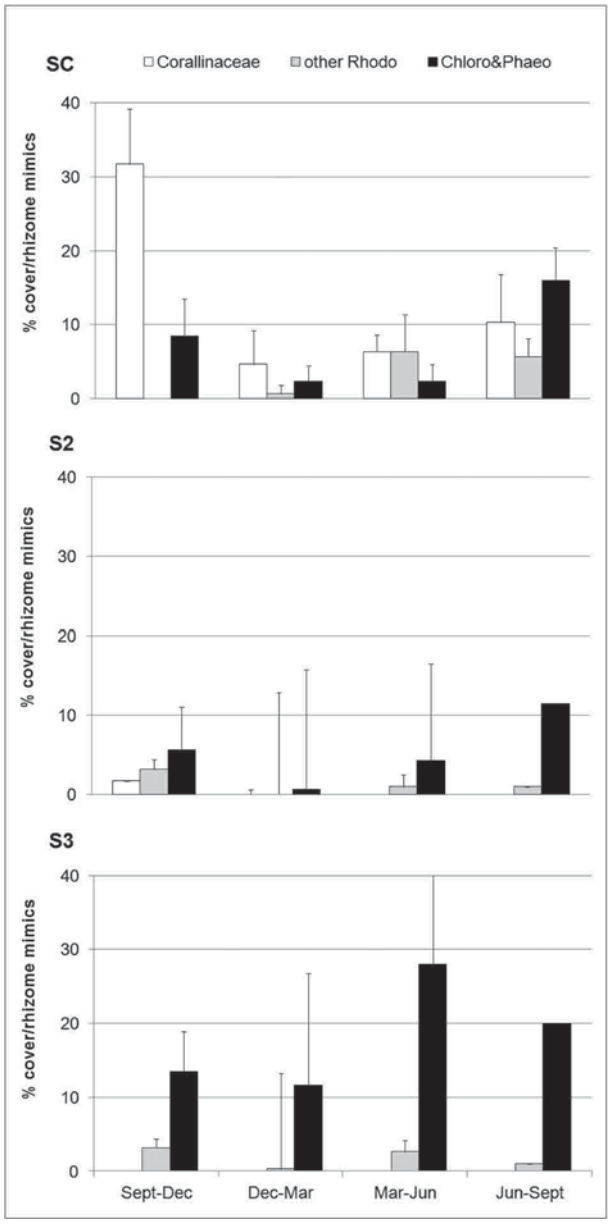

Fig. 6: Trend of cover (%) of the main macroalgal forms in the rhizome mimics of *P. oceanica* in the period from Sept. 2009 - Sept. 2010 along the gradient, in the south side of Castello Aragonese. Bars represent standard deviations.

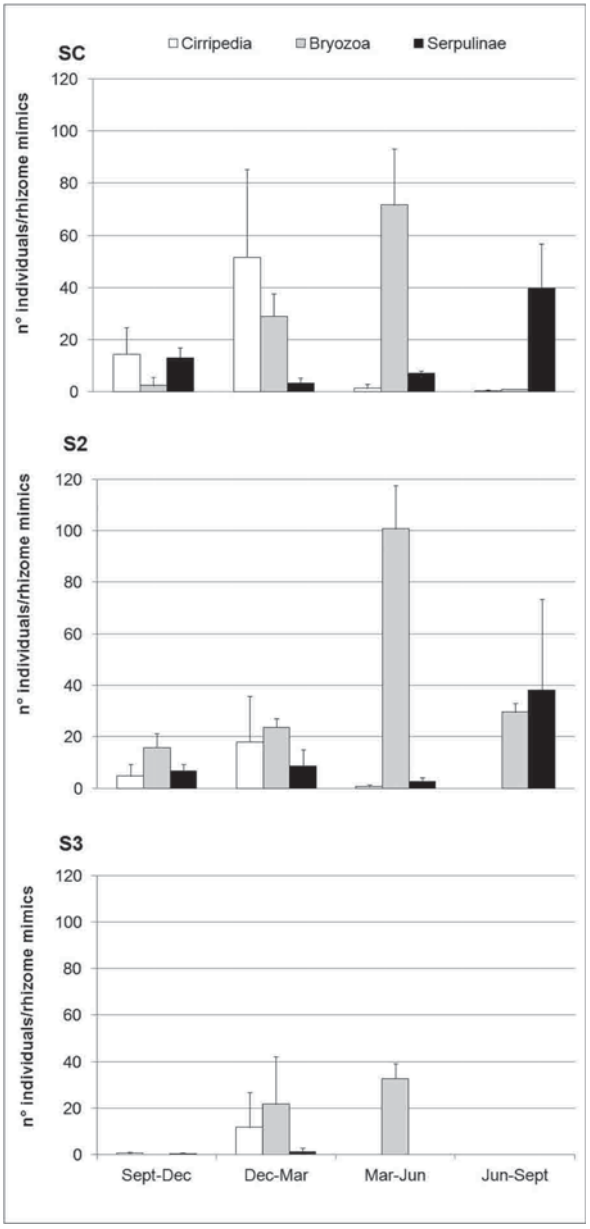

Fig. 7: Trend of the abundance of the main sessile invertebrates in the rhizome mimics of *P. oceanica* in the period from Sept. 2009 - Sept. 2010 along the gradient, in the south side of Castello Aragonese. Bars represent standard deviations.

Table 2A. One-way ANOVA of the main macroalgal forms in the artificial leaves, among stations (SC - S3), every three months (December 2009, March, June and September 2010), and in the period (Sept 09 - Sept 10).
df: degree of freedom, p: probability with ns (p>0.05), * (p<0.05), ** (p<0.01); *** $P < 0.001$.

Factors	Hydro/Pneo			Titanoderma			Chloro/Phaeo		
	***F* values**	**df**	**p**	***F* values**	**df**	**p**	***F* values**	**df**	**p**
Stations SC - S3									
Sept - Dec 09	74.11	2	***	0.85	2	n.s.	523.16	2	**
Dec 09 - Mar 10	35.03	2	***	0.57	2	n.s.	104.63	2	**
Mar - June 10	131.27	2	***	0.03	2	n.s.	20.23	2	***
June - Sept 10	149.90	2	***	6.47	2	**	36.30	2	***
Period									
Sept 09 - Sept 10	10.59	3	***	4.08	3	**	4.63	3	**
Post Hoc Test	June - Sept 10			June - Sept 10			Dec 09 - Mar 10		

Table 2B. One-way ANOVA of epibiont on the rhizome mimics among stations (SC - S3), every three months (December 2009, March, June and September 2010), and in the period (Sept 09 - Sept 10).

Factors	Corallinaceae			Rhodo			Chloro/Phaeo			Cirripedia			Bryozoa			Serpulinae			Spirorbinae		
	F values	df	p	*F* values	df	p	*F* values	df	p	*F* values	df	p	*F* values	df	p	*F* values	df	p	*F* values	df	p
Stations SC-S3																					
Sept - Dec 09	74.33	2	***	11.64	2	**	13.57	2	**	4.72	2	*	20.18	2	***	19.03	2	***	8.11	2	**
Dec 09 - Mar 10	21.77	2	n.s.	0.60	2	n.s.	1.83	2	n.s.	2.48	2	n.s.	0.25	2	n.s.	4.38	2	n.s.	31.97	2	***
Mar - June 10	22.56	2	**	2.18	2	n.s.	11.48	2	**	1.50	2	n.s.	13.16	2	**	48.14	2	***	20.61	2	**
June - Sept 10	2.83	2	n.s.	3.86	2	n.s.	1.00	2	n.s.	0.37	2	n.s.	10.03	2	*	1.04	2	n.s.	2.79	2	n.s.
Period																					
Sept 09 - Sept 10	2.15	3	n.s.	2.61	3	n.s.	1.60	3	n.s.	6,32	3	**	21,53	3	***	11,63	3	***	9,16	3	***
Post Hoc Test										Dec 09 - Mar 10			Mar - June 10			June - Sept 10			Mar - Sept 10		

stations mainly in Dec-Mar 2010 (ANOVA, Table 2B); the Bryozoa were scarce in S3 in all periods, and showed a clear peak in Mar-June 2010 in intermediate and control stations. The bryozoans represent the most diverse group of epibionts found on the mimics, with at least eleven species some of which have been found also in low pH; Serpulinae, always absent in S3 in all periods, showed in the other stations the greater settlement in June-Sept 2010. Finally, Spirorbinae (graph not shown) almost absent in S3 in all periods, showed in the other stations a long settlement period, although wth strong time fluctuations, with minima in Dec-Mar (mean number of individuals: 79 per mimics in SC and 35 in S2) and maxima in Jun-Sept (mean number of individuals: 669 per mimic in SC and 637 in S2) (ANOVA, Table 2B).

Discussion

In the present study *Posidonia* mimics were exposed to a natural acidification gradient due to volcanic CO_2 emissions. The results show that mimics highlight well the differences between leaves and rhizomes in epibiont colonization which occur in the natural shoots, although we have not yet compared the mimic epibiont composition of both rhizome and artificial leaves with the natural epiphytic communities. In natural *Posidonia* shoots, leaves always show reduced epiphyte diversity represented by more specialized taxa, respect to rhizomes assemblages (Chimenz *et al.,* 1989; Balata *et al.,* 2008; Gambi & Morri, 2008). The single study related to natural leaf epiphytes along the pH gradient at the Castello south side (Martin *et al.,* 2008) highlighted the dominance of coralline algae and some bryozoans at the control stations, and a strong reduction of all the calcareous organisms at the acidified sites. So our results related to artificial leaves are very consistent with those occurring in natural shoots.

The artificial leaves show a clear reduction of all encrusting calcareous Corallinaceae and animal taxa at the acidified stations both on the north and south sides (N3, S3) and, on the contrary, an increase of the filamentous algae (Chloro/Phaeo). These results are consistent with data reported in previous studies which highlight the sensitivity of coralline algae to the low pH level (Jokiel *et al.,* 2008; Martin *et al.,* 2008; Porzio *et al.,* 2011). Filamentous algae and thick biofilm occurring at acidified stations can explain the grazing traces visible on some artificial leaves due to radular scraping by gastropods (e.g. *Gibbula* spp.) (Mazzella & Russo, 1989), given the fact that artificial leaves lack the phenolic compound that has been shown to prevent or limit grazing (Agostini *et al.,* 1998; Dumay *et al.,* 2004).

The rhizome mimics' analysis shows a community with a higher diversity of organisms, compared to the artificial leaves. Indeed, different serpulid and spirobid species, two barnacle species, and eleven bryozoan species, in addition to the algal species, are present on the mimics. The distribution of both plant and sessile animals on the rhizome mimics along the pH gradient is consistent with a significant reduction of the calcareous organisms on the leaf mimics at the stations with very low pH, and an increase of the filamentous algae. This trend is detectable at both north and south side stations, and the pH is the factor that influences the similarity of the community at the stations as a whole, as summarized by the multivariate analysis model nMDS and ANOSIM test.

The colonization and settlement on rhizome mimics in time shows temporal trends diversified among the different sessile organisms. According to Cocito *et al.* (2012) who studied bryozoan settlement on mimics in a deep *Posidonia* meadow off Ischia, bryozoan settlement mainly occurred in spring time, thus confirming observations on bryozoans' recruitment peak on mimics. It is worth to note that the obliged epiphytic species of *Posidonia*, *Electra posidoniae*, is present only on natural leaves and never on the artificial leaves, a pattern observed also in a different study using artificial leaves (Michel, 2011). This fact supports once more that natural leaves are not a simple substrate for epiphytes but exert an attractive or repulsive action due to specific compounds. For both serpulids and spirorbids recruitment peaks in summer are consistent with the actual knowledge on the reproductive biology, at least for the Mediterranean species (Bianchi,

1981).

Overall, every analyses of leaves' and rhizomes' mimics show a similar trend represented by a reduction or total disappearance of calcifying forms in the low pH stations. This is consistent with what observed in studies on benthic plant components (Porzio *et al.,* 2011) and animals of hard substrata studied in the same vent area (Hall-Spencer *et al.,* 2008; Kroeker *et al.,* 2011). These findings confirm that most calcifying organisms are particularly sensitive to increased seawater acidification, and may be less competitive for space than non-calcifying organisms in areas with very low pH levels. However, some of them may still persist in low pH due to their mineralogy and calcification features. In fact, cellular wall in coralline algae is impregnated with deposits of calcium carbonate in the form of calcite, but with a variable amount of magnesium in the crystal lattice (with variable concentrations 3.5-6% Mg; Milliman, 1974). Similarly, the ability of some bryozoans to persist at least for short period in low pH could be explained by the diverse skeletal mineralogies. Most bryozoan species are calcitic, but some have aragonitic or bimineralic skeletons, and the complex mineralogies can vary among species, within a single colonies and sometimes transition from one mineralogy to another can be observed within the same modular unit (Smith *et al.,* 2006; Taylor *et al.,* 2008, 2009). Recent experiments conducted on bryozoans transplanted in the same volcanic vent area off Ischia where this study was conducted, revealed different responses depending on organic components and mineralogy of the species, with possible reallocation of energy resources within the colonies when exposed to unfavourable conditions such as low pHs (Lombardi *et al.,* 2011a, b). The presence of calcifying bryozoans species along a pH gradient, as those observed on the rhizome mimics, could be explained by the possibility of the larvae to settle and tolerate low pH at least for few weeks or species could be potentially able to 'adapt' to the changing chemistry (pH) conditions. The Cyclostome *Patinella radiata,* for example, has been found on natural *Posidonia* leaves in Ischia growing along a pH gradient revealing its potential to settle, live and reproduce in below normal pH environments (Lombardi C., personal observation).

Overall community simplification and altered succession dynamics have been highlighted on hard bottoms off the Castello area, using artificial substrates (volcanic tiles) by Kroeker *et al.* (2013a), and on natural rocky substrates (Kroeker *et al.,* 2013b). As for the *Posidonia* system, considering that mimics resulted a good proxy of the natural epiphytic community, this profound difference in epibiont communities in areas with low and very low pH levels, showed also by the natural leaf community (Martin *et al.,* 2008), has certainly cascading effects on the food web of the meadow, and must influence the functioning of the system. Studies on both epiphytes and motile fauna of these acidified *Posidonia* stands are in progress to highlight the effects of acidification on the whole community associated to this important ecosystem of the Mediterranean Sea.

Acknowledgements

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REVIEW ARTICLE

Cephalopods in neuroscience: regulations, research and the 3Rs

Graziano Fiorito · Andrea Affuso · David B. Anderson · Jennifer Basil · Laure Bonnaud · Giovanni Botta · Alison Cole · Livia D'Angelo · Paolo De Girolamo · Ngaire Dennison · Ludovic Dickel · Anna Di Cosmo · Carlo Di Cristo · Camino Gestal · Rute Fonseca · Frank Grasso · Tore Kristiansen · Michael Kuba · Fulvio Maffucci · Arianna Manciocco · Felix Christopher Mark · Daniela Melillo · Daniel Osorio · Anna Palumbo · Kerry Perkins · Giovanna Ponte · Marcello Raspa · Nadav Shashar · Jane Smith · David Smith · António Sykes · Roger Villanueva · Nathan Tublitz · Letizia Zullo · Paul Andrews

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Abstract Cephalopods have been utilised in neuroscience research for more than 100 years particularly because of their phenotypic plasticity, complex and centralised nervous system, tractability for studies of learning and cellular mechanisms of memory (e.g. long-term potentiation) and anatomical features facilitating physiological studies (e.g. squid giant axon and synapse). On 1 January 2013, research using any of the about 700 extant species of "live cephalopods" became regulated within the European Union by Directive 2010/63/EU on the "Protection of Animals used for Scientific Purposes", giving cephalopods the same EU legal protection as previously afforded only to vertebrates. The Directive has a number of implications, particularly for neuroscience research. These include: (1) projects will need justification, authorisation from local competent authorities, and be subject to review including a harm-benefit assessment and adherence to the 3Rs principles (Replacement, Refinement and Reduction). (2) To support project evaluation and compliance with the new EU law, guidelines specific to cephalopods will need to be developed, covering capture, transport, handling, housing, care, maintenance, health monitoring, humane anaesthesia, analgesia and euthanasia. (3) Objective criteria need to be developed to identify signs of pain, suffering, distress and lasting harm particularly in the context of their induction by an experimental procedure. Despite diversity of views existing on some of these topics, this paper reviews the above topics and describes the approaches being taken by the cephalopod research community (represented by the authorship) to produce "guidelines" and the potential contribution of neuroscience research to cephalopod welfare.

Keywords Cephalopods · Directive2010/63/EU · Animal welfare · 3Rs · Neuroscience

Graziano Fiorito, Jennifer Basil, Frank Grasso, Michael Kuba, Nadav Shashar and Paul Andrews have contributed equally to this work.

Disclaimer Cephalopod research, housing and mantainace is an often controversial and little studied subject; therefore, not all the authors and participants of the meeting necessarily agree with all points raised in the present paper.

G. Fiorito · A. Affuso · F. Maffucci · D. Melillo · A. Palumbo
Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy

G. Fiorito (✉) · G. Ponte · P. Andrews
Associazione Cephalopod Research 'CephRes', ONLUS,
Via dei Fiorentini 21, 80133 Naples, Italy
e-mail: cephres@cephalopodresearch.org

A. Affuso
Animal Model Facility, BIOGEM SCARL,
Via Camporeale Area PIP, Ariano Irpino, AV, Italy

D. B. Anderson
Midlothian Innovation Centre, Pentland Management Systems,
Pentlandfield, Roslin EH25 9RE, UK

J. Basil
Biology Department, CUNY Graduate Center, Brooklyn College, 2900 Bedford Avenue, Brooklyn, NY 11210, USA

L. Bonnaud
Muséum National d'Histoire Naturelle, DMPA, Lab. BOREA,
UMR MNHN CNRS 7208-IRD 207-UPMC, Paris Cedex, France

L. Bonnaud
Université Paris Diderot, Sorbonne Paris Cité, Paris, France

G. Botta
Ministero della Salute, Via G. Ribotta 5, 00144 Rome, Italy

Introduction

Cephalopods are a numerically small but significant taxon of invertebrates (phylum Mollusca) whose richness of behavioural capabilities (Borrelli and Fiorito 2008) fascinate the public and researchers alike, but that also represent a very important resource for human consumption (Jereb et al. 2005). The class Cephalopoda is considered the most complex one in the phylum Mollusca and arguably amongst all other invertebrate phyla as reflected in the use of the term "advanced invertebrate" or "exceptional invertebrate class" (sensu Zullo and Hochner 2011). It includes exclusively marine living species considered to have rivaled fishes during evolution (e.g. Packard 1972; but see also Kröger et al. 2011). Cephalopods demonstrate a refined and extraordinary ability to adapt their morphology (Kröger et al. 2011) and behavioural repertoire to their niche; this may have contributed greatly to their success (Hochner et al. 2006; Hochner 2008, 2012; Borrelli and Fiorito 2008). Amongst the several notable expressions of phenotypic plasticity in cephalopods (Hanlon and Messenger 1996; see also Barbato et al. 2007) is the capability to display environmentally cued phenotypes, i.e. body patterns (Borrelli et al. 2006). The complex behavioural and learning capabilities of cephalopods (Hanlon and Messenger 1996; Borrelli and Fiorito 2008; Huffard 2013) correspond to a highly sophisticated nervous system that appears to be correlated with their lifestyle (Nixon and Young 2003; Borrelli 2007). The flexibility of the behavioural repertoire of cephalopods is supported by evident cellular and synaptic plasticity at the level of the central and peripheral nervous system and of the neuromuscular junctions (review in Brown and Piscopo 2013). Cephalopods are well known amongst neuroscientists for their contribution to fundamental understanding of the nervous system (Young 1985; Abbott et al. 1995; but see also Brown and Piscopo 2013).

This paper is prompted by the recent inclusion of "all live cephalopods" in Directive 2010/63/EU that regulates the use of animals for scientific purposes (European Parliament and Council of the European Union 2010).

Regulation of scientific uses of cephalopods

National legislation regulating experimentation on living animals began to appear in several European countries in the late nineteenth century and made a division between vertebrates and invertebrates, with only vertebrates being regulated (i.e.: United Kingdom, 1876; Germany, 1883; Denmark, 1891; see Smith et al. 2013 for references).[1]

One species of cephalopod, *Octopus vulgaris*, was included in a revision of the UK legislation (Animals [Scientific Procedures] Act 1986), but no studies have ever been conducted under the legislation. Cephalopods have been included in various national codes of practice and legislation covering research in several countries outside the EU, for example: Canada, 1991; New Zealand, 1999; Australia, 2004; Switzerland, 2011; Norway, 2011; see Smith et al. (2013) for details and references.

Animal experimentation involving all vertebrates has been regulated at EU level since 1986 (Directive 86/609/

A. Cole
BIOGEM SCARL, Via Camporeale Area PIP,
Ariano Irpino, AV, Italy

L. D'Angelo · P. De Girolamo
Department of Veterinary Medicine and Animal Productions,
University of Naples Federico II, Naples, Italy

N. Dennison
Home Office, Animals in Science Regulation Unit,
Dundee DD1 9WW, Scotland, UK

L. Dickel
Groupe Mémoire et Plasticité Comportementale, EA4259, GdR
CNRS 2822 Ethology, University of Caen Basse-Normandy,
Caen, France

A. Di Cosmo
Department of Biology, University of Naples Federico II,
Naples, Italy

C. Di Cristo
Department of Biological and Environmental Sciences,
University of Sannio, Benevento, Italy

C. Gestal
Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain

R. Fonseca
Center for GeoGenetics, University of Copenhagen,
1350 Copenhagen, Denmark

F. Grasso
Department of Psychology, BioMimetic and Cognitive Robotics,
Brooklyn College, CUNY, Brooklyn, NY 11210, USA

T. Kristiansen
Institute of Marine Research, 5817 Bergen, Norway

M. Kuba
Max Planck Institute for Brain Research,
60438 Frankfurt, Germany

[1] The vertebrate species commonly covered were those utilised in experiments (primarily physiology) at the time (e.g. frogs, cats, dogs). The wording of the UK 1876 Cruelty to Animals Act perhaps gives an additional insight into the basis for "the division" between invertebrates and vertebrates. The Act permits "the advancement of new discovery of physiological knowledge by experiments calculated to *give pain*" (our italics), implying that the authors of the Act may have taken the view that whilst vertebrates may experience pain invertebrates do not (but see Fiorito 1986; and Andrews 2011a; Andrews et al. 2013 for details).

EEC) and Directive 2010/63/EU (European Parliament and Council of the European Union 2010), which we will refer to here as the "Directive" is a major revision intended to make the regulation "more stringent and transparent" as well as recognising advances in research techniques, improved understanding and assessment of animal welfare (see: Broom 1991a, b, 2011 for an introduction to the issues) and developments in ethical review of animal experimentation (Smith et al. 2013) particularly in relation to invertebrates (Mather and Anderson 2007; Moltschaniwskyj et al. 2007; Horvath et al. 2013). The Directive also places particular emphasis on application of the "3Rs" principles of Replacement, Reduction and Refinement formulated by Russell and Burch (1959) and discussed in detail below in relation to neuroscience research.

For invertebrate research in the EU, Directive 2010/63/EU which implemented on 1 January 2013 marks a paradigm shift by covering the use of an entire class of Molluscs, namely "live cephalopods" (i.e. hatched juveniles and adults) in the legislation covering experimental procedures likely to cause pain, suffering, distress or lasting harm (EFSA Panel on Animal Health and Welfare 2005; European Parliament and Council of the European Union 2010; Smith et al. 2013). This means that, under the Directive and transposed national laws, cephalopods have the same legal status as vertebrates in relation to their experimental use in research and testing (Smith et al. 2013).

It should be noted that drafts of the Directive also included decapod Crustacea (e.g. crabs, lobsters). Although decapod crustaceans were not included in the adopted Directive, it is likely that this issue will be revisited because of the continuing debate about their pain perception (Gherardi 2009; Magee and Elwood 2013; Horvath et al. 2013) and also because as was the case with cephalopods there is interest in this issue from animal welfare and animal rights groups (Advocates for Animals 2005).

The decision to include cephalopods was based primarily upon the recommendations of a scientific panel which concluded that there was "scientific evidence of their ability to experience pain, suffering, distress and lasting harm" (i.e. PSLDH; Directive 2010/63/EU: Recital 8, European Parliament and Council of the European Union 2010). However, note that this view is not universally shared by the global research community. In essence, much of the evidence for inclusion of cephalopods in the Directive is based upon various aspects of neuroscience research on cephalopods and the criteria used, as well as additional recent studies, are reviewed by Andrews et al. (2013).

It is anticipated that the Directive will provide a stimulus to cephalopod neuroscience research, as ensuring the highest welfare standards requires answers to a number of questions some of which are summarised in Table 1.

The Directive will impact upon scientific work using any of the approximately 700 extant species of cephalopods, but in practice within the EU the species most commonly used are the coleoid cephalopods: the cuttlefish *Sepia officinalis*; the squids *Loligo vulgaris* and *Loligo forbesi*; and the octopuses *O. vulgaris*, *Eledone cirrhosa and Eledone*

A. Manciocco
Institute of Cognitive Sciences and Technologies, CNR, Via Aldovrandi 16b, Rome, Italy

F. C. Mark
Integrative Ecophysiology, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany

D. Osorio · K. Perkins
School of Life Sciences, University of Sussex, Brighton, E Sussex BN1 9RH, UK

M. Raspa
European Mouse Mutant Archive (CNR-EMMA), Consiglio Nazionale delle Ricerche, Campus A. Buzzati-Traverso, Viale E. Ramarini, 32, 00015 Monterotondo Scalo, Roma, Italy

N. Shashar
Department of Life Sciences, Ben-Gurion University of the Negev, Eilat Campus, 84105 Beer-Sheva, Israel

J. Smith
The Boyd Group, Hereford, UK

D. Smith
Federation for Laboratory Animal Science Associations (FELASA), London, UK

A. Sykes
Centre of Marine Sciences (CCMAR), Universidade do Algarve, 8005-139 Faro, Portugal

R. Villanueva
Renewable Marine Resources Department, Institut de Ciències del Mar (CSIC), Barcelona, Spain

N. Tublitz
Department of Biology, University of Oregon, Eugene, OR 97403, USA

L. Zullo
Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Genoa, Italy

P. Andrews
Division of Biomedical Sciences, St George's University of London, Cranmer Terrace, London SW17 0RE, UK

Table 1 Possible areas of biological and neuroscience research expected to contribute to increasing knowledge of cephalopod welfare as stimulated by Directive 2010/63/EU

Optimal conditions of care and maintenance of animals also aimed to increase well-being
Evidence of the capacity for cephalopods to experience pain
Search for receptors sensitive to noxious stimuli
Functional analysis of "brain centres"
Analysis of nervous pathways connecting the nociceptive system to higher "brain centres"
Search for receptors for opioid, cannabinoid and analgesic steroid substances
Studies on analgesia and animals' responses
Behavioural and functional analysis of animals' response to painful stimuli
Search of objective signs of pain, suffering and distress
Physiological indicators of pain
Humane end points in cephalopod studies
General anaesthesia for cephalopods
Establishment of objective criteria for assessing depth of general anaesthesia
Methods for maintenance of general anaesthesia and facilitation of recovery
Methods for production of local anaesthesia and systemic analgesia
Methods for humane killing
Physiological analysis and evaluation of stress, suffering or pain, including evaluation of biomarkers of immune response linked to diseases and distress
Noninvasive approaches to characterise physiological function of organs and systems and monitoring effects of experimental treatments

For review and further discussion see Andrews (2011a, b), Andrews et al. (2013) and Smith et al. (2013). See also: Borrelli and Fiorito (2008), Ponte and Fiorito (2011, 2013), Boal (2011), Margheri et al. (2011b), Ponte et al. (2013)

moschata. The shelled cephalopod *Nautilus pompilius* is also used occasionally but is imported from tropical waters.

Compliance with the new EU legislation will be challenging for many areas of cephalopod research, especially neuroscience; some concern has already been expressed regarding the applicability of "mammal-centric" regulations to cephalopods (Nosengo 2011). Yet, the legislation by itself is not aimed to be "mammal-centric", as the law applies equally to fish, amphibians and birds as well as mammals, and the principles are the same for all species!

Some implications of the Directive for research on cephalopods

The inclusion of cephalopods in the Directive has a number of implications for different groups:

1. *Researchers* All researchers who use cephalopods in their research will need to ascertain whether the intended experiments are covered by the Directive and if so an application will need to be submitted to the appropriate National Competent Authority (NCA;[2] e.g. Home Office in the UK; Ministère de l'Enseignement Supérieur et de la Recherche in France; Ministero della Salute in Italy) and approval obtained prior to starting the project. The authorisation process involves impartial evaluation of the project by the NCA including examination of the purpose of the research procedures (permitted purposes are listed in Article 5 of the Directive), compliance with 3Rs, severity classification of procedures and a harm-benefit analysis of the project (Voipio et al. 2004; for details and examples see: Smith et al. 2013). Researchers should consult their NCA to obtain details of the authorisation process as although the principles are common throughout the EU, the way in which the Directive is transposed into national legislation may differ. It should also be noted that in addition to covering the experiments themselves, the Directive also regulates the place where experiments are undertaken, the standards of housing and care of animals used for research and methods of euthanasia. Researchers will also need to ensure that their project is authorised and that their whole team is familiar with the national law covering their experiments are appropriately trained and competent to perform the procedures (Article 23 of the Directive) and, if required by the national legislation, that the project and personnel are covered by appropriate licences (e.g. in the UK Home Office Project and Personal Licences). A checklist of what is needed in the case of conducting cephalopod research in the EU is summarised in Smith et al. (2013).
2. *Animal technologists, veterinarians and regulators* The Directive places the care and welfare within a legal framework requiring documented monitoring and compliance. Research on cephalopods, under the Directive, is likely to be performed in the same places where research is currently undertaken, so those currently responsible for care and welfare will be hopefully familiar with the expected requirements. Nonetheless, it is likely that some training will be needed even for those familiar with maintenance of cephalopods in the laboratory. In addition, veterinarians or other suitable qualified experts with responsibility for laboratory animal facilities will need to become familiar with all aspects of health and welfare of the cephalopod species in their care. Although there

[2] A list of NCA is available at http://ec.europa.eu/environment/chemicals/lab_animals/ms_en.htm.

are reviews covering cephalopod health (e.g.: Boletzky and Hanlon 1983; Hochberg 1990; Hanlon and Forsythe 1990a, b; Boyle 1991; Castellanos-Martinez and Gestal 2013), there are few aquatic medicine courses covering invertebrates (see for example: Virginia-Maryland Regional College of Veterinary Medicine, http://www.vetmed.vt.edu/research/aquatic/education.html).

One aspect of monitoring compliance with the Directive involves "regular inspections" of establishments, of which "an appropriate proportion" is to be carried out "without prior warning" (Directive Article 34). Monitoring may involve inspection of the place where the animals are kept, observations of procedures and inspection of experimental records. The records must include the source of the animals, whether they were purpose bred, what they were used for and by whom, and their fate at the end of the study (Directive Article 30). Those responsible for monitoring compliance with the Directive will need training to become familiar with this newly regulated class of animal.

3. *Funders* Most grant funding agencies and charities already require that grant applications involving research on vertebrates certify that, if required, appropriate authorisation (normally including "ethical" review) to conduct the proposed studies is in place. As cephalopods are now covered by the same legislation as vertebrates, grants involving particular types of research concerning their regulated use will need to ensure that the proposed studies comply with the Directive and any national Codes of Practice related to care and welfare.
4. *Journal editors and reviewers* The editors and reviewers of Journals will need to be made aware of the change in the regulation within the EU to ensure that papers submitted for publication if appropriate make reference to compliance with the Directive. This may be difficult for a short period as although the Directive was implemented on 1 January 2013, some EU states have not yet transposed it into national legislation (http://ec.europa.eu/environment/chemicals/lab_animals/transposition_en.htm). Although not part of the Directive, several journals (e.g. Nature, PLoS)[3] have voluntary adopted the ARRIVE (Animal Research: Reporting of in vivo Experiments: http://www.nc3rs.org.uk/page.asp?id=1357) guidelines for reporting experiment (Kilkenny et al. 2010). These guidelines provide checklists of information that should be included in published papers, particularly in the methods sections. Whilst many papers involving cephalopods already contain much of this information, key information is lacking in others. For example, only in the 40 % of papers published in the 2010 ($n = 65$; source WoK: ISI Web of Knowledge), mention the conditions in which cephalopods are maintained. However, only half of those (13 out 26 papers) provide details on tank and lighting. Further analysis reveals that for the five cases in which octopuses were utilised, tanks ranged from 200 to 7,000 L and for cuttlefishes ($n = 7$) a wider range of tank sizes was utilised (from 30 to 20,000 L). It is remarkable that a justification for such a diversity of approach for accommodating animals is missing in the papers. Finally, no indication of the stocking density of animals is provided in the great majority of studies here considered.

   The lack of such information makes it difficult to undertake systematic analysis of housing conditions in order to derive guidelines reflecting the consensus in the literature. In addition, lack of critical information on sex, body weight, feeding, tank size, lighting, handling and euthanasia methodology can compromise assessment of results. Based upon studies in vertebrates, the outcome of neuroscience studies, and in particular studies of behaviour in cephalopods, is most likely to be sensitive to environmental factors.
5. *The public* Although cephalopods are frequently portrayed as creatures of nightmares in films and literature (e.g.: Muntz 1995; Ellis 1998), people are nevertheless fascinated by these animals in display aquaria and they make frequent appearances in natural history documentaries and the media. In contrast to mice, rats and rabbits, the public do not make an immediate association between cephalopods and "animal experimentation", but this may change as the knowledge of their inclusion in the Directive becomes more widely known and researchers should be aware that their studies may come under public and media scrutiny.

Neuroscience research and the impact of the Directive

Cephalopods are a large group of marine predators whose major aspects of biology, behaviour, and ecology provide a backdrop against which their neurobiology can be interpreted. Special features of their reproduction (Rocha et al. 2001), camouflage, motor control, memory, learning, and behavioural ecology may be considered as special cases of convergent evolution with vertebrates (Packard 1972; Borrelli and Fiorito 2008; Huffard 2013).

[3] For an updated list see Journals that have incorporated ARRIVE in their Instructions for Authors at: http://www.nc3rs.org.uk/page.asp?id=1796.

Table 2 A selected summary of cephalopod neuroscience and neurobehavioural research [for review see also: Borrelli and Fiorito (2008), Brown and Piscopo (2013), Huffard (2013)]

Squid giant axon and giant synapse

Physiology of resting membrane potential and action potential [consider also the Nobel Prize to Eccles (Hodgkin and Huxley1952)]

Giant axon-Schwann cell signalling

Physiology and pharmacology of synaptic transmission

Axoplasmic transport

Consider also recent studies on

The effect of mutant SOD1 implicated in Lou Gehrig disease in humans

Effect of human tau-protein implicated in Alzheimer's disease

Relevant references: Young (1938), Bullock (1948), Hodgkin and Huxley (1952), Bloedel et al. (1966), Coles and Abbott (1996), Moreno et al. (2011), Song et al. (2012)

Behavioural studies and the search for their neural correlates

Behavioural plasticity, learning and memory

Sleep-like states

Consciousness

Physiology and pharmacology of long-term potentiation (LTP)

Relevant references: Sanders (1975), Fiorito et al. (1990), Young (1991, 1995), Fiorito and Scotto (1992), Robertson et al. (1994, 1995, 1996), Fiorito and Chichery (1995), Boal 1996, Boal and Gonzalez (1998), Boal and Golden (1999), Boal et al. (2000), Agin et al. (2001), Vinogradova et al. (2002), Agin et al. (2003), Hochner et al. (2003, 2006), Karson et al. (2003), Darmaillacq et al. (2004, 2006), Boal (2006), Agin et al. (2006), Brown et al. (2006), Langridge et al. (2007), Hochner (2008), Shomrat et al. (2008, 2010), Mather (2008), Edelman and Seth (2009), Zullo et al. (2009), Zylinski et al. (2011), Shomrat et al. (2011), Tricarico et al. (2011), Zullo and Hochner (2011), Edelman (2011), Osorio and Zylinski (2011), Gutnick et al. (2011a, b), Josef et al. (2012), Hochner (2012), Frank et al. (2012)

Neurotransmitters (sensu lato)

Relevant references: Florey (1963), Loe and Florey (1966), Florey and Winesdorfer (1968), Tansey (1978, 1979), Budelmann and Bonn (1982), Williamson (1989), Cornwell et al. (1993), Messenger (1996), Palumbo et al. (1999), Loi and Tublitz (2000), Lima et al. (2003), Di Cosmo et al. (2004, 2006, 2007), Fiore et al. (2004), Scheinker et al. (2005), Di Cristo et al. (2007), Boyer et al. (2007), Wollesen et al. (2008, 2010a, b, 2012), Bardou et al. (2009, 2010), Shomrat et al. (2010), Ponte (2012), Conti et al. (2013)

Nociception

Relevant references: Crook and Walters (2011), Crook et al. (2011, 2013), Hague et al. (2013), Andrews et al. (2013), but see also: Wells et al. (1965), Wells (1978), Hanlon and Messenger (1996), Mather and Anderson (2007)

Regeneration

Regeneration of appendages following damage (wild and experimental)

Nerve regrowth

Relevant references: Lange (1920), Sereni and Young (1932), Sanders and Young (1974), Féral (1988), Rohrbach and Schmidtberg (2006), Florini et al. (2011), Fossati et al. (2013)

Table 2 continued

Neuromotor control

Motor and sensory control of arm movements

Arm use preference and functioning (including suckers)

Octopus arm as a bio-inspired robotic model

Control of chromatophores and body patterning

Relevant references: Kier (1982, 1985, 1991), Kier and Smith (1985), Hanlon and Messenger (1996), Kier and VanLeeuwen (1997), Mather (1998), Loi and Tublitz (2000), Messenger (2001), Sumbre et al. (2001, 2005, 2006), Borrelli et al. (2006), Gutfreund et al. (2006), Byrne et al. (2006a, b), Grasso and Setlur (2007), Barbato et al. (2007), Grasso (2008), Kier and Schachat (2008), Zullo et al. (2009), Mattiello et al. (2010), Calisti et al. (2011), Margheri et al. (2011a, b, 2012), Mazzolai et al. (2012), Laschi et al. (2012)

Physiology of the sensory systems

Visual and chemo-tactile systems

Statocyst and oculomotor systems

Relevant references: Bullock (1965), Williamson (1986, 1989, 1995), Budelmann (1995), Abbott et al. (1995), Lucero and Gilly (1995), Budelmann et al. (1997), Williamson and Chrachri (2004)

Development and functional organisation of the "brain" and muscles

Relevant references: Young (1991, 1995), Gutfreund et al. (1996), Shigeno et al. (2001a, b, 2008a, b), Callaerts et al. (2002), Shigeno and Yamamoto (2002), Lee et al. (2003), Hartmann et al. (2003), Nixon and Young (2003), Grimaldi et al. (2004), Borrelli (2007), Navet et al. (2008), Lee et al. (2009), Baratte and Bonnaud (2009), Navet et al. (2009), Wollesen et al. (2009), Zullo et al. (2009), Zullo and Hochner (2011), Hochner (2012), Mattiello et al. (2012)

An annotated bibliography on classical contributions to cephalopod' biology and physiology is also provided by Ponte et al. (2013). References to relevant studies included are given as examples

Neuroscience research involving brain and behaviour is particularly prominent because of the perceived status of cephalopods as "advanced invertebrates". Cephalopods are model organisms for a diverse range of neuroscience areas, and their anatomical features provide unique opportunities for research (see examples in Table 2). Neuroscience research studies may be particularly impacted by the Directive as they cover a diversity of experimental techniques ("procedures") which are often invasive and may cause pain, suffering, distress and lasting harm. This aspect is discussed in detail hereunder with examples of the types of study likely to fall within the scope of the Directive and which will need to be authorised by the appropriate national competent authority. Although researchers should be familiar with all the requirements of the Directive in relation to routine care and welfare, it is the aspects of the Directive covering procedures and their impact upon the health and welfare of the animal that are likely to have the greatest impact upon their use in research.

Care and welfare of cephalopods in neuroscience research and the need for guidelines

The inclusion of all live cephalopods (i.e. larval and adult forms) in the new EU Directive has a number of practical implications for those undertaking research involving cephalopods, irrespective of the subject area. Guidelines for the general care and welfare for vertebrate laboratory species such as mammals (Sikes and Gannon 2011) and fishes (DeTolla et al. 1995; Hawkins et al. 2011a) are well developed, and specific guidelines are available for the welfare of vertebrates used in particular types of research such as cancer (Workman et al. 2010). For vertebrates in general and mammals specifically, objective criteria for identification and assessment of pain, suffering, distress and lasting harm are well researched (e.g.: Morton and Griffiths 1985; Bateson 1991) and protocols for surgery, anaesthesia, analgesia and humane euthanasia established. However, for cephalopods such knowledge is relatively rudimentary and maybe further hampered by lack of specific veterinary expertise; as in contrast to vertebrates, cephalopods are not common companion animals, although they are often found in display aquaria and knowledge gained in this setting is making a useful contribution to understanding their general welfare requirements.

The Directive is likely to stimulate research in the above areas so as to facilitate development of evidence-based guidelines for optimal care and welfare (Moltschaniwskyj et al. 2007; Louhimies 2011; see for example: Andrews 2011a, b; Goncalves et al. 2012; Sykes et al. 2012; Andrews et al. 2013; Smith et al. 2013).

The text of the Directive does not provide specific guidance on the above aspects for cephalopods, and at present, there are no national codes of practice for care and use of cephalopods under the terms of the Directive. In view of this, the cephalopod research community initiated a project to develop guidelines for the Care and Welfare of Cephalopods in Research. This project is an initiative[4] between the Federation of European Laboratory Animal Science Association (FELASA: www.felasa.eu), the Boyd Group (http://www.boyd-group.demon.co.uk/) and CephRes (www.cephalopodresearch.org). The guidelines are being developed based upon structured discussions amongst 30 active cephalopod researchers drawn from 26 research institutes in 11 countries including from outside the EU. The discussions also included national and EU legislators and regulators, as well as researchers with expertise in vertebrate animal welfare (i.e. Giovanni Botta, Italy; Paolo De Girolamo, Italy; Ngaire Dennison, UK; Tore Kristiansen, Norway; Marcello Raspa, Italy; Jane Smith, UK; David Smith, UK). Some of the main points arising from these discussions, with particular impact upon neuroscience research, are discussed below. It must be emphasised that these only provide an overview, and there are still many areas of contention. More detailed reviews of specific aspects and species should be consulted for more practical information.

Table 3 summarises some of the main reviews and topics in this area. However, given that there are more than 700 known living species of cephalopods of which a wide variety are used for scientific purposes, care should be taken to meet the particular requirements of individual species involved in experiments or other scientific procedures. Species-specific guidelines will need to be developed, and for many aspects of care and welfare, this will require research, but here we focus on the more generic

Table 3 Summary of resources relevant to implementation and compliance with specific aspects of Directive 2010/63/EU in relation to cephalopods

Area covered by the Directive	References
Biology including normal behaviour and physiology	Bullock (1965), Wells (1962, 1978), Hanlon and Messenger (1996), Norman (2000), Boyle and Rodhouse (2005), Borrelli et al. (2006), Boal (2011)
Overview of Directive requirements and project ("ethical") review	Smith et al. (2013)
List of what needs to be done if you are a researcher	
Ethics of cephalopod research and invertebrates in general	Mather and Anderson (2007), Moltschaniwskyj et al. (2007), Andrews (2011a), Horvath et al. (2013)
3Rs principles in relation to cephalopod research including worked examples of project review	Smith et al. (2013)
Various aspects of general maintenance, handling, rearing and culture of a number of cephalopod species	Grimpe (1928), Walker et al. (1970), Boletzky and Hanlon (1983), Boal (2011), Sykes et al. (2012)
Pain, suffering and distress in cephalopods	Crook and Walters (2011), Crook et al. (2011, 2013), Andrews et al. (2013)
Approaches to objective measurement of cephalopod health and welfare	
General anaesthesia	Gunkel and Lewbart (2008), Pagano et al. (2011), Lewbart and Mosley (2012), Goncalves et al. (2012), Gleadall (2013), Andrews et al. (2013)
Euthanasia	Boyle (1991), Demers et al. (2006), Andrews et al. (2013)

[4] Developing guidelines for the care and welfare of Cephalopods under European directive 2010/63/EU. Available at: http://www.felasa.eu/announcements/felasa-collaboration-on-cephalopods.

issues relating to the cephalopod species most commonly used in the EU, as a baseline for future Guideline development.

Care and welfare of cephalopods: an introduction

This section discusses some of the key areas covered by the Directive and which we believe impact particularly upon neuroscience research involving cephalopods.

Sources of animals

Cephalopods used in research are currently commonly taken from the wild (for review on fishing methods see: Lane 1960; Boyle and Rodhouse 2005) mainly because of the difficulties of laboratory breeding of many but not all species. Recent exceptions are, for example, *S. officinalis*, *Octopus bimaculoides*, *Euprymna scolopes* (review in Albertin et al. 2012). However, the Directive (Article 9) prohibits capture in the wild *unless* an exemption has been granted by the NCA. In practice, this means that animals may still be obtained from the wild provided that this can be justified to the Competent Authority. In addition, capture must be undertaken by competent persons using methods which do not cause pain, suffering, distress or lasting harm.

Wild caught animals may be obtained from approved suppliers (including authorised laboratories specialising in cephalopod research or specialist importers as in the case of *Nautilus*), but they must also obtain approval for the capture from the NCA.

Depending upon the research project, one potential issue with using research animals from the wild is that it may be harder to ensure "standardised" groups of animals both within a study continuing over several years and to permit comparison between research groups in different locations. This inherent variability may lead to the use of a larger number of animals than in other studies to demonstrate statistically significant effects, particularly in behavioural studies, and this could become an issue in project evaluation and authorisation where factors taken into account include animal numbers (estimates may include power calculation) and experimental design (including statistical analysis) to ensure that the minimum number of animals are used to achieve the scientific objective (see instructions and citations included in Animal Behaviour 2012).

Transport, quarantine and acclimatisation

Transport of animals should be minimised, and where possible the researcher should travel to study the animals not vice versa. A solution is to transport eggs rather than animals (e.g. cuttlefish) and to culture these; however, as mentioned above, this is not possible for most cephalopod species. Transport of animals should always be in sea water. The levels of available oxygen and accumulation of metabolites in a limited volume are important considerations for transport of living cephalopods, as recommended in the classic work by Grimpe (1928).

When animals are transported, the potential impact upon their health and welfare will need to be assessed and careful consideration given to the time required for adaptation before experimentation. On arrival in the laboratory, all animals should be closely inspected for overt signs of illness and if necessary advice sought from the person with legal responsibility for the care of animals (e.g. veterinarian or other appropriately qualified expert) on action to be taken.

Quarantining the animals is good practice whether they come from the wild or an authorised breeder/supplier as it reduces the risk of introducing infectious agents or parasites that could spread to other animals. It also gives time for diseases to manifest before animals are assigned to a research project requiring long-term study.

Irrespective of their origin, animals will need some time to acclimatise to their novel home or experimental environment (review in: Grimpe 1928; Borrelli 2007; Borrelli and Fiorito 2008) before any experimental procedures can be contemplated, although the nature of the study may affect the duration of acclimatisation. Research is needed to identify objective measures of acclimatisation.

Acclimatisation also needs to be considered when moving animals from one tank to another within the laboratory especially if the animal has been moved out of water even for brief periods. For example, in *E. cirrhosa* Malham et al. (2002) showed that 5-min exposure to air produced a significant increase in plasma noradrenaline lasting up to 30 min and in reactive oxygen species lasting 2 h. The experimenter should be aware of potential handling and relocation stress, and their possible impacts upon their study. For instance, the skin of cuttlefishes and squids is delicate and may be harmed if they are removed from the water with nets; nautiluses are particularly sensitive to exposure to air (J. Basil, personal communication), and this should be avoided if possible by transporting them in vessels containing sea water; for octopuses, it is acceptable to use wet nets with a fine mesh (but see Walker et al. 1970). Movement of animals should be minimised.

Environment and its control

Water supply and quality

As a minimum, sea water salinity, dissolved oxygen, pH, nitrogenous compounds and temperature must be

monitored and maintained within physiological ranges reported for each species.

Cephalopod housing systems currently are predominantly based on open systems where a continuous supply of fresh sea water from a nearby location is available. More recently, efficient and relatively easily maintained closed aquarium systems have been developed (Toonen 2003; Gutnick et al. 2011b). In open sea water systems, water flow and exchange should be high enough to maintain water quality comparable to natural conditions. In a closed system, sea water salinity, dissolved oxygen, pH, nitrogenous compounds and temperature must be monitored and maintained within physiological ranges reported for each species.

Commercially available artificial seawater preparations are considered adequate and contain all the necessary substances and trace elements to keep cephalopods in good health (e.g. any mixture designed for marine invertebrates and corals but not fish is recommended). Trace elements, in particular strontium and calcium, should be monitored and added, if necessary.

Cephalopods are reported to accumulate (e.g.: Storelli et al. 2005; Seixas et al. 2005; Seixas and Pierce 2005; Raimundo et al. 2005; Napoleao et al. 2005; Raimundo and Vale 2008; Lacoue-Labarthe et al. 2008; Bustamante et al. 2008; Raimundo et al. 2009, 2010b; Pernice et al. 2009; Lourenco et al. 2009; Galitsopoulou et al. 2009; Pereira et al. 2009; Cirillo et al. 2010; Lacoue-Labarthe et al. 2012), and be sensitive to heavy metals (Raimundo et al. 2010a; de Polo and Scrimshaw 2012; Semedo et al. 2012), so care should be taken to ensure these are monitored and maintained within normal ambient ranges.

It is important to keep water and tanks clean of animal waste, uneaten food or inedible components (e.g. crab shells).

Light requirements

Photoperiod and light intensity should be maintained according to the natural living habits and possibly the geographical origin of the animal. A simulated dusk and dawn period is desirable. In the great majority of cases, cephalopods will adapt to changes in the lighting conditions in captivity (see for example: Fiorito et al. 1990; Borrelli 2007; Sykes et al. 2011). A number of studies have been carried out to analyse the circadian rhythm of several species (Houck 1982; Meisel et al. 2003, 2006; Brown et al. 2006; Frank et al. 2012). Recent studies also revealed an effect of light regimes on the growth of cuttlefish (Sykes et al. 2013). However, further studies are required to assess whether significant deviations in light intensity or photoperiod from the natural environment negatively impact animal welfare.

The use of a weak ambient light (e.g. moonlight lamp) or a specific red light illumination reduces the risk of disturbance when observation of the animal is required at night (e.g. Allen et al. 2010).

Noise and vibration

Recent evidence provides preliminary information on the impact of sound on cephalopods well-being (Guerra et al. 2007; André et al. 2011; Fewtrell and McCauley 2012). Noise, vibration and other sources of disturbance should be avoided; those originating from aquarium systems should be minimal, and preferably pumps and any other noise sources should be placed in a separate room.

Assessment and maintenance of health and welfare

Animals must be inspected at least once a day by a competent person, and a record kept of their conditions (Directive Annex III requirement). Signs of health and illness in cephalopods vary with species (for a review of possible signs due to diseases see: Hochberg 1990; Hanlon and Forsythe 1990a, b). Signs based upon appearance, behaviour and physiology which could be used as part of health monitoring programme are summarised below. Criteria for identification of well-being and illness are closely related to the development of signs of pain, suffering, distress and lasting harm (PSDLH) required for assessment of the impact of regulated procedures and development of humane end points[5] for studies (Andrews 2011a; Andrews et al. 2013) including assessment of the effect of surgical procedures or drug treatments on the animals. Signs of illness and PSDLH also need to be capable of some quantification to assess their magnitude and duration for implementing humane end points and reporting data (a requirement under the Directive, for publication by the EU) on the actual severity of effects caused by procedures (e.g. mild, moderate, severe) in comparison with that anticipated at the time of project evaluation. This is an area requiring considerable research, and the criteria outlined below should be viewed as a starting point, from which more detailed guidance is being developed (for details see Andrews et al. 2013).

For each of the categories below, consideration needs to be given to grading the signs to link to the assessment of severity. For example, what degree of weight loss would be considered mild, moderate and severe?

[5] I.e. the predetermined criteria which if they are reached result in termination of the procedure or require treatment/euthanasia of the animal.

Indicators based on appearance of the animal

- Abnormal body colouration and body patterning, skin texture including swellings (bruising or oedema) and compromised skin integrity (erosion and ulceration); for examples see figures included in Hochberg (1990) and Hanlon and Forsythe (1990a, b). Skin lesions should always be closely monitored and if possible treated. For example, *E. cirrhosa* housed long term in the laboratory died within 2–4 days of the skin ulcerating (Boyle 1981, 1991).
- Abnormal morphology or damage to cuttlebone or shell.
- Abnormal body posture or position in the water column.
- Abnormal appearance as a result of body weight loss, possibly secondary to reduced food intake.

Indicators based upon the behaviour of the animal

- Reduced or absent food intake and a reluctance to feed or to attack (consider that reduced feeding during acclimatisation should be expected).
- Reduced or absent social interaction in social species and refusal or reluctance to leave a shelter in solitary housed species (e.g. *O. vulgaris*); lack of response to external stimuli or a sluggish response and in octopus a lack of natural curiosity.
- Stereotypic or repetitive behaviours.
- Reduced or excessive grooming and guarding behaviour of a body part.
- Abnormal motor or locomotor coordination.
- Autophagy or automutilation normally indicated by removal of one or more arms (Reimschuessel and Stoskopf 1990; Budelmann 1998).
- Excessive, uncontrolled or inappropriate inking.

Clinical signs

- Abnormal change (increase or decrease) in ventilation defined by rate, depth and coordination.
- Reduction in body weight over specific periods of time.
- Biomarkers such as phagocytes and catecholamines in the blood may increase due to several causes (e.g.: Malham et al. 1998a, b, 2002).[6]

[6] Note there is also a possibility of measuring faecal steroids (e.g. cortisol) as well as reproductive hormones (Larson and Anderson 2010) to assess welfare with the advantage that such techniques are noninvasive.

Causes of illness in cephalopods

Knowledge of the causes and diagnosis of illness (taken here to be any deviation from normal functionality) in cephalopods is rudimentary in comparison with fish and other vertebrates. The Directive's requirement for health monitoring should act as a stimulus to research in this area and in particular systematically collection, collation and exchange data. It will be important to distinguish between illness acquired in the wild, acquired in the laboratory and congenital defects. Causes of illness can be summarised under the following headings, but each cause requires detailed research, as do treatments.

1. *Physical trauma* This includes skin, shell, pen or gladius and cuttlebone damage during capture of wild caught animals or by collision with a transport or holding tank wall (Grimpe 1928; Boyle 1981). Bites and limb amputation are also commonly observed in wild caught animals (e.g. *O. vulgaris*, Florini et al. 2011) but may also occur during fighting, cannibalistic behaviour (Ibáñez and Keyl 2010) or by autophagy/automutilation (Reimschuessel and Stoskopf 1990; Budelmann 1998).
2. *Parasites, bacteria and viruses* Host defence mechanisms in cephalopods have been reviewed by Ford (1992) and recently by Castellanos-Martinez and Gestal (2013). In the words of Boyle, "Cephalopods carry a wide variety of parasites and symbionts which include viruses, bacteria, fungi, sporozoans, ciliates, dicyemids (mesozoa), monogeneans, digeneans, cestodes, acanthocephalans, nematodes, polychaetes, hirudineans, branchiurians, copepods and isopods" (Boyle 1991, p. 133). However, there appear to be few data on the health impact (if any) of these various organisms, although it is likely that bacteria and viruses are causal agents of illness particularly in senescing animals with compromised defences (Anderson et al. 2002; Pascual et al. 2010). The cases provided below represent only few examples.

   Bacteria have been isolated from skin lesions in octopus and squid (e.g.: Hanlon et al. 1984; Pascual et al. 2006) and infection of *Octopus joubini* with *Vibrio alginolyticus* induced skin ulceration in 2 days (as reported by Boyle 1991) and in *E. cirrhosa* a related *Vibrio* sp. (obtained from the diet) impaired skin would healing (Polglase et al. 1983; Bullock et al. 1987).

   The gastrointestinal coccidian parasite *Aggregata octopiana* is found in *O. vulgaris* and produces malabsorption syndrome impacting growth (Castellanos-Martinez and Gestal 2011; but see also Castellanos-Martinez and Gestal 2013). A related organism

(*A. andresei*) has been identified in the flying squid, *Martialia hyadesi* (Gestal et al. 2005). Larval nematodes including *Ascaris* and *Anisakis* (commonly found in fish) and larval and adult trematodes are reported in many cephalopod species (Hochberg 1990; Pascual and Hochberg 1996), but the health impact (if any) on the cephalopod is not known (for review see also Castellanos-Martinez and Gestal 2013).

The bacterium *Vibrio fischeri* has been studied extensively as it is a symbiont of the bobtail squid, *E. scolopes* (McFall-Ngai 1994; Nyholm et al. 2009; McFall-Ngai et al. 2010; Rader and Nyholm 2012; Collins et al. 2012; Nyholm and Graf 2012) and should not be considered as a disturbance.

3. *Toxic substances* As reviewed by Smith (2008) and Smith et al. (2008) toxic agents may originate from food and seawater. Food, particularly crustacea and bivalves, are a potential source of a number of neurotoxins including paralytic, diarrhoeic and neurotoxic shellfish toxins (Watkins et al. 2008; for review see for example: Wang 2008; see also Paredes et al. 2011). Although the clinical effects of these toxins on humans are clear, the impact (if any) on cephalopods is not known, but—in view of the number of brain behavioural studies in which cephalopods are used—studies of the potential effect of the neurotoxic substances (including amnesia inducing toxins reported in cephalopods; e.g.: Costa et al. 2005; Costa and Pereira 2010; Lage et al. 2012; Braid et al. 2012) are needed to assess whether this could be a confounding factor in some research studies. Sea water may become toxic from excess levels of heavy metals and environmental pollutants. Little is known of the sensitivity of cephalopods to specific agents (e.g.: Raimundo et al. 2010a; Semedo et al. 2012). Measurements of antioxidant enzyme activity (catalase, superoxide dismutase, and glutathione *S*-transferases) in the digestive gland have been shown to be markers of oxidative stress induced by metal accumulation in *O. vulgaris* (Semedo et al. 2012). Recent studies have also shown that ingested nanoparticles induce immune responses in the octopus (Grimaldi et al. 2013). Sea water may also become toxic if oxygen, carbon dioxide, pH and nitrogenous waste products are outside normal limits (e.g.: Gutowska et al. 2010a, b; Hu et al. 2011) particularly if accompanied by elevated temperature.

Age estimation and senescence

Age estimation in cephalopods is essentially based upon direct methods (Semmens et al. 2004) and analysis of increments in internal structures (e.g.: Choe 1963; Bettencourt et al. 1996; Perez et al. 1996; Le Goff et al. 1998; Jackson and Moltschaniwskyj 1999; Bettencourt and Guerra 2000; Arkhipkin 2005; Hall et al. 2007; Ikeda and Kobayashi 2010; Hermosilla et al. 2010; Canali et al. 2011a, b; Lei et al. 2012; Arkhipkin and Shcherbich 2012; Raya et al. 2013). Further research is recommended to estimate age in cephalopods in vivo.

With age, cephalopods undergo the natural process of senescence, a process where the body appears to "shut down" in females after brooding (review in Rocha et al. 2001) and the animal begins to die. The clinical signs of animals in senescence include reduced or absent drive to eat, cloudy eyes and changed behaviour (Chichery and Chichery 1992a, b; Dumont et al. 1994; for review see also Anderson et al. 2002). Good record keeping of age may help to differentiate between animals that are affected by diseases or simply show signs of senescence.

The predictable onset of senescence in some species of cephalopods post-reproduction (Rocha et al. 2001; but see also Anderson et al. 2002) and the modulation of the process by the secretions from the optic gland (Wodinsky 1977) may make cephalopods a model for investigating the impact of senescence on the brain (see also: Chichery and Chichery 1992a, b; Dumont et al. 1994) and provide insights in neuroprotective mechanisms. Such studies would need to be justified in the project evaluation process and in particular the potential welfare issues regarding the care of senescent animals carefully considered (see Smith et al. 2013 for discussion).

Housing and care

Tank specification and location

Tank requirements (for review see also: Grimpe 1928; Hanlon et al. 1983; Boletzky and Hanlon 1983; Borrelli 2007) vary tremendously between species as do stocking densities. In some benthic species, the available bottom surface area is an important requirement, whilst in others the volume of water is of more relevance. Shape and size of tanks should accommodate the natural behaviour of the animals. For example, Nautiloids need to be provided with vertical space, but benthic cephalopods need to be given large surface areas rather than deep tanks, and pelagic species need sufficient space to swim. Smooth, curved walls are recommended at least for cuttlefish and squid. Annex III of the Directive requires that "All animals shall be provided with space of sufficient complexity to allow expression of a wide range of normal behaviour. They shall be given a degree of control and choice over their environment to reduce stress-induced behaviour". Animals

should be provided with dens and shelters based upon their natural requirements. Use of gravel as a substrate for benthic species is highly recommended, but not mandatory. Environmental enrichment is already part of the best practice in cephalopod care for experimental purposes (e.g.: Fiorito et al. 1990; Mather and Anderson 1999; Dickel et al. 2000; Anderson and Wood 2001; Poirier et al. 2004; Borrelli 2007; Borrelli and Fiorito 2008; Boal 2011). It is interesting to note that in the classic work by Grimpe (1928) gravel, pebbles and stones are recommended to facilitate self-construction of a refuge by animals. In addition, other species, such as medium-sized sea stars, should be accommodated in the tanks to facilitate reduction in remains of food and faeces (Grimpe 1928). This would provide the enriched type of environment considered to be good welfare practice.

Cephalopods can be kept in shared water systems and rooms with different cephalopods species or other marine organisms. In principle, there is no need for separate rooms for experimental treatments and housing, but this will depend upon the type of study. For example, it is strongly recommended that a standardised dedicated room is used for behavioural experiments, and it is not good practice to perform surgical procedures and euthanasia in the same room where animals are housed. Moreover, animals subjected to surgical lesions should not be placed in a tank where there is a possibility that any chemical signal can be detected by un-operated animals.

Note that Directive Annex III, section A, includes general requirements pertaining to all species and also section B, for fish, where most principles might also apply to cephalopods.

Animal stocking

Solitary animals (e.g. *O. vulgaris*) should be kept separately. Annex III of the Directive states that social animals must be socially housed in stable groups of compatible individuals (e.g. squids), but interactions should be monitored and animals separated if there is evidence of non-compatibility. Some animals such as *Nautilus* are primarily solitary in the wild, but may be housed together at low densities. The social structures of many species (e.g. *S. officinalis*) are not yet known, but captive bred European cuttlefish adults, as well as hatchling and young of all sources can be kept in groups (A. Sykes, pers. communication).

Routine animal care and maintenance

Animal care includes routine maintenance, husbandry, and animal handling. Handling procedures should be standardised within the laboratory (and field) to minimise experimental variability produced by different handlers, also taking into account that some animals may learn to anticipate handling procedures (Boycott 1954). As with any live animal, handling and human interaction should be kept to the minimum needed to meet daily care and experimental requirements, standardised and performed by trained staff only, to minimise stress. Handling and all human interactions should be recorded, as the amount, frequency and nature of the interactions can influence husbandry and the outcome of experiments (for a general review see: Davis and Balfour 1992). For octopuses, the effects of rough handling on the skin may not be apparent until several days (Wells 1962), and as mentioned above, skin lesions may be fatal (as reviewed in Boyle 1991) so this could have major consequences if the animal had been assigned to a study requiring long-term survival. Even for commonly used laboratory mammals, the effects of different handling techniques are still being discovered; for example, Hurst and West (2010) compared commonly used techniques of handling laboratory mice and showed marked differences in biomarkers of anxiety. For cephalopods, optimal handling protocols need to be identified for each species to minimise adverse effects, which can be a confounding factor in experiments.

Feeding

Feeding regimes should fit the lifestyle, natural diet, and developmental stages of the animals (see reviews in: Boletzky and Hanlon 1983; Borrelli 2007; Sykes et al. 2011, 2012). Cephalopods are carnivorous and, with the exception of the *Nautilus*, are predatory, and therefore, the use of live food can be essential, although may require justification (Smith et al. 2013). There are many examples of species and life stages where live prey is the only food accepted, and the benefits outweigh the risk of disease from the food. Efforts are underway to develop artificial diets. Daily feeding is common practice, and higher frequencies might be needed for young animals. Over feeding is preferred as long as excess food is removed in a time frame fitting the feeding habits of the species (Oestmann et al. 1997) and does not overwhelm the capacity of the filter system of the tank. Cuttlefish and squid are especially sensitive to lack of food; dead food can be used as alternative to live in some species (e.g.: Domingues et al. 2004; Ferreira et al. 2010).

Research is needed to identify optimal nutritional requirements that ensure health and welfare of each of the common laboratory species of cephalopod at key life stages. In addition, studies are needed to understand the physiological impact of a reduction in food intake because of illness, as a consequence of a surgical procedure or pharmacological intervention and as part of a training protocol for example when food may be used as a positive

reinforcement. The impact of a particular experimental protocol upon food intake is likely to be a key question in harm-benefit evaluation of a project because of the high metabolic rate of cephalopods.

Identification and marking techniques

Most studies identify animals using individual housing, but some studies are done with groups of animals. In general, marking soft parts of cephalopods may have a deleterious effect on health and welfare and should be avoided. When scientifically necessary, individual marking may be performed, under anaesthesia, using for example fluorescent elastomer tags (Zeeh and Wood 2009; e.g. Sepioteuthis sp.: Ikeda et al. 2009; e.g. Octopus sp.: Barry et al. 2011; Brewer and Norcross 2012) or integrated archival tags including implanted microchips (in *O. vulgaris*: Estefanell et al. 2011; in *S. officinalis*: Wearmouth et al. 2013). For *Nautilus*, individual shell marking is preferred and can be done without anaesthesia (J. Basil, pers. communication).

In non-shelled cephalopods, there have been some reports of the use of unique natural patterns of individual animals as a means of identification (Huffard et al. 2008). The application of noninvasive methods for identification of individuals is important in the interests of animal welfare.

Procedures

A procedure within the Directive (Article 3, 1) is defined as "Any use, invasive or noninvasive, of an animal for experimental or other scientific purposes, with known or unknown outcome, or educational purposes, which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice". Objective criteria will need to identified by which it is possible to determine whether a particular procedure causes pain, suffering, distress or lasting harm equivalent or higher than that caused by the skilled introduction of a needle. In addition, the Directive also makes specific references to humane methods of killing (Article 6) and the use of anaesthesia and analgesia (Article 14). The potential impact upon many aspects of cephalopod research in general and the broad range of neuroscience research in particular is considerable. To illustrate this, examples of published studies are listed in Table 4, which are now likely to be regulated under the scope of the Directive if performed in the EU. In this section, we focus on some specific aspects to illustrate some challenges to neuroscience research presented by the above aspects of the Directive.

Pain, suffering, distress and lasting harm (PSDLH)

One of several drivers for the inclusion of cephalopods in the Directive was a review of the evidence relating to their ability to perceive pain (EFSA Panel on Animal Health and Welfare 2005). The criteria used in the EFSA report have recently been reviewed in detail (Andrews et al. 2013) as has nociception in invertebrates (Crook and Walters 2011). At the time of the EFSA report (2005), evidence for the existence of nociceptors in cephalopods was largely circumstantial. Recently, afferents with the characteristics of nociceptors sensitive to mechanical stimulation have been described in a squid and evidence provided for long-term sensitisation (Crook et al. 2013). However, there are major gaps in our knowledge of the central processing of the information arising from the nociceptors in invertebrates in general and cephalopods specifically (Crook and Walters 2011; Andrews et al. 2013). The anatomy of the afferent projections from the arms and various lobes of the brain has been described for *O. vulgaris*, *S. officinalis* and *L. vulgaris* (Budelmann and Young 1985, 1987), but again neurophysiological studies are needed to understand the central processing of information from well-characterised nociceptors. Until such studies are performed, "pain perception" (i.e. what the animal might "feel" as a result of nociceptor activation) in cephalopods will remain a contentious issue. However, from an animal welfare perspective, researchers should be mindful of stimuli likely to activate nociceptors in their experimental protocols and either justify their use or take action to mitigate the impact. Neurophysiological studies in combination with behavioural studies will also be required to identify substances with analgesic effects that can be used postoperatively and to identify the mechanism(s) by which substances with presumed general anaesthetic actions in cephalopods act.

In addition to the physiology and pharmacology of pain perception in cephalopods, objective criteria for the identification and measurement of pain are required as part of welfare assessment and in particular to assess the impact of any experimental intervention. Although a great emphasis is rightly placed upon pain, equal consideration needs to be given to other ways in which an animal may suffer, be in distress or be caused lasting harm in an experimental setting and ways in which they can be identified and measured. Examples of "non-painful" types of suffering could include isolation in social species, housing in a tank of inappropriate size or with no refuge or being caused fear and anxiety (see Hawkins et al. 2011b for other examples). A preliminary approach to monitoring PSDLH in cephalopods has been recently described (see Table 1 in Andrews et al. 2013) based upon the types of criteria that have been developed over

Table 4 Examples of published research on cephalopods which if carried out in the EU would now be likely to come within the scope of Directive 2010/63/EU

Research topic or technique	References
Implantation of electromyographic electrodes under anaesthesia in cuttlefish fin muscle and recording from unanaesthetised animals	Kier et al. (1989)
Removal of optic glands under anaesthesia followed by recovery (study effect on senescence)	Wodinsky (1977)
Sampling of haemolymph usually under anaesthesia	Malham et al. (1998a), Collins and Nyholm (2010), Grimaldi et al. (2013), Locatello et al. (2013)
Implantation of a catheter into the dorsal aorta for administration of drugs to the brain	Andrews et al. (1981)
Investigation of the efficacy of different anaesthetic techniques and mechanisms of anaesthesia	Andrews and Tansey (1981), Messenger et al. (1985), Seol et al. (2007), Sen and Tanrikul (2009), Pagano et al. (2011), Goncalves et al. (2012), Gleadall (2013)
Implantation of electrodes for recording or stimulation into the brain under anaesthesia followed by investigation of the effects in the conscious animal	Chichery and Chanelet (1976), Brown et al. (2006), Shomrat et al. (2008), Zullo et al. (2009), Mooney et al. (2010), Shomrat et al. (2011)
Removal of an arm or a tentacle with or without anaesthesia to investigate regeneration or the acute tissue and behavioural response to injury	Lange (1920), Crook et al. (2011), Fossati et al. (2013), Tressler et al. (2013)
Administration of substances into the circulation via the branchial hearts or intramuscular routes or directly into the brain	Agnisola et al. (1996), Fiorito et al. (1998), Agin et al. (2003), Graindorge et al. (2008)
Tracing nerve pathways using marker injection under anaesthesia followed by recovery to allow marker transport	Gaston and Tublitz (2004), Tublitz et al. (2006)
Implantation of electronic tags for tracking movement in the wild	Wearmouth et al. (2013)
Noninvasive measurement of brain size and arm morphology under anaesthesia with or without recovery	Grimaldi et al. (2007), Margheri et al. (2011b)
Killing animals (including hatchlings) to remove tissue (e.g. arm, brain), for study in vitro (e.g. brain slices), histological and molecular studies particularly if the study involves "nonstandard" methods	Kier et al. (1989), Westermann et al. (2002), Hochner et al. (2003), Kier and Stella (2007), Mackie (2008), Hague et al. (2013)
Brain or peripheral nervous system lesions under anaesthesia followed by recovery	Fiorito and Chichery (1995), Sumbre et al. (2001), Graindorge et al. (2006, 2008)
Use of aversive stimuli (e.g. electric shock, bitter taste) in training protocols	Robertson et al. (1994, 1995, 1996), Darmaillacq et al. (2004), Borrelli (2007)
Deprivation of food for 5 days, feeding with barium sulphate labelled shrimps, constraint of the animal and exposure to X-rays for imaging gut contents	Westermann et al. (2002)
Exposure of an animal to a potentially "stressful" environment/stimulus as an experimental procedure; examples include a large moving shape, a larger conspecific, a predator, air or sea water with temperature or oxygen partial pressure outside the normal aquarium range or manipulation of natural photoperiod/light intensity. Noninvasive immobilisation (confinement) may also constitute a stressful stimulus. The intensity, duration and exposure frequency are all factors which need to be considered	Malham et al. (2002), Cole and Adamo (2005), King and Adamo (2006), Adamo et al. (2006), Kuba et al. (2006), Canali et al. (2011a)
Production of hatchlings with deleterious phenotypes/genotypes by exposure of the eggs to a harmful environment or mutagen or genetic manipulation	Rosa et al. (2012)

Note that not all examples relate to invasive or surgical procedures (see also Ponte et al. 2013 for other resources). Papers have been selected to illustrate the diversity of studies likely to be regulated, and no comment is made about whether a particular study would now be permitted by a particular national competent authority

many years for mammals (e.g. Morton and Griffiths 1985).[7]

[7] For severity assessment see also: http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Consensus%20doc%20on%20severity%20assessment.pdf.

General anaesthesia

General anaesthesia is required for performing surgical procedures followed by recovery (e.g. selective brain or nerve lesions, implantation of telemetry devices) for some types of in vivo physiological study (e.g. reflex control of

the cardio-respiratory system, investigation of somatosensory processing) and to permit handling for veterinary investigation and treatment. Over the last century, a diverse range of substances has been used to induce general anaesthesia in cephalopods (Pagano et al. 2011; Goncalves et al. 2012; Gleadall 2013; Andrews et al. 2013), but there have been relatively few studies utilising objective criteria to define the anaesthetic state or the mechanism and site of action and little consideration has been given to the procedures used from a welfare perspective (e.g. how aversive are the agents used?). Recently, isoflurane as been tested as an anaesthetic in *O. vulgaris* (Di Cosmo, pers. communication), but more investigation is required.

All current techniques use immersion in sea water containing the anaesthetic agent. Magnesium chloride and ethanol, used either separately or in combination, are the most commonly used agents. Following Andrews et al. (2013), criteria for assessment of general anaesthesia in cephalopods include: (1) depression of ventilation and in some cases cessation, probably accompanied by reduced cardiac activity; (2) decrease in chromatophore tone (indicative of reduced drive to or from the sub-oesophageal chromatophore lobes); (3) reduced arm activity, tone and sucker adhesion (particularly octopus); (4) loss of normal posture and righting reflex; (5) reduced or absent response to a noxious stimulus. The last needs to be used with some care as in *O. vulgaris* arms removed from the body withdraw in response to a noxious stimulus (Hague et al. 2013). Studies are urgently required to understand the way in which the putative anaesthetic agents act on the nervous system to produce the above effects and to render the animal into a presumed state of insensibility and unconsciousness. The site and mechanism of action of general anaesthetics has been studied extensively in mammals (e.g. Angel 1993), but there are few studies in cephalopods (e.g.: Andrews and Tansey 1981; Messenger et al. 1985), although with their high degree of encephalization combined with a brain organised in a fundamentally different way from vertebrates studies of general anaesthesia may provide novel insights into mechanisms of consciousness.

Humane methods of killing

The Directive requires that if it is necessary to kill an animal (e.g. at the end of project, to obtain tissue for an in vitro study, because a humane end point is reached), it must be done "with the minimum of pain, suffering and distress" (Article 6). Acceptable methods should comply with the general principles of humane animal euthanasia set out in Demers et al. (2006) and Annex IV of the Directive.

Identification of humane methods for killing is a particular challenge for neuroscience as physical destruction of the brain is a commonly used method and maybe acceptable if the method used can be demonstrated to be humane, but it is obviously not suitable when the brain is the subject of study. Similarly, overdose of general anaesthetic is often used, but again could be argued to compromise subsequent studies of brain function because of the residual pharmacological effect of the agent used and the effects of asphyxia caused by the prolonged (usually >30 min) immersion in anaesthetic needed to kill the animal. Such constraints may encourage investigation of electrical euthanasia methods similar to those used for crustaceans (Neill 2010). Annex IV of the Directive also includes methods for confirmation of death, and these are discussed in relation to cephalopods in Andrews et al. (2013). It should also be noted that the requirement for humane killing also applies to hatchlings. In this last case, killing by direct immersion in fixative would not now be considered acceptable in the EU, although it might be possible to obtain permission to use this as a method if it could be justified to the NCA.

Humane killing methods for both hatchlings and developmental stages through adult cephalopods require additional research, but in the *interim* it is proposed that animals are either anaesthetised prior to mechanical destruction of the brain (this may be difficult in nautiloids) or if the brain is required that animals (including hatchlings) are killed by prolonged immersion in anaesthetic, recognising that the impact upon the brain will need to be considered in the light of the scientific objectives and that a shorter period of anaesthesia followed by decapitation when the animal is insensible may need to be considered (Andrews et al. 2013).

Replacement, refinement and reduction and cephalopod research

The principles of Replacement, Refinement and Reduction ("3Rs") developed by Russell and Burch (1959) as key elements of humane experimentation involving sentient animals are at the heart of the Directive (Article 4), and project evaluation prior to authorisation requires an assessment of how the 3Rs are addressed in the proposed study (Article 38). Replacement "of the use of a regulated living animal" is often used in the context of pharmaceutical research to describe replacing a test (e.g. for drug efficacy) in a living animal with one using a microorganism, human tissue or in silico methods. Superficially, "replacement" may not appear to apply to most cephalopod research as many researchers are undertaking the research because they have a specific interest in an aspect to cephalopod biology, but "replacement" requires researchers to consider a priori whether they need to use a "living animal" (of a species covered by the Directive) to

answer the specific research question or whether the same research question could be tackled in another way which could e.g. include in vitro studies of tissue take from the same species provided that the animal is killed using an approved humane method.

Most researchers will already be applying the principle of "reduction" to their studies as this relates to the use of the minimum number of animals required to achieve the scientific objectives of the project and is an inherent part of good experimental and statistical design. Although not strictly an example of "reduction" within the meaning of the 3Rs the Directive also makes a specific point (Article 18) about sharing organs and tissues from killed animals. This could contribute to a reduction in the overall number of animals used within an institute by coordinating the killing animals at the end of a procedure with in vitro studies requiring fresh living tissue and/or banking tissue for molecular or histological studies. In the case of the latter, tissue could be shipped to other institutions.

Refinement is the "R" most likely to impinge upon current cephalopod research by requiring that experimental procedures, housing, husbandry and all aspects of care are "refined" so that they cause the minimum possible pain, suffering, distress or lasting harm throughout the life of the animal being used. Refinement of current best practice in the care and welfare assessment of the various cephalopod species will evolve by research to provide evidence to support changes of approach and technique that reduce adverse effects and maybe informed by approaches to refining procedures commonly carried out on laboratory vertebrates (Hawkins et al. 2011a). For experimental procedures, refinement requires the researcher to carefully examine their protocols and see where changes in can be made in any aspect likely to cause PSDLH to reduce adverse effects whilst achieving the scientific outcome. For example, it might be asked whether the number of haemolymph samples taken or number of drug doses given each day could be reduced; or whether positive reinforcement could be used instead of negative ones in training protocols; and whether induction of general anaesthesia could be made more humane by exposing the animal to a gradually rising concentration of anaesthetic rather than direct immersion in a fully effective concentration. All three are examples of approaches accepted and used to refine procedures in vertebrates. Additional examples using hypothetical research projects involving cephalopods are discussed in Smith et al. (2013).

Conclusion

Directive 2010/63/EU is a milestone for invertebrate research in the EU because it is the first time particular types of research involving an entire class of invertebrates, the cephalopods, will be regulated in the same way as scientific projects involving vertebrates. Although regulation presents challenges, there are several areas where neurophysiological and behavioural neuroscience research could be useful to address key questions related to cephalopod care and welfare discussed above. Most researchers already recognise the relationship between good welfare and good science, but the development of consensus Guidelines for Care and Welfare of Cephalopods led by the research community will facilitate the dissemination and adoption of good practice. Guidelines are being developed based upon literature review and discussion meetings, but they are only an initial step and evolution of such guidelines will rely upon capturing the experience and knowledge of the cephalopod research community. It is hoped that this review will prompt readers to investigate some of the neuroscience questions posed and to contribute to the future development of guidelines for optimal care and welfare of cephalopods via publication and contributions to online research fora (e.g. CephRes: www.cephalopodresearch.org; CephSeq: http://cephseq.org/; Cephalopod International Advisory Council: http://www.abdn.ac.uk/CIAC/).

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Guidelines for the Care and Welfare of Cephalopods in Research –A consensus based on an initiative by CephRes, FELASA and the Boyd Group

Graziano Fiorito[1,2], Andrea Affuso[1,3], Jennifer Basil[4], Alison Cole[2], Paolo de Girolamo[5,6], Livia D'Angelo[5,6], Ludovic Dickel[7], Camino Gestal[8], Frank Grasso[9], Michael Kuba[10], Felix Mark[11], Daniela Melillo[1], Daniel Osorio[12], Kerry Perkins[12], Giovanna Ponte[2], Nadav Shashar[13], David Smith[14], Jane Smith[15] and Paul LR Andrews[16,2]

Abstract

This paper is the result of an international initiative and is a first attempt to develop guidelines for the care and welfare of cephalopods (i.e. nautilus, cuttlefish, squid and octopus) following the inclusion of this Class of ~700 known living invertebrate species in Directive 2010/63/EU. It aims to provide information for investigators, animal care committees, facility managers and animal care staff which will assist in improving both the care given to cephalopods, and the manner in which experimental procedures are carried out. Topics covered include: implications of the Directive for cephalopod research; project application requirements and the authorisation process; the application of the 3Rs principles; the need for harm-benefit assessment and severity classification. Guidelines and species-specific requirements are provided on: *i.* supply, capture and transport; *ii.* environmental characteristics and design of facilities (e.g. water quality control, lighting requirements, vibration/noise sensitivity); *iii.* accommodation and care (including tank design), animal handling, feeding and environmental enrichment; *iv.* assessment of health and welfare (e.g. monitoring biomarkers, physical and behavioural signs); *v.* approaches to severity assessment; *vi.* disease (causes, prevention and treatment); *vii.* scientific procedures, general anaesthesia and analgesia, methods of humane killing and confirmation of death. Sections covering risk assessment for operators and education and training requirements for carers, researchers and veterinarians are also included. Detailed aspects of care and welfare requirements for the main laboratory species currently used are summarised in Appendices. Knowledge gaps are highlighted to prompt research to enhance the evidence base for future revision of these guidelines.

[1]Stazione Zoologica Anton Dohrn, Villa Comunale, Napoli, Italy
[2]Association for Cephalopod Research 'CephRes', Italy
[3]Animal Model Facility - BIOGEM S.C.A.R.L., Ariano Irpino (AV), Italy
[4]Biology Department, Brooklyn College - CUNY Graduate Center, Brooklyn, NY, USA
[5]Department of Veterinary Medicine and Animal Productions - University of Naples Federico II, Napoli, Italy
[6]AISAL - Associazione Italiana per le Scienze degli Animali da Laboratorio, Milano, Italy
[7]Groupe mémoire et Plasticité comportementale, University of Caen Basse-Normandy, Caen, France
[8]Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain
[9]BioMimetic and Cognitive Robotics, Department of Psychology, Brooklyn College - CUNY, Brooklyn, NY, USA
[10]Max Planck Institute for Brain Research, Frankfurt, Germany
[11]Integrative Ecophysiology, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany
[12]School of Life Sciences, University of Sussex, Sussex, UK
[13]Department of Life Sciences, Eilat Campus, Ben-Gurion University of the Negev, Beer, Sheva, Israel
[14]FELASA, Federation for Laboratory Animal Science Associations
[15]The Boyd Group, Hereford, UK
[16]Division of Biomedical Sciences, St George's University of London, London, UK

Corresponding author:
Graziano Fiorito, Research – CephRes, via dei Fiorentini 21, 80133 Napoli Italy.
Email: cephres@cephalopodresearch.org

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Contributors

People listed here provided data, information and comments, and contributed to different extents during the preparation of this work.

The following list is arranged by country in alphabetical order; different contributors are merged by Institution.

France

Christelle Alves, Cecile Bellanger, Anne-Sophie Darmaillacq, Céline Gaudin
Groupe mémoire et Plasticité comportementale, EA4259, University of Caen Basse-Normandy, Caen, France
Joël Henry
Physiologie de la reproduction des Mollusques, University of Caen Basse-Normandy, Caen, France

Germany

Tamar Gutnick
Max Planck Institute for Brain Research, Frankfurt, Germany

Italy

Anna Di Cosmo
Department of Biology - University of Naples Federico II, Napoli, Italy
Carlo Di Cristo
Department of Biological and Environmental Sciences - University of Sannio, Benevento, Italy
Viola Galligioni
CIBio - Centre for Integrative Biology, Trento, Italy & Association for Cephalopod Research 'CephRes', Italy
Anna Palumbo
Stazione Zoologica Anton Dohrn, Napoli, Italy
Perla Tedesco
Department of Biological and Environmental Science and Technologies - University of Salento, Lecce, Italy
Letizia Zullo
Istituto Italiano di Tecnologia, Department of Neuroscience and Brain Technologies, Genoa, Italy

Portugal

António Sykes
C. Mar – Centre of Marine Sciences, Universidade do Algarve, Faro, Portugal

Spain

Roger Villanueva López
Renewable Marine Resources Department - Institut de Ciències del Mar, Barcelona, Spain

United Kingdom

Ngaire Dennison
Home Office, Animals in Science Regulation Unit, Dundee, Scotland, UK
Penny Hawkins
RSPCA Research Animals Department, Southwater, West Sussex, UK

United States of America

Gregory J. Barord, Heike Neumeister, Janice Simmons, Roxanna Smallowitz
Biology Department, Brooklyn College - CUNY Graduate Center, Brooklyn, NY, USA
Jean Geary Boal
Biology Department, Millersville University, Millersville, PA, USA
Roger Hanlon, William Mebane
Marine Resources Center, Marine Biological Laboratory, Woods Hole, MA, USA
Judit R Pungor
Hopkins Marine Station of Stanford University, Pacific Grove, CA, USA
James B. Wood
Waikiki Aquarium, University of Hawaii-Manoa, Honolulu, HI, USA

1. Introduction

Cephalopods (i.e. nautilus, cuttlefish, squid and octopus) have been used for diverse scientific purposes across Europe for over 100 years.[1,2] However, until recently, scientific procedures involving cephalopods have not been covered by EU regulations, with

the exception of procedures using *Octopus vulgaris* in the United Kingdom (see discussion in Smith et al.[3]).

The inclusion of 'live cephalopods' (Article 1, 3b) in EU Directive 2010/63/EU on the 'protection of animals for scientific purposes' represents a landmark. It is the first time that an entire class of invertebrates, covering approximately 700 known species,[4,5] has been included in laboratory animal legislation throughout the EU. The decision was largely based upon a review of the evidence for sentience and capacity to experience pain, suffering, distress and lasting harm (PSDLH) in cephalopods[6] (see also Table 1) which is now supported by more recent circumstantial (for reviews see[7,8]) and objective evidence[9–11] for the existence of nociceptors in cephalopods. Annexes III and IV to the EU Directive provide general guidance on care and accommodation requirements and methods of humane killing for all species covered by the Directive, but specific guidance is restricted to vertebrates, and there are no specific details for cephalopods.

Prompted by the need for guidelines on these and other matters covered by the Directive, members of the international cephalopod research community have met on several occasions over the past 3 years and have produced publications aimed at cephalopod researchers, on: *i*. requirements of the EU Directive, implementation, ethics and project review;[3] *ii*. PSDLH, anaesthesia and humane killing;[8] and *iii*. implications for neuroscience research and the Three Rs, i.e. Replacement, Reduction, Refinement.[2]

This work has led to the development of a set of consensus Guidelines for the Care and Welfare of Cephalopods in Research which aim to assist researchers in complying with the Directive, and are the subject of this paper. These guidelines have been developed as a joint initiative between CephRes (www.cephalopodresearch.org), FELASA (www.felasa.eu) and the Boyd Group UK (http://www.boyd-group.demon.co.uk/).

The Guidelines for the Care and Welfare of Cephalopods in Research, which should be regarded as a starting point for future developments, begin with a set of general principles of good practice, representing the present state of knowledge that may reasonably be applied to all cephalopods. These are followed by a tabulated set of specific guidelines (see Appendices) for typical cephalopod species, currently used in EU laboratories, which also reflect well-established principles.

1.1 What is a cephalopod?

For the purpose of these guidelines, cephalopods are defined as all living species that are members of the molluscan class Cephalopoda.[4,5,12] The term 'live cephalopod' is not defined in the Directive, but guidance indicates that these animals are covered by the Directive from 'when they hatch'.[13,14]

Cephalopods are characterised by bilateral body symmetry, a prominent head and a set of arms, including tentacles in Decapods, which are considered as muscular hydrostats and derived from the primitive molluscan foot.[15–21] The class contains two, only distantly related, living subclasses: Nautiloidea (represented by *Nautilus* and *Allonautilus*) and Coleoidea, which includes cuttlefish, squid and octopuses.[20,22] In the Nautiloidea, the external shell, common to the molluscan *Bauplan,* still exists, whereas in the Coleoidea it has been internalised or is absent. The variety of species that compose the taxon is reflected in the diversified habitats they have adapted to: oceans, benthic and pelagic zones, intertidal areas and deep sea, polar regions and the tropics.[23–26]

Understanding the requirements of a particular species in relation to its natural habitat is fundamental in maintaining healthy laboratory populations of cephalopods. Assumptions for housing, care and use of these animals based on fish, whilst appropriate in some circumstances, should be made with great caution as the evolutionary convergence between fish and cephalopods[24,27] does not reflect the actual requirements of different species.

Generally, cephalopods have a high metabolic rate, grow rapidly and are short-lived.[28,29] These animals are exothermic, highly adapted to the marine aquatic environment and are therefore unlikely to tolerate rapid or significant changes in the quality or temperature of the water they are housed in. They react rapidly to environmental changes/external stimuli with immediate physiological consequences that can be relatively long lasting. Such changes, as well as having potential welfare implications, will also impact upon experimental results.

Cephalopods are considered among the most 'advanced' invertebrates, having evolved many characteristic features such as relatively large, highly differentiated multi-lobular brains, a sophisticated set of sensory organs, fast jet-propelled locomotion, and complex and rich behavioural repertoires.[25,30–37]

1.2 What the Directive 2010/63/EU means for cephalopod research

The entry into force of the Directive 2010/63/EU (hereafter referred to as 'the Directive')[38,39] means that, from 1st January 2013, scientific research and testing involving 'live cephalopods' is regulated by a legal framework at both EU and Member State levels, and as a consequence all scientific projects that cross the threshold set for regulation (i.e. involve procedures that may cause PSDLH equivalent to, or higher than that caused by the insertion of a hypodermic needle in line with good veterinary practice) will require authorisation by

Table 1. Summary of evidence for the capacity of cephalopods to experience pain based upon the criteria used by the EFSA 2005 panel (as the basis for recommending the inclusion of cephalopods in revision of Directive 86/609/EEC), and here updated with more recent studies. See also Andrews et al. for review[8] and additional references.

Criterion used by EFSA 2005 panel	Judgement	Comment and references
Presence of receptors sensitive to noxious stimuli, located in functionally useful positions on (or) in the body, and	YES	Circumstantial evidence, e.g. cutaneous free nerve endings[491] available at time of EFSA report. Recent neurophysiological afferent recording studies[10,11] have provided direct evidence for presence of mechano-nociceptors in both squid and octopus.
Connected by nervous pathways to the 'lower' parts of the nervous system	LIKELY (but not proven)	Evidence that peripheral afferent axons project to brain from the arms and mantle,[492,493] but modality not identified although likely to include nociceptors if present.
Possession of higher brain centres [in the sense of integration of brain processing], especially a structure analogous to the human cerebral cortex	YES	Most complex brain structure amongst invertebrates and clear hierarchical organisation.[34,35]
		Vertical lobe approximates to the hippocampus in mammals and is unique in invertebrates in having gyri.[26,142]
		Studies in progress investigating self-awareness and consciousness, as discussed in Edelman and coworkers.[494,495]
Possession of nervous pathways connecting the nociceptive system to the higher brain centres	LIKELY (but not proven)	Evidence for ascending afferent projections from 'lower' to 'higher' brain regions including the vertical lobe, but no neurophysiological studies showing projection of signals from nociceptors.[492,493]
		Indirect evidence from behavioural studies for projection of signals from nociceptors to higher brain regions (see below).
Receptors for opioid substances found in the central nervous system especially the brain	LIKELY (but not proven)	Not studied directly. Opioid system is highly conserved in evolution.[496] Limited evidence for presence of enkephalins[497,498] and opioid receptors.[497–502]
Analgesics modify the animal's response to stimuli that would be painful for a human	Not studied	Investigation of candidate substances is required using a combination of neurophysiological recording from nociceptive afferents and behavioural studies.
An animal's response to stimuli that would be painful for a human is functionally similar to the human response (that is, the animal responds so as to avoid or minimise damage to its body)	YES	Good evidence of learned avoidance of punishment (e.g. electric shock) but assumes that this stimulus activates nociceptors and not some other afferent modality that evokes an aversive but non-painful sensation (e.g.[26,503]). Limited supportive evidence from behavioural studies of predatory behaviour.[26,36,72,504–507]
		Equivocal evidence for wound-directed behaviours.[9,10,189]
An animal's behavioural response persists and it shows an unwillingness to resubmit to a painful procedure; the animal can learn to associate apparently non-painful with apparently painful events.	YES	Evidence for: peripheral mechano-nociceptor sensitisation (at least 48 h) following injury to either an arm or fin; contralateral afferent sensitisation (but see Alupay et al.[10]); hyper-responsiveness to visual stimuli following arm injury.[9,11]

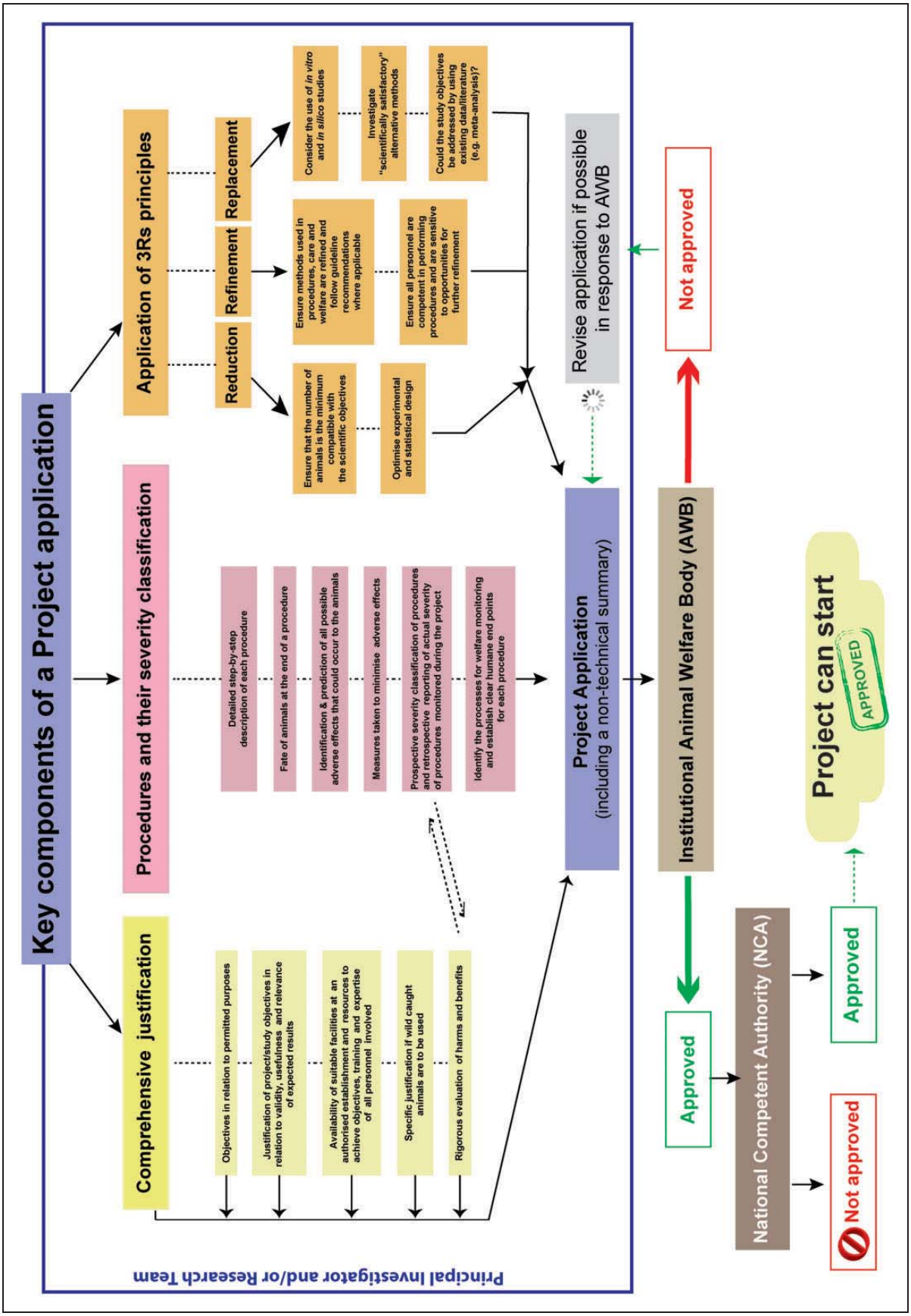

Figure 1. Schematic overview summarising the major components of a project application and stages of project approval under Directive 2010/63/EU. Note that the details of the project approval process may differ across member states. For details see text and review in Smith et al[3].

Text Box 1. Definition of key terms utilised in Directive 2010/63/EU.

A **project** is a programme of work with a defined scientific objective involving one or more *procedures*, which can run for a term of up to 5 years, after which authorisation must be renewed.
A **procedure** is any use of an animal covered by the Directive for experimental or other scientific or educational purposes, which 'may cause the animal pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle in accordance with good veterinary practice'. This can include procedures that do not involve any 'invasive' technical acts such as administration of substances or surgery, but which cause psychological distress (such as anxiety) above the threshold level of suffering defined above. Unless specifically justified as part of the authorisation process, procedures may only be carried out at authorised user establishments.
Authorisation is limited to the procedures and purposes described in the application. If, during the life of the project, there is need for any **amendments** to the project plans that may have a negative impact on animal welfare, these must also be authorised.
A **Competent Authority** is a body responsible for implementing a specific task (or tasks), laid down by the Directive, within a Member State; for example, project evaluation and/or project authorisation. Member States must designate one or more competent authorities to fulfil these tasks.

Text Box 2. Definitions of the levels of severity of procedures according to Directive 2010/63/EU, Annex VIII; see also Lindl et al.[490]

Mild: *Procedures on animals as a result of which the animals are likely to experience short-term mild pain, suffering or distress, as well as procedures with no significant impairment of the well-being or general condition of the animals shall be classified as 'mild'.*
Moderate: *Procedures on animals as a result of which the animals are likely to experience short-term moderate pain, suffering or distress, or long-lasting mild pain, suffering or distress as well as procedures that are likely to cause moderate impairment of the well-being or general condition of the animals shall be classified as 'moderate'.*
Severe: *Procedures on animals as a result of which the animals are likely to experience severe pain, suffering or distress, or long-lasting moderate pain, suffering or distress as well as procedures, that are likely to cause severe impairment of the well-being or general condition of the animals shall be classified as 'severe'.* Note that the Competent Authority will require retrospective assessment of projects involving 'severe' procedures.
Non-recovery: *Procedures which are performed entirely under general anaesthesia from which the animal shall not recover consciousness.*

the National Competent Authority (see the list available at: http://ec.europa.eu/environment/chemicals/lab_animals/ms_en.htm).[3]

Three key aspects of the project that will need to be considered by researchers and those responsible for animal care and welfare are outlined below (see also Figure 1). Specific topics for inclusion and consideration are listed in Appendix 1, and a more detailed overview of issues relating to implementation of the Directive for cephalopods can be found in Smith et al.,[3] which also includes some hypothetical worked examples of project review, particularly in relation to opportunities for implementing the 3Rs (see 2.2.1 below).

2. Project application requirements and authorisation process

2.1 Application requirements

The key requirements of project authorisation are outlined here to provide a background to the technical sections, which aim to show how these requirements can be fulfilled specifically for cephalopods (Figure 1).

Before they can begin, all projects involving live cephalopods must be authorised by a competent authority appointed by the Member State in which the project is to take place (see Text Box 1 and Text Box 2 for definitions of key terms in the Directive). This will involve 'comprehensive project evaluation', 'taking into account ethical considerations' and 'implementation of principles of reduction, refinement and replacement' of the use of animals, the 3Rs (Recital 38 and 39).

An application for project authorisation must include, as a minimum, the project proposal and the items listed in Appendix 1. A non-technical project summary will be required unless waived by the National Competent Authority.

Applications must also include specific scientific justification for any requests for exemptions from certain requirements of the Directive (where permitted – see also Appendix 1). This will include requests for permission to: *i.* use an endangered cephalopod species

where it falls within the criteria laid out in Article 7.1 of the Directive; *ii*. use cephalopods taken from the wild (Article 9, see also section 3.3 below); *iii*. carry out procedures in a place that is not an authorised users' establishment (Article 12); *iv*. re-use (in a different procedure) animals that have already undergone a procedure (Article 16, see also section 8.10 below); *v*. use drugs, such as neuromuscular blocking agents, that could stop or restrict an animal's ability to show pain, without an adequate level of anaesthesia or analgesia (Article 14§3); *vi*. depart from any of the general standards of animal care and accommodation outlined in Section A of Annex III of the Directive (Article 33).

For species other than cephalopods, specific justification is also required for killing animals by a method not listed in Annex IV of the Directive, or for departing from species-specific standards of animal care and accommodation outlined in Section B of Directive Annex III. However, at present, cephalopods are not included in either of these Annexes.

Once a project is authorised and underway, it should continue to be critically evaluated by the Principal Investigator and all members of the project team, using the factors listed in Appendix 1, so as to ensure that ethical considerations and opportunities for implementing the 3Rs are identified and addressed in an ongoing process for the entire duration of the project, not only at the start.

2.2 Factors to be evaluated in project authorisation and project operation

The Directive sets out the factors that must be evaluated during project authorisation and throughout the lifetime of authorised projects. These factors are listed below as a series of action points and associated questions for consideration.

2.2.1 Implement the 3Rs to minimise the harms caused to the animals. The Three Rs (3Rs) principles were first described by Russell and Burch[40] and are now internationally accepted as an essential requirement for the ethical and humane conduct of scientific studies involving animals (Recital 11; Articles 4 and 38 §2b). In addition, it is widely recognised that implementation of the principles can enhance scientific quality.[41–44,45]

The following are examples of questions that will need to be addressed:

Replacement

- What on-going efforts will you make to identify 'scientifically satisfactory' alternative methods that could replace the use of some or all animals? (Article 4§1)
- Could you avoid the use of animals by asking different type of question, or making better use of existing data or literature to address the scientific objectives?
- Could *in vitro* studies or *in silico*-modelling be used to replace some or all of the animals?

Reduction

- How will you ensure that the number of animals used in the project, and in individual studies within the project, is 'reduced to a minimum without compromising the scientific objectives'? (Article 4§2)
- Could any further reductions be made, e.g. by taking expert statistical advice to help optimise experimental and statistical design?

Refinement

- How have you refined the 'breeding, accommodation and care of the animals' and the 'methods used in procedures', so as to 'reduce to the minimum any possible pain, suffering, distress or lasting harm to the animals' throughout their lives? (Article 4§3)
- Have you considered and implemented all the possibilities for refinement described elsewhere in these guidelines?
- How will you ensure that all relevant personnel working on the project are adequately educated and trained, and are supervised until they have demonstrated their competence in the procedures?

2.2.2 Assess and assign the severity classification of the procedures used in the project. Each procedure outlined in a project application must be classified according to the severity of its adverse effects on the animals (Article 38§2c). This *prospective* severity classification sets an upper limit on the level of suffering that an individual animal undergoing the procedure is allowed to experience.[46,47] The categories are: 'non-recovery' (for procedures carried out entirely under general anaesthesia from which the animal does not recover consciousness), 'mild', 'moderate' and 'severe' (Article 15§1 and Annex VIII; see also section 8 below, and Text Box 2).

The following points must be considered for all projects, in order to fully address the 3Rs and meet the requirements of the project evaluation process:

- Have you tried to identify and predict all possible adverse effects that could be caused to the animals in the project? Include any pain, distress, lasting harm and other forms of suffering, such as hunger, anxiety, boredom and osmotic or thermal stress caused to the animals, which may occur at any time during

the animals' lifetime; for example, a result of capture and transport to the laboratory, routine handling, housing and husbandry, or method of killing, as well as the effects of the procedures themselves.
- Have you taken steps to minimise each of these adverse effects as far as possible, by: *i.* applying the 3Rs in the design of procedures (see above), *ii.* using these guidelines, other relevant literature, advice from colleagues and from the institution's Animal Welfare Body to assist you?
- Based on the above, have you set prospective severity classifications (see section 8) and clear humane end-points for all procedures?
- How will you monitor the welfare of the animals used in the project, and when and how will you intervene to ensure that the animals do not suffer beyond the upper limit of severity needed to achieve the objectives of any particular procedure?

Member States must also collect *retrospective* information on the *actual* severity of procedures, after they have ended, and must make this publicly available on an annual basis (Article 54§2). The assessment of actual severity is based on day-to-day observations of the animals, and the 'most severe' severity experienced by the animal is reported to the National Competent Authority. For example, if records of observations indicate that suffering is moderate at the beginning of a procedure and then mild for the remainder, actual severity for reporting purposes is 'moderate'.

Annex VIII of the Directive further explains the severity categories (see also section 8), and the European Commission has put together a series of examples* to illustrate the process of prospective severity classification, day-to-day observation and monitoring of animals and actual severity assessment.

2.2.3 Weigh the harms and benefits of the project and the individual studies within it. Taking into account all the points listed above:

- What is the basis for your overall assessment that 'the harm to the animals in terms of suffering, pain and distress is justified by the expected outcome, taking into account ethical considerations, and may ultimately benefit human beings, animals or the environment'? (Article 38§2d)
- Have you explained this evaluation in your project application and, where relevant, your non-technical project summary (Article 43) – the latter using language suitable for the general public?
- How will you ensure that the 'weighing of harms and benefits' is an on-going process throughout the project – i.e. part of the day-to-day practice of 'ethical science', and not just a one-off event at the time of authorisation?

A detailed examination of procedures for harm-benefit analysis in animal research and testing is available in a document from the Animal Procedures Committee in the UK.[48]

3. Supply, capture and transport

3.1 Source of animals

Article 9§1 of the Directive requires that animals must *not* be taken from the wild for use in procedures, unless an exemption has been granted by the relevant National Competent Authority, based on 'scientific justification to the effect that the purpose of the procedure cannot be achieved by the use of an animal that has been [purpose-] bred for use in procedures'.

This means that, in principle, cephalopods used for experimental or other scientific purposes should be bred and reared in captivity. However, there are significant difficulties in captive-breeding most cephalopod species (for exceptions, see[2,49,50]) and, therefore, this may not be feasible at the time of writing.

Development of more successful, standardised breeding procedures is urgently required. Article 38§1c indicates that projects must be designed 'to enable procedures to be carried out in the most humane and environmentally sensitive manner possible'.

Where there is scientific justification for using animals taken from the wild, animals may be captured 'only by competent persons using methods which do not cause the animals avoidable pain, suffering, distress or lasting harm' (Article 9§3).[2] All those involved must observe a strict ethic of respectful treatment of animals, take into account their conservation status (section 3.2 below), and minimise the impact on the local ecosystem (section 3.3).

Care should be taken to prevent physical injury and stress to cephalopods at all stages in the supply chain, including capture (section 3.3), transportation (section 3.4), acclimatisation to laboratory conditions (3.5) and quarantine where required (3.6). It is also important to check local requirements for transport of animals in all countries along the route.

3.2 Cephalopod species commonly used in research and conservation status

An analysis of cephalopod species used in EU laboratories and the types of research undertaken can be found in Smith et al.[3] Species from the main taxa of

*http://ec.europa.eu/environment/chemicals/lab_animals/pdf/examples.pdf

Table 2. Summary of major species of cephalopod used in research together with their source. Eggs may either wild caught or captive bred (e.g. for *S. officinalis*). See also reviews in Smith et al. (2013)[3] and Fiorito et al. (2014).[2] The possibility of obtaining animals through a given source is indicated by (√), and (**X**) indicates this source is considered possible, but not currently available. For details and comments on welfare issues see also text.

	Source		
	Wild caught	Eggs	Captive bred animals
Nautiloid	√		
Cuttlefish	√	√	**X**
Sepiolid	√	**X**	**X**
Squid	√	**X**	**X**
Octopus	√	**X**	**X**

cephalopods used in research are shown in Table 2. Note that in some cases, e.g. *Nautilus* sp. or *Idiosepius* sp., animals are mainly available by importation and this requires permits and documentation from both exporting and importing countries[†].

At the time of writing, species of the Class Cephalopoda have not yet been assessed for possible inclusion in the IUCN Red List of Threatened Species[‡] and hence none is listed as endangered. However, concerns are being raised for some rare species, based on local evidence and experience. Examples of locally protected species are: *Euprymna scolopes* in Hawaii; *Octopus cyanea, Sepia elongata, Sepia pharaonis* and *Sepia prashadi* in Israel (N. Shashar, pers. comm.); and some Mastigoteuthidae species in New Zealand.[51] Indeed, assessments are now underway for cephalopods[§] under the Sampled Red List Index (SRLI) initiative**, which indicates the relative rate at which the conservation status of certain species groups changes over time, and aims to broaden the taxonomic coverage of the IUCN Red List.

3.3 Capture methods

Commonly used cephalopod species are listed in Table 2 together with information on currently available sources (e.g. wild, captive bred, eggs), and Table 3 indicates possible capture methods.

Several reviews describe commercial capture methods for cephalopod species.[29,52] Current methods include, but are not limited to, nets, traps and pots (see Table 3).[53,54] Environmentally destructive methods (e.g. trawling) should be avoided wherever possible. Hand-jigging is considered the 'best' method for capturing squid, but may not be appropriate for all squid species.[51,55]

Whichever method is used for capturing animals for research, it must not cause 'avoidable pain, suffering, distress or lasting harm' (Article 9). As noted above, animals may be captured only by competent persons. Moreover, researchers (and institutions) should only accept animals from suppliers who use appropriate capture and transportation methods; and the competence of third party providers should be evaluated based on the condition and survival of the animals supplied (see also section 3.4).

3.4 Transport (local, national and international)

Transport of cephalopods should always be in well-oxygenated seawater.[56]

Whenever possible and applicable to the research project, transport of eggs is the simplest, most successful, and, hence, preferable approach. Details of methods for egg transport are available for some cephalopod species.[56,57]

When juvenile and adult cephalopods are transported, high survival rates should be achieved through careful selection of container type, maintenance of seawater quality in appropriate volumes and consideration of other measures to support animal welfare, such as food deprivation and cooling. The following discussion outlines general principles for transport of cephalopods, along with variations according to the duration of transport.

In the following sections, *short-duration* (i.e. short distance) transport is defined as requiring less than 2 hours; and *long-duration* (i.e. long distance) transport is for any longer period. These working definitions are based upon the consumption of available oxygen and detrimental changes in water chemistry (e.g. accumulation of ammonia and carbon dioxide and depletion of oxygen) as the duration of confinement for transport increases.

Since no specific systematic studies of transport methods for cephalopods are currently available, it is recommended that transport requirements should be based on the FAO guidelines for fish,[58–60] paying particular attention to oxygen-sensitive species as they are considered to be comparable to cephalopods in their metabolic rate. Transport should also comply with the European Convention on the Protection of Animals during International Transport (ETS no 65, ETS no 193).

[†]http://ec.europa.eu/environment/cites/info_permits_en.htm
[‡]www.iucnredlist.org
[§]http://www.zsl.org/blogs/wild-science/animals-in-the-red
[**]http://www.zsl.org/science/indicators-and-assessments-unit/the-sampled-red-list-index

Table 3. Schematic overview of the most common methods utilised for capture of cephalopod species for research purposes. See text for comments about welfare and environmental issues assocated with each method.

	Capture methods						
	Collection of eggs	Hand net	Traps	Pots	Hand jigging	SCUBA	Trawling
Nautiloid			√			√	
Cuttlefish	√	√	√			√	√
Sepiolid	√						
Squid	√	√	√		√	√	√
Octopus	√	√	√	√		√	√

3.4.1 Transport containers. Since seawater is crucial for the survival of the animals, steps must be taken to ensure that water will not leak at any time (e.g. by containing animals in double bags and placing bags within a sealed container). It should be noted that several cephalopod species (e.g. octopuses) are occasionally reported to cut and bite through thin plastic bags (M. Kuba, pers. comm.), hence stronger transport containers should be used for them. In any case, the external container should be able to contain the entire water volume even if all inside containers/bags rupture.

Short-duration transport does not necessarily require animals to be placed into plastic bags, and other appropriate containers (large plastic bucket or box with a lid) may be preferred containing sufficient pre-oxygenated seawater to allow the animal to be completely immersed. Animals should be kept in dim or dark conditions, and movement and vibration of containers should be minimised.

For *long-duration transport,* similar to the protocol for transporting fishes[58] and depending on body size, one animal should be placed with 1/3 pre-oxygenated seawater and 2/3 oxygen-enriched air in double common aquarium bags (see also description in[61–63]). Once the bag is aerated, and the animal placed within it, it should be properly sealed (e.g. twisted at the top and folded over) and doubly secured (e.g. two rubber bands or cable grips). For transport periods over 12 hours, aeration and oxygenation may be necessary during transport, but care must be taken to use methods which do not add adverse conditions (i.e. bubbling) that may cause distress the animals.

Sealed holding bags (transparent to facilitate inspection if required) containing oxygenated seawater should be placed into insulated boxes (e.g. Styrofoam) to ensure that a temperature appropriate to the species is maintained during transport. The transport box should be darkened with a secure lid to keep the animals in darkness during transport, which reduces their stress. Bags should be packed with cushioning material (e.g. paper, Styrofoam pellets) to ensure they do not move during transport.

The use of specialised aerating and insulated transport containers is not yet common for cephalopods. However, the development of specialised transport containers should be encouraged (for reviews on fish transport containers see[58,60]).

3.4.2 Other factors for consideration. Food deprivation before shipping may be desirable, where appropriate, dependent on *i.* shipping distance, *ii.* the species and *iii.* size of animal. It is common practice among cephalopod researchers to withdraw food before shipping. This is to help prevent fouling of the water and ammonia build-up during transport. The duration of food deprivation should be based upon a consideration of normal feeding frequency, oro-anal transit time and renal ammonium ion excretion. However, food deprivation may require authorisation by the National Competent Authority.

Food deprivation before transport, alongside lowered water temperature, can also help to avoid build-up of toxic ammonia and carbon dioxide.[64,65]

With the aim of lowering metabolic rate before shipping it has been suggested that pre-cooled seawater at 2–3°C above the thermal tolerance minimum of an individual species should be used, ensuring that the temperature stays at that level for the duration of transport (see e.g. for the giant octopus[66]). However, the impacts of this method on animal welfare are not yet known.

Sedation is not essential and is not recommended for transport of most cephalopods. However, sedation methods (i.e. cold water[67] or 'chemical' methods[68]) has been used during transport in some instances with cephalopods.

It is interesting to note that in 1928 Grimpe suggested that very long duration transportation, i.e. requiring more than 2 days, should be achieved in

steps allowing 'resting' periods in appropriate locations.[56]

3.5 Acclimatisation after transport

Transport inevitably causes animals stress. Therefore it is important to allow time for them to recover from transport-related effects, to acclimatise to the new conditions including possible differences in water quality, temperature, illumination, diet, and the shape and arrangement of the environment (i.e. the tank). Allowing time for acclimatisation is vital for both animal welfare and science, as stress can confound scientific results.

Almost all cephalopods are highly stenohaline and stenotherm, and care should be provided to avoid any difference in salinity and water temperature of the container utilised for the transport and the tank where the animals will be placed. In the case of significant difference in water temperature, an adjustment of the different 'media' after transport (e.g. placing the container inside the final tank for slow adaptation of the temperature to the final one) should be considered.

Cephalopods arriving in a facility should be examined for injuries or other health issues, and treated and/or quarantined (see section 3.6) or humanely killed (see section 8.11) where necessary. It is also recommended that NH_4 and CO_2 levels in the transport water are measured. Together, these observations can help in assessing the quality of transport methods and suppliers.

