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Ecophysiological behaviour of different diatoms in response to copepod signals

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RUNNING TITLE

Diatom responses to copepod signals

ABSTRACT

Phytoplanktonic communities have been moulded from a higher level in the food web by zooplankton predation. Diatoms, which are the major primary producers in present day oceans, have evolved many strategies to thrive in the presence of copepods, their predators. Chemical signals are released in the aquatic environment by microarthropods and elicit morphological, behavioural and/or physiological modifications in diatom cells which allow them to cope with predators. Other than the most studied effects of copepod signals on the release of toxins by diatoms, the role of chemical cues on growth and biology of non-toxic diatoms has been little investigated, although it may broaden our knowledge of co-evolutive physiological mechanisms. To examine the response of diatoms to copepod-derived signals, *Phaeodactylum tricornutum*, *Chaetoceros muelleri* and *Cylindrotheca fusiformis* (Bacillariophyceae) were grown in the presence of copepod cues. Physiological modifications occurred and differed in sign and magnitude between species: i) in *P. tricornutum* cell density was higher, and dry weight lower, suggesting energy allocation into cell division at the expense of lipid and protein quotas; ii) *C. muelleri* showed a very homeostatic behaviour; and iii) in *C. fusiformis* cell density was lower and lipid content higher, showing energy allocation into lipids, which likely act as deterrent to feeders. Copepod cues are here suggested to have ruled diatom species composition in the ocean by affecting species-specific growth performance and the energy allocation into macromolecules.

KEYWORDS

Chemical cues; Defence strategy; Energy allocation; Growth; Morphology

INTRODUCTION

Among the interactions occurring in the trophic chain, one of the main causes of death for phytoplankton is predation (Sommer *et al*. 2002; Rigby & Selander 2021). It has been estimated that, on average, predators remove 56.9% of oceanic and 40.6% of coastal marine primary producers; one half and a quarter of them, respectively, are consumed only by mesozooplanktonic predators (Duarte & Cebrián 1996; Calbet 2001). Diatoms are the most diverse group of phytoplankton and play an essential role in the carbon and silicon cycles, being responsible for 45% of primary production in the ocean and exporting 240 Tmol/y Si (Mann 1999; Armbrust 2009; Treguer *et al.* 2018; Behrenfeld *et al.* 2021; Bi *et al.* 2021). Copepods are among the major mesozooplanktonic predators of diatoms and other micro-phytoplankton organisms present in the ocean (Amato *et al.* 2018).

Such long-term interaction with predators deeply influenced radiation trajectories in diatoms (Turner 2004; Hamm & Smetacek 2007; Giordano *et al.* 2018) resulting in several strategies to cope with copepods (Pančić & Kiørboe 2018; Johnson *et al.* 2020; Lürling 2021): morphological defences (e.g. colony formation, thick silica shell) and physiological defences (e.g. toxicity). In particular, pennate diatoms are known to produce either apo-fucoxanthinoids and/or domoic acid, which act as feeding deterrents to predators (Shaw *et al.* 1995; El Hattab *et al.* 2008; Vidoudez & Pohnert 2008; Tammilehto *et al.* 2015; Cabrera *et al.* 2020; Smodlaka Tanković *et al.* 2022). To date, the literature is furnished with evidence demonstrating the effect of allelopathic signals released by microalgae in response to the presence of predators and the effect deterrents have on them (Chu 1942; Shaw *et al.* 1995; Pohnert 2005; Li *et al.* 2008; Ianora & Miralto 2010; Ianora *et al.* 2011; Saha *et al.* 2019).

On the other hand, calanoid copepods are responsible for the production of copepodamides, polar lipids recently discovered and known to induce paralytic shellfish toxin production as well as bioluminescence in dinoflagellates (Selander *et al.* 2015; Lindström *et al.* 2017). Regarding diatoms, copepodamides trigger domoic acid overproduction in the genus *Pseudo-nitzschia* and chain shortening in non-toxic colony-forming diatoms (Selander *et al.* 2019). Since cues are potentially related to the global phenomenon of harmful algal blooms (HABs) (Glibert *et al*. 2005; Granéli *et al.* 2008; Lundholm *et al.* 2018; Selander *et al.* 2019; Rigby & Selander 2021; Olesen *et al.* 2022), their effect on toxic algae and diatoms is deeply investigated. However, the effect of cues has been little addressed in non-toxic diatoms, which represent most of the community and may mirror the complex and wide co-evolutive interaction through physiological mechanisms other than strictly defence.

