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Toxin content of *Ostreopsis* cf. *ovata* depends on bloom phases, depth and macroalgal substrate in the NW Mediterranean Sea

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Abstract :

Over the last fifteen years, blooms of the genus Ostreopsis have been reported more frequently and at higher abundances in the Mediterranean area. Ostreopsis cf. ovata is known to produce ovatoxins (OVTXs), structural analogues of palytoxin, which is one of the most potent non-polymeric toxins. However, the production of OVTXs is poorly characterized in situ. The present study focuses on toxin content and profile according to the bloom phase during summer 2017 in Villefranche-sur-Mer, France (NW Mediterranean Sea), depth (from 0.5 to 5 m) and three different macroalgal substrates of this epiphytic dinoflagellate (Padina pavonica, Dictyota spp. and Halopteris scoparia). Ovatoxin quantification of all samples was performed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The bloom started at the end of June and declined in mid-July, showing the typical seasonal pattern of the NW Mediterranean Sea area. The peak was observed on the 10 July with 1.8 × 106 cells/g FW and 1.7 × 104 cells/L for benthic and planktonic cells, respectively. Total toxin content of cells, collected using artificial substrates, increased during the exponential and stationary growth phases. After reaching a maximum concentration of 9.2 pg/cell on 18 July, toxin concentration decreased and remained stable from 25 July until the end of monitoring. A decreasing trend of the abundance and of the associated total toxin content was noted with depth. Finally, the decreasing order of maximal epiphytic concentration of O. cf. ovata was: Dictyota spp. (8.3 × 105 cells/g FW), H. scoparia (3.1 × 105 cells/g FW) and P. pavonica (1.6 × 105 cells/g FW). Interestingly, the highest OVTX quota was obtained in cells present on Halopteris scoparia, then on Dictyota spp. and Padina pavonica. This suggests that the nature of the macroalgal substrate influences both growth and toxin production of O. cf. ovata and further work will be required to understand the underlying mechanisms (e.g., competition for nutrition, pH or allelopathic interaction). However, the toxin profiles (i.e., the proportion of each ovatoxin analogue) were not affected by any of the studied parameters (bloom phase, depth, macroalgae or artificial substrates).

Highlights

► *Ostreopsis* cf. *ovata* bloomed from late June to August 2017 at Villefranche-sur-Mer, France, Mediterranean Sea. ► Ovatoxins contents were higher during the exponential and stationary growth phases. ► Highest abundance was on *Dictyota* spp. but highest toxin quota on *Halopteris scoparia*. ► Ovatoxin profile was not affected by the bloom phase, the depth or the macroalgae.

Keywords : Dinoflagellate, Benthic HABs, Ostreopsis cf. ovata, Ovatoxins, Mediterranean Sea

1 Introduction

The benthic dinoflagellate *Ostreopsis*, originally found in tropical areas (Ballantine et al., 1988; Morton et al., 1992; Faust et al., 1996; Parsons and Preskitt, 2007), has now spread markedly in temperate areas, as observed on the coasts of the Mediterranean or New Zealand (Mangialajo et al., 2011; Rhodes, 2011; Parsons et al., 2012; Vassalli et al., 2018).

In the Mediterranean Sea, *Ostreopsis* has been reported in Spain, France, Italy, Croatia, Greece, Cyprus, Lebanon and Tunisia (e.g., Aligizaki and Nikolaidis, 2006; Turki et al., 2006; Totti et al., 2010; Mangialajo et al., 2011; Amzil et al., 2012; Accoroni et al., 2016; Vassalli et al., 2018) and three species have been identified: *O.* cf. *ovata*, *O.* cf. *siamensis* and *O. fattorussoi* (Penna et al., 2010; Accoroni et al., 2016).

Different time windows of blooms can be observed in the northwest Mediterranean Sea from late June to August in southeast France and the Ligurian Sea (Mangialajo et al., 2008; Biré et al., 2013; Cohu et al., 2013; Brissard et al., 2014; Giussani et al., 2017; Meroni et al., 2018) and from July to mid-October on Catalan coasts (Carnicer et al., 2015; Vila et al., 2016), while blooms in the Adriatic Sea tend to occur from the end of July to October (Monti et al., 2007; Totti et al., 2010; Accoroni et al., 2011; Mangialajo et al., 2011; Accoroni et al., 2012; Pfannkuchen et al., 2012; Accoroni et al., 2015a).

Regarding vertical spatial distribution, *Ostreopsis* has mainly been observed between depths of 0.5 and 1 m (Cohu and Lemée, 2012;

Brissard et al., 2014) and, although predominantly epiphytic, cells were found on both biotic and abiotic substrates (e.g., macroalgae, seagrass, sediment, rocks or pebbles) without any clear preference, as well as in the water column (Bomber et al., 1989; Vila et al., 2001; Totti et al., 2010; Accoroni et al., 2011; Hachani et al., 2018).

Blooms of *O.* cf. *ovata* have been associated with mortality and a possible reprotoxic effect in marine organisms (Simoni et al., 2003; Accoroni et al., 2011; Faimali et al., 2012; Guidi-Guilvard et al., 2012; Neves et al., 2018; Pavaux et al., 2019) as well as various mild health symptoms in humans, including skin irritation, respiratory distress, or a cough caused by direct contact with water or exposure to marine aerosols (e.g., Gallitelli et al., 2005; Kermarec et al., 2008; Vila et al., 2008; Tichadou et al., 2010; Tubaro et al., 2011; Pfannkuchen et al., 2012).

Toxicity may result from the production of palytoxin-like molecules, a family of highly potent marine toxins (Poli et al., 2018) including isobaric palytoxin (isob-PLTX) and more than a dozen ovatoxins (OVTX-a to -l) (Ciminiello et al., 2008; Ciminiello et al., 2010; Ciminiello et al., 2012a; Ciminiello et al., 2012b; Brissard et al., 2015; García-Altares et al., 2015; Tartaglione et al., 2016; Tartaglione et al., 2017). OVTXs are produced by both *O.* cf. *ovata* and *O. fattorussoi*, but OVTX-j1, -j2 and -k have only been reported in the latter (Tartaglione et al., 2016; Tartaglione et al., 2017).

