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1 Efficient, fast and inexpensive bioassay to monitor benthic 2 microalgae toxicity: Application to *Ostreopsis* species

3 Anne-Sophie Pavaux^{1*}, Eva Ternon², Louison Dufour¹, Sophie Marro¹, Marin-Pierre Gémin³,
4 Olivier P. Thomas⁴ and Rodolphe Lemée¹

5 ¹ Sorbonne Université, CNRS, Laboratoire d'Océanographie de Villefranche, LOV, F-06230,
6 Villefranche-sur-Mer, France ;

7 ² Université Côte d'Azur, CNRS, OCA, IRD, Géoazur, 250 rue Albert Einstein, 06560 Valbonne,
8 France ;

9 ³ IFREMER - Laboratoire Phycotoxines (PHYC), Rue de l'Île d'Yeu, BP 21105, F- 44311 Nantes,
10 France ;

11 ⁴ Marine Biodiscovery, School of Chemistry and Ryan Institute, National University of Ireland
12 Galway, University Road, H91 TK33 Galway, Ireland ;

13 * Correspondence: annesophie.pavaux@gmail.com;

14
15 **Abstract:** Even though HPLC-MS is commonly used to quantify the toxin content of *Ostreopsis*
16 spp. cells, there is a need to develop easy-to-use toxicological tests to set thresholds during
17 *Ostreopsis* spp. blooms. The crustacean *Artemia* has been widely used to evaluate the presence
18 and toxicity of chemicals and biological contaminants and we anticipated that it could also be
19 useful to test *Ostreopsis* spp. toxicity. Its relevance was first assessed by investigating the
20 variability of the toxic effects among *Ostreopsis* spp. strains and throughout the dinoflagellate life
21 cycle in combination with chemical analyses of the toxic content by UHPLC-HRMS. After testing
22 the toxicity of fractions prepared from *Ostreopsis* spp. cells, the known ova- and paly-toxins were
23 not the only toxic metabolites to *Artemia franciscana*, indicating that other toxic compounds
24 synthesized by *Ostreopsis* spp. still remain to be identified. To extend the bioassay to *in situ*
25 monitoring, the toxicity of the benthic microalgal consortium was tested during a natural bloom
26 of *Ostreopsis* cf. *ovata* in the NW Mediterranean Sea. The results highlight the accuracy and
27 sensitivity of the ecotoxicological assay with *Artemia franciscana* to assess the toxicity of
28 *Ostreopsis* spp. blooms.

29
30 **Keywords:** *Ostreopsis* spp. ; *Artemia franciscana* ; bioassay ; ecotoxicity ; HPLC-HRMS

31
32
33 **1. Introduction**

34 *Ostreopsis* spp. blooms become more intense in temperate areas and regularly occur in the
35 Mediterranean sea during summer (Accoroni et al., 2011; Accoroni and Totti, 2016; Battocchi et
36 al., 2010; Mangialajo et al., 2011, 2008; Totti et al., 2010; Vila et al., 2001). The species *Ostreopsis*
37 cf. *ovata* is well known to cause adverse effects on human health (Durando et al., 2007; Gallitelli et
38 al., 2005; Kermarec et al., 2008; Tichadou et al., 2010; Vila et al., 2016) associated with some
39 hospitalization of hundreds of affected patients, like in Genova in 2005 (Brescianini et al., 2006)
40 and in Algeria in 2009 (Illoul et al., 2012). Toxic effects on vertebrates and invertebrates have also
41 been documented (Accoroni et al., 2016b; Blanfune et al., 2012; Faimali et al., 2012; Prato et al.,
42 2011; Privitera et al., 2012): reprotoxicity on benthic copepods (Guidi-Guilvard et al., 2012; Pavaux
43 et al., 2019) and sea urchins (Migliaccio et al., 2016; Pagliara and Caroppo, 2012, Privitera et al.,
44 2012), as well as harmful effects on the polychaete *Dinophylus gyrociliatus* (Simonini et al., 2011)
45 and the jellyfish *Aurelia* sp. (Giussani et al., 2016 ;). The toxicity of *Ostreopsis* cf. *ovata* has been
46 attributed to the production of palytoxin (PLTX) and its analogs named ovatoxins (OVTX-a, -b, -c, -
47 d, -e, -f, -g (Ciminiello et al., 2012a, 2012b, 2010)), known to be strong Na⁺/K⁺ ATPase pump
48 inhibitors (Poli et al., 2018; Rouzair-Dubois and Dubois, 1990).

49 Due to these sanitary and ecological effects, the monitoring of *Ostreopsis* cf. *ovata* toxicity
50 has been the focus of most research the past decades (Battocchi et al., 2010; Casabianca et al.,
51 2014, 2013). Analytical chemistry (HPLC-MS) constitutes the most widespread approach to
52 monitor OVTX concentrations in the ecosystem , despite its various constrains: (1) cutting edge
53 facilities (HPLC-HRMS) and skills are needed; (2) relatively high quantification limit (2.5 µg
54 PLTX/mL) (3) involve time- and solvent-consuming analysis. Therefore, there is an urgent need to
55 develop an efficient (i.e rapid, simple, inexpensive and more sensitive than HPLC-MS) bioassay to
56 quickly assess the toxicity of *Ostreopsis* species and strains, and further improve the early
57 detection of toxic events in concerned areas.

58 In the middle of the 20th century , the brine shrimp *Artemia* sp. was proposed as a bio-
59 indicator for chemical contamination (Michael et al., 1956; Vanhaecke et al., 1981) owing to its
60 sensitivity to sodium lauryl sulfate and various insecticides. Up to now *Artemia* sp. has been widely
61 used to test fungal toxins (Harwig and Scott, 1971), plant extract toxicity (Meyer et al., 1982),
62 heavy metals (Martínez et al., 1999; Sarabia et al., 2008), cyanobacteria toxins (Jaki et al., 1999;
63 Tokodi et al., 2018) and pesticides (Comeche et al., 2017; Gambardella et al., 2018; Jawahar et al.,
64 2018). *Artemia* sp. was also shown to be useful for marine natural products screening as shown by
65 the work of Carballo et al. (Carballo et al., 2002) who compared the results of this bioassay with
66 the cytotoxicity of the specialized metabolites produced by 20 marine organisms (mostly
67 invertebrates) against 2 human cell lines (lung carcinoma A-549 et colon carcinoma HT29).

68 Therefore, *Artemia franciscana* bioassay may constitute an excellent way to quickly assess the
69 toxicity of *Ostreopsis* spp. blooms.

70 A protocol investigating the variability of the toxicity between *Ostreopsis* species and strains
71 and throughout their life cycles was first set up using living cells of two Mediterranean species (*O.*
72 *cf. ovata* and *O. fattorusoi*). Then, the compounds responsible for the toxicity of *Ostreopsis* spp.
73 were investigated using both chemical extracts of *O. cf. ovata* cells coming from a culture and pure
74 PLTX and OVTX. Finally, the implemented bioassay was successfully used to monitor the toxicity of
75 a natural bloom of *O. cf. ovata* in 2018 in N.W. Mediterranean Sea (South of France).

76

77 **2. Materials and methods**

78 2.1. Bioassays

79 Four experiments were performed and referred as Experiment 1 to 4 hereinafter. Experiment
80 1 investigated the variability of the toxicity on *A. franciscana* among species and strains of
81 *Ostreopsis* at a given stage of growth, using two French *O. cf. ovata* and two Lebanese *O.*
82 *fattorusoi* strains; Experiment 2 assessed the toxicity on *A. franciscana* throughout the growth of
83 an *O. cf. ovata* culture; Experiment 3 investigated the compound responsible for the toxicity using
84 cell extracts of *O. cf. ovata* and pure OVTX and PLTX; Experiment 4 evaluated the use of *A.*
85 *franciscana* as a tool for biomonitoring the toxicity of a natural bloom in the N.W. Mediterranean
86 Sea (Villefranche; South of France).