Trying to fulfil this gap in knowledge, three distinct diatoms known not to be responsible for toxin production were exposed to calanoid copepod signals: two phytoplankters (*Chaetoceros muelleri* Lemmermann, *Cylindrotheca fusiformis* Reimann & J.C. Lewin) and *Phaeodactylum tricornutum* Bohlin, a thycoplankter actively adapted to the planktonic environment (Sabir *et al.* 2018). Their physiological response was assessed in terms of growth performance, organic and inorganic composition, and morphological changes. By investigating the signalling pathway in prey-predator interaction, we intend to shed light on co-evolutive relationships ruling diatom species composition and affecting the entire pelagic ecosystem and marine food web.

MATERIAL AND METHODS

Harvesting of copepod cues

Calanoid copepods were sampled offshore in the Adriatic Sea using a horizontal plankton net (mesh width 200 μm). Zooplankton was collected through phototactic attraction, 120 specimens were isolated using a stereomicroscope, gently washed and transferred to three flasks (40 each) containing 500 ml of AMCONA medium, sterile artificial seawater (Fanesi *et al.* 2014). Animals were starved for 72 h in culture chambers with temperature of 20°C, and illuminated with white, fluorescent lamps at 70 μmol photons m^{-2} s^{-1} and 12:12 h light:dark cycle. At the end of the starvation period, the medium was filtered through 0.30 mm to remove copepods and subsequently at 0.22 μm for sterilization. Filtered medium was kept at +4°C until use.

Testing presence and activity of copepod cues

Filtered culture medium containing copepod signals was analysed through FTIR spectroscopy. Aliquots of 50-µl filtered medium were transferred to silica holders and dried at 80°C; aliquots of AMCONA standard medium were used as control. FTIR spectra on culture media were acquired by Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany). All the spectra were normalized with OPUS 6.5 software (Bruker Optics).

Growth, cell volume and dry weight

Four biological replicates of diatoms *Cylindrotheca fusiformis* NEPCC417 (https://www.ccap.ac.uk/), *Chaetoceros muelleri* CCAP 1010/3 and *Phaeodactylum tricornutum* DCG 0981 (https://bccm.belspo.be/about-us/bccm-dcg) were established in 250-ml flasks filled with 80 ml AMCONA medium as control (CTR, control) and other four flasks filled with 80 ml of filtered medium containing copepod signals (TR, treatment). The initial inoculum of 1×10^5 cells ml^{-1} was grown in culture chambers at 20°C, illuminated with white, fluorescent lamps at 70 µmol photons m^{-2} s^{-1} and 12:12 h light:dark cycle, and manually shaken twice a day. Daily counts and cellular volume measurements of growing cells were carried out through CASY® counter (Casy TT cell counter, Roche Innovatis) as described by Petrucciani *et al.* (2022b). The specific growth rate (µ) was calculated according to Monod (1949):

$$\mu = \frac{ln(N_t/N_0)}{t} \qquad \text{Eq. 1,}$$

where t represents time (d), N_0 is the initial cell density (cells ml^{-1}) at time 0, and Nt is the cell density at the considered time t. To determine the cell dry weight, empty tubes were weighed before and after adding 50 ml of washed diatom cells in exponential phase; cells were dried at 80°C until a stable cell weight was reached.

Morphological characterization

Imaging Flow Cytometer (IFC) FlowSight® (Amnis Corp., Seattle, Washington, USA) using the INSPIRE software package (Amnis Corp.) was used to assess morphological characteristics of diatoms collected during exponential phase as detailed by Petrucciani and *et al.* (2023). Post-acquisition data analysis was performed using the IDEAS software package following the procedure illustrated by Petrucciani *et al.* (2022a). Morphological features used for further PCA analysis were the average values of each feature and were calculated on at least 50,000 cells for each biological replicate.

Macromolecular composition

Protein content per diatom cell collected during the exponential phase was determined following the Lowry protocol with the modifications introduced by Peterson (1977). All the measurements were carried out on four biological replicates.

Ratios in macromolecular pools (carbohydrates:proteins; carbohydrates:lipids; lipids:proteins) were obtained by FTIR spectroscopy analysis. Samples of diatoms harvested during the exponential phase were prepared following the procedure described by Petrucciani *et al.* (2022b); absorbance spectra on the whole cell were collected between 4,000 and 400 cm^{-1}. All the measurements were carried out on four biological replicates. Cellular pools were derived after band assignments as reported in Giordano *et al.* (2001), and the band integrals of deconvolved spectra were used to calculate the relative abundances of lipids, carbohydrates and proteins using OPUS 6.5 software (Bruker Optics GmbH, Ettlingen, Germany). As already reported in Petrucciani *et al.* (2022b), the integrated value at *c.* 1,150 cm^{-1} was used as proxy for carbohydrates, as silica absorbance (*c.* 1,075 cm^{-1}) masks some of the typical carbohydrate bands in diatoms.

