Cyclodextrin polymers as passive sampling materials for lipophilic marine toxins in *Prorocentrum lima* cultures and a *Dinophysis sacculus* bloom in the NW Mediterranean Sea

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Abstract

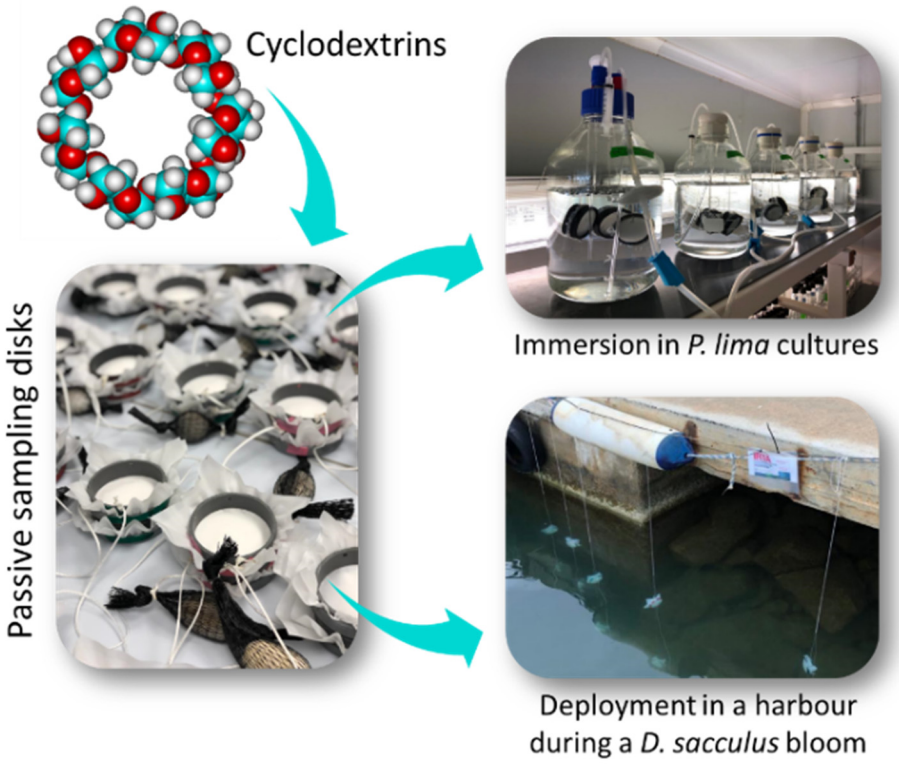
Cyclodextrins, cyclic oligomers that form a conical structure with an internal cavity, are proposed as new and sustainable materials for passive sampling of lipophilic marine toxins. Two applicability scenarios have been tested. First, disks containing *β*-cyclodextrin-hexamethylene diisocyanate (*β*-CD-HDI) and *β*-cyclodextrin-epichlorohydrin (*β*-CD-EPI) polymers were immersed in *Prorocentrum lima* cultures for different days (2, 12 and 40). LC-MS/MS analysis showed capture of free okadaic acid (OA) and dinophysistoxin-1 (DTX1) by cyclodextrins at contents that increased with immersion time. Cyclodextrins resulted more efficient in capturing DTX1 than OA. In a second experiment, disks containing *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI and *γ*-CD-EPI were deployed in harbor waters of El Masnou (NW Mediterranean Sea) during a *Dinophysis sacculus* bloom in February 2020. Free OA and pectenotoxin-2 (PTX2) were captured by

cyclodextrins. Toxin contents were higher at sampling points and sampling weeks with higher *D. sacculus* cell abundance. In this case, PTX2 capture with cyclodextrins was more efficient than OA capture. Therefore, cyclodextrins have provided information regarding the toxin profile of a *P. lima* strain and the spatial and temporal dynamics of a *D. sacculus* bloom, proven efficient as passive sampling materials for environmental monitoring.

Keywords

Cyclodextrin, okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxin-2 (PTX2), *Prorocentrum lima*, *Dinophysis sacculus*.

Graphical abstract



1. Introduction

Harmful algal blooms (HABs) are increasing in geographical expansion, frequency and severity, some of the possible reasons being ocean warming, eutrophication and globalization (Wells et al., 2020). HABs represent a threat for food safety and consumers, since toxins produced by toxic microalgae are accumulated by shellfish and fish. Regarding shellfish safety, monitoring programs involve sampling of shellfish for analysis of marine toxins as well sampling of seawater for phytoplankton identification and counting. Early warning of HABs and shellfish contamination would be a useful approach to facilitate management and protect human health.

Solid-phase adsorption toxin tracking (SPATT) was conceived by MacKenzie in 2004 (MacKenzie et al., 2004). The technique involves the passive adsorption of toxins on porous synthetic resins for the subsequent extraction and analysis. These resins are encased into different types of frames and are deployed into the water column, where they can adsorb the toxins released by toxic microalgae. Passive samplers have been proposed as an early warning tool to forecast shellfish contamination or at least as complementary tool in monitoring programs. The main advantages of passive samplers are simplicity, low cost, low matrix effects when analyzing the resins, spatially and temporally integrated responses, and accumulative capacity. However, some issues still need to be resolved, such as optimal deployment times, saturation limits, lack of calibration and standardization, and the insufficient knowledge about the correlation with toxin contents in shellfish. Additionally, a major limitation is that they allow the monitoring of dissolved toxins only. Nevertheless, passive samplers already have an undeniable application in environmental monitoring and research, since they can provide information on HABs such as geographical and temporal distribution, environmental persistence and toxin dynamics.

Diaion® HP-20 has been the adsorbent substrate most commonly used in passive samplers (Roué et al., 2018) both in microalgae cultures (Fux et al., 2008; Li et al., 2011; Kudela, 2017) and field studies (MacKenzie et al., 2004; Zendong et al., 2015; Kudela, 2017). Unlike this resin, which adsorbs toxins, cyclodextrins (CDs) can capture organic compounds by supramolecular

63 interactions, which may result in different affinity, kinetics and saturation behaviors.
64 Cyclodextrins are cyclic α-1$\rightarrow$4-linked glucose oligomers that form a conical structure with an
65 essentially hydrophobic internal cavity filled with disordered water molecules and two external
66 hydrophilic rims decorated with hydroxyl groups. The number of glucose units in the most
67 common cyclodextrins, 6 in α-CD, 7 in *β*-CD and 8 in *γ*-CD, dictates the size of the cavity, which
68 allows the inclusion of a variety of organic molecules of appropriate size, shape and polarity
69 (Villalonga et al., 2007). They have been exploited in different fields, such as in targeted therapy
70 as drug carriers (Ramirez et al., 2006a, 2006b, 2007), and in biosensors for electrode surface
71 modification and signal amplification (Ortiz et al., 2011a, 2011b, 2011c, 2011d, 2012, 2014; Wajs
72 et al., 2014, 2016). Nevertheless, until now they had never been used for marine toxin tracking.