87

88 2.1.1. Micro-algal culture

89 The four strains used in this study were obtained from the Mediterranean Culture Collection
90 of Villefranche (MCCV, <http://www.embrc-france.fr>). *Ostreopsis cf. ovata* (MCCV 054 and MCCV
91 055) were collected at the Rochambeau site in 2014 (Bay of Villefranche-sur-Mer, N-W
92 Mediterranean, 43°41'35.64''N-7°18'31.54''E) and *Ostreopsis fattorusoi* (MCCV 057 and MCCV
93 058) were isolated in 2015 from Bat 14 site (Lebanon, Batroun, 34°15'9.0''N-35°39'41.3''E) and
94 were respectively grown in L1 and L1/2 medium (Guillard and Hargraves, 1993) prepared with
95 autoclaved aged and filtered seawater, adjusted to a salinity of 38. Cultures were maintained at
96 24°C, under a 14:10 light/dark cycle with a light intensity of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

97

98 2.1.2. Molecular Taxonomic Identification of *Ostreopsis* species by a PCR Based Assay

99 A volume of 5 mL of each MCCV clonal culture was centrifuged for 6 min at 2000 rpm and 4°C.
100 The supernatant was removed and the cell pellet was resuspended with 1 mL of sterile water
101 (autoclaved MilliQ) and centrifuged again for 2 min to remove salt and media. The cell pellet was
102 kept and homogenized with 50 μL of sterile water and a fraction of 10 μL was further transferred

103 to 0.2 μ L polymerase chain reaction (PCR) tubes. Then, PCR tubes were stored at -20°C until
104 further analysis. Approximately 300 base pairs of the internal transcribed spacer region (ITS1-
105 5.8s-ITS2) rDNA were amplified by PCR using the primer 329-F (5'-GTGAACCTGCRGAAGGATCA-3')
106 which is the inverse complementary sequence of the universal eukaryote primer 329-R (Moon-van
107 der Staay et al., 2001) and the designed primer D1R-R (5'-TATGCTTAAAATTCAGCAGGT-3') which is
108 the inverse complementary sequence of the primer DIR-F (Scholin et al., 1994). A mix of 1 μ L of
109 each primer, 1 μ L of dNTP at 10 mM, 5 μ L of 10X Advantage 2 PCR Buffer, 31 μ L of sterile water and
110 1 μ L of 50X Advantage 2 Taq polymerase (Clontech) was added in each PCR tube containing the cell
111 pellet. The PCR was performed using a MasterGradient thermocycler (Eppendorf) with the
112 following conditions: one initial denaturation at 94°C for 10 min followed by 35 cycles each
113 consisting of 1 min at 94°C , 1 min at 53°C , 1 min at 68°C and a final elongation for 10 min at 68°C .
114 The PCR products were purified using QIAGEN MinElute PCR Purification kit according to the
115 recommendations. After sequencing by Genewiz, sequences were treated using BioEdit software
116 and compared to NCBI database using BLASTn tool to ensure the taxonomy of the strains.

117

118 2.1.3. Biomass production of *Ostreopsis*

119 • Cultures

120 All strains used in Experiment 1, 2 and 3 were grown in triplicates, in 150-mL or 300-mL flasks,
121 at an initial cell concentration of $1\ 000\ \text{cell}\cdot\text{mL}^{-1}$. A volume of 12 mL was sampled and fixed with
122 acidic lugol (4% v/v) for cell counting at day 10 in Experiment 1 (150 mL-flasks) and every other day
123 during 14 days in Experiment 2 (300 mL flasks). Cell counting was performed in triplicates using a
124 liquid particle counter (©HIAC/Royco 9703, Pacific Scientific Instruments) following a size range of
125 2-80 μm (Stramski et al., 2002). Aliquots of living cells from each flasks were subsequently diluted
126 using L1 medium to a final volume of 20 mL and a cell concentration of $20\ 000\ \text{cell}\cdot\text{mL}^{-1}$ to perform
127 *Artemia* bioassays.

128

129 • *In situ*

130 Living cells used in Experiment 4 were sampled on artificial substrates (Jauzein et al., 2016)
131 during summer 2018 (27th of June to 23rd of August) to cover the bloom of *Ostreopsis cf. ovata* .
132 Three screens composed by a rigid frame with a 1 mm mesh were placed in the morning at a
133 depth of 50 cm at 3 sampling sites, separated from each other by few meters, in Rochambeau (Bay
134 of Villefranche). After 24 hours, the screens were carefully collected in 1 L plastic bottles
135 containing seawater and gently homogenized in order to resuspend microalgae. For each screen, a
136 volume of 20 mL was subsampled in triplicates fixed with acidified lugol (4% v/v) and observed
137 under microscope using Sedgewick Rafter counting chamber to measure both the abundance and

138 the diversity of microalgae. After counting, each sample obtained from the screens was diluted
139 with the appropriate culture medium to reach a final concentration of 20,000 cells.mL⁻¹ prior to
140 the *Artemia* bioassays. It is noteworthy that these samples are natural samples and therefore
141 contain a broad benthic micro-algal assemblage, including *Ostreopsis* cells. Nevertheless, during
142 the peak of the bloom, the proportion of diatoms represented less than 25 % of the total number
143 of cells tested (e.g 2.7 to 17% for *Cocconeis* sp., 6.9 to 25% for *Navicula* sp., 0.5 to 18% for
144 *Gyrosigma* sp., and 20% for *Bleakeleya* sp.) and any biological effect held by those species was
145 considered negligible in regards to *O. cf. ovata*.

146

147 2.1.4. Obtention of *Ostreopsis* extracts, fractions and pure ovatoxins

148 A volume of 1.4 L of a culture of *Ostreopsis cf. ovata* (MCCV 054) of 10 days of age was
149 centrifuged at 600 g at 21°C for 10 min. The supernatant was discarded and the cell pellet flash
150 frozen using liquid nitrogen to stop the cells metabolism, and further stored at -20°C before being
151 freeze dried. An amount of 85 mg of dried cells was extracted using 4 mL of a mixture MeOH:H₂O
152 (80:20) in an ultrasonic bath (Elma Transonic T420) for 10 min. The extract was centrifuged at
153 1,000 g during 10 min and the supernatant was collected and transferred into a glass vial. The
154 procedure was repeated two more times to obtain 12 mL of extract. The extract was then
155 evaporated under a gentle flow of N₂ at 35°C until full evaporation of the MeOH (leaving about 2-3
156 ml of water). The aqueous extract was deposited on a C18 SPE cartridge (500 mg, Supelco)
157 previously washed with MeOH (6 mL) and equilibrated with MilliQ-water (6 mL). Before elution,
158 the cartridge was rinsed with 6 mL of MilliQ-water. A step gradient elution was thus applied to the
159 cartridge, from H₂O:MeOH (100:0) to H₂O:MeOH (0:100), using steps of 20% and volumes of 6 mL.
160 An additional elution with 6 mL of acetonitrile (ACN 100%) was performed, yielding seven fractions
161 (F1 to F7, from the most polar to the non-polar metabolites). Aliquots of 100 µL sampled from
162 each fraction were evaporated to dryness under a N₂ stream (35°C) to assess their respective
163 weight. A volume of 1 mL of dimethylsulfoxide (DMSO) was added to the remaining fractions
164 before evaporation of the volatile organic solvents (MeOH, ACN) and water. Based on the fractions
165 weight, the volume for dilution were further adjusted using DMSO to reach a concentration of 3
166 mg.mL⁻¹. All fractions were stored at -20°C until further bioassays and chemical analyses.

167 A purified pool of ovatoxins was obtained following a protocol previously published (Brissard
168 et al., 2015). The cell pellet (75 g of *O. cf. ovata* - MCCV 054 - after 21 days of growth) and
169 fractionated by size-exclusion chromatography (Sephadex LH20). Methanolic fractions containing
170 OVTXs (assessed by HPLC-MS) were pooled, concentrated and purified by semi-preparative
171 chromatography (Uptisphere C18-TF, 250 mm × 4.6 mm, 5 µm; λ= 233 and 266 nm). A pool of
172 OVTXs was used as the separation of the analogues was not straightforward. The final pool of

173 OVTX was concentrated in DMSO and was proven to contain a mixture of OVTX-a (61 %), -b (25 %),
174 -c (3.4 %), -d (5.3 %), -e (3.9 %) and -f (1.1 %) at 7.8 mg/mL, based on LC-MS/MS quantification
175 (Gémin et al., 2020). Pure palytoxin was purchased from ©Wako Chemicals, GmbH, Neuss,
176 Germany. All extracts/toxins were solubilized in DMSO at a concentration of 12 µg/mL that would
177 allow an addition of 5 µl for the *Artemia* bioassay without exceeding 0.1% of DMSO in final volume
178 of seawater.

