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

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Abstract :

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

Highlights

► *Artemia* bioassay is relevant to evaluate *Ostreopsis* spp. Toxicity ► *Ostreopsis* spp. produce other toxic compounds than known ovatoxins ► *Artemia* bioassay could be used for bio-guided fractionation approach

Keywords : *Ostreopsis* spp., *Artemia franciscana*, HPLC-HRMS, bioassay, ecotoxicity

1. Introduction

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

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

In the middle of the 20th century , the brine shrimp *Artemia* sp. was proposed as a bio-indicator for chemical contamination (Michael et al., 1956; Vanhaecke et al., 1981) owing to its sensitivity to sodium lauryl sulfate and various insecticides. Up to now *Artemia* sp. has been widely used to test fungal toxins (Harwig and Scott, 1971), plant extract toxicity (Meyer et al., 1982), heavy metals (Martínez et al., 1999; Sarabia et al., 2008), cyanobacteria toxins (Jaki et al., 1999; Tokodi et al., 2018) and pesticides (Comeche et al., 2017; Gambardella et al., 2018; Jawahar et al., 2018). *Artemia* sp. was also shown to be useful for marine natural products screening as shown by

the work of Carballo et al. (Carballo et al., 2002) who compared the results of this bioassay with the cytotoxicity of the specialized metabolites produced by 20 marine organisms (mostly invertebrates) against 2 human cell lines (lung carcinoma A-549 et colon carcinoma HT29). Therefore, *Artemia fransciscana* bioassay may constitute an excellent way to quickly assess the toxicity of *Ostreopsis* spp. blooms.

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

2. Materials and methods

2.1. Bioassays

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

2.1.1. Micro-algal culture

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

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

A volume of 5 mL of each MCCV clonal culture was centrifuged for 6 min at 2000 rpm and 4°C. The supernatant was removed and the cell pellet was resuspended with 1 mL of sterile water (autoclaved MilliQ) and centrifuged again for 2 min to remove salt and media. The cell pellet was kept and homogenized with 50 µL of sterile water and a fraction of 10 µL was further transferred to 0.2 µL polymerase chain reaction (PCR) tubes. Then, PCR tubes were stored at -20°C until further analysis. Approximatively 300 base pairs of the internal transcribed spacer region (ITS1-5.8s-ITS2) rDNA were amplified by PCR using the primer 329-F (5'-GTGAACCTGCRGAAGGATCA-3') which is the inverse complementary sequence of the universal eukaryote primer 329-R (Moon-van der Staay et al., 2001) and the designed primer D1R-R (5'-TATGCTTAAAATTCAGCAGGT-3') which is the inverse complementary sequence of the primer DIR-F (Scholin et al., 1994). A mix of 1 µl of each primer, 1 µl of dNTP at 10 mM, 5 µl of 10X Advantage 2 PCR Buffer, 31 µl of sterile water and 1 µl of 50X Advantage 2 Taq polymerase (Clontech) was added in each PCR tube containing the cell pellet. The PCR was performed using a MasterGradiant thermocycler (Eppendorf) with the following conditions: one initial denaturation at 94°C for 10 min followed by 35 cycles each 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. The PCR products were purified using QIAGEN MinElute PCR Purification kit according to the recommendations. After sequencing by Genewiz, sequences were treated using BioEdit software and compared to NCBI database using BLASTn tool to ensure the taxonomy of the strains.

2.1.3. Biomass production of *Ostreopsis*

- Cultures

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

- *In situ*

Living cells used in Experiment 4 were sampled on artificial substrates (Jauzein et al., 2016) during summer 2018 (27th of June to 23rd of August) to cover the bloom of *Ostreopsis* cf. *ovata* . Three screens composed by a rigid frame with a 1 mm mesh were placed in the morning at a depth of 50 cm at 3 sampling sites, separated from each other by few meters, in Rochambeau (Bay

of Villefranche). After 24 hours, the screens were carefully collected in 1 L plastic bottles containing seawater and gently homogenized in order to resuspend microalgae. For each screen, a volume of 20 mL was subsampled in triplicates fixed with acidified lugol (4% v/v) and observed under microscope using Sedgewick Rafter counting chamber to measure both the abundance and the diversity of microalgae. After counting, each sample obtained from the screens was diluted with the appropriate culture medium to reach a final concentration of 20,000 cells.mL^{-1} prior to the *Artemia* bioassays. It is noteworthy that these samples are natural samples and therefore contain a broad benthic micro-algal assemblage, including *Ostreopsis* cells. Nevertheless, during the peak of the bloom, the proportion of diatoms represented less than 25 % of the total number of cells tested (e.g 2.7 to 17% for *Cocconeis* sp., 6.9 to 25% for *Navicula* sp., 0.5 to 18% for *Gyrosigma* sp., and 20% for *Bleakeleya* sp.) and any biological effect held by those species was considered negligible in regards to *O.* cf. *ovata*.

