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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 perspective. 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<sup>-1</sup>. 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.

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

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## 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 interactions 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 allelochemicals 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 physiological effects of microalgal allelochemicals is also insufficient although recent findings showed a disruption of osmoregulation, photosynthesis, or lipid biosynthesis 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 *Karodinium 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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123 It has been shown recently that while distant allelopa-  
124 thy of *O. cf. ovata* through a membrane is not detri-  
125 mental to competitors (Ternon et al. 2018), the filtrate  
126 of *O. cf. ovata* cultures does inhibit the growth and  
127 the maximum quantum yield (photosystem II) of sev-  
128 eral competitors (Monti and Cecchin 2012; García-  
129 Portela et al. 2016; Ternon et al. 2018). These first  
130 observations are more in favour of a contact interac-  
131 tion that could be mediated through the mucus layer  
132 well described for this species, but experimental data  
133 were needed to confirm this assumption.

134 Contact interactions play an important role in  
135 aquatic chemical ecology due to the dilution factors  
136 acting at a longer distance (Jonsson et al. 2009). The  
137 present study aimed to give some insights into the  
138 contact allelopathy of *O. cf. ovata* with co-occur-  
139 ring microalgae. Co-culture experiments allowing  
140 cell–cell contacts were carried out on four benthic  
141 microalgal species sharing the same habitat *Proro-*  
142 *centrum lima*, *Coolia monotis*, *Licmophora para-*  
143 *doxa* and *Navicula arenaria*. Bioassays assessing  
144 the impact of ovatoxins on the same set of competi-  
145 tors were also carried out. In an attempt to identify  
146 the allelochemicals responsible for the observed  
147 effects, both targeted and un-targeted metabolomics  
148 analyses of the culture medium were finally per-  
149 formed by ultra-high-performance liquid chromatog-  
150 raphy coupled to high-resolution mass spectrometry  
151 (UHPLC-HRMS).

## 152 Materials and methods

153 Co-culture experiments and the bioassay were per-  
154 formed on four microalgal species known to occupy  
155 the same ecological niche as *O. cf. ovata* (Accoroni  
156 et al. 2016; Marro et al. 2019; Ninčević Gladan et al.  
157 2019): the diatoms *Licmophora paradoxa* and *Navic-*  
158 *ula arenaria*; and the dinoflagellates *Prorocentrum*  
159 *lima* and *Coolia monotis*. All strains were monoclonal  
160 and obtained from the MCCV (Mediterranean Culture  
161 Collection of Villefranche, MCCV33, 109, 47 and  
162 112, respectively, and 54 for *O. cf. ovata*) and grown  
163 in L1 medium (Guillard and Ryther 1962) prepared  
164 with autoclaved aged and filtered seawater adjusted to  
165 a salinity of 38. Nitrate and phosphate concentrations  
166 in L1 medium are 882  $\mu\text{M}$ , and 36.2  $\mu\text{M}$ , respec-  
167 tively. Algae cultures were maintained at 22 °C,

under a 14:10 light/dark cycle with a light intensity of 168  
250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . 169

## Co-culture experiments 170

The co-culture experiments allowed cell–cell contact 171  
between *O. cf. ovata* and one competitor, as a dual 172  
interaction. All competitors were initially grown in 173  
separate 75-mL flasks, *O. cf. ovata* in one 300-mL 174  
Fernbach, and were used to seed the co-cultures after 175  
10 days of growth (end of the exponential phase). 176  
To mimic natural conditions, the ratios of cells of 177  
*O. cf. ovata*/competitors observed in the field at the 178  
peak of the 2016 bloom of the Rochambeau site (43° 179  
41' 35.64" N–7° 18' 31.54" E, Northwestern Medi- 180  
terranean Sea) were used to seed the co-cultures (*O.* 181  
*cf. ovata*: competitor = 5:1). 182

Before seeding the co-cultures, an aliquot of 1 mL 183  
was sampled from the parent cultures and immedi- 184  
ately fixed with Lugol's iodine solution (4% v/v) for 185  
cell counting under microscope. After determination 186  
of the cell abundance, a small volume (< 1 mL) of 187  
the parent culture of the four competitors was used 188  
to seed the co-culture flasks of 50 mL containing 189  
L1 media at the desired cell concentration. For each 190  
competitor, six flasks were prepared among which 191  
three received cells of *O. cf. ovata* at a concentration 192  
of 1.50E+02 cell mL<sup>-1</sup> and three were control flasks 193  
(Fig. 1 SI). Three additional 50-mL control flasks 194  
were set for *O. cf. ovata* alone using the same cell 195  
concentration and volume of L1 media. All mono- 196  
and co-cultures were maintained for 17 days, and, 197  
every 3–4 days, an aliquot of 1 mL was sampled from 198  
each flask after scraping the bottom of the flask to 199  
detach the cells. The aliquots were fixed with Lugol's 200  
iodine solution to monitor the cell concentration. 201  
Additionally, 15 mL was sampled in sterile conditions 202  
at days 14 and 17 from all flasks and filtered on 0.2- 203  
 $\mu\text{m}$  syringe filters to remove cells. These filtrates were 204  
stored at – 20 °C until analyses of nitrate and nitrite 205  
concentrations. 206

## Cell counting 207

Samples were stored in the fridge (4 °C) and ana- 208  
lysed within a month. All counting was performed 209  
under microscope using Sedgewick rafter counting 210  
chamber. 211

212 *Nutrients measurements*

213 Nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) concentrations  
 214 were estimated to follow the microalgae uptake and  
 215 nutrient availability. Measurements were performed  
 216 using an Auto-analyser II Technicon as described by  
 217 Bendschneider and Robinson (Bendschneider and  
 218 Robinson 1952).

219 *Chemistry of *Ostreopsis cf. ovata**220 *Extraction of the intra- and extra-cellular metabolites*  
221 *from *O. cf. ovata* monocultures*

222 In order to assess the chemical diversity produced and  
 223 released throughout the growth of the *O. cf. ovata*  
 224 strain used in this study (MCCV54), the chemical  
 225 content of the cells and the medium from monocul-  
 226 tures was analysed at three time points (3, 10 and  
 227 17 days of growth).