O. cf. *ovata* therefore shows high chemodiversity. Indeed, four different toxin profiles were reported for strains isolated from the Mediterranean Sea, differing qualitatively (i.e., in the number of OVTXs) and thus in terms of analogue proportions (Tartaglione et

al., 2017). The most common strain contains OVTX-a as the major analogue, followed by OVTX-b (Brissard et al., 2014; Ciminiello et al., 2014; García-Altares et al., 2015; Carnicer et al., 2016; Ninčević Gladan et al., 2019). The two other profiles were restricted to only four strains out of the fifty-five that were screened including non OVTX-producing strains (Tartaglione et al., 2017). Effects of growth and OVTX-modulating factors on *O.* cf. *ovata* toxin content have been largely studied in laboratory experiments (Guerrini et al., 2010; Pistocchi et al., 2011; Nascimento et al., 2012; Pezzolesi et al., 2012; Brissard et al., 2014; Carnicer et al., 2016). However, data on OVTX content from field studies (e.g., to look at how content depends on bloom phases and biotic or abiotic factors) are scarcer (Pfannkuchen et al., 2012; Accoroni et al., 2017; Ninčević Gladan et al., 2019). Most monitoring studies have focused only on cell abundances during blooms (Brissard et al., 2014; Carnicer et al., 2015; Giussani et al., 2017; Meroni et al., 2018), depending on the depth (Totti et al., 2010; Richlen and Lobel, 2011; Cohu and Lemée, 2012; Cohu et al., 2013; Brissard et al., 2014; Accoroni et al., 2015b; Hachani et al., 2018) or on the macrophyte species used as substrate (Vila et al., 2001; Accoroni et al., 2015b). Toxin content was not analysed in those studies although total OVTX content in field samples shows high variability, with concentrations ranging from undetectable to 75 pg/cell (Accoroni et al., 2011; Pfannkuchen et al., 2012; Brissard et al., 2014; Ninčević Gladan et al., 2019).

This study therefore focused on toxin content and profile at different bloom phases and depths and on different macroalgal host species

(substrates) in Villefranche-sur-Mer, France (NW Mediterranean Sea) during the bloom in summer 2017.

2 Material and methods

2.1 Sampling area and methods

Ostreopsis cf. *ovata* cells were sampled in the small creek at Rochambeau, Villefranche-sur-Mer, France (Figure 1), on the French Mediterranean coast (43°41′34.83″ N; 7°18′31.66″ E) where blooms have been observed regularly since 2006 (Mangialajo et al., 2011; Cohu et al., 2013; Jauzein et al., 2016; Jauzein et al., 2018). This site is a sheltered environment with a rocky shore and a macroalgal community dominated by *Padina pavonica*, *Halopteris scoparia*, *Dictyota* spp. (Phaeophyceae) and *Ellisolandia elongata* (Florideophyceae). The monitoring of *Ostreopsis* cf. *ovata* was realized at three different sampling points, A, B and C, on the Rochambeau shore (Figure 1).

The monitoring of planktonic and benthic cells of *O.* cf. *ovata* was conducted between 6 June and 30 August 2017. For planktonic cell counts, water was sampled 20 cm above the most abundant macroalgae of the station, with a 250-mL plastic bottle. For benthic cell counting, 5–10 g from one individual of the most abundant macroalga located at 0.5 m depth were carefully collected in 250-mL plastic bottles. The sampling was done each week at the three stations (A, B and C), which are situated 10–30 m apart. Depending on the station and time, the most abundant macroalgae was either *Padina*

pavonica, *Halopteris scoparia*, *Dictyota* spp. or *Ellisolandia elongata*. *Dictyota* species were limited to genus determination in order to avoid species misidentification because large intraspecific morphological plasticity of this genus can lead to ambiguous morphological identification criteria (Tronholm et al., 2010).

The methods for sampling, cell collection and counting were as described in Jauzein et al. (2018). Briefly, cells on the macroalgae or artificial substrate were vigorously shaken in the sampling bottle, rinsed twice with 100 mL filtered seawater then filtered through a 500 µm mesh. A 50-mL sub-sample was fixed with acidic Lugol's solution (1% final concentration) before counting. Enumeration was performed using a 1-mL Sedgewick Rafter Counting Cell and a light microscope. For planktonic cells, a sedimentation chamber (50 or 100 mL depending on the abundances) was used before enumeration according to Utermöhl's method (Utermöhl, 1958).

In order to quantify total toxin content per cell during the bloom period, artificial substrates were used to collect cells from 23 June to 17 August 2017. This method, standardized by Jauzein et al. (2016), consists in collecting cells of *O.* cf. *ovata* from the water column on a rectangular piece of window screen (20 × 20 cm and 1.3 mm porosity) fixed on a plastic frame at 50 cm depth and immersed for 24 h. Artificial substrates proved to be particularly suited as they made it possible to obtain a high concentration of *Ostreopsis* cells, with less other microalgae (including diatoms or other dinoflagellates).

To investigate the effects of the macroalgal species on cell abundance and OVTX content, *P. pavonica*, *H. scoparia* and *Dictyota* spp. were

collected in triplicate on 7 July 2017 at 0.5 m depth in the same rocky area (station B), and in less than 1 square metre in order to reduce effects of other biotic and abiotic parameters.

Finally, the importance of depth effect on both cell abundance and OVTX content was assessed by collecting *Ostreopsis* cells from *P. pavonica* at six different depths (0.5, 1, 2, 3, 4 and 5 m from the surface), in triplicate samples for each depth, along a rocky slope on 11 July 2017 (see the transect on Figure 1). *Padina pavonica* was the only macroalga found in abundance along this transect.

To assess the effects of macroalgal substrate and depth on *Ostreopsis* cf. *ovata* abundance, the same methods as described above were used for sampling, cell collection and counting (Jauzein et al., 2018).

2.2 Toxin extraction and analysis by LC-MS/MS

Ostreopsis cf. *ovata* cells were filtrated on GF/F glass microfibre filters (diameter 47 mm, Whatman). Filtrate volume and cell concentration were determined to allow the calculation of toxin content per cell.

Ovatoxins were extracted according to Brissard et al. (2014), with slight modifications. Cells on the filter were extracted with 5 mL MeOH/H_2O (1:1, v/v) with an ultrasonic probe (Vibra Cell 75115, Bioblock Scientific) for 5 min in an ice bath at 30% amplitude (10 sec on, 5 sec off). After centrifugation (4000 *g* for 5 min at 4 °C), the resulting pellet was extracted again and both supernatants were

pooled. A 150-µL aliquot was ultrafiltered (0.2 µm, Nanosep MF, Pall) for toxin quantification by LC-MS/MS.