73 In this work, several insoluble cyclodextrin polymers (*β*-cyclodextrin-hexamethylene
74 diisocyanate (*β*-CD-HDI), *β*-cyclodextrin-epichlorohydrin (*β*-CD-EPI), *γ*-cyclodextrin-
75 hexamethylene diisocyanate (*γ*-CD-HDI) and *γ*-cyclodextrin-epichlorohydrin (*γ*-CD-EPI)) (Fig. 1)
76 have been immersed in *Prorocentrum lima* cultures and deployed in harbor waters during a
77 *Dinophysis sacculus* bloom for their evaluation as new and sustainable passive sampling
78 materials. The commercial Diaion® HP-20 has been used as a control. Results have been useful
79 to obtain information about the toxin profile of the *P. lima* strain and to elucidate the toxin
80 production, development and dynamics of the *D. sacculus* HAB.

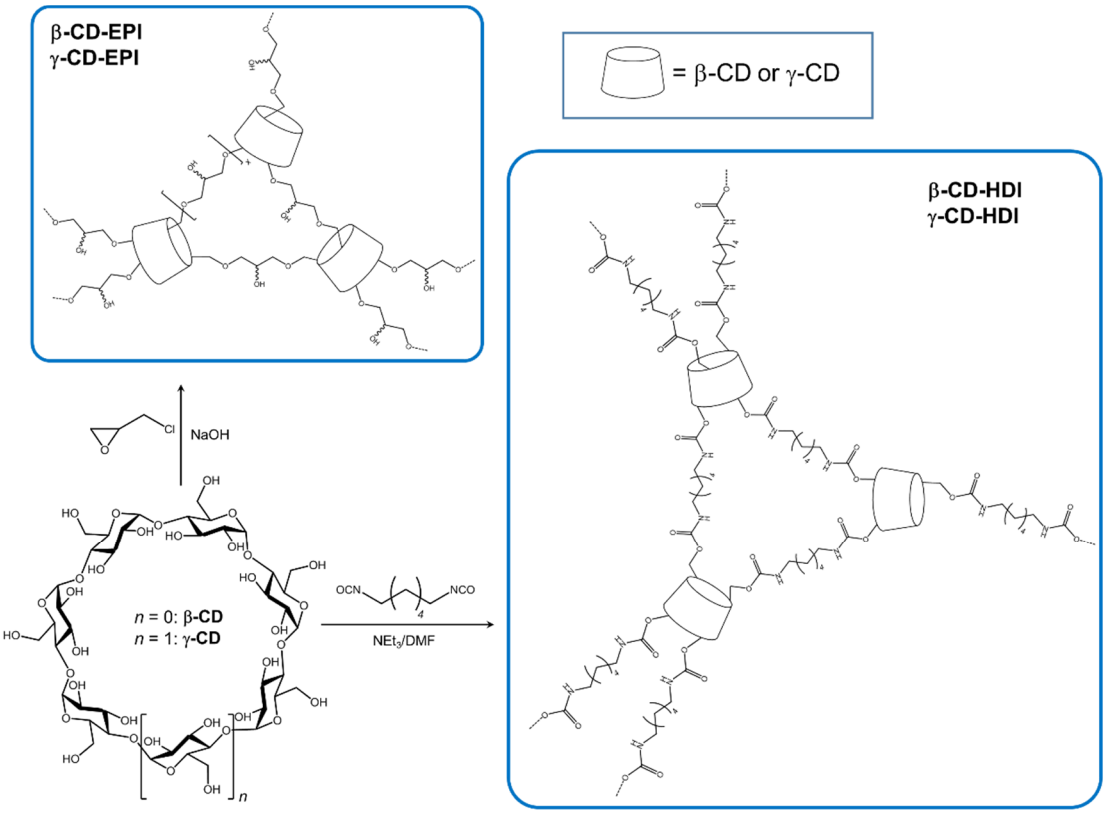


Figure 1. Schematic representations of *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI and *γ*-CD-EPI and their syntheses from native *β*-CD and *γ*-CD.

2. Materials and methods

2.1. Reagents and materials

β-CD-HDI and *γ*-CD-HDI were synthesized by crosslinking the native dried CDs with hexamethylene diisocyanate (1:7 and 1:8 molar ratio, respectively) in dimethylformamide containing triethylamine (Mohamed et al., 2011). *β*-CD-EPI and *γ*-CD-EPI were prepared by reaction of the native CDs with epichlorohydrin (1:14 and 1:16 molar ratio, respectively) in NaOH (Crini et al., 1998) (Fig. 1). The products were purified by Soxhlet extraction with EtOH and water. Diaion® HP-20 Supelco resin was obtained from VidraFoc (Barcelona, Spain). Certified reference material of okadaic acid (OA) (15.56 µg mL^{-1} in MeOH) was obtained from CIFGA (Lugo, Spain). Dinophysistoxin-1 (DTX1) (8.52 µg mL^{-1} in MeOH) and pectenotoxin-2 (PTX2) (4.40 µg mL-*1* in MeOH) were obtained from the National Research Council of Canada (NRC, Halifax, Canada).

Passive sampling disks were constructed by placing 1 g (cultures) or 10 g (harbor) of *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI, *γ*-CD-EPI or Diaion® HP-20 between two layers of 1 µm nylon mesh (Sefar Maissa S.A.U., Cardedeu, Barcelona, Spain), clipped between two cylindrical PVC rings (4-cm diameter for immersion in cultures and 7-cm diameter for deployment in a harbor) (Fig. 1SA). The passive sampling disks to be deployed in the harbor were provided with a counterweight to ensure stability. Cyclodextrins and resin were activated by soaking the disks in MeOH for 15 min and rinsing them with milli-Q water.

2.2. Immersion of cyclodextrins in *Prorocentrum lima* cultures

Clonal cultures of *P. lima* strain IRTA-SMM-17-47 (GenBank accession number: MW328564) from IRTA collection were grown in modified ES medium (Provasoli, 1968), first in Nunclon™ cell culture polystyrene flasks (Thermo Fisher Scientific) and afterwards in glass bottles. Modified ES medium was prepared with sterile aged seawater obtained from L'Ametlla de Mar (Spain), Mediterranean Sea (40.8465° N; 0.77243° E) at 10 m depth, which was passed through an activated carbon-PTFE membrane filter (Thermo Fisher Scientific) and a 0.22-µm cellulose acetate filter (Merck KGaA, Germany). The salinity was adjusted to 36 with milli-Q water. Cultures were maintained at 24 ± 0.5 °C under a light intensity of 110 µmol photons m^{-2} s^{-1} with a 12:12 h light:dark regime. Passive sampling disks containing of *β*-CD-HDI, *β*-CD-EPI or Diaion® HP-20 were immersed into *P. lima* cultures (3 disks per glass bottle) at day 0, and collected at day 2, day 12 and day 40 (Fig. 1SB) (at the time of the experiment, *γ*-CD-HDI and *γ*-CD-EPI were not available). A culture with no passive sampling disks was used as a control to evaluate if their presence had any effect on the culture growth. Two aliquots of each culture were taken every few days, fixed with 3% Lugol's iodine, and cells were counted in duplicate using a Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain). Cultures (~4 L) were harvested at day 40 through vacuum filtration using a 5-µm nylon mesh (Sefar Maissa S.A.U., Spain).