228 Twelve flasks were filled with 300 mL of L1  
 229 medium, and nine of them were further seeded with  
 230 *Ostreopsis cf. ovata* at an initial concentration of  
 231  $1.10\text{E}+03 \text{ cell mL}^{-1}$ , the three remaining flasks being  
 232 the blanks (only L1 medium, no cells added, Fig. 2  
 233 SI). All flasks were placed in an incubator at  $24^\circ\text{C}$   
 234 avoiding any shading between them. At each sam-  
 235 pling day (days 3, 10 and 17), one blank and three  
 236 *O. cf. ovata* flasks were removed from the incuba-  
 237 tor. After subsampling of 1 mL for cell counting, the  
 238 flasks' content was transferred to 50-mL falcon tubes  
 239 for centrifugation at 900 g and  $21^\circ\text{C}$  for 10 min. The  
 240 supernatant was collected and filtered upon  $0.2 \mu\text{m}$  of  
 241 a glass filtering device, and the cell pellet was flash-  
 242 frozen using liquid nitrogen and further stored at  
 243  $-20^\circ\text{C}$ . The sterile supernatants were immediately  
 244 extracted using C18 discs (47 mm diameter, Supelco)  
 245 under low vacuum ( $<5 \text{ mm Hg}$ ), following the  
 246 method described in Ternon et al. (2018). Briefly, the  
 247 discs were activated using  $3 \times 5 \text{ mL}$  of MeOH, equi-  
 248 librated with 3 mL of MQ water. After sample load-  
 249 ing, the discs were rinsed with 3 mL of MQ water and  
 250 eluted with  $3 \times 3 \text{ mL}$  of MeOH. The organic extracts  
 251 were evaporated to  $250 \mu\text{L}$  under a stream of nitrogen  
 252 before being stored at  $-20^\circ\text{C}$  until analysis. The cell  
 253 pellets were freeze-dried and extracted three times  
 254 with 5 mL of MeOH/ $\text{H}_2\text{O}$  (v/v 80/20) in an ultra-  
 255 sonic bath for 10 min. The extract was further centri-  
 256 fuged at 1000 g for 12 min, and the supernatant was

collected. All extracts were evaporated to dryness, 257  
 weighed, and resuspended in MeOH to reach a con- 258  
 centration of  $3 \text{ mg mL}^{-1}$ . 259

260 *Preparation of the ovatoxins*

261 A mixture of five ovatoxins (OVTX-a to OVTX-e) 261  
 was obtained from pellets of *O. cf. ovata* MCCV 54 262  
 cultivated in L1 medium. The toxins were extracted 263  
 from 5 g pellets (75 g in total) with 20 mL of MeOH/  
 $\text{H}_2\text{O}$  (1/1, v/v) by vortex-mixing during 1 min. After 264  
 centrifugation (4000 g, 5 min), the supernatant was 265  
 collected, and the pellet rinsed twice with the same 266  
 volume of mixture of solvents. All supernatants were 267  
 pooled and concentrated at  $30^\circ\text{C}$  under a stream of 268  
 nitrogen until a final volume of 1.2 mL. These concen- 269  
 trated extracts were further fractionated by size 270  
 exclusion chromatography (Sephadex LH-20, column 271  
 of  $70 \times 1.5 \text{ cm}$ ) according to Brissard et al. (Brissard 272  
 et al. 2015) by using 300 mL of pure MeOH for the 273  
 elution and collections of 10 mL fractions. The ova- 274  
 toxin content of each fraction was determined by 275  
 LC-MS/MS as in Gémin et al. (Gémin et al. 2020). 276  
 The OVTX-containing fractions were pooled and 277  
 concentrated before purification by semi-prep chro- 278  
 matography according to Brissard et al. (2015) using 279  
 a liquid chromatographic system (Agilent 1160) 280  
 coupled to a DAD detector (1260 Infinity II DAD 281  
 WR) ( $\lambda=233$  and  $\lambda=263 \text{ nm}$ ). Ovatoxins were puri- 282  
 fied through the semi-preparative column Uptisphere 283  
 $\text{C}_{18}\text{-TF}$  (Interchim,  $250 \text{ mm} \times 10 \text{ mm}$ ,  $5 \mu\text{m}$ ) at  $25^\circ\text{C}$ . 284  
 A linear elution gradient using  $\text{H}_2\text{O}$ /acetic acid 0.2% 285  
 (A) and ACN/ $\text{H}_2\text{O}$ /acetic acid 0.2% (95:5 v/v) (B) 286  
 started from 80:20 (v:v) to 0:100 (v:v) over 30 min 287  
 followed by an isocratic during 5 min 0:100 (v:v). 288  
 Fractions corresponding to the pool of OVTXs were 289  
 collected in polypropylene tubes and finally con- 290  
 centrated at  $30^\circ\text{C}$  under a flux of nitrogen in 1 mL 291  
 DMSO. 292  
 293

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

297 An analysis of the chemical content of the endo- 297  
 and the exometabolome of the monocultures of *O.* 298  
*cf. ovata* was performed by UHPLC-HRMS/MS 299  
 using an Agilent 1290 system (Agilent Technolo- 300  
 gies, USA) equipped with a diode array detector and 301



302 coupled to an Agilent 6540 QTOF mass spectrometer  
 303 (Agilent Technologies, USA). Analyses were per-  
 304 formed in full scan positive mode (ESI+). A volume  
 305 of 10  $\mu\text{L}$  of each sample was injected and analysed  
 306 in a reverse phase column (Acquity UPLC HSS T3  
 307 1.8  $\mu\text{m}$ , 2.1 mm $\times$ 100 mm, Waters) using a linear  
 308 elution gradient over 15 min with  $\text{H}_2\text{O}/\text{MeOH}/$  for-  
 309 mic acid 0.1% from 90:10 (v:v, isocratic from 0 to  
 310 2 min) to 0:100 (v:v, isocratic from 12 to 13 min) at  
 311 a flow rate of 0.4  $\text{mL min}^{-1}$ . Collision energies (CE)  
 312 of 40 eV were applied to obtain the MS/MS spectra.  
 313 UV spectra were extracted at 210, 233 and 263 nm.  
 314 The capillary voltage of the MS spectrometer was set  
 315 at 4500 V (positive mode), and the nebulizing param-  
 316 eters were set as follows: nebulizing gas ( $\text{N}_2$ ) pressure  
 317 at 0.4 bar, drying gas ( $\text{N}_2$ ) flow at 11  $\text{L min}^{-1}$ , dry-  
 318 ing temperature at 300  $^\circ\text{C}$  and vaporizer/sheath gas  
 319 temp, 350  $^\circ\text{C}$ . A quantification of the ovatoxins was  
 320 performed using commercial palytoxin as a reference  
 321 (Wako Chemicals, GmbH, Neuss, Germany) consid-  
 322 ering similar MS responses for all the analogues. A  
 323 calibration curve for palytoxin was built using con-  
 324 centrations between 1 and 10  $\mu\text{g mL}^{-1}$  and the major  
 325 triply charged ion  $[\text{M}+3\text{H}-4\text{H}_2\text{O}]^{3+}$  at  $m/z$  870.1571  
 326 for quantification. The same adduct was used to quan-  
 327 tify ovatoxin a ( $m/z$  859.4956) in the samples, consid-  
 328 ering other analogues in the pool of ovatoxins negli-  
 329 gible (Brissard et al. 2014).