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

A volume of 1.4 L of a culture of *Ostreopsis* cf. *ovata* (MCCV 054) of 10 days of age was centrifuged at 600 g at 21°C for 10 min. The supernatant was discarded and the cell pellet flash frozen using liquid nitrogen to stop the cells metabolism, and further stored at -20°C before being freeze dried. An amount of 85 mg of dried cells was extracted using 4 mL of a mixture MeOH:H_2O (80:20) in an ultrasonic bath (Elma Transonic T420) for 10 min. The extract was centrifuged at 1,000 g during 10 min and the supernatant was collected and transferred into a glass vial. The procedure was repeated two more times to obtain 12 mL of extract. The extract was then evaporated under a gentle flow of N_2 at 35°C until full evaporation of the MeOH (leaving about 2-3 ml of water). The aqueous extract was deposited on a C18 SPE cartridge (500 mg, Supelco) previously washed with MeOH (6 mL) and equilibrated with MilliQ-water (6 mL). Before elution, the cartridge was rinsed with 6 mL of MilliQ-water. A step gradient elution was thus applied to the cartridge, from H_2O:MeOH (100:0) to H_2O:MeOH (0:100), using steps of 20% and volumes of 6 mL. An additional elution with 6 mL of acetonitrile (ACN 100%) was performed, yielding seven fractions (F1 to F7, from the most polar to the non-polar metabolites). Aliquots of 100 µL sampled from each fraction were evaporated to dryness under a N_2 stream (35°C) to assess their respective weight. A volume of 1 mL of dimethylsulfoxide (DMSO) was added to the remaining fractions before evaporation of the volatile organic solvents (MeOH, ACN) and water. Based on the fractions weight, the volume for dilution were further adjusted using DMSO to reach a concentration of 3 mg.mL^{-1}. All fractions were stored at -20°C until further bioassays and chemical analyses.

A purified pool of ovatoxins was obtained following a protocol previously published (Brissard et al., 2015). The cell pellet (75 g of *O.* cf. *ovata* - MCCV 054 - after 21 days of growth) and

fractionated by size-exclusion chromatography (Sephadex LH20). Methanolic fractions containing OVTXs (assessed by HPLC-MS) were pooled, concentrated and purified by semi-preparative chromatography (Uptisphere C18-TF, 250 mm × 4.6 mm, 5 µm; λ= 233 and 266 nm). A pool of OVTXs was used as the separation of the analogues was not straightforward. The final pool of OVTX was concentrated in DMSO and was proven to contain a mixture of OVTX-a (61 %), -b (25 %), -c (3.4 %), -d (5.3 %), -e (3.9 %) and -f (1.1 %) at 7.8 mg/mL, based on LC-MS/MS quantification (Gémin et al., 2020). Pure palytoxin was purchased from ©Wako Chemicals, GmbH, Neuss, Germany. All extracts/toxins were solubilized in DMSO at a concentration of 12 µg/mL that would allow an addition of 5 µl for the *Artemia* bioassay without exceeding 0.1% of DMSO in final volume of seawater.

2.1.5. Experimental bioassays

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

Artemia franciscana larvae's mortality was measured every hour during 12 hours, then at 24 and 48 h exposure times (only at 24 h for chemical fraction, Experiment 3). Larvae were considered dead when there was no movement during 10 seconds and no reaction to strong light. A negative control was also performed for all experiments, using seawater + fresh L1 medium, 80: 20 (v:v) for Experiments 1, 2, 3 and 4 as well as 0.1% DMSO (final volume) for Experiments 3.

Bioassays are considered consistent when mortalities rate in the control condition are lower than 10% as described in Artoxkit (1990).

2.2. Toxin profiles

2.2.1. Cell extraction

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

All flasks content (50 mL and 300 mL) were transferred to 50 mL Falcon tubes before being centrifuged at 600 g and ambient temperature, for 10 min. After removal of the supernatant, the cell pellets were flash frozen using liquid nitrogen and further stored at -20°C. A volume of 4 mL of MeOH: H_2O (80:20, v/v) was poured in the falcon tubes before placing them in ultra-sonic bath for 10 min cooled in an ice batch. The falcon tubes were then centrifuged again at 2,500 g for 10 min at ambient temperature and the supernatants were transferred in 20 mL vials.

These steps (organic extraction, ultra-sonic bath and centrifugation) were repeated two more times and the resulting extracts were evaporated under a gentle stream of N_2 until full evaporation of the MeOH. To avoid dryness of the samples, 500 µL of DMSO was then added to the extracts and the remaining H_2O further evaporated. All extracts were stored at -20°C until UHPLC-HRMS analysis.

2.2.2. UHPLC-HRMS

UHPLC-DAD-HRMS experiments were performed using an Agilent 1290 system (Agilent Technologies, USA) equipped with a diode array detector and coupled to an Agilent 6540 Qtof mass spectrometer (Agilent Technologies, USA). Mass analyses were carried out in positive mode (ESI +) between 60 to 3000 Da. A volume of 10 µL of each samples were injected and separated on a T3 column (Acquity UPLC HSS T3 1.8µm, 2.1mm x 100mm, Waters) using a linear elution gradient of H_2O/MeOH (80:20, v:v) and MeOH containing 0.1mM of ammonium formate and 0.1% formic 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 mL.min^{-1} flow rate. Collision energies (CE) of 30, 70 and 110 eV were applied to confirm the presence of OVTXs. UV detection was set at 210, 233 and 263 nm. The spectrometer analyzer

parameters were set as follows: nebulizer sheath gas, N_2 (35 psig); drying gas, N_2 (11 L.min^{-1}); Gas Temperature, 300°C; capillary, 4.129 µA; Vaporizer/Sheat Gas Temp, 350°C.

