Universitat Autònoma de Barcelona

MÁSTER UNIVERSITARIO EN ZOONOSIS Y UNA SOLA SALUD

Cyclodextrins as capture agents of lipophilic marine toxins

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ABSTRACT

Seafood contamination with marine toxins due to harmful algal blooms (HABs) is a global public health issue on the rise. Most countries have monitoring programs for the detection of toxins in shellfish of toxic phytoplankton in seawater to prevent consumer intoxications. The use of solid phase adsorbent and toxin tracking (SPATT) technology as toxin detection straight from the aquatic environment could complement the labour-intensive traditional monitoring methods. In this work, several types of cyclodextrins (cyclic oligomers with a conical structure and an internal cavity) have been evaluated as novel materials for SPATT. Cyclodextrins were tested at Masnou harbour (Catalonia, NW Mediterranean) during a *Dinophysis* sp. bloom. The cyclodextrins and the commercial Diaion (HP-20) were deployed twice for a 1-week period at five different locations of Masnou harbour. At the time of the experiment, *Dinophysis* sp. reached abundances as high as 91 341 cells /L.
Successful accumulations of the lipophilic marine toxins okadaic acid (OA) and pectenotoxin-2 (PTX2) were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Higher levels of PTX2 were found in all cyclodextrins whereas OA and PTX2 contents were similar in the commercial resin. Accumulation of OA was higher in the commercial resin than in cyclodextrins, but these last proved best for PTX2 adsorption. A clear correlation between cell abundance and toxin accumulation was observed.

1. Introduction

In the last decades harmful algal blooms (HABs) have been increasing and spreading with more frequency and geographically (McCarthy et al., 2014; Gobler et al., 2020). Different oceanographic and environmental conditions are affecting the proliferation of phytoplankton, such as water temperature, salinity, sunlight, nutrients, wind and current direction. Climate change combined with anthropogenic pressure including tourism and fishery activities are also contributing to these phenomena (Fan et al., 2014; Gobler et al., 2017; Roué et al., 2018)

Marine toxins are secondary metabolites produced by toxic phytoplankton that may bioaccumulate in fish and shellfish, specially in filter feeding bivalves such as mussels, oysters, clams and scallops rendering them toxic for human consumption (Pizarro et al, 2013). Additionally, they are also a danger to the wildlife (Rundberget et al., 2007). Lipophilic marine toxins are produced by dinoflagellates of the genus *Dinophysis* spp. and *Prorocentrum* spp. (Wang et al., 2020). Some of them, such as okadaic acid (OA) (Valdiglesias et al., 2013) and its derivatives dinophysistoxins (DTXs) are responsible for diarrhetic shellfish poisoning (DSP) in humans (Fux et al., 2008; Pizarro et al., 2009; Rodríguez et al., 2015). *Dinophysis* spp. are also producers of pectenotoxin-2 (PTX2) (Pizarro et al., 2008) According to the Global legislation of marine toxins, both OA and PTX2 (Fig. 1) have the same EU limit of consumption (160 μg/kg) (EURLMB). Even though PTX2 has no recorded cases of human intoxication (Li Z. et al., 2010), keeping in mind that in cases like gastrointestinal illnesses such as DSP, clinical testing is rarely done and the illness is easily mistaken by a bacterial or viral infection, it is safe to say that there is an underestimation of intoxication cases due to the lack of clinical testing.

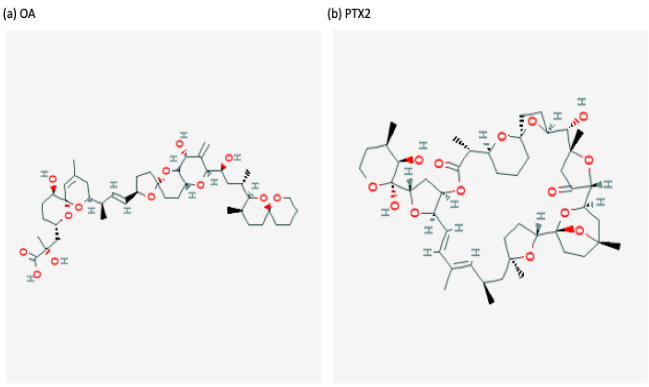


Fig . 1. Structures of (a) okadaic acid (OA) and (b) pectenotoxin-2 (PTX2)

Contamination of seafood by marine toxins is an international public health issue as well as an issue for the seafood industry (Rundberget et al., 2009; James et al., 2010). Therefore, the presence and contents of these toxins in shellfish have been regulated in the EU, as well as the methods to be used for their control. Besides, many countries have implemented monitoring programs based on toxic phytoplankton counts. In some countries and regions, toxins in shellfish flesh and toxic phytoplankton in seawater are monitored in parallel (Fux et al., 2009; Fernández et al., 2019). Thus, the availability of sensitive early warning monitoring techniques is imperative for both consumer health protection and reduction of economic loss (Li A. et al., 20011; Estevez et al., 2019). Solid phase adsorption and toxin tracking (SPATT) technology has shown potential as an alternative monitoring tool and early warning system (MacKenzie et al., 2004). The SPATT passive samplers remove disadvantages and fast-track the toxin identification process. Direct capturing adsorption of toxins from the water column with SPATT technology provides a time, cost and labour efficient method.
In this study, cyclodextrins were evaluated as novel adsorbents and potential passive samplers for the accumulation of marine lipophilic toxins. Cyclodextrins are cyclic oligosaccharides composed of six (α), seven (β) or eight (γ) glucose units linked by glucosidic bonds (Fig. 2) (Crini et al., 2018), their hydrophobic inner cavity allowing guest molecules to enter and be captured within these macromolecules. Four different cyclodextrins were compared (β-epichlorohydrin (β-EPI), γ-epichlorohydrin (γ-EPI), β-hexamethylene diisocyanate (β-HDI), γ-hexamethylene diisocyanate (γ-HDI)) as well as the commercially available Diaion® (HP-20).

