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Assessment of the allelochemical activity of *Ostreopsis* cf. *ovata* and the ovatoxins towards competitive benthic microalgae

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Abstract :

Recurrent blooms of the toxic dinoflagellate Ostreopsis cf. ovata are frequently reported in the Northwestern Mediterranean Sea. The impact of these proliferations on other microalgal species inhabiting the same habitats is of interest from an ecological prospective. In vitro experiments were carried out to investigate the influence of O. cf. ovata on the growth of the co-occurring benthic diatoms Licmophora paradoxa, Navicula arenaria and the benthic dinoflagellates Prorocentrum lima and Coolia monotis. Overall, O. cf. ovata exhibited weak allelopathic effects towards these microalgal species, with a reduction in the cell abundance for L. paradoxa and P. lima only. Interestingly, dead cells of L. paradoxa and N. arenaria were observed embedded in the thick mucus surrounding O. cf. ovata cells, suggesting that the mucous layer could act as a toxic phycosphere, especially for non-motile cells. All competitors were further exposed for 24 h to ovatoxins, the major toxins produced by O. cf. ovata, and the maximum quantum yield efficiency of L. paradoxa, N. arenaria and P. lima was affected at a minimum concentration of 10 µg mL−1. We then hypothesized that the diffusion of solubilized ovatoxins in the culture medium affects only moderately the competitors' growth, whereas their accumulation in the mucus would yield deleterious effects. More precisely, the competitors' sensitivity to ovatoxins was enhanced in their stationary phase of growth and resulted from a rapid inhibition of an uncharacterized photosynthetic step downstream photosystem II. Altogether, these results emphasize the predominant role of the O. cf. ovata's mucus in driving ecological interactions and suggest that it can affect the growth of several benthic microalgae by accumulating the potent ovatoxins.

Keywords : Ostreopsis cf. ovata, Allelochemistry, Competition, Ovatoxins, Metabolomics

Introduction

Allelopathy is being increasingly highlighted as an important trait in all microalgal clades for inter-specific competition (Schwartz et al. 2016; Brown et al. 2019). Indeed, the release of allelochemicals by microalgae can exhibit an influence on competitors by modifying their nutrient uptake (Lyczkowski and Karp-Boss 2014), motility (Lim et al. 2014; Fernán-dez-Herrera et al. 2016), photosynthetic efficiency (Poulson-Ellestad et al. 2014), growth (Wang et al. 2017a) or even cell integrity (Tillmann et al. 2007, Poulson-Ellestad et al. 2014, Wang et al. 2017b, Poulin et al. 2018). Even though chemical interac-tions occur at the cellular scale, they may then induce cascade effects at the ecosystem level (Hattenrath-Lehmann and Gobler 2017). However, and despite recent efforts on the characterization of allelochemi-cals produced by microalgae, only a limited number of these metabolites have been structurally identified (Pohnert 2005; Gillard et al. 2013; Selander et al. 2015; Gallo et al. 2017). Our knowledge on the physi-ological effects of microalgal allelochemicals is also insufficient although recent findings showed a disrup-tion of osmoregulation, photosynthesis, or lipid bio-synthesis in some cases (Poulson-Ellestad et al. 2014; Poulin et al. 2018; Long et al. 2018a).

Dinoflagellates are known to be a rich source of bioactive metabolites that can exhibit negative effects on other marine organisms but also on humans (Botana 2014; Shimizu 1993; Simon et al. 2009). Even though some dinoflagellate metabolites have been identified as toxins, their ecological effects especially on competitive microalgae have rarely been studied (Tillmann and John 2002, Kubanek et al. 2005, Tillmann and Hansen 2009, Poulson-Ellestad et al. 2014). Intriguingly, microalgal toxins are only scarcely associated with allelochemistry (Granéli and Hansen 2006; Prince et al. 2010), with the exception of karlotoxins (Place et al. 2012) produced by strains of the dinoflagellate *Karlodinium veneficum* and involved in prey capture. Usually, allelochemicals in microalgae are more described by a variety of chemical structures (Brown et al. 2019), with small and non-toxic metabolites like sterols, polyunsaturated aldehydes or fatty acids being known to mediate chemical interactions in diatoms (Pohnert 2005; Gallo et al. 2017; Ianora et al. 2004). A wide range of effects are associated with these allelochemicals towards other microalgal species, spanning from a weak reduction in cell concentration (Kubanek et al. 2005, 2007) to cell lysis (Tillmann and John 2002; Tillmann and Hansen 2009). In particular, effects of dinoflagellate allelochemicals on diatoms have been increasingly reported during the past decade (Hakanen et al. 2014, Lim et al. 2014, Lyczkowski and Karp-Boss 2014, Poulson-Ellestad et al. 2014, Sala-Pérez et al. 2016, Long et al. 2018a)

Most studies on dinoflagellate allelochemistry focused on planktonic species (Poulson-Ellestad et al. 2014; Long et al. 2018a; Tillmann and John 2002; Kubanek et al. 2005; Tillmann and Hansen 2009), while benthic microalgae have been largely understudied (Monti and Cecchin 2012; García-Portela et al. 2016; Ternon et al. 2018). Blooms of the benthic and toxic dinoflagellate *Ostreopsis* cf. *ovata* frequently occur in the northern Mediterranean Sea (Mangialajo et al. 2011), and the increase in the frequency and intensity of these blooms have become health (Tichadou et al. 2010; Vila et al. 2016) and ecological (Faimali et al. 2012; Simonini et al. 2011; Pavaux et al. 2019) hazards in the last decades. The main metabolites described for this species are structural analogues of the potent palytoxin and named ovatoxins (Ciminiello et al. 2008; Brissard et al. 2015), and they are believed to induce toxic effects on humans. The impact of the blooms of *O.* cf. *ovata* on other microalgae of the benthic consortium has been insufficiently investigated, and the involvement of these toxins in allelochemistry remains understudied.

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It has been shown recently that while distant allelopathy of *O.* cf. *ovata* through a membrane is not detrimental to competitors (Ternon et al. 2018), the filtrate of *O.* cf. *ovata* cultures does inhibit the growth and the maximum quantum yield (photosystem II) of several competitors (Monti and Cecchin 2012; García-Portela et al. 2016; Ternon et al. 2018). These first observations are more in favour of a contact interaction that could be mediated through the mucus layer well described for this species, but experimental data were needed to confirm this assumption.

Contact interactions play an important role in aquatic chemical ecology due to the dilution factors acting at a longer distance (Jonsson et al. 2009). The present study aimed to give some insights into the contact allelopathy of *O.* cf. *ovata* with co-occurring microalgae. Co-culture experiments allowing cell–cell contacts were carried out on four benthic microalgal species sharing the same habitat *Prorocentrum lima, Coolia monotis, Licmophora paradoxa* and *Navicula arenaria*. Bioassays assessing the impact of ovatoxins on the same set of competitors were also carried out. In an attempt to identify the allelochemicals responsible for the observed effects, both targeted and un-targeted metabolomics analyses of the culture medium were finally performed by ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS).