LC-MS/MS analysis was performed following García-Altares et al. (2015) for the chromatography and following Brissard et al. (2014) for the MS/MS detection on an LC system (UFLC XR, Shimadzu) coupled to a hybrid triple quadrupole/linear ion-trap mass spectrometer (API 4000 Qtrap, AB SCIEX) equipped with an ESI turbospray interface. Detection was carried out by Multiple Reaction Monitoring using the positive mode of acquisition. Three transitions per analogue were monitored for OVTX-a to -f and isob-PLTX: $[M+2H]^{2+}$, $[M+2H-H_2O]^{2+}$ and $[M+3H-H_2O]^{3+}$ → fragment A (Brissard et al., 2014), with a declustering potential of 56 V, collision energy of 31 and 47 eV for doubly and triply charged ions, respectively, and a dwell time of 180 ms. Toxins were separated on a Poroshell 120 EC-C18 column (100 × 2.1 mm, 2.7 µm, Agilent) with its pre-column maintained at 25°C with a flow rate of 0.2 mL/min. Mobile phases consisted of water (A) and acetonitrile/water (95:5, v/v) (B), both containing 0.2% acetic acid. The gradient used was modified from García-Altares et al. (2015): 28–29% B over 10 min, 29–30% B over 5 min, 30–100% B for 1 min and maintained 5 min, then 100–28% B over 1 min, held 4 min for equilibration. Injection volume was 5 µL. In the absence of standards for OVTXs, these were quantified relative to a palytoxin standard (Wako Chemicals GmbH), assuming similar molar response, and expressed in PLTX equivalents. Limit of detection (LOD) and of quantification (LOQ) of the system were 20 and 40 ng/mL, respectively. A minimum of 6.0×10^4 cells per artificial substrate was necessary for toxin quantification

(i.e., to obtain results >LOD with the sensitivity of our LC-MS/MS method).

2.3 Statistical analysis

SigmaPlot (version 14.0) software was used to perform statistical analyses. Normality and homoscedasticity were ascertained by the Shapiro-Wilk and Brown-Forsythe tests. Significant differences of cell abundances and toxin content according to macroalgae and depth were estimated by the analysis of variance (ANOVA) followed by multiple comparison Tukey's HSD post hoc tests.

3 Results

3.1 *Ostreopsis* cf. *ovata* bloom development and cellular toxin content

The benthic bloom (Figure 2A) showed an increase of cell abundance between 6 and 27 June, corresponding to the exponential growth phase. It then entered a stationary phase divided into two sub-phases, the first corresponding to a plateau with maximal cell abundance between 10 July ($9.2 \pm 7.5 \times 10^5$ cells/g FW) and 18 July. Then, because of a heavy swell event (>1 m, see Discussion), abundances decreased and remained low but quite stable ($3.7\text{-}8.0 \times 10^3$ cells/g FW) until 30 August.

Planktonic abundance mirrored benthic abundance, increasing from 6 June to reach $6.0 \pm 7.3 \times 10^3$ cells/L on 27 June. Planktonic

abundance gradually decreased after 12 July and remained quite low until 30 August from 33 ± 30 to 100 ± 34 cells/L.

Total toxin content (Figure 2B) increased linearly from the beginning of monitoring on 23 June until 18 July, thus during the exponential phase and the first 3 weeks of the stationary growth phase of both benthic and planktonic cells. The maximum concentration was 9.2 pg/cell on 18 July, i.e., before the decrease in cell abundances. Then, total toxin content decreased to 3.2 ± 0.7 pg/cell on 25 July and remained low from 1.5 to 3.2 pg/cell until the end of monitoring.

It should be noted that OVTXs were not detected (<LOD) on the 21 July, as the cell abundance on the three artificial substrates was insufficient. Similarly, OVTXs could only be quantified in cells of one replicate on 18 July, and 9 and 17 August, which is the reason that no standard deviation is given for these dates.

3.2 Variability of *Ostreopsis* toxin content with the depth

The depth experiment was performed on 10 July, i.e., during the stationary phase (Figure 2A and B). Cellular abundance (Figure 3A) was found to decrease from 0.5 to 5 m depth ($2.8 \pm 1.8 \times 10^4$ to $1.9 \pm 1.2 \times 10^3$ cells/g FW). Three toxin levels were observed depending on the depth. Maximal abundance was observed at 0.5 m depth, sharply decreased at 1 m depth and remained stable between 1 and 3 m ($1.3 \pm 0.9 \times 10^4$, $1.2 \pm 1.5 \times 10^4$, $1.4 \pm 1.0 \times 10^4$ cells/g FW at 1, 2 and 3 m, respectively). Finally, the cell abundance decreased slightly at 5 m depth to reach $1.9 \pm 1.2 \times 10^3$ cells/g FW.

The trend of total toxin content (Figure 3B) decreased with depth from 25 ± 18 pg/cell at 0.5 m to 14 ± 4.1 pg/cell at 4 m, while no OVTX was detected at 5 m (<LOD), although the large standard deviations mean that no significant difference was observed.

The OVTX profile did not change with depth (Figure 4) except at 2 and 4 m where the quantity of cells was too low for the detection of all analogues (<LOD). The main OVTX produced was OVTX-a followed by OVTX-b (48–56% and 16–32% respectively). Between 0.5 and 3 m, OVTX-c varied from 16 to 9%, OVTX-d from 22 to 9% and OVTX-e from 10 to 17%, without any clear trend. OVTX-c and OVTX-d were not quantifiable (<LOQ) at 2 and 4 m depth. The concentration of all toxins was below LOD at 5 m depth. Neither isob-PLTX nor OVTX-f were detected (<LOD).

3.3 Variation of *Ostreopsis* toxin content with macroalgal substrate

The abundance of epiphytic *O.* cf. *ovata* cells (Figure 5A) on *Halopteris scoparia* and *Padina pavonica* was significantly lower than on *Dictyota* spp. ($3.1 \pm 2.5 \times 10^5$ and $1.6 \pm 0.6 \times 10^5$ vs. $8.3 \pm 2.4 \times 10^5$ cells/g FW, respectively, *p-values = 0.01* and *0.04*). No significant difference was observed between the abundances of epiphytic *O.* cf. *ovata* cells on *Halopteris scoparia* and *Padina pavonica* (*p-value = 0.53*).