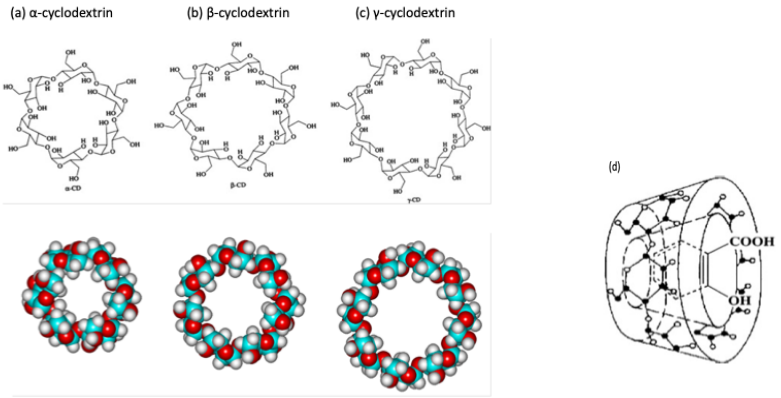


Fig. 2. (a-c) Cyclodextrins per size (α, β, γ) and d) the hydrophobic inner cavity of the conical cyclodextrin seen from the side. Note that in this study α-cyclodextrin has not been used.

The SPATT discs were deployed at the Masnou harbour where weekly phytoplankton monitoring is carried out and with a history of *Dinophysis* spp. blooms, common dinoflagellates found in the Mediterranean sea as well as around the European part of the Atlantic ocean (Cañete et al., 2008).

Identification of the toxins was conducted by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). For decades method development was slowed down due to the complex nature of the toxins produced by microalgae and the lack of reference material. Nevertheless, since 2011 the LC-MS/MS has become the EU reference method for the analysis of marine lipophilic toxins (No 15/2011) (García-Altares et al., 2013; Zedong et al., 2015).

The objectives of the study were to evaluate the behaviour of cyclodextrins under natural parameters, to evaluate the potential of these novel cyclodextrins as a monitoring tool and early warning system for lipophilic marine toxins and to better understand their behaviour when introduced into the natural environment. Specific objectives were to observe toxin accumulation differences depending on the cyclodextrins and their size, to understand the accumulation capabilities depending on toxins, to confirm if toxin esters were present, and finally, to evaluate if matrix effects were involved in the analysis by the LC-MS/MS.

2. Materials and methods

2.1. Chemicals, reagents and standards

Certified reference standard solutions of okadaic acid (OA: 14.3 µg/mL) dinophysistoxin-2 (DTX2: 15.1 µg/mL) pectenotoxin-2 (PTX2: 8.6 µg/mL) obtained from the Institute for Marine Bioscience of the National Research Council (NRC) from Halifax (Canada). HPLC grade methanol (MeOH), LC-MS grade MeOH and LC/MS hypergrade acetonitrile (ACN) 99.9% were obtained from Merck (Darmstadt, Germany). Ammonium hydroxide (NH_4OH) 67 mM 25% and sodium hydroxide (NaOH) 2.5 M were obtained from Riedel-de Haën (Seelze, Germany). Hydrochloric acid (HCl) 2.5 M was purchased from Sigma-Aldrich (St. Louis, MO). Milli-Q purified water produced in-house, quality at 18 MΩ/cm (Millipore, Bedford, MA).

2.2. Adsorbent resins

Cyclodextrins evaluated: β-epichlorohydrin (β-EPI), γ-epichlorohydrin (γ-EPI), hexamethylene diisocyanate (HDI), β-hexamethylene diisocyanate (β-HDI) and γ-hexamethylene diisocyanate (γ-HDI). The cyclodextrins were obtained through a collaboration with Dr. Fragoso and Dr. Torréns from Universitat Rovira i Virgili (Tarragona, Spain). The DIAION® HP20 was obtained from Sigma-Aldrich (Tres Cantos, Madrid, Spain).

2.3. SPATT design and deployment

In the first field trial (W1), 50 SPATT discs were deployed at Masnou harbour from 14.2.2020 until 21.02.2020 for a period of 1 week at 5 different points (P1, P2, P3, P4 and P5) at two different depths, in the second field trial (W2), 50 SPATT discs were deployed from 21.2.2020 until 28.02.2020 under the same conditions. In Fig. 3, the sampling locations are placed in a map and in Fig. 4 . the SPATT discs and their deployment are shown.

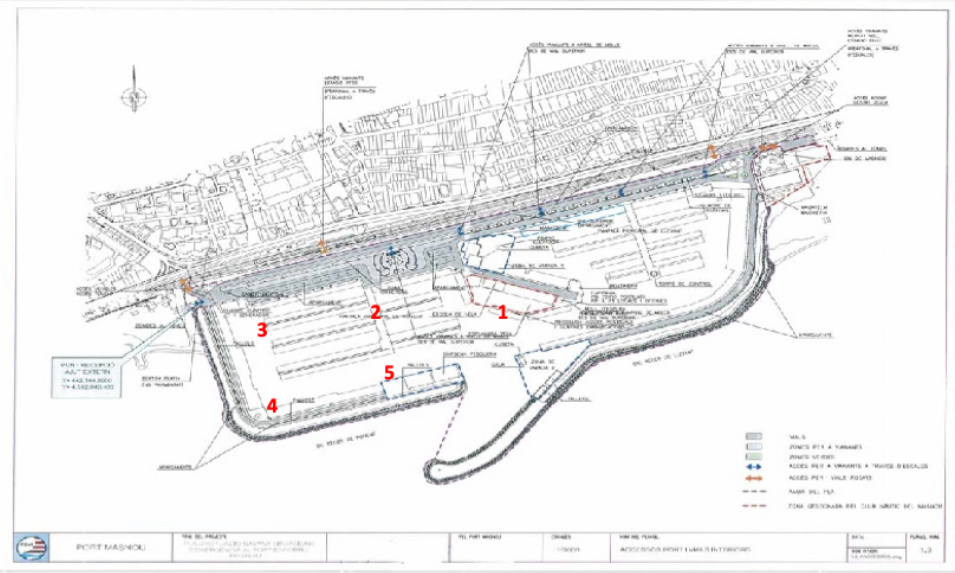


Fig. 3. Masnou harbour with each point (P) marked (points 1, 2, 3, 4 and 5).