Materials and methods

Co-culture experiments and the bioassay were performed on four microalgal species known to occupy the same ecological niche as *O.* cf. *ovata* (Accoroni et al. 2016; Marro et al. 2019; Ninčević Gladan et al. 2019): the diatoms *Licmophora paradoxa* and *Navicula arenaria*; and the dinoflagellates *Prorocentrum lima* and *Coolia monotis*. All strains were monoclonal and obtained from the MCCV (Mediterranean Culture Collection of Villefranche, MCCV33, 109, 47 and 112, respectively, and 54 for *O.* cf. *ovata*) and grown in L1 medium (Guillard and Ryther 1962) prepared with autoclaved aged and filtered seawater adjusted to a salinity of 38. Nitrate and phosphate concentrations in L1 medium are 882 µM, and 36.2 µM, respectively. Algae cultures were maintained at 22 °C, under a 14:10 light/dark cycle with a light intensity of 250 µmol m^{-2} s^{-1}.

Co-culture experiments

The co-culture experiments allowed cell–cell contact between *O.* cf. *ovata* and one competitor, as a dual interaction. All competitors were initially grown in separate 75-mL flasks, *O.* cf. *ovata* in one 300-mL Fernbach, and were used to seed the co-cultures after 10 days of growth (end of the exponential phase). To mimic natural conditions, the ratios of cells of *O.* cf. *ovata*/competitors observed in the field at the peak of the 2016 bloom of the Rochambeau site (43° 41′ 35.64″ N–7° 18′ 31.54″ E, Northwestern Mediterranean Sea) were used to seed the co-cultures (*O.* cf. *ovata*: competitor = 5:1).

Before seeding the co-cultures, an aliquot of 1 mL was sampled from the parent cultures and immediately fixed with Lugol's iodine solution (4% v/v) for cell counting under microscope. After determination of the cell abundance, a small volume (< 1 mL) of the parent culture of the four competitors was used to seed the co-culture flasks of 50 mL containing L1 media at the desired cell concentration. For each competitor, six flasks were prepared among which three received cells of *O.* cf. *ovata* at a concentration of 1.50E+02 cell mL^{-1} and three were control flasks (Fig. 1 SI). Three additional 50-mL control flasks were set for *O.* cf. *ovata* alone using the same cell concentration and volume of L1 media. All mono- and co-cultures were maintained for 17 days, and, every 3–4 days, an aliquot of 1 mL was sampled from each flask after scraping the bottom of the flask to detach the cells. The aliquots were fixed with Lugol's iodine solution to monitor the cell concentration. Additionally, 15 mL was sampled in sterile conditions at days 14 and 17 from all flasks and filtered on 0.2-µm syringe filters to remove cells. These filtrates were stored at − 20 °C until analyses of nitrate and nitrite concentrations.

Cell counting

Samples were stored in the fridge (4 °C) and analysed within a month. All counting was performed under microscope using Sedgewick rafter counting chamber.

Nutrients measurements

Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were estimated to follow the microalgae uptake and nutrient availability. Measurements were performed using an Auto-analyser II Technicon as described by Bendschneider and Robinson (Bendschneider and Robinson 1952).

Chemistry of Ostreopsis cf. *ovata*

Extraction of the intra- and extra-cellular metabolites from O. cf. ovata monocultures

In order to assess the chemical diversity produced and released throughout the growth of the *O.* cf. *ovata* strain used in this study (MCCV54), the chemical content of the cells and the medium from monocultures was analysed at three time points (3, 10 and 17 days of growth).

Twelve flasks were filled with 300 mL of L1 medium, and nine of them were further seeded with *Ostreopsis* cf. *ovata* at an initial concentration of 1.10E+03 cell mL^{-1}, the three remaining flasks being the blanks (only L1 medium, no cells added, Fig. 2 SI). All flasks were placed in an incubator at 24 °C avoiding any shading between them. At each sampling day (days 3, 10 and 17), one blank and three *O.* cf. *ovata* flasks were removed from the incubator. After subsampling of 1 mL for cell counting, the flasks' content was transferred to 50-mL falcon tubes for centrifugation at 900 g and 21 °C for 10 min. The supernatant was collected and filtered upon 0.2 µm of a glass filtering device, and the cell pellet was flash-frozen using liquid nitrogen and further stored at − 20 °C. The sterile supernatants were immediately extracted using C18 discs (47 mm diameter, Supelco) under low vacuum (< 5 mm Hg), following the method described in Ternon et al. (2018). Briefly, the discs were activated using 3 × 5 mL of MeOH, equilibrated with 3 mL of MQ water. After sample loading, the discs were rinsed with 3 mL of MQ water and eluted with 3 × 3 mL of MeOH. The organic extracts were evaporated to 250 µL under a stream of nitrogen before being stored at − 20 °C until analysis. The cell pellets were freeze-dried and extracted three times with 5 mL of MeOH/H_2O (v/v 80/20) in an ultrasonic bath for 10 min. The extract was further centrifuged at 1000 g for 12 min, and the supernatant was collected. All extracts were evaporated to dryness, weighed, and resuspended in MeOH to reach a concentration of 3 mg mL^{-1}.

Preparation of the ovatoxins

A mixture of five ovatoxins (OVTX-a to OVTX-e) was obtained from pellets of *O.* cf. *ovata* MCCV 54 cultivated in L1 medium. The toxins were extracted from 5 g pellets (75 g in total) with 20 mL of MeOH/H_2O (1/1, v/v) by vortex-mixing during 1 min. After centrifugation (4000 g, 5 min), the supernatant was collected, and the pellet rinsed twice with the same volume of mixture of solvents. All supernatants were pooled and concentrated at 30 °C under a stream of nitrogen until a final volume of 1.2 mL. These concentrated extracts were further fractionated by size exclusion chromatography (Sephadex LH-20, column of 70 × 1.5 cm) according to Brissard et al. (Brissard et al. 2015) by using 300 mL of pure MeOH for the elution and collections of 10 mL fractions. The ovatoxin content of each fraction was determined by LC–MS/MS as in Gémin et al. (Gémin et al. 2020). The OVTX-containing fractions were pooled and concentrated before purification by semi-prep chromatography according to Brissard et al. (2015) using a liquid chromatographic system (Agilent 1160) coupled to a DAD detector (1260 Infinity II DAD WR) ($\lambda = 233$ and $\lambda = 263$ nm). Ovatoxins were purified trough the semi-preparative column Uptisphere C_{18}-TF (Interchim, 250 mm × 10 mm, 5 µm) at 25 °C. A linear elution gradient using H_2O/acetic acid 0.2% (A) and ACN/H_2O/acetic acid 0.2% (95:5 v/v) (B) started from 80:20 (v:v) to 0:100 (v:v) over 30 min followed by an isocratic during 5 min 0:100 (v:v). Fractions corresponding to the pool of OVTXs were collected in polypropylene tubes and finally concentrated at 30 °C under a flux of nitrogen in 1 mL DMSO.