Toxin quantification (Figure 5B) showed that OVTX content was significantly higher in epiphytic cells found on *H. scoparia* with 29 ± 6.1 pg/cell (*p-value = 0.002* and *0.01* when compared with *P. pavonica* and *Dictyota* spp., respectively). The cells collected on *P.*

pavonica and *Dictyota* spp. contained 19 and 4% less OVTXs, respectively, and no significant difference was found between these two macroalgae (*p-value = 0.29*).

For all samples, the main toxin quantified was OVTX-a (74–87%) followed by OVTX-b and -e (Figure 6). Traces of ovatoxin-c and -d were only detected for the epiphytic cells on *H. scoparia* and *Dictyota* spp. (2.5 and 2.7%, 1.9 and 2.0% for *Dictyota* spp. and *H. scoparia*) while no OVTX-c and -d were detected for cells on *P. pavonica.* Neither isob-PLTX nor OVTX-f were detected (<LOD).

4 Discussion

In the present study, the high variability of results (shown by the large standard deviations) could be explained by the extremely patchy benthic distribution of *Ostreopsis,* affecting the replicability of sampling and ultimately the results (i.e. counts, toxin content per cell, statistical analysis) (Mangialajo et al., 2008; Cohu et al., 2013; Jauzein et al., 2018). Indeed, Jauzein et al. (2018) observed that *Ostreopsis* abundance could vary by up to a factor of four among replicates collected on the same macroalgal species in the same 1 m² of seabed.

4.1 Temporal development of the bloom

Ostreopsis has been described as having a clear seasonality pattern in the Mediterranean area (Rhodes et al., 2000; Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Pistocchi et al., 2011; Cohu et al., 2013). In the present study, the benthic bloom was initiated in late

June and reached a maximum benthic and planktonic cell concentration on 10 July 2017.

Rochambeau is one of the most monitored locations on French coasts where *Ostreopsis* blooms have regularly been observed (Mangialajo et al., 2011; Biré et al., 2013; Cohu et al., 2013; Brissard et al., 2014; Jauzein et al., 2016). During the last decade, blooms have seemed to follow the same seasonal pattern each year. Indeed, between 2009 and 2015, the peaks occurred during the last two weeks of July (Biré et al., 2013; Brissard et al., 2014; Biré et al., 2015; Vassalli et al., 2018). The bloom of 2017 was therefore somewhat early and was also observed in Genoa (Ligurian Sea, Italy) the same year with maximal abundance (2.9×10^6 cells/g FW) recorded on 4 July (Meroni et al., 2018). Similarly, both blooms declined after mid-July, thus during the expected bloom peak period. Meroni et al. (2018) observed the same decrease in Genoa and suggested that the decrease could be linked to a stormy event. Indeed, hydrodynamism is known to adversely affect the bloom development (Mangialajo et al., 2008; Totti et al., 2010; Accoroni et al., 2012; Meroni et al., 2018) and a turbulent sea (i.e., swell > 1 m) was observed in Rochambeau on 21 July (i.e., bloom decline).

In the present case, there was a good correlation between planktonic and benthic abundances ($R^2 = 0.86$, see Supplementary data S1) as regularly observed in previous studies (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008; Mangialajo et al., 2011; Cohu et al., 2013; Jauzein et al., 2018).

During the bloom of summer 2017, the OVTX content increased during both exponential and stationary phases until the 18 July,

which is similar to several laboratory studies (Guerrini et al., 2010; Vanucci et al., 2012; Brissard et al., 2014). This was not, however, in agreement with Mendes et al. (2017) who reported no difference. Contrasting results were also observed in field studies. Indeed, Accoroni et al. (2017) reported a maximum toxin concentration before the bloom peak, and Pfannkuchen et al. (2012) observed a constant concentration during the bloom. Variation of toxin content according to bloom phase is still not clear from either laboratory or field studies, suggesting that some other factors could modulate the production of OVTXs.

4.2 Variability of *Ostreopsis* abundances and total toxin content with depth

In the present study, the highest cell concentration was observed at the shallowest depth (0.5 m), in agreement with previous studies in Rochambeau (Cohu and Lemée, 2012; Cohu et al., 2013; Brissard et al., 2014) and on Italian (Totti et al., 2010) and Tunisian coasts (Hachani et al., 2018). The decrease in *Ostreopsis* abundance below 3 m depth seems to be common as this trend has already been reported by several authors (Totti et al., 2010; Cohu and Lemée, 2012; Cohu et al., 2013). This trend was also reported in the Caribbean Sea for *Ostreopsis* spp. by Boisnoir et al. (2018) who found a 14-fold difference between 0.5 and 1 m. Then, between 3 and 10 m, no cells were observed on some of the sites.

Brissard et al. (2014) showed a decrease in toxin content with depth in cells sampled on *Padina pavonica*. They reported 23, 11 and 9 pg/cell of OVTXs at 0.5, 1 and 3 m depth, although no similar

decreasing trend was observed with depth in the present study. Nevertheless, the total toxin content was still of the same order, with average concentrations varying from 12 to 35 pg/cell. Differences in *Ostreopsis* abundance and toxin content from 0.5 to 5 m depth may be caused by differences in temperature or light availability (Granéli et al., 2011; Cohu and Lemée, 2012; Scalco et al., 2012). In the bay of Villefranche-sur-mer, Cohu and Lemée (2012) reported photosynthetically available radiation values ranging from around 950 and 400 µmol photon/m^2/s between the sub-surface and 5 m depth. Moreover, they noted that the water column was stratified with the first thermocline between 3 and 8 m depth.

4.3 Variation in *Ostreopsis* toxin content with macroalgal substrate

The benthic cells of *Ostreopsis* cf. *ovata* can be found on sediments, rocky substrates, molluscs, macroalgae or seagrass, meaning that this dinoflagellate is not an obligate epiphyte (Bomber et al., 1989; Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Totti et al., 2010). Benthic blooms are routinely monitored on macroalgae and a huge diversity of species has been sampled to date among the Phaeophyceae, Florideophyceae and Chlorophyceae classes.

Some studies investigated substrate preferences of *O.* cf. *ovata* in detail (e.g., Aligizaki and Nikolaidis, 2006; Totti et al., 2010; Blanfuné et al., 2015; Yong et al., 2018); however, to our knowledge, there are as yet no publications examining the impact of the substrate on OVTX production. The present study therefore focused on the three most common macroalgae encountered in Rochambeau

(Villefranche-sur-Mer, France) in July 2017, namely the Phaeophyceae *Dictyota* spp., *Halopteris scoparia* and *Padina pavonica*, which have different morphologies, to study the effect of substrate species on cellular abundance and OVTX content and profile.