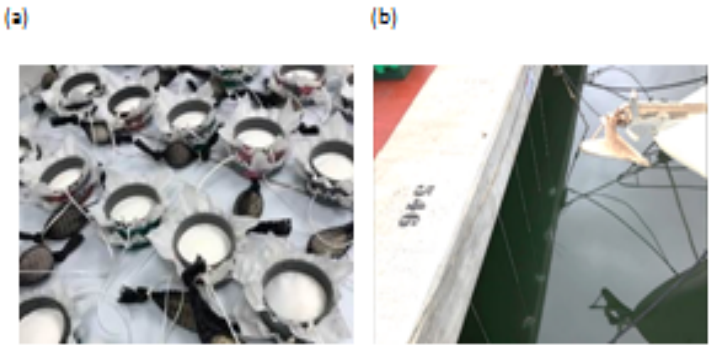


Fig. 4. (a) Image of SPATT discs and (b) SPATT discs immersed in the water at Masnou harbour, five lines are seen where each resin is located at two depths (1m and 1.5m)

Solid phase adsorbent and toxin tracking (SPATT) discs were prepared from five different resins (β-EPI, γ -EPI, β-HDI, γ -HDI and Diaion). Each disc containing 10 g of resin (Zendong et al., 2016) was placed between two sheets of nylon mesh 1 µm in pore size, the discs were held together using embroidery rings. Two SPATT discs were placed on a string and deployed at two different depths, 1 m and 1.5 m from the surface. The SPATT passive samplers were deployed at the port of Masnou, coordinates 41º 28' 30" N / 02º 18' 47" E where known *Dinophysis* spp. blooms occur and weekly HAB monitoring is carried out. At the end of the field trials all SPATT devices were immersed in Milli-Q water in plastic bottles for their transport to the laboratory for their extraction.

2.4. Sample extraction and preparation

After the removal of the SPATT discs immersed in the sea, the discs were rinsed in 500 mL Milli-Q water during 30 min while stirring vigorously for removal of any salts or debris adhered to the nylon mesh. Carefully opening the SPATT discs, the resin was removed and stored in 10 mL of MeOH for all resins except for the Diaion where 20 mL of MeOH was used. All samples were stored at -20°C until extraction.
For desorption of the toxins, resins and methanol (HPLC grade) were transferred into a beaker with a ratio 1:8 mL/g resin to solvent. Avoiding resin sedimentation, the extracts were left stirring for 2 h (Fux et al. 2008). Then resins were poured into low frequency polyvinyl chloride (LPVC) (100 mL) plastic filtration columns containing a filtration mesh of 1 µm pore size fixed on the manifold applying vacuum (Vac-Elut SPE vacuum manifold (Varian, Harbor City, CA, USA)).
Another 20 mL of MeOH was added and the extracts were collected in 100 mL glass bottles. The final volume of each extract was 100 mL with methanol HPLC grade. *Note: cyclodextrins required an extra 20 mL of MeOH for a collection of 100 mL final extract. The samples were evaporated at 90 °C for 2-4 hours (depending on the resin) until 1.5 mL using a Syncore Buchi (Flawil, Switzerland). All extracts were adjusted to 4 mL with MeOH. These extracts were filtered by 0.2-µm PTFE syringe filters and stored in ambar vials at -20 ºC until analysis.

2.5. LC-MS/MS analyses

Prior to LC-MS/MS analysis, all extracts were transferred to LC-MS/MS vials properly labelled. Toxins were separated on a Waters X-BridgeTM C8 (guard column 2.1 mm×10 mm, 3.5 m particle size, column 2.1 mm×50 mm, 3.5 µm particle size; Waters, Milford, MA) in an Agilent 1200 LC System (Agilent Technologies, Santa Clara, CA) coupled with 3200 QTRAP triple quadrupole mass spectrometry through TurboV electrospray ion source (Applied Biosystems, Foster City, CA).
A triple quadrupole 3200 QTRAP® mass spectrometer (MS) equipped with a TurboV electrospray ion source (Applied Biosystems, Foster City, CA). The MS was operated in the multiple reaction monitoring (MRM) mode, selecting two product ions per toxin to allow quantification (the most intense transition) and confirmation (the second intense transitions). The MS/MS conditions were based on the recommended values in the EURLMB SOP for a 3200 QTRAP® MS. Mass spectrometric detection was performed in both negative (−ESI) and positive polarity (+ESI).
For LC-MS/MS analysis of lipophilic marine toxins, a binary gradient was programmed with water (mobile phase A) and acetonitrile/water (mobile phase B), both containing 6.7 mM of ammonium hydroxide. All Mobile phases were filtrated through 0.2-µm nylon-membrane filters. All runs were carried out at 30 °C using a flow rate of 500 µL/min. The injection volume was 10 µL and the autosampler was set at 4 °C. A total run time of 12 min was used. These toxins were analysed in both negative (-ESI) and positive polarity (+ESI) (García-Altares et al., 2013), selecting two product ions per toxin to allow quantification (the most intense transition) and confirmation. Identification was supported by toxin retention time and MRM ion ratios. Fragmentation conditions for OA were: 803.5 > 255.0 *m/z* (MRM1) and 803.5 > 113.0 *m/z* (MRM2) and for PTX2: 876.5>213.3 *m/z* (MR1) and 876.5>823.5 *m/z* (MRM2). Calibration curve set in the range of 2 ng/mL - 40 ng/mL for OA and 5ng/mL – 50 ng/mL for PTX2 at six calibration levels. Preparation of the calibration curve was performed using a multi-stock solution of 200 ng/mL for OA and 250 ng/mL for PTX2. Calibration curve

linearities analysed before and after each sample set, whereas the quantification curve correlation coefficients (r^2) had to exceed 0.98 and slope deviations should be below 25%.