179

180 2.1.5. Experimental bioassays

181 A mass of 0.15 g of dehydrated *Artemia franciscana* cysts (© Ocean Nutrition, Belgium) were
182 hatched in 1 L of filtered and aged sea water at a salinity of 38, and a temperature of 20°C under a
183 continuous light of intensity 250 µmol.m⁻².s⁻¹ and a continuous and strong aeration. After 40 hours
184 of incubation, all larvae moulted to metanauplii stage (2-3 stages), considered as the most
185 sensitive stages to be used in ecotoxicological bioassay (Nunes et al., 2006; Sorgeloos et al., 1978).
186 For each experiments (1 to 4), *Artemia* larvae were isolated from empty cysts using a Pasteur
187 Pipette and transferred in 6-well plates (5 larvae per well); each well containing 4 mL of 0.2 µm
188 filtered and aged sea-water adjusted to salinity 38 and kept at a temperature of 24°C (except for
189 Experiments 3 that contained 5 mL of seawater). All tests were then performed using 30
190 metanauplii larvae per condition (5 larvae per well, in a 6-well plate). Experiments 1, 2, 3 and 4
191 were conducted using the four following cellular concentrations: 4, 40, 400 and 4000 cells.mL⁻¹. In
192 addition to these four cellular concentrations, experiment 3 was also performed using four
193 concentrations of ovatoxins and palytoxin (0.012, 0.12, 1.2 and 12 µg/mL) corresponding to
194 theoretical concentrations that would be reached with 0.4, 4, 40 and 400 cells.mL⁻¹ of *Ostreopsis*
195 cells (Brissard et al., 2014; Nascimento et al., 2012; Pistocchi et al., 2011). Also, all seven chemical
196 fractions F1-F7 obtained from cell pellet were added in the wells at a final concentration of 10
197 µg/ml.

198 *Artemia franciscana* larvae's mortality was measured every hour during 12 hours, then at 24
199 and 48 h exposure times (only at 24 h for chemical fraction, Experiment 3). Larvae were
200 considered dead when there was no movement during 10 seconds and no reaction to strong light.
201 A negative control was also performed for all experiments, using seawater + fresh L1 medium, 80:
202 20 (v:v) for Experiments 1, 2, 3 and 4 as well as 0.1% DMSO (final volume) for Experiments 3.
203 Bioassays are considered consistent when mortalities rate in the control condition are lower than
204 10% as described in Artoxkit (1990).

205

206 2.2. Toxin profiles

207 2.2.1. Cell extraction

208 To assess the variability of the toxin content (i) among strains and species, and (ii) throughout
209 the growth phase, chemical extractions of MCCV 054, 055, 057 and 058 were performed. For inter-
210 strain variability, each strain was inoculated with 1 000 cell.mL⁻¹ in triplicate 50-mL flasks and
211 grown during 10 days before harvesting the whole culture. For growth-dependent variability, the
212 MCCV 055 was inoculated at 1 000 cell.mL⁻¹ in twenty-one 300-mL flasks and grown for fourteen
213 days. Every other day, three flasks were sacrificed for cell abundance (subsample of 1.2 mL) and
214 chemical extraction (about 300 mL). Samples for cell abundance were fixed and counted as
215 previously described.

216 All flasks content (50 mL and 300 mL) were transferred to 50 mL Falcon tubes before being
217 centrifuged at 600 g and ambient temperature, for 10 min. After removal of the supernatant, the
218 cell pellets were flash frozen using liquid nitrogen and further stored at -20°C. A volume of 4 mL of
219 MeOH: H₂O (80:20, v/v) was poured in the falcon tubes before placing them in ultra-sonic bath for
220 10 min cooled in an ice batch. The falcon tubes were then centrifuged again at 2,500 g for 10 min
221 at ambient temperature and the supernatants were transferred in 20 mL vials.

222 These steps (organic extraction, ultra-sonic bath and centrifugation) were repeated two more
223 times and the resulting extracts were evaporated under a gentle stream of N₂ until full
224 evaporation of the MeOH. To avoid dryness of the samples, 500 µL of DMSO was then added to
225 the extracts and the remaining H₂O further evaporated. All extracts were stored at -20°C until
226 UHPLC-HRMS analysis.

227

228 2.2.2. UHPLC-HRMS

229 UHPLC-DAD-HRMS experiments were performed using an Agilent 1290 system (Agilent
230 Technologies, USA) equipped with a diode array detector and coupled to an Agilent 6540 Qtof
231 mass spectrometer (Agilent Technologies, USA). Mass analyses were carried out in positive mode
232 (ESI +) between 60 to 3000 Da. A volume of 10 µL of each samples were injected and separated on
233 a T3 column (Acquity UPLC HSS T3 1.8µm, 2.1mm x 100mm, Waters) using a linear elution gradient
234 of H₂O/MeOH (80:20, v:v) and MeOH containing 0.1mM of ammonium formate and 0.1% formic
235 acid from 90:10 (v:v, isocratic from 0 to 2mins) to 0:100 (v:v, isocratic from 12 to 13 mins) at a 0.4
236 mL.min⁻¹ flow rate. Collision energies (CE) of 30, 70 and 110 eV were applied to confirm the
237 presence of OVTXs. UV detection was set at 210, 233 and 263 nm. The spectrometer analyzer
238 parameters were set as follows: nebulizer sheath gas, N₂ (35 psig); drying gas, N₂ (11 L.min⁻¹); Gas
239 Temperature, 300°C; capillary, 4.129 µA; Vaporizer/Sheat Gas Temp, 350°C.

240 The quantification of the OVTXs in the samples was performed using commercial PLTX as a
241 reference, considering identical ionization of these compounds. A calibration curve for PLTX was

242 set using concentrations between 1 and 10 mg.mL⁻¹, using the tricharged adduct [M+3H-4H₂O]³⁺
243 for quantification. This same adduct was used to quantify the OVTX a in all samples.

244

245 2.3. Statistical analysis

246 The statistical significance of the mortality variation between the different experimental
247 conditions tested was evaluated using Kruskal-Wallis analyses. Dunn's post hoc tests were used *a*
248 *posteriori* to identify which experimental conditions differed from the others when significant
249 differences were showed by the Kruskal-Wallis test (p<0.05). These tests were realized using the
250 PAST software (Hammer et al., 2001). The R package ecotoxicology was used to assess the median
251 lethal concentration (LC₅₀) at 24 and 48 hours of exposition.

252

253 3. Results

254 3.1. Molecular analyses

255 PCR amplification based assay of the strains using genus and species-specific primers
256 confirmed the morphological analysis. The sequences obtained after PCR analysis showed a
257 perfect alignment between the strains MCCV 054/MCCV 055 and a perfect match (100% of
258 confidence) with *Ostreopsis cf. ovata* (E-value: 2e-157, accession number: MG551865.1). Similarly,
259 the strains MCCV 057/MCCV 058 were identified as *Ostreopsis fattorussoi* (level of confidence
260 100%, E-value: 1e-129, accession number: LT220222.1 ; Accoroni et al., 2016a). These sequences
261 were deposited in GenBank to the accession number: MT270691, MT270692, MT271779 and
262 MT271780 for the strains MCCV 054, MCCV 055, MCCV 057 and MCCV 058 respectively.

263

264 3.2. Inter-strain variability of the toxicity - Experiment 1

265 Mortality rates in controls were lower than 10% throughout the experiment, meeting the
266 acceptability threshold of this bioassay as defined by the Artoxkit (1990). [A final concentration of
267 0.1% of DMSO used in the bioassays did not significantly influence the outcome of the
268 experiments, since not exceeding the lethal concentration of 1.25% determined by Thong et al.,
269 2013.](#) Mortality was significantly higher (p-value <0.05) in presence of all *Ostreopsis* cells
270 suggesting that all strains were toxic to *Artemia franciscana* larvae, whilst slight variability owed to
271 the time of exposure, the cell concentration and the strain used were found (Figure 1).