On 7 July (i.e., during the first part of stationary growth phase of the 2017 bloom in Rochambeau), *O.* cf. *ovata* was significantly more abundant on *Dictyota* spp. as on *P. pavonica* (around 3–5 times), and twice as abundant on *H. scoparia* . In some studies of *Ostreopsis* sp., however, no clear substrate preference was reported (Vila et al., 2001; Cohu et al., 2013; Shah et al., 2013; Selina et al., 2014). The results of the present study confirm the ability of *Dictyota* spp., and to a lesser extent *H. scoparia*, to support a high abundance of *O.* cf. *ovata* on the eastern part of the French Mediterranean coastline during summer blooms (Cohu et al., 2013; Blanfuné et al., 2015).

In the rest of the Mediterranean Sea, contrasting results have been reported with, for example, the highest abundance of *Ostreopsis* spp. on *H. scoparia* on the Catalan coast of Spain (Vila et al., 2001) or a higher abundance on *P. pavonica* than on *Dictyota dichotoma* in the Gulf of Tunis (Hachani et al., 2018). These differences may have been caused by the experimental protocols, the presence of an *Ostreopsis* species that is not *O.* cf. *ovata* (e.g., *O.* cf. *siamensis* in Penna et al. (2005), according to Pistocchi et al. (2011)), different assemblages of macroalgae or hydrodynamism in distinct regions of the Mediterranean Sea, or the spatial (i.e., from local to regional scale) and temporal variability of *Ostreopsis* blooms (Vila et al., 2001; Mangialajo et al., 2011; Cohu et al., 2013; Blanfuné et al.,

2015). Indeed, in this last case, Cohu et al. (2013) did show a significant interaction between macroalgae and time, meaning that abundances of *O*. cf. *ovata* varied according to the sampling period. Another factor that may contribute to controversies is the level and confidence of species identification within the *Dictyota* genera. This was highlighted by Blanfuné et al. (2015), who observed negative correlations depending on the species (i.e., two species of *Dictyota dichotoma* and *Dictyota fasciola*) when analysing the abundance of *Ostreopsis* on macroalgae.

Factors controlling the settlement of *Ostreopsis* on macroalgae are still poorly understood (Blanfuné et al., 2015). Some authors have suggested that *Ostreopsis* prefers macroalgae with branched, three-dimensional thalli and a high surface/volume ratio over non- or less branched macrophytes (Lobel et al., 1988; Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Totti et al., 2010; Selina et al., 2014). The present study confirms this hypothesis as there was a higher abundance of *Ostreopsis* on the two branched macroalga compared with *P. pavonica*, which displays fan-shaped thalli. Allelopathic interactions have also been suggested to play a role (Accoroni et al., 2015b; Blanfuné et al., 2015) and may explain the significant difference of OVTX content among cells collected on the three Phaeophyceae on the same day and in the same area in this study. Indeed, OVTX concentration was higher for epiphytic cells on *H. scoparia* than on *Dictyota* spp. and *P. pavonica*, with concentrations of 29, 11 and 5.1 pg/cell, respectively. It should be noted that three days later (i.e., when studying the impact of depth at the bloom peak), OVTX content in cells collected on *P. pavonica* at 0.5 m depth

was found to reach 25 pg/cell. On 10 June, cells on artificial substrates contained five times less toxin than those on *P. pavonica*, supporting the idea that other factors could influence toxin production. We hypothesized that, as the artificial substrates caught *Ostreopsis* cells coming from a diversity of macroalgae (which can have different OVTX contents), this may have increased the variability and difference in toxin content per cell compared with levels in *Ostreopsis* from only one species of macroalgae.

Comparison of the variation of OVTX content among substrates with previous research is hampered by the scarcity of available data and the diversity of macroalgae sampled. A wide range of concentrations was reported, from undetected to 75 pg/cell (Accoroni et al., 2011; Pfannkuchen et al., 2012; Brissard et al., 2014; Accoroni et al., 2017; Ninčević Gladan et al., 2019). The only study that used one of the macroalgae sampled here (Brissard et al. (2014) found a toxin concentration of 23 pg/cell on *Padina pavonica* in 2011 in Rochambeau (i.e., a level of concentration similar or up to five times higher than the current study). In order to improve assessments of the way substrate effects OVTX production, quantification of OVTXs should be systematic in future studies.

Allelopathy is a natural and complex phenomenon to study *in situ* due to the effect of other environmental parameters (e.g., nutrient competition, pH, light availability, temperature or hydrodynamism) that could interfere or hide the allelopathic effect (Keating, 1977). However, Phaeophyceae are known to produce algicidal molecules, e.g., diterpens production by *Dictyota dichotoma* (Kim et al., 2014), phlorotannins by *Ecklonia* (Nagayama et al., 2003) or glycerolipids

by *Ishige sinicola* (Hirao et al., 2012), with the last two classes of molecules showing algicidal activity on five different HAB species. Interestingly, in a controlled laboratory co-culture experiment, Accoroni et al. (2015b) showed that *Dictyota dichotoma* induced a complete algicidal effect on *Ostreopsis* cf. *ovata*. This bioactivity cannot explain the maximal abundance observed on *Dictyota* spp. in the current study. However, the species or variety of *Dictyota* present in Rochambeau may differ from the one growing in the Adriatic Sea. It was also hypothesized that both the changing of environmental conditions affecting the health of the host (e.g., epiphytes, herbivores and pathogens) and *Dictyota* life stage may change the bioactive compounds released, allowing a suitable environment for epiphyte growth or not (Parsons et al., 2011).

The overall divergent results on *Ostreopsis* preferred substrates show that interactions between macroalgae and *Ostreopsis* are still unclear, suggesting that other parameters than morphology of the macroalgae and allelopathic interaction should be studied, including predation (Fraga et al., 2012; Blanfuné et al., 2015). Ultimately, the chemical ecology between *Ostreopsis* and its substrates should be investigated further.