2.6. Alkaline hydrolysis

The alkaline hydrolysis of the samples was performed by adding 125µL of NaOH 2.5 M in 1250 µL of extract in a HPLC vial, homogenizing in vortex for 0.5 min then heating aliquots at 76 °C for 40 min. Then cooling samples at room temperature and neutralisation with 125 µL of HCl 2.5M per vial, vortex for 0.5 min for homogenisation. Filtering samples through methanol compatible 0.2-µm PTFE syringe filter.
A small experiment was performed with the resin extracts in order to detect the possible presence of OA esters in our samples. The first 12 SPATT disc extracts were subjected to hydrolysis for the detection of any esters present. All extracts analysed in this study showed that OA esters were not present in our samples.

2.7. Confirmation of OA by spiking evaluation

A spiking test was performed in order to confirm the presence of OA in our extract samples due to some of the OA peaks in selected results presented with higher retention times. Okadaic acid and its isomer DTX2 have the same MRM1 and MRM2 and you can only differentiate them by the retention time. The lipophilic method requires that the resolution between both peaks should be at least 1.5. To calculate this resolution, the following equation is used:

$$Rs = 2(tR(DTX\text{-}2) - tR(OA)) / (W(OA) + W(DTX2))$$

Pure methanol and several selected samples were spiked with known levels of OA (8 ng/mL) and DTX2 (8 ng/mL).

2.8. Ion suppression evaluation

A recovery and ion suppression evaluation was performed for all the different resins. For ion suppression evaluation, five samples from each resin extracts were chosen and spiked after their extraction with known concentrations of OA and PTX2, 20ng/mL and 25 ng/mL, respectively. These samples were analysed by LC-MS/MS in order to observe the presence of ion suppression or ion enhancement due to the matrix effect. The following equation was used:

% R = ((ng/ml) calculated/ (ng/ml) theoretical)*100
% R crm = Recovery calculation as obtained per analysis of spiked extract

2.9. Extraction recovery evaluation (in vitro exposure to the resin)

For recovery evaluation, quantification of OA and PTX2 and the recovery percentage was measured by spiking of the cyclodextrins and the Diaion with a concentration of 100 ng/mL of OA and 100 ng/mL of PTX2. The protocol as follows was using 50 mg of cyclodextrins (β-EPI, γ-EPI, β-HDI, γ-HDI) and 50 mg of Diaion in a 1 mL OA solution

of 100 ng/mL in sterile seawater, 1 mL PTX2 solution of 100 ng/mL in sterile seawater and 1 mL OA+PTX2 solution of 100 ng/mL in sterile seawater. Samples were incubated overnight, centrifuged for 2 min and the supernatant was removed. For the extraction of the samples, 1 mL of MeOH (LC-MS quality) was added followed by a 2-h incubation, then centrifuged for 2 min and the supernatant was collected and transferred into 2-mL Eppendorf tubes. The second sequential extraction was carried out by adding 1 mL of MeOH, incubating for another 10 min and again centrifuging for 2 min. The supernatant was removed and added to the first supernatant. The crude extracts of 2 mL/per sample were filtered through a 0.22-um PTFE membrane syringe filters into glass vials. A dilution of ½ was performed, 250 µL of sample and 250 µL of MeOH was added in LC-MS vials for analysis. These results were used in order to verify the adoption potency, interaction and the affinity of a toxin to a specific resin.

2.10. Data analysis

Statistical calculations were obtained using the IBM SPSS statistics V.26. The ANOVA three-way analysis of variance was used to differentiate OA and PTX2 among resin, point and time, followed by the Tukey post-hoc test.

3. **Results and discussion**

3.1. Confirmation of toxins by HPLC-MS/MS

Certified multi-toxin standards for lipophilic toxins were used to construct calibration curves and subsequently perform quantification of toxins present in the samples. Analysis by LC-MS/MS confirmed presence OA and PTX2 in the samples (EURLMB). Same characteristics of OA in negative ionization mode (transition *m/z* MRM1: 803.5>255.2, MRM2: 803.5>113.1) and PTX2 in positive ionization mode (transition *m/z* MRM1: 876.5>213.3, MRM2: 876.5>823.5) were obtained. Some samples showed two peaks in the negative ionization mode on the chromatogram which gave suspicion of the presence of the OA isomer dinophysistoxin-2 (DTX2). To confirm this, spiking was done. All suspicious samples were spiked with OA (8 ng/mL) and DTX2 (8 ng/mL). In Fig. 5 (a) the red peak shows the OA present in the samples and the blue peak shows the OA spiking, and it is clearly seen that the interrogative peak is overlaid. Thus, the presence of OA is confirmed. In Fig.5 (b) the same sample is spiked with DTX2. In the chromatogram we clearly see the overlay of the first peak which indicates OA and a second peak appeared, which is the spiked DTX2. Therefore it can be confirmed that DTX2 was not present in the samples.