Total protein content assessed as described before was used to elaborate FTIR absorbance ratio and to obtain a semiquantification of carbohydrates and lipid pools according to Palmucci *et al.* (2011).

C and N quotas and isotopic fractionation

C and N abundance and stable isotopic fractionation ($\delta^{13}C$ and $\delta^{15}N$) were carried out with an elemental analyser (ECS 4010, Costech, Italy) connected to the ID Micro EA isotope ratio mass spectrometer (Compact Science Systems, Lymedale Business Centre, Newcastle-under-Lyme, United Kingdom). Diatoms collected during the exponential phase were used to prepare samples following the procedure illustrated by Petrucciani *et al.* (2022b). Data acquisition and analysis were performed with the software EA IsoDelta (Compact Science Systems). All the measurements were carried out on four biological replicas.

Quantification of elements other than C and N was carried out with a total reflection X-ray fluorescence spectrometer (S2 PICOFOX, Bruker AXS Microanalysis, Berlin, Germany) according to Petrucciani *et al.* (2022b). Diatoms sampled during the exponential phase were prepared for the analysis as described in Petrucciani *et al.* (2022b). Spectra 6.1 software (Bruker AXS Microanalysis) was used for spectral deconvolution and quantification of elemental abundances. All the measurements were carried out on four biological replicas.

Statistical analysis

Statistical analysis of data was carried out with GraphPad Prism software v8.0.1 by entering the original values of the four biological replicas. The significance was set at 5%. Comparison of dependent variables (e.g. growth rates, elemental composition, macromolecular pools) between cells in control condition (CTR) and those exposed to copepod signals (TR) in the filtered medium (independent variable) was carried out with a two-tailed Student’s *t*-test.

Factoextra and prcomp packages in R studio software 4.0.2 were used to perform Principal Component Analysis (PCA). Morphological features (i.e. Circularity, Area, Width, Height, Perimeter, Elongatedness (length:width ratio), Diameter, Compactness, Length,) measured with Imaging Flow Cytometer were used as dependent variables for PCA analysis, comparing cells grown in control medium (CTR) with those in filtered medium (TR).

Regarding media FTIR spectra, the range of absorbance comprised between 1,800 and 950 cm^{-1} was analysed through the Principal Component Analysis function of The Unscrambler® X 10.4 (64-bit) software (CAMO Process AS, Oslo, Norway) (Deore *et al.* 2021).

RESULTS

Preliminary assessment of both presence and activity of copepod cues

The presence of soluble molecules in the filtered medium obtained from copepod starvation was verified by PCA analysis of FTIR absorbance spectra in the range 950–1,800 cm^{-1} in comparison with control medium (Figs 1, S1). Axes PC1 + PC2 represented 83% of the total variance; the filtered medium containing copepod signals was differentiated from the control medium mostly according to PC1.

Growth, cell volume and dry weight

Cultures of *P. tricornutum* had a higher maximum cell density and smaller weight when exposed to copepod signals compared to those found in control condition, without any significant change in the growth rate (Fig. 2; Table 1). On the other hand, *C. muelleri* growth, cell volume and dry weight were not affected by copepod cues (Fig. 2; Table 1). *Cylindrotheca fusiformis* showed a significantly lower maximum cell density and slightly bigger cell volume when exposed to copepod signals as compared to the values in control condition (Fig. 2), and the growth rate did not change (Table 1).

Morphological characterization

Morphological features of diatoms (Circularity, Area, Width, Height, Perimeter, Diameter, Compactness, Length; Length:Width) are presented in Fig. 3, through a PCA. In *P. tricornutum* PC1 + PC2 explained 78.7% of the total variation contained in the data matrix with PC1 accounting for 57.4% and PC2 for 21.4%. In *C. muelleri*, PCA first two axes explained 98.3% of the total variation (85.8% + 12.5%, PC1 + PC2) while in *C. fusiformis* they explained 81.4% of the total variation (64.6% + 16.8%, PC1 + PC2). Except from a slight change in cell morphology observed in *C. muelleri*, great differences between cells grown in the control medium and cells exposed to copepod signals were not recorded.

Macromolecular composition

The semiquantitative analysis of macromolecular pools showed a species-specific pattern in response to copepod cues: *P. tricornutum* had a significant lower content of lipids and proteins in the presence of signals, in *C. muelleri* the macromolecular composition did not change while in *C. fusiformis* the lipid content was significantly higher in cells exposed to copepod signals as compared to the control condition. No difference in the carbohydrate pool was ever recorded in response to copepod signals (Fig. 4).