4.4 Toxin profiles

In this study, the OVTX content was assessed according to bloom phase and to the depth and species of the hosting macroalgae. The toxin profiles of cells collected on artificial substrates and macroalgae were typical, with a major proportion of OVTX-a (48–75%), followed by OVTX-b (12–26%), OVTX-e (5–17%), OVTX-d

(2–9%) and OVTX-c (2–8%). In some cases (e.g., profiles for cells on *P. pavonica* sampled at 2 and 4 m depth on 7 July, Figure 4 and profile for cells on *P. pavonica* sampled on 10 July, Figure 6), OVTX-c, -d or -e could not be quantified due to either the low amount of cells or a combination of low amount of cells and low OVTX content. Such an issue has already been reported (Accoroni et al., 2017) and may bias comparisons of OVTX profiles if not properly taken into consideration.

In a valuable study by Tartaglione et al. (2017), the chemodiversity of OVTXs in the Mediterranean Sea was well characterized and the strains clustered into four profiles. The profile obtained here corresponds to the "profile 1" described by Tartaglione et al. (2017), which seems predominant for both lab-cultured strains (67% of the strains screened in Tartaglione et al., 2017) and cells collected *in situ*, including from Villefranche-sur-mer (Accoroni et al., 2011; Pfannkuchen et al., 2012; Brissard et al., 2014; Ninčević Gladan et al., 2019).

However, the proportion of each analogue was different between cells collected on *P. pavonica* on 10 July (Figure 4) and on *Dictyota* spp. and *H. scoparia* on 7 July (Figure 6). The first profile was in good agreement with "profile 1" defined by Tartaglione et al. (2017) but the second was somewhat different, with a higher proportion of OVTX-a compared with other analogues (i.e., mean of 78% vs. 56% in Tartaglione et al. (2017)).

Some other differences were noted between the profiles obtained here and those previously reported. Indeed Brissard et al. (2014) observed two analogues (isob- PLTX and OVTX-f) in cells from

Rochambeau that were screened for but not detected in the present work. OVTX-f is not common, only being observed in minor amounts in some strains (Brissard et al., 2014; Tartaglione et al., 2017); it is therefore not surprising that it was not detected here. Concerning isob-PLTX, previous *in situ* studies reported concentrations of less than 1% (Pfannkuchen et al., 2012) and up to 15% (Accoroni et al., 2011; Brissard et al., 2014; Accoroni et al., 2017).

The factors influencing OVTX profiles and explaining these discrepancies and potential differences between cells growing in a natural environment compared with the lab should therefore be investigated more thoroughly.

Toxin profiles did not vary significantly among cells collected during the bloom (i.e., on artificial substrates, see Supplementary data S2), among cells collected on *P. pavonica* according to depth or among cells attached to the three macroalgae. Similar observations have previously been made *in situ* (Accoroni et al., 2011; Pfannkuchen et al., 2012; Brissard et al., 2014) and in controlled experiments (Pezzolesi et al., 2012; Vanucci et al., 2012; Pezzolesi et al., 2014; Carnicer et al., 2016; Pezzolesi et al., 2016; Mendes et al., 2017). Taken together, these past and present results suggest that OVTX profiles may be constitutive, as neither abiotic nor biotic factors studied so far modify them, although this hypothesis requires further investigation.

5 Conclusion

The present study confirmed the seasonal pattern of recurrent *Ostreopsis* blooms in the NW Mediterranean area even though the bloom of 2017 in Rochambeau appeared earlier, with a proliferation from the end of June to August in 2017. Cells collected using artificial substrates accumulated OVTXs during exponential and stationary phases of the bloom. Concerning the effect of depth, *Ostreopsis* mainly grows in shallow areas with a highest toxin content near the surface. The comparison of macroalgae highlighted that when monitoring a bloom, a change in macroalgal substrate (e.g., due to disappearance or mortality) may have a significant impact on both cellular abundance and OVTX content, with up to a six-fold change observed in this study. Alternatively, the deployment of artificial substrates proved to be a highly robust and representative method for bloom monitoring (Jauzein et al., 2018) and for total toxin content analysis in this study. Overall, relative proportions of each analogue seemed not to be affected by the bloom phase, depth or macroalgal substrate, suggesting that OVTXs might be constitutive.

Further investigations will be needed to understand the influence of macroalga species on toxin content and to evaluate the risk arising from different macroalgal assemblages in the Mediterranean Sea. Moreover, the present work provides new toxin content data depending on the bloom phase that may help decision-makers make better assessments of exposure-derived risks during blooms that coincide with the tourist season.

6 Conflict of Interest

The authors declare that there are no conflicts of interest.

7 Author contributions

Conception and design of the experiments: MPG, VS, SB, RL, ZA. Experiments: MPG, FH, ASP, MT, RL. Data analysis: MPG, DR, RL. Supervision of experimental work: VS, SB, RL, ZA. Writing of the manuscript: MPG, DR. All authors amended and corrected the paper.

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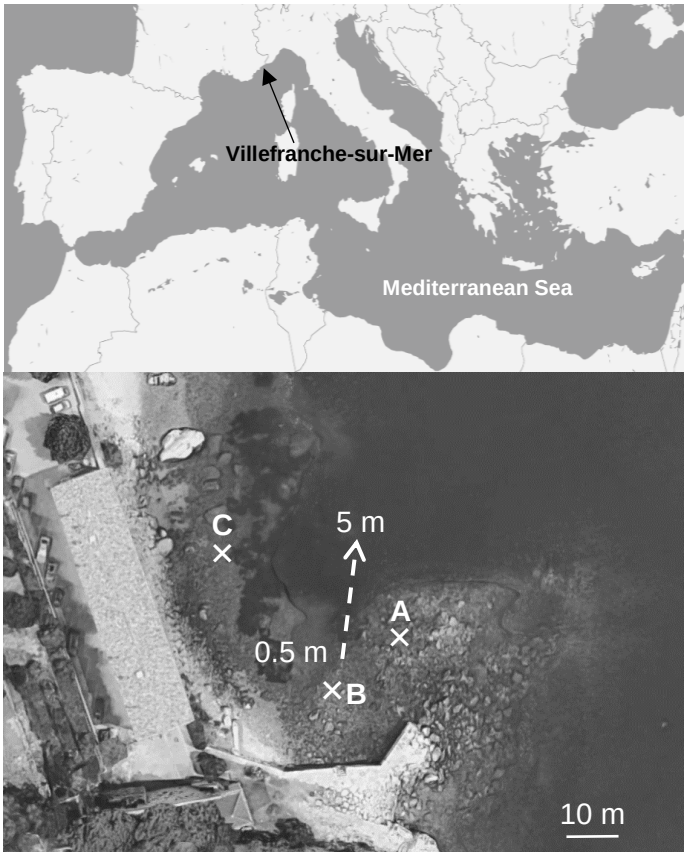


Figure 1: Sampling was performed at three stations (A, B, and C) at Rochambeau, Villefranche-sur-Mer, France (NW Mediterranean Sea). Dotted line shows the slope where *Padina pavonica* was sampled from 0.5 m to 5 m depth.