330 To perform untargeted metabolomics on the exo-  
 331 metabolome, a random injection of the samples on  
 332 the UHPLC-HRMS/MS was performed in two separ-  
 333 ate batches to avoid systematic errors. Five qual-  
 334 ity control samples (QCs) were prepared by mix-  
 335 ing all the samples at equimolar concentrations and  
 336 were injected every seven samples. Analytical blanks  
 337 were also prepared and injected at the beginning and  
 338 the end of the analyses. Raw UHPLC-HRMS data  
 339 were analysed using mass hunter qualitative analy-  
 340 sis, converted into mzXML files using msConvert  
 341 (Holman et al. 2014), and processed for mass detec-  
 342 tion, building chromatogram, deconvolution, align-  
 343 ment, isotope finding and annotation using the open-  
 344 source MZmine (version 2.37.corr17.7\_kai\_merge2).  
 345 The resulting list of features was filtered using three  
 346 successive steps (signal/noise using blanks, coef-  
 347 ficient of variation using QCs samples) using an in-  
 348 house script run on R. These variables were normal-  
 349 ized by the quantile,  $\log_{10}$ -transformed, auto-scaled  
 350 and analysed by partial least-square discriminant

analysis (PLS-DA) using the MetaboAnalyst 3.5  
 online resource (Xia and Wishart 2016). Subse-  
 quently, an annotation of analogues for identified bio-  
 markers was tried out using molecular networking on  
 the GNPS platform (Wang et al. 2016).

## Bioassays

Three sets of bioassays were carried out to investi-  
 gate 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 station-  
 ary (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  $\text{mL}^{-1}$   
 for each species. In total, 10 assays were performed  
 for each species—ovatoxins 1 and 10  $\mu\text{g mL}^{-1}$  and  
 a control—and all assays were performed in tripli-  
 cates. DMSO solutions of 3 and 0.3  $\text{mg mL}^{-1}$  were  
 prepared for the ovatoxins, and 10  $\mu\text{L}$  was added to  
 the corresponding well to reach a concentration of  
 10 and 1  $\mu\text{g mL}^{-1}$ . For control conditions, 10  $\mu\text{L}$  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  $^\circ\text{C}$  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 pre-  
 viously (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 saturat-  
 ing 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 calcu-  
 lated as  $(F_m - F_0)/F_m$ , where  $F_0$  is the fluorescence  
 of a dark-adapted sample and  $F_m$  is measured after

396 application of a saturation pulse of light (intensity  
397  $431 \mu\text{E m}^{-2} \text{s}^{-1}$ , 300 ms duration). Curve fitting  
398 software provided with the instrument (PAMwin  
399 V3.20 W) was used to obtain  $F_v/F_m$ . All curve fits  
400 and fluorescence transients were manually inspected  
401 in real time. An activity of the toxins was calcu-  
402 lated as a percentage based on the  $F_v/F_m$  of the PSII  
403 using the following equation:

$$404 \quad \% = \left( 1 - \frac{\left( \frac{F_v}{F_m} \right)_{\text{treatment}}}{\text{Average} \left( \frac{F_v}{F_m} \right)_{\text{control}}} \right) \times 100$$

405

#### 406 Photosynthetic performance

407 The effect of ovatoxins on the photosynthetic per-  
408 formance of *L. paradoxa* was measured using a Jol-  
409 iot-type spectrometer (JTS-10, Biologic, Grenoble,  
410 France). Maximum quantum yield of PSII ( $F_v/F_m$ )  
411 was calculated as explained before. The PSII quantum  
412 yield in light-adapted ( $800 \mu\text{E m}^{-2} \text{s}^{-1}$ ) samples of  
413 PSII ( $\Phi_{\text{PSII}}$ ) was calculated as  $\Phi_{\text{PSII}} = (F_m' - F_{\text{stat}})/F_m'$ ,  
414 where  $F_{\text{stat}}$  is the fluorescence of the sample adapted  
415 to the actinic light (6 min,  $800 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and  $F_m'$   
416 the fluorescence when a saturating pulse is applied  
417 on light-adapted sample. The photochemical rate  
418 (PSI+PSII activity) was calculated as described  
419 in Bailleul et al. (2010). In brief, the electro-chro-  
420 mic shift of photosynthetic pigments was followed  
421 at 532 nm under steady-state illumination ( $800 \mu\text{E}$   
422  $\text{m}^{-2} \text{s}^{-1}$ ) and at the offset of light. The slope of ECS  
423 was measured for the first 5 ms after light offset and  
424 then normalized by a saturating laser flash-induced  
425 ECS increase, providing an expression of the photo-  
426 chemical rate in charge separations per photosystem  
427 per second (charge sep.  $\text{PS}^{-1} \text{s}^{-1}$ ).

#### 428 Statistics

429 A Shapiro test was performed to check the normal-  
430 ity of the data, and a Mann–Witney test was subse-  
431 quently performed on R 3.4.0 to obtain significant  
432 differences in cell abundance in the co-culture experi-  
433 ments. A *t* test was used to assess statistical signifi-  
434 cance from photosynthetic performance experiments.

## Results

435

### Co-culture experiments

436

#### Growth of the competitors of *O. cf. ovata*

437

In monocultures of the two diatoms *Licmophora* 438  
*paradoxa* and *Navicula arenaria*, a standard growth 439  
curve with an exponential phase lasting for 7 to 440  
10 days and a stationary phase between day 10 and 441  
day 17 was observed (Fig. 1A, B). Monocultures of 442  
the dinoflagellates *Prorocentrum lima* and *Coolia* 443  
*monotis* did not reach the stationary phase after 444  
17 days (Fig. 1C, D), indicating a slower growth than 445  
diatoms on the same culture medium. Similar obser- 446  
vations were made for the co-culture flasks. 447

Although all cultures were inoculated with a 448  
cell concentration of the same order of magnitude 449  
( $\sim 1\text{E}+02 \text{ cell mL}^{-1}$ ), the final cell concentration in 450  
control flasks was species-dependent. The stationary 451  
phase was reached at  $9.24 \text{ E}+03 \text{ cell mL}^{-1}$  for the 452  
diatom *N. arenaria* and  $6.22 \text{ E}+04 \text{ cell mL}^{-1}$  for the 453  
diatom *L. paradoxa*, which showed the highest cell 454  
concentration of all competitors. The two dinoflagel- 455  
lates were still in their exponential phase at the end 456  
of the experiment with cell concentrations of  $4.04$  457  
 $\text{E}+04 \text{ cell mL}^{-1}$  and  $9.4 \times 10\text{E}+02 \text{ cell mL}^{-1}$  for *C.* 458  
*monotis* and *P. lima*, respectively. 459