2.3. Deployment of cyclodextrins in a harbor during a *Dinophysis sacculus* bloom

Two consecutive deployments lasting 7 days (from 14/02/2020 to 21/02/2020, and from 21/02/2020 to 28/02/2020) were performed at 5 sampling points of El Masnou harbor (NW Mediterranean Sea) during a *D. sacculus* bloom (Fig. 2S). Passive sampling disks containing of *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI, *γ*-CD-EPI or Diaion® HP-20 (in duplicate) were deployed at 1.2 m depth and left for 1 week (Fig. 1SC). Phytoplankton cells were counted under an inverted light microscope (Leica DMIL, Spain), following the Utermöhl method (Utermöhl, 1931).

2.4. Toxin extraction

The passive sampling disks were soaked in milli-Q water for 30 min. Afterwards, the embroidery hoop was opened, and the *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI, *γ*-CD-EPI or Diaion® HP-20 were transferred to beakers and incubated with MeOH (40 mL when using 1 g (cultures) and 80 mL when using 10 g (harbor)) for 2 h. Cyclodextrins and resin were then transferred to low frequency polyvinyl chloride (LPVC) plastic filtration columns containing 1 µm nylon mesh filters and frits, vacuum was applied with a Vac-Elut SPE vacuum manifold (Varian, Harbor City, CA, USA), and the MeOH was collected. Rinsing was performed with additional MeOH (20-30 mL approx.), which was also collected. The total volume of eluate was evaporated to dryness in a Syncore Buchi (Flawil, Switzerland) and redissolved in 0.5 (when using 1 g) or 4 mL (when using 10 g) of MeOH.

Prorocentrum lima culture media (0.5 L) were filtered through Empore™ C18 SPE Disks (Supelco, Sigma-Aldrich, Tres Cantos, Madrid, Spain). Disks were first conditioned with 10 mL of MeOH and 10 mL of milli-Q water. Then, culture media were loaded, vacuum was applied, and the collected media were discarded. Samples were then eluted with 20 mL of MeOH. *Prorocentrum lima* cultures filters were sonicated 3 times in 150 mL of MeOH for 30 min. The three extracts were joined and centrifuged at 3,000 rpm for 10 min, and the supernatant was kept.

To investigate the possible presence of fatty acid acyl esters, alkaline hydrolysis of the extracts was performed the same day of analysis by adding 125 µL of 2.5 M NaOH in 1.25 mL of extract

in a HPLC vial (the same ratio was maintained when hydrolyzing extracts coming from 1 g of cyclodextrin or resin), vortexing for 0.5 min, and heating at 76 °C for 40 min. Samples were then cooled at room temperature, neutralized with 125 µL of 2.5 M HCl, and vortexed for 0.5 min.

All extracts were passed through 0.2-µm PTFE syringe filters and stored at -20 °C until LC-MS/MS analysis.

2.4. LC-MS/MS analysis

LC-MS/MS analyses were conducted on a 1200 LC system (Agilent Technologies, Santa Clara, CA) coupled with a 3200 QTRAP triple quadrupole mass spectrometer through a TurboV electrospray ion source (Applied Biosystems, Foster City, CA), using a previously described methodology (García-Altares et al., 2016; Leonardo et al., 2018). Samples were analyzed on an XBridge BEH C8 column, 2.5 µm, 2.1 × 50 mm and an XBridge BEH C8 Prep Guard cartridge, 2.5 µm, 2.1 x 5 mm (Waters, Milford, MA, USA). A binary gradient was programmed with ultrapure milli-Q water (mobile phase A) and 90:10 *v:v* acetonitrile:water (mobile phase B), both containing 6.7 mM of ammonium hydroxide. Mobile phases were filtered through 0.2-µm nylon membrane filters (Whatman, Springfield Mill, UK). Chromatographic separations were performed at 30 °C using a flow rate of 500 µL min^{-1}. The elution gradient started at 20% B, reached 100% B in 8 min, held for 1 min, then back to 20% B in 1 min and equilibrated for 2 min before the next run started. The injection volume was 10 µL and the auto-sampler was set at 4 °C. A total run time of 12 min was used. Lipophilic toxins were analyzed in both negative (-ESI) and positive (+ESI) mode, selecting two product ions per toxin to allow quantification (the most intense transition) and confirmation (the second intense transitions). Identification was supported by toxin retention time and multiple reaction monitoring (MRM) ion ratios. Monitored transitions of the detected toxins were 803.5>255.0 *m/z* (MRM1) and 803.5>113.0 *m/z* (MRM2) for OA, 817.5>255.2 *m/z* (MRM1) and 817.5>113.1 *m/z* (MRM2) for DTX1, and 876.5>213.3 *m/z* (MRM1) and 876.5>823.5 *m/z* (MRM2) for PTX2. Calibration curves were performed in the range of 2 ng mL^{-1}

– 40 ng mL^{-1} for OA and DTX1, and 5 ng mL^{-1} – 50 ng mL^{-1} for PTX2, at six calibration levels. Calibration curve linearities were confirmed before and after each sample set. Curve correlation coefficients (r^2) had to exceed 0.98 and slope deviations had to be below 25% to pursue toxin quantifications. Limits of detection (LODs, signal/noise > 3) were 1.3 ng/mL for OA and DTX1, and 1.7 ng/mL for PTX2. Limits of quantification (LOQs, signal/noise > 10) were 4 ng/mL for OA and DTX1, and 5 ng/mL for PTX2. Samples were analyzed in duplicate.