C and N quotas and isotopic fractionation

In *P. tricornutum*, major changes in elemental composition were observed in cells exposed to copepod signals as compared to cells grown in the control condition: C and N contents per cell were lower; $\delta^{13}C$ value was higher (Fig. 5). The cellular iron was less abundant (Table S2). Cells of *C. muelleri* accumulated significantly more C and N when exposed to cues, whereas $\delta^{15}N$ was significantly lower (Fig. 5). *Cylindrotheca fusiformis* did not show any change in C and N quotas (Fig. 5) but significantly decreased carbon fractionation (higher $\delta^{13}C$ values) when grown in the presence of copepod signals as compared to the control.

DISCUSSION

Results highlighted that among non-toxic diatoms distinct species have developed different responses to the presence of predators that is perceived through molecular signals released in the environment (no physical interaction).

Phaeodactylum tricornutum coping with copepod signals

Phaeodactylum tricornutum was the only species showing a greater maximum cell density in the presence of cues (Fig. 3). Increasing the population size almost by 23% may perhaps be an algal strategy to increase survival chances in the presence of predators. A similar hypothesis was also proposed by Irigoien *et al.* (2005) and by Li & Ismar (2018): copepod cues on *P. tricornutum* caused an upregulation of genes involved in cell mitosis, enhancing DNA replication and maximum number of cells (Li & Ismar 2018). Implementing this kind of response allowed *P. tricornutum* not to rely on strategies already described in literature, such as size-based avoidance (since cell morphology did not change; Fig. 3; Table 1) or protection through thicker silicified armour (since the Si content per cell did not change, as shown in Table S2, and cell weight was reduced, as shown in Table 1) (Moller 2005; Pondaven *et al.* 2007; Pančić *et al.* 2019; Petrucciani *et al.* 2022a). It is worth noticing that this strategy was implemented by the only tychoplankter and not by the planktonic experimental species (Fig. 2).

Moreover, *P. tricornutum* showed metabolic changes consistent with a higher population density in the presence of the same nutrient availability. When exposed to copepod cues, *P. tricornutum* cells had lower dry weight, less C and N quotas and reduced C allocation into lipids and mainly into proteins (Fig. 3), as demonstrated by a significantly higher C to N ratio (Table S5), even without affecting pigment composition (Table S1). The energy not invested into the synthesis of costly organic molecules (Gerotto *et al.* 2020) appears to have fueled the activation of a CO_2-concentrating mechanism (CCM) as shown by the C fractionation (Fig. 5). Since a more positive fractionation value occurs when more HCO_3^- contributes as inorganic C source to photosynthesis (Vuorio *et al.* 2006; Brutemark *et al.* 2009; Doi *et al.* 2010; Petrucciani *et al.* 2022b, 2023) the observed change suggests CCM induction to support C fixation and cell growth, inorganic carbon availability between control and treated cultures being equal. The metabolic tuning exerted by copepod cues enabled the allocation of resources among a larger number of cells while maintaining the same assimilated C and biomass per unit of volume and time present in the CTR cultures (Table

S5). Lastly, cells with a lower protein content are supposed to become less palatable (Palmucci *et al.* 2011; Ratti *et al.* 2013) likely reducing predation pressure and increasing *P. tricornutm* competitiveness.

Chaetoceros muelleri coping with copepod signals

Chaetoceros muelleri, the only centric species in this data set, did not show significant changes in terms of growth (Fig. 2; Table 1), morphology (Fig. 3) and organic composition (Fig. 4; Tables 2, S1) in response to copepod signals. The overall palatability of cells was constant (Ratti *et al.* 2013). Summing up, *C. muelleri* showed homeostatic behaviour in response to the presence of predator signals. As proposed by Giordano (2013), a homeostatic response to external perturbations may be an energetically and competitively preferable option, especially when the perturbation is of moderate intensity, because it requires no alteration of the expressed proteome and eliminates the requirement for reverse acclimation, upon cessation of the perturbation. The low energy consuming metabolism was evident in a steeper N fractionation suggesting that cells relied on more reduced (less costly) N species when signals were perceived (Needoba *et al.* 2003; Needoba & Harrison 2004). Consequently, biomass was characterized by a slightly higher N quota whose assimilation required more C skeletons as demonstrated by a slightly higher C quota and a similar C to N ratio (Fig. 5; Table S5).

