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Baseline

Lipophilic marine toxins in sediments from Arrábida marine protected area, Portugal (NE Atlantic)

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ABSTRACT

During the development and senescence of harmful algal blooms (HAB), most of the algae cells not ingested by grazers or filter-feeding organisms sink to the bottom, making sediments important reservoirs of algae toxins. In this study, lipophilic marine toxins were determined in the sediments collected from depths ranging from 5 to 145 m depth in the marine protected area of Arrábida (southwest Portuguese coast). Sediments were characterized in terms of granulometry, water and organic matter content. The toxins were determined by liquid chromatography with tandem mass spectrometry. Okadaic acid (OA), dinophysistoxin-2 (DTX2), and azaspiracid-2 (AZA2), reaching concentrations up to 3.4, 1.3, and 0.13 ng/g, respectively, were found. A trend in the occurrence of DTX2 and AZA2 with sediment water and organic matter content was observed, as well as with AZA2 and depth. This study highlights the need to further investigate sediment deposition of toxins and their availability for bottom-dwelling organisms and its contamination.

Marine toxins produced by harmful algal blooms (HAB) are well characterized in shellfish due to their threats to seafood safety. Shellfish and particularly bivalve molluscs are filter-feeding organisms that accumulate high levels of marine toxins during the seasonal HAB occurrence thus representing a toxic vector for human shellfish consumers. To minimize the risk of acute intoxications, most European coastal countries conduct a monitoring program and shellfish harvesting is closed whenever safety limits are exceeded (Cruz et al., 2022). The most common hazardous algal toxins in Europe, including Portugal, are those causing diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), and amnesic shellfish poisoning (ASP) (Braga et al., 2023).

While the mechanisms behind the HAB development and seafood contamination have been extensively studied, little is known concerning the fate of the pool of toxins generated during HABs other than accumulation in shellfish. Over the course of a HAB event and their senescence, toxins may be released into dissolved fraction, due to cell lysis or excretion, and be adsorb by suspended particles that sink to the bottom. The toxins remaining within the algal cell, if not ingested by biota, may reach the bottom via cell deposition (Sekula-Wood et al., 2011). Faecal pellets of plankton grazers, such as copepods, also represent a major pathway of toxins to the bottom (Kuuppo et al., 2006). Finally, several benthic microalgae species are known to produce marine toxins (Durán-Riveroll et al., 2019) increasing the load of natural contaminants available to bottom-dwelling communities (Costa et al., 2005; Mafra Jr et al., 2015).

Among the wide variety of marine toxins, those with hydrophobic chemical properties are expected to remain longer in the environment and to easily adsorb to particles. The lipophilic toxins that include the okadaic acid group, also known as diarrhetic shellfish poisoning (DSP) toxins, are among the most common toxins globally. The growing concerns of their presence in surface sediments have been particularly investigated in China, namely in the Yellow Sea, Bohai Sea, East China Sea and South China Sea (Chen et al., 2017, 2018; Liu et al., 2019, 2021; Wang et al., 2015). These studies highlighted marine sediments as relevant reservoir for DSP toxins and other lipophilic toxins, such as pectenotoxins, azaspiracids, yessotoxins and cyclic imines. In Europe, deposition of DSP toxins in sediments traps was only investigated in the

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Baltic Sea, pointing out the relevant role of grazers on the transport of toxins to bottom sediments (Kuuppo et al., 2006).

Brevetoxins (BTX), that seriously affect humans and marine wildlife causing neurotoxic and respiratory illnesses, have been characterized in sediments from Florida coastline, USA (Hitchcock et al., 2012; Javaruski et al., 2022; Mendoza et al., 2008). These lipid soluble polyether compounds were initially screened in surficial sediments (Mendoza et al., 2008), and shown to persist for up to 8 months after blooms of the toxin-producing dinoflagellate *Karenia brevis* (Hitchcock et al., 2012). Recently, Javaruski et al. (2022) highlighted the possibility of reconstructing the occurrence of *K. brevis* in sediment cores by means of radiometry dating samples using ^{210}Pb.

Cyclic imines are another class of lipid soluble toxins appearing in sediments in China and New Zealand. More specifically, gymnodimine was determined in sediments from Daya Bay, South China Sea, and Pinnatoxin-F, -E and —D have been determined in Rangaunu Harbour sediments, New Zealand (Liu et al., 2019; MacKenzie et al., 2011).

The present study aims to assess, for the first time, lipophilic marine toxins in sediments at the NE Atlantic region.