2.5. Statistical analysis

Differences in toxin concentration (*i.e.*, OA and PTX2) among passive sampling disks (*i.e.*, *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI, *γ*-CD-EPI and Diaion® HP-20), sampling points, and between weeks were analyzed with analysis of variance (three-way ANOVA). In addition to *P* values, partial eta squared (η_p^2) was used as a measure of effect size (*i.e.*, the importance of factors). Similar to r^2, partial η_p^2 is the proportion of variation explained for a certain effect, and does not depend on the number of sources of variation used in the ANOVA, thus it could be compared among different designs (Tabachnick and Fidell, 2001). In contrast to *P* value, η_p^2 has the advantage of allowing the proper comparison of treatments, whereas a lower *P* value does not necessarily mean that a factor has stronger effect (see *e.g.*, Alcaraz et al., 2008). Adjusted (or marginal) means of a dependent variables are the means for each level of the factor adjusted for the other variables (see *e.g.*, Alcaraz et al., 2015), and were used to describe differences. Student's *t* test was used to analyze differences in toxin concentration (OA *vs.* PTX2) for each passive sampling disk. Quantitative variables were log-transformed prior to analysis because homoscedasticity and linearity were clearly improved. All statistical analyses were performed with SPSS 26.0.

3. Results

3.1. Analysis of cyclodextrins immersed in *Prorocentrum lima* cultures

Toxins captured by *β*-CD-HDI, *β*-CD-EPI and Diaion® HP-20 immersed in *P. lima* cultures were analyzed by LC-MS/MS, which revealed the presence of free OA and DTX1 in all of them (Fig. 2). Although it is evident that Diaion® HP-20 provided the highest values, both cyclodextrins showed OA and DTX1 capture. The OA contents at the different days were 7, 35 and 24% for *β*-CD-HDI and 17, 38 and 30% for *β*-CD-EPI, compared to Diaion® HP-20. The DTX1 contents were 20, 44 and 56% for *β*-CD-HDI and 31, 47 and 17% for *β*-CD-EPI, compared to Diaion® HP-20. OA and DTX1 contents showed exponential trends with culture day.

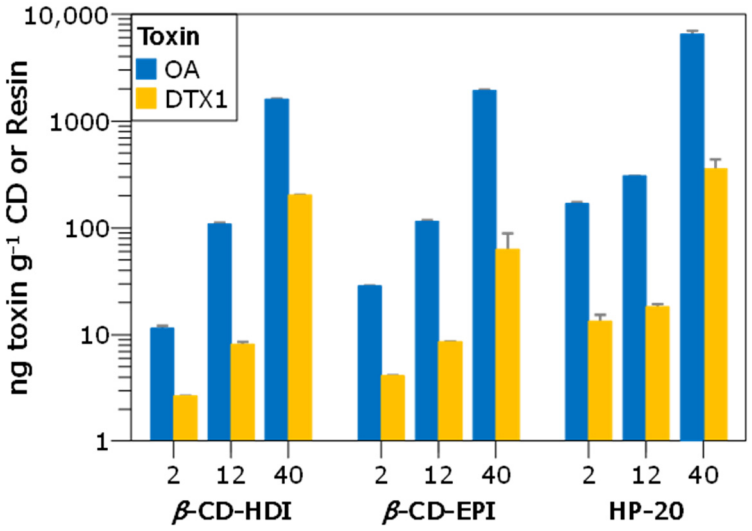


Figure 2. Free OA and DTX1 captured by the passive sampling disks immersed in *Prorocentrum lima* cultures and collected at day 2, day 12 and day 40. Each bar corresponds to 1 disk analyzed twice.

Prorocentrum lima culture media and the corresponding microalgal cells at the moment of harvesting were also analyzed (Fig. 3). The control culture, with neither cyclodextrins nor resin, showed the highest toxin levels in the culture media. Nevertheless, it is interesting to mention that the total number of cells at harvesting in the control culture was higher than in the cultures with passive sampling disks, where the number of cells reached 54-65% that of the control culture (Fig. 3S and Table 1S). The culture media with Diaion® HP-20 showed the lowest OA contents (23% that of the control), followed by *β*-CD-EPI (29%) and finally *β*-CD-HDI (50%) (Fig. 3A), trend that was the opposite of that observed in the passive sampling materials (Fig. 2). Even

normalizing to the number of microalgal cells, the trend was the same. DTX1 contents were also lower in the culture media with Diaion® HP-20 than in the culture media with *β*-CD-EPI. However, this difference and the lack of DTX1 in the culture media with *β*-CD-HDI may be simply due to the fact that all DTX1 concentrations were very close to the LOD. When observing the toxin contents in the microalgal cells, no clear trends were observed (Fig. 3B).

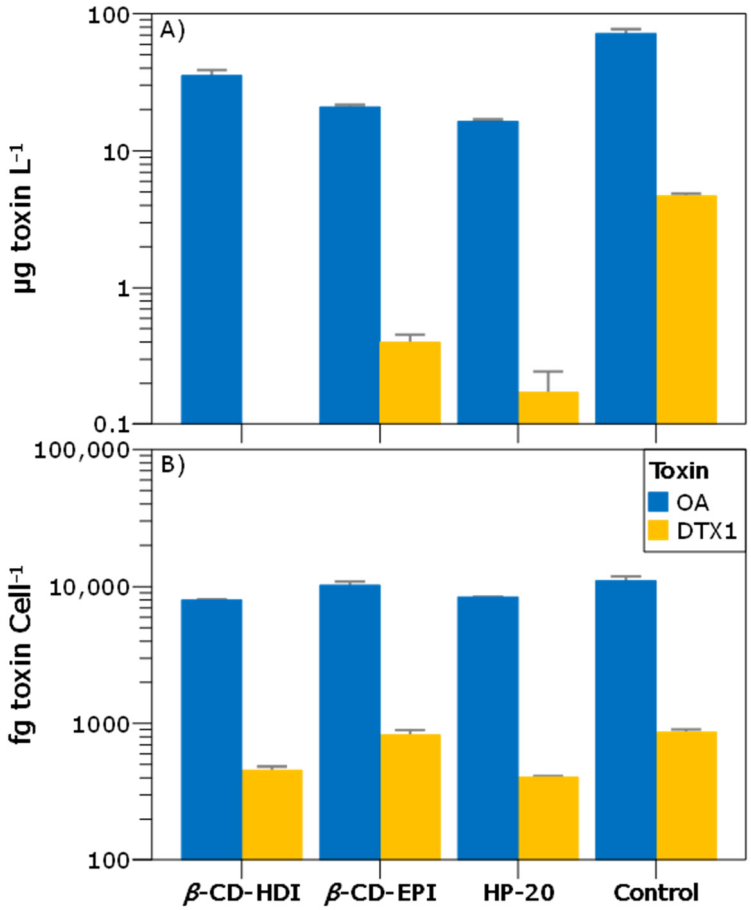


Figure 3. Free OA and DTX1 present in the *P. lima* culture media (A) and in the microalgal cells (B) at day 40.