Overall, cell densities between monocultures and 460  
co-cultures were not statistically different for all four 461  
microalgal competitors. No dramatic decrease in their 462  
cell abundance was observed in co-cultures with *O.* 463  
*cf. ovata*. Yet, the cell abundances for the diatom *L.* 464  
*paradoxa* and the dinoflagellate *P. lima* were lower 465  
in the co-culture flasks at the end of the experiments. 466  
The optimal growth rate revealed an inhibition of the 467  
cell division for *L. paradoxa* and *P. lima* in the pres- 468  
ence of *O. cf. ovata* ( $\mu_{\text{max}}$ , Table 1). A second obser- 469  
vation was that cell division was completed faster for 470  
*N. arenaria*, which entered its stationary phase at day 471  
7 instead of day 10 in the mono-culture. 472

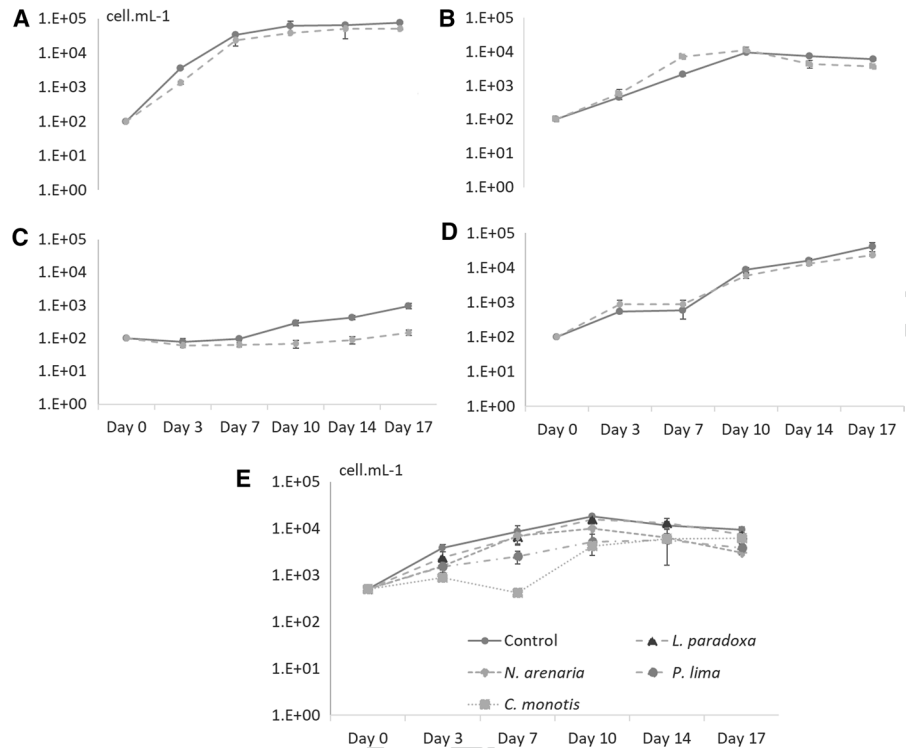
#### *O. cf. ovata* growth

473

In both mono- and co-culture flasks, the growth 474  
curve of *O. cf. ovata* followed a similar trend, with 475  
an exponential phase between day 3 and 10, followed 476  
by a stationary/senescent phase towards the end of the 477  
experiment (Fig. 1E). The maximal cell concentration 478



**Fig. 1** Cell abundance of the competitors **A** *Licmophora paradoxa*, **B** *Navicula arenaria*, **C** *Pro-rocentrum 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 ( $\mu_{max}$ ,  $d^{-1}$ ) and mean concentration of nitrates ( $NO_3^-$ ,  $\mu M$ ) and nitrite ( $NO_2^-$ ,  $\mu M$ ) with their standard error for the competitors and *O. cf. ovata* in mono- and co-cultures

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

The  $\mu_{max}$  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

479 of  $1.8E+04$  cell  $mL^{-1}$  was reached in control flasks  
 480 on day 10. Overall, no beneficial effects from being  
 481 co-cultured with other species were observed on *O.*  
 482 *cf. ovata*'s cell abundance (Fig. 1E). Conversely, a  
 483 reduction in the cell abundance of *O. cf. ovata* was  
 484 observed when co-cultured with all competitors but  
 485 *L. paradoxa* (Fig. 1E). *O. cf. ovata* cell division was

486 slowed by the presence of *N. arenaria*, *P. lima* and  
 487 *C. monotis* and this effect was particularly marked for  
 488 the two dinoflagellates with  $\mu_{max}$  of *O. cf. ovata* rang-  
 489 ing from 0.20 to 0.21  $d^{-1}$  against  $0.36 d^{-1}$  in control  
 490 flasks ( $\mu_{max}$ , Table 1).

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

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

#### 500 Nutrients availability

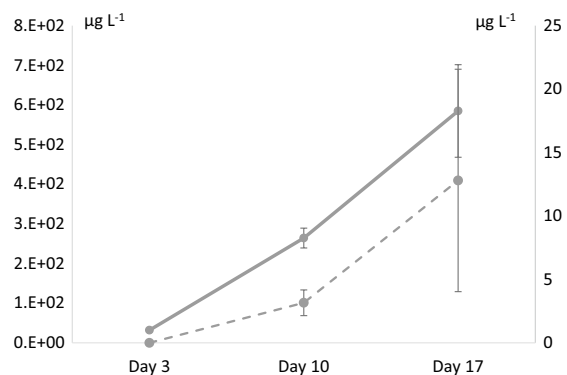
501 The initial  $\text{NO}_3^-$  concentration was 882  $\mu\text{M}$ , and even  
 502 if a decrease was observed for all species,  $\text{NO}_3^-$  con-  
 503 centrations were  $> 20 \mu\text{M}$  for the first 14 days in both  
 504 mono- and co-cultures. At the end of the experiment,  
 505  $\text{NO}_3^-$  concentration decreased to lower values, but  
 506 was always  $> 2 \mu\text{M}$  in the co-culture chambers. The  
 507 highest rate of  $\text{NO}_3^-$  consumption was observed for  
 508 monocultures of *O. cf. ovata* and led to concentra-  
 509 tions as low as  $2.05 \pm 1.2 \mu\text{M}$  at day 17 (Table 1).  
 510 In competitors' monocultures,  $\text{NO}_3^-$  concentrations  
 511 were high until the end of the experiment, with val-  
 512 ues ranging from 62 to 793  $\mu\text{M}$  (Table 1). When com-  
 513 petitors were present, the  $\text{NO}_3^-$  consumption was  
 514 lower than in *O. cf. ovata* monocultures. It led to  
 515 concentrations that ranged between  $5.42 \pm 3.25$  and  
 516  $34 \pm 2 \mu\text{M}$  at the end of the experiment, depending on  
 517 the competitive species (Table 1).