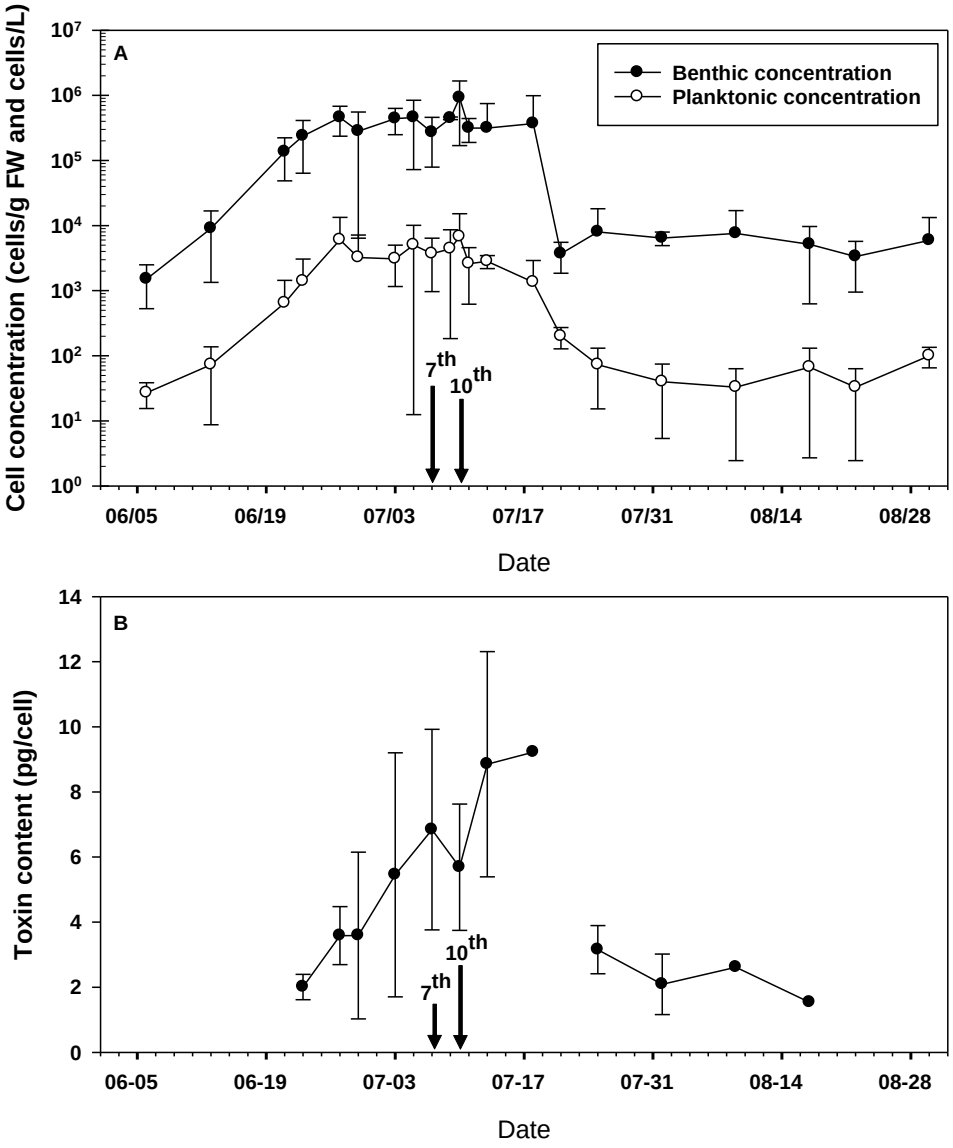


Figure 2: (A) Monitoring *Ostreopsis* cf. *ovata* blooms during summer 2017 in Rochambeau. Means stations A, B and C were expressed in cells/g FW and cells/L for benthic and planktonic abundances. (B) Mean total toxin content of epiphytic cells sampled on artificial substrate (pg/cell). Error bars represent standard deviations (n = 3). Arrows show the day of sampling to study the importance of the macroalgae (7^{th}) and the depth (10^{th}) on abundance and toxin content.

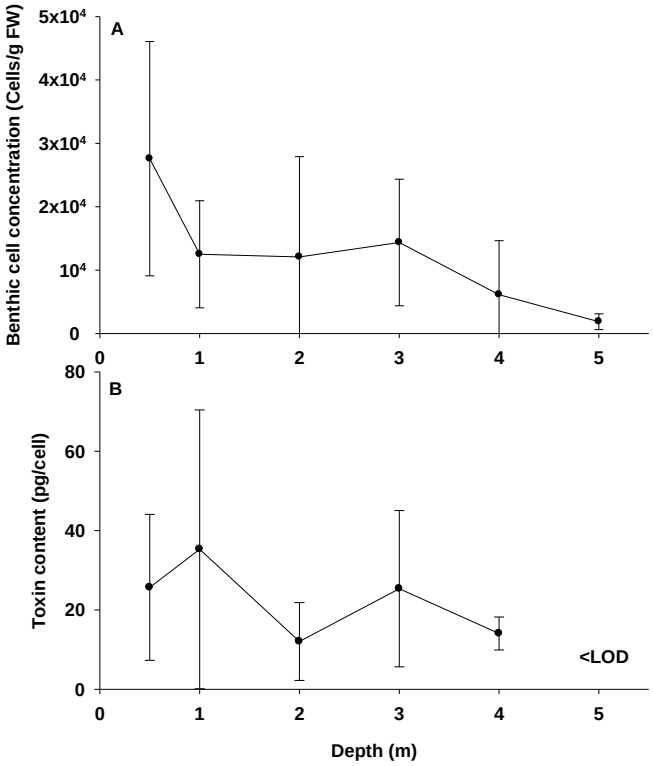


Figure 3: (A) Mean benthic cell abundance and (B) mean OVTX content of the epiphytic *O.* cf. *ovata* cells on *P. pavonica* from 0.5 m to 5 m depth. No significant difference was observed (*p-value = 0.30* and *0.20*, n = 3, for toxin and cell concentration). Error bars represent standard deviations.