Cylindrotheca fusiformis coping with copepod signals

Cylindrotheca fusiformis growth was negatively affected in terms of maximum cell density by the presence of copepod signals (Fig. 2; Table 1), showing an opposite behaviour as compared to *P. tricornutum*. A possible explanation may consist in the considerable size of *C. fusiformis* and a lower growth rate, in control condition, as compared to those of the other experimental diatoms (2.2× and 1.4× slower than in *P. tricornutum* and *C. muelleri*, respectively; Table 1). Due to a slow cell metabolism the enhancement of population size in order to survive may not be feasible. On the other hand, the higher lipid content in *C. fusiformis* cells (Fig. 4) could instead be consistent with a higher production of allelopathic lipophilic substances playing the role of predator chemical mediator such as PUAs (Miralto *et al.* 1999; Brugnano *et al.* 2016). Producing allelopathic substances to influence the predator's habits (i.e. reproduction, ingestion rate and feeding preference) is known to be a good strategy to increase the chances of survival (Chu 1942; Shaw *et al.* 1995; Pohnert 2005; Li *et al.* 2008; Ianora & Miralto 2010; Franzè *et al.* 2018). The $\delta^{13}C$ signature (Fig. 5) was a sign of metabolic shift towards energy consuming mechanisms to support C fixation and allocation into lipids at the expense of growth and population size (Fig. 2; Table 1).

CONCLUSIONS

Studying the perception of molecules coming from predators highlighted response strategies to predation that differ in sign and magnitude even among species of the same taxonomic group, and are not strictly related to toxin production. Cues released by copepods apparently did not cause severe cell damage to preys, never altered growth rates nor modulated their silicification but elicited important metabolic changes. In pennate diatoms grown in presence of predator cues a shift in $\delta^{13}C$ was involved in resource and energy allocation to make new cells at the expense of the C storage pools and vice versa, while in the centric diatom a homeostatic response was implemented. Mainly affecting species growth in terms of population size, copepod signals can be considered a powerful selective pressure ruling species composition in diatom communities.

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TABLES

Table 1. Growth rates (d^{-1}), maximum cell density (cells ml^{-1}), cell volume (fl) and dry weight (pg $cell^{-1}$), of *Phaeodactylum tricornutum*, *Chaetoceros muelleri* and *Cylindrotheca fusiformis* grown in control medium (CTR) and filtered medium (TR).

		P. tricornutum	*C. muelleri*	*C. fusiformis*
μ (d^{-1})	CTR	0.64 ± 0.02	0.39 ± 0.01	0.29 ± 0.01
	TR	0.63 ± 0.04	0.39 ± 0.02	0.26 ± 0.02
Max Density (10^6 cells ml^{-1})	CTR	5.75 ± 0.67^a	6.25 ± 0.11	1.84 ± 0.12^a
	TR	7.43 ± 0.28^b	6.26 ± 0.18	1.55 ± 0.12^b
Cell volume (fl)	CTR	77 ± 10	191 ±5	349 ± 4^a
	TR	69 ± 9	190 ± 7	394 ± 5^b
Dry weight (pg $cell^{-1}$)	CTR	13.8 ± 0.1^a	24.3 ± 1.1	58.8 ± 10.7
	TR	11.3 ± 0.9^b	26.4 ± 0.6	55.5 ± 10.8

Data are expressed as average ± *s* calculated on four biological replicas. Letters represent significant difference between the conditions in the same species ($p < 0.05$).

LEGENDS FOR FIGURES

Fig. 1. PCA of absorbance spectra obtained from culture media. Grey dots represent filtered medium containing copepod signals (CUES) while black dots represent control medium (AMCONA).

Fig. 2. Growth curves of *Phaeodactylum tricornutum*, *Chaetoceros muelleri* and *Cylindrotheca fusiformis* grown in control medium (CTR, solid line, dark circles) and filtered medium (TR, dashed line, white triangles). Error bars show standard deviation (*s*).

Fig. 3. PCA analysis of morphological characteristics recorded in diatoms (*Phaeodactylum tricornutum*, *Chaetoceros muelleri* and *Cylindrotheca fusiformis*) grown in control condition (●) and filtered medium (○).

Fig. 4. Semi-quantification of carbohydrates, lipids and proteins in cells grown in control medium (CTR) and filtered medium (TR). Error bars show standard deviation while asterisks indicate significant difference between condition in the same species (* $p < 0.05$, ** $p < 0.01$).

Fig. 5. Carbon and nitrogen content (pg cell^{-1}), $\delta\,^{13}C$ and $\delta\,^{15}N$ values in cells grown in control medium (CTR) and filtered medium (TR). Error bars show standard deviation while asterisks represent significant difference between the conditions in the same species (* $p < 0.05$, ** $p < 0$).