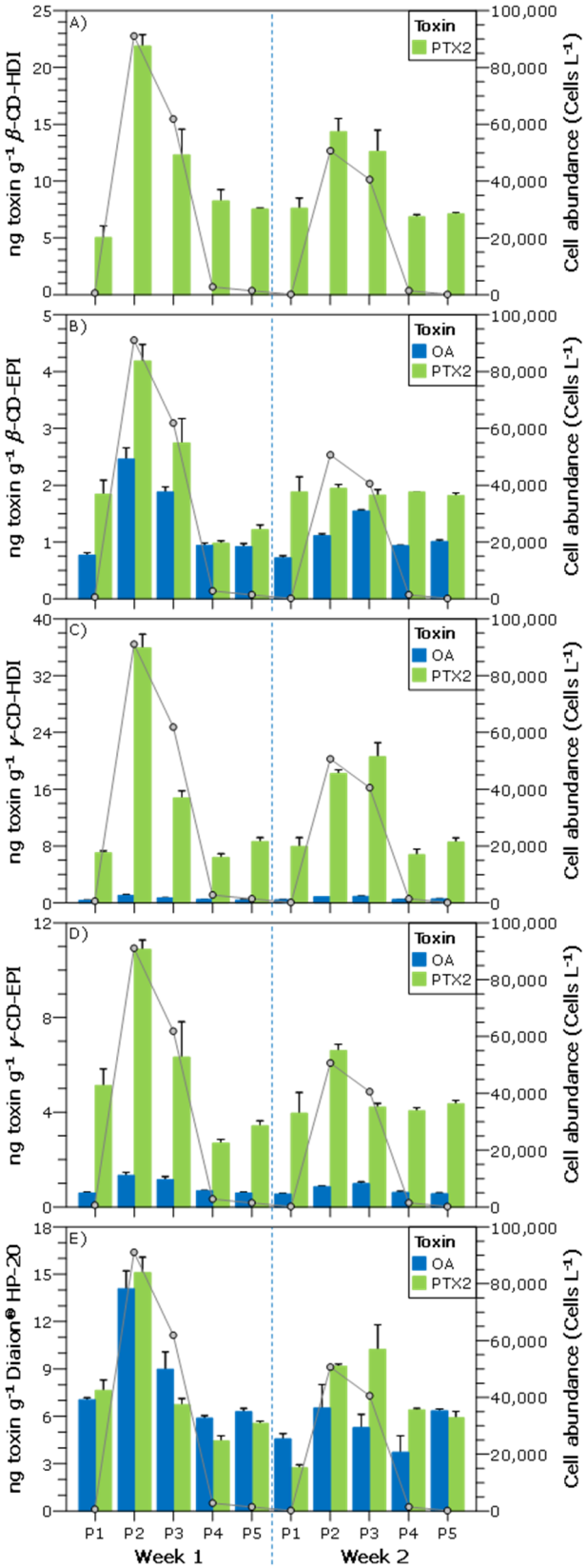
The analysis of hydrolyzed extracts showed fatty acid acyl OA esters in all cell pellets and small amounts of fatty acid acyl DTX1 esters in the cell pellets corresponding to the culture with *β*-CD-EPI (Fig. 4S). No esters were found in the culture media. Regarding the passive sampling disks, OA and DTX1 esters were detected in *β*-CD-EPI even after only 2 days, and OA esters were present in all cyclodextrins and the resin at the last sampling.

3.2. Analysis of cyclodextrins deployed in a harbor during a *Dinophysis sacculus* bloom

Toxins captured by *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI, *γ*-CD-EPI and Diaion® HP-20 deployed at 5 sampling points of El Masnou harbor (NW Mediterranean Sea) during two consecutive weeks of a *D. sacculus* bloom were analyzed by LC-MS/MS. The analysis revealed the presence of OA and PTX2 in all of them, sometimes at very different levels (Fig. 4, Fig. 5 and Fig. 6). Unlike the experiment in *P. lima* cultures, DTX1 and esters were not found. It is important to mention that although the LC-MS/MS analysis of extracts from *β*-CD-HDI revealed presence of OA, the chromatographic peaks did not fulfil the analytical standard criteria and thus, quantification was not possible. This effect was probably due to the presence of matrix compounds that interfere in the analysis for this specific sampling material. Further work would be necessary to remove this interference and quantify OA.

In general, considerable differences were observed between sampling points (ANOVA, $P < 0.0001$ for OA, and $P < 0.0001$ for PTX2, *see* Table 2S), P2 showing the highest toxin contents, followed by P3, and with P1, P4 and P5 showing much lower toxin contents (Table 2S, Fig. 5S and 6S). This trend is in accordance with the *D. sacculus* cell abundance distribution, which in the first sampling week was 91,341 cells L^{-1} in P2, 62,195 cells L^{-1} in P3 and between 880 and 3,040 cells L^{-1} in P1, P4 and P5. Additionally, in P2 and P3, toxin contents in sampling week 1 were higher than in sampling week 2 (Table 2S, Fig. 7S and 8S), also following the temporal variation of *D. sacculus* cell abundance, which decreased at the second deployment (e.g. to 50,949 cells L^{-1} in P2 and 40,851 cells L^{-1} in P3).

The global effect of type of cyclodextrin can be observed in Fig. 7, where data points have been merged. In general terms, the trend for OA contents was Diaion > *β*-CD-EPI > *γ*-CD-EPI > *γ*-CD-HDI and the trend for PTX2 contents was *γ*-CD-HDI > *β*-CD-HDI > Diaion > *γ*-CD-EPI > *β*-CD-EPI. The OA contents were 17, 12 and 9% for *β*-CD-EPI, *γ*-CD-EPI and *γ*-CD-HDI, respectively, compared to Diaion® HP-20. The PTX2 contents were 182, 144, 70 and 27% for *γ*-CD-HDI, *β*-CD-HDI, *γ*-CD-EPI and *β*-CD-EPI, respectively, compared to Diaion® HP-20. Therefore, although Diaion® HP-20 is better to capture OA, CD-HDIs are more efficient for PTX2.



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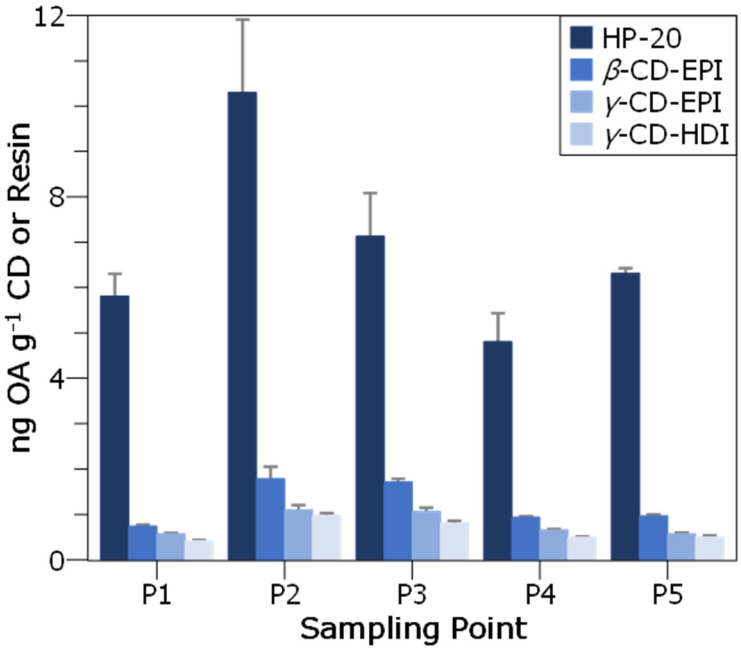
254 **Figure 4.** Left axis: OA and PTX2 captured by the passive sampling disks deployed El Masnou harbor (NW
255 Mediterranean Sea). Right axis: *Dinophysis sacculus* cell abundance in the water column (average from
256 deployment and collection days). Each bar corresponds to the mean of 2 disks (duplicates) analyzed
257 twice.