Metabolomic analyses of the exometabolome in monocultures of O. cf. ovata by UHPLC-HRMS/MS

An analysis of the chemical content of the endo- and the exometabolome of the monocultures of *O.* cf. *ovata* was performed by UHPLC-HRMS/MS using an Agilent 1290 system (Agilent Technologies, USA) equipped with a diode array detector and

coupled to an Agilent 6540 QTOF mass spectrometer (Agilent Technologies, USA). Analyses were performed in full scan positive mode (ESI+). A volume of 10 µL of each sample was injected and analysed in a reverse phase column (Acquity UPLC HSS T3 1.8 µm, 2.1 mm × 100 mm, Waters) using a linear elution gradient over 15 min with H_2O/MeOH/ formic acid 0.1% from 90:10 (v:v, isocratic from 0 to 2 min) to 0:100 (v:v, isocratic from 12 to 13 min) at a flow rate of 0.4 mL min^{-1}. Collision energies (CE) of 40 eV were applied to obtain the MS/MS spectra. UV spectra were extracted at 210, 233 and 263 nm. The capillary voltage of the MS spectrometer was set at 4500 V (positive mode), and the nebulizing parameters were set as follows: nebulizing gas (N_2) pressure at 0.4 bar, drying gas (N_2) flow at 11 L min^{-1}, drying temperature at 300 °C and vaporizer/sheath gas temp, 350 °C. A quantification of the ovatoxins was performed using commercial palytoxin as a reference (Wako Chemicals, GmbH, Neuss, Germany) considering similar MS responses for all the analogues. A calibration curve for palytoxin was built using concentrations between 1 and 10 µg mL^{-1} and the major triply charged ion $[M+3H\text{-}4H_2O]^{3+}$ at m/z 870.1571 for quantification. The same adduct was used to quantify ovatoxin a (m/z 859.4956) in the samples, considering other analogues in the pool of ovatoxins negligible (Brissard et al. 2014).

To perform untargeted metabolomics on the exometabolome, a random injection of the samples on the UHPLC-HRMS/MS was performed in two separate batches to avoid systematic errors. Five quality control samples (QCs) were prepared by mixing all the samples at equimolar concentrations and were injected every seven samples. Analytical blanks were also prepared and injected at the beginning and the end of the analyses. Raw UHPLC-HRMS data were analysed using mass hunter qualitative analysis, converted into mzXML files using msConvert (Holman et al. 2014), and processed for mass detection, building chromatogram, deconvolution, alignment, isotope finding and annotation using the open-source MZmine (version 2.37.corr17.7_kai_merge2). The resulting list of features was filtered using three successive steps (signal/noise using blanks, coefficient of variation using QCs samples) using an in-house script run on R. These variables were normalized by the quantile, $\log_{10}$-transformed, auto-scaled and analysed by partial least-square discriminant analysis (PLS-DA) using the MetaboAnalyst 3.5 online resource (Xia and Wishart 2016). Subsequently, an annotation of analogues for identified biomarkers was tried out using molecular networking on the GNPS platform (Wang et al. 2016).

Bioassays

Three sets of bioassays were carried out to investigate the effects of the ovatoxins on the physiology of each of the four competitors at three different stages of their growth, determined beforehand: initiation (IN, day 3), exponential (EX, day 10) and stationary (ST, day 17) phases of growth. Each competitor was grown beforehand in a 150-mL mother flask, for 20 days. At days 3 (IN), 10 (EX) and 17 (ST), a volume of 1 mL was sampled in sterile conditions from this mother flask and added to 2 mL of fresh L1 medium in 12-well plates to reach 200 cell mL^{-1} for each species. In total, 10 assays were performed for each species—ovatoxins 1 and 10 µg mL^{-1} and a control—and all assays were performed in triplicates. DMSO solutions of 3 and 0.3 mg mL^{-1} were prepared for the ovatoxins, and 10 µL was added to the corresponding well to reach a concentration of 10 and 1 µg mL^{-1}. For control conditions, 10 µL of DMSO only was added yielding a final non-toxic concentration of 0.3% of DMSO each well. The well plates were further incubated at 22 °C for 24 h until assessment of the photosynthetic efficiency of the competitors.

Pulse-amplitude-modulated (PAM) measurement

The method used in this study was described previously (Ternon et al. 2018). Briefly, after being incubated 24 h with the mixture of ovatoxins, the well plates containing the competitors were placed in the dark for 15 min before being transferred to a 2-mL glass cuvette immediately moved to a MC-PAM (Multi-Color Pulse-Amplitude-Modulated, Heinz Walz Gmbh, Effeltrich, Germany) equipped with a blue LED (440 nm) as a source for the actinic light and a white LED used for the saturating pulses. The F_v/F_m (maximum quantum yield) of the photosystem II (PSII) was used as a proxy of the microalgae physiological state. It was calculated as $(F_m - F_0)/F_m$, where F_0 is the fluorescence of a dark-adapted sample and F_m is measured after

application of a saturation pulse of light (intensity 431 µE m^{-2} s^{-1}, 300 ms duration). Curve fitting software provided with the instrument (PAMwin V3.20 W) was used to obtain F_v/F_m. All curve fits and fluorescence transients were manually inspected in real time. An activity of the toxins was calculated as a percentage based on the F_v/F_m of the PSII using the following equation:

$$\% = \left(1 - \frac{\left(\frac{F_v}{F_m}\right)\text{treatment}}{\text{Average}\left(\frac{F_v}{F_m}\right)\text{control}}\right) \times 100$$

Photosynthetic performance

The effect of ovatoxins on the photosynthetic performance of *L. paradoxa* was measured using a Joliot-type spectrometer (JTS-10, Biologic, Grenoble, France). Maximum quantum yield of PSII (F_v/F_m) was calculated as explained before. The PSII quantum yield in light-adapted (800 µE m^{-2} s^{-1}) samples of PSII (Φ_{PSII}) was calculated as $\Phi_{PSII} = (F_m' - F_{stat})/F_m'$, where F_{stat} is the fluorescence of the sample adapted to the actinic light (6 min, 800 µE m^{-2} s^{-1}) and F_m' the fluorescence when a saturating pulse is applied on light-adapted sample. The photochemical rate (PSI + PSII activity) was calculated as described in Bailleul et al. (2010). In brief, the electro-chromic shift of photosynthetic pigments was followed at 532 nm under steady-state illumination (800 µE m^{-2} s^{-1}) and at the offset of light. The slope of ECS was measured for the first 5 ms after light offset and then normalized by a saturating laser flash-induced ECS increase, providing an expression of the photochemical rate in charge separations per photosystem per second (charge sep. PS^{-1} s^{-1}).

Statistics

A Shapiro test was performed to check the normality of the data, and a Mann–Witney test was subsequently performed on R 3.4.0 to obtain significant differences in cell abundance in the co-culture experiments. A *t* test was used to assess statistical significance from photosynthetic performance experiments.

Results

Co-culture experiments

Growth of the competitors of O. cf. ovata

In monocultures of the two diatoms *Licmophora paradoxa* and *Navicula arenaria*, a standard growth curve with an exponential phase lasting for 7 to 10 days and a stationary phase between day 10 and day 17 was observed (Fig. 1A, B). Monocultures of the dinoflagellates *Prorocentrum lima* and *Coolia monotis* did not reach the stationary phase after 17 days (Fig. 1C, D), indicating a slower growth than diatoms on the same culture medium. Similar observations were made for the co-culture flasks.