The quantification of the OVTXs in the samples was performed using commercial PLTX as a reference, considering identical ionization of these compounds. A calibration curve for PLTX was set using concentrations between 1 and 10 mg.mL^{-1}, using the tricharged adduct $[M+3H-4H_2O]^{3+}$ for quantification. This same adduct was used to quantify the OVTX a in all samples.

2.3. Statistical analysis

The statistical significance of the mortality variation between the different experimental conditions tested was evaluated using Kruskal-Wallis analyses. Dunn's post hoc tests were used *a posteriori* to identify which experimental conditions differed from the others when significant differences were showed by the Kruskal-Wallis test ($p<0.05$). These tests were realized using the PAST software (Hammer et al., 2001). The R package ecotoxicology was used to assess the median lethal concentration (LC_{50}) at 24 and 48 hours of exposition.

3. Results

3.1. Molecular analyses

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

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

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

A positive correlation between mortality and exposure time ($p < 0.001$) was observed, and 12 hours of exposure was sufficient to induce significant mortality of *A. franciscana* ($p < 0.05$). Mortality reached its maximum after 48 hours (96,6%) for all strains and concentrations tested. Several concentrations of *Ostreopsis* spp. cells were tested to explore their effects on *A. fransiscana*. Despite inter-strains variability, a dose-dependent response was observed with a strong increase of mortality until a plateau of 96% of mortality was reached at 400 cells.mL^{-1} at all exposure time and for most strains. Inter-strains variability was mostly observed for low exposure times (12 and 24 hours) and low cell concentrations (4 to 40 cell.mL^{-1}). Using a concentration of *Ostreopsis* spp. cells of 4 cell.mL^{-1}, the toxicity of the strains could be ranked as follows: MCCV 054<055=058<057 (Cf. $LC_{50\text{-}24h}$ values – Table 1). By increasing the concentration of *Ostreopsis* spp. cells, this distribution is smoothed and no significant differences are observed between strains for 400 and 4 000 cell.mL^{-1} (except for MCCV 058 that shows the most toxic behaviour, 96,6 %). The differences in the $LC_{50\text{-}24h}$ observed between strains of a same species is somehow important and may be attributed to the natural variability of microalgae metabolism (Meyer and Pohnert, 2019), and in particular the production of toxic metabolites (Tartaglione et al., 2017).

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

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

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

Interestingly, the variability of the toxin content did not reflect the pattern of mortality observed for *Artemia franscicana* (Figure 3 a, b and c). No clear trend emerged from the mortality measured after 12 hours of exposure, although it increased with cell concentrations. Likewise, at

high cell concentration of *O.* cf. *ovata* (40 and 400 cell.mL^{-1}) and for an exposure time of 48 hours, mortality varied little ($p>0.05$) and was close to 100 % during the whole duration of the experiment, precluding drawing any relationship between mortality and the growth phase. Only exposure times of 24 and 48 hours at low concentrations of *O.* cf. *ovata* cells (4 cell.mL^{-1}) allowed drawing a pattern of mortality for *Artemia* larvae that followed both the cellular growth and the toxin content (Figure 3 a) with a minimum between days 2 and 4 (10 % and 43 % for 24 h and 48 h respectively, 0.40 pg eq. PLTX/cell, 1,436 cells.mL^{-1}) and a maximum reached at day 10 (26 % and 83 % for 24 h and 48 h, respectively, 13.1 pg eq. PLTX/cell, 6,389 cells.mL^{-1}).

3.4. Metabolites toxic for *Artemia* – Experiments 3

The nature of the toxic metabolite(s) responsible for the toxicity of *O.* cf. *ovata* cells on *Artemia* was further investigated comparing the toxic behaviour of (i) living *O.* cf. *ovata* cells, (ii) chemical fractions obtained from *O.* cf. *ovata* cells extracts, (iii) purified OVTX, and (iv) commercial PLTX. Interestingly, living cells induced an earlier response than equivalent amount of dissolved PLTX and OVTX (mortality of 46 %, 13 % and 13 %, respectively after 12 h of exposure at a concentration of 400 cells.mL^{-1}), although these differences tend to be reduced with increasing exposure time (Figure 4). After 48 h, the mortality was significantly higher with the dissolved toxins (100 % for both OVTXs and PLTX and 77 % for living cells at a cellular concentration of 4 cells.mL^{-1}), as also confirmed by the LC_{50} values given in Table 1. To test whether the OVTX and PLTX are the only toxic metabolites produced by *O.* cf. *ovata*, the activity of seven fractions of different polarities obtained from *O.* cf. *ovata*, was assessed over 24 hours on *Artemia* larvae (Figure 5). No toxicity was found for fractions 1, 2 and 3 whereas all fractions from 4 to 7 were toxic towards the larvae, showing mortalities of 13%, 30%, 33% and 71% for fractions 6, 4, 7 and 5, respectively.

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

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

Since *Artemia franciscana* larvae mortality was shown to be a good proxy for the toxicity of *Ostreopsis* spp. cells from lab cultures, the application of the *Artemia* bioassay to *in situ*

monitoring of *Ostreopsis* cf. *ovata* blooms was further investigated. Cells sampled *in situ* were tested for their toxicity throughout the bloom in July 2018. The bloom of *Ostreopsis* cf. *ovata* lasted a month (June 27th to July 24th) with a peak the 13th of July. The cell concentration increased from 100,000 cells.mL^{-1} to 800,000 cells.mL^{-1} before plummeting to 8,000 cells.mL^{-1} at the end of the bloom (Figure 6).