518 Part of the nitrogen taken up as  $\text{NO}_3^-$  was released  
 519 as  $\text{NO}_2^-$  (nitrite) by the cells. Nitrite excretion was  
 520 easily noticeable on day 14, with  $\text{NO}_2^-$  concentrations  
 521 in the medium even exceeding 20  $\mu\text{M}$  for co-cultures  
 522 with *L. paradoxa*, *N. arenaria* and *C. monotis*. The  
 523  $\text{NO}_2^-$  concentration then decreased at concentrations  
 524 below 7  $\mu\text{M}$  on day 17 (Table 1).

#### 525 Targeted and untargeted analysis of the endo- 526 and the exo-metabolome of *O. cf. ovata*

527 The metabolic contents of *O. cf. ovata* cells (endo-  
 528 metabolome) and the culture media (exometabo-  
 529 lome) were analysed in triplicate monocultures of  
 530 the dinoflagellate harvested at days 3, 10 and 17  
 531 by un-targeted and targeted metabolomics analysis  
 532 UHPLC-HRMS/MS.

533 The targeted analysis first revealed an increase  
 534 in the total cellular ovatoxin content in the flask  
 535 from 31.88 to 584.80  $\mu\text{g L}^{-1}$  throughout the growth  
 536 (Fig. 2). In the meantime, the toxin content in the  
 537 culture medium positively correlated with the



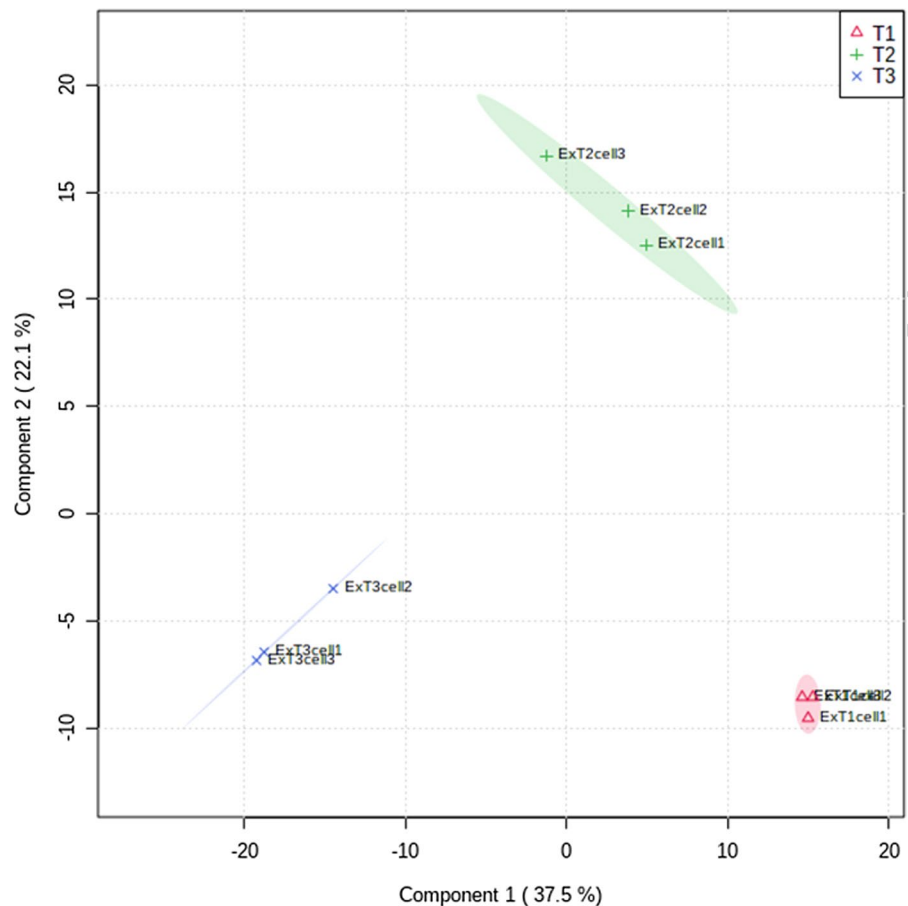
**Fig. 2** Intra- (plain line, left axis) and extra-cellular (dashed line, right axis) mean concentration of ovatoxin obtained from monocultures of *O. cf. ovata* ( $n=3$ ) harvested after 3, 10 and 17 days of growth

intracellular concentration of ovatoxins, starting from 538  
 0 to  $12.8 \pm 8.8 \mu\text{g L}^{-1}$  at day 17. It is worth noting 539  
 that if the total cellular toxin content increased over 540  
 time until day 17, the maximal concentration of 541  
 toxin per *O. cf. ovata* cell was reached at day 10 with 542  
 $35.2 \pm 7.4 \text{ pg equivalent pltx cell}^{-1}$ . 543

The untargeted approach performed on the same 544  
 exometabolome content from monocultures of *O. cf.* 545  
*ovata* led to 736 features after filtering. A clear separa- 546  
 tion between the different sampling time points is 547  
 displayed by the PLS-DA plot (Fig. 3), mainly on 548  
 the first component (37.5%), highlighting a signifi- 549  
 cant (95% interval of confidence is not overlapping) 550  
 modification of the metabolites produced and exuded 551  
 by *O. cf. ovata* over time. Chemical differences at day 552  
 10 are highlighted by the second component (22.1% 553  
 of the total variance), indicating additional diversity 554  
 of the metabolites biosynthesized and released at the 555  
 end of the exponential phase. The first 18 Variable 556  
 Importance in Projection (VIP) responsible for the 557  
 distribution of the samples on the PLS-DA plot (VIP 558  
 score  $> 1.5$ ) are listed in Table 2. The identification of 559  
 the metabolites was very limited, but some proposi- 560  
 tions can be made. 561

Only VIP16 was identified as 3-Amino-4-methyl- 562  
 hexanoic acid by comparing its fragments with the 563  
 MassBank data. Due to a poor fragmentation, the 564  
 annotation of the other VIP was only putative. The 565  
 VIP1 was a penta-charged ion at  $m/z$  1010.61859 566  
 eluting at 5.27 min (Fig. 4), corresponding to a 567  
 $[\text{M}+\text{H}]^+$  mono-charged ion at  $m/z$  5053.0929, with 568  
 a tetra-charged ion detected at  $m/z$  1258.07. The 569