Surface sediments were collected in the Marine Park *Professor Luiz Saldanha* and adjacent areas, at the mouth of the Sado estuary (south-west coast of mainland Portugal) which affects this region in terms of phytoplankton community structure (Santos et al., 2022) (Fig. 1). The Marine Park, a marine protected area included in the Arrábida Natural Park, was created in 1998 as an extension of the already existing land-based protected area (Ministério do Ambiente, 1998) (Decree 23/98) and was later included in the EU Nature 2000 network (PTCON0010) with the aim of improving the ecosystem conservation status and to reduce the fisheries impact in the region. Only low-impact fisheries are allowed within the marine protected area boundaries, except for restricted zones where any maritime usage is interdict, if not intended for research and monitoring. Thus, Arrábida marine protected area can act as a natural laboratory to investigate the presence of toxins in surficial sediments in a low disturbed area.

Sediment samples were collected from 30 stations (Fig. 1) within or near the boundaries of the marine protected area at depths ranging from 5 to 145 m onboard the Research Vessel Diplodus (see full characteristics at https://www.ipma.pt/en/navios/diplodus/). Samples were collected using a Smith McIntyre grab with a sampling area of 0.1 m^2. Once the grab was recovered on board, a sediment subsample was stored in plastic bags at −20 °C until analyses.

Granulometry analysis was carried out by dry sieving, following the procedure described in (Gaudêncio et al., 1991). The percentage of the sediment fractions were calculated according to Folk's classification (Folk, 1954). The total organic matter (TOM) content of the sediment samples was estimated by mass Loss on Ignition using a muffle furnace. Samples were pre-weighed, oven-dried at 100 °C, mashed up, and then ignited at 450 °C to constant weight. After cooling in a desiccator at room temperature, they were weighed again. The TOM content was calculated by the difference between the weight of the oven-dried samples and the combusted ones. The water content was determined by the ratio between the weight of samples oven-dried at 100 °C until constant weight and their wet weight, according to Bale and Kenny (2005). The percentage of water, organic matter and granulometry obtained for each sample is presented in Table S1. (Supplementary Material).

Sediment samples devoted to toxin analyses were freeze dried, grounded, sieved through a 2 mm mesh and stored at −20 °C until toxin extraction. Toxins were extracted using ultrasonic-assisted extraction as in Liu et al. (2017, 2019, 2021), with modifications based on the standard protocol for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS (EURLMB- European Union Reference Laboratory for Monitoring of Marine Biotoxins, 2015). Briefly, 10 g of sediment were extracted with 10 ml of MeOH by vortexing it for 1 min. The mixture was ultrasonicated for 10 min and centrifuged at 2000*g* for 10 min. The supernatant was transferred to a new 30 ml centrifuge tube, and the extraction was repeated with another 10 ml of MeOH. After the two sequential extractions, the supernatants were combined. Five ml of