Based upon species-specific requirements (i.e. individual or group living; see also Appendix 2) animals should be placed in a holding tank until they are habituated to it. The requirements and duration of this practice are species-specific (generally from 1 to 5 days) and for experimental reasons may be reduced to a minimum (e.g. when studying individual preferences towards a prey item or a stimulus), because evidence is available for contextual learning to occur in most cephalopods.[26,69–71]

To facilitate the required 'habituation' to the captive situation, it is best if the holding tanks are designed according to the same principles as the maintenance/experimental tanks for the species. Where food has been withdrawn during transport, a slow reintroduction is also recommended.

In the classic literature on cuttlefish and octopus, an adequate predatory performance is considered a sign of acclimatisation to a holding tank.[26,36,72] In addition, while excessive inking upon introduction to a new tank is a sign of stress, low swimming rates, reduced likelihood of inking in response to a small disturbance near a tank, can serve as indications of successful acclimatisation (N. Shashar, pers. comm.).

When moving animals from one tank to another within the laboratory a standardised, minimally stressful protocol should be applied. In these cases, only a brief acclimatisation time may be needed (in the order of minutes to few hours). However, the experimenter should be aware of potential handling and relocation stress and its physiological consequences that may impact on the research.

3.6 Quarantine

The purpose of quarantine after reception of animals is to isolate cephalopods from the main population accommodated in the facility to allow observation and testing until animals are assessed as healthy and free from potential infectious diseases. Individuals identified as ill should either be separated for treatment, if the cause can be identified and treatment is available (see section 7), or humanely killed (see section 8.11) and autopsied (see section 6.3).

Quarantine is also useful to isolate individuals that become sick while being maintained in the facility, allowing time for sanitary measures to be put in place and ensure appropriate containment of organisms and waters.

Currently, quarantine is not the general practice in the cephalopod research community. However, further studies are required based on recent research of cephalopod diseases and diffusion of parasites.[73]

The duration of quarantine should be sufficient to assure health of the individual animals. The needs of individual quarantined animals vary according to the biology and behaviour of the species (e.g. group holding maybe appropriate for gregarious species, but others may require individual accommodation; see also Appendix 2).

Quarantine should involve complete separation between animals to be quarantined and the current laboratory population; this should be achieved either by using separate rooms or equipping facilities with plastic screens to separate quarantine tanks from others. In addition, water supply should also be separate, to prevent diffusion of any potential harm to the water circulation and/or the environment. Similarly, equipment (e.g. nets) should not be shared between tanks.

During the quarantine period, animals should be monitored closely for unusual clinical signs or behaviours (see also sections 6 and 7), and detailed examinations (including autopsy; see section 6.3) made of any individuals who are considered to be 'abnormal'. In the cases of identification of diseased animals present in the laboratory holding facility, this should be regarded as a possible indicator of disease in the entire stock/holding group, and hence particular attention should be provided, and eventually they should all be treated or humanely killed.

4. Environment and its control

4.1 Seawater supply and quality

Both natural and artificial seawater (see also below) are suitable for the maintenance of cephalopods. For fish, Annex III of Directive 2010/63/EU requires that 'an adequate water supply of suitable quality [is] provided at all times', and that 'at all times water quality parameters' are 'within the acceptable range that sustains normal activity and physiology for a given species and stage of development' and such requirements apply equally well to cephalopods.

4.1.1 Types of seawater circulation system. There are two principal seawater systems for keeping cephalopods: *closed systems* which recycle a reservoir of seawater, and *open systems* which either draw a continuous supply of water from the ocean (*flow-through systems*), or pump seawater into a reservoir and regularly replenish it with fresh seawater (*semi-closed systems*).

Closed systems have the advantage of enabling control of all parameters of the environment, but are more costly due to the need for additional environmental monitoring and control equipment.

Open systems rely on fresh seawater being drawn from the ocean. While this has some advantages (especially not needing expensive filters), it limits the facility to keeping animals that can live within the given water parameters. For example, non-native species cannot be kept in this type of system without considerable efforts purifying and sterilising the reflow. Naturally open systems are also limited to areas close to the shore.

Closed systems: Efficient and relatively easy-to-maintain closed aquarium systems have been developed for cephalopods.[74–78] Commercially available artificial seawater preparations are considered adequate, provided they contain the necessary substances and trace elements to meet the physiological needs of the particular cephalopod species, and for this reason mixtures designed for marine invertebrates and corals should be preferred.

Water flow in recirculating systems or filtration within enclosures should be sufficient to remove suspended waste and to ensure that water quality parameters are maintained within acceptable levels. Filtering systems in recycling/recirculating seawater should be adequately planned and maintained.[79,80] Appropriate processes for monitoring water parameters should be implemented, and alarms in place to ensure flow and seawater levels are adequate.

Open systems: seawater drawn from the ocean should be tested for contaminants and pathogens, and treated to remove them. The water supply should also be evaluated to ensure that there is sufficient capacity, including ability to cope with periods of maximum demand and emergency situations. To protect animals from potential contaminants, other measures, such as appropriate filtering or a reverse osmosis system, may be required.[79,80]

Tests to determine the chemical composition and presence of contaminants/toxins will determine the treatment necessary to make the water suitable for use. Seasonal factors such as phyto- or zoo-plankton blooms, tidal cycles, and seasonal seawater mass turnovers can have periodic effects (on a scale of hours, days or months) for seawater and these should be anticipated.

*All systems: w*ater flow should enable cephalopods to maintain normal locomotion and behaviour. Cephalopods can use rapid expulsion of water through the funnel to power jet propulsion, which results in swift movement. Cuttlefish and squid have fin-like structures on the mantle to assist in locomotion. Squid are in continuous motion due to their pelagic nature, thus water flow[75,77] needs to be sufficient to ensure appropriate life-style requirements, and adequate water quality including quick removal of ink (if released).

4.1.2 Water quality. As for other aquatic species, water quality is the most important factor in maintaining the health and well-being of cephalopods. Insufficient water quality will cause stress and disease. Water-quality parameters should at all times be within the acceptable range that sustains normal activity and physiology for a given species and individual (see Appendix 2); and should remain stable, unless the life style of a given species requires changes (e.g. because of large vertical migrations during a day or seasonal changes)[‡]. Optimum conditions vary between species (e.g. deep-sea benthic octopuses are especially sensitive to changes), between life-stages (e.g. paralarvae, juveniles, and adults) and according to physiological status of the individual (e.g. females preparing to lay eggs).

Most cephalopods show little adaptability to changing water-quality conditions, and so when animals need to be moved between tanks or systems, it is important to ensure that water parameters are mirrored and maintained. If this is not possible, gradual acclimatisation will be needed (see section 3.5), as for other marine invertebrates and fishes.

Dissolved oxygen, pH, carbon dioxide, nitrogenous compounds and salinity should be monitored and maintained according to the appropriate range for each species. Appendix 2 provides a list of water quality criteria for optimum health and welfare of cephalopods; for more detailed discussion of monitoring water quality see below.

[‡]but this may be difficult to achieve in laboratory facilities.

4.2 Monitoring water quality (O_2, pH, CO_2, nitrogenous material, salinity and metals)

Seawater parameters should be monitored (continuously by specific electrodes or intermittently by chemical methods) and recorded at an appropriate frequency (at least daily), thus allowing proactive, rather than reactive, management of water quality. Parameters that need to be measured and the frequency of measurement vary (see also Appendix 2), depending on whether the system is open or closed. For example, while there may be no need to measure nitrites/nitrate in a high volume flow-through system (depending on the source of the water), such measurements are critical with recirculation systems.

At a minimum, environmental monitoring systems should provide information on water flow, oxygen saturation and water temperature. Parameters measured should also be relevant to the health and welfare of the particular species housed in the facility (see Appendix 2). In general, recirculation systems should be monitored for a larger number of parameters, including, but not restricted to, dissolved oxygen, pH, nitrogenous material, salinity, total dissolved salts and temperature (see below). As a minimum, water quality analysis should be carried out at times of greatest demand on the system (usually after feeding) to identify potential problems.

Water and tanks should be kept clean particularly of faeces and uneaten food. In semi-open and closed systems, water should be treated to reduce potential pathologies, for example, using UV light or ozone. If ozone is used, measurements of ozone concentrations and/or redox potential of the reflow entering the system are necessary to avoid toxicity.

Alarm and notification values must be set and their significance as potential indicators of problems in the system explained to all relevant personnel. There must be an agreed, clear protocol for contacting those responsible for the facility when problems are identified outside of normal working hours.

The monitored parameters should be recorded and the information stored for at least 5 years. For all parameters considered below and for techniques of keeping animals information is also available through a recent compilation of research on the culture of cephalopods.[50]

4.2.1 Oxygen. Cephalopods have high metabolic rates, so oxygen concentration should generally be kept high (close to saturation); and where tank inflow is not sufficient, supplementary aeration of the water must be provided. However, supersaturating the water is not advisable as it may cause gas bubbles to become trapped in the mucus layer of the animals, thus limiting gas exchange at the gills (for octopus: G. Fiorito, pers. comm.; for several cephalopod species: J. Rundle, pers. comm.).

Information on oxygen consumption in some cephalopods is provided by Winterstein[81] (see also[82,83,83–87]) and reccomended requirements are summarised in Appendix 2.

It is important to note that monitoring oxygen levels is not informative of the oxygen available to the cephalopod unless it is combined with measurements of pH (see next section).

4.2.2 pH. Due to the effects of pH on the carriage of oxygen by blood pigments, cephalopods tolerate low pH poorly.[88–96] Regular measurement and careful maintenance of pH is therefore critical. Acceptable pH levels depend on many water quality factors, for example, carbon dioxide (see below), and calcium; as a consequence, control of soluble gases and water solutes is important. This may be a particular issue for establishments that use synthetic marine salts rather than natural seawater and closed filtration systems, especially where water changes are limited. Acceptable pH values for keeping cephalopods are summarised in Appendix 2.

4.2.3 Carbon dioxide. Carbon dioxide is produced during respiration and dissolves in water to form carbonic acid, thus lowering the pH. Since stability of pH is very important, accumulation of carbon dioxide should be avoided. Situations that may increase carbon dioxide levels include high stocking density and poor aeration.

Care should be taken that water supply systems, particularly in the case of groundwater-based systems, do not introduce harmful quantities of carbon dioxide to the enclosures.

4.2.4 Nitrogenous material. As cephalopods are carnivores, hence requiring a high protein diet, the accumulation of potentially toxic nitrogenous compounds can be a problem, particularly in closed systems. It is also necessary to avoid accumulations of ink (especially when keeping cuttlefish, that ink in large volumes). Timely removal of uncomsumed food, use of adequate protein skimmers and suitable water flow rates, along with careful water filtering in closed systems, will help to reduce levels of organic waste, including ammonia, nitrites and nitrates. It is also beneficial if the flow rate in the tanks can be adjusted to different situations (e.g. increased after inking). Additional water changes can provide a supplementary means of removal of waste products and substances such as ink.

Build up of nitrogenous compounds may lead to behavioural changes and/or changes in skin

colouration in cephalopods. For example, at nitrate levels >80 mg/l cuttlefish become very agitated, will ink profusely and their skin tone may be dark; and larvae and hatchlings are more vulnerable to bacterial disease (A. Sykes, pers. comm.).

Levels of nitrogen compounds tolerable by different species are reviewed in Appendix 2 (see also Iglesias et al.[50]).

4.2.5 Salinity and metals in seawater. As cephalopods are marine organisms, maintaining an appropriate salt concentration is vital. The salinity should match the natural habitat of the animals.

Commercially available artificial seawater preparations and especially any mixture designed for marine invertebrates and corals are considered adequate as they contain all the necessary substances and trace elements to keep cephalopods in good health. However, in accordance with instructions for the different brands of salt, some trace elements, in particular strontium and calcium should be monitored and added if necessary.

Copper and its alloys are considered to affect the salinity and 'poison' the seawater[56] and therefore should be avoided in any system holding cephalopods.

Cephalopods are reported to accumulate and be sensitive to heavy metals so care should be taken to ensure these are monitored and maintained within normal ambient ranges for the species, to limit potential damage (see above and also[2] for relevant literature).

4.3 Lighting control

Light influences, either directly or indirectly, almost all physiological and behavioural processes in cephalopods, including growth, development and reproduction. Lighting requirements vary between cephalopod species, and both wavelengths and intensity of lighting should 'satisfy the biological requirements of the animals', where these are known (Directive, Annex III, section 2.2a). The natural history of the species, in particular the normal living depth, can provide clues to help meet the species lightling preferences: for example there are many cephalopods that prefer very little light. There is limited specific knowledge on wavelength perception for almost all cephalopod species.[97] However, it is estimated that simulated sun-light equivalent to that normally experienced at 3–8 m depth at sea should be acceptable for the majority of cephalopod species commonly used as laboratory animals (but see further information in Appendix 2).

Photoperiod should also be maintained according to the natural requirements of the species.[98–103] However, there is evidence that some cephalopods may easily adapt to changes in day/night regime.[26,104–106]

Where task lighting is needed for people working in the room, it should be restricted in its dispersion, and/or be placed below the level of the tank surface, to reduce disturbance to the animals. Use of automated dimmer controls that allow light intensity to be gradually increased is important and recommended (G. Fiorito, pers. comm.; see also[107]). For example, for decapods and nocturnal octopuses sudden changes in light level may cause escape reactions, and in some cases inking, thus a simulated dusk and dawn period is desirable. Care should also be taken to ensure that animals are not disturbed by night-time security lighting entering through windows in the holding facilities. The output of fluorescent lights can be diminished by using dummy bulbs to reduce light levels.

4.4 Temperature control

Water temperature should be controlled within the natural range for the species; and, where necessary, appropriate chilling/heating equipment must be used to ensure the optimal temperature range for the animals.

Cephalopod species vary in their sensitivity to changes in water temperature. In general, higher water temperatures create problems for animals from temperate climates like octopus and the cuttlefish. Transitions of temperature should not be sudden.[56,108] Where water changes are performed on larger scales, temperature spikes, which may cause adverse effects, should be controlled and avoided.

4.5 Noise and vibration control

Background noise, and vibration from housing systems, such as pumps or ventilation units, should be minimised as they are likely to impact on cephalopod welfare.

Several studies suggest that cephalopods can detect sounds even at low frequencies,[109–115] and other recent work shows that cephalopods are as likely as other marine organisms, to suffer from low-frequency noise traumas.[116–118]

In common with other aquatic species, cephalopods dislike vibrations, such as drilling or banging on tank sides, and some species, such as cuttlefish, may respond by inking. Therefore, the most important aspect of sound reduction is to minimise disruption and avoid sudden noises, which could startle the animals.

4.6 Aquatic life support systems and emergencies

Tanks should be built so that complete drainage is impossible when they are inhabited (although ability to drain tanks may be required for cleaning purposes).

Two independent sources of water movement/oxygen supply are also recommended, for example, pumps for water circulation plus extra air sources to provide additional aeration.

Electronic alarm systems help to ensure that problems in a system are detected promptly. All facilities must have an emergency plan in place should problems arise (including out-of-hours), with clear actions that are understood by all and effectively communicated to everyone. There must be a backup system to enable an appropriate response to the worst case scenario of a complete system failure, and so avoid circumstances in which animals would have to be humanely killed due to suffering from anoxia or a build up of organic waste.

5. Accommodation and care

5.1 Background and requirements of the Directive

About 50 species of cephalopod have been kept successfully in aquaria (M. Kuba, pers. comm.; see also[108]). These range from small species such as bobtail squid (*E. scolopes*) to larger pelagic squid (e.g. *Loligo vulgaris, Doryteuthis pealeii*), octopuses (e.g. *O. vulgaris*, *Eledone cirrhosa*) and cuttlefishes (*Sepia officinalis*), and the giant pacific octopus (*Enteroctopus dofleini*).

At the Stazione Zoologica di Napoli, considered to be the first large-scale facility for the maintenance of cephalopods[56] (mostly for cuttlefishes and octopuses, A. Droesher and G. Fiorito, pers. comm.), outdoor tanks were preferred to indoor rooms, to allow animals to be kept in natural light with seasonal daylength. However, shading was provided to reduce direct sunlight to the animals. In the following years, indoor tanks were installed and artificial lighting was introduced to supplement natural illumination (A. Droesher and G. Fiorito, pers. comm.).

The knowledge accumulated in various laboratories around the world, with a variety of cephalopod species, supplemented the original studies at Stazione Zoologica and facilitated the design of closed systems for maintenance of species which adapt less readily to laboratory housing such as squid (for review and methods see[75]).

Annex III of Directive 2010/63/EU sets out requirements for care and accommodation of animals. Section A lists general requirements pertaining to all species and section B lists species-specific requirements, for all vertebrate classes, including brief guidance for fish (but with no distinction between the different classes of fish). Some of these Section B requirements for fish might also apply to cephalopods,[2] but cephalopods are not specifically mentioned in Annex III.

5.2 Holding facilities for cephalopods

Planning design and maintenance of new accommodation facilities for cephalopods should take into consideration key points outlined by the Committee for the Update of the Guide for the Care and Use of Laboratory Animals.[119]

Access to the facility should be allowed only to people who have received relevant training and have a legitimate need for access. Movements of personnel inside the facility should also be monitored and controlled to minimise disturbance to the animals and ensure biosecurity, which may require measures such as physical barriers and access restriction/control.

Walls of holding rooms should generally be of dark neutral and continuous colours. However, very dark colours may make it difficult to identify dirty areas, so specific evaluation of the appropriate colour may be required.

Cephalopods require large volumes of seawater. All facilities should have an emergency contingency capacity, capable of maintaining aerated and filtered seawater should normal systems fail. Monitoring systems including remote alarm notification should be designed and used in cephalopod facilities.

Noise should be minimised to avoid disturbing animals in both housing and experimental rooms. When applying sound-attenuating material to the ceiling or walls, always consider that it has to be sanitisable. All vibration sources (e.g. mechanical equipment, electrical switches, through ground-borne transmission) should be identified and vibration isolation methods should be used to reduce noise (e.g. by placing equipment on rubber pads). Noise-producing support functions, such as tank and filter washing, should be separated from housing and experimental areas, wherever possible.

Fire and environmental-monitoring alarm systems should be selected and positioned to minimise potential disturbance to animals.

All procedures and other manipulations of living animals should be carried out inside the facility to minimise stress to the animals, unless there is scientific justification for doing otherwsise. Therefore, a typical cephalopod facility should have available: *i.* adult animal housing/holding room(s) divided by species if possible, and breeding/'hatching' room(s); *ii.* quarantine room(s) (if needed); *iii.* an area for acclimatisation of animals; *iv.* procedure rooms separated from holding and breeding rooms, for experimental techniques, including regulated procedures (e.g. surgery, behavioural experiments, imaging, clinical treatment, humane killing, necropsy, etc.); *v.* separate 'service' rooms for storage of food, supplies, chemicals, etc., and for waste (including biological material) storage before incineration or removal.

Shared facilities, where cephalopods are kept in water systems and rooms hosting a range of other

types of marine organisms, are not recommended. Additionally prey species should never be accommodated in the same tank as their predators.

In designing holding facilties for cephalopods and selecting the construction materials, it is recommended that guidelines developed for fish are followed (for review see[79]).

Materials used to build aquatic facilities should be non toxic. Any unavoidable use of material with the potential to be toxic should be reduced to the minimum, recorded and the information made available to staff, veterinarians and inspectors. In particular, materials that may release specific ions, chemicals or corrosion by-products from their surfaces should be avoided. The use of metals requires consideration of their interactions with seawater, and the potential effects of that interaction on the animals.

Special attention should also be given to the behavioural needs of the animals. For example, non-gregarious animals or animals that might show aggressive interactions (e.g. males during mating season) may require housing out of sight of others. Attention must also be paid to species-specific differences in terms of the level of disturbance that may be acceptable; for example, *O. vulgaris* appears to be quite resilient whereas cuttlefish or squid react more strongly to unfamiliar and sudden movements.

5.3 Housing

Cephalopods are strictly marine, and all require high-quality sea-water, but their varying habitats, social behaviour and especially nature and level of locomotion determine how they should be housed. Aquarium size and stocking density should be based on the physiological and behavioural needs of the individual species, and requirements for their health and welfare (see Appendix 2).

Section 3§3b of Annex III of the Directive indicates that all animals, including cephalopods, 'shall be provided with space of sufficient complexity to allow expression of a wide range of normal behaviour', including social behaviour, locomotion and feeding, and 'shall be given a degree of control and choice over their environment to reduce stress-induced behaviour'.

Stocking density will vary depending on the animals' natural history and behaviour, water flow, size, age and health. Water quality is critical (see section 4.1.2 above).

Most octopuses are solitary and should be kept in isolation. *Nautilus* are primarily solitary in the wild but may be housed together at low densities.

The social structures of many species, including the European cuttlefish (*S. officinalis*) are not known, but in general social animals including many squid, are best kept in groups. However, social interactions should be monitored to check for adverse welfare effects; animals should be grouped according to age to avoid fighting and possible cannibalism, particularly in the breeding season or where there could be territorial antagonism. Such measures should not alter the overall welfare of the animal, and, in general, should be respectful of the behavioural needs of each individual species.

Depending on the species, individuals may require dens, shelters and other devices (mostly for bottom-living cephalopods).

Enriched environments must be provided, to allow the animals to express their normal behaviour (see further discussion of enrichment in section 5.11 below).

5.3.1 Tanks. Tank requirements and stocking density vary among cephalopod species and ages (see Appendix 2 for a summary of requirements). For example, for benthic species, like *O. vulgaris* and *S. officinalis*, the available bottom area is an important requirement, while for pelagic species this is represented by the volume of water; the depth of the tank should be considered for species with known diel (diurnal) vertical migration (e.g. *Nautilus pompilius*).

All cephalopods should be kept in opaque tanks of neutral colour. *O. vulgaris* and *S. officinalis* may also be kept exceptionally in transparent aquaria, as long as the floor of the tank is opaque (and/or covered by sand). Tanks can be of rectangular or of any other shape, but for decapod cephalopods they should have rounded corners to minimise potential injuries (see Appendix 2).

In general (and especially for *Sepia* and other decapods), sharp objects and rough surfaces that can cause skin damage must be avoided. Jetting can lead to collisions with the walls of the tank if animals are startled, or there is insufficient space for escape reactions.

Tanks can be made of PVC, fibreglass, glass or any non-toxic material capable of being adapted to achieve appropriate shapes and allowing a smooth internal surface, and which is easy to clean and sterilise or decontaminate as necessary. As potentially toxic materials might have been used during initial tank assembly (e.g. silicone-based adhesives and sealants), it is recommended to wash the tank thoroughly, leave it filled with water for at least 24 hours, and then rinse with seawater, before animals are introduced.

Tanks should be equipped with a covering (e.g. tank net or rigid transparent covering) that prevents animals escaping. Lids also serve as a barrier against the accidential introduction of any foreign objects, animals or chemicals. Tanks lids may be constructed of materials such as plexiglass or clear acrylic to allow visual inspection. The distance between the water surface and lid should be enough to minimise the risk of damage, for

example, in the case of squid which are capable of leaving the water using their 'jet-propulsion'.

5.3.2 Tank labelling. Tanks must be carefully labelled, to identify and record the histories of individuals or groups of animals.

Labels should include detailed information for each individual, including origin, first dates in captivity and arrival in the laboratory, sex and morphometric measurements if possible (e.g initial body weight, dorsal mantle length), along with the number of animals in the tank (which may be an estimate depending on developmental stage).

For animals undergoing procedures, the label should identify: *i*. the procedures being performed (e.g. the label could refer to a detailed protocol filed for easy access by all relevant staff); *ii*. the date when the procedures were started; *iii*. the person responsible for the animals (e.g. the Principal Investigator). Records of any adverse effects shown by the animals should be also be easily accessible 'tankside' and should be carefully maintained (see below for further discussion). Taken together, all these points should make it is easy for animal care staff and scientists to identify animals showing signs of welfare compromise, determine the likely cause of the adverse effects (e.g. whether procedures applied, such as recent anaesthesia) could explain the abnormalities, and take action to mitigate them.

5.4 Cleaning of tanks

Water quality should be monitored daily as a minimum (see also section 4). When water changes are necessary, the smallest possible amount should be removed.

Tanks should be free of organic waste (e.g. uneaten food or faeces), otherwise water quality, and thus animal health will be harmed.

Open systems: tanks should be regularly drained and cleaned to prevent fouling and reduced water exchange. There should be no risk of back-flushing, and consequent fouling of enclosure water. The sides and bottom of enclosures should be cleaned regularly to avoid the accumulation of detritus.

Closed systems: waste material should be removed as soon as possible after feeding. Total water replacement and whole tank cleaning should be avoided, as the biochemistry and flora that develop in a mature system are essential to well-being, as known in common practice for acquaria keeping. Depending on the size (i.e. number of tanks/system) care should be given to facilitate the most appropriate conditions at equilibrium. Where complete draining out of a system is required for decontamination reasons, the system must be allowed to remature after the addition of clean seawater, prior to adding animals.

When cleaning of tanks occupied by animals is necessary, the process should be designed to minimise disturbance and distress; in most cases animals will need to be removed from the tank during cleaning. Capture and transfer methods should conform to the principles outlined in these Guidelines, and the time spent in a holding tank should be minimised.

Disinfectants should be used with extreme caution and only in dry tanks, which are then rinsed with clean water. Detergents should be avoided and substitutes are preferred.[120] Animals must not be exposed to any substance used for cleaning of tanks.

5.5 Methods for individual identification and marking of cephalopods

Depending on stocking density, it can be difficult to identify individual cephalopods. Marking or tagging, other than in species with external shells such as *Nautilus*, is difficult, owing to vulnerability to tissue damage. Individual cephalopods may have unique natural markings, and whenever possible these should be used for identification.[121,122] Several marking methods have been successfully applied to different species of cephalopods (for examples see review in[123]). Methods used with success, but which require anaesthesia for their application – and hence scientific justification and approval from the National Competent Authority – have included implanted fluorescent elastomer tags in squid and octopus,[124,125] subcutaneous dye injection into the arm of octopus,[123,126] and external tagging of cuttlefish, octopus and other species.[127–132]

Careful consideration of harms, benefits and justification is therefore needed for invasive tagging, and development of minimally invasive individual marking methods for cephalopods is an important goal.[31,133]

5.6 Food and feeding for adult cephalopods

Most cephalopods are carnivorous and active predators,[134–136] hunting their prey using a range of strategies (review in[134]). However, nautiloids are scavengers and some species of octopus will eat dead food items.

For many cephalopod species at different life-stages, live prey is the only known method of feeding. This prey may be fish or invertebrates, such as crustaceans, which need to be treated ethically and legally,[137,138] and the feeding regime should suit the lifestyle, natural diet and developmental stage of the animals.

The duration of digestion (food intake to elimination) is 6–15 hours in the common laboratory species of cephalopods and is slower at lower temperatures in a given species,[139–141] so feeding frequency (and appetite) may alter with season (temperature) in open systems.

Data on the richness of cephalopod diets in their natural habitats is limited, but known to include, amongst others, zooplankton, molluscs (including other cephalopods), polychaete worms, crustaceans, chaetognaths, sea urchins, fishes and jellyfish.[26,142] An estimation of the relative breadth of diet has been attempted for some cephalopod species, including species most frequently used as laboratory animals,[26] and shows that some species' 'natural' diets are restricted to certain prey items (i.e specialists), such as *Spirula spirula*, which feeds on detritus and zooplankton,[143] whilst others are more opportunistic species (i.e. generalists) such as *S. officinalis* or *O. vulgaris* (for review see[26]). However, estimation of diet variety is substantially biased by research effort.

In laboratory conditions, animals usually adapt to prey on several different types of food.[36,108]

Nautilus requires food with a high level of calcium carbonate, such as shrimp with carapace, lobster moult shells or fish heads. Most cephalopods have a higher metabolic rate than fish, and their daily food intake which is rich in protein can be considerable: for example, 3–10% body weight per day.[144] The feeding regime, palatability and method of food presentation should ensure that animals are adequately fed. Young and/or wild caught animals need particular attention. In some cases, enrichement with favoured foods and touching the animals' arms with food may trigger feeding. Refusal to eat can be an early sign of illness (see also section 6.1.1).

Cuttlefish and squid are especially sensitive to inadequate nutrition; the most evident signs include: protruding eyes, poor body condition and floating (especially in juveniles). Consequently, in general over-feeding is preferred, as long as excess food is removed in an appropriate time-frame for the feeding habits of the species.[145] However, *ad libitum* feeding of *S. officinalis* may cause buoyancy problems, so this is not advised (K. Perkins, unpublished data).

Artificial diets have been developed for cuttlefish and other species[108] and are continuing to be explored in aquaculture research (Table 4; see also[50]). However, whilst an artificial diet may be ethically preferable and carries reduced risk of infection, studies to date indicate that growth and possibly welfare of the animals is reduced.[146,147]

The frequency of feeds is important and depends on the species and water temperature in the tank. The duration of digestion also depends on the species, and other factors including the animals' size, maturity and the type of food (for review see[148]). In *O. vulgaris,* gut transit times are quite rapid (about 12 hours at 18–19°C) suggesting that crop capacity is not great and so daily feeding should be the norm.[141,149,150] Daily feeding is also common practice for most coleoid cephalopods. However, other evidence from adult

Table 4. Use of alternatives and/or artificial/synthetic food to natural prey for rearing of some cephalopod species. The table is based on an overview of recent literature (most representative papers are included) mostly for aquaculture purposes (unless otherwise stated). For review see also Iglesias et al.[50]

Species	Artificial food	Food item	References
Sepia officinalis	Yes	Pellet Surimi	508–510
	No	Shrimps	e.g.[511]
	Yes	Lysine diet 4	151
	No	Natural frozen diet	512
Octopus maya	Yes	CPSP[a]	513
	Yes	Purina @ 51%	514
	Yes	Shrimp pellet + CPSP[a]	515
Octopus vulgaris	Yes	Moist pellets (fish and prawn mixed with alginate or gelatin as binders)[b]	508,516–518
	Yes	CPSP[a,b]	519
	Yes	Diet S (50% water, 20% gelatin, 10% egg yolk, 5% *S. aurita,* 15% *T. sagittatus*)	520
	No	Crustaceans; aquaculture by-products	521–527

[a]CPSP: namely 'Concentrés de Protéines Solubles de Poisson' is a concentrated fish hydrolysate currently trademark of COPALIS (http://www.copalis.fr/en/home/products-and-applications/animal-nutrition/aquaculture-breeding.html); see also Kristinsson and Rasco[528]
[b]This study is also aimed to the understanding of nutritional requirements of octopus

cephalopods, particularly cuttlefish and octopus, suggests that they may not need to eat every day.[146,147,151]

5.7 Food and feeding for larvae and hatchlings

Evidence is provided that different dietary needs are required for cephalopod species during the early stages post-hatching.[28,152,153] For example, hatchlings of *S. officinalis* often have a yolk sac which provides nutrition until they start feeding a few days later; in contrast, *O. vulgaris* paralarvae need to feed immediately in the water column. However, not all taxa of cephalopods have been successfully reared in laboratories, and so information on the dietary requirements of hatchlings is limited (for review see[50]). Evidence available for some species suggests that embryonic development often requires trace nutrients that are present in natural seawater,[154,155] in which case development might be hindered in closed artificial systems. It has also been suggested that 'dissolved gases and nutrients may also contribute to metabolic and nutritional requirements via absorption through the epidermis'.[144,156] In addition, a close relationship between the fatty acid profile of the dietary components and of the individual at early stages after hatching has been reported.[157] This emphasises the importance of improving understanding the nutritional needs of juveniles, especially if artificial diet is being considered for rearing purposes.

5.8 Handling and moving cephalopods in the laboratory

Handling procedures should be carried out only by competent, trained personnel using techniques that minimise the potential for injury and reduce stress to the animals (see also section 10). It is recommended that laboratory coats and gloves should not be of white/pale colours, as the handler can be mistaken for a 'predator'.

The skin of cephalopods acts as an organ[158] and is very delicate and so every effort should be made to minimise handling and removal of animals from the water. It is especially beneficial to standardise handling procedures, as anecdotal evidence indicates that cephalopods can habituate to handling.

It is preferable to move the animals in water using containers where they can be gently restricted before moving from one tank to another or any other location. Training animals to enter a container, possibly using small rewards, may reduce stress and habituate them to the transfer.

Cuttlefish and squid should be immersed at all times and a dark net should only be used to coax the animal into a container.

Nautilus is particularly sensitive to air, and repeated air exposure is anedoctally reported to have negative effects on the health of the animals (R. Smallowitz, unpublished data).

Octopuses can be moved using nets (suggested dark nylon 2-mm mesh) with a long sleeve to reduce the risk of escape; exposure to air should be minimised. A container method has been developed for *O. maya*[159] and, although not currently in use, represents a useful approach indicative of methods that should be developed for animal transfer within a facility.

Nets and containers should be clean, disinfected and rinsed before use. Agitation during moving should be minimised, as all cephalopods have a sensitive statocyst system.[160]

Handling and other human interactions should be monitored and recorded, as the frequency and nature of the interactions can influence behavioural performance of individual animals.

5.9 Environmental enrichment

Environmental enrichment should not compromise the need for adequate levels of hygiene and the ability to observe the animals' health (section 6 below) without causing too much disruption. The impact on health and welfare of environmental enrichment should be evaluated objectively,[161–163] particularly to avoid the application of 'environmental changes' which may be detrimental to the animal well-being, and to ensure health or water quality are not compromised.

Section 3§3b of Annex III of the Directive states that 'Establishments shall have appropriate enrichment techniques in place, to extend the range of activities available to the animals and increase their coping activities including physical exercise, foraging, manipulative and cognitive activities, as appropriate to the species. Environmental enrichment in animal enclosures shall be adapted to the species and individual needs of the animals concerned.' The same section also states that 'the enrichment strategies in establishments shall be regularly reviewed and updated'.

These provisions require on-going consideration of the effects of laboratory housing on animal welfare and efforts to enhance well-being wherever possible. Exemptions from these, and other, requirements outlined in Annex III have to be approved by the National Competent Authority, and must be for scientific, animal welfare or animal health reasons.

Environmental enrichment aims to enhance the well-being of animals in captive conditions, by identifying and providing stimuli that enable animals to express as wide a range of their normal behaviours as possible.[164,165] Enrichment is proven to be effective for

many species, including fishes,[166–168] cephalopods and other invertebrates.[138,169–175]

Enrichment may be accomplished through changes in the tank environment, for example, by varying factors, such as the shape of the tank, flow of water, variety of live prey items (if these are essential), conspecifics and environmental complexity; and also by providing opportunities for animals to engage in specific activities and exercise some choice.

Enrichment strategies should be tailored to the needs of the particular species concerned. For example, open-water species may require large but less complex environments. Social animals should be housed in groups. Benthic cephalopods are better kept in complex environments with suitable substrates (sand, gravel or pebbles) and dens.

Nautiluses should have access to vertical space for movements and attachment at a variety of levels, thus meeting their natural habit of daily vertical migrations.[176,177] However, not too many vertical attachments should be added, as nautiluses naturally swim up and down whilst circling around the perimeter of tanks and require space to do so. Adding texture (artificial coral reef) to at least one wall of the tank may make it more attractive to the animal (and may promote egg laying; G. Barord, pers. comm.).

In octopuses, interaction with objects is a common form of enrichment and is recommended; providing a den as refuge is not considered to be enrichment, as it is a basic requirement for octopuses, and for all benthic species that use refuges in the wild. Artificial shelters can take the form of many different objects (e.g. bricks, ceramic pots, plastic jars), but dark and opaque dens are preferred over clear ones.

Suggestions for the type of objects (artificial and/or natural) to be added in tanks as enrichment for most common cephalopod species are provided by Grimpe.[56] Recent systematic studies are missing and data available are mostly anedoctical.

Caution should be taken to avoid objects added to holding tanks that could harm or limit full expression of the behavioural repertoire of the animal. Mirrored surfaces should be avoided, since may create agonistic reactions expressed by some individuals towards the 'ghost' reflected image (G. Fiorito, pers. comm.). Accounts of tank design for coastal and reef squid species provide also information on environmental enrichment for these animals.[76,178]

6. Assessment of health and welfare

Annex III, Article 3.1 of the Directive requires that establishments have a strategy to ensure that the state of health of the animals safeguards animal welfare and meets scientific requirements. This should include regular health monitoring and plans for dealing with health breakdowns (see section 7). The starting point for fulfilling these requirements is objective monitoring and recording of the health and welfare of the animals and recognition of the factors likely to cause deviations from optimal status.

The primary factors that could cause a decline in health and welfare and which require monitoring are effects of:

- environmental and housing conditions (including: capture, transport, handling, stocking density, tank design); nutrition; variations in: water temperature, oxygen levels, pH, salinity and water contaminants (sections 4 and 5);
- infectious disease (section 7); and
- experimental (regulated) procedures (section 8).

Irrespective of the cause, objective criteria for assessment of the overall health and welfare status of animals are required to:

(i) ensure that animals arriving in the laboratory are healthy;
(ii) ensure that housing and care are adequate for the maintenance of good health and welfare;
(iii) assess the impact of experimental procedures (section 7) in terms of severity and identification of pre-established humane end-points;
(iv) identify and implement measures to rectify health and welfare problems and enhance the well-being of animals, and refine procedures so that they cause less harm to the animals; and
(v) monitor the efficacy of any therapeutic interventions.

As for other animals, the key parameters used to assess the health and welfare of cephalopods are behaviour and appearance, supplemented in some cases by measurement of a number of physiological 'biomarkers'.

Animals should be inspected immediately on arrival in the laboratory and at least daily thereafter; a consistent method should be used for recording observations, evaluation and actions to be taken modified as required for each species. An example of the types of observation that could be made is shown in Table 5 and provides a starting point for the development of species specific observation sheets. Oestmann et al.[145] recommend that general behaviour, indicative of well-being, in cephalopods is assessed 2–4 times per day; and G. Fiorito (pers. comm.) recommends at least twice a day.

For animals that are being used in an experimental (regulated) procedure (section 8), the observation

Table 5 A-C. Potential *generic indicators* of health and welfare in cephalopods (primarily cuttlefish, squid and octopus) that could be used in daily assessment and adapted for monitoring animals following a procedure (see section 7). For each sign (see section 5 for details and references) a guide is given to show how each can be graded (indicated from green to red) to indicate an escalation of aspects of PSDLH from mild to moderate and severe. The table makes no assumptions about the underlying cause or what the animal is actually experiencing. The table utilises the principles of health and welfare assessment developed by Morton and Griffiths[385,387] and widely adopted for welfare assessment,[485] adapted for cephalopods by Andrews et al.[8] and incorporated into Directive 2010/63/EU severity assessment framework (Expert Working Group 2012: http://goo.gl/DozPKi). It should be appreciated that for practical implementation this table will need to be adapted for each species, validated by research in multiple laboratories and revised accordingly. Note that the table is included here to prompt consideration of the challenges involved in objective assessment of PSDLH in cephalopods and to stimulate research.

Table 5A. Appearance (Physical state) - see text section 6.1.2 for details.

Types of sign	Positive welfare status ('health and good welfare')	Monitor animal with increased frequency of observation depending upon parameter; Additional checks of water quality	Monitor for signs of resolution or increased severity; Seek advice and treat where possible	Requires immediate action (including euthanasia) when observed or at the end of a defined monitoring period
Skin colour *(arms, head and dorsal and ventral surfaces of mantle and arms [suckers])*	Normal skin colour, pattern and reflectance/iridescence, appropriate prompt changes to external stimuli (prey, threat, conspecific).	Occasional inappropriate flashing, wandering clouds, deimantic display in absence of an overt stimulus; transient pallor such as that seen during general anaesthesia (reversible*)*; unusual skin markings or colouration should always be monitored for changes with time.	Frequent abnormal displays; uncoordinated colour changes between arms, head or mantle; some continuously pallid areas or areas with an unchanging colour or pattern (often associated with a swelling or skin lesion).	Entire animal pale and fails to change colour when challenged.
Skin texture *(dorsal and ventral surfaces)*	Skin smooth with a thin mucus layer except when there is a stimulus appropriate display of papillae; no swellings.	Small swelling (relative to size of animal), not in a location that interferes with vision or feeding and with no breach of the skin. Occasional behaviourally unrelated display of papillae.	Continuous display of papillae possibly indicative of an aroused state. Larger swelling suddenly appearing; a small swelling increasing in size; a swelling interfering with vision or ability to feed (buccal area); swelling with signs of infection (e.g. fluid filled cyst); excessive skin mucus production.	Swelling associated with breach of the skin; gas filled swelling likely to interfere with posture.
Skin integrity	Skin intact (no underlying muscle visible) over entire body (dorsal and ventral surfaces).	Small, punctate breaches on arms or mantle (often caudal regions-'butt burn') showing a distinct adherent wound edge indicative of healing; no overt signs of infection.	Larger and more numerous breaches especially with irregular detached edges; small breaches that increase in size or develop a stable colouration distinct from adjacent skin.	Full thickness (muscle visible and possibly penetrated) skin lesions in multiple parts of body (arms and mantle) covering >10% of apparent surface; wound dehiscence following a surgical procedure especially cranial or mantle if liver capsule opened because of gut herniation risk; externalised portion of cuttle bone or gladius.

(continued)

Table 5A. Continued

Types of sign	Positive welfare status ('health and good welfare')	Monitor animal with increased frequency of observation depending upon parameter; Additional checks of water quality	Monitor for signs of resolution or increased severity; Seek advice and treat where possible	Requires immediate action (including euthanasia) when observed or at the end of a defined monitoring period
Abnormal body morphology	Normal positional relationship between arms, head and mantle appropriate to location in tank.	In octopus arms unaligned with mantle during jetting; in cuttlefish pendulous/dangling arms during jetting.	In octopus an arm with a permanent acute angle indicative of a muscle trauma. Dangling pallid arms in cuttlefish.	Tentacles un-retracted in cuttlefish and squid; Mantle deformation in cuttlefish indicative of damaged cuttle bone; dorsal ridge on cuttlefish.
Eyes	Normal prominent position, clear cornea and pupil diameter and orientation appropriate for light level and cranial axis.	Unilateral clouding of cornea; nystagmus.	Eyes sunken indicative of weight loss; pupils incorrectly orientated in relation to head; exophthalmos.	Bilateral clouding of corneas (functionally blind-unresponsive to visual stimuli); fixed constricted or dilated pupils unresponsive ambient light change; absence of consensual pupil response but see section 6.1.2 b.
Number of arms or tentacles	All arms, tentacles and suckers present and intact and no indication of regeneration.	Part or all of one arm missing with signs of wound healing.	Loss of one tentacle club (cuttlefish, squid).	Loss of both tentacle clubs (cuttlefish, squid) and >3 arms in octopus.
Animal found dead		N/A	N/A	If an animal is found dead in the tank especially following a procedure the cause should be investigated immediately (including autopsy) An assessment should be made of the degree of suffering prior to death as this will be required for the report of 'actual severity' of the procedure.

Table 5B. Behaviour (psychological state) - see text section 6.1.1 for details.

Types of sign	Positive welfare status ('health and good welfare')	Monitor animal with increased frequency of observation depending upon parameter; Additional checks of water quality	Monitor for signs of resolution or increased severity; Seek advice and treat where possible	Requires immediate action (including euthanasia) when observed or at the end of a defined monitoring period
Unprovoked behaviours				
Apathetic and/or withdrawn	Animals normally explores tank, is curious about novel objects in tank.	Reluctance to leave den/refuge area; rarely seen exploring tank.	Has not been observed to leave den/refuge on two consecutive days; adopts defensive posture in den (*Octopus*).	Does not leave den/refuge even when challenged.
Abnormal body position in the tank or the water column	Animal able to maintain a position in the tank/water column with ease and to move in relation to a stimulus (e.g. food, light change, conspecific).	Animal continually swimming and appears to experience difficulty in maintaining a stable position in the water.	Squid located near/on floor of tank for extended periods; cuttlefish spending prolonged periods at/near surface; octopus with prolonged periods with part/all of the body out of water.	Squid that do not move from the floor of tank; cuttlefish that do not move from near the water surface; octopus in a fixed location with most or all the body out of water.
Stereotypic behaviour	Normal diversity of behaviour with no indication of repetitive, overtly purposeless activity.	Occasional.	Daily but not continuous.	Present continuously (irrespective of when the animal is observed)
Abnormal motor or locomotor coordination	Locomotion and other motor activity (e.g. prey capture) is precisely coordinated.	Inability to coordinate arms/tentacles during attack on 2 consecutive occasions; inability to maintain a straight line; persistent tremor/twitching in limbs.	Further deterioration or resolution in 48hours; stiff movement; bradykinesia; ataxia.	Convulsions, seizures or extensive muscle spasms.
Cleaning/grooming	Cleaning behaviour is most obvious in octopus that can reach all parts of the body with arms and is a normal period activity. Sucker rings floating in the water may be a surrogate marker for grooming.	Animal spends progressively more time demonstrating grooming behaviour or signs that grooming is reducing leading to a deterioration in skin condition or obvious rings hanging from suckers.	Continues grooming when presented with food or a distraction; mucus accumulation; skin infection or algal deposits may be a marker of significantly reduced grooming.	Absent grooming or continuous grooming as indicated by continuous wiping of mantle by the arms in octopus.
Wound/lesion directed behaviour	The existence of this behaviour is controversial and likely to be most relevant following an invasive procedure (injection site, surgery).	In octopus probes wound occasionally in first 24 hours post procedure; with an arm lesion may examine arm with mouth; in cuttlefish attempts to reach dorsal mantle with an arm.	One or more arms continuously in contact with wound or attempting to reach wound in 24 hours post procedure.	One or more arms continuously in contact with wound in 24 hours post procedure and wound shows signs of dehiscence or infection.
Changes in social interactions with conspecifics	For social species interaction (e.g. display) is a normal behaviour.	Animal becomes withdrawn from the group on occasions and does not always respond to signals from conspecifics.	Animal withdraws for 24 h from all normal social interactions.	Withdrawal from all normal social interactions on consecutive days; Inappropriate and persistent aggression.
Autophagy	Not a normal behaviour so any occurrence of autophagy should be investigated promptly.	Removal of a few suckers or a skin lesion on the arm may indicate incipient autophagy.	Removal of distal 50% of an arm.	Removal of an entire arm.

(continued)

Table 5B. Continued

Types of sign	Positive welfare status ('health and good welfare')	Monitor animal with increased frequency of observation depending upon parameter; Additional checks of water quality	Monitor for signs of resolution or increased severity; Seek advice and treat where possible	Requires immediate action (including euthanasia) when observed or at the end of a defined monitoring period
Feeding				
Changed urge/speed to attack prey and possibly time to subdue live prey	Rapidly approaches and captures/takes food. Attack latency within 1 SD of the normal range established for a given lab/species/prey type; for live prey attack should be coordinated and prey subdued quickly.	Reduced urge; increased time to attack.	Progressive increase in attack latency and uncoordinated attack; misses target.	No desire to attack or unable to subdue live prey.
Provoked behaviours				
Defaecation	Defaecation may be triggered by handling (cf. mammals) but the significance as an index of 'stress' in cephalopods is not known.	***?***	***?***	***?***
Inking	Inking is part of a defensive response but the threshold for induction in relation to stimuli likely to cause PSDLH is unknown. Threshold differs amongst individuals exposed to the same stimulus (e.g. handling).	On consecutive days animal inks when the tank is opened or a human is visible (wearing dark clothing); signs of ink in the tank.	Inking behaviour/signs of ink in tank continues for a third day; signs of ink leaking uncontrollably from ink duct/anal canal (loss of control).	Persistent inking in the absence of an overt stimulus; continuous leakage of ink from ink duct/anal canal.
Response to humans and non-food items placed in the tank (see also withdrawal/apathy above)	Cephalopods are naturally curious and will usually interact with humans without aggression. Objects placed in the tank are usually explored and octopus may use them for den construction.	Fails to respond to presence of human or novel non-food object on consecutive days. Apparent aggressive behaviour indicated by directed squirting at a human; withdraws to den on appearance of human.	Continuation of behaviours for a third day.	Behaviours continue for a fourth day.

Table 5C. Clinical signs (physiological/biochemical state) - see text section 6.1.3 for details.

Types of sign	Positive welfare status ('health and good welfare')	Monitor animal with increased frequency of observation depending upon parameter; Additional checks of water quality	Monitor for signs of resolution or increased severity; Seek advice and treat where possible	Requires immediate action (including euthanasia) when observed or at the end of a defined monitoring period
Digestive *Food intake (criteria critically depend upon species, body weight and age)*	Cephalopods are ready feeders with a relatively high metabolic rate so any reduction in appetite should be monitored carefully.	Fails to take food on two consecutive days (assumes a daily or every two days feeding schedule) or fails to completely eat a normal size meal.	Fails to take any food on 3 consecutive days including an attempt with a novel food or live prey if animals not normally given live prey.	Fails to take any food on 4 consecutive days including when pieces of food are placed in the arms /near mouth.
Faecal output (consider in combination with food intake)	No data on faecal weight/composition and normal frequency of defaecation. Likely to be a large normal range. Faeces may be hard to detect/collect especially fresh.	Reduced; presence of parasites or cysts; cytological markers of epithelial damage.	Very reduced.	Absent (if animal has fed recently or is still feeding may indicate gut obstruction).
Vomiting/regurgitation	Not normally present so any occurrences should be a cause for concern but controversy over existence.	Rare.	Often occurs following ingestion of food.	Always occurs following ingestion of food.
Rates *Ventilation*	Ventilation is normally regular, clearly inflates the mantle (depth), has a steady frequency and is coordinated with siphon opening and closing. Frequency can be measured and depth estimated in both conscious and anesthetised animals by an observer.	Small rate change (increase or decrease), but remains coordinated; no indication of laboured breathing.	Sustained rate change; periods of tachypnoea /apnoea/dyspnoea/hyperpnoea; uneven depth (augmented breaths).	Slow, shallow, poorly coordinated (mantle/siphon); frequent periods of apnoea/dyspnoea/hyperpnoea.
Heart rate (bradycardia and tachycardia)	Heart rate appropriate for behaviour but may be affected by drug treatments including anaesthesia. Currently no telemetric methods available for HR monitoring in unrestrained conscious animals but can be monitored in anaesthetised animals to check physiological status.	**?**	**?**	**?**
Blood biomarkers *Increased concentration of catecholamines and phagocyte number/type*	Utility requires 'normal' values to be established utilising methods which do not result in changes and which allow good temporal resolution.	Transient increase in catecholamine (<24hours) indicates exposure to a mild stressor.	Large increase in phagocytes maintained for 48 h indicates an infection/illness/sustained stressor exposure.	Increase in phagocytes unresponsive to treatment and accompanied by other signs of an infection/illness.

(continued)

Table 5C. Continued

Types of sign	Positive welfare status ('health and good welfare')	Monitor animal with increased frequency of observation depending upon parameter; Additional checks of water quality	Monitor for signs of resolution or increased severity; Seek advice and treat where possible	Requires immediate action (including euthanasia) when observed or at the end of a defined monitoring period
Body weight *Reduction in body weight (or other external morphological indicator) over specific time periods*	Maintenance of normal growth depending on food availability, season, species, age, reproductive status.	Reduced rate of growth.	10% loss of body weight over 1 week?	20% over 1 week?
Free observations *Observations of other behaviour not anticipated in the checklist and which may have a negative impact upon welfare.*				

frequency may be increased depending upon the nature of the procedure and its anticipated impacts on animal welfare. Factors to be considered are described in detail below, but most require further validatation, including consideration of their severity (see section 2.2.3 and 8.2).

It should be noted that prompt identification of problems is essential so that *i*. action can be taken to reduce and preferably eliminate any suffering and *ii*. in the case of procedures, to ensure that humane end-points are promptly implemented where appropriate and that severity limits are not exceeded (sections 2.2.3 and 8).

6.1 Objective assessment of health and welfare

Proposed parameters are described in detail below and summarised in Table 5. Welfare assessments should be performed in the animals' home tank and without removal of the animal from the water wherever possible. Each element of the assessment will ideally require some form of quantification to enable recognition of points at which particular parameters reach a pre-set humane end-point and to enable actual severity of a procedure to be reported.

Observation and evaluation of the following criteria can help to determine whether 'something is wrong' with the animals, and, considering the overall pattern of observations, can help decide strategies for rectifying any health and welfare problems.

6.1.1 Observation of spontaneous and provoked animal behaviour

a) *Feeding*. The common laboratory species of cephalopod have voracious appetites and eat relatively large amounts of food in relation to body weight, reflecting a relatively high metabolic rate.
Therefore, altered feeding behaviour manifests as a reluctance to take food or an increase in the time to attack or subdue live prey, and is usually the first and most apparent behavioural indicator that there could be a health or welfare problem. For example, in octopuses fed on crabs or mussels, the tank should be checked for empty carapaces and shells to ensure the animals are ingesting prey and not just attacking them. In *O. vulgaris* the willingness to attack can be tested equally well with an artificial crab as with a live crab.[179]
b) *Location in the tank and water column*. Each species normally locates in a characteristic place in the tank and water column.
Nautiluses spend much of their time attached to the sides of tanks, and undergo daily vertical migrations

(see above), so animals spending large amounts of time at the bottom of the tank other than when engaged in feeding are exhibiting abnormal behaviour.

Cuttlefish alternate between hovering/swimming in the water column and resting on the bottom, partially covered by the substrate, and an animal spending a considerable period of time at or near the surface of the tank should be inspected closely for signs of physical damage to the mantle. Such changes have been observed following a pharmacological treatment, as described by Agin et al.,[180] as a consequence of cycloheximide injections.

Squid rarely rest on the bottom of the tank, so their presence there for an extended period should be regarded as abnormal, as should extended periods spent at the surface.

For octopuses, a 'problem' in the tank may be indicated if the animals spend excessive time clinging to the lid of the tank with most of the body out of the water.

c) *Swimming and locomotor activity and coordination*. Each species has a characteristic method of moving in the tank: by walking, swimming or a combination of both.[31] Repetitive locomotion in cephalopods, such as jetting backwards continuously, or in the case of octopuses performing swimming motions while attached to a tank wall, can be a sign of stress. Any abnormalities of coordination should be noted. For example, a defect in the statocyst leads to an inability to control orientation during swimming. This 'spinner' behaviour has been reported in species of cuttlefish, squid and octopus.[181] Although there are likely to be minute-to-minute changes in the level of locomotor activity (see e.g. Figure 1 in Boyle[182]), each species has its own overall daily pattern of activity cued by the photoperiod.[98,100,130,183–186] Rest/sleep-like–activity cycles are documented in *S. officinalis*,[102] *O. vulgaris*[101] and *Octopus macropus*;[103] nocturnal vertical migration is known to occur in *N. pompilius*.[29,177] Changes could be an indication that 'something is wrong'.

d) *Use of arms and tentacles*. The behavioural repertoire of arm movements in cephalopod species is reviewed and described in Borrelli et al.[31] A taxonomy of arm movements for octopuses is provided by Mather.[187] An animal spending an extended amount of time with the arms curled over the body (a defensive posture), either in the den or the corner of the tank, should be monitored for other indications of distress. In *Nautilus* withdrawal of all tentacles into the shell with the opening obstructed by the hood is a defensive behaviour.[188] There are scattered reports of octopus using an arm to 'guard' an injured part of the body (I. Gleadall personal observation cited in;[8] reports of animals 'guarding' the mantle or cranium post-surgery by G. Fiorito, unpublished data, and also[189]), but this behaviour has not been systematically investigated and could also be linked to facilitation of healing by secreted antimicrobial peptides. Wound-directed behaviour was not observed in a study of tentacle amputation in the squid *Loligo pealeii*,[9] although in two species of cuttlefish (*S. officinalis* and *S. pharaonis*), the use of a partially (80–90%) amputated arm for prey manipulation and body posturing was avoided for up to 3 days post lesion.[190]

In cuttlefish, reaching over the dorsal mantle ('scratching-like' behaviour) has been observed a few days after a transient rise in ammonia concentration leading to skin damage.[145] Tentacles in cuttlefish and squid should be retracted except when the animal is engaged in an attack.

The arms are used for skin cleaning in octopus; excessive cleaning activity and/or frequent presence of sucker-rings in the tank could be indicative of abnormality. In all cephalopods, a loss of adhesion in the suckers should be a cause for concern.

e) *Interactions with humans and conspecifics*. While cephalopods, in general, are very responsive to any novel features introduced in their tank, octopuses are especially curious about their environment. Healthy octopuses acclimatised to laboratory housing will often leave their den when the tank is inspected and will interact with a hand placed below the surface of the water (for an historical account see[191]).[36,192] Reluctance to interact with humans should be a cause for concern. It should also be noted that there is some evidence that at least one species of octopus (*E. dofleini*) may recognise individual humans;[193] therefore, care should be taken to ensure that staff who are involved in any procedure likely to be aversive should not be involved in routine feeding or inspection as there is a possibility of inducing a conditioned aversion/avoidance.

If visually exposed to conspecifics, octopuses may alter their predatory response due to agonistic interactions, but habituation resulting in a resumption of normal behaviour has been observed under controlled laboratory conditions.[194,195] Squid and cuttlefish are social species and changes in social interactions with conspecifics again may indicate a welfare/health problem.

f) *Squirting, inking, defaecation and regurgitation*.

Squirting: all cephalopods use expulsion of water from the mantle via the siphon in breathing and locomotion; and this is particularly noticeable in the jetting escape reaction. Squid and cuttlefish

may direct jets of water at a person attempting to capture them, and this behaviour is particularly notable in *O. vulgaris* where jetting is also a component of the deimatic display.[31,134] Water jets directed at an observer is indicative of a moderate aversive reaction; in some cases this can also be a sign of 'recognition'.[195]

Inking: is a defensive response in cephalopods (apart from nautiloids which do not have an ink sac), so inking should always be taken as an indication that the animal perceives a threat or is stressed. However, there is individual variability in the threshold for induction of inking as some *O. vulgaris* will ink profusely in response to handling (M.G. Valentino and P. Andrews, unpublished data) that does not evoke the same response in other individuals (G. Fiorito, pers. comm.). In addition, inking does not necessarily result from the animal receiving a presumed noxious stimulus, such as an electric shock (I. Gleadall personal observation cited in;[8] G. Fiorito, pers. comm.). Therefore, absence of inking should not be interpreted as an absence of anxiety or distress. A continuous trickle of ink from the animal should be investigated as it may indicate a problem with the neural control mechanism or with ink duct sphincter competence. Animals should not be allowed to remain in a closed tank in which inking has occurred, and care should be taken to ensure that ink does not enter other tanks as it is an alarm signal.[196] Intramantle inking has been reported as a post-transport stress behaviour in *Octopus bimaculoides*.[197]

Defaecation: there is insufficient knowledge of defaecation patterns and their control in cephalopods to determing whether any changes may be linked to pain, anxiety or stress, as is the case in many vertebrates. Although faecal ropes may emerge in octopus exposed to general anaesthetics, this could be due to loss of anal sphincter control. The production of faecal ropes in octopus is a useful indicator of normal digestive tract functioning in a feeding animal, but it is not known whether disease can alter the faecal fluid content or defaecation frequency (constipation/diarrhoea). However, chemical and cytological examination of fresh faecal samples can provide important insights into the health of the animal and as collecting faecal samples is non-invasive, its utility in cephalopod health monitoring should be explored.

Regurgitation: there are two isolated observational reports of regurgitation/vomiting of upper digestive tract contents one in *E. dofleini* (I. Gleadall personal observation cited in[8]) and the other in *Sepioteuthis sepioidea*.[198] The location of the beak within the crown of arms would make this behaviour very difficult to detect. However, if the ability to regurgitate/vomit upper digestive tract contents is confirmed then it should be added to the list of possible indicators of illness, as is the case in vertebrates.[199]

6.1.2 Appearance

a) *Skin colour, pattern and texture*. Skin colour and pattern are primarily regulated by motorneurones from the suboesophageal chromatophore lobes of the brain, with contributions from reflecting cells, depending upon the location on the body and the species.[134,200–202]
   At the time of writing, there is no evidence to show that changes in the colour or pattern of the skin in any cephalopod species are specifically associated with changes in health or welfare of the animals. Oestmann and coworkers[145] caution that normally functioning chromatophores and iridiocytes may mask underlying skin defects. In *Nautilus* discoloration of the mantle (with loss of buoyancy) is a sign of poor health.[203] However, loss of ability to match substrate or background (see e.g.[204–207]) or sustained pallor with loss of normal patterning should be taken as an indication of a problem, as should excessive or inappropriate flashing in squid[208] and wandering clouds in coleoid species.[209] Note should also be taken of colour changes in response to a provocative stimulus, such as the deimantic display often observed in response to a perceived threat. Skin texture in octopuses and cuttlefish can be changed by the formation of papillae, particularly prominent above the eyes and on the mantle and is indicative of an aroused or vigilant animal.[31]
b) *Skin and external shell integrity*. Any breach to the skin of a cephalopod is potentially problematic because of the possibility of bacterial infection (section 7.2.2) causing systemic sepsis, preventing healing, and local inflammation causing hyperalgesia. Bleeding from wounds may not be readily apparent as, although oxygenated haemolymph is pale blue (extracellular haemocyanin), it will be rapidly diluted in the tank and deoxygenated haemolymph is colourless.
   Healing of small wounds in octopuses, such as transection of the distal 10% of the length of the arm, appears to be rapid, with the exposed area in some animals being almost completely covered by skin in about 24 hours (T. Shaw and P. Andrews, unpublished data), but larger wounds and particularly those to the mantle appear to take longer to heal even without infection.[210] Damage to the skin most frequently occurs at the distal part of the mantle in

animals (particularly squid and cuttlefish) that frequently impact the wall of the tank ('butt-burn': J. Rundle, pers. comm.), with four impacts per hour being recorded in a study investigating the long-term health of cultivated cuttlefish in soft-sided tanks.[211] In cuttlefish and octopus, the ventral surface of the mantle contacting the substrate should also be examined, and anecdotal evidence that ill or senescent octopuses avoid rough substrates may indicate that the skin in this region is particularly sensitive to damage. Animals showing signs of healed skin damage should be inspected closely to ensure that it is healing, as death may ensue rapidly if the lesion increases in size and penetrates the underlying muscle.[212]

Breaks in the shell of nautiloids may compromise their buoyancy mechanism and so require treatment.

The cause of any breach in skin or shell occurring after an animal's arrival in the laboratory should be identified and action taken to prevent recurrence (e.g. carefully examine the tank and items in it for sharp edges, deep clean and disinfect the tanks, see section 5.6).

c) *Eyes*. The eyes should be inspected to ensure that the cornea and lens are transparent, as opacity is one of the signs of natural senescence (see below). Pupil diameter should decrease over a few seconds in response to a sudden increase in illumination (for *Nautilus* see;[213] for cuttlefish and octopus;[214] for squid[215]), although there is some evidence (*S. officinalis* and *E. cirrhosa*) that the response is not consensual.[214] The classic contributions of Beer[216] and Magnus[217] should be also considered in this framework. The slit-like pupil remains close to horizontal irrespective of the position of the body, and this is particularly noticeable in octopus.[150] Both the pupil diameter and statocyst–ocular responses (nystagmus) are mediated by the brain and hence give an insight into central nervous system functionality.[181,218–220]

d) *Body posture*. Two aspects need to be considered: *i.* the relationship between the mantle, head and arms/tentacles (i.e. the overall appearance of the animal), and *ii.* the orientation of the animal in relation to the floor and sides of the tank.

In *Nautilus* the shell should be vertical; however, air bubbles can become trapped in the eyes and under the hood, leading to adverse health effects.[221,222] Information on treatments related to poor health conditions is available in Barord et al.[223] Trapped air can be released by slowly turning the animal, laterally from side to side.

All cephalopods have well-developed statocyst systems to maintain body posture and coordinate body position with eye orientation[224] so an abnormal body posture may indicate a nervous system problem or a physical defect that the animal cannot compensate for (e.g. a broken cuttlebone, a fluid-filled chamber in *Nautilus*, gas trapped in the distal mantle of octopus). Damage to arms can also affect posture as Tressler et al.[190] reported unbalanced swimming (body axis tilted to the lesioned side) lasting up to 3 days in cuttlefish in which 80–90% of the length of third right arm was removed. The head in octopuses is particularly mobile and a raised head, particularly if moving from side to side or bobbing has been regarded as sign of 'agitation' in *O. vulgaris* by Boyle.[182] However, head bobbing and similar behavioural patterns are indicative of increased arousal, as reviewed by Borrelli and coworkers.[31]

6.1.3 Biomarkers

a) *Body weight*. The optimum frequency with which animals can be handled for routine weighing, taking into account that anaesthesia may be necessary, is not known. It has been suggested[225] that frequent handling may impede growth, but this requires systematic investigation. Cephalopods, particularly when young, increase body weight daily (assuming sufficient food) so failure to increase weight or a loss of weight following an experimental procedure may be the earliest objective measurement of declining health or welfare, but the potential additional harms of frequent weighing will need to be assessed.

Dorsal mantle length (DML) is also frequently used as an index of body size in cephalopods although the relationship to body weight (TBW) is not linear. The *K*-Fulton condition index, which combines length and body weight measurement and is used in fish, has been adapted for cephalopods ($K = (\mathrm{TBW}/\mathrm{DML}^3) \times 100$).[226] In *O. vulgaris* infected with *Aggregata octopiana,* the *K*-Fulton condition index decreased as the sporocyst counts in the caecum increased.[226] Consideration should be given to using this index as part of routine growth and health and welfare monitoring in cephalopods, as applied to many species of fish,[227–233] and in other circumstances to other vertebrates (see, e.g.[234,235]).

In stock animals, weekly measurement of body weight may also be a useful index of health and welfare status, providing this can be done with minimal distress to the animal (e.g. in seawater), but the percentage loss of weight over time that is indicative of illness is not known. The digestive gland has a

lipid reserve;[236] Mangold and Bidder estimated as 9–13% of digestive gland weight in *S. officinalis*.[148] However, the impact of food deprivation on this is not known.

Growth data based upon body weight are species and laboratory specific with the latter depending upon food type and feeding frequency, water temperature, stocking density, animal age, activity level (influenced by tank size and photoperiod) and parasite load. There are limited growth curve data for representative cephalopod species; for example for: *Nautilus*,[237] cuttlefish and squid,[238] and octopus.[225,239]

b) *Ventilation (breathing) frequency*. Ventilation frequency can be monitored by an observer provided that the animal is not disturbed, but a video system may provide an alternative in the absence of other non-invasive methodology, as standardised by Borrelli.[26] Although an increase in ventilation frequency may be an indication of physiological stress (e.g. particularly a fall in inspired pO_2[84,240,241]), it is also indicative of arousal to innocuous stimuli and ventilation frequency also correlates positively with activity levels, as for *S. officinalis* and *O. vulgaris*.[182,242] Observation of breathing should also note whether the pattern is even or is interspersed with periods of apnoea/tachypnoea.

Although frequency is relatively easy to monitor,[26] some assessment should also be made of depth (mantle stroke volume) as again excessively deep, shallow or laboured breathing may also indicate a problem and Smith et al. comment that maintenance of oxygen uptake (in *O. vulgaris*) relies more on stroke volume than increasing ventilation frequency.[240]

Deep/forceful breathing may manifest as currents in the water or ripples in the surface if the animal is close. In the coleoids, the way in which the mantle distends during inspiration should be noted to ensure it is bilaterally symmetrical, that the entire mantle is involved, and inspiration and expiration are coordinated with the closing and opening of the siphon respectively. During general anaesthesia, ventilation frequency, depth and coordination all become suppressed.[243] Therefore, similar changes in a non-anaesthetised animal are likely to reflect depression of brain drive and should be investigated immediately.

Stress state in *Nautilus* is usually expressed as a 'rocking behaviour' (which reflects hyperventilation), with the animal clearly rocking from front to back.

c) *Cardiovascular parameters*. Currently there is no established non-invasive method for routine measurement of heart rate or blood pressure in an unanaesthetised cephalopod (but see[244,245]). In addition, studies of animals with indwelling catheters show that both parameters are very labile[150] suggesting that, even with appropriate methodology, they may not be helpful as indices of health or welfare. In particularly compliant individual *O. vulgaris*, it is possible to observe the beating of the systemic and branchial hearts in the mantle without anaesthesia, and to use Doppler ultrasound to investigate cardiac function and image the viscera (G. Ponte, pers. comm.). The resolution achieved is much improved from previous attempts with cephalopods.[244,246–248] This potentially represents a revolution for future physiological studies with these animals (D. Fuchs and G. Ponte, unpublished data; Vevo 2100 Visualsonics, The Netherlands). Such methodology may be suitable for detailed investigation of animals showing signs of illness and could be useful for monitoring some physiological functions during general anaesthesia.

d) *Other biomarkers: analysis of blood*. There are no validated blood biomarkers indicative of the health or welfare status of a cephalopod, and the development of such markers is hampered by difficulty in obtaining blood samples using minimally invasive techniques comparable to those available for vertebrates (but see also Table 8). Descriptions of blood sampling in the literature employ some form of general anaesthesia as, for example, done in the bobtail squid *E. scolopes*.[249] In animals sedated for investigation using a low concentration of a general anaesthetic (see section 8.5.5), blood sampling and analysis may be a helpful aid to diagnosis and treatment but the relative harms and benefits of undertaking this procedure solely for welfare assessment need to be considered.

The following parameters should be considered.

Haemocytes. Blood samples enable culture for bacteria and examination of smears by electron microscopy for viruses. The utility of haemocyte counts and morphology in general and phagocytes specifically as indicators of infection or stress is limited because sampling methods (especially repeated sampling involving anaesthesia) themselves seem to increase haemocyte concentration,[250,251] although the concentration is increased further by bacterial infection the effect is transient (i.e. present at 4 but not 24 hours in *E. cirrhosa*).[126] The increase in haemocyte counts in response to intramuscular injection of *Escherichia coli* lipopolysaccharides in *O. vulgaris* begins within 4 hours of injection and is returning to control levels by 24 hours.[251] Vehicle injection (phosphate buffered saline) produced a smaller increase in

haemocytes indicating that a rise in haemocytes may be a useful indicator of generalised stress as well as of infection.

Chemistry. Levels of the respiratory pigment haemocyanin can be measured by the haemolymph copper concentration.[252] Routine measurements of the common inorganic ions (e.g. Na^+, K^+, Ca^{++}, Mg^{++}, Cl^- and SO_4^{++}) and protein[253–257] would be helpful in establishing their utility as parameters for diagnosing disease. For example, in *O. vulgaris* a decrease in most inorganic elements and/or in haemocyte concentrations is observed when infection by the gastrointestinal parasite *A. octopiana* increases.[258] Finally, measuring activity levels of respiratory enzymes and total protein concentration may provide additional information on the health status of an animal.[259,260]

Humoral agents. Plasma noradrenaline and dopamine increase transiently (5 min) in response to stress induced by air exposure in *E. cirrhosa*.[255] However, it is difficult to envisage how such transient changes could be applied in routine health monitoring. Circulating levels of a number of hormones may give insights into health (e.g. steroids) and reproductive status, but normal ranges need to be established before these could be useful for health and welfare monitoring. Other molecules that may be of relevance for health monitoring include complement system molecules, anti-microbial peptides (AMPs) and other innate immunity-related proteins released by haemocytes, as they increase rapidly in infectious disease.[261–266]

e) *Other biomarkers: analysis of faeces*. One of the most promising methods to evaluate the physiological conditions of animals in laboratory settings is through the examination of faeces. Samples may be utilised to estimate various biomarkers including steroids (e.g. corticosteroids, estrogens, testosterone[267]), as well as to evaluate, for example, the digestibility of alternative diets as in the case of fish,[267–270] or to identify possible parasite infections or cytological indicators of intestinal damage.[271]

We recommend the development of faecal analysis methods to assist in evaluation of health and welfare of cephalopods in laboratory settings.

6.2 Health and welfare of ageing cephalopods: a special case?

It is very difficult to determine the age of living cephalopods. Age is a parameter that is known for almost all other species used in research and should be included in the methods section of published papers (see ARRIVE Guidelines[272]), but which is rarely known in studies of cephalopods, unless they are laboratory reared.

Within a wild-caught population of a particular cephalopod species, in a circumscribed location and time of year, cephalopods of higher body weight are likely to be older, but the relationship between body weight and age is not linear, particularly in octopuses.[238,273,274] The absence of precise age data complicates experimental design.

While there are variations due to the ecological niche of individual species, cephalopods generally live for about a year. With the exception of nautiloids, cephalopods undergo an exponential early growth phase during which they mature to adult size rapidly. However, this growth phase can be influenced by many factors, such as temperature, food availability and space, which makes body size a poor indicator of an animal's age.[238,274–276] The age of sexual maturity is variable and also appears to depend on the ecological niche of the species. As reviewed by Rocha et al., some cephalopods (e.g. *Loligo opalescens* among squid and *O. vulgaris* among octopods) are semelparous (i.e. breed and then die soon after) while others (e.g. *Nautilus* sp., *S. officinalis* among cuttlefishes, *L. vulgaris* among squid and *Octopus chierchiae* among octopods) are iteroparous (i.e. breed multiple times, generally with longer lifespan).[277] For a summary of reproductive strategies of some cephalopods species refer to Appendix 3.

In light of these considerations, ageing is relevant in the context of physical senescence (i.e. ageing changes in animals over time/after breeding, especially in females, once their eggs have hatched), but also when experimental procedures are applied to animals and age could influence the results.

Possible signs of cephalopods in senescence include reduced/absent drive to eat, poor skin quality, cloudy eyes, and changed activity pattern and behaviour.[278–280] It may be difficult to distinguish this state from an animal that is showing similar signs due to disease. Good record keeping of time kept in the laboratory and age whenever possible, alongside general health records of individuals may help to differentiate the two situations.

It is unknown whether cephalopods experience any form of pain or suffering during senescence, but the precautionary principle should be applied when determining humane end-points (see section 8.3) for studies involving senescent cephalopods.

The senescent state makes animals more susceptible to a number of problems which, if they occurred in non-senescent animals, would be regarded as indicators of illness.[145,278,280] These include: skin breaches

including ulceration; primary and secondary cutaneous (e.g. *Aeromonas* sp., *Vibrio* sp. and *Staphylococcus* sp) and systemic (e.g. *Flexobacter*, *Vibrio*) bacterial or fungal (e.g. *Labyrinthula* sp., *Cladosporium* sp.) infections; increased parasite load.[281,282]

The senescent animals are not only more susceptible to infections, particularly of skin,[283] but they also appear to have a reduced ability to recover once infected.

In general, animals showing signs of senescence should be humanely killed, unless there is sound scientific or animal welfare justification for keeping them alive.

A discussion of ethical aspects of both caring for and using senescent cephalopods in research is available in Smith et al.[3]

Careful routine monitoring of the physical condition of captive cephalopods at all life stages is essential for their proper care.

6.3 Post-mortem evaluation

Post-mortem evaluation of cephalopods is often a neglected aspect of health and welfare monitoring. It enables thorough inspection, revealing abnormalities not readily visible when the animal was living, the cause of death can be confirmed or ascertained, histological samples collected, and a database of findings can be gathered to support future post mortem evaluations.

The overall aim of such detailed evaluation is to facilitate better health and welfare assessments, and implementation of humane end-points, in future studies. Tissues such as the beak, statoliths and vestigial shells can also be collected, which may provide information on the age of the animal (for example for: *O. vulgaris*;[273,284] *Sepioteuthis lessoniana*;[285,286] other cephalopod species[287–300]).

Cephalopod tissues are rich in protease enzymes, which cause rapid tissue autolysis *post mortem*.[301,302] Autopsies should be performed immediately after humane killing an animal, for example, on welfare grounds when humane end-points have been reached, or at the end of a novel procedure and/or when the cause of welfare effects is uncertain; or as soon as an animal is found dead (see section 8.11), but only once death is confirmed (see section 8.12).

Cephalopods do not exhibit *post mortem* rigidity so *rigor mortis* cannot be used to confirm death, and other methods need to be employed (see section 8.12).

Autopsy findings should be reported in the first instance to the person responsible for overseeing the welfare and care of the animals, and any actions needed to safeguard animal welfare in future should be agreed, recorded and implemented.

Information on likely cause of death may be required for consideration by the local Animal Welfare Body or the National Competent Authority, especially if there is unexpected mortality following a procedure but note that mortality should never be used as an end-point for a procedure.

Steps to be considered for inclusion in *post mortem* evaluations include:

Haemolymph sampling: when animals are humanely killed for welfare reasons, it will be possible to collect haemolymph immediately surgical anaesthesia is achieved, but before death ensues (see section 8.5.5 for techniques). A bacterial septicaemia is suspected when the haemocytes have aggregated into visible clumps. Systemic bacterial infections should be confirmed by bacterial culture of the haemolymph.

Skin examination: external lesions should be blotted to remove excess mucus, then, aseptically, samples obtained using swabs and submitted for bacterial culture. Smears obtained from the swab or skin scrapings should be air dried and stained for bacteria or fungi.

Anatomical examination: descriptions of the gross internal anatomy of the main cephalopod species can be found in the following classic references: *N. pompilius*;[303] *S. officinalis*;[304] *L. vulgaris*;[305] *O. vulgaris*;[306] *E. cirrhosa*.[307]

In brief, following a detailed external inspection including skin breaches, abnormal colouration, damage to appendages and deformities the mantle cavity is opened by an incision, following the anatomical approach that gives best accessibility in each species.

The viscera are examined visually and particular note taken of the state of the hearts, gills and the digestive gland (hepatopancreas), which is the largest and the main metabolic organ. Organs should be inspected for abnormal colour (particularly hepatopancreas), shape, size, texture (e.g. oedema, hard lump caused by a cyst or tumour), and presence of parasites (particularly intestine) or foreign bodies.

The presence, or not, of food (digested/undigested) in the crop, stomach and caecum/intestine should be noted as well as faecal ropes in the rectum to assess gastrointestinal tract functionality. Digestive tract samples should be analysed for the presence of parasites.

The degree of filling of the ink sac should be noted as an empty one may indicate that the animal has inked profusely in the tank prior to death, which might not otherwise be apparent if the animal was found dead in a tank with circulating seawater.

Haemorrhage is impossible to detect as the blood is colourless when deoxygenated, and oedema may be hard to detect without histology.

Tissue samples can be fixed by immersion in neutral-buffered 10% formalin, and standard histo-

pathological techniques applied although fixation in buffered glutaraldehyde will be required for ultrastructural studies and for identification of viruses.

Creation of a repository of data and/or reports on cephalopod pathology would provide an important resource in the effort to ensure good health and welfare in captive cephalopods used in laboratories. This is a current project of the non-profit Association for Cephalopod Research (see www.cephalopodresearch.org/projects), which is also included as goal of the COST Action FA1301 (Cephs*In*Action; http://www.cost.eu/COST_Actions/fa/Actions/FA1301; www.cephsinaction.org).

7. Disease: causes, prevention and treatment

The major known risks to health and welfare in cephalopods are environmental parameters, and especially water quality issues, physical injury and infection (especially due to parasites), all of which may be interrelated. In addition, the psychological well-being of the animals should be considered (for a preliminary discussion see:[308,309]), as presented in section 6.

7.1 Introduction to the issues related to diseases of cephalopods

7.1.1 Environmental influences on disease. Host-pathogen interactions can be strongly influenced by the environment in which an animal lives. In addition, stressful conditions deriving from inadequate physico-chemical parameters (e.g. increased or decreased temperature, presence of undesirable chemicals, low oxygen saturation) may result in impaired defense responses against pathogens (review in[310]), thus increasing the probability of a disease outbreak.

Maintenance of water quality is essential for minimising infectious diseases and tank design (including enrichment) is important for avoiding self-induced physical trauma (e.g. 'butt-burn', see 6.1.2b above) and reducing general 'stress'.

Close attention to these factors should make animals more resistant to infection as well as improving psychological well-being.

7.1.2 Effects of physical trauma. Injuries may be inflicted by some methods of capture (e.g. tentacle loss by squid jig;[311] see also[312]) and handling without appropriate care (e.g. skin damage by net reported in[313]), or during transport as, for example, for *O. vulgaris* that may fight if transported together and eventually bite or cannibalise each other (see description of fighting in[31]).

Skin damage makes the animal susceptible to secondary infections (particularly bacterial) which can be fatal if untreated.[314]

7.1.3 Disease caused by feeding live food. Although feeding with live food may be preferred to an artificial diet (see section 4.2), it is important to avoid the use of species that are recognised for their role as hosts of important pathogenetic parasites. For example, coccidian *Aggregata* sp.[258,315,316] (for review see[73]) or viruses[317–319] may infect cephalopods through food items such as crustaceans. In the case of the use of crabs as a prey item, special attention should be given to distinguish between those carrying parasites and to remove them from cephalopod facilities.

It is also noteworthy that penetrative injury to the brain has been reported to occur in *O. vulgaris*[320] due to the setae of ingested polychaete scaleworms (*Herminone hystrix*) so their presence in laboratory tanks should also be avoided.

See also section 7.2 below for discussion on infectious diseases of cephalopods.

7.1.4 Action points when disease is suspected. Animals showing signs of illness should be placed in quarantine (see section 3.6) to reduce the potential spread of the causal agent, and any animals found dead removed and autopsied (see section 6.3). Water from tanks of quarantined animals should not contaminate water supplies to other animals or the environment, and personnel handling potentially infectious animals should wear protective clothing (see section 9). Seriously ill animals not responding to treatment (where treatment is possible) should be humanely killed (see section 8.11) and autopsied immediately (see section 6.3).

Investigation of disease outbreaks should not only include identification of the immediate cause (e.g. infectious agent), but also underlying origin such as adverse water quality, contamination of food or effects caused by other animals. Failure to correct such factors will often result in further outbreak of disease.

Careful records must be kept of all occurrences of illness or mortality irrespective of identification of cause, so that patterns can be identified over time (e.g. there might be higher mortality/infection rates from certain suppliers).

7.2 Infectious agents in cephalopods

Immunity in cephalopods differs from vertebrates due to the absence of an adaptive immune response.[321,322] However, these animals do have an innate (non-specific) immune response, mediated by both humoral (e.g. haemagglutinin) and cellular (haemocyte) mechanisms.[73,252,323–325]

As in other molluscs, circulating haemocytes are responsible for infiltration, aggregation, encapsulation, cytotoxic reactions and phagocytosis of foreign particles. Cowden and Curtis estimated that the phagocytic capacity of octopus haemocytes was low;[326] while high phagocytosis of carbon particles has been described in *E. cirrhosa*.[327] Phagocytic capacities of the haemocytes of the common octopus, *O. vulgaris,* challenged *in vitro* using zymosan as a test particle,[325] and those of the haemocytes of *E. dofleini* (see citations in[328]) have been reported. Recently, an extensive analysis of octopus haemocytes at morphological, flow cytometry and functional level (including phagocytic capability as well as reactive oxygen species (ROS) and nitric oxide production) after challenging with different stimuli has has been carried out by C. Gestal and coworkers.[73,329] In addition, several biologically active molecules likely to be involved in responses to infection and injury are known to be present in the haemolymph of cephalopods, such as lectins, proteinases, including antiprotease and lysozyme activities.[126,266,330–332]

The immunobiological system in cephalopods is quite effective, as reflected by the scarce reporting of illness in captivity for this class over many years, but this low incidence could also reflect under-diagnosis, particularly of systemic disease that may not have an external manifestation, or under-reporting.

7.2.1 Viruses. Viruses are the most abundant component of aquatic microbial communities (for review refer to[333–335]). However, there are few records of virus-inducing pathologies in cephalopods as reviewed in Hanlon and Forsythe.[336] The first known evidence of viral infections in cephalopods was provided in specimens of *O. vulgaris* and in the cuttlefish *S. officinalis*.[337,338] In the octopus, oedematous, modular tumors embedded in arm musculature and tissue degeneration were observed in animals showing anorexia, apathy and often autophagy.[337,339] The lesions were linked to the presence of viral particles suggested to belong to the group of iridovirus, according to their size, morphology and location.[340] In *S. officinalis,* virus-like particles were identified in the gastric epithelium and described as similar to reoviruses of vertebrates, but details on the symptoms induced are not provided.[338] Virus-like particles have been also reported in the epithelial cells of the tubules of the digestive gland of *Loligo pealei*, and in the renal appendages of several octopod species.[336]

More recently, Gregory and coworkers[341] reported another possible infection of iridovirus in cephalopods (i.e. *Nautilus* sp.). Intracytoplasmic inclusion bodies were observed in tissues from an animal found dead in aquarium without premonitory signs of disease.

Furthermore, *Todarodes pacificus* (utilised as a food item in aquaculture) have been reported to be positive for *Betanodavirus*,[342] which is the aetiologic agent of a serious viral disease known as VER (viral encephalopathy and retinopathy) that has been detected in a wide range of vertebrate and invertebrate hosts worldwide and caused severe mass mortalities in both farmed and wild marine organisms.[343] Betanodavirus was also identified in skin lesions, in the eye and in the branchial heart of *O. vulgaris*.[344] Squid have been suggested also as possible vectors of zoonotic viral agents such as *Norovirus*.[345–347]

Infections from viruses may sometimes be asymptomatic (see examples from fishes:[348,349]) but pathogenicity may be higher if temperature increase, as in the case of global environmental changes.[350] In fishes, symptoms of viral infection may include clouding of the eye, anorexia, changes in body colour and uncoordinated swimming.

7.2.2 Bacteria. In cephalopods, pathogenic bacterial infections are caused by several microbes; for an overview see Table 6. These include various species of Gram-negative *Vibrio* (review in[336,351]). However, *Vibrio* bacteria can also be symbiotic, as for the case of the Hawaiian bobtail squid (*E. scolopes*) where *Vibrio fischeri* is a mutualist in the light organ,[352,353] as well as *Psedomonas* sp. and other bacteria that are symbionts in *Nautilus* sp.[354]

Secondary bacterial infections in skin lesions have been reported in squid,[355] cuttlefish[356] and octopus,[357] and skin lesions are considered to be the most common conditions in which infections occur.[351] Bacterial infections may spread to conspecifics sharing the tank.[77] In addition, bacteria may cause skin ulcers on mantle, head and arms, hyperplasia of the epidermis and increased mucus production (e.g. in *Lolliguncula brevis*;[355] in *O. joubini* and *O. briareus*;[357] for review see[336,358]).

While infections occurring on the skin are most commonly reported, they are not the only tissues susceptible to bacterial infection, since Rickettsiales-like organisms have been found in the gills of laboratory reared *O. vulgaris*, observed as basophilic intracytoplasmatic microcolonies within epithelial cells, on which they cause hypertrophia and occasionally necrosis. No significant harm has been observed in the host, but under conditions of stress or intensive husbandry, it has been suggested that these bacteria may have a detrimental effect on the host's respiratory gaseous exchange although this has not been shown experimentally.[359]

Gram-negative bacteria *Vibrio lentus* have been also identified in the branchial heart of wild *O. vulgaris* and reported to induce mortality in 50% of octopuses in the first six hours, with lesions showing a typical round pattern on the arms or head.[360]

Table 6. Most common bacteria reported in cephalopods. Information included hereunder is deduced from various works and reviews[336,355,357,359–361,363,530].

	Sepia officinalis	*Loligo forbesi*	*Loligo pealei*	*Lolliguncula brevis*	*Sepioteuthis lessoniana*	*Octopus vulgaris*	*Octopus briareeus*	*Octopus bimaculoides*	*Octopus joubini*	*Octopus maya*	*Enteroctopus dofleini*
Acinetobacter anitratus											✓
Aeromonas cavia									✓		
Aeromonas sp.				✓							
Micrococcus sp.		✓									
Myxobacteria spp.			✓								
Pseudomonas sp.		✓		✓	✓			✓			✓
Pseudomonas stutzeri	✓						✓		✓		
Rickettsia sp.						✓					
Vibrio parahaemolyticus							✓				
Vibrio alginolyticus									✓		
Vibrio anguillarum					✓						
Vibrio carchariae					✓			✓		✓	
Vibrio damsela							✓		✓		
Vibrio harveyi					✓						
Vibrio lentus						✓					
Vibrio pelagius	✓										
Vibrio sp.		✓		✓				✓			
Vibrio splendidus	✓				✓						

Finally, cloudy-to-opaque corneal tissue as well as opaque lenses in *Loligo forbesi* and *S. lessoniana* have been reported due to infection with Gram-positive bacteria (*Micrococcus* sp.) found in the vitreous-induced swelling of the infected eye and causing opacity of the cornea.[361]

7.2.3 Fungi. Reports of fungal infections in cephalopods are scarce and mostly relate to eggs and embryos. Hanlon and Forsythe[336] refer to infection by *Labyrinthula* sp. in adult *O. vulgaris;* in these animals grey patches of inactivated chromatophores appeared followed by progressively larger and whiter patches in which the entire epidermis and dermis was missing. Thraustochytrid and labyrinthulid fungi have also been isolated from skin lesions in *E. cirrhosa*, but it is not clear whether these organisms are causal agents or secondary infections.[362]

Harms et al.[282] reported a case of mycotic infection in adult captive cuttlefish showing skin lesions in the dorsal mantle. Cytology revealed hemocyte granulomas surrounding fungal hyphae, and culture yelded *Cladosporium* sp. Infection from the same organism was also reported by Scimeca and Oestmann (1985, cited in[351]) in octopus, while *Fusarium* sp. has been found infecting the chambered nautilus, *N. pompilius*.[351]

7.2.4 Parasites. Most wild cephalopods host parasites include protozoans, dicyemids and metazoans. Generally, these are found in skin, gills, digestive tract, digestive gland and kidneys.[73,363–370]

Among the protozoans, one of the main parasites infecting both wild and cultured cephalopods is the gastrointestinal coccidian of the genus *Aggregata*, which produces severe disease in cuttlefishes and octopuses, by causing a malabsorption syndrome, diminishing nutrient absorption and reducing the immune response capability.[226,258] In addition, the parasite may produce behavioural alterations in the infected host inducing excitation, impaired ability to camouflage and aggressive behaviour.[316] Mortality has been attributed to the infection, and it has been reported that a few days before dying an infected octopus became inactive in the shelters and unresponsive to stimuli.[316]

Dicyemids are endosymbionts that inhabit the renal sacs of cephalopods including cuttlefish, loliginid squid and octopuses.[370–372] No host damage has been reported due to dicyemids, but a possible contribution to ammonium ion elimination from the host urine has been proposed[370]. However, dicyemids could be a problem if the parasite load is elevated enough to cause physical obstruction of the renal sac.

Cephalopods are intermediate or parathenic hosts for a variety of metazoan parasites, namely trematodes, digenea, cestodes and nematodes transmitted via the food chain.[370,373]

Amongst these, one of the most abundant and frequent parasites are anisakid nematodes, which have been reported to cause important pathological effects in several cephalopod host species.[374–376] Larvae of *Anisakis simplex* are pathogenic to humans when raw, under-cooked or lightly marinated fish or squid are ingested.[377] Crustaceans, such as copepods and isopods, also parasitise the gills and mantle cavity of cephalopods, affecting the body condition of the host.[378]

Apart from the potentially pathogenic organisms (e.g. *Vibrio*, *Aeromonas*, *Pseudomonas* and *Flavobacterium* sp.; ciliates and dicyemids), none of these diverse organisms is known to cause severe health problems in captive cephalopods. However, as some of the mechanisms exploited by parasites to produce changes in host's behaviour would seem to be highly conserved throughout the evolution of both vertebrates and invertebrates,[379–382] it would be unwise to exclude such interactions in cephalopods without specific research.

7.3 Antibiotic treatment of infectious diseases

Antibiotics have been utilised in some instances to treat cephalopods in laboratory experiments as reviewed in Table 7. Several routes of administration have been used (i.e. oral, parenteral or tank/bath immersion); in addition Berk and coworkers[383] have suggested a technique for gavage in squid that could be adapted to octopus and cuttlefish. Intramuscular injections of antibiotics have been given at the base of the arms taking special care to avoid the axial nerve cord.[358] Sherrill et al.[356] have suggested the use of oral, parenteral, or tank/bath immersion prophylactic antibiotics as reasonable for captive cuttlefish subjected to physiological stress, since this treatment may delay disease progression and improve longevity. This method should be avoided unless there are exceptional, scientifically justified circumstances, as it is clearly preferable to identify and remove the source of the stress.

Despite published evidence, caution should be applied when using oral or parental routes of administration since these are stressful for animals and may be difficult to perform safely.

In any case, prophylactic use of antibiotics is not recommended because of the risk of promoting bacterial and fungal resistance, masking infection and allowing secondary infection. It should not be used to 'prop up' poor tank hygiene.

Cephalopods used for scientific purposes should be maintained free from infections and contact with infection sources avoided. To this end, the use of high-performing filtration systems (i.e. combining mechanical, biological and physical filtrations) is highly recommended in combination with careful

Table 7. Substances given to some cephalopods to treat infection, but not recommended for routine use in laboratory facilities; see text for details. The table summarises for each species the treatment (i.e. dosage, route and duration) and substances tested. All drugs included in this table belong to the class of pharmaceuticals utilised as antibacterial agents, unless otherwise stated. The studies cited refer to treatment of infections, with the exception of Gore et al.[530] who investigated a pharmacokinetics. For a short discussion see also Scimeca[351] and Forsythe et al.[358] Therapeutic interventions should be discussed with a veterinarian.

Drug	Dosage	Route[a]	Duration	Species				References
				Sepia officinalis	*Sepioteuthis lessoniana*	*Lolliguncula brevis*	*Octopus joubini*	
Chroramphenicol	40 mg/kg	*PO*	7 d	√	√			145
	75–100 mg/kg	*PO/IM*	twice* 6 d				√	358b
Enrofloxacin	5 mg/kg	*IM/IV*	8–12 h	√				530
	10 mg/kg	*PO*	8–12 h	√				530
	2.5 mg/L	*Imm*	5 h/d* 7–10 d	√				530
Gentamicin	20 mg/kg	*IM*	7 d	√	√			145
Tetracycline	10 mg/kg	*PO*		√				531
Furazolidone	50 mg/L	*Imm*	10 min* 2 d	√				Gore et al. 2004, cited in[351]
Nitrofurazone	2 mg/L	*Imm*	1 h* 2 d			√		355
	2 mg/L	*Imm*	72 h	√	√			145
	25 mg/L	*Imm*	1 h* 2 d	√				Gore et al. 2004, cited in[351]
Metronidazole[c]	100 mg/L	*Imm*	16 h	√				Gore et al. 2004, cited in[351]

[a]Administration route: *PO, per os* (i.e. provided through food items); *Imm,* immersion in a solution; *IM,* intramuscular injection; IV, intravenous injection.
[b]But see also Table 1–3 in Hanlon and Forsythe.[336]
[c]Antiprotozoal agent.

screening of animals entering the facility and efficient quarantine procedures. The importance of tank design in minimising the potential for skin damage and subsequent increased probability of infection should not be overlooked.

8. Scientific procedures, severity and harm-benefit assessment, anaesthesia and humane killing

8.1 Definition of a 'procedure'

Directive 2010/63/EU defines a regulated 'procedure' as, 'Any use, invasive or non-invasive, of an animal [e.g. living cephalopod] for experimental or other scientific purposes, with known or unknown outcome, or education purposes, which may cause the animal a level of pain, suffering or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice'.

It should be noted that this definition is not confined to procedures that induce pain, but also includes procedures that cause other forms of suffering, such as anxiety, fear, stress and distress. Table 8 illustrates this point by listing some studies which include procedures that are likely to be subject to regulation under the Directive.

8.2 Identifying and reducing the adverse effects of procedures

All *adverse effects* that could be caused to animals by particular scientific procedures must be identified and predicted at the project planning stage (prospective assessment), then adequately monitored throughout

Table 8. Selected examples of research in cephalopods which involves an intervention that it is considered would come within the definition of a procedure (see also section 8 of this work) within the Directive. Studies published recently have been selected where possible to show that the Directive will impact on current research areas. It should be noted that non-surgical interventions that may induce PSDLH fall within the definition of a procedure. The fact that a particular technique has been used in a previously published study does not guarantee that the same technique would now be permitted by the national competent authority under the Directive.

Research topic or technique and species studied	References
Amputation of a portion of an arm under general anaesthesia followed by recovery *Octopus vulgaris*, *Sepia officinalis*, *S.pharaonis*, *Doryteuthis pealeii*	10,190,431
Deprivation of 'sleep' for 48 h by continuous visual stimulation *Sepia officinalis*	102
Administration of *E. coli* lipopolysaccharide by intramuscular injection into the arm under general anaesthesia followed by recovery and subsequent repeated sampling of haemolymph *Octopus vulgaris*	251
Investigation of the efficacy of different general anaesthetic substances techniques and mechanisms of anaesthesia *Sepia officinalis*	440
Production of hatchlings with deleterious phenotypes/genotypes by exposure of the eggs to: a harmful environment, or mutagen, or genetic manipulation *Loligo vulgaris*	532
Implantation of electrodes for either recording or stimulation into the brain under anaesthesia followed by investigation of the effects in the conscious/sedated animal *Octopus vulgaris, Doryteuthis pealeii*	208,533
Removal of samples of haemoloymph from the dorsal aorta under anaesthesia with recovery *Euprymna scolopes*	249
Administration of drugs to modify nervous system functionality *Doryteuthis pealeii, Sepia officinalis*	396,402
Implantation of temperature and depth-logging archival tags under general anaesthesia with recovery and monitoring for up to 5 months *Sepia officinalis*	130,132
Non-invasive measurement of brain and arm morphology under anaesthesia with recovery *Octopus vulgaris*	246,247
Immobilisation of animal and exposure to light stimuli to investigate the pupillary reflex *Lolliguncula brevis*	215
Food deprivation for 7 or more days *Sepia officinalis*	534,535
Aversive training paradigms to test acquisition, consolidation and memory recall *Sepia officinalis, Octopus vulgaris*	26,420,504,506,507

the procedure. Steps must be taken to: i. refine each procedure, so as to minimise and preferably eliminate its adverse effects, and ii. alleviate any animal suffering that occurs during the conduct of procedures or whilst animals are recovering.

This is a legal, as well as ethical, requirement under Directive 2010/63/EU, which requires implementation of *replacement, reduction and refinement (3Rs) strategies* (section 2) wherever possible, with the aim of 'eliminating or reducing to a minimum any possible pain, suffering, distress or lasting harm' [PSDLH] caused to the animals. Note that 3Rs strategies should be implemented whenever feasible, at all times from birth to death of the animals including: sourcing, transport, housing and care, handling, and fate of animals, as well as the procedures themselves.

8.3 Monitoring animals undergoing procedures and setting humane end-points

Assessment of the severity of adverse effects must be carried out before, during and after procedures (see also details in section 2.2 above, which describes requirements for setting *prospective* 'severity limits' and *retrospective* reporting of the severity of procedures, along with questions for consideration).

Schemes for monitoring adverse effects during the procedure should cover the criteria outlined in section 6 and Table 5 for routine daily assessment of animal welfare; these should be supplemented with any other, *specific,* adverse effects that might be caused by the *particular* procedure(s). Criteria for assessment, and frequency and timing of observations, should be agreed before studies commence; then regularly reviewed as studies progress, and, wherever necessary, added to or amended.

A *humane end-point* must also be defined for each procedure, to describe (in terms of indicators of the nature and degree of suffering) the earliest point at which a specific intervention must be made to end an animal's suffering, e.g. by: *i*. removing the animal from the study, *ii*. providing analgesia, *iii*. humanely killing the animal and/or terminating the study. The use of 'score sheets' for monitoring can be particularly helpful in determining when humane end-points have been reached, and when severity limits are being approached.[384] To be effective, this monitoring requires a team approach, with good planning, and appropriate training for all involved. Methods for the observation and assessment of adverse effects are relatively well developed for many vertebrate species including fish.[384–387]

There is a need for further development of objective criteria for assessing severity that can be used by the entire EU cephalopod community to ensure consistency. This is a current project of the non-profit Association for Cephalopod Research (see www.cephalopodresearch.org/projects).

Working on a consensus view for severity assessment of procedures is also a goal of the FA1301 COST Action (Cephs*In*Action; http://www.cost.eu/COST_Actions/fa/Actions/FA1301; www.cephsinaction.org). An equivalent initiative has been set up for describing characteristics of laboratory mice (http://www.mousewelfareterms.org/doku.php),[8] and and for severity classification classification of scientific procedures involving fish.[384]

8.4 Harm-benefit assessment

Of course, one way of eliminating animal suffering is not to carry out the procedure at all. In this context, it must be remembered that 'procedures may only be carried out within the framework of a [authorised] project' (Directive, Article 12.2), which is subject to a *harm-benefit analysis*, 'to assess whether the harm to the animals in terms of suffering, pain and distress is justified by the expected outcome taking into account ethical considerations, and may ultimately benefit human beings, animals or the environment'.

Hence, for legal as well as moral reasons, investigators should carry out a harm-benefit analysis, as described above, prior to conducting any procedure.

In the following discussion, we cite published studies that provide evidence about possible harms caused by common procedures. These are included as examples that will help to predict and identify harms in future projects, but we are not suggesting that these studies would necessarily be considered justified according to the harm-benefit analysis conducted under the new EU Directive.

8.5 Some common procedures in cephalopod research

The following sections summarise current knowledge of the regulatory status, adverse effects and possibilities for refinement of some common procedures. It is evident that this knowledge is patchy and there is need for further work, especially to help refine procedures. Nevertheless, where possible, provisional recommendations for good practice are made.

8.5.1 Behavioural studies. Cephalopods have been used extensively for a variety of behavioural studies, as reviewed in several works.[2,25,36,175,192,195,388]

Classical behavioural studies have used a variety of aversive stimuli (e.g. electric shock;[72] acid solutions;[389] bitter;[390] mechanical[391]) as part of training protocols. Because of their potential to cause distress and possible suffering, such studies would certainly fall within the definition of a regulated procedure under the Directive and should be avoided wherever possible. For example, electric shock or application of acid solutions should be unacceptable, and these and other harmful stimuli should be replaced with reward-based conditioning and, at the least, stimuli that do not cause pain.

Exposure of an animal to a stimulus known to evoke an escape response (i.e. inking, jetting locomotion, dymantic display) could also be argued to cause distress, especially if the exposure is repeated, and hence is likely to be a regulated procedure. Deprivation of food for prolonged periods, deliberate exposure to elevated noise or adverse change in water temperature, pH or chemistry, and direct exposure to a predator would also be likely to fall within the definition of a procedure, depending on the severity/degree of change.

Table 9. Routes used for haemolymph sampling and drug administration in exemplar species of laboratory cephalopods species. Species are listed on the basis of the most commonly utilised for such procedures. Abbreviations: NS, not stated; NA, not applicable; R, sample removed; A, drug administered; EtOH, ethanol; SW, seawater.

Species	Body weight (g)	Anaesthetic when injection given	Site	Needle size	Volume removed [R] Administered (A)	Substance injected Fluid removed	Reference
Octopus vulgaris	98–1268 (*mean 533*)	55 mM $MgCl_2$ + 1% EtOH	Branchial hearts	30G	R 70–100 µl	Haemolymph	251
Octopus vulgaris	200–500	Cold water No anaesthetic	Branchial heart	Microlance 3	A 500 µl	Filtered seawater	395
Octopus vulgaris	98–1268 (*mean* 533)	No anaesthetic	Arm (×2)	NS	A 1 ml/kg divide between two arms	Phosphate buffered saline or Lipopolysaccharide (15 mg/kg)	251
Octopus vulgaris	200–400	No anaesthetic	Branchial heart	Microlance 3	A 1 ml/kg	Filtered seawater or scopolamine (2 mg/kg)	536
Octopus vulgaris	290–1040	NA	Implanted cannula in dorsal aorta or afferent branchial vessel	NA	R 40% of blood volume in total (~20 ml in a 1Kg animal)	Haemolymph	537
Octopus vulgaris	200–1500	No anaesthetic	Intramuscular (site NS)	25G × 5/8 in.	NS	L-NAME (75 mg/kg), D-NAME, artificial seawater	503,538,539
Octopus vulgaris[a]	NS	No anaesthetic	Intramuscular at base of arm	NS	A 400–600 µl 300 µl	Reserpine (4 mg/kg) Pargylline hydrochloride (100 mg/kg)	540
Octopus vulgaris	200–500	No anaesthetic	Arm in the region of the brachial nerve to produce nerve block	NS	NS	2% xylocaine	243
Octopus vulgaris[b]	200–500	NA	Implanted cannula in dorsal aorta	NA	A 100 µl substance + 100 µl SW	Acetylcholine, carbachol, dopamine,encephalin, noradrenaline, GABA, 5-HT, kainic acid, L-glutamate, methysergide, nicotine, nor-adrenaline, pentagastrin, taurine, tubocurarine (doses: from 10–100 µg)	401,456
Eledone cirrhosa	>250	2.5% EtOH	Branchial vessel	21G × 1.5 in	R 300 µl/100 g	Haemolymph	250
Eledone cirrhosa	500–800	2.5% EtOH	Branchial vessel	26G × 0.5in	R 1 ml/animal	haemolymph	255

(continued)

Table 9. Continued

Species	Body weight (g)	Anaesthetic when injection given	Site	Needle size	Volume removed [R] Administered (A)	Substance injected Fluid removed	Reference
Eledone cirrhosa	493–1050	2.5% EtOH	Web tissue at arm base	21G × 1.5in	A (NS)	1% Alcian Blue for marking	250
Eupymna scolopes	NS	2% EtOH	Cephalic vessel between eyes	26.5G	R 10–20 µl (for multiple sampling) 50–100 µl (for single sample)	haemolymph	249
Sepia officinalis	590–900	No anaesthetic	Into the side of the neck at a depth of 10 mm	NS	A 1 ml/kg	Cycloheximide (10 mg/kg) Octopressin (3–60 µg/kg) Cephalotocin (3–60 µg/kg) 150 mM NaCl	180,396
Sepia officinalis	220	3% EtOH	Cephalic vein	25G × 9 mm	R 500 µl	haemolymph	282
Sepia officinalis	220 ?	3% EtOH ?	Cephalic vein	NS	A NS	Enrofloxacin (10 mg/kg) Enrofloxacin (5 mg/kg)	282,530
Sepia officinalis	NS	No anaesthetic	Intramuscular at base of arm	NS	A NS	Chloramphenicol (40 mg/kg, daily) Gentamicin (20 mg/kg, daily)	145
Sepia officinalis	NS (DML 4–8 cm)	~1% EtOH	Fin nerve branch		A 2 µl	5% Texas red dextran	541
Sepia officinalis	200–1200	2%EtOH + 17.5‰ $MgCl_2$	Brain vertical lobe	Microcannula (OD: 125 µm)	A 2 µl over 3 min	Kainic acid (25–100 mM) L-Glutamate (100–800 mM) (injections also contained methylene blue and DiI for site marking)	398
Doryteuthis pealeii	~65	No anaesthetic	Crop/stomach	Tygon tube (OD: 760 µm))	A 250 µl	T-817 3.6 mM (a neuroprotective agent)	383,402
Doryteuthis pealeii[c]	200–400	NA	Anterior vena catheter previously impanted under anaesthesia	PE50 cannula extruded	R NS	Haemolymph for measurement of pH, PCO_2, [$HCO3^-$]	417
Doryteuthis pealeii	68.5 (mean of N = 3)	No anaethetic	Intramuscular, head, arms or mantle	NS	A 1 ml/kg	Gallamine triethiodide (2.37 mmol)	208

[a]Intramuscular injection of reserpine at the same dose also studied in *Eledone cirrhosa* and *Sepia officinalis*[542]
[b]Similar but more limited studies also performed in *Eledone cirrhosa*, *Sepia officinalis* and *Alloteuthis* sp.[401]
[c]Similar studies performed also using *Illex illecebrosus*[417]

Protocols using positive reinforcement (for example see:[104,392–394]) should be used wherever possible.[2]

8.5.2 Administration of substances. Administration of substances can cause harms to animals as a result of the route of administration and/or the substance itself. Table 9 lists commonly-used routes of administration, but does not recommend a particular technique since, despite the number of examples included, there have been no systematic studies investigating the optimal size of needles or injection sites and volumes in cephalopods. In addition, although adverse effects of injections have not been reported, in view of the limited literature this needs further investigation from a welfare perspective.[251,395]

Cephalopods do not have readily accessible large superficial blood vessels; therefore, in general experimental agents and therapeutic drugs are given to unanaesthetised animals by *bath application* (assuming absorption via skin and gills), or injection via subcutaneous and intramuscular routes.

The efficacy of *subcutaneous injections* is not known, but drug absorption may be slow because of a low capillary density. However, this is not based on pharmacokinetic studies and only a limited number of substances have been studied.[123,395]

Locations for *intramuscular injections* include the arms (particularly proximal parts in octopus) and 'neck' (in cuttlefish) reported to have a high vascular density.[123,180,208,251,396] Care should be taken not to damage arm nerve cords and ganglia by injection in the arms (especially in octopus), nor the cuttlebone or gladius in cuttlefish and squid respectively. The use of ultrasound may be helpful in directing injections to avoid vulnerable structures.[246,247]

Substances have been injected into the branchial hearts of *O. vulgaris*, but this requires eversion of the mantle. The animals recover rapidly from this procedure and no adverse events have been reported; however, although behaviour rapidly (<1 hour) returned to normal,[395] a systematic study of welfare of the animal has not been fully carried out.

8.5.3 Other methods. It should be noted that the welfare effects of the following methods has not been assessed. *Intravenous* (including vena cava) and *intradorsal aortic* routes have been used for substance administration by direct injection.e.g.[282] Direct administration into the brain has been successfully attempted in a number of studies. e.g.[397,398]. Finally, surgical implantation of vascular catheters has been used as a method of drug administration and for pressure recording.[399–401]

A method for gavage administration of drugs to cephalopods has been described by Berk et al.[383] and used to investigate the efficacy of a putative neuroprotective agent in blocking the effects of human tau protein on transmission in the squid giant synapse.[402] This method requires restraint of the animal and may require removal from the water and sedation. As the brain in cephalopods encircles the oesophagus care should be taken to use the narrowest gauge tube as possible, and it should also have a round end to minimise the chances of puncturing the gut particularly at the oesophageal/crop junction. Substances administered by this route will reach the crop and stomach directly.

Drugs and other substances such as nanoparticles have also been administered *per os* (i.e. by inclusion in the food,;[403] G. Ponte, unpublished data). Further experiments may help in better refine this approach.

8.5.4 Administration of drugs as investigational agents. When undertaking studies using pharmacological agents, the wider effects of the drug must also be considered in assessing the overall impact of a treatment on the welfare of the animal, and any unexpected side-effects should be recorded and minimised/avoided where possible. For example, Agin et al.[180] used cycloheximide, a protein synthesis inhibitor, to investigate the machinery involved in long-term memory in the cuttlefish. However, it induced positive buoyancy that interfered with animals' ability to catch prey.

Relatively little is known about the pharmacology of central and peripheral neurotransmitter systems.[1,404,405] In addition, very limited is the knowledge on the pharmacological characteristics of drug receptors in cephalopods. Therefore, caution should always be exercised when drugs, whose pharmacological properties have been defined in mammalian systems, are used as investigational agents in cephalopods.

If such studies are undertaken in group housed species, it will be necessary to identify individual animals (see section 4.1.2) so that individual variations in drug response can be identified. Additionally in group-housed species, special attention should be paid to agents that may increase aggression (e.g. by alteration of brain neurotransmitters) or that may impair the ability of an individual to escape or defend itself and action taken to reduce or eliminate these adverse effects.

8.5.5 Haemolymph (blood) collection. It should be noted that cephalopod blood does not clot, but haemocytes aggregate and vessels constrict to prevent blood loss.[150,406] However, as haemolymph is pale blue (oxygenated) or colourless (deoxygenated), haemorrhage may be very difficult to detect.

A summary of routes used in several studies for haemolymph sampling is presented in Table 9. There are no systematic data on the welfare impacts of blood sampling methods in cephalopods, but in several

studies behaviour was reported to return to normal following the sampling.[249,251,395]

In large cephalopods (i.e. *E. dofleini*), cuttlefish and *E. scolopes,* small blood samples have been obtained under anaesthesia via a needle inserted into the cephalic vein dorsal to the funnel.[249,282,407,408] This method has also been successfully applied for haemolymph sampling in *O. vulgaris* (G. Ponte and G. Fiorito, pers comm.). In addition, small single samples of haemolymph can be taken directly from the branchial hearts in *O. vulgaris* (~250 µl) and *E. cirrhosa* (<300 µl/100 g body weight; 1 ml/animal in animals of 500–800 g, as shown in[250]) using a hypodermic needle, although this requires general anaesthesia to permit manipulation of the mantle to expose the hearts.[250,251,255,403] In the bobtail squid (*E. scolopes*) single haemolymph samples of 50–100 µl can be withdrawn from the dorsal aorta by direct needle puncture under anaesthesia.[249] Where frequent sampling of blood is necessary, it is likely that this will require implantation of catheters.[240,407,408] Again, the welfare impact of this procedure has not been fully assessed.

The maximum volume of blood which can be collected at a single sampling in particular cephalopods has not been assessed from a welfare perspective. In mammals, it is recommended that no more than 10% of the blood volume is removed at any one time.[409] For fish the Canadian Council on Animal Care (CCAC)[‡‡] recommends a maximum withdrawal of 1 ml blood/kg weight.

Blood volume in octopus is estimated at 5–6% of body weight,[410,411] hence using the mammalian values as a guide, a maximum of 5–6 ml blood/kg weight would be advised, but, as noted above, only 1 ml/kg according to CCAC guidelines for fish.

However, in publications where multiple sampling has been used, authors either collected a reduced volume on each occasion (e.g. 10–20 µl for multiple sampling *vs* 50–100 µl for single samples in bobtail squid) as done by Collins and Nyholm[249] or have set a time for recovery between samples (e.g. 4 hours in *E. cirrhosa*).[126]

8.6 Surgery

Cephalopods have been subjected to a broadly similar range of surgical procedures as have been performed in vertebrates, but in general surgical techniques are not described in detail in publications; this is no longer considered acceptable and we strongly recommend following ARRIVE Guidelines on reporting.[272]

Surgical approaches and techniques, along with understanding of their welfare effects, are not as advanced in cephalopods when compared with vertebrates, particularly in relation to: *i.* intra-operative monitoring and maintenance of physiological parameters (e.g. blood pressure, PO_2, PCO_2, pH, temperature; see also section 8.8.4); *ii.* identification and control of haemorrhage; *iii.* controlled general anaesthesia and analgesia (see section 8.8); *iv.* optimal techniques for the repair of muscle and skin tissue and wound closure (see section 8.6.2) and healing in general; *v.* post-operative monitoring and special care that may be needed if, for example, feeding is transiently impaired; *vi.* infection risk (intra- and post-operatively) and requirement for prophylactic antibiotic cover (see section 7.3).

Although it will be some time before there is welfare guidance on all the above topics, anyone contemplating undertaking a surgical procedure in cephalopods will need to consider all of the factors, and submit relevant information, as part of the project evaluation and authorisation process, and during the conduct of any authorised surgical procedures. This will include:

(i) ethical 'justification' for the procedures, in terms of harm *vs.* benefit of the outcomes;
(ii) identification of potential adverse effects and the steps taken to refine the procedures, so as to minimise the adverse effects;
(iii) processes for intra- and post-operative monitoring of the animals;
(iv) timely application of methods to minimise post-operative suffering, e.g. analgesia and other specific care; and
(v) clearly defined humane end-points (section 8.3) to set an upper limit to the suffering that an animal experiences.

Some general principles for performing surgery on aquatic animals can be found in the fish literature, and these should be adapted where possible to cephalopods.[412–414]

As for all other procedures, anyone attempting any surgery in cephalopods must be 'adequately educated and trained' in the principles and practical techniques of surgery, and must be 'supervised in the performance of their tasks, until they have demonstrated the requisite competence' (Directive Article 23§2), for example, to the designated veterinarian or other competent person (see section 10 for further discussion).

A full description and evaluation of surgical techniques in cephalopods is outside the scope of this document but the following four specific aspects are highlighted, along with a detailed discussion of anaesthesia in section 8.8 below.

[‡‡]see: http://www.ccac.ca/Documents/Education/DFO/4_Blood_Sampling_of_Finfish.pdf

8.6.1 The operating room and environment. Surgical procedures should be performed in a dedicated room located close to the animals' home tanks to minimise animal transport, and induction of anaesthesia should take place in the surgical procedure room. Good surgical lighting is essential, but care should be taken that this does not cause localised heating of the animal.