272 A positive correlation between mortality and exposure time (p < 0.001) was observed, and 12
273 hours of exposure was sufficient to induce significant mortality of *A. franciscana* (p < 0.05).
274 Mortality reached its maximum after 48 hours (96,6%) for all strains and concentrations tested.

275 Several concentrations of *Ostreopsis* spp. cells were tested to explore their effects on *A.*
276 *franciscana*. Despite inter-strains variability, a dose-dependent response was observed with a

277 strong increase of mortality until a plateau of 96% of mortality was reached at 400 cells.mL⁻¹ at all
 278 exposure time and for most strains. Inter-strains variability was mostly observed for low exposure
 279 times (12 and 24 hours) and low cell concentrations (4 to 40 cell.mL⁻¹). Using a concentration of
 280 *Ostreopsis* spp. cells of 4 cell.mL⁻¹, the toxicity of the strains could be ranked as follows: MCCV
 281 054<055=058<057 (Cf. LC_{50-24h} values – Table 1). By increasing the concentration of *Ostreopsis* spp.
 282 cells, this distribution is smoothed and no significant differences are observed between strains for
 283 400 and 4 000 cell.mL⁻¹ (except for MCCV 058 that shows the most toxic behaviour, 96,6 %). The
 284 differences in the LC_{50-24h} observed between strains of a same species is somehow important and
 285 may be attributed to the natural variability of microalgae metabolism (Meyer and Pohnert, 2019),
 286 and in particular the production of toxic metabolites (Tartaglione et al., 2017).

287 Interestingly, the toxin content determined by UHPLC-HRMS did not support the toxicity
 288 pattern highlighted by the *Artemia* bioassay since no ovatoxins were found in neither MCCV 057
 289 nor 058. On the other hand, an amount of 18.0 (± 4.9) and 23.7 (± 9.2) pg PLTX eq/cell were
 290 measured for MCCV 054 and MCCV 055, respectively, therefore matching the level of toxicity
 291 evidenced in the *Artemia* bioassay.

292

293 **Table 1** : LC₅₀ values and confidence limits obtained exposing *Artemia franciscana* larvae
 294 to different strains of *Ostreopsis* sp. and dissolved toxins (n=6). All values are expressed
 295 in cells.mL⁻¹ to facilitate comparison across experimental treatment. A theoretical cell
 296 concentration was calculated for both OVTX and PLTX in link with the cells toxin content
 297 (estimating that one cell of *Ostreopsis* cf. *ovata* contain in average 30 pg.cell⁻¹ – Accoroni
 298 et al., 2011; Accoroni et al., 2012; Brissard et al., 2014; Ciminiello et al., 2010; Ciminiello
 299 et al., 2012; Guerrini et al., 2010; Nascimento et al., 2012; Pistocchi et al., 2011). NaN =
 300 Not a Number.

Treatment	LC _{50-24h} (cells.mL ⁻¹)	LC _{50-48h} (cells.mL ⁻¹)
MCCV 054	81.96 (± 63.33)	NaN
MCCV 055	0.25 (± 0.90)	NaN
MCCV 057	0.0012 (± 0.00033)	NaN
MCCV 058	0.023 (± 0.039)	NaN
OVTX	5.96 (± 2.12)	0.42 (± 0.092)
PLTX	48.42 (± 14.23)	0.42 (± 0.099)
Living cells	9.01 (± 3.30)	1.16 (± 0.40)

301

302 3.3. *Ostreopsis* cf. *ovata* toxicity throughout growth - Experiment 2

303 After inoculation, *Ostreopsis cf. ovata* (MCCV 055) showed a linear growth during nine days,
304 corresponding to an increase of cells concentration by a factor 2, up to 6 621 cells.mL⁻¹ at day 10
305 (growth rate of 0.24 day⁻¹), before declining on day 14 (3 637 cells.mL⁻¹; Figure 2). Meanwhile, the
306 toxin content quickly stabilized at around 10 pg eq. PLTX/cell between day 4 and 10, followed by a
307 decrease towards the end of the experiment (Figure 2). The high concentrations obtained at day 0
308 were likely influenced by the fact that the extracted cells had been sampled in a culture of age 10
309 days.

310 Interestingly, the variability of the toxin content did not reflect the pattern of mortality
311 observed for *Artemia franciscana* (Figure 3 a, b and c). No clear trend emerged from the mortality
312 measured after 12 hours of exposure, although it increased with cell concentrations. Likewise, at
313 high cell concentration of *O. cf. ovata* (40 and 400 cell.mL⁻¹) and for an exposure time of 48 hours,
314 mortality varied little ($p>0.05$) and was close to 100 % during the whole duration of the
315 experiment, precluding drawing any relationship between mortality and the growth phase. Only
316 exposure times of 24 and 48 hours at low concentrations of *O. cf. ovata* cells (4 cell.mL⁻¹) allowed
317 drawing a pattern of mortality for *Artemia* larvae that followed both the cellular growth and the
318 toxin content (Figure 3 a) with a minimum between days 2 and 4 (10 % and 43 % for 24 h and 48 h
319 respectively, 0.40 pg eq. PLTX/cell, 1,436 cells.mL⁻¹) and a maximum reached at day 10 (26 % and
320 83 % for 24 h and 48 h, respectively, 13.1 pg eq. PLTX/cell, 6,389 cells.mL⁻¹).

321

322 3.4. Metabolites toxic for *Artemia* – Experiments 3

323 The nature of the toxic metabolite(s) responsible for the toxicity of *O. cf. ovata* cells on
324 *Artemia* was further investigated comparing the toxic behaviour of (i) living *O. cf. ovata* cells, (ii)
325 chemical fractions obtained from *O. cf. ovata* cells extracts, (iii) purified OVTX, and (iv) commercial
326 PLTX. Interestingly, living cells induced an earlier response than equivalent amount of dissolved
327 PLTX and OVTX (mortality of 46 %, 13 % and 13 %, respectively after 12 h of exposure at a
328 concentration of 400 cells.mL⁻¹), although these differences tend to be reduced with increasing
329 exposure time (Figure 4). After 48 h, the mortality was significantly higher with the dissolved
330 toxins (100 % for both OVTXs and PLTX and 77 % for living cells at a cellular concentration of 4
331 cells.mL⁻¹), as also confirmed by the LC₅₀ values given in Table 1. To test whether the OVTX and
332 PLTX are the only toxic metabolites produced by *O. cf. ovata*, the activity of seven fractions of
333 different polarities obtained from *O. cf. ovata*, was assessed over 24 hours on *Artemia* larvae
334 (Figure 5). No toxicity was found for fractions 1, 2 and 3 whereas all fractions from 4 to 7 were
335 toxic towards the larvae, showing mortalities of 13%, 30%, 33% and 71% for fractions 6, 4, 7 and 5,
336 respectively.

337 The analysis of the chemical content of each fraction by UHPLC-HRMS revealed that
338 only fractions 5 and 6 contained detectable amounts of OVTX (29 and 7.5 μg , respectively),
339 yielding a final concentration in the wells of 305 and 15 ng eq. PLTX.mL⁻¹, respectively. Taken
340 together, these results indicated that *Artemia* larvae are sensitive to amount of OVTX and
341 PLTX as low as 12 ng.mL⁻¹ but also to other compounds produced by *O. cf. ovata* and present
342 in fractions 4 and 7.

343

344 3.5. Application to *in situ* monitoring - Experiment 4

345

346 Since *Artemia franciscana* larvae mortality was shown to be a good proxy for the toxicity of
347 *Ostreopsis* spp. cells from lab cultures, the application of the *Artemia* bioassay to *in situ*
348 monitoring of *Ostreopsis cf. ovata* blooms was further investigated. Cells sampled *in situ* were
349 tested for their toxicity throughout the bloom in July 2018. The bloom of *Ostreopsis cf. ovata*
350 lasted a month (June 27th to July 24th) with a peak the 13th of July. The cell concentration increased
351 from 100,000 cells.mL⁻¹ to 800,000 cells.mL⁻¹ before plummeting to 8,000 cells.mL⁻¹ at the end of
352 the bloom (Figure 6).