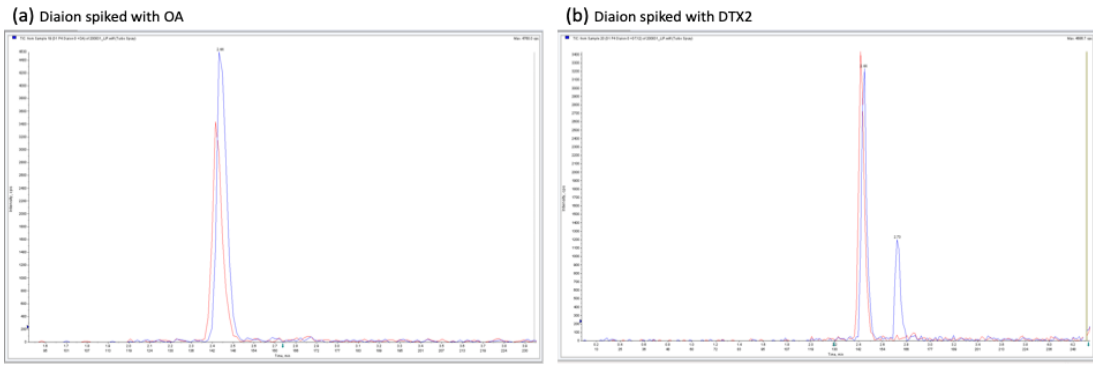
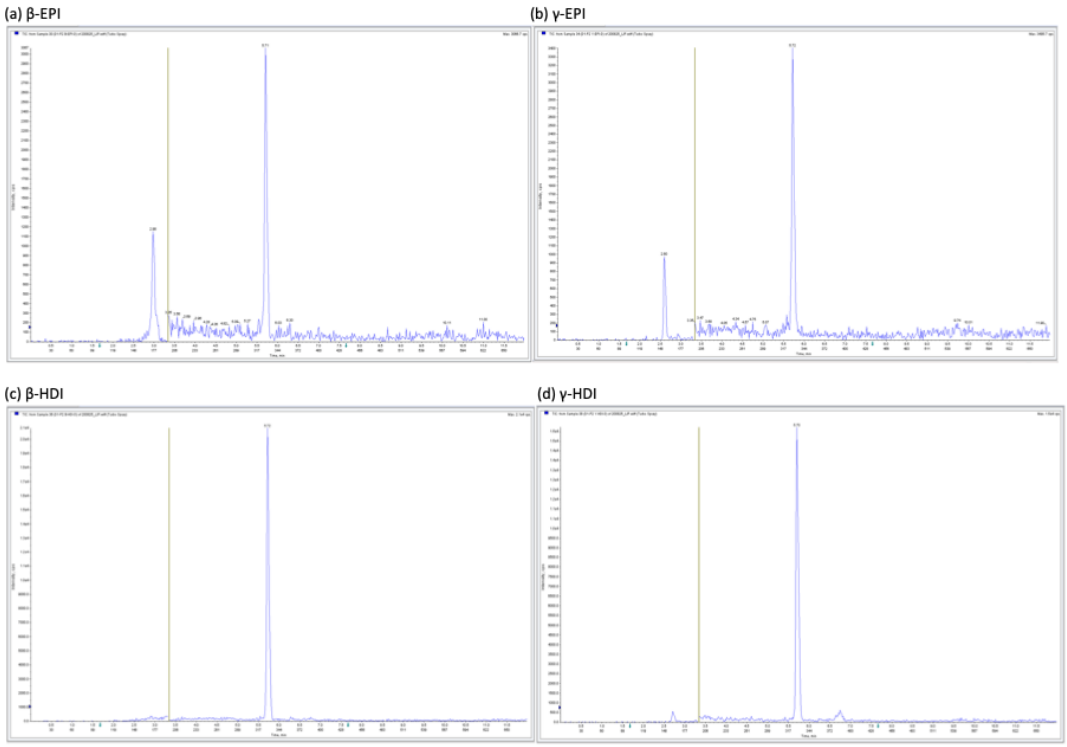


Fig. 5. Spiking of the Diaion (8ng/mL) (a), the peak shows clear overlay and confirms OA as the present toxin.. Spiking of DTX2 (8ng/mL) (b), a new peak appears indicating the absence of DTX2 in the sample.

In Fig. 6, a chromatography of adsorbed toxins of each resin from the same week (W1) and the same point (P2) is represented. β-EPI (Fig. 6 a) shows a clear peak of OA in negative ionization mode and PTX2 in positive ionization mode. A shift in the retention time (RT) at 2.98 min for the OA was observed for this resin, this could be due to a resin matrix effect, PTX2 (RT 5.71 min). γ-EPI (Fig. 6 b) shows normal RT for OA at 2.60 min and a clear peak for PTX2 (RT 5.72 min). β-HDI (Fig. 6 c) shows no quantifiable data on OA, PTX2 however (RT 5.72 min) shows a clear peak with a high intensity. In the chromatogram for γ-HDI (Fig. 6 d) we can see a peak for OA (RT 2.70 min) and PTX2 (RT 5.70 min) is captured at high intensity. The Diaion (Fig. 6 e) shows a clear peak for OA (RT of 2.56) and PTX2 (RT 5.72 min).



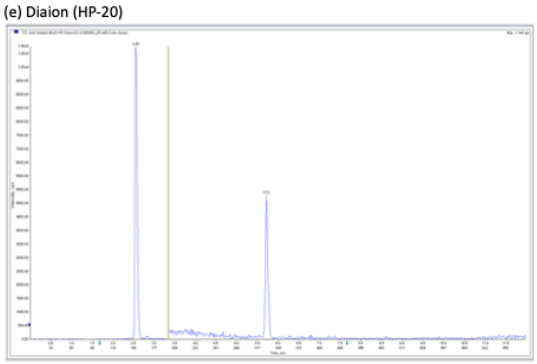


Fig. 6. Chromatograms from the 3200 TRAP triple quadrupole mass spectrometer. Results correspond to week 1 point 2 for the DSP toxins found in the SPATT passive samplers using β-EPI (a) , γ-EPI (b), β-HDI (c), γ-HDI (d) and the Diaion (HP-20) (e).First peak seen in the chromatogram shows the OA in negative ionization mode and second peak shows the PTX2 in positive ionization mode.

3.2. Toxin recovery

Ion suppression evaluation performed by spiking of the samples as per materials and methods section 2.8 where spiking of the samples with known concentrations of OA and PTX2 was measured and calculated. Shortly, spiking of the examples OA and PTX2, 20ng/mL and 25ng/mL respectively, the detected concentrates were calculated using the calibration curves and a recovery value was calculated in relation to the spiked concentrates. This recovery value was obtained in the ion suppression study and was applied to all values obtained by the LC-MS/MS in this study. Each resin had its individual recovery and it should be applied on the corresponding resin through all the study. All the concentrations in the results had this recovery applied (Table. 1).