Although a *sterile operating environment* is highly desirable to prevent cross-contamination from human to cephalopod (and potentially *vice versa*, see section 9), and to ensure a consistent scientific baseline, maintenance of sterility is not practical unless: sterile seawater and anaesthetic solutions are used throughout the operation, and the surgeon and any assistants are fully trained in sterile technique and are wearing appropriate clothing. General information on asceptic surgical techniques can be found in a LASA 2010 report.[415]

As a minimum, it is recommended that:

i. all apparatus or surgical instruments contacting the animal during surgery should be sterilised or at a minimum cleaned thoroughly with anti-bacterial and viricidal agents (e.g. diluted Halamid[§§] or Virkon[***], F. Mark, pers. comm.);
ii. all material contacting the wound site such as surgical swabs and sutures should be sterile; and
iii. all personnel contacting the animal in the surgical environment should have cleaned their hands in accordance with aseptic technique and/or use sterile surgical gloves (blue nitrile gloves are recommended for handling animals, to avoid pale colours but these are not sterile and so should not be used for surgery);
iv. the surgical site should be cleaned; but at present there is no information on the effect of commonly used skin cleaning preparations on cephalopod skin integrity and healing;
v. the surgical site should be isolated as far as possible from any source of contamination (e.g. seawater/anaesthetic solution).

8.6.2 Maintaining physiological function. Water used to irrigate cephalopod gills during surgery should be circulated, treated and monitored to maintain appropriate anaesthetic levels, oxygen, pH, temperature and salinity, and to remove particulates. Pörtner et al. note that the sensitivity to hypoxia is greater in squid than either cuttlefish or octopus.[417]

Consideration should be given to using a cooled operating table (temperature monitored) in combination with mantle perfusion with a chilled anaesthetic solution, as cooling can act as an adjuvant to anaesthesia,[243,418] but should *not* be used as an anaesthetic alone. Cooling will also reduce metabolic rate, which may be advantageous when cardiorespiratory function is impaired as a result of anaesthesia.

The skin must be kept moist and this can be done with a sterile surgical drape moistened with sterile seawater. Care should be taken to minimise the potential for seawater to enter the wound site. Although anaesthesia to a depth sufficient to markedly suppress or stop ventilation may not be desirable from a physiological perspective, surgery involving incision of the mantle muscle and suturing may be very difficult in the absence of such marked suppression.

Whilst the efficacy of mantle perfusion with gassed anaesthetic/seawater solution for maintenance of blood gases may be questioned when there is severe suppression of cardiac function by the anaesthetic/anaesthesia, such perfusion may still be advisable to reduce anaesthetic wash out from the blood/tissues, to prevent desiccation of visceral structures and prevent/reduce hypoxic damage to critical tissues such as branchial hearts and gills. This may not be necessary for short duration (<10 min) minor surgical procedures, provided that full surgical anaesthesia can be assured.

8.6.3 Wound closure and healing. There are no studies assessing optimal materials to use for repair/closure of skin, muscle, connective tissue (e.g. hepatopancreas capsule), blood vessels or cranial cartilage in cephalopods, and publications do not often detail the materials used. The inclusion of such information in future publications is essential.

Closure techniques should follow normal practice for surgery in vertebrates, using round-bodied needles to reduce tissue trauma. Braided non-absorbable silk[123,243], polyglyconate sutures[282] and cyanoacrylate adhesives[132,401,419–422] have been utilised. Several authors report that cephalopods will try to remove sutures with their arms (F. Mark, unpublished data),[240] although this is not a universal finding and may reflect differences in surgical technique. Sutures sealed with cyanoacrylate have been used, but in some instances they may cause skin irritation (possibly due to being inflexible) and may not maintain integrity for longer periods of time in seawater.

An evaluation of five commonly used suture materials including braided silk, monofilament nylon and poliglecaprone has been undertaken in the mollusc *Aplysia californica*.[423] All materials induced similar adverse skin and subcutaneous tissue reactions, but the authors recommended the use of braided silk because it induced a less intense granuloma reaction. This result was unexpected as in studies of aquatic vertebrates monofilament sutures are less reactive than braided silk.[423]

[§§]N-chloro tosylamide, sodium salt; see: http://www.halamid.com/Aquaculture-desinfection.htm, last visited August 2014.
[***]Peroxygenic acid; see Hernndez et al.[416] and http://www2.dupont.com/Virkon_S/en_GB/, last visited August 2014.

We would encourage anyone undertaking surgery in cephalopods to undertake histology on the wound site at the end of study, and include findings in any publications, with the aim of generating a database of good surgical practice. In addition to suture material, the most appropriate suture patterns (e.g. discontinuous, continuous) and needle size for skin closure in cephalopods has yet to be established, and this is knowledge especially important as the skin is particularly delicate in cephalopods.

Studies involving chronic implantation of devices such as archival ('data logging') tags[185,417,424–426] need to consider possible chronic reaction of the tissues to the device and the impact that this may have on the normal behaviour of the animal. Mounts implanted in cuttlefish to support archival depth and temperature tags were well tolerated over a period of up to 5 months although post-mortem tissue thickening (possibly fibrosis) was observed around the implant and this increased in thickness with the duration of implantation.[130,132] Chronic tissue reactions are also a potential issue for implanted identification tags (see section 5.7).

Descriptions of the natural course of wound healing following skin damage to the dorsal mantle in *E. cirrhosa* can be found in Polglase et al.[189] Authors also found that bacterial infection of a mantle wound impairs healing.[210] Healing and regeneration of the fin dermis in *S. officinalis* is described by Yacob et al.[427] and regeneration of the mantle connective in several species of octopus by Sereni and Young.[428]

The arms in cephalopods have a remarkable capacity to heal and regenerate when a segment is removed by transection with the histological changes described in detail in *O. vulgaris*, *E. cirrhosa*, *S. officinalis* and *S. pharaonis*[190,429–431] In *O. vulgaris* following transection of the distal 10% of the length of the arm as part of study to investigate regeneration, the exposed area was almost completely covered by skin in about 24 hours in some animals (T. Shaw and P. Andrews, unpublished data).

Cephalopods frequently injure arms, tentacles and fins in the wild;[311,312,432–435] therefore a well-developed healing and regeneration mechanism is perhaps expected. This suggests that, provided sufficient time is allowed, surgical wounds, appropriately repaired, should heal successfully provided they do not become infected.

In addition to general anaesthesia surgery also requires the use of analgesics and this is discussed in section 8.9 below.

8.7 Tissue biopsy

Tissue biopsies for DNA isolation and PCR analysis for genotyping cephalopods have been obtained by taking small samples from the tip of an arm or tentacle with a sharp, sterile blade.[436,437]

The harms *vs* benefits of performing this procedure under general anaesthesia have not been assessed. We propose that irrespective of the use of general anaesthesia the arm tip should be treated with a local anaesthetic (in the absence of systemic analgesics). Only the absolute minimum amount of tissue should be taken (taking into account animal size) especially in cuttlefish as removal of large amounts of arm tissue transiently interferes with food manipulation and results in abnormal body posture.[190] In squid and cuttlefish, the fins may provide an alternative site for biopsy as they also appear to heal rapidly and regrow.[427] Although arms, fins and other tissues will regenerate, attention should be provided to avoid unnecessary sampling.

8.8 General anaesthesia

The Directive requires that 'procedures are carried out under general or local anaesthesia' unless anaesthesia is judged to be 'more traumatic to the animal than the procedure itself' and/or 'is incompatible with the purpose of the procedure' (Article 14§2). 'Analgesia or another appropriate method' must also be 'used to ensure that pain, suffering and distress are kept to a minimum' (Article 14§1), for example, peri- and post-operatively (see section 8.9 for further discussion). Furthermore, drugs that stop or restrict the ability of an animal to show pain (e.g. neuromuscular blocking agents) must not be used without an adequate level of anaesthesia or analgesia. When such agents are used, 'a scientific justification must be provided, including details on the anaesthetic/analgesic regime' (Article 14§3). After completion of the procedure requiring anaesthesia 'appropriate action must be taken to minimise the suffering of the animal', for example, by use of analgesia and special nursing care (Article 14§5).

During and after general anaesthesia and surgery, animals should be carefully and regularly monitored using a welfare monitoring scheme, and a record kept of observations and interventions to reduce or alleviate adverse effects. Of particular relevance is wound-directed behaviour but other potential welfare indicators are listed in Table 5. It is currently believed that operated animals should be housed individually at least until full recovery from general anaesthesia is assured, but this will clearly need particular consideration in gregarious species.

Many scientific procedures in cephalopods will require the use of general anaesthesia but despite more than 60 years of literature detailing studies involving anaesthesia, covering at least 17 agents, information is lacking on which agents in which species provide the most effective, humane general anaesthesia (i.e. which definitely blocks nociception and pain perception,

generates no aversion, enables rapid induction and allows animals to recover quickly without adverse effects).

Recent studies have reviewed the use of agents claimed to have anaesthetic properties in cephalopods and criteria for evaluation of anaesthesia.[8,61,131,418,438–440] In a recent contribution, isofluorane has been utilised to induce 'deep anesthesia' in *O. vulgaris*.[441] However, more detailed studies are required to assess the application of this agent to induce anaesthesia in cephalopods.

In some cases, a range of behavioural responses is reported to occur during exposure of the animals to anaesthetic agents (i.e. inking, jetting, escape reactions, increased ventilation);[208,243,438] however, it should be noted that most studies report immersing the animal directly in the anaesthetic at the final anaesthetic concentration, and this may not be the best technique to minimise trauma (see below). It should be also noted that criteria for general anaesthesia are not consistent between studies and that behaviours observed during induction and to assess depth may differ between species.[439]

Moreover, studies of general anaesthesia in cephalopods have investigated single agents and the familiar concept of 'balanced anaesthesia' involving more than one agent, used in mammalian studies (for reference see[409,442]) has not been explored in cephalopods (but see exceptions in Packard[200,443]).

8.8.1 General anaesthetic agents. Detailed descriptions of the commonly used anaesthetic agents (magnesium chloride, ethyl alcohol and clove oil) and their advantages and disadvantages in the common laboratory species can be found in recent reviews,[8,439,440] and key features are described in Table 10.

Although a large number of agents have been investigated for anaesthetic efficacy in cephalopods, some are now considered unacceptable on either welfare or safety grounds (e.g. urethane),[444] and so are not considered here. Similarly, we are not considering those utilised in a single experiment, and pending more evidence they are not considered herein.

Magnesium chloride ($MgCl_2$) is the most extensively studied and used agent, probably because it appears to be the least aversive. However, it disturbs haemolymph Mg^{2+} levels, and so may not always be appropriate, for example, when blood samples are required to investigate normal magnesium ion levels.

Furthermore, there has been a recurrent concern that $MgCl_2$ may be acting at least in part as a neuromuscular blocking agent;[441,445] but see comments in Graindorge et al.[398] However to date there is no direct experimental evidence to support this concern in cephalopods, as reviewed in Andrews et al.[8] The supposition may have arisen from the original use of magnesium chloride to relax small invertebrates prior to fixation.[446]

Messenger et al.[447] concluded that $MgCl_2$ exerts an effect on the central nervous system, and subsequent studies (G. Ponte, M.G. Valentino and P.L.R. Andrews, unpub. obs.) showed that electrical stimulation of efferent nerves in 'anaesthetised' *O. vulgaris* (as per criteria below) with $MgCl_2$ (3.5%) evoked chromatophore contraction (arm and mantle), arm extension and mantle contraction showing a lack of effect at peripheral sites of motor control.

However, more recently, Crook et al. have shown that local subcutaneous and intramuscular injection of isotonic $MgCl_2$ suppressed the afferent nerve activity in nociceptors activated by crushing a fin.[11] This demonstrates that should such high local concentrations occur in animals immersed in $MgCl_2$, it may have analgesic/local anaesthetic effects. Further studies of its mechanism of action are urgently required.

Ethanol is widely used as an anaesthetic agent in cephalopods, but it is not clear whether it blocks pain perception and/or is aversive. Some variability is reported in the response of animals exposed to it (see Table 10A), but this could reflect impurities in the different sources of ethanol used[439] and other factors such as temperature.

For example, ethanol is considered effective in *O. vulgaris*, but has been reported to produce inking and escape reactions,[243] suggesting that it is aversive. However, these reactions are not noted if the temperature is below 12°C.[448] Moreover, ethanol has been reported to be ineffective in cold water octopus species, as cited in Lewbart and Mosley following a personal communication from I.G. Gleadall.[418] However, this observation seems contradicted by other personal experience on Antarctic octopods (F. Mark, pers. comm.).

Clove oil has been the subject of limited study so it is difficult to make a judgment about its use especially as there appear to be marked species differences in the response (see Table 10D). Further studies with clove oil, and its active constituent eugenol, are required to fully assess its utility as an anaesthetic for cephalopods.

Both $MgCl_2$ and ethanol have been reported to induce general anaesthesia as defined in section 8.8.2 below to a level sufficient to perform relatively short duration (~30 min; see Table 10A,B) surgical or invasive procedures (see also Tables 8 and 9). However, it must again be emphasised that the analgesic, aversive and amnesic effects of these agents have not been studied in any detail and the molecular mechanism of their general anaesthetic action in cephalopods has not been elucidated.

In addition to further research on the agents themselves, it is also apparent from the limited data in Table 10 that there is emerging evidence of

Table 10A-D. Examples of studies using ethyl alcohol (EtOH), magnesium chloride ($MgCl_2$) alone or in combination, or clove oil for induction and maintenance of general anaesthesia in exemplar cuttlefishes, squid and octopuses. Only publications with primary data are included; for recent reviews of other agents and species see: Andrews et al.[8], Gleadall[438], Sykes et al.[61] Studies utilising the same agents solely for euthanasia are not included.
For each case we report: species, temperature at which the study have been carried out (T), body size (expressed in grams, unless otherwise stated), concentration of the agent utilised (expressed in %, unless otherwise stated), time to anaesthesia (in seconds), criteria for time to anaesthesia, duration of anaesthesia (in seconds, unless othewise stated), purpose of the study, recovery time (in seconds or minutes, as required), references and comments, if available. Number of subjects utilised and their gender are reported, if available from the original work, with body size. Abbreviations: *F*, females; *M*, males; NS, not stated; NA, not applicable; DW, distilled water; SW, seawater. See original works for full description. Note that other criteria for time to anaesthesia are also used to identify onset of general anaesthesia and are discussed in the text.

Table 10A. Ethanol as an agent.

Species	T (°C)	Body size	Concentration (%)	Time to anaesthesia (s)	Criteria for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comments
S. officinalis	19.3 ± 1.4	35.1 ± 8.3[a] 42.2 ± 9.4[a] 42.7 ± 6.6[a]	1.0 2.0 3.0	434.0 ± 192.3 88.3 ± 41.2 73.3 ± 29.1	Body colour, swimming behaviour, funnel suction intensity[b]	180	Investigation of anaesthetics (no procedures applied)	64.7 ± 26.2 s 91.2 ± 36.7 s 101.7 ± 49.3 s	[440] Aim of the study was to sedate animals for handling not for surgery
S. officinalis	NS	NS *M* (N = 7) *F* (N = 6)	2.0	NS	Cessation of arm movement and no righting response	NS	Branchial heart injection	NS	[543,544]
S. officinalis	21	DML: 4–8 cm (N = 6)	~1	NS	NS	NS	Fin dye injection	NS	[541]
S. officinalis		220 *F*	3% for 1 min then 1.5%	NS	NS	44 min	Mantle granuloma excision	NS	[282]
S. officinalis	20–22	200–400 (N = 3)	1.5	NS	NS	NS	Mantle cannulation	NS	[545]
S. officinalis	10–14	DML: 2–5 cm	2	NS	NS	NS	Arm tip removal	NS	[430]
S. officinalis	22–23	DML: 5.2–6.4 cm (see[c])	0.8–1.0 % (in gradual increments)	NS	No response to: light, pinch by forceps; pallor	NS	Arm tip removal	60–300 s	[190]
S. pharaonis		DML: 5.1–5.8 cm (see[d])							
E. scolopes	NS	adult	2	600	Cessation of swimming + unresponsive to touch (note: ventilation + chromatophore activity continues)	NS	Haemolymph sampling from cephalic blood vessel	<30 min	[249]

(continued)

Table 10A. Continued

Species	T (°C)	Body size	Concentration (%)	Time to anaesthesia (s)	Criteria for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comments
D. pealeii	14	68.6[e]	1 3	696 240	Unresponsive to handling, immobile, loss of righting response	43.1 min	Investigation of anaesthetics (no procedures applied)	NS	[208] Animals attach to container, jetting behaviour, unusual colour/patterns; 0% mortality
S. sepioidea	NS	42.2–290.9[f]	1–3	<120 s	Absence of body hardness, flexible tentacles, pallor	90 (handling)	Anaesthetic efficacy study	~4–12 min (proportional to exposure time and concentration)	[198] 'no pernicious side effects'
S. lessoniana	NS	3.2–16.9 cm[g]	1.0 1.5 2.0	30 s	Immobility + transparent with dark band on mantle and head	~120 s	Implantation of Visible Implant Fluorescent Elastomer tags	20–30 s	[547] Higher mortality amongst young animals with 2%
O. vulgaris	NS	200–350 (N = 15)	1.0–2.0	NS	NS	NS	Electrode implantation in brain	NS	[101]
O. vulgaris	20–24	673–1369 (N = 8)	2.5	NS	NS	NS	Anterior and posterior basal lobe removal	NS	[156]
O. vulgaris	20–22	500–1500 (N = 6)	2	NS	NS	NS	Mantle cannulation	NS	[545]
O. vulgaris		290–1040	2.5	NS	NS	NS	Branchial vessel cannulation	NS	[537]
O. vulgaris	15	1000–1500 (N = 28)	2.5 (in cold seawater)	NS	Total relaxation and mantle manipulable to access branchial vasculature	NS	Branchial vessel sampling	NS	[325]
O. vulgaris	22–25	150–500[h]	2.0	234 ± 33	Cessation of ventilation	Up to 10 min	Branchial heart injection, brain lesion, dorsal aorta catheter implantation, pallial nerve section	27 ± 13 s	[456]

(continued)

Table 10A. Continued

Species	T (°C)	Body size	Concentration (%)	Time to anaesthesia (s)	Criteria for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comments
O. vulgaris	22.3 ± 0.5	1268 ± 291[i]	1.0 1.5 2.0	from 240 to 50	Skin pallor	NS	PIT tag implantation	180–360 s	[131] At 1.5% (considered optimal) showed correlation between body weight (700 g-1130 g) and time (~60–100 sec) to anaesthesia
O. vulgaris	18	500–800 *F* (N = 6)	2	~300	Body pattern change	NS	Arm tip amputation	120–300 s	[431]
O. vulgaris	25–26	730–817 (N = 2)	1	420–480	Unresponsive to external stimuli, loss of righting reflex, cessation of ventilation[j]	1 min	Mixture of surgical interventions (not described)[k]	180–420 s	[439] shallow anaesthesia at 1%; also provides information on other octopus species
	19–21	150–490 (N = 7)	2	90–600		14 to 20 min (and more)		300	
	21	335 (N = 1)	3	360		>14 min		NS	
E. cirrhosa	10–11	350 (mean) (N = 12)	2	NS	NS	NS	Dorsal mantle skin lesion	NS	[189]
E. cirrhosa	14–15	500–800	2.5	NS	NS	NS	Branchial vessel haemolymph sampling	NS	[255]

[a]Juveniles (N = 6 for each concentration).
[b]Detailed description of stages of anaesthesia provided in the original study.
[c]10–12 weeks post hatch; N = 9.
[d]13–14 weeks post hatch; N = 4.
[e]DML: 18.5 cm (mean of N = 5).
[f]DML: 7.6– 17.1 cm.
[g]Young and sub-adult; N = 46.
[h]*M* + *F*, N = 10.
[i]N = 3 for each concentration.
[j]Additional detailed description in the original study.
[k]A study of anaesthesia only.

Table 10B. Magnesium chloride as an agent.

Species	T (°C)	Body size	Concentration (% or g/L)	Time to anaesthesia (s)	Criteria used for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comments
S. officinalis	15	365–890[a]	7.5[b]	300–720	Pallor, arm flaccidity, cessation of ventilation, loss of righting response, unresponsive to noxious stimulus	0–25 min	Surgery (no details)	120–1200 s	[447] for use to implant fin EMG electrodes see [548] $MgCl_2$ reported to be 'more reliable' than EtOH
S. officinalis	NS	DML: 122–240 mm (adult animals)	1.9	~600	Floating at surface, skin pallor, cessation of breathing and medial fin motion, unresponsive to gentle mantle pressure	<5 min	Implantation of data storage tag-includes skin incision and drilling cuttlebone	~20 min (full recovery)	[132]
S. officinalis	NS	DML: 170–205 mm (adult animals)	1.9	9–19 min *12.1 ± 3.25 min N = 9*	Floating at surface, skin pallor, cessation of breathing and medial fin motion, unresponsive to gentle mantle pressure	<5 min	Implantation of data storage tag-includes skin incision and drilling cuttlebone	NS recovery time longer for sub -adults	[130] In sub-adults using 1.9% $MgCl_2$ time to anaesthesia was 29–58 min (mean 36.7 ± 14.17 min, N = 4). Note: the sub-adults were caught in October but the adults were caught in May, therefore season could be a confounding factor
		DML: 173–457 132–180 mm (sub adults)	3.3	4–8 min *5.9 ± 1.2 min N = 10*					
S. officinalis	19.3 ± 1.4	48.2 ± 5.1 (N = 6; juveniles)	20 g/L	468.7 ± 88.9	Body colour, swimming behaviour, funnel suction intensity. Detailed description of stages in paper	180 (handling)	Investigation of anaesthetics (no procedures applied)	381.2 ± 62.1 s	[440] Study aimed to sedate animals for handling not for surgery. $MgCl_2$ considered the 'best' agent of the six studied
		Juveniles 62.9 ± 12.9 (N = 6)	27g/L	368.7 ± 77.8				353.2 ± 101.7 s	
L. forbesi	13	DML: 210 mm *M*	7.5[b]	90	Pallor, arm flaccidity, cessation of ventilation, loss of righting response, unresponsive to noxious stimulus.	300	Anaesthetic efficacy	98 s	[447]
D. pealeii	14	54.6[c]	30.5 g/L (0.15 mol)	186	Unresponsive to handling, immobile, loss of righting response	162.8 min (max 302 min)	Anaesthetic efficacy and implantation of electrodes in statocyst nerves	NS	[208] 3.8% mortality Repeated induction investigated; sedation time increased with induction number

(continued)

Table 10B. Continued

Species	T (°C)	Body size	Concentration (% or g/L)	Time to anaesthesia (s)	Criteria used for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comments
S. sepioidea	NS	42.2–290.9[d]	1.5–2	~300–420	Absence of body hardness, flexible tentacles, pallor	90 handling	Anaesthetic efficacy study	4–18 min (proportional to exposure time and concentration)	[198] Inking and mantle colouration changes on initial exposure. Also investigated $MgSO_4$ at 3–4% (similar to $MgCl_2$)
I. illecebrosus	8–15	300–500	7.5[e]	A few minutes	Muscle relaxation	NS	Cannula implantation in vena cava	300–420 s	[417]
O. vulgaris	22	120 *F*	7.5%[b]	780	Pallor, arm flaccidity, cessation of breathing, loss of righting response, unresponsive to noxious stimulus.	NS	Anaesthetic efficacy	480 s	[447] Study also investigated 20% $MgSO_4$ reported to be 'slightly less effective'
O. vulgaris	NS	58–1532 *M* (N = 71) *F* (N = 78)	3.5%	~900	Lack of spontaneous movement, complete relaxation and cessation of breathing	NS	Brain ultrasonography	NS	[246f]
O. vulgaris	17	NS	7.5%[b]	NS	NS	NS	Removal of arm segment	NS	[549]
O. vulgaris	NS	500–1000	7.5%[b]	NS	NS	NS	Brain lesion	NS	[539]
O. vulgaris	NS	158–428 (N = 17)	3.5% (in SW)	~1200	Lack of spontaneous movement, complete relaxation and cessation of breathing	~20 min	Removal of distal 10% of one arm	15–30 min	T. Shaw et al., pers. comm.
E. cirrhosa	14 15	301 (*F*) 845 (*F*)	7.5%[b]	1200 900	Pallor, arm flaccidity, cessation of ventilation, loss of righting response, unresponsive to noxious stimuli	0 min 3 min	Anaesthetic efficacy	460 s 420 s	[447]

[a]DML: 145–180 mm, *M* (N = 7).
[b]Isotonic solution in DW with an equal volume of SW (i.e. 3.75%).
[c]DML: 14.8 cm, average of N = 26.
[d]DML: 7.6–17.1 cm.
[e]In DW mixed 1:1 with SW; final concentration 3.75%.
[f]See also Margheri et al. for similar study of arm ultrasonography.[247]

Table 10C. Magnesium chloride and ethyl alcohol mixture as agents. In the column 'Concentration' values for EtOH and $MgCl_2$ are indicated with their respective concentrations.

Species	T (°C)	Body size	Concentration (EtOH + $MgCl_2$)	Time to anaesthesia (s)	Criteria used for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comment
S. officinalis	NS	200–1200	2% + 17.5 ‰	80–90	NS	NS	Drug microinjection in brain; electrolytic lesion	NS	[398] Addition of $MgCl_2$ is to 'prevent any muscular contraction during surgery'
O. vulgaris	24 ± 0.5	166–1268 *M* (N = 20) 89–1256 *F* (N = 15)	1% + 55 mM	~900	NS	NS	Haemolymph sampling; injection of LPS/PBS into arm	NS	[251]
O. vulgaris	NS	200–500	1% + 55 mM	~25–45 min	NS	NS	Tetanisation (Vertical lobe)	A few minutes	See supplementary data in Shomrat et al. [420]

Table 10D. Clove oil as an agent. Clove oil is diluted in SW unless otherwise stated. For the four studies with clove oil it must be noted that only two cite the source.

Species	T (°C)	Body size	Concentration	Time to anaesthesia (s)	Criteria used for time to anaesthesia	Duration of anaesthesia (s)	Purpose of anaesthesia	Recovery time	References and comment
S. officinalis[a]	19.4 ± 1.4	46.5 ± 9.0 31.1 ± 8.4	0.05 ml/L[b] 0.15 ml/L[b]	Anaesthesia not achieved	Body colour, swimming behaviour, funnel suction intensity[c]	NA	Investigation of anaesthetics-no procedures	NA	[440] Study aimed to sedate animals for handling not for surgery. Clove oil obtained from Omya Peralta, Germany
D. pealeii	14	83.5 (DML: 190 mm)	1 ml/L	Anaesthesia not achieved	Unresponsive to handling, immobile, loss of righting response	NA	Anaesthetic efficacy and implantation of electrodes in statocyst nerves	NA	[208] Traumatic reaction; died within 4 min. Clove oil source NS
O. vulgaris	22.3 ± 0.5	NS	20 mg/L 40 mg/L 100 mg/L	Anaesthesia not achieved	Skin pallor	NA	PIT tag implantation	NA	[131] Surgical anaesthesia not achieved at any concentration tested; exposure times not given. Clove oil source NS
O. minor[d]	25	NS	50 mg/L 100 mg/L 150 mg/L 200 mg/L 250 mg/L 300 mg/L	559 ± 52.6 317 ± 32.8 277 ± 17.5 265 ± 23.4 240 ± 24.0 230 ± 15.9	Pallor, arm inactivity, loss of suction, cessation of breathing	NA	Study of anaesthetic efficacy	586 ± 53.6 s 620 ± 60.8 s 678 ± 65.2 s 724 ± 60.3 s 797 ± 70.8 s 964 ± 72.5 s	[449] Mortality at >550 mg/L Also studied efficacy at 15 and 20°C and showed that, as temperature decreased, induction and recovery times increased. 200 mg/L considered optimal. Clove oil from Sigma USA

[a]Juvenile animals were utilised in this study.
[b]N = 6 for each concentration.
[c]Detailed description of stages of anaesthesia provided in the original study.
[d]Diluted in 95% EtOH and then 1:10 in SW; N = 10 for each concentration.

differences in 'efficacy' of these agents according to age[102] and body weight[131] of animals; and temperature of the solution.[449]

It should also be noted that the majority of systematic studies of anaesthesia in cephalopods involve the commonly used laboratory species, and particular care should be taken when attempting to anaesthetise other species, as responses to the same agent may differ markedly. For example, benzocaine (ethyl *p*-amino benzoate) produces a violent reaction followed by death in the squid *D. pealeii*,[208] but violent reactions are not reported in the octopus *E. dofleini* where it has been used for euthanasia.[450]

8.8.2 Criteria for general anaesthesia. A detailed discussion of criteria for general anaesthesia in cephalopods, based on a review of recent studies on cuttlefishes, squid and octopuses is now available.[8,440] They will only be outlined here, and further work is needed to define species-specific criteria for general anaesthesia.

Quantification of the physical (externally visible) parameters listed below might enable assessment of *putative* 'stages' or 'planes' of anaesthesia, and studies in cuttlefish[440] and octopuses[131,243,449] illustrate this approach. In particular, ventilation depth and frequency and chromatophore activity are the parameters most amenable to continuous real time quantification to monitor the onset of 'anaesthesia'. Currently there are no studies of electroencephalogram (EEG) activity in cephalopods under general anaesthesia, and all the parameters illustrated below rely on the assumption that the agents used to induce anaesthesia do not act only as neuromuscular blocking agents.

(i) *Decreased or absent response to a noxious stimulus*. This is the most important test that the animal is sufficiently anaesthetised for surgery or other procedures to commence. Without blocking pain perception general anaesthesia cannot be considered to have been achieved. Studies have used a mechanical stimulus (e.g. a pinch) applied to the arm, mantle or supraorbital skin as a test of insensibility to a noxious stimulus.[132,243,447] The selection of this type of challenge appears appropriate, as it is now known that cephalopods possess mechano-sensitive peripheral nociceptors,[10,11] and in future this knowledge will enable the identification of better-defined stimuli to test for insensibility. Assuming that there is evidence to suggest that the animal is insensible, the following criteria should also be evaluated.

(ii) *Depression of ventilation*. The initial reaction to exposure to the anaesthetic may be an increase in depth and frequency of mantle contractions with attempts to eject the solution via the siphon. Subsequently the frequency decreases progressively with time after exposure, and the coordination between the mantle and siphon become uncoordinated. With prolonged exposure, mantle and siphon contractions cease.

(iii) *Decreased chromatophore tone*. Although animals become pale overall with increasing time of exposure to the anaesthetic, flashing colour changes have been reported on initial exposure to anaesthetic agents (e.g. in *D. pealeii*[208] or in *S. officinalis*[440]). The overall paling of the animal is indicative of a decrease in the central nervous system drive to the chromatophore motorneurones.[201]

(iv) *Decreased locomotor activity, arm/tentacle tone and sucker adhesiveness*. The initial reaction to anaesthetic exposure may be an increase in activity (i.e. agitation, for review see Gleadall[439]), but activity gradually decreases, including swimming activity and fin movement, as for example in cuttlefish and squid.[132,451] Octopuses will tend to settle on the bottom of the tank as the arms and suckers begin to lose tone and adhesion, but anaesthetised cuttlefish may float near the surface of the tank.[132] The arms should be flaccid and readily manipulated.[449]

(v) *Loss of normal posture and righting response*. As animals (e.g. squid and cuttlefish) become deeply 'anaesthetised' they lose the ability to maintain a normal position in the water column or adopt an abnormal position with the arms, head and mantle at angles not normally seen in conscious animals. For example, in squid and cuttlefish the arms and head may appear unsupported by the mantle collar muscles; octopuses adopt a flattened appearance on the floor of the tank rather than the usual posture with the head raised. In addition, animals placed on their dorsal surface make no attempt to right themselves. The righting response returns after ventilation and chromatophore activity return,[243] and this is probably a good indicator of overall recovery from anaesthesia, as both effects require complex coordination of neuromuscular activity.

(vi) *Absence of a response to light*. The absence of a reaction to a strong light has been used as one sign of general anaesthesia (e.g. in Messenger et al.[447]), but this is poorly characterised.

8.8.3 Induction. A common practice for induction is to immerse the animal in the anaesthetic solution (made up in seawater) at its final concentration. However, to

minimise trauma it is preferable to expose the animal to a rising concentration of the agent (as for example in Yacob et al.[427]), which will also allow any adverse reaction to be quickly identified.

The animal should remain completely immersed in the anaesthetic solution for rapid effect. In addition, the use of a specialised closed anaesthetic chamber should be considered. The chamber could also be used as a transport box from the home tank to the operating room, and it may be possible to habituate at least some species (e.g. octopus and cuttlefish) to the box and train them to enter. This will reduce stress to the animals, and a closed chamber will prevent octopus escaping. As a general rule, animals should always be transported in seawater and movement should be minimised.

Anaesthetic solutions should always be freshly made, using filtered seawater, which is gassed (preferably with oxygen rather than air) and equilibrated to home tank temperature before immersing the animal. It is not good practice to anaesthetise an animal in a solution that has been used to anaesthetise another animal, as the water may contain chemical alarm signals. Animals must not be left immersed in an anaesthetic solution in which they have inked.

Although limited in scope, the pre-anaesthetic sedation technique applied by Packard[200] has not been followed by any other systematic study of methods to minimise the stress of general anaesthesia in cephalopods, as has been done for fishes.[452]

Moreover, there is no general agreement about whether cephalopods should be deprived of food prior to anaesthesia. Some studies remove food for 24 hours[440] while others do not.[208]

We are aware of only one report of food regurgitation by *S. sepiodea* during 'anaesthesia' in magnesium sulphate,[198] although defaecation is relatively common.

In view of the above, the anaesthetic protocol for cephalopods requires careful planning, including considering whether or not to withdraw food and, if so, for how long.

8.8.4 Maintenance and monitoring. Once the animal is fully anaesthetised (see above for criteria), it will usually be necessary to remove it from the anaesthetic chamber to perform a procedure. Anaesthesia must be maintained for the entire duration of the procedure, and physiological functions supported.[282,453,454]

Several authors describe apparatus (adapted from fish anaesthetic apparatus) for maintenance of anaesthesia during surgery (e.g. for cuttlefish:[132,418,454] for squid:[417,451]), and these could also be better tested, and also adapted for octopus.

Marked suppression or cessation of ventilation (indicated by mantle/siphon contraction) is a common feature of general anaesthesia in cephalopods, so it is essential that the mantle is perfused with oxygenated seawater/anaesthetic. Such perfusion will only be effective if the branchial and systemic hearts continue to function, but little is known of cardiac function under anaesthesia. However, it has been observed that heart rate is very low in *O. vulgaris* anaesthetised with $MgCl_2$ (M.G. Valentino and P.L.R. Andrews, unpublished observations) and in animals immersed in cold water.[243]

Monitoring of physiological function under anaesthesia and during surgery is clearly an area requiring research so that the extent of hypoxia/hypercapnia is known and its impact on post-operative recovery and procedures can be assessed. However, *O. vulgaris* appears to be able to recover rapidly from protracted periods of apnea[150] and Pörtner et al.[417] comment that cuttlefish and octopus are less sensitive to hypoxia than squid (as might be expected from their different lifestyles). Doppler ultrasound (e.g. as in D. Fuchs and G. Ponte, unpublished observations; Vevo 2100 Visualsonics, The Netherlands) offers the best technique for monitoring cardiovascular function, but non-invasive methods for real time monitoring of blood gases (such as oximetry) and metabolic status (e.g. NMR spectroscopy as in Melzner et al.[455]) need further development.

8.8.5 Recovery. The mantle should be flushed of residual anaesthetic solution and the animal then placed in clean aerated/oxygenated seawater. Ventilation can often start without intervention (depending upon the duration of anaesthesia), but gentle massage of the mantle is frequently used in cuttlefish, squid and octopods until spontaneous ventilation, as indicated by mantle and siphon movements, restarts. Other functions (sucker adhesion, chromatophore tone, righting) recover after ventilation recommences, usually in the reverse order to which they were lost.

Many studies monitor the time at which various functions return, but the time taken for full recovery of normal function from particular anaesthetic protocols is not known and, as most studies of anaesthesia do not involve surgery, the impact of surgery upon recovery is not known, but see Shomrat et al.[420] As currently assessed, recovery appears rapid (less than 15 mins, e.g. in[243,420,440]) and dependent on the procedure(s) performed, animals will usually take food quickly after 'anaesthesia' when returned to their home tank.[395,420] However, further monitoring criteria are needed to ensure that animals have fully recovered from the anaesthesia and any surgical procedure. If recovery from anaesthesia is as rapid as appears to be, and the anaesthetic agents used do not have residual analgesic properties, it is vital that suitable analgesics are administered – which at this time of writing may best be done

by infiltrating surgical sites with local anaesthetic (see next section below).

8.9 Analgesia and local anaesthesia

Directive 2010/63/EU, Article 14§4, requires 'an animal which may suffer pain once general anaesthesia has worn off, shall be treated with pre-emptive and post-operative analgesics or other appropriate pain-relieving methods, provided that it is compatible with the procedure'.

At the time of writing, there is no information on the efficacy of any analgesics in cephalopods, although both ketoprofen and butorphanol have been recommended.[445]

As nociceptors have been recently studied in cephalopods,[10,11] it should be possible to investigate the efficacy of systemically administered substances for potential analgesic activity. In addition to identification of mechano-nociceptors, Crook et al. also showed that injury to a fin in squid induced spontaneous activity and sensitisation at sites distant from the lesion including the contralateral body;[11] similar sensitisation of both the wound site and at distant sites has been reported in octopus.[10] This implies that potentially surgery at any surface site (but possibly anywhere including the viscera) could evoke a more general sensitisation of nociceptors. If this is the case, it is essential that suitable analgesic agents are quickly identified.

In the absence of the availability of systemic analgesics, it is recommended that local anaesthetics are used to produce localised analgesia either by infiltration into a wound site or local nerve block. Xylocaine (2%) and mepivacaine (3%) have both been shown to be effective in producing a block of transmission in the arm nerve cord lasting at least 1 hour (G. Ponte, M.G. Valentino and P.L.R. Andrews, unpub. obs.).[149,456] It should be noted that local anaesthetics acting on the fast tetrodotoxin (TTX) sensitive voltage gated sodium channels may not be effective in species such as the blue-ringed octopus which possesses endogenous TTX.[457]

Selective nerve block with infiltration of a local anaesthetic should also provide an interim means of preventing more generalised nociceptor sensitisation (see above), as it has been demonstrated that afferent nerve block by injected isotonic $MgCl_2$ prevented both local and distant sensitisation.[11]

8.10 Fate of animals at the end of a procedure

At the end of a procedure as defined in section 8.1 above, a decision about the fate of the animal is required. The Directive identifies three possibilities:

Humane killing: Article 17 requires that 'at the end of a procedure' an animal must 'be killed when it is likely to remain in moderate or severe pain, suffering, distress or lasting harm'. Other animals should also be humanely killed at the end of procedures, unless 'a decision to keep an animal alive' has been taken 'by a veterinarian or another competent person', when the possibilities described below should be considered. Methods of humanely killing cephalopods are discussed in section 8.11 below.

Release or re-homing: An animal 'may be... returned to a suitable habitat or husbandry system appropriate to the species', provided that: 'its state of health allows it; there is no danger to public health, animal health or the natural environment; and appropriate measures have been taken to safeguard [its] well-being' (Article 19). This could include release to the animals' natural environment, transfer to public aquaria, educational or other competent holding facilities. However, in general, cephalopods should not be returned to the wild, except in studies where, following a procedures, animals are released immediately at the exact location where they were captured, during a relevant season for migratory species, having been certified fit by a veterinarian or other competent person (Articles 17§2 and 19). Any requirements of other national and international legislation regarding the release of animals to the wild must also be met.

Re-use: Animals may also be considered for re-use, provided certain conditions are met.

Article 16 defines re-use as 'use of an animal already used in one or more procedures, when a *different* animal on which no procedure has previously been carried out *could also be used*. By definition, re-use applies to situations in which the objectives of the first and second procedures are *unrelated*.[†††]

Reuse is only permitted when:

- 'the actual severity of the previous procedure(s) was 'mild' or 'moderate';
- the animal's 'health and wellbeing has been fully restored;
- the further procedure is classified as 'mild', 'moderate' or 'non-recovery' (see section 8.1); and
- the proposed re-use 'is in accordance with veterinary advice, taking into account the lifetime experience of the animal'.[‡‡‡]

[†††]This distinguishes reuse from situations where the scientific objective can *only* be achieved by using the same animal in more than one procedure (this is known as *continuing use*, although the term is not used in the Directive).

[‡‡‡]For further discussion and examples, see pp. 8–10 in EU National Competent Authorities endorsed document (2011), available at: http://ec.europa.eu/environment/chemicals/lab_animals/interpretation_en.htm

8.11 Methods of humane killing

The ultimate fate of cephalopods in the majority of studies will be humane killing. All personnel involved in humane killing should be trained and be familiar with the principles of good practice, such as those set out by Demers et al.[458]

Article 6 requires that, whenever animals are killed:

- it should done with minimum pain, suffering and distress by a competent person; and
- one of the methods of killing listed in Annex IV (section 3) of the Directive should be used, followed by confirmation of death, using one of the methods listed in Annex IV section 2.

However, whilst methods listed for fish might be applied to cephalopods, Annex IV offers no specific guidance on methods for humanely killing cephalopods. Although the CCAC[459] take the view that the priority is a rapid loss of consciousness, these guidelines concur with the view of Hawkins et al. from a discussion of CO_2 killing in vertebrates that 'it is more important to avoid or minimise pain and distress than it is to ensure rapid loss of consciousness' (p. 2).[460]

We have described some possible methods below, but the efficacy, and level and nature of any suffering caused, have not been comprehensively evaluated for all of these techniques, and further research is needed. Animals should not be killed in the rooms used to house other animals nor within sight of conspecifics. It must also be ensured that blood or chemical alarm signals cannot be detected by other animals (e.g. by entering the water system). Whichever method is used in a given species, the potential impacts of factors such as body weight, age, sex, season and water temperature on the efficacy of the method must be considered.

8.11.1 Chemical methods. Chemical methods for killing cephalopods are based on an overdose of anaesthetic agents, by using either a higher concentration and/or longer exposure time than that needed for anaesthesia. As also outlined above, these methods all have the potential to cause adverse effects prior to unconsciousness, such as skin or eye irritation, or sensation of asphyxia. To reduce and avoid any such suffering, animals should be exposed to a gradually rising concentration of the anaesthetic, and *not* directly immersed in a solution at the full concentration needed to cause death. In tropical and temperate species, cooling may be used as an adjuvant to the anaesthetic for humane reasons, and could also reduce *post-mortem* tissue damage if tissue is required for *in vitro* studies.

A previous review of this topic proposed the following protocol as suitable for *S. officinalis*, *D. pealei, O. vulgaris* and *E. cirrhosa*: 'At least 15 minutes immersion in $MgCl_2$, with a rising concentration [optimal rates to be determined], ending with a final concentration of at least 3.5% in the chamber used for humane killing; possibly enhanced by using chilled solutions or with the clove oil's active ingredient eugenol, followed by immediate mechanical destruction of the brain' (Andrews et al., p. 61).[8]

It was further proposed that if the brain was needed, the immersion period should be extended to more than 30 minutes to ensure unconsciousness as required in Directive 2010/63/EU (Annex IV, 1(a)) prior to removal of the brain. If the brain is not removed for study, confirmation of permanent cessation of circulation (see 8.12 below) is also considered as a possible method for completing killing according to Directive 2010/63/EU (Annex IV, 2(a)).

8.11.2 Mechanical methods. When carried out by highly skilled operators, death by mechanical destruction of the brain takes only a few seconds, but the nature and degree of any suffering is unknown. For this reason we take the view that cephalopods should not be killed by this, or any other, mechanical method without prior sedation/anaesthesia. However, it may be possible to utilise a 'mechanical method' if it can be justified and is authorised by the National Competent Authority as a specific regulated procedure within a project application.

Electrical methods such as 'Crustastun' used for humanely killing crustacea, such as lobsters and crabs,[461] might also be considered and evaluated for their suitability in terms of animal welfare. It will be particularly challenging to develop humane methods for use when the brain is required intact, but where the use of anaesthesia may be a confounding factor.

8.12 Confirmation of death

Use of a method for confirming death following humane killing is mandatory, and options are listed in Annex IV§2 of the Directive. Two of the methods listed, i.e. 'dislocation of the neck' and 'confirmation of onset of *rigor mortis*' are impossible for cephalopods – the latter because it does not occur in cephalopods.

This leaves three possible methods: *i.* confirmation of permanent cessation of the circulation; *ii.* destruction of the brain; or *iii.* exsanguination.

Confirmation of permanent cessation of the circulation and exsanguination. Octopuses, cuttlefish and squid have two branchial hearts that move blood through the capillaries of the gills.[462] A single systemic heart (the only one in nautiloids) then pumps the oxygenated blood through the rest of the body. The heart(s) may continue beating for some time after permanent cessation of breathing, so transection of the dorsal aorta/vena cava may be used. Transection of the dorsal aorta/vena cava will be effective in inducing exsanguination if the systemic heart is able to

pump effectively (i.e. the anaesthetic used does not supress cardiac function); also note that the systemic heart is distension sensitive.[463] Finally, the possibility of transection of the branchial aorta afferent to the heart, at the level of the auricle, should be further explored considering the easy access to them through the mantle cavity nearby the gills (G. Ponte and G. Fiorito, pers. comm.).

The effectiveness of exsanguination as a method of killing is not known.

Freezing (below −18°C for several hours) after killing may be a further means of confirming cessation of circulation and hence death that does not necessarily entail destruction of the body.

Destruction of the brain may be difficult to ensure in some species because of the location and relatively small size (e.g. *Nautilus* sp.) although this can be overcome by training and a detailed knowledge of the cranial anatomy of the relevant species.

The methods used for humane killing and confirmation of death should always be included in publications.

Article 18 of the Directive requires member states 'to facilitate the sharing of organs and tissues of animals killed' where appropriate. Researchers should be encouraged to use tissue from animals killed in other projects for *in vitro* research (e.g. tissue bath pharmacology), rather than killing an animal specifically/only to obtain tissues; and consideration should be given to setting up banks of frozen and fixed tissue to optimise animal use.

9. Risk assessment for operators

This section will focus on the potential risks from the direct handling of cephalopods in a laboratory setting, but will not cover the more generic risks associated with working in either a laboratory (e.g. tissue fixatives, reagents) or a marine aquarium environment (e.g. tank cleaning agents, slipping, electricity in a wet environment). However, all personnel involved in research should be appropriately educated, trained and competent to perform any task relevant to the research. Personnel should be actively involved in risk assessment and management, and incident reporting encouraged.

9.1 Personnel to consider

The following personnel should be considered and their risks assessed:

a. Fishermen, divers or others responsible for the capture of cephalopods in the wild. Although such people may not be employees of the institution/facility, as far as possible, the institution/facility should be assured that safe practices are being employed. The Directive specifies that, in cases where justification is provided, to obtain animals from the wild 'competent persons' (Article 9) should be involved. As part of competency assessment issues related to health and safety practices of cephalopod capture should be explored.
b. Animal carers and technicians. This includes anyone involved in cleaning and feeding animals; cleaning animal rooms and equipment (e.g. tanks, filters).
c. Animal technologists or research laboratory technicians. These are personnel who may be involved in manipulating animals during experiments, sampling biological fluids, euthanasia and necropsies.
d. Principal and other Investigators: All personnel involved in performing research experiments including *in vivo* regulated procedures and *in vitro* handling of live tissue.
e. Designated veterinarian (or other suitably qualified expert).
f. Personnel responsible for the disposal of animal remains.

9.2 Risk identification, prevention and protection

9.2.1 Physical risk. Bites of cephalopods are produced by the hard beak-like jaws associated with powerful musculature of the buccal mass located at the center of the arm crown. Such bites do not always penetrate the skin of human beings (e.g. see p. 68 in Wells).[150] The effects of penetrating bites are exacerbated by enzymes, venoms, other bioactive substances and microorganisms in the saliva. The possibility of injury should not be overlooked even when animals are transported in a plastic bag.[464]

The arms of all cephalopods are relatively strong, and this is especially the case in larger octopuses where the grip is enhanced by the numerous suckers. Areas of erythema may be induced if attempts are made to detach an animal by pulling in air, rather than allowing it to leave the arm naturally under water. Special precautions should be used if handling members of the teuthoid family as their arms/tentacles have hook-like appendages. Staff should be trained in how to remove animals that have become attached to their arms using the minimum of force and without inducing the animal to bite.

The use of Personal Protective Equipment (PPE), such as gloves or gauntlets resistant to penetration, may be suggested, but care should be taken to ensure that the gloves do not harm the delicate skin of cephalopods and that safe handling is not impaired by wearing gloves. In addition, the typical behaviour of the animals should be well recognised and mostly for signs of imminent aggression, escape attempt and other putative abnormal behaviours.

9.2.2 Chemical risk

Direct contact with mucus, faeces and biological fluids. Biological materials can represent a risk of allergy, intolerance and/or toxicity particularly with repeated exposure. Exposure to mucus, faeces and ink may occur during routine cleaning and handling and other fluids during autopsy. Faeces and blood (haemolymph) are also potential routes of infection. We are not aware of reports of reactions to these biological fluids, but in case of doubt the use of waterproof gloves is recommended; in addition, in case of inadvertent contact with any biological substances, hands should be properly washed immediately.

Handlers and those undertaking autopsies should also be aware of any experimental procedures previously undertaken involving the administration of potentially hazardous substances (e.g. infectious agents, radioactive material, drugs, nanoparticles) to the animals so that assessment of potential risks can be undertaken.

Venom, enzymes and other pharmacologically active substances. The secretion from the posterior salivary glands of coleoid cephalopods (see Table 4.1 in Wells[150]) is injected into prey via the salivary papilla to immobilise and digest it with a mixture of venoms (i.e. cephalotoxin[465]), enzymes (e.g. chitinase, carboxypeptidase, hyaluronidase, phospholipase A2) and other biologically active substances (e.g. 5-hydroxytryptamine, dopamine, substance P) as reviewed by Ruder et al.[466]

A localised or systemic response could be induced by one or more of these substances particularly in sensitive individuals, but documented examples of systemic reactions to bites are rare except in the case of the blue-ringed octopus.

In fact, the venom of blue-ringed octopuses (*Hapalochlaena* spp.) can be fatal[467] unless there is prompt medical attention.[467,468] The toxin involved is the potent sodium channel blocker TTX[469] that is found in the posterior salivary glands, skin, branchial hearts, gills and Needham's sac,[470] so care should also be taken with handling these animals *post mortem*. Recently, TTX has been found in the eggs with the levels increasing after laying;[471] therefore, the risk with this species does not only come from adults. Other data show that the venom is produced by symbiotic bacteria (*Aeromonas*, *Bacillus*, *Pseudomonas* and *Vibrio*) found in the salivary glands.[472] Clinically, the bite of the blue-ringed octopus is most often painless but freely bleeding. Erythema and edema at the bite site usually occurs, but the most important effects are those that are systemic. Most severe envenomations are characterised by generalised weakness, slurred speech, circumoral paraesthesias, respiratory difficulty and dysphagia. Such symptoms may last for 12–24 hours. There is no antivenom, therefore treatment includes pressure immobilisation and immediate transport to a medical facility while monitoring respiratory and neurologic status.[473]

No reactions specifically attributable to the venoms from other species of cephalopod have been described, but there are several reports of localized reactions to bites from cephalopods although these seem rare. Wells reports having been bitten himself 10 or 20 times without event, but also describes a reaction to an *O. vulgaris* bite on the forearm in a student who had previously never been exposed to cephalopods.[150] The response included swelling of the forearm (comparable to a bee sting) and overnight pain, both of which resolved the next day. A local skin reaction probably related to proteolytic activity has been described after red octopus (*O. rubescens*) bite.[474] Haemolytic activity against mammalian red cells has been reported *in vitro* with low concentrations of saliva from *E. cirrhosa*,[475] but we are not aware of any evidence for haemolysis *in vivo*, although caution should be exerted.

Reactions to a bite will depend upon the sensitivity of the individual and this may be a particular issue with atopic individuals or those who have become sensitised by repeated exposure. The reaction to a bite may not be immediate as indicated by a case report of giant cell-rich granulomatous dermatitis/panniculitis one month after a bite from an octopus (species not given) on the wrist.[476] Bites are also a source of infection (see below) and again the reaction may be delayed.

Precautions should be the same as for the protection against bites (see above). All occurrences of bites by cephalopods should be recorded in the laboratory safety book, medical/paramedical advice sought particularly in cases where the skin is broken and the clinical outcome monitored. Anderson et al. reported anecdotally that immediate hot water treatment was effective in neutralising the localised effects of the bite of *O. rubescens*.[474]

Seawater and ink. Cephalopods in general and octopuses in particular can forcibly squirt seawater and/or ink directly at handlers. In addition to melanin, ink contains an array of bioactive substances including enzymes (e.g. tyrosinase, tyrosine hydroxylase) and other chemicals (e.g. dopamine, 5,6-dihydroxyindole).[477,478] There is a theoretical risk that the seawater may be contaminated by pollutants or infectious agents in open systems but seawater or ink in the eye could cause irritation and also distract the handler from their task increasing the risk of a bite or animal escape. Wearing eye or face protection should be considered when handling cephalopods.

9.2.3 Biohazards

The animals. Animals, especially those taken from the wild, can transmit infectious agents (zoonoses) to humans and as cephalopods host a number of bacteria (Gram + and −), viruses and parasites; this is a potential risk for anyone in contact with cephalopods. The major risk of transmission is via accidental ingestion or a bite.

This section will not cover the potential pathogenicity of different agents, however, mention should be given to, for example, *Anisakis* and *Aggregata* that are known to be the cause of the zoonotic disease, as reviewed by Yang et al.[479]

Most information regarding infection with *Anisakis* comes from human consumption of uncooked cephalopods, but poor hand hygiene and laboratory practice – especially when undertaking autopsies of fresh animals – means that hand-to-mouth transmission is a possibility. An additional potential source of parasites (and possibly bacteria, viruses and toxins) is the fish, crustacea and molluscs used as food for cephalopods so wearing gloves and hand hygiene should also be considered.

The main documented risk is bacterial infection from bites although the bacteria may originate from contaminated water as well as the animal itself.[480–482]

Particular attention should be paid when handling cephalopods with skin lesions as these wounds are often infected (see section 4.4).

Seawater. Seawater itself is a potential reservoir and transmission vehicle for infectious organisms, chemical pollutants and toxins (e.g. from algae) particularly in open systems. In seawater, many pathogens can be responsible for infections, e.g. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Mycobacterium marinum*, *Vibrio vulnificus*, *Erysipelothrix rhusiopathiae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Prototheca wickerhamii*.[473]

These represent a potential hazard for both animals and humans who have contact with the seawater but the risk should be minimal if the water quality is monitored and maintained within strict limits. Additional safety precautions may need to be put in place if there is a breakdown in water quality management especially for those who may need to decontaminate and clean the tanks.

Allergens. Repeated exposure to animals, chemicals (e.g. some disinfectants) and some disposables can result in the appearance of several clinical forms of allergic reaction (e.g. contact dermatitis or urticaria). To minimise the allergy risk operators should have a medical assessment before starting work and at regular intervals. Cephalopod mucus can be an irritant and has been occasionally described as an allergen (A. Affuso, pers. comm.). Since there are cases of an acquired allergy to cephalopod eggs, it is recommended that they are handled wearing gloves. Arginine kinase from *Octopus fangsiao* has been shown to react with IgE in the serum of octopus-allergenic subjects,[483] emphasising the importance of identifying individuals who may be especially sensitive to cephalopods before they begin work so that the risk can be assessed and managed.

9.3 A summary of practical advice

9.3.1 Assess and manage the potential risks. Each person involved with cephalopods should be assessed, and monitored for potential risks, notes taken of the species involved and the work to be undertaken before any work assigned. For example, the risks in moving a potentially lethal blue–ringed octopus or a 30 kg *E. dofleini* between tanks are different from moving a 200 g cuttlefish. Protocols of good laboratory practice (GLP) – Standard Operating Procedures (SOPs), should be developed in conjunction with the Health and Safety officer or other person, incorporated into training programmes (see section 9) and common protocols (e.g. handling, cleaning, humane killing) displayed or kept in a file in the facility and in the room dedicated to procedures. Risk assessment will also need to comply with local 'out of hours' policies. All working areas should be equipped with required PPE (overalls, hypoallergenic gloves, safety glasses, eye wash) and have a telephone with an emergency number displayed.

9.3.2 Operator health. Most staff undergo some form of health assessment at the beginning of employment. Anyone working with cephalopods should be asked if they are allergic to these animals or if they are atopic so that risks can be managed. All staff with regular exposure to cephalopods should be monitored regularly for signs of sensitisation.

9.3.3 Recording incidents. All incidents (including 'near misses') should be recorded partly to facilitate development of improved protocols. For example, skin-penetrating bites must be recorded and reported immediately to the relevant person so that appropriate action can be taken and any delayed reactions (e.g. infections) documented.

The 'incident book' should be reviewed regularly and protocols and policies modified as required. Lessons for the wider cephalopod community should be posted on the CephRes web site (www.cephalopodresearch.org) and if possible published.

9.3.4 Dealing with incidents. Staff should be familiar from training and laboratory SOPs/GLP with action to be taken in particular circumstances. The most likely incident requiring immediate action is a bite in which the skin is broken and the wound infiltrated with

secretions from the posterior salivary glands and/or seawater. A protocol for this and other eventualities should be drawn up in consultation with the trained on-site first responder and a medical practitioner.

10. Education and training: carers, researchers and veterinarians

The Directive requires that all staff involved in regulated research are adequately educated and trained for tasks they are required to perform (Article 23) including: *i*. carrying out procedures on animals; *ii*. designing procedures and projects; *iii*. taking care of animals; or *iv*. humane killing of animals.

Education and training therefore includes all staff involved in daily care (including veterinarians; since courses on marine invertebrate medicine are rare), researchers performing procedure and principal investigators designing studies. Anyone having direct contact with the animals will need to be able to demonstrate that they are practically competent in performing tasks or regulated procedures and have this competence assessed periodically. There may also be an argument for providing limited theoretical training for members of an institutional project/ethical review committee about novel species.

Trained individuals should understand and be able to demonstrate the importance of the animal welfare regulations and guidelines for housing, care and use, assessment of animal welfare including signs of illness, PSDLH and their palliation or treatment.

Developing training programmes to meet these requirements is a particular challenge for cephalopods as although knowledge of general care and welfare is relatively well developed (as reviewed in Fiorito et al.[2]), knowledge of PSDLH and their application to assessment of animal welfare aspects of a project are less well established.[3,8] In addition, there are multiple species of cephalopod with a variety of care (see also Appendix 2) and welfare requirements. Many aspects of training will need to be delivered by animal technologists, researchers and veterinarians who have gained experience prior to the regulation of research involving cephalopods, but it would be desirable to involve some trainers with expertise in working with aquatic vertebrates (including expertise in ethical review).

Courses will need to align with specific national requirements, and will need to be recognised by the National Competent Authority (http://ec.europa.eu/environment/chemicals/lab_animals/ms_en.htm) of Member States as fulfilling the requirements of the Directive 2010/63/EU for all persons involved in the use, care and breeding of cephalopods for scientific procedures. Recognition at EU level will facilitate movement of personnel between member states. The content and delivery of the modules should be validated by a university or other competent awarding body, and linked to Continuing Professional Development (CPD) programmes of professional bodies.

Training modules should be designed taking into account of the EU Commission working document of a development of a common education and training framework to fulfil the requirements under the Directive.

Ideally courses should be offered at EU level but as many aspects of training are 'hands-on' courses will need to be based in facilities with aquaria and access to several species. It is proposed that a training course should be structured into three modules; basic training, a species-specific module, procedures, PSDLH assessment and management module. These modules cover the key welfare assessment competencies and welfare training topics outlined in Tables 12 and 13 of Hawkins et al.[484]

Delivery and assessment of modules are not discussed here.

10.1 Indicative content of modules

10.1.1 Basic module: an introduction to cephalopods in research. This should cover: *i*. national and EU legislation on protection of animals used for scientific purposes; *ii*. a brief introduction to cephalopod biology; *iii*. why cephalopods were included in the Directive; *iv*. the philosophy of the institution regarding animal care and use; v. the requirement to comply with all national regulations and institutional guidelines; *vi*. the key differences between undertaking research in a legally regulated and an unregulated environment; *vii*. record keeping; *viii*. the requirement to respond immediately to any PSDLH issues; *ix*. reporting animal care and use concerns at institutional and national levels; *x*. health, safety, risk assessment and security; *xi*. roles of the institution and project ('ethical') review committee, veterinarian (or other competent expert), animal care, and research staff in the animal care and use programme; *xii*. public engagement.

10.1.2 Species-specific module. This focuses on the species utilised for research in a particular institution and should include the following species-specific topics: *i*. biology and behaviour; *ii*. supply, capture and transportation (including any additional permits and regulations); *iii*. environment (tanks, water, enrichment) and control; *iv*. signs of health, welfare and disease; *v*. assessing when an animal should be killed humanely; *vi*. specialised techniques for identification of individuals; *vii*. anaesthesia and humane killing; *viii*. tagging and marking; *ix*. genotyping; *x*. analgesia/anaesthesia/euthanasia/confirmation of death.

10.1.3 Procedures, PSDLH assessment and management module. This module has three main elements:

a) Project/licence and ethical committee applications. This will cover: *i*. experimental design from a 3Rs perspective (see section 2.2.2; see also Smith et al.[3]); *ii*. principles of harm-benefit analysis (e.g. Bateson cube); *iii*. principles of severity assessment (prospective, actual and retrospective); *iv*. setting humane endpoints; *v*. writing a lay summary of the project/ethical application; *vi*. public engagement.

b) Recognising PSDLH and their management. This component deals with these aspects in depth as this module is intended for those designing projects or performing procedures some of which may never have been performed in a cephalopod previously. The following topics will be covered: *i*. evidence for the capacity of cephalopods to experience PSDLH (see review and discussion in Andrews et al.[8]); *ii*. recognising PSDLH and techniques to minimise and treat them in the context of a regulated procedure; *iii*. special considerations regarding senescent cephalopods.

c) Procedures. This covers: *i*. what is a procedure within the meaning of the Directive?; *ii*. an introduction to basic surgical techniques and post-operative monitoring and care; *iii*. non-surgical procedures, techniques and assessing their impact on the animal.

d) Reporting studies under the Directive. Annual statistical return (Article 54) and an introduction to the ARRIVE Guidelines (www.nc3rs.uk/ARRIVE;[272]) and the Gold Standard Publication Checklist.[485,486]

Education and training of all personnel involved in the research programme at whatever level is essential to ensure the optimal care and welfare of animals and for the standards to improve with time by identification and dissemination of examples of good practice. These Guidelines and recent publications[1,2,487] establish the core material required for the delivery of the modules outlined here as a basis for education and training of personnel involved in research now regulated by the Directive. The next step will be to develop the above outlines into a document that can be used for accreditation of a course compliant with FELASA recommendations for laboratory animal science education and training, as outlined by Nevalainen et al. and recently updated.[488,489]

11. Concluding comment

This paper represents the first attempt by members of the international cephalopod community to develop guidelines for Care and Welfare of cephalopods utilised in scientific research. Although the guidelines primarily address the requirements of Directive 2010/63/EU, we anticipate they will also be utilised by the wider cephalopod research community outside the EU. It is recognised that in contrast to equivalent guidelines for vertebrates the evidence base for some aspects of these guidelines is not strong.

It is hoped that this paper will prompt research directed specifically at the Care and Welfare of cephalopods in the laboratory to provide a solid evidence base for future revisions of these guidelines.

Disclaimer

These Guidelines represent a consensus view, but inclusion in the authorship or contributor list does not necessarily imply agreement with all statements.

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Appendix 1.
Summary of key information required in a project application under the Directive (see for further detailed information Smith et al., 2013)[1]

Information required in applications for project authorisation

Directive 2010/63/EU Annex VI and Article 38(2)e

1. Relevance and justification of the following:
   (a) use of animals including their origin, estimated numbers, species and life stages;
   (b) procedures.
2. Application of methods to replace, reduce and refine the use of animals in procedures.
3. The planned use of anaesthesia, analgesia and other pain relieving methods.
4. Reduction, avoidance and alleviation of any form of animal suffering, from birth to death where appropriate.
5. Use of humane end-points.
6. Experimental or observational strategy and statistical design to minimise animal numbers, pain, suffering, distress and environmental impact where appropriate.
7. Re-use of animals and its accumulative effect on the animals.
8. The proposed severity classification of procedures.
9. Avoidance of unjustified duplication of procedures where appropriate.
10. Housing, husbandry and care conditions for the animals.
11. Methods of killing.
12. Competence of persons involved in the project

Purposes of procedures permitted

Directive 2010/63/EU Article 5

Procedures may be carried out for the following purposes only:

(a) basic research;
(b) translational or applied research with any of the following aims:
   (i) the avoidance, prevention, diagnosis or treatment of disease, ill-health or other abnormality or their effects in human beings, animals or plants.
   (ii) the assessment, detection, regulation or modification of physiological conditions in human beings, animals or plants.
   (iii) the welfare of animals and the improvement of the production conditions for animals reared for agricultural purposes
(c) for any of the aims in (b), in the development, manufacture or testing of the quality, effectiveness and safety of drugs, food- and feed-stuffs and other substances or products;
(d) protection of the natural environment in the interests of the health or welfare of human beings or animals;
(e) research aimed at preservation of the species;
(f) higher education, or training for the acquisition, maintenance or improvement of vocational skills;
(g) forensic inquiries.

Questions that should be addressed in relation to the Three Rs in writing a project application, and in the project review and approval process

Replacement

(i) What on-going efforts will you make to identify 'scientifically satisfactory' alternative methods that could replace the use of some or all animals? (Article 4§ 1)
(ii) Could you avoid the use of animals by asking different type of question or making better use of existing data or literature to address the scientific objectives?
(iii) Could *in vitro* studies or *in silico* (computer)-modelling be used to replace some or all of the animals?

Reduction

(iv) How will you ensure that the number of animals used in the project, and in individual studies within the project, is 'reduced to a minimum without compromising the scientific objectives'? (Article 4§2)
(v) Could any further reductions be made, e.g. by taking expert statistical advice to help optimise experimental and statistical design?

Refinement

(vi) How have you refined the 'breeding, accommodation and care of the animals' and the 'methods used in procedures', so as to 'reduce to the

[1]For full reference to the cited document see Reference List (#3) at the end of the paper.

minimum any possible pain, suffering, distress or lasting harm to the animals' throughout their lives? (Article 4§3)

(vii) Have you considered and implemented all the possibilities for refinement described elsewhere in these guidelines? (see Smith et al. 2013 for examples of refinement in the context of specific procedures).

(viii) How will you ensure that all relevant personnel working on the project are adequately educated and trained, and are supervised until they have demonstrated their competence in the procedures? (see section 10)

Appendix 2. Recommended species-specific minimal requirements for care and management of cephalopods under Directive 2010/63/EU. The appendix is organized into sub-sections providing information about housing, environmental parameters, transport, and feeding.

Appendix 2A (Housing) Recommended species-specific minimal requirements for housing for the establishment, the care and accomodation of cephalopods.[a] Data for different body size/life stages are included when available.

	Nautilus sp.	Cuttlefishes		Squids		Octopuses
Life stages	Juveniles, adults	Juveniles	Adults[b]	Juveniles[c]	Adults	Juveniles, adults
Structural materials	Fibreglass, PVC, acrylic, glass or any non-toxic material	Polycarbonate, glass or any non-toxic materials	Polycarbonate, glass or any non-toxic materials	Fibreglass, PVC, acrylic, glass or any non-toxic material	Fibreglass, PVC, Acrylic, glass or any non-toxic material	PVC or glass (for glass tanks see recommendations in main text)
Pipe materials	Copper and heavy metal free, PVC[d]	Copper and heavy metal free	Copper and heavy metal free	Copper and heavy metal free	Copper and heavy metal free	Copper and heavy metal free, PVC[c]
Recommended tank design	Cylindrical (height/width > 1)	Maximal horizontal surface area, rounded ends	Maximal horizontal surface area, rounded ends	Circular/elliptical	Circular/elliptical	Any shape
Recommended internal tank colour	Dark	Opaque	Opaque	Opaque; Contrasted or dark sides so that the squid are able to detect	Opaque; Contrasted or dark sides so that the squid are able to detect	Opaque-grey; Mirrored surfaces should be avoided[e]
Tank internal surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Minimum suggested size		0.005 square metre per animal	0.48 square metre per animal			0.30 square metre per animal is recommended
Lid cover	No (to control light levels a dark plastic drape or a solid lid with a few holes drilled in the drape/lid to allow dappled light into the tank and communicate the daily light cycle)	No (but one shelter per animal in the tank)	No (but one shelter per animal in the tank)	No	No	Yes. Transparent covers, firmly secured to the tank
Drain cover	Yes	No	No	Suspended drains with netting		Yes
Mesh size	No mesh	120–160 mm	120–160 mm	Small	Small	No mesh
Light source	Standard fluorescent lighting is acceptable	Halogen lamps are recommended. Standard fluorescent lighting should be avoided.	Halogen lamps are recommended. Standard fluorescent lighting should be avoided.	Can be overhead lighting via normal strip lights or by 400w halide (10–100 lx)	Can be overhead lighting via normal strip lights or by 400w halide (10–100 lx)	Halogen lamps are recommended. Standard fluorescent lighting should be avoided.
Recommended minimum water volume	30 L/animal	2 L/animal	80 L/animal	220 L	1500 L	100 L/animal (depending from the body size of the animal)

(continued)

Appendix 2A (Housing) Continued

	Nautilus sp.	Cuttlefishes		Squids		Octopuses
Life stages	Juveniles, adults	Juveniles	Adults[b]	Juveniles[c]	Adults	Juveniles, adults
Recommended minimum water high	1.5 m	0.05 m	0.4 m	0.6 m for a 1.8 m tank	0.6 m for a 1.8 m tank	0.40 m
Group rearing	Yes	Possible but it is preferable individual housing	Possible but it is preferable individual housing	Yes	Yes	Depending on species (not recommended for *Octopus vulgaris*)
Recommended maximum stocking density	Few individuals	200/square metre	2/square metre	Dependent on size and volume of tank; e.g in a 2-m circular tank, 10–15 individuals of *Loligo* species (150 to 250 mm ML). No less than 1 squid per 58 L		Individual rearing, preferred
Enrichment	Yes (Few smooth vertical PVC pipes attached to the side of the tank to allow the animal for its natural movement). Adding texture (artificial coral reef) to at least one wall of the tank may make it more attractive to the animal.	A shadow area should be provided in each tank. A fine layer of gravel/pebbles where cuttlefish can settle is recommended in each tank. Sand can be provided in the tanks		Substrate can be placed on bottom but be aware that it needs to be maintained to reduce bacterial problems. Fake seaweed and overhead shelter to provide shadow may also be used to give areas of shelter		Each tank should be provided with a den for the animal and a layer of sand

[a]These requirements do not take into account those adopted and suggested for rearing cephalopods for purposes of aquaculture (for review see Iglesias et al.[1]).
[b]Sexually mature females and males should be in distinct tanks to avoid fighting.
[c]Schooling behaviour in *Sepioteuthis lessoniana* occurs around 20 days after hatching and that synchronisation of individuals into shoaling increased over time.[2]
[d]Indicated as suggested material.
[e]Although recommendation to avoid mirroring surfaces in the tank is available only for *O. vulgaris*, we suggest the same precaution will be utilized for all cephalopod species.

Appendix 2. Recommended species-specific minimal requirements in relation to environmental parameters[a] to be applied for the establishment, the care and accomodation of cephalopods.

Appendix 2B (Environmental parameters)

	Nautilus sp.	Cuttlefishes	Squids	Octopuses
Temperature range	14–26°C (preferably 14–17°C) In accordance with the species of which are being held	12–25°C In accordance with the species of which are being held	In accordance with the species of which are being held	16–26°C In accordance with the species of which are being held
Salinity range	34–36 ppt	29–33 ppt	30–36 ppt	32–35 ppt
pH range	8.0–8.5 (preferably 8.2) (within the range of the natural fishing site)	7.8–8.1 (within the range of the natural fishing site)	7.8–8.3 (within the range of the natural fishing site)	7.9–8.3 (within the range of the natural fishing site)
Max [NH_4]	<0.10 mg/L	<0.5 mg/L	<0.01 mg/l	<0.10 mg/L
Max [NO_2]	<0.10 mg/L	<0.2 mg/L	<0.1 mg/l	<0.10 mg/L
Max [NO_3]	<20 mg/L	<80 mg/L	<40 mg/l	<20 mg/L
Min [O_2]	8 ppm (saturated)[b]	7.0 ppm	Saturated	8 ppm (saturated)
Recommend light wave length	450–650 nm	No specific requirements	Normal overhead light so yellow/white	Natural light at 3–5 m depth (for deep living species, blue light or shadow is recommended)
Recommended light intensity at water surface	Dim[c]	<350 lux	100–200 lux	200–400 lux
Photoperiod	According to the natural geographical location of the animal	According to the natural geographical location of the animal. A dimming period is recommended	According to the natural geographical location of the animal. A dimming period is recommended	According to the natural geographical location of the animal. Add crepuscular effect as enrichment[d]
Noise level and vibration	Avoid vibration	Avoid vibration	Avoid vibration	Avoid vibration

[a]These requirements do not take into account those adopted and suggested for rearing cephalopods for purposes of aquaculture (for review see Iglesias et al.[1A]).
[b]Care should be taken that the source of oxygenation be kept away from the animals (in a separate tank or sump). Nautiluses are attracted to and can retain air bubbles in their eyes, and under their hood leading to adverse health effects. *Nautilus* is particularly sensitive to air and any exposure should be avoided.
[c]Tanks should allow for both dark refuges and dim light.
[d]It is reported that crepuscular light prevent from premature hatching of paralarvae from octopus females caring eggs in tanks.

Appendix 2 Recommended species-specific minimal requirements for short-duration transport for the establishment, the care and accomodation of cephalopods. Data for different body size/life stages are included when available.

Appendix 2C (Short-duration transport)

	Nautilus sp.	Cuttlefishes		Squids		Octopuses		
Life stages		Eggs, Juveniles	Adult	Eggs	Adults	Eggs	Paralarvae	Juveniles, Adults
Source	Wild	Wild and captive bred	Wild and captive bred	Wild and captive bred	Wild	Wild; Captive bred	Captive bred	Wild
Transport container	A bucket that allows the animal to move freely and attach to the side of the bucket	Large buckets	Large buckets	Plastic bags	Plastic bags[a]	250–500 ml tissue culture flask Plastic bags	Plastic bags	Large buckets
Water quantity/quality[b]	Water covering the bucket surface and at least 2 shell depths beneath	1 L oxygenated seawater (Hatchlings, max 10)	Maximum 10 animals in 50 L oxygenated seawater	Eggs should be transported in their natural seawater (if from wild)	15 L oxygenated seawater/ animal (but depends on size of squid and distance at which it is travelling)	50% water or embryo media and 50% air. 1–2 per ml of sterile water. Methylene blue (0.5 mg/L or 0.5 ppm) can also be added to the solution to reduce fungal growth	Seawater (1/3) and O_2 (2/3) at a density up to 3000 individuals	1–5 animals/ 15 L oxygenated seawater.
Water temperature	14–17°C	12–18°C	12–18°C	17–19°C	17–19°C	Around 15°C	Around 15°C	15–24°C
Food deprivation	No	No	No	No	No	No	No	No
Acclimatisation after transport	Yes, brief[c]	Yes, brief	Yes, brief	Yes, brief	Yes, brief	Yes, brief	Yes, brief	Yes, brief
Quarantine	Not required	Not required	Not required	Not required	Not required	Not required	Not required	Not required

[a]During transport within lab squid may be transported in plastic containers to awaiting larger containers such as an ice chest or bucket which can be submerged in the recipient tank. In some cases it may be necessary to anaesthetize the animal with $MgCl_2$.
[b]No special requirements for quality, except the normal clean seawater utilized in establishments.
[c]To equilibrate pH and temperature values and let the animal adapt to the new environment.

Appendix 2 Recomended species-specific minimal requirements for long-duration transport for the establishment, care and accomodation of cephalopods. Data for different body size/life stages are included when available.

Appendix 2D (Long-duration transport)

	Nautilus sp.[a]	Cuttlefishes		Squids		Octopuses		
Life stages		Eggs, Juveniles	Adult	Eggs	Adults	Eggs	Paralarvae	Juveniles, adults
Transport container	Double-bagged common aquarium bags placed into styrofoam boxes. The external container should be able to carry and hold the entire water volume in it even if all inside containers/bags are raptured.	Dark bags	Dark containers (50 to 100 liters) with opaque covers.	Recommend either horizontal cylindrical tank or rectangular horizontal tank with closed top		Double-bagged common aquarium bags placed into styrofoam boxes. The external container should be able to carry and hold the entire water volume in it even if all inside containers/bags are ruptured.		
Water quantity/quality	1/3 pre-oxygenated seawater and 2/3 air (1 animals per bag)	1/3 Oxygen 2/3 seawater Max density: 3000/L	1/3 Oxygen 2/3 seawater Max 10 animal/ containers	Depending on size of squid and distance at which it is traveling	15 L oxygenated seawater/animal (depending on size of squid and distance at which it is traveling)	To be developed	To be developed	Octopus (BW 50–600 g) should be double-bagged in a good quality plastic fish bag at a density of about 1 octopus per 2 L seawater + 2/3 of oxygen AmQuel (a commercially available ammonia sequestrator) can be added to bind any ammonia that is produced during transport.
Water temperature[b]	Should never exceed 23°C	12–18°C	12–18°C	Should match that of where the squid naturally occurs	Should match that of where the squid naturally occurs	13–19°C	13–19°C	15–22°C
Food deprivation	Yes (2–3 days)	Yes (24 h)	Yes (24 h)		Only if possible (animals not caught from the wild)	Not Applicable	Not Applicable	No For *O. dofleini* it is suggested 10–12 days.

(continued)

Appendix 2D (Long-duration transport) Continued

	Nautilus sp.[a]	Cuttlefishes		Squids		Octopuses		
Life stages		Eggs, Juveniles	Adult	Eggs	Adults	Eggs	Paralarvae	Juveniles, adults
Sedation	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended
Acclimatisation after transport	Yes (approximately 1 week)	Yes (3–4 h)	Yes (3–4 h)[c]	Yes	Yes	Criteria to be developed	Criteria to be developed	Yes
Quarantine	Yes (only for ill or injured animals)	If applicable a diagnosis of the levels of parasite infection and of haemocyte count may be carried out by the animal care Staff to determine if quarantine is required.	If applicable a diagnosis of the levels of parasite infection and of haemocyte count may be carried out by the animal care staff to determine if quarantine is required.	Separate quarantine tanks should be available if squid is damaged and needs veterinary attention	Separate quarantine tanks should be available if squid is damaged and needs veterinary attention	Criteria to be developed	Criteria to be developed	Yes (only for ill or injured animals)

[a]It is important to check local requirements for the transport of animals in all countries along the route while transporting live cephalopods. Nautiluses are particularly sensitive to exposure to air and this should be avoided if possible by transporting them in vessels containing seawater.
[b]Should match that of where the species naturally occurs.
[c]Change of environment, particularly slight changes in temperature, can induce egg laying.

Appendix 2 Recommended species-specific minimal requirements for feeding[a] to be provided to animals as applied to the establishment, care and accomodation of cephalopods. Data for different body size/life stages are included when available.

Appendix 2E (Feeding)

	Nautilus sp.	Cuttlefishes			Squids		Octopuses
Life stages		Hatchlings	Post hatchlings	Juveniles, Adults	Juveniles	Adults	
Food items	Dead food only. Nautilus requires food with a high level of Calcium Carbonate, such as shrimp with carapace, lobster moult shells or fish heads.	No food for 1–7 days	Shrimp-like prey	Different prey items. Frozen food is acceptable (live preys preferred)[b]	Live larvae such as artemia, mysis or fish	Live or dead fish	Live prey. Crabs, mostly utilized *Carcinus mediterraneus*.[c]
Feeding regimes	Daily-2 times/week	Daily, *ad libitum*	Daily, *ad libitum*	Daily, *ad libitum*	Three to five times a day	Three to five times a day	At least every other day. Providing food once a day should reccomended.

[a]Information on nutritional requirements of cephalopods species reared for purposes of aquaculture is reviewed in Iglesias et al.[1A].
[b]They can accept frozen food from 2 months of age, after familiarisation.
[c]A list of potential live prey traditionally utilized for octopuses is also given by Borrelli.[3A] Remove any solids after the last feeding.

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Article

The Effect of Polyunsaturated Aldehydes on *Skeletonema marinoi* (Bacillariophyceae): The Involvement of Reactive Oxygen Species and Nitric Oxide

Alessandra A. Gallina [†], Christophe Brunet, Anna Palumbo and Raffaella Casotti *

Stazione Zoologica Anton Dohrn di Napoli, Villa Comunale, Napoli I80121, Italy;
E-Mails: christophe.brunet@szn.it (C.B.); palumbo@szn.it (A.P.)

[†] Current Address: Department of Biology, Colorado State University, Fort Collins, CO 80523, USA;
E-Mail: Alessandra.Gallina@colostate.edu

* Author to whom correspondence should be addressed; E-Mail: raffaella.casotti@szn.it;
Tel.: +39-081-5833235; Fax: +39-081-7641355.

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Abstract: Nitric oxide (NO) and reactive oxygen species (ROS) production was investigated in the marine diatom, *Skeletonema marinoi* (SM), exposed to 2*E*,4*E*/*Z*-decadienal (DECA), 2*E*,4*E*/*Z*-octadienal (OCTA), 2*E*,4*E*/*Z*-heptadienal (HEPTA) and a mix of these last two (MIX). When exposed to polyunsaturated aldehydes (PUA), a decrease of NO was observed, proportional to the PUA concentration (85% of the initial level after 180 min with 66 µM DECA). Only OCTA, HEPTA and MIX induced a parallel increase of ROS, the highest (2.9-times the control) with OCTA concentrations twice the EC_{50} for growth at 24 h (20 µM). The synthesis of carotenoids belonging to the xanthophyll cycle (XC) was enhanced during exposure, suggesting their antioxidant activity. Our data provide evidence that specific pathways exist as a reaction to PUA and that they depend upon the PUA used and/or the diatom species. In fact, *Phaeodactylum tricornutum* (PT) produces NO in response to DECA, but not to OCTA. We advance the hypothesis that SM perceives OCTA and HEPTA as intra-population infochemicals (as it produces PUA), while PT (non-PUA producing species) perceives them as allelochemicals. The ability to produce and to use PUA as infochemicals may underlie ecological traits of different diatom species and modulate ecological success in natural communities.

Keywords: octadienal; heptadienal; infochemical; xanthophylls; signaling; competition

1. Introduction

Marine diatoms are one of the most successful groups of eukaryotic photosynthetic organisms on Earth and are major players in global marine primary production by representing most of the organic carbon that is at the base of the marine food web [1]. About 30% of the marine diatoms tested so far have been found to produce polyunsaturated aldehydes (PUA) as secondary metabolites derived from fatty acid metabolism [2]. PUA, together with a plethora of other different metabolites derived from the same biosynthetic pathway, all belonging to oxylipins, play key roles in chemically-mediated plankton interactions (for a review, see [3]).

Since their first identification in marine diatoms [4], PUA have been demonstrated to have negative effects on copepod development and reproduction, as well as on other marine invertebrates [5–8]. PUA do not act only as a chemical defense against grazers, but also as signals, determining the fate of the pelagic microbial community structure. Indeed, PUA affect bacterial community composition, with some groups being more sensitive and others highly resistant [9,10]. They also inhibit the growth of phytoplankton species belonging to different taxonomical groups, thereby possibly acting as allelochemicals [11,12]. Thus, it is speculated that a stress surveillance system might exist in diatoms in response to PUA, which leads to population-level cell death and bloom termination at specific PUA concentrations [13]. In the diatom, *Phaeodactylum tricornutum*, this stress-surveillance system induces the production of nitric oxide (NO) and a transient rise in intracellular Ca^{2+} that later activates a gene cascade involved in programmed cell death [13,14].

NO is a versatile molecule involved in many different processes in animals, such as neurotransmission, vasodilatation and defense against pathogens (e.g., [15,16]). In plants, NO regulates a number of different genes involved, for instance, in photosynthesis, cell death and basic cellular metabolism (for a review, see [17]). Compared to animals and higher plants, studies of NO production in marine phytoplankton are limited. NO is reported to regulate physiological processes and stress responses in the chlorophycean, *Scenedesmus obliquus* [18,19], and ichthyotoxicity in raphidophycean flagellates [20]. In addition, NO production by the dinoflagellate, *Symbiodinium* spp., occurs during coral bleaching [21,22]. NO is also involved in sensing PUA-derived stress [13], in regulating gene expression and subsequent cell death under light stress [23], in controlling the adhesion of benthic diatoms to different substrates [24] and in inhibiting biofilm formation in response to PUA [25]. In addition, NO is produced during normal growth in different phytoplankton species [26,27], suggesting that it can function as a growth-regulating factor.

Apart from NO, other molecules are involved in the stress response of different organisms. Reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are natural byproducts of cell metabolism and are commonly produced in response to different stress agents, including heat, UV radiation, chemicals and, more generally, to any stressful change in environmental conditions [28]. In phytoplankton, CO_2 limitation [29], viral infection [30], high pH and iron limitation [31], prolonged darkness [32], as well as cadmium [33] and paraquat exposure [34] are

reported to induce an increase in ROS. Furthermore, compounds involved in biotic interactions are associated with ROS production, such as thiol proteases excreted by ageing cells [35], the cyanobacterial toxin nodularin [36] and other macrophytes-derived allelochemicals [37].

The concomitant production of NO and ROS (hydrogen peroxide) is, indeed, reported to be involved in the wound-activated response of the macroalga, *Dasycladus vermicularis* [38]. In this case, their production is also partly co-regulated, supporting the conclusion that a signaling relationship exists between ROS and reactive nitrogen species (RNS), as previously suggested in algae and plants [39,40].

NO and ROS are toxic and reactive molecules causing strong damage to many cell components. To cope with the related oxidative stress and to prevent or limit irreversible damages, cells have developed several mechanisms. Among the observed responses, there is the increase of the two antioxidative enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) [36], and the accumulation of carotenoids [41]. In the freshwater *Haematococcus pluvialis*, the carotenoid, astaxanthin, is accumulated during the encystment process triggered by paraquat exposure, with a negative linear relationship with ROS [34]. In the marine diatom, *Thalassiosira weissflogii* (TW), photoprotective xanthophyll carotenoid pigments increase when cells are exposed to PUA, suggesting that they play a role as antioxidants independently of light [11].

The general aim of this study is to investigate the responses of the diatom, *Skeletonema marinoi* (SM), to PUA in terms of NO and ROS production, to test the hypothesis that these responses underlie an adaptive advantage leading to ecological success. Opposite from *Thalassiosira weissflogii* (TW) [11] and *Phaeodactylum tricornutum* (PT) [13], which do not produce PUA [2], SM produces high amounts of PUA, mainly 2*E*,4*E*/*Z*-octadienal, 2*E*,4*E*/*Z*-heptadienal and 2*E*,4*E*/*Z*,7-octatrienal [42]. Moreover, SM is a successful species in nature, being widespread worldwide and forming spring or autumn blooms in many coastal areas [43]. Our study focuses on NO and ROS production (and their interrelationship) in response to PUA and on the activation of the xanthophyll cycle (XC) as a protection mechanism to maintain photosynthetic performance. The differences between PT and SM in terms of NO production are analyzed comparatively to infer their involvement in the ecological success of SM and interpreted as an adaptive response of diatoms to chemical cues in general.

2. Results

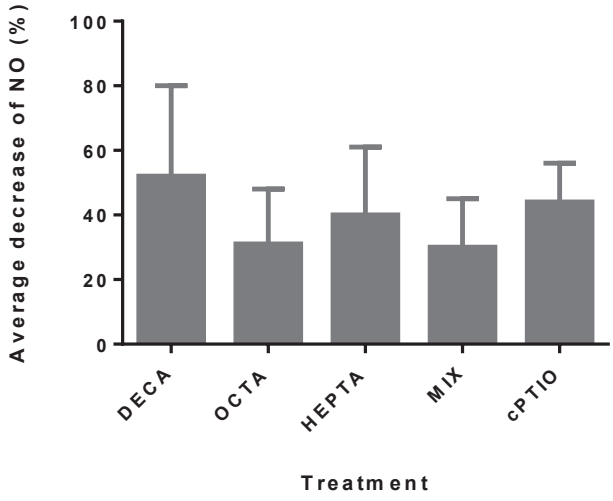
2.1. Nitric Oxide Production

SM cultures were inoculated with different concentrations of three PUA (2*E*,4*E*/*Z*-decadienal (DECA), 2*E*,4*E*/*Z*-octadienal (OCTA), 2*E*,4*E*/*Z*-heptadienal (HEPTA)) or a 1:1.4 mix of OCTA and HEPTA (MIX). Values were normalized by the values of the controls measured at the same time. Controls are samples treated exactly like the others, except for the PUA (zero-PUA). The normalization is necessary in order to avoid the natural variability in physiological NO levels hiding the new NO production. DECA has been chosen because it is a commonly used PUA in toxicity tests on several model and non-model organisms (e.g., [25,44]). OCTA and HEPTA are, instead, the PUA commonly produced by SM, as well as by other diatoms [2]. The concentrations used were multiples of the EC_{50} concentration for growth at 24 h for SM, as reported in [12] (*i.e.*, 2.48 $\mu mol \cdot L^{-1}$ for DECA,

8.94 μmol·L^{-1} for OCTA and 18.17 μmol·L^{-1} for HEPTA), and the concentrations reported to elicit NO production in PT (33 μM and 66 μM DECA) [13]. The mix of OCTA and HEPTA reflected the ratio of these two PUA in SM cultures, as reported by [42].

When exposed to DECA, no increase in 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA)-green fluorescence was observed, indicating the absence of NO production. Rather, a NO decrease was observed after 180 min of exposure with all the concentrations tested (Table 1). The decrease, although less striking, was also observed in cultures exposed to the other PUA (Figure 1 and Table 1), and it was also due to a consumption of the basal levels of NO, as also confirmed by the comparable effects obtained with the NO scavenger carboxy-PTIO, (cPTIO) (Figure 1). The average decrease of NO after 180 min was 52% (SD ± 28%) with DECA, 31% (SD ± 17%) with OCTA and 40% (SD ± 21%) with HEPTA (Figure 1). After 180 min, fluorescence values were significantly different than the controls and, among them, for all the DECA concentrations tested (Table 1). With OCTA, a significant difference in NO relative to the control was evident starting from OCTA concentrations of 1 μM (Table 1), while for HEPTA, NO reduction at 180 min started to be significant at 5 μM. When SM was exposed to the MIX, no synergistic effect was observed, as the NO values were similar to the ones elicited by OCTA alone (30%, SD ± 15%) (Figure 1), and a significant reduction in NO was evident only with 10 μM OCTA + 14 μM HEPTA and 20 μM OCTA + 28 μM HEPTA.

Figure 1. The average decrease of NO in *Skeletonema marinoi* (SM) expressed as the percent (%) of decrease relative to the control (zero-PUA, 100%) after 180 min of exposure to the different PUA treatments (the average of all concentrations tested). Data are the means ± SD, n = 6. cPTIO is a NO scavenger (carboxy-PTIO, negative control), inoculated at the same concentration as the highest concentration used for the PUA tested (see Table 1). DECA, 2*E*,4*E*/*Z*-decadienal; OCTA, 2*E*,4*E*/*Z*-octadienal; HEPTA, 2*E*,4*E*/*Z*-heptadienal; MIX, mix of OCTA and HEPTA.



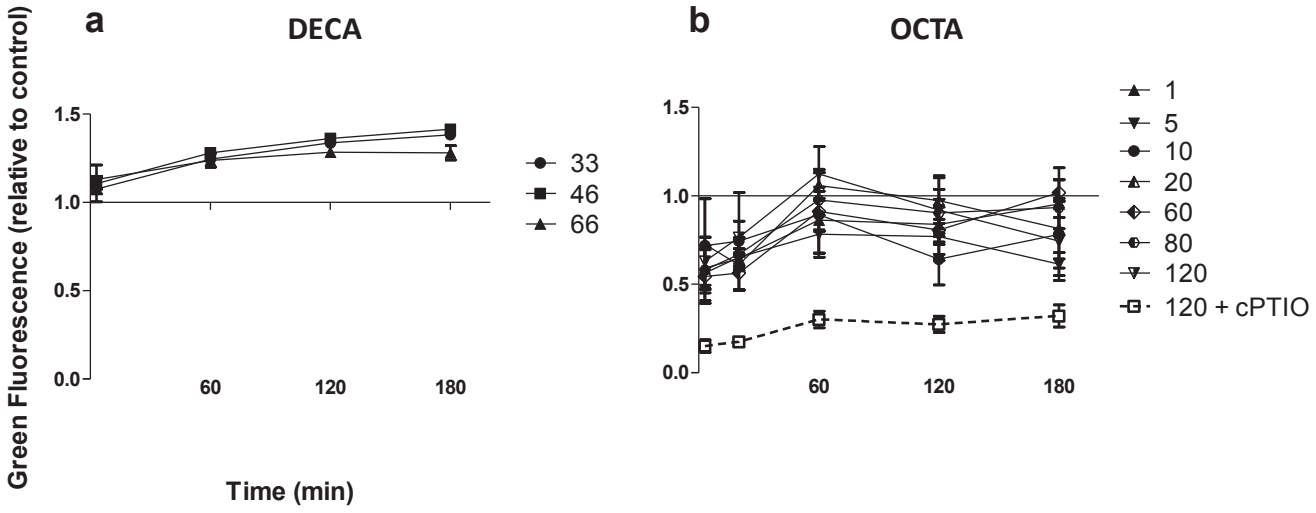
Since NO was previously reported to be produced by the diatom, PT, in response to DECA [13], we tested NO production in PT upon exposure to DECA, in order to exclude an artifact in our experiments, due, for instance, to different culture conditions or methodological procedures. NO production in PT upon exposure to DECA was indeed confirmed (Figure 2a). When PT was exposed to

OCTA, instead (not tested in [13]), NO was not produced (Figure 2b), and a decreasing gradient of DAF-FM-NO fluorescence was observed, similar to SM, showing, indeed, a differential response to different PUA in PT. The NO scavenger, cPTIO (negative control), dramatically reduced green fluorescence from DAF-FM-NO, further supporting this interpretation.

Table 1. The significance of the NO decrease after 180 min of exposure to the different PUA. cPTIO was used as an NO scavenger (negative control) inoculated at the same concentration as the highest concentration used for the PUA tested in each experiment. The MIX is a 1:1.4 mix of OCTA and HEPTA, and the concentration indicated is the one of the OCTA. The significance is calculated using the Student's *t*-test; (*) $p < 0.05$; ns: not significant; $n = 3$.

Concentration (µM)	DECA	OCTA	HEPTA	MIX
0.05	*	ns	ns	ns
0.1	*	ns	ns	ns
1	*	*	ns	ns
5	*	*	*	ns
10	-	*	*	*
20	-	*	*	*
33	*	-	-	-
40	-	-	*	-
66	*	-	-	-
cPTIO	*	*	*	*

Figure 2. NO production in *Phaeodactylum tricornutum* (PT) exposed to DECA and OCTA. (**a**) DECA; (**b**) OCTA. Concentrations in the legends are expressed in µM. Data are fluorescence values normalized to the control value (zero PUA, equal to one) and are the means ± SD, $n = 3$ (biological replicates). cPTIO was used as an NO scavenger (negative control) inoculated at the same concentration as the highest concentration used for the PUA tested in each experiment.



2.2. ROS Production

The hypothesis that reactive species other than NO are involved in the stress response of SM to PUA was tested, using flow cytometry and the ROS-sensitive dye, dihydrorhodamine 123 (DHR).

When exposed to DECA, SM did not show any significant increase in ROS (Figure 3a), as contrary to the other PUA and the MIX (Figures 3b and 4a,b). In fact, these induced a peak 20 min after exposure to concentrations equal to or higher than 5 μM OCTA ($p < 0.05$, $n = 3$) and 20 μM HEPTA ($p < 0.01$, $n = 3$), respectively. The highest increase in ROS (2.9 times the control, $p < 0.001$, $n = 3$, Figure 4a) was induced by 20 μM OCTA. When inoculated together, OCTA and HEPTA elicited an increase in ROS comparable to OCTA alone (2.9-times the control with 20 μM OCTA plus 28 μM HEPTA, $p < 0.001$, $n = 3$, Figure 4b), therefore excluding, again, a synergistic effect of the two PUA when inoculated together. Preliminary tests included acetaldehyde as a control, in order to exclude general toxicity due to the aldehydic group. This was then excluded from further tests, as no effect was observed. In all of these experiments, cells inoculated with PUA together with the ROS scavenger, Tempol, showed a lower DHR-ROS-derived green fluorescence, confirming that the observed increase in fluorescence was really due to ROS production (Figures 3 and 4).

Figure 3. ROS production in SM exposed to different PUA. (**a**) DECA; (**b**) HEPTA. Concentrations in the legends are expressed in μM. Data are fluorescence values normalized to the control value (basal dihydrorhodamine 123 (DHR) fluorescence, zero-PUA) and are the means ± SD, $n = 3$ (biological replicates). Tempol was used as an ROS scavenger (negative control) inoculated at the same concentration as the highest concentration used for the PUA tested in each experiment.

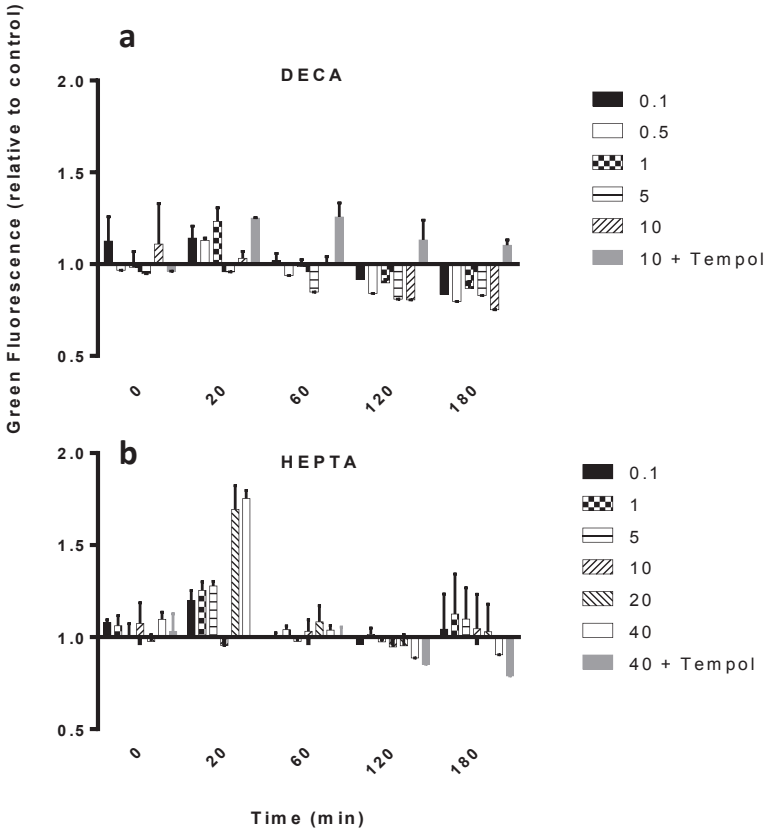
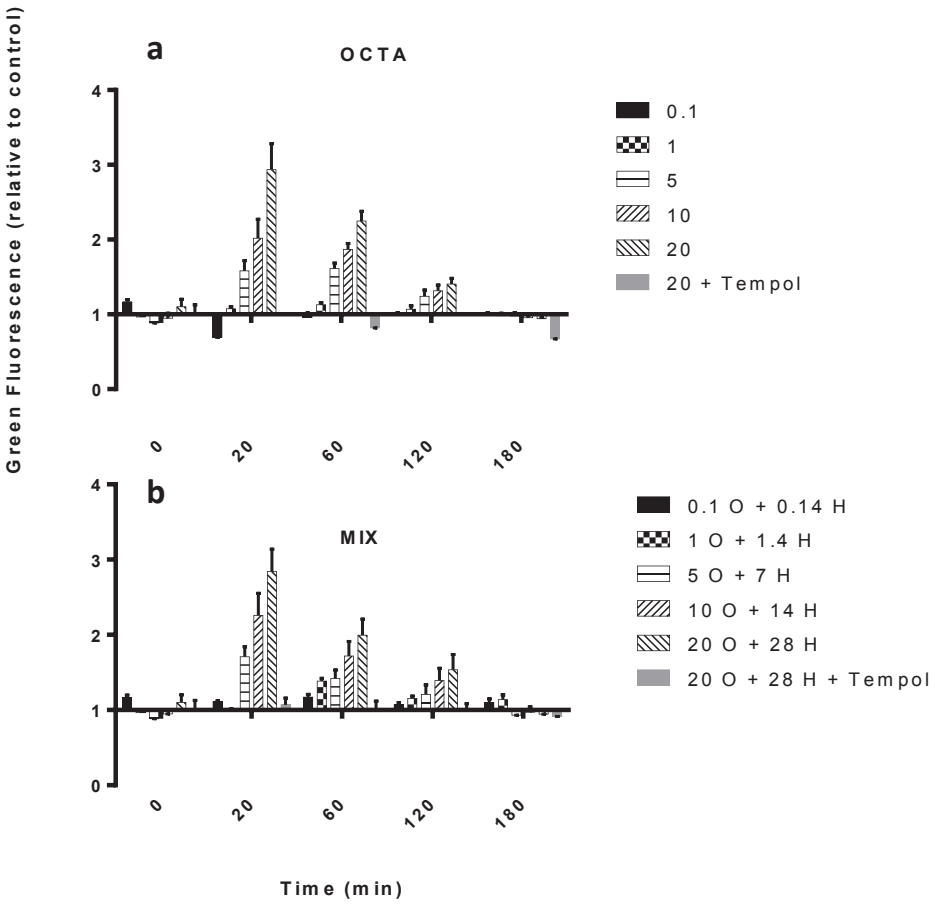


Figure 4. ROS production in SM exposed to different PUA. (**a**) OCTA; (**b**) MIX (O = OCTA, H = HEPTA). Concentrations in the legends are expressed in μM. Data are fluorescence values normalized to the control value (basal DHR fluorescence) and are the means ± SD, $n = 3$ (biological replicates). Tempol was used as a ROS scavenger (negative control) inoculated at the same concentration as the highest concentration used for the PUA tested in each experiment.



2.3. Xanthophyll Cycle Activation and Photosynthetic Efficiency

The xanthophyll cycle (XC) in diatoms involves the enzymatic removal of the epoxy groups from xanthophyll diadinoxanthin (Ddx) to form the de-epoxidized xanthophyll diatoxanthin (Dtx). The XC is activated by high light conditions, playing a key role in photoprotecting the photosystems and being responsible for energy dissipation by non-photochemical quenching (NPQ), a mechanism to reduce the amount of energy that reaches the photosynthetic reaction centers. It has been already shown that the XC might be also activated by chemical stress [11].

In OCTA-exposed SM cultures, an increase in Dtx (Table 2) was observed at all the concentrations tested, after one hour of exposure, and this increase was significant for the concentrations >10 μM ($p < 0.05$, $n = 3$). Since also Ddx increased, we can conclude that this was a response of the whole xanthophyll cycle, with the cells activating the photoprotective biochemical pathway, which leads to an increase of Ddx and, consequently Dtx. Indeed, β-carotene, a xanthophyll upstream precursor of XC pigments, decreased in the first 20 min after exposure and subsequently increased up to 24 h in cultures exposed to 20 μM OCTA, indicating its involvement in the antioxidant response to PUA

(Table 2). Interestingly, the de-epoxided state (DES: Dtx/(Ddx + Dtx), Table 2) started to increase after 20 min of exposure, highlighting the fast de-epoxidation activity after chemical exposure. The highest increase in Dtx and DES was observed with 20 µM OCTA after 3 h (Table 2).

Table 2. Xanthophyll cycle (XC) pigments (de-epoxidized xanthophyll diatoxanthin (Dtx), xanthophyll diadinoxanthin (Ddx) and the de-epoxided state (DES) = Dtx/(Ddx + Dtx)), β-carotene, non-photochemical quenching (NPQ) and growth rates (µ) in SM exposed to different concentrations of OCTA and monitored for 48 h. Data are the means ± SD, $n = 3$ (biological replicates). Values are normalized to the control, except for NPQ and the growth rates (day^{-1}). Values discussed in the text are highlighted in bold.

Treatment	Time (h)	Ddx	Dtx	DES	β-carotene	NPQ	µ (day^{-1})
5 µM OCTA	0	1.07 ± 0.14	0.95 ± 0.18	0.91 ± 0.23	1.07 ± 0.05	-	-
	0.3	0.51 ± 0.09	0.79 ± 0.10	1.52 ± 0.27	**0.63 ± 0.08**	**0.95 ± 0.04**	-
	1	1.29 ± 0.11	1.51 ± 0.25	1.30 ± 0.31	1.00 ± 0.09	**0.88 ± 0.03**	-
	3	2.36 ± 0.51	1.57 ± 0.51	0.95 ± 0.49	1.30 ± 0.48	**0.88 ± 0.02**	-
	24	0.59 ± 0.07	1.20 ± 0.36	1.86 ± 0.63	**0.70 ± 0.07**	**1.10 ± 0.04**	**0.42 ± 0.08**
	48	-	-	-	-	-	−0.19 ± 0.12
10 µM OCTA	0	0.84 ± 0.03	0.89 ± 0.13	1.05 ± 0.17	1.00 ± 0.06	-	-
	0.3	0.48 ± 0.11	0.80 ± 0.15	1.61 ± 0.17	**0.61 ± 0.11**	**0.92 ± 0.03**	-
	1	1.37 ± 0.14	1.77 ± 0.04	1.50 ± 0.30	0.95 ± 0.12	**0.95 ± 0.00**	-
	3	1.21 ± 0.11	2.14 ± 1.52	1.78 ± 0.64	1.20 ± 0.30	**1.00 ± 0.03**	-
	24	1.23 ± 0.58	1.76 ± 1.17	2.37 ± 0.42	**1.08 ± 0.25**	**1.25 ± 0.04**	0.11 ± 0.15
	48	-	-	-	-	-	**−0.12 ± 0.13**
20 µM OCTA	0	0.75 ± 0.12	0.84 ± 0.07	1.12 ± 0.20	0.89 ± 0.01	-	-
	0.3	0.63 ± 0.22	1.10 ± 0.01	1.90 ± 0.46	**0.63 ± 0.16**	**0.90 ± 0.17**	-
	1	0.82 ± 0.10	2.19 ± 0.05	1.80 ± 0.37	0.87 ± 0.16	**1.01 ± 0.04**	-
	3	2.28 ± 0.46	4.51 ± 1.57	1.41 ± 0.38	1.56 ± 0.46	**1.05 ± 0.00**	-
	24	1.71 ± 0.49	**5.83 ± 1.60**	2.88 ± 0.17	**2.04 ± 0.23**	**1.32 ± 0.02**	**−0.29 ± 0.08**
	48	-	-	-	-	-	**−0.10 ± 0.10**

OCTA = 2*E*,4*E*/*Z*-octadienal; Ddx = diadinoxanthin; Dtx = diatoxanthin; NPQ = non-photochemical-quenching, µ = growth rate; DES = de-epoxidated state. In bold, values specifically discussed in the text.

Without a doubt, the activation of the XC was independent of excess light, as also confirmed by the constant NPQ values measured over the different conditions and during all of the experiment. Indeed, the NPQ value of *ca*. one has been observed within the first 3 h and throughout the 24 h of exposure (Table 2), suggesting that the activation of the XC was not involved in excess light energy dissipation, but, rather, in protecting the cells from another source of oxidative stress (*i.e.*, PUA-induced ROS production). Additionally, Fv/Fm values (where Fv is variable fluorescence and Fm maximum fluorescence), which measure photochemical efficiency of photosystem (PS) II quantum efficiency, did not vary in response to OCTA exposure and remained constantly high (*ca*. 0.66), indicating that the photosynthetic system was not being impaired by the oxidative stress induced by PUA (data not shown).

2.4. Growth and Recovery from PUA Stress

The growth rate after 24 h decreased from 0.42 ± 0.08 day^{-1} with 5 μM OCTA (virtually no effect compared to the control) to −0.29 ± 0.08 day^{-1} with 20 μM. After 48 h, the growth rate was as low as −0.12 ± 0.13 day^{-1} and −0.10 ± 0.10 day^{-1} with 10 μM and 20 μM (Table 2).

In order to verify the reversibility of PUA effect, OCTA-exposed cultures were resuspended in fresh medium with no OCTA added and sampled for 48 h. Twenty four hours after resuspension, the growth rate of cultures exposed to 5 μM and 10 μM had resumed to 0.69 ± 0.1 day^{-1} and 0.57 ± 0.04 day^{-1}, respectively, thus comparable to that of the control (0.48 ± 0.05 day^{-1}, Table 3). On the contrary, the growth rate of cultures previously exposed to the highest OCTA concentration (20 μM) increased to only 0.09 ± 0.07 day^{-1} after 24 h, but had increased to 0.47 ± 0.11 day^{-1} after 48 h.

Diatoxanthin content had decreased 24 h after resuspension for all of the OCTA concentrations (Table 3). This was particularly evident in cultures exposed to 20 μM OCTA, for which Dtx decreased 43% (from 5.83 ± 1.60-times the control 24 h after inoculation, to 3.30 ± 0.12-times the control 24 h after resuspension) (Tables 2 and 3, respectively). Concomitantly to a decrease in Dtx, an increase in Ddx (39% increase for 20 μM OCTA) was evident, also parallel to a decrease in the DES (Table 3). β-carotene showed, instead, a different pattern, decreasing its concentration in the first 20 min of OCTA exposure at the highest OCTA concentration (Table 2) and then increasing again both during the remaining exposure time and 24 h after resuspension (Tables 2 and 3). For the other OCTA concentrations tested (5 μM and 10 μM), β-carotene values did not vary significantly after the resuspension (Tables 2 and 3).

Since no change from the original value of 0.66, close to the value indicated as optimal by Falkowsky and Raven [45], was detected in Fv/Fm or NPQ after 24 h of exposure, these parameters were not further measured after the resuspension of cultures in fresh medium.

Table 3. XC pigments Dtx, Ddx and DES = Dtx/(Ddx + Dtx), β-carotene and growth rates in SM following resuspension in fresh medium with no PUA following 24 h after exposure to different concentrations of OCTA and monitored up to 48 h. Data are the means ± SD, *n* = 3 (biological replicates). Values are normalized to the control, except for growth rates (day^{-1}). Values discussed in the text are highlighted in bold.

Treatment	Time (h)	Ddx	Dtx	DES	β-carotene	μ (day^{-1})
5 μM OCTA	24	0.80 ± 0.25	0.82 ± 0.53	0.92 ± 0.38	0.88 ± 0.18	**0.69 ± 0.10**
	48	-	-	-	-	0.34 ± 0.12
10 μM OCTA	24	1.22 ± 0.76	1.20 ± 0.76	0.98 ± 0.04	0.92 ± 0.27	**0.57 ± 0.04**
	48	-	-	-	-	0.46 ± 0.08
20 μM OCTA	24	2.79 ± 0.26	**3.30 ± 0.12**	1.00 ± 0.17	2.34 ± 0.28	**0.09 ± 0.07**
	48	-	-	-	-	**0.47 ± 0.11**

OCTA = 2*E*,4*E*/*Z*-octadienal; Ddx = diadinoxanthin; Dtx = diatoxanthin; μ = growth rate; DES = de-epoxidated state. In bold, values specifically discussed in the text.

3. Discussion

3.1. Protective Responses

The fact that we could not detect an increase in NO production in SM exposed to PUA is surprising as NO appears to be involved in different stress responses in marine macro- and micro-algae [22,38,46], including diatoms [13]. In *Skeletonema costatum* (now replaced as *S. tropicum* [47]), Chung *et al.* [23] observed NO production in response to light stress, and this was also confirmed in SM [48]. This suggests that NO production in SM is stress-type dependent. Interestingly, the PUA DECA induces an increase of NO in another diatom, PT, revealing that the response to PUA is species specific and suggesting that different stress-signaling pathways are elicited by DECA in the two species. Moreover, not all PUA induce the same response, since PT produces NO only for DECA and not for the other PUA tested.

Despite being the most used PUA in toxicological experiments, DECA is not the most common PUA present in marine phytoplankton. In a survey of 51 species of marine diatoms, DECA was the least detected PUA [2], in agreement with what it has been observed at sea during diatom blooms [49]. DECA is reported *in situ* only during blooms of the prymnesiophyte, *Phaeocystis pouchetii* [50,51]. In PT, which does not produce any PUA [2], the response elicited by DECA should be attributable to its toxicity only. This reinforces the hypothesis that commonly present PUA, such as OCTA and HEPTA, act as infochemicals.

It is important to point out that not only was there no increase in NO in response to PUA exposure observed in SM, but, indeed, NO-related fluorescence decreased in both SM (exposed to all PUA) and PT (exposed to OCTA), as also confirmed by the effect of the NO scavenger. This suggests an impairment of physiological NO production, probably related to growth inhibition, which has already been shown to be an effect of PUA [11,12]. The difference in NO content in these two diatom species might be related to their different growth capabilities in culture, much higher in PT than in SM, which is confirmed by the stronger scavenging effect of cPTIO (Figure 2b).

In the Antarctic chlorophyte, *Chlorella* sp., a peak in NO marks the passage from lag to the exponential phase of growth, suggesting that NO is involved in growth regulation/modulation [26]. Additionally, a correlation is found between NO production and growth in different marine phytoplankton species, including *S. costatum*, with a peak in NO occurring right before the highest cell density [46]. This also implies that NO is normally present in the cells during growth and has a role in key passages of the physiological regulation of growth. Instead, it is hypothesized that PT might react differently to OCTA, because this PUA is believed to be more common in nature and consequently recognized as a signal. This observation calls for further studies comparing the reaction of different diatom species to PUAs and also a deeper understanding of the complex phenomena underlying the different steps involved. Among these, it cannot be excluded that intermediate chemical products are responsible for the observed effects, rather than the PUAs themselves. This does not reduce the value of the data presented, as PUAs have been identified as the main molecules inducing different effects in different model and non-model organisms [3].

The decrease in NO levels upon exposure to PUA may be due to a shift of the stress response pathway towards the production of different N or O reactive species or a plethora of them. Among these, we have chosen to investigate ROS.

Our results show that when SM is exposed to PUA other than DECA, ROS production occurs, with a peak 20 min after exposure and a threshold at half the EC_{50} for growth at 24 h for OCTA and the EC_{50} for growth at 24 h itself for HEPTA. This agrees with [13], reporting that in PT, a threshold exists in cells sensing a PUA, although its value was not quantified. The difference in the values of this threshold between OCTA and HEPTA is probably related to the stronger activity of OCTA [12], due to its relatively longer C chain.

Remarkably, DECA does not elicit ROS production in SM. This suggests that the response mediated by PUA is not simply due to the toxicity of the aldehydic group, but indeed, it is a specific reaction to specific PUA that the cells recognize as self-produced. It is therefore possible that ROS have a similar role as NO in DECA-exposed PT and that ROS (and not NO) are involved in the intra-population stress signaling pathway in SM. The ROS downstream response is likely to activate genes involved in either alternate signaling pathways or a cell death cascade, depending on the PUA concentration. In the congeneric *S. tropicum* (previously S. *costatum*) a cell death-specific gene (*ScDSP*) is involved in the cascade leading to cell death by apoptosis [52]. The expression of this gene is enhanced by the NO donor, diethylamine nitric oxide (DEANO) [23], and even more by hydrogen peroxide [53] . Additionally, in *S. tropicum*, PUA have also been shown to both induce ROS production and *ScDSP* expression, indicating a possible role of PUA in the cell death cascade in a congeneric diatom [53]. This suggests that ROS have an important role in the molecular cascade following PUA exposure, and this has strong implications for population dynamics, such as, for instance, during the later phases of diatom blooms, when cell lysis increases and PUA are released [54], allowing for a higher PUA concentration to be present. ROS production has been reported in other phytoplankton species in response to different stresses. Vardi *et al.* [35] reported that in the dinoflagellate, *Peridinium gatunense*, a thiol protease excreted by ageing cells was able to induce ROS production and concomitant cell death at the population level. Additionally, the cyanobacterial toxin, nodularin, has been found to induce an increase oxidative stress in the red alga, *Furcellaria lumbricalis* [36], and different allelochemicals produced by submerged freshwater macrophytes were reported to increase ROS production in both green algae and cyanobacteria [37].