353 Based on the results obtained from experiments 1 and 2, we decided to only monitor the
354 mortality of *Artemia* larvae after a 24-hours exposure to *Ostreopsis* cells (at a cellular
355 concentration of 4 cells.mL⁻¹). The mortality of *A. franciscana* correlated with the concentration of
356 *O. cf. ovata* ($r=-0.64$, $p<0.05$) along the bloom (Figure 6). The mortality of *Artemia* larvae exhibited
357 a pattern that followed the bloom of *O. cf. ovata*, with a minimum at the end of the bloom
358 (between 10 to 25 % after the 3rd of August) and a maximum at the peak of the bloom (87% on the
359 14th of July – Figure 6). Interestingly, an increase of mortality was observed in August while low
360 abundance of *O. cf. ovata* was detected (from 8th of August to 22th of August; Figure 6). A quick
361 survey of the diversity of the collected microalgae showed that several species of diatoms
362 (*Licmophora paradoxa*, *Naviculaceae* sp. and *Cylindrotheca closterium*) were also blooming at that
363 time (data not shown).

364

365 4. Discussion

366 4.1. *Artemia franciscana* as a relevant model to evaluate *Ostreopsis* sp. toxicity

367 To ensure monitoring of Harmful Algal Blooms and Human Health protection, the usefulness
368 of biological assays has long been recognized for toxicity assessment of samples (Botana, 2014).
369 For instance, mouse bioassay (MBA) has been widely used as primary tool for HABs monitoring,
370 notably for the shellfish toxicity (Delaney, 1984). However, this biological assay presents a lack of
371 sensitivity, is prone to interferences with various metals and salts resulting in false positives and

372 negatives, and often raises major ethical and political concerns (McCulloch et al., 1989; Wiberg
373 and Stephenson, 1961). Hence, developing more sensitive and ethical bioassays for HABs
374 monitoring is currently of high relevance. The crustacean species *Artemia* has been shown of
375 interest to evaluate the toxicity of diverse biological and chemical contaminants in the marine
376 environment (Michael et al., 1956; Nunes et al., 2006; Vanhaecke et al., 1981) and its relevance for
377 assessing the toxicity of *Ostreopsis* spp. cells was successfully investigated in this study. First, the
378 replicability of this bioassay was satisfactory with coefficients of variations between 0 and 6.1%. All
379 strains from the two *Ostreopsis* species (*O. cf. ovata* and *O. fattorussoi*) induced significant
380 mortality of the *Artemia* larvae. The lethal concentration (LC_{50-24h}) obtained with the MCCV 054
381 ($81.96 \pm 63 \text{ cell.mL}^{-1}$) was comparable to the effect concentration (EC_{50}) observed after 24 hours
382 using a strain isolated from the Italian Marche Region ($80 \pm 7 \text{ cell.mL}^{-1}$; Pezolesi et al., 2012). And
383 in agreement with our findings, a concentration of 250 cell.mL^{-1} of *Ostreopsis siamensis* induced
384 the mortality of all *artemia* larvae after 24 hours of exposure (Rhodes et al., 2000). Our results
385 confirm the suitability of this bioassay to monitor the toxicity of *Ostreopsis* species from any origin.

386 The *Artemia* larvae were shown to be extremely sensitive to the presence of *Ostreopsis* spp.
387 cells since concentrations as low as $0.4 \text{ cell. mL}^{-1}$ induced significant mortality (Experiment 3). In
388 the context of *in situ* biomonitoring, this sensitivity could greatly facilitate an early detection of
389 blooms. Nevertheless, it should be considered that differences in toxicity exist according to the
390 growth stage of the dinoflagellate (Experiment 2), following a pattern that correlated with the
391 cellular growth: toxicity was maximal at the end of the exponential phase (43%, day 10) and
392 minimal at the beginning of the growth (83%, day 3). Thus, for standardization aspects, our data
393 suggest that the toxicity of newly isolated strains/species should be tested using cells that reached
394 the end of their exponential phase (~10 days after inoculation).

395 Several exposure time (12, 24 and 48 hours) and cell concentrations (0.4, 4, 40, 400 and 4000
396 cell.mL^{-1}) were tested in Experiments 1, 2 and 3 to evaluate the best conditions that should be
397 chosen to run the *Artemia* bioassay. Exposure lower than 12 hours is not relevant whereas
398 exposure over 24 hours provides the best accuracy (Experiment 1 and 2 – Figure 1 and 3) and
399 allows the determination of LC_{50} values. Also, cell concentrations ranging from 0.4 to 400 cell.mL^{-1}
400 give the best results (Experiment 1 and 3 – Figure 1b and 4b).

401 However, results obtained in the experiment 3 highlighted earlier action of living cells
402 compared to dissolved toxins that would stay undetected with only one check. Standardized
403 conditions that should be used to run an *Artemia* bioassay in the context of evaluating the toxicity
404 of an *Ostreopsis* species are summarized in the Figure A.1 and A2 of the supplementary
405 information.

406

407 4.2. Compounds responsible for *Ostreopsis* sp. toxicity on *Artemia franciscana*

408 Even though no OVTX was quantified, the *O. fattorussoi* species was surprisingly highly toxic
409 to the *Artemia* larvae (93,3%), suggesting the production of other toxic compounds by this
410 *Ostreopsis* species (Experiment 1). Although the production of OVTXs by *O. fattorussoi* has been
411 already reported (Accoroni et al., 2016a), the experimental conditions used in this study did not
412 provide any detection of OVTXs. This result underlines the variability in the toxin production
413 among species (already documented for *O. cf. ovata* : Accoroni et al., 2011; Brissard et al., 2014;
414 Ciminiello et al., 2012b; Hwang et al., 2013) and the potential production of alternative toxic
415 compounds to OVTXs. Likewise, several fractions of the cells of the *ovata* species were also shown
416 to be toxic although devoid of OVTX (e.g. 4 and 7, Experiment 3), and supporting the production of
417 unknown toxic metabolites by *Ostreopsis cf. ovata*. Historically, the quantification of toxic
418 compounds produced by the Mediterranean *Ostreopsis* species were undertaken by LC-MS,
419 searching for known metabolites of the palytoxin family (Ciminiello et al., 2011, 2008, 2006).
420 However, distinct metabolites have been identified from *Ostreopsis* species (Accoroni et al.,
421 2016a; Hwang et al., 2013; Rhodes et al., 2002; Ternon et al., 2018), underlining the importance of
422 bio-guided fractionation and the isolation and characterization of toxic molecules using usual
423 natural product chemistry. The OVTX are nevertheless likely to be the most toxic metabolites
424 produced by *O. cf. ovata* given the high mortality reached when testing the most OVTX-
425 concentrated fraction (F5). The activity of both OVTX and PLTX was further confirmed by testing
426 purified compounds on *Artemia* larvae using concentrations related to the cell concentration 0.4,
427 4, 40 and 400 cells.mL⁻¹. This dose-dependent experiment highlighted the extreme sensitivity of
428 the *Artemia* larvae to the presence of both OVTX and PLTX since concentrations as low as 12 pg
429 induced 100% mortality. Therefore, the *Artemia* bioassay allows the detection of the OVTX
430 230,000 times more sensitive than UHPLC-HRMS, and could greatly help the early detection of
431 toxic events.