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

4. Discussion

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

To ensure monitoring of Harmful Algal Blooms and Human Health protection, the usefulness of biological assays has long been recognized for toxicity assessment of samples (Botana, 2014). For instance, mouse bioassay (MBA) has been widely used as primary tool for HABs monitoring, notably for the shellfish toxicity (Delaney, 1984). However, this biological assay presents a lack of sensitivity, is prone to interferences with various metals and salts resulting in false positives and negatives, and often raises major ethical and political concerns (McCulloch et al., 1989; Wiberg and Stephenson, 1961). Hence, developing more sensitive and ethical bioassays for HABs monitoring is currently of high relevance. The crustacean species *Artemi*a has been shown of interest to evaluate the toxicity of diverse biological and chemical contaminants in the marine environment (Michael et al., 1956; Nunes et al., 2006; Vanhaecke et al., 1981) and its relevance for assessing the toxicity of *Ostreopsis* spp. cells was successfully investigated in this study. First, the replicability of this bioassay was satisfactory with coefficients of variations between 0 and 6.1%. All strains from the two *Ostreopsis* species (*O.* cf. *ovata* and *O. fattorussoi*) induced significant mortality of the Artemia larvae. The lethal concentration ($LC_{50\text{-}24h}$) obtained with the MCCV 054 (81.96 ± 63 cell.mL^{-1}) was comparable to the effect concentration (EC_{50}) observed after 24 hours using a strain isolated from the Italian Marche Region (80 ± 7 cell.mL^{-1}; Pezzolesi et al., 2012). And

in agreement with our findings, a concentration of 250 cell.mL^{-1} of *Ostreopsis siamensis* induced the mortality of all artemia larvae after 24 hours of exposure (Rhodes et al., 2000). Our results confirm the suitability of this bioassay to monitor the toxicity of *Ostreopsis* species from any origin.

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

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

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

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

Even though no OVTX was quantified, the *O. fattorussoi* species was surprisingly highly toxic to the *Artemia* larvae (93,3%), suggesting the production of other toxic compounds by this *Ostreopsis* species (Experiment 1). Although the production of OVTXs by *O. fattorussoi* has been already reported (Accoroni et al., 2016a), the experimental conditions used in this study did not provide any detection of OVTXs. This result underlines the variability in the toxin production among species (already documented for *O.* cf. *ovata* : Accoroni et al., 2011; Brissard et al., 2014; Ciminiello et al., 2012b; Hwang et al., 2013) and the potential production of alternative toxic compounds to OVTXs. Likewise, several fractions of the cells of the *ovata* species were also shown to be toxic although devoid of OVTX (e.g. 4 and 7, Experiment 3), and supporting the production of unknown toxic metabolites by *Ostreopsis* cf. *ovata*. Historically, the quantification of toxic

compounds produced by the Mediterranean *Ostreopsis* species were undertaken by LC-MS, searching for known metabolites of the palytoxin family (Ciminiello et al., 2011, 2008, 2006). However, distinct metabolites have been identified from *Ostreopsis* species (Accoroni et al., 2016a; Hwang et al., 2013; Rhodes et al., 2002; Ternon et al., 2018), underlining the importance of bio-guided fractionation and the isolation and characterization of toxic molecules using usual natural product chemistry. The OVTX are nevertheless likely to be the most toxic metabolites produced by *O*. cf. *ovata* given the high mortality reached when testing the most OVTX-concentrated fraction (F5). The activity of both OVTX and PLTX was further confirmed by testing purified compounds on *Artemia* larvae using concentrations related to the cell concentration 0.4, 4, 40 and 400 cells.mL^{-1}. This dose-dependent experiment highlighted the extreme sensitivity of the *Artemia* larvae to the presence of both OVTX and PLTX since concentrations as low as 12 pg induced 100% mortality. Therefore, the *Artemia* bioassay allows the detection of the OVTX 230,000 times more sensitive than UHPLC-HRMS, and could greatly help the early detection of toxic events.

Despite a high similarity in their molecular formulae, PLTX and OVTX have been previously shown to display different toxic activity on HaCaT cell lines and rats (Pelin et al., 2016; Poli et al., 2018), which is not in agreement with the results obtained with the *Artemia* larvae at all concentrations and exposure times. Interestingly, living cells induced a higher mortality than pure compounds at low exposure times (80 and 93% after respectively 12 and 24 hours of exposition). One reason could be that living cells are ingested by *A. fransciscana*, as previously described by Faimali et al. (2012) when dissolved toxins affected the larvae by direct contact. These findings also confirm the toxic role of other metabolites than OVTXs produced by *O.* cf. *ovata* as suggested by all the experiments of this study. For that matters, several metabolites of high molecular weight displaying the same ionization pattern than OVTX (tri-charged ions) are known to be produced by *O.* cf. *ovata* (Brissard et al., 2015; Ternon et al., 2018). . Altogether, these results highlight the complementarity of the *Artemia* assay and the chemical analysis since together they offer a rapid and highly sensitive screening of toxic fractions prior to the identification of the toxic metabolites.