FIGURE 4 COLORFUL ONLY ON WEB

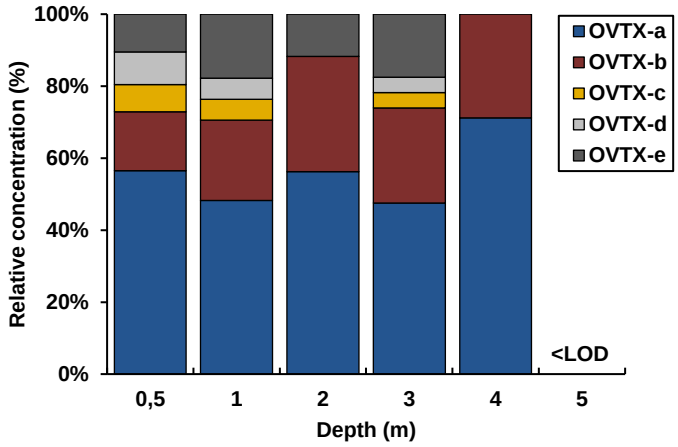


Figure 4: Relative concentration (%) of OVTX-a to -e for each depth (isob-PLTX and OVTX-f <LOD).

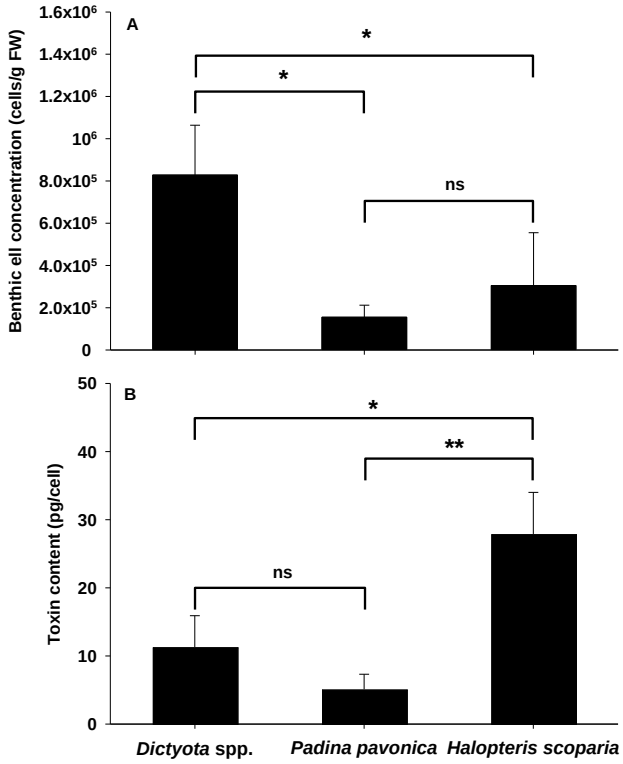


Figure 5: (A) Mean epiphytic cell abundance (cells/g FW) sampled on *Dictyota* spp., *Padina pavonica* and *Halopteris scoparia* (n = 3) and (B) mean total toxin content of the associated *Ostreopsis* cells (n = 3) on 7 July 2017. ns: non-significant, * p<0.05, ** p<0.01. Error bars represent standard deviations.

FIGURE 6 COLORFUL ONLY ON WEB

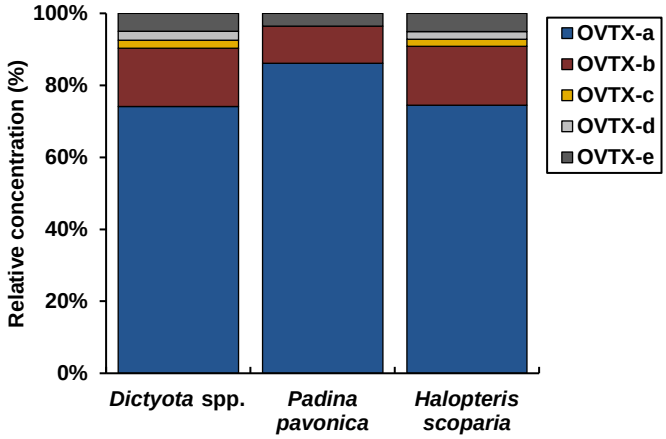
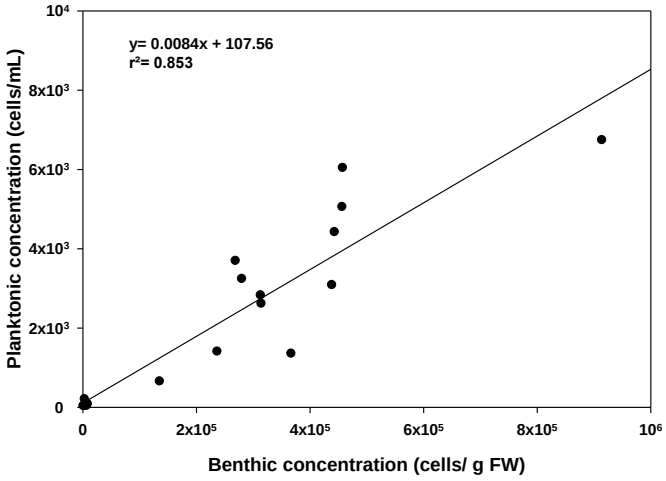
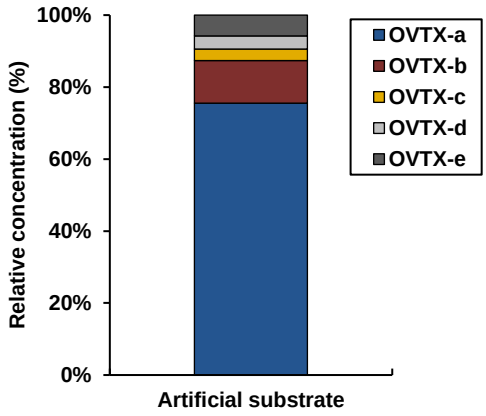


Figure 6 : Relative concentration (%) of OVTX-a to -e for cells epiphytic collected on *Dictyota* spp., *P. pavonica* and *H. scoparia* (isob-PLTX and OVTX-f <LOD).



Supplementary data S1: Linear regression between mean of benthic and planktonic abundances of cells sampled on site A, B and C in Rochambeau.



Supplementary data S2: Relative concentration (%) of OVTX-a to -e for cells collected on artificial substrates (isob-PLTX and OVTX-f <LOD).