432 Despite a high similarity in their molecular formulae, PLTX and OVTX have been previously
433 shown to display different toxic activity on HaCaT cell lines and rats (Pelin et al., 2016; Poli et al.,
434 2018), which is not in agreement with the results obtained with the *Artemia* larvae at all
435 concentrations and exposure times. Interestingly, living cells induced a higher mortality than pure
436 compounds at low exposure times (80 and 93% after respectively 12 and 24 hours of exposition).
437 One reason could be that living cells are ingested by *A. franciscana*, as previously described by
438 Faimali et al. (2012) when dissolved toxins affected the larvae by direct contact. These findings
439 also confirm the toxic role of other metabolites than OVTXs produced by *O. cf. ovata* as suggested
440 by all the experiments of this study. For that matters, several metabolites of high molecular weight
441 displaying the same ionization pattern than OVTX (tri-charged ions) are known to be produced by

442 *O. cf. ovata* (Brissard et al., 2015; Ternon et al., 2018). . Altogether, these results highlight the
443 complementarity of the *Artemia* assay and the chemical analysis since together they offer a rapid
444 and highly sensitive screening of toxic fractions prior to the identification of the toxic metabolites.

445 4.3. Application to *in situ* monitoring of *Ostreopsis cf. ovata*

446 The efficiency of the *Artemia* bioassay to monitor a real bloom of *Ostreopsis cf. ovata* was
447 investigated in 2018 at the Rochambeau site (Villefranche-sur-mer, France). Consistently with the
448 results obtained *in vitro*, the *Artemia* mortality was closely correlated with the concentration of
449 *Ostreopsis* cells. Although the toxin content was not analyzed during this experiment, previous
450 monitoring studies showed that the OVTX concentrations is maximal at the peak of the bloom at
451 this site (e.g. highest cell concentration; Gémin et al., 2020). Various toxin profiles have been
452 obtained in other Mediterranean areas, suggesting a variability in the species/strains and
453 environmental parameters, that could easily be evidenced by the *Artemia* bioassay. Indeed, this
454 simple monitoring tool is relevant to evaluate the toxicity due to the OVTX but also other
455 metabolites of *Ostreopsis* cells in the field. Interestingly, a slight increase of mortality of *Artemia*
456 larvae was measured in August while *Ostreopsis* exhibited a low cell abundance. Other benthic
457 microalgae could also induce a mortality of *Artemia* larvae such as other Dinoflagellates or
458 Diatoms

459 The high sensitivity of this test, its high reproducibility but also its ease of use and its low
460 cost are major assets in favour of its use as a routine evaluation of the toxicity of *Ostreopsis* sp. in
461 the field. It could also be potentially applied to other toxic dinoflagellates, but also as a tool to
462 investigate the presence of toxins in microalgae using bio-guided fractionation approach. This
463 would greatly facilitate the implementation of this bioassay for the assessment of HABs species
464 toxicity by stakeholders, water and coastal managers. The main limitation of the *Artemia* bioassay
465 we could identify is that the information is not specific.

466

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478 **Appendices:**

479 **Figure A.1:** Hatching conditions used in all the toxicity experiments

Incubation	Glassware	Media	Volume	Light	Temperature	Salinity	Oxygen	Cysts (g)	Food
40 hours	Erlenmeyer 500 mL	Filtered autoclaved sea water	300 mL	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	20°C	38	Continuous aeration	0.15 g	No food

480

481 **Figure A.2:** Experimental conditions for toxicity assay

	Experiment 1: Strain/species comparison	Experiment 2: Growth vs toxicity	Experiment 3: PLTX/OVTX/living cells	Experiment 4: <i>In situ</i> bloom
Experimental treatment	MCCV 054/055/057/058 4,40,400,4 000 cells .mL ⁻¹	MCCV 055 every 2 days during 14 days	PLTX/OVTX/MCCV055 living cells	<i>In situ Ostreopsis cf. ovata</i> cells / 1 toxicity assay per week
Time exposition	48 hours	48 hours	48 hours	48 hours
Time observation	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time
Temperature	24°C	24°C	24°C	24°C
Light	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$
Salinity	38	38	38	38
Glassware	6 well plates	6 well plates	6 well plates	6 well plates
Replicates	30 (5 larvae per well / 6 wells per experimental condition)	30 (5 larvae per well / 6 wells per experimental condition)	30 (5 larvae per well / 6 wells per experimental condition)	30 (5 larvae per well / 6 wells per experimental condition)
Medium	Filtered and autoclaved sea water + cells culture (80:20, v:v)	Filtered and autoclaved sea water + cells culture (80:20, v:v)	Filtered and autoclaved sea water + cells culture (80:20, v:v) Sea water + DMSO (0.1% final volume)	Filtered and autoclaved sea water + cells culture (80:20, v:v)
Volume	5 mL	5 mL	5 mL	5 mL
Control	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)
Quality Control	Mortality < 10%	Mortality < 10%	Mortality < 10%	Mortality < 10%

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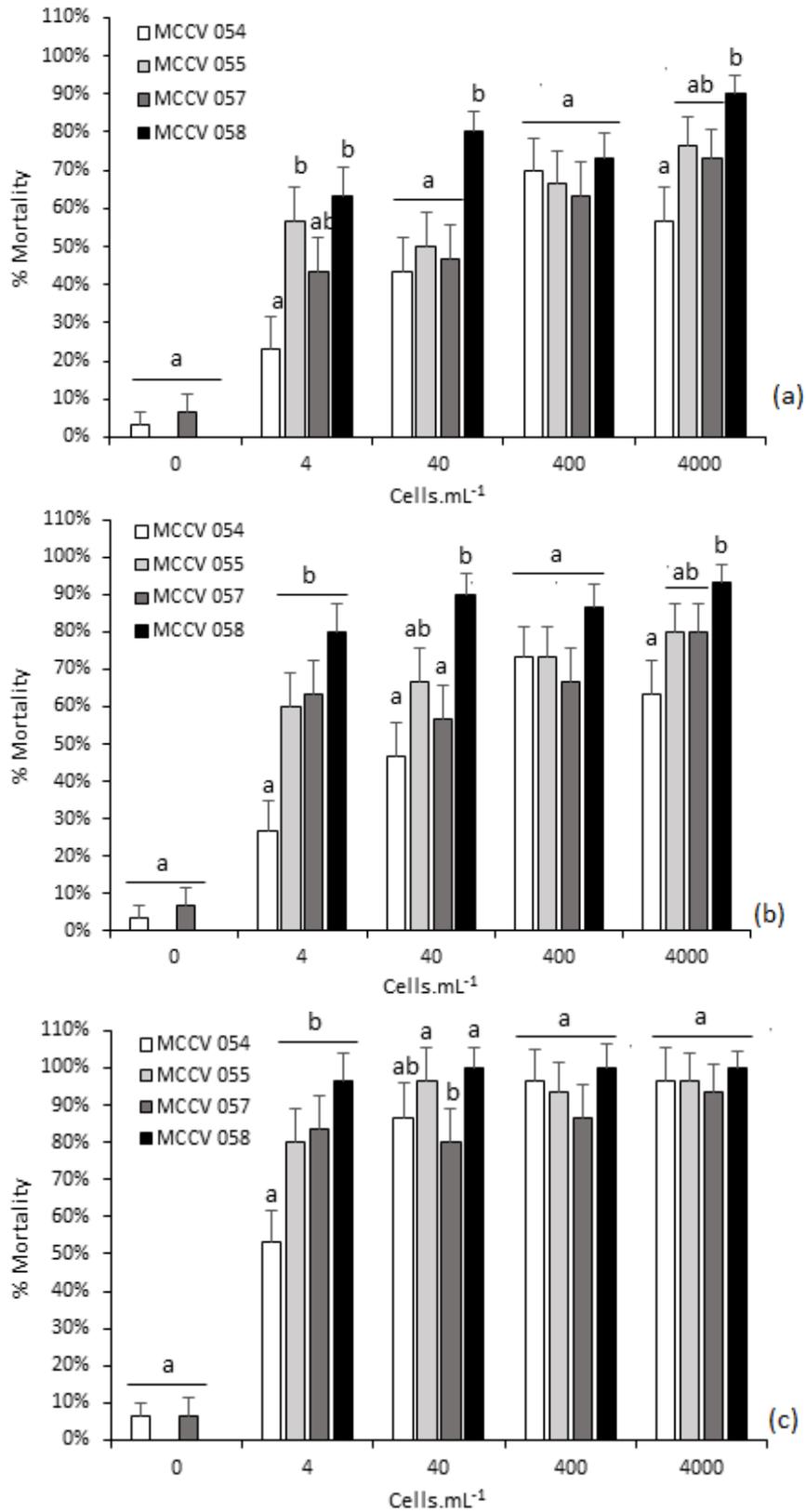


Figure 1 : Percentage of mortality (Mean \pm SE) after 12 h (a), 24 h (b) and 48 h (c) of *Artemia franciscana* larvae exposed to various concentrations of Mediterranean strains of *Ostreopsis cf. ovata* (MCCV 054 and 055) and *Ostreopsis fattorussoi* (MCCV 057 and 058). Letters formulate significant differences: groups that have no common letter differ significantly.