**Fig. 3** PLS-DA plot obtained for the exo-metabolome at three stages of the growth of monocultures of *O. cf. ovata* (T1 = Day 3, T2 = Day 10 and T3 = Day 17)



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

#### 580 Evaluation of the ecotoxicity of the ovatoxins

581 The effect of the ovatoxins on the four competitors  
 582 was tested at three different stages of their growth:  
 583 initial (IN, day 3), exponential (EX, day 10) or sta-  
 584 tionary (ST, day 17) phases. While long-term (24 h)  
 585 exposure to  $1 \mu\text{g mL}^{-1}$  of ovatoxins did not affect the  
 586 PSII of any competitors (Fig. 5), a concentration of  
 587  $10 \mu\text{g mL}^{-1}$  (or approximately  $3.77 \mu\text{M}$ ) did inhibit

the PSII of both diatoms (*L. paradoxa* and *N. are-*  
*naria*) and one dinoflagellate (*P. lima*). The sensitiv-  
 ity 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 maxi-  
 mum quantum yield of the PSII ( $F_v/F_m$ ) after short-  
 term exposure (5 min) to  $10 \mu\text{g mL}^{-1}$  of ovatoxins  
 (Fig. 6A). However, if the same sample was then illu-  
 minated, steady state of photosynthesis was signifi-  
 cantly affected. A decrease by  $48 \pm 25\%$  of the quan-  
 tum yield of the PSII ( $\Phi_{\text{PSII}}$ ) 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<sup>2</sup> fragments

VIP no.	m/z	RT	Formula	Err (ppm)	Score	Fragments	Annotation
1	1010.6185	4.98	C <sub>258</sub> H <sub>446</sub> N <sub>6</sub> O <sub>104</sub>	nd	nd	–	Ovatoxin-a dimer*
2	532.2806	5.04	C <sub>29</sub> H <sub>35</sub> N <sub>6</sub> O <sub>4</sub>	– 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 <sub>31</sub> H <sub>31</sub> N <sub>6</sub>	– 0.37	76.78	87.04; 89.06; 103.04	nd
4	577.2299	4.72	C <sub>20</sub> H <sub>32</sub> N <sub>4</sub> O <sub>8</sub>	0.28	68.74	139.08; 267.13; 447.17; 577.24	nd
5	439.26	4.62	C <sub>18</sub> H <sub>36</sub> NO <sub>11</sub>	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 <sub>29</sub> H <sub>34</sub> N <sub>3</sub> O <sub>7</sub>	0.08	88.64	479.26	nd
9	200.0788	1.83	C <sub>8</sub> H <sub>11</sub> N <sub>2</sub> O <sub>4</sub>	1.78	47.01	–	C6-homoserine lactone*
10	576.3183	5.28	C <sub>32</sub> H <sub>41</sub> N <sub>5</sub> O <sub>5</sub>	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 <sub>7</sub> H <sub>15</sub> O <sub>2</sub> S	– 8.42	80.67	66.02	L-ethionine*
13	463.0986	4.90	C <sub>20</sub> H <sub>18</sub> N <sub>2</sub> O <sub>11</sub>	0.11	82.55	–	Chrysoeriol-7-O-glucoside*
14	246.1446	7.40	C <sub>10</sub> H <sub>19</sub> N <sub>3</sub> O <sub>4</sub>	2.76	78.30	–	Leu-Gly-Gly*
15	620.2859	5.47	C <sub>21</sub> H <sub>43</sub> N <sub>6</sub> O <sub>15</sub>	0.47	95.47	73.06; 87.04; 89.05; 103.3; 113.05; 133.07	nd
16	146.1226	4.61	C <sub>7</sub> H <sub>15</sub> NO <sub>2</sub>	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  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 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  $36 \pm 12\%$  (Fig. 6C).