Although all cultures were inoculated with a cell concentration of the same order of magnitude (~ 1E+02 cell mL^{-1}), the final cell concentration in control flasks was species-dependent. The stationary phase was reached at 9.24 E+03 cell mL^{-1} for the diatom *N. arenaria* and 6.22 E+04 cell mL^{-1} for the diatom *L. paradoxa*, which showed the highest cell concentration of all competitors. The two dinoflagellates were still in their exponential phase at the end of the experiment with cell concentrations of 4.04 E+04 cell mL^{-1} and 9.4 × 10E+02 cell mL^{-1} for *C. monotis* and *P. lima*, respectively.

Overall, cell densities between monocultures and co-cultures were not statistically different for all four microalgal competitors. No dramatic decrease in their cell abundance was observed in co-cultures with *O.* cf. *ovata*. Yet, the cell abundances for the diatom *L. paradoxa* and the dinoflagellate *P. lima* were lower in the co-culture flasks at the end of the experiments. The optimal growth rate revealed an inhibition of the cell division for *L. paradoxa* and *P. lima* in the presence of *O.* cf. *ovata* (μ_{max}, Table 1). A second observation was that cell division was completed faster for *N. arenaria*, which entered its stationary phase at day 7 instead of day 10 in the mono-culture.

O. cf. ovata growth

In both mono- and co-culture flasks, the growth curve of *O.* cf. *ovata* followed a similar trend, with an exponential phase between day 3 and 10, followed by a stationary/senescent phase towards the end of the experiment (Fig. 1E). The maximal cell concentration

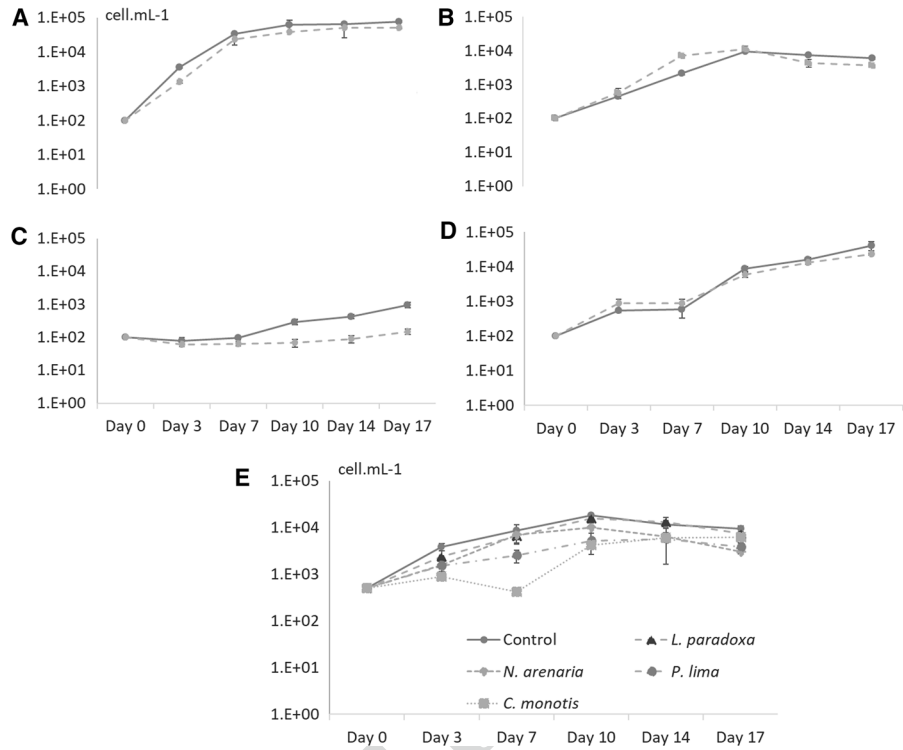

Fig. 1 Cell abundance of the competitors **A** *Licmophora paradoxa*, **B** *Navicula arenaria*, **C** *Prorocentrum lima*, **D** *Coolia monotis* in mono-culture flasks (plain line) and when co-cultured with *Ostreopsis* cf. *ovata* (dashed line). Cell abundance of **E** *O.* cf. *ovata* in mono-culture (plain line) and when co-cultured with its competitors (dashed line). A log scale is used for the ordinates to improve the visualization of the exponential phase of growth

Table 1 Mean optimal growth rate (μ_{max}, d^{-1}) and mean concentration of nitrates (NO_3^-, µM) and nitrite (NO_2^-, µM) with their standard error for the competitors and *O.* cf. *ovata* in mono- and co-cultures

	O. cf. *ovate*	*L. paradoxa*	*N. arenaria*	*P. lima*	*C. monotis*
Monoculture					
μ_{max}	0.36 ± 0.005	0.83 ± 0.008	0.45 ± 0.008	0.23 ± 0.02	0.44 ± 0.016
[NO_3^-]	2.05 ± 1.2	247.93 ± 14.33	103.40 ± 25.79	793.7 ± 5.82	62.07 ± 20.22
[NO_2^-]	0.05 ± 0.02	2.8 ± 1.4	2.95 ± 2..95	0.93 ± 0.93	6.99 ± 4.44
		L. paradoxa	*N. arenaria*	*P. lima*	*C. monotis*
Co-culture					
μ_{max} competitor		0.76 ± 0.05	0.60 ± 0.01	0.12 ± 0.06	0.40 ± 0.02
μ_{max} *O.* Cf. *ovata*		0.34 ± 0.02	0.29 ± 0.03	0.20 ± 0.06	0.21 ± 0.008
[NO3$^-$]		5.42 ± 2.30	17 ± 14.3	34 ± 22.6	13 ± 1.89
[NO2$^-$]		0.17 ± 0.05	0.39 ± 0.18	4.16 ± 3.5	2.21 ± 1.87

The µmax was calculated over the exponential phase, which is species and condition dependent. The mean concentration of nitrogen species was measured at the end of the experiment (day 17) for all conditions and species

of 1.8E+04 cell mL^{-1} was reached in control flasks on day 10. Overall, no beneficial effects from being co-cultured with other species were observed on *O.* cf. *ovata*'s cell abundance (Fig. 1E). Conversely, a reduction in the cell abundance of *O.* cf. *ovata* was observed when co-cultured with all competitors but *L. paradoxa* (Fig. 1E). *O.* cf. *ovata* cell division was slowed by the presence of *N. arenaria*, *P. lima* and *C. monotis* and this effect was particularly marked for the two dinoflagellates with μ_{max} of *O.* cf. *ovata* ranging from 0.20 to 0.21 d^{-1} against 0.36 d^{-1} in control flasks (μ_{max}, Table 1).

Microscopic observations of the co-cultures showed an accumulation of dead diatom cells for both

L. paradoxa and *N. arenaria*, embedded in the thick mucus produced by *O.* cf. *ovata*. *P. lima* cells present in the thick brown mucus could not be distinguished unambiguously from those of *O.* cf. *ovata* due to similar cell colour and morphology, but we assumed a similar behaviour for the three species. No such observation was made for *C. monotis*.