In the field of chemical ecology, an important aspect to be taken into consideration is the ecological relevance of the concentrations used in laboratory studies and how they relate to naturally-occurring and, therefore, ecologically-relevant concentrations. In the case of PUA, only a few studies have addressed the important issue of measurements of PUA concentrations directly at sea [54,55]. In a field study in the Northern Adriatic Sea (Italy), Vidoudez *et al.* [54] reported the patchy distribution of PUA associated with a spring bloom of the diatom, *S. marinoi*, which was found to be the major contributor to the total PUA detected. Dissolved HEPTA and OCTA concentrations were found to be in the nanomolar range. Similarly, another recent study by Ribalet *et al.* [55] reported the presence of nanomolar concentrations of PUA in the Northern Adriatic Sea following diatom cell lysis. Even though these concentrations are considerably lower compared to the ones used in the present study (which are in the micromolar range), it must be considered that the methodology used to estimate PUA concentrations at sea averages values over liters of sea water, while smaller local patches of PUA at a

much higher concentration are expected to be produced in the immediate surroundings of diatom cells when lysing. In fact, Ribalet *et al.* [55] argued and proposed calculations showing that concentrations produced in the field could induce similar effects as those observed in culture, since bulk measurements of PUA in seawater do not reflect the concentrations in the proximity of PUA-releasing cells in the natural environment. In general, our understanding of PUA release and dynamics at sea is currently very limited, and also, methodologies to detect PUAs at sea are not as refined.

In diatoms, an increase of Dtx is usually related to high light stress and the need to protect the photosynthetic machinery from photoinhibition, Dtx being responsible for the major part of the excess energy dissipation through non-photochemical-quenching (NPQ) ([56] and the references therein). However, the role of the XC in the antioxidant response has been putatively assumed by other authors in response to nutrient limitation [57] or starvation [58], Cd exposure [59] and viral infection [60]. In TW, an increase in Dtx was also observed in reaction to DECA, reflected in a decrease in photosynthetic efficiency [11]. In our experiments, light remained constant, and the increase in Dtx did not couple with variations in NPQ values, indicating that in PUA-exposed SM cultures, the activation of the XC was providing protection against oxidative stress, likely derived from PUA-induced ROS production. Opposite from TW, the photosynthetic efficiency of OCTA-exposed SM cultures was not affected, indicating that the cells were able to cope with PUA maintaining their photosynthetic capability, allowing the cells to efficiently recover after PUA removal, even after 24 h of exposure.

The activation and functioning of the XC is PUA-concentration dependent. Low OCTA concentrations induce a gradual increase of Dtx from 1 h after exposure, parallel to a decrease of Ddx, which was likely converted into Dtx already in the first 20 min. Instead, under the highest OCTA concentration (20 μM), Dtx almost doubled between 20 min and 1 h without any visible Ddx decrease. This indicates that a rapid and strong *de novo* synthesis of both Ddx and Dtx occurred, probably as a protective mechanism against oxidative stress. This XC response has been already observed in *S. costatum* following a drastic change in light [61]. Indeed, the decrease of XC pigments during recovery from PUA stress suggests that cells were in a good physiological state and, thus, that the protective mechanism was very effective.

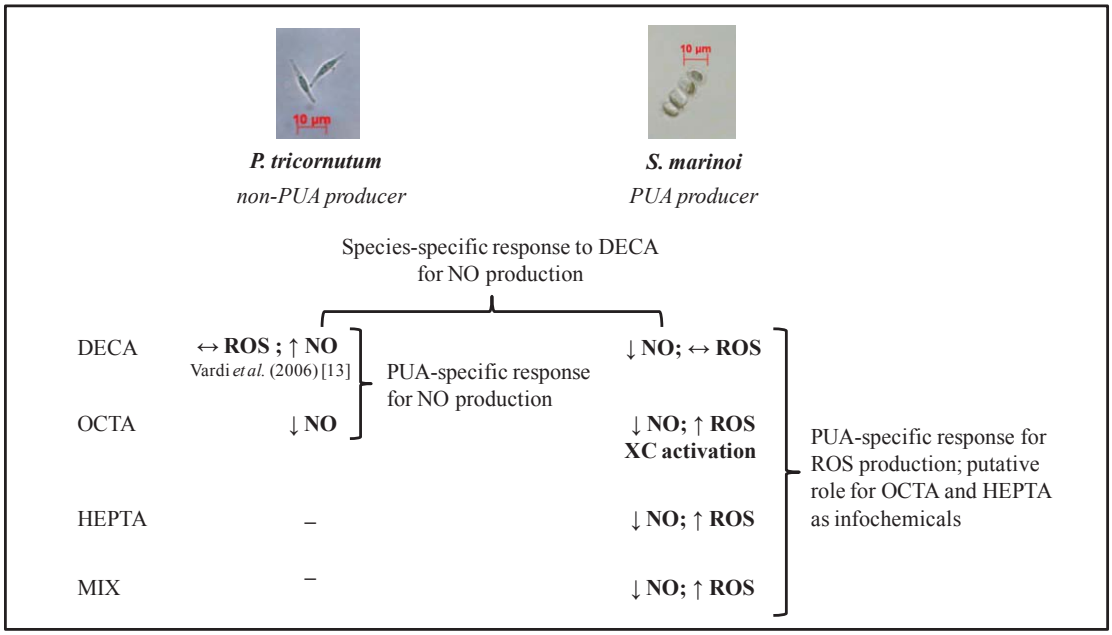
Together with the XC pigments, β-carotene was also involved in the antioxidant defense of SM, as shown by its increase from 3 to 24 h, in the cultures exposed to the highest OCTA concentration. This pigment is an upstream XC pigments precursor [62], as well as an antioxidant molecule, even in marine phytoplankton [60,63–65]. Its later increase indicates that it is involved in a later response than the XC [56] and also when oxidative stress is more severe, involving a more intense modification of the membrane apparatus structure.

A scheme summarizing the different NO and ROS dynamics in PT and SM and the hypotheses proposed in this study is presented in Figure 5.

Finally, it is possible that the green fluorescence detected in SM using DHR was also partly due to peroxynitrite ($ONOO^-$) formed by the reaction of NO with an excess of the ROS superoxide anion. This would also be consistent with the observed concomitant decrease of NO and is supported by the strong effect of the ROS scavenger used in our experiments (Tempol), which is also known to attenuate peroxynitrite [66]. Accordingly, preliminary experiments [48] indicate a major involvement of superoxide anion (O_2^-) and superoxide dismutase (SOD) activity with consequent H_2O_2 formation (and H_2O_2-dependent downstream ROS generation) in PUA-exposed SM cells. The data were obtained

using two SOD inhibitors, 2ME (2-methoxyestradiol) and DETC (sodium diethyldithiocarbamate trihydrate) and suggest an involvement of the H_2O_2-dependent oxidation of DHR123, as previously reported [67–69]. Both inhibitors induced a significant decrease of DHR-derived green fluorescence with respect to PUA-treated samples, suggesting that the production of H_2O_2 from O_2^- via SOD was prevented. In contrast, the use of the NO scavenger, cPTIO, elicited the opposite response, resulting in an increase of DHR-derived green fluorescence. It has been previously suggested that inhibition of NO synthesis in combination with DHR staining may lead to unexpected results [67]. In fact, by blocking or scavenging NO, the resulting unreacted superoxide can induce a (higher) H_2O_2 formation and consequent dye oxidation via a peroxidase- or metal- dependent pathway [67]. However, if superoxide is directed to other cellular targets and, consequently, will not form either $ONOO^-$ or H_2O_2, then the DHR-derived fluorescence should not change [67]. These preliminary results show that NO scavenging induces an increase in DHR-derived green fluorescence, and it is therefore unlikely that the excess of superoxide is being directed away from the production of H_2O_2 and H_2O_2-derived products.

Figure 5. Schematic representation summarizing the results for NO and ROS dynamics in PT and SM (both from this study and available in the literature). PUA: polyunsaturated aldehydes; NO: nitric oxide; ROS: reactive oxygen species; XC: xanthophyll cycle; DECA: 2*E*,4*E*/*Z*-decadienal; OCTA: 2*E*,4*E*/*Z*-octadienal; HEPTA: 2E,4*E*/*Z*-heptadienal; MIX: combination of OCTA and HEPTA. (↑) increase; (↓) decrease; (↔) no change.



3.2. Growth

Only 20 µM OCTA (twice the EC_{50} for growth at 24 h) induced total decline of the culture (Table 2), whereas 5 µM and 10 µM inhibited growth in a concentration-dependent way without leading to the complete decline of the culture. When cells were removed from PUA, those that had been exposed to the lower concentrations (5 µM and 10 µM OCTA) were able to resume growth rapidly with faster rates than the control (Table 3). This suggests that they maintained all of their ability to divide and grow, as confirmed by their unaffected photosynthetic efficiency (Table 2).

Cultures exposed to the highest OCTA concentration (20 μM) resumed growth at a slower pace and fully recovered only 48 h later (Table 3). This suggests that OCTA concentrations around the EC_{50} for growth at 24 h affect key components of the growth machinery in SM, but not irreversibly, as opposed to TW, which was unable to recover cell division or metabolic activity when exposed to DECA for 24 h, in a threshold- and time-dependent manner [11]. It is also suggested that the blockage in the G1 phase of the cell cycle previously observed in TW [11] might be reversible in SM, probably related to the putative role of PUA as modulators and regulators of population size in this latter species.

4. Experimental Section

4.1. Culture Conditions and Experimental Design

Skeletonema marinoi (SM) (Sarno and Zingone), strain CCMP 2092, and *Phaeodactylum tricornutum* Bohlin (PT), strain CCMP 632, were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Booth Bay Harbor, Maine, USA). Natural filtered seawater (FSW), amended with f/2 nutrients [70], was used as the medium. PT and SM were cultured in a thermostated growth chamber (Hereus Holding GmbH, Hanau, Germany). PT was cultured in polystyrene flasks (Corning Inc., Corning, NY, USA) and SM in 2-L polycarbonate bottles with air bubbling, in order to avoid long chain formation, so as to be amenable to flow cytometry analyses. The axenicity of SM was confirmed before and after every experiment by inoculating 1 mL of culture in a sterile solution of peptone in autoclaved FSW (1 $mg \cdot mL^{-1}$). The cultures were maintained at 20 °C on a 12 h light,12 h dark cycle under a photon flux density of 110 μmol photons$\cdot m^{-2} \cdot s^{-2}$. All experiments used exponentially growing cultures, from at least 6 generations, at cell densities from 1 to 2×10^5 cells$\cdot mL^{-1}$ in biological triplicates. Each experiment was performed in triplicates and replicated at least twice. NO and ROS experiments lasted for 180 min, and samples were collected at times 0, 20, 60, 120 and 180 min after inoculation of PUA.

For the recovery experiment, cultures were resuspended in fresh medium without PUA after 24 h of exposure and samples taken after 24 and 48 h for cell counts, XC pigment, NPQ and photosynthetic efficiency analyses.

Cell concentrations were checked by microscopy (Zeiss Axioskop 2, Jena, Germany) from at least 200 cells at 200× magnification using an Axioskop 2 microscope (Carl Zeiss GmbH, Jena, Germany) and Sedgewick-Rafter counting chambers. Growth rates were calculated according to:

$$\mu = \ln[(N_1/N_0)/t] \quad (1)$$

where N_0 and N_1 represent cell density at the start and the end of the growth period and t is the time between measurements (in days).

The Student's *t*-test was used to compare average values using Excel software (Microsoft, Redmond, Washington, DC, USA).

4.2. PUA

The PUA used in these experiments were: 2*E*,4*E*/*Z*-decadienal (DECA), 2*E*,4*E*/*Z*-octadienal (OCTA), 2*E*,4*E*/*Z*-heptadienal (HEPTA) and a combination of OCTA and HEPTA (MIX) (all from

Sigma Aldrich Inc., Milan, Italy). PUA working solutions were prepared by dissolving them in absolute methanol (MeOH) (JT Baker, Phillipsburg, NJ, USA) at RT. The effective PUA concentration of the working solution was assessed spectrophotometrically (Hewlett-Packard 8453, Hewlett-Packard Company, Palo Alto, CA, USA) by using a 274-nm wavelength and a specific molar absorption coefficient of 31,000 [71].

4.3. NO and ROS Detection

Staining protocols were modified from [13] for NO detection and from [33] for ROS detection.

Optimal loading times and concentrations were determined empirically for SM. For endogenous NO detection *in vivo*, the fluorescent dye, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA), was used. DAF-FM DA is a membrane-permeable ester derivative of DAF-FM. Once inside the cell, the compound DAF-FM DA is first deacetylated by intracellular esterases to become DAF-FM and then further converted into its fluorescent triazole derivative (DAF-FM T) upon reaction with the NO oxidation product, N_2O_3 [72]. It has been used on cells from a variety of different organisms, including higher plants, marine invertebrates and phytoplankton [13,20,73,74]. Twenty milliliters of culture were spun and the pellet incubated in the dark in 50 μM DAF-FM DA for 30 min with agitation at 20 °C. Cells were rinsed twice with growth medium and incubated with PUA. DAF-FM DA-green fluorescence was detected by flow cytometry. In order to account for basal levels of NO production, control samples consisted of DAF-treated samples without the addition of PUA, and DAF-fluorescence data of treated samples are therefore represented as increased fluorescence relative to the control. No quenching on NO fluorescence was observed when only MeOH was added to control cultures (treated with DAF), suggesting that the observed decrease in green fluorescence was consistently generated by NO and not due to other artifacts caused by sample manipulation.

For ROS production, dihydrorhodamine 123 (DHR, Molecular Probes, Leiden, NL, USA) (5 mM stock in DMSO) was used. DHR is oxidized by different reactive oxygen species, including H_2O_2 and peroxynitrite ($ONOO^-$), to form the fluorescent derivative, rhodamine 123 [67]. DHR has been utilized for the detection of ROS in different cell types [29,33,34]. Samples were incubated with both 10 μM DHR and PUA in the dark at RT. Green fluorescence from the dye was assessed by flow cytometry. Carboxy-PTIO (Vinci-Biochem, Florence, Italy) and Tempol (Sigma-Aldrich Inc., Milan, Italy) were used as scavengers (negative controls) for NO and ROS, respectively, at a final concentration of 100 μM and 5 mM (in FSW). cPTIO is a water-soluble and stable nitric oxide radical scavenger that shows an antagonistic action against NO both in chemical and biological systems via a radical reaction [75], while Tempol is a nitroxide compound, which has been reported to be a general redox cycling agent acting as a catalase (CAT)-like agent [76]. Samples with the scavengers were incubated for 20 min before PUA inoculation. Controls consisted of dye-loaded samples processed like all treatments, except for PUA inoculation.

4.4. Flow Cytometry

A Becton-Dickinson Biosciences (Palo Alto, CA, USA) FACScalibur flow cytometer equipped with a 488-nm Ar laser as the excitation source was used. The flow rate was kept constant at 65 $\mu L \cdot min^{-1}$. Red fluorescence was used as a trigger, with a threshold at Channel 52. Red fluorescence

from chlorophylls was collected through a 650 long-pass filter, while green fluorescence from DAF-FM DA and DHR was collected through a 530/30 BP filter. FSW was used as a sheath, and 3.7 μm beads (Coulter Flowset Fluorospheres, Beckman Coulter, Fullerton, CA, USA) were used as internal standards. Data acquisition (10^4 cells on average for each sample) was performed using CellQuest software (Becton-Dikinson, Palo Alto, CA, USA), while data analysis was performed using FCS4 Express (De Novo Software, Los Angeles, CA, USA). All data are relative units to the beads and are expressed as ratios of control values.

4.5. XC Pigments and Photosynthetic Performance

For pigment concentration analyses, 10 mL of culture were filtered through 47-mm GF/F filters (Whatman, Maidstone, UK) and immediately frozen in the dark at −80 °C. Pigments were analyzed by high performance liquid chromatography (HPLC) (Hewlett Packard, series 1100, Kennett Square, PA, USA) shortly after the experiments (1 week at the latest). Filters were extracted in 100% methanol (Sigma-Aldrich, Milan, Italy), and 500 μL of 1 $mol \cdot L^{-1}$ ammonium acetate (final concentration 0.33 $mol \cdot L^{-1}$) were added to the 1-mL pigment extract 5 min before the analysis. A 3-μm C8 BDS column (100 mm × 4.6 mm) was used, and the procedure was the same as described in [77]. The mobile phase was composed of two solvent mixtures: A, methanol/aqueous ammonium acetate, 70:30 vol/vol; and B, methanol. The gradient between the two solvents was programmed as follows: 75% A (0 min), 50% A (1 min), 0% A (15 min), 0% A (18.5 min), 75% A (19 min). Pigments were detected at 440 nm using a photodiode array detector (model DAD series 1100, Hewlett Packard), which gives the 400- to 700-nm spectrum for each detected pigment. A fluorometer (series 1100, Hewlett Packard) allowed the detection of fluorescent pigments, with a 410-nm excitation wavelength and a 665-nm emission wavelength. Identification and quantification of single pigments were realized using chlorophyll (chl) and carotenoid standards obtained from the VKI (Water Quality Institute, Horsholm, Denmark) International Agency for 14C Determination (Horsholm, Denmark).

The photochemical efficiency of photosystem (PS) II was estimated by a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Triplicate variable fluorescence analysis was performed on 15-min dark-acclimated samples, to measure the maximal photochemical efficiency (Fv:Fm, dark-acclimated samples). Fm was measured after a saturating pulse of red light (2400 μmol $photons \cdot m^{-2} \cdot s^{-1}$, lasting 450 ms), causing a complete reduction of the PSII acceptor pool.

The non-photochemical quenching of fluorescence (NPQ) was quantified using the Stern–Volmer equation, where Fm and Fm′ are the maximal PSII fluorescence yield for dark-adapted and illuminated cells, respectively:

$$NPQ = (Fm:Fm') - 1 \quad (2)$$

5. Conclusions

Implications and Ecological Hypotheses

It is believed that chemically-mediated interactions have driven the evolution of certain organisms by selecting those individuals that had the ability to either resist, exploit or avoid external metabolites

from close-by cells [78]. It is also claimed that this is only expected if chemically-mediated interactions were sporadic, as happens during bloom events [79].

Our data highlight a wide variability of responses to PUA. The type of response depends both on the concentration of PUA to which cells are exposed and the time of exposure. Moreover, different pathways are activated in response to the PUA to which a diatom is exposed, depending also on the diatom species.

From an ecological point of view, our results suggest that diatom species perceive and are able to discriminate the PUA that they are exposed to, probably depending on their adaptive traits. SM is a widespread and bloom-forming marine diatom [43], whereas PT is not very well represented in the natural environment, and it has not been reported to form blooms in nature [80]. SM is a PUA producer, especially of OCTA and HEPTA, which are the most common PUA represented in marine diatoms and found dissolved in seawater after a bloom [49]. On the other hand, PT does not produce any PUA [2]. We therefore suggest that SM recognizes OCTA and HEPTA as intracellular signaling molecules and uses them as infochemicals, while PT reacts to PUA as external deleterious stimuli (probably allelochemicals) [81]. Tests on other diatom species and/or other taxa are needed before concluding that this is a general rule in diatoms and phytoplankton.

We can speculate that the physiological responses documented in this paper are expected to occur at sea during a SM bloom, when nutrients become limiting and senescent cells lyse [82], releasing and increasing the local concentrations of PUA. The response of a PUA-producing diatom to released PUA is modulated by and highly dependent upon the time of exposure and the PUA concentration, both in terms of ROS production and antioxidant defense, leading to increased resistance or death. At intermediate PUA concentrations, cells able to protect themselves are likely to slow down their growth rates, maintaining at the same time their photosynthetic performance. This is expected to allow a quick recovery in case the chemical stress is removed, e.g., by mixing or physical advection or degradation. In this case, the population modulates its size to the new limiting conditions and assures a recovery, increasing its competitiveness as soon as it experiences better environmental conditions.

In conclusion, we suggest that the interactions between chemical signals and reactive pathways underlie the functional diversity of species and their ability to cope with the environment. Indeed, the physiological responses to stimuli and biological interactions are intertangled and can shape ecosystems in a dynamical way, determining the ecological success of a species and its role.

Author Contributions

RC and AAG conceived and designed the experiments. AAG performed the experiments. CB analyzed and interpreted the xanthophyll cycle data. AP provided advice on experiments and the reagents to be used. AG and RC wrote the paper and all authors contributed for the respective field of expertise.

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Conflicts of Interest

The authors declare no conflict of interest.

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The Velocity of Light Intensity Increase Modulates the Photoprotective Response in Coastal Diatoms

Vasco Giovagnetti[1], Serena Flori[1¤], Ferdinando Tramontano[1], Johann Lavaud[2], Christophe Brunet[1]*

1 Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy, **2** Littoral Environnement et Sociétés, Unité Mixte de Recherche 7266, CNRS-Université de La Rochelle, La Rochelle, France

Abstract

In aquatic ecosystems, the superimposition of mixing events to the light diel cycle exposes phytoplankton to changes in the velocity of light intensity increase, from diurnal variations to faster mixing-related ones. This is particularly true in coastal waters, where diatoms are dominant. This study aims to investigate if coastal diatoms differently activate the photoprotective responses, xanthophyll cycle (XC) and non-photochemical fluorescence quenching (NPQ), to cope with predictable light diel cycle and unpredictable mixing-related light variations. We compared the effect of two fast light intensity increases (simulating mixing events) with that of a slower increase (corresponding to the light diel cycle) on the modulation of XC and NPQ in the planktonic coastal diatom *Pseudo-nitzschia multistriata*. During each light treatment, the photon flux density (PFD) progressively increased from darkness to five peaks, ranging from 100 to 650 µmol photons m^{-2} s^{-1}. Our results show that the diel cycle-related PFD increase strongly activates XC through the enhancement of the carotenoid biosynthesis and induces a moderate and gradual NPQ formation over the light gradient. In contrast, during mixing-related PFD increases, XC is less activated, while higher NPQ rapidly develops at moderate PFD. We observe that together with the light intensity and its increase velocity, the saturation light for photosynthesis (Ek) is a key parameter in modulating photoprotection. We propose that the capacity to adequately regulate and actuate alternative photoprotective 'safety valves' in response to changing velocity of light intensity increase further enhances the photophysiological flexibility of diatoms. This might be an evolutionary outcome of diatom adaptation to turbulent marine ecosystems characterized by unpredictable mixing-related light changes over the light diel cycle.

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* Email: christophe.brunet@szn.it

¤ Current address: University de Grenoble Alpes, CNRS (UMR5168), INRA (USC1359), CEA, IRTSV, Grenoble, France

Introduction

Photosynthetic organisms have evolved a set of interconnected mechanisms of photoacclimation and photoprotection in order to efficiently regulate light harvesting, and prevent the impairment of photosynthesis and biomass production [1]. The non-photochemical fluorescence quenching (NPQ) is a photoregulative mechanism that rapidly and efficiently operates to mold photochemistry under changing light. NPQ dissipates excess light energy as heat and occurs in the light-harvesting complex antennae (LHC) of photosystem (PS) II [1–3]. Three major components are commonly identified in NPQ, on the basis of their different kinetics of formation and relaxation: the energy-dependent (qE), the state-transitions (qT), and the photoinhibitory (qI) quenching [1–3]. While the importance of each NPQ component varies among photosynthetic lineages, qE is essential for photoprotection in most of them and is mainly controlled by the build-up of a transthylakoidal proton gradient (ΔpH) and the inter-conversion between epoxidized and de-epoxidized forms of xanthophyll carotenoids during the so-called xanthophyll cycle (XC) [1,3–5].

Several studies have demonstrated that the capacity of phytoplankton to efficiently regulate photosynthesis is functionally related to their adaptation to the underwater light environment [5–9]. Light fluctuations can indeed either limit the rate of photosynthesis (low light), or cause photo-oxidative stress due to the generation of reactive oxygen species in the photosynthetic apparatus (high light) [1]. Furthermore, when compared to terrestrial habitats, aquatic ecosystem mixing adds further unpredictability to light variations along the water column, which are either cyclic (i.e. diurnal/seasonal cycles) or irregular/stochastic (i.e. absorption and scattering due to dissolved substances and suspended particles in the water column, and intermittent cloud cover) [10,11]. Cells therefore experience variations in the velocity of light intensity increase, from predictable diel cycle-related light changes to faster and unpredictable mixing-related ones. Changes in phytoplankton photophysiology have been observed during daylight in the field [12–14]. Moreover, major physiological processes and growth rate are differently affected in relation to the fluctuating light regimes tested and phytoplankton groups/species under investigation [15–

17]. However, the effects of varying velocities of light intensity increase on phytoplankton capacity to photoprotect are unknown.

Among phytoplankton, diatoms constitute the most diversified group populating marine and freshwater ecosystems [18,19], due to their plasticity to changing conditions, a feature that has been often related to their evolutionary origin [19–21]. Their ecological and biological success has largely influenced both the structure and biogeochemistry of contemporary oceans [20], where they contribute to approximately 40% of the oceanic primary production [19,22]. Diatoms are known to have a remarkable capacity to cope with the variable underwater light environment [2,3,5]. They possess fucoxanthin (Fuco) chlorophyll (Chl) *a/c* binding proteins (FCP) as peripheral light-harvesting proteins and their antenna is organized in oligomeric complexes with groups−/species-dependent oligomeric state differences [2,3].

NPQ in diatoms mainly relies on qE, that is triggered by (i) the light-dependent generation of a ΔpH, (ii) the presence of specific light-harvesting complex stress-related proteins (LhcSR), termed Lhcx, and (iii) the XC [2–4,23]. In diatoms, qT seems to be missing [24], while the origin of qI – the most slowly forming and relaxing NPQ component that was originally ascribed to the photoinhibition of PSII reaction centre (RC) – is unclear, although the involvement of XC pigments is likely [2,3].

High light induces the de-epoxidation of the epoxy-xanthophyll, diadinoxanthin (Dd), into the epoxy-free xanthophyll, diatoxanthin (Dt), while the epoxidation from Dt back to Dd occurs in low light or darkness [2–4]. Recently, it has been shown that the exposition to gradually increasing light intensities can result in a partial Dt epoxidation under moderate and high light in different Chl *a/c*-containing phytoplankton species [25]. Dt molecules are spatially and functionally segregated among several pools in the thylakoid membrane of diatoms [2,3,26,27]. Under prolonged high light, Dd and Dt (as well as Lhcx proteins) can be *de novo* synthesized [2–4,28], while Dt does not necessarily enhance NPQ [29,30], but can fulfil an antioxidant function in the thylakoid membrane [27]. The violaxanthin (Vx) cycle, which is found in higher plants and green algae, is also present in diatoms, and consists of the de-epoxidation of Vx into zeaxanthin (Zx) via the intermediate xanthophyll, antheraxanthin (Ax), and reverse epoxidation [4,31]. In diatoms, Vx serves as precursor pigment in the biosynthesis of Dd and their main FCP light-harvesting pigment, Fuco [31–33].

The aim of our study is to investigate if the photoprotective mechanisms activated by coastal diatoms under an unpredictable and fast mixing-related photon flux density (PFD) increase differ from those in response to the predictable and slower diel cycle-related PFD increase. Here we address the effect of three velocities of light intensity increase on XC and NPQ modulation in the marine planktonic coastal diatom *Pseudo-nitzschia multistriata* (Takano) Takano, a toxic diatom known to form blooms in the Gulf of Naples (Mediterranean Sea) [34,35], where it was isolated. *P. multistriata* photophysiological responses to each light kinetics were studied by subjecting cells to light intensities that progressively increased from darkness to five peaks, ranging from 100 to 650 μmol photons m^{-2} s^{-1} (Fig. 1).

Our results suggest that both the light intensity and the velocity of its increase control the regulation of photoprotection in *P. multistriata*. A slow light increase that resembles the light diel cycle enables a strong XC activation and moderate NPQ formation, through which cells can photoprotect against and photoacclimate to high light. Instead, velocities of light increase greater than that experienced during the light diel cycle lead to a flexible coupling between rapidly forming NPQ and XC operation, the former being enhanced, while the latter decreasing.

Materials and Methods

Ethics Statement

No specific permission was required for the isolation of the diatom *Pseudo-nitzschia multistriata* (strain SY416, Bacillariophyceae), which was carried out in the framework of the long-term ecological research MareChiara (LTER-MC, Stazione Zoologica Anton Dohrn, Naples, Italy), a research program conducted in coastal waters of the Gulf of Naples (Mediterranean Sea). No endangered or protected species has been used in this work.

Culture Conditions

The coastal diatom *Pseudo-nitzschia multistriata* (Takano) Takano (strain SY416) was isolated (Gulf of Naples, Mediterranean Sea) and provided by SVM Tesson (Laboratory of Ecology and Evolution of Plankton, Stazione Zoologica Anton Dohrn, Naples, Italy). Cultures were grown non-axenically at 20°C in f/2 medium [36] made with locally obtained and sterilized seawater, using 225 cm^2 polystyrene canted neck flasks (Corning Flask, Corning Inc., NY, USA). Cells were cultured under a sinusoidal light regime set to peak at the photon flux density (PFD) of 100 μmol photons m^{-2} s^{-1} (preacclimation light, PL), during two weeks before experiments, in a 11 hours (h) light/13 h dark photoperiod (Fig. 1A). Cells were gently and continuously flushed with sterile air, and maintained in exponential phase by daily and semi-continuous dilution. Temperature and pH were checked daily using an HI-9214-Stick pH meter (Hanna Instruments, Woonsocket, RI, USA). Light was provided using the Advanced Control Lighting System (ACLS) and Infinity XR4 pendant reflector (Aquarium Technologies, Sfiligoi S.r.l., Italy). Infinity XR4 was equipped with a HQI metal halide lamp (400 W, 10000 K). Photosynthetically available radiation (PAR) intensity was measured using a laboratory PAR 4 π sensor (QSL 2101, Biospherical Instruments, San Diego, CA, USA), while lamp spectral composition (PAR(λ)) was measured at light peak using a radiometer (Hyper OCR I, Satlantic, Halifax, CA).

Experimental Design

After preacclimation (PL, Fig. 1A), *P. multistriata* cells in the exponential growth phase were shifted to the experimental light conditions before the light was switched on. Three experiments were performed in triplicate, testing three gradually increasing light treatments, namely the 5 h, 3 h and 2 h kinetics of light increase (Fig. 1B−D, respectively). During each of these three experimental kinetics of light increase, five light conditions were applied, characterized by light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 μmol photons m^{-2} s^{-1} (Fig. 1). Note that the 5 h kinetics of light increase peaking at 100 μmol photons m^{-2} s^{-1} was identical to PL (Fig. 1A−B). Samples were taken at three sampling time points during light increase (dots in Fig. 1B−D). Cultures were sampled 15 minutes (min) before light started to increase. Then, after 3 h (5 h kinetics), 2 h (3 h kinetics), and 1.5 h (2 h kinetics), samples were taken at the PFD of 42, 123, 150, 164 and 280 μmol photons m^{-2} s^{-1} for the light condition peaking at 100, 250, 350, 500 and 650 μmol photons m^{-2} s^{-1}, respectively. The last sampling was carried out at the PFD peak (Fig. 1B−D). At each sampling time point, aliquots of 20–30 mL of culture were rapidly collected to measure Chl *a* fluorescence yield and non-photochemical fluorescence quenching (NPQ), and pigment content. Cell concentration and absorption spectrum were measured once a day during the first sampling time point.

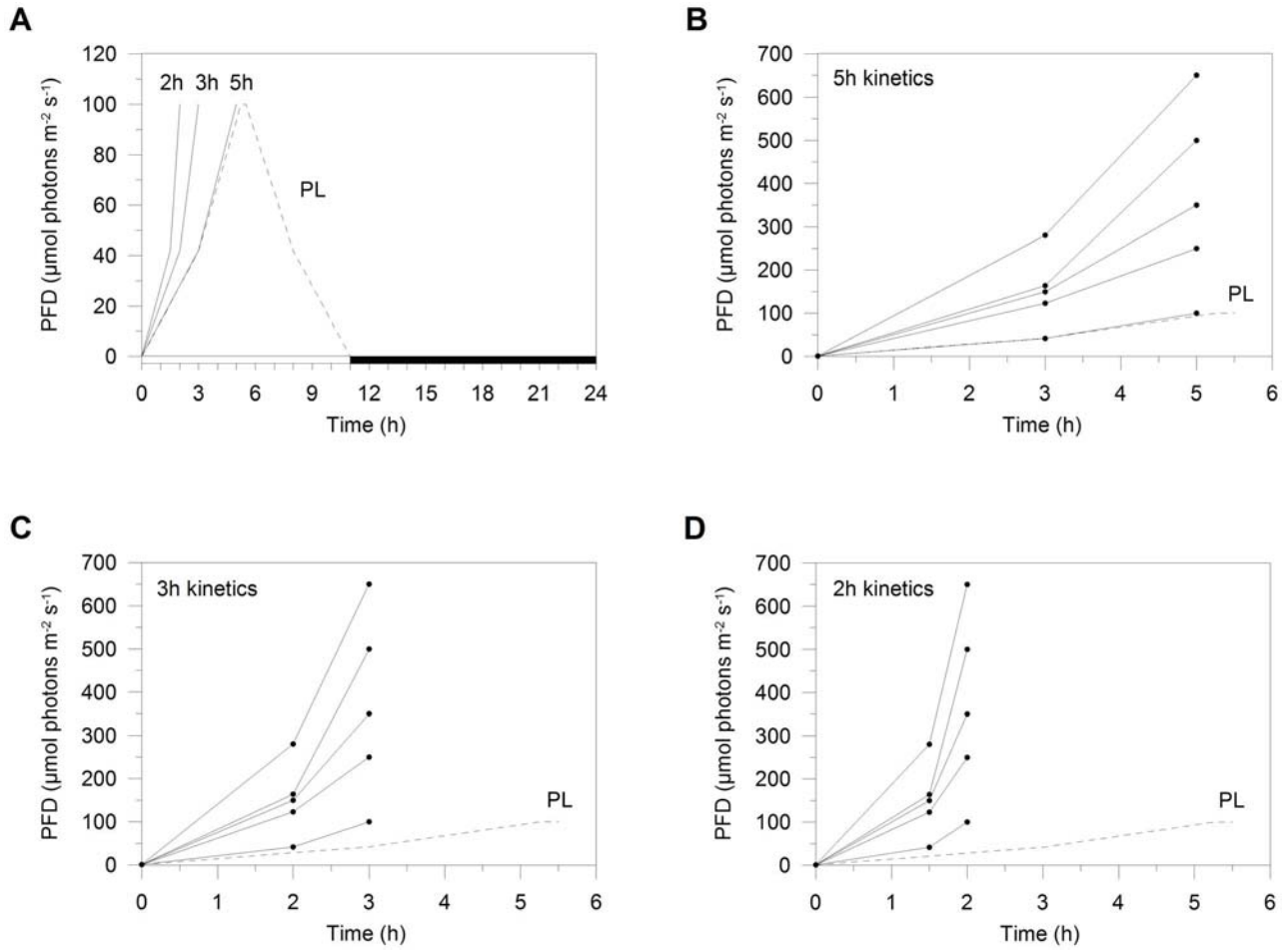

Figure 1. Preacclimation and experimental light conditions. (A) *Pseudo-nitzschia multistriata* cells were grown under a sinusoidal light regime set to peak at the PFD of 100 µmol photons m^{-2} s^{-1} (preacclimation light, PL; dashed line). After two weeks of preacclimation, cells in the exponential growth phase were shifted to three experimental light treatments, the 5 h (diel cycle-related PFD increase; B), 3 h and 2 h kinetics of light increase (mixing-related PFD increases; C and D, respectively), each characterized by light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}. In each panel, experimental light increases (solid lines) are compared to PL (dashed line). Triplicate samples were taken at three sampling time points during light increase (dots, B–D). Firstly, cultures were sampled in darkness. Then, after 3 h (5 h kinetics), 2 h (3 h kinetics), and 1.5 h (2 h kinetics), samples were taken at the PFD of 42, 123, 150, 164 and 280 µmol photons m^{-2} s^{-1} for the light condition peaking at 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, respectively. Lastly, cultures were sampled at PFD peaks.
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Cell Growth

During the preacclimation and the day in which each experiment was performed, growth was monitored by cell counting performed daily on triplicate sub-samples, using a Zeiss Axioskop 2 Plus microscope. Aliquots of 1 mL of algal culture were used to fill Sedgewick Rafter cell counting chambers. Growth rate was estimated from cell concentration measurements using the following equation, $\mu = \ln [N_{t2}/N_{t1}]/[t_2-t_1]$, where μ is the growth rate (day^{-1}) and N_t is the mean cell concentration at time t, and t_1 and t_2 correspond to the morning sampling times of days 1 and 2, respectively. The growth rate (μ) of *P. multistriata* cells grown under PL was 0.76 ± 0.10 d^{-1} ($n = 9$, Table 1), and did not change during experiments, ranging between ~0.68 and ~0.90 (Table 1). Cell concentration ranged between ~4.2 and $\sim 9.7\times10^4$ cells mL^{-1}, during preacclimation and experiments.

Pigment Analysis

High performance liquid chromatography (HPLC) was performed to analyse pigment content. Aliquots of 10 mL of algal culture were filtered onto GF/F glass-fibre filters (Whatman, Maidstone, UK) and immediately stored in liquid nitrogen until further analysis. Triplicate samples were taken during each sampling time point. Pigments were extracted by mechanical grinding during 3 min in 2 mL of a 100% methanol solution. Then, the homogenate was filtered onto Whatman 25 mm GF/F glass-fibre filters (Whatman, Maidstone, UK) and the volume of the extract was accurately measured. Prior to injection into the loop of the HPLC system, 250 µL of an Ion Pairing Agent (ammonium acetate 1 mol L^{-1}, final concentration 0.33 mol L^{-1}) were added to 0.5 mL of the pigment extract and incubated for 5 min in darkness at 4°C. This extract was then injected in the 50 µL loop of the Hewlett Packard series 1100 HPLC system (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (2.6 µm diameter C_8 Kinetex column, 50 mm×4.6 mm; Phenomenex, USA). The temperature of the column was steadily maintained at 20°C and the flow rate of the mobile phase was set up at 1.7 mL min^{-1}. The mobile phase was composed of eluent A, a solvent mixtures of methanol and aqueous ammonium acetate (70/30, v/v), while eluent B was methanol. During a 12 min-lasting elution, the gradient between the solvents

Table 1. Photosynthetic and physiological properties, and photosynthetic pigment content of *Pseudo-nitzschia multistriata*.

Parameters	Light conditions	Mean values ± SD
$_{rel}ETR_{max}$	Preacclimation	0.99±0.04
Ek	Preacclimation	246±12
μ	Preacclimation and 5 h, 3 h, 2 h kinetics	0.76±0.10
F_v/F_m	Preacclimation	0.71±0.01
Chl *a* cell^{-1}	5 h, 3 h, 2 h kinetics	4.63±1.14
Chl c_1 Chl a^{-1}	5 h, 3 h, 2 h kinetics	3.97±0.77
Chl c_2 Chl a^{-1}	5 h, 3 h, 2 h kinetics	6.46±0.94
Chl c_3 Chl a^{-1}	5 h, 3 h, 2 h kinetics	7.41±2.01
Fuco Chl a^{-1}	5 h, 3 h, 2 h kinetics	67.63±6.75

The measurement of photosynthetic and physiological properties was performed on cells in the exponential growth phase, during preacclimation, the day before the experiments started. The growth rate did not change during experiments. $_{rel}ETR_{max}$, maximal relative electron transport rate (in mol e^- g Chl a^{-1} h^{-1}); Ek, saturation light for photosynthesis (in μmol photons m^{-2} s^{-1}); μ, growth rate (in d^{-1}); F_v/F_m, photosystem II maximal photochemical efficiency. Values are means ± SD ($n = 9$). Chlorophyll *a* cellular content (Chl *a*, in 10^{-16} mol Chl *a* cell^{-1}) and photosynthetic accessory pigments Chl a^{-1} content (in mol pigment/100 mol Chl *a*) measurements were performed during experiments. Fuco, fucoxanthin: Chl *c*, chlorophyll $c_{1, 2, 3}$. Pigment data are means ± SD of the all data set ($n = 135$).
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was programmed: 75% A (0 min), 50% A (1 min), 0% A (8 min), 0% A (11 min), 75% A (12 min). Pigments were detected spectrophotometrically at 440 nm using a model DAD, Series 1100 Hewlett-Packard photodiode array detector. Fluorescent pigments were detected in a Hewlett-Packard standard FLD cell series 1100, with excitation and emission wavelengths set at 407 and 665 nm, respectively. For determination and quantification of pigments, calibration curves were obtained using pigment standards from Danish Hydraulic Institute (DHI) Water & Environment (Hørsholm, Denmark).

Absorption Spectrum

Aliquots of 10 mL of algal culture were filtered onto Whatman GF/F filters (Whatman, Maidstone, UK) and immediately frozen. Absorption spectrum measurements were performed as previously described, and correction factors (e.g. due to filter absorption enhancement) were applied accordingly [37]. Absorption was measured between 280 and 800 nm with 1-nm increments on a spectrophotometer (Hewlett-Packard HP-8453E) equipped with an integrating sphere RSA-HP-53 (Labsphere Inc., North Sutton, NH, USA). The mean integrated absorption value (a^*) was thus normalized by the chlorophyll (Chl) *a* concentration to obtain the Chl *a*-specific absorption coefficient (a^*_{ph}; m^2 mg Chl a^{-1}). The number of absorbed photons Chl a^{-1} integrated over time (expressed in mol photons mg Chl a^{-1}) was calculated as the product of PAR (λ, 400–700 nm) and a^*_{ph} (λ, 400–700 nm) integrated over the time course of the experiments.

Chl *a* Fluorescence Yield and Non-Photochemical Fluorescence Quenching (NPQ)

Photochemical efficiency of photosystem (PS) II was estimated by pulse amplitude fluorescence (PAM) measurements, using a PHYTO-PAM fluorometer (Heinz Walz, Effeltrich, Germany). F_0 and F_m are defined as the minimum PSII fluorescence yield and the maximum PSII fluorescence yield measured on 15 min dark-acclimated cells, while being termed F_0' and F_m' when measured on light-acclimated cells. F_m or F_m' were measured after a saturating pulse of red light (2400 μmol photons m^{-2} s^{-1}, lasting 450 ms), causing a complete reduction of the PSII acceptor pool. The maximum photosynthetic efficiency of PSII is calculated as the ratio F_v/F_m, where F_v is the variable fluorescence emission and is equal to $F_m - F_0$.

The electron transport rate (ETR) *versus* irradiance (E) curves were performed on 15 min dark-acclimated samples by applying 10 stepwise increasing actinic irradiances (E, from 1 to 1500 μmol photons m^{-2} s^{-1}), at intervals of 2 min each. The maximal relative rate of linear electron transport, normalized by Chl *a* concentration ($_{rel}ETR_{max}$, expressed in mol e^- g Chl a^{-1} h^{-1}), was calculated as $_{rel}ETR_{max} = (F_v'/F_m') \times PFD \times (a^*_{ph}/2)$, where F_v' and F_m' are PSII variable and maximal fluorescence yield, respectively, for illuminated cells (measured at the end of the 2 min lasting actinic light), and PFD is the incident irradiance (expressed in μmol photons m^{-2} s^{-1}). The Chl *a*-specific absorption coefficient a^*_{ph} (see above) was divided by two, assuming that the excitation energy is evenly distributed between the two photosystems. The photosynthetic parameters, maximal relative electron transport rate ($_{rel}ETR_{max}$) and saturation light for photosynthesis (Ek) were retrieved from the ETR-E curves [38].

Non-photochemical fluorescence quenching (NPQ) was measured on 15 min dark-acclimated cells. Actinic light was fixed at 480 μmol photons m^{-2} s^{-1} and the cells were illuminated for 10 min, and the maximum fluorescence yield was estimated each min. Actinic light intensity during the measurement was chosen in order to saturate photosynthesis in control cultures and ensure maximal NPQ amplitude. NPQ was quantified by the 'Stern-Volmer' expression, $NPQ = (F_m/F_m') - 1$, where F_m' is the maximum PSII fluorescence yield of light-acclimated cells [39].

A sustained light-acclimated NPQ (NPQ_{sl}) was calculated as $(F_{mt0}/F_m') - 1$ [40]. F_{mt0} corresponds to F_m measured from the dark-acclimated cells sampled during the first sampling time point. F_m' is measured at each sampling time point on light-acclimated cells. Differently from the sustained phase of NPQ (NPQ_s) estimated in [40], NPQ_{sl} represents the overall NPQ, i.e. the fraction that rapidly relaxes and its more sustained components, totally accumulating during the light increase.

Statistical Analysis

Student's *t*-test analysis for comparison of means and Spearman correlation were performed using the software Statistica (StatSoft, OK, USA).

Results and Discussion

Photoacclimation to gradual increases of PFD

Growth rate (μ), photosystem (PS) II maximal photochemical efficiency (F_v/F_m), and maximal relative electron transport rate ($_{rel}ETR_{max}$; see Table 1) confirmed the healthy physiological state of *P. multistriata* cells grown under preacclimation light (PL, i.e. sinusoidal light peaking at the PFD of 100 μmol photons m^{-2} s^{-1}; Fig. 1A). From the measured $_{rel}ETR_{max}$ (0.99 ± 0.04 mol e^- g Chl a^{-1} h^{-1}), an oxygen evolution rate of ~250 μmol O_2 mg Chl a^{-1} h^{-1} was estimated. The saturation light for photosynthesis (Ek) was ~250 μmol photons m^{-2} s^{-1} in *P. multistriata* cells under PL (Table 1). This means that the Ek value was higher than the maximal PFD reached during preacclimation, probably indicating that this species could not decrease Ek to values below ~250 μmol photons m^{-2} s^{-1} when subjected to PL. Interestingly, similar results have been reported on three different Chl *a*/*c*-containing species (belonging to the class of Bacillariophyceae [9] and Pinguiophyceae [41]), grown under the same light conditions provided by the same light system in this study. It should be noted that the light system we applied mainly provides blue wavelengths, which are known to be more efficiently used by diatoms than red or green wavelengths [42,43].

For each kinetics of light increase, our experimental design allowed us to test the photophysiological regulation of *P. multistriata* under three increasing light conditions that reached PFD peaks higher than Ek and two conditions reaching PFD peaks lower than or similar to Ek. Whatever was the condition of light increase, and regardless of the kinetics of light increase, the Chl *a* cellular content and photosynthetic pigment Chl a^{-1} content did not change significantly over time ($p>0.05$, $n=15$; Table 1), with concentrations of Chl *a* significantly correlated to those of Fuco, Chl c_1, c_2, and c_3 ($p<0.005$, $n=45$). Fuco was the main accessory pigment, with its pool size being approximately ten- and seventeen-fold higher than that of Chl c_2/c_3 and c_1, respectively (Table 1). The presence of Chl c_3, which is a pigment rarely found in diatoms, agrees with previous findings on the same species ([42] and references therein).

The absence of a photoacclimative response involving variation in the photosynthetic pigment content contrasts with the results generally observed in previous studies (e.g., [44−46]). Some authors [46] showed that the exposure to high light (500 μmol photons m^{-2} s^{-1}) of *Phaeodactylum tricornutum* cells caused a rapid down-regulation of Chl *a* biosynthesis and transcripts encoding putative light harvesting antenna proteins, as well as an immediate decline in Fuco cellular content and the subsequent decrease in Chl *a* and *c* cellular content. The reason of such a difference with our results is linked to the gradual light increase applied in our study, in contrast to the sudden light increase that is often applied (e.g., [46]). Indeed, the use of an abrupt light increase activates regulative and photoacclimative strategies related to a stress-response, which might involve a prompt rearrangement of the light harvesting system and consequent decrease in photosynthetic pigment pool size, together with XC/NPQ induction (e.g., [45,46]). In contrast, a "naturally occurring" gradual increase of light allows cells to progressively modulate the photoprotective process. In this framework, the modulation of XC not only acts as short-term photoprotective process controlling NPQ formation, but also enables cells to photoacclimate to gradual increases of light without significantly changing the light-harvesting capacity of the photosynthetic antenna. This confirms previous results obtained in a study conducted on different Chl *a*/*c*-containing species [25], in which the authors also show that the epoxidation of Dt to Dd can take place under moderate and high light in some species when cells undergo a gradual light increase. Overall, these results are a further proof that the experience of a gradual light increase enables cells to efficiently regulate their photophysiological properties by properly balancing photoacclimation and photoprotection.

However, results on photoacclimation and photoprotection regulation should be considered in the context of light adaptation [25] and nutrient availability ([47,48] and references therein). Indeed, Dimier et al. [25] showed different photoresponses to PFD increase in high light-, low light- and variable light-adapted phytoplankton species, such as the coastal diatom *P. multistriata*, on the basis of their XC characteristics. Since it is known that light history influences photoregulation [8], it should be underlined that preacclimation light (PL, 100 μmol photons m^{-2} s^{-1}; Fig. 1) corresponds to PAR values measured at a depth range of 7–12 m in the mixed layer of the coastal waters of the Gulf of Naples (Mediterranean Sea; Brunet, unpublished data).

Furthermore, nutrient supply controls phytoplankton cellular response in the field, modifying the balance between light-harvesting processes and those that generate and utilize energy sources (adenosine 5′-triphosphate, ATP, and reduced nicotinamide adenine dinucleotide phosphate, NADPH), hence modulating cell photoacclimation/photoprotection dynamics [47,48]. Therefore, our results refer to nutrient-replete conditions, such as those found during the onset of the spring bloom.

One of the main aspects addressed by this study is the role played by light increase velocity on the photoregulation capacity of *P. multistriata*. Figure 2A depicts the number of absorbed photons Chl a^{-1} integrated over time that characterizes the three tested light treatments, simulating diel cycle-related (5 h kinetics) and mixing-related PFD increase conditions (3 h and 2 h kinetics; Fig. 1). Over the gradient of the time-integrated absorbed photons per Chl *a*, the faster kinetics of light increase (3 h and 2 h kinetics) distinctively affect the sustained light-acclimated NPQ (NPQ_{sl}, which is the overall NPQ that totally accumulates during the light increase; Fig. 2B) and the de-epoxidation state (DES = Dt/[Dd+Dt]; Fig. 2C), when compared to the slowest condition (5 h kinetics). These results reveal that changes in the kinetics of light increase influence XC/NPQ modulation (see next subsections), thus probably impacting the productivity of the mixed layer.

XC and NPQ responses to a diel cycle-related PFD increase

In the 5 h kinetics of light increase, Dt synthesis exponentially increased over the light range and Dt Chl a^{-1} reached the highest value measured among the tested light treatments (26.5 ± 1.4 mol Dt/100 mol Chl *a*; Fig. 3A). The augment in Dt pool size largely relied on Dd *de novo* synthesis as revealed by the significant and positive correlation between Dd and Dt when PFD was ≤ 350 μmol photons m^{-2} s^{-1} ($R^2=0.68$, $p<0.005$, $n=39$; black dots in Fig. 3B). In contrast, when PFD was ≥500 μmol photons m^{-2} s^{-1}, the relationship between the two xanthophylls was inverse ($R^2=0.87$, $p<0.025$, $n=6$; white dots in Fig. 3B), showing a further (almost three-fold) increase in Dt pool size through Dd pool depletion.

DES linearly increased over the light gradient ($R^2=0.92$, $p<0.005$) reaching the maximal value of 78%. The strong activation of the XC in *P. multistriata* is fostered by an efficient enhancement of the carotenoid biosynthetic pathway, as demonstrated by the significant correlation found between either Vx or Zx Chl a^{-1} and Dt Chl a^{-1} (when Vx cycle xanthophylls were detected, $R^2=0.48$, $p<0.01$, $n=25$, and $R^2=0.59$, $p<0.05$, $n=9$, respectively; Fig. S1A and S1B), as well as between β carotene (β-Car) and Dd Chl a^{-1} ($R^2=0.52$, $p<0.005$, $n=44$;

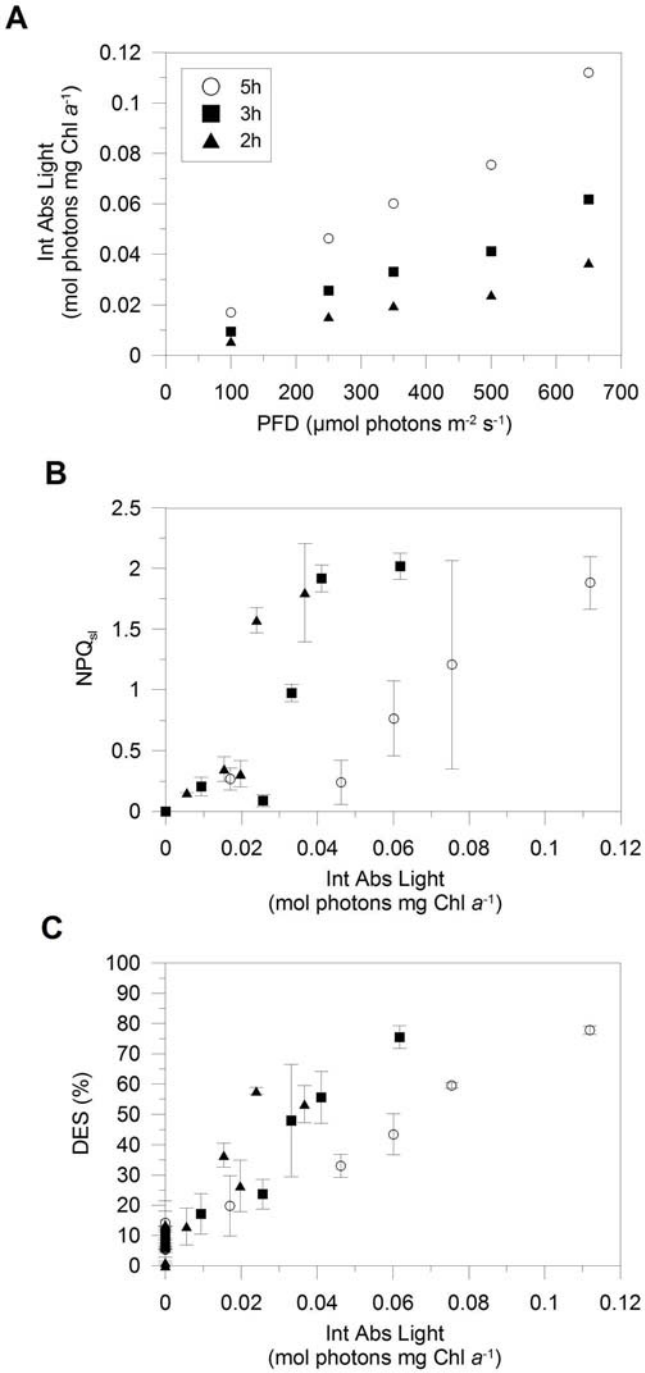

Figure 2. Influence of the kinetics of light increase on the photoprotection modulation. (A) Evolution of the number of absorbed photons per Chl *a* integrated over time (integrated absorbed light, Int Abs Light; expressed in mol photons mg Chl a^{-1}) over the light gradient, at the PFD peaks of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, during the 5 h (white dots), 3 h (black squares) and 2 h kinetics of light increase (black triangles). Induction of the sustained light-acclimated NPQ (NPQ_{st}; B) and evolution of the de-epoxidation state (DES = Dt/[Dd+Dt]; C) *versus* Int Abs Light during the 5 h (white dots), 3 h (black squares) and 2 h kinetics of light increase (black triangles). Values are means ± SD ($n = 3$).
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Fig. S2A). These results further confirm the role of Vx cycle pigments as biosynthesis precursors of Dd and Fuco [31,33], a feature that has been regarded as metabolically advantageous in order to poise photoprotection and light harvesting in Chl *a*/*c*-containing phytoplankton groups [25,31,33]. Indeed, while Ax was not found in our study [31], both Vx and Zx were detected all along the light range (Table 2), with their pool size especially increasing as PFD was ≥280 µmol photons m^{-2} s^{-1} (Table 2), i.e. close to the Ek value (Table 1). This finding makes Ek a key parameter in controlling the photoprotective response development at the pigment content level, and not only the limiting/optimal light switch for photosynthesis.

Despite cells activated the strongest Dt synthesis in this condition (Fig. 3A), NPQ was the lowest among light treatments

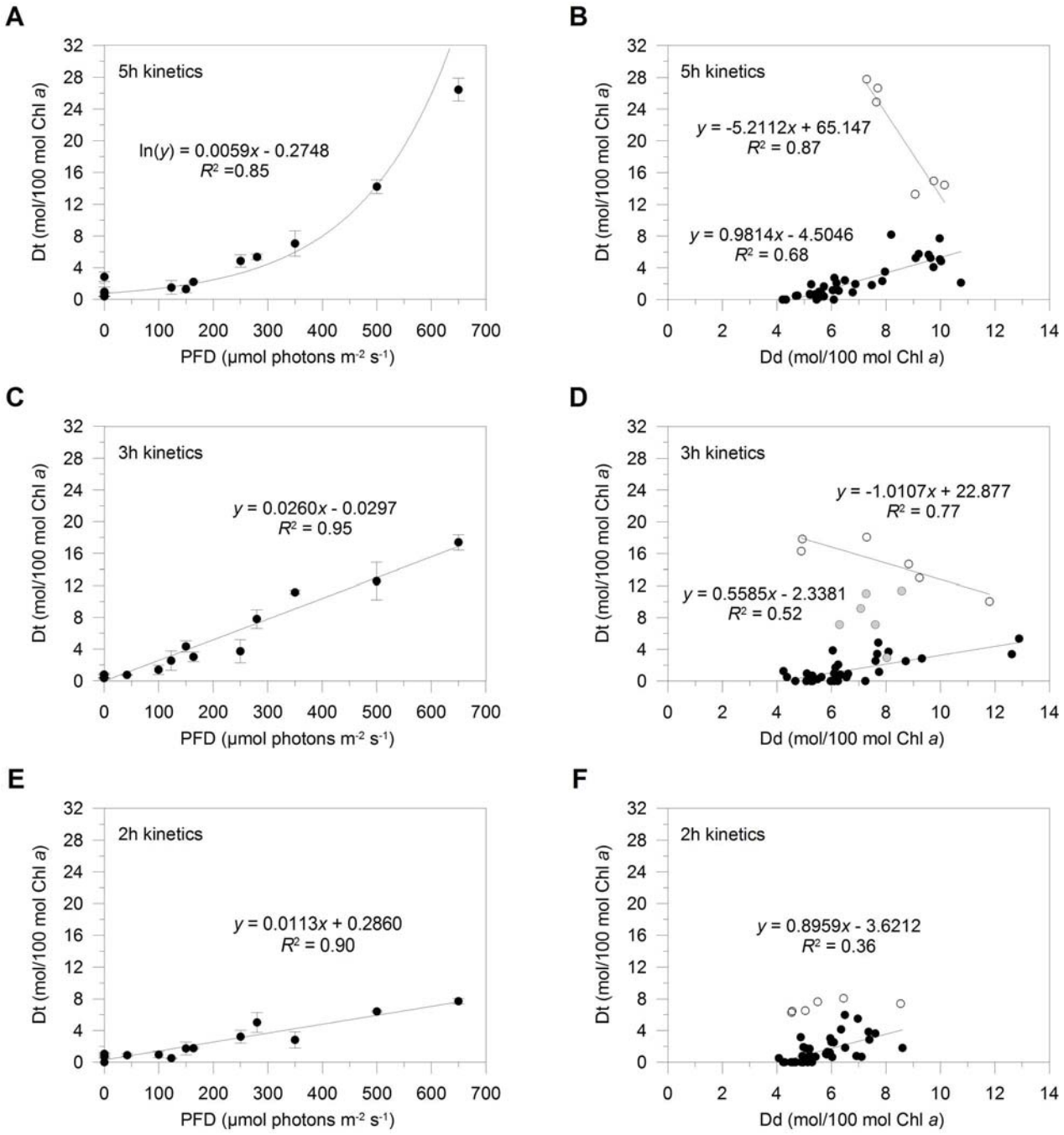

Figure 3. Xanthophyll cycle modulation. Evolution of diatoxanthin (Dt)/chlorophyll (Chl) *a* (in mol Dt/100 mol Chl *a*) over the light gradient, in *Pseudo-nitzschia multistriata* cells experiencing light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, during the 5 h (A), 3 h (C) and 2 h kinetics of light increase (E). Values are means ± SD ($n = 3$). Relationship between Dt and diadinoxanthin (Dd)/Chl *a* (in mol pigment/100 mol Chl *a*), during the 5 h (B), 3 h (D) and 2 h kinetics of light increase (F). In (B) and (F) data measured at PFD ≤350 µmol photons m^{-2} s^{-1} (black dots, $n = 39$) and ≥500 µmol photons m^{-2} s^{-1} (white dots, $n = 6$) are discerned. In (D) data measured at PFD ≤250 µmol photons m^{-2} s^{-1} (black dots, $n = 33$), at 280 and 350 µmol photons m^{-2} s^{-1} (grey dots, $n = 6$), and at PFD ≥500 µmol photons m^{-2} s^{-1} (white dots, $n = 6$) are discerned.
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(Fig. 4A). NPQ gradually increased over the light gradient and reached the maximal value of 0.81±0.17 at 500 µmol photons m^{-2} s^{-1}, after which it remained stable (Fig. 4A). This suggests that the Dt pool size synthesized to cope with a diurnal light increase is not entirely involved in NPQ formation, as also reported in other diatom species [26,29,30]. Although NPQ was weakly induced, its development was significantly correlated to Dt Chl a^{-1} ($R^2 = 0.70$, $n = 45$, $p<0.005$; Fig. 4B) and DES ($R^2 = 0.65$, $n = 45$, $p<0.005$). Intriguingly, the linear relationship between NPQ formation and Dt synthesis, which is commonly reported (e.g., [29,49,50]), was only found for PFD ≤ Ek (precisely for PFD ≤280 µmol photons m^{-2} s^{-1}, $R^2 = 0.78$, $n = 36$, $p<$

Table 2. Carotenoid content of *Pseudo-nitzschia multistriata* cells.

Pigments		5 h kinetics	3 h kinetics	2 h kinetics
β-Car Chl a^{-1}	All data	4.17±0.83 ($n=44$)	4.56±0.60 ($n=45$)	4.71±0.65 ($n=44$)
Vx Chl a^{-1}	<280 µmol photons m^{-2} s^{-1}	0.34±0.04 ($n=15$)	0.31±0.09 ($n=14$)	0.45±0.08 ($n=6$)
Vx Chl a^{-1}	≥280 µmol photons m^{-2} s^{-1}	0.53±0.14 ($n=10$)	0.33±0.08 ($n=10$)	0.45±0.09 ($n=3$)
Zx Chl a^{-1}	<280 µmol photons m^{-2} s^{-1}	0.36±0.11 ($n=3$)	0.28±0.08 ($n=13$)	0.00
Zx Chl a^{-1}	≥280 µmol photons m^{-2} s^{-1}	0.46±0.16 ($n=6$)	0.44±0.20 ($n=7$)	0.44±0.03 ($n=3$)

β-carotene (β-Car), violaxanthin (Vx) and zeaxanthin (Zx)/chlorophyll (Chl) *a* (in mol pigment/100 mol Chl *a*) of *Pseudo-nitzschia multistriata* cells experiencing light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, during the 5 h, 3 h and 2 h kinetics of light increase (see Fig. 1B–D). Pigment values are means ± SD.

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0.005; black dots in Fig. 4B). For PFD greater than Ek, NPQ only slightly increased and poorly relied on the further synthesis of Dt (white dots in Fig. 4B). Such a discrepancy in the expected Dt/NPQ linear relationship might be related to the spatial and functional heterogeneity of Dt pools in the thylakoid membrane of diatoms [26,27,51]. Dt molecules might be located among the monogalactosyl-diacylglycerol (MGDG) molecules of the lipid shield that surrounds the FCPs, instead of being bound to FCP specific antenna polypeptides [27,28,51]. These Dt molecules are likely to prevent lipid peroxidation [27] instead of effectively participating to NPQ, which needs the so-called 'activation' of Dt molecules through the protonation of some FCP binding sites during the ΔpH build-up (Δ522 nm fingerprint) [26,49,52]. The weak development of NPQ is also in line with the fact that Dt molecules dissolved in MGDG shield are not able to interact excitonically with Chl *a* [27], hence decreasing the light energy that is channelled to the PSII RC.

NPQ_{sl} almost gradually increased over the light range and reached its maximal value at 650 µmol photons m^{-2} s^{-1} (1.9±0.2, Fig. 5A). Differently from what we observed in the two faster kinetics of light increase (see below), NPQ_{sl} values were low and quite stable for PFD ≤ Ek, while increasing more steeply when PFD was>Ek in the 5 h kinetics (Fig. 5A).

XC and NPQ response to mixing-related PFD increase

The faster was the kinetics of light increase, the less activated was the XC (Fig. 3C and E), leading to a decrease of the maximal Dt Chl a^{-1} from ~27 mol Dt/100 mol Chl *a* (5 h kinetics, Fig. 3A) to 17.4±1.0 (3 h kinetics, Fig. 3C) and 7.7±0.4 mol Dt/100 mol Chl *a* (2 h kinetics, Fig. 3E). Dt synthesis linearly increased ($p<0.005$) during the two fast kinetics of light increase, in a stronger manner in the 3 h than the 2 h kinetics (Fig. 3C and E). These results demonstrate that XC operation is not exclusively driven by the light intensity increase, but also by the velocity at which cells undergo such an increase of light intensity. It is interesting to note that light intensity and velocity of its increase affect XC modulation in opposite ways.

During the 3 h kinetics, Dt and Dd Chl a^{-1} were positively correlated for PFD ≤ Ek (until 250 µmol photons m^{-2} s^{-1}, $R^2=0.52$, $p<0.005$, $n=33$; black dots in Fig. 3D). For PFD similar to or greater than Ek (at 280 and 350 µmol photons m^{-2} s^{-1}; grey dots in Fig. 3D), no correlation was observed between the two xanthophylls and the Dt pool size further increased through Dd pool depletion (at 280 µmol photons m^{-2} s^{-1}) and subsequent Dd *de novo* synthesis (until 500 µmol photons m^{-2} s^{-1}). The decrease in Dd Chl a^{-1} at 280 µmol photons m^{-2} s^{-1} indicates that the rate of Dd de-epoxidation was faster than that of its replenishment, consistently with a greater requirement of Dt synthesis at PFD~Ek (see below). When PFD was ≥500 µmol photons m^{-2} s^{-1}, Dt and Dd Chl a^{-1} were instead negatively correlated, revealing that Dt pool size continued to increase again by depleting the Dd pool ($R^2=0.77$, $p<0.05$, $n=6$; white dots in Fig. 3D).

During the 2 h kinetics, the increase in Dd Chl a^{-1} was the least strong among the tested light kinetics (Fig. 3F), which might in part explain the weakest Dt synthesis in this condition (Fig. 3E). Dt and Dd Chl a^{-1} were positively correlated until PFD was ≤ 350 µmol photons m^{-2} s^{-1} ($R^2=0.36$, $p<0.025$, $n=39$; black dots in Fig. 3F). When PFD became ≥500 µmol photons m^{-2} s^{-1}, Dt pool size slightly increased without correlating to Dd pool variations (white dots in Fig. 3F).

Taken together, these results reveal that XC operation is light increase kinetics-dependent and its efficiency decreases when the velocity of light intensity increase is too fast. Furthermore, they highlight a new feature of XC functioning: even though XC is rapidly activated in response to light changes, it seems to be best fitted to cope with slow light increases, as the case of the light diel cycle or low mixing. This feature probably relates to the time needed to activate the carotenoid biosynthetic pathway for XC pigment pool replenishment. Indeed, during both mixing-related PFD increases, Vx Chl a^{-1} was lower than the values measured in the 5 h kinetics and stable regardless of the light intensity (Table 2), with no correlation between Vx and Dt Chl a^{-1} (Fig. S1C and S1E). Whereas in the 3 h kinetics Zx Chl a^{-1} mean values were similar to those found in the 5 h kinetics (Table 2) and a significant correlation was found between Zx and Dt Chl a^{-1} (when Zx was detected, $R^2=0.56$, $p<0.01$, $n=20$; Fig. S1D), Zx was almost never detected in the 2 h kinetics (Table 2 and Fig. S1F). Concomitantly, β-Car Chl a^{-1} was quite stable among light treatments (Table 2), and β-Car and Dd Chl a^{-1} were not correlated in both mixing-related conditions (Fig. S2B and S2C), in contrast to what we observed during the diel cycle-related one (Fig. S2A).

Although XC is not efficiently activated in cells subjected to light increases faster than the predictable light diel cycle, mixing-related PFD increases enhance NPQ, when compared to the diel cycle-related one (Fig. 4). Intriguingly, we measured NPQ maxima at moderate PFD.

During the 3 h kinetics, NPQ most steeply increased until PFD reached Ek (Fig. 4C) and relied on a rapid and strong Dt synthesis ($R^2=0.62$, $p<0.005$, $n=36$; black dots in Fig. 4D). This is demonstrated by the almost two-fold greater amount of Dt (up to ~9 mol Dt/100 mol Chl *a* at 280 µmol photons m^{-2} s^{-1}; Fig. 4D) than that measured during the 5 h kinetics (up to ~5 mol Dt/100 mol Chl *a*; Fig. 4B), which relied on the greatest Dd Chl a^{-1} value at 250 µmol photons m^{-2} s^{-1} (up to ~13 mol

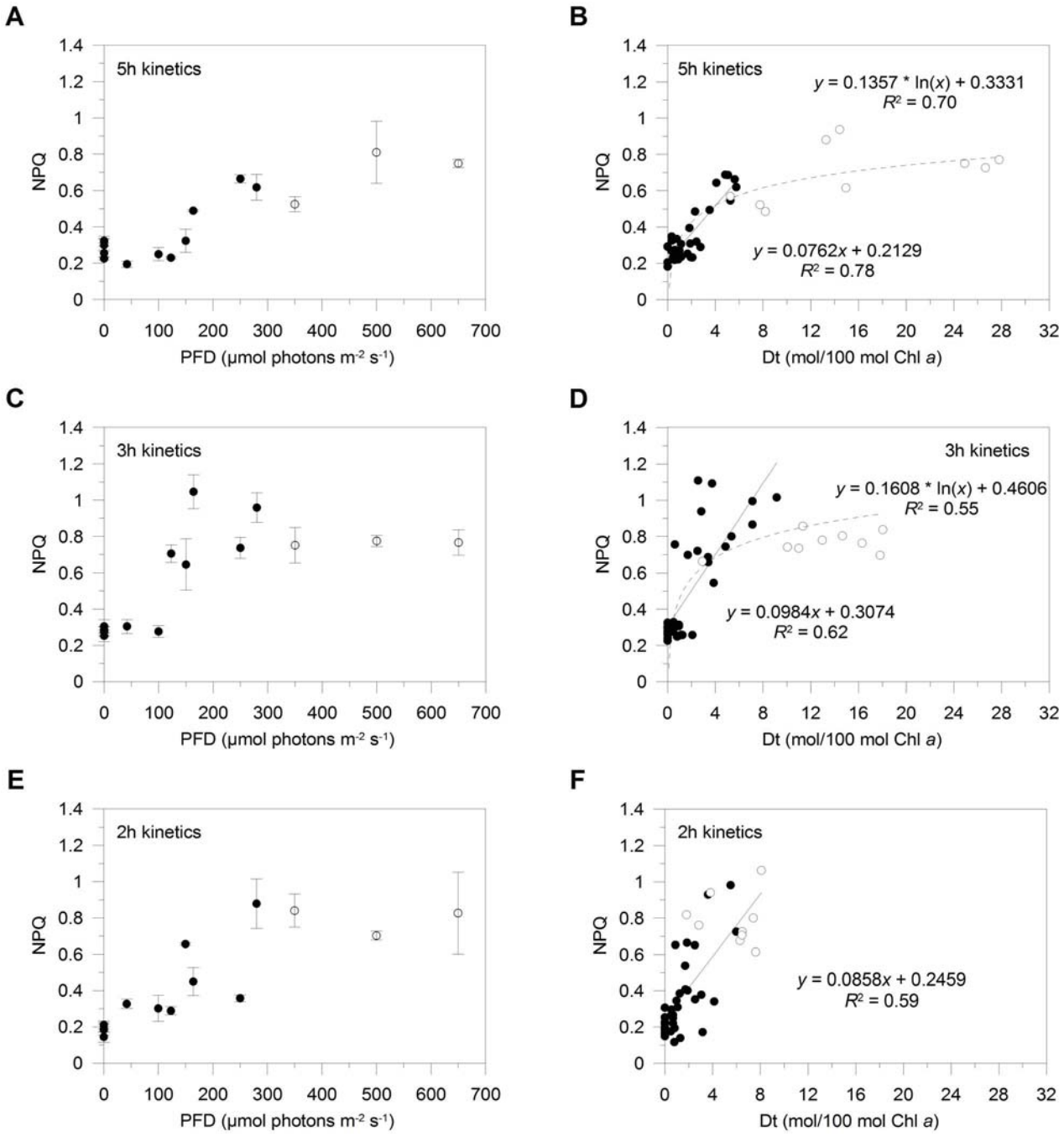

Figure 4. Non-photochemical fluorescence quenching (NPQ), and relationship between NPQ formation and diatoxanthin (Dt) synthesis. Induction of NPQ over the light gradient in *Pseudo-nitzschia multistriata* cells experiencing light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, during the 5 h (A), 3 h (C) and 2 h kinetics of light increase (E). Values are means ± SD ($n = 3$). Relationship ($n = 45$) between NPQ and Dt Chl a^{-1} (in mol Dt/100 mol Chl *a*) in *P. multistriata* cells during the 5 h (B), 3 h (D) and 2 h kinetics of light increase (F). Black and white dots are data measured at PFD ≤280 and ≥350 µmol photons m^{-2} s^{-1}, respectively.
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Dd/100 mol Chl *a*) and Dd depletion at 280 µmol photons m^{-2} s^{-1} (Fig. 3D). When PFD was >280 µmol photons m^{-2} s^{-1}, NPQ was instead lower and stable, despite Dt content almost doubled (up to ~20 mol Dt/100 mol Chl *a*; white dots in Fig. 4D). In this condition, an enhanced synthesis of Dt molecules that functionally participate to NPQ [52,53] might relate to the harsher build-up of ΔpH caused by the greater PFD change per unit time than during the light diel cycle. Thus, a prompt and efficient regulation of XC functionally drives a rapid NPQ formation, despite the lower accumulation of Dt molecules than during a diel cycle-related PFD increase. These results also indicate that the fastest and strongest NPQ induction serves as first photoprotective defense to cope with a rapid increase of light. Therefore, *P. multistriata* cells are able to modulate the functional link between NPQ formation and XC operation in relation to light intensity and velocity of its increase probably via the intensity-

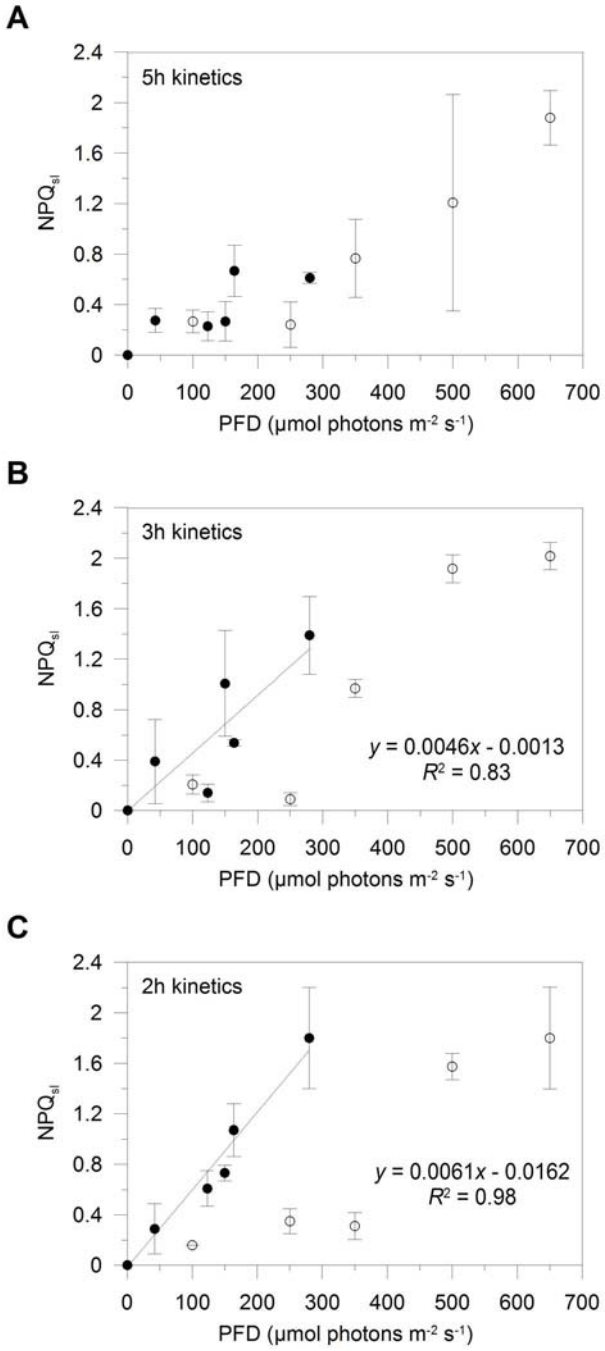

Figure 5. Sustained light-acclimated non-photochemical fluorescence quenching (NPQ_{sl}). Induction of NPQ_{sl} over the light gradient, in *Pseudo-nitzschia multistriata* cells experiencing light gradual increases peaking at the PFD of 100, 250, 350, 500, and 650 µmol photons m^{-2} s^{-1}, during the 5 h (A), 3 h (B) and 2 h kinetics of light increase (C). Black dots are values estimated for the first and second sampling time point, white dots are values estimated for the last sampling time point. Values are means ± SD ($n = 3$).
doi:10.1371/journal.pone.0103782.g005

dependent electron transport rate (ETR) and the coupled transthylakoidal proton gradient [26,54].

During the 2 h kinetics, the relationship between NPQ and Dt Chl a^{-1} was linear ($R^2 = 0.59$, $p<0.005$, $n = 45$; Fig. 4F) and did not change over the full range of PFD, in contrast to the 5 h and 3 h kinetics (Fig. 4B and D). The highest NPQ was measured for PFD ≥ Ek, i.e. when PFD becomes saturating for photosynthesis, although these NPQ values were lower than those measured in the 3 h kinetics. These evidences suggest that a very fast light intensity increase, as the case of the 2 h kinetics, is too rapid for an efficient modulation of NPQ and XC in *P. multistriata* cells.

In contrast to the 5 h kinetics, in the two mixing-related PFD increases, both light intensity and time affected the NPQ_{sl} dynamics over the light gradient (Fig. 5B and C). In the 3 h kinetics, until PFD was ~Ek, NPQ_{sl} was higher when similar PFD values were reached more rapidly (<2 h, black dots *versus* 3 h, white dots; Fig. 5B). Same results were obtained in the 2 h kinetics (compare ≤1.5 h, black dots *versus* 2 h, white dots; Fig. 5C). A strong difference between the three experiments also concerned the value of NPQ_{sl} developed in the faster response when PFD was ~Ek: 0.61±0.04, 1.39±0.31 and 1.80±0.40 in the 5 h, 3 h and 2 h kinetics, respectively (black dots in Fig. 5). We might therefore speculate that NPQ components independent of Dt activation and rapidly induced are more developed the faster is the mixing-related increase of light (i.e. in the 2 h than in the 3 h kinetics), to compensate the impaired Dt synthesis. Diatoms can indeed develop a diverse set of mechanisms of Dt-independent NPQ, such as the PSII electron transfer cycle [7,55], the conformational changes in the core of PSII [56], and the aggregation of FCPs functionally-detached from PSII [51,57,58]. The capacity to form functionally disconnected FCP complexes can partially explain the degree of amplification of the Dt-dependent quenching among different diatom species and strains [26]. Interestingly, NPQ_{sl} values measured at 500 and 650 µmol photons m^{-2} s^{-1} were almost similar among the three kinetics of light increase (~1.9, Fig. 5). This result emphasizes the fact that the overall NPQ (i.e. NPQ_{sl}) is enhanced when cells experience mixing events until they reach a photosynthesis-saturating PFD, above which similar NPQ_{sl} values are developed regardless of time. Below the photosynthesis-saturating PFD, the NPQ/XC coupling is strongly dependent on the velocity of the light intensity increase through interactions between ETR, the ΔpH build-up, the lumen pH-dependent activation of the Dd de-epoxidase and 'activation' of Dt molecules in the NPQ process [26,54]. Once diatoms establish the NPQ component that is triggered by the fast net accumulation of Dt, the breakdown of the proton gradient does not lead to its direct relaxation, which rather depends on the efficiency of the epoxidation of Dt to Dd [49] and removal of Dt from its FCP-binding sites [3]. Since mixing seems to complementarily activate Dt-dependent and Dt-independent NPQ components possibly characterized by different kinetics of induction and relaxation, we might hypothesize that their interplay is crucial to dissipate excess light energy and modulate diatom photosynthesis in the mixed layer.

Conclusions

Our results show that during a diel cycle-related PFD increase, a strong and prolonged activation of XC is the main photoprotective response developed by the diatom *P. multistriata*. XC operation triggers gradual NPQ formation and strong accumulation of Dt molecules over the light range, through an effective regulation of the carotenoid biosynthesis that involves changes in β-Car and Vx cycle xanthophyll pool size. In this condition, the photosynthetic machinery is able to progressively acclimate to the diurnal light increase and balance all photosynthetic regulatory partners, thus preventing a strong NPQ formation. The weak development of NPQ also highlights the photoprotective efficiency of the synthesis of Dt in coping with a predictable diel cycle-related PFD increase. In contrast, mixing-related velocities of light increase favour NPQ development, and do not allow an efficient XC activation. Indeed,

the carotenoid biosynthetic pathway is only partially activated under mixing regimes, causing a limited synthesis of Vx cycle xanthophylls. In case of mixing events, Dt-independent NPQ components seem to be more induced to compensate the impairment of the Dt synthesis. This flexible coupling between NPQ and XC in relation to predictable/unpredictable changes in light environment fits with the outstanding photophysiological plasticity of diatoms, possibly reflecting an evolutionary adaptation they acquired thriving in turbulent waters.

During the applied gradual light increases, we found the highest development of NPQ at moderate light, i.e. when PFD becomes saturating for photosynthesis. Moreover, the whole photoprotective response is activated before cells undergo light conditions that saturate photosynthesis. These results therefore suggest that the saturation light for photosynthesis (Ek) plays a relevant role on the modulation of the photoprotective processes, XC and NPQ, together with the velocity of light increase.

This study gives new insights into the role of water mixing on the photophysiology of coastal diatoms and the importance of NPQ formation/XC operation in coping with light variability. Furthermore, it highlights the necessity of conducting experiments in which phytoplankton are submitted to gradual light increase conditions, in order to gain a better understanding of their ecophysiological plasticity in the field, which in turn might improve mathematical models of phytoplankton growth and succession [59–61].

Supporting Information

Figure S1 Violaxanthin (Vx) cycle xanthophylls *versus* diatoxanthin (Dt) amount. Relationship between Vx and Dt/chlorophyll (Chl) *a* (in mol pigment/100 mol Chl *a*), and between zeaxanthin (Zx) and Dt/Chl *a* (in mol pigment/100 mol Chl *a*) in *Pseudo-nitzschia multistriata* cells experiencing light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, during the 5 h (A and B), 3 h (C and D) and 2 h kinetics of light increase (E and F).
(TIF)

Figure S2 β-carotene (β-Car) *versus* diadinoxanthin (Dd) amount. Relationship between β-Car and Dd/chlorophyll (Chl) *a* (in mol pigment/100 mol Chl *a*, $n = 45$) in *Pseudo-nitzschia multistriata* cells experiencing light gradual increases peaking at the PFD of 100, 250, 350, 500 and 650 µmol photons m^{-2} s^{-1}, during the 5 h (A), 3 h (B) and 2 h kinetics of light increase (C).
(TIF)

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Author Contributions

Conceived and designed the experiments: VG CB. Performed the experiments: VG SF FT CB. Analyzed the data: VG SF CB. Contributed to the writing of the manuscript: VG JL CB.

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Invertebrate Neuroscience

Invertebrate Neuroscience and CephsInAction at the Mediterranean Society for Neuroscience Meeting Cagliari 2015

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Corresponding Author:	Lindy Holden-Dye, PhD University of Southampton Southampton, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Southampton
Corresponding Author's Secondary Institution:	
First Author:	Lindy Holden-Dye, PhD
First Author Secondary Information:	
Order of Authors:	Lindy Holden-Dye, PhD
	Graziano Fiorito
	Giovanna Ponte
Order of Authors Secondary Information:	
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Abstract:	Invertebrate neuroscience, and in particular cephalopod research, is well represented in the Mediterranean region. Therefore the recent meeting of the Mediterranean Society for Neuroscience in Santa Margherita di Pula, Sardinia (June 12th-15th, 2015) provided an excellent opportunity for invertebrate contributions. Furthermore, the Chair of an EU COST Action for cephalopod research (FA1301; www.cephsinaction.org), Giovanna Ponte, together with Graziano Fiorito from the Stazione Zoologica, Naples, aligned a meeting of research groups working in the field of cephalopod neurophysiology from across Europe to coincide with the neuroscience meeting. This provided an exciting forum for exchange of ideas. Here we provide brief highlights of both events and an explanation of the activities of the COST Action for the broader invertebrate neuroscience community.

Invertebrate Neuroscience and Cephs*In*Action at the Mediterranean Society for Neuroscience Meeting Cagliari 2015

Lindy Holden-Dye[1], Graziano Fiorito[2] and Giovanna Ponte[3]

1 Centre for Biological Sciences, Building 85, Highfield Campus, Southampton UK SO17 1BJ

2 Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy

3 Cephalopod Research – CephRes, via dei Fiorentini, 80133 Napoli, Italy

Corresponding author: Lindy Holden-Dye

Email: lmhd@soton.ac.uk

Abstract

Invertebrate neuroscience, and in particular cephalopod research, is well represented in the Mediterranean region. Therefore the recent meeting of the Mediterranean Society for Neuroscience in Santa Margherita di Pula, Sardinia (June 12th-15th, 2015) provided an excellent opportunity for invertebrate contributions. Furthermore, the Chair of an EU COST Action for cephalopod research (FA1301; www.cephsinaction.org), Giovanna Ponte, together with Graziano Fiorito from the Stazione Zoologica, Naples, aligned a meeting of research groups working in the field of cephalopod neurophysiology from across Europe to coincide with the neuroscience meeting. This provided an exciting forum for exchange of ideas. Here we provide brief highlights of both events and an explanation of the activities of the COST Action for the broader invertebrate neuroscience community.

Meeting report

A beautiful seaside location was a fitting venue for a meeting of cephalopod, other invertebrate and vertebrate neuroscience research. The 5th Mediterranean Society for Neuroscience Meeting (MNS2015, http://mnsmeeting2015.it/) spanned 4 days with 389 delegates from 34 countries (Australia, Canada and USA included) and provided 21 travel grants to early career researchers.

The 40 symposia were heavily influenced by the important societal problems being addressed by neuroscience with an emphasis on psychopathology and psychopharmacology. The meeting was also marked by six high profile plenary contributions on mechanisms for drugs addiction, cognition enhancing drugs, TRP channels, hippocampal neurogenesis, and motor behaviour. Two COST Actions

merged with the MNS2015 programme: CMST COST Action CM1103 '*Structure-based drug design for diagnosis and treatment of neurological diseases: dissecting and modulating complex function in the monoaminergic systems of the brain*' and FA COST Action FA1301 '*A network for improvement of cephalopod welfare and husbandry in research, aquaculture and fisheries (CephsInAction)*'. Professor Laura Della Corte and Dr Graziano Fiorito, respectively, introduced the two COST Actions during the Welcome and Introductory session.

There were 24 invertebrate contributions (including 6 poster presentations) at the meeting, counting for about 10% out the overall number of scientific presentations delivered.Talks on *Drosophila melanogaster*, *Caenorhadbitis elegans* and *Lymnaea stagnalis* were integrated into sessions based on their topic, whilst there was a dedicated session for talks on octopus and their allies, the cephalopods.

Elia Di Schiavi described the power of *C. elegans* as a genetic system to understand dopamine signalling and in particular its interaction with nicotinic receptor signalling as an excellent starting point for a symposium dedicated to 'Emerging concepts in dopaminergic system development and regulation'. The tractability of the pond snail *Lymnaea stagnalis* for defining neural substrates of learning and memory was ably demonstrated in a presentation by Ildiko Kemenes in her talk on 'Evolutionary conserved mechanisms in associative learning' in which she described a highly tractable paradigm for appetitive conditioning in which periods of 'forgetting' appear to be important for long-term memory consolidation. In another session concerning 'reward pathways' there were contributions from both *L. stagnalis* (George Kemenes; "A two-neuron system for goal-directed decision-making in the defined feeding network of *Lymnaea stagnalis*") and *C. elegans* (Lindy Holden-Dye; "A multimodal framework for optimising *C. elegans* feeding behaviour to changing food availability"), further showing how simple invertebrate preparations can provide novel insight into fundamental neural mechanisms regulating behaviour. In the latter two talks there was a compelling similarity between the neural framework underpinning feeding behaviour for the mollusc and the nematode further suggesting evolutionary conservation of some basic principles.

The dedicated session on cephalopods was organised by Giovanna Ponte and Graziano Fiorito in the framework of the annual activities of the FA1301 COST Action WG3. This working group on *'Neurophysiology, Anaesthesia and Humane end-points*' deals with the requirements of experimental procedures considered to potentially induce distress and painful experience to animals to fit with principles stated in Directive 2010/63/EU. It also aims to promote basic physiological, including neuroscience, approaches to the study of cephalopod biology and welfare. The current WG3 leaders are B. Hochner (Israel, leader) and P. Grigoriou (Greece, vice-leader , the former of whom was present at the meeting. The session, and the following one-day meeting, was coordinated also by, coordinators of CephRes (http://www.cephalopodresearch.org/), a non-profit association that promotes the advancement of biological research with particular reference to cephalopods.

By way of introduction Lindy Holden-Dye reflected on the ground-breaking advances in neuroscience that have been made using invertebrates, not least the squid with its giant axons and synapses that permitted first insight into the mechanisms of neural signalling. The talk prompted a further discussion on the part invertebrate systems have to play in addressing new global challenges of climate change and food security and highlighted the importance of understanding the behavioural plasticity of invertebrates, both beneficial and pest species. This was followed by four presentations

that focused on octopus and provided an excellent insight into its neurobiology and contributions to the field of neuroscience. Tal Shomrat's (Israel) talk entitled "Conservation and convergence in the evolution of the octopus neural system mediating learning and memory" described the organisation of the cephalopod vertical lobe and the phenomenon of convergence-divergence or 'fan-in fan-out' connectivity which is resonant with the organisation of artificial classification networks. He discussed paradigms for reward learning and evidence that octopamine and serotonin are important negative and positive reinforcers, respectively, in this system (Shomrat et al. 2015). This was followed by Shuichi Shigeno (Japan and USA) who provided a fascinating talk detailing the comparative organisation of the human and octopus brain "Identifying molecular and connectivity architecture shared in mammalian and octopus brains". He considered the extent to which common design principles for neural systems have been deployed through evolution from a connectivity through to a molecular perspective encompassing dye labelling and pathway tracing and an analysis of conserved transcription factors e.g. *pax-6* and provided evidence for somatotopic maps in the octopus brachial system. The main message was that this experimental approach in octopus can underpin powerful advances in the design of artificial intelligence. Giovanna Ponte's talk (Italy) "Octopamine in Octopus brain: a long history of mapping a 'neglected' neuromodulator" explained that although the biogenic amine neuromodulator was first discovered in octopus in 1948, isolated from the salivary gland, and hence the name, we still have a very sketchy understanding of its functional role in cephalopods. Her research is providing the first information on the central organisation of this important neuromodulator in octopus, and evidence that it may be involved in visual and chemo-tactile sensory-motor processing. Benny Hochner (Israel) wrapped the session up with an excellent account of octopus motor control and how ideas from robotics (e.g. "embodied organisation") can explain "Why motor control in the octopus is full of surprises". He described current understanding of the mechanisms that have evolved in octopus so that it can cope with the challenge of moving its eight long and flexible arms rapidly, efficiently and independently through central activation of autonomous motor programmes in the arm (Levy et al.).

The following day a satellite meeting of the EU COST Action (FA1301) which supports a "network for improvement of cephalopod welfare husbandry in research, aquaculture and fisheries" was held at the same venue. This provided an opportunity for several of the groups involved in the network to share ideas on the neurophysiology and behaviour of the cephalopods.

Fabio de Sio (Germany) provided a very informative review of the history of octopus and its adoption by JZ Young and Brian Boycott as an intriguing model system (De Sio 2001). His extensive research into the historical aspects of octopus, funded by the Wellcome Trust, has demonstrated amongst other things, that octopus research was at the forefront of interdisciplinary experimental approaches with an involvement of cybernetics from the early 1950s with a view to cross-referencing brain and machine to improve understanding of the former and refinement of the latter. Joachim Pflüger (Germany) provided a comprehensive review of octopamine as a neuromodulator in locust and discussed its role in stress response which was of interest to the session given the apparently extensive octopaminergic innervation in octopus. He showed that stressors trigger the conversion of tyramine to octopamine and the latter in turn is required to increase the efficacy of transmission at the neuromuscular junction whilst at the same time elevating the levels of fructose 2,6-bisphosphate in flight muscle. Overall, octopamine would appear to be involved in shifting the behaviour of the animal from tonic, resting activity to a dynamic state that supports sustained flight (Pflüger and Duch 2011).

Carlo di Cristo (Italy) focused on the neural mechanisms regulating the sexual maturation of the octopus in his presentation entitled 'Nervous control in *Octopus vulgaris*; a physiologist's point of view'. In this regard the behaviour of octopus is complex and very poorly understood. This talk provided an elegant example of how recent transcriptomic data, mapping of pathways and behavioural analysis is providing important insight into key mechanisms. There is a particularly pivotal role for the optic gland. This is needed for the production of yolk protein for the eggs, but in addition directs complex behaviours encompassing the engagement of the octopus in parental care for the eggs, the suppression of feeding during this period, and her subsequent death. However, the identity of the hormonal signal or signals from the optic gland that regulate these behaviours has yet to be revealed. The talk highlighted the potential for data mining of transcriptomic data to reveal candidates (Di Cristo 2013). Nir Nesher (Israel) described a very interesting phenomenon, clearly showing that each of the eight arms of the octopus is capable of minute-to-minute autonomous control, and that this is under the overarching regulation of the central nervous system. He described the observation that the suckers of an amputated octopus arm will readily and strongly attach to any surface they are in contact with, with the exception of octopus skin. The activation of the suckers is inhibited by an extract of octopus skin neatly illustrating that local chemical signals prevent the octopus from effectively tying itself up in knots. The nature of this chemical signal has yet to be resolved. This inhibitory phenomenon is not observed in intact octopus which can still grab the arm of another octopus, or indeed an amputated arm. Remarkably, this interaction of an intact octopus with an amputated arm shows a level of self-recognition as the octopus is more likely to treat the arm as food if it is from another octopus rather than their own. Clearly there is some fascinating biology to be probed here in terms of chemical communication (Nesher et al. 2014). Moreover, the solution that has evolved in octopus, to regulate the movement of its eight arms in an autonomous fashion, is elegantly simple in sparing the brain from computational load and provides an excellent example of biology evolving simple elegant solutions to complex problems.

A very important aspect of the COST Action in terms of improving cephalopod welfare is improving the basic understanding of nociception and pain. In her talk, Giulia di Cristina (Italy) described the progress she is making with her research 'Mapping the putative sensory nociceptive inputs in the suckers of *Octopus vulgaris'*. She is conducting a careful analysis of the morphological characteristics of the sensory receptors in the suckers and using molecular markers to characterise their neurochemical properties with a view to identifying candidate nociceptors.

Finally, Frederike Hanke (Germany) and Christelle Alves Jozet (France) each took a much more behavioural perspective investigating perception and behaviour in cephalopod through some elegant experimental design which looked at preference for symmetry and perception of laterality, respectively.

Overall, this was an excellent format for a meeting of invertebrate neuroscience. The contribution to the success of the conference served to reinforce the importance of the invertebrate community being proactive in engaging with national, regional and international societies through one off communications and co-ordinated themed sessions. The next meeting of the MSN is in Malta in 2017, and provides a great opportunity to build on the excellent precedent set in 2015 and for 'invertebrates' to gather again by the beautiful blue Mediterranean sea.

Acknowledgments

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ORIGINAL PAPER

Depth perception: cuttlefish (*Sepia officinalis*) respond to visual texture density gradients

Noam Josef · Ofri Mann · António V. Sykes · Graziano Fiorito · João Reis · Steven Maccusker · Nadav Shashar

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Abstract Studies concerning the perceptual processes of animals are not only interesting, but are fundamental to the understanding of other developments in information processing among non-humans. Carefully used visual illusions have been proven to be an informative tool for understanding visual perception. In this behavioral study, we demonstrate that cuttlefish are responsive to visual cues involving texture gradients. Specifically, 12 out of 14 animals avoided swimming over a solid surface with a gradient picture that to humans resembles an illusionary crevasse, while only 5 out of 14 avoided a non-illusionary texture. Since texture gradients are well-known cues for depth perception in vertebrates, we suggest that these cephalopods were responding to the depth illusion created by the texture density gradient. Density gradients and relative densities are key features in distance perception in vertebrates. Our results suggest that they are fundamental features of vision in general, appearing also in cephalopods.

N. Josef (✉) · O. Mann · S. Maccusker · N. Shashar
Ben-Gurion University of the Negev, Department of Life Sciences, Eilat Campus, 84105 Beer Sheva, Israel
e-mail: Bigblue2810@gmail.com

N. Josef · O. Mann
H. Steinitz Marine Biology Laboratory, Interuniversity Institute for Marine Sciences, P.O.B. 469, 88103 Eilat, Israel

A. V. Sykes · J. Reis
CCMAR-CIMAR L.A., Centro de Ciências do Mar do Algarve, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

G. Fiorito
Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

Keywords Cephalopods · Visual illusions · Distance processing · 3D comprehension

Background

The way animals perceive the world around them has long captured people's imagination. What does the deep blue sea look like to an octopus? Moreover, we take vision and the perception of our surroundings for granted, and we fail to appreciate the sophisticated processes underlying this ability. Visual illusions are those stimuli that exist at the extremes of what our system has evolved to handle. Created from the uncertainties inherent to images and the assumptions made by the visual system, illusions occasionally represent active recalibrations of the image that lead to a misinterpretation of the scene (Eagleman 2001). These uncertainties show that visual systems do not report impartial information but instead create elaborate interpretations based on refined detective work. However, carefully structured visual illusions have served over the years as useful and robust tools in neuroscience research (Eagleman 2001). As examples, the Zöllner illusion taught us that the visual cortex enhances orientation contrast by making similar orientations seem to tilt away from each other. The "Hermann Grid" (Cohen et al. 1970) contributed to the neural theory of lateral interaction between nerve cells. Thus, illusions can provide a window into the neurobiology of vision, and onto the path by which the brain interprets visual information (Gunderson et al. 1993; Nealey and Edwards 1960; Reid and Spetch 1998; Eagleman 2001).

One such interpretation relates to the three-dimensional (3D) perception of the surroundings. The 3D environment in which we live is visually represented as a two-

dimensional (2D) image on the retina, yet we are able to transform this 2D information back into 3D perception. Interpreting depth-related information occurs through a combination of experience and cognitive processing. Nearly all so-called pictorial depth cues that are known have been used by artists over the years (Kaufman 1974). Gibson's seminal work (Gibson 1950) described the key features of such transformations. As an example, to detect a sudden drop in height such as from a cliff, the light arriving to an animal's eyes must provide it with the information needed to differentiate the drop-off from the surface on which it is situated. This information should provide an indication as to the presence of an edge and, ideally, of a gradation of depth below the edge. If two surfaces, which are textured or patterned similarly, are positioned at different heights or distances from a sensor, a difference in the density of the optical texture will be present at the level of the sensor (Gibson 1950). This density difference can be used to estimate the relative distance to the surfaces.

In later studies (Marr and Nishihara 1978), computational challenges imposed on the visual system while interpreting a given scene were used to describe the specific cues and cognitive processes involved in depth perception. Human visual depth cues are well known (Palmer 1999). Within the scope of the present study, we will not elaborate on all known parameters; however, it is important to emphasize that when several of these cues are available simultaneously, the visual system attempts to combine them into a single solution without presenting alternative options (Palmer 1999). Indeed, although information from multiple cues is combined to provide the viewer with a unified estimation of depth and shape, the combination process can fail, leading to the illusion of a characteristic, such as depth that does not actually exist. One way to combine different depth cues from a scene is to calculate discrete approximations of depth based on each depth cue individually. The separate depth estimates can then be averaged into an overall depth map of the scene. This method of combination is called *weak fusion*, and it differs from *strong fusion*, in which the observer determines (often unconsciously) the most probable three-dimensional interpretation of the scene, given the current visual data, often favoring one source of data over another (Landy et al. 1995).

Numerous studies of cephalopods have used both 2D and 3D pictorial displays to examine the animals' visual cues in the contexts of dynamic coloration, body-shape, and cognition processes (Barbosa et al. 2007, 2011; Chiao et al. 2005; Pronk et al. 2010; Chiao and Hanlon 2001; Kelman et al. 2008). Evidence of depth perception in cuttlefish has been found by investigating their camouflaging reaction to substrates at different distances (Kelman et al. 2008; Chiao et al. 2005, 2007). The question remains, however, as to which pictorial characteristics are used by non-human animals to decipher depth information in 3D scenes. Infant macaque monkeys are responsive to pictorial depth cues of linear texture gradient and relative size, while the praying mantis uses mostly binocular vision (Gunderson et al. 1993; Reid and Spetch 1998). Following human-like experiments, rats noticed and avoided a visual drop-off, while pigeons discriminated 3D from 2D pictures of an object (Nealey and Edwards 1960; Maldonado and Rodriguez 1972; Gunderson et al. 1993; Reid and Spetch 1998). In the current study, we address one of the previously mentioned depth cues, *texture density gradient*, in which texture density (the number of texture elements per unit area) is used for depth estimation (Frisby and Stone 2010).

Texture density gradient is the systematic change in the size and shape of small elements that often occur as a function of depth or distance. In particular, we address gradients of spacing and density. The basic physical rule is that objects at a greater distance appear smaller in an inverse ratio to their relative distance. The exact way in which texture density differs within an image depends on the relative inclination of the surface—for a surface with a large inclination, the image texture in the background is more densely packed than the texture in the foreground. More importantly, texture density systematically changes across an image in relation to the surface distance and inclination.

To examine whether cephalopods respond to texture density gradients, we monitored the behavior of cuttlefish on surfaces with different types of optical patterning. As benthic animals are a very common prey among various fish, swimming over a dark, unfamiliar crevasse may pose a potential danger to a stray cuttlefish.

Methods

Visual stimuli treatments

Sixteen European cuttlefish (*Sepia officinalis*) with a mantle length of 4.0–6.5 cm were examined in two visual stimuli setups. The animals came from a captive population (F4) reared from eggs, in the Ramalhete Aquaculture Station (Ria Formosa, Southern Portugal) according to the methodology described in Sykes et al. (2006). A uniformly light blue-colored aquarium (125 × 33 × 17 cm; water depth 13 cm), with running sea water, was used as the study arena. Note that cuttlefish are color blind so that they presumably perceive this light blue background as being light gray (Marshall and Messenger 1996; Mathger et al. 2006). A light meter (PeakTech 5025) was used to ensure the study area was evenly illuminated (335–350 lux),

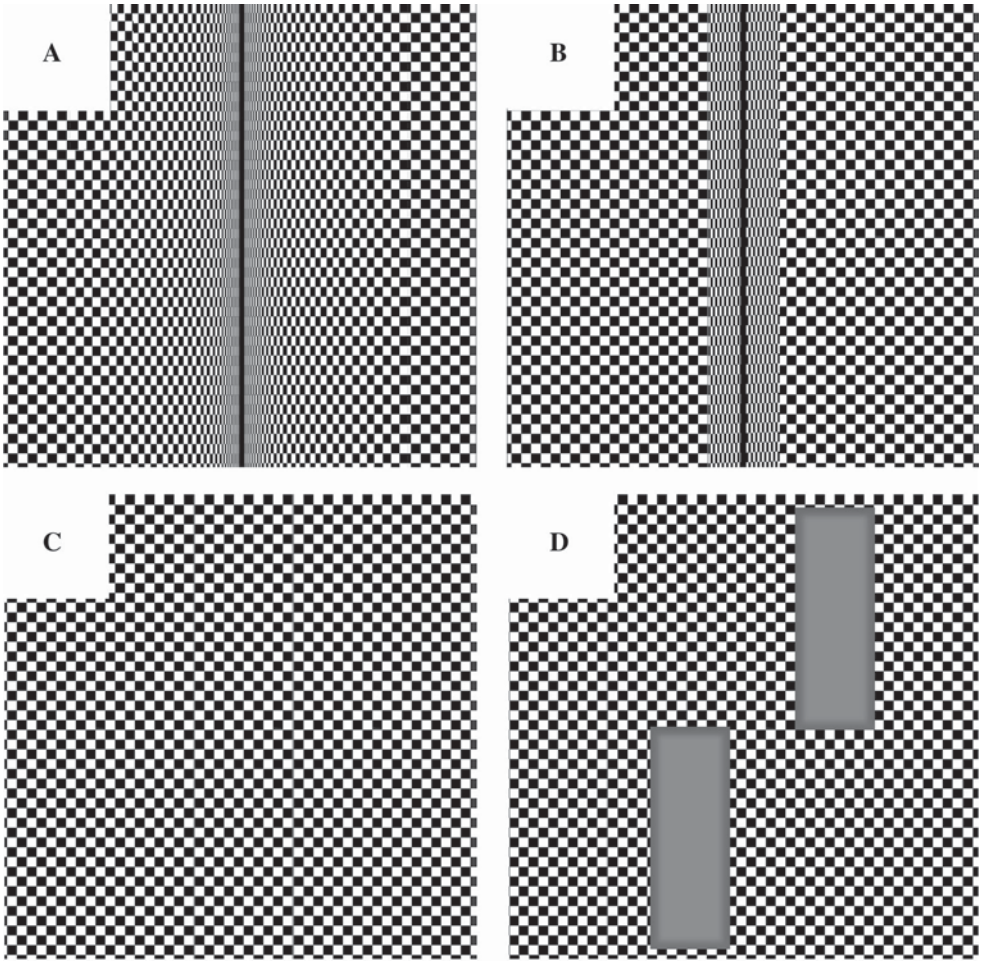

Fig. 1 Patterns used in this study. **a** Gradual texture with a false-depth element in the *shape* of a deep crevasse. **b** A non-gradual pattern with spatial elements similar to those in pattern "A" but lacking the continuous size gradient that creates the illusion of depth. **c** A uniform checkerboard pattern used as a negative control. **d** A uniform checkerboard pattern with two deep sections cut into it—marked as *gray rectangles*. All patterns were fixed to the *bottom* of the aquarium

except for a somewhat higher illumination at the acclimatization section (see supplemental link for images of the experimental setup: http://www.youtube.com/watch?v=UyyNhWPSvwE). A removable partition was placed 25 cm from one end of the aquarium to hold the animals before the experiments. We used two computer-generated patterns as the two stimulus treatments: a gradual texture (Fig. 1a) and a non-gradual texture (Fig. 1b). The treatment patterns included a texture gradient appearing to humans as a false-depth scene in the form of a deep crevasse (Fig. 1a) when viewed from 15° to 90° (perpendicular) inclinations, and an alternative pattern with comparable spatial elements that were located in two distinct sections, therefore not creating an illusion of depth (Fig. 1b). Each pattern (33 × 33 cm) was printed on a negatively buoyant adhesive plastic which was then fastened to the bottom of the tank with Velcro strips. The dimensions of the illusionary crevasse area were marked starting after measuring a 20 % change in the original checkerboard. One-ninth of the area covered by each stimulus pattern comprised a dense texture pattern of smaller black and white squares. The patterns were printed in such a way that they were either centered width-wise in the tank, or situated half-way between the center and the edge of the tank. In each treatment, the two patterns were aligned: one centered visual obstacle and one offset obstacle to one side (of the same type), both lined up end-to-end along the length of the tank. This design hindered, but did not prevent the animal from bypassing the obstacles while crossing the tank. To eliminate the animal's potential trajectory preference, the patterns were randomly aligned in four conformations: center-right, center-left, right-center, and left-center. As an example, the right-center conformation is presented in Fig. 3.

The sixteen experimental animals were placed, one at a time, in the partitioned section of the aquarium for acclimatization. Once the cuttlefish had ceased erratic movement (i.e., settled on the tank bottom for more than 1 min), the partition barrier was removed and it was motivated to swim to the other side of the tank by human presence and minor gestures if needed; hence, the animal could roam across the whole tank. The entire test area was videotaped with a Canon Powershot Ixus 8515 digital camera (frame resolution of 640 × 480 pixels). In an effort of avoiding habituation, each animal was presented once with each of the treatments (gradual texture and non-gradual texture).

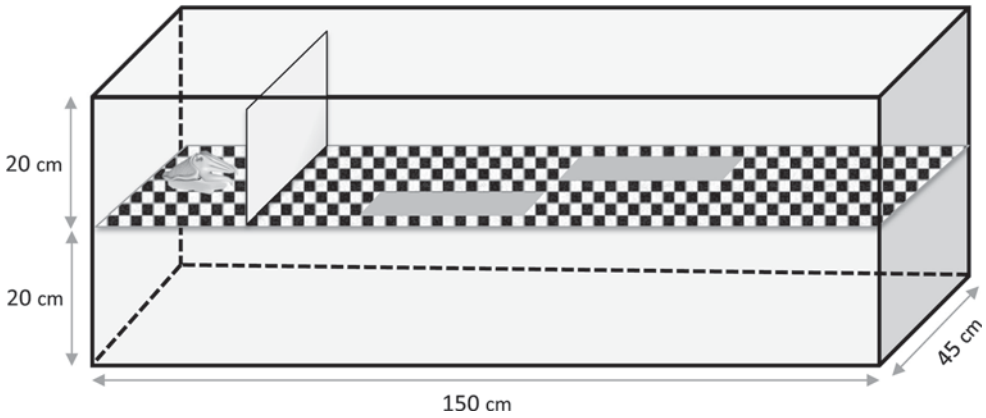

Fig. 2 The control experiments arena. Negative control was designed as a uniform checkerboard substrate, allowing the animal to roam freely in the tank. As a positive control, two sectors (marked as *rectangular gray* sections) were cut from the pattern revealing a transparent *bottom* and a 20-cm drop to a swimming cuttlefish

Due to technical problems (one film came out badly and another was filmed out of focus), 14 of the 16 animals' videos were analyzable. To avoid observational bias, we analyzed each video using an automatic computerized trajectory analysis code.

Control and baseline behavior experiments

Nine naïve cuttlefish (*S. officinalis*) (mantle length 6.0–10.5 cm) were examined for baseline behavior in the presence or absence of a deep obstacle (positive and negative controls, respectively). All nine cuttlefish used for the control experiments were held for 2 days of acclimatization prior to the experiments. The control experiments' arena tank (150 × 40 × 45 cm) was constructed in such a way that a transparent horizontal surface could rest 20 cm above the tank bottom (Fig. 2).

For negative control, a uniform checkerboard pattern (square size 1.8 × 1.8 cm) covered the transparent horizontal surface to create a continuous textured bottom (Fig. 1c).

For positive control, two transparent sections were exposed (4.5 × 36 cm) with a 10 cm gap between them (Fig. 2). The horizontal surface now held a checkerboard pattern with two 20-cm-deep sectors functioning as deep visual obstacles (Fig. 1d). In both cases, animals were introduced one at a time into the elongated tank, allowing them to swim to the other side of the tank in any trajectory.

The entire control tank was videotaped with a SONY HDR-CX110 digital HD video camera (frame resolution of 1,080 × 1,440 pixels), and the animal's trajectory and depth avoidance behavior were examined and analyzed with the same trajectory analysis code as before.

Analysis of trajectory

The animals' trajectories in the tanks were analyzed with a dedicated MATLAB^{TM} code, which quantified the characteristics of movement and path (velocity, location, and trajectory plotting). The cuttlefish movements were analyzed from the time the animal began to move at one end of the tank until the moment it stopped moving at the other end of the tank. To avoid biasing results if an animal settled over a pattern, elements of cuttlefish movement such as hovering and stopping (for more than three frames) were not included in the analysis. The MATLAB^{TM} analytical process involved obtaining a video file, marking the aquarium arena and the obstacles it contained, identifying the cuttlefish with the aid of human input, defining the cuttlefish starting position, and tracking the cuttlefish movements in three-frame intervals. Each movement resulted in a line that indicated the path of the animal, and velocity was calculated and graphically represented by applying different colors to the trajectory line (Figs. 3, 4). Movements were categorized into two groups:

'Movements over the uniform checkerboard' (UC)
'Movements over the visual obstacle' (VO).

Since the visual obstacles were only 1/9th of the entire pattern, for comparison we normalized the results of the background area by multiplying the number of VO by eight. Using the normalized movements over the visual obstacle (NVO), the percentage of movement over either checkerboard or the visual obstacle normalized to the area was calculated.

As an example, let us say an animal's total movement duration (VO + UC) is 3.1 s, i.e., it spent 3 s over the checkerboard and 0.1 s over the visual obstacle. For normalization purposes, we multiply VO by eight, resulting in NVO = 0.8 s. The new total duration is now 3.8 s, indicating that the animal spent 21 and 79 % of its time over the obstacle and the checkerboard, respectively.

Statistical analysis

A nonparametric Wilcoxon paired signed rank test, with a 95 % confidence interval, was used to distinguish UC and NVO movements in both treatment textures. The Kruskal–Wallis one-way analysis of variance was used to compare

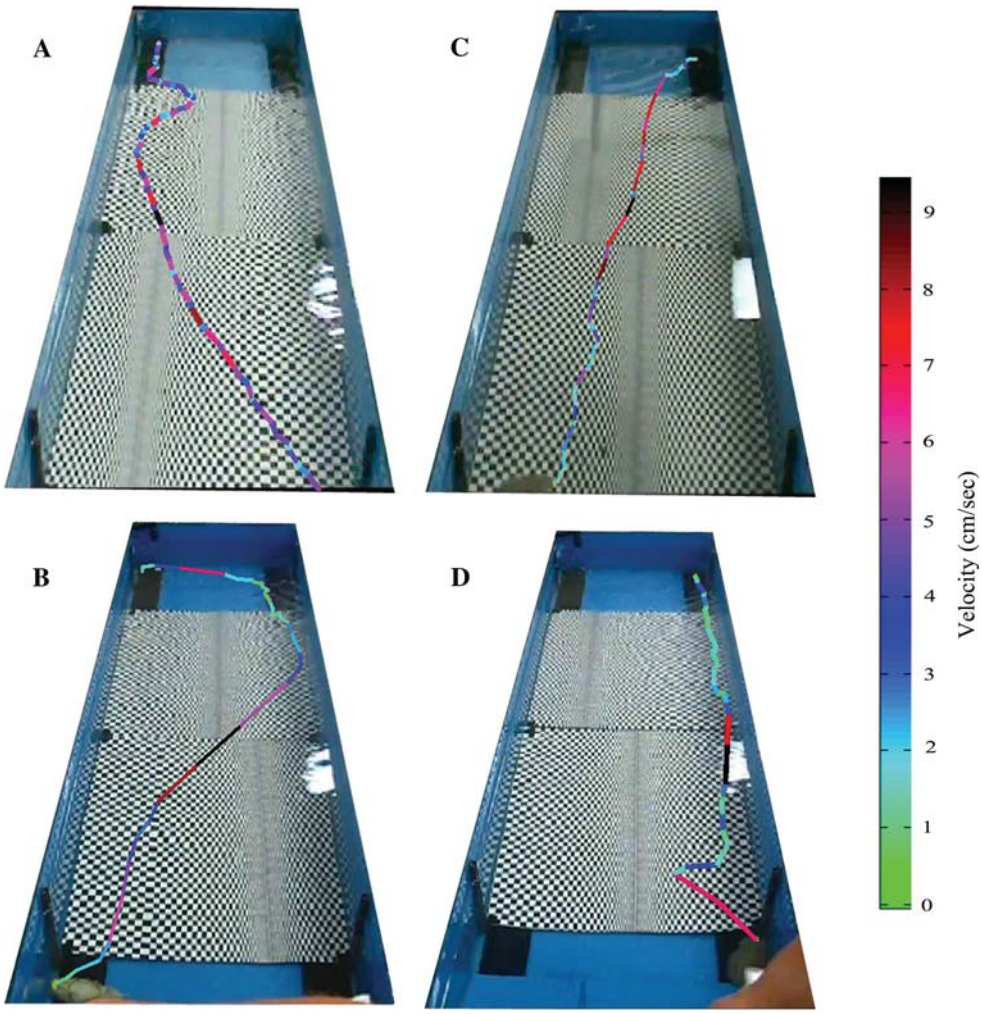

Fig. 3 Gradual density texture trajectory *lines* (*color coded* for speed) of four tested cuttlefish. The animals' paths indicate that they might be trying to avoid the visual obstacles, moving at high velocity while crossing the gap. Pattern configuration: **a** *left-center*, **b** *center-left*, **c** *right-center*, **d** *center-left*

the time spent over the different textures (Sokal and Rohlf 1969). This test allowed us to compare animal behavior over all patterns and to determine whether any of the patterns caused some significantly different behavior. Applying a post hoc test to discriminate which pattern, if any, caused different behavior, we used the Mann–Whitney U multiple comparison with the Bonferroni–Holm correction ($\alpha = 0.016$).