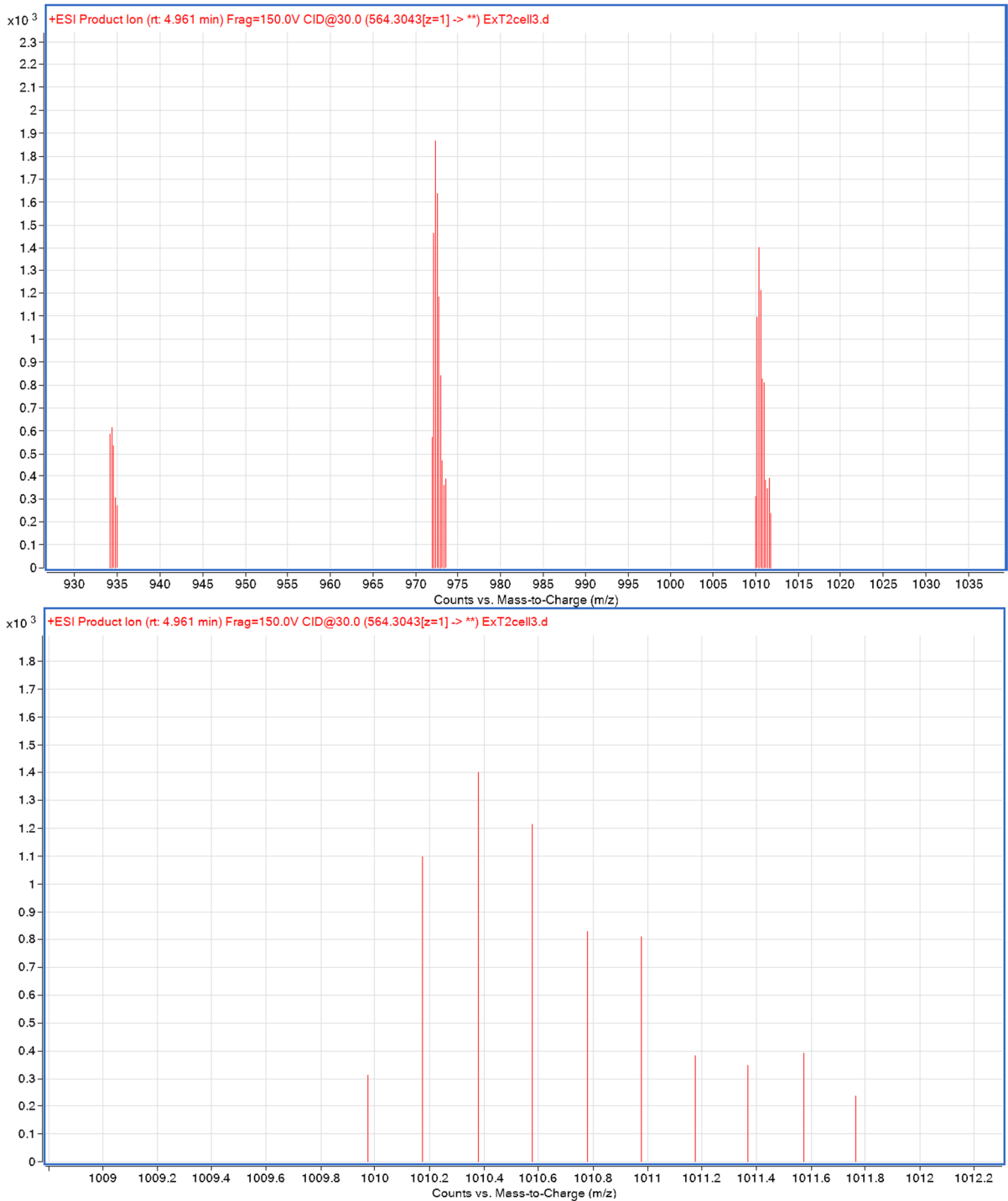
## Discussion

The interactions observed in the present study are not driven by nutrient limitation given the  $\text{NO}_3^-$  concentrations at the end of the experiment ( $> 2 \mu\text{M}$ ), 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

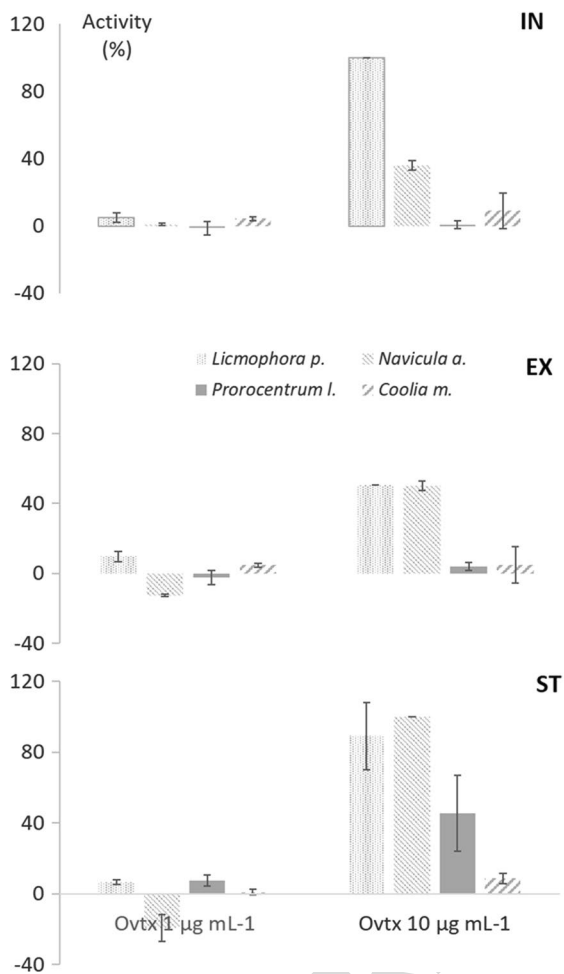
The effects of *O. cf. ovata* cells on the benthic diatoms *N. arenaria* and *L. paradoxa* or the dinoflagellates *P. lima* and *C. monotis* were not as dramatic as those reported for toxic species of the genera

*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.



**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  $\mu\text{g mL}^{-1}$  and an exposure time of 24 h. The sensitivity of the competitors was tested at three stages of growth (IN, EX and ST)

651 Dead competitors (particularly *L. paradoxa*) were  
 652 only observed when the cells were embedded in the  
 653 mucus produced by *O. cf. ovata*, confirming the central  
 654 role played by contact interactions promoted by  
 655 the dense mucus (Ternon et al. 2018; Giussani et al.  
 656 2015). By trapping competitors or concentrating  
 657 allelochemicals, the mucus may form a toxic phycosphere.  
 658 And indeed, competitors' motility is believed  
 659 to influence the effects of *O. cf. ovata* on their cellular  
 660 growth. The strains of *L. paradoxa* and *P. lima* were  
 661 the less motile and the most sensitive to the presence  
 662 of *O. cf. ovata* in co-cultures. No impact on the very  
 663 motile *C. monotis* and a contrasted impact on the

664 moderately motile *N. arenaria* were noted. Motility  
 665 enables microalgae to navigate across environments  
 666 as shown for benthic diatoms in sediments (Con-  
 667 salvey et al. 2004) or when attracted by pheromones  
 668 (Gillard et al. 2013; Bondoc et al. 2019) and could  
 669 allow them to escape the toxic phycospheres such as  
 670 *O. cf. ovata*'s mucus.

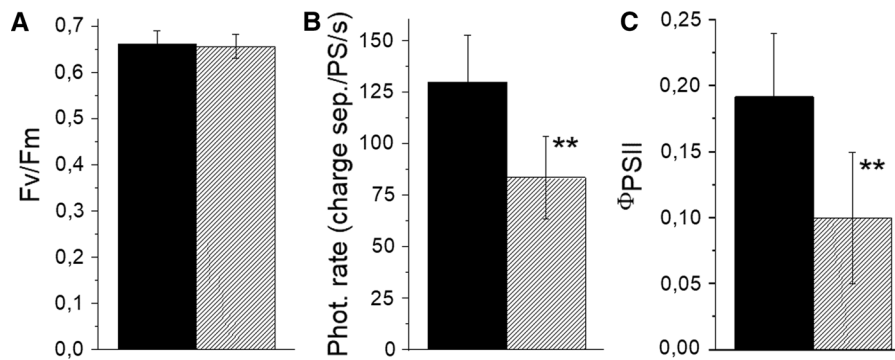
#### Effects of the ovatoxins

671  
 672 The toxicity of the ovatoxins varies with the tested  
 673 species, and also with the growth phase of the micro-  
 674 algae (IN, EX and ST), highlighting species-specific  
 675 response. Cell lysis of *L. paradoxa* and *N. arenaria*  
 676 occurred when exposed to 10  $\mu\text{g mL}^{-1}$  of ovatoxins,  
 677 confirming the deleterious effects of ovatoxins on two  
 678 diatoms largely distributed in the same ecological  
 679 niche. Yet, the maximal free extra-cellular concentra-  
 680 tion of ovatoxins was only  $12.8 \pm 8.8 \text{ ng mL}^{-1}$  at  
 681 day 17. Whether competitive interactions have been  
 682 shown to stimulate the production and the release of  
 683 ovatoxins by a factor 2 (Ternon et al. 2018), the active  
 684 concentrations of the ovatoxins would still be lower  
 685 by a factor thousand than the active concentration of  
 686 10  $\mu\text{g mL}^{-1}$ .