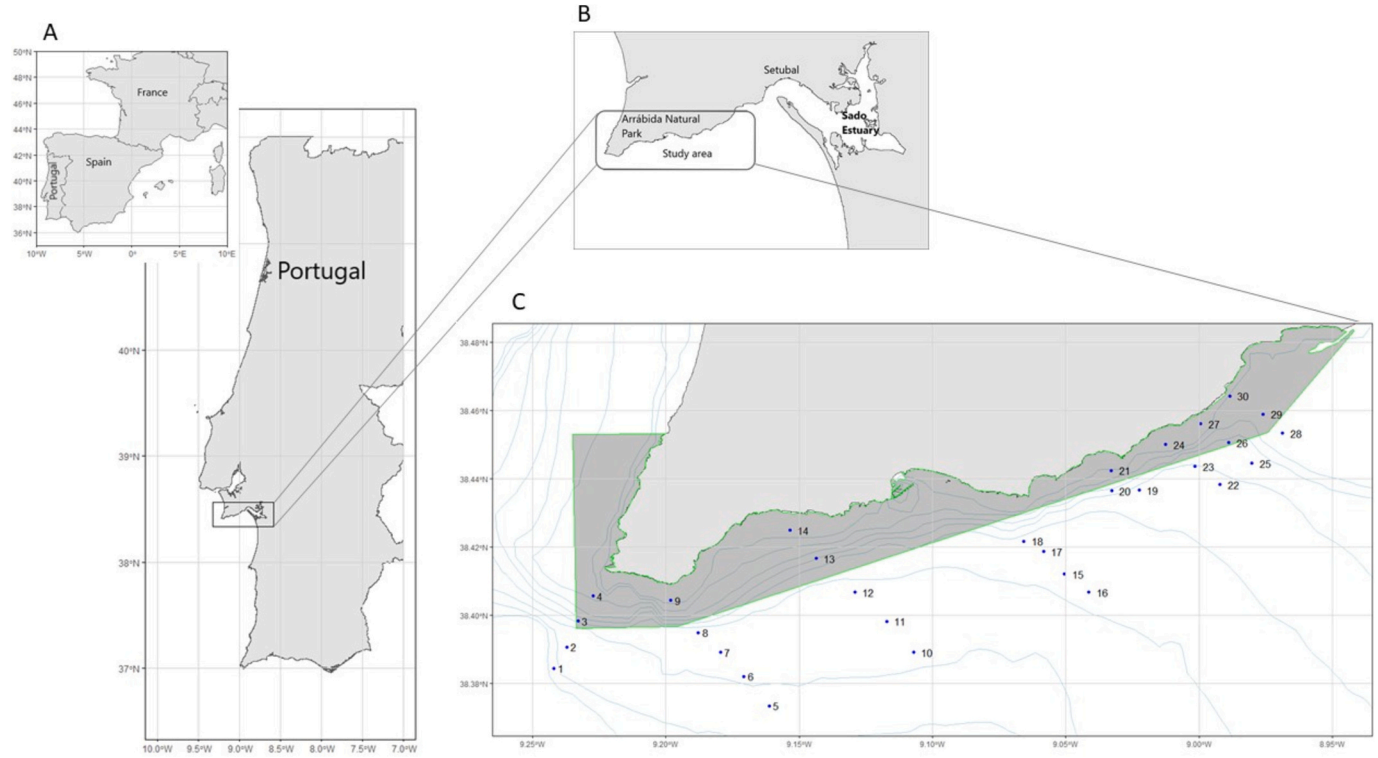

Fig. 1. A, B. Location of the study area. C. sampling stations where sediment samples were collected. The shaded area within the green line represents the boundaries of the Arrábida marine protected area. Bathymetric lines are displayed every 20 m depth. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the methanolic extract were diluted in 10 ml deionised water to obtain a 30 % (*v*/v methanol/water) solution for the Solid-phase extraction (SPE). SPE Strata-X cartridges (3 ml, 60 mg, Phenomenex, Milford, MA, USA) were activated and equilibrated with 6 ml MeOH followed by 6 ml deionised water. The diluted sample was loaded into the cartridge and washed with 3 ml of methanol/water (20:80, *v*/v). The cartridge was dried under mild vacuum, and the toxins were then eluted with 1 ml methanol containing 0.3 % (*v*/v) ammonium hydroxide. Again the cartridge was dried under vacuum to collect residual MeOH. Before analysis, the sample was filtered with a syringe RC membrane (0.22 μm).

Lipophilic toxins were determined by LC-MS/MS analysis using a 1290 Infinity chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6470 triple quadrupole mass spectrometer. The chromatographic separation was conducted with a Zorbax SB-C8 RRHT column (2.1 × 50 mm, 1.8 μm), protected with a guard column (2.1 × 5 mm, 1.8 μm). The mobile phase A was water with 2 mM ammonium formate and 50 mM formic acid, and the mobile phase B was 95 % acetonitrile with 2 mM ammonium formate and 50 mM formic acid. An elution gradient at a flow rate of 0.4 ml/min was used as follows: 0–3 min, gradient from 88 to 50 % eluent A; 3–6.5 min gradient 50 to 10 % eluent A; 6.5–8.9 min 10 % eluent A; 8.9–10 min, gradient 10 to 88 % eluent A. The detection of the toxins was carried out in multiple reaction monitoring (MRM) acquisition mode. Two MRM transitions were monitored one for quantification and the other for confirmation (Table S2, 3). A five point calibration curve with a correlation >0.990 was set up for quantification using certified OA, DTX1, DTX2, YTX and hYTX reference standards purchased from CIFGA (Lugo, Spain), and AZA1–3, Gym, SPX1 and Pntx-G purchased from NRC Canada. The limit of quantification varied from 0.02 ng/g (AZA-2) to 0.4 ng/g (OA and DTX2). Statistical analyses, as well as data visualization and maps were performed with R program (R Core Team, 2021) using the following packages: dplyr (Wickham et al., 2023) ggplot (Wickham, 2016), sf (Pebesma, 2018), rnaturalearth (Massicotte and South, 2024) and marmap (Pante and Simon-Bouhet, 2013). Bathymetric data was imported from NOAA (NOAA National Centers for Environmental Information, 2004) and the marine area polygon was obtained from (Flanders Marine Institute, 2024).

Okadaic acid (OA), dinophysistoxin-2 (DTX2) and azaspiracid-2 (AZA2) were detected in the analysed sediments (Fig. 2). No other toxins, namely yessotoxins and cyclic imines were found.

Toxins in sediments with detectable levels averaged 2.1 ± 0.9 ng/g for OA, 0.8 ± 0.3 for DTX-2 and 0.08 ± 0.02 ng/g for AZA-2. OA was detected in 13 out of the 30 stations (43 %), while DTX-2 was found in 33 % of the sampled stations. Both toxins were present mainly in stations 10 to 24 (Fig. 3 A, B). In contrast, most of sampled stations (70 %) contained AZA-2 although at low concentrations (Fig. 3).