Results

The animal moved in the tank at a velocity ranging from 0 to 10 cm per second, with an average of 4.5 cm/sec, crossing the tank from one side to the other at an average of 13.9 s.

Control and baseline behavior experiments

All nine animals presented with the negative control roamed the tank freely, and once they started a trajectory toward the other side, they kept a straight line in any section of the tank.

When facing a deep obstacle in the positive control setup, seven out of nine cuttlefish presented avoidance trajectory—spending 94.26 and 5.74 % (normalized to 89.84 and 10.16 %) of the durations of their movement over the checkerboard and gradual texture obstacle, respectively, spending significantly less time over the latter ($W = 0$, $n = 9$, $P < 0.001$, Fig. 5), suggesting that the animal refused to swim over the deep section.

Visual stimulus treatments

When presented with the illusionary-gradual texture, the cuttlefish spent 99.14 and 0.86 % (normalized to 94.5 and 5.5 %) of the duration of their movement over the checkerboard and gradual texture obstacle, respectively, spending significantly less time over the latter ($W = 0$, $n = 14$, $P < 0.001$, Fig. 5). However, when presented with the non-gradual texture, the cuttlefish spent 84.42 and 15.58 %

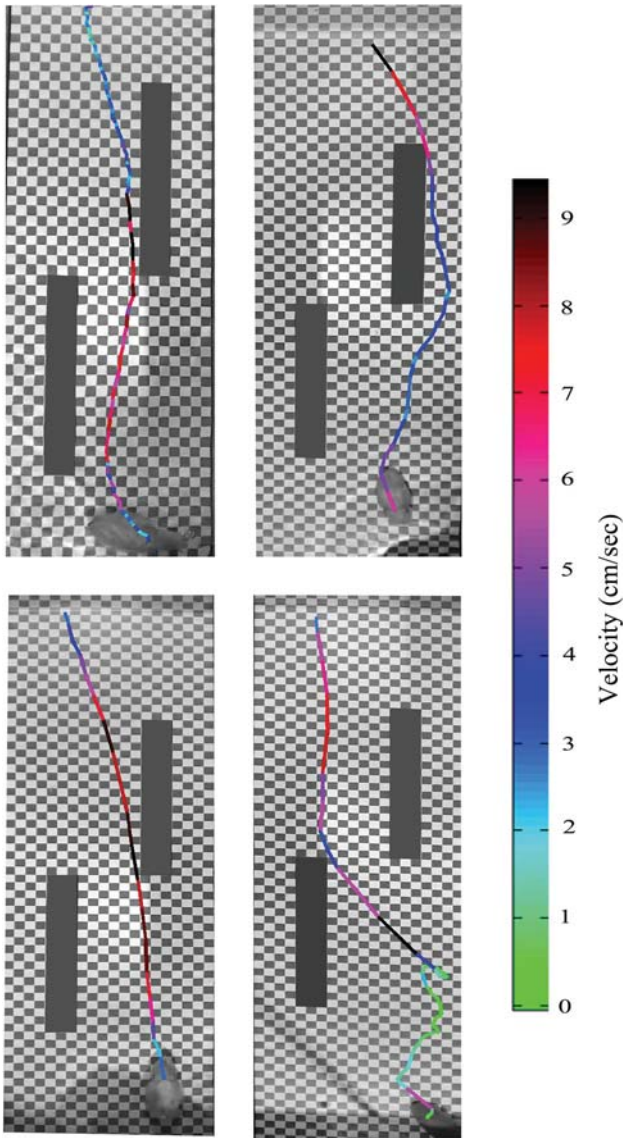

Fig. 4 Positive control trajectory *lines* (*color coded* for speed) of four control cuttlefish. The animals' paths indicate they might be trying to avoid the visual obstacles, moving at high velocity while crossing the gap

(normalized to 59 and 41 %) of the duration of their movement over the checkerboard and the visual obstacle, respectively, showing no significant difference between both textures ($W = 41$, $n = 14$, $P = 0.534$, Fig. 5).

Facing the illusionary-gradual texture, ten animals swam across the gap between the visual obstacles, trying to avoid swimming over it. On the other hand, only two animals presented this behavior when facing the non-illusionary texture.

The Kruskal–Wallis test conducted on the four patterns (illusionary pattern; checkerboard surrounding the illusionary pattern; non-illusionary pattern; checkerboard surrounding the non-illusionary pattern) showed a significant difference ($\chi^2 = 26$, $df = 3$, $n = 14$, $P < 0.001$), and the post hoc Mann–Whitney U showed a significant difference between the illusory crevasse and all other three patterns ($n = 14$, $P < 0.001$), although no such difference was present between the non-gradual texture and the control surface. As shown in Fig. 5, matching time spent over all patterns and control surfaces showed significant differences between both gradual texture and positive control and all other patterns. This finding indicates that the animals tried to minimize the time they lingered over the gradual texture and the deep positive control, but swam without distinction over the other checkerboard and non-illusionary obstacles.

Discussion

The study of illusions has led to the refinement of our understanding as to the role played by specific visual cues in image processing (Gregory 1997; Hermann 1870; Mariotte 1668). Regarding animal perception, examining their behavior when faced with different visual cues is essential for understanding how they perceive a scene. As in our case, the study of visual illusions can increase our knowledge regarding the processes involved in acquiring depth- and distance-related information in a given scene.

In this experiment, the cuttlefish avoided passing over a visually deep section in the tank even though it was covered by a transparent bottom. When presented with a uniform checkerboard, the animals roamed freely in the test tank traveling in straight line trajectories. Similarly, the cuttlefish actively avoided the density gradient texture which provided an illusion of depth, but ignored the non-gradual pattern. Furthermore, eight out of ten animals that eventually passed over the test patterns did so rapidly, mainly over the small gaps between the illusionary obstacles (Fig. 3). We therefore conclude that the *S. officinalis* cuttlefish are responsive to the pictorial cues of a texture gradient.

Taking into account how humans and other vertebrates relate to gradient textures (Gunderson et al. 1993), and the responses of the cuttlefish to the non-illusionary pattern, the present results suggest that cuttlefish interpret the test pattern as representing a drop in depth. However, it should be stressed that the setup of the current study included a one-dimensional increase in texture density that accounted only for the width of the pattern while neglecting its length. This increase is enough to fool human perception and presumably also that of cuttlefish.

Previous studies that examined spatial learning in cuttlefish (Alves et al. 2007; Karson et al. 2003) did not find a trajectory preference based on topographic elements (except for obstacles). The present results suggest that the cuttlefish trajectory is influenced by the visual shape,

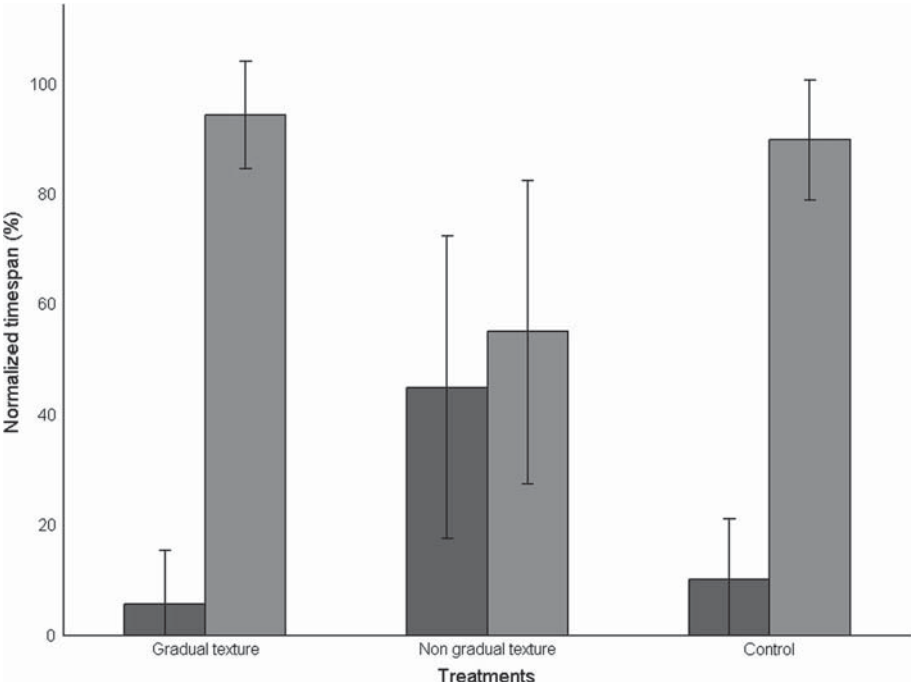

Fig. 5 Cuttlefish time span when swimming over different textures. The *dark columns* represent the visual objects, while the *bright columns* represent the simple checkerboard, and the *error-bars* show standard error. The cuttlefish spent significantly shorter amounts of time over the gradual texture than over the simple checkerboard ($P < 0.001$), yet they showed no difference in behavior when presented with the non-illusionary pattern. There was no significant difference between the positive control and the gradient texture

texture, and topographic elements of the seabed. Clearly, the tendency to avoid swimming over a gradient texture and to traverse the texture by swimming between the visual obstacles justifies the claim that these animals choose their trajectories according to topographic markers; however, this claim calls for further investigation.

Cephalopods are highly visual invertebrates that possess a complex visual system (Hanlon and Messenger 1998). Therefore, it is interesting to examine whether their image processing path follows a course similar to that in evolutionary distant animals, such as vertebrates. Although a conclusion to this question lies beyond the scope of the present study, our results and those of Zylinski et al. (2012) suggest that some of the ways they perceive and process pictorial information are indeed comparable and that it is possible that the rise in density of visual information combined with the increase in distance is a fundamental physical characteristic of natural scenes. Hence, the perception of depth using such a fundamental attribute should develop in any image processing system. A likely scenario is that visual convergence in spatial vision is coupled with a convergence in visual problem solving. Hence, the evolution of animals facing similar challenges will likely converge toward the most effective solution. Additional experiments should be performed to test whether texture density gradients can be used to observe whether cuttlefish can evaluate the depth/distance of objects or distinguish between different depths/distances in the environment.

Age-related studies in humans show that depth perception appears in early developmental stages (Schwartz et al. 1973; Richards and Rader 1983; Timney 1981). Cuttlefish obtain chemical and visual information about their environment before they hatch (Darmaillacq et al. 2006; Dickel et al. 2000).Thus, an examination of the ontogenetic development of depth perception in cuttlefish is therefore required.

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Camouflage during movement in the European cuttlefish (*Sepia officinalis*)

Noam Josef[1,2*], Igal Berenshtein [1,2], Graziano Fiorito[3], António V. Sykes[4] and Nadav Shashar[1]

[1] Eilat Campus, Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva, 84105, Israel

[2]H. Steinitz Marine Biology Laboratory, Interuniversity Institute for Marine Sciences, P.O.B. 469, Eilat 88103, Israel

[3]Stazione Zoologica Anton Dohrn, Villa Comunale 80121, Napoli, Italy
[4]CCMAR-CIMAR L.A., Centro de Ciências do Mar do Algarve, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

Abstract

A moving object is considered conspicuous because of the movement itself. Once moving from one background to another, even dynamic camouflage experts such as cephalopods, should sacrifice their extraordinary camouflage. Therefore, minimizing detection at this stage is crucial and highly beneficial. In this study we describe a background-matching mechanism during movement, which aids the cuttlefish to downplay its presence throughout movement. In situ behavioural experiments using video and image analysis, revealed a delayed, sigmoidal, colour-changing mechanism during movement of *Sepia officinalis* across a uniform black and grey backgrounds, which we describe below. This is a fist and important step in understanding dynamic camouflage during movement, while the new behavioural mechanism may be incorporated and applied to any dynamic camouflaging animal or man-made system on the move.

Introduction

Crypsis is a common behavioural-morphological adaptation aimed at minimizing detection by predators or prey (Caro, 2005a; Caro, 2005b; Ruxton et al., 2004). Visually active organisms are skilled at detecting movement, which often indicates the existence of a potential danger or of an object of interest (Cronin et al., 2014; Land and Nilsson, 2012). As a result, cryptic animals tend to keep still in various situations (Cott; Poulton, 1890; Zhang and Richardson, 2007), e.g. a prey which ‘freezes’ upon detecting a distant predator (Broom, 2005; Eilam, 2005), and "sit-and-wait" or ambush predators that do no move while waiting for their prey (Thery, 2004).Therefore, camouflage is traditionally linked to motionlessness, making it a widespread example of coevolution between behaviour and morphology (Ioannou and Krause, 2009). This linkage has led to the common belief that it is difficult to move and stay camouflaged at the same time. The obvious advantage of camouflaging during movement is enabling the approach of a predator to a prey without it noticing. This and other advantages have led to the development of several strategies that allow animals to maintain at least partial camouflage and remain undetected while moving. There seem to be three schemes in which this may occur: motion signal minimization; optic flow mimicry; and motion disruption (Troscianko et al., 2009). Camouflage through motion signal minimization is associated with the prevention of low-level detectors indicating motion activity. Camouflage through optic flow mimicry is associated with an attempt to mimic the background or surrounding motion so that, although the motion is detected, it does not provide a cue for

segmentation. Motion disruption involves a breaking or misrepresentation of motion cues to distort the perception of that motion. Dragonflies and hoverflies, for example, perform “motion camouflage” by keeping their narrow cross-section faced towards the target even when flying sideways in relation to it (Mizutani et al., 2003). The observation that certain unrelated species resemble one another has long been reported (Darwin and Wallace, 1958; Ruxton et al., 2004), describing strategies such as mimicry, stealth and deceptive resemblance (Cott; Randall, 2005), but evidence to their use and effectiveness in the animal kingdom is lacking.

Cephalopods are considered masters of rapid adaptive camouflage. Using their multi-layered skin and neural controlled chromatophores they can change colour, pattern, texture and reflectance in a fraction of a second (Barbosa et al., 2012; Hanlon et al., 2009; Marshall, 1996; Mäthger and Hanlon, 2007; Mathger et al., 2006; Mäthger et al., 2009; Messenger, 1974; Zylinski and Johnsen, 2011) making them hard to detect by both prey and predator. The cephalopods' camouflage has been investigated through qualitative and quantitative experiments, pattern catalogues and behavioural studies (Barbosa et al., 2012; Borrelli et al., 2006; Darmaillacq et al., 2014; Josef et al., 2012; Kelman et al., 2008; Zylinski et al., 2011). Recent works have shown that cephalopods can use camouflage to minimize detection when moving over different background patterns (Hanlon et al., 1999; Shohet et al., 2006; Zylinski et al., 2009). Some octopuses perform the ‘moving rock’ manoeuvre (Hanlon et al., 2008; Hanlon et al., 1999) mimicking a rock as they move, some mimic other fish as they swim (Norman et al., 2001) and the work by Stauding et al. show that cuttlefishes adapt it’s cryptic behavior according to the presence of various teleost predators (Staudinger et al., 2013). Multiple camouflaging techniques and anti-predator behaviours, such as the ‘moving rock' are synergistically combined to yield the best cryptic result (Norman et al., 2001; Stevens et al., 2011). However, the question of camouflage and background matching during motion remains open. In other words, how can a cuttlefish motion be camouflaged and which of the mentioned schemes does it use? In the current study we examined the cuttlefish's ability to alter their mantle's reflectance while crossing between two highly contrasted backgrounds.

Theoretically speaking, any dynamic camouflager facing a change in background, may choose to match its background from selection of schemes. A camouflaging cuttlefish facing a change in background may choose to modify its mantle pattern either instantaneously or gradually (Fig. 1). In the first approach (Fig. 1 A), the animal does not change its colour and remains cryptic as long as it is moving over the first background. Once moving onto a new background, the animal instantaneously becomes highly conspicuous. A step-like change (Fig. 1 B) is achievable, assuming the animal operates a dynamic and very quick camouflage, as many cephalopods do. Clearly, instantly matching the background is the utmost camouflaging ability, yet physiological constraints drive animals to find alternative approaches. In a gradual approach (Fig. 1 C-F), an animal modifies its reflectance over a certain distance and time. Such an approach moderates the change in the observer’s field of view, at the cost of becoming partially conspicuous over both backgrounds. Finally, A gradual modification may also occur in the rate of change and in the position in which it takes place (Fig. 1 D-F), as change can start and end before (Fig. 1 D), during (Fig. 1E) or well after (Fig. 1 F), passing from one background to the other. As is illustrated in Fig 1, camouflage during motion includes in many cases (all but Fig. 1 B) a period of time in which the animal may be conspicuous in relation to the background. This potential mismatching is considered another reason for avoiding camouflage during motion.
In this study we wanted to study the time related process of such camouflage during motion.

Fig 1 near here

Results

Eight naive common European cuttlefish (*Sepia officinalis),* were placed in an elongated tank with either a control pattern (complete 18% reflectance grey; Fig 7 A), or a dichromic pattern composed of three areas: grey, black and grey again (3% Black and 18% grey; Fig 7 B). The swimming cuttlefish were tracked and their mantle reflectance was continuously monitored. In describing the results, the following acronyms are used (as discussed in the Methods section): (TBC) Time-Before-Crossing and Time-Post-Crossing (TPC).

In the uniform grey control tank, all eight animals maintained their overall light and uniform body coloration, matching the background throughout their movement (Fig 2 A). However, when swimming over the changing background, all animals became darker as they swam over the dark section and then lighter as moved back to the grey section (Fig 2 B-C).

In all cases, the cuttlefish's mantle changed simultaneously, without having the front or back half of the animal showing different reflectance as the animal passes the boundary.

Fig 2 near here

Comparing the different features of movement and camouflage did not reveal any difference between crossings from a grey to a black background vs. crossing a black to a grey background. For example, comparing the mean reflectance change, change duration, and TBC versus TPC resulted in no significant difference between the two (Wilcoxon rank test with 95 % confidence interval, $p=0.1$, $p=0.29$, $p=0.18$ respectively). Therefore, from here on, we only address reflectance change behaviour without distinguishing whether it was from grey to black or from black to grey. An example of such reflectance change can be found in Fig 3. TBC, TPC and total time of change did not correlate to the average velocity during transition (pairwise correlation test coefficient of 0.216, 0.325 and 0.42 respectively).

Fig 3 near here

The reflectance values within different mantle segments were measured. Measuring frame by frame, indicated a uniform change along the animal's axis, $F(9,220)=53.8$, $p=0.002$, with no gradual change recorded between the different segments, indicating that the entire mantle changed simultaneously (Fig 4).

Animals varied in their levels and duration of reflectance changes. Normalizing each transition to the animal's maximal reflectance and total transition duration, revealed a sigmoidal trend in the

reflectance change of all animals (Fig 5). Each of the 30 transitions could be fitted with a sigmoidal curve, with an r^2 not smaller than 0.85 (See example in Fig 5A).

Fig 5 near here

The mean duration of reflectance changes was 1.59 $\pm$0.96 seconds. In 27 out of the 30 transitions, animals started changing their body reflectance before they crossed the background boundary (Fig 6). However, the greatest part of the changing process took place after crossing onto the new background. The TBC varied between individuals (mean TBC of 0.47$\pm$0.58 seconds), corresponding to ~30% of the total time of change. Yet, most of the transition occurred after switching backgrounds (TBC vs. TPC, $p<<0.01$, n=30, Wilcoxon rank test with 95 % confidence interval).

Fig 6 near here

Discussion

We present here, for the first time in the study of animal's camouflage, characteristics of background-matching during motion, reporting the animal's competence to alter its mantle reflectance and match a changing background, maximizing its crypsis, while still on the go (Fig 3, video in the Supplementary material). Our results demonstrate that swimming cuttlefishes alter their mantle reflectance according to changes in their background, that a sigmoidal reflectance change is favoured and that animals start their change in advance but finish their change well after crossing to the new background (Fig 1E).

Dynamic camouflage is a multidimensional task. Matching the brightness, texture, and pattern to different backgrounds requires an advanced visual system, processing capabilities and proper skin physiology, which may include specific photoreceptors in it (Ramirez and Oakley, 2015). Changing colour and reflectance while chasing a prey or hiding from a predator, requires that the cephalopod possess a high level of visual information processing and the control of the skin chromatophores and irridophores, without interfering in the activity it is engaged in.

It should be noted that background matching should fit the visual system of the predators and not necessarily that of the cuttlefish. In this experiment we examined background matching on black and grey patterns, which should be similar to most types of observers .Yet a detailed study as to how potential predators view cephalopods camouflage awaits to be performed (Siddiqi et al., 2004; Stuart-Fox et al., 2008). Preliminary observations as well as discussions with Prof. Roger T. Hanlon suggest that octopuses may also be able to camouflage during motion. This report, along with the current study, brings into focus the need for a deeper understanding of camouflage during movement in various organisms, its limitations, and the controlling mechanisms.

A moving camouflaging animal may change its properties in many ways. Our results indicate that in most cases, the cuttlefish were anticipating the upcoming background (Fig 6) and changed their reflectance in a sigmoidal fashion (Fig 5). To achieve this, an animal has to estimate

in advance (1) the time it reaches the new background and (2) the reflectance of the approaching background. Following the optic flow mimicry scheme, such prediction and early response are beneficial, together with the gradual change in reflectance, in order to avoid unwelcome attention from nearby observers. In primates and cats, neurons sensitive to motion are already found in the primary visual cortex (Hubel and Wiesel, 1959; Hubel and Wiesel, 1962); in other species (i.e. rabbits and frogs) they may be found within retinal processing (Barlow et al., 1964; Finkelstein and Grusser, 1965). The high sensitivity of many animals to drastic changes in the visual field (Borst and Egelhaaf, 1989) reviewed in (Hildreth and Koch, 1987), may alert bystanders and break crypsis. In an effort to minimize such drastic changes, camouflaged organisms tend to remain motionless or move as slowly as possible. These behaviours, together with our current results, raise the question of whether there is a speed limit, at which all camouflage fails, and then there is a switch to other evasive manoeuvres.

In our case, no correlation was found between camouflage properties and the animal's velocity, which indicates a highly dynamic mechanism, regulating the general appearance of the animal during the phase of reflectance change. The dynamics of the change, following a general sigmoidal change vs. time relationships, suggests an early slow phase, possibly showing the end of detecting the approaching background, a rapid phase and a slow, fine-tuning phase. This raises the question of whether dynamic camouflage is completely cognitive or whether it consists of a passive/reflex-like component. Such a mechanism may derive from the requirement to complete the transition in the shortest time possible.

We would like to stress that in the wild, a clear transition between two uniform backgrounds is a rare scene, while most natural scenes include a blending phase comprising complex backgrounds affecting each other. This reason alone may drive the development of such a gradual and sigmoidal trend in reflectance change. In Fig 5 B, it seems like there is a potential biological constraints for the reflectance change, suggesting upper and lower limitations to the animal's background matching. Such limitations might represent the animal's point of view, chromatophores change rate, processing time, or a combination of all three.

Although all animals changed their reflectance while moving between the two uniform backgrounds, in several cases the overall match in reflectance was partial and limited (Michelson contrast of ranged between 0.15 and 0.76). This is possibly due to biological and physical constraints when matching an artificial uniform background (i.e. the cuttlefish could produce neither a perfectly uniform 18% grey nor a 3% black pattern). Since the uniform backgrounds elicited a uniform display in all the cuttlefish, we address the overall reflectance of the mantle without dividing it into the common skin components (Barbosa et al., 2007; Hanlon and Messenger, 1988). An interesting continuum to this study would be to further investigate mantle reflectance change while moving over complex backgrounds.

Changing one's reflectance to match a changing background provides a period of time during which crypsis is compromised, at least to some extent. Since camouflage is sacrificed, one has to choose when to do so: before or after changing backgrounds. Each timing preference enfolds slightly different benefits and drawbacks. Since the tested animals performed most of the change process after crossing onto the new background, we suggest that in most cases, minimizing

detection over the current background while compromising crypsis over the next is favoured. Although it is out of this manuscript scope, these results might also suggest that dynamic camouflage behaviour requires time to integrate visual input and respond accordingly.

Static camouflage is a widespread adaptation strategy, known in many animal taxa. Background matching via colour changing is a widespread phenomenon appearing on various time scales, from several months, to weeks, minutes and even seconds (e.g. rock ptarmigan *(Lagopus muta),* Spider crab (*Misumena vatia),* Common chameleon (*Chamaeleo chamaeleon*), and Snubnose emperor (*Lethrinus borbonicus*) respectively). Given the benefits of dynamic camouflage, one would expect it to develop many times throughout the evolution process. Yet, few species are known to obtain dynamic camouflage capabilities.

The fast and adaptive camouflaging system of cephalopods enables the changing of colour in less than a second (Hanlon et al., 2011). Therefore, these rapidly camouflaging animals are facing an unprecedented dual challenge - staying as cryptic as possible during motion and altering their colour in a manner that will not attract undesired attention. As far as we know, the described camouflaging scheme is the first to address such a challenge. To address this challenge, cuttlefish anticipate the upcoming background, start changing colour in advance and change it in a sigmoidal manner. This type of camouflaging strategy may be beneficial for other fast moving animals or man-made objects, trying to maintain crypsis while moving. When designing a camouflaging mechanism or algorithms, one should take into consideration all possible background-matching scheme, including when and where should the modification take place. Our current study emphasis the importance of background-matching anticipation and gradual colour change in dynamic camouflaging– which may also apply to advanced dynamic camouflaging technologies. While we stress the importance and application of studying dynamically changing patterns, automated camouflage pattern quantification and classification are not yet fully understood and still undergoes many changes. Therefore, we here stated by studying uniform backgrounds and analysing the mechanism of background matching using well-defined factors and descriptors. We expect that further in-depth studies may include the study of changing patterns during motion using more complex backgrounds.

In conclusion, we described here the camouflaging behaviour of a moving cuttlefish (*Sepia officinalis*) when crossing from one uniform background to another. Following the optic flow mimicry scheme and minimizing changes in the optic flow, the cuttlefish altered their mantle's reflectance in a sigmoidal fashion, while preforming most of the mantle-matching well after crossing onto the new background (as described in Fig 1F). The cuttlefish have proven to be an exciting model for investigating evolutionary and development processes, in general (Bassaglia et al., 2013), and camouflage and motion, in particular. Indeed, our analysis is applicable to a broader examination of camouflage patterning and may be used for both hypothetical and practical applications in the development of man-made dynamic camouflage systems (Yu et al., 2014). In a moving yet camouflaging system, one should take into consideration that camouflage is compromise to some extent and use, within it’s physio-mechanical constrains, the most effective scheme of background matching. We suggest that even when given a fast changing dynamic mechanism, an advantage would arise from a gradual change, preferably over the new background. Future studies may include quantification of shifts in body-patterns and examining the use of papillae during movement.

Material and methods

(a) Animals

Eight naive common European cuttlefish (*Sepia officinalis)*, mantle size of 7.2-12.3 cm, were collected from the Gulf of Naples, Italy and were held in separate tanks with running seawater at the Stazione Zoologica Anton Dohrn, in Italy, for two days of acclimatization. The cuttlefish were fed with live crabs, and maintained under a 12:12 (D: L) light regime. When experiments ended, all animals were returned to the Gulf of Naples. The animals' maintenance and the experimentation were in compliance with EU directive 2010/ 63 on the protection of animals used for scientific purposes, and following the recommendations of the 3Rs (Fiorito et al., 2014).

(b) Experimental design

Experiments were conducted in a secluded room, with a dividing curtain surrounding the set-up to minimize visual cues and external stressors. An elongated tank (200×40 cm, water level 45 cm) was coloured in a uniform 18% reflectance grey (Fig 7 A); the reflectance throughout this article is based on a standard 18% grey card, photographed inside the elongated tank, where 0 to 100% represents black and white respectively. In the dichromic pattern, a dark section (64×40 cm, average reflectance 6±1) at the center of the bottom of the tank was added (Fig 7 B). Since tactile information is a potential camouflage signal, all textures were uniform and smooth.

(c) Illumination across the tank was fairly homogeneous (350±5 lux – measured with a PeakTech 5025 light meter), to avoid shaded areas or light reflections. The water in the experimental tank was replaced prior to each trial.

Fig 7 near here

(d) Testing procedure

Animals were tested separately. After being placed at one end of the experimental tank each animal was left to settle for at least 5 minutes. We then waited until two conditions were met: (1) The animal remained motionless on one side of the tank; (2) The body colour became uniform and generally matched the grey background, and remained stable for at least 2 min. The animals were then observed and recorded using video, as they moved in the tank, mostly crossing it along its length. If the animals did not move within 15 min. of observation, they were motivated to cross the tank either by simply standing at one end of the tank, , or by providing a shelter at the opposite side of the tank. Under no circumstances, were the animals scared or strongly motivated to minimize stress. Both in control and dichromic conditions, animals were recorded crossing the tank, mantle first, from one side to the other (hereafter: ′Full-cross′). Swimming mantle-first did not effect the results, since cuttlefish posses both anterior and posterior binocular visual fields(Watanuki et al., 2000), which allow them to

clearly see and plan their route while swimming forward or backwards. In the control situation, a full crossing of the tank provided information on the animals' changes in body colours during motion, when the background remained constant, while the dichromic pattern involved one grey-to-black and one black-to-grey background transition. Two ′full-crossings′ (back and forth) were recorded for each of the 8 animals resulting in 16 full crossings and 32 background transitions: 16 grey-to-black and 16 black-to-grey. Due to technical limitations (one ink cloud occluding an animal's position and one fast-jetting animal), two background changes were excluded from the analysis, consequently leaving 30 background changes. In the control background, each of the eight animals swam across the tank once.
Experiments were run during daytime (9:00-17:00).

(e) Data acquisition

The movements of the animals were recorded by a SONY HDR-CX110 digital video camera, mounted vertically above the tank, providing a top-down view. The camera's field of view covered the entire width and 70% of the tank's length, filming 1440×1080 pixels video files. To achieve high-resolution frames for analysis, the camera was set so it photographed only 140 cm out of the 200 cm tank's length. Hence we have no record of the last 30 cm at each end of the elongated tank.

(f) Data analysis

Cuttlefish possess a single, mid-wavelength visual pigment, making them essentially colour-blind (Hanlon and Messenger, 1998; Marshall, 1996; Mathger et al., 2006). Moreover, most of the changes in the background and the cuttlefish display are monochromatic in nature, so we chose to look only at changes in reflectance and not in color. Therefore, videos were grey-scale transformed, using the green channel alone. Videos were analysed using a designated MATLAB™ code (Matlab version 7.14, MathWorks Inc., Natick, MA, USA). The outline of the code is: loading a video file, transforming each frame into a grey-scale intensity image, balance each frame according to the 18% grey standard, manually tracking the animal in $\frac{1}{10}$ of a second intervals, and measuring the animal′s mantle reflectance (an average of the mantle section), velocity and relative position in relation to the next background. Cuttlefish can present three types of body patterns- uniform, mottle, or disruptive (Chiao et al., 2007; Cott; Hanlon and Messenger, 1998). In our set-up, due to the uniform background, the animals elicited a uniform body pattern in all cases. Therefore, we used the average values of 1000 (40X25) pixels surrounding the center of the mantel (Fig 7 D). To characterize the trends in each case, we extracted and analysed each section separately, paying special attention to the start and end points of each transition. Transition start and ending, were determined by manually selecting points which marked the beginning or ending of drastic change in reflectance. A starting or ending point was only chosen if the trend was maintained for at least three consecutive measurements. Once we marked the beginning and ending points of each session, we calculated the 'time before crossing' (TBC) and the 'time post crossing' (TPC). During the short reflectance-changing sections animals swam at a rather constant speed (average acceleration/deceleration $0.022 \pm 0.01 \frac{m}{sec^2}$). Therefore, in this setup, time and distance are linearly related.

Since each animal began and completed their transitions in various locations and reflectence's values, we normalized our results to the entire transition change (100%). The percentage was calculated as the portion out of the entire change in place and in reflectance.

The percentage we used in Fig 5 represent the change out of the entire transformation. Meaning, at the Y-axis it is the percent in change out of the whole reflectance change, while in the X-axis it is the displacement percentage out of the total animal movement during the transition. After plotting the normalized transitions (Fig 5) we used Matlab Curve Fitting Tool to fit the Gaussian trend line (using 2 terms). To eliminate possible learning and habituating factors, we analysed the first and second transitions independently and compared them to each other. Validating a normal distribution (using the Kolmogorov-Smirnov test), a paired-sample t-test with 95 % confidence interval was preformed to check for differences in behaviours between the first and the second transition. No significant differences were found between the first and the second transition in terms of average velocity, mean change duration, and total reflectance ($p=0.028$, 0.034, 0.021 respectively). Therefore, data from both transitions were combined in the analysis.
To measure the reflectance uniformity within the mantle, we divided the mantle length into 10 segments, where 1 is the posterior and 10 is the anterior side. Each segment was measured for its average reflectance, during the cuttlefish swim over changing backgrounds.
Then, we tested the ten segments during transformations using a two-way ANOVA to verify whether the mantle was changed in a uniform or a gradual manner.

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Author contributions

Noam Josef: Experimental design, performed experiments, data analysis and manuscript preparation.

Igal Berenshtein: Data analysis and edited the manuscript.

Graziano Fiorito: Experimental design and data acquisition.

António V. Sykes: Experimental design and data acquisition.

Nadav Shashar: Experimental design, performed experiments and manuscript preparation.

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Figures

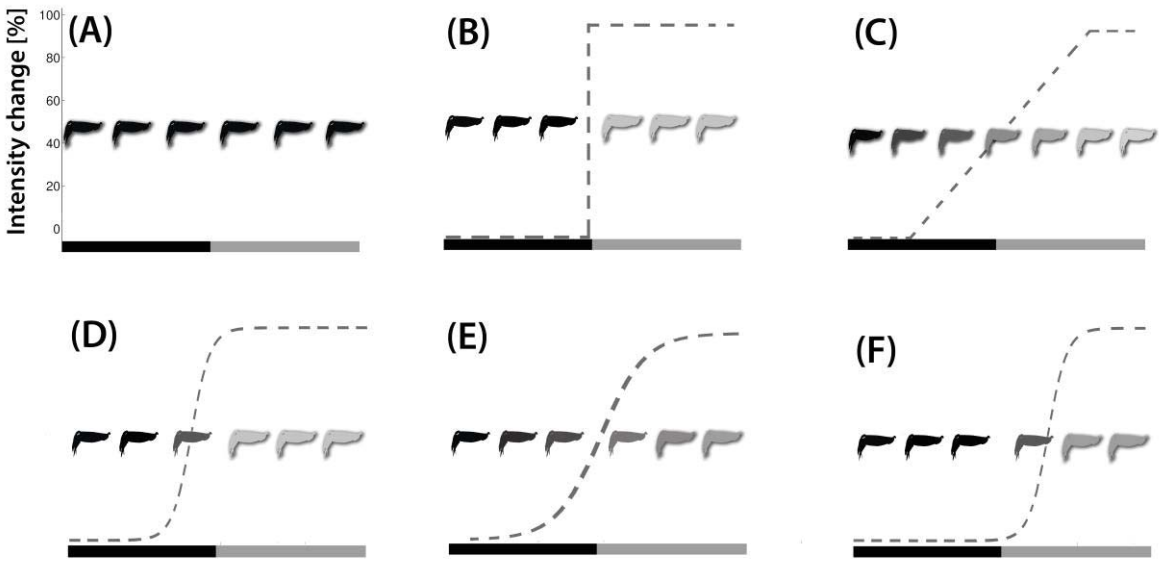

Fig 1: Theoretical illustration of a cuttlefish swimming from left to right, passing from a black to a grey background, presenting optional camouflage schemes. The dashed line represents the border between backgrounds. (A) An animal may retain its body reflectance throughout its course, forgoing camouflage altogether. (B) It may gradually alter its body pattern- in this case its reflectance. The gradual change can be expressed as linear (dashed black line), sigmoidal (solid black line) or by other functions. Alternatively (C), an animal may change its reflectance in an instantaneous step-like manner, with very short transition between the two displays. (D) An animal may start and finish changing its mantle reflectance in advance, matching its reflectance prior to the upcoming background. (E) It may change its mantle reflectance precisely when crossing the border between backgrounds. (F) Animals may perform the entire reflectance change, after crossing to the new background. Or (G) they may start changing their mantle reflectance in advance, while finishing the alteration well after crossing to the upcoming background.

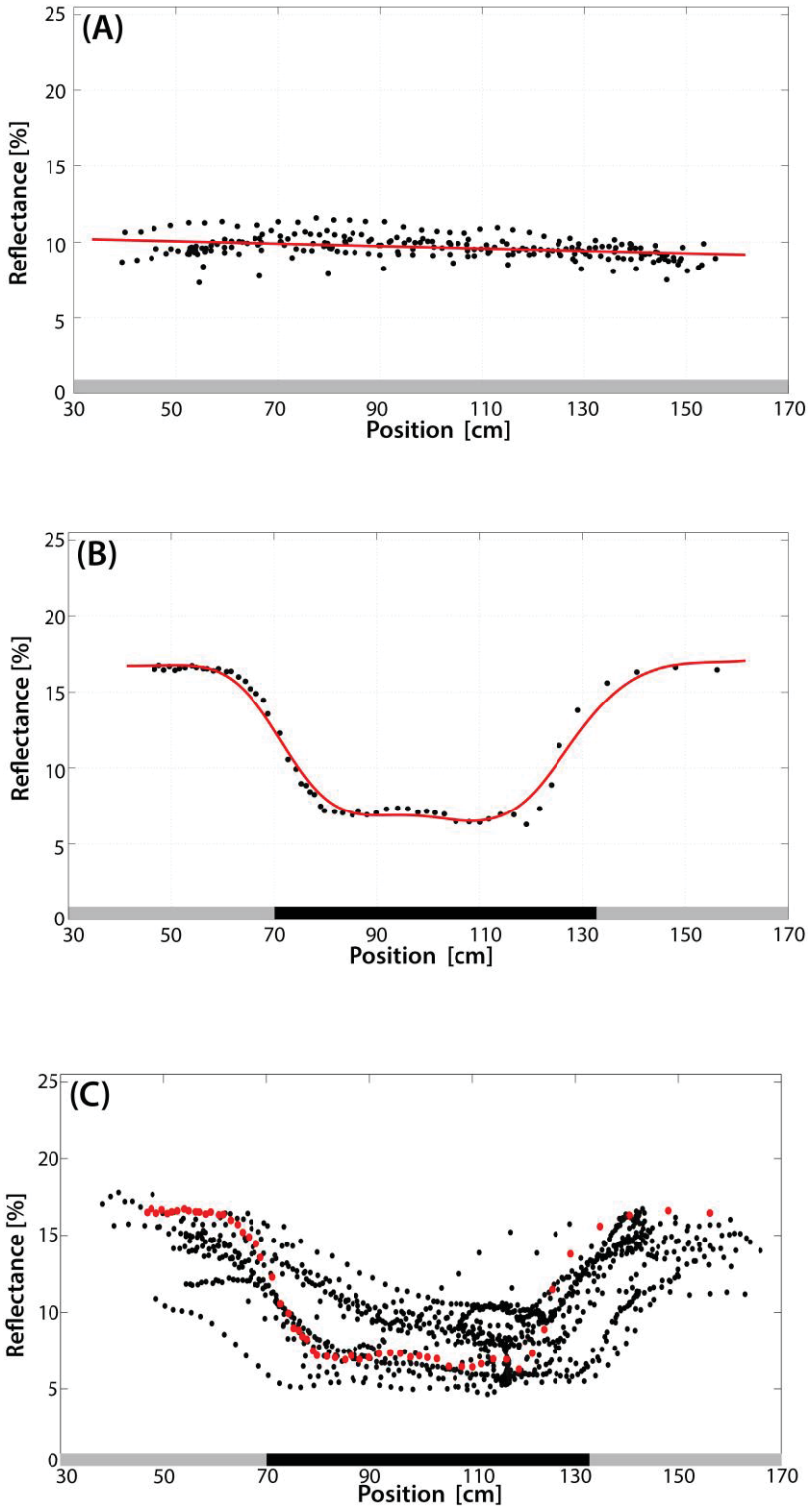

Fig 2: Body reflectance (on a 0-100% scale) of the cuttlefish as they crossed the experimental tank. (A) crossing the control grey tank; none of the eight cuttlefish drastically changed its reflectance while crossing the uniform grey tank. The nearly horizontal linear regression line demonstrates the overall constant reflectance (B) Mantle reflectance of a single animal swimming from left to right over a changing background, matching its mantle reflectance to the background as it swam across the tank. The smooth trend line (smoothing spline parameter: 1.05x10-5) demonstrates the reflectance matching along its path. (C) All runs (N=30) superimposed illustrating the persistent reflectance matching behaviour over a changing background. The red dots describe the single animal reflectance, as in (B).

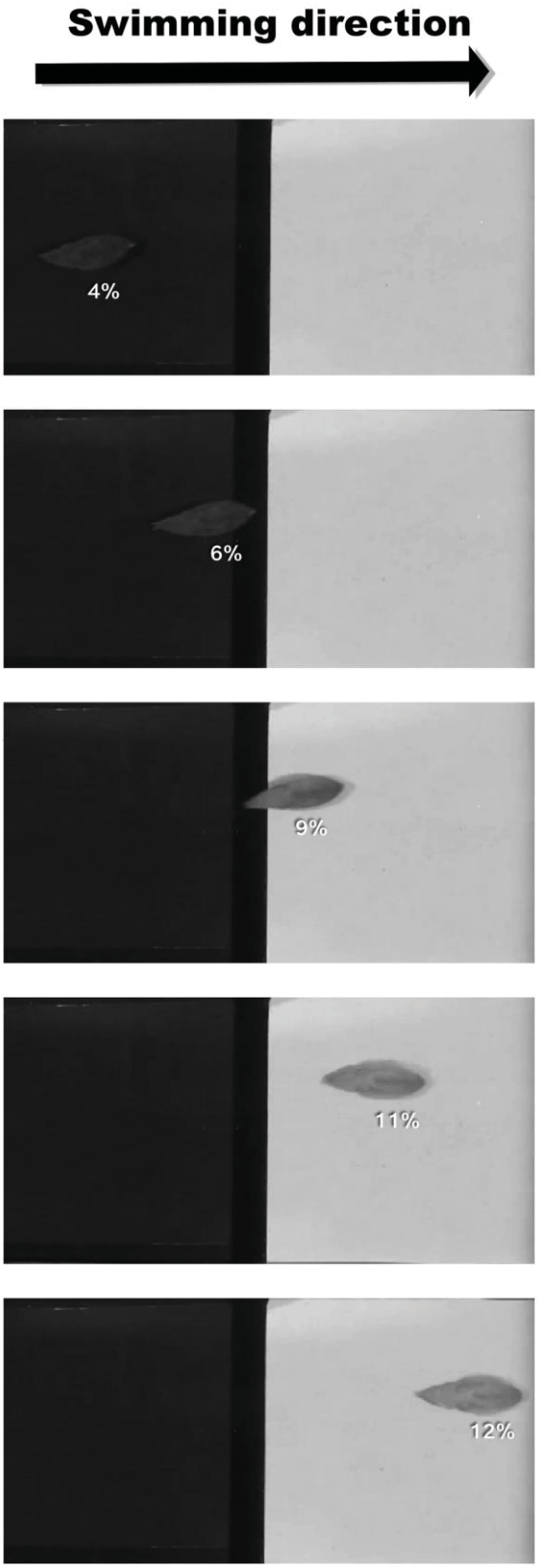

Fig 3: From up down - a cuttlefish matching its background as it swims from a black to a grey background. The white number represents the average cuttlefish's mantle reflectance values along it's movement.

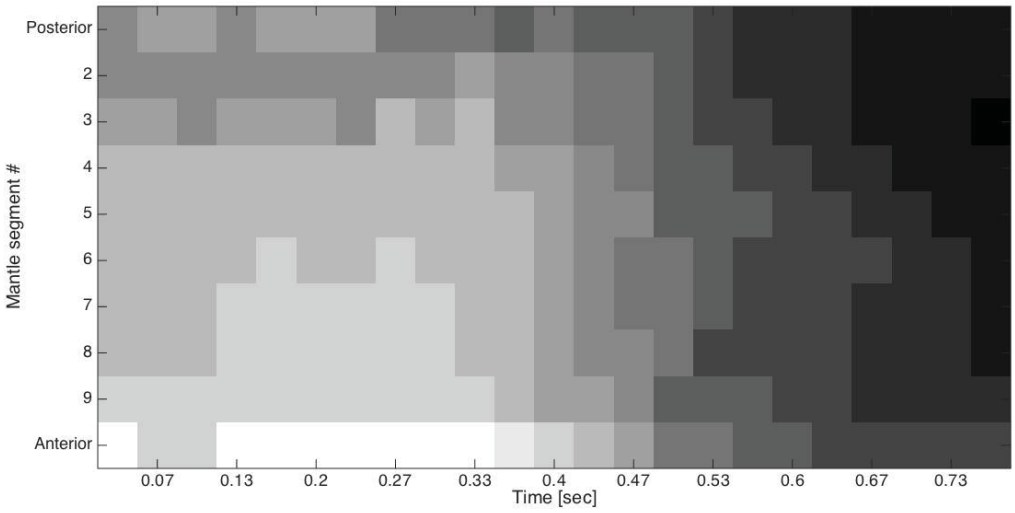

Fig 4: A representative reflectance map of the 10 mantle segments (Y axis) during a change in background. As can be seen, each column segments changed almost simultaneously with no evident of a gradual change.

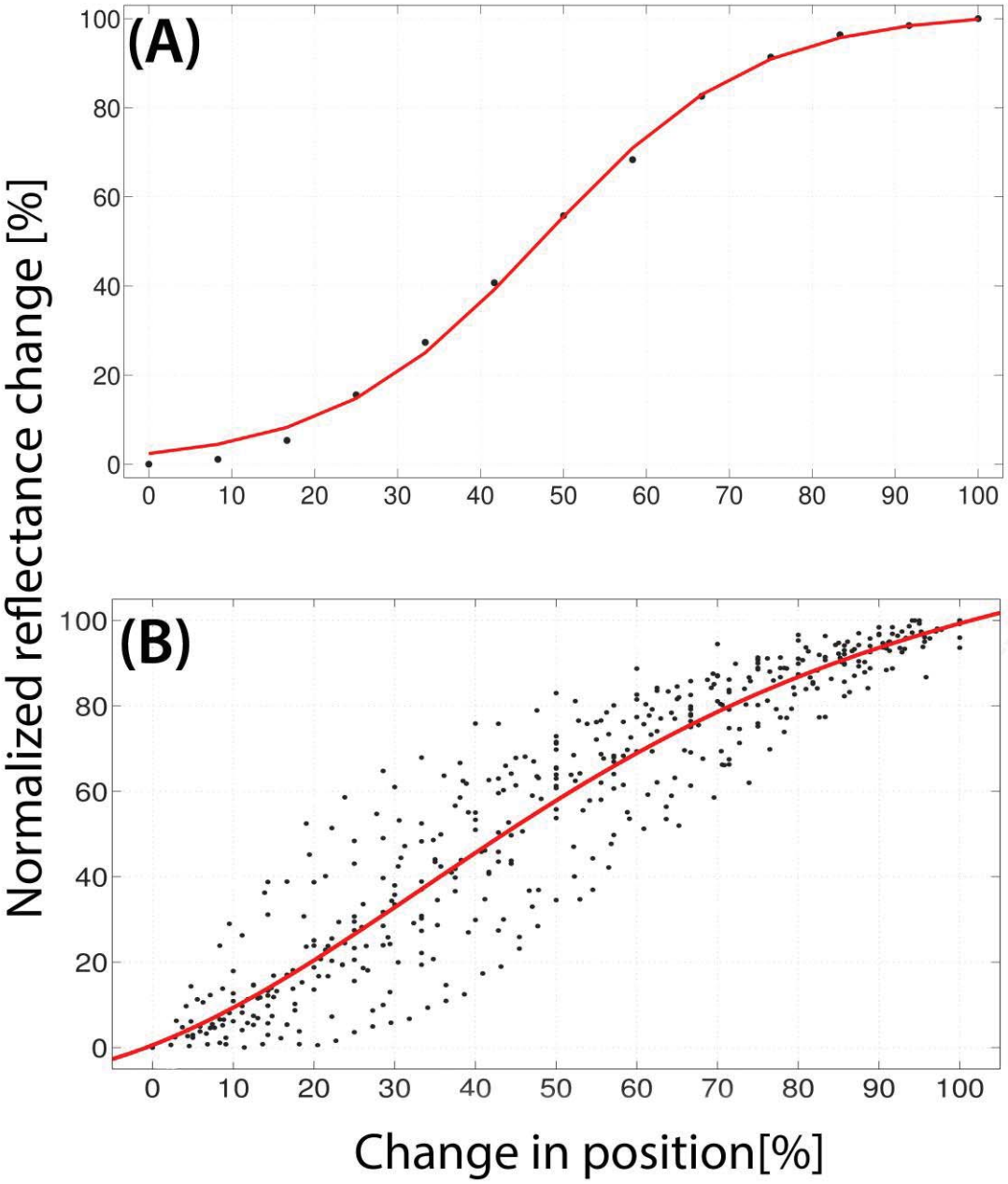

Fig 5: Normalised reflectance transitions of cuttlefishes as they crossed from one background to another. (A) in a single transition event, displaying a sigmoidal trend, which was consistent in all transitions (r^2=0.992). (B) Superimposing all 30 background-crossing episodes. The red sigmoidal fitting is the averaged overall Gaussian sigmoidal trend, complying with the single transition in (A) (r^2=0.9).

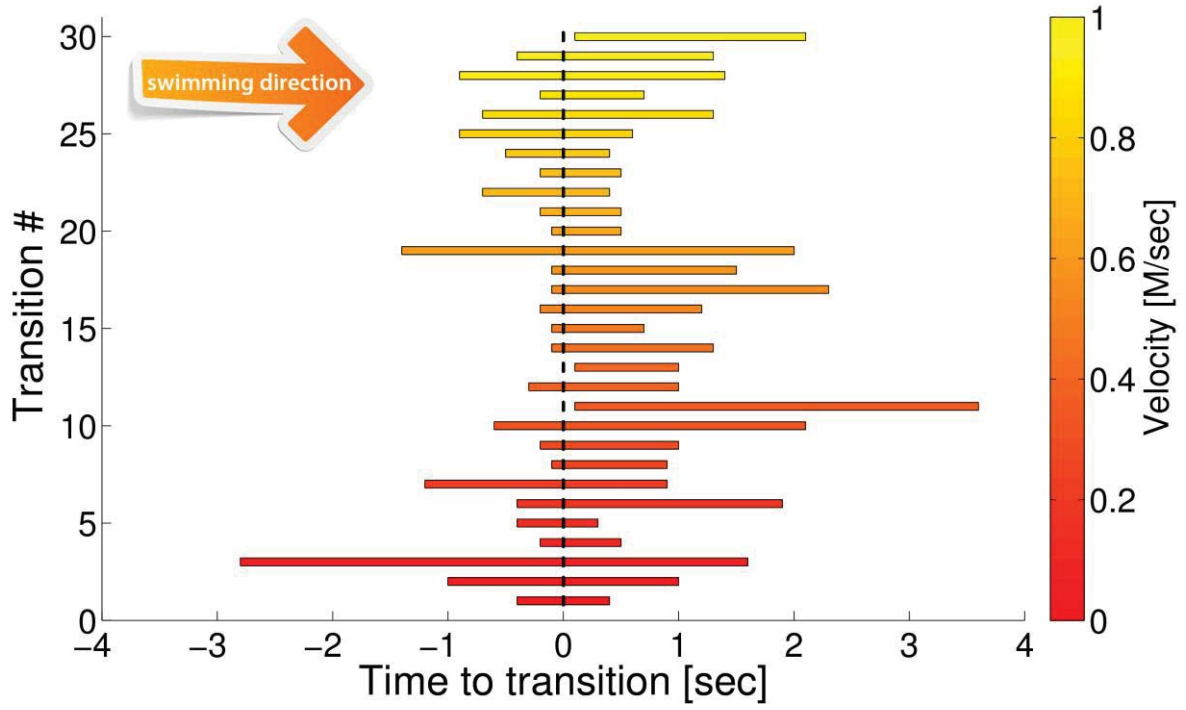

Fig 6: The duration and timing of each reflectance change relative to the background transition line. Swimming from left to right, the bars represent the time in which the animal changed its body pattern. "0" is the set time of crossing between backgrounds. Coloured bars represent the average velocity of the animal during this period.

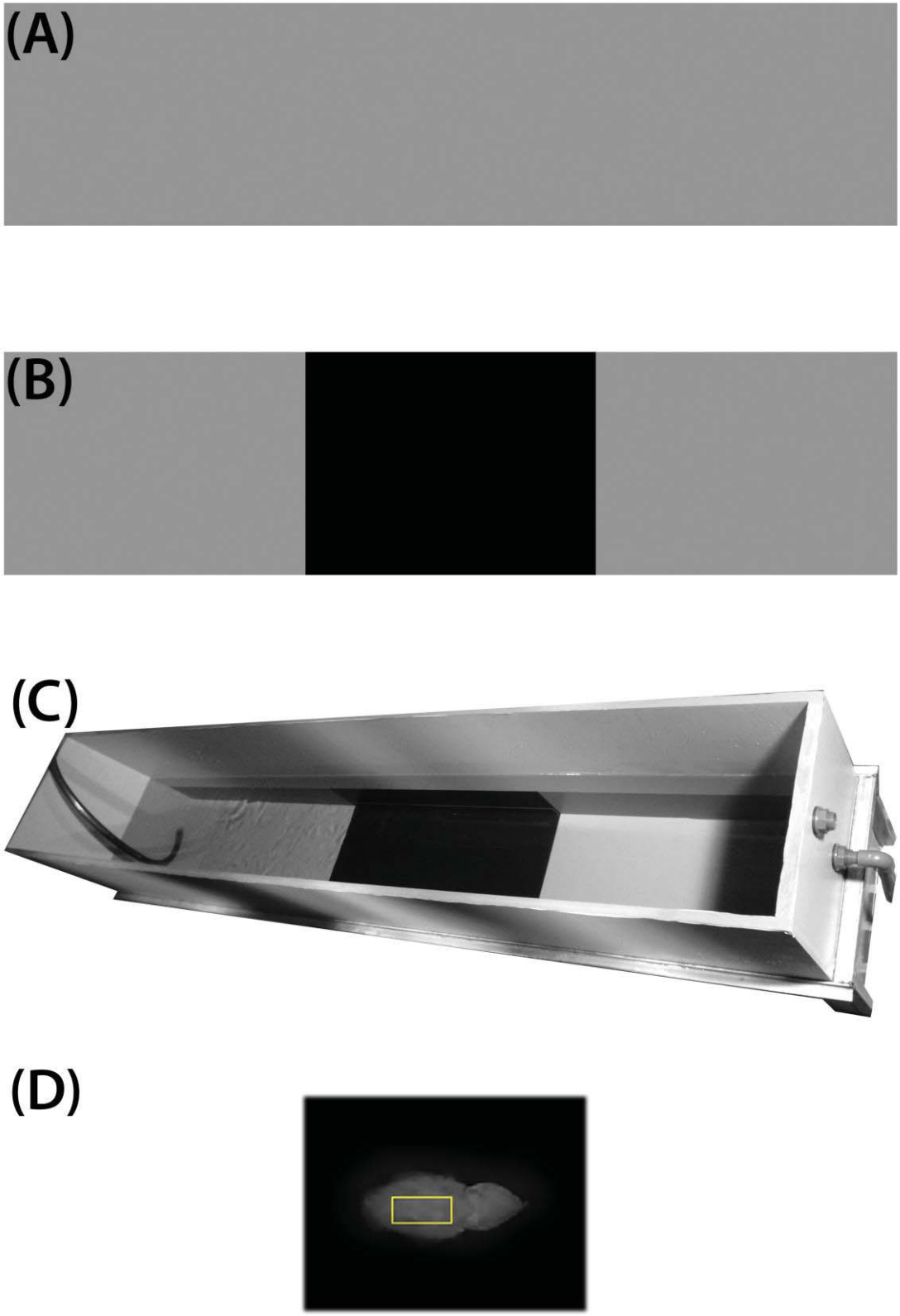

Fig 7: Experimental set up. (A) Control background –uniform 18% grey (B) Dichromic background- providing a change in background reflectance (C) Experimental tank, holding the dichromic pattern. (D) An example of a cuttlefish mantle with a 1000 pixels rectangular sample (yellow).

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Specificity of Lipoxygenase Pathways Supports Species Delineation in the Marine Diatom Genus *Pseudo-nitzschia*

Nadia Lamari[1¤☯], Maria Valeria Ruggiero[2*☯], Giuliana d'Ippolito[1], Wiebe H. C. F. Kooistra[2], Angelo Fontana[1], Marina Montresor[2]

1 Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy, **2** Laboratory of Ecology and Evolution of Plankton, Stazione Zoologica Anton Dohrn, Napoli, Italy

Abstract

Oxylipins are low-molecular weight secondary metabolites derived from the incorporation of oxygen into the carbon chains of polyunsaturated fatty acids (PUFAs). Oxylipins are produced in many prokaryotic and eukaryotic lineages where they are involved in a broad spectrum of actions spanning from stress and defense responses, regulation of growth and development, signaling, and innate immunity. We explored the diversity in oxylipin patterns in the marine planktonic diatom *Pseudo-nitzschia*. This genus includes several species only distinguishable with the aid of molecular markers. Oxylipin profiles of cultured strains were obtained by reverse phase column on a liquid chromatograph equipped with UV photodiode detector and q-ToF mass spectrometer. Lipoxygenase compounds were mapped on phylogenies of the genus *Pseudo-nitzschia* inferred from the nuclear encoded hyper-variable region of the LSU rDNA and the plastid encoded *rbc*L. Results showed that the genus *Pseudo-nitzschia* exhibits a rich and varied lipoxygenase metabolism of eicosapentaenoic acid (EPA), with a high level of specificity for oxylipin markers that generally corroborated the genotypic delineation, even among genetically closely related cryptic species. These results suggest that oxylipin profiles constitute additional identification tools for *Pseudo-nitzschia* species providing a functional support to species delineation obtained with molecular markers and morphological traits. The exploration of the diversity, patterns and plasticity of oxylipin production across diatom species and genera will also provide insights on the ecological functions of these secondary metabolites and on the selective pressures driving their diversification.

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* E-mail: mariavaleria.ruggiero@szn.it

☯ These authors contributed equally to this work.

¤ Current address: Environmental Toxicology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland

Introduction

Oxylipins are low-molecular-weight cyclic or acyclic products derived from the incorporation of oxygen into the carbon chains of polyunsaturated fatty acids (PUFAs), mediated by lipoxygenase (LOX) enzymes. These secondary compounds occur in many photosynthetic organisms including plants, algae and cyanobacteria (e.g., [1]), as well as in animals [2], [3] [4], [5], [6]) and fungi [7]. In higher plants, these molecules have evolved as primary defense in responses to physical damage by animals or insects, stress and attack by pathogens, and as regulators of growth and development [8], [9] [10], [11], [12], [13], [14]). In algae, LOX products have been reported in brown and red algae ([15], [16] [17]) and seem to be intermediates for innate immunity [18], [12]. Oxylipins have been reported also in diatoms [19], [20], [21]. These compounds are suggested to impair the reproductive biology of copepods, the principal plankton grazers, and to mediate intracellular communication (e.g. [22], [23], [24], [25], [26], [27], [28]). These studies also showed a high level of species-specific variability of diatom oxylipins that might indicate genetic [29] and physiological control of their biosynthesis [30], [31], [32], [26].

The planktonic pennate diatom genus *Pseudo-nitzschia* is encountered in marine environments all over the world and includes ca. 30 taxonomically described species [33]. *Pseudo-nitzschia* cells are needle-shaped and form stepped-chains by aligning tip-to-tip. Species identification is based on cell shape and width, cell overlapping pattern in the chain, and

ultrastructural features of the siliceous frustule [34]. Several groups of morphologically highly similar or even identical species occur, which can only be told apart using rapidly evolving DNA-markers such as the internal transcribed spacer region (ITS) and the hyper-variable domain (D1-D3) of the large subunit (LSU) in the nuclear ribosomal RNA cistron [33]. For instance, the morpho-species *P. delicatissima* consists of at least four genetically distinct species: *P. delicatissima* '*sensu stricto*', *P. arenysensis, P. decipiens*, and *P. dolorosa* [35], [36] [37]. Many of these species occur in sympatry in our study area, the Gulf of Naples in the Mediterranean Sea [37].

Recently we described a novel protocol for rapid characterization of oxylipins in lipid extracts of diatoms by reverse phase liquid chromatography and tandem quadrupole-ToF mass spectrometry (qToF LC-MS/MS) [21]. In addition to traditional metabolite profiling, the method permits to deduce the positional specificity of lipoxygenation by MS/MS fragmentation of hydroxy-epoxy PUFA derivatives, generically named epoxy-alcohols, which are among the major classes of oxylipins so far reported in diatoms. Thus, the qToF LC-MS/MS analysis provides a powerful tool to investigate distribution of LOX activities among different diatom samples. The aims of the present study were to assess: i) the spectrum of oxylipins produced by different *Pseudo-nitzschia* species, i.e., if one or more compounds are produced per monoclonal strain; ii) if oxylipin composition changes among strains within the same genetically defined species; and iii) if a match exists between phylogenetic relationship and LOX profiles, i.e., if closely related species produce the same compounds and more distantly related species different ones, and in addition, if phylogenetically closely related *Pseudo-nitzschia* species produce more similar oxylipins than more distantly related species. Strains were genetically identified based on the hyper-variable region (ca. 700 base pairs) at the 5'-end of their nuclear-encoded LSU rDNA and on the plastid-encoded large subunit of their ribulose-1,5-bisphosphate carboxylase/ oxygenase (*rbc*L). These markers have been shown to differ among biologically distinct species in *Pseudo-nitzschia* [31], [38].

Materials and Methods

Diatom cultures

Pseudo-nitzschia strains were isolated at the Long Term Ecological Research Station MareChiara in the Gulf of Naples (Tyrrhenian Sea, Mediterranean Sea) (Table S1). *Pseudo-nitzschia multiseries* strain NWFSC 316 was isolated in Clam Bay, Puget Sound, USA (Table S1). Clonal cultures were established by isolating single cells or short chains from phytoplankton net samples collected in the surface layer of the water column. Cultures were grown in sterile filtered oligotrophic seawater amended with f/2 nutrients [39] and maintained at a temperature of 20°C, a 14: 10h light: dark cycle, and with a photon flux of 100 µmol photons m^{-2} s^{-1}.

Molecular characterization

A 25 ml subsample was collected from each culture in exponential growth phase and genomic DNA was extracted following [40]. DNA was amplified by PCR using the primers D1R and D3Ca [41], [42] for the hypervariable domains D1 and D3 of the LSU rDNA and two degenerated primers, rbcL1 and rbcL7 [37] for the *rbc*L. PCR conditions and sequence analyses were carried out as reported in [37]. The accession number of the sequences produced within this investigation is reported in Table S1. Additional LSU rDNA and *rbc*L sequences of other *Pseudo-nitzschia* species were downloaded from GenBank, aligned by eyeball and used to build Maximum Likelihood phylogenies. Strain codes and GenBank accession numbers are reported in Table S1. Maximum-likelihood trees were inferred based on the substitution models selected through the Akaike information criterion option implemented in jModeltest [43] using MEGA5 [44], with 100 bootstrap replicates. Best fit models were GTR + I + G (I= 0.59; γ = 0.2266) for *rbc*L and GTR + I + G (I= 0.69; γ = 0.2180) for LSU.

Chemical analyses

To obtain the biomass required for chemical analyses, cultures were grown in Erlenmeyer flasks filled with 1 L f/2 medium at the conditions illustrated above. Cell concentration was monitored over time by collecting a 2-ml subsample that was used for estimating cell numbers with a Sedgewick-Rafter counting chamber utilizing a Zeiss Axiophot microscope (Karl Zeiss, Oberkochen, Germany). Cells were harvested at the stationary phase by centrifugation at 1200 g for 10 min at 5°C. The cell pellets were collected in 50 ml Falcon tubes, immediately frozen in liquid nitrogen, and kept at -80°C until chemical analysis was performed. Each strain was treated as an independent entry in the chemical analysis, irrespective of its taxonomic assignment; some strains have been grown and analysed on different occasions to test for constancy of their oxylipin pattern (Table S1). When possible, replicates of the chemical analyses were also carried out.

Cell pellets were dissolved in distilled water (1 $ml \cdot g^{-1}$ of wet pellet) and sonicated for 30 sec at 4°C prior to extraction with methanol (equal volume with water). The internal standard, 16-hydroxyhexadecanoic acid (15 $\mu g \cdot g^{-1}$ of pellet) was added before the extraction, and the resulting suspension was centrifuged at 2000 g for 5 min at 5°C. The organic extracts were dried under reduced pressure with a rotary evaporator (Buchi, Rotavapor R-200) and then methylated with ethereal diazomethane in diethyl ether (0.4 ml per 10 mg extract) for 1 hr at room temperature. After removing the organic solvent under nitrogen stream, the raw extract was used for analysis of non-volatile oxylipins by a Waters Alliance liquid chromatography equipped with UV photodiode detector (PDA) and Micromass *micro*q-ToF (qToF) mass spectrometer (Waters, Milford, MA, USA) as described in [21]. Identification of individual products was supported by matching of primary analytical indicators (PAIs), (retention time, UV spectrum, molecular weight and mass/mass fragmentation) with those experimentally determined (standards) or calculated [21]. Determination of lipoxygenase positional specificity is based on mass/mass analysis of epoxy-alcohols. Oxylipin levels were established by normalizing the component peak area by the peak area of the internal standard (16-hidroxyhexadecaoic acid methyl ester).

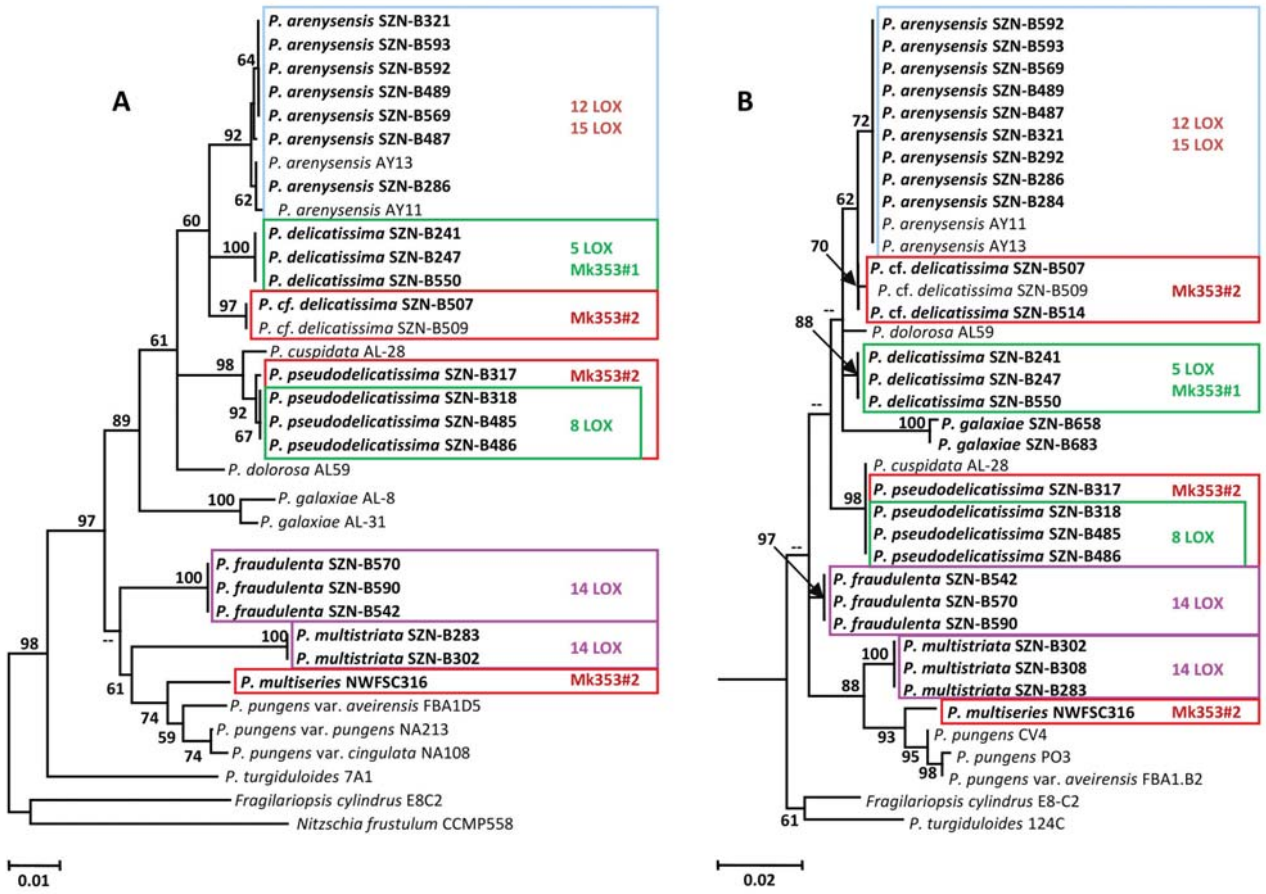

Figure 1. Molecular phylogeny of *Pseudo-nitzschia* species. Maximum Likelihood tree constructed with *rbc*L (A) and LSU (B) sequences. The strains in bold have been analysed for oxylipin production. The position specificity of the LOX activity detected for the different strains and the still unidentified compounds are mapped with different colours on the trees.

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Results

Molecular phylogeny

Maximum likelihood inference of the *rbc*L sequences provided a well-resolved phylogeny (Figure 1A). *Pseudo-nitszchia turgiduloides* was recovered at a basal position and the remainder *Pseudo-nitzschia* species grouped into three main clades. One of these included all *P. fraudulenta* strains, the second one contained a clade with strains of *P. multistriata* as sister to a clade with strains of *P. multiseries* and *P. pungens*, and a third one contained a clade with stains of *P. galaxiae* as sister to a polytomy comprising species specific clades with strains of *P. pseudodelicatissima* and all species of the *P. delicatissima* complex (*P. delicatissima*, *P. dolorosa, P. arenysensis*). Within this third clade, strains SZN-B507 and SZN-B509 grouped together. The two strains showed morphological and ultrastructural characters very similar to those of *P. delicatissima* (D. Sarno, personal communication) and their sequences did not match with any sequence deposited in GenBank. We thus considered these strains as a new species, and provisionally designated it as P. cf. *delicatissima* new genotype (D. Sarno, MV Ruggiero and collaborators, manuscript in preparation). The *rbc*L marker separated *P. pseudodelicatissima* from *P. cuspidata*. Intraspecific genetic differences were evident among strains of *P. arenysensis* and *P. pseudodelicatissima* in the form of single nucleotide differences. Remarkably large genetic diversity was instead evident within the *P. galaxiae* clade.

The LSU rDNA tree (Figure 1B) revealed the same terminal clades as the *rbc*L tree, with the following exceptions; the marker did not discriminate *P. pseudodelicatissima* from *P. cuspidata* and did not show differences amongst *P. arenysensis* strains. In addition, *Fragilariopsis cylindrus* grouped with *P. turgiduloides*, though with a low bootstrap support. Relationships among the terminal clades were less well resolved than in the *rbc*L tree as shown by low or lacking bootstrap support and by the recovery of polytomies. Nonetheless, the topologies between the two trees showed no conflicts. We did not obtain *rbc*L sequences for the two *P. galaxiae* strains analysed for oxylipin composition. However,

we included in the phylogenetic analysis the *rbc*L sequences of two other *P. galaxiae* strains whose LSU sequences were identical to LSU sequences of the analysed strains (see Table S1). The *rbc*L sequences of those *P. galaxiae* strains showed a remarkably higher level of nucleotidic divergence with respect to LSU.

Chemical analyses

Of the eight *Pseudo-nitzschia* species tested (Table S1), only the two strains of *P. galaxiae* did not show unequivocal presence of oxylipins, although traces of molecules possibly derived from C18- and C20- polyunsaturated fatty acids, were detected. LC-MS/MS profiles were generally consistent amongst different strains (Table S1) belonging to the same species as well as between replicates of chemical analyses carried out on the same samples, whereas they differed significantly among species. Figure 2 reports the oxylipins identified in the diatom strains during this study. All these compounds derive from eicosapentaenoic acid (EPA) and can be grouped in two major families containing hydroxy-eicosapentaenoic acids (HEPEs) and hydroxy-epoxy eicosatetraenoic acids (HEpETEs). Only a few samples of *P. arenysensis* also revealed presence of 15-oxo-5*Z*,9*E*,11*E*,13*E*-pentadecatetraenoic acid (15-OXO) [26]. Thus, the diversity of oxylipin profile in the *Pseudo-nitzschia* samples stemmed only from changes in lipoxygenation site of EPA. This feature is associated to positional specificity of LOX enzymes, which was inferred by analysis of the fragmentation pattern of HEpETEs in MS/MS analysis (Figure 3) [21]. According to these results, marked variations were detected among the *Pseudo-nitzschia* species (Figures 1, 4). Within the *P. delicatissima* complex, all *P. arenysensis* strains clustered for the presence of 15- and 12-LOX-derived compounds, namely 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-eicosapentaenoic acid (15-HEPE), 13-hydroxy-14-epoxy-5*Z*,8*Z*,11*Z*,17*Z*-eicosatetraenoic acid (13,14-HEpETE), 15-OXO, 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaenoic acid (12-HEPE) and 10-hydroxy-11-epoxy-5*Z*,8*Z*,14*Z*,17*Z*-eicosatetraenoic acid (10,11-HEpETE). These products generally co-occurred in the species even if only 15-LOX activity was detected in strains SZN-B593, SZN-B321 and SZN-B487, as well as only oxylipins derived from 12-LOX metabolism were found in strain SZN-B489. On the contrary, the three samples of *P. delicatissima* exhibited metabolites derived from the 5-LOX pathway: 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (5-HEPE) and 7-hydroxy-5-epoxy-8*Z*,11*Z*,14*Z*,17*Z*-eicosatetraenoic acid (7,5-HEpETE). This species also produced a pair of isomeric oxylipins (R_t= 24.2 and 21.6 min) with MS molecular ion at 353 ($M+Na^+$). Both compounds showed UV λ_{max} at 248 nm in agreement with a conjugated keto C-20 oxylipin but, since the amount of the sample did not allow completion of the structure assignment, they have been designed as undefined chemical markers of group 1 (Mk353#1). Two other oxygenated fatty acids (R_t= 21.2 and 18.4 min) with molecular mass of *m/z* 353 characterized the MS profiles of *P. pseudodelicatissima* strains. In analogy with Mk353#1, these data suggested the presence of another pair of isomeric keto derivatives of EPA but their structures remain not fully determined and these compounds are thus reported as undefined chemical markers of group 2 (Mk353#2). This last pair of products was also detected in the new genotype of P. cf. *delicatissima* and in the distantly related *P. multiseries*.

P. multistriata and *P. fraudulenta*, which form a grade in Figure 1, contained 14-LOX activity that was inferred from MS/MS fragmentation of 16-hydroxy-14-epoxy-5*Z*,8*Z*,11*Z*,17*Z*-eicosatetraenoic acid (16,14-HEpETE). With the exception of strain SZN-B317, that differed from others strains in *rbc*L sequence, the MS data of *P. pseudodelicatissima* were fully consistent with the occurrence of 8-LOX products, namely 8-hydroxy-5*Z*,9*E*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (8-HEPE) and 10-hydroxy-8-epoxy-5*Z*,11*Z*,14*Z*,17*Z*-eicosatetraenoic acid (10,8-HEpETE).

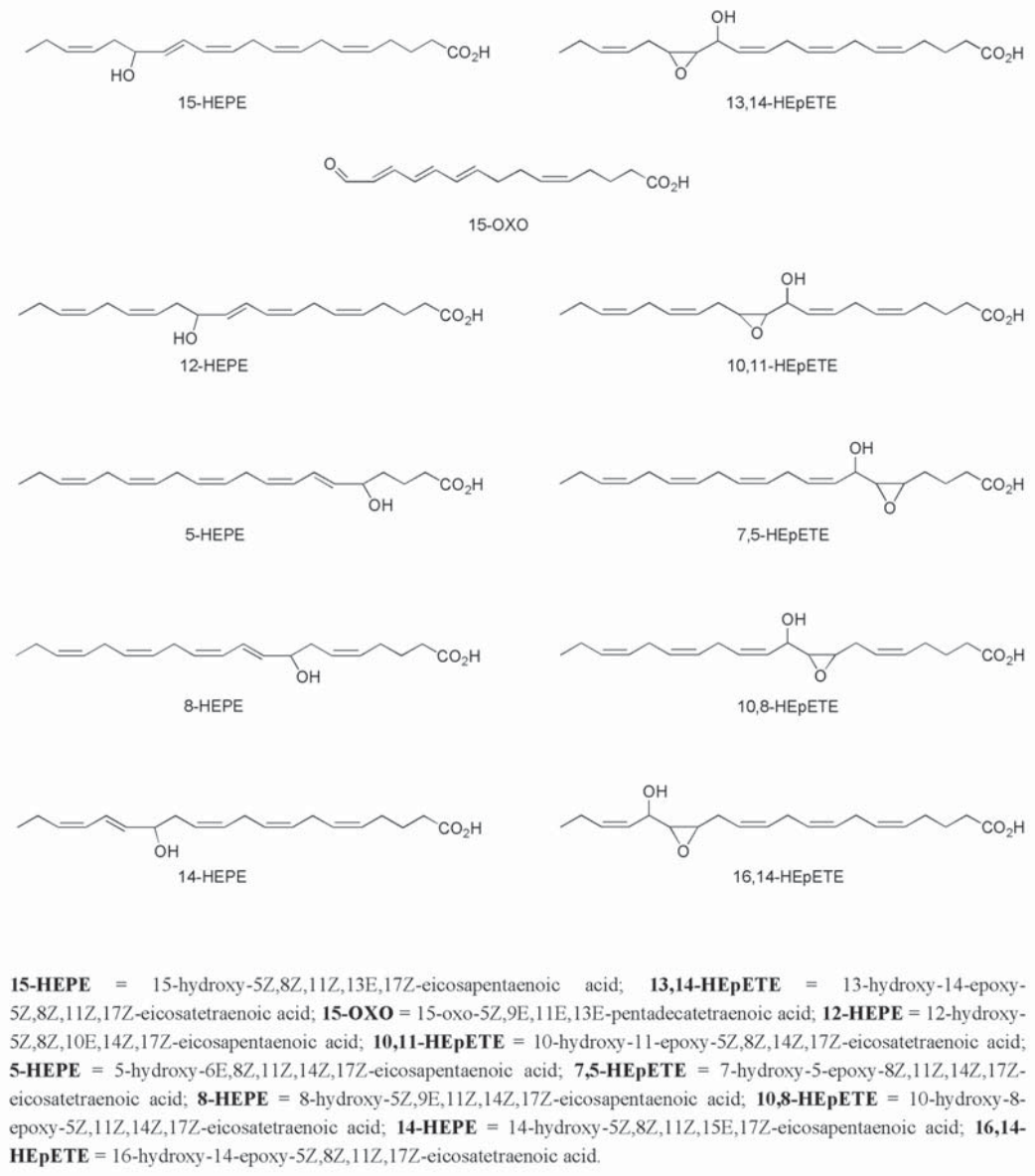
Discussion

Species specificity of LOX activity

The results of the present study show that the genus *Pseudo-nitzschia* exhibits a rich and varied LOX metabolism of eicosapentaenoic acid (EPA). As shown in Figure 4 that reports occurrence and relative abundance of individual oxylipin in the diatom samples undergone to qToF LC-MS/MS analysis, LOX positional specificity is suggestive of at least four chemotypes corresponding to 15- and 12-LOX in *P. arenysensis*, 5-LOX in *P. delicatissima*, 8-LOX in *P. pseudodelicatissima* and 14-LOX in *P. fraudulenta* and *P. multistriata*. Other possible chemotypes are associated to the presence of the unidentified compounds of group 1 and 2 (Mk353#1 and Mk353#2).

The species-specificity of these chemotypes was generally in good agreement with the molecular phylogenies based on *rbcl* and LSU, though not in all cases. For instance, the strains belonging to the distinct species *P. fraudulenta* and *P. multistriata* form a grade in the *rbc*L tree. Yet, they share 14-LOX, rendering the production of this compound probably a shared ancestral feature. Moreover, the fact that compound Mk353#2 is shared amongst different, not particularly closely related, species, suggests that the ability to produce this compound has been acquired at least twice, and possibly three times independently, or it has been acquired once and, subsequently, has gone lost in several species. In addition, each species produces just one or a few oxylipins. A notable exception is *P. galaxiae*, for which only traces of undefined oxygenated derivatives of fatty acids were detected. Our results support those of previous studies [26,45] that *Pseudo-nitzschia* lacks a distinctive trait of other planktonic diatoms, namely the LOX oxidation of C16 polyunsaturated fatty acids [46], [47], [48], [49], [45], [25].

Two LOX pathways appear to co-exist in *P. arenysensis*. Grouping of strains according to the minor *rbc*L differences amongst them did not corroborate a grouping according to 12- and 15-LOX products. Since LOX activity in diatoms seems to be modulated along the growth phase when maintained in culture [26], differences in LOX patterns could be due to physiological differences among the strains at the time of harvesting. Alternatively, this strain differentiation could be the result of genetic variation of LOX enzyme. In fact, single mutations of the peptide chain has been reported to induce



15-HEPE = 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid; **13,14-HEpETE** = 13-hydroxy-14-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid; **15-OXO** = 15-oxo-5Z,9E,11E,13E-pentadecatetraenoic acid; **12-HEPE** = 12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid; **10,11-HEpETE** = 10-hydroxy-11-epoxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid; **5-HEPE** = 5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid; **7,5-HEpETE** = 7-hydroxy-5-epoxy-8Z,11Z,14Z,17Z-eicosatetraenoic acid; **8-HEPE** = 8-hydroxy-5Z,9E,11Z,14Z,17Z-eicosapentaenoic acid; **10,8-HEpETE** = 10-hydroxy-8-epoxy-5Z,11Z,14Z,17Z-eicosatetraenoic acid; **14-HEPE** = 14-hydroxy-5Z,8Z,11Z,15E,17Z-eicosapentaenoic acid; **16,14-HEpETE** = 16-hydroxy-14-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid.

Figure 2. Major EPA-derived oxylipins characterized in *Pseudo-nitzschia* species during this study. Compounds have been identified on the basis of comparison of primary analytical indicators (retention time, UV spectrum, molecular weight and mass/mass fragmentation) with those experimentally determined or calculated in agreement with [21].
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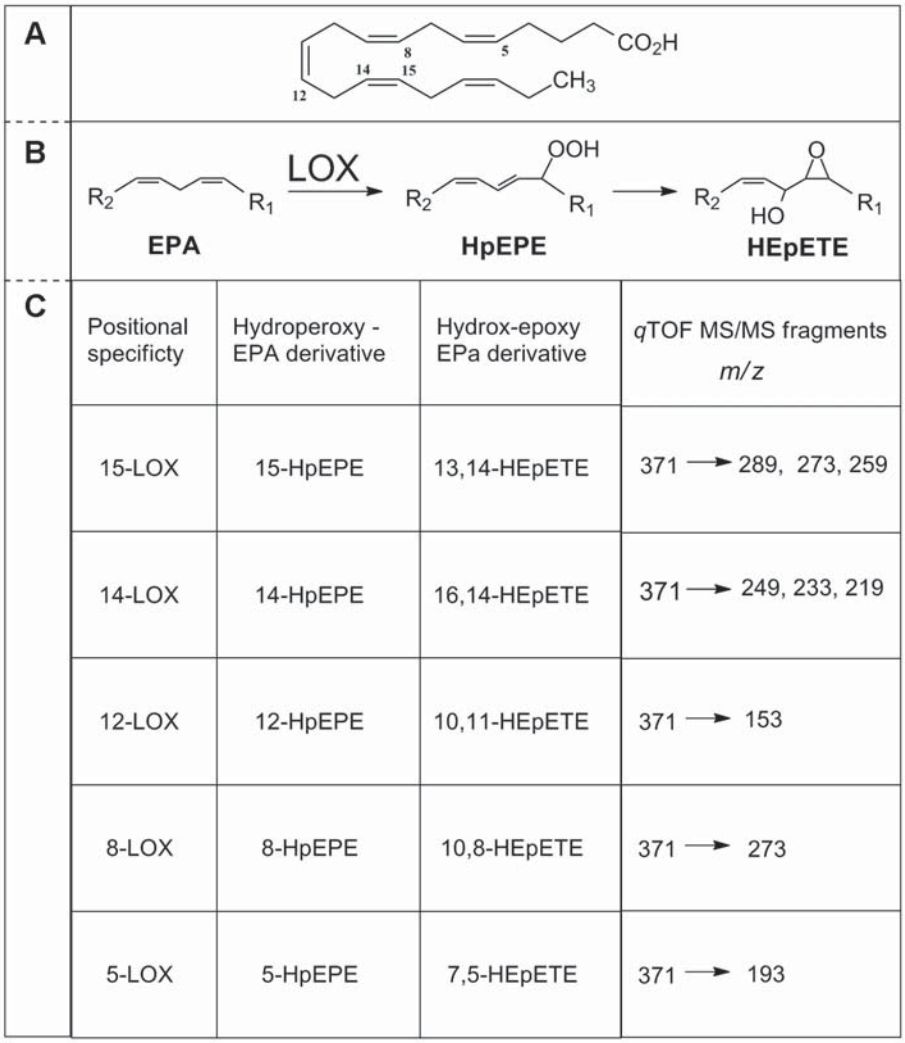
modification of the fatty acid binding pocket, converting a 15-lipoxygenating enzyme into a 12-lipoxygenating enzyme and *vice versa* [50] [51] [52] (for a general discussion see [53]). The observation that some strains possess both compounds and others just one of these could then be explained by the fact that diatoms are diploid and that some of the strains are 12-LOX or 15-LOX homozygous and others 12-LOX + 15-LOX heterozygous.

Also *P. pseudodelicatissima* strains show minor differences in the *rbc*L sequences. Notably, the strain SZN-B317 that lacks 8-HEPE and 10,8-HEpETE also differs in the *rbc*L signature from the three strains that possess 8-LOX activity. In addition to the products reported in Figure 2, some of the species show compounds that were not conclusively characterized, but correspond to two sets of undisclosed LOX products (MK353#1 and MK353#2) with identical molecular weight. These products characterize all species of the apical *rbc*L clade grouping *P. pseudodelicatissima, P*, cf. *delicatissima* - new genotype, and *P. delicatissima*, but appear to have gone lost in *P. arenysensis*. Interestingly, the compound MK353#2 also



Positional specificty	Hydroperoxy - EPA derivative	Hydrox-epoxy EPa derivative	*q*TOF MS/MS fragments *m/z*
15-LOX	15-HpEPE	13,14-HEpETE	371 → 289, 273, 259
14-LOX	14-HpEPE	16,14-HEpETE	371 → 249, 233, 219
12-LOX	12-HpEPE	10,11-HEpETE	371 → 153
8-LOX	8-HpEPE	10,8-HEpETE	371 → 273
5-LOX	5-HpEPE	7,5-HEpETE	371 → 193

Figure 3. Lipoxygenase positional specificity in the genus *Pseudo-nitzschia*. Identification is inferred on MS/MS fragmentation of hydroxy-epoxy eicosatetraenoic acids (HEpETEs) derived from LOX-mediated metabolism of EPA. (**a**) EPA and position of LOX oxidation. (**b**) Biochemical mechanism leading to specific transformation of primary LOX product (hydroperoxy-eicosapentaenoic acid, HpEPE) to HEpETE. For simplicity, the polyunsaturated chain of EPA is represented by the 1,3-pentadiene moiety that undergoes to enzymatic oxidation. R_1 and R_2 are variable alkyl residues to complement the structure of EPA. (**c**) Diagnostic ions of different HEpETEs generated by MS/MS fragmentation.

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characterizes *P. multiseries*, which is - according to the *rbc*L tree - not the sister of the abovementioned species. Previous studies on *P. multiseries* have established the presence of C20-derived lipoxygenase products, namely bacillariolides, that can be structurally related to the uncharacterized molecules reported here ([54] [55]).

Oxylipins as identification markers of *Pseudo-nitzschia* species

Traditionally, identification and delineation of unicellular microalgal species is based on phenotypic features such as cell size and shape and fine ultrastructural characters of their cell wall. Nucleotide markers have increased the precision of this identification process and have uncovered numerous cases of several genetically distinct lineages existing within single phenotypically delineated species (e.g. [56]). Also in diatoms, morphological differentiation has not held equal pace with differentiation into genetically and biologically distinct species, as shown by the discovery of numerous cases of genetically and biologically distinct species that are either morphologically indistinguishable (cryptic species) or can be separated only by minor ultrastructural details of their siliceous frustule (pseudo-cryptic species) (e.g. [57], [37] [58], [38],).

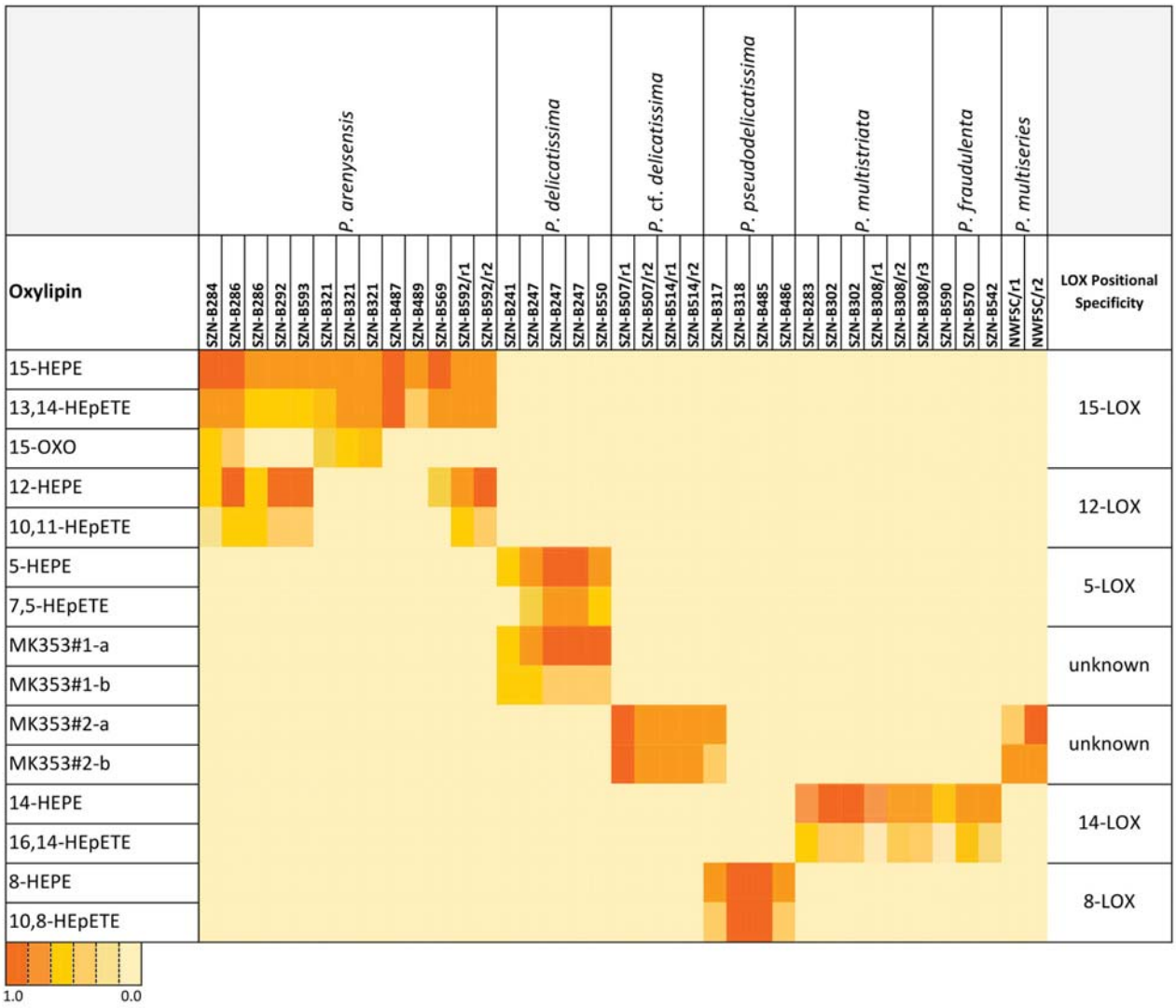

Figure 4. Heat map representation of oxylipin signals in the *Pseudo-nitzschia* strains. A semi-quantitative estimate of the individual oxylipins (rows) recorded in the analyzed strains (columns). Color scale represents the ratio between peak areas of individual oxylipins and internal standard (see Materials and Methods). Suffix '/r' followed by number indicates biological replicates of the same strain. Chemical abbreviations are in agreement with Figure 2.

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Differences in lipoxygenase products among a series of strains within *Pseudo-nitzschia* corroborate their genotypic delineation, even among genetically closely related cryptic species within the *P. delicatissima* complex, suggesting that these products can aid identification of species more precisely than morphological characters. This is particularly relevant when the presence of different epoxy-alcohols allows the rational deduction of the enzymatic dioxygenation site of the EPA polyunsaturated chain and, consequently, the classification according to the positional specificity of the putative LOXs. The three species within the complex (*P. arenysensis, P, delicatissima, P*, cf. *delicatissima* new genotype) are indistinguishable in light microscopy and present only minor morphometric differences in the density of the poroids on their siliceous frustule visible in EM ([37], D Sarno, MV Ruggiero and co-authors, manuscript in preparation for *P*, cf. *delicatissima* new genotype). The fact that closely related species show different LOX positional specificity seems to confirm that genetic changes accumulate rapidly in the enzymes of this pathway in *Pseudo-nitzschia*.

Several studies tested the Biological Species Concept sensu [59] in diatoms by carrying out mating experiments between sympatric strains of the same species as defined with morphological and molecular characters (e.g. [60], [37], [61]). The approach has been deployed also to delineate several of the *Pseudo-nitzschia* species included in the present

investigation [37]. The corroboration of oxylipin distribution, LOX positional specificity and species assignment of the strains used in the present study suggests that LC-MS/MS profiling constitutes a promising support for molecular identification of *Pseudo-nitzschia* species. However, more data, including analyses of strains from other geographic regions, need to be screened for their oxylipin profiles to test if these profiles are species specific.

Secondary metabolites have been used successfully as markers for species delimitation in fungi [62] [63] or in ciliates [64]. Chemotaxonomic approaches in fungi are based both on the use of one specific metabolite or on metabolite profiles that allow the identification of distinct chemotypes. At times, chemotypes matched with groups based on sequence data, at times not, giving support to the hypothesis that chemical profiling is useful for species recognition, but not for inferring phylogenies [63]. Oxylipin profiling and related MS/MS expansion can assist in the species identification of *Pseudo-nitzschia* strains, but they cannot be used as sole taxonomic identification tool, as is illustrated by the different LOX activities among the *P. arenysensis* strains and among the *P. pseudodelicatissima* strains as well as by the sharing of the same LOX profiles among different species. Clearly, no single marker constitutes the univocal criterion for species recognition, as markers may not accurately reflect species diversity. Different nucleotide markers can provide conflicting evidence about species boundaries [65]. Moreover, the very process of speciation implies an *interim* phase during which species delineation is, by definition, impossible. A way forward to strive for accurate species delineation - and to identify cases where that is impossible - is to deploy different and complementary types of identifiers. Examples of this approach provide promising results. Sympatric pseudo-cryptic species have been detected in the benthic estuarine diatom *Navicula phyllephyta* based on morphological and molecular investigations; these species showed different growth responses along a salinity gradient, thus suggesting that they occupy distinct ecological niches [66]. Also the planktonic diatom morpho-species *Chaetoceros socialis* conceals pseudo-cryptic genotypes with apparently different geographic ranges [67]. Qualitative and quantitative differences of the metabolomic profiles of the two genotypes suggest that they evolved distinct adaptive capabilities to the contrasting temperature characteristics of their environment [68].

The fact that *Pseudo-nitzschia* species produce one or two isomeric forms of oxylipins and not a broad spectrum of these compounds suggests the existence of specific metabolic pathway to produce them. The distinct oxylipin profiles among closely related species indicate that the profiles change rapidly and suggest that selective pressure drives the change. The nature of such a selective pressure depends on the roles of these compounds and on their specific targets [69]. Diatom oxylipins may function as species-specific mediators of bloom control [24], [26]. Species-specific chemical communication requires species-specific compounds and species-specific receptors. Hence, the receptors need to evolve in concert with the compounds. In case the oxylipins function as signaling tools amongst specimens within the same *Pseudo-nitzschia* species then different species producing the same compounds should not co-occur, or they should produce additional compounds that, together, function as species-specific communication. Moreover, the observation that strains belonging to the same species produce different LOX-profiles suggests that some compounds could serve for purposes other than intraspecific communication. In fact, in addition or alternatively, LOX pathways may play a key role in producing defence chemicals in the arms race against grazers [70]. Such a function favours rapid changes in the types of oxylipins produced to outwit the copepods' detoxification systems. The ensuing arms race is then expected to result in differences in LOX products among closely related species, or even between geographical populations of the same species. If the compounds serve as grazer deterrent, then intraspecific differences and interspecific identity do not pose any problem.

Supporting Information

Table S1. *Pseudo-nitzschia* strains analysed for oxylipin production. Species name and strain code, date in which the chemical characterization was carried out, LSU rDNA GenBank accession number, *rbc*L GenBank accession number. When LSU and *rbc*L sequences were identical to sequences already deposited in GenBank, the reference number of the deposited sequences is provided.
(DOCX)

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Author Contributions

Conceived and designed the experiments: AF MM. Performed the experiments: NL MVR. Analyzed the data: NL MVR GdI WHCFK AF. Contributed reagents/materials/analysis tools: MM AF. Wrote the manuscript: NL MVR GdI WHCFK AF MM.

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Chapter 3

Immunohistochemical Analysis of Neuronal Networks in the Nervous System of *Octopus vulgaris*

Giovanna Ponte and Graziano Fiorito

Abstract

Here we present two protocols developed to investigate the spatial distribution and relationship of neuroactive substances in the nervous tissues of cephalopod molluscs. The protocols are designed for frozen and vibratome sections of the *Octopus vulgaris* brain, but are easily transferable to other cephalopod species and tissues, and are also specifically designed to detect small molecules such as monoamines. One of the two protocols has been adjusted to process paraformaldehyde-fixed tissues, while the second is designed for tissues that require glutaraldehyde fixation.

Key words Immunohistochemistry, Immunofluorescence, Neurotransmitters, Biogenic amines, Cephalopods, *Octopus vulgaris*

1 Background and Historical Overview

Cephalopods are classic examples of "special" laboratory animals due to their phylogenetic position and the extraordinary richness of their behavioral repertoire [1–3]. They constitute a well-differentiated class belonging to the phylum Mollusca, clade Lophotrochozoa, comprising more than 700 living species. Cephalopods are all predators, widely distributed around the world occupying almost all marine niches. These animals evolved during the Cambrian (~530 Ma) from a putative monoplacophoran-like mollusc; during their evolution a general tendency of reduction of the external (molluscan) shell reflects the trend toward more active modes of life and the acquisition of more complex behaviors [4]. During evolution dramatic modifications occurred in the molluscan *Bauplan*; cephalopods are unique in morphology, physiology, complexity of the neural system and behavior [1, 5, 6].

The molluscan nervous system varies greatly in complexity and in the number of neurons among taxa [7] and reaches its highest degree in cephalopods. Ganglia become fused together forming "brains" whose size (relative to body weight) is comparable to that

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of the vertebrate brains and positions of cephalopods just below higher vertebrates [8]. In these large brains, for example in the octopus, 200 million cells are accommodated [9]. Differently from the typical molluscan design, in the cephalopod brain, the ganglia are fused together and clustered around the most anterior part of the esophagus. The agglomeration of the ganglia, which happened by the shortening of the connectives and commissures, forms three almost distinct parts: the supra- and the subesophageal masses and a pair of optic lobes lateral to the supraesophageal mass and positioned just behind the eyes. This provides cephalopods with the highest degree of centralization compared with any other mollusc. Such nervous system continues to have basic invertebrate organization: layers of cell bodies distributed externally around an inner neuropil. Gross morphology, neuroanatomy, and organization of the cephalopod brain have been extensively described [10].

Cephalopods greatly contributed to the modern biology and are emerging "models" for biology, physiology, genomics, neuroscience, cognition, and robotics [3, 11–14].

It is this "complexity" and the resemblance with vertebrates, that provided enough justification for the inclusion of the entire class in the Directive 2010/63/EU. They are, in fact, the sole representatives among invertebrates to be included in the list of species that from the first of January 2013 are regulated by the European Directive on the protection of animals used for scientific purposes (*Directive 2010/63/EU*: 15, 16).

While "peripheral" nervous system preparations of cephalopods greatly contributed to neuroscience (6, e.g., *giant axon*: 17–20), the "central brain" has been thoroughly explored to search for neural correlates of plasticity of behavioral response and learning [2, 21–23].

From the functional point of view, the nervous system of an octopus is characterized by a distributed organization due to the neural anatomy and elaborated sensory motor processing of the "peripheral" system, the latter being wired to a "central" system where coordination and decision-making units are present (*review in*: 2, 3, 22). In addition, the high degree of "autonomy" of the arms of an octopus is achieved, pending a hierarchical functional control of the higher motor centers, through highly stereotyped movements even in the absence of higher-order inputs [3, 24–27].

The analysis of hundreds of lesion experiments conducted mainly on octopuses (*review in* 28) and of several dozens of serial histological sections of the brains of the animals allowed Young and coworkers to describe the functional anatomy of the nervous system by identifying a "circuitry" leading to their visual and tactile processing: a circuit where learning and memory is achieved by a series of intersecting matrices [2, 22].

The most recent electrophysiological studies in the octopus confirm the view that convergent evolution has led to the selection of similar networks and synaptic plasticity in remote taxa (i.e., cephalopods and higher vertebrates), contributing to the production of complex behavior and learning capabilities [23]. A similar architecture and physiological connectivity of the vertical lobe system of the octopus with that of the mammalian hippocampus, together with the large number of small neurons acting as interneurons, suggest a typical structure with high redundancy of connections working with en passant innervations. This makes the octopus brain capable to create large-capacity memory associations [23, 29]. However, the analogy between the octopus and mammalian systems is not complete, the major differences being in the morphological organization and biophysical characteristics (6; *for review see* 23).

The great detail of knowledge available on the morphological and functional organization of the "brain" has been also complemented by ultrastructural studies. These provided strong evidence that the great majority of synapses in the central nervous system of cephalopods are chemical [30].

Sixteen years ago Dr. J.B. Messenger, one of the most representative "students" of J.Z. Young, published a review entitled "Neurotransmitter of cephalopods" [31] where a detailed overview is given for the existence of acetylcholine, catecholamines (dopamine and noradrenaline), indolamines (histamine, 5-HT), octopamine (OA), purines, amino acids, nitric oxide, substance P, somatostatin, FMRF-amide, and other peptides in the central and peripheral nervous systems, sensory organs, and viscera of cephalopods. Data presented by Messenger are deduced by more than 50 experimental papers and a number of dissertations published between 1935 and 1995. According to the author, the large number of presumed transmitters and modulators in the brain of cephalopods is also evidence of the presence of many "loops" that control the system [31, 32]. In accounting for such a variety of transmitters/modulators, Messenger drafted a table on their distribution in the brain of cephalopods based essentially on data from *Sepia officinalis*, *Octopus vulgaris*, *Eledone cirrhosa*, and other few species. Such body of knowledge allows providing general indication on the regional distribution of different neuromodulators in the central nervous system of *Octopus vulgaris* (*see* Fig. 5 in 33).

Despite the accuracy of the original works, the data reviewed by Messenger [31] reveal only limited regional differences among, for example, biogenic amines, and therefore a precise distribution of individual molecules within brain lobes in several species of cephalopods is not possible and still lacking (*for review see* 33). Despite the research efforts accounted by Tansey [34, 35] and Messenger [31], the statement "the chemical identity of the transmitter is not known for any synapse in the cephalopod brain" (34: *p 173*) appeared to be still true at the end of the 1990s [31, 36].

In order to explore how much was added over the last 30 years, we carried out a short pilot survey of published research involving cephalopods linked to the molecules listed in Messenger's account. The survey was based on full original papers (not reviews or abstracts) indexed by Web of Science and PubMed and published between 1996 and August 2012. The query returned 316 papers focusing, for example, on acetylcholine, dopamine, serotonin (5-HT), GABA, and glutamate. It is noteworthy to report that the use of these search criteria did not originate any study published on OA after 1996. Each record was then annotated to count the number of papers for each of the molecules considered [37].

1.1 Immunohistochemistry and Cephalopods

Several studies accounting for the presence and/or role of different molecules in cephalopods published over the last 20 years [37] are based on immunohistochemistry.

Although the working principle for immunohistochemistry existed since the 1930s, it is only in the 1941 that the first study using this approach appeared [38].

Despite this long-standing use in science, it is only after more than 40 years of the original study [38] that immunohistochemistry was applied to study the distribution of the 5-HT in the brain of octopus [39].

Table 1 summarizes the most representative studies that utilized an immunohistochemical approach in different species of cephalopods. The works included herein have been published after the monumental review by Messenger [31], with the sole exception of a study on 5-HT [39]. The chronological account utilized in Table 1 is preferred to other sorting criteria, since it is out of the aims of this chapter to review such information and to provide an overview of the distribution of different modulators and other proteins studied in different tissues in several cephalopod species. The table only serves as an annotated index of the available literature.

Despite the studies carried out in recent years, a precise localization of modulators and small molecules within the brain of cephalopod species is still missing. In this chapter, we present two different protocols developed to investigate the spatial distribution and relationship of neuroactive substances in the nervous tissues of cephalopods. These are designed for frozen and vibratome sections of *Octopus vulgaris* brain but may be applied with little variations to other species/tissues and are also specifically designed to detect small molecules such as monoamines.

One of the two protocols presented here has been adjusted to process paraformaldehyde-fixed tissues while the second for tissues that require glutaraldehyde fixation since the primary antibodies utilized are developed with immunogens obtained by coupling small molecules (e.g., neurotransmitters) with carrier proteins (*for details see* 40). The techniques described herein are based on those

Table 1
Chronological overview of studies carried out on cephalopods that have utilized immunohistochemical methods (not exhaustive list)

Species	Samples	Antibodies	Conjugation	Methods	Fixatives and processing	Refs.
Octopus vulgaris	Brain	Anti-serotonin (p)	BTG or BSA with FA	PAP	PFA 4 %; PF	[39]
Alloteuthis subulata, *Loligo vulgaris*, *Lolliguncula brevis*	Skin	Anti-l-glutamate (p) Anti-serotonin (p)	BSA with GA BSA with PFA	PAP	1 % PFA and 1 % GA; WM	[48]
Octopus vulgaris	Brain	Anti-FMRF-amide (p) Anti-GnRH (p)[a] Anti-mammalian GnRH (m)		ABC; FLUO	Bouin; PF	[49]
Sepia officinalis	Brain	Anti-rat neuronal NOS (p)[b] Anti-NMDAR1 (p) Anti-NMDAR2/3 (p) Anti-rat neuronal NOS (p)[c]	OVA KLH	ABC	Bouin; PF	[50]
Octopus vulgaris	Optic lobes	Anti-galanin (p) Anti-5-HT (p)		ABC; FLUO	PFA 4 % and 0.2 % PA; CT	[51]
Sepia officinalis	Brain	Anti-rat neuronal NOS (p)[b]	OVA	ABC	Bouin; PF	[52]
Sepia officinalis	Brain, fin	Anti-FMRF-amide (p) Anti-glutamate (m)		ABC; FLUO	PFA 4 %; CT	[53]
Idiosepius paradoxus	Hatchlings, juveniles, adults	Anti-acetylated α-tubulin (m)		ABC; FLUO	PFA 4 % or Bouin; WMe and PF	[54]
Octopus vulgaris	Ovary, oviducts, hepatopancreas	Anti-estradiol-17B receptor (p)		ABC; FLUO	PFA 4 % or PFA 4 % with 0.5 % GA; CT and PF	[55]

(continued)

Table 1
(continued)

Species	Samples	Antibodies	Conjugation	Methods	Fixatives and processing	Refs.
Sepia officinalis	Optic lobes	Anti-FMRF-amide (p)		ABC	PFA 4 %; (ND)	[56]
Sepia officinalis	Central heart	Anti-5-HT (p)		PAP	PFA 4 %; PF	[57]
Octopus vulgaris	CNS, heart, oviducal gland, oviduct	Anti-ovGnRH (p)		FLUO	PFA 4 %; CT	[58]
Sepia officinalis	Ink gland	Anti-DA (p)		PAP	5 % GA with 1 % SMB; CT	[59]
Sepia officinalis	Brain	Anti-mammalian NKA A (p) Anti-5-HT (m)	HSA BSA	FLUO	PFA 4 %; CT	[41]
Octopus vulgaris	Brain	Anti-calretinin (p)[d]		PAP	Bouin; PF	[60]
Idiosepius notoides	Brain	Anti-FMRF-amide (ND)		FLUO	PFA 4 %; VT	[61]
Loligo opalescens, *Octopus vulgaris*, *Octopus rubescens*	Embryos, hatchlings	Anti-tubulin[e] Anti-FMRF-amide Anti-5-HT Anti-SCP (m)[f] Anti-LHRH[g] Anti-TGnRH (p)[h]		FLUO	PFA 4 % or ice-cold methanol; WM and (ND)	[62]
Octopus vulgaris	Eyes, optic lobes	Anti-cChAT (p)[i]		ABC	PFA 4 % and 0.2 % PA; CT	[63]
Loligo vulgaris, *Sepia officinalis*, *Argonauta argo*, *Idiosepius notoides*, *Euprymna scolopes*	Embryos, hatchlings, brain of adults	Anti-acetylated α-tubulin (m) Anti-F-actin (ND) phalloidin Anti-FMRF-amide (p)	Alexa Fluor 488	FLUO	PFA 4 %; VT	[64]

Octopus vulgaris	Optic lobes	Anti-NMDAR 2A and anti-NMDAR 2B (p) Anti-oct-GnRH (p)		FLUO	Bouin; PF	[65]
Sepia officinalis	Brain	Anti-oct-GnRH (p)		PAP; FLUO	Bouin; PF	[66]
Sepia officinalis	Brain	Anti-oxytocin Anti-Arg8-vasopressin	BSA BSA	FLUO	PFA 4 %; CT	[67]
Euprymna scolopes	Juvenile	Anti-C3 (p) [j]	OVA	FLUO	PFA 4 %; WM and (ND)	[68]
Sepia officinalis	Embryos	Anti-acetylated α-tubulin (m) Anti-TH (m)		ABC	3.7 % PFA; WM	[69]
Idiosepius notoides	Embryos, brain of adults	Anti-5-HT (p) Anti-acetylated α-tubulin (m)		FLUO	PFA 4 %; WM and VT	[70]
Loligo vulgaris, *Sepia officinalis*	Embryos, hatchlings	Anti-Na+/K+-ATPase α1 (p)[k]		FLUO	Bouin; PF	[71]
Octopus vulgaris, *Argonauta hians*	Embryos, hatchlings	Anti-5-HT (p) Anti-FMRF-amide (p) Anti-acetylated α-tubulin (m)		FLUO	PFA 4 %; VT	[72]
Idiosepius notoides, *Euprymna scolopes*, *Sepioteuthis australis*, *Loligo vulgaris*, *Octopus vulgaris*	Embryos, hatchlings, adults	Anti-VD1/RPD2 α1-peptide (p)[l] Anti-acetylated α-tubulin (m)		FLUO	PFA 4 %; WM and VT	[73]
Octopus vulgaris	Brain	Anti-cChAT (p)[i]		ABC	PFA 4 % and 0.2 % PA; CT	[74]
Sepia pharaonis	Optic lobes	Anti-NR2A (p)[n]		PAP	4 % PFA and 5 % GA; CT	[75]

(continued)

Table 1
(continued)

Species	Samples	Antibodies	Conjugation	Methods	Fixatives and processing	Refs.
Sepioteuthis lessoniana	Brain	Anti-GAD 65 and 67		ABC	10 % formaldehyde; PF	[76]
Loligo pealei	Stellate ganglia, embryos, hatchlings	Anti-FMRF-amide (p)		ABC	4 % PFA; PF	[77]
Octopus vulgaris	Arm	Anti-cChAT (p)[i] Anti-pChAT (p)[o]		ABC	PFA 4 % and 0.2 % PA; CT	[78]

Species, tissues utilized (samples), antibodies and their conjugation, and information on methods (i.e., PAP) and fixation, and samples processing are provided. *Abbreviations utilized*: *p* polyclonal, *m* monoclonal, *ABC* avidin biotin complex, *BSA* bovine serum albumin, *BTG* bovine thyroglobulin, *FA* formaldehyde, *GA* glutaraldehyde, *HSA* human serum albumin, *KLH* keyhole limpet hemocyanin, *OVA* ovalbumin, *PFA* paraformaldehyde, *PAP* peroxidase anti-peroxidase method, *FLUO* direct or indirect immunofluorescence method, *PF* paraffin, *PA* picric acid, *WM* whole mount, *CT* cryostat, *VT* vibratome, *ND* not described

[a]As polyclonal antibodies authors utilized, chicken I (cGnRH-I), chicken II (cGnRH-II), salmon (sGnRH)

[b]Synthetic peptide C-terminal sequence

[c]Synthetic peptide N-terminal sequence

[d]Human recombinant calretinin

[e]Antisera 12G10 and E7

[f]Gastropod small cardioactive peptide

[g]Human luteinizing hormone-releasing hormone

[h]Tunicate gonadotropin-releasing hormone

[i]Rat common type of ChAT peptide encoded by exons 7–8

[j]For anti-C3 a 13-aa synthetic peptide was utilized to produce the antigen

[k]Internal region of Na+/K+-ATPase α1 of human origin

[l]Amino acid sequence: CDMYEGLAGRCQHHPRNCPGFN

[n]C-terminal synthetic peptide rat glutamate receptor subunit

[o]Peripheral type ChAT

outlined by [41, 42] and adapted to cephalopods according to Ponte [33]. These protocols have been developed to detect small molecules (i.e. biogenic amines). The small changes required for the use for detection of other molecules (e.g.: enzymes, receptors) are not discussed herein.

2 Equipment, Materials, and Setup

2.1 General

- Anesthetic solution: $MgCl_2$ 3.5 % in seawater [43].
- Dissecting microscope.
- Dissecting tools.
- Seawater (filtered).
- Phosphate-buffered saline (PBS) 0.1 M, pH 7.4.
- Disposable embedding molds.
- Superfrost™ Plus Microscope Slides (Thermo Fisher Scientific Inc., Waltham, MA).
- Histology slide tray.
- Orbital shaker.
- PapPen.
- Filter paper.
- Primary antibody (ies) against antigenic protein(s) of interest.
- Normal goat serum (NGS) or blocking serum from the species where primary antibodies were raised.
- Species-specific fluorescent secondary antibodies.
- Tween® 20.
- DAPI stock solution: 14.3 mM DAPI in dH_20.
- Cover glasses.
- Fluoromount™ (Sigma Chemicals, St. Louis, MO).
- Transparent nail polish.
- Fluorescence microscope or confocal laser microscope.