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

The efficiency of the *Artemia* bioassay to monitor a real bloom of *Ostreopsis* cf. *ovata* was investigated in 2018 at the Rochambeau site (Villefranche-sur-mer, France). Consistently with the results obtained *in vitro*, the *Artemia* mortality was closely correlated with the concentration of *Ostreopsis* cells. Although the toxin content was not analyzed during this experiment, previous monitoring studies showed that the OVTX concentrations is maximal at the peak of the bloom at this site (e.g. highest cell concentration; Gémin et al., 2020). Various toxin profiles have been

obtained in other Mediterranean areas, suggesting a variability in the species/strains and environmental parameters, that could easily be evidenced by the *Artemia* bioassay. Indeed, this simple monitoring tool is relevant to evaluate the toxicity due to the OVTX but also other metabolites of *Ostreopsis* cells in the field. Interestingly, a slight increase of mortality of *Artemia* larvae was measured in August while *Ostreopsis* exhibited a low cell abundance. Other benthic microalgae could also induce a mortality of *Artemia* larvae such as other Dinoflagellates or Diatoms

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

Author statement

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 1 : Percentage of mortality (Mean ± 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.

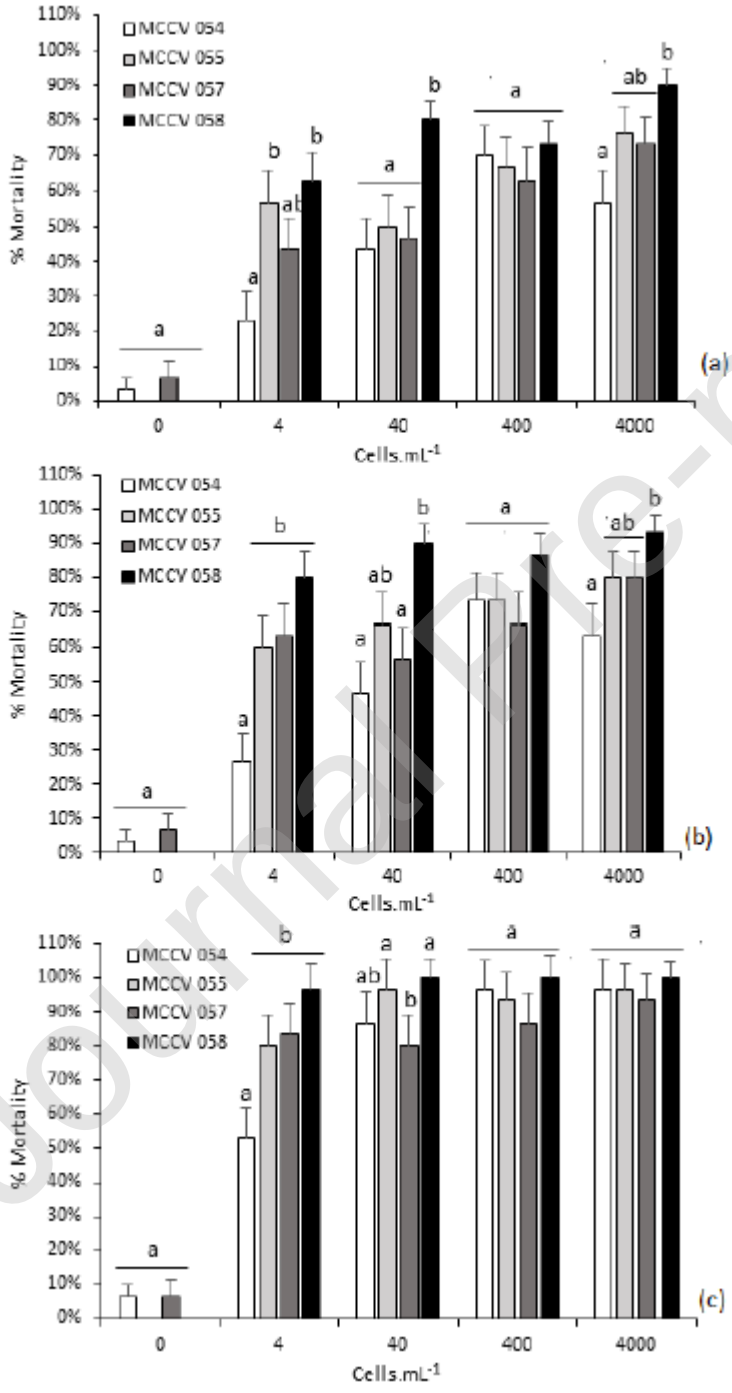


Figure 2: Toxin concentration (Mean ± SE) in pg eq. PLTX/cell (black line) depending on the growth phase of *Ostreopsis* cf. *ovata* (MCCV 055 – grey bars).