Resin	OA (%)	PTX2 (%)
B-EPI	**223**	**70**
Y-EPI	**210**	**34**
B-HDI	*	**81**
Y-HDI	**154**	**49**
Diaion	**201**	**41**

Table 1. Toxin recovery calculations in percentage (%) per OA and PTX2

3.3. Quantitative analysis

From the naturally contaminated SPATT discs, the obtained concentrations of OA and PTX2 are shown by resin (β-EPI, γ-EPI ,β-HDI ,γ-HDI and Diaion) in Fig.7**.** A clear correlation was observed between the contents of toxins in all resins and the *Dinophysis* spp. cell abundances during each week. A maximum concentration of OA (40 ng/mL) obtained by Diaion and a maximum PTX2 concentration (97 ng/mL) obtained by γ-HDI

occurred during the peak of the *Dinophysis* spp. bloom (91 341 cells/L) week 1 at point 2. SPATT discs were deployed at two different depths, top (T), deployed at 1 m below surface and bottom (B), deployed at 1.5 m below surface. However, no trends were observed in toxin accumulation regarding preference of depth, the phytoplankton count at the location of the passive samplers was calculated as an integrated column, therefore no clear cell count per depth has been established.

Fig . 7. SPATT toxin profiles of OA and PTX2 per resin (a) β-EPI (b) γ-EPI (c) β-HDI (d) γ-HDI and (e) Diaion top (T) and bottom (B) during the 2-weeks of field trials. Left scale shows *Dinophysis* spp. cell abundance. (* indicates the non-quantifiable position of OA in the β-HDI resin).

In Fig. 8. a and b, clear higher toxins contents are observed during week 1, where *Dinophysis* spp. cell abundance was at its maximum, and a clear pattern of decrease in the toxin contents can be observed as *Dinophysis* spp. cell abundance decreases in the second week. This result was different to that found by M. Garcia-Altares et al., (2016), where an increasing tendency of the toxins was observed towards the end of the phytoplankton bloom.

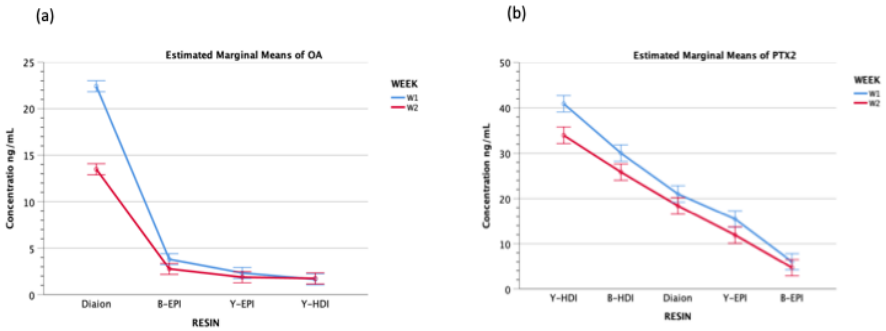


Fig. 8. Average concentration of (a) OA and (b) PTX2 per resin based on weekly adsorption

Each point at Masnou harbour showed some difference in toxin accumulation as seen in Fig. 9. Point 2 and point 3 had higher toxin concentrates throughout the 2-week experiment in comparison to points 1, 4 and 5. Both OA and PTX2 abided the highest concentrations with significant differences in comparison with the other points throughout the experiment. Therefore, the following graphs will be merged into 2 points where point 2 and 3 (P2 and P3) are grouped together as high concentration points and 1, 4 and 5 (P1, P4 and P5) are grouped together as low concentration points, in order to facilitate statistical treatment.

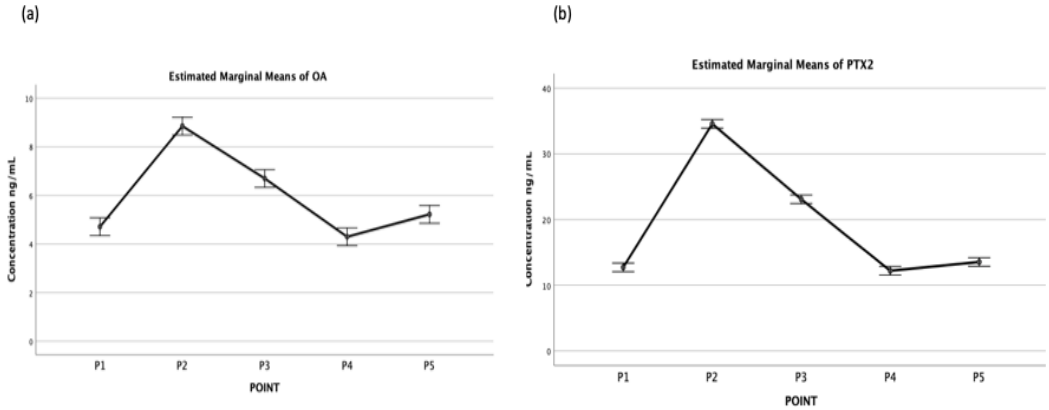


Fig. 9. Average concentrations based for the period of 2 weeks for (a) OA and (b) PTX2 detected at each deployment point: P1, P2, P3, P4 and P5.

In Fig. 10. we can see the temporal scale of *Dinophysis* spp. correlating with toxin abundance for each week (W1 and W2). Highest phytoplankton counts were seen at point 2 and 3 during the first week and the points remained the highest during the second week. The OA and PTX2 contents correlate with the *Dinophysis* spp. dynamics during both weeks. These results correlate with the observations from Fig. 8.