Nutrients availability

The initial NO_3^- concentration was 882 µM, and even if a decrease was observed for all species, NO_3^- concentrations were > 20 µM for the first 14 days in both mono- and co-cultures. At the end of the experiment, NO_3^- concentration decreased to lower values, but was always > 2 µM in the co-culture chambers. The highest rate of NO_3^- consumption was observed for monocultures of *O.* cf. *ovata* and led to concentrations as low as 2.05 ± 1.2 µM at day 17 (Table 1). In competitors' monocultures, NO_3^- concentrations were high until the end of the experiment, with values ranging from 62 to 793 µM (Table 1). When competitors were present, the NO_3^- consumption was lower than in *O.* cf. *ovata* monocultures. It leaded to concentrations that ranged between 5.42 ± 3.25 and 34 ± 2 µM at the end of the experiment, depending on the competitive species (Table 1).

Part of the nitrogen taken up as NO_3^- was released as NO_2^- (nitrite) by the cells. Nitrite excretion was easily noticeable on day 14, with NO_2^- concentrations in the medium even exceeding 20 µM for co-cultures with *L. paradoxa*, *N. arenaria* and *C. monotis*. The NO_2^- concentration then decreased at concentrations below 7 µM on day 17 (Table 1).

Targeted and untargeted analysis of the endo- and the exo-metabolome of O. cf. *ovata*

The metabolic contents of *O.* cf. *ovata* cells (endometabolome) and the culture media (exometabolome) were analysed in triplicate monocultures of the dinoflagellate harvested at days 3, 10 and 17 by un-targeted and targeted metabolomics analysis UHPLC-HRMS/MS.

The targeted analysis first revealed an increase in the total cellular ovatoxin content in the flask from 31.88 to 584.80 µg L^{-1} throughout the growth (Fig. 2). In the meantime, the toxin content in the culture medium positively correlated with the

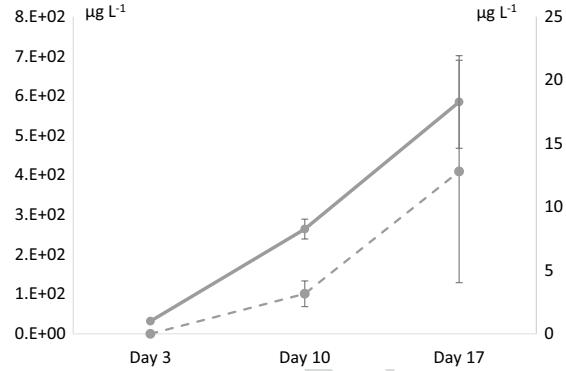

Fig. 2 Intra- (plain line, left axis) and extra-cellular (dashed line, right axis) mean concentration of ovatoxin obtained from monocultures of *O.* cf. *ovata* ($n = 3$) harvested after 3, 10 and 17 days of growth

intracellular concentration of ovatoxins, starting from 0 to 12.8 ± 8.8 µg L^{-1} at day 17. It is worth noting that if the total cellular toxin content increased over time until day 17, the maximal concentration of toxin per *O.* cf. *ovata* cell was reached at day 10 with 35.2 ± 7.4 pg equivalent pltx $cell^{-1}$.

The untargeted approach performed on the same exometabolome content from monocultures of *O.* cf. *ovata* led to 736 features after filtering. A clear separation between the different sampling time points is displayed by the PLS-DA plot (Fig. 3), mainly on the first component (37.5%), highlighting a significant (95% interval of confidence is not overlapping) modification of the metabolites produced and exuded by *O.* cf. *ovata* over time. Chemical differences at day 10 are highlighted by the second component (22.1% of the total variance), indicating additional diversity of the metabolites biosynthesized and released at the end of the exponential phase. The first 18 Variable Importance in Projection (VIP) responsible for the distribution of the samples on the PLS-DA plot (VIP score > 1.5) are listed in Table 2. The identification of the metabolites was very limited, but some propositions can be made.

Only VIP16 was identified as 3-Amino-4-methylhexanoic acid by comparing its fragments with the MassBank data. Due to a poor fragmentation, the annotation of the other VIP was only putative. The VIP1 was a penta-charged ion at m/z 1010.61859 eluting at 5.27 min (Fig. 4), corresponding to a $[M+H]^+$ mono-charged ion at m/z 5053.0929, with a tetra-charged ion detected at m/z 1258.07. The

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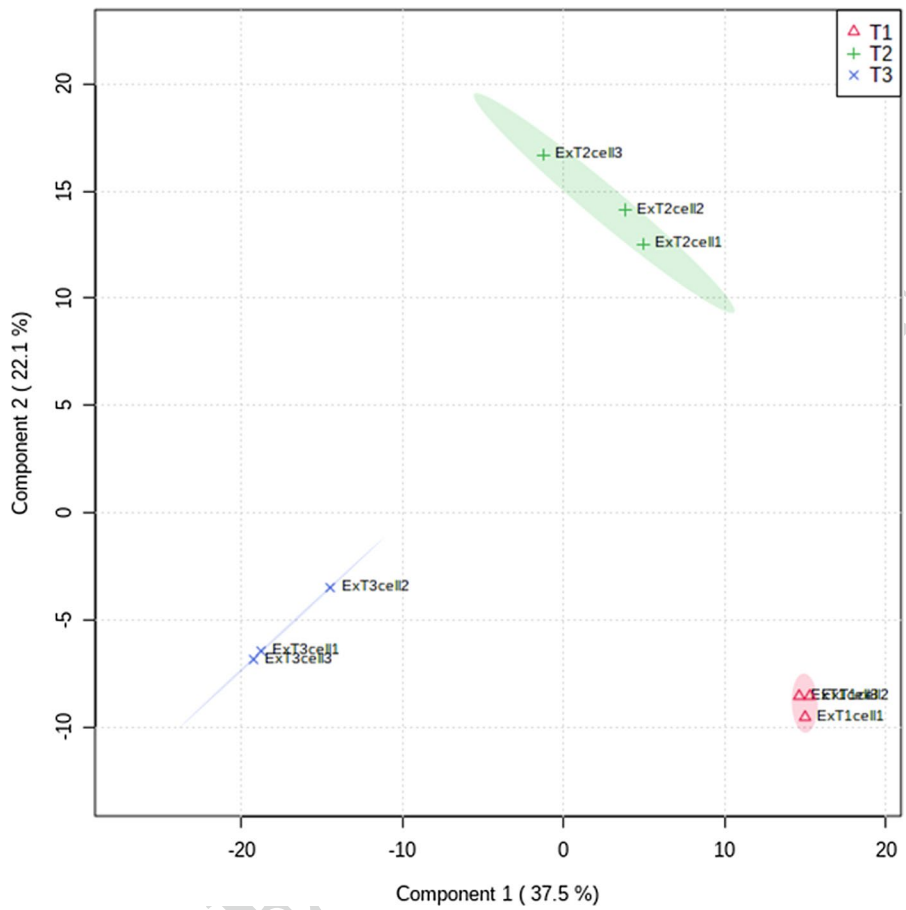

Fig. 3 PLS-DA plot obtained for the exo-metabolome at three stages of the growth of mono-cultures of *O.* cf. *ovata* (T1 = Day 3, T2 = Day 10 and T3 = Day17)

high molecular mass together with a similarity with the ovatoxin-a isotopic pattern suggests a structurally related compound. This ion may result from the dimerization of the ovatoxin-a as the number of carbons is multiplied by 2 in the proposed molecular formula. The ovatoxin-a monomer, eluting at 8 min, was not listed as VIP for the exo-metabolome. Lastly, no identification of the other VIP was successful, but both VIP 6 and 9 are found to be produced by *O.* cf. *ovata* cells (data not shown).