2.2 Immunofluorescence of Frozen Sections

- Fixative solution: 4 % paraformaldehyde (PFA) in filtered seawater. *See* **Note 1**.
- 30 % sucrose in PBS.
- Falcon™ tubes (Thermo Fisher Scientific Inc.).
- Optimum cutting temperature (OCT) mounting medium, e.g., Sakura Tissue-Tek® OCT Compound (Gentaur, San Jose, CA).
- Dry ice.
- Cryostat, including knife holder, glass anti-roll guide, disposable microtome knives, and specimen discs. *See* **Note 2**.

- BD Falcon™ Square BioDish XL (Thermo Fisher Scientific Inc.).
- PBST: PBS +0.1 % Tween® 20.
- DAPI working solution: DAPI stock solution 1:1,000 in PBST.

2.3 Immunofluorescence of Vibratome Sections

- Fixative solution: 6.25 % glutaraldehyde, 75 % picric acid, 5 % glacial acetic acid, and 1 % sodium metabisulfite (SBM) in distillated water.
- SMB solution: SBM 1 % in dH_2O.
- Tris–HCl: 0.1 M Tris–HCl buffer pH 7.6
- Tris–HCl-SMB: 0.1 M Tris–HCl buffer pH 7.6, containing 0.45 % SMB.
- Tris–HCl-Triton®: 0.1 M Tris–HCl buffer pH 7.6, containing 1 % Triton® X-100.
- Tris–HCl-SMB- Triton®: 0.1 M Tris–HCl buffer pH 7.6, containing 1 % Triton X®-100 and 0.45 % SMB.
- Bleaching solution: Tris–HCl-SMB containing 1 % Na-borohydride.
- Mixture of 100 % ethanol and methyl salicylate (1:1 v/v).
- Blocking solution: Tris–HCl-Triton® containing 10 % NGS.
- Antibody diluent solution: Tris–HCl-Triton® containing 0.05 % sodium azide (NaN_3) and 5 % NGS with primary antibody. *See* **Note 3**.
- DAPI working solution: DAPI stock solution 1:1,000 in Tris–HCl-Triton®.
- Fine-tipped paintbrush.
- Ethanol series: 100°, 95°, 70°, and 50° (in dH_2O).
- Vibratome including blade holder, disposable double-edged razor, and magnetic specimen holders. S*ee* **Note 2**.
- Super glue.
- 4 % agarose in Tris–HCl-SMB.
- 24-well culture plate.
- Iced water.

3 Procedures

3.1 Immunofluorescence on Frozen Sections

3.1.1 Tissue Preparation

Euthanize the animal with anesthetic solution [43].

Dissect brain masses and ganglia, fix them for 1–2 h (*see* **Note 4**) in fixative solution at 4 °C, and wash samples in PBS several times at room temperature or at 4 °C overnight. Then incubate tissues in 30 % sucrose in PBS at 4 °C for 1–2 days. *See* **Note 5**.

After sucrose infiltration, dip samples into OCT for at least 5 min and transfer them to an embedding mold containing OCT. Orient samples as desired (*see* **Note 6**) and freeze in dry ice-containing ethanol. Store frozen specimen blocks at –80 °C. *See* **Note 7**.

Transfer block samples at –20 °C the day before cutting. Attach a block to the specimen disc and cut sections at –20 °C in the cryostat. *See* **Note 2**.

Slides can then be placed in a slide box and stored at –80 °C for several days until further processing.

3.1.2 Immunostaining

All passages are carried out at room temperature unless otherwise stated.

First Day

Prepare a humidified chamber or a Falcon BioDish with filter paper and place slides inside. Allow sections to equilibrate at room temperature at least 1 h, then outline specimens with a PapPen, and allow drying. Wash sections with PBST 3 times for 10 min each and incubate with blocking solution for 90 min, followed by overnight incubation at 4 °C in primary antibodies diluted at optimal titer with antibody diluent solution (*see* **Note 3**) in humid atmosphere.

Second Day

Wash sections with PBST 6 times for 10 min each and incubate with the secondary antibodies 1:250 in PBST for 90 min. Protect slides from light from this step on. Wash sections with PBST three times for 10 min each and then incubate with DAPI working solution for 15 min. Finally, wash sections with PBST six times for 10 min each and mount slides with Fluoromount™. Once mounted remember to seal each slide with nail polish.

3.2 Immunofluorescence on Vibratome Sections

3.2.1 Preparation of Tissues

Euthanize the animal with anesthetic solution.

Dissect brains and/or ganglia and fix for 3 h in fixative solution (*see* **Note 4**). Wash two times for 5 min each in SMB solution and dehydrate in an ascending ethanol series (50°, 70°, 90°, 100° 10 min each). Permeabilize in ethanol/methyl salicylate for 5 min and then rehydrate in a descending ethanol series (100°, 90°, 70°, 50° 10 min each). Wash 6 times for 5 min each in Tris–HCl-SMB (*see* **Note 8**) and dry samples on filter paper.

Assemble the embedding mold, fill the ring mold with agarose solution warmed at 62 °C, allow cooling for about 2 min, and transfer tissue into the agarose solution. Use forceps to orient the tissue in agarose so that it is suspended halfway between the top and bottom of the mold. Incubate at 4 °C until the agarose solidifies.

Use super glue to attach the sample to the sectioning block and let it dry for several min. Place the sectioning block with the attached sample into the vibratome and fill the buffer reservoir with Tris–HCl-SMB and the ice bath with iced water.

Cut specimens by adjusting section thickness to 50 μm, speed to 0.4 mm/s, and blade amplitude to 1.8. *See* **Note 9**.

With a paintbrush transfer each tissue section to a 24-well culture plate well containing ice-cold Tris–HCl-SMB. *See* **Note 8**.

3.2.2 Immunostaining

All passages are carried out at room temperature unless otherwise stated.

First Day

Wash free-floating sections in Tris–HCl-SMB for 15 min and treat them for 10 min with the bleaching solution. Then wash with Tris–HCl-SMB-Triton® for 10 min and incubate in blocking solution overnight at 4 °C with gentle shaking.

Second Day

Remove the blocking solution and incubate with primary antibodies diluted at optimal titer in diluent solution for 3 days at 4 °C with gentle shaking. *See* **Note 3**.

Sixth Day

Remove the primary antibody and wash six times for 20 min each in Tris–HCl-Triton®. Then incubate with secondary antibody diluted at optimal titer in diluent solution overnight at 4 °C with gentle shaking. *See* **Note 3**.

Seventh Day

Remove the secondary antibody solution and wash 6 times for 20 min each in Tris–HCl-Triton®. Incubate with DAPI working solution for 30 min, and wash extensively 6×20 min each using Tris–HCl- Triton®. *See* **Note 8**.

Mount the slices on Superfrost™ Plus slides, cover with Fluoromount™, and seal slides with nail polish.

4 Typical/Anticipated Results

The presented protocols have been successfully applied in *Octopus vulgaris* [33, 44, 45], and in this section we briefly illustrate examples of the results obtained.

Using the first protocol (frozen sections), samples have been fixed in paraformaldehyde since haptens used for production of the antibodies (i.e., anti-GABA and anti-5-HT) have been conjugated to BSA (carrier protein) via paraformaldehyde. Figure 1 *Top* shows a detail of the brachial lobe (part of the subesophageal mass) with cell bodies and fibers positive to 5-HT immunoreactivity.

Using the second protocol (vibratome sections), hapten has been conjugated to BSA via glutaraldehyde (i.e., OA-G-BSA), and thus tissue fixation requires glutaraldehyde for an in situ conjugation. In this case, we utilized a modified Bouin fixative solution containing 6.25 % of glutaraldehyde. In addition, since OA is a biogenic amine, the addition of SMB is required to prevent oxidation. Figure 1 *Bottom* shows evident OA-immunoreactivity.

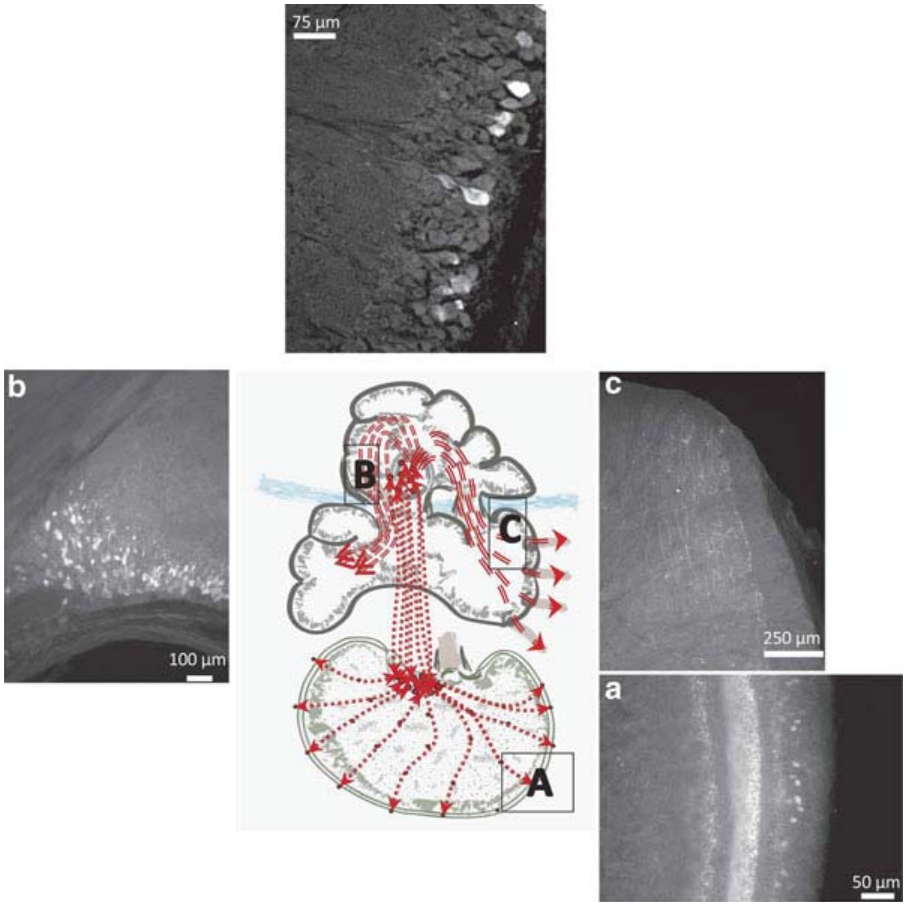

Fig. 1 Results of the immunohistochemistry protocols for localization of 5-HT and octopamine in the CNS of *Octopus vulgaris. Top*: 5-HT-IR in the brachial lobe (subesophageal mass); several positive cells and fibers are visible. *Bottom*: octopamine-IR cells and fibers in various districts of the octopus brain. (**a**) Optic lobe, (**b**) basal lobe (supraesophageal mass), (**c**) brachial lobe (subesophageal mass). Drawing: exemplified circuitry of octopaminergic modulation

According to previous findings, the peduncle and the basal lobes exhibit a similar cytoarchitecture and the presence of monoamines (i.e., 5-HT), thus letting these two structures be considered as functional analogues of the vertebrate cerebellum [46, 47]. In our results numerous cell bodies, having various sizes and shapes, were positively stained for OA in several districts of the octopus brain. This allows us to identify a circuit involved in the modulation of motor patterns possibly following the contribution of OA (Fig. 1 *Bottom*).

5 Notes and Troubleshooting

1. Make fresh 4 % paraformaldehyde each time. Preparation should be carried out inside a fume hood.
2. See manufacturer's manual for details.

3. Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. Often, a manufacturer recommends dilution ranges compatible with other variables, such as method, incubation time, and temperature. However, it is often useful to determine the optimal concentration by carrying out a series of dilutions.
4. The fixation timing should be optimized for each tissue individually.
5. Tissue has to sink in 30 % sucrose in PBS for optimal cryoprotection.
6. Standard orientation planes for sections are sagittal, transverse, and frontal.
7. Avoid storing samples for long time as this increases autofluorescence in the tissue (e.g., lipofuscin content).
8. If the time is not enough, you can store the section at 4 °C and continue the day after with the next step.
9. The setting should be optimized for each tissue individually.

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Diversity and temporal pattern of *Pseudo-nitzschia* species (Bacillariophyceae) through the molecular lens

Maria Valeria Ruggiero *, Diana Sarno, Lucia Barra [1], Wiebe H.C.F. Kooistra, Marina Montresor, Adriana Zingone

Department of Ecology and Evolution of Plankton, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy

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ABSTRACT

In diatoms, as in other organisms, many genetically distinct and reproductively isolated species may show identical or highly similar morphological features. Such groups of species are defined as cryptic and pseudo-cryptic species, respectively. The difficulty of discriminating them with optical means impairs the study of their temporal patterns and geographic ranges. This is also the case for *Pseudo-nitzschia*, a worldwide distributed planktonic diatom genus which includes several toxigenic species. Using a *Pseudo-nitzschia*-specific pair of large sub-units ribosomal DNA (LSU rDNA) primers, we generated clone libraries from 19 samples collected at the Long Term Ecological Station MareChiara (LTER-MC) in the Gulf of Naples (GoN) from 2009 to 2010 and compared sequence records with light microscopy (LM) counts from the same samples. Our aim was to elucidate the diversity and the seasonal patterns of taxa within *Pseudo-nitzschia*. Most of the *Pseudo-nitzschia* species already known from the GoN were identified within the 1643 obtained sequences. In addition, two species known from elsewhere and three un-described ribotypes were detected. Several cryptic species showed distinct temporal patterns of occurrence, with most species confined to restricted periods and only a few present year-round. Microscopic and molecular results generally concurred for species recognizable using LM, while clone libraries tended to overestimate the relative abundance of some of the species. Due to its high resolution and detection power, the DNA-barcoding approach used in our study is an optimal tool to trace the distribution of cryptic and toxigenic *Pseudo-nitzschia* species and the diversity of this key diatom genus in the natural environment.

1. Introduction

Diatoms are among the best known marine protists. Their silica investment, the frustule, permits a variety of shapes and ornamentations, which has facilitated species identification based on morphology since the introduction of the first microscopes (Agardh, 1830–1832; Ehernberg, 1838). Morphology-based studies on diatom diversity and distribution patterns have flourished over the last century along with studies on their ecology and evolution (reviewed in Mann, 1999; Mann and Chepurnov, 2004). On the other hand, molecular phylogenetic studies have mainly focused on the overall topology of the diatom trees and rarely have they included multiple strains within the same morphologically defined species. Recent studies integrating molecular phylogenetics, morphological, ultrastructural and biological information have uncovered numerous cases of genetically distinct and at times reproductively isolated groups of strains that could not be distinguished easily or at all with microscopy (e.g. Sarno et al., 2005; Amato et al., 2007; Nanjappa et al., 2013). Such groups of specimens indeed deserve the status of species and are designated as cryptic or pseudocryptic species in case of null or subtle morphological differences, respectively. At the same time, evidence is accumulating that such cryptic species can exhibit remarkable physiological and ecological differences (e.g. Vanelslander et al., 2009; Degerlund et al., 2012; Huseby et al., 2012; Mann and Vanormelingen, 2013). Molecular approaches constitute the most straightforward way to identify these taxa and elucidate their distribution over space and time. In addition, molecular

Abbreviations: ASP, amnesic shellfish poisoning; DA, domoic acid; EM, electron microscopy; GoN, Gulf of Naples; ITS, internal transcribed spacer; LM, light microscopy; LSU, large sub-unit; LTER-MC, Long Term Ecological Research Station MareChiara; rDNA, ribosomal DNA.

* Corresponding author. Tel.: +39 081 5833 2121; fax: +39 0817641355.
E-mail address: mariavaleria.ruggiero@szn.it (M.V. Ruggiero).
[1] Present address: Institute of Biosciences and Bioresources (CNR-IBBR), Via Università 133, 80055 Portici, Italy.

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approaches permit the investigation of the actual genetic diversity among and within species, tracing phylogenetic relationships and shedding light on evolutionary patterns and speciation mechanisms.

The planktonic pennate diatom *Pseudo-nitzschia* (Heterokonta, Bacillariophyceae) is a cosmopolitan genus commonly found in neritic and oceanic waters. It represents a typical case in which the number of genetically distinct lineages is markedly higher than the number of taxa recognizable in LM. The genus includes at present 43 species of which 39 have been genetically characterized. At least 12 of these produce domoic acid (DA), a neurotoxin responsible for amnesic shellfish poisoning (ASP) mainly in mammals and birds (Lelong et al., 2012; Trainer et al., 2012). In LM, *Pseudo-nitzschia* species can only be differentiated based on cell shape and size and on the shape of frustule ends in cell chains. In EM, a wealth of ultrastructural details of the frustule have permitted a far finer resolution of diversity, generally concurring with differences detected with nucleotide markers such as the nuclear encoded rDNA internal transcribed spacer region (ITS1 and ITS2 and the hyper-variable domains D1–D3 of the LSU; e.g. Hasle and Syvertsen, 1997; Lundholm et al., 2003, 2006; Amato et al., 2007). In addition, an ever-increasing number of cryptic and pseudo-cryptic species have been found within most of the morphologically delineated species recognizable in LM (Amato et al., 2007; Quijano-Scheggia et al., 2009; Lim et al., 2012, 2013; Lundholm et al., 2012; Trainer et al., 2012; Orive et al., 2013; Teng et al., 2014), prompting the use of these molecular markers as DNA barcode (Evans et al., 2007; Moniz and Kaczmarska, 2010; Hamsher et al., 2011). Besides its value in ecological and biogeographic studies, correct identification of *Pseudo-nitzschia* species has relevant implications for monitoring and management purposes, considering that DA-producing species in the genus may be morphologically similar or identical to non-toxigenic ones.

The genetic diversity of *Pseudo-nitzschia* in the Gulf of Naples (GoN, Tyrrhenian Sea, Mediterranean Sea), has been studied extensively by means of culturing techniques, combined with LM and electron microscopy observations and mating compatibility experiments. These studies have resulted in the description of new species and have provided insight in the genetic composition of blooms (Orsini et al., 2004; Amato et al., 2007; Amato and Montresor, 2008). DNA barcoding approaches based on clone libraries, which by-pass underestimation due to cultivation biases and species rarity, was first tested on a limited number of samples from the GoN to assess *Pseudo-nitzschia* genetic diversity (McDonald et al., 2007), while microarrays provided contrasting results, highlighting the challenges of appropriate probe design and hybridization conditions (Barra et al., 2013). At the Long Term Ecological Research Station MareChiara (LTER-MC, GoN), *Pseudo-nitzschia* species are present basically year round, with concentrations up to 10^6 cells L^{-1} (Ribera d'Alcalà et al., 2004). Different species or species-complexes, as recognized by LM, bloom at different times of the year, while some species show multiple peaks over the year (Zingone et al., 2003, 2006; Cerino et al., 2005).

In the present study we investigated the genetic diversity of *Pseudo-nitzschia* in environmental clone libraries from 19 plankton samples collected over 16 months at the LTER-MC station using genus-specific LSU rDNA primers (McDonald et al., 2007) and compared the molecular identification results with those obtained by LM cell counts on the same dates. The aims of this DNA-barcoding study were: (i) to study the diversity of *Pseudo-nitzschia*, possibly detecting taxa unknown to date in the GoN or anywhere; (ii) to unveil patterns of intraspecific diversity in the species in the genus; (iii) to depict the seasonal patterns for the different cryptic species in the genus.

2. Materials and methods

2.1. Study site and sample collection

Water samples were collected at the LTER-MC station (40°48.5′ N, 14°15′ E; bottom at −75 m) in the GoN (Fig. S1). The station is located 2 nautical miles off the coast of the city of Naples, at the boundary between the eutrophic coastal zone and the oligotrophic Tyrrhenian Sea waters (Ribera d'Alcalà et al., 2004). The samples were collected at 0.5 m depth with a 12 L Niskin bottle mounted on an automatic Carousel sampler on 19 sampling dates from September 2009 to December 2010 (Table 1). Temperature and salinity values in surface waters varied between 13.39 °C (16 March 2010) and 28.92 °C (20 July 2010), and between 36.58 (20 July 2010) and 38.20 (28 September 2010), respectively. Average values of nutrient concentrations in the upper layer (0.5–5 m depth) were 0.09 ± 0.07 μM for phosphates, 2.93 ± 1.91 μM for silicates and 2.88 ± 2.72 μM for inorganic nitrogen ($NH_4 + NO_3 + NO_2$); unpublished results, courtesy of MECA service).

Supplementary Fig. 1 related to this article can be found, in the online version, at doi:10.1016/j.hal.2014.12.001.

Table 1
Pseudo-nitzschia diversity per sample. Sample = denomination of the LTER-MC sample; date = sampling date; N = number of sequences; N_h = number of ribotypes; H_d = gene diversity; Ds^{-1} = inverse Simpson index; Chao1 = Chao index; C = coverage; cells L^{-1} = light microscopy cell counts. Seasons are defined according to the Gregorian calendar.

Season	Sample name	Date	N	N_h	H_d	Ds^{-1}	Chao1	C	Cells L^{-1}
Summer 2009	MC878	08 September 2009	112	12	0.20	1.26	34.5	0.89	4.41×10^4
Autumn 2009	MC882	06 October 2009	94	16	0.71	3.47	34.0	0.83	2.60×10^5
	MC883	13 October 2009	60	6	0.33	1.49	6.0	0.90	3.59×10^5
	MC886	04 November 2009	86	19	0.86	7.18	26.2	0.78	9.88×10^3
	MC890	02 December 2009	81	13	0.79	4.81	15.5	0.84	2.96×10^4
Winter 2010	MC895	12 January 2010	89	15	0.74	3.81	20.3	0.83	9.88×10^3
	MC899	09 February 2010	96	20	0.84	6.30	35.0	0.79	5.01×10^4
	MC903	09 March 2010	86	15	0.72	3.56	18.3	0.83	1.12×10^5
	MC904	16 March 2010	74	15	0.85	6.67	25.5	0.80	4.22×10^5
Spring 2010	MC908	13 April 2010	92	20	0.67	3.00	39.5	0.78	8.05×10^5
	MC909	20 April 2010	78	10	0.56	2.28	15.0	0.87	1.22×10^5
	MC913	18 May 2010	75	9	0.58	2.39	24.0	0.88	0.00
	MC917	15 June 2010	82	16	0.77	4.35	71.0	0.80	6.65×10^4
Summer 2010	MC921	13 July 2010	101	12	0.34	1.51	40.0	0.88	1.52×10^6
	MC924	03 August 2010	98	14	0.33	1.50	18.7	0.86	5.14×10^6
	MC930	14 September 2010	100	14	0.72	3.51	17.8	0.86	1.58×10^5
Autumn 2010	MC934	12 October 2010	95	13	0.61	2.54	15.5	0.86	1.91×10^5
	MC938	11 November 2010	71	17	0.87	7.58	26.3	0.76	0.00
	MC941	14 December 2010	73	17	0.84	6.21	22.6	0.77	0.00
Total dataset[a]			1643	84	0.86	7.36	84.0	0.95	

[a] N_h, H_d, Ds^{-1}, Chao1 and coverage calculated over the whole dataset.

For genetic analyses, 2–5 L of seawater was filtered on cellulose-ester filters (47 mm diameter, 1.2 μm pore size, EMD Millipore, USA). Filters were cut immediately into two halves, frozen in liquid nitrogen and stored at −80 °C until further processing.

2.2. Light microscopy cell counts

Water samples for phytoplankton cell enumeration were collected at the same depth and fixed with 0.8% neutralized formaldehyde. One to 50 mL of fixed sample was allowed to sediment in an Utermöhl chamber. Phytoplankton cells were identified and enumerated using an inverted light microscope (LM, Zeiss Axiovert, Carl Zeiss, Oberkochen, Germany) at 400× magnification. Counts were performed generally on two transepts and the volume of seawater inspected ranged from 0.02 to 1.52 mL. Only few of the species present in our samples could be identified at the species level in LM. This was the case of *Pseudo-nitzschia multistriata* (Orsini et al., 2002) and *Pseudo-nitzschia galaxiae*, for which small and medium-large morphotypes were distinguished (Cerino et al., 2005). *Pseudo-nitzschia fraudulenta* cannot be distinguished from *Pseudo-nitzschia subfraudulenta* (Trainer et al., 2012). Cells with a transapical diameter ≤3 μm were assigned to the *Pseudo-nitzschia pseudodelicatissima*- or *P. delicatissima*-complex (hereafter indicated as *P.* cf. *pseudodelicatissima and P.* cf. *delicatissima*) based on the presence of pointed or rounded/truncated ends when seen in girdle view, respectively.

2.3. DNA extraction and PCR amplification

Total genomic DNA was extracted from half filters using CTAB extraction buffer as described in McDonald et al. (2007). The *Pseudo-nitzschia* LSU primers D1-186F and D1-548R (McDonald et al., 2007) were used to amplify a 348 bp fragment within the nuclear-encoded LSU rDNA from the total genomic DNA extracted from the environmental samples. In detail, total reaction volume of 50 μl contained approximately 40 ng environmental DNA, 200 μM deoxynucleoside triphosphates, 1 μM of each primer and 2.5 U Taq polymerase in 1× enzyme buffer with $MgCl_2$ added (Roche Diagnostics GmbH, Mannheim, Germany). The PCR conditions were 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, primer annealing at 62 °C for 35 s and elongation at 72 °C for 1 min 20 s, with a final elongation step at 72 °C for 5 min.

In three cases in which no visible PCR product was obtained (samples MC913, MC938 and MC941), a nested PCR approach was used: total genomic DNA was used as template for amplification of the D1–D3 LSU fragment using universal primers D3Ca and DIR (Lenaers et al., 1989; Scholin et al., 1994); the purified PCR-product was then used as a template for the amplification of the 348 bp fragment (as in McDonald et al., 2007).

The amplified LSU-fragment is able to distinguish all *Pseudo-nitzschia* species described to date, with two exceptions: *Pseudo-nitzschia micropora* and *Pseudo-nitzschia dolorosa*, which are identical for this fragment, and *Pseudo-nitzschia cuspidata* and *Pseudo-nitzschia pseudodelicatissima*, which are identical for the whole D1–D3 LSU rDNA marker (ca 800 bp), commonly used for species discrimination. While *P. cuspidata* has been previously identified in the GoN based on other markers (Amato et al., 2007), *P. micropora* has never been reported in the Mediterranean Sea. The primers selected also amplify LSU sequences of the genus *Fragilariopsis*, which is recovered as a clade inside *Pseudo-nitzschia* (Lundholm et al., 2002a).

2.4. Clone libraries

PCR fragments were purified using a QIAquick PCR purification kit (Qiagen Ltd., Venlo, The Netherlands) and cloned using TA Cloning® kit (Invitrogen™ Life Technologies, Carlsbad, California). Approximately 100 clones were manually picked for each sample. Plasmids were purified using the Millipore Montage Plasmid Miniprep Kit (Millipore Corporate, 290 Concord Road, Billerica, MA 0182, USA) and a robotic station, Beckman Coulter's Biomek1 FX Laboratory Automation Workstation, equipped with ORCA1 robotic arm (Beckman Coulter, Fullerton, CA). Single strand sequences were obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA), using a standard T7 primer, and purified in automation using the Millipore Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore Corporate) and the Biomek1 FX. Products were run on an Automated Capillary Electrophoresis Sequencer 3730 DNAAnalyzer (Applied Biosystems). These sequence data have been submitted to the GenBank database (accession numbers in Table S1).

Supplementary Table 1 related to this article can be found, in the online version, at doi:10.1016/j.hal.2014.12.001.

2.5. Data analyses

Sequence chromatograms were inspected in Bioedit (Hall, 1999). ClustalW (Thompson et al., 1994) implemented in Bioedit was used to align sequences. Putative chimeras were identified as generating different hits for different portions of the sequence in the Blast analysis, further checked by eye and finally eliminated from the dataset. Singletons over the whole dataset were considered as *Taq* errors and eliminated from the dataset.

2.5.1. Sample diversity

MOTHUR (Schloss et al., 2009) was used to calculate sample diversity and richness. The inverse Simpson diversity index (Ds^{-1}) takes into account the number of species present and their relative abundance (Simpson, 1949). The non-parametric estimator Chao1 (Chao, 1984) was employed to assess ribotype richness (95% CI), correcting for abundance of rare ribotypes, i.e. counted once or twice (Hughes et al., 2001). Coverage of each clone library and of total samples was calculated as $C = 1 - (N_h/N)$, where N_h is the number of observed ribotypes and N is the total number of sequences in that sample (Romari and Vaulot, 2004). Gene diversity (H_d, Nei, 1987) was calculated by means of the software DNAsp (Librado and Rozas, 2009); this parameter takes into account both the number and the frequency of each ribotype. Sampling dates were assigned to seasons according to the Gregorian calendar.

2.5.2. Species diversity

Taxonomic assignation was performed by blasting each ribotype against the GenBank database (NCBI BLAST version 2.2.9, Altschul et al., 1997) and attributing the ribotypes to the species whose reference sequence in GenBank was showing the highest matching score. (Table S1). Within *Pseudo-nitzschia galaxiae*, ribotypes were assigned to ribogroups corresponding to the LSU clades identified in McDonald et al. (2007). In order to assess within-species diversity and temporal patterns of occurrence, all ribotypes assigned to species were gathered from all sampling dates. Gene diversity H_d and pairwise nucleotide diversity (π, Nei, 1987) were calculated by means of the software DNAsp.

3. Results

3.1. Diversity

Of the19 clone libraries obtained, 16 were from samples in which different abundance values of *Pseudo-nitzschia* species were recorded by LM counts and 3 from samples in which no *Pseudo-nitzschia* were detected by LM counts. (Fig. 1; Table 1). Of the 1885 sequences

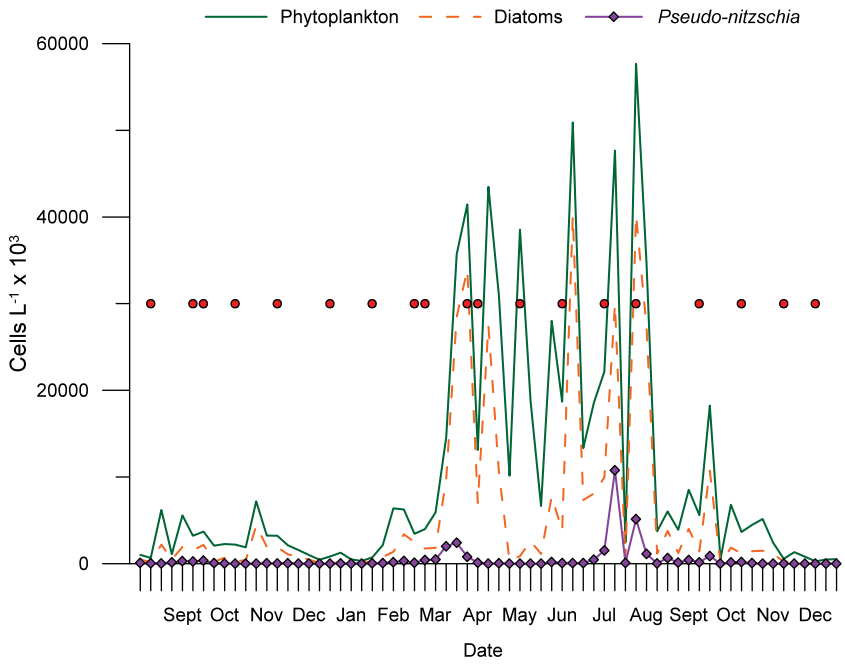

Fig. 1. Cell counts from LTER-MC dataset over the whole sampling period (67 dates) for total phytoplankton, diatoms and *Pseudo-nitzschia* spp. Dots indicate the dates for which a clone library was obtained.

obtained, 242 (12.9%) consisted of unreliable reads, chimeras or sequences containing single base changes. The coverage value for each clone library was generally high; the maximum value was obtained over the complete dataset, indicating that the genetic diversity of *Pseudo-nitzschia* in the GoN was sampled exhaustively in this study. Gene diversity was correlated ($R^2 = 0.779$) with the Inverse Simpson Index. The Chao1 index ranged between 6 and 71. Diversity indices for the samples generated through a nested PCR did not differ markedly from the average of all other samples.

Blast results of the ribotypes revealed 15 taxa, among which 12 were assigned to taxonomically described species. The ribotypes with the highest number of occurrences were generally identical to a reference sequence of a described species (Table S1).

In the *P. delicatissima* species-complex, three known cryptic or pseudo-cryptic species were retrieved, namely *Pseudo-nitzschia arenysensis*, *P. delicatissima* and *Pseudo-nitzschia dolorosa*. In addition, 259 sequences (ribotypes #14–20, Table S1) were attributed to a taxon herein called *P. delicatissima* IV, already known from the GoN (McDonald et al., 2007, as "*P. delicatissima* new genotype", Lamari et al., 2013, as *P.* cf. *delicatissima*). Another group of sequences formed a clade close to the reference sequence of *P. delicatissima* in a phylogeny inferred from the D1–D3 domain of the LSU (Fig. S2). These sequences included one ribotype (#12) identical to a sequence of a Namibian strain (SZN-B341) attributed to *P. delicatissima* and a sequence from an Australian strain CHB named *Pseudo-nitzschia arenysensis* in Ajani et al. (2013), as well as two ribotypes (#7 and #11) close to those Namibian/Australian sequences (Table S1 and Fig. S1). These sequences are attributed to a new taxon henceforth referred to as *P. delicatissima* V. The whole *P. delicatissima* species-complex accounted for 29% of the total number of sequences, of which about half was close or identical to the sequence of *P. delicatissima* IV, which showed the highest gene and nucleotide diversity (Table 2).

Supplementary Fig. 2 related to this article can be found, in the online version, at doi:10.1016/j.hal.2014.12.001.

Within the *Pseudo-nitzschia pseudodelicatissima* species-complex, environmental sequences identical or close to reference sequences of four cryptic species were retrieved, namely those of *Pseudo-nitzschia calliantha*, *Pseudo-nitzschia mannii*, *Pseudo-nitzschia hasleana* and *P. pseudodelicatissima*/*cuspidata*. These sequences accounted for 10% of the total, about half of which belonging to *P. calliantha*. Each species except *P. hasleana* was represented by two ribotypes. Within both *P. mannii* and *P. calliantha* two different ribotypes were found; in both cases, the two ribotypes matched different GenBank accessions attributed to each species. Among the species in this complex, *P. calliantha* showed the lowest gene and nucleotide diversity (Table 2).

Sequences belonging to *Pseudo-nitzschia galaxiae* were the most abundant (49% of the total) and showed the highest genetic diversity (Table 2), with 45 distinct ribotypes forming four ribogroups (following McDonald et al., 2007): ribogroup I (closest reference sequence: *P. galaxiae* SM1); ribogroup II (*P. galaxiae* SM26), ribogroup III (*P. galaxiae* SM10), and ribogroup IV (*P. galaxiae* 220604_F04). Ribogroup II included the vast majority of the ribotypes (36), while ribogroup IV harbored the highest ribotype, gene and nucleotide diversity (Table 2).

Pseudo-nitzschia multistriata (5% of the total sequences) included six different ribotypes, showing moderately high gene and nucleotide diversity (Table 2). *Pseudo-nitzschia fraudulenta* (3% of the total sequences) showed high gene diversity (0.45) but only moderate nucleotide diversity. Its sister species, *Pseudo-nitzschia subfraudulenta*, was present with 2 sequences of a single ribotype. *Pseudo-nitzschia linea* was detected as 10 sequences of a single ribotype.

Nine sequences (ribotypes #22, #23 and #24) were similar to a sequence from South Africa (Table S1) and clustered in a single ribogroup, herein called *Pseudo-nitzschia* sp., with high gene and nucleotide diversity (Table 2).

One ribotype (#21) was similar to *Neodenticula seminae* (96.24%) and *Fragilariopsis curta* (95%) and was excluded from further analyses.

3.2. Seasonality

Almost all species, and the 4 ribogroups of *Pseudo-nitzschia galaxiae*, were recorded in two or more consecutive seasons. The species found in single seasons were *Pseudo-nitzschia subfraudulenta*, only found in winter, and *Pseudo-nitzschia mannii* and the three ribotypes not attributable to any described species yet

Table 2
Intraspecific diversity within the genus *Pseudo-nitzschia*; N = number of sequences; N_h = number of ribotypes; S = number of segregating (polymorphic) sites; H_d = gene diversity; π = pairwise nucleotide diversity. Cell counts per species, species complex or morphotypes are summed over sampling dates. na = not applicable.

	N	N_h	S	H_d	π	Species complex/morphotype	Cells L^{-1}
Pseudo-nitzschia delicatissima species-complex							
P. arenysensis	150	2	2	0.04	0.0002		
P. delicatissima	67	4	2	0.41	0.0012	*Pseudo-nitzschia* cf. *delicatissima*	1.92×10^6
P. dolorosa	8	1	na	na	na		
P. delicatissima IV	251	6	5	0.10	0.0003		
P. delicatissima V	44	3	3	0.46	0.0033		
Pseudo-nitzschia pseudodelicatissima species-complex							
P. pseudodelicatissima/cuspidata	19	2	2	0.20	0.0011		
P. calliantha	89	2	1	0.04	0.0001	*Pseudo-nitzschia* cf. *pseudodelicatissima*	5.42×10^5
P. mannii	33	2	1	0.22	0.0006		
P. hasleana	29	1	na	na	na		
Pseudo-nitzschia galaxiae							
Ribogroup I	176	5	3	0.19	0.0006	*P. galaxiae* small morphotype	1.28×10^5
Ribogroup II	613	36	32	0.34	0.0016		
Ribogroup III	7	1	na	na	na	*P. galaxiae* medium-large morphotype	6.59×10^6
Ribogroup IV	8	3	3	0.69	0.0034		
P. galaxiae tot	804	45	38	0.58	0.0025		
P. fraudulenta	51	4	2	0.45	0.0013	*P. fraudulenta/subfraudulenta*	5.27×10^3
P. subfraudulenta	2	1	na	na	na		
P. multistriata	75	6	7	0.27	0.0011		1.16×10^5
P. linea	10	1	na	na.	na	na	na
Pseudo-nitzschia sp.	9	3	7	0.72	0.0097	na	na
Neodenticula/Fragilariopsis[a]	2	1	na	na	na	na	na
Total diversity	1643	84	68	0.86	0.0178		

[a] Ribotype not attributable to the genus *Pseudo-nitzschia*, not included in following analyses.

(herein grouped as *Pseudo-nitzschia* sp., see above), only found in autumn (Figs. 2 and 3).

Pseudo-nitzschia galaxiae was present in all samples and seasons (Figs. 2A and B and 3). Among the different morphotypes known for the species (Cerino et al., 2005) the small-sized cells were present in winter-early spring and the medium-large sized in late spring-summer (Fig. 2A). Accordingly, ribogroup I, corresponding to the small morphotype (McDonald et al., 2007), reached the highest abundance in winter while ribogroup II (medium and large morphotypes) dominated in summer and was substituted again by ribogroup I in autumn (Fig. 2B). Ribogroups III and IV, also corresponding to medium and large morphotypes, were much less abundant and were observed from autumn to spring and in summer/autumn, respectively.

Three peaks of *P.* cf. *delicatissima* were observed by LM (Fig. 2C), in the autumns 2009 and 2010 and in spring 2010. Based on clone library results, *P. delicatissima* and *Pseudo-nitzschia arenysensis* overlapped to a large extent in their occurrences, being the main contributors to the spring peak of this morphotype (Fig. 2D). *P. arenysensis* appeared and decreased later than *P. delicatissima*. *Pseudo-nitzschia dolorosa* was present in low abundances in early spring, while *P. delicatissima* IV dominated in the early autumn samples (Fig. 2D), both in 2009 and 2010. *Pseudo-nitzschia delicatissima* V occurred in low numbers in all seasons.

Within the *Pseudo-nitzschia pseudodelicatissima* species-complex (Fig. 2E–F), the spring peak recorded by LM was mainly due to *Pseudo-nitzschia calliantha* with minor contributions of *Pseudo-nitzschia hasleana* and *P. pseudodelicatissima/cuspidata*. *P. calliantha* was also abundant in autumn (Fig. 2F); *P. hasleana* and *Pseudo-nitzschia mannii* were detected both in spring and autumn, the latter species more abundant in 2010 than in 2009. *P. pseudodelicatissima/cuspidata* and *P. hasleana* were present in winter), when no *P. pseudodelicatissima* morphotype was detected by LM.

Three peaks of *Pseudo-nitzschia multistriata*, in December, June and September, were detected in both cell counts and clone libraries (Fig. 2G–H). *Pseudo-nitzschia fraudulenta/subfraudulenta* was observed by LM on a single date in winter 2010, while in clone libraries it was also detected in low abundance in December 2009, January, March and June 2010 (Fig. 2I–J). *P. subfraudulenta* was only present in December 2010, while *P. linea* was detected from March to April (Fig. 2K). The three ribotypes of *Pseudo-nitzschia* sp. were all detected in autumn.

4. Discussion

4.1. Pseudo-nitzschia *diversity at LTER-MC*

Our dataset of 1643 partial LSU sequences collected over 16 months covered the specific and intraspecific diversity of the genus *Pseudo-nitzschia* in the GoN with unprecedented resolution. As a complement to a series of studies on *Pseudo-nitzschia* diversity in the GoN genotyping several hundred strains (Orsini et al., 2004; Amato et al., 2007; Cerino et al., 2005; Tesson et al., 2014), clone libraries on 6 dates (175 sequences, McDonald et al., 2007) and a microarray study over one year sampling (Barra et al., 2013), the present study expands the known generic diversity in the GoN, adding new species and ribotypes never before found there. These include *P. linea*, described from the East Atlantic coasts (Gulf of Mexico and Narragansett Bay, Lundholm et al., 2002b) and reported from the Western Mediterranean (Catalan coasts, Quijano-Scheggia et al., 2010), which was probably overlooked in previous observations of the GoN plankton because of its small size (ca 10 μm length), and solitary and epiphytic habit. Indeed following its detection in the clone libraries *P. linea* was also observed in net samples as epiphyte on *Chaetoceros* sp. *Pseudo-nitzschia hasleana*, described from coastal waters of Washington State (Eastern Pacific Ocean, Lundholm et al., 2012) and detected along Greek and East Mediterranean coasts (Moschandreou et al., 2012), was also found for the first time in the GoN. Other newly found ribotypes, probably belonging to un-described species,

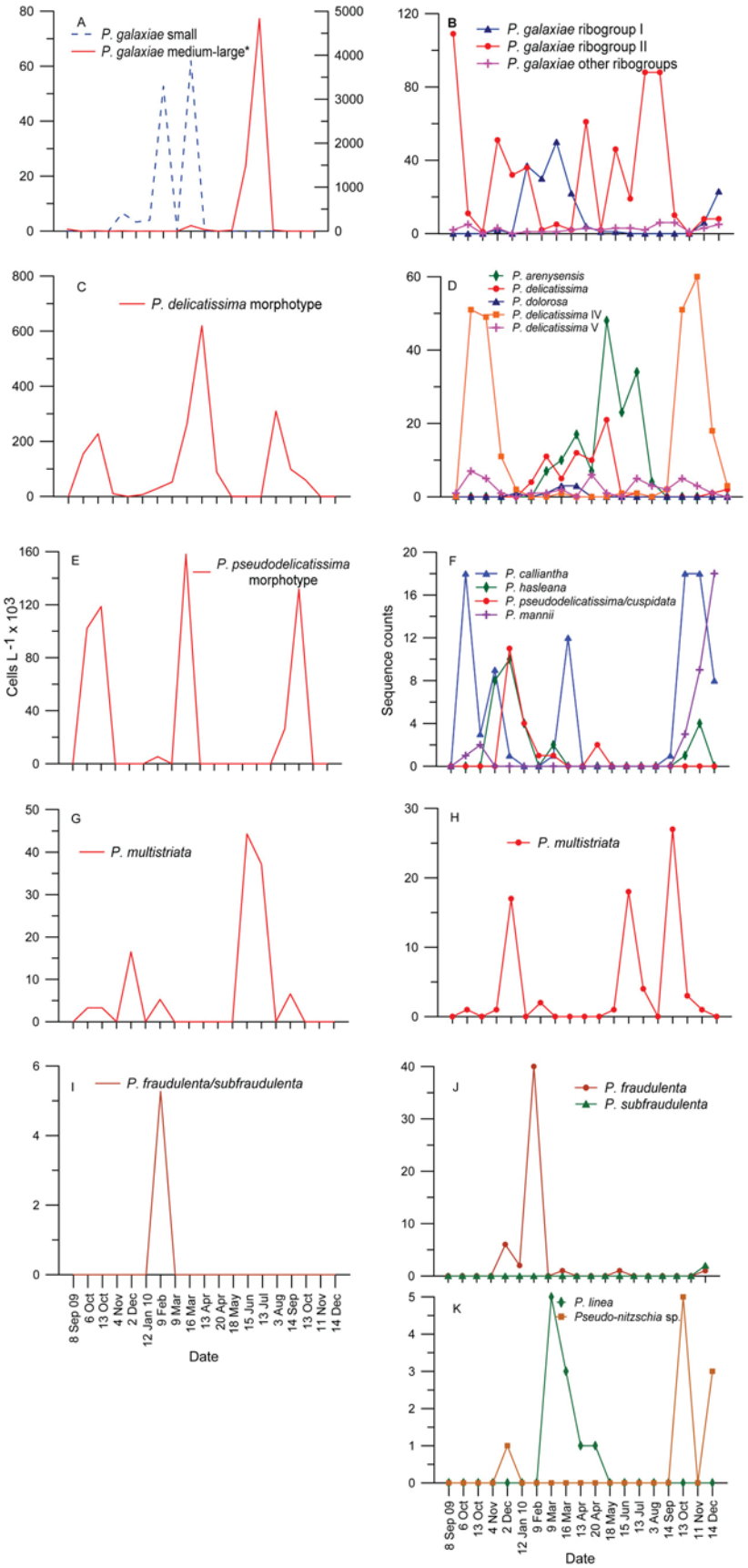

Fig. 2. Abundance of *Pseudo-nitzschia* species and sequence counts from clone libraries (CL). A: *P. galaxiae* (* = right axis), LM; B: *P. galaxiae*, CL; C: *P. delicatissima*-complex, LM; D: *P. delicatissima*-complex, CL; E: *P. pseudodelicatissima*- complex, LM; F: *P. pseudodelicatissima*- complex, CL; G: *P. multistriata*, LM; H: *P. multistriata*, CL; I: *P. fraudulenta*/*subfraudulenta*, LM; J: *P. fraudulenta* and *P. subfraudulenta, CL*; K: *P. linea* and *Pseudo-nitzschia* sp., CL.

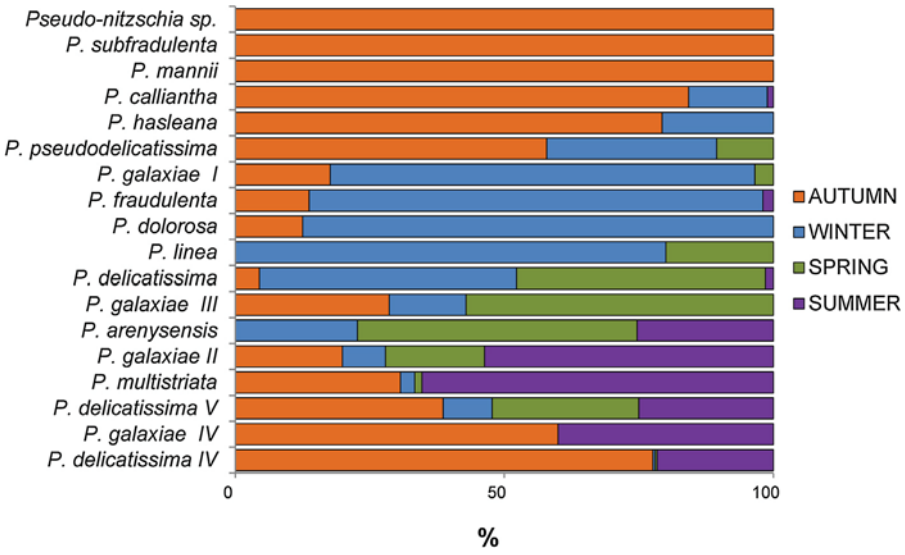

Fig. 3. Seasonal signal for *Pseudo-nitzschia* species and ribogroups in the Gulf of Naples as detected by clone libraries. Bars indicate the proportional abundance of the sequences for each species/ribogroup in the different seasons (as defined in Table 1).

include *P. delicatissima* V, similar to Namibian and Australian strains, and *Pseudo-nitzschia* sp., close to a strain from South-African waters. These species may simply have been overlooked by the LM-monitoring because of their apparent rarity. Alternatively, the new findings of genotypes previously detected in regions remote from the GoN (Namibian, South African and Australian waters) might indicate a recent introduction into the Mediterranean Sea through anthropic-driven (ballast waters) routes as well as natural routes such as migratory birds, aerosols, hydrodynamic processes *etc*. The hypothesis of a man-mediated introduction has been proposed for *Pseudo-nitzschia multistriata* (Zenetos et al., 2010) a species now fairly common, but only observed since 1996 in the GoN and initially only known from Japanese waters.

Some species previously found in the GoN did not appear in this study.

Pseudo-nitzschia subpacifica, identified by LM and EM, was common at station LTER-MC in the spring period from the beginning of the long-term series (1984) until 1990, but it has never been seen again since. Two strains of *Pseudo-nitzschia caciantha* were isolated in a previous study (Amato et al., 2007) but this species was not detected in the previous clone library-based study (McDonald et al., 2007), nor in any other isolation and observation efforts in the GoN. Finally, this study did not find a genotype close to *Pseudo-nitzschia inflatula* (strain B26), only encountered once and misidentified as *Pseudo-nitzschia pseudodelicatissima* in Orsini et al. (2002) based on the resemblance in poroid ultrastructure with taxa in the *P. pseudodelicatissima* complex. It is difficult to assess whether *P. caciantha* and *P.* cf. *inflatula* are still present but extremely rare in the GoN, or if they represent a case of local extinction, like *Pseudo-nitzschia subpacifica*.

Four other *Pseudo-nitzschia* species known from other localities in the Mediterranean Sea (*Pseudo-nitzschia pungens*, *Pseudo-nitzschia brasiliana*, *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*), have never been recorded in the phytoplankton counts at our LTER in the GoN and were absent in our clone libraries as well. At least one of them, *P. pungens* is quite frequent and abundant in the Adriatic Sea (Penna et al., 2013) and in Greek waters (Moschandreou et al., 2012) and has been found recently also in the nearby Gulf of Gaeta (unpublished data). The other three species have been recorded only rarely and basically at single sites in the Mediterranean.

With 16 taxonomically recognized species retrieved so far (considering *Pseudo-nitzschia cuspidata* separately from *Pseudo-nitzschia pseudodelicatissima*) and three additional ones that are only delineated genetically, the *Pseudo-nitzschia* diversity in the GoN is among the highest recorded so far at any site. Considering only the recent recognitions, mainly based on strain genotyping and microscopy observations, eight species were recovered from the Bilbao estuary (Spanish Atlantic coasts, Orive et al., 2013), 10 from the Australian coasts (Ajani et al., 2013), eleven from the North western Pacific (Stonik et al., 2011), and 12 from Greek waters (Moschandreou et al., 2012). Twenty-two species were reported in a study based on electron microscopy (Teng et al., 2013) in Malaysian waters, which however comprise a much more extensive area compared to the LTER in the GoN. However, the higher diversity observed in the GoN could simply be due to the fact that the genus *Pseudo-nitzschia* has been investigated more intensively here than in other areas of the world (e.g. Ribera d'Alcalà et al., 2004; McDonald et al., 2007; Amato et al., 2007; Orsini et al., 2004).

Highly diverse phytoplankton assemblages are reported for the GoN in the winter season, despite only occasional increases in the autotrophic biomass (Zingone et al., 2010). Our results also show a reverse relationship between abundance and genetic diversity. This is not surprising, as blooming ribotypes may saturate the sample, masking the presence of rare ribotypes. In the absence of dominating species, as in the case of winter and autumn samples, low abundance species have a higher chance to be detected. As a matter of fact, the samples with a few *Pseudo-nitzschia* cells or none at all by LM were the most diverse in clone libraries. *Pseudo-nitzschia* species do not form resting benthic stages (Montresor et al., 2013), and hence each species could persist in low densities in the water column at any time outside their bloom optimum.

At the intraspecific level, the most striking case of diversity is that of *Pseudo-nitzschia galaxiae*, which harbors at least five groups of LSU-ribotypes (McDonald et al., 2007). Cerino et al. (2005) first highlighted the high morphological variability within this species. In addition to the type material, constituted by lanceolate cells, 25–41 μm long (Lundholm and Moestrup, 2002c), named as medium morphotype, Cerino et al. (2005) recognized two more morphotypes: one larger (<82 μm) than the type material, with parallel valve sides named large morphotype, and another much smaller (down to 10 μm) non-chain forming, named small

morphotype. Although the medium and large morphotypes share the same LSU, the three morphotypes are distinct in ITS (McDonald, 2006) and rbc*L* (unpublished data). The observed phenotypic diversity is thus apparently supported by a genetic basis in this species, possibly highlighting a case of incomplete lineage sorting which would identify *P. galaxiae* as a species-complex. This hypothesis could be tested collecting data on sexual compatibility between isolates belonging to the different ribogroups.

The high genetic diversity in *Pseudo-nitzschia galaxiae* could be due to its larger sample size in terms of sequence counts for this species; the larger the number of sequences obtained for a species, the higher the probability to catch less common ribotypes and hence to have a more exhaustive picture of the genetic diversity of the species. This explanation, however, does not apply to other species such as *Pseudo-nitzschia fraudulenta*, which is also remarkably diverse despite being the least represented species in the clone libraries, and *Pseudo-nitzschia hasleana*, which is much less polymorphic than comparably well represented species. Considering that populations in recently colonized areas are often less polymorphic than source populations (Allendorf and Lundquist, 2003), the low diversity of *P. hasleana* supports the idea of a recent introduction in the GoN. A recent introduction is also suggested by the fact that the species was never recorded during multiple isolations with subsequent genotyping carried out in the area since the beginning of the 2000 s.

The considerable differences in nucleotide and gene diversity observed even among cryptic sister species (e.g. in the *P. delicatissima* species-complex) could also be the effect of intragenomic variations. rDNA is a multi-copy region with ribosomal genes arranged in tandemly repeated cistrons (18S-5.8S-28S intermingled by ITS1 and ITS2) and the number of copies can vary highly among species (Weider et al., 2005; Song et al., 2012). The more the copies, the more likely that concerted evolution fails to homogenize the paralogues within individuals (Alvarez and Wendel, 2003; Thornhill et al., 2007). The presence of intragenomic variation at different regions of the rDNA cistron has been described in several phytoplankton species (Behnke et al., 2004; Alverson and Kolnick 2005; Pillet et al., 2012), including *Pseudo-nitzschia* species (McDonald et al., 2007; D'Alelio et al., 2009).

4.2. Seasonal dynamics and bloom composition

The temporal dynamics that generate patterns of species abundance and community structure constitute an important question in ecology (Magurran and Henderson, 2010). However, only a few studies have dealt with temporal changes in diversity in unicellular microalgae, mainly based on isolates from single species (Evans et al., 2005; Rynearson et al., 2006; Godhe and Härnström, 2010), or focused on the total phytoplankton sample (Behnke et al., 2010) or on size-selected planktonic groups (Massana et al., 2004; Romari and Vaulot, 2004; McDonald, 2006; Piwosz and Pernthaler, 2010). More recently, some studies based on phylochips have dealt with temporal patterns of occurrence in several harmful algae (Dittami et al., 2013; Edvardsen et al., 2013; Kegel et al., 2013), including *Pseudo-nitzschia* (Barra et al., 2013).

The clone library approach used in the present work allowed disclosing the actual composition of the blooms of cryptic and pseudo-cryptic species as well as the seasonal patterns of the individual species. As expected, clone libraries showed a far higher detection power than that achievable by means of LM observations, recording the presence of any *Pseudo-nitzschia* species observed by LM but also of species not observed in corresponding samples. However, the relative proportion of the species present in environmental samples often diverged from LM observations. For example, in comparison with LM cell counts, *Pseudo-nitzschia galaxiae* was often over-represented in the clone libraries, while *Pseudo-nitzschia pseudodelicatissima*-complex was under-represented. This mismatch can be due to several factors, including the exponential nature of the PCR technique (Gonzalez et al., 2012), the variability of the extraction efficiency and the different numbers of rDNA copies in the genomes of the different species. Indeed, the rDNA copy number in *Pseudo-nitzschia* can vary not only among species but even within a single species sampled at different times, suggesting a relationship with physiological activity and/or adaptive strategies of the strains (Penna et al., 2013).

Interestingly the seasonal patterns revealed by the clone library approach basically match results obtained in previous studies (Orsini et al., 2004; Cerino et al., 2005; Zingone et al., 2006; D'Alelio et al., 2009) and confirm that most species, including the cryptic ones, tend to occur in specific periods of the year. For morphologically distinct species, i.e. *Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia multistriata*, clone library results matched those obtained by LM. Regarding cryptic species, this study confirms the high diversity of the *P. delicatissima* complex in spring (Orsini et al., 2002; McDonald et al., 2007), mainly formed by *Pseudo-nitzschia arenysensis* and *P. delicatissima*, accompanied by much less abundant *Pseudo-nitzschia dolorosa* and another ribotype new for the area (*P. delicatissima* V). In contrast, it was mainly *P. delicatissima* IV to form the late summer-early autumn bloom in both 2009 and 2010, matching previous records of this taxon in late summer in 2004 (McDonald et al., 2007, as "*P. delicatissima* new genotype"). These results highlight the regular seasonality pattern of this cryptic species and support the hypothesis that *P. delicatissima* IV can indeed be the species found to undergo a massive sexual reproduction in September 2006 (Sarno et al., 2010). In the *Pseudo-nitzschia pseudodelicatissima* complex, *Pseudo-nitzschia calliantha*, which produces domoic acid (Trainer et al., 2012), appeared to be the main contributor to the autumn and spring peaks, while the other species, including *P. pseudodelicatissima sensu stricto*, increased in the clone libraries in late autumn-winter, when the corresponding morphotypes were virtually absent from cell counts. Neither morphotypes nor sequences of this group were detected in late spring-summer in 2010, which indeed is the period of low abundance of *P.* cf. *pseudodelicatissima* in the LTER-MC time series. However in a few years (1996, 2000 and 2006–2008) there were increases of *P.* cf. *pseudodelicatissima* in late May–June, with the highest density (3×10^6 cells L^{-1}) for the whole time series in June 2007 (D.S. and A.Z., unpublished results). This irregular behavior reminds us that several years of observations are needed to fully cover the seasonal pattern of a species.

The peculiar seasonality of the different morphotypes of *Pseudo-nitzschia galaxiae* described by Cerino et al. (2005) was also confirmed by our clone library results which show that the ribogroup I, corresponding to the small morphotype, was responsible for the early spring increase, while the ribogroup II, encompassing the medium and large morphotypes, was found in late spring and in summer. This last ribogroup was found to produce domoic acid in the GoN (Cerino et al., 2005).

The present study shows that distinct but closely related cryptic diatom species may be present in different seasons, over a broad range of environmental conditions in terms of light, turbulence, temperature and nutrients (Ribera d'Alcalà et al., 2004). Seasonal occurrence of species appears not to be constrained by nutrient levels because their regularity strongly contrasts with the chemical variability in coastal waters (Ribera d'Alcalà et al., 2004 and other unpublished results). This is particularly true for species that contribute little to the total phytoplankton abundance and even less to total phytoplankton biomass. Other factors, such as temperature and photoperiod, which are linked to astronomical cycles, may be related to species occurrence over the seasons,

possibly through a direct effect, but also as triggers of endogenous clocks (Anderson and Kaefer, 1987). Advection processes could, in theory, explain the alternation of different blooming species during the year. Yet, the persistence of the blooms over periods exceeding the time-scales of the complex surface hydrodynamics of the GoN (Uttieri et al., 2011) suggests that such short-term hydrographic events do not affect species succession.

The alternation of closely related species along seasons raises questions about the mechanisms of the speciation process at sea. In fact, populations of closely related lineages can display different ecophysiological characteristics (Rynearson et al., 2006; Degerlund et al., 2012; Huseby et al., 2012), which may reflect adaptations to particular ecological niches. A reduction of the gene flow due to different heritable reproductive times (isolation-by-time, Hendry and Day, 2005) could be an important speciation driver and could be advocated to explain the remarkable number of cryptic, closely related lineages in unicellular microalgae.

5. Concluding remarks

Based on our results *Pseudo-nitzschia* confirms to be among the most diverse diatom genus in the GoN plankton. The molecular lens used in this study highlighted differences in the seasonal and relative abundance patterns among cryptic sister species and in some cases among ribotypes belonging to the same species. Although we did not investigate their driving factors, either environmental or endogenous, these patterns point at differences in the ecological/seasonal niches of cryptic species, stimulating studies on their ecophysiological characteristics as well as on their mode of speciation. In addition, our approach can represent a useful tool for management purposes, revealing the presence of potentially toxic species and indicating the most probable periods for their occurrence in the plankton.

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THE DYNAMICS OF SEXUAL PHASE IN THE MARINE DIATOM *PSEUDO-NITZSCHIA MULTISTRIATA* (BACILLARIOPHYCEAE)[1]

Eleonora Scalco, Krzysztof Stec, Daniele Iudicone, Maria Immacolata Ferrante, and Marina Montresor[2]

Stazione Zoologica Anton Dohrn, Villa Comunale, Naples 80121, Italy

Sexual reproduction represents a fundamental phase in the life cycle of diatoms, linked to both the production of genotypic diversity and the formation of large-sized initial cells. Only cells below a certain size threshold can be sexualized, but various environmental factors can modulate the success of sexual reproduction. We investigated the role of cell density and physiological conditions of parental strains in affecting the success and timing of sexual reproduction in the marine heterothallic diatom *Pseudo-nitzschia multistriata*. We also studied the dynamics of the sexual phase in still conditions allowing cell sedimentation and in gently mixed conditions that keep cells in suspension. Our results showed that successful sexual reproduction can only be achieved when crossing parental strains in the exponential growth phase. Evidence was provided for the fact that sexual reproduction is a density-dependent event and requires a threshold cell concentration to start, although this might vary considerably amongst strains. Moreover, the onset of the sexual phase was coupled to a marked reduction in growth of the vegetative parental cells. The crosses carried out in physically mixed conditions produced a significantly reduced number of sexual stages as compared to crosses in still conditions, showing that mixing impairs sexualization. The results of our experiments suggest that the signaling that triggers the sexual phase is favored when cells can accumulate, reducing the distance between them and facilitating contacts and/or the perception of chemical cues. Information on the progression of the sexual phase in laboratory conditions help understanding the conditions at which sex occurs in the natural environment.

Key index words: **density-dependent; diatoms; heterothallic; mixing; *Pseudo-nitzschia multistriata*; sexual reproduction**

Sexual reproduction represents a fundamental phase in the life cycle of diatoms: it provides genotypic diversity through meiotic recombination occurring when gametes are produced (Tesson et al. 2013), and restores the maximum size through the formation of a specialized zygote, called the auxospore (Chepurnov et al. 2004). In fact, diatoms have a distinctive life cycle characterized by a progressive cell size reduction as vegetative division proceeds. This is due to the architecture of diatom cells that are surrounded by a rigid siliceous cell wall constituted by two unequal halves fitting together as a box and its lid. Upon mitotic division, the new hypovalves of the daughter cells are synthesized within the mother cell, thus producing one cell with the size of the mother cell and a slightly smaller one (Round et al. 1990).

In the ancestral centric diatoms, male (motile sperm) and female (immotile egg) gametes can be produced within the same clonal strain (homothallic life cycle), whereas in the vast majority of pennate diatoms strains of opposite mating type have to be co-cultured to induce the sexual phase (heterothallic life cycle). The sexual phase can be induced only in cells below a species-specific cell size threshold (Chepurnov et al. 2004). However, the reach of a critical cell size threshold is an obligate requirement but it is not the sole factor necessary for the induction of sex, because external cues can further regulate the process. Changes in salinity, light quantity and quality, or shifts in the composition of the growth medium, have been shown to induce sexualization in centric diatoms (e.g., Schultz and Trainor 1968, Drebes 1977, Schmid 1995, Godhe et al. 2014), while the mixing of two sexually compatible strains seems to be sufficient for the induction of sexuality in heterothallic pennate diatoms (e.g., Davidovich and Bates 1998, Amato et al. 2005, Mann and Pouličkovà 2010, Fuchs et al. 2013). Nevertheless, the success of the sexual phase in terms of production of gametes, auxospores and initial cells can be tuned by day length, irradiance or temperature in pennate diatoms, and the effect of these environmental factors varies considerably amongst species (Mizuno and Okuda 1985, Davidovich 1998, Hiltz et al. 2000, Mouget et al. 2009). To our knowledge, little or no information is available on the link between cell concentration and the onset of the sexual phase in planktonic diatoms and generally in other unicellular microalgae (e.g., Sandgren and Flanagin 1986 in Chrysophyceae).

A density-dependent mechanism could explain the production of sexual pheromones recently reported for two benthic diatoms (Sato et al. 2011, Gillard et al. 2013). In the araphid pennate *Pseudostaurosira*

[1]Received 21 January 2014. Accepted 6 July 2014.
[2]Author for correspondence: e-mail marina.montresor@szn.it.
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trainorii, sexualization is a two-step process in which female cells secrete a sex pheromone that induces the formation of gametes in the male strain, which in turn, secretes a sex pheromone that induces gametogenesis in the female strain. A sex pheromone has been identified in *Seminavis robusta*, where MT^- cells release l-diproline that is capable of attracting MT^+ cells (Gillard et al. 2013).

Most of our knowledge on life cycle features of diatoms derives from detailed observation of strains in laboratory conditions and from observations carried out on natural population of freshwater, mostly benthic, species (reviewed in Edlund and Stoermer 1997, Chepurnov et al. 2004). In fact, notwithstanding the key-role of sexual reproduction in the life cycle of diatoms, there are only a handful of reports of sexual stages for planktonic species in the marine environment (Crawford 1995, Assmy et al. 2006, Holtermann et al. 2010, Sarno et al. 2010). These findings have been gained in markedly different environmental conditions, spanning from open and well-mixed waters in the Southern Ocean (Crawford 1995, Assmy et al. 2006), to the surface layer of coastal waters (Sarno et al. 2010), to the very shallow surf zone (Holtermann et al. 2010). The frequency, success and environmental conditions that might regulate the occurrence of sexual reproduction are important factors to consider for explaining and eventually modeling population dynamics, genetic structure and persistence of diatom species in the natural environment. As an example, if the conditions for sex are not met for consecutive years, a heterothallic diatom species might risk local extinction due to the fact that the population is not rejuvenated by the formation of large-sized cells (D'Alelio et al. 2010).

We present the results of experiments aimed at elucidating the dynamics of sexual reproduction in the marine planktonic diatom *Pseudo-nitzschia multistriata* (Takano) Takano. This species has been recorded in different coastal sites worldwide (reviewed in Lelong et al. 2012) and is capable of producing the neurotoxin domoic acid (Orsini et al. 2002). *Pseudo-nitzschia multistriata*, as the vast majority of congeneric species (Lelong et al. 2012), has a heterothallic life cycle and sexual reproduction is induced when cells are below 55 μm in apical length (D'Alelio et al. 2009). We tested the role of physiological conditions and the density of parental strains in affecting the success and timing of sexual reproduction. We also assessed the success and timing of sexual reproduction in time-course experiments carried out in still conditions and under gentle constant mixing, with the hypothesis that physical mixing would impair the sexual phase by increasing the distance between cells.

MATERIAL AND METHODS

Culture isolation and maintenance. Single cells or short chains of *Pseudo-nitzschia multistriata* were isolated with a micropipette from net samples collected at the Long-Term Ecological Research station Mare Chiara (LTER-MC) in the Gulf of Naples, Mediterranean Sea (40°47′ 33″ N, 14°11′ 18″ E; Table S1 in the Supporting Information). The cultures were grown in f/2 culture medium (Guillard 1975) at a temperature of 18°C, a photoperiod of 12:12 h L:D, and a photon flux density of 60 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ provided by cool white fluorescent tubes (Philips TLD 36W/950, Philips, Amsterdam, The Netherlands). Before carrying out each experiment, the apical axis of 20 cells per strain were measured at 400× magnification using a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) equipped with an ocular micrometer.

Crosses of parental strains at different cell densities and growth phases. Experiments were carried out with different pairs of strains of compatible mating type (Table S1). The mating type of the strains used for the experiments was assessed by crossing them with reference strains of known mating type. The strain that was bearing the auxospores was defined "Pm−," i.e., female, and the other one "Pm+," i.e., male.

A first experiment (Experiment #1) was aimed at estimating the timing and success of sexual reproduction in crosses carried out with strains at different growth phases and cell densities (Fig. S1 in the Supporting Information). Two flasks, one per parental strain, containing 700 mL of f/2 medium were inoculated with cells at a final concentration of about 300 cells $\cdot$ mL^{-1} and placed at a temperature of 18°C, a photoperiod of 12:12 L:D h and an irradiance of 110 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$. Cell concentration of the two parental strains was estimated on day 0 (T0), 2 (T2), 4 (T4), 5 (T5), 6 (T6) and 7 (T7). At each time point, 50 mL of culture for each parental strain were mixed in a flask and aliquots of 4 mL were dispensed, after careful mixing, in two 6-wells culture plates (Fig. S1a). Plates were incubated at 18°C, a photoperiod of 12:12 L:D h and an irradiance of 60 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$. Culture plates were inspected every day at an inverted microscope to check for the presence of gametes. Starting from the day at which gametes were first observed, and for four consecutive days, the content of three wells was fixed with formaldehyde solution at a final concentration of 1.6%. One mL of each triplicate was placed in a Sedgewick–Rafter counting slide and vegetative cells, gametes or zygotes (the two stages cannot always be differentiated in light microscopy), auxospores, initial cells (large cells still surrounded by the perizonium), and F1 generation cells (long vegetative cells) were enumerated. A control was run with the parental strains in monoculture. Each strain was inoculated in a 500 mL Costar culture flask containing 200 mL of f/2 to reach a concentration of about 300 cells $\cdot$ mL^{-1}. Four mL aliquots were dispensed into 6-wells culture plates and grown at the experimental temperature and light conditions illustrated above for the crosses. Cell concentration was estimated every 2 d as illustrated above.

The highest percentage of sexual stages was obtained in crosses started with parental strains in exponential growth phase, at a cell concentration of about 5,000 cells $\cdot$ mL^{-1}. A second experiment was designed to test if parental strains in late- or post-exponential phase could become again competent for sex (Experiment #2; Table S1, Fig. S1b). The set up was the same as Experiment #1, with the only difference that parental strains collected at times 4 (T4), 5 (T5), 6 (T6), and 7 (T7) of the growth curve were diluted back, when co-cultures were started, to ~5,000 cells $\cdot$ mL^{-1} with fresh f/2 medium. A third experiment was conducted to assess the concentration of parental strains at which sexual stages were first produced in co-cultures started from exponentially growing parental strains collected at the same time point of their growth curve (Experiment #3; Table S1). The experimental set up used for Experiments # 1 and 2 was followed, with

the difference that parental strains were collected on day 3, when in exponential growth phase, centrifuged at 900 *g* and re-suspended in 15 mL of medium. This concentrated stock was used to inoculate a series of culture plates at cell concentration (values for both parental strains) of 400 (1), 4,000 (2), 6,000 (3), 8,000 (4) cells · mL^{-1}. The concentration of vegetative parental cells, gametes/zygotes, auxospores and initial cells was monitored every day until the formation of initial cells was confirmed. To estimate growth rate of the individual parental strains and nutrient (NO_3, NO_2, NH_4, PO_4, and SiO_4) concentration, one flask for each parental strain, filled with 500 mL of f/2 medium, was inoculated with cells from the concentrated stock at a final concentration of 300 cells · mL^{-1}. Every 2 d, 4 mL of culture were sub-sampled in duplicate and used to estimate cell concentration. On the same days, 20 mL of culture were sub-sampled in duplicate, filtered through a 0.22 μm pore size MILLEX-GS filter unit and stored at −20°C until the analysis. Nutrient concentration was analyzed with a Systea Flowsys autoanalyzer (AxFlow, Ealing, London, U.K.) equipped with five continuous flux channels, following Hansen and Grasshoff (1983).

To rule out the possibility that the change in growth dynamics observed between the mono-cultures of parental stains and the co-cultures undergoing sexual reproduction could depend on the interaction between different strains independently from their mating type, we tested the growth curve of co-cultures of *P. multistriata* strains of the same mating type (Experiment #4, Table S1). We used strains of different cell size to distinguish them when co-cultured. One culture flasks was inoculated with 150 cells · mL^{-1} for each Pm+ strain and the other with the same concentration of Pm− strains. Subsamples of 4 mL were placed in 6-well culture plates and incubated at the same experimental conditions illustrated above. Every 2 d and for a period of 10 d, the culture material of 2 wells for each couple were fixed with formaldehyde solution at a final concentration of 1.6%. Cell concentration of the two co-cultured strains was estimated using a Sedgewick–Rafter counting slide.

Crosses carried out in still and mixed conditions. In this experiment (Experiment #5), parental strains differing in cell size were used to follow the growth and behavior of the individual strains (Table S1). Two 500 mL flasks, one for each parental strain, were filled with 240 mL of f/2 medium and inoculated with cells at final concentration of ~3,000 cells · mL^{-1}. Aliquots of 30 mL were dispensed, after gentle mixing, in eight 70 mL culture flasks. The stock co-culture of the two parental strains was prepared in a flask filled with 800 mL of f/2 filtered medium and inoculated with cells at final concentration of about 1,500 cells · mL^{-1} for each parental strain (3,000 cells · mL^{-1} in total). Aliquots of 30 mL were dispensed, after careful mixing, in twenty-six 70 mL flasks. For each parental strain and for the crosses, half of the flasks were placed on a rotating wheel (RW) and the other half were placed on a shelf (SH). The RW and the SH were located in a walk-in climatic chamber at a temperature of 18°C and a photoperiod of 12:12 L:D. The integrated irradiance at which cultures were exposed on the RW was 60 μmol photons · m^{-2} · s^{-1} (110 μmol photons · m^{-2} · s^{-1} at the top; 35 μmol photons · m^{-2} · s^{-1} at the bottom), which was the same as the one on the SH. The RW was set at a 0.1 rpm; this rotation velocity caused a gentle and constant mixing of the cultures.

To monitor the growth rate of the individual parental strains at the two different conditions (RW and SH), 2 mL of culture were sub-sampled from each parental strain every 2 d from one randomly selected flask on the RW and from one randomly selected flask on the SH, they were placed in Eppendorf vials and fixed with formaldehyde at a final concentration of 1.6%. To monitor the growth rate and the production of sexual stages in the crosses, one flask from the RW and one from the SH were randomly collected every day. From each flask, two subsamples of 3.5 mL were fixed with formaldehyde solution at a final concentration of 1.6% and stored at 4°C.

For each parental strain, 1 mL of fixed culture was counted using a Sedgewick–Rafter counting slide and live vegetative cells (cells with cytoplasm content) were enumerated using an Axiophot light microscope. For the cross samples, one mL of fixed culture, in triplicate, was placed in a Sedgewick–Rafter counting slide and the following stages were enumerated: live vegetative cells (cells with cytoplasm content), gametes/zygotes, auxospores, initial cells, and large F1 generation cells. Growth rate, expressed as divisions per day, was estimated by calculating linear regression over the exponential portion of the curve (Guillard 1980). Statistical significance between the different treatments was estimated by using Student's *t*-test.

The theoretical distance (D) between cells in flasks incubated on the RW was calculated applying the formula (1) and assuming that cell distribution in the flask was homogeneous:

$$D = 1\ \mu m^3 - [v \times (N \times 30\ mL)/V]/[(N \times 30\ mL)/V] \quad (1)$$

where N = cell concentration (cells · mL^{-1}); v = average volume of a single cell; V = volume of all cells in the 30 mL sample.

The theoretical distance (D) between cells incubated on the SH was calculated applying the formula (2), assuming that cells were sinking in a thin layer (TL) at the bottom of the flask. This layer was considered 10 μm-high and estimates were calculated assuming variable percentages of sinking cells, i.e. 80%, 60% and 40% of the total, respectively.

$$D = 1\ \mu m^3 - [v \times (N \times 30\ mL \times (\%))/V]/[(N \times 30\ mL \times (\%))/V] \quad (2)$$

where (%) = percentage of sinking cells.

RESULTS

The physiological state and the density of parental strains affect the success of sexual reproduction. To define whether the occurrence and success of sexual reproduction in *Pseudo-nitzschia multistriata* could be dependent on the physiological state of the cells, we carried out crosses with parental strains collected at different time points of the growth curve, from day 0 (T0) to day 7 (T7) (Experiment #1, Fig. S1). Sexual reproduction occurred in all crosses but with variable timing and success (Fig. 1). In *P. multistriata,* the sexual phase starts with the pairing of cells of the opposite mating type along their longitudinal axis (Fig. S2a in the Supporting Information). Each gametangium produces two gametes (Fig. S2b). Upon fertilization of the Pm− gametes by the Pm+ ones, two auxospores (the zygotes) develop attached to the empty frustule of the Pm− gametangium (Fig. S2c). A large-size initial cell is produced within each auxospore (Fig. S2d). In the very initial phase of development, auxospores are round and can be confused with gametes. Because it was not always possible to discriminate between gametes and early

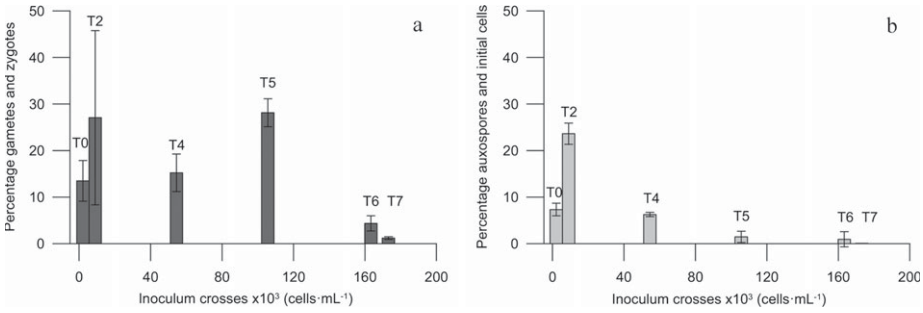

FIG. 1. Success of sexual reproduction in *Pseudo-nitzschia multistriata* as related to the physiological state of parental strains (Experiment #1). Crosses were started with parental strains at different time points of their growth curve. Percentage of gametes and zygotes (a) and percentage of auxospores and initial cells (b) recorded when starting the co-cultures with parental strains at different inoculum concentrations (cells · mL^{-1}), i.e., at different phases of their growth curve. Each bar represents the average of 3 replicate values; vertical lines represent SD. The time points of the growth curve at which parental strains were collected to start the co-cultures are reported above the bars.

stage auxospores (the zygotes), we reported their sum in our graphs. The average maximum percentage of gametes and early stage zygotes was comprised between 13.5% ± 4.37% and 28.1% ± 3.01% SD in crosses carried out with parental strains in the exponential growth phase (i.e., before day 5), when the inoculum concentration was comprised between 300 and 100,000 cells · mL^{-1} (Fig. 1a). In crosses started with parental strains in post-exponential or stationary growth phase, i.e., on day 6 and 7, the percentage of gametes and early stage auxospores decreased considerably to 4.3% ± 1.63% and 1.2% ± 0.29% SD, respectively (Fig. 1a). Gametes were first recorded on day 6 in crosses started at T0, and their timing of appearance gradually decreased in the crosses carried out in the following days, which were started with progressively higher cell concentrations. In crosses started on T2, gametes were detected on day 4 and in all the other crosses on day 3. In crosses carried out from T0 to T4, sexual reproduction was successful. Auxospores and large initial cells were produced with percentages ranging between 6.3% ± 0.46% and 23.6% ± 2.28% SD (Fig. 1b). Very few auxospores and no initial cells were instead retrieved in the crosses started with parental strains inoculated in the late and post-exponential growth phase (Fig. 1b). No sexual stages were observed in the control test, where parental strains were grown in monoculture.

A second experiment was carried out with a similar set up but, starting from the inoculum carried out on day 4 (T4), the co-cultures of parental strains were diluted to <5,000 cells · mL^{-1} to bring them in exponential growth (Experiment #2, Fig. S1). The maximum percentage of gametes and early stage zygotes produced in all crosses carried out between T0 and T7 was very similar (Student's *t*-test: t_3 = 6.2–7.6 for the 15 combinations, $P > 0.1$) and was ranging between 7.6% ± 5.84% and 14.1% ± 1.99% SD (Fig. 2a). Sexual reproduction was successful in all crosses, where auxospores and initial cells were produced in percentages spanning from 5.6% ± 1.20% to 12.5% ± 4.02% SD (Fig. 2b). Gametes were first recorded on day 6 in the crosses started at T0, on day 4 in those started at T2 and on the third day in all the other crosses. These results indicate that cells have to reach a minimum concentration before they can enter the sexual phase; the cell density at which gametes were first observed in Experiment #2 spanned between 2,989 ± 619 and 5,150 ± 220 cells · mL^{-1} (Fig. 3).

Experiment #3, conducted with parental strains collected at the same time point of the growth phase, was carried out with a different pair of strains that produced a lower number of sexual stages as compared to the previous experiments. Also in this experiment, sexual stages were produced with a different timing, when cell concentration was at about 8,000 cells · mL^{-1} (Fig. S3 in the Supporting Information). Gamete production lasted for 2–3 d and was followed by the formation of auxospores and initial cells (Fig. S3). The maximum percentage of gametes and early stage zygotes was comprised between 1.4% and 15.25%.

In Experiment #3, nutrient concentration along the growth curve of the parental strains was measured (Table S2 in the Supporting Information). The average nutrient consumption over the exponential growth phase was $4.2 \cdot 10^{-4}$ μmol · $cell^{-1}$ for total nitrogen, 1.0×10^{-4} μmol · $cell^{-1}$ for phosphate, and 3.3×10^{-4} μmol · $cell^{-1}$ for silicate. Applying these values to the cross experiments started with parental cell inocula of different concentration (Fig. S3), the amount of nutrients consumed over the duration of the experiment ranged between 20.52 and 22.62 μmol · L^{-1} for total nitrogen, between 4.83 and 5.33 μmol · L^{-1} for phosphate and between 16.21 and 17.87 μmol · L^{-1} for silicate, thus showing that nutrient limitation was not occurring.

Vegetative growth is reduced when sexual reproduction takes place. The two parental strains grown in monoculture reached similar maximum cell concentration

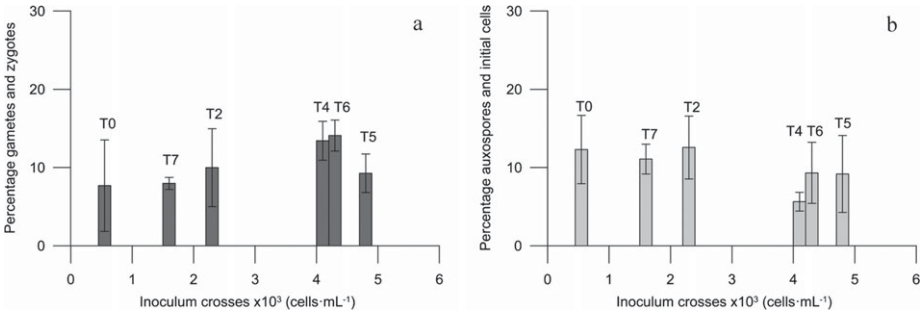

FIG. 2. Success of sexual reproduction in *Pseudo-nitzschia multistriata* as related to the inoculum cell density of parental strains (Experiment #2). Crosses were started with parental strains collected at different time points of their growth curve, but diluted back to the exponential growth phase (see text for explanation). Percentage of gametes and zygotes (a) and percentage of auxospores and initial cells (b) recorded when starting the co-cultures after dilution. Each bar represents the average of 3 replicate values; vertical lines represent SD. The time points of the growth curve at which parental strains were collected to start the co-cultures are reported above the bars.

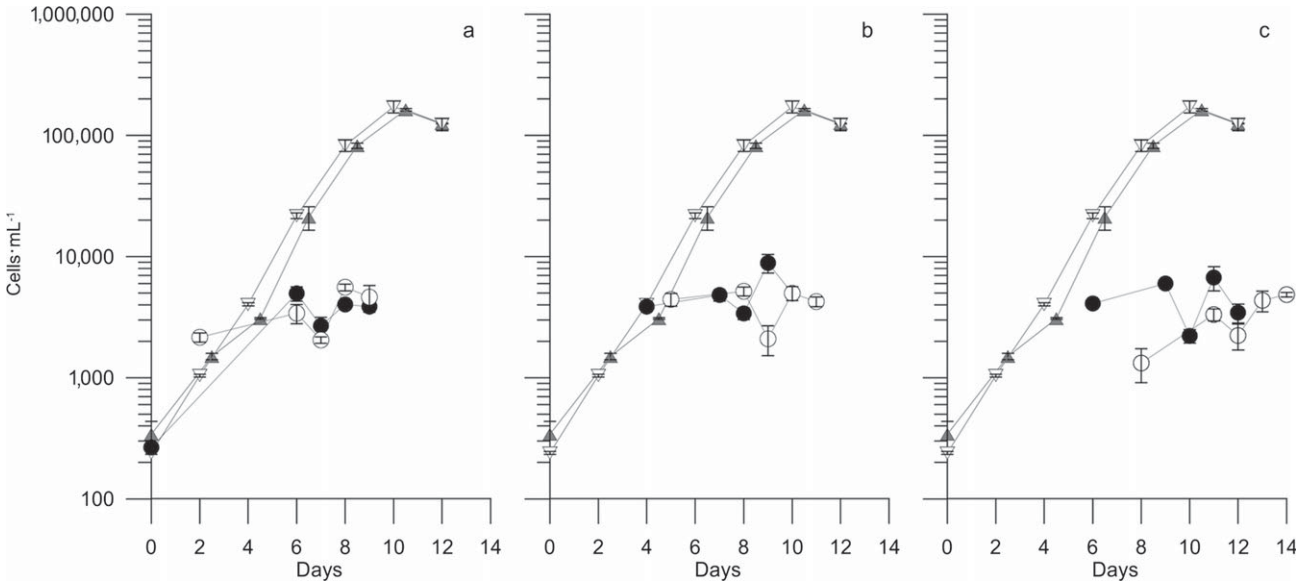

FIG. 3. Growth curves of co-cultures of *Pseudo-nitzschia multistriata* started with parental strains collected at different time points along their growth curve (T0→T7), but diluted back to the exponential growth phase (data of Experiment #2 illustrated in Fig. 2). Circles represent the total number of cells: parental vegetative cells, sexual stages, large F1 cells. For comparison, the growth curve of the monoclonal parental strains (Pm+, gray up-triangles; Pm−, white down-triangles) is included in each panel. (a) Crosses carried out at T0 (black circles) and T2 (white circles). (b) Crosses carried out at T4 (black circles) and T5 (white circles). (c) Crosses carried out at T6 (black circles) and T7 (white circles). For each cross are represented: the initial cell concentration (cells · mL^{-1}) and the total cell concentration estimated for 4 d, starting from the day in which gamete production was first observed. Symbols represent the average value of duplicate (parental strains) or triplicate (crosses) counts; vertical bars represent maximum and minimum values (parental strains) or standard deviation (crosses).

(121,420 ± 4,900 cells · mL^{-1} for the Pm+ strain and 131,562 ± 562 cells · mL^{-1} for the Pm− strain), with maximum growth rates of 1.52 and 1.50 divisions per day for the Pm+ and Pm− strains, respectively (Fig. 3). When comparing the time course of cell concentration of monocultures of parental strains with that of cells and sexual stages recorded in the crosses performed in Experiment #2, a much lower cell number was detected in the latter experimental set up, suggesting that an arrest of cell growth occurred in concomitance with the sexual phase. Crosses started with a low inoculum of parental cells (experiments at T0 and T2, Fig. 3a) had an increase in cell numbers up to the day in which gametes were first recorded, but the number of cells leveled out in the subsequent 3 d in which their concentration was monitored. The formation of sexual stages does not account for the observed lack of increase in cell number, since they represented only 3%–20% of the total (Fig. 2, a and b). In the crosses started with higher cell concentration, when gametes were recorded on the third day, a similar trend was observed (Fig. 3, b and c). Vegetative growth was observed only up to the day in which sex started and then cell number did not increase at a rate comparable to that recorded in the clonal strains. A similar trend was observed also in Experiment #3, where co-cultures at different

concentration of parental strains were started with inocula collected at the same time point of the growth curve (Fig. S4 in the Supporting Information).

We tested the growth curve of co-cultures of *P. multistriata* strains of the same mating type, to rule out the possibility that the change in growth dynamics could depend from the interaction between different strains, independently from their mating type (Experiment #4). To this end, we used strains of different cell size, so to distinguish them when co-cultured. The two pairs of co-cultured strains of identical mating type had similar growth curves and growth rates (0.99 and 0.9 divisions per day for the two Pm− strains and 1.33 and 1.49 divisions per day for the two Pm+ ones), thus showing that there was no interaction between them (Fig. 4).

Mixing affects cell growth and patterns of sexual reproduction. The results of the previous experiments showed that a threshold concentration has to be reached to allow the onset of sex in the planktonic species *P. multistriata*. We thus designed an experiment in which the time course of parental cells and sexual stages was monitored in two different settings that reproduce (i) a condition in which cells are allowed to aggregate and/or sink and (ii) a condition in which cells are kept in continuous suspension. In the latter setting, a set of replicate bottles with either parental strains in monoculture or crosses was incubated on a RW that kept the cultures in constant slow mixing, while a second set of replicate bottles was incubated on a SH, without mixing. In this experiment we used parental strains of different cell size, so to monitor cell concentration of the two mating types in co-culture over time.

When co-cultures were started, parental strains showed a decrease in cell concentration during the first 2 d, more marked in the RW set up (Fig. 5). In the latter experimental condition, the Pm− strain grew exponentially from day 2 to day 8 (max growth rate 1.56 divisions per day ±0.12 SD) reaching maximum cell concentration 543,613 ± 67,363 SD cells · mL^{-1} on day 10. The Pm+ strain showed a lower growth rate (1.15 divisions per day ±0.11 SD from day 2 to day 8) and reached a much lower cell concentration on day 10 (an average of 34,293 cells · mL^{-1}). In this set up, gametes were first recorded on the second day, auxospores on the third day, albeit at very low concentration (from an average of 20.6 to 4.1 cells · mL^{-1} on day 2 and 5, respectively) corresponding to extremely low percentages (0.04% and 5.2% over the total number of cells, Fig. 5a and Fig. S5a in the Supporting Information). Gamete production was followed by their transformation into auxospores. Initial cells, i.e., cells of the maximum size and still wrapped in the perizonium, were observed on day 4 and 5 (Fig. S5a). Large F1 cells increased their number exponentially (maximum growth rate 1.79 divisions per day ±0.14) till day 8, reaching a maximum average concentration of >14,000 cells · mL^{-1} on day 10 (Fig. 5a).

In the co-cultures incubated on the SH, a condition in which cells tend to sink and aggregate at the bottom of the flasks, the Pm− strain grew exponentially (max growth rate 0.81 divisions per day ±0.07 SD) until day 8 and reached an average maximum cell concentration of 58,157 cells · mL^{-1} on day 12, at the end of the observation period (Fig. 5b). Also in this experimental set up, both maximum growth rate (0.57 divisions per day ±0.03 SD) and maximum cell concentration (5,051 divisions per day ±634 SD) of the Pm+ strain on day 7 were lower as compared to those of the Pm− strain. Gametes and auxospores were found starting from the 2nd day

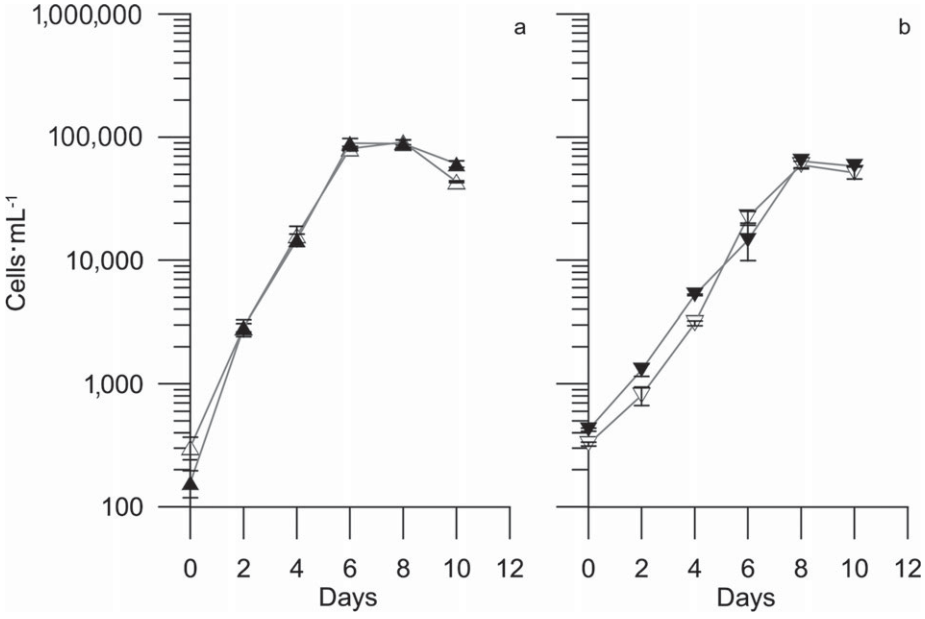

FIG. 4. Growth curve of co-cultures of *Pseudo-nitzschia multi striata* strains of the same mating type (Experiment #4). (a) Co-culture of two male Pm+ strains: large strain Sy668 (white up-triangles) and small strain Sy373 (black up-triangles). (b) Co-culture of two female Pm− strains: large strain Sy800 (white down-triangles) and small strain Sy379 (black down-triangles).

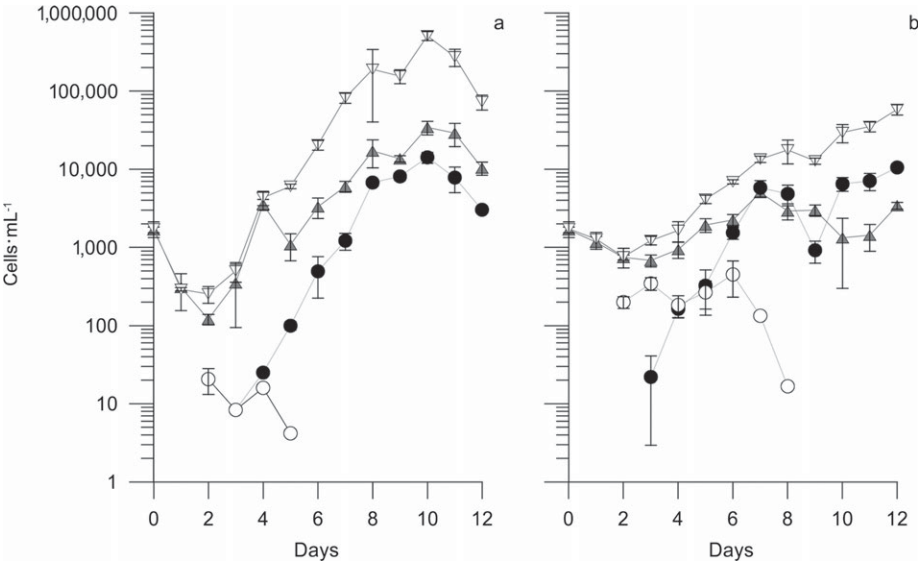

FIG. 5. Dynamics of sexual reproduction in *Pseudo-nitzschia multistriata* co-cultures grown in mixed (a) versus still (b) conditions (Experiment #5). Cell concentration (cells · mL^{-1}) of the two parental strains (gray up-triangles for Pm+ and white down-triangles for Pm−), sexual stages (white circles for the sum of zygotes, auxospores, and initial cells), and large F1 cells (black circles). Each symbol represents the average value of triplicate counts; SD is represented with vertical lines.

while initial cells were observed from day 3 until day 6 (Fig. S5b). The total number of sexual stages, i.e., gametes, auxospores and initial cells was higher as compared to the RW and reached values of 450 cells · mL^{-1} ±218 SD, corresponding to an average percentage of 5.4% ± 2.5% over the total number of cells on day 6. Large F1 cells appeared on day 2, increased their number exponentially until day 7 and reached a maximum cell concentration of 10,533 cells · mL^{-1} ±1,290 SD on day 12 (Fig. 5b).

The two parental strains grown in monoculture showed a different growth dynamics. Cell concentration of the small Pm+ strain peaked on day 8 and maximum growth rate was 1.01 and 0.79 divisions per day on the RW and in still conditions, respectively (Fig. 6). The larger Pm− strain reached the maximum cell concentration on day 4 in both conditions with maximum growth rate of 1.69 and 1.99 divisions per day on mixed and still conditions, respectively (Fig. 6). When comparing the growth curves of the parental strains in mono-culture with the growth curve of vegetative cells and sexual stages in co-culture, it is evident that, also in this experiment, the growth dynamics was different (Fig. 6). This difference is particularly noticeable in the experiment carried out in still conditions, over the time interval in which sexual stages were produced.

We have estimated the theoretical distance between cells (Pm+, Pm− and F1 generation when present) in mixed and in still conditions assuming – in this latter setting – that either 40% or 60% of cells sink on the bottom of the culture flask, in a TL 10 μm high. The difference in distance was significant (Fig. S6 in the Supporting Information; Student's *t*-test: 40%, $t_{18} = 26.3$, $P < 0.00005$; 60%, $t_{18} = 11.6$, $P < 0.00005$). On the second day after the inoculum, when the first sexual stages were observed, the theoretical distance between parental cells was 1,354.3 μm ±87.3 SD in mixed conditions, while it was much lower (113 μm ±6.3 SD) in still conditions.

DISCUSSION

The experiments carried out to elucidate the time-course of sexual reproduction in the marine planktonic diatom *Pseudo-nitzschia multistriata* provided novel and interesting results on the conditions at which sex occurs and on the progression of the sexual phase in laboratory conditions. Sexual reproduction is density-dependent and requires a threshold cell concentration to occur. The formation of sexual stages is reduced in mixed conditions, indicating that the cell-cell signaling that triggers the sexual phase is favored when cells can accumulate, reducing the distance between them and facilitating contacts and/or the perception of chemical cues. We also provided experimental evidence for the fact that successful sexual reproduction can only be achieved when parental strains are in the exponential growth phase. Finally, we showed that the onset of the sexual phase is coupled to a significant reduction in growth of the vegetative parental cells.

Short-term dynamics. Although the mode of sexual reproduction has been studied in many pennate diatoms (reviewed by Chepurnov et al. 2004), detailed quantitative information on the time-course progression of the sexual phase has been reported only for a few species (Gillard et al. 2013, Vanormelingen et al. 2013). Prolonged incubation in the dark, a treatment that in several species has been shown to synchronize cell cycle (e.g., Brzezinski et al. 1990, Gillard et al. 2008), was effective in synchronizing the progression of the sexual phase of both *Seminavis robusta* (Gillard et al. 2013) and *Cylindrotheca closterium* (Vanormelingen et al. 2013), with the formation of high percentages of gametes within a

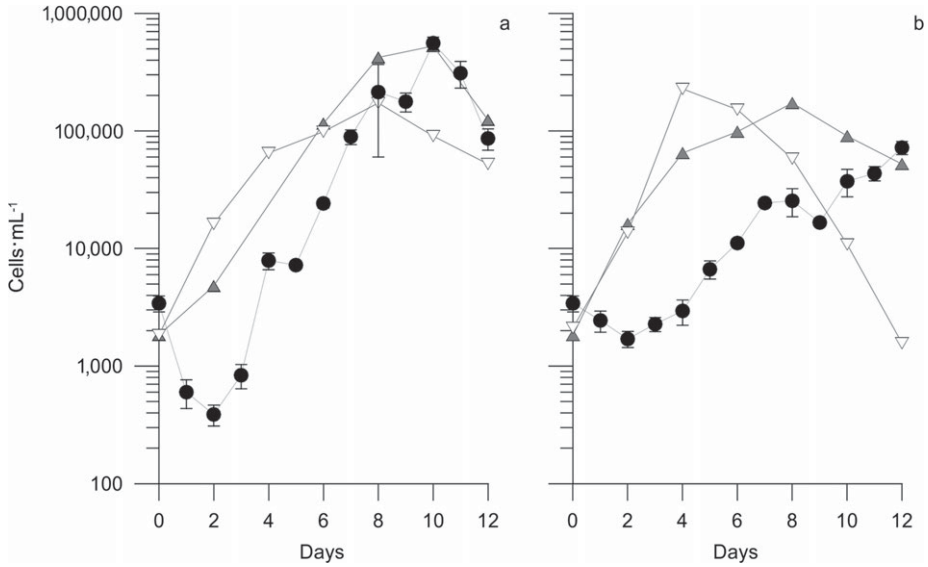

FIG. 6. Growth curves of *Pseudo-nitzschia multistriata* parental strains in monoculture and in co-cultures grown in mixed (a) *versus* still (b) conditions (Experiment #5). Cell concentration (cell · mL^{-1}) of the two parental strains (gray up-triangles for Pm+ and white down-triangles for Pm−) and the average cumulative abundance of cells (parental vegetative cells, sexual stages, F1 cells; data of Fig. 5) in co-culture (black circles).

short time interval. In *S. robusta,* DNA quantification proved that, after a prolonged dark treatment, cells were arrested in the G1 phase of the cell cycle (Gillard et al. 2008), a phase in which cells are prone to the reception of the pheromone that induces the attraction of cells of the opposite mating type (Gillard et al. 2013). In both *S. robusta* and *C. closterium* higher sexualization percentages were reported as compared to the values recorded for *P. multistriata.* This difference might be due to the fact that we did not apply a prolonged dark treatment to synchronize the cells, but carried out our experiments under the same photoperiod conditions – 12:12 h light:dark – at which strains were routinely maintained in culture. However, preliminary experiments suggest that a prolonged dark treatment, while effective in blocking cells in G1 phase, is not effective in significantly increasing gamete production in *P. multistriata* (M. I. Ferrante and S. Patil unpublished data). In *P. multistriata,* we do not observe an obvious attractive behavior of one strain upon the other, rather cells from both mating types move actively and explore the environment until they find a cell to pair with (E. Scalco, unpublished data). These observations suggest that differences between the two species might be substantial, and this makes sense considering the different habits –benthic and planktonic, respectively – of the two species.

Gametogenesis appears as a rather synchronous process in *P. multistriata* and gametes are detected in the co-cultures only for a couple of days and immediately followed by the formation of auxospores and large initial cells. In the dark-synchronized *C. closterium,* a massive gamete production was largely confined within the first 14 h after re-exposure to the light cycle (Vanormelingen et al. 2013) and a synchronous production of auxospores was observed also in the centric diatom *Skeletonema marinoi* within 3–4 d from the application of the environmental cue inducing gametogenesis (Godhe et al. 2014). This suggests that the production of gametes is linked to the perception of a chemical cue that synchronizes the process, once cells of opposite mating type perceive each other.

An interesting result of our experiments was the different growth dynamics observed in monocultures of single strains and in co-cultures of strains of opposite mating type in which sexual reproduction was in progress. In the latter conditions, the progression of sexual reproduction was coupled to a decrease in vegetative growth. This was evident in both the experiments carried out in small-volume culture plates (Experiments #2 and #3) and when comparing the growth dynamics of monocultures of the individual parental strains and the crosses in Experiment #5 (comparison between still and mixed conditions). In this latter experiment, where significantly (Student's *t*-test: $t_3 = 23$, $P < 0.001$) higher growth rates of parental strains were detected in the co-cultures with extremely low percentages of sexual stages, i.e., in the mixed condition. This result, together with the more marked reduction in growth in the co-cultures of Experiment #2, might suggest that the reduction in growth is proportional to the percentage of gamete formation. The arrest of the cell cycle in correspondence with the onset of the sexual phase mediated by sex pheromones has been reported for unicellular fungi (Bardwell 2004, Cote and Whiteway 2008) and the finding of a similar response in *P. multistriata* could indicate that a similar signaling mechanism also occurs in diatoms. This endogenous control of cell growth linked to the interaction of cells of different mating type might play an important role in regulating bloom dynamics in the natural environment. Phytoplankton blooms are accumulation of biomass due to rapid asexual cell division. The increase in cell concentration will facilitate encounter rates and/or the

perception of chemical signals between cells. If gametogenesis – induced during exponential growth – is accompanied by a reduction in growth of the parental cells, the growth dynamics of the population will be negatively impaired. This peculiar aspect of diatom life cycle, might in fact further amplify the "cost of sex" for these unicellular microalgae (Lewis 1983), which would not only be due to the investment of biomass into gamete formation and the risk of finding the partner, but would also have the counter-effect of impacting the growth of vegetative cells.

Cell density threshold for sex. Although the need to reach a threshold cell density should be a fundamental requirement to allow the perception of chemical signals and/or encounter and conjugation of gametes, this aspect has been seldom addressed in planktonic microalgae and – to our knowledge – never in diatoms. In the heterothallic chrysophyte *Synura petersenii,* sexual reproduction has been shown to be density-dependent (Sandgren and Flanagin 1986) and in dinoflagellates the formation of gametes and subsequent encystment were not observed when containers were shaken, thus inhibiting cell clustering at a microscale (Persson et al. 2008). The fact that a threshold cell concentration has to be reached in the planktonic *P. multistriata* for sex to occur supports the fact that chemical cues are responsible for the induction of sexuality, as recently reported for two benthic diatoms (Sato et al. 2011, Gillard et al. 2013). Preliminary experiments show that gamete formation is indeed induced in *P. multistriata* strains when exposed to the culture medium conditioned by the growth of strains of the opposite mating type (E. Scalco, unpublished data). Moreover, the fact that a higher percentage of sexual stages was recorded in the experiments carried out in physically undisturbed conditions, as compared to the set up in which bottles were constantly mixed, provides additional evidence that cell aggregation is needed to trigger sexual reproduction. Visual inspection of undisturbed culture vessels indeed shows that a variable percentage of cells is not suspended in the water but sinks to the bottom of the vessel. The different results obtained in the two experimental settings with and without mixing can thus be attributed to the fact that cells exchange diffusible signals and the perception of these signals increases when cells reach a threshold density. We have calculated the theoretical distance between cells growing in mixed and still conditions and found that the estimated distance between cells is one order of magnitude lower in the TL at the bottom of the culture flask (~100 μm when gametes were first detected) than in mixed conditions. The markedly reduced distance between cells favors the perception of chemical signals and facilitates encounters between gametangia of opposite mating type. An alternative or complementary hypothesis is the presence of a quorum sensing-like mechanism that activates the production of sex pheromones only beyond a critical cell threshold. This mechanism has been reported for bacteria, where two antagonistic molecules regulate the transfer of antibiotic resistance through conjugation only within defined threshold concentrations of the donor cells (e.g., Lyon and Novick 2004, Chatterjee et al. 2013). This would optimize the investment of energy in the production of chemical cues to the conditions in which they will have the maximum chance of being effective.

The cell density at which gamete production was first detected varied between experiments carried out with different pairs of *P. multistriata* strains. All strains used in the present investigation have been isolated in the same geographic area and have been characterized with microsatellite markers proving that they belong to the same genetic population (data not shown). These results thus suggest that there is a notable intraspecific difference in the competence for sex. Similar results have been obtained for the centric diatom *Skeletonema marinoi,* where both sexual and asexual auxospores production differed amongst strains (Godhe et al. 2014). A considerable intraspecific diversity in other life cycle traits, such as production of resting stages and their dormancy length has been reported in dinoflagellates (e.g., Figueroa et al. 2005, 2006).

Two of the few reports of sexual reproduction in the natural environment deal with *Pseudo-nitzschia* species. In both cases, the sexual event involved two different species at the same time: *P. australis* and *P. pungens* along the NW Pacific coast (Holtermann et al. 2010), and *P.* cf. *pseudodelicatissima* and *P.* cf. *calliantha* in the Gulf of Naples, Mediterranean Sea (Sarno et al. 2010). The density of vegetative cells during the sexual event ranged from 187 to 929 cells · mL^{-1}, the cumulative cell concentration of both *P. australis* and *P. pungens* (Holtermann et al. 2010), to 700 cells · mL^{-1} for *P.* cf. *calliantha,* up to 9.1×10^3 cells · mL^{-1} for *P.* cf. *pseudodelicatissima* (Sarno et al. 2010). The range of cell concentrations spanned over one order of magnitude, but it is in the range recorded in the laboratory experiments carried out with *P. multistriata.* We have shown that in our experimental model species the formation of gametes started when parental cell concentration was comprised between 2,600 and 5,000 cells · mL^{-1} in the experiments carried out using small-volume culture plates, between 1,199 and 1,666 cells · mL^{-1} in the experiment conducted at still conditions, and between 325 and 450 cells · mL^{-1} in the experiment at constantly mixed conditions. While the values recorded in mixed bottles might be assimilated to a well-mixed water column, the values recorded in still conditions might be assimilated either to very high cell concentrations during a bloom or to values recorded in accumulation layers along the water column. The high concentration of *P. cf. delicatissima* (millions of

cells · L^{-1}) might represent an example of the first case, when sexual stages were indeed recorded in correspondence with one of the highest concentrations of this species ever recorded at the LTER-MC station (D. Sarno, pers. comm.). The formation of TLs due to physical processes such as vertical gradients of horizontal velocity due to shear, advection, or gradients in temperature and/or salinity can be produced along the water column (Durham and Stocker 2012). TLs can have a thickness in spanning from centimeters to a few meters, can extend horizontally for kilometers and persist for days. The small vertical structure that characterizes TLs makes them difficult to detect by conventional sampling and profiling instruments (McManus et al. 2008). In several cases, *Pseudo-nitzschia* species have been recorded in TLs, with cell concentrations comprised between 5×10^4 and 3.3×10^6 cells · L^{-1}, which were about 3 times higher than outside the accumulation layers (Rines et al. 2002, Ryan et al. 2005, McManus et al. 2008, Velo Suárez et al. 2008). It is thus reasonable to assume that the short-term TLs represent a place where sexual reproduction in *Pseudo-nitzschia* species can occur at sea. Model simulations have also shown that the formation of clusters of cells can also occur along the water column as a result of passive sedimentation under calm hydrodynamic conditions (Botte et al. 2013). Our time-course experiments, in which non-synchronized strains were used, showed that the formation of gametes can occur within a very short time interval (a few days), which is compatible with the life-time of TLs (Durham and Stocker 2012). The clustering of phytoplankton cells in TLs or their clustering due to passive sinking in low turbulence environments can facilitate the perception of chemical signals and/or increase the probability of mating success, due to higher cell concentration.

Sex in optimal conditions. The observation that optimal growth conditions are required for the onset of the sexual phase in pennate diatoms has been reported in several publications (e.g., Davidovich and Bates 1998, Amato et al. 2005, Chepurnov et al. 2005), but this statement was not supported by an experimental approach. We have provided quantitative experimental evidence showing that the timing and success of sexual reproduction in *P. multistriata* is related to the physiological condition of parental strains. Successful completion of the sexual phase with formation of auxospores and initial cells was in fact obtained only when crossing parental strains in their exponential growth phase. When crossing strains in the late exponential or in the post-exponential phase, the formation of a few gametes can still be recorded but the production of auxospores and initial cells does not take place. Sex is a costly event requiring energy for meiosis, production of gametes, synthesis of the auxospore, and de novo synthesis of the frustule of initial cell (Lewis 1983). Moreover, initial cells give rise to new cohorts of cells in the population, which require energy to increase in number. From a "demographic" perspective, it is thus expected that sex is related to positive growth conditions in the natural environment (Flatt and Heyland 2011). Plants and animals invest in offspring production when environmental conditions are favorable for growth and when food is available. However, in other microalgae such as dinoflagellates and chlorophytes, sex is generally induced by environmental conditions unfavorable for growth. Either phosphorous or nitrogen deficiency are required to induce the sexual phase in dinoflagellates (Pfiester and Anderson 1987), while shifts to considerably high temperature trigger sexualization in *Volvox* (Nedelcu and Michod 2003) and nitrogen starvation induces gamete differentiation and subsequent syngamy in *Chlamydomonas* (Beck and Haring 1996). Amongst diatoms, a link between low nutrient concentration and auxosporulation has been demonstrated only for *Leptocylindrus danicus* (French and Hargraves 1985). In the above mentioned taxa, sexual reproduction is generally followed by the transformation of the zygote into a resting stage, which might explain the role of adverse exogenous cues in triggering sex. Nevertheless, in chrysophyceans – where the sexual phase is also followed by the formation of a resting statospore – sexual reproduction takes place during periods of active population growth (Sandgren and Flanagin 1986). Although information on the proximate role of external cues in controlling sexual reproduction in different microalgal taxa is still too limited to allow generalizations, it is evident that different selective pressures shaped the structure of life cycle in these unicellular organisms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Schematic drawings illustrating the experimental set up of crosses carried out with *Pseudo-nitzschia multistriata* parental strains collected at different cell densities and growth phases.

Figure S2. Light micrographs of different life cycle stages of *Pseudo-nitzschia multistriata*.

Figure S3. Growth curves of *Pseudo-nitzschia multistriata* co-cultures started with parental strains collected at the same time points of their growth curve and diluted at different inoculum concentration (Experiment #3).

Figure S4. Growth curves of *Pseudo-nitzschia multistriata* co-cultures started with parental strains collected at the same time points of their growth curve and diluted at different inoculum concentration (Experiment #3).

Figure S5. Cell concentration (cells · mL^{-1}) of *Pseudo-nitzschia multistriata* parental strains (black squares), the sexual stages (yellow circles: gametes; green circles: auxospores; violet circles: initial cells) and the large F1 cells (light blue circles) in co-cultures incubated in mixed (a) and in still (b) conditions (Experiment #5). Each point represents the average of triplicates counts; SD is represented with vertical lines.

Figure S6. Estimated distance between adjacent *Pseudo-nitzschia multistriata* cells (Pm+, Pm−, and F1 generation are included) when cultures were incubated in mixed (a) and still (b) experimental conditions; note the different scale between (a) and (b).

Table S1. Strains used in the different experiments: strain code and mating type (in parenthesis), isolation date and the average length of the apical axis (n = 20 cells) when experiments were carried out.

Table S2. Nutrient concentrations (μmol · L^{-1}) measured at different time points along the growth curve of Pm− strain MVR171.8 and Pm+ strain MVR171.1. For each time point, two replicate measurements are reported.

Protoplasma
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ORIGINAL ARTICLE

The sexual phase of the diatom *Pseudo-nitzschia multistriata*: cytological and time-lapse cinematography characterization

Eleonora Scalco[1] · **Alberto Amato**[1] · **Maria Immacolata Ferrante**[1] · **Marina Montresor**[1]

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Abstract *Pseudo-nitzschia* is a thoroughly studied pennate diatom genus for ecological and biological reasons. Many species in this genus, including *Pseudo-nitzschia multistriata*, can produce domoic acid, a toxin responsible for amnesic shellfish poisoning. Physiological, phylogenetic and biological features of *P. multistriata* were studied extensively in the past. Life cycle stages, including the sexual phase, fundamental in diatoms to restore the maximum cell size and avoid miniaturization to death, have been well described for this species. *P. multistriata* is heterothallic; sexual reproduction is induced when strains of opposite mating type are mixed, and proceeds with cells producing two functionally anisogamous gametes each; however, detailed cytological information for this process is missing. By means of confocal laser scanning microscopy and nuclear staining, we followed the nuclear fate during meiosis, and using time-lapse cinematography, we timed every step of the sexual reproduction process from mate pairing to initial cell hatching. The present paper depicts cytological aspects during gametogenesis in *P. multistriata*, shedding light on the chloroplast behaviour during sexual reproduction, finely describing the timing of the sexual phases and providing reference data for further studies on the molecular control of this fundamental process.