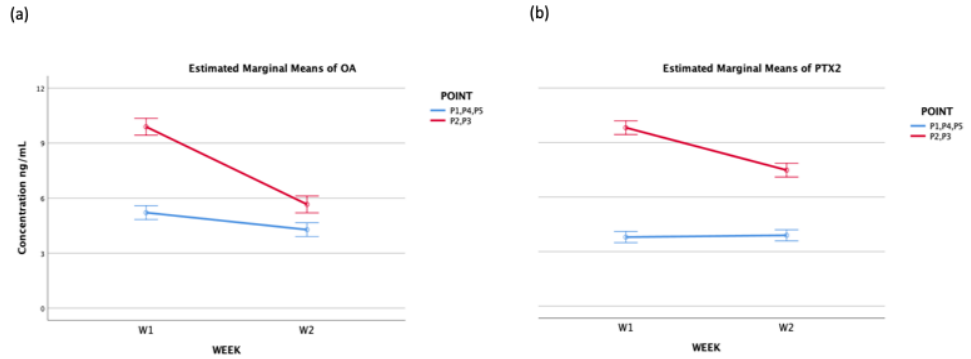


Fig. 10. Average concentrations for (a) OA and (b) PTX2 based on weekly toxin adsorption per point where blue indicates low concentration points (P1, P4 and P5) and red indicated high concentration points (P2 and P3)

The accumulation of OA at low concentrations follows the same trend as accumulation at high contents as, as it can be seen in Fig. 11 (a). The Diaion was by far the best resin in accumulating OA, and not high differences were observed between cyclodextirns. Interestingly, in Fig. 11 (b) it can be observed that the resins show significant differences in PTX2 uptake at high concentrations, the γ-HDI being the best. However, at low concentrations, the differences were not as evident.

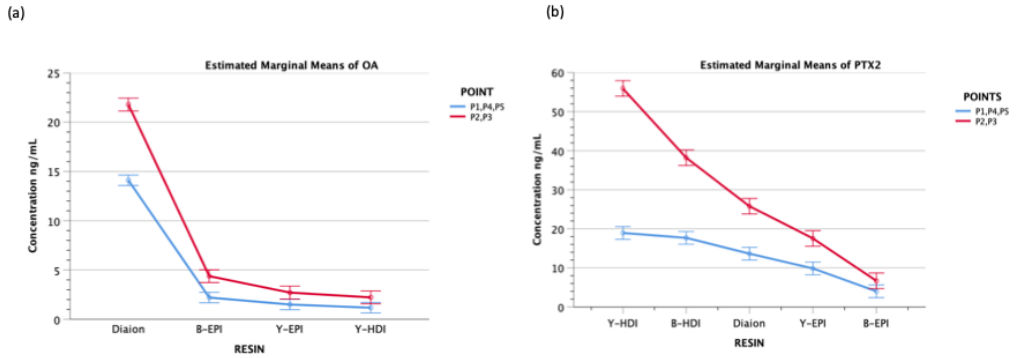


Fig. 11. Average concentrations for (a) OA and (b) PTX2 per resin based on points where blue (Point: 1,4 and 5) indicates points with low concentrations (Point:1, 4 and 5) and red indicated points with high concentrations (Point: 2 and 3)

The trends in Fig. 11 can be also observed in Fig 12, where all points have been merged. The accumulation of OA and PTX2 depends on the resin, and it is clear that the Diaion is the predominant adsorber of OA (17 ng/mL) while no significant differences between the cyclodextrins were observed. OA accumulated by the cyclodextrins did not show significant differences between the β-EPI, γ-EPI and γ-HDI, mean concentrations being 3 ng/mL, 2 ng/mL and 2 ng/mL, respectively (Fig. 12 a). OA uptake by β- HDI on the other hand was not quantifiable by the LC-MS/MS, certainly due to a matrix effect. In Fig. 12 b, a different pattern of toxin adsorption capacity is observed, where predominant accumulation of PTX2 by the γ-HDI (34 ng/mL) can be seen, followed by β-HDI (26 ng/mL), HP-20 (18 ng/mL), γ-EPI (13 ng/mL) and β-EPI (5 ng/mL). In contrast to OA accumulation in the EPI cyclodextrins where no significance between β- and γ- were seen, the accumulation of PTX2 in the EPI cyclodextrin showed significantly greater toxin accumulation in γ-EPI compared to β-EPI, contents being 13 ng/mL and 5 ng/mL respectively. Significant adsorption differences are seen between the resins and the resin sizes, since in both cyclodextrins the γ-resin has greater PTX2 accumulation.

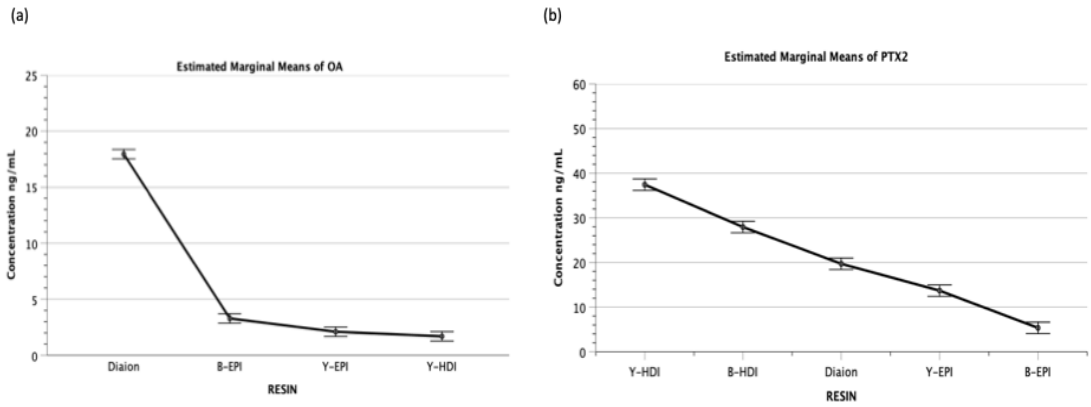


Fig. 12. The average toxin concentration of (a) OA and (b) PTX2 per resins over a period 2-weeks.

It is important to remember that the cyclodextrins vary in size, whereas β- and γ- consists of 7 and 8 glucose units, respectively. Differences between the resins as well as variances in the toxin contents depending on the resin size was observed. In short, as it can be seen in Fig. 13. the HDI cyclodextrins showed better adsorption potential of PTX2 compared to the EPI cyclodextrins, higher toxin contents were obtained by γ-HDI compared to β-HDI, being 34 ng/mL and 26 ng/mL, respectively. OA adsorbed by the EPI cyclodextrins showed no significant difference in comparison to γ-HDI. The Diaion showed good toxin accumulation for OA and PTX2 with no significant differences in toxin contents.