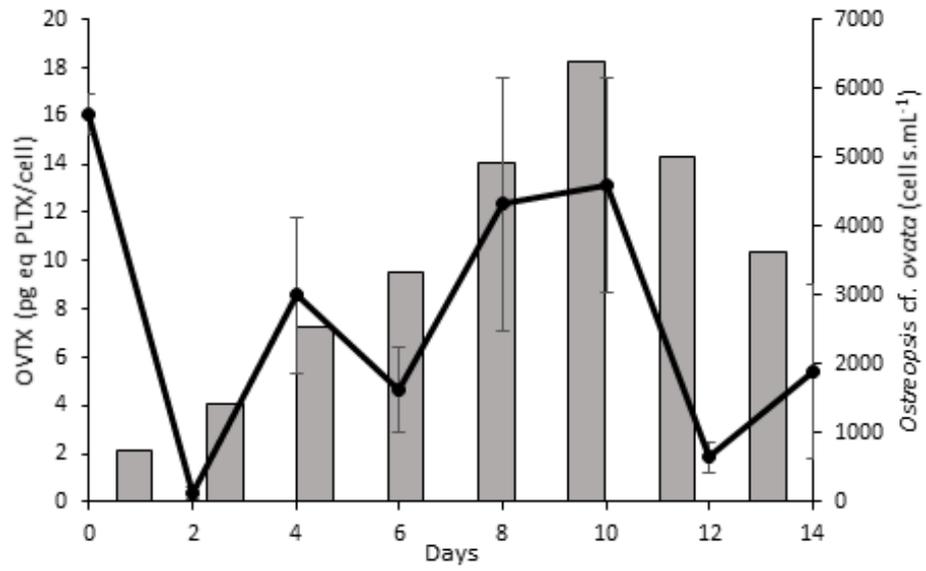


Figure 2: Toxin concentration (Mean \pm SE) in pg eq. PLTX/cell (black line) depending on the growth phase of *Ostreopsis cf. ovata* (MCCV 055 – grey bars).

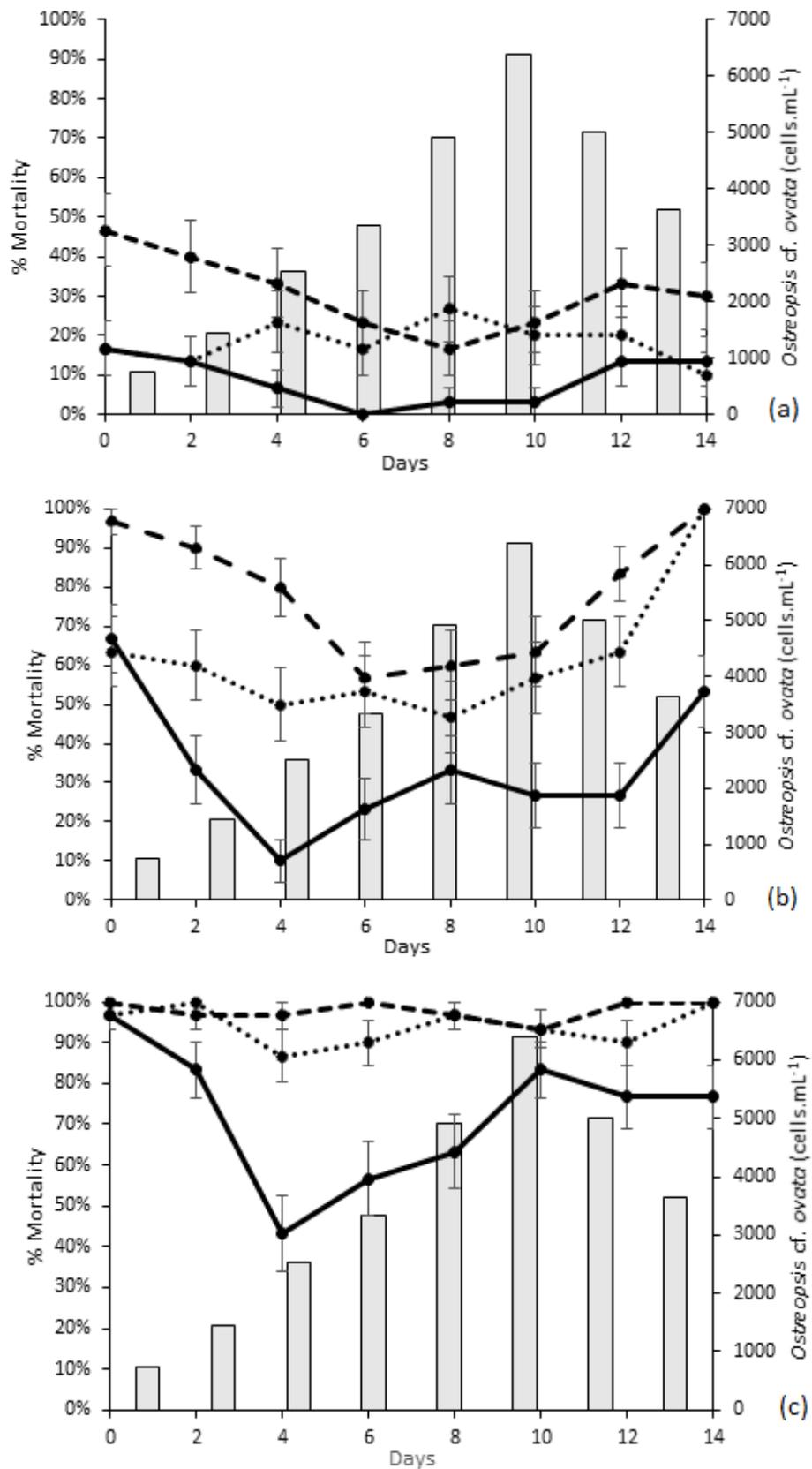


Figure 3: Percentage of mortality (Mean \pm SE) of *Artemia franciscana* larvae exposed to 4 (black lines), 40 (dotted lines) and 400 cells.mL⁻¹ (dashed lines) of *Ostreopsis cf. ovata* (MCCV

055) after (a) 12 hours, (b) 24 hours and (c) 48 hours exposure time. The growth curve of *O. cf. ovata* (MCCV 055) cultured in 300 mL flasks is also represented in grey bars.