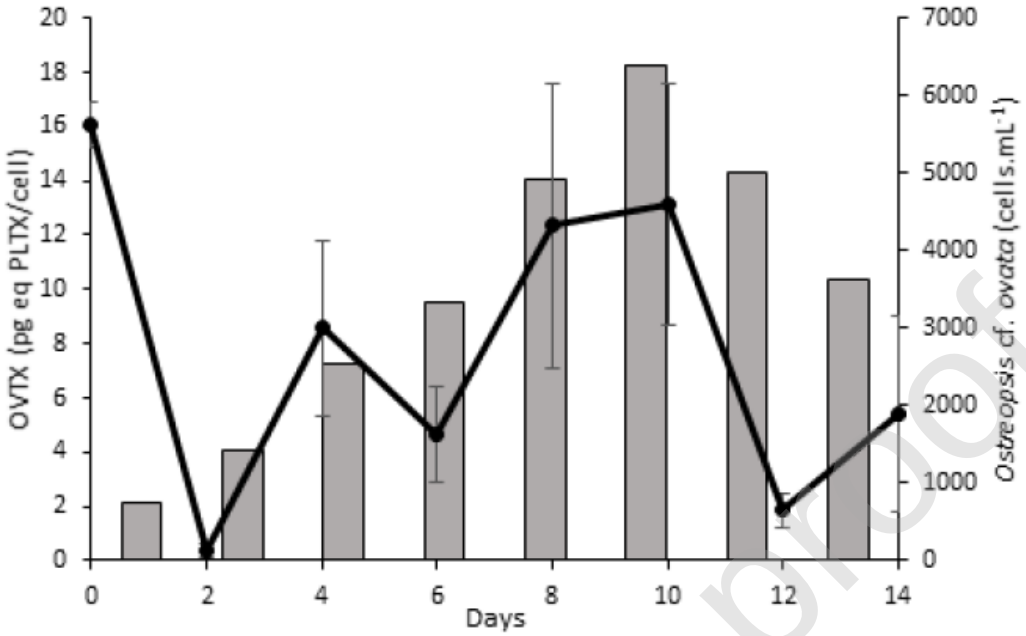


Figure 3: Percentage of mortality (Mean ± SE) of *Artemia franciscana* larvae exposed to 4 (black lines), 40 (dotted lines) and 400 cells.mL^{-1} (dashed lines) of *Ostreopsis* cf. *ovata* (MCCV 055) after (a) 12 hours, (b) 24 hours and (c) 48 hours exposure time. The growth curve of *O.* cf. *ovata* (MCCV 055) cultured in 300 mL flasks is also represented in grey bars.

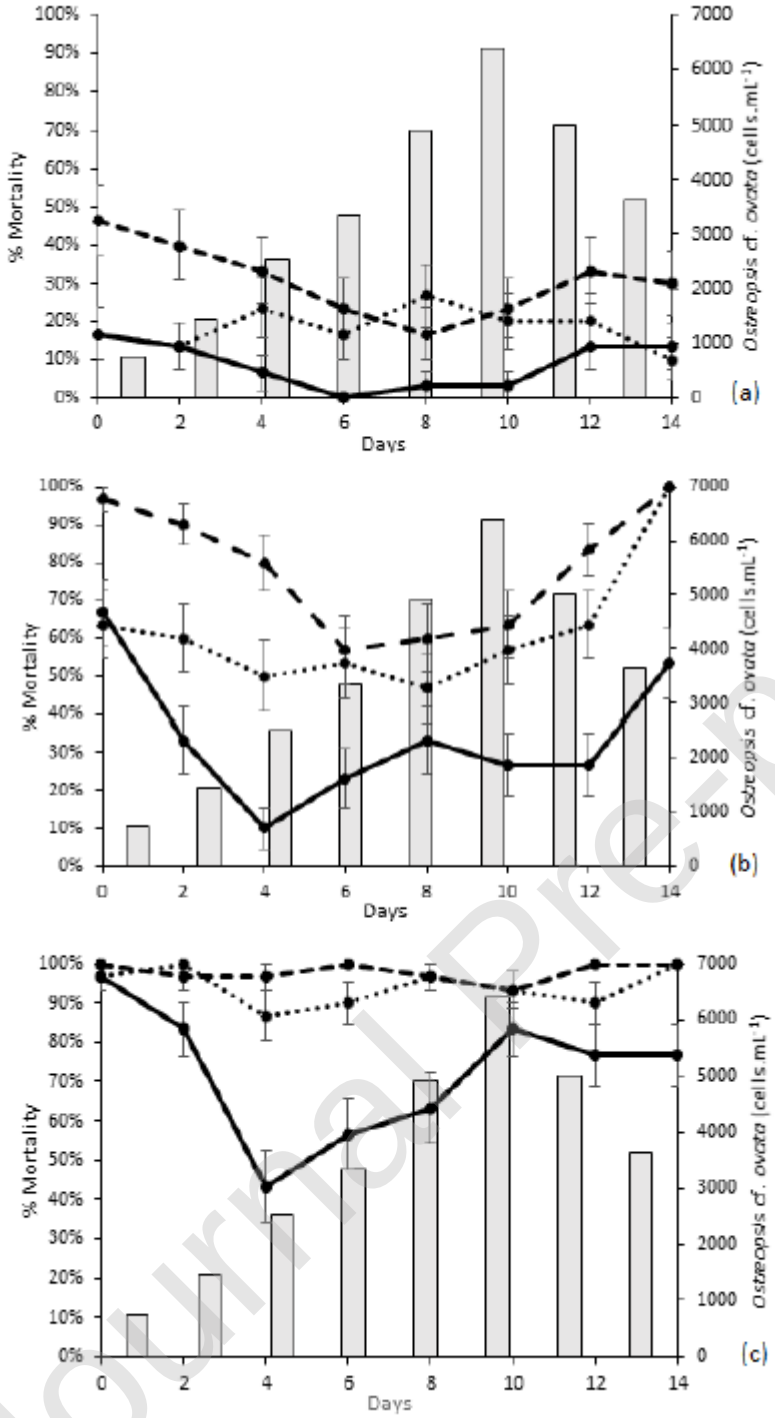


Figure 4: Mortality (Mean ± SE) after 12 h (a), 24 h (b) and 48 h (c) of *Artemia franciscana* larvae exposed to increased concentrations of dissolved PLTX and OVTX (from 0.012, 0.12, 1.2 and 12 µg/mL) and living cells at various cell-equivalent concentrations. Letters formulate significant differences: groups that have no common letter differ significantly ($p < 0.05$).