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✉ Eleonora Scalco
eleonora.scalco@szn.it

[1] Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

Keywords Chloroplasts · Diatoms · Life cycle · *Pseudo-nitzschia multistriata* · Sexual reproduction · Time-lapse cinematography

Introduction

The genus *Pseudo-nitzschia* includes 45 species of marine planktonic diatoms that are important members of the phytoplankton communities in both coastal and open oceanic waters (Trainer et al. 2012; Teng et al. 2014). A considerable amount of information has been gathered in the last decades on the distribution of the different species, their physiology, toxicology and genetic diversity, making them one of the best known genera of marine phytoplankton (see reviews by Lelong et al. 2012; Trainer et al. 2012). This interest stems from the fact that some *Pseudo-nitzschia* species produce domoic acid (Mos 2001), a neurotoxin responsible for the amnesic shellfish poisoning syndrome (Pulido 2008). Since 1989, when the first paper describing the life cycle of a *Pseudo-nitzschia* species was published (Davidovich and Bates 1998), information has been gained on the life cycle features of 14 different species and 1 variety (reviewed in Lelong et al. 2012). Almost all the investigated species have a heterothallic life cycle; i.e. sexual reproduction was obtained only when strains with opposite mating type get in contact (Fig. S1). Up to now, the only documented exception is *Pseudo-nitzschia brasiliana* Lundholm, Hasle and Fryxell, where sexual stages were observed in clonal cultures (Quijano-Scheggia et al. 2009). The basic mode of the sexual phase of the life cycle is conserved among *Pseudo-nitzschia* species (Lelong et al. 2012). Upon mixing two strains of compatible mating type and in the cell size window for sexualization, some cells align side to side and differentiate into gametangia. Two gametes are produced within each gametangium; one gametangium produces *active*

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(+) gametes that migrate towards the *passive* (−) partners and conjugate. The zygote transforms into an auxospore, which is not surrounded by the rigid frustule, and within the auxospore, a large-sized initial cell is produced (Fig. S1).

To date, various aspects of the life cycle of these pennate diatoms have been investigated, spanning from the description of the sexual phase in different species (e.g. Amato et al. 2005; Amato and Montresor 2008; D'Alelio et al. 2009) to investigations on mating compatibility to test the biological species concept (Amato et al. 2007; Casteleyn et al. 2008; Quijano-Scheggia et al. 2008; Amato and Orsini 2015) and to studies on the variability of toxin production among F1 generation strains (Amato et al. 2010) and chloroplast inheritance mode (Levialdi Ghiron et al. 2008). Experimental studies have addressed the effect of different light/dark cycles on the success of sexual reproduction (Hiltz et al. 2000) and the dynamics of the sexual phase in relation to different concentrations of the parental strains (Scalco et al. 2014). Evidence for sexual events involving *Pseudo-nitzschia* species in the natural environment has been provided (Holtermann et al. 2010; Sarno et al. 2010), and the timing of sexual events has also been estimated by following cell size patterns of natural populations over time (D'Alelio et al. 2010).

We investigated the progression of the sexual phase of the marine pennate diatom *Pseudo-nitzschia multistriata* (Takano) Takano by means of light microscopy (LM), confocal laser scanning microscopy (CLSM) and time-lapse microscopy (TLM). TLM and CLSM technologies represent powerful tools to decipher cytological features of sexual reproduction and cell cycle in diatoms (Sato et al. 2011; Laney et al. 2012; Edgar et al. 2014). This technique was used to follow sexual events in other organisms, e.g. in the red alga *Bostrychia* Montagne to track nuclear fate and plasmogamy during sexual reproduction (Pickett-Heaps and West 1998), or to study the formation and release of gametes in *Ulva* Linnaeus (Wichard and Oertel 2010). Here, we focus on gametogenesis and conjugation and describe the nuclear behaviour during meiosis. Time-lapse cinematography allowed to estimate the time required for the formation of gametes, to describe their conjugation and to illustrate chloroplast division and segregation in the two gametes produced within the gametangial cell. *P. multistriata* has been successfully genetically transformed using a biolistic method (Sabatino et al. 2015), enabling a more detailed follow-up of the present study using e.g. fluorescent protein fusions with markers of different subcellular compartments to follow each organelle during meiosis, gametogenesis and plasmogamy/karyogamy. A de novo genome sequencing project is in progress for this species, and a number of transcriptomes are already available (M.I. Ferrante, unpublished data). These resources and techniques applied on a species whose sexual cycle is well established and easily manipulated will enable functional studies and further investigations on life cycles which are not currently possible in other diatom model species.

Material and methods

Six strains of *P. multistriata* were used for the experiments and the observations in time-lapse microscopy (Table S1). Strains were isolated and cultured as illustrated in Scalco et al. (2014).

Time course of sexual reproduction and confocal laser scanning microscopy

Two independent mating experiments were carried out with one couple of strains of opposite mating type. The average cell size of Pm^- and Pm^+ strains was different but always below the threshold size for sexualization in order to monitor parental cells of the two mating types (Table S1). For each experiment, a culture flask containing 100 mL of F/2 culture medium (Guillard 1975) was inoculated with both parental strains at a final concentration of 5×10^3 cells mL^{-1} each. Aliquots of 4 mL were dispensed in three 6-well culture plates, for a total of 18 wells. Plates were incubated in a growth chamber at the same conditions at which strains were grown (18 °C, 60 μmol photons m^{-2} s^{-1}, 12:12 h dark/light photocycle). Every 12 h and for a total of six sampling points, the content of 3 wells was fixed with neutralized formaldehyde at a final concentration of 1.6 % *v/v*. In order to visualize the nuclear behaviour, the samples were stained with SYBR Green I (S7567, liquid; Molecular Probes, Leiden, The Netherlands) to a final dilution of 1:10,000 for 15 min in the dark at room temperature. The concentration of the different life cycle stages (i.e. large and small parental cells), meiotic cells with segregated cytoplasm, round gametes with one nucleus, round zygotes with two nuclei, auxospores and initial cells was estimated using the Utermöhl method (Edler and Elbrächter 2010) using the Zeiss Axiovert 200 epifluorescence microscope equipped with the FS09 filter (exCitation, 450 to 490 nm; emission, 515 nm) at ×400 magnification.

A parallel experiment, at the same conditions and with the same strains, was run in glass bottom Petri dishes (WillCo Wells B.V., Amsterdam, The Netherlands) for CLSM. Petri dishes were inspected at a laser excitation of 488 nm, with 1–2 % of laser intensity, and a BP 500–550 acquisition filter was used. Images of the different life cycle stages were captured at different magnifications with by Zeiss LSM 510 META CLSM (Carl Zeiss, Oberkochen, Germany).

Time-lapse microscopy

Microcinematography was carried out in bright field (BF) with a Leica DMI 6000B time-lapse microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica

DFC360 FX photocamera. Two pairs of strains, differing in their average apical axis length, were used (Table S1), and co-cultures of the two parental strains in glass bottom Petri dishes were started at the same cell concentration as above. Time-lapse sessions were carried out at different time points, i.e. starting from 24 and 48 h after the inoculum of the parental strains and lasting 16 h. A frame frequency of 1.33 frames min^{-1} (one frame every 45 s) and the *best focus* option were selected.

Two controls were set up: (i) one Petri dish was kept in the growth chamber to confirm that strains underwent sexual reproduction with the expected timing and (ii) another Petri dish was placed in the microscope room at the moment of time-lapse experiment but was wrapped in aluminium foil to avoid exposition to the light flashes of the microscope; this control was meant to confirm that the conditions in the microscope room did not impair the success and timing of sexual reproduction. Control Petri dishes were inspected using a Zeiss Axiovert 200 light microscope (Carl Zeiss, Oberkochen, Germany) at ×400 magnification to check for the presence of sexual stages.

Results

Sexual reproduction: time course

The results of two parallel experiments in which parental strains of opposite mating type were co-cultured and monitored for three consecutive days are shown in Fig. 1. Within each pair, strains of opposite mating type differed in cell size, in order to track them in the culture vessels. The concentration of parental strains remained almost constant during the whole experiment. Paired gametangia were observed 12 h after the inoculum. At the same time, paired gametangia showing cleavage of the cytoplasm (meiotic cells) were observed, when vegetative cell concentration was $\geq 5 \times 10^3$ cells mL^{-1} (Fig. 1a). Meiotic cells reached a maximum concentration of 287.1±234.5 cells mL^{-1} after 60 h from the inoculum with an average maximum percentage of 3.24±3.3 % (Fig. 1a). Observation in CLSM of samples stained with the nuclear stain SYBR Green I after 24 and 36 h provided evidence that these are stages where cells undergo meiotic division (Fig. 2a–f). Meiosis occurred asynchronously in the two mating types, with the Pm^+ mating type (in this case, the smaller cell) starting first (Fig. 2a–f). In Fig. 2a, b, two paired gametangia are shown: the nucleus of the larger Pm^- cell on the left had lost its rounded shape and had begun to expand, approaching the early stage of meiotic prophase. The smaller Pm^+ gametangium on the right contained two protoplasts, each of them with a nucleus. Meiosis I was completed in Pm^+ and cytokinesis that is visible as a cleavage separating the two portions of the gametangial cell took place. During the second meiotic division, only karyokinesis occurred in the Pm^+ gametangium, with the formation of two haploid nuclei within each protoplast (Fig. 2c, d). The small Pm^+ gametangium shown in Fig. 2e, f contained two protoplasts with one nucleus each, suggesting that pyknosis, i.e. the degeneration of one of the two nuclei originating from meiosis II, had already taken place, while the large Pm^- gametangium had completed meiosis II and had two haploid nuclei in each protoplast. Cells with cleaved cytoplasm, i.e. gametangia undergoing meiosis, were detected till the end of the experiment (Fig. 1a). Round gametes with one nucleus encased in the gametangial frustule (Fig. 2g) were first detected 24 h after the inoculum (Fig. 1b), while zygotes and auxospores were observed after 36 h (Fig. 1b, c). At times, gametangia detached from each other and/or gametes detached from their gametangium were then observed free in the medium. Round stages with two nuclei and generally attached to the gametangial frustule were identified as zygotes. Zygotes had a well-defined rounded shape due to the presence of the primary zygote wall. Gametes and zygotes reached the maximum average percentage of 15.2±9.6 and 3.06±1.9 %, respectively, after 48 h from the inoculum (Fig. 1b, c). The conjugation of gametes of opposite mating type (see the following section for details) produced a zygote, called auxospore (Fig. 2h), with two haploid nuclei that fused only after completion of auxospore elongation. The maximum auxospore concentration (>1×10^3 cells mL^{-1}, 7.13 %) was observed after 60 h from the inoculum (Fig. 1c), at the same time when initial cells were detected (Fig. 2i, j).

A triploid zygote and a tetraploid auxospore were also observed, the former bearing six chloroplasts and three nuclei (Fig. S2a-b), the latter with eight chloroplasts and four nuclei (Fig. S2c-d).

Time-lapse microscopy

In order to follow the behaviour of Pm^+ and Pm^- during mate search and the first steps of gametogenesis, mating experiments with strains considerably different in apical axis length were carried out (Table S1). The longer strain, regardless of the mating type, seemed more active and moved more rapidly than the shorter one. Thus, in experiments involving longer Pm^-, these scanned the environment in search for the Pm^+. In experiments involving longer Pm^+, it was the opposite (Movie S1).

In the paired gametangia, cytoplasmic movement was evident during the process of gametogenesis, which included the two meiotic divisions illustrated above as well as the rearrangement of cytoplasmic organelles (Movie S2). The observations in time-lapse cinematography (Movies S3 and S4) allowed following the movement of chloroplasts, which is also illustrated in Fig. 3, prepared with frames extracted from Movie S3 and with schematic drawings (Fig. 4) that help to follow the process. At the beginning of meiosis I, plastokinesis occurred and four daughter chloroplasts were clearly visible in each gametangium (Figs. 3c, d and 4b). After completion of meiosis I and before

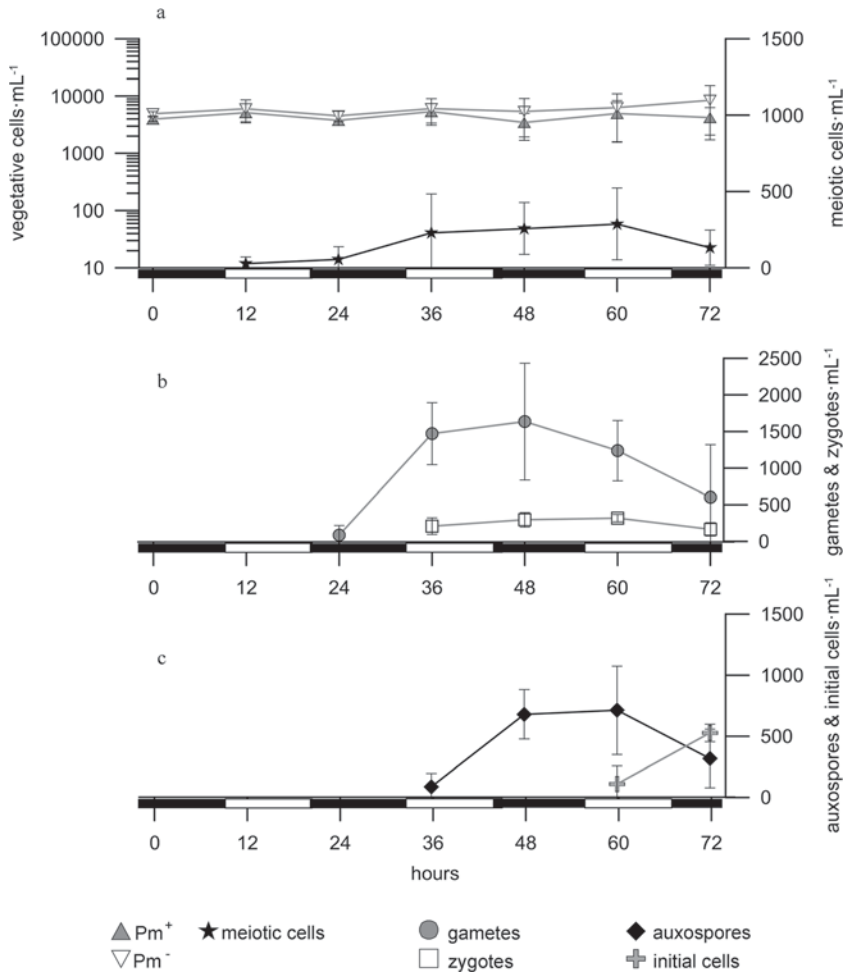

Fig. 1 Time course of sexual reproduction in *P. multistriata* and cell concentration during the different stages: *a* parental cells (*left axis*, log scale; Pm^+, *grey up-triangles*; Pm^-, *white down-triangles*) and meiotic cells (*right axis*; *black stars*); *b* gametes, *grey circles*, and zygotes, *white squares*; and **c** auxospores, *black diamonds*, and initial cells, *grey crosses. Each point* represents the average of six replicated counts± standard deviation. *The black and white bars on the x-axis* indicate the light and dark periods

plasmokinesis, each chloroplast slid along the frustule in concert with the other chloroplasts: the two daughter chloroplasts on the left side slid upwards and the other two on the right, downwards (Figs. 3e–j and 4c). Plasmokinesis occurred that is visible as a cleavage in the middle portion of the gametangial cell (Fig. 3k–m). The two portions of cytoplasm, i.e. the two gametes, thus contained two daughter chloroplasts, each of them deriving from one mother chloroplast of the gametangial cell (Figs. 3n–p and 4d, Movies S3 and S4) and one nucleus that will subsequently undergo meiosis II (Fig. 2c, d). The duration of gametogenesis at the selected experimental conditions was estimated as the time elapsing from the first evidence of cytoplasmic movement in the paired gametangia and the complete formation of two round gametes in each gametangium and lasted, on average, 1 h and 59 min and 24 s±26 min and 51 s (data gained from 5 time-lapse movies). Observations in TLM showed that gametogenesis always started in the Pm^+ gametangium.

Once gamete formation was completed, the gametangial frustules opened up and set the active gametes (Pm^+) free to join the passive ones (Pm^-) (Fig. 4e, Movie S5). Active gametes always conjugated with the passive gametes located on the opposite gametangium, type IA2 functional anisogamy sensu Geitler (1973), in a cross fashion (Fig. 4e, f). The conjugation process yielded one zygote bearing four chloroplasts. Within each zygote, all the four plastidial genomes of the parental cells were thus represented (Figs. 3 and 4f). The duration of the conjugation process at the selected experimental conditions was estimated as the time elapsing from the completion of gamete formation and the completion of the conjugation process of the two pairs of gametes and lasted, on average, 1 min and 35±47 s (data gained from 12 time-lapse movies).

The zygote expanded in a bipolar way to produce the auxospore (Fig. 4g, Movie S2). The two haploid nuclei, which remained separated until the completion of expansion, were close to each other and located in the central portion of the auxospore (Fig. 2h). In the expanded auxospore, the four chloroplasts became very elongated (Movie S2). The entire auxospore elongation was never followed because the individual movies only lasted 16 h. However, an approximate calculation of auxospore elongation was possible using different clips. At the beginning, the elongation was faster with 4.78 μm h^{-1} while, at the end the elongation speed, dropped

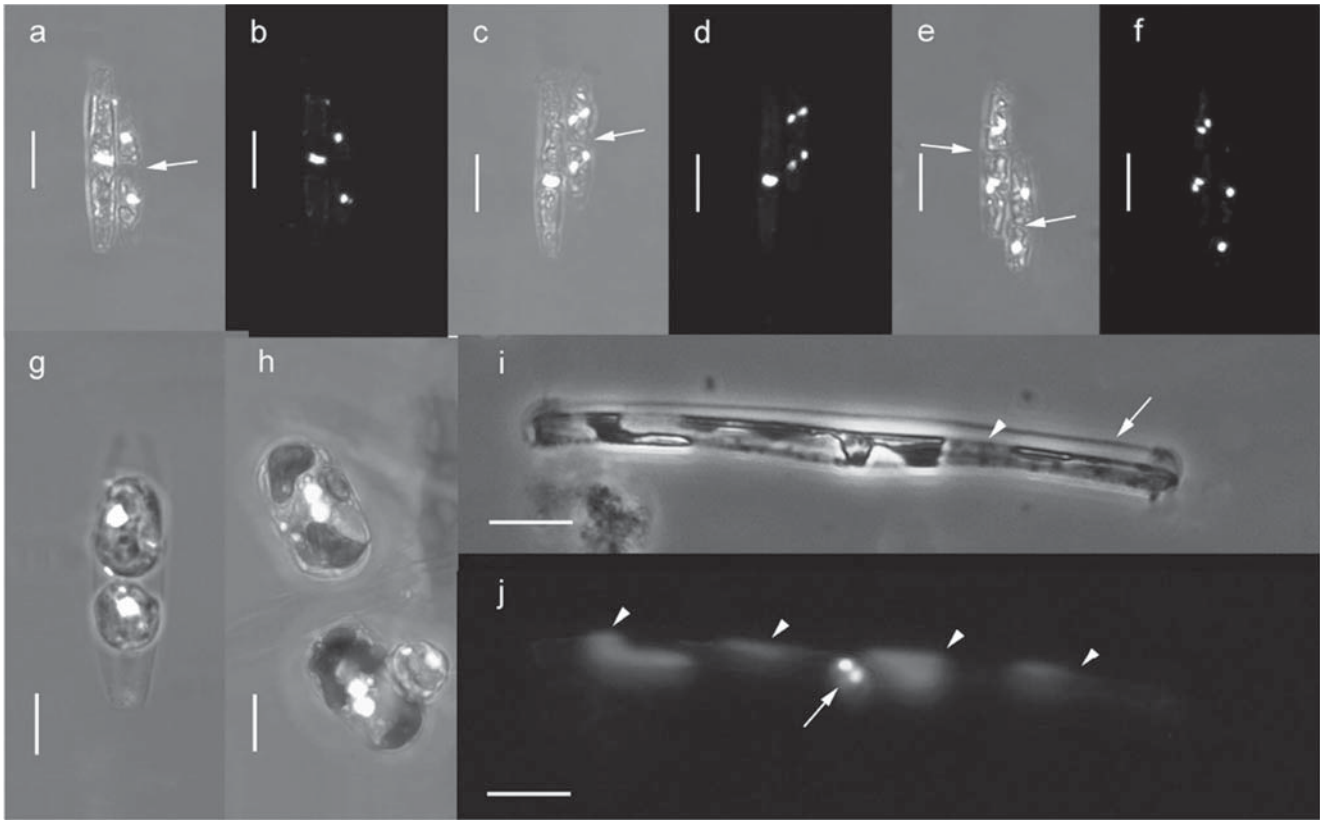

Fig. 2 Confocal Z-stack projections (**a**–**h**) and LM micrographs (**i**, **j**) of different sexual stages of *P. multistriata*. Nuclei are stained with SYBR Green I. Gametogenesis (**a**–**f**), each pair of pictures includes the merge of a bright-field image with the corresponding 488 nm excitation image (**a**, **c**, **f**) and the 488 nm argon/2 laser excitation image only (**b**, **d**, **e**). **a**, **b** The small gametangium on the right has gone through meiosis I and has one nucleus inside each protoplast; the cytoplasm segregation is *arrowed* in **a. c**, **d** The small gametangium on the right has completed meiosis II and has two nuclei inside each protoplasm; the cytoplasm segregation is *arrowed* in **c. e**, **f** The small gametangium on the right has completed meiosis II, and one of the two nuclei in each protoplasm has degenerated; the large gametangium on the *left* has completed meiosis II, and two nuclei are still present in each protoplasm. The cytoplasm segregation in both gametangia is *arrowed* in **e. g** Two round gametes with one nucleus each. **h** Two early auxospores, each one with two nuclei. **i**, **j** Formation of the initial cell inside the auxospore. **i** The new epi-valve (*arrowhead*) has been synthetized inside the perizonium (*arrow*), phase contrast micrograph. **j** The same cell in epifluorescence illumination, with the four chloroplasts (*arrowheads*) and the two nuclei (*arrow*). *Scale bars*=10 μm (**a**–**f**, **i**, **j**) and 5 μm (**g**, **h**)

to 1.5 μm h^{-1}, with an average of 3.9±2.4 μm h^{-1} (data gained from 15 time-lapse movies). It took about 20.5 h for a complete auxospore expansion. When the auxospore expansion was completed, the two nuclei fused to produce the diploid nucleus. The frustule of the initial cell, which contained four chloroplasts, was subsequently deposited within the auxospore (Fig. 2i, j), and the initial cell escaped the perizonium (Fig. 4h). At the first mitotic division that is not accompanied by plastokinesis, the four chloroplasts segregated into the two post-initial cells. The first mitotic division took place when the initial cell was still surrounded by the perizonium or after the initial cell hatched.

Reorganization into gametes of cell content is a crucial process for a correct gametogenesis to occur. A failure in this precisely orchestrated process impairs gametogenesis and, eventually, conjugation. Evidence for these failures was provided by the observation of chloroplast displacement (about 30 % of the total observation), where the two daughter chloroplasts from one mother chloroplast took the wrong direction in the gametangium. In the example illustrated with schematic drawings in Fig. 5 and shown in Movies S6 and S7, one of the two daughter chloroplasts (indicated in white, Fig. 5) went upwards while the other one went downwards (Fig. 5c). This produced the formation of three gametes instead of two (Fig. 5d, Movies S6 and S7). The gamete in the central part of the gametangium contained two daughter chloroplasts (one black and one white in Fig. 5), while on opposite poles of the gametangium, two smaller protoplasts were produced containing one daughter chloroplast each (Fig. 5d). Nuclear behaviour was not followed in this experiment, but it can be hypothesized that the central gamete contained one nucleus and one of the two apical gametes contained the other one. One alternative possibility is that the central gamete contained both nuclei and the two apical protoplasts were akaryotic. The central gamete conjugated with one gamete of the opposite gametangium and produced an apparently

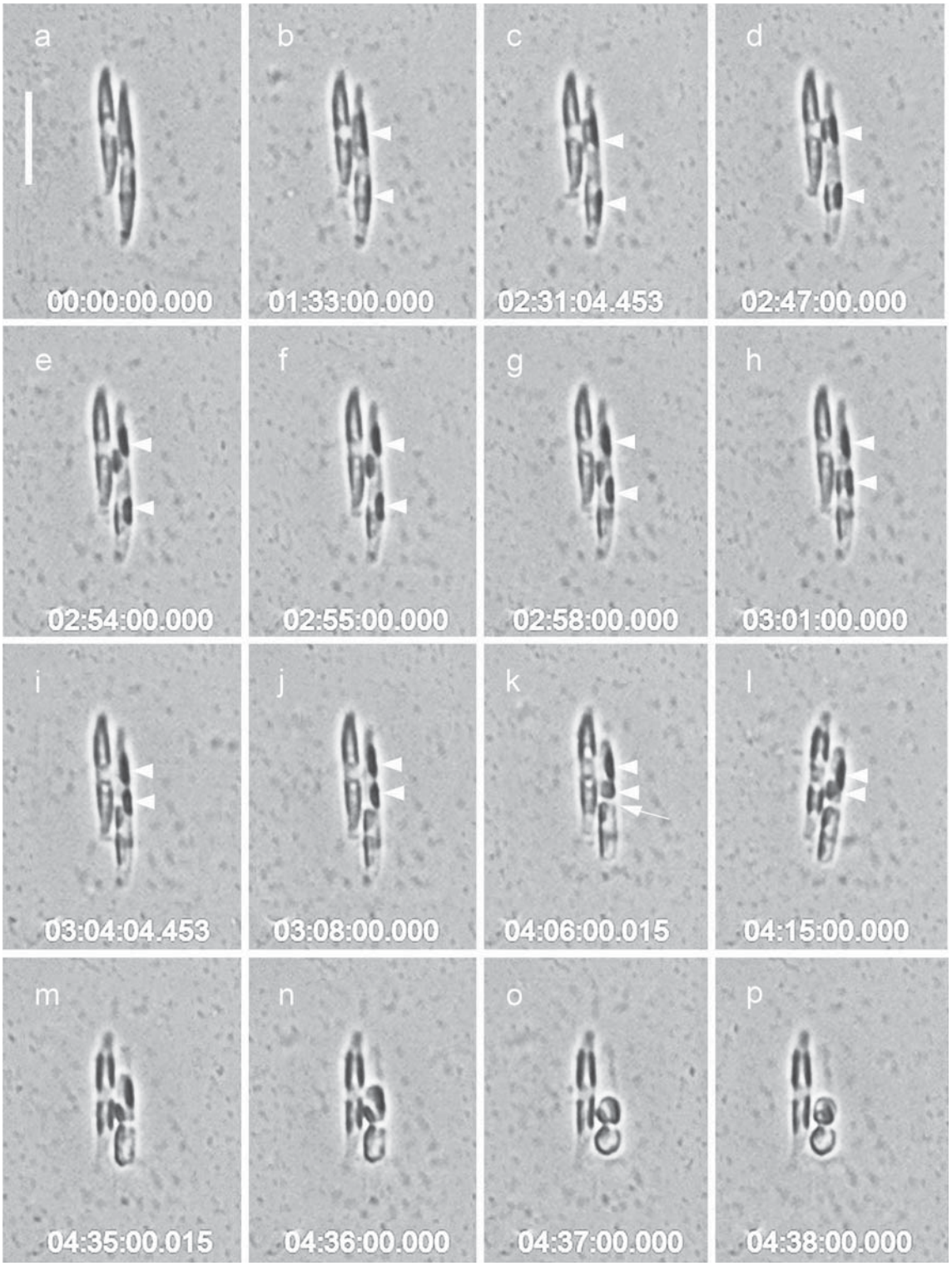

Fig. 3 Chloroplast translation and gametogenesis in *P. multistriata*. Pictures are selected frames of Movie S3. Gametogenesis is not synchronous in *P. multistriata* and occurs first in the gametangial cell on the *right*. *Arrowheads* indicate the two chloroplasts on the *right side* of this gametangium. On the *bottom* of each picture, the time elapsed from the beginning of the time-lapse experiment is reported. The schematic drawings in Fig. 4 help the reader to follow the main steps of the processes. **a** Two parental cells of opposite mating type aligned side to side (Fig. 4a). Note that the chloroplasts already started dividing. **b** Chloroplast division was completed, and each cell contains four daughter chloroplasts. **c**, **d** In the cell on the *right*, chloroplasts contract to prepare translation (Fig. 4b). **e** Chloroplast translation starts; the chloroplasts on the *right-hand side* will slide upwards (*arrowheads*), while the other couple will slide downwards. **f**–**j** The process of chloroplast translation (Fig. 4c) is accomplished in 14 s [from 2 min and 54 s (**e**) to 3 min and 8 s (**j**)]. **k**–**m** Plasmokinesis occurs, marked with an *arrow* on **k. n**–**p** Gametes round up (Fig. 4d); each gamete contains two chloroplasts, deriving from the division of the parental chloroplasts. *Scale bar*=20 μm

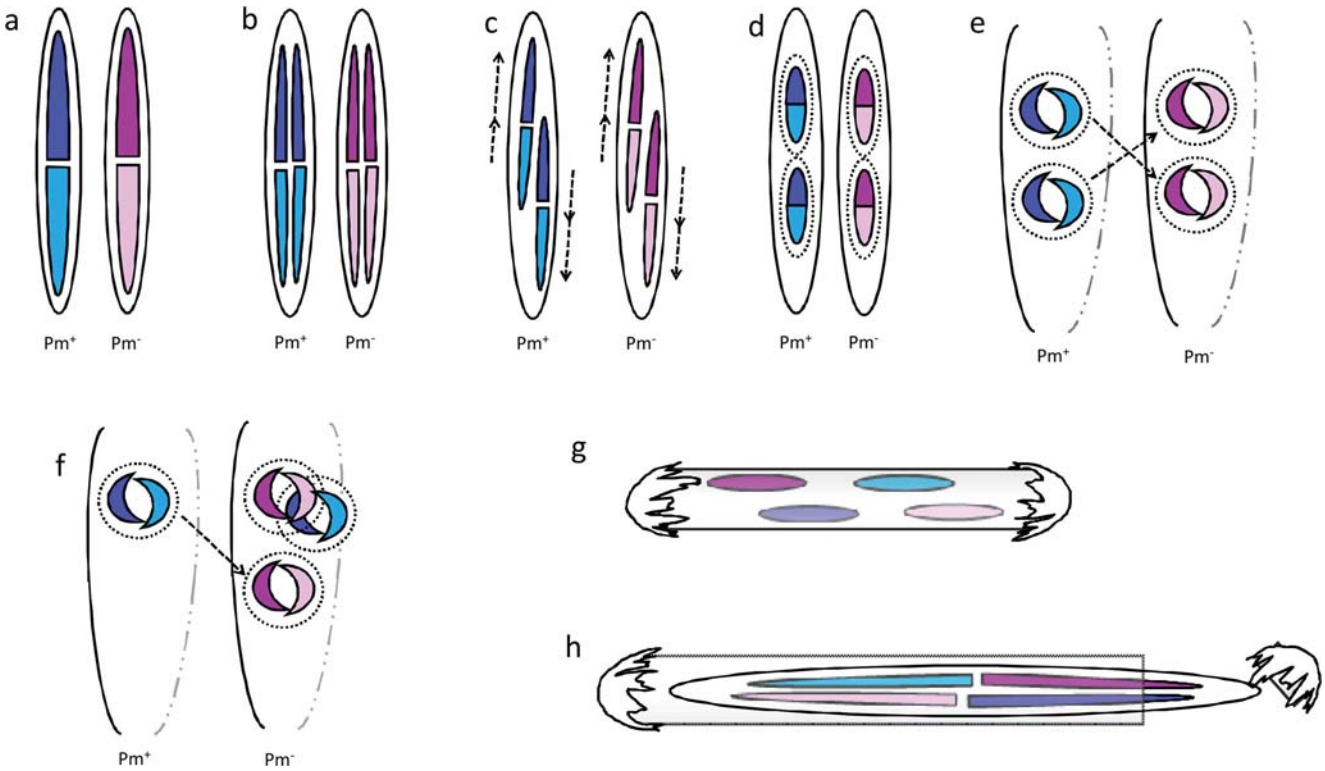

Fig. 4 Schematic drawings of the sexual stages of *P. multistriata* with focus on chloroplast behaviour. Pm^+ and Pm^- indicating the different mating types. **a** Paired gametangia with two chloroplasts each. **b** Chloroplast division in each gametangium. **c** Chloroplasts migration in opposite directions indicated with *arrows*. **d** Cytoplasm cleavage and formation of two round gametes, each of them with two chloroplasts. **e** The frustule opens, with the *arrows* indicating the movement of the gametes and chloroplast. **f** Conjugation of the first two gametes. **g** The elongated auxospore with four chloroplasts. **h** The initial cell escapes from the perizonium

normal zygote with four chloroplasts, while one apical gamete degenerated immediately after formation. The conjugation of the other apical gamete led to a zygote with only three chloroplasts.

One case of automixis was observed, where two gametes from the same gametangium conjugated and produced a zygote that, soon after, degenerated (Movie S8).

Discussion

Sexual reproduction: time course experiment and time-lapse cinematography

The results of the two short-term time course experiments showed that parental cells did not significantly increase in number when sexual stages were produced, thus confirming previous observations (Scalco et al. 2014): vegetative division is inhibited when the two strains of opposite mating type get in contact. The arrest of the cell cycle in conjunction with the onset of the sexual phase has been reported also in fungi, where it has been interpreted as a response to pheromone signalling (Cote and Whiteway 2008; Garcia-Muse et al. 2003).

The examination of samples stained with the nuclear stain SYBR Green I allowed to distinguish gametangia in the process of producing gametes and early-stage zygotes. Experiments started with a cell concentration of 5×10^3 mL^{-1} for each parental strain, and the first appearance of gametes was recorded after 24 h from co-culturing, corroborating previous findings (see Fig. 2 relative to experiment 2 in Scalco et al. 2014). Gametangia undergoing meiosis were detected earlier, 12 h after co-culturing started, and were still present after 2 days, albeit in lower concentrations. The various sexual stages appeared in sequence: the gametes after 24 h, reaching their maximum number after 48 h and subsequently decreasing; the first auxospores after 36 h and constantly increasing till 60 h; and the first initial cells after 60 h. A standardized experimental set-up is of fundamental importance to address questions related to the regulation of the life cycle, e.g. the mechanisms that trigger gamete attraction or the presence of molecular checkpoints during the formation of sexual stages.

The observations in time-lapse microscopy of co-cultures of strains of opposite mating type showed an active behaviour of the large-sized strains that seem to actively search for cells of the opposite mating type. This behaviour is apparently not

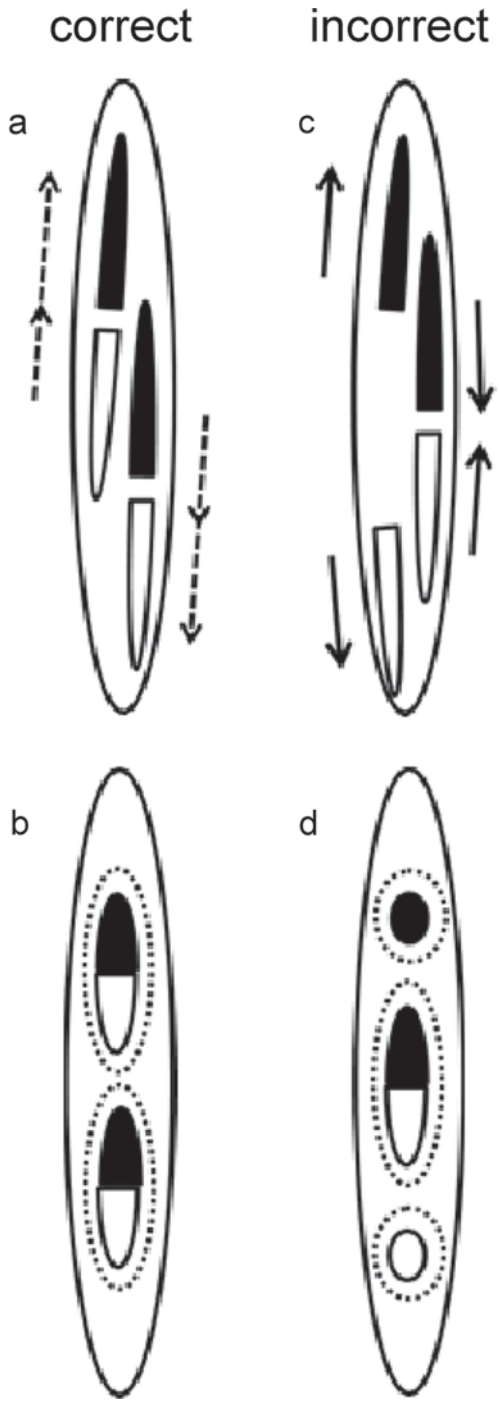

Fig. 5 Schematic drawings illustrating the correct (*a*, *b*) and the incorrect (*c*, *d*) chloroplast movement during gametogenesis in *P. multistriata*: *a* correct chloroplast behaviour and *b* formation of two gametes with two chloroplasts each; *c* anomalous chloroplast behaviour and *d* formation of one gamete with two chloroplasts and two round bodies with one chloroplast each

related to the mating type but to cell size and markedly differs from what is reported for the benthic pennate raphid diatom *Seminavis robusta* Danielidis and Mann, where MT^+ cells actively moved around an attracting MT^- cell (Gillard et al. 2013).

We have estimated by time-lapse microscopy the time required for the formation of gametes (between 1 h and 19 min and 2 h and 23 min), and the very fast conjugation process only lasted a couple of minutes. This raises the question of how planktonic cells can perceive each other and pair (i.e. gametangial pairing) in the water column. It has been hypothesized that sex might occur in thin layers of physical discontinuity (Rines et al. 2002), where density gradients can facilitate encounters (Amato et al. 2005; Scalco et al. 2014) or that hydrodynamic interactions at low Reynolds number between sinking cells or chains might favour contacts between them (Botte et al. 2013).

Chloroplast arrangement during gametogenesis

In *P. multistriata*, like in other diplastidic biraphid pennate diatoms (Mann 1996; Round et al. 1990), chloroplasts divide along the apical axis of the cell during mitotic division and segregate in the two daughter cells. In several pennate diatoms, chloroplasts can rotate before mitosis in order to reach the proper position at the moment of cytokinesis (chloroplast translation).

A series of observations of co-cultures of opposite mating type in time-lapse microscopy allowed tracking the behaviour of chloroplasts during gametogenesis. In the gametangial cell, the two chloroplasts divide and two sibling plastids of each parental chloroplast migrate at the opposite poles of the cell. When cytokinesis occurs after the first meiotic division, each gamete thus inherits sibling plastids from both chloroplasts present in the gametangial cell (biparental inheritance). We observed abnormal chloroplast translation that yielded one central gamete with two daughter chloroplasts and two residual bodies at the poles of the cell with one chloroplast each. We could follow the conjugation of the normal gamete and one of the two apical bodies with the two gametes of the opposite gametangium but could not assess if the zygotes were viable. These irregular movements of chloroplasts (and, most probably, other organelles, including nuclei) and consequent production of malfunctioning gametes were observed in culture; if this also occurs in natural populations, it might represent an additional cost of sex for diatoms (Lewis 1983).

The pattern of chloroplast segregation into gametes has implications for the inheritance of plastidial genomes. In higher plants, a considerable diversity of plastid inheritance patterns exists and both uniparental and biparental models are known (Greiner et al. 2015). Few are the studies on chloroplast inheritance patterns for micro- and macro-algae. In the green micro-algae *Chlamydomonas reinhardtii* Dangeard and *Volvox* Linnaeus (Chlorophyta), plastids are inherited from the *maternal* mating type (Miyamura 2010). In brown algae that, together with diatoms, belong to Stramenopiles, inheritance of chloroplasts is biparental in isogamous species while it is

maternal in oogamous ones (Motomura et al. 2010). The male flagellate gametes of centric diatoms do not have chloroplasts or have very reduced ones (Jensen et al. 2003), whose fate in the zygote remains, however, unknown. The chloroplast inheritance in diatoms depends on two processes: their segregation pattern during gamete formation and the segregation pattern that occurs during the first mitotic division of the initial cell. Establishing how the two steps occur is critical, especially for diplastidic diatoms, where each gamete can inherit one of the two chloroplasts of the maternal cell or copies of both of them. Time-lapse microscopy observations provided, for the first time, information on chloroplast transmission mode in the gametes of the pennate diatom *P. multistriata*. Levialdi Ghiron et al. (2008) took advantage of sexually compatible strains of *Pseudo-nitzschia arenysensis* Quijano-Scheggia, Garcés and Lundholm (*Pseudo-nitzschia delicatissima* (Cleve) Heiden in the paper) with a distinct rbcL ribotype to examine the inheritance pattern in several cultures established from the isolation of large F1 cells. It was shown that chloroplasts segregate stochastically during the first mitotic division of the initial cell. We add to this study the information that each of the two auxospores bears an identical chloroplast assortment; i.e. it has chloroplasts originating from both parental cells. This inheritance mechanism guarantees the highest diversity. Evolution is driven by mutations and fixation: therefore, inheriting two different chloroplast genomes can boost evolution but the presence of a backup chloroplast reduces the risks of fixing a deleterious mutation.

During gametogenesis, we observed daughter chloroplast translation that places the four newly divided plastids in the proper position for gametogenesis. Conversely, during vegetative division, no chloroplast displacement was observed. In other pennate diatoms, chloroplasts move within the cell at mitosis (Mann 1996) while *P. multistriata* does not show such movements at mitosis but at gametogenesis, which occurs very rarely (D'Alelio et al. 2010). Minimizing chloroplast displacement could play a role in optimizing the cellular energetic budget during cell cycle.

Polyploidization and automixis

When observing samples with stained nuclei in CLSM, we could detect a triploid zygote and a tetraploid auxospore in *P. multistriata* co-cultures. They should be the product of the conjugation of three and four gametes, respectively, or of the conjugation between gametes in which pyknotic degeneration of nuclei after the second meiotic division did not occur. The production of polyploid auxospores and initial cells was reported for *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle (Chepurnov et al. 2005), *Dickieia ulvacea* Berkeley ex Kützing (Mann 1994), *S. robusta* (Chepurnov et al. 2002) and other diatoms (reviewed in Chepurnov et al. 2004). Only for *D. ulvacea* the viability of these polyploid stages was assessed, but their capability to undergo sexual reproduction has not been determined (Mann 1994). Evidences of polyploidization in diatoms are scarce (Kociolek and Stoermer 1989; von Dassow et al. 2008; Koester et al. 2010), but the production of anomalous sexual stages might represent a mechanism through which genome content duplicates and polyploid lineages arise.

Time-lapse microscopy provided also evidence for automixis, i.e. conjugation of gametes produced within the same gametangium, in *P. multistriata*. Zygotes, though, rapidly degenerated after their formation. This fits with the fact that sexual stages were never observed in monoclonal cultures of *P. multistriata* (D'Alelio et al. 2009; Scalco et al. 2014), and suggests that automixis is a very sporadic event in this species.

A very intriguing question to be addressed would be the definition of the intricate cytological and molecular machinery regulating chloroplast displacement.

Microscopy studies focusing on diatom life cycles date back to more than 150 years ago (e.g. Thwaites 1847), and since then, a large amount of information has been produced describing different cell processes in many species. Advanced tools in microscopy, and most importantly emerging molecular tools, are now allowing to leap forward in the description and understanding of key phases in diatom life cycles. *P. multistriata*, with the extensive information available in the literature and because of the recent development of molecular tools (Sabatino et al. 2015) and genomic resources, represents a promising model species to address different questions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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PLOS ONE

RESEARCH ARTICLE

Temporal Changes in Population Structure of a Marine Planktonic Diatom

Sylvie V. M. Tesson*¤, Marina Montresor, Gabriele Procaccini, Wiebe H. C. F. Kooistra

Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

*sylvie.tesson@gu.se

¤ Current address: Department of Chemistry and Molecular Biology, University of Gothenburg, PO Box 462, SE-405 30 Gothenburg, Sweden

Abstract

A prevailing question in phytoplankton research addresses changes of genetic diversity in the face of huge population sizes and apparently unlimited dispersal capabilities. We investigated population genetic structure of the pennate planktonic marine diatom *Pseudo-nitzschia multistriata* at the LTER station MareChiara in the Gulf of Naples (Italy) over four consecutive years and explored possible changes over seasons and from year to year. A total of 525 strains were genotyped using seven microsatellite markers, for a genotypic diversity of 75.05%, comparable to that found in other *Pseudo-nitzschia* species. Evidence from Bayesian clustering analysis (BA) identified two genetically distinct clusters, here interpreted as populations, and several strains that could not be assigned with ≥90% probability to either population, here interpreted as putative hybrids. Principal Component Analysis (PCA) recovered these two clusters in distinct clouds with most of the putative hybrids located in-between. Relative proportions of the two populations and the putative hybrids remained similar within years, but changed radically between 2008 and 2009 and between 2010 and 2011, when the 2008-population apparently became the dominant one again. Strains from the two populations are inter-fertile, and so is their offspring. Inclusion of genotypes of parental strains and their offspring shows that the majority of the latter could not be assigned to any of the two parental populations. Therefore, field strains classified by BA as the putative hybrids could be biological hybrids. We hypothesize that *P. multistriata* population dynamics in the Gulf of Naples follows a meta-population-like model, including establishment of populations by cell inocula at the beginning of each growth season and remixing and dispersal governed by moving and mildly turbulent water masses.

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Introduction

Marine planktonic organisms can grow extremely fast. Such fast growth, sustained by plentiful resources and temporally relaxed predation pressure, can lead to episodic, rapid and vast increases in their population sizes. The huge numbers of individuals and the moving and mixing water masses they inhabit are expected to foster large-scale population genetic homogeneity. Yet, a series of recent studies demonstrated that geographic structuring can occur in marine planktonic organisms [1–3]. In the case of the jellyfish *Aurelia,* trans-oceanic populations exist genetically in isolation-by-distance because the restricted lifespan of its planktonic medusa-stage prohibits gene flow across such extensive tracts of ocean [1].

Unicellular phytoplankton species usually show high genotypic diversity and in cases where genetically distinct populations are observed, they are often correlated with hydrographic or geographic features [1, 4–10]. Marine eukaryotic microalgae grow by means of mitotic division, but in contrast to daughter cells in macrophytes and animals, microalgal daughter cells disconnect and drift apart in their mildly turbulent environment, thus forming widely distributed clones. Episodic sexual reproduction in a population composed of large numbers of clones generates huge numbers of F1 cells with distinct genotypes, each of which in its turn can form a clone [11]. Therefore, the likelihood of sampling multiple individuals belonging to the same clone in a large phytoplankton population is very small, given the sample sizes normally deployed in population genetic studies [12].

Although the emergence of genetic differentiation without geographic barriers remains highly controversial, speciation can occur in sympatry [13] that is, if populations reproduce in distinct temporal windows, and/or have distinct ecological niches [14–15]. In phytoplankton, Casteleyn *et al.* [3] demonstrated that strains of the diatom *Pseudo-nitzschia pungens* established from cells collected in Belgian, Danish and Irish waters grouped into two genetically distinct, but apparently sympatric populations. Such genetic distinctness could merely be temporal, for instance resulting from contemporary establishment of founder populations from distinct sources, to be homogenized if sexual reproduction can still occur amongst them. In fact, marine habitats are among the most heavily invaded systems on Earth [16] and this is not necessarily restricted to invasions of alien species, but also to alien populations of resident species (e.g., [17]). Alternatively, mate preference and/or slightly offset bloom windows may keep these sympatric populations genetically segregated. If this is the case, then subtly different performance, e.g., different growth rates and environmentally governed differential mating success, could explain radical shifts in their proportions from one growth season to the next.

Few studies have addressed the structure of planktonic microalgal species over a temporal scale. A considerable genetic differentiation was detected over two

consecutive years for the dinoflagellate *Alexandrium fundyense* in a coastal pond, where different populations were detected even amongst samples collected after 7 days. These highly diverse and dynamic patterns contrast with the constant genetic structure of the diatom *Skeletonema marinoi,* where samples composed of strains resulting from the germination of up to 100-years old resting stages collected from laminated sediment cores were found to belong to the same population as samples composed of strains obtained from the present-day plankton from the same site [18].

In the present study we explore population genetic structure of the planktonic, chain-forming diatom *Pseudo-nitzschia multistriata* at the Long Term Ecological Research station MareChiara (LTER-MC) in the Gulf of Naples (GoN, Tyrrhenian Sea, Mediterranean Sea, **Fig. 1**). The species has been recorded here from summer until late autumn of every year since its first appearance in the GoN, in 1996 [19–21].

Pseudo-nitzschia multistriata, as almost all diatoms, possesses rigid siliceous cell wall elements. During cell division each daughter cell inherits a parental cell wall element and makes a new one that fits precisely inside the parental one. Hence average cell size diminishes with ongoing mitotic division and the variance around the average increases. Populations of clonal cell lines escape from this miniaturization trap through sexual reproduction, upon which large cells (82–72 μm long) are formed within the zygote, thus reestablishing the initial large cell size. Yet, cells can be sexualized only within a restricted window of cell sizes [22]. A life cycle model of *P. multistriata* in the GoN by D'Alelio *et al.* [21] predicts that sexual reproduction in this species occurs once every two years. If the model is correct, then only six or seven sexual reproduction events have occurred between its first appearance in the GoN in 1996 and the onset of our population genetic sampling, in 2008.

To investigate intraspecific genetic diversity and population genetic structure of *P. multistriata*, we obtained seven polymorphic microsatellite markers [23] and used these to genotype strains generated from single cells, or clonal chains of cells, isolated from 22 net samples obtained during the species' seasonal appearance at the LTER-MC station over four consecutive years (September 2008 to July 2011). The genotype data were analyzed utilizing Bayesian clustering software to assess the most probable number of genetically distinct populations. Evidence emerged for the occurrence of multiple populations in the GoN, and therefore we assessed if these were separated in time, i.e., by sampling date, season or year, and if there were signs of them merging, e.g., by the increasing proportion of hybrids. In order to support the idea that sampled strains assigned as putative hybrids resulted from crossings between strains assigned to different populations, we included the genotypes of F1 offspring strains and their parental lineages, generated in Tesson *et al.* [11] in our analysis.

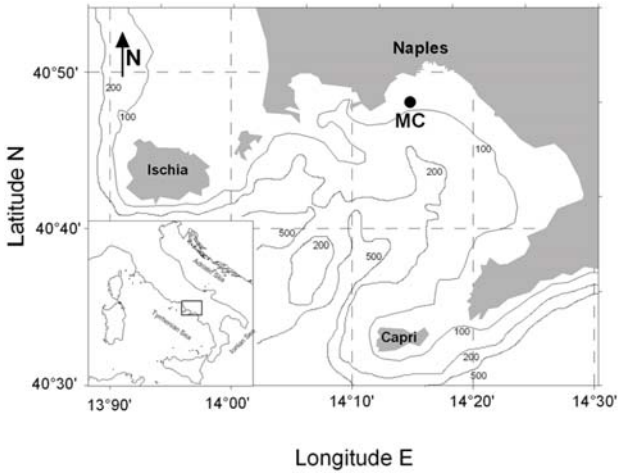

Fig. 1. The Gulf of Naples (GoN) and the location of the Long Term Ecological Research station MareChiara (LTER-MC; 40˚48.5′N, 14˚15′E).

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Materials and Methods

Ethics Statement

The present study on the planktonic diatom *Pseudo-nitzschia multistriata* was performed in accordance with the Italian laws and did not investigate endangered or protected species. No specific permissions were required to collect surface phytoplankton samples at the LTER-MC (40˚48.5′N, 14˚15′E, Fig. 1) in the Gulf of Naples.

Sampling site

The Gulf of Naples (GoN; Fig. 1) is an embayment open to the Tyrrhenian Sea (Western Mediterranean) and connected to the nearby Gulfs of Gaeta and Salerno (North-west and South-east, respectively) by slow seasonal cyclonic and anti-cyclonic eddies and offshore currents [24]. The sampling site, the LTER-MC, is located two miles offshore in the Gulf and is characterized by a surface temperature between 14˚C in February–March and 26˚C in August, a salinity of 37.5–38.0 ($\pm$0.1) and the presence of a seasonal thermocline from April to September [19].

Cell collection, identification and culture maintenance

Surface phytoplankton samples were collected weekly using a rosette sampler equipped with Niskin bottles. Water samples for cell counts were fixed with neutralized formaldehyde at a final concentration of 0.8%. Depending on the total phytoplankton cell abundance of the samples, between 1 and 50 ml were allowed to settle in combined sedimentation chambers and cell concentration was estimated at 400x magnification [25].

For the isolation of living cells and chains, phytoplankton samples were collected with a standard phytoplankton net (20 µm mesh size). Cells and chains of *Pseudo-nitzschia multistriata* were isolated from 21 net samples collected between September 2008 and September 2010, and from a single net sample taken July 2011 (Table 1). Isolated cells were incubated each in 2 ml f/2 filtered medium at 22℃, *ca.* 80 µmol photons·m^{-2}·s^{-1}, and a photoperiod 12L:12D hour (Light : Dark). After a few days, isolation success and purity of the cultured strains were checked using an inverted light microscope; average cell size was determined by measuring cell length over the apical axis of at least 5 cells per strain at 200x magnification. After one week of growth, the obtained strains were transferred into culture bottles (Corning Flask, Corning Inc., NY, USA) containing 25 mL of f/2 medium to reach a concentration of about 10^3 cell·ml^{-1}. A total of about 1100 single cells or short chains were isolated, and of these, 735 (66.8%) were grown successfully in culture. Strain isolation success was comparable among sampling dates.

DNA extraction and strain genotyping using microsatellite markers

Genomic DNA of *P. multistriata* was extracted as described in Tesson *et al.* [23]. DNA-purity and quantity were assessed using the NanoDrop Spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA) and agarose gel electrophoresis (1.5%). Pure DNA was diluted to 25 ng·$µl^{-1}$ and 2 µl of the dilution was added to a well in a 96-well-plate containing the polymerase chain reaction (PCR) mix [23]. Seven microsatellite markers (*PNm1, PNm2, PNm3, PNm5, PNm6, PNm7,* and *PNm16*) were amplified in multiplexes by PCR, purified and combined for microsatellite identification as described in Tesson *et al.* [23] using forward labeled primers. Microsatellite reactions were prepared in automation with a robotic station Biomek FX (Beckman Coulter, Fullerton, CA) and analyzed on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Life technologies, 5791 Van Allen Way Carlsbad, CA 92008 USA). Electropherogram analysis was performed using Peak Scanner (version 1.0, Applied Biosystems). Peak assignment was refined manually in order to avoid scoring mistakes. In the few cases where stutter bands were present and interpretations were not univocal, samples were re-run up to three times to confirm scoring.

Microsatellite data analysis

Micro-Checker (version 2.2.3 [26]) was applied to calculate the frequency of null-alleles (i.e., non-amplification of alleles) for each of the loci under the assumption that the loci are in HWE (S1 Table). A Brookfield-1 estimation was applied following recommendation by the program manual. Tests of presence of stutter bands due to slight changes in microsatellite length and low efficiency of large alleles amplification (large allele dropout) in PCR were performed as described in the manual (S1 Table).

Table 1. Genetic diversity of the strains of *Pseudo-nitzschia multistriata* sampled within the 22 field samples collected at the Long Term Ecological Research station MareChiara.

Sample Nr.	Sample date	N	G	G/N	A/I	Na	Na7	SE	PA	He	Ho	$F_{IS} \pm SE$
1	170908	21	16	76.2	4.43	31	22.830	0.077	2	0.566	0.687	-0.19 ± 0.26
2	230908	53	40	75.5	5.57	39	21.733	0.076	2	0.536	0.717	-0.31 ± 0.20
3	300908	44	39	88.6	6.57	46	23.314	0.088	5	0.552	0.750	-0.31 ± 0.19
4	141008	18	13	72.2	3.43	24	19.859	0.046	0	0.521	0.730	-0.38 ± 0.24
5	041108	7	6	85.7	3.29	23	-	-	0	0.509	0.714	-0.39 ± 0.14
6	111108	7	5	71.4	2.86	20	-	-	0	0.501	0.735	-0.40 ± 0.27
7	181108	7	6	85.7	3.57	25	-	-	1	0.539	0.673	-0.25 ± 0.11
8	300609	24	24	100.0	4.86	34	24.678	0.070	3	0.535	0.524	0.03 ± 0.10
9	070709	35	33	94.3	4.71	33	23.034	0.054	1	0.527	0.531	0.09 ± 0.13
10	140709	18	14	77.8	4.14	29	22.879	0.065	2	0.559	0.587	0.02 ± 0.25
11	220909	8	8	100.0	3.14	22	21.627	0.015	0	0.551	0.607	-0.07 ± 0.13
12	290909	57	39	68.4	5.43	38	22.554	0.085	1	0.544	0.624	-0.05 ± 0.21
13	061009	24	14	58.3	4.71	33	22.711	0.113	0	0.540	0.601	-0.03 ± 0.25
14	201009	14	10	71.4	3.00	21	18.568	0.053	0	0.454	0.510	-0.09 ± 0.16
15	271009	13	6	46.2	3.14	22	18.892	0.045	1	0.464	0.626	-0.25 ± 0.20
16	150610	9	8	88.9	3.29	23	21.845	0.021	0	0.477	0.571	-0.23 ± 0.12
17	060710	14	11	78.6	3.57	25	20.519	0.053	0	0.491	0.561	-0.19 ± 0.15
18	100810	33	33	100.0	5.71	40	26.834	0.074	14	0.609	0.628	-0.04 ± 0.13
19	070910	48	47	97.9	6.57	46	25.157	0.076	4	0.578	0.598	-0.03 ± 0.12
20	140910	32	29	90.6	4.29	30	21.847	0.070	0	0.571	0.554	0.05 ± 0.16
21	210910	26	18	69.2	3.86	27	21.544	0.060	2	0.576	0.484	0.13 ± 0.22
22	100711	13	13	100.0	4.71	33	25.332	0.043	2	0.553	0.593	0.01 ± 0.21

Sampling dates are expressed as DDMMYY. Included in the table are the number of strains analyzed (N), the number of genotypes (G), the genotypic diversity (G/N, in %), the average number of alleles per locus (A/I), the total number of alleles (Na), the average number of alleles when resampled 1000 times for N=7 as smallest sample size (Na7) and associated Standard Error (SE), the number of private alleles (PA), the expected (He) and observed (Ho) heterozygosity, and the fixation index ($F_{IS} \pm SE$) over the seven microsatellite loci.

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Average number of alleles per locus, expected and observed heterozygosity, the inbreeding coefficient F_{IS}, the fixation index F_{ST}, and the G_{ST} index of diversity across population were calculated for groups of strains using GenAlEx (version 6 [27]). F_{IS} is defined here as the proportion of the genetic variance in a group of strains contained in a sample. A significant positive F_{IS} implies a significant inbreeding/homozygote over-representation, whereas a significant negative value, significant outbreeding/heterozygote over-representation; F_{ST}, is defined here as the proportion of the total genetic variance contained in a group of strains relative to the total genetic variance among all of the strains; G_{ST}, is a relative measures of F_{ST} that takes into the account differences in sample size and the amount of genetic variation among populations versus all populations.

Allelic richness was re-calculated for each sample using GenClone [28]. In order to compare groups (i.e., samples, sampling years) with different numbers of strains, allelic richness was estimated in each group, with a resampling procedure, performing 1000 permutations for all possible numbers of sampling units (from 1

to N). Values obtained for the same number of samples were taken for comparison. Genotypic diversity (G/N) within samples was estimated using GIMLET (version 1.3.3 [29]).

The number of genetically distinct populations and their occurrence through time were inferred using a Bayesian cluster analysis performed with STRUCTURE (version 2.1 [30]) The number of clusters (K; populations) was estimated following Evanno *et al.* [31] using the web-based program STRUCTURE HARVESTER [32]. Population structure was obtained applying a burn-in of 15,000 iterations, with 75,000 MCMC repetitions from K=1 to K=23. The parameters used to infer genetic structure were an ancestry model with admixture, along with an independent frequency model (infer λ estimated to 0.1414). The LOCPRIOR assumption was not applied. The Assignment Probability (AP) threshold for a strain to a population assigned by STRUCTURE was set at 90%. A separate STRUCTURE analysis with the same settings was performed also including the genotypes of a set of parental strains and their F1 progeny obtained from Tesson *et al.* [11]. The existence of a bottleneck effect in the two populations and in consecutive years was tested using the Wilcoxon test implemented in the software BOTTLENECK [33], after 1000 iterations and hypothesizing the two-phased model (TPM) of mutation.

GENETIX (version 4.05.2 [34]) was used to identify linkage disequilibrium (10,000 permutations and a significance level of 0.05) within the whole data set. Factorial Correspondence Analysis was also performed with GENETIX, using genotypes assigned to populations as inferred from STRUCTURE and those not assigned to any of them. Principal Component Analysis (PCA) based on single strains as well as on groups of strains was performed using GENALEX and the pairwise matrix of Nei genetic distance. Statistical analysis and graphical representation of *P. multistriata* abundance and frustule size distribution - of cells observed in formalin fixed field samples as well as of cells from which the genotyped strains were raised - were performed using R [35].

Results

Pseudo-nitzschia multistriata was detected in the surface layer at the LTER-MC from June/July until October/November of the four sampled years (2008–2011), with cell abundances up to 465×10^3 cells l^{-1} (**Fig. 2**). A total of 525 strains out of the 735 successfully grown in culture were successfully genotyped from 22 of the net-samples in which the species was present (157 strains in samples 1–7 in 2008; 193 in samples 8–15 in 2009; 162 in samples 16–21 in 2010, and 13 in sample 22 in 2011; **Table 1**). Twelve of these strains belonged to the frustule size class ≥55 μm, in which sexual reproduction cannot be induced, whereas the remainder belonged to the 55–30 μm size-interval, i.e. the size window in which sex can be induced (**Fig. 3**). We assessed cell size distribution of *P. multistriata* in a limited number of samples collected between 2008 and 2010 (**Fig. 3**). The cell size ranges observed in

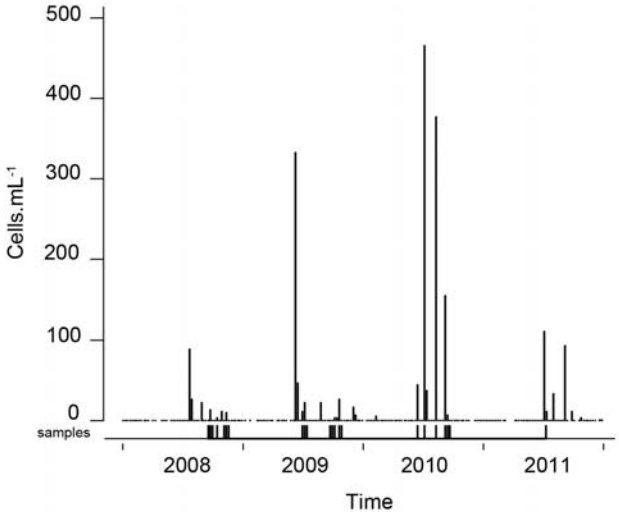

Fig. 2. Cell concentration of *Pseudo-nitzschia multistriata* in the surface samples collected at LTER-MC from January 2008 to December 2011. All LTER-MC samples are indicated with dots along the x-axis. The LTER-MC samples from which *P. multistriata* cells have been isolated for genotyping are indicated with small vertical bars under the sample dots (for sample numbers see Table 1).

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these natural samples matched those observed among the cells from these samples (Fig. 3).

Genetic diversity within samples and sample years

The 525 strains generated a clearly readable microsatellite fingerprint without any apparently missing values for any of the seven loci (S2 Table). All loci were polymorphic in all of the 22 samples, except locus *PNm3*, which was monomorphic in three samples (data not shown). The observed heterozygosity (Ho) was higher than the expected (He) – and often markedly so - in all samples except 8, 20 and 21. The inbreeding coefficient F_{IS} was significantly negative for almost all of the samples from 2008 (samples 2–7) and for samples 15 and 16, implying significant outbreeding. The average inbreeding coefficient (F_{IS}) over all 22 sampling dates was negative (-0.131 ± 0.039) indicating outbreeding and overall heterozygosity excess (Table 1).

The number of alleles per locus ranged from 7 to 21. All loci, except *PNm1* and *PNm5*, showed heterozygosity excess. No allele-drop out was detected in any of the loci (S1 Table). Only two loci (*PNm2-PNm5*) were in linkage disequilibrium within the whole data set (data not shown).

The strains exhibited 394 distinct genotypes (G). The genotypic diversity (G/N) ranged from 46.2% to 100% across the individual samples (Table 1), from 66.2% for 2008, to 87.0% for 2010, with an average of 75.5% over all 22 samples (Table 2). No clear relationship was detected between sample date and genotypic diversity, also because sample size varied markedly among sample dates.

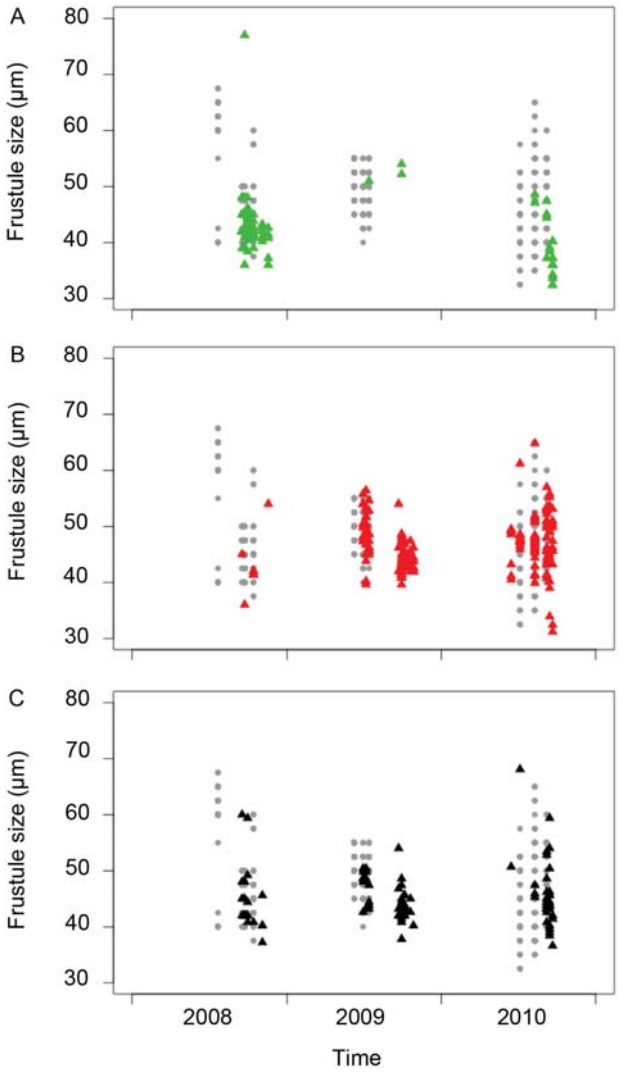

Fig. 3. Cell length of *Pseudo-nitzschia multistriata* strains assigned to A) POP_A (green), B) POP_B (red), and C) putative hybrids (black), as determined by STRUCTURE. The grey dots in the background represent the cell size classes recorded in the natural environment.

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The total number of alleles (Na) per sampling date, over the seven loci considered, ranged from 20 (sample 111108) to 46 (samples 300908 and 070910), with an average value of 30 alleles ($\pm$8.1) (Table 1). The normalized average number of alleles (Na7; resampling with N=7, i.e., the smallest sample size) ranged between 18.568 (sample 201009) and 26.834 (sample 100810) (Table 1). The number of private alleles per sample (PA) ranged from 0 (at many sampling dates) to 14 (sample 100810) (Table 1). The total number of alleles (Na) per year was 54, 53, 58 and 33, in 2008, 2009, 2010 and 2011, respectively (Table 2). The number of private alleles (PA) per year ranged from 12 (2009) to 33 (2010), whereas the 13 strains sampled in 2011 showed two private alleles (Table 2).

Table 2. Genetic diversity of *Pseudo-nitzschia multistriata* per year of sampling (2008–2011).

Samples	Sample year	N	G	G/N	A/I	Na	Na157	SE	PA	He	Ho	$F_{IS} \pm SE$
1–7	2008	157	104	66.2	7.71	54	-	-	20	0.547	0.722	-0.285 ± 0.20
8–15	2009	193	137	71.0	7.57	53	50.688	0.043	12	0.554	0.580	0.038 ± 0.17
16–21	2010	162	141	87.0	8.28	58	57.567	0.022	33	0.610	0.572	0.045 ± 0.13
22	2011	13	13	100.0	4.71	33	-*	-*	2	0.553	0.593	0.01 ± 0.21

For sampling dates see Table 1.
Included in the table are the number of strains analyzed (N), the number of genotypes (G), the genotypic diversity (G/N, in %), the average number of alleles per locus (A/I), the total number of alleles (Na), the average number of alleles when resampled 1000 times for N=157 as smallest sample size (Na157) and associated Standard Error (SE), the number of private alleles (PA), the expected (He) and observed (Ho) heterozygosity, and the fixation index ($F_{IS} \pm SE$) over the seven microsatellite loci. Resampling for the smallest sampling size on the samples grouped per year was not performed for the 2011 sample (*).

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Population genetic patterning

Results of the independent runs of K=1 to 22 for assessing Bayesian population clustering as implemented in STRUCTURE, showed that the existence of two groups (K=2; mean LnP(K)=−8683.8; SD LnP(K)=1.78; ΔK=206.7) was by far the most likely (S1 Figure). The inferred groups are denoted from here on as populations POP_A and POP_B (green and red, respectively in Fig. 4, S2 Table). Strains assigned to POP_A dominated in 2008 and in the single sample of 2011, whereas the ones assigned to POP_B dominated in 2009 and 2010 (Table 3). Since STRUCTURE results were obtained using the ancestry model with admixture, strains that could not be assigned to any of the two populations with a probability above the 90%-threshold were considered to be putative hybrids. These assigned hybrids constituted 15%, 27% and 24% of the strains sampled in 2008, 2009 and 2010, respectively, whereas no putative hybrids were assigned among the 2011-samples. The proportion of assigned hybrids differed significantly among years (p=0.02861 χ^2-test (χ^2=7.11, df=2)). The proportion of assigned hybrids did not differ significantly between 2008 and 2010 (p-value=0.012), but both of these differed from that in 2009 (p-value>0.05).

Factorial Correspondence Analysis (FCA) of the strains' genotypes resulted in a plot in which all the strains were distributed along a stretched-out swarm without any apparent subdivision (Fig. 5). Strains assigned to POP_A grouped towards the right side, those assigned to POP_B towards the left side, and those classified as putative hybrids, lay in-between. PCA mapping of the 22 samples resulted in a plot (Fig. 6) in which the location of the samples corroborated the findings of STRUCTURE (Fig. 4). In addition, patterns not captured by STRUCTURE were uncovered. The 2008-samples, composed of strains mostly assigned to POP_A, clustered together on the lower right of Fig. 6, whereas sample 22 from 2011 (also POP_A) was also placed on the positive side of axis 1 but away from this cluster, to the top-far right. The 2009- and 2010-samples, composed of strains assigned mainly to POP_B, clustered on the left side. Samples 11 (2009), 19, 20 and 21 (2010) (enclosed in the ellipse in Fig. 6) were still dominated by POP_B strains, but, contained a minority of strains assigned to POP_A and others classified as

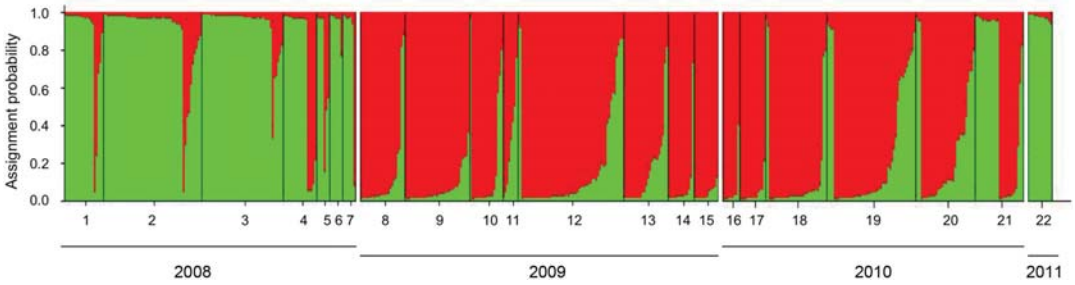

Fig. 4. Genetic structure of the strains of *Pseudo-nitzschia multistriata* within each sample, as defined by STRUCTURE. Samples are presented in ranking order (1–22) along the x-axis. Each vertical bar represents one strain. The y-axis indicates the proportion of a strain's genotype assigned by STRUCTURE to a population (i.e., K-cluster); the two populations identified by STRUCTURE are indicated POP_A (green), POP_B (red). Strains in each sample are ranked along the x-axis based on their assignment probabilities. Strains not assignable to any of the two populations above the 0.9-probability-level, are indicated to the right side of each sample. Samples have been grouped by year of collection: samples 1–7 in 2008, samples 8–15 in 2009, samples 16–21 in 2010 and sample 22 in 2011.

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putative hybrids, and were recovered on the right side of the POP_B-cluster, towards the POP_A-cluster.

Genetic diversity of the two populations

If all the strains analyzed by STRUCTURE are taken together, then a total of 103 distinct genotypes were found among the 164 strains assigned to POP_A (G/N=62.8%, Table 3), whereas 182 distinct genotypes were detected among the 246 strains assigned to POP_B (G/N=74.0%). A total of 110 genotypes was identified (G/N=95.7%) among the 115 strains that did not obtain an assignment probability ≥90% to POP_A or POP_B (Table 3). We classified these strains as putative hybrids. The genotypic diversity among POP_A strains was comparable between 2008 (G/N=59.5%; N=126) and 2010 (G/N=54.6%; N=22), while it was higher in the other two years (G/N=100%), where only 3 individuals (in 2009) and 13 individuals (in 2011) were present. Differences in POP_A G/N values among years are not statistically comparable, since the population is represented by few strains in 2009, 2010 and 2011. The same accounts for POP_B in 2008 and 2011 (Table 3). The genotypic diversity among POP_B strains was

Table 3. Genotypic diversity of the inferred *Pseudo-nitzschia multistriata* populations POP_A, POP_B and the putative hybrids as identified by STRUCTURE over the four years of study.

	N				G			G/N		
Year	POP_A	POP_B	Hybrids	Total	POP_A	POP_B	Hybrids	POP_A	POP_B	Hybrids
2008	126	7	24	157	75	5	24	59.5	*71.4*	*100*
2009	3	138	52	193	3	85	49	*100*	61.6	94.2
2010	22	101	39	162	12	92	37	54.6	91.1	94.9
2011	13	0	0	13	13	0	0	*100*	-	-
Total	164	246	115	525	103	182	110	62.8	74	95.7

Included in the table are the number of strains (N), the number of genotypes (G) and the genotypic diversity (G/N).

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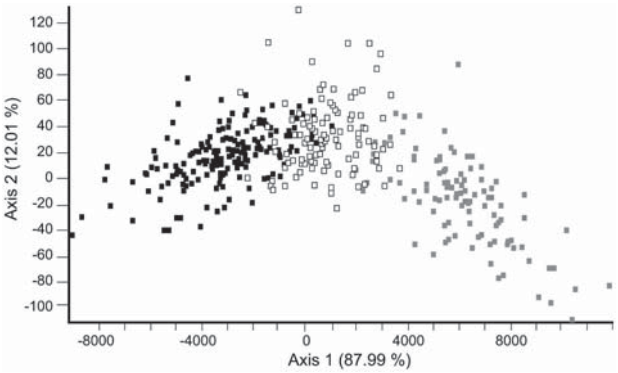

Fig. 5. Placement of the individual *Pseudo-nitzschia multistriata* strains along the first two FCA axes (GENETIX); specimens assigned by STRUCTURE to POP_A are indicated with grey squares, those to POP_B with black squares and those classified as putative hybrids (HYBR) with white squares.

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significantly lower in 2009 (G/N=61.6%; N=138) than in 2010 (G/N=91.1%; N=101) (χ^2=24.89, df=1, p-value<0.01). Fifteen genotypes in POP_A and 16 in POP_B were shared by different strains within the same year, but not between different years (Table 4). Amongst the putative hybrids, five genotypes were shared; only one of these was present in different years (i.e. 2008 and 2010; Table 4). Neither of the two populations (POP_A and POP_B) was in HWE ($p<0.05$ and χ^2-test >50). However, the fixation-index not significantly different from zero in both populations indicated random mating within populations (F_{IS}-POP_A=-0.149 ± 0.202 and F_{IS}-POP_B=-0.083 ± 0.090). POP_A and POP_B exhibited a similar effective number of alleles (POP_A: 18.86 alleles and POP_B: 18.55 alleles), and a similar number of private alleles (POP_A: 24 and POP_B: 23).

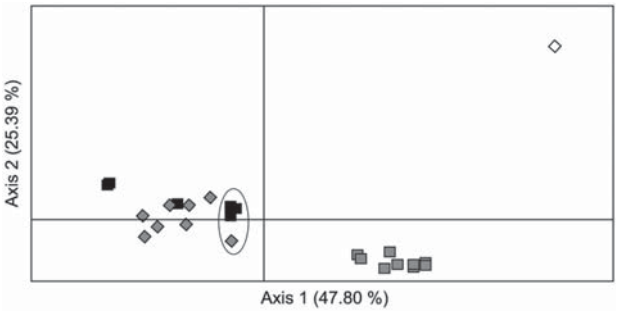

Fig. 6. Placement of the 22 samples along the first two PCA axes (GENALEX); samples of *P. multistriata* gathered at LTER_MC in 2008 are indicated with grey squares; those in 2009 with grey diamonds, those in 2010 with black squares, and the one in 2011 with a white diamond. Samples 11 (2009), 19, 20, and 21 (2010), which showed a higher abundance of POP_B strains, but contained a minority of strains assigned to POP_A and others assigned as putative hybrids, are enclosed within an ellipse (see text). The two PCA axes capture 73.2% of the variability.

doi:10.1371/journal.pone.0114984.g006

Table 4. Number of single, multiple and shared genotypes in the inferred populations, POP_A, POP_B and the putative hybrids, as identified by STRUCTURE over the four years of the present study.

	POP_A		POP_B		Hybrids	
Year	Ns	Gs	Ns	Gs	Ns	Gs
2008	64	13	3	1	1	1#
2009	0	0	64	11	5	2
2010	12	2	13	4	5	2#
2011	0	0	0	0	0	0
Total	76	15	80	16	11	5
Shared per year	0	0	0	0	2	1

Included in the table are the number of strains that shared a common genotype (Ns) and the number of shared genotypes (Gs). # indicates that genotypes are shared between different years.

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Pseudo-nitzschia multistriata showed a marked seasonal cycle with high cell abundances restricted to a relatively short period. Results of the Wilcoxon test of each of the two populations sampled in consecutive years were negative, revealing no significant likelihood ($p<0.05$) for the existence of bottlenecks. Results were likewise after pooling all strains within single years.

Genetic structure of the F1 samples resulting from crosses

A Bayesian clustering analysis was carried out on the genotypes of the 525 strains from the field and the F1 strains generated from crossing experiments conducted in the study of Tesson *et al.* [11]. A cross between parental strains SY017 (POP_A) and SY278 (hybrid; cross 1 in Fig. 7, S2 Table) generated an F1 in which most strains were assigned to POP_A and a minority were classified as putative hybrids. A cross between parents SY017 (POP_A) and SY138 (POP_B; cross 2 in Fig. 7, S2 Table) generated an F1 in which most strains were classified as putative hybrids (i.e. 82.7% of F1). Crosses between parents SY138 (POP_B) and SY378 (POP_B) and parents SY138 (POP_B) and SY379 (POP_B; crosses 3 and 4 in Fig. 7, S2 Table) generated an F1 of which most strains were classified as POP_B and only a few as hybrids (i.e. 0 out of 25 F1 and 7 out of 62 F1 in crosses 3 and 4, respectively).

Discussion

Results of the Bayesian analysis and the FCA of the microsatellite genotypes obtained from the *Pseudo-nitzschia multistriata* strains uncovered two genetically distinct populations (denoted POP_A and POP_B) in the Gulf of Naples. A substantial proportion of strains could not be assigned with a ≥90%-probability to either of these populations and may represent hybrids between them. POP_A dominated in 2008, was largely replaced by POP_B in 2009 and 2010, and apparently returned to dominance in 2011. This latter observation is based on only a single sample with 13 strains, but all of these are assigned to POP_A.

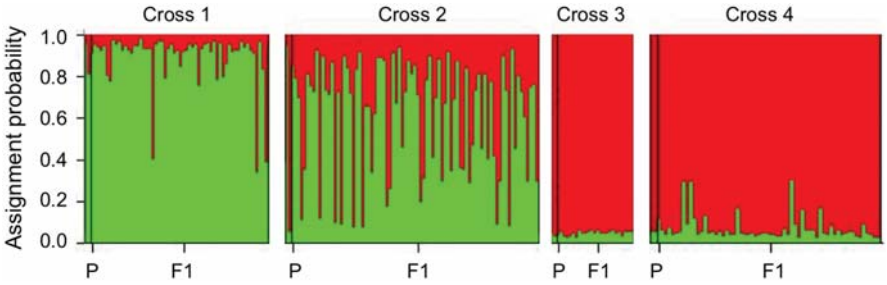

Fig. 7. Population genetic structure of *Pseudo-nitzschia multistriata* parental strains and samples of their F1 offspring [21] as identified by STRUCTURE. For each cross, parental and F1-strains are grouped in boxes, ordered along the x-axis. Each vertical bar in a box represents one strain; the first two bars represent the parental strains, the remainder, the F1 strains. The y-axis indicates the proportion of a strain's genome assigned by STRUCTURE to POP_A (green) and POP_B (red). Strains not assignable to one of these populations at or above the 0.9-probability-level are considered putative hybrids based on the STRUCTURE results. Parental strain assignment according to STRUCTURE: Cross 1: SY017 (POP_A; green) × SY278 (hybrid); Cross 2: SY017 (POP_A; green) × SY138 (POP_B; red); Cross 3: SY138 (POP_B; red) × SY378 (POP_B; red); and Cross 4: SY138 (POP_B; red) × SY379 (POP_B; red).

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Samples dominated by strains assigned to one population usually included a minority of strains assigned to the other, as well as strains classified as putative hybrids, suggesting that the two populations persisted in sympatry throughout the four years of our study. Crosses between parents assigned to the two populations produced viable progeny with a genetic fingerprint by and large comparable to that of field strains classified by STRUCTURE as putative hybrids.

Genotypically distinct populations occur in sympatry

Genetically distinct populations in the same or overlapping areas, i.e., in sympatry or parapatry, have been reported before in planktonic species of diatoms (*Pseudo-nitzschia pungens, Ditylum brightwellii, Skeletonema marinoi*) [3, 6, 36, 37, 8] and dinoflagellates (*Alexandrium fundyense* and *A. minutum*) [9, 10, 38]. Our observation of two apparently sympatric populations among the Neapolitan strains of *P. multistriata* resembles results by Casteleyn *et al.* [3], who observed genotypically distinct populations within one of the three clades of *Pseudo-nitzschia pungens* co-occurring at their western European sample sites. Their sympatric populations exhibited an F_{ST} value comparable to that between the two Neapolitan *P. multistriata* populations. Remarkably high genotypic difference was detected between populations of *P. pungens* along the Pacific Northwest (F_{ST}=0.418; [6]), but these populations were attributed subsequently to different clades and are likely to belong to different cryptic species [36]. Similarly, four genetically distinct populations detected in *Ditylum brightwellii* [37] probably also represented closely related species [39]. In our case, results demonstrate that strains of the two *P. multistriata* populations can interbreed, and therefore, belong to a single biological species.

When genetically distinct populations are detected within a species, environmental or biological factors can usually be identified that keep these populations

apart. For instance, a population of the diatom *Skeletonema marinoi* in a Swedish fjord differed genetically from one in the nearby open sea (F_{ST} ranging from 0.200 to 0.267) [8]. In this case, a shallow sill limits water exchange between the fjord and the open sea, maintaining environmental differentiation [8]. In addition, the species produces resting spores that can survive in the sediments for decades [18], thus permitting these populations to persist in their distribution areas [8, 18]. Similarly, populations of the cyst-forming dinoflagellate *Alexandrium fundyense* from the open Gulf of Maine differed genetically from those in adjacent coastal ponds [10], although genetic differentiation was also recorded along the development of a single bloom [38]. In the case of *P. multistriata,* however, the two populations co-existed in a coastal area without physical barriers. Resting stages are not known for this species [40], nor do we have any evidence for gradual seasonal succession of POP_A and POP_B, thus excluding such explanations for the co-occurrence of genetically distinct populations in the Gulf of Naples.

Both *P. multistriata* populations appeared to persist over the entire growth season and throughout the four years of our study. Their relative proportions changed between consecutive years, but remained, by and large, similar across the growth season within years. The Gulf of Naples is an open system, exhibiting complex surface hydrodynamics and marked exchange with adjacent gulfs along the southwestern Italian coast and with the open Tyrrhenian Sea [41]. So, the detection of similar proportions between POP_A and POP_B throughout a growth season, or even between two consecutive growth seasons, leads us to conclude that these populations co-occur in the same proportions far beyond our sampling point.

Population structure may change in consecutive years

This study is the first in *Pseudo-nitzschia* - or in any phytoplankton species as far as we are aware of - revealing a turnover of populations between consecutive years, i.e., the dominant population becoming a minority and *vice versa.* Apparently, the turnover happens when the species is not observed in the plankton. Since benthic resting stages are unknown for *Pseudo-nitzschia* [40], the species must persist in the plankton outside the growth season either below the detection threshold for routine LM-observation or beyond the depth range at which we sample the plankton.

Periods of extremely low densities could render populations prone to bottleneck effects. However, no such a bottleneck was evident from the results of the Wilcoxon tests. The genetic difference between POP_A in 2008 and POP_B in 2009 cannot be explained assuming a bottleneck since the alleles present in POP_B (2009 and 2010) do not represent a sub-set of the alleles present in POP_A (2008); POP_B exhibits several alleles absent in POP_A (2008). Therefore, other factors must be responsible for the turnover, possibly ones related to the life cycle or to mortality, affecting the populations differently.

Possible effects of the life cycle on population structure

The virtual disappearance of POP_A after 2008 could be a consequence of the episodic nature of sexual reproduction in *P. multistriata* and a differential success of sexual reproduction in the two populations. We do not have direct observations of the sexual phase of this species in the field. Yet, a model developed by D'Alelio *et al.* [21] from patterns of cell size distribution in field samples over time predicts that sexual reproduction occurs in the autumn of every second year. This peculiar timing is made possible because cell size in *P. multistriata*, as in most other diatom species, is a function of time due to a gradual reduction of average cell size (length) with on-going mitotic division. Once the size-range for sexual maturity has been reached (ca. 55 μm, after two years in *P. multistriata*), cells can reproduce sexually, giving rise to large F1 cells (82–72 μm) [22].

The model predicts that towards the end of the growth season of every odd calendar year, all cells in the populations belong to a single, two-years old cohort of short cells (55–40 μm) that have reached the size-range for sexual maturity and can give rise to an F1 cohort of very long, juvenile cells towards the end of the growth season. The following growth season (i.e., in an even calendar year) shows a bimodal cell size distribution with short cells (ca. 50–30 μm) belonging to a now three years old remainder of the parental cohort, and a juvenile, one-year old cohort of long cells (70–55 μm) that are not yet sexually mature. The latter will reach sexual maturity only at the end of the next growth season (i.e., an odd year; see figure 3 in [21]).

The model [21] was inferred from cell size distributions observed in the field until 2006, whereas our observations span the years 2008–2011. If the model is extrapolated to our sample period, then only one cohort is expected during the growth seasons of 2009 and 2011, with sex occurring towards the end of these seasons, and two cohorts are expected in the years 2008 and 2010 without sex. The distribution of cell lengths in the natural samples (grey dots in **Fig. 3**) was indeed bi-modal in 2008. In 2009, the distribution was narrow and unimodal, representing the now sexually mature, two years old cohort. In 2010, the distribution was continuous but very broad, representing in the lower part the short cells belonging to the remainder of the 2007 cohort and in the upper part the long cells of the juvenile 2009 cohort.

In 2008, the cells sampled for genetic analyses (triangles in **Fig. 3**) belonged basically all to the remainder of the cohort that underwent sex in 2007 and was destined to perish between the growth seasons of 2008 and 2009. The vast majority of this cohort belonged to POP_A (green triangles, **Fig. 3**). In 2009, the sampled cells belonged to a single, now sexually mature cohort, with the vast majority of cells being assigned to POP_B (red triangles, **Fig. 3**) or classified as putative hybrids (black triangles, **Fig. 3**). In 2010, POP_B and putative hybrids dominated again, both among the three-years old remainder of the 2007-cohort of short cells as well as among its offspring - the one-year old 2009-cohort of long cells.

The virtual disappearance of POP_A between 2008 and 2009 could be due to a low success of sexual reproduction within POP_A versus that within POP_B at the end of the growth season of 2007. If this is correct, then we expect the larger cells in 2008 to belong mainly to POP_B. Unfortunately, we did not sample this cohort well; only three cells sampled in 2008 were long. The dominance of POP_B in both 2009 and 2010 is in accordance with expectations, because in 2009 a sexually mature cohort dominated by POP_B and hybrid cells must have generated a new cohort of large cells detected in the following year, belonging mainly to POP_B or classified as putative hybrids.

Thus, different success of sexual reproduction of the two populations in 2007 and a comparable success in 2009 could explain the observed patterns until the end of 2010. However, this explanation requires that the two populations use different cues or triggers to commence sexual reproduction, or that they have a mating preference for partners belonging to the same population, for neither of which we have any evidence. Moreover, a different reproductive success cannot explain the dominance of POP_A in the only sample available to us in 2011 and neither does it explain the marked rise in the proportion of POP_A at the end of the 2010 growth season. Therefore other factors must be at work to explain these transitions.

Persistence of genetic structure in the face of hybridization

The persistence of POP_A and POP_B in large proportions over the four years is unexpected in the face of the large proportion of strains classified as putative hybrids and the apparent lack of reproductive barriers between POP_A and POP_B strains. At least under controlled laboratory conditions, cells of the opposite mating type undergo sexual reproduction and their F1 exhibit microsatellite genotypes largely in accordance with Mendelian inheritance rules [11]. Most F1 strains resulting from crossing POP_A and POP_B cells (cross 2) are not assigned to either one of these populations above the 90% probability threshold by STRUCTURE and are therefore classified as putative hybrids, suggesting that field strains not assigned to either one of these populations above the 90% probability threshold are hybrids in a biological sense as well. Such hybrids are fertile because a cross of a strain classified as hybrid with a POP_A-strain (cross 1) generated a perfectly viable F1, which in its turn is fertile as well (unpublished results). Results of the mating experiments in the lab show no evidence for mating barriers between the populations, though in these mating experiments partners were not offered a choice.

Our results indicate that hybridization is common between POP_A and POP_B also in the field. First, the results of the FCA reveal a continuum from POP_A *via* the assigned hybrids to POP_B, and even if the assigned hybrids are ignored, there exists marked gene flow between POP_A and POP_B. Second, many strains identified as putative hybrids by STRUCTURE shared alleles seen otherwise only among POP_A-strains with those observed otherwise exclusively among POP_B-strains. Third, samples composed of strains of two reproductively isolated

populations should reveal a Wahlund effect, i.e., a lower number of heterozygotes than expected if the samples were composed of a single population in Hardy-Weinberg equilibrium [42]. Instead, almost all samples show heterozygote over-dominance. Such a pattern is typical for populations connected by substantial gene flow [42].

Interestingly, some F1-strains of cross 1 (hybrid × POP_A) were classified as putative hybrids and others as belonging to POP_A, indicating that a strain assigned to POP_A does not necessarily need to be derived from POP_A parents. Likewise, several F1-strains resulting from a cross between two POP_B parents were classified as putative hybrids. Two explanations can be given for these observations. First, assignment probability of a strain to, e.g., POP_B depends on the presence of private alleles for POP_B and on alleles found with higher frequency in POP_B strains. If an F1 strain happens to inherit from its POP_B parents alleles not very specific for POP_B, then its assignment probability to POP_B can fall below the 90% and hence, it is classified as a hybrid. Second, the precision of assignment probability improves with the number of loci, the number of individuals, and the F_{ST} between the two populations. The smaller these values, the larger the imprecision around an assignment. In the case of our data, the total number of strains is high but the number of loci and the F_{ST} are modest, suggesting that some of the assignments could be inaccurate. This argument does not explain why in the face of hybridization the majority of the strains remain assignable to POP_A or POP_B throughout the years of our sample campaign.

A reason why the two populations remain to be encountered in sympatry as genetically distinct entities in the face of hybridization might be that they both are relatively new arrivals. However, the species appeared in the Gulf of Naples in 1996 and must have gone since then through eight periods of sexual reproduction until 2011, according to the model by D'Alelio *et al.* [21]. If the two populations arrived simultaneously and remained confined in the Gulf of Naples ever since, then these eight phases of sexual reproduction sufficed to merge them entirely. However, this has not happened.

Towards a meta-population explanation

Periodical appearance and disappearance of populations have been observed in meta-population structures of organisms living in fragmented habitats, where single local populations crash and the site is re-populated by other, distinct, but not completely disjoint, populations [43]. Apparently, *P. multistriata* could follow such a meta-population-like structure, with distinct but connected populations blooming in different regions. This scenario could explain away the sudden rise in the proportion of POP_A strains sampled at the end of the 2010 growth season, or any other change in the proportions. We lack information about the population genetic structure of *P. multistriata* in other coastal regions in the Mediterranean Sea and in other basins, though the species is known to occur elsewhere along the Tyrrhenian coastline [44] and is, in fact, distributed globally (see references in [45]). Casteleyn *et al.* [3] demonstrated the existence of multiple genetically

distinct, geographically distant populations in *Pseudo-nitzschia pungens* clade I, with samples taken at each of the geographically distant locations containing one or a few immigrants, or members of minor resident populations, exhibiting genotypes typical for populations dominating elsewhere. Our results show that within the PCA plot in Fig. 6, the 2011-sample dominated by POP_A strains was recovered distantly from the cluster of 2008 samples dominated by POP_A, indicating genetic changes over time, possibly resulting from exchange with yet unknown populations elsewhere. Extensive genotyping of *P. multistriata* samples from geographically distant places will reveal if such a patchwork of populations exists also in *P. multistriata*.

In conclusion, we hypothesize that *P. multistriata* population dynamics in the Gulf of Naples follows a meta-population-like model, which includes establishment of populations by cell inocula from previous populations at the beginning of each growth season as well as remixing and dispersal governed by water masses that host these populations. Further multiannual studies of population genetic diversity and structure will help clarifying the environmental and internal factors that govern the evolution and fate of the populations of unicellular microalgae.

Supporting Information

S1 Figure. The number of clusters (K; populations) as estimated following Evanno *et al.* [29] using the web-based program Structure Harvester [30]. Independent runs were performed for K=1 to 22; results for K>5 have been pruned from the figure.
doi:10.1371/journal.pone.0114984.s001 (TIF)

S1 Table. Genetic parameters of the seven microsatellite loci of *Pseudo-nitzschia multistriata*.
doi:10.1371/journal.pone.0114984.s002 (DOCX)

S2 Table. Microsatellite multilocus genotypes of *Pseudo-nitzschia multistriata* strains, including parental and F1 strains from crossing experiments. Footnotes for S2 Table: First column: code of 759 strains tested including 525 strains sampled at LTER-MC research station (SYxxx isolated in 2008–2010, and esxxx and mmxxx isolated in 2011), the two parental and the F1 strains isolated from the four crossing experiments. Second column: 1–22: the 22 sampling dates (Table 1) during which *P. multistriata* were isolated; 23, 25, 27, 29: the parental strains used in each crossing-experiment (SY017 × SY278, SY017 × SY138, SY138 × SY378 and SY138 × SY 379, respectively) and 24, 26, 28, 30: the four respective crossing-experiments. Third column: 1–3: genetic population (POP) as assigned by STRUCTURE software (1: POP_A, 2: POP_B and 3: putative Hybrids). Columns 4–17: allele size at each of the seven loci tested.
doi:10.1371/journal.pone.0114984.s003 (XLSX)

S1 Data. Matrix_GenAlex_MC_POP.txt. Matrix_GenAlex_MC_POP.txt has to be opened in Excel. The file is the input matrix that has been utilized for running

the software GenAlex on Excel. It contains the microsatellite multilocus genotypes for the diatom species *Pseudo-nitzschia multistriata*, sampled at the LTER-MC research station in the Gulf of Naples, Italy, in 22 sampling dates along years 2008, 2009, 2010 and 2011. First row: the number of microsatellite loci amplified; the total number of strains; the number of samples and the number of strains in each sample. Second row: name of the analysis. Third row: names of the seven microsatellite loci amplified. First column: code of 525 strains tested and sampled at LTER-MC research station (SYxxx for 2008–2010 strains and esxxx and mmxxx for 2011 strains). Second column: 1–22: the 22 sampling dates during which *P. multistriata* were isolated. Third column: 1–3: the genetic population as assigned by STRUCTURE software (1: POP_A, 2: POP_B and 3: putative Hybrids). Columns 4–18: allele size at each of the seven loci tested.
doi:10.1371/journal.pone.0114984.s004 (TXT)

S2 Data. Matrix_Structure_plot_MCPF1.txt. Matrix_Structure_plot_MCPF1.txt is the input matrix that has been utilized for running the software STRUCTURE. Results are shown in Fig. 7. It contains the microsatellite multilocus genotypes for the diatom species *Pseudo-nitzschia multistriata*, sampled at the LTER-MC research station in the Gulf of Naples, Italy, in 22 sampling dates along years 2008, 2009, 2010 and 2011, the parents utilized in four crossing experiments and the relative F1 strains obtained. First row: name of the seven microsatellite loci amplified. First column: code of 759 strains tested: 525 strains sampled at LTER-MC research station (SYxxx isolated in 2008–2010, and esxxx and mmxxx isolated in 2011), the two parental and the F1 strains isolated from the four crossing experiments. Second column: 1–22: the 22 sampling dates during which *P. multistriata* were isolated; 23, 25, 27, 29: the parental strains used in each crossing-experiment (SY017 × SY278, SY017 × SY138, SY138 × SY378 and SY138 × SY 379, respectively) and 24, 26, 28, 30: the four respective crossing-experiments. Columns 4–17: allele size at each of the seven loci tested.
doi:10.1371/journal.pone.0114984.s005 (TXT)

S3 Data. Matrix_Structure_qplot_MC1-22.txt. Matrix_Structure_qplot_MC1-22.txt is the input matrix that has been utilized for running the software STRUCTURE. Results are shown in Fig. 4. It contains the microsatellite multilocus genotypes for the diatom species *Pseudo-nitzschia multistriata*, sampled at the LTER-MC research station in the Gulf of Naples, Italy, in 22 sampling dates along years 2008, 2009, 2010 and 2011. First row: names of the seven microsatellite loci amplified. First column: code of 525 strains tested and sampled at LTER-MC research station (SYxxx isolated in 2008–2010 and esxxx and mmxxx isolated in 2011). Second column: 1–22: the 22 sampling dates during which *P. multistriata* were isolated. Columns 4–17: allele size at each of the seven loci tested.
doi:10.1371/journal.pone.0114984.s006 (TXT)

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Author Contributions

Conceived and designed the experiments: SVMT GP WHCFK. Performed the experiments: SVMT. Analyzed the data: SVMT GP. Contributed reagents/materials/analysis tools: MM WHCFK. Wrote the paper: SVMT MM GP WHCFK.

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OCEAN PLANKTON

Environmental characteristics of Agulhas rings affect interocean plankton transport

Emilie Villar,[1]* Gregory K. Farrant,[2,3]† Michael Follows,[11]† Laurence Garczarek,[2,3]† Sabrina Speich,[5,23]¶ Stéphane Audic,[2,3] Lucie Bittner,[2,3,4]‡ Bruno Blanke,[5] Jennifer R. Brum,[6]** Christophe Brunet,[7] Raffaella Casotti,[7] Alison Chase,[8] John R. Dolan,[9,10] Fabrizio d'Ortenzio,[9,10] Jean-Pierre Gattuso,[9,10] Nicolas Grima,[5] Lionel Guidi,[9,10] Christopher N. Hill,[11] Oliver Jahn,[11] Jean-Louis Jamet,[12] Hervé Le Goff,[13] Cyrille Lepoivre,[1] Shruti Malviya,[4] Eric Pelletier,[14,15,16] Jean-Baptiste Romagnan,[9,10] Simon Roux,[6]** Sébastien Santini,[1] Eleonora Scalco,[7] Sarah M. Schwenck,[6] Atsuko Tanaka,[4]§ Pierre Testor,[13] Thomas Vannier,[14,15,16] Flora Vincent,[4] Adriana Zingone,[7] Céline Dimier,[2,3,4] Marc Picheral,[9,10] Sarah Searson,[9,10]|| Stefanie Kandels-Lewis,[17,18] *Tara* Oceans Coordinators¶ Silvia G. Acinas,[19] Peer Bork,[17,20] Emmanuel Boss,[8] Colomban de Vargas,[2,3] Gabriel Gorsky,[9,10] Hiroyuki Ogata,[1]# Stéphane Pesant,[21,22] Matthew B. Sullivan,[6]** Shinichi Sunagawa,[17] Patrick Wincker,[14,15,16] Eric Karsenti,[4,18]* Chris Bowler,[4]* Fabrice Not,[2,3]*†† Pascal Hingamp,[1]*†† Daniele Iudicone[7]*††

Agulhas rings provide the principal route for ocean waters to circulate from the Indo-Pacific to the Atlantic basin. Their influence on global ocean circulation is well known, but their role in plankton transport is largely unexplored. We show that, although the coarse taxonomic structure of plankton communities is continuous across the Agulhas choke point, South Atlantic plankton diversity is altered compared with Indian Ocean source populations. Modeling and in situ sampling of a young Agulhas ring indicate that strong vertical mixing drives complex nitrogen cycling, shaping community metabolism and biogeochemical signatures as the ring and associated plankton transit westward. The peculiar local environment inside Agulhas rings may provide a selective mechanism contributing to the limited dispersal of Indian Ocean plankton populations into the Atlantic.

The Agulhas Current, which flows down the east coast of Africa, leaks from the Indo-Pacific Ocean into the Atlantic Ocean (*1*). This leakage, a choke point to heat and salt distribution across the world's oceans, has been increasing over the last decades (*2*). The influence of the Agulhas leakage on global oceanic circulation makes this area a sensitive lever in climate change scenarios (*3*). Agulhas leakage has been a gateway for planetary-scale water transport since the early Pleistocene (*4*), but diatom fossil records suggest that it is not a barrier to plankton dispersal (*5*). Most of the Agulhas leakage occurs through huge anticyclonic eddies known as Agulhas rings. These 100- to 400-km-diameter rings bud from Indian Ocean subtropical waters at the Agulhas Retroflection (*1*). Each year, up to half a dozen Agulhas rings escape the Indian Ocean, enter Cape Basin, and drift northwesterly across the South Atlantic, reaching the South American continent over the course of several years (*1*, *6*). During the transit of Agulhas rings, strong westerly "roaring forties" winds prevalent in the southern 40s and 50s latitudes cause intense internal cooling and mixing (*7*).

We studied the effect of Agulhas rings and the environmental changes they sustain on plankton dispersal. Plankton such as microalgae, which produce half of the atmospheric oxygen derived from photosynthesis each year, are at the base of open-ocean ecosystem food chains, thus playing an essential role in the functioning of the biosphere. Their dispersal is critical for marine ecosystem resilience in the face of environmental change (*8*). As part of the *Tara* Oceans expedition (*9*), we describe taxonomic and functional plankton assemblages inside Agulhas rings and across the three oceanic systems that converge at the Agulhas choke point: the western Indian Ocean subtropical gyre, the South Atlantic Ocean gyre, and the Southern Ocean below the Antarctic Circumpolar Current (Fig. 1).

Physical and biological oceanography of the sampling sites

The Indian, South Atlantic, and Southern Oceans were each represented by three sites sampled between May 2010 and January 2011 (Fig. 1 and table S1). A wide range of environmental conditions were encountered (*10*). We first sampled the two large contiguous Indian and South Atlantic subtropical gyres and the Agulhas ring structures that maintain the physical connection between them. On the western side of the Indian Ocean, station TARA_052 was characterized by tropical, oligotrophic conditions. Station TARA_064 was located within an anticyclonic eddy representing the Agulhas Current recirculation. Station TARA_065 was located at the inner edge of the Agulhas Current on the South African slope that feeds the Agulhas retroflection and Agulhas ring formation (*3*). In the South Atlantic Ocean, station TARA_070, sampled in late winter, was located in the eastern subtropical Atlantic basin. Station TARA_072 was located within the tropical circulation of the South Atlantic Ocean, and Station TARA_076 was at the northwest extreme of the South Atlantic subtropical gyre. Two stations (TARA_068 and TARA_078) from the west and east South Atlantic Ocean sampled Agulhas rings. Three stations (TARA_082, TARA_084, and TARA_085) in the Southern Ocean were selected to sample the Antarctic Circumpolar Current frontal system. Station TARA_082 sampled sub-Antarctic waters flowing northward along the Argentinian slope, waters that flow along the Antarctic Circumpolar Current (*11*) with characteristics typical

[1]Aix Marseille Université, CNRS, IGS UMR 7256, 13288 Marseille, France. [2]CNRS, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France. [3]Sorbonne Universités, Université Pierre et Marie Curie UPMC, Université Paris 06, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France. [4]Ecole Normale Supérieure, Institut de Biologie de l'ENS (IBENS), and Inserm U1024, and CNRS UMR 8197, F-75005 Paris, France. [5]Laboratoire de Physique des Océans (LPO) UMR 6523 CNRS-Ifremer-IRD-UBO, Plouzané, France. [6]Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA. [7]Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy. [8]School of Marine Sciences, University of Maine, Orono, ME, USA. [9]Sorbonne Universités, UPMC Université Paris 06, Observatoire Océanologique, F-06230 Villefranche-sur-Mer, France. [10]INSU-CNRS, UMR 7093, LOV, Observatoire Océanologique, F-06230 Villefranche-sur-Mer, France. [11]Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA. [12]Université de Toulon, Laboratoire PROTEE-EBMA E.A. 3819, BP 20132, 83957 La Garde Cedex, France. [13]CNRS, UMR 7159, Laboratoire d'Océanographie et du Climat LOCEAN, 4 Place Jussieu, 75005 Paris, France. [14]Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Institut de Génomique, Genoscope, 2 Rue Gaston Crémieux, 91057 Evry, France. [15]CNRS, UMR 8030, CP5706, Evry, France. [16]Université d'Evry, UMR 8030, CP5706, Evry, France. [17]Structural and Computational Biology, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany. [18]Directors' Research, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany. [19]Department of Marine Biology and Oceanography, Institute of Marine Sciences (ICM), CSIC, Passeig Marítim de la Barceloneta, 37-49, Barcelona E08003, Spain. [20]Max-Delbrück-Centre for Molecular Medicine, 13092 Berlin, Germany. [21]PANGAEA, Data Publisher for Earth and Environmental Science, University of Bremen, Bremen, Germany. [22]MARUM, Center for Marine Environmental Sciences, University of Bremen, Bremen, Germany. [23]Department of Geosciences, Laboratoire de Météorologie Dynamique (LMD) UMR 8539, Ecole Normale Supérieure, 24 Rue Lhomond, 75231 Paris Cedex 05, France. ***Corresponding author. E-mail: villar@igs.cnrs-mrs.fr (E.V.); not@sb-roscoff.fr (F.N.); hingamp@igs.cnrs-mrs.fr (P.H.); iudicone@szn.it (D.I.); karsenti@embl.de (E.K.), cbowler@biologie.ens.fr (C.B.)** †These authors contributed equally to this work. ‡Present address: CNRS FR3631, Institut de Biologie Paris-Seine, F-75005 Paris, France; Sorbonne Universités, UPMC Université Paris 06, Institut de Biologie Paris-Seine (IBPS), F-75005 Paris, France. §Present address: Muroran Marine Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan. ||Present address: CMORE, University Hawaii, Honolulu, USA. ¶*Tara* Oceans coordinators are listed at the end of this paper. #Present address: Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto, 611-0011, Japan. **Present address: Department of Microbiology, Ohio State University, Columbus, OH 43210, USA. ††These authors contributed equally to this work.

of summer sub-Antarctic surface waters and are stratified by seasonal heating. Station TARA_084 was located on the southern part of the Antarctic Circumpolar Current, in the Drake Passage between the Polar Front and the South Antarctic Circumpolar Current front (*11*). Station TARA_085 was located on the southern edge of the South Antarctic Circumpolar Current front with waters typical of polar regions.

We compared overall plankton community structures between the three oceans using imaging and genetic surveys of samples from the epipelagic zone of each station (*12*). Prokaryote, phyto-, and zooplankton assemblages were similar across Indian and South Atlantic Ocean samples but different from Southern Ocean samples (Fig. 2A). In the Indian and South Atlantic Oceans, zooplankton communities were dominated by Calanoida, Cyclopoida (Oithonidae), and Poecilostomatoida copepods (*12*); phytoplankton communities were mainly composed of chlorophytes, pelagophytes, and haptophytes (*12*). In contrast, Southern Ocean zooplankton communities were distinguished by an abundance of *Limacina* spp. gastropods and Poecilostomatoida copepods. Southern Ocean phytoplankton were primarily diatoms and haptophytes. The divergence was even more conspicuous with respect to prokaryotes, in that picocyanobacteria, dominant in the Indian and South Atlantic Oceans, were absent in the Southern Ocean. The Southern Ocean had a high proportion of Flavobacteria and Rhodobacterales (*12*). Virus concentrations in the <0.2-μm size fractions were significantly lower in the southernmost Southern Ocean station (*13*). Viral particles were significantly smaller in two of the three Southern Ocean sampling sites, and two Southern Ocean viromes had significantly lower richness compared with the South Atlantic and Indian Oceans (*13*). Although nucleocytoplasmic large DNA viruses were similarly distributed in the South Atlantic and Indian Oceans (*12*), two Southern Ocean sites contained coccolithoviruses also found in the TARA_068 Agulhas ring but not in the other Indian and South Atlantic stations.

Biological connection across the Agulhas choke point

Genetic material as represented by ribosomal RNA gene (rDNA) sequences showed exchange patterns across the oceans (shared barcode richness) (*14*). Despite a smaller interface between the Indian and South Atlantic Oceans than either have with the Southern Ocean, more than three times as much genetic material was in common between the Indian and South Atlantic Oceans than either had with the Southern Ocean (Fig. 2B) (*15*). Indeed, the Indian–South Atlantic interocean shared barcodes richness (32 ± 5%) was not significantly different from typical intraocean values (37 ± 7%, Tukey post hoc, 0.95 confidence). Shared barcode richness involving the Southern Ocean was significantly lower (9 ± 3%) (Fig. 2C). We found that the proportion of whole shotgun metagenomic reads shared between samples, both intraoceanic and Indian–South Atlantic interocean similarities, were in the 18 to 30% range, whereas interocean similarities with Southern Ocean samples were only 5 to 6% (*16*). The statistically indistinguishable Indo-Atlantic intra- and interocean genetic similarities revealed a high Indo-Atlantic biological connection despite the physical basin discontinuity.

Nonetheless, differences on either side of the Agulhas choke point were evident. We found that prokaryote barcode richness was greater in the South Atlantic than in the Indian Ocean (Fig. 3A) (0.2- to 3-μm size fraction). The opposite trend characterized eukaryotes larger than 20 μm in size. We cannot rule out the possibility that the higher prokaryote diversity observed in the South Atlantic Ocean might be due to a protocol artifact resulting from a difference in prefiltration pore size from 1.6 μm (Indian Ocean) to 3 μm (South Atlantic and Southern Oceans). As also evident from the panoceanic *Tara* Oceans data set (*17*), smaller size fractions showed greater eukaryote diversity across the Agulhas system. In all size fractions that we analyzed, samples from the Southern Ocean were less diverse than samples from the South Atlantic Ocean and Indian Ocean (Fig. 3A).

When rDNA barcodes were clustered by sequence similarity and considered at operational taxonomic unit (OTU) level (*14*), more than half (57%) of the OTUs contained higher sub-OTU barcode richness in the Indian Ocean than in the South Atlantic Ocean, whereas less than a third (32%) of OTUs were richer in the South Atlantic Ocean, leaving only 11% as strictly cosmopolitan (Fig. 3B). Taken together, these 1307 OTUs represented 98% of the barcode abundance, indicating that the observed higher barcode richness within OTUs in the Indian Ocean was not conferred by the rare biosphere. Certain taxa displayed unusual sub-OTU richness profiles across the choke point. Consistent with their relatively large size, Opisthokonta (mostly copepods), Rhizaria (such as radiolarians), and Stramenopiles (in particular diatoms) had much higher sub-OTU barcode richness in the Indian Ocean, whereas only small-sized Hacrobia (mostly haptophytes) showed modest increased sub-OTU barcode richness in the South Atlantic Ocean. The plankton filtering that we observed in fractions above 20 μm through the Agulhas choke point might explain the reduction of marine nekton diversity from the Indian Ocean to the South Atlantic Ocean (*18*) by propagating up the food web (*19*).

In situ sampling of two Agulhas rings

To understand whether the environment of Agulhas rings, the main transporters of water across the choke point, might act as a biological filter between the Indian Ocean and the South Atlantic Ocean, we analyzed data collected in both a young and an old Agulhas ring. The young ring sampled at station TARA_068 was located in the Cape Basin, west of South Africa, where rings are often observed after their formation at the Agulhas Retroflection (*7*, *20*). It was a large Agulhas ring that detached from the retroflection about 9 to 10 months before sampling. This ring first moved northward and then westward in the Cape Basin while interacting with other structures (red track in Fig. 1) (*21*). Ocean color data collected by satellite showed that surface chlorophyll concentrations were higher in the Cape Basin than at the retroflection, suggesting that vigorous vertical

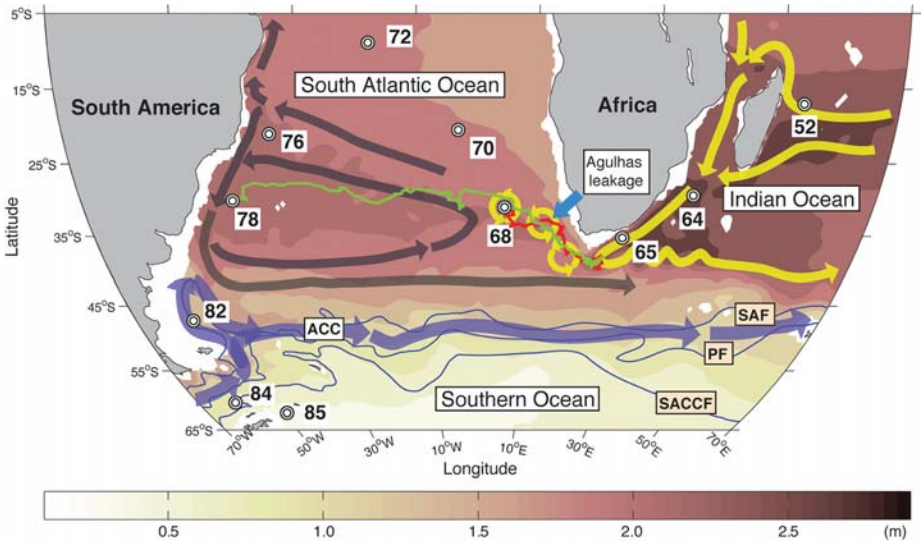

Fig. 1. The oceanic circulation around the Agulhas choke point and location of *Tara* Oceans stations. The map shows the location of sampling stations, together with trajectories of the young and old Agulhas rings (TARA_068 and TARA_078, red and green tracks, respectively). The stations here considered as representative of the main basins are (i) TARA_052, TARA_064, and TARA_065 for Indian Ocean; (ii) TARA_070, TARA_072, and TARA_076 for the South Atlantic Ocean, and (iii) TARA_082, TARA_084, and TARA_085 for the Southern Ocean. The mean ocean circulation is schematized by arrows (currents) and background colors [surface climatological dynamic height (0/2000 dbar from CARS2009; www.cmar.csiro.au/cars)] (*70*). Agulhas rings are depicted as circles. The Antarctic Circumpolar Current front positions are from (*13*).

mixing might have occurred in the Cape Basin (*22*). At the time of sampling, the anticyclonic Agulhas ring was 130 to 150 km in diameter, was about 30 cm higher than average sea surface height, and was flanked by a 130- to 150-km cyclonic eddy to the north and a larger (>200 km) one to the east (Fig. 4A) (*23*). Thermosalinograph data showed that filaments of colder, fresher water surrounded the young ring core (Fig. 4A) (*23*). To position the biological sampling station close to the ring core, a series of conductivity-temperature-depth (CTD) casts was performed (*23*, *24*). The young Agulhas ring had a surface temperature and salinity of 16.8°C and 35.7 practical salinity units (PSU), respectively, and the isopycnal sloping could be traced down to CTD maximal depth (900 to 1000 m). The core of the ring water was 5°C cooler than Indian Ocean subtropical source waters at similar latitudes (TARA_065) (table S1), typical for the subtropical waters south of Africa (17.8°C, 35.56 PSU, respectively) (*25*). The mixed layer of the young ring was deep (>250 m) compared with seasonal cycles of the mixed layer depths in the region (50 to 100 m) (Fig. 4C), typical of Agulhas rings (*26*). At larger scales (Fig. 4B) (*24*), steep spatial gradients were observed, with fresher and colder water in the Cape Basin than in the Agulhas Current because of both lateral mixing with waters from the south and surface fluxes. This confirms that the low temperature of the young Agulhas ring is a general feature of this Indian to South Atlantic Ocean transitional basin. Air-sea exchanges of heat and momentum promoted convection in the ring core, which was not compensated by lateral mixing and advection. The core of the Agulhas ring thus behaved as a subpolar environment traveling across a subtropical region.