Evaluation of the ecotoxicity of the ovatoxins

The effect of the ovatoxins on the four competitors was tested at three different stages of their growth: initial (IN, day 3), exponential (EX, day 10) or stationary (ST, day 17) phases. While long-term (24 h) exposure to 1 µg mL^{-1} of ovatoxins did not affect the PSII of any competitors (Fig. 5), a concentration of 10 µg mL^{-1} (or approximately 3.77 µM) did inhibit the PSII of both diatoms (*L. paradoxa* and *N. arenaria*) and one dinoflagellate (*P. lima*). The sensitivity of the competitors to the ovatoxins was dependent on the cultures growth phase: *Licmophora paradoxa* was more sensitive at the IN and ST phases (100 and 90% of activity of the toxins) than during the EX one (50%). The sensitivity of *N. arenaria* increased from IN to ST phases, as shown by the increase in the toxin activity from 35 to 100%. *Prorocentrum lima* was hampered by the ovatoxins only at the ST phase (45%).

A side experiment on the diatom *L. paradoxa* revealed an absence of the variation of the maximum quantum yield of the PSII (F_v/F_m) after short-term exposure (5 min) to 10 µg mL^{-1} of ovatoxins (Fig. 6A). However, if the same sample was then illuminated, steady state of photosynthesis was significantly affected. A decrease by 48 ± 25% of the quantum yield of the PSII (ΦPSII) was measured after the samples were exposed to high light (six minutes

Table 2 Variable of importance projection (VIP) obtained from the PLS-DA plot with their characteristics (m/z, RT) and a putative annotation based on their MS^2 fragments

VIP no.	m/z	RT	Formula	Err (ppm)	Score	Fragments	Annotation
1	1010.6185	4.98	$C_{258}H_{446}N_6O_{104}$	nd	nd	–	Ovatoxin-a dimer*
2	532.2806	5.04	$C_{29}H_{35}N_6O_4$	− 2.11	74.89	87.04; 89.06; 103.04; 104.04; 113.06; 133.09; 147.06	nd
3	488.2688	4.72	$C_{31}H_{31}N_6$	− 0.37	76.78	87.04; 89.06; 103.04	nd
4	577.2299	4.72	$C_{20}H_{32}N_4O_8$	0.28	68.74	139.08; 267.13; 447.17; 577.24	nd
5	439.26	4.62	$C_{18}H_{36}NO_{11}$	nd	nd	313.16; 314.17	nd
6	492.2557	7.79	nd	nd	nd	77.03; 91.04; 93.04; 117.05	nd
7	1042.5519	5.27	nd	nd	nd	975.20	nd
8	537.2470	5.04	$C_{29}H_{34}N_3O_7$	0.08	88.64	479.26	nd
9	200.0788	1.83	$C_8H_{11}N_2O_4$	1.78	47.01	–	C6-homoserine lactone*
10	576.3183	5.28	$C_{32}H_{41}N_5O_5$	0.58	86.93	87.03; 89.05; 103.03; 104.03	nd
11	1024.5404	5.45	nd	nd	nd	93.07	nd
12	164.0880	1.45	$C_7H_{15}O_2S$	− 8.42	80.67	66.02	L-ethionine*
13	463.0986	4.90	$C_{20}H_{18}N_2O_{11}$	0.11	82.55	–	Chrysoeriol-7-O-glucoside*
14	246.1446	7.40	$C_{10}H_{19}N_3O_4$	2.76	78.30	–	Leu-Gly-Gly*
15	620.2859	5.47	$C_{21}H_{43}N_6O_{15}$	0.47	95.47	73.06; 87.04; 89.05; 103.3; 113.05; 133.07	nd
16	146.1226	4.61	$C_7H_{15}NO_2$	nd	nd	69.03; 81.07; 85.06; 109.10; 123.11; 139.11; 151.11; 169.12	(3R,4S)-3-Amino-4-methyl-hexanoic acid

*means putative annotation not confirmed by MS2 data

of 800 µE m^{-2} s^{-1}, Fig. 6B). Furthermore, under the same irradiance, the exposure to ovatoxins decreased the photochemical rate, which measures the combined activities of photosystem I (PSI) and PSII, by 36 ± 12% (Fig. 6C).

Discussion

The interactions observed in the present study are not driven by nutrient limitation given the NO_3^- concentrations at the end of the experiment (> 2 µM), but are rather hypothesized to be chemically mediated based on previous results obtained on the bioactivity of axenic cell-free filtrates from *O.* cf. *ovata*'s cultures (Ternon et al. 2018).

Weak allelopathic effects by O. cf. *ovata on its competitors*

The effects of *O.* cf. *ovata* cells on the benthic diatoms *N. arenaria* and *L. paradoxa* or the dinoflagellates *P. lima* and *C. monotis* were not as dramatic as those reported for toxic species of the genera *Alexandrium*, *Prymnesium* and *Chrysochromulina* (Tillmann and John 2002; Tillmann and Hansen 2009), that lead to a strong induction of cell lysis or reduced motility of their competitors. A reduction in the cell concentration and the growth rate of some competitors was, however, observed and appeared to be quite specific, just as the effects caused by the dinoflagellate *Karenia brevis* on other phytoplanktonic species (Poulson-Ellestad et al. 2014; Kubanek et al. 2005). Weak allelopathic effects of *O.* cf. *ovata* towards the benthic dinoflagellates *P. minimum*, *C. monotis* (Monti and Cecchin 2012), *G. excentricus* and *P. hoffmannianum* (García-Portela et al. 2016) have previously been reported. Although weak, the existence of an allelopathic effect on *C. monotis* (Monti and Cecchin 2012; García-Portela et al. 2016) is, however, not in agreement with our results and could be attributed to metabolic variability between strains of a same species (Meyer and Pohnert 2019; Pavaux et al. 2020). Even if not drastic, a decrease in the cell concentration and adhesion (García-Portela et al. 2016) of other competitive species should benefit *O.* cf. *ovata* in colonizing the substrate.