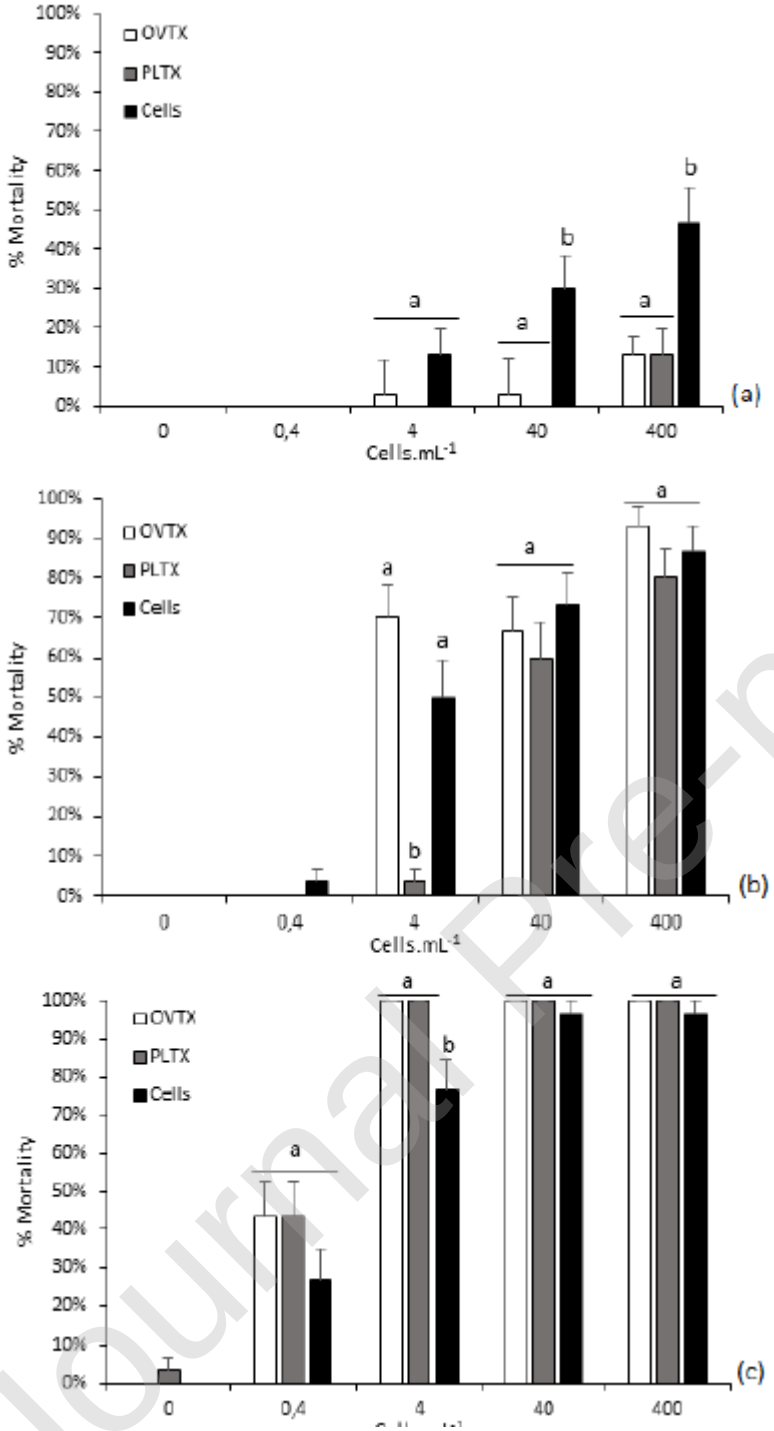


Figure 5: Mortality (Mean ± SE) of *Artemia franciscana* larvae after being exposed 24 hours to fractions of *Ostreopsis* cf. *ovata* obtained from MCCV 054 extracts. The concentration of all fractions was previously adjusted to 3 mg.ml^{-1} using DMSO and MilliQ. The polarity of the metabolites decreases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