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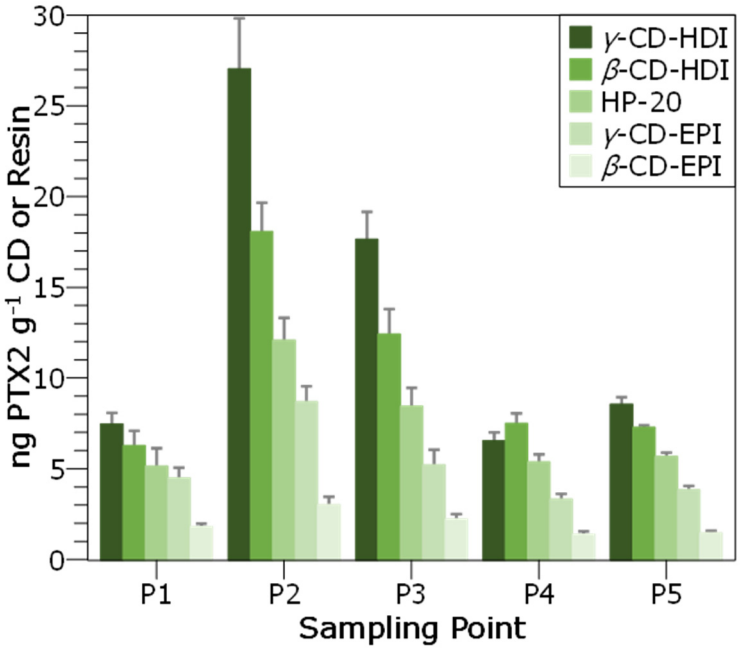
259 **Figure 5.** OA captured by the passive sampling disks per cyclodextrin/resin and sampling point (P1 to P5)
260 of El Masnou harbor (NW Mediterranean Sea). Each bar corresponds to the mean of 4 disks (duplicates
261 and 2 weeks) analyzed twice.



262

263 **Figure 6.** PTX2 captured by the passive sampling disks per cyclodextrin/resin and sampling point (P1 to
264 P5) of El Masnou harbor (NW Mediterranean Sea). Each bar corresponds to the mean of 4 disks
265 (duplicates and 2 weeks) analyzed twice.

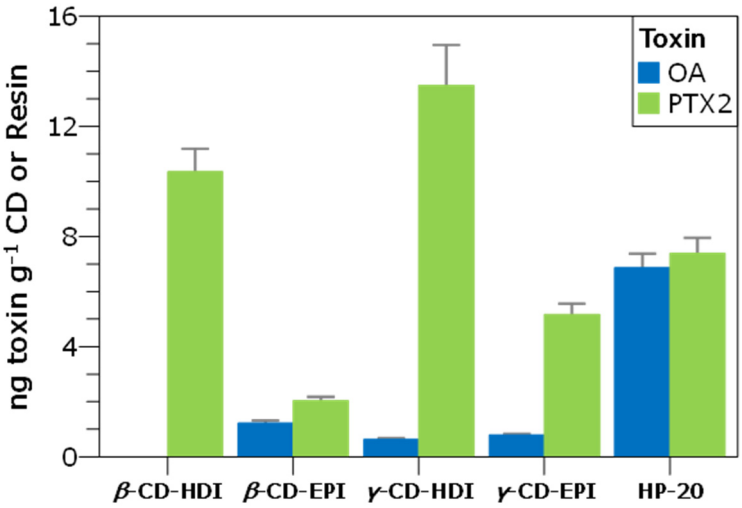


Figure 7. Total OA and PTX2 captured by the passive sampling disks per cyclodextrin/resin deployed at El Masnou harbor (NW Mediterranean Sea). Each bar corresponds to the mean of 20 disks (duplicates, 2 weeks and 5 points) analyzed twice. Error bars are the standard error of the means.

4. Discussion

4.1. Cyclodextrins immersed in *Prorocentrum lima* cultures

The immersion of *β*-CD-HDI, *β*-CD-EPI and Diaion® HP-20 in *P. lima* cultures for different days provided information about the toxin production of the strain combined with the toxin capture efficiency of the different passive sampling materials. LC-MS/MS analysis revealed the presence of OA and DTX1 in all extracts even at day 2 and, as expected, toxin contents showed an exponential increase trend with culture day. Although Diaion® HP-20 provided the highest toxin contents, both cyclodextrins also showed OA and DTX1 capture. Except for DTX1 at day 40, *β*-CD-EPI was slightly more efficient in capturing toxins than *β*-CD-HDI, and this observation was more evident at day 2 than at days 12 and 40, suggesting a faster capture rate of the former cyclodextrin. The percentage decrease of DTX1 in *β*-CD-EPI at day 40 (17%) could be due to different experimental parameters, such as a lower DTX1 production at that stage of the culture or the saturation of the passive sampling material. In general terms, although DTX1 values were around 10-fold lower than OA values, DTX1 capture with cyclodextrins was more efficient than OA capture compared to Diaion® HP-20. The additional methyl moiety in one of the extremes of

DTX1 (Fig. 9S) makes it less polar than OA, characteristic that could be favoring the interaction with the cyclodextrins, as previously observed for Diaion® HP-20 (Li et al., 2011).

When making all these comparisons, it is important to keep in mind that *β*-CD-HDI, *β*-CD-EPI and Diaion® HP-20 were immersed in different glass bottles during culture, in order to avoid competition between them. Therefore, differences in microalgae growth and toxin composition of the media cannot be discarded. Analysis of the culture media and the corresponding microalgal cells contributed to better characterize the system. The analysis of *P. lima* culture media at the moment of harvesting revealed lower dissolved toxin contents in the cultures with passive sampling disks than in the control. However, the number of *P. lima* cells was also lower. A possible explanation is that the presence of the disks may have inhibited the culture growth (Table 1S), by decreasing light exposure or capturing culture media components (e.g. nutrients, vitamins and metals). For those cultures with passive sampling disks, the trend of dissolved OA content in the media was inversely proportional to that in the passive sampling material, observation that supports the capture efficiency previously observed, which is Diaion® HP-20 > *β*-CD-EPI > *β*-CD-HDI. Regarding the toxin contents in microalgal cells, no clear trends were observed among passive sampling materials and no significant differences with the control. This seems to indicate that the passive sampling disks, although are affecting culture growth, may not be affecting toxin production per cell.