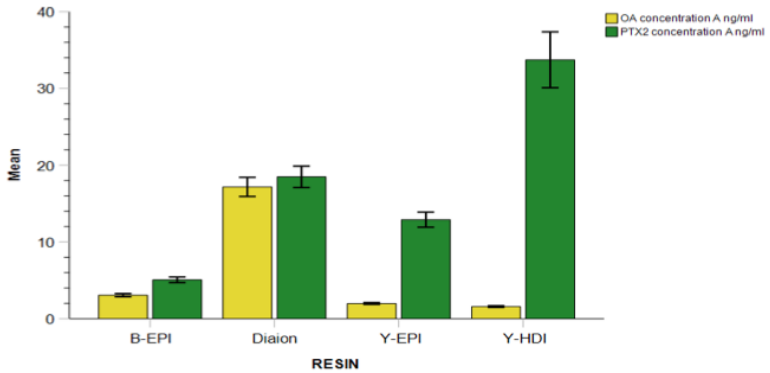


Fig. 13. Average concentration of PTX2 per resin based on weekly adsorption

Results could indicate a higher amount of PTX2 present, toxin profiling of *Dinophysis sacculus* studied by Riobo et al. (2013), suggested PTX2 as the main toxin of *Dinophysis sacculus* with the following intracellular concentrations of PTX2 and OA: 13.2 pg/cell and 7.8 pg/cell respectively. However, in the work by M. Garcia-Altares et al., (2016), higher contents of OA (94 ng/g) in comparison to PTX2 (42 ng/g) were found in the SPATTs. Consequently, further efforts should be made to better understand and elucidate the dynamics and toxin production of the bloom, as many different experimental parameters (Fux et al., 2010; Zendong et al., 2016; Onofrio et al., 2020) (wind, current, temperature, salinity, oxygen, co-occurrence with other microalgae, etc.), some of them under control, may be playing significant roles.

3.4. Spiked seawater

As large differences of OA and PTX2 adsorption was observed in the cyclodextrins, an *in vitro* toxin spiking experiment was performed to verify if competition occurred between toxins for the resins. Each resin was exposed to spiked seawater for a period of 24 h after which the samples were extracted to determine toxic accumulation per resin. All resins showed acceptable recoveries for both OA and PTX2 (Fig. 14). HP-20 and β-EPI were the most suitable for the OA with 80 and 77% recoveries, respectively. β- HDI and γ-HDI showed best recoveries for the accumulation of PTX2 with 82 and 76%, respectively. All resins were able to accumulate OA and PTX2 at good efficiencies, the poorest passive sampler as an OA adsorber was γ-HDI with a recovery of 52% and poorest PTX2 adsorber was β-EPI with a recovery of 46%. Differing from our field studies, it was possible to quantify OA contents in the β-HDI, no matrix effects were present in the *in vitro* experiment (suggesting that the matrix effects observed in the deployment experiment were due to some parameters intrinsic to the natural bloom and environment). Competition between toxins was not observed. Thus, the differences observed with the field experiments may indicate that the field samples are much more complex and several phenomena, some of them not fully elucidated or understood, may be occurring at the same time.

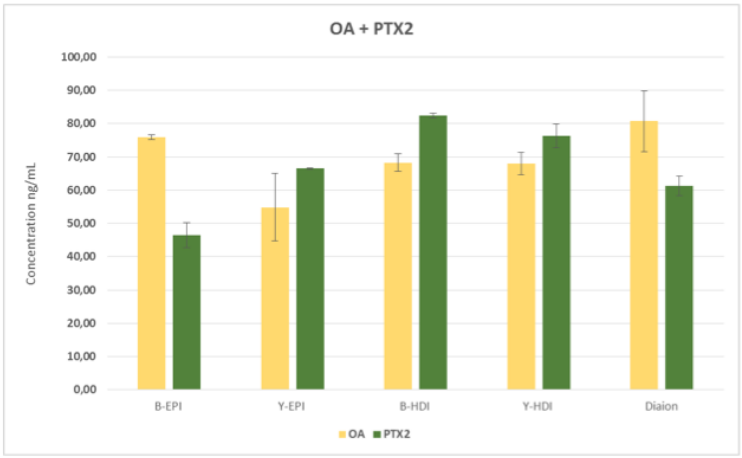


Fig. 14. Average concentration of OA and PTX2 per resin in the *in vitro* experiment

4. Conclusions

The work provides an extensive study on the cyclodextrins as novel material for SPATT technology and as a first study of investigating cyclodextrins in the environment. This work has shown that cyclodextrins are able to accumulate lipophilic marine toxins from the water column. Different efficiencies of toxin accumulation depending on the cyclodextrin material and size are reported. HDI cyclodextrins showed best adsorption capacities for PTX2 where the γ-HDI was the predominant resin. γ-HDI also accumulated OA. However, accumulation of OA in the β-HDI was not quantifiable. On the other hand, both β- and γ-EPI accumulated OA at equal contents. γ-EPI showed higher PTX2 accumulation in comparison to β-EPI. The commercial Diaion showed equal toxin accumulation profiles for both OA and PTX2. Additionally, good correlation between phytoplankton abundances and the toxin contents was observed, in terms of both temporal

(week 1 and week 2) and locational (P1, P2, P3, P4 and P5) sampling, indicating that cyclodextrins could be used as time efficient and reliable tools for early warning in SPATT technology.
Cyclodextrins have proven very sensitive to PTX2 accumulation. However, more research is needed for a better analysis of the accumulation of OA where matrix effect was observed.
Further studies of interest should provide the relationship between toxin accumulation in cyclodextrins and toxin accumulation in shellfish.
The study has given good insight on the potential of cyclodextrins, it has also opened a broader view for future investigations.

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