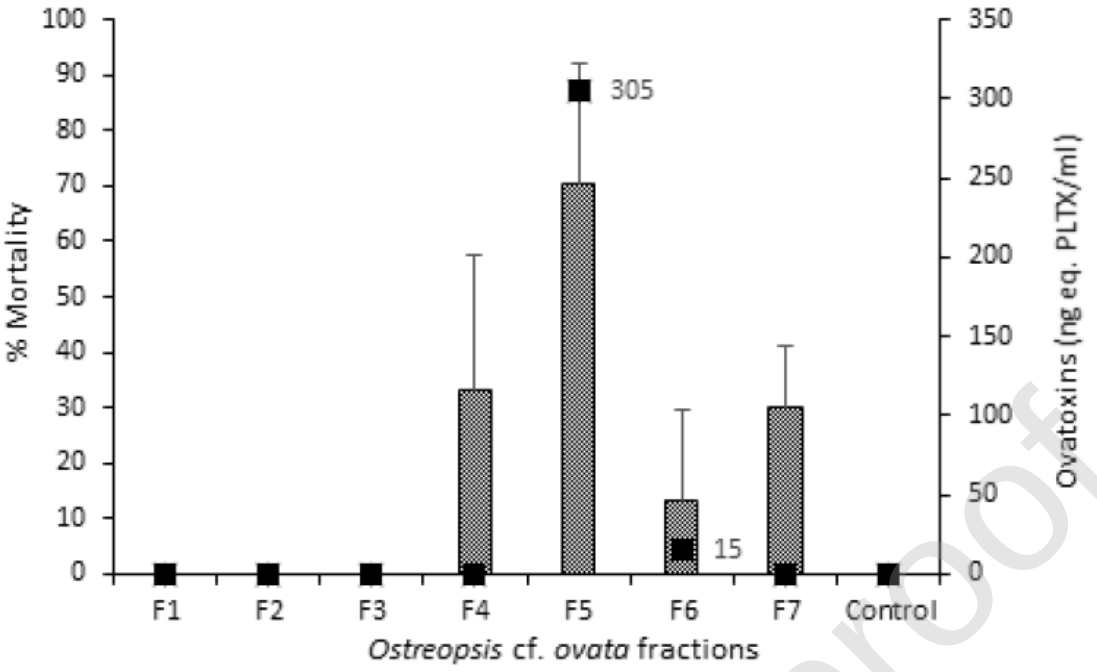
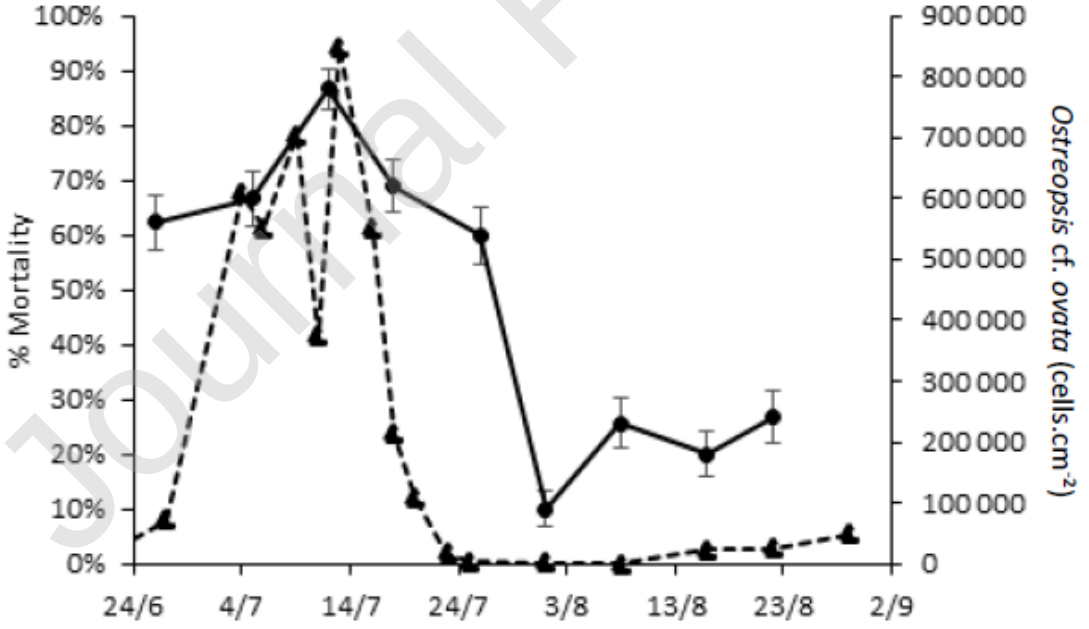


Figure 6: Evolution of the mortality of *Artemia franciscana* (continuous line – Mean ± ES) after 24 hours exposition to 4 cells.mL^{-1} of cells of *Ostreopsis* cf. *ovata* sampled along the bloom in July 2018. The cellular concentration of *O.* cf. *ovata* on artificial substrates is represented in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



Appendices:

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 µE.m^{-2}.s^{-1}	20°C	38	Continuous aeration	0.15 g	No food

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: *In situ* 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	*In situ Ostreopsis* cf. *ovata* 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 µE.m^{-2}.s^{-1}	250 µE.m^{-2}.s^{-1}	250 µE.m^{-2}.s^{-1}	250 µE.m^{-2}.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%

Table 1 : LC*50* values and confidence limits obtained exposing *Artemia franciscana* larvae to different strains of *Ostreopsis* sp. and dissolved toxins (n=6). All values are expressed in cells.mL^{-1} to facilitate comparison across experimental treatment. A theoretical cell concentration was calculated for both OVTX and PLTX in link with the cells toxin content (estimating that one cell of *Ostreopsis* cf. *ovata* contain in average 30 pg.cell^{-1} – Accoroni et al., 2011; Accoroni et al., 2012; Brissard et al., 2014; Ciminiello et al., 2010; Ciminiello et al., 2012; Guerrini et al., 2010; Nascimento et al., 2012; Pistocchi et al., 2011). NaN = 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)