Regarding to absolute values and compared to other works, much lower toxin contents are obtained herein (e.g. 168 ng OA g^{-1} and 13 ng DTX1 g^{-1} for the Diaion® HP-20 after 48 h in front of 982 ng OA g^{-1} and 846 ng DTX1 g^{-1} found by Fux et al., 2008), differences probably due to the strain, its age and the culture experimental conditions.

Prorocentrum lima is a well-known producer of OA, DTX1, DTX2 and several types of esters, the toxin profile and contents depending on the strain and growing conditions (Pan et al., 1999, Bravo et al., 2001, Nascimento et al., 2005, Paz et al., 2007, Morton et al., 2009, Vale et al., 2009,

311 Li et al., 2012, Hu et al., 2017). However, no esters or only trace amounts are usually found in
312 culture media (Pan et al., 1999, Nascimento et al., 2005), probably because they are hydrolyzed
313 before their release from the cells (Hu et al., 2017). In the current work, esters were not found
314 in the culture media either. Regarding their presence in passive samplers, a previous work
315 showed that OA esters were detected in SPATT bags with Diaion® HP-20 (Mackenzie et al., 2011).
316 In the current work, OA and DTX1 esters were detected in some passive sampling disks, mainly
317 in those from the last sampling. The lipophilic character of the fatty acid acyl ester derivatives
318 makes them suitable to be captured by the cyclodextrins and the Diaion® HP-20 resin. Therefore,
319 these passive sampling materials could be pre-concentrating esters once released from the cells,
320 allowing their detection better than in the media.

321 **4.2 Cyclodextrins deployed in a harbor during a *Dinophysis sacculus* bloom**

322 The deployment of *β*-CD-HDI, *β*-CD-EPI, *γ*-CD-HDI, *γ*-CD-EPI and Diaion® HP-20 passive samplers
323 during a *D. sacculus* bloom revealed the capture of OA and PTX2. However, there is not a clear
324 indication about which was the most predominant toxin, since different passive sampling
325 materials provide different trends (Fig. 4). Additionally, in the current work, toxin levels in the
326 cyclodextrins (or in the commercial resin used as a control) did not reach the values found in
327 other works, which may suggest an overall lower toxin production from the bloom. The toxin
328 concentration and profile of *D. sacculus* may vary depending on the strain, its geographical
329 origin, the experimental parameters of the culture and, of course, multiple environmental
330 conditions in the case of field samples. In the Mediterranean, *D. sacculus* blooms have been
331 associated to OA (Cañete et al., 2008; Garibo et al., 2014; García-Altares et al., 2016; Leonardo
332 et al., 2018; Giacobbe et al., 2000), PTX2 (Cañete et al., 2008; García-Altares et al., 2016; and
333 sometimes DTX1, to a lesser extent (Giacobbe et al., 2000). In a previous study performed by our
334 group during a *D. sacculus* bloom in Alfacs Bay (NW Mediterranean Sea), 200 km to the south of
335 Masnou harbor, PTX2 was the main component in the toxin profiles of phytoplankton aggregates
336 (up to 668 pg PTX2 $cell^{-1}$ in front of 461 pg OA $cell^{-1}$), while OA was the most concentrated toxin

in Diaion® HP-20 SPATTs (94 ng OA g^{-1} in front of 42 ng PTX2 g^{-1}) (García-Altares et al., 2016). In another work performed with a culture of a *D. sacculus* strain from Galicia (NE Atlantic Ocean), PTX2 was also the main toxin in the cells (13 pg $cell^{-1}$), followed by OA (8 pg $cell^{-1}$), whereas OA was more abundant in the medium (28 ng OA mL^{-1} in front of 23 ng PTX2 mL^{-1}) (Riobó et al., 2013). In that work, only traces of DTX1 were observed in the cell pellet extract, but not in the medium. OA contents in SPATT discs deployed during a *D. acuta* event in the west coast of Ireland (Atlantic Ocean) were also much higher than PTX2, followed by DTX2 (maximum of 5645 ng OA g^{-1}, 1265 ng PTX2 g^{-1} and 533 ng DTX2 g^{-1}) (Fux et al., 2009). The deployment of passive samplers in Galicia (NE Atlantic Ocean) during a bloom where several *Dinophysis* species were present (mainly *D. ovum* and *D. acuminata*, but also *D. acuta* and *D. caudata*, depending on the day) also followed this pattern (maximum 4495 ng OA g^{-1}, 2705 ng PTX2 g^{-1} and 1876 ng DTX2 g^{-1}) (Pizarro et al., 2013). On the contrary, toxin contents found in SPATT discs deployed during another *D. acuta* bloom near the south-west coast of Ireland (Atlantic Ocean) were higher for PTX2 (between 1.1 and 4.5 µg g^{-1}), followed by DTX2 (between 0.9 and 3.7 µg g^{-1}) and finally OA (between 0.8 and 2.9 µg g^{-1}) (Fux et al., 2010), and the deployment of SPATT bags during a *D. acuminata* bloom in New Zealand also resulted in higher levels of PTX2 compared to OA and DTX1, which were 1.73 µg PTX2 g^{-1} and 0.11 µg OA/DTX1 g^{-1} when the maximum cell numbers of *D. acuminata* were observed. All these works underline the complexity in the understanding and elucidation of toxin profiles.

Nevertheless, results provided herein showed interesting and informative trends. Regarding location distribution, the highest toxin contents, for both OA and PTX2, were detected in the sampling points with the highest *D. sacculus* cell abundance (Fig. 4). When looking at the geographical position of the sampling points (Fig. 2S), P2 is in an interior corner of the harbor, followed by P3, and finally by P4, P5 and P1. Thus, a correlation with the spatial *D. sacculus* bloom dynamics is observed, *D. sacculus* cells accumulating in the most confined part of the harbor. Additionally, apart from the *D. sacculus* cell abundance, the diffusion of the dissolved

363 toxins may be playing a role. Regarding temporal distribution, data from P2 and P3 clearly
364 demonstrate that toxin contents correlate with the temporal variation of *D. sacculus* cell
365 abundance. Another important issue is that OA and PTX2 were detected in almost all samples,
366 even at very low *D. sacculus* cell abundances (as low as 400 cells L^{-1}), which indicated how
367 common traces of these toxins are in seawater and how stable they are (already demonstrated
368 at least for OA, Blanco et al., 2018), and also how sensitive the SPATT strategy is.