687 Other dinoflagellates are known to release toxins  
 688 in cell-to-cell contact during micro-predation interac-  
 689 tions, leading to prey cell lysis (e.g. *Karlodinium* spe-  
 690 cies) (Place et al. 2012). In co-cultures, cell lysis was  
 691 mostly observed within the mucus, suggesting that  
 692 deleterious concentrations of ovatoxins may only be  
 693 reached in this phycosphere.

694 Besides, their amphiphilic character confers to the  
 695 ovatoxins surfactant-type properties that may lead to  
 696 potential dimerization (as proposed in the untargeted  
 697 metabolomic study) or binding to the mucus as sug-  
 698 gested by Giussani et al. (2015). Therefore, the ova-  
 699 toxin pool embedded into the mucus may result from  
 700 a combination of dimeric ovatoxins and mucus-inter-  
 701 acting ovatoxins.

702 It is difficult to conclude on the allelochemicals  
 703 responsible for the observed weak inhibition of com-  
 704 petitors' growth in the co-cultures. Altogether, the  
 705 data and observations suggest a central role played  
 706 by the mucus, by either accumulating the ovatoxins  
 707 or other unknown allelochemical, to toxic concentra-  
 708 tions, or by trapping competitors. Nevertheless, the  
 709 absence of sensitivity of *P. lima* to the ovatoxins at  
 710 most growth stages indicates that other metabolites



**Fig. 6** Photosynthetic response of *Licmophora paradoxa* to short-term exposures to  $10 \mu\text{g mL}^{-1}$  of ovatoxins: **A** Maximum quantum yield of PSII ( $F_v/F_m$ ) in dark-adapted cells, **B** quantum yield of PSII ( $\Phi\text{PSII}$ ) under  $800 \mu\text{E m}^{-2} \text{s}^{-1}$  in light illumination, **C** photochemical rate under  $800 \mu\text{E m}^{-2} \text{s}^{-1}$  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  $\pm$  S.D of four independent biological samples is shown. Paired sample *t* test was used for statistical analysis (\*\* $p < 0.05$ )

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

#### 720 Mode of action of the toxins and other 721 allelochemicals

722 Although growth inhibition was observed for three  
723 of the four species studied when exposed to *O. cf.*  
724 *ovata*, this does not evidence an induction of cell  
725 lysis as strong as the one recorded for other genera  
726 like *Alexandrium* (Tillmann and John 2002; Tillmann  
727 and Hansen 2009). Other types of interactions might  
728 be involved and cell membranes can be disrupted by  
729 dinoflagellate allelochemicals, even though cell lysis  
730 does not occur [*K. brevis* (Prince et al. 2008)]. In  
731 the present study, no measurement of the membrane  
732 permeability was performed, but an inhibition of the  
733 PSII efficiency was detected for the two diatoms  
734 *L. paradoxa* and *N. arenaria* and for the dinoflagel-  
735 late *P. lima*, in response to ovatoxins. Inhibition of  
736 PSII efficiency is commonly proposed as a mechanism  
737 for allelopathy (Prince et al. 2008; Tilney et al.  
738 2014; Long et al. 2018b). However, the results of the

additional experiment performed with *L. paradoxa* 739  
suggest that the decrease in photosynthetic activ- 740  
ity stems from a rapid inhibition of an uncharacter- 741  
ized photosynthetic step downstream PSII. There- 742  
fore, inhibition of PSII is rather a consequence of the 743  
stress cascade induced by allelopathy. Recent pieces 744  
of evidence of disruption of the membrane polarity, 745  
permeability and associated lipids in phytoplankton 746  
by dinoflagellate allelochemicals (Poulin et al. 2018; 747  
Long et al. 2021) are indeed supporting the hypothe- 748  
sis of a symptomatic maximum quantum yield inhibi- 749  
tion. Therefore, the lack of inhibition of the PSII effi- 750  
ciency in the dinoflagellate *P. lima* may not indicate 751  
that cells are not stressed when exposed to ovatoxins, 752  
but this species may rather present other undeter- 753  
mined symptoms. 754

#### 755 Implication for natural blooms

756 The results obtained from the co-cultures are mir- 757  
roring observations of the natural microalgae con- 758  
sortium made during *O. cf. ovata* blooms. Field sur- 759  
veys conducted over *O. cf. ovata* blooms reported 760  
unchanged abundances of the dinoflagellate *C.*  
761 *monotis* (Marro et al. 2019; Ninčević Gladan et al.  
762 2019), a decrease in the dinoflagellate *P. lima*  
763 (Marro et al. 2019; Vila et al. 2001) and in diatoms  
764 abundance (Accoroni et al. 2016; Marro et al.  
765 2019), particularly the non-motile ones [i.e. *Lic-*  
766 *mophora paradoxa*; (Accoroni et al. 2016)]. Thus,

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

771 The extent of this control must, however, be miti-  
772 gated by the retro-control that can be applied by  
773 the competitors on *O. cf. ovata* abundance. Indeed,  
774 despite a mixotrophic character (Jauzein et al.  
775 2017), *O. cf. ovata* does not benefit from being co-  
776 cultured with its competitors. Conversely, a decrease  
777 in cell concentration is observed in the presence of  
778 *C. monotis*, *N. arenaria* and *P. lima*. This inhibition  
779 is in agreement with previous observations with *C.*  
780 *monotis* (García-Portela et al. 2016), even though  
781 no morphological changes or encystment in *O. cf.*  
782 *ovata* was noticed by the authors. A control on *O.*  
783 *cf. ovata* growth by its competitors could also be  
784 mediated by chemicals. Both dinoflagellates and  
785 diatoms are known to produce a large array of small  
786 molecules that can act as allelochemicals (Pohnert  
787 2005; Gillard et al. 2013; Long et al. 2018a; Ma  
788 et al. 2011), and some of them have been shown to  
789 inhibit the growth of dinoflagellates (Wang et al.  
790 2017a; Tillmann and John 2002; Hakanen et al.  
791 2014; Ternon et al. 2018; Xu et al. 2015, 2019).

792 The chemical impact of *O. cf. ovata* on ecologi-  
793 cal succession may also depend on several factors  
794 such as the intensity and the duration of the blooms  
795 (i.e. variations in biosynthesized chemicals, includ-  
796 ing the toxins) and the competitor species (adaptive  
797 ecophysiological traits like motility). Moreover,  
798 other parameters could influence the outcome of  
799 this control in natural ecosystems, such as the avail-  
800 ability in nutrients that can modulate allelopathic  
801 interactions (Grover and Wang 2013), the intra-  
802 specific variability of the individuals (Meyer and  
803 Pohnert 2019) and eventually the considerable com-  
804 plexity of natural assemblages that involve several  
805 parties (Bigalke and Pohnert 2019).

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