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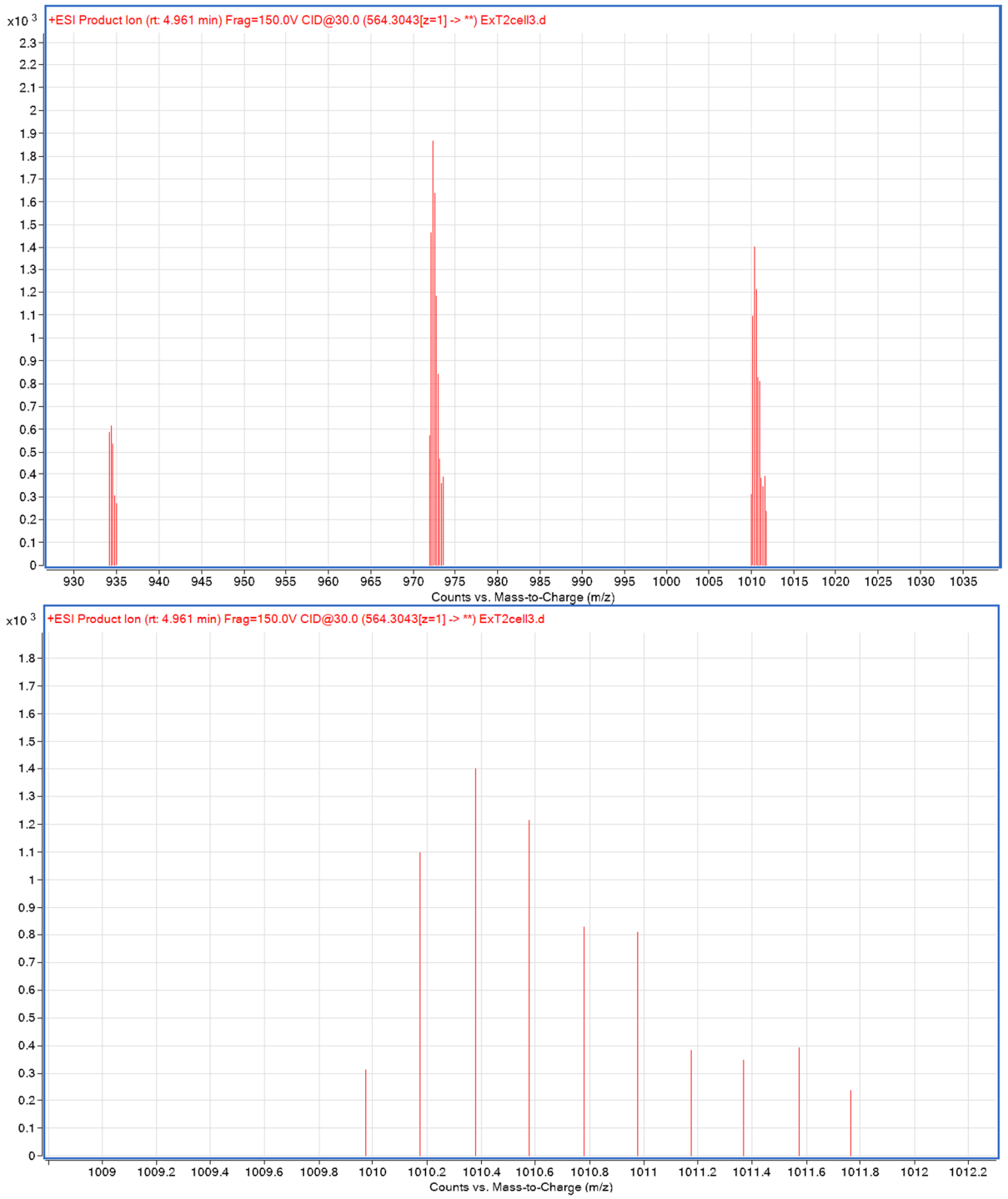

Fig. 4 Mass spectra exhibiting **i** the m/z 1010. 61,859 and two other ions of similar isotopic pattern at m/z 972.55 and m/z 934.55 (upper panel), and **ii** a focus on the mass spectra of the m/z 1010. 61,859 indicating its quinta-charged state (lower panel)

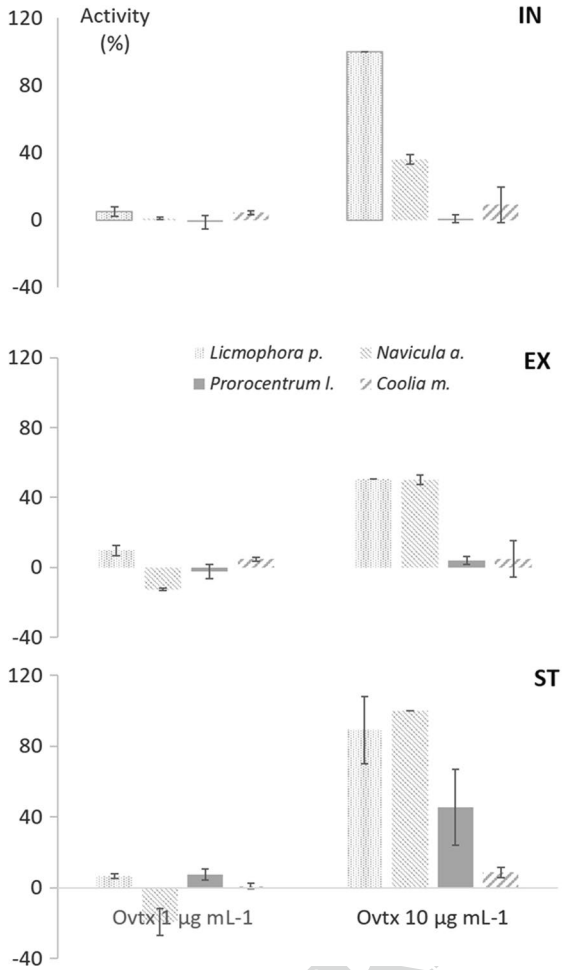

Fig. 5 Activity (%) of the ovatoxins (calculated from the F_v/F_m, see Methods) on each competitor, testing concentrations of 1 and 10 µg mL^{-1} and an exposure time of 24 h. The sensitivity of the competitors was tested at three stages of growth (IN, EX and ST)

Dead competitors (particularly *L. paradoxa*) were only observed when the cells were embedded in the mucus produced by *O.* cf. *ovata*, confirming the central role played by contact interactions promoted by the dense mucus (Ternon et al. 2018; Giussani et al. 2015). By trapping competitors or concentrating allelochemicals, the mucus may form a toxic phycosphere. And indeed, competitors' motility is believed to influence the effects of *O.* cf. *ovata* on their cellular growth. The strains of *L. paradoxa* and *P. lima* were the less motile and the most sensitive to the presence of *O.* cf. *ovata* in co-cultures. No impact on the very motile *C. monotis* and a contrasted impact on the moderately motile *N. arenaria* were noted. Motility enables microalgae to navigate across environments as shown for benthic diatoms in sediments (Consalvey et al. 2004) or when attracted by pheromones (Gillard et al. 2013; Bondoc et al. 2019) and could allow them to escape the toxic phycospheres such as *O.* cf. *ovata*'s mucus.

Effects of the ovatoxins

The toxicity of the ovatoxins varies with the tested species, and also with the growth phase of the microalgae (IN, EX and ST), highlighting species-specific response. Cell lysis of *L. paradoxa* and *N. arenaria* occurred when exposed to 10 µg mL^{-1} of ovatoxins, confirming the deleterious effects of ovatoxins on two diatoms largely distributed in the same ecological niche. Yet, the maximal free extra-cellular concentration of ovatoxins was only 12.8 ± 8.8 ng mL^{-1} at day 17. Whether competitive interactions have been shown to stimulate the production and the release of ovatoxins by a factor 2 (Ternon et al. 2018), the active concentrations of the ovatoxins would still be lower by a factor thousand than the active concentration of 10 µg mL^{-1}.

Other dinoflagellates are known to release toxins in cell-to-cell contact during micro-predation interactions, leading to prey cell lysis (e.g. *Karlodinium* species) (Place et al. 2012). In co-cultures, cell lysis was mostly observed within the mucus, suggesting that deleterious concentrations of ovatoxins may only be reached in this phycosphere.