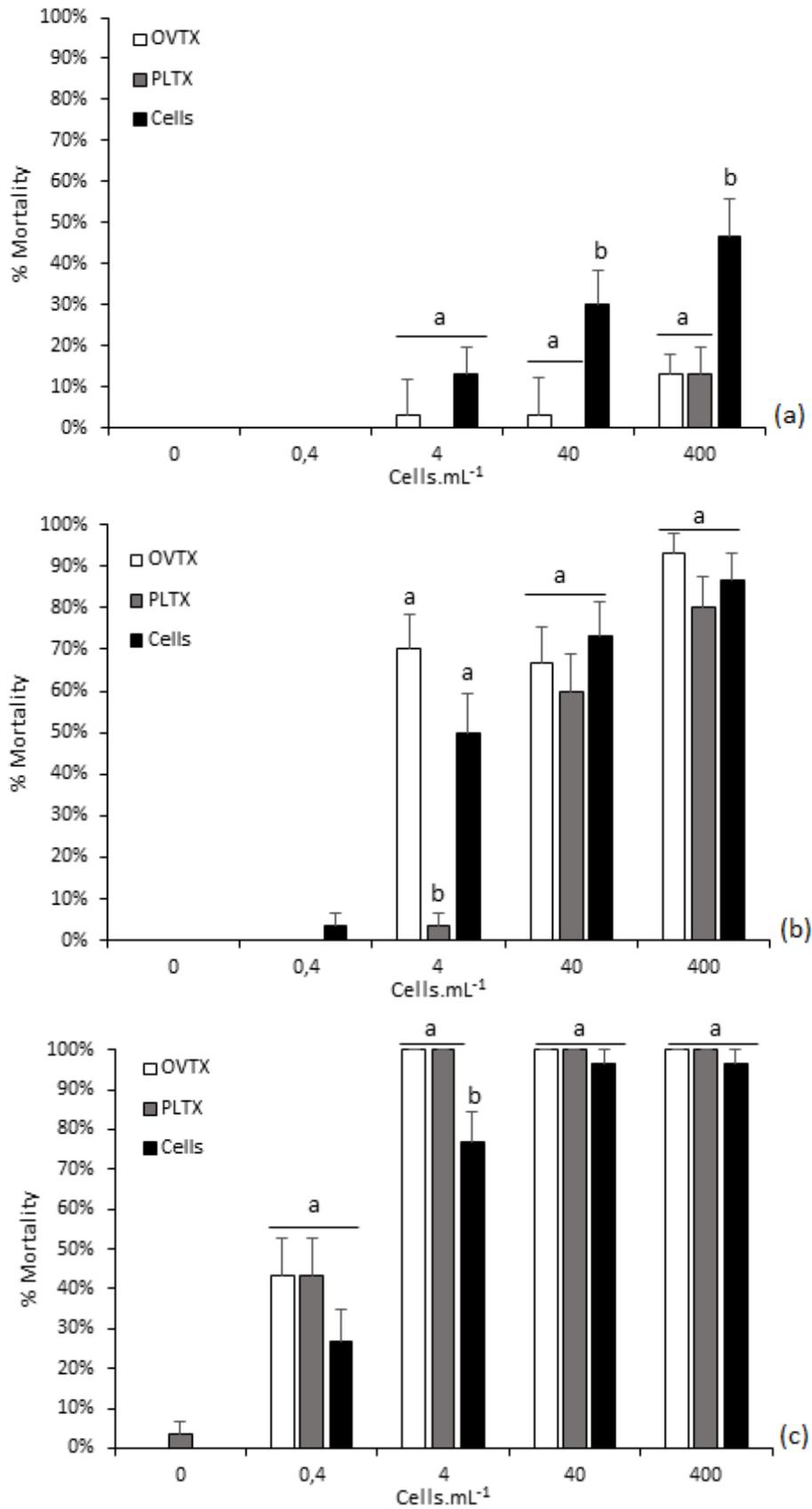


Figure 4: Mortality (Mean \pm SE) after 12 h (a), 24 h (b) and 48 h (c) of *Artemia franciscana* larvae exposed to increased concentrations of dissolved PLTX and OVTX (from 0.012, 0.12, 0.4, 4, 40, and 400 Cells.mL⁻¹).

1.2 and 12 $\mu\text{g}/\text{mL}$) and living cells at various cell-equivalent concentrations. Letters formulate significant differences: groups that have no common letter differ significantly ($p < 0.05$).

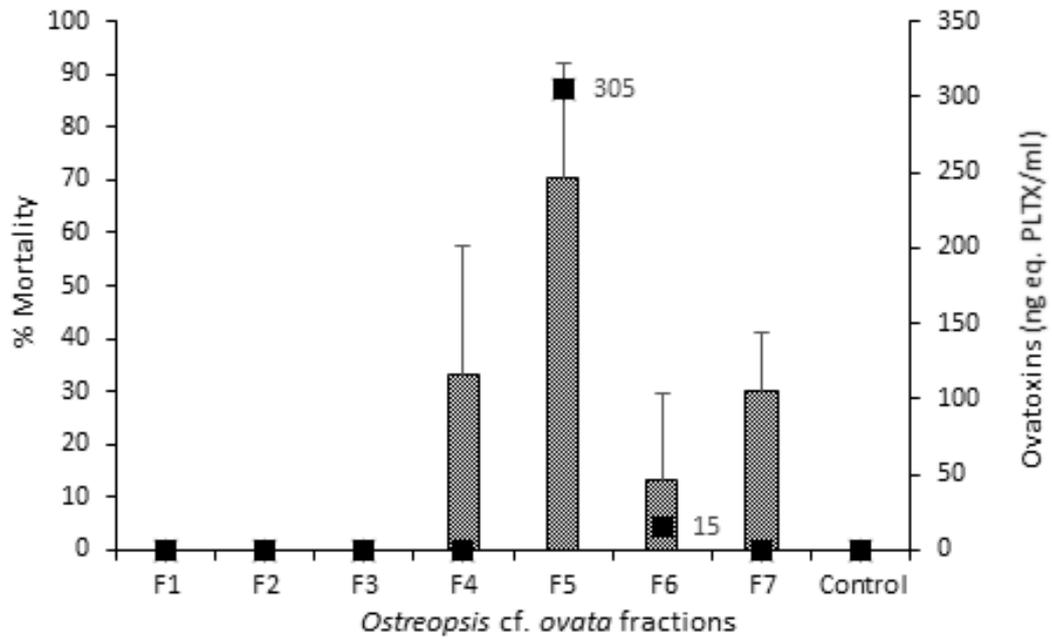


Figure 5: Mortality (Mean \pm SE) of *Artemia franciscana* larvae after being exposed 24 hours to fractions of *Ostreopsis cf. ovata* obtained from MCCV 054 extracts. The concentration of all fractions was previously adjusted to 3 mg.ml⁻¹ using DMSO and MilliQ. The polarity of the metabolites decreases from Fraction 1 to 7 (F1-F7). The content of OVTXs in each fraction is also represented (black squares).

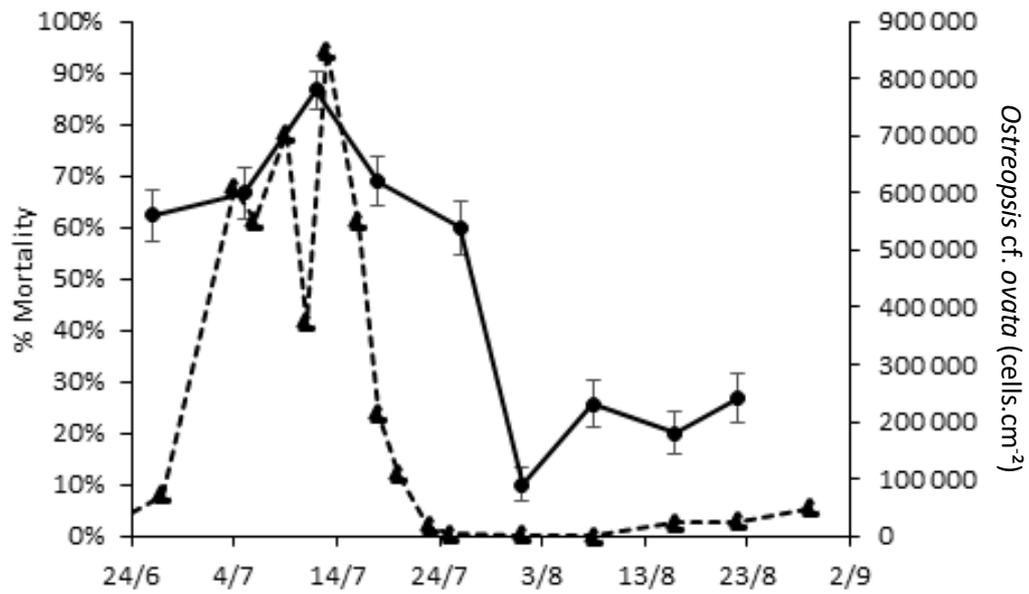


Figure 6: Evolution of the mortality of *Artemia franciscana* (continuous line – Mean ± ES) after 24 hours exposition to 4 cells.mL⁻¹ of cells of *Ostreopsis cf. ovata* sampled along the bloom in July 2018. The cellular concentration of *O. cf. ovata* on artificial substrates is represented in dashed line.