At station TARA_078, we sampled a second structure whose origins were in the Agulhas Retroflection, likely a 3-year-old Agulhas ring. This old ring, having crossed the South Atlantic Ocean, was being absorbed by the western boundary current of the South Atlantic subtropical gyre. The structure sampled at station TARA_078 was characterized by a warm salty core (*27*). As for the young Agulhas ring sampled, the old ring also had a 100-m-deeper pycnocline than surrounding waters, typical of large anticyclonic structures.

The plankton assemblage of both Agulhas rings most closely resembled the assemblages found in Indian and South Atlantic samples (Fig. 2A). At higher resolution, barcodes (Fig. 2, B and C) and metagenomic reads (*16*) shared between the Agulhas rings and the Indian or South Atlantic samples showed that the young ring was genetically distinct from both Indian and South Atlantic samples,

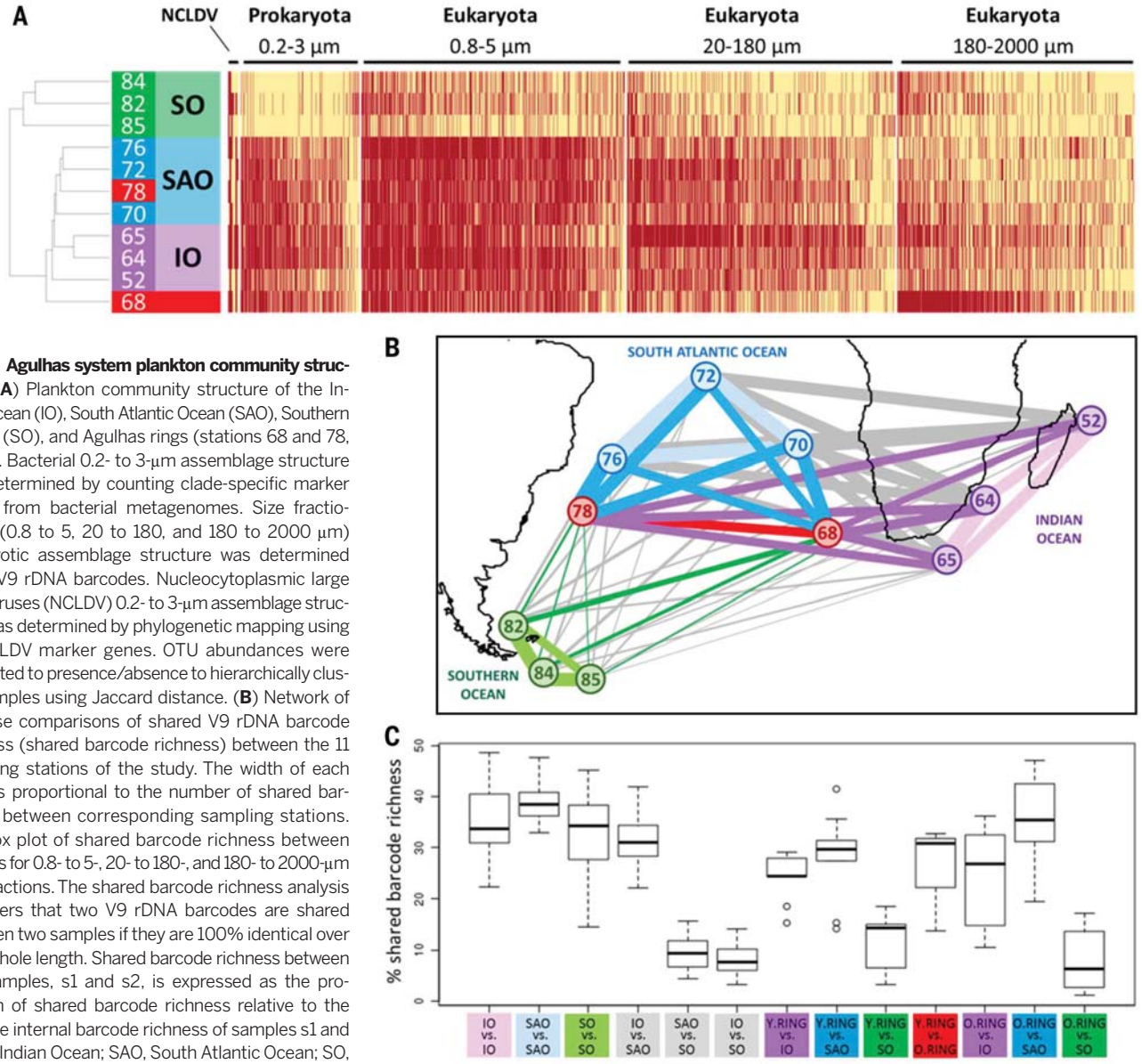

Fig. 2. Agulhas system plankton community structure. (**A**) Plankton community structure of the Indian Ocean (IO), South Atlantic Ocean (SAO), Southern Ocean (SO), and Agulhas rings (stations 68 and 78, in red). Bacterial 0.2- to 3-µm assemblage structure was determined by counting clade-specific marker genes from bacterial metagenomes. Size fractionated (0.8 to 5, 20 to 180, and 180 to 2000 µm) eukaryotic assemblage structure was determined using V9 rDNA barcodes. Nucleocytoplasmic large DNA viruses (NCLDV) 0.2- to 3-µm assemblage structure was determined by phylogenetic mapping using 16 NCLDV marker genes. OTU abundances were converted to presence/absence to hierarchically cluster samples using Jaccard distance. (**B**) Network of pairwise comparisons of shared V9 rDNA barcode richness (shared barcode richness) between the 11 sampling stations of the study. The width of each edge is proportional to the number of shared barcodes between corresponding sampling stations. (**C**) Box plot of shared barcode richness between stations for 0.8- to 5-, 20- to 180-, and 180- to 2000-µm size fractions. The shared barcode richness analysis considers that two V9 rDNA barcodes are shared between two samples if they are 100% identical over their whole length. Shared barcode richness between two samples, s1 and s2, is expressed as the proportion of shared barcode richness relative to the average internal barcode richness of samples s1 and s2. IO, Indian Ocean; SAO, South Atlantic Ocean; SO, Southern Ocean; Y.RING, young ring; O.RING, old ring.

whereas the old ring was similar to its surrounding South Atlantic samples (Tukey post hoc, 0.95 confidence). Light microscopy analyses revealed some plankton groups specific to the young Agulhas ring, such as *Pseudo-nitzschia* spp., which represented 20% of the phytoplankton counts but less than 10% in all other stations (*12*). Other potentially circumstantial plankton characteristic of the young Agulhas ring included the tintinnid *Dictyocysta pacifica* (*12*), the diatom *Corethron pennatum* (*12*), and the dinoflagellate *Tripos limulus* (*12*). A tiny (less than 15 μm long) pennate diatom from the genus *Nanoneis,* which we saw only in the young Agulhas ring and Indian Ocean stations around the African coasts (*28*), was an example of the Indo-Atlantic plankton diversity filtering observed at rDNA barcode level and corroborated by microscopy. OTU clustered barcodes revealed a variety of young Agulhas ring sub-OTU richness patterns compared with source and destination oceans (Fig. 5A). Among Copepoda, *Gaetanus variabilis* and *Corycaeus speciosus* were the more cosmopolitan species (Fig. 5B), whereas *Bradya* species found in the young ring were mainly similar to those from the Indian Ocean. *Acartia negligens* and *Neocalanus robustior* displayed high levels of barcode richness specific to each side of the Agulhas choke point. Bacillariophyceae were heavily filtered from Indian to South Atlantic Oceans (Fig. 5C), and most OTUs (17 out of 20) were absent in the young ring, suggesting that diversity filtering could take place earlier in the ring's 9-month history. Consistent with the observed particularities of the plankton in the young ring, continuous underway optical measurements showed that the ring core photosynthetic community differed from surrounding waters (*29*–*31*). Intermediate size cells, and relatively low content of photoprotective pigments, reflected low growth irradiance and suggested a transitional physiological state. Thus, the plankton community in the young Agulhas ring had diverged from plankton communities typical of its original Indian waters but, even 9 months after formation, had not converged with its surrounding South Atlantic waters.

Deep mixing in Agulhas rings promotes plankton bloom

The upper water column of the young ring showed a high nitrite concentration (>0.5 mmol m^{-3}) (Fig. 4D) (*32*). This observation, along with its particularly deep mixed layer (>250 m), suggested that as Agulhas rings proceed westward in the Cape Basin, vigorous deep mixing of their weakly stratified waters may have entrained nitrate and stimulated phytoplankton blooms. Typically, fresh organic material would then either be exported as sinking particles or locally recycled, sustaining heterotrophic production of ammonium that would, in turn, be consumed by photoautotrophs in the euphotic layer but nitrified below. The resulting nitrite, eventually oxidized to nitrate, might remain evident at subsurface as observed in the nitrite anomaly of the young ring detected here. This hypothesis was supported by numerical simulations of the Massachusetts Institute of Technology General Circulation Model (*33*), which resolved Agulhas rings, their phytoplankton populations, and associated nutrient cycling (Fig. 6A). We tracked 12 Agulhas rings in the ocean model and characterized their near-surface biogeochemical cycles (Fig. 6B) (*34*). As the rings moved westward, storms enhanced surface heat loss, stimulating convection and the entrainment of nitrate. In the model simulations, proliferation of phytoplankton generated subsurface nitrite, which persisted because phytoplankton were light-limited at depth and because nitrification was suppressed by light at the surface (*35*). The associated blooms were dominated by large opportunistic phytoplankton and nitrate-metabolizing *Synechococcus* spp. analogs, whereas populations of *Prochlorococcus* spp. analogs dominated the quiescent periods (*34*). Each of the 12 simulated Agulhas rings exhibited this pattern in response to surface forcing by weather systems, and all rings maintained a persistent

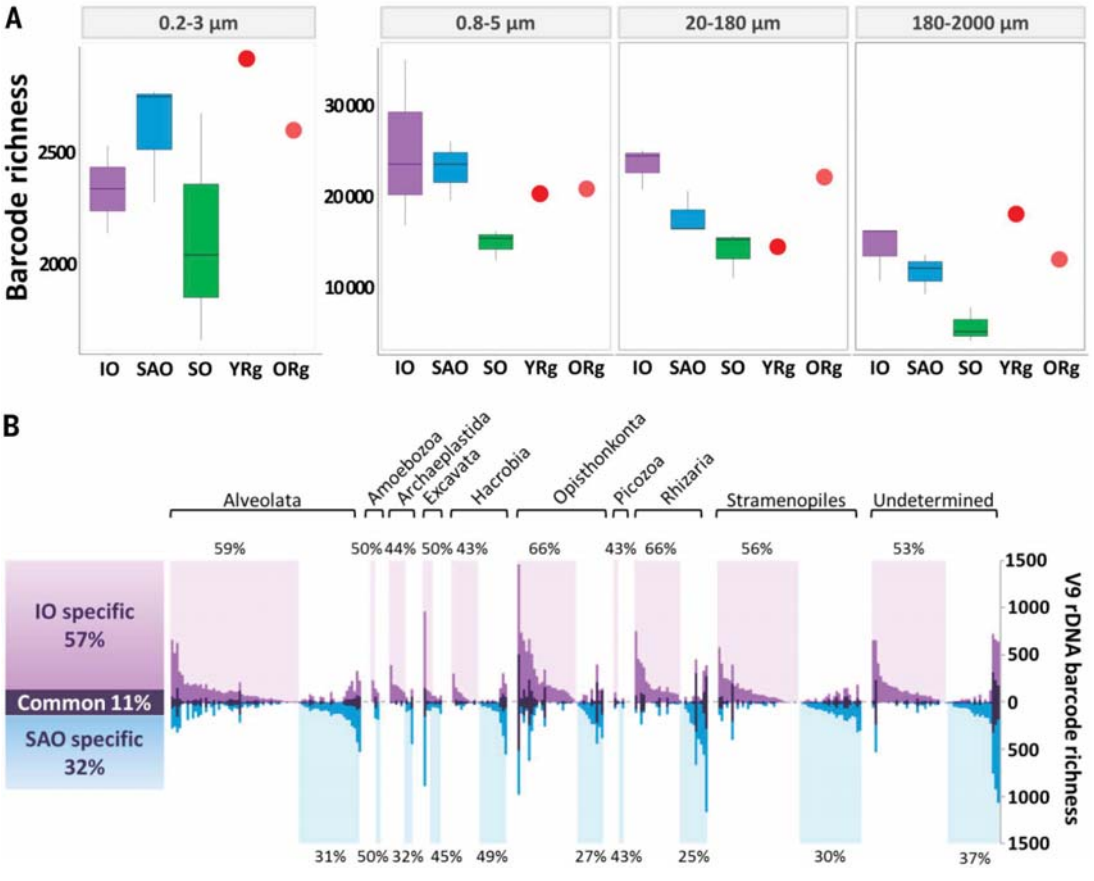

Fig. 3. Diversity of plankton populations specific to Indian and Atlantic Oceans. (**A**) Box plot of 16S (0.2 to 3 μm) and V9 rDNA barcodes richness (0.8- to 5-, 20- to 180-, and 180- to 2000-μm size fractions). Each box represents three sampling stations combined into Indian, South Atlantic, and Southern Ocean. Single Agulhas ring stations are represented as red (young ring) and orange (old ring) crosses. (**B**) Plankton sub-OTU richness filtering across the Agulhas choke point. Each vertical bar represents a single eukaryotic plankton OTU, each of which contains >10 distinct V9 rDNA barcodes (*14*). For each OTU are represented the number of distinct barcodes (sub-OTU richness) found exclusively in the South Atlantic Ocean (blue), exclusively in the Indian Ocean (pink), and in both South Atlantic Ocean and Indian Ocean (gray). OTUs are grouped by taxonomic annotation (indicated above the bar plot). For each taxonomic group, the percentage of OTUs with higher sub-OTU richness in the Indian Ocean (shaded in pink) or in the South Atlantic Ocean (shaded in blue) is indicated, respectively, at the top and bottom of the bar plot. A total of 1307 OTUs are presented, representing 98% of total V9 rDNA barcode abundance.

subsurface nitrite maximum in the region, as observed in TARA_068 and in other biogeochemical surveys (*36*).

The nitrite peak observed at TARA_068 in the young Agulhas ring was associated with a differential representation of nitrogen metabolism genes between the ring and the surrounding South Atlantic and Indian Oceans metagenomes derived from 0.2- to 3-μm size fractions (Fig. 7) (*37*). Agulhas ring overrepresented KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs (KOs) were involved in both nitrification and denitrification, likely representing the overlap between plankton assemblages involved in the conversion of nitrate to nitrite on the one hand and in denitrification of the accumulating nitrite on the other. Distinct KOs involved in successive denitrification steps were found to be encoded by similar plankton taxa. For instance, KO10945 and KO10946 (involved in ammonium nitrification) and KO00368 (subsequently involved in nitrite to nitrous oxide denitrification) appeared mostly encoded by Nitrosopumilaceae archaea. KO00264 and KO01674 (involved in ammonium assimilation) were mostly assigned to eukaryotic Mamiellales, whereas the opposite KO00367 and KO00366 (involved in dissimilatory nitrite reduction to ammonium), followed by KO01725 (involved in ammonium assimilation), were encoded by picocyanobacteria. In the specific case of the picocyanobacteria, metagenomic reads corresponding to *nirA* genes showed that the observed young Agulhas ring KO00366 (dissimilatory nitrite reduction) enrichment was mainly due to the overrepresentation of genes from *Prochlorococcus* (Fig. 8B). This enrichment was found to be associated with a concomitant shift in population structure from *Prochlorococcus* high-light II ecotypes (HLII, mostly lacking *nirA* genes) to codominance of high-light I (HLI) and low-light I (LLI) ecotypes. Indeed, among the several *Prochlorococcus* and *Synechococcus* ecotypes identified based on their genetic diversity and physiology (*38*, *39*), neutral marker (*petB*) (Fig. 8A) recruitments showed that dominant clades in the Indian Ocean upper mixed layer were *Prochlorococcus* HLII and *Synechococcus* clade II, as expected given the known (sub)tropical preference of these groups (*40*). Both clades nearly completely disappeared (less than 5%) in the mixed cold waters of the young ring and only began to increase again when the surface water warmed up along the South Atlantic Ocean transect. Conversely, young ring water was characterized by a large proportion of *Prochlorococcus* HLI and LLI and *Synechococcus* clade IV, two clades typical of temperate waters. Besides temperature, the *Prochlorococcus* community shift from HLII to HLI + LLI observed in the young ring was likely also driven by the nitrite anomaly. Indeed, whereas most *Synechococcus* strains isolated so far are able to

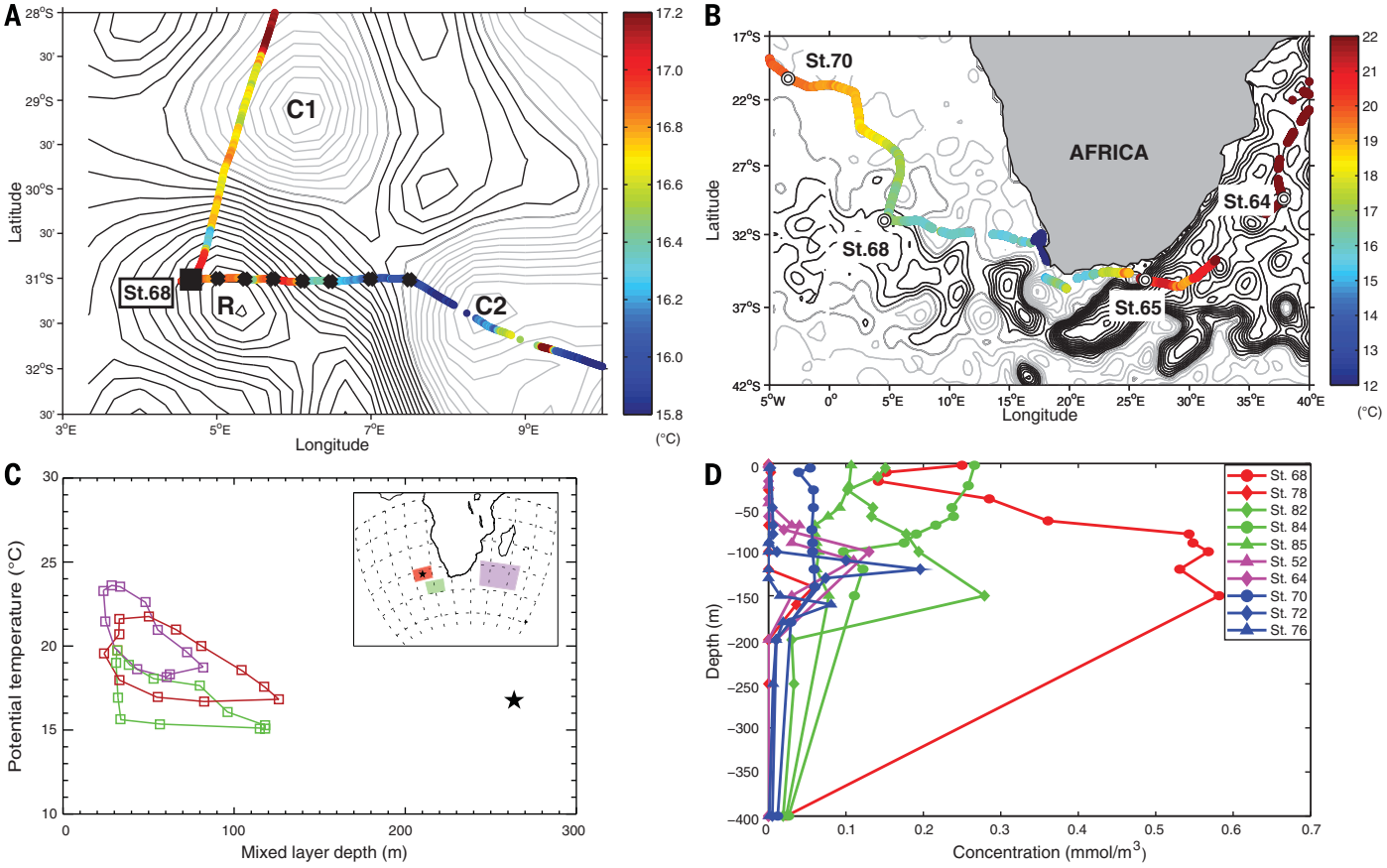

Fig. 4. Properties of the young Agulhas ring (TARA_068). (**A**) Daily sea surface height around young Agulhas ring station TARA_068 [absolute dynamic topography (ADT) from www.aviso.altimetry.fr]. R, C1, and C2, respectively, denote the centers of the Agulhas ring and two cyclonic eddies. The contour interval is 0.02 dyn/m. The ADT values are for 13 September 2010. Light gray isolines, ADT < 0.46 dyn/m. The crosses indicate the CTD stations, and the square symbol indicates the position of the biological station TARA_068. The biological station coincides with the westernmost CTD station. ADT is affected by interpolation errors, which is why CTD casts were performed at sea so as to have a fine-scale description of the feature before defining the position of the biological station (*23*). Superimposed are the continuous underway temperatures (°C) from the on-board thermosalinograph. (**B**) Same as (A) but at the regional scale. Round symbols correspond to biological sampling stations. The contour interval is 0.1 dyn/m. (**C**) Seasonal distribution of the median values of the mixed layer depths and temperatures at 10 m (from ARGO) provided by the IFREMER/LOS Mixed Layer Depth Climatology L2 database (www.ifremer.fr/cerweb/deboyer/mld) updated to 27 July 2011. The mixed layer is defined using a temperature criterion. The star symbol represents the young ring station TARA_068. (Inset) Geographic position of the areas used to select the mixed layer and temperature data. The mixed layer depth measured at TARA_068 is outside the 90th percentile of the distribution of mixed layer depths for the same month for both the subtropical (red and magenta) regions. The temperature matches the median for the same month and region of sampling. (**D**) Nitrite (NO_2) concentrations from CTD casts at different sampling sites (expressed in mmol/m^3).

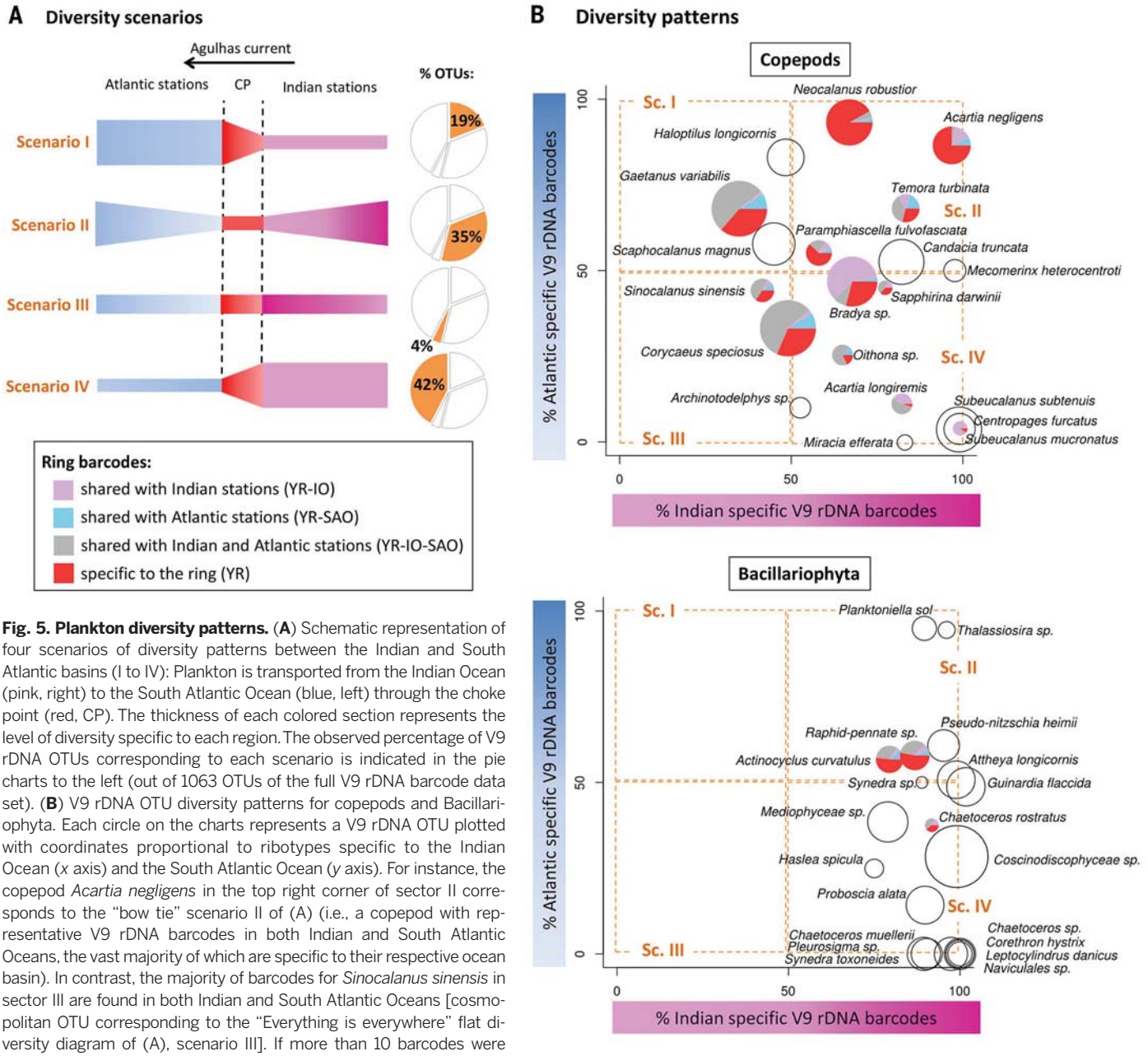

Fig. 5. Plankton diversity patterns. (**A**) Schematic representation of four scenarios of diversity patterns between the Indian and South Atlantic basins (I to IV): Plankton is transported from the Indian Ocean (pink, right) to the South Atlantic Ocean (blue, left) through the choke point (red, CP). The thickness of each colored section represents the level of diversity specific to each region. The observed percentage of V9 rDNA OTUs corresponding to each scenario is indicated in the pie charts to the left (out of 1063 OTUs of the full V9 rDNA barcode data set). (**B**) V9 rDNA OTU diversity patterns for copepods and Bacillariophyta. Each circle on the charts represents a V9 rDNA OTU plotted with coordinates proportional to ribotypes specific to the Indian Ocean (*x* axis) and the South Atlantic Ocean (*y* axis). For instance, the copepod *Acartia negligens* in the top right corner of sector II corresponds to the "bow tie" scenario II of (A) (i.e., a copepod with representative V9 rDNA barcodes in both Indian and South Atlantic Oceans, the vast majority of which are specific to their respective ocean basin). In contrast, the majority of barcodes for *Sinocalanus sinensis* in sector III are found in both Indian and South Atlantic Oceans [cosmopolitan OTU corresponding to the "Everything is everywhere" flat diversity diagram of (A), scenario III]. If more than 10 barcodes were found in the young Agulhas ring (TARA_068), their distribution is indicated in a pie chart (colors are coded in the legend inset); otherwise, the OTU is represented by an empty circle. Circle sizes are proportional to the number of considered barcodes for each OTU. The Bacillariophyta OTU defined as *Raphid pennate* sp. likely corresponds to the *Pseudo-nitzschia* cells observed by light microscopy.

use nitrate, nitrite, and ammonium, only the *Prochlorococcus* LLI and IV and some populations of HL clades, having acquired the *nirA* gene by lateral gene transfer, are able to assimilate nitrite. In the young ring, overrepresentation of cyanobacterial orthologs involved in nitrite reduction could thus have resulted from environmental pressure selecting LLI (87% of the *nirA* recruitments) and HL populations (13%) that possessed this ability. Because the capacity to assimilate nitrite in this latter ecotype reflects the availability of this nutrient in the environment (*41*), these in situ observations of picocyanobacteria indicated that the nitrogen cycle disturbance occurring in the young ring exerts community-wide selective pressure on Agulhas ring plankton.

Discussion

We found that whether or not the Agulhas choke point is considered a barrier to plankton dispersal depends on the taxonomic resolution at which the analysis is performed. At coarse taxonomic resolution, our observations of Indo-Atlantic continuous plankton structure—from viruses to fish larvae—suggested unlimited dispersal, consistent with previous reports (*5*, *42*). However, at finer resolution, our genetic data revealed that the Agulhas choke point strongly affects patterns of plankton genetic diversity. As anticipated in (*5*), the diversity filtering by Agulhas rings likely escaped detection using fossil records because of the limited taxonomic resolution afforded by fossil diatom morphology (*42*). The community-wide evidence presented here confirms observations on individual living species (*43*, *44*), suggesting that dispersal filters mitigate the panmictic ocean hypothesis for plankton above 20 μm.

The lower diversity we observed in the South Atlantic Ocean for micro- and mesoplankton (>20 μm) may be due to local abiotic/biotic pressure or to limitations in dispersal (*33*, *45*). Biogeography emerging from a model with only neutral drift (*46*) predicts

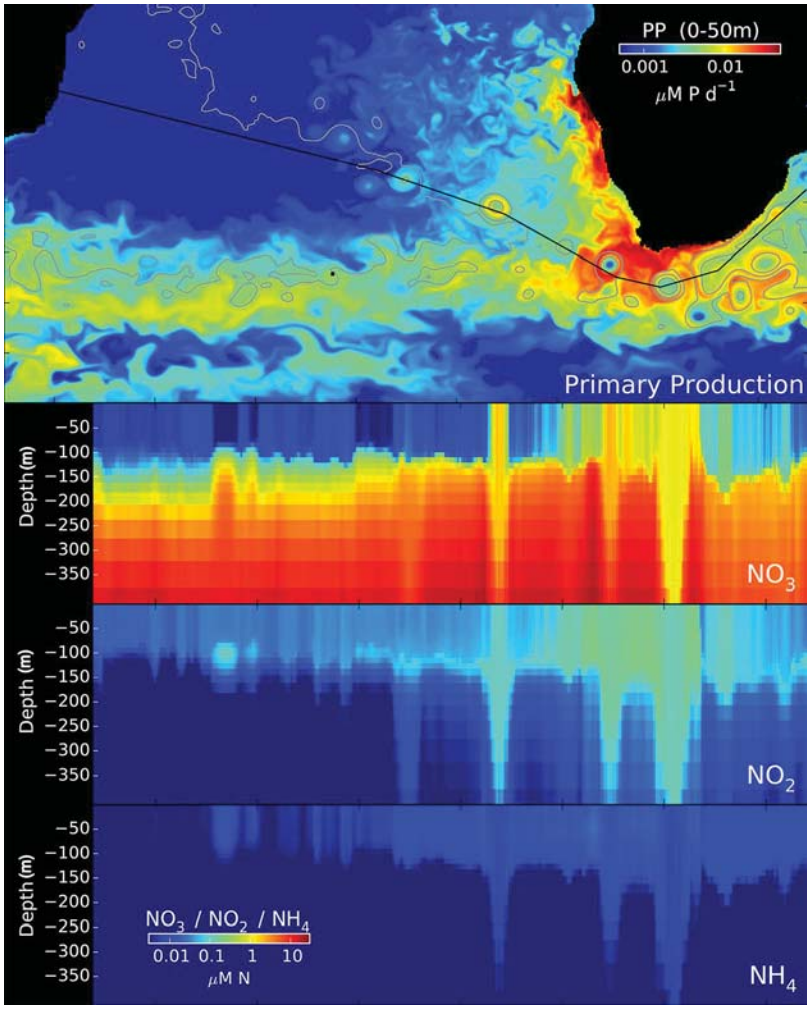

Fig. 6. Modeled nitrogen stocks along Agulhas ring track. (**Top**) Simulated primary production (PP) in the Agulhas system using the MIT-GCM model. The solid black line shows the average northwesterly path of 12 distinct virtual Agulhas rings tracked over the course of the simulation. Color scale for PP is given in the top right inset, with warmer colors indicating higher PP. (**Bottom**) Modeled profiles of NO_3, NO_2, and NH_4 along the Agulhas ring average track (*x* axis) presented in (A). The *y* axis is the depth (in meters) in the water column. The color scale is given in the bottom left inset, with warmer colors indicating higher concentrations of nitrogen compounds.

basin-to-basin genetic differences that are qualitatively consistent with our data. However, the increased proportion of *Prochlorococcus* HL populations carrying the *nirA* gene in the young Agulhas ring indicates that selection is at work in Agulhas rings. Based on our analysis of two Agulhas rings, we propose that environmental disturbances in Agulhas rings reshape their plankton diversity as they travel from the Indian Ocean to the South Atlantic Ocean. Such selective pressure may contribute to the South Atlantic Ocean plankton diversity shift relative to its upstream Indo-Pacific basin. Thus, environmental selection applied at a choke point in ocean circulation may constitute a barrier to dispersal (*47*, *48*). Furthermore, we show that taxonomic groups were not equally affected by the ring transport, both within and between phyla, with a noticeable effect of organism size. The differential effects due to organism size highlight the difficulty in generalizing ecological and evolutionary rules from limited sampling of species or functional types.

Considering the sensitivity of Agulhas leakage to climate change (*1*, *49*), better understanding of the plankton dynamics in Agulhas rings will be required if we are to understand and predict ecosystem resilience at the planetary scale. Considering the breadth of changes already observed in the 9-month-old Agulhas ring, it would be interesting to acquire samples from specific Agulhas rings tracked from early formation to dissipation. Finally, our data suggest that the abundance of Indian Ocean species in South Atlantic Ocean sedimentary records, used as proxies of Agulhas leakage intensity (*4*), may actually also depend on the physical and biological characteristics of the Agulhas rings.

Materials and methods

Sampling

The *Tara* Oceans sampling protocols schematized in Karsenti *et al.* (*9*) are described in Pesant *et al.* (*50*); specific methods for 0.8- to 5-, 20- to 180-, and 180- to 2000-μm size fractions in de Vargas *et al.* (*17*); for 0.2- to 3-μm size fractions in Sunagawa *et al.* (*51*); and for <0.2-μm size fraction in Brum *et al.* (*52*). Due to their fragility, 1.6-μm glass fiber filters initially used for prokaryote sampling were replaced by more resistant 3-μm polycarbonate filters from station TARA_066 onward. In the present text, both 0.2- to 1.6-μm and 0.2- to 3-μm prokaryote size fractions are simply referred to as 0.2 to 3 μm.

Data acquisition

A range of analytical methods covering different levels of taxonomic resolution (pigments, flow cytometry, optical microscopy, marker gene barcodes, and metagenomics) were used to describe the planktonic composition at each sampled station. Viruses from the <0.2 μm size fraction were studied by epifluorescence microscopy, by quantitative transmission electron microscopy, and by sequencing DNA as described in Brum *et al.* (*52*). Flow cytometry was used to discriminate high-DNA-content bacteria (HNA), low-DNA-content bacteria (LNA), *Prochlorococcus* and *Synechococcus* picocyanobacteria, and two different groups (based on their size) of photosynthetic picoeukaryotes, as described previously (*53*). Pigment concentrations measured by high-performance liquid chromatography (HPLC) were used to estimate the dominant classes of phytoplankton using the CHEMTAX procedure (*54*). Tintinnids, diatoms, and dinoflagellates were identified and counted by light microscopy from the 20- to 180-μm lugol or formaldehyde fixed-size fraction. Zooplankton enumeration was performed on formol fixed samples using the ZOOSCAN semi-automated classification of digital images (*55*). Sequencing, clustering, and annotation of 18*S*-V9 rDNA barcodes are described in de Vargas *et al.* (*17*). Metagenome sequencing, assembly, and annotation are described in Sunagawa *et al.* (*51*). NCLDV taxonomic assignations in the 0.2- to 3-μm samples were carried out using 18 lineage-specific markers as described in Hingamp *et al.* (*56*). Virome sequencing and annotation are described in Brum *et al.* (*52*). Samples and their associated contextual data are described at PANGAEA (*57*–*59*).

Data analysis

Origin of sampled Agulhas rings

Using visual and automated approaches, the origins of the TARA_068 and TARA_078 stations were traced back from the daily altimetric data (Fig. 1) (*21*). The automated approach used either the Lagrangian tracing of numerical particles initialized in the center of a given structure and transported by the geostrophic velocity field calculated from sea surface height gradients, or the connection in space and time of adjacent extreme values in sea level anomaly maps.

V9 rDNA barcodes

To normalize for differences in sequencing effort, V9 rDNA barcode libraries were resampled 50 times for the number of reads corresponding to the smallest library in each size fraction: 0.8 to 5 μm, 776,358 reads; 20 to 180 μm, 1,170,592 reads; and 180 to 2000 μm, 767,940 reads. V9 rDNA barcode counts were then converted to the average number

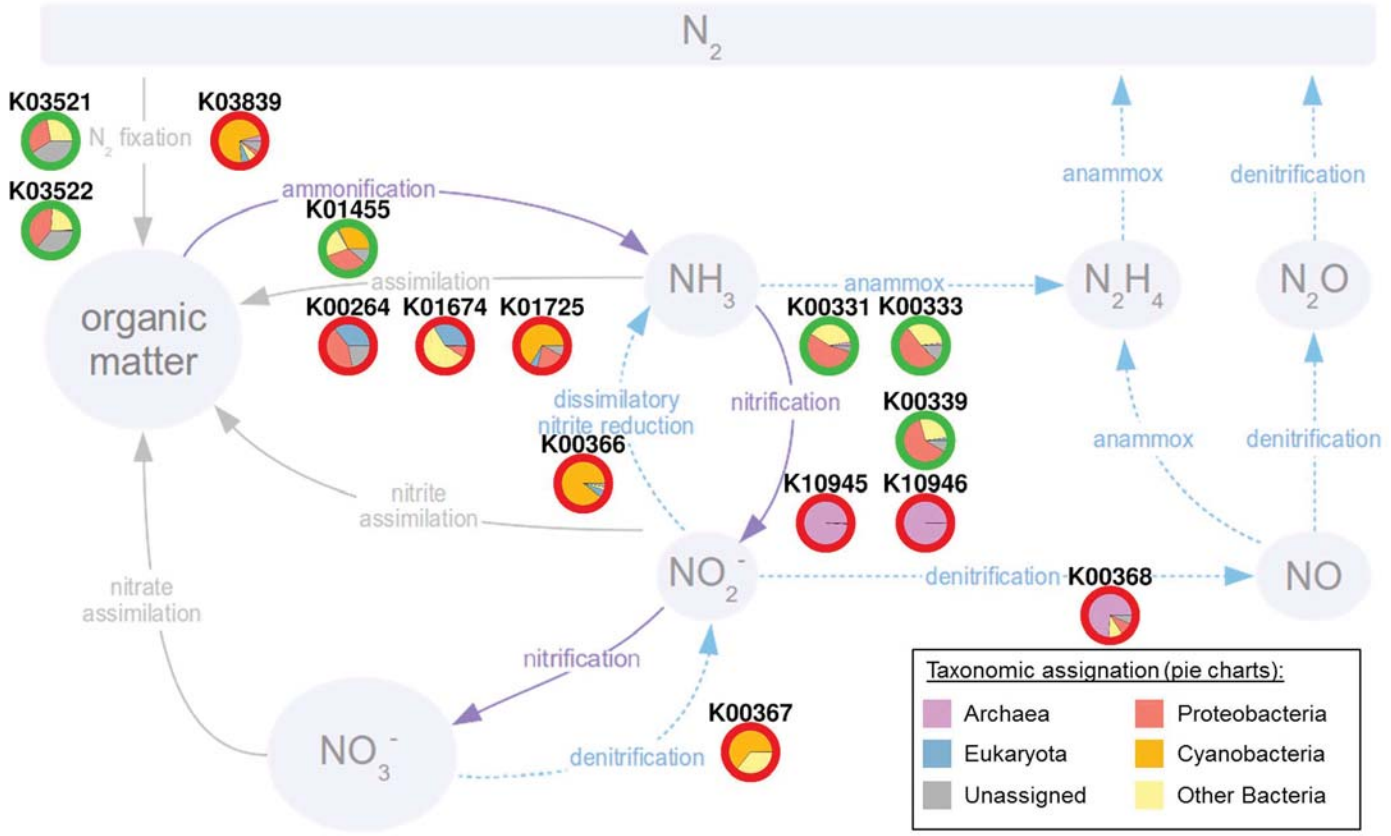

Fig. 7. Nitrite anomaly in the young Agulhas ring is accompanied by shifts in nitrogen pathway–related genes. Metagenomic over- and underrepresented nitrogen pathway genes in young Agulhas ring. Over- (red circles) and under- (green circles) represented metagenome functional annotations (KEGG Orthologs, KO#) involved in the nitrogen pathway in the young ring compared to Indian and South Atlantic Oceans reference stations, at surface and deep chlorophyll maximum depth. Pie charts inside circles represent the taxonomic distribution for each ortholog.

of times seen in the 50 resampling events, and barcodes with less than 10 reads were removed as potential sequencing artifacts. We used down-sampled barcode richness (number of distinct V9 rDNA barcodes) as a diversity descriptor because using V9 rDNA barcode abundances to compare plankton assemblages would likely be biased due to (i) technical limitations described in de Vargas *et al.* (*17*) and (ii) seasonality effects induced by the timing of samplings (table S1). Barcode richness was well correlated with Shannon and Simpson indexes (0.94 and 0.78, respectively). The shared barcode richness between each pair of samples (*14*) was estimated by counting, for the three larger size fractions (0.8 to 5, 20 to 180, and 180 to 2000 μm), the proportion of V9 rDNA barcodes 100% identical over their whole length. V9 rDNA barcodes were clustered into OTUs by swarm clustering as described by de Vargas *et al.* (*17*). The sub-OTU richness comparison between two samples s1 and s2 (*14*) produces three values: the number of V9 rDNA barcodes in common, the number of V9 rDNA barcodes unique to s1, and the number of V9 rDNA barcodes unique to s2. These numbers can be represented directly as bar graphs (Fig. 3B) or as dot plots of specific V9 rDNA barcode richness (Fig. 5).

Metagenomic analysis

Similarity was estimated using whole shotgun metagenomes for all four available size fractions (0.2 to 3, 0.8 to 5, 20 to 180, and 180 to 2000 μm). Because pairwise comparisons of all raw metagenome reads are intractable given the present data volume, we used a heuristic in which two metagenomic 100–base pair (bp) reads were considered similar if at least two nonoverlapping 33-bp subsequences were strictly identical (Compareads method) (*60*). For prokaryotic fractions (0.2 to 3 μm), taxonomic abundance was estimated using the number of 16*S* $_{mi}$tags (*51*). The functional annotation, taxonomic assignation, and gene abundance estimation of the panoceanic Ocean Microbial Reference Gene Catalog (OM-RGC) (243 samples, including all those analyzed here) generated from *Tara* Oceans 0.2- to 3-μm metagenomic reads are described in Sunagawa *et al.* (*51*). Gene abundances were computed for the set of genes annotated to the nitrogen metabolism KO (*61*) group by counting the number of reads from each sample that mapped to each KO-associated gene. Abundances were normalized as reads per kilobase per million mapped reads (RPKM). Gene abundances were then aggregated (summed) for each KO group. To compare abundances between the young ring (TARA_068) and other stations, a *t* test was used. KOs with a *P* value <0.05 and a total abundance (over all stations) >10 were considered as significant (*37*). *Prochlorococcus* and *Synechococcus* community composition was analyzed in the 0.2- to 3-μm size fraction at the clade level by recruiting reads targeting the high-resolution marker gene *petB*, coding for cytochrome b_6 (*62*). The *petB* reads were first extracted from metagenomes using Basic Local Alignment Search Tool (BLASTx+) against the *petB* sequences of *Synechococcus* sp. WH8102 and *Prochlorococcus marinus* MED4. These reads were subsequently aligned against a reference data set of 270 *petB* sequences using BLASTn (with parameters set at -G 8 -E 6 -r 5 -q -4 -W 8 -e 1 -F "m L" -U T). *petB* reads exhibiting >80% identity over >90% of sequence length were then taxonomically assigned to the clade of the best BLAST hit. Read counts per clade were normalized based on the sequencing effort for each metagenomic sample. A similar approach was used with *nirA* (KO 00366) and *narB* genes (KO 00367), which were highlighted in the nitrogen-related KO analysis (Fig. 7). Phylogenetic assignment was realized at the highest possible taxonomic level using a reference data set constituted of sequences retrieved from Cyanorak v2 (www.sb-roscoff.fr/cyanorak/) and Global Ocean Sampling (*41*, *63*) databases.

Nitrogen cycle modeling

Numerical simulations of global ocean circulation were based on the Massachusetts Institute of Technology General Circulation Model (MIT-GCM) (*64*), incorporating biogeochemical and ecological components (*65*, *66*). It resolved mesoscale

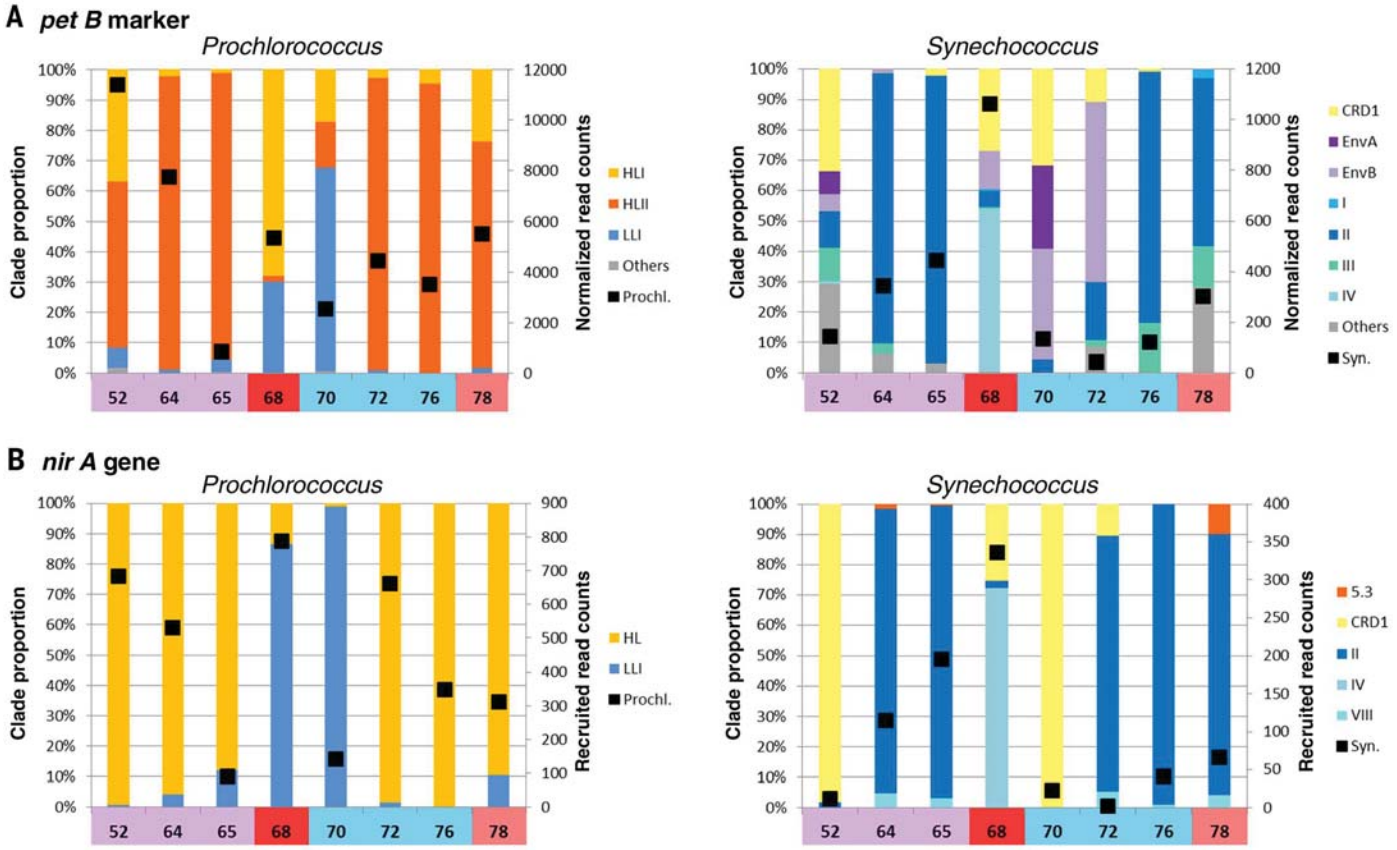

Fig. 8. Picocyanobacterial clade shift in the young Agulhas ring. (**A**) Relative abundance of *Prochlorococcus* and *Synechococcus* clades, estimated by *petB* read recruitments from 0.2- to 3-μm metagenomes. Solid squares correspond to read counts normalized based on the sequencing effort (right axis). (**B**) Relative abundance of *nirA* gene from *Prochlorococcus* and *Synechococcus* clades estimated by number of reads recruited from 0.2- to 3-μm metagenomes. The bar colors correspond to cyanobacterial clades indicated in the inset legends for each panel. Solid squares correspond to the number of reads recruited (right axis). Data are shown for stations TARA_052 to TARA_078 only, because too few cyanobacteria were found in Southern Ocean stations TARA_082, TARA_084, and TARA_085.

features in the tropics and was eddy-permitting in subpolar regions. The physical configurations were integrated from 1992 to 1999 and constrained to be consistent with observed hydrography and altimetry (*67*). Three inorganic fixed nitrogen pools were resolved—nitrate, nitrite, and ammonium—as well as particulate and dissolved detrital organic nitrogen. Phytoplankton types were able to use some or all of the fixed nitrogen pools. Aerobic respiration and remineralization by heterotrophic microbes was parameterized as a simple sequence of transformations from detrital organic nitrogen, to ammonium, then nitrification to nitrite and nitrate. In accordance with empirical evidence (*35*), nitrification was assumed to be inhibited by light. Nitrification is described in the model by simple first-order kinetics, with rates tuned to qualitatively capture the patterns of nitrogen species in the Atlantic (*66*).

Continuous spectral analysis

A continuous flow-through system equipped with a high-spectral-resolution spectrophotometer (AC-S, WET Labs, Inc.) was used for data collection during the *Tara* Oceans expedition, as described previously (*68*). Phytoplankton pigment concentrations, estimates of phytoplankton size γ, total chlorophyll a concentration, and particulate organic carbon (POC) are derived from the absorption and attenuation spectra (*69*) for the 1-km^2-binned *Tara* Oceans data set available at PANGAEA (http://doi.pangaea.de/10.1594/PANGAEA.836318).

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31. Companion Web site, figure W11; available at http://www.igs.cnrs-mrs.fr/Tara_Agulhas/#FigW11
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***Tara* Oceans Coordinators**

Silvia G. Acinas,[1] Peer Bork,[2] Emmanuel Boss,[3] Chris Bowler,[4] Colomban de Vargas,[5,6] Michael Follows,[7] Gabriel Gorsky,[8,9] Nigel Grimsley,[10,11] Pascal Hingamp,[12] Daniele Iudicone,[13] Olivier Jaillon,[14,15,16] Stefanie Kandels-Lewis,[2,17] Lee Karp-Boss,[3] Eric Karsenti,[4,17] Uros Krzic,[18] Fabrice Not,[5,6] Hiroyuki Ogata,[19] Stephane Pesant,[20,21] Jeroen Raes,[22,23,24] Emmanuel G. Reynaud,[25] Christian Sardet,[26,27] Mike Sieracki,[28] Sabrina Speich,[29,30] Lars Stemmann,[8] Matthew B. Sullivan,[31] Shinichi Sunagawa,[2] Didier Velayoudon,[32] Jean Weissenbach,[14,15,16] Patrick Wincker[14,15,16]

[1]Department of Marine Biology and Oceanography, Institute of Marine Science (ICM)-CSIC, Pg. Marítim de la Barceloneta, 37-49, Barcelona E08003, Spain. [2]Structural and Computational Biology, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany. [3]School of Marine Sciences, University of Maine, Orono, Maine, USA. [4]Ecole Normale Supérieure, Institut de Biologie de l'ENS (IBENS), and Inserm U1024, and CNRS UMR 8197, F-75005 Paris, France. [5]CNRS, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France. [6]Sorbonne Universités, UPMC Univ Paris 06, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France. [7]Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA. [8]CNRS, UMR 7093, LOV, Observatoire Océanologique, F-06230 Villefranche-sur-Mer, France. [9]Sorbonne Universités, UPMC Univ Paris 06, UMR 7093, LOV, Observatoire Océanologique, F-06230 Villefranche-sur-Mer, France. [10]CNRS UMR 7232, BIOM, Avenue du Fontaulé, 66650 Banyuls-sur-Mer, France. [11]Sorbonne Universités

Paris 06, OOB UPMC, Avenue du Fontaulé, 66650 Banyuls-sur-Mer, France. [12]Aix Marseille Université CNRS IGS UMR 7256, 13288 Marseille, France. [13]Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy. [14]CEA, Institut de Génomique, GENOSCOPE, 2 rue Gaston Crémieux, 91057 Evry, France. [15]CNRS, UMR 8030, CP5706, Evry, France. [16]Université d'Evry, UMR 8030, CP5706, Evry, France. [17]Directors' Research, European Molecular Biology Laboratory, Heidelberg, Germany. [18]Cell Biology and Biophysics, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany. [19]Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto, 611-0011, Japan. [20]PANGAEA, Data Publisher for Earth and Environmental Science, University of Bremen, Bremen, Germany. [21]MARUM, Center for Marine Environmental Sciences, University of Bremen, Bremen, Germany. [22]Department of Microbiology and Immunology, Rega Institute, KU Leuven, Herestraat 49, 3000 Leuven, Belgium. [23]Center for the Biology of Disease, VIB, Herestraat 49, 3000 Leuven, Belgium. [24]Department of Applied Biological Sciences, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium. [25]Earth Institute, University College Dublin, Dublin, Ireland. [26]CNRS, UMR 7009 Biodev, Observatoire Océanologique, F-06230 Villefranche-sur-Mer, France. [27]Sorbonne Universités, UPMC Univ Paris 06, UMR 7009 Biodev, F-06230 Observatoire Océanologique, Villefranche-sur-Mer, France. [28]Bigelow Laboratory for Ocean Sciences, East Boothbay, USA. [29]Department of Geosciences, Laboratoire de Météorologie Dynamique (LMD), Ecole Normale Supérieure, 24 rue Lhomond 75231 Paris Cedex 05, France. [30]Laboratoire de Physique des Océans, UBO-IUEM, Place Copernic, 29820 Plouzané, France. [31]Department of Ecology and Evolutionary Biology, University of Arizona, 1007 East Lowell Street, Tucson, AZ 85721, USA. [32]DVIP Consulting, Sèvres, France.

SUPPLEMENTARY MATERIALS

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Table S1

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Environmental characteristics of Agulhas rings affect interocean plankton transport

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ORIGINAL ARTICLE

Phylogeographic study on the chub mackerel (*Scomber japonicus*) in the Northwestern Pacific indicates the late Pleistocene population isolation

Shuai Yan[1], Gaetano Catanese[2], Christopher L. Brown[3], Ming Wang[1,4], Chaopin Yang[1] & Tingbao Yang[1]

1 State Key Laboratory of Biocontrol and Center for Parasitic Organisms, School of Life Sciences, Sun Yat-sen University, Guangzhou, China
2 Stazione Zoologica Anton Dohrn, Napoli, Italy
3 National Oceanic and Atmospheric Administration, Aquaculture and Enhancement Division, The Milford Laboratory, Milford, CT, USA
4 Guangdong Institute of Standardization, Guangzhou, China

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Correspondence
Tingbao Yang, State Key Laboratory of Biocontrol and Center for Parasitic Organisms, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China.
E-mail: lssytb@mail.sysu.edu.cn

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Abstract

In the present study, the population genetic structure and historical demography of the chub mackerel, *Scomber japonicus*, in the Northwestern Pacific were examined based on the full-length sequences of the mitochondrial control region and cytochrome *b* gene. A total of 320 individuals was sampled from 11 localities along the coast of China and Japan from August 2011 to May 2013. Two main clades representing Chinese and Japanese populations, respectively, were detected, suggesting population isolation of *S. japonicus* during the late Pleistocene era. The Chinese clade was further divided into two small clades and the distribution of haplotypes were not related to sampling locality, which may be a signature of secondary contact following past division. Analyses of molecular variance and pairwise F_{ST} revealed significant genetic differentiation between the Chinese and Japanese populations, but a lack of genetic structure for the populations along the coast of China. Both neutrality tests and mismatch distribution analysis suggested that the populations along the coast of China experienced population expansion during the late Pleistocene. Historical events, biological characteristics and other extrinsic forces such as ocean currents may all be associated with the current phylogeographic pattern of *S. japonicus* in the Northwestern Pacific.

Introduction

The genetic structure of fish populations has attracted considerable interest, not only because of a fundamental interest in biotic evolution (Tudela *et al.* 1999), but also because of its importance in fisheries management (Bailey 1997; Roldán *et al.* 2000). Determination of population genetic structure is essential to underpin resource recovery and to aid delineation and monitoring of populations for fishery management (Roldán *et al.* 2000). The population genetic structures of marine species are influenced by both historical events and contemporary conditions.

Historical processes associated with climatic oscillations in the Pleistocene ice ages are among the most important determinants of the current distribution of species. A number of contemporary factors, such as dispersal ability of larvae and adult organisms, local adaptation, oceanographic currents, sea temperature, discontinuities of salinity and isolation by distance, have also been recognized as important factors in generating intra-specific genetic structure (Schulte 2001; Fauvelot & Planes 2002). Genetic homogeneity across large geographic scales is often attributed to the connections generated by sea-surface currents. In the ocean waters along the coast of China, the China Coastal Current, Yellow

Sea Warm Current, Taiwan Warm Current and South China Sea Warm Current collectively facilitate contact among the three marginal seas of China: the Yellow Sea, East China Sea and South China Sea.

The chub mackerel (*Scomber japonicus*) is a widespread fish in the warm and temperate transition coastal areas and the adjacent seas of the Atlantic, Pacific and Northwest Indian oceans (Collette & Nauen 1983; Zeng *et al.* 2012). This and related mackerel species are prized for human consumption because of their oily meat, and the health benefits of their abundant omega-3 fatty acids (Nettleton 1995). *Scomber japonicus*, a pelagic, migratory and large-shoal-forming species (Lockwood 1988), is one of the most important fishery resources of China (Cheng & Lin 2004). In addition to its important economic value for fisheries, it figures prominently in marine ecosystems as a key component of the diet of large predatory pelagic fishes and sea mammals (Cha *et al.* 2010). Although the fisheries' yield of chub mackerel has been maintained at a high level, the population has been under pressure from overfishing, and is thought to be recovering from a population collapse in the mid-1960s (NOAA 2013).

The protection of fish resources and the avoidance of severe future declines in fish stocks is dependent on effective management, which relies on knowledge of population structure, gene flow levels, and genetic diversity within and among populations of commercially harvested species (Utter 1991; Cha *et al.* 2010). In the present study, we sequenced the full length of the mitochondrial (mt) DNA non-coding control region of 300 samples along the coast of China, and 20 samples from the Japanese coast, to investigate the genetic diversity and population genetic structure of *S. japonicus* in the Northwestern Pacific. This genetic marker has been shown to be particularly sensitive in detecting population genetic structures of marine fishes. We also examined another molecular marker, the cytochrome *b* gene, from 30 samples along the coast of China to verify the results obtained from the control region. In addition, the population history of *S. japonicus* was estimated in order to reflect how this species is likely to have responded to the severe climatic oscillations in the late Pleistocene era. These results have potential value in the development of a knowledge base that is essential for appropriate conservation and sustainable harvest plans for this economically important fish species.

Material and Methods

Sample collection

A total of 300 *Scomber japonicus* individuals was collected at 10 localities along the coast of China from August 2011 to May 2013. Sampling locations were as follows: Huludao (HLD, 40°50′ N, 121°05′ E), Dandong (DD, 39°45′ N, 124°08′ E), Qingdao (QD, 36°04′ N, 120°20′ E) and Rudong (RD, 32°20′ N, 121°11′ E) in the Yellow Sea (YS); Taizhou (TZ, 28°68′ N, 121°43′ E) and Xiamen (XM, 24°28′ N, 118°06′ E) in the East China Sea (ECS); Huizhou (HZ, 23°05′ N, 114°24′ E), Zhanjiang (ZJ, 21°27′ N, 110°35′ E), Beihai (BH, 21°20′ N, 108°47′ E) and Sanya (SY, 18°25′ N, 109°50′ E) in the South China Sea (SCS). An additional sample of 20 individuals previously collected from the coast of Kochi prefecture (KC, 33°33′ N, 133°31′ E), Japan, for a comparative study was also included in our analyses (Catanese *et al.* 2010). Geographic locations and sample size are presented in Fig. 1 and Table 1, respectively. All individuals were identified based on morphological characteristics, and muscle samples were collected and preserved in 95% ethanol before DNA extraction.

DNA extraction, PCR amplification and sequencing

Genomic DNA was isolated from muscle tissue samples using DNA extraction kits (Sangon, Shanghai, China) following the manufacturer's instructions. PCR primers were designed based on the complete mitochondrial DNA sequence of this species (GenBank accession no. AB102724) to amplify the full length of the mtDNA control region and the cytochrome *b* gene. The primer pairs were as follows: SjCRF, 5′-TCCACCCCTAACTCCCAAAG-3′ (forward), and SjCRR, 5′-TTTTCAGGGCCCATCTTAA CAT-3′ (reverse), for the control region and SjCbF, 5′-CATAAGTCATAATTCCTGCC-3′ (forward), and SjCbR, 5′-TTGAAGCAAAAGGGAGG-3′ (reverse), for the cytochrome *b* gene. Each PCR reaction was performed in a volume of 50 μl containing 20–50 ng template DNA, 5 μl of 10× reaction buffer, 5 μl $MgCl_2$ (25 mM), 1 μl deoxynucleotide triphosphates (10 mM), 10 pM of each primer and 2.5 units Ex Taq DNA polymerase (TaKaRa, Dalian, China). The PCR amplification was carried out in a Biometra thermal cycler under the following conditions: 5 min initial denaturation at 94 °C, and 35 cycles of 30 s at 94 °C for denaturation, 30 s at 50 °C for annealing, 70 s at 72 °C for extension and a final extension at 72 °C for 8 min. PCR products were purified using a UNIQ-10 Spi Column PCR Product Purification Kit (Sangon) and subjected to automated DNA sequencing (BGI, Shenzhen, China) with the same primers as used for amplification.

Data analyses

Sequences were edited and aligned by MEGA 6.0 (Tamura *et al.* 2013) and further verified by visual examination. Molecular diversity indices, such as the number of haplotypes (H), polymorphic sites (S), transitions, trans-

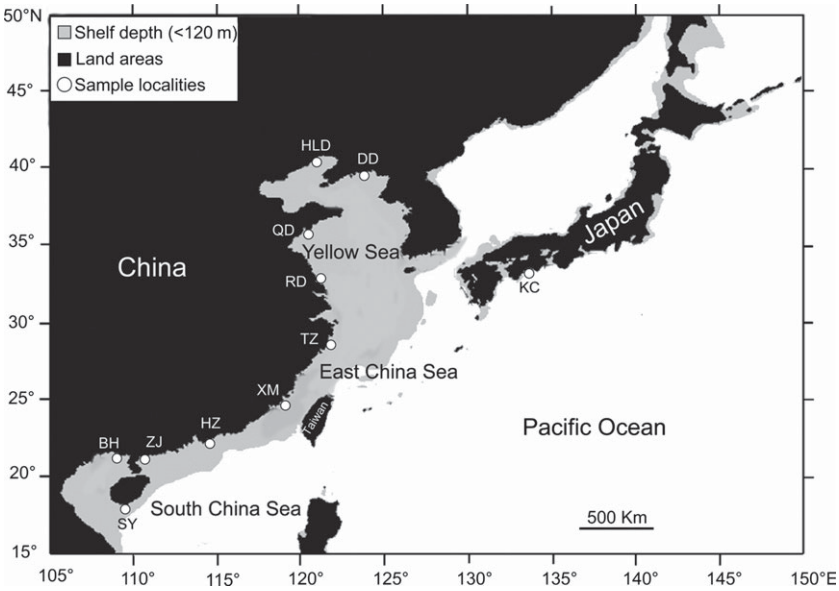

Fig. 1. Map showing sample localities for *Scomber japonicus*. Shaded areas indicate continental shelves that would have been exposed during periods of low sea level. HLD = Huludao; DD = Dandong; QD = Qingdao; RD = Rudong; TZ = Taizhou; XM = Xiamen; HZ = Huizhou; ZJ = Zhanjiang; BH = Beihai; SY = Sanya; KC = Kochi. This figure was modified after Liu *et al.* (2006).

Table 1. Sampling localities, grouped regions and descriptive statistics of genetic diversity of *Scomber japonicus* based on control region sequence data.

region and site	abbr.	n	H	S	h	π	k
Yellow Sea	YS	120	60	46	0.9476 ± 0.0119	0.010204 ± 0.005248	8.816681 ± 4.094755
Huludao	HLD	30	20	28	0.9494 ± 0.0266	0.010601 ± 0.005582	9.159195 ± 4.333632
Dandong	DD	30	20	26	0.9586 ± 0.0216	0.010226 ± 0.005398	8.835283 ± 4.191115
Qingdao	QD	30	21	30	0.9586 ± 0.0216	0.010164 ± 0.005367	8.781476 ± 4.167438
Rudong	RD	30	22	29	0.9517 ± 0.0279	0.010328 ± 0.005448	8.923771 ± 4.230052
East China Sea	ECS	60	39	35	0.9582 ± 0.0168	0.010154 ± 0.005268	8.772801 ± 4.104456
Taizhou	TZ	30	21	28	0.9287 ± 0.0391	0.009617 ± 0.005100	8.309431 ± 3.959682
Xiamen	XM	30	23	28	0.9747 ± 0.0172	0.010125 ± 0.005349	8.748385 ± 4.152876
South China Sea	SCS	120	60	44	0.9591 ± 0.0095	0.010049 ± 0.005173	8.682284 ± 4.036871
Huizhou	HZ	30	19	26	0.9425 ± 0.0272	0.009414 ± 0.005000	8.133766 ± 3.882350
Zhanjiang	ZJ	30	21	30	0.9494 ± 0.0276	0.010842 ± 0.005699	9.367237 ± 4.425155
Beihai	BH	30	19	27	0.9609 ± 0.0204	0.009166 ± 0.004879	7.919624 ± 3.788064
Sanya	SY	30	23	29	0.9747 ± 0.0172	0.010116 ± 0.005344	8.740579 ± 4.149441
Kochi	KC	20	11	18	0.9263 ± 0.0338	0.005641 ± 0.003206	4.873580 ± 2.479864
total samples	total	320	133	71	0.9580 ± 0.0062	0.010742 ± 0.005475	9.281109 ± 4.276263

abbr. = abbreviation; n = sample size; H = number of haplotypes; S = number of polymorphic sites; h = haplotype diversity (±SD); π = nucleotide diversity (±SD); k = mean pairwise difference (±SD). Yellow Sea: Huludao, Dandong, Qingdao, Rudong; East China Sea: Taizhou, Xiame; South China Sea: Huizhou, Zhanjiang, Beihai, Sanya.

versions and indels, were calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010). Haplotype diversity (h; Nei 1987), nucleotide diversity (π; Nei 1987) and the average numbers of pairwise nucleotide differences (k; Tajima 1983) were obtained as well. Pairwise and overall distances among haplotype sequences were calculated in MEGA 6.0 (Tamura *et al.* 2013).

MODELTEST 3.7 (Posada & Crandall 1998) was used to determine the best substitution model for both the control region and cytochrome *b* gene based on the Akaike information criterion and to calculate the gamma correction for heterogeneity of mutation rates among sites. The models and parameters suggested were then used for the analyses of molecular variance (AMOVA) and of phylogenetic relationships.

Both neighbor-joining (NJ) and Bayesian inference (BI) methods were adopted to reconstruct the phylogenetic relationships, using MEGA 6.0 with 1000 bootstrap replicates and MrBayes v. 3.2.1 (Ronquist *et al.* 2012), respectively, with *Scomber colias* as an outgroup. Another 56 haplotypes of *Scomber japonicus* sampled from three localities around Taiwan Island (downloaded from

GenBank with accession nos EF508372–EF508461) were also included in this analysis. For well-supported clades, the divergence time was estimated from net divergence by the formula $t = d_A/2\mu$ (Nei & Li 1979), where μ is mutation rate of the specific gene and d_A was obtained by MEGA using the suggested model given by MODELTEST. As the intra-specific phylogenetic relationships were often revealed more clearly and accurately by haplotype network analysis (Zhao *et al.* 2008), a network based on the control region data was constructed by NETWORK 4.6.1.1 (http://www.fluxus-engineering.com) using the median-joining network approach (Bandelt *et al.* 1999) with the maximum parsimony calculation (Polzin & Daneshmand 2003).

Population structure was measured with an AMOVA using F-statistics in three geographic hierarchies: the proportions of variations among population of different regions (F_{CT}), among populations within regions (F_{SC}) and within populations (F_{ST}). The four regions subdivided represent the Kochi of Japan and three marginal seas of China. Another AMOVA test for two groups, the Chinese group including 10 samples from the Chinese coast and the Japanese group from Kochi, Japan, was also conducted. Genetic differentiation between pairs of population samples was evaluated by the pairwise fixation index (F_{ST}), which includes information on mitochondrial haplotype frequency and genetic distances. The significances of F_{ST} and of the co-variance components associated with the different possible levels of genetic structure were tested with 5000 permutations. Both AMOVA and F_{ST} calculations were performed in ARLEQUIN 3.5. Given that the Hasegawa-Kishino-Yano (HKY) model suggested by MODELTEST for the control region is not available in ARLEQUIN, genetic distances between haplotypes were corrected for multiple hits using the Tamura and Nei model (Tamura & Nei 1993) of nucleotide substitution with a gamma ($G = 0.6174$) correction for heterogeneity of mutation rates. The null hypothesis of population panmixia was also tested in ARLEQUIN 3.5 using an exact test of the differentiation of haplotypes among populations. The exact test of population differentiation of haplotypes aims to test if the observed distribution of frequencies coincides with the expectation under panmixia (Raymond & Rousset 1995). Probabilities were estimated by permutation analyses using 10,000 randomly permuted r (populations) × k (different haplotypes) contingency tables of haplotype frequencies.

The historical demographic pattern of *S. japonicus* was examined using mismatch distribution analysis (Rogers & Harpending 1992). The frequency distribution of pairwise differences among all haplotypes was tested under the sudden expansion model of Rogers (1995). The distribution is usually multimodal in samples drawn from populations at demographic equilibrium, but is usually unimodal in populations that have undergone recent demographic and range expansions (Slatkin & Hudson 1991; Rogers & Harpending 1992; Ray *et al.* 2003; Excoffier 2004). Past demographic parameters, including tau value (τ; time since expansion expressed in units of mutational time), were estimated (Rogers & Harpending 1992). Deviations from the estimated demographic model were evaluated using the tests of Harpending's raggedness index (Harpending 1994) and the sum of squared differences (SSD) with a parametric bootstrapping approach using 10,000 replicates. Given that mismatch distributions have been found to be very conservative (Ramos-Onsins & Rozas 2002), two neutrality tests for mutation-drift equilibrium were performed. First, Tajima's D test compares two estimators of the mutation parameter θ, Watterson's estimator θs and Tajima's estimator θπ; significant D values can be attributed to population expansion, bottlenecks or selection (Tajima 1989). Fu's F_S test compares the number of haplotypes observed with that expected in a random sample under the assumption of an infinite-sites model without recombination (Fu 1997). F_S is sensitive to demographic expansion, which generally leads to a significant negative value. Both mismatch analysis and neutrality tests were performed in ARLEQUIN 3.5.

The values of τ generated by mismatch distribution analysis were transformed to estimate the expansion time with the equation $\tau = 2ut$ (Rogers & Harpending 1992), in which u is the mutation rate per sequence per generation and is calculated by the equation $u = 2\mu k$, where μ is the mutation rate per nucleotide and k is the number of nucleotides of the fragment analysed. Finally, the approximate time of expansion in years was calculated by multiplying t by the generation time of *S. japonicus*. As female and male chub mackerel mature at different sizes and ages [between 1 and 3 years, respectively (Love 1996; Tzeng *et al.* 2007)], a generation time of 2 years was used.

A molecular clock for the mitochondrial control region has not been accurately determined for marine fishes, and seems to vary among major taxonomic groups. A divergence rate of 2%·million years $(MY)^{-1}$ has widely been adopted for the cytochrome *b* locus in many bony fishes (Bermingham *et al.* 1997; Bowen *et al.* 2001). In reference to a previous relevant study (Zardoya *et al.* 2004), the mutation rate (11–16%·MY^{-1}) of the control region sequences of *S. japonicus* was determined from the generally used divergence rate of cytochrome *b* and the divergence time of the two Chinese clades on the NJ trees based on the cytochrome *b* and control region sequences in the present study.

Results

Genetic diversity

The complete control region sequence of 864 bp was obtained from each of 320 individuals of *Scomber japonicus* sampled from 11 geographic populations (GenBank accession nos KM199869–KM200001; Fig. 1). A total of 71 polymorphic sites, including 43 parsimony informative ones, was detected, with 64 transitions, 12 transversions and no indels. Sequence divergence (Tamura and Nei distance) among haplotypes ranged from 0.1% to 2.4%, with an average of 1.2%. The average base composition was as follows: A = 33.18%, T = 29.40%, C = 22.06%, G = 15.37%. A total of 133 haplotypes was identified from 320 individuals. The most common haplotype, H5, was shared by 50 individuals scattered throughout all sampling localities along the coast of China. Among all the haplotypes, 98 were unique, 30 were shared by more than one individual sampled from different localities and five were possessed by individuals only from one locality (Kochi).

Genetic diversity indices are presented in Table 1. The haplotype diversity (h) was high for all populations, with an overall value of 0.9580, but the values of nucleotide diversity (π) were generally low (0.010742 for the total samples).

The 1141-bp complete cytochrome *b* sequence was determined for 30 individuals (three for each sample site along the coast of China; GenBank accession nos KM200002–KM200012). A total of 10 polymorphic sites was observed, which defined 11 haplotypes in *S. japonicus*, giving an overall haplotype diversity of 0.7379 and nucleotide diversity of 0.001088.

Phylogenetic analysis

Based on the sequences of the control region, the NJ tree reconstructed using the Tamura and Nei model with a gamma distribution of 0.6174 was divided into two main clades (Fig. 2). One clade contained all the individuals sampled along the coast of China including those from around Taiwan, while the other consisted of individuals from Kochi. The Chinese clade was further divided into two small clades with low support, which are not related to sampling localities. Except for the slight differences in some small branches, the topology of the BI tree was similar to the NJ tree which was presented. The posterior probabilities were provided for the main clades in bold font on the NJ tree (Fig. 2). Network analysis revealed similar results to those in the phylogenetic tree. Three clusters corresponded perfectly to the KC clade and two Chinese clades defined in the NJ tree were present (Fig. 3). Both of these Chinese clusters exhibited a star-like pattern with large numbers of unique haplotypes surrounding some dominant haplotypes, such as H5, H7 and H8, which is indicative of population expansion.

The NJ tree based on the sequences of the cytochrome *b* gene also revealed two clades along the coast of China (Fig. 4). The compositions of the two clades were unrelated to the geographic distribution of samples, which is consistent with the composition of the two clades representing samples from the Chinese coast as seen on the NJ tree based on the control region sequences.

Net average genetic distance (Tamura and Nei with gamma correction) between the Chinese and Japanese lineages was 0.7% for the control region. Using the divergence rate of 11–16%·MY^{-1}, it was estimated that the divergence between these two lineages occurred during the late Pleistocene era, about 22,000–32,000 years ago. As for the two clades within the Chinese lineage, it was suggested the divergence occurred around 25,000–36,000 years ago (also in the late Pleistocene) based on net average genetic distances of 0.8% for the control region and 0.1% for cytochrome *b*.

Population genetic structure

Genetic differentiation among populations was assessed using F_{ST} pairwise comparisons. As shown in Table 2, F_{ST} values between Chinese and Japanese populations were high and significant, while insignificant pairwise F_{ST} values were obtained among populations along the coast of China. These results revealed that significant genetic differentiation existed between the Chinese and Japanese populations, but that no pattern of significant genetic structure was seen in the range investigated along the coast of China. The same conclusion was also supported by hierarchical AMOVA tests (Table 3). A two-group AMOVA indicated that the genetic variation between the Chinese and Japanese populations was 43.88%, while that among populations within the Chinese group was 0.23%. A four-group AMOVA indicated that the genetic variation among four regions (Yellow Sea, East China Sea, South China Sea and Japan) was 11.06% and among populations within each of the marginal seas was 1%. Moreover, the exact test of population differentiation (non-differentiation exact P-values) showed significant differences between the Chinese and Japanese populations while the P-values among the 10 Chinese populations were insignificant (Table 2).

Historical demography

The demographic history of *Scomber japonicus* was investigated using mismatch distributions. The mismatch

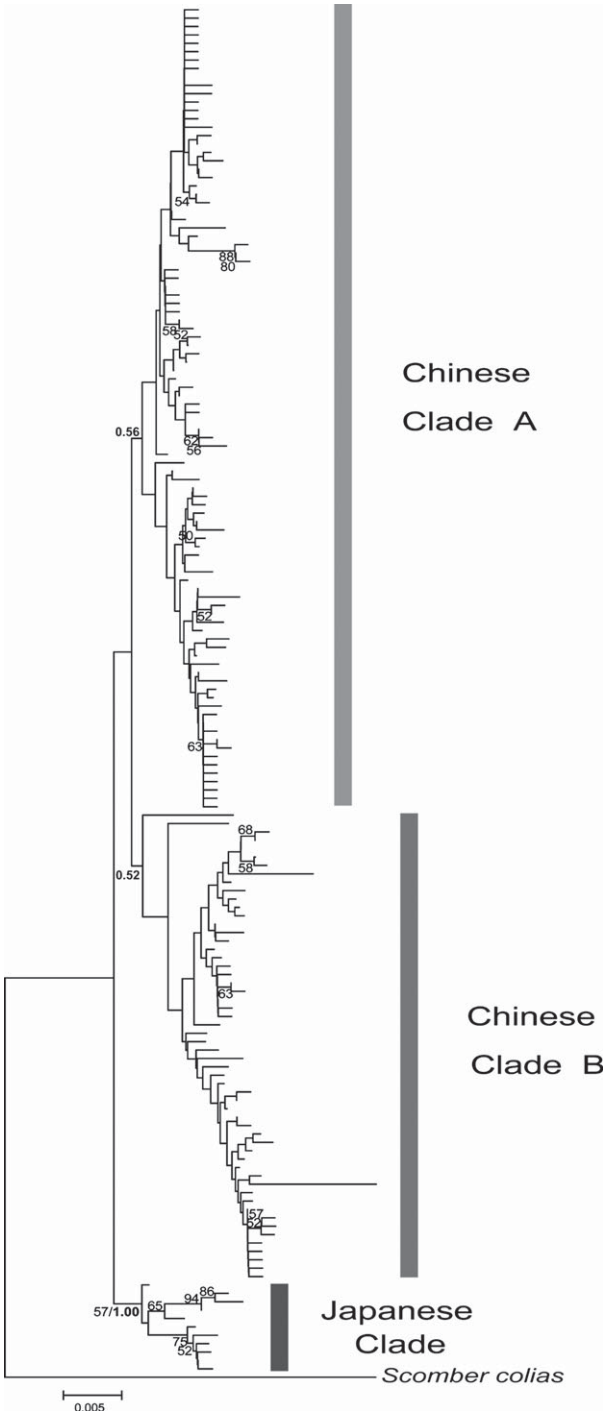

Fig. 2. Neighbor-joining tree for control region haplotypes of *Scomber japonicus*. Bootstrap supports of those >50% are shown at nodes. Bayesian posterior probabilities (1000 replications) are provided for main clades in a bold font.

distributions for the Yellow Sea, East China Sea, South China Sea, Chinese clade A and Chinese clade B were not exactly unimodal; however, the observed values approximately matched the expected distributions under the sudden expansion model (Fig. 5). A goodness-of-fit test confirmed that no mismatch distributions for the groups mentioned above deviated significantly ($P > 0.05$) from predicted values under the sudden expansion model of Rogers & Harpending (1992) (Table 4). For the KC population, the mismatch distribution was multimodal and obviously deviated from the expected value under the sudden expansion model (Fig. 5). Fu's Fs test and Tajima's D test agreed well with the mismatch analyses (Table 4). The Fs values of the three marginal seas and two Chinese clades were negative and statistically significant ($P < 0.05$). Tajima's D values were low and insignificant. For the KC population, the neutrality tests for both Tajima's D and Fs were not significant. These results collectively suggest that *S. japonicus* along the coast of China experienced population expansion in the past, but that the KC population did not.

The tau value (τ), which reflects the location of the mismatch distribution crest, provides a rough estimate of the time when rapid population expansion started. The observed values of the age expansion parameters (τ) were 8.30 and 5.21 for Chinese clade A and clade B, respectively, allowing us to estimate that the past population expansion started approximately 30,000–43,700 years ago for clade A and 19,000–27,400 years ago for clade B.

Discussion

Historical demography and genetic diversity

Both the mismatch distribution analysis and the neutrality tests hinted that population expansion occurred for *Scomber japonicus* along the coast of China, but not for the population along the coast of Kochi prefecture, Japan. This conclusion was also supported by the P_{SSD} and raggedness tests. The shallow bimodal or multimodal mismatch distributions of the populations in the three marginal seas and the two Chinese clades present in the NJ tree suggest that the *S. japonicus* populations along the coast of China had multiple ancestral sources and experienced historical re-mixture of separated populations. In general, population expansion and a heterogeneous mutation rate have opposite effects on Tajimas' D statistics, the former leading to consistent and large negative values, and the latter resulting in small and variable negative values (Aris-Brosou & Excoffier 1996). Thus, the insignificant Tajima's *D* might be attributed to the gamma distribution shape with the parameter of 0.6174 for *S. japonicus* , which indicated moderate mutation rate heterogeneity among sites. In addition, the haplotype network was characterized by a star-like phylogeny, which is characteristic of a population experiencing expansion

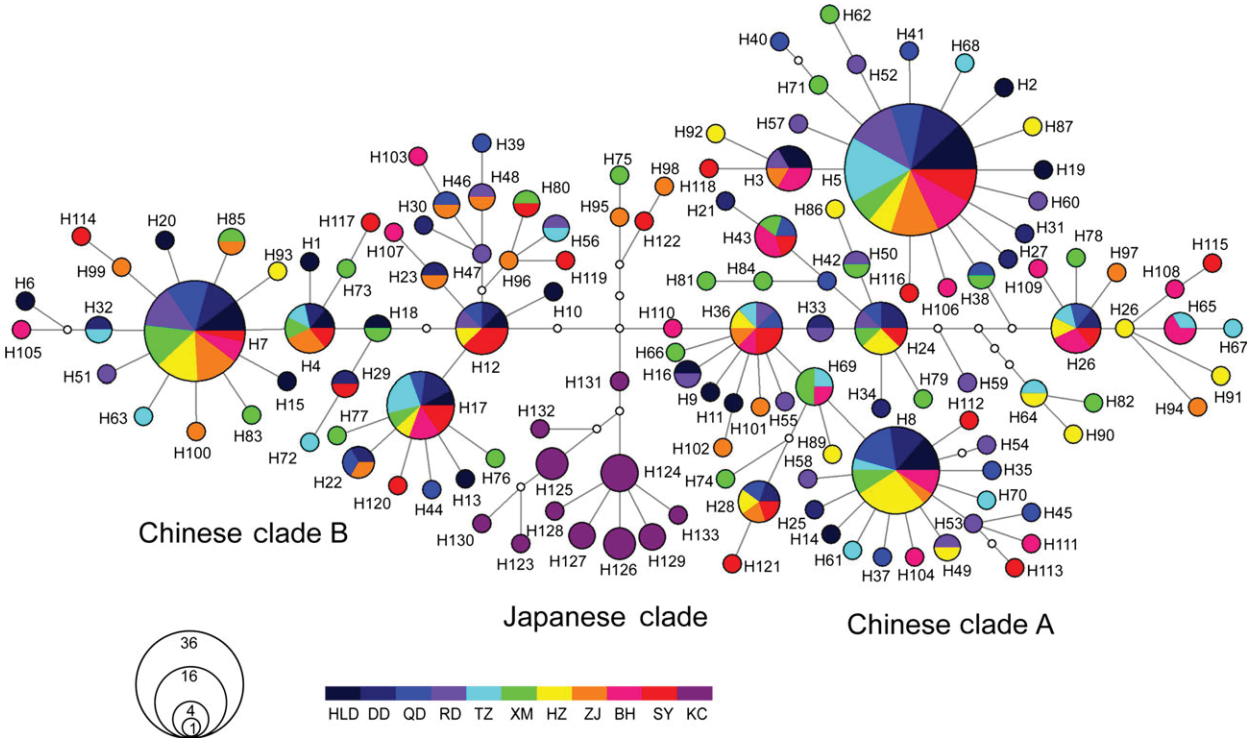

Fig. 3. Median-joining network for control region haplotypes of *Scomber japonicus*. The sizes of the circles are proportional to haplotype frequency and the colours represent the population (HLD = Huludao; DD = Dandong; QD = Qingdao; RD = Rudong; TZ = Taizhou; XM = Xiamen; HZ = Huizhou; ZJ = Zhanjiang; BH = Beihai; SY = Sanya; KC = Kochi) to which they belong; small hollow circles represent missing haplotypes.

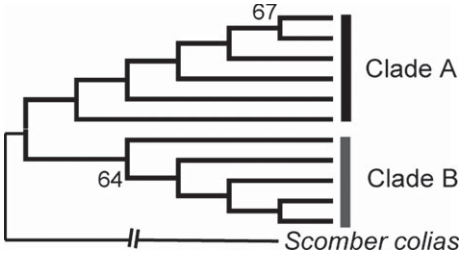

Fig. 4. Neighbor-joining tree for cytochrome b haplotypes of *Scomber japonicus*. Bootstrap supports of >50% are shown at nodes.

after bottleneck effects (Avise 2004). Previous studies on *S. japonicus* off Taiwan and in the Mediterranean Sea also suggested the occurrence of historical population expansion (Zardoya *et al.* 2004; Tzeng *et al.* 2007).

The present study suggests that population expansion occurred approximately 30,000–43,700 years ago for Chinese clade A, and 19,000–27,400 years ago for clade B, both in the late Pleistocene era. The late Quaternary period (the past 1,000,000 years) was punctuated by a series of large glacial–inter-glacial cycles (Imbrie *et al.* 1992), resulting in the rise and fall of sea levels and producing changes in temperatures, sea current patterns, upwelling intensity and the displacement, or even eradication, of coastal habitats (Kennett & Ingram 1995; Bond *et al.* 1997). During the Last Glacial Maximum (LGM) of the Pleistocene period, sea levels have been estimated to be approximately 120–140 m lower than their current depth, which is believed to have led to severe deterioration of marine habitats (Lambeck *et al.* 2002). These events could have resulted in the extinction of numerous coastal marine species or their redistribution to different glacial refuges. As for the Western Pacific marginal seas, the entire Bohai Gulf and the YS were exposed (Fig. 1), and the ECS was reduced into an elongated trough (Boggs *et al.* 1979; Wang & Sun 1994). The SCS became a semi-enclosed gulf connected with the Pacific Ocean mainly through the Bashi Strait (Wang 1999). Most of the habitats of marine species in these regions became unavailable, resulting in a decrease in the abundance of *S. japonicus* along the coast of China. The recolonization and distribution expansion of surviving *S. japonicus* populations from different refuges probably took place during the glacial retreat and the rise in sea levels. Therefore, late Pleistocene glaciations probably had a very substan-

Table 2. Pairwise F_{ST} (below diagonal) and P-values for exact test of population differentiation (above diagonal) among populations of *Scomber japonicus*.

	HLD	DD	QD	RD	TZ	XM	HZ	ZJ	BH	SY	KC
HLD		0.86172	0.89397	0.78014	0.29451	0.80977	0.53035	0.90949	0.67535	0.28468	0.00000*
DD	–0.02126		1.00000	0.59705	0.56085	0.89358	0.97989	0.85260	0.56498	0.78824	0.00000*
QD	–0.00909	–0.02161		0.54955	0.16218	0.96173	0.99869	0.89897	0.76653	0.62715	0.00000*
RD	–0.00835	–0.01827	–0.01961		0.19849	0.44716	0.18788	0.95712	0.27865	0.51636	0.00000*
TZ	0.01524	0.00946	0.00972	–0.00900		0.18250	0.04049	0.21647	0.72282	0.55756	0.00000*
XM	–0.01067	–0.01456	–0.00021	0.00580	0.05324		0.64624	0.79082	0.58673	0.41232	0.00000*
HZ	0.01655	–0.00013	–0.01854	–0.01194	0.01326	0.02081		0.20936	0.28920	0.21989	0.00000*
ZJ	–0.01810	–0.00633	0.01442	0.01213	0.04994	–0.01307	0.05151		0.37676	0.34509	0.00000*
BH	0.01368	0.00560	–0.00486	–0.01176	–0.02256	0.04468	–0.00043	0.04847		0.44981	0.00000*
SY	–0.01747	–0.02322	–0.01414	–0.00224	0.02758	–0.01285	0.01837	–0.01032	0.01755		0.00000*
KC	0.47339*	0.48064*	0.47645*	0.47508*	0.51846*	0.46967*	0.50253*	0.45204*	0.51155*	0.47696*	

HLD = Huludao; DD = Dandong; QD = Qingdao; RD = Rudong; TZ = Taizhou; XM = Xiamen; HZ = Huizhou; ZJ = Zhanjiang; BH = Beihai; SY = Sanya; KC = Kochi.
*P < 0.01.

Table 3. Summary of hierarchical analysis of molecular variances for *Scomber japonicus*.

	degrees of freedom		percentage of variation		F-statistics	
source of variation	four-group	two-group	four-group	two-group	four-group	two-group
among groups	3	1	11.06	43.88	F_{CT} = 0.11058	F_{CT} = 0.43876
among populations within groups	7	9	1.00	0.23	F_{SC} = 0.01121	F_{SC} = 0.00413
within populations	309	309	87.94	55.89	F_{ST} = 0.12055*	F_{ST} = 0.44108*

four-group: Kochi population and the three Chinese marginal seas.
two-group: Chinese group and Japanese group.
*P < 0.01.

tial effect on the demographic history of *S. japonicus*, including population isolation and subsequent expansion and remixing. Previous studies conducted in the Northwestern Pacific region have also suggested population expansion of many marine species, such as Japanese Spanish mackerel, *Scomberomorus niphonius*, grey mullet, *Mugil cephalus*, redlip mullet, *Chelon haematocheilus* and spotted sea bass, *Lateolabrax maculates* (Liu *et al.* 2006, 2007, 2009; Shui *et al.* 2009). Climatic oscillations of this sort during the late Pleistocene glaciation are generally recognized to be among the most important factors affecting the demographic history of marine species.

Regarding the *S. japonicus* population in the coastal Kochi prefecture, Japan, the mismatch distribution analysis, and the Tajima's D and Fu's Fs values suggested that it has not experienced population expansion and that it is at a state of population equilibrium, which differs from the demographic pattern of *S. japonicus* along the coast of China. This pattern may be the result of moderate oceanographic changes along the Japanese coast on the Pacific side during the LGM compared with those along the coast of China, which did not cause substantial changes to the *S. japonicus* population (Fig. 1; Liu *et al.* 2006; Han *et al.* 2008a). However, it is also possible that the small sample size from Kochi prefecture, Japan (only 20 individuals), may have concealed expansion events within this population.

By contrast with the high haplotype diversity (0.9580), low nucleotide diversity (0.010742) was found in the mitochondrial control region among *S. japonicus* samples. According to the four basic scenarios proposed by Grant & Bowen (1998), the high h and low π for the *S. japonicus* population along the Chinese coast may be the result of rapid population expansion after a period of low effective population size. Similar results – high haplotype diversities coupled with medium to low nucleotide diversities – have also been demonstrated for populations of other fish species (Vaughan *et al.* 1992; Fauvelot *et al.* 2003; Chen *et al.* 2004; Zhang *et al.* 2006).

Phylogeographic patterns and genetic structure

The two distinct lineages representing Chinese and Japanese *Scomber japonicus* populations likely reflect the isolation

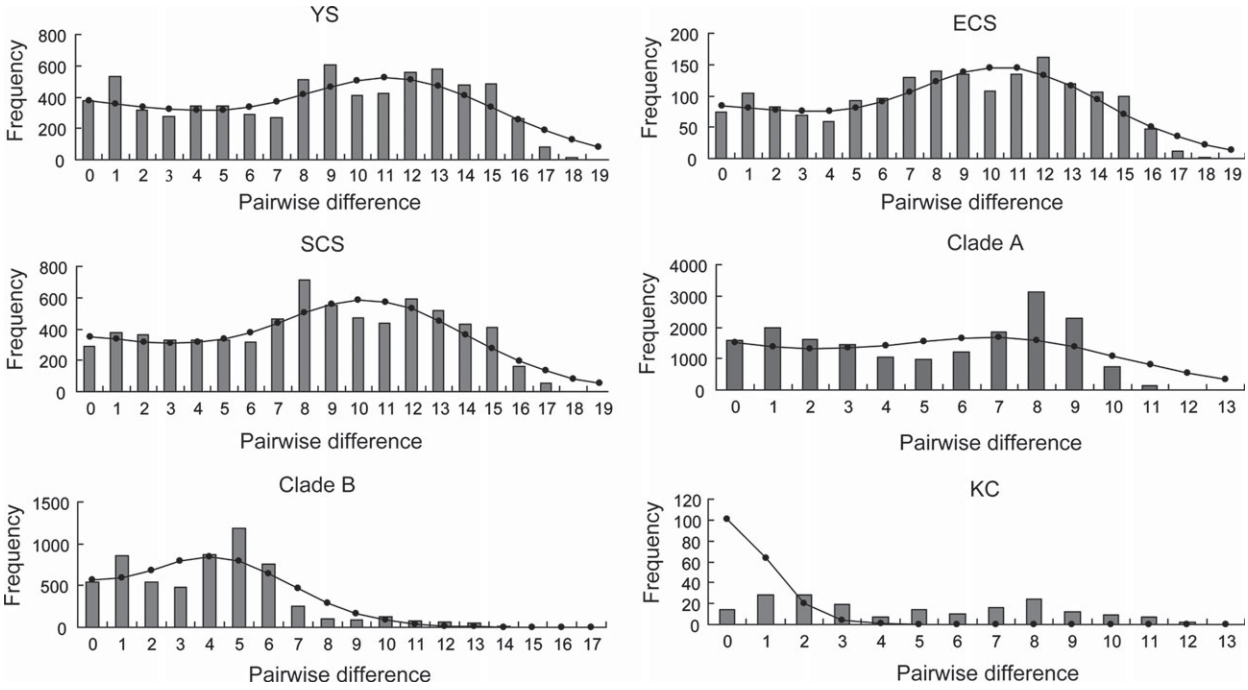

Fig. 5. Mismatch distribution of control region haplotypes for four regions (YS = Yellow Sea; ECS = East China Sea; SCS = South China Sea; KC = Kochi) and the two Chinese clades (Chinese Clade A and Chinese Clade B) of *Scomber japonicus*. The observed pairwise differences are shown as bars and the expected values under the sudden expansion model are solid lines.

Table 4. Tajima's D, Fu's F_S statistics, mismatch distribution parameter estimates and the corresponding P-values for *Scomber japonicus* based on the control region sequence data.

	Tajima's D		Fu's Fs			goodness-of-fit tests			
	D	P	Fs	P	τ	SSD	P	HRI	P
HLD	0.89840	0.85780	–4.82079	0.04850	13.24219	0.00895	0.66880	0.01359	0.75160
DD	1.07874	0.89600	–5.05693	0.04160	12.17188	0.00394	0.88390	0.00851	0.93590
QD	0.43649	0.72570	–6.31690	0.01920	11.88086	0.00321	0.95640	0.00822	0.94140
RD	0.63916	0.79520	–7.54815	0.00900	13.44141	0.00559	0.87220	0.01123	0.88880
TZ	0.49393	0.74520	–6.76503	0.01370	11.83398	0.00413	0.94240	0.00870	0.95080
XM	0.71136	0.81700	–9.24981	0.00300	11.32031	0.00378	0.89590	0.00713	0.95910
HZ	0.71713	0.81400	–4.52414	0.05380	7.45312	0.01109	0.44420	0.02509	0.35250
ZJ	0.69975	0.80680	–5.81759	0.02760	14.25195	0.00895	0.53620	0.01559	0.73260
BH	0.44641	0.72480	–4.71339	0.05050	9.36719	0.00728	0.78110	0.01337	0.81610
SY	0.56333	0.76400	–9.25252	0.00190	10.92188	0.00496	0.76020	0.00921	0.86090
KC	–0.24195	0.44740	–1.91448	0.18580	0.63672	0.29079	0.00560	0.02139	1.00000
YS	–0.02302	0.57200	–24.59483	0.00020	12.54492	0.00317	0.87700	0.00601	0.90120
ECS	0.42931	0.72430	–20.37297	0.00000	11.57812	0.00198	0.95550	0.00398	0.98960
SCS	0.07167	0.60490	–24.61335	0.00000	11.20508	0.00263	0.88370	0.00475	0.94570
clade A	–1.35310	0.05910	–25.15229	0.00000	8.29297	0.01623	0.25180	0.01870	0.52200
clade B	–1.13069	0.11630	–25.83096	0.00000	5.20508	0.01243	0.31270	0.02535	0.45280

SSD = sum of squared differences; HRI = Harpending's raggedness index; HLD = Huludao; DD = Dandong; QD = Qingdao; RD = Rudong; TZ = Taizhou; XM = Xiamen; HZ = Huizhou; ZJ = Zhanjiang; BH = Beihai; SY = Sanya; KC = Kochi; YS = Yellow Sea; ECS = East China Sea; SCS = South China Sea.

between the Chinese coast and Pacific Ocean during Pleistocene low sea-level episodes, as the control region sequence data suggested the divergence of the two lineages as about 22,000–32,000 years ago, in the late Pleistocene era. This species is thought to be particularly sensitive to environmental alterations (NOAA 2013). As mentioned above, during

the LGM, the entire Bohai Gulf and the Yellow Sea were exposed and the East China Sea together with the South China Sea were almost isolated from the Pacific Ocean during Pleistocene glaciation events due to the shallow sills (120–140 m lower than the current levels; Wang 1999; Kimura 2000; Liu *et al.* 2007; Han *et al.* 2008a). As a result of these environmental changes, the *S. japonicus* populations might have been isolated in the marginal seas of China and the Pacific side of Japanese coastal water respectively, finally resulting in the genetic divergence of these two populations. As for the two poorly supported clades in the Chinese lineage, neither the NJ tree nor the haplotype network revealed any correlation with the geographic distribution of the samples, indicating high levels of gene flow among the three marginal seas of China. Our results also suggest that the divergence of these two clades occurred 25,000–36,000 years ago, late in the Pleistocene era, followed by secondary contact from different glacial refuges with the rise in sea level during the postglacial period. This explanation was also supported by the mismatch distribution analysis.

Previous studies on other fishes, including the redlip mullet, *Chelon hematocheilus*, and white croaker, *Pennahia argentata*, in the Northwestern Pacific Ocean have also revealed significant genetic breaks between Chinese and Japanese populations (Liu *et al.* 2007; Han *et al.* 2008a). Similarly, fluctuating sea levels during the Pleistocene era were also assumed to have caused strong genetic divergence between marine organisms from the Indian and Pacific Oceans in South-East Asia, such as the starfish *Linckia laevigata*, the coconut crab *Birgus latro* and the mantis shrimp *Haptosquilla pulchella* (Lavery *et al.* 1996; Williams & Benzie 1998; Barber *et al.* 2002). Furthermore, numerous marine organisms exhibit a phylogeographic break between the Atlantic and the Mediterranean basins (Bargelloni *et al.* 2003; Duran *et al.* 2004; Baus *et al.* 2005). These genetic breaks mentioned above in the Northwestern Pacific, South-East Asia and Atlantic–Mediterranean areas have been attributed mainly to the isolation caused by the low sea level during Pleistocene glaciations (Han *et al.* 2008a).

Marine fishes generally show low levels of genetic differentiation over vast geographic regions due to the high dispersal potential of planktonic egg, larval and adult stages, coupled with the absence of physical barriers to movement between ocean basins or adjacent continental margins (Palumbi 1994; Grant & Bowen 1998; Hewitt 2000). In our study, the presence of two distinct lineages, and the results of the pairwise F_{ST} analyses, hierarchical AMOVA and exact tests all suggested significant genetic differentiation of *S. japonicus* populations in Chinese and Japanese coastal waters, indicating limited gene flow between the two *S. japonicus* populations in these regions.

On the contrary, little population genetic structure was revealed for the *S. japonicus* population in the area investigated along the coast of China, suggesting that this population is subject to panmixia, and consequently implying that this population has an elevated rate of gene exchange. Based on all of these results, it appears likely that the *S. japonicus* population in KC has been separated from its conspecifics along the coast of China since the late Pleistocene, which allowed these two groups to accumulate genetic differentiation independently in relatively stable environments. The relatively low level of genetic differentiation of *S. japonicus* populations along the coast of China may be the result of their own particular biological characteristics and the influences of other extrinsic forces, such as ocean currents. Both the pelagic eggs and larvae of *S. japonicus* may be broadly dispersed by sea currents prior to metamorphosis, which occurs at 22–29 days of age (Hunter & Kimbrell 1980; Scoles *et al.* 1998); this scattering of eggs and young fish may facilitate the mixing of *S. japonicus* among the YS, ECS and SCS regions. Furthermore, adult *S. japonicus* migrate seasonally over long distances (Froese & Pauly 2014). Both the passive transport of larvae and the active migrations of adults therefore appear to contribute to the flow and mixing of genes, resulting in a high degree of genetic similarity throughout the *S. japonicus* populations along the coast of China. Similarly, populations of many other marine species along the coast of China have been found to lack genetic structure (Han *et al.* 2008a,b; Shui *et al.* 2009).

Conclusions

The present study provides evidence for strong genetic divergence among *Scomber japonicus* in Chinese and Japanese coastal populations and suggests high gene flow within the Chinese population. These results should figure importantly in the development of an appropriate conservation strategy for this economically important fish species. The populations along the coast of China experienced population expansion in the late Pleistocene era, which confirms that climatic oscillations play an important role in affecting the demographic history of marine species. Further studies based on more genetic data such as microsatellite DNA analysis and nuclear markers would advance the understanding of the population genetic structure of *S. japonicus* and contribute to the development of sustainable management plans for the species.

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Learning and memory in *Octopus vulgaris*: a case of biological plasticity

Ilaria Zarrella[1], Giovanna Ponte[1,2], Elena Baldascino[1] and Graziano Fiorito[1]

Here we concisely summarize major aspects of the learning capabilities of the cephalopod mollusc *Octopus vulgaris*, a solitary living marine invertebrate. We aim to provide a backdrop against which neurobiology of these animals can be further interpreted and thus soliciting further interest for one of the most advanced members of invertebrate animals.

Addresses
[1] Stazione Zoologica Anton Dohrn, Villa Comunale, Napoli, Italy
[2] Association for Cephalopod Research 'CephRes', Italy

Corresponding author: Fiorito, Graziano (graziano.fiorito@szn.it)

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The octopus: a 'model' of the brain

About fifty years ago an English zoologist and neuroanatomist, John Zachary Young, published 'A model of the brain' [1]. The book is an account of decades of studies on predatory responses and learning abilities of several species of cephalopods, mainly the common octopus, *Octopus vulgaris*. In its aim, J.Z. Young tried to answer to the question 'how do brains work'. He adopted the approach utilized by 'communication engineers' and cybernetics: the 'brain' is acting as the computer of a homeostat [1].

In the model, a mnemon (i.e. a visual/tactile feature with associated memory value resulting from experience; [2,3••]) is activated by a given input (visual and/or chemo-tactile) to a specific set of classifying cells and switched on/off on the basis of other inputs that depend on the taste-pain circuits. The output of these units is summed up to produce an overall attack strength (i.e. predatory response), in contrast to the opposite inputs that build a retreat. These 'strengths' are combined to determine the final attack/retreat responses.

The 'model' is the result of hundreds of experiments where the predatory response of *O. vulgaris* has been dissected to deciphering its neural control ([4]; for review see also: [5,6••,7,8,9,10•,11,12,13]). It is noteworthy to mention that in several occasions the 'model' found its cybernetic application [14–16]. The mnemon model developed by Clymer [14] is based on a visual feature with associated memory value resulting from experience that is activated by a given visual input to a specific set of classifying cells and switched on/off on the basis of other inputs that depend on the taste-pain circuits. The output of these units, corresponding to the attack command, is further summed-up to produce an overall attack strength, in contrast to the opposite units (retreat command) that in a similar way build an overall retreat strength. These values, or strengths, are then combined and determine the final attack/retreat response [14]. Interestingly, the results produced by Clymer's model are comparable to those obtained from proper experiments with live animals, including the responses resulting from short- and long-term changes in behavior and interference on learning performance when spacing between trials is reduced in time [14]. In a similar way, Myers developed a modified 'cybernetic circuit' based on octopus' mnemon taking into account findings on neural networks and learning in simulated environments [15].

The octopus: a cephalopod

The common octopus is one of the most famous representatives of the class Cephalopoda (i.e. nautilus, cuttlefish, squid and octopus), a numerically small but ecologically and scientifically significant taxon of invertebrates belonging to the phylum Mollusca. The richness of behavioral capabilities of these animals fascinates human beings since the antiquity [6••,17]. Together with other cephalopod species, octopuses also represent a very important resource for human consumption [18].

The class Cephalopoda includes about 700 exclusively marine-living species considered to have rivaled fishes during evolution [19••,20•]. Cephalopods demonstrate a refined and extraordinary ability to adapt their morphology and behavioral repertoire to their living environment [6••,20,21]. Examples among many are: (i) special locomotion including fast jet propulsion, bipedal and tiptoeing [22–24]; (ii) active changes of body patterning achieving crypsis, polyphenism, mimicry and communication including hidden channels [5,25–27]; (iii) special

physiological, neural and behavioral characteristics acquired during evolution (e.g. [19••]). The extraordinary adaptive/plasticity of their physiology and behavior may have contributed greatly to their success [6••,28,29].

Cephalopods are also well known amongst neuroscientists for their contribution to fundamental understanding of the nervous system functioning [3••,30,31,32,33•]. These animals are also emerging models for biology, genomics, neuroscience, cognition and robotics [34•,35,36,37,38].

The octopus: a regulated 'laboratory animal'

Octopuses and their allies have been included from 1st January 2013 in the Directive 2010/63/EU that regulates the use of animals for scientific purposes [39,40]. As a consequence, the invertebrate research in the EU experienced a paradigm shift. In fact, the Directive covers the use of 'live cephalopods' (i.e. hatched juveniles and adults) in the legislation regulating experimental procedures likely to cause pain, suffering, distress or lasting harm [41•,42•]. Under the Directive 2010/63/EU in all Member States cephalopods have the same legal status as vertebrates in relation to their experimental use in research, testing and education [41•,42•,43,44,45]. The outstanding position of cephalopods among the list of regulated animals derives from the assumptions (see Table 1) advocated by the EFSA Panel at the time of the revision of the former EU regulatory document [46,47].

Directive 2010/63/EU is a milestone for invertebrate research because it is the first time particular types of research involving an entire class of invertebrates are regulated in the same way as scientific projects involving vertebrates. As reviewed by Fiorito [42•], although regulation presents obvious challenges, there are several areas where neurophysiological and behavioral neuroscience research is required to address key questions. Current efforts at the international scale are provided with the aim to strengthen the scientific community, and to facilitate the dissemination and adoption of a consensus on the best practices. Such important approach may result in an international coordination of research projects and in facilitating the growth of a 'network for improvement of cephalopod welfare and husbandry in research, aquaculture and fisheries' (Cephs*In*Action; COST Action FA1301: http://www.cost.eu/COST_Actions/fa/Actions/FA1301).

The octopus: a learning animal

Learning and memory appear to occur in all cephalopod species and has been studied with various details in some key species such as the cuttlefish and the octopus (review for example in [6••,48•]). This is considered to be one of the most advanced examples of behavioral plasticity among invertebrates [6••,21,49•]. Sophisticated behavioral repertoire (e.g. individual and social learning, behavioral syndromes, problem solving, communication through hidden channels) and its plasticity parallel those of higher vertebrates; these are related with a highly sophisticated nervous system that–despite the molluscan design–achieves vertebrate-like functional complexity. The neural system organization seems to be also correlated with species-specific lifestyle [20•,50,51]. Finally, the flexibility of the behavioral repertoire of cephalopods is supported by evident cellular and synaptic plasticity at the level of the central and peripheral nervous system, and of the neuromuscular junctions (review in [32•]).

As mentioned above, learning has been studied for decades in the octopus ([6••]; for review see for example [52•]).

Table 1

Summary of judgments on the capacity of experience of pain and distress in cephalopods as assessed by the Scientific Panel on Animal Health and Welfare on the Revision of the Directive 2010/63/EU. These assumptions are based on several scientific contributions [44] and extended by recent works (for review see [42•]). References included in the Notes are provided as further support to the statements provided. Most of the data provided herein comes from octopus and in some cases from cuttlefish.

Criterion	Judgement	Notes
Higher brain centres (c.f. cerebral cortex)	YES	e.g. Vertical lobe in octopus is considered comparable to limbic lobe [52•,70]. Studies are in progress investigating self-awareness and consciousness ([21,35••]; e.g.: [71,72])
Presence of nociceptors	Likely (but not proven)	Neurophysiological afferent recording studies in progress
Nociceptors project to higher neural centres	Likely (but not proven)	Require development of *in vivo* brain recording techniques (but see: [73,74,75])
Behavioral responses	YES	Avoidance of electric shock and other noxious (not necessarily painful stimuli ([57];e.g. [66]).
Receptors for opioids found in the nervous system	Likely (very limited data)	Enk-like peptides, peripheral δ receptors; Opioid growth factor receptor-like protein 1*; Kappa-type opioid receptor*. No direct 'pain' studies are currently available.
Action of analgesics	Not studied	Requires investigation and objective criteria, plus studies on drug delivery

* Gene sequences identified in G. Fiorito laboratory at the Stazione Zoologica Anton Dohrn (Italy) by assembling and annotating *O. vulgaris* transcripts coming from several sources (RNA-seq experiments and [76•,77,78]).

Various forms of learning have been demonstrated in cephalopods, from simple sensitization, to associative learning and problem solving, to more complex forms such as spatial and social learning and tool use (review in [6••]). In essence, a large number of the entities proposed by Moore [53••] in his cladogram of learning processes have been shown (e.g. habituation, classical and instrumental conditioning, associative and spatial learning, perceptual processes in visual learning [6••]) in some cephalopod species. Associative learning paradigms have been used in laboratory experiments of cephalopod learning. In *O. vulgaris*, consolidated long-term memory is controlled by the vertical lobe (a lobe in the brain [54,55•]), while short-term memory is stored in more distributed neural networks, a vertebrate-like pattern of separate memory storage sites [56•,57].

Learning can be also critical to the survival of juvenile forms [58–60]. Vicarious learning, i.e. the capability of learning from conspecifics, has been shown for *O. vulgaris* in the laboratory [61•] and appears to be somehow modulated by neural centers such as the vertical lobe [62]. Despite criticisms proposed to the original finding, the capability of learning from others is documented in octopuses and also recently in other cephalopod species [63•]. Octopuses and cuttlefishes can demonstrate conditional learning [64,65]. As reviewed by Huffard [49•], male octopuses in the wild exhibit mating tactics consistent with their size-based chances of winning agonistic contests, suggesting learning of rank followed by conditional use of mate guarding. In these field observations, mate guarding is not exhibited by small males and thus unlikely to win contests in the local population. By contrast, males appear to mate, guard and maximize mating opportunities only if they are large enough to win contests with other nearby individuals.

Table 2 summarizes several training paradigms successfully utilized with *O. vulgaris* to test learning and memory recall capabilities in this species (for review see also: [6••,21]).

Some of these have been also applied in learning studies aimed to decipher the biological machinery involved in the modulation of *O. vulgaris* behavioral plasticity [66]. In particular, the relationship between learning processes and gene expression in octopus has been evaluated by analyzing changes of some genes (e.g.: *stathmin: Ov-stm*, *tyrosine hydroxylase: Ov-TH*, *dopamine transporter: Ov-dat*, *octopressin: Ov-OP*, *cephalotocin: Ov-CT*) in response to fear conditioning (learned fear) and social interaction (innate fear). A differential pattern on down-regulation and up-regulation of gene expression in different regions of the octopus central nervous system resulted in these studies as a consequence of either innate or learned fear.

Table 2

Training paradigms utilized with *Octopus vulgaris* to assess learning and memory recall capabilities [6••,49•,51,52•,57,65,79,80].

	Training paradigm and/or Stimuli
Habituation	e.g. jar
Sensitization	Artificial and natural prey items
Classical conditioning	Plastic spheres having different brightness as stimuli; discriminanda differing in orientation, brightness, size or shape (i.e. rectangle, circle, square, diamond); barrel-shaped objects
Avoidance learning	Discriminanda having several shapes including plastic spheres
Spatial learning	Mazes
Problem solving	Mazes, jar and boxes with simple and multiple openings
Social learning	Discriminanda, problem solving
Perceptual processes	Discriminanda that differed in orientation, brightness, roughness, size or shape (rectangle, circle, square, diamond)

For example, in response to learned fear an increase of the expression of *stathmin* and *Ov-TH* was observed, while *Ov-stm* was the sole to increase significantly as the consequence of the 'innate fear'. In parallel, *Ov-stm* and *Ov-dat* decreased their expression in the subesophageal mass (i.e. center of motor control) in response to fear conditioning, while no effect appears to be caused by social interaction. Finally, learned and innate fear paradigms induced an increased expression of *Ov-stm* in the optic lobes (i.e. centers of visual-sensory processing). Instead, *Ov-dat* and *Ov-TH* exhibit an opposite pattern in response to fear conditioning and social interaction.

The increased expression of *Ov-stm* in octopuses subjected to innate and learned fear suggests that in octopus this gene plays a role similar to what is known in vertebrate brain. In mammals, it is known that amygdala enriched stathmin is required for the expression of innate fear and the formation of memory for learned fear [67–69]. Interestingly, *Ov-stm* undergoes in the octopus to a negative regulation in response to fear conditioning. This suggests that the synaptic architecture may be able to change, and that these changes could be related to variations in microtubule dynamics. This result opens the way to a fascinating working hypothesis that requires further studies to understand the relationship between microtubule dynamics, synapse formation, and plasticity of neurons in the octopus.

Conclusive remark

This review does not aim to provide a detailed description of the most recent results obtained by the application of several learning paradigms for studying the biological and

cellular mechanisms underlying learning in *O. vulgaris* and other cephalopod species.

As reviewed by Borrelli and Fiorito [6••] and despite the considerable number of studies published on the extent of learning and memory recall, and on the effects of its impairment induced by experimental interference, very little is known about the ability of cephalopods to encode and retrieve information. From the classic works of Young and co-workers, it is known that cuttlefishes and octopuses are capable of short-term and long-term memory. In many cases the memory trace was reported to last for a very long time: in octopus for weeks to months (review in [6••]). A systematic analysis of the memory phases, the time course of retention and memory consolidation, and possible reconsolidation in octopuses and cephalopods still appear insufficient when compared with the knowledge currently available for other taxa. Whether the memory recall observed in cephalopods corresponds to a more phylogenetically conserved consolidation mechanism or to a characteristic of the neural-network is an issue that has to be tested.

We hope to have provided enough elements to facilitate the continuous regrowth of interest for these fascinating highly 'flexible' animals.

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