Besides, their amphiphilic character confers to the ovatoxins surfactant-type properties that may lead to potential dimerization (as proposed in the untargeted metabolomic study) or binding to the mucus as suggested by Giussani et al. (2015). Therefore, the ovatoxin pool embedded into the mucus may result from a combination of dimeric ovatoxins and mucus-interacting ovatoxins.

It is difficult to conclude on the allelochemicals responsible for the observed weak inhibition of competitors' growth in the co-cultures. Altogether, the data and observations suggest a central role played by the mucus, by either accumulating the ovatoxins or other unknown allelochemical, to toxic concentrations, or by trapping competitors. Nevertheless, the absence of sensitivity of *P. lima* to the ovatoxins at most growth stages indicates that other metabolites

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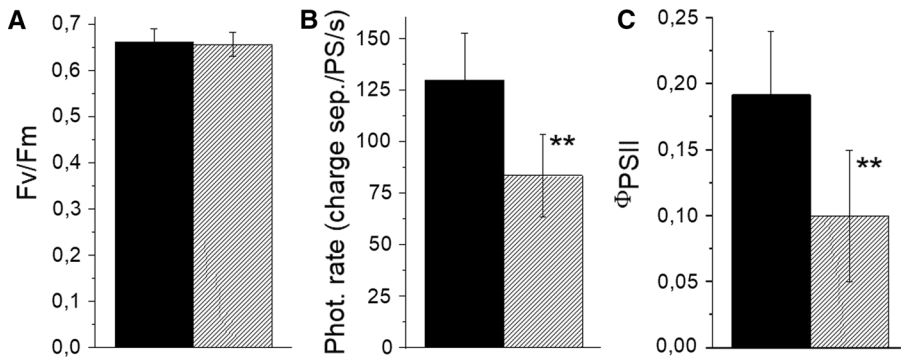

Fig. 6 Photosynthetic response of *Licmophora paradoxa* to short-term exposures to 10 µg mL^{-1} of ovatoxins: **A** Maximum quantum yield of PSII (F_v/F_m) in dark-adapted cells, **B** quantum yield of PSII (ΦPSII) under 800 µE m^{-2} s^{-1} in light illumination, **C** photochemical rate under 800 µE m^{-2} s^{-1} light illumination. Black bars represent the control (0.6% of DMSO final volume), and grey bars represent the ovatoxin-treated samples. After exposure to ovatoxins (or DMSO alone), cells were dark-adapted for 1 min before F_v/F_m was measured and then illuminated till photosynthesis reached a steady state (~6 min). Mean value ± S.D of four independent biological samples is shown. Paired sample *t* test was used for statistical analysis (**$p < 0.05$)

are involved in its growth inhibition. The bacteria of the Rhodobacteraceae clade (*Oceanicaulis*, *Dinoroseobacter* and *Roseovarius*), known to live in close association with *O.* cf. *ovate* (Guidi et al. 2018), may have also contributed to the observed interactions by either producing some of the secondary metabolites highlighted by the metabolomics analysis or by recycling or transforming metabolites produced by *O.* cf. *ovata*, including the toxins (Smith et al. 2002).

Mode of action of the toxins and other allelochemicals

Although growth inhibition was observed for three of the four species studied when exposed to *O.* cf. *ovata*, this does not evidence an induction of cell lysis as strong as the one recorded for other genera like *Alexandrium* (Tillmann and John 2002; Tillmann and Hansen 2009). Other types of interactions might be involved and cell membranes can be disrupted by dinoflagellate allelochemicals, even though cell lysis does not occur [*K. brevis* (Prince et al. 2008)]. In the present study, no measurement of the membrane permeability was performed, but an inhibition of the PSII efficiency was detected for the two diatoms *L. paradoxa* and *N. arenaria* and for the dinoflagellate *P. lima*, in response to ovatoxins. Inhibition of PSII efficiency is commonly proposed as a mechanism for allelopathy (Prince et al. 2008; Tilney et al. 2014; Long et al. 2018b). However, the results of the additional experiment performed with *L. paradoxa* suggest that the decrease in photosynthetic activity stems from a rapid inhibition of an uncharacterized photosynthetic step downstream PSII. Therefore, inhibition of PSII is rather a consequence of the stress cascade induced by allelopathy. Recent pieces of evidence of disruption of the membrane polarity, permeability and associated lipids in phytoplankton by dinoflagellate allelochemicals (Poulin et al. 2018; Long et al. 2021) are indeed supporting the hypothesis of a symptomatic maximum quantum yield inhibition. Therefore, the lack of inhibition of the PSII efficiency in the dinoflagellate *P. lima* may not indicate that cells are not stressed when exposed to ovatoxins, but this species may rather present other undetermined symptoms.

Implication for natural blooms

The results obtained from the co-cultures are mirroring observations of the natural microalgae consortium made during *O.* cf. *ovata* blooms. Field surveys conducted over *O.* cf. *ovata* blooms reported unchanged abundances of the dinoflagellate *C. monotis* (Marro et al. 2019; Ninčević Gladan et al. 2019), a decrease in the dinoflagellate *P. lima* (Marro et al. 2019; Vila et al. 2001) and in diatoms abundance (Accoroni et al. 2016; Marro et al. 2019), particularly the non-motile ones [i.e. *Licmophora paradoxa*; (Accoroni et al. 2016)]. Thus,

the weak allelopathic effect of the dinoflagellate *O.* cf. *ovata* on some of its competitors may still have the ability to shape microalgae species succession in the benthic ecosystem.

The extent of this control must, however, be mitigated by the retro-control that can be applied by the competitors on *O.* cf. *ovata* abundance. Indeed, despite a mixotrophic character (Jauzein et al. 2017), *O.* cf. *ovata* does not benefit from being co-cultured with its competitors. Conversely, a decrease in cell concentration is observed in the presence of *C. monotis*, *N. arenaria* and *P. lima*. This inhibition is in agreement with previous observations with *C. monotis* (García-Portela et al. 2016), even though no morphological changes or encystment in *O.* cf. *ovata* was noticed by the authors. A control on *O.* cf. *ovata* growth by its competitors could also be mediated by chemicals. Both dinoflagellates and diatoms are known to produce a large array of small molecules that can act as allelochemicals (Pohnert 2005; Gillard et al. 2013; Long et al. 2018a; Ma et al. 2011), and some of them have been shown to inhibit the growth of dinoflagellates (Wang et al. 2017a; Tillmann and John 2002; Hakanen et al. AQ4 2014; Ternon et al. 2018; Xu et al. 2015, 2019).

The chemical impact of *O.* cf. *ovata* on ecological succession may also depend on several factors such as the intensity and the duration of the blooms (i.e. variations in biosynthesized chemicals, including the toxins) and the competitor species (adaptive ecophysiological traits like motility). Moreover, other parameters could influence the outcome of this control in natural ecosystems, such as the availability in nutrients that can modulate allelopathic interactions (Grover and Wang 2013), the intraspecific variability of the individuals (Meyer and Pohnert 2019) and eventually the considerable complexity of natural assemblages that involve several parties (Bigalke and Pohnert 2019).

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