369 It is evident that PTX2 capture with cyclodextrins was more efficient than OA capture (Fig. 7).
370 This can be explained considering the structures of both toxins and cyclodextrin polymers and
371 the interactions involved in the capture process. The Diaion® HP-20 resin is known to capture a
372 wide range of organic molecules from aqueous solution through a hydrophobic binding
373 mechanism involving its styrene-divynilbenzene matrix. That is why it showed no significantly
374 different OA and PTX2 contents (Student's *t*-test, $P = 0.53$) and is not selective to any of the toxins
375 as compared with the cyclodextrin-based materials that show higher contents for PTX2
376 (Student's *t*-test, $P < 0.0001$ in all cases). Unlike OA and DTX1, OA and PTX2 have very different
377 structures with different overall hydrophilic/hydrophobic balances (Fig. 9S). PTX2 is a neutral
378 molecule with a ring structure composed by oxolane and oxane rings connected by aliphatic
379 spacers (O'Rourke et al., 2017). This makes PTX2 a polar but essentially hydrophobic molecule,
380 which could favor its capture by cyclodextrins and may explain the higher capture efficiency of
381 the cyclodextrin polymers over Diaion® HP-20. On the other hand, OA is also composed by
382 oxolane and oxane rings but in a linear structure and possesses an ionizable carboxylic acid group
383 at the A ring resulting in a higher polarity and solubility in aqueous solution (Mackenzie et al.,
384 2004). This may explain, in general terms, its lower capture by the cyclodextrins as compared to
385 the commercial resin.

386 In the case of the cyclodextrin materials, their capture efficiency should be a combination of the
387 cavity size and the nature of the bridging units. Regarding OA and cavity size, capture efficiencies
388 with *β*-CD-EPI, consisting of seven glucopyranose units, were only slightly higher (Table 2S and

Fig. 10S) than with *γ*-CD-EPI, consisting of eight glucopyranose units (it is important to mention that absolute OA contents were in general very low). Therefore, the cavity size plays a negligible effect, if any, in the capture process of OA. Nevertheless, when looking at the PTX2 results, *γ*-CD-HDI and *γ*-CD-EPI provided significantly higher capture efficiencies than their *β*-CD counterparts (Table 2S and Fig. 11S), suggesting that the larger internal diameter (0.95 nm for *γ*-CDs in front of 0.78 nm for *β*-CDs (Szejtli, 1998)) could be playing a role in accommodating the PTX2 molecule, most likely through the D/E rings.

On the other hand, the role of the bridging units was more evident in the case of PTX2 than OA. Both CD-EPIs, which contain polar hydroxyalkyl groups of different lengths depending on the degree of polymerization, were slightly more efficient than CD-HDI in capturing the more polar OA (Table 2S and Fig. 10S). On the contrary, CD-HDIs were much more efficient in capturing the more lipophilic PTX2 than CD-EPIs (Table 2S and Fig. 11S). The explanation could rely on the higher hydrophobicity of the HDI spacer (which contains six CH_2 groups connected to the CD by O(C=O)NH groups) compared to EPI that provides a more hydrophobic environment for PTX2, as well as the occurrence of hydrogen bond interactions between the amido and OH groups of the capture polymer with the polar groups of the toxin. Therefore, the nature of the bridging units is certainly playing a more decisive role than the cavity size in this case as evidenced by the trend *γ*-CD-HDI > *β*-CD-HDI > Diaion > *γ*-CD-EPI > *β*-CD-EPI. Another important point to consider is that, in contrast to Diaion® HP-20, *γ*-CD-HDI and *β*-CD-HDI are not porous materials and the capture of PTX2 occurs mainly on the surface of the particles. Hence, the high capture efficiency showed by materials with a lower active surface such as both cyclodextrin polymers is indicative of the high strength of the intermolecular interactions involved.

An experiment was performed in the lab, with seawater spiked with the two toxins at equimolar concentrations, to investigate if competition of OA and PTX2 for the cyclodextrin cavities could explain the results obtained in the *in-situ* deployment experiment. However, all cyclodextrins were able to capture both toxins with recovery values of about 80%, and no competition was

observed. In fact, although the saturation of the cyclodextrins was not evaluated, it is evident that toxin contents in the cyclodextrins from the *in-situ* deployment experiment were far from saturation (much lower toxin contents than those reached in the culture experiment). Thus, what is happening in nature is much more complex and other environmental parameters are certainly playing a significant role. Since cyclodextrins are not specific for marine toxins, capture and competition of other compounds from seawater (e.g. chemical contaminants, organic matter, micro/nanoplastics and demucilaged seeds) cannot be discarded.

4.3 General discussion

The cyclodextrin-based materials tested in this work have provided useful information regarding the toxin profile of a *P. lima* strain and the spatial and temporal dynamics of a *D. sacculus* bloom. It is necessary to take into account that these passive sampling materials are capturing dissolved toxins from the culture media or the seawater, during a time interval, and with different efficiencies. Therefore, as observed in this work, discrepancies may arise among them. Although more work is required to better understand the results and to fully characterize the two case studies, multiple experimental parameters are not under control (e.g. diffusion of dissolved toxins, competition of toxins with other compounds that can also be captured by cyclodextrins, stability of dissolved toxins, environmental conditions, etc.). Nevertheless, this work is a step forward to understand what is happening in nature.

Compared to other passive sampling materials, cyclodextrins have the advantages of being easy to manufacture, cheap and ecologically sustainable, and their chemical and structural versatility could allow a rational synthesis according to the type of toxin to be captured. Therefore, they could be useful as early warning tools in monitoring programs. To this purpose, toxin contents in shellfish from specific regions should be determined, tailor-made cyclodextrins should be tested, and correlation between both should be established.

5. Conclusions

Summarizing, the results of the experiments described herein demonstrate the potential of cyclodextrin polymers as new materials for passive sampling of lipophilic marine toxins. In the culture experiment, OA and DTX1, and related esters at a lower extent, have been captured along *P. lima* culture time, the first signals being detected even after only 2 days. In the *in-situ* deployment experiment, toxin contents were significantly higher in the sampling points and dates where *D. sacculus* cell abundance was also higher, the effect being more evident for PTX2 than for OA. While the exact capture mechanism and the role of cavities and spacers is currently under study, the evaluated cyclodextrin-based materials have already proven efficient in providing integrated contents of toxins released into culture media and the environment. Further investigation is underway to evaluate the capture and equilibrium rates, saturation, regeneration and reusability, to remove matrix effects and to establish the correlations with shellfish contamination with the aim to develop novel passive sampling materials able to satisfy the environmental monitoring demands of specific geographic regions.

Sample CRediT author statement

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