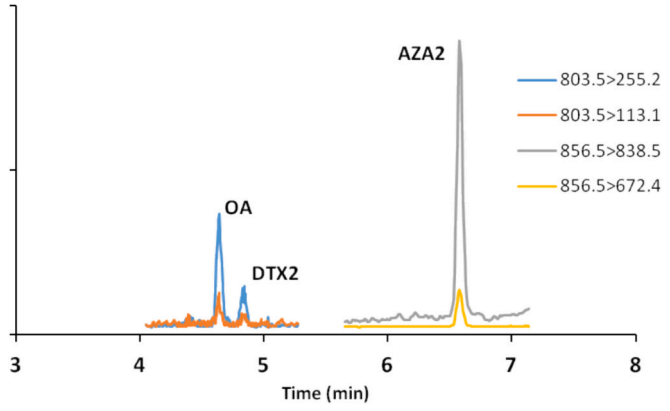

Fig. 2. LC-MSMS dynamic multiple reaction monitoring (dMRM) chromatograms of okadaic acid (OA), dinophysistoxin-2 (DTX2) and azaspiracid-2 (AZA2) found in sediment sample 24 collected in the Arrábida marine protected area, southwest Portuguese coast, in September 22th, 2020.

These results are in line with those carried out in China, where OA derivatives showed the highest concentrations in sediment samples, while AZA-2 were the most ubiquitous (Liu et al., 2019, 2020). The levels found here for OA fall in the range of those reported previously in that country (Chen et al., 2017, 2018; Liu et al., 2019) but are higher than those reported for Laizhou Bay (Wang et al., 2015), Daya Bay (South China Sea) (Liu et al., 2020), Pearl River Estuary (Li et al., 2022) and Sishili and Rongcheng Bays (Sheng et al., 2023), though lower than OA quantified in Jiaozhou Bay (Liu et al., 2021). DTX-2 was also detected in Pearl River Estuary, yet at lower levels than those found in Portugal (Li et al., 2022). The sensitivity of the LC-MS/MS method allowed for the detection of AZA-2 in most sampling sites at very low concentration, following a pattern similar to that observed in other studies with moderate AZA-2 concentrations but high occurrence (Li et al., 2022; Liu et al., 2019; Sheng et al., 2023). On the other hand, lipophilic toxins, other than OA, DTX-2 and AZA-2, such as DTX-1, PTX-2, homo-YTX and GYM were not detected in the present work. Differences in microalgae species occurrence may be behind the differences in toxin distribution in sediments from Portugal.

The coast of Arrábida is an important harvesting area, where clams, mussels, cockles, sea urchins and limpets are collected for trade or self-consumption. Marine biotoxins are routinely monitored in Portugal, according to European Regulations, which set the thresholds for lipophilic toxins in bivalves at 160 μg/kg for both OA and AZA toxins groups (Regulation (EC) No 853/2004) (European Union, 2004). Lipophilic toxins and their producing dinoflagellates are occasionally reported in the area and the neighbouring Sado estuary (https://www.ipma.pt/pt/bivalves/fito/index.jsp). OA and DTX-2 are related toxins produced by dinoflagellate species of the genera *Dinophysis* and *Prorocentrum* (Hu et al., 2010; Reguera et al., 2012), while AZA-toxins are synthesized by the genera *Azadinium* and *Amphidoma* (Tillmann et al., 2017). This could explain the co-occurrence of OA and DTX-2 in most samples and the higher correlation between both toxins compared to AZA-2 (Fig. 3). *D. acuta*, *D. acuminata* and *D. caudata* are regularly detected in Portuguese coast along with *Prorocentrum* spp. (e.g. *P. lima*) (Bresnan et al., 2021; Santos et al., 2022), and can be predominant at the mouth of Sado estuary during summer and autumn (Santos et al., 2022). In contrast, species of the genus *Azadinium*, despite being present in Portugal (Danchenko et al., 2019), are rarely detected, likely because of the difficulties in their monitoring due to their small size and accurate identification that can lead to cell loss and misidentifications (Bresnan et al., 2021). Similarly, Sheng and collaborators (2023) detected AZA-2 in most sediment samples, even though toxin producing dinoflagellates were not observed in their area of study. Indeed, AZA-2 was not detected in seawater but occurred in sediments and in suspended matter of the studied areas (Chen et al., 2018; Sheng et al., 2023). The high hydrophobic nature of AZA-2 may favour their sinking rate and accumulation in sediments, although their stability on sedimentary systems is unknown. On the other hand, OA has been observed to be stable in sediments after 23 days, being estimated that 1 % of the toxin could remain for >28 years (Blanco et al., 2018). Resting cysts can also act as a source of toxins on the seafloor (Blanco et al., 2018; Pizarro et al., 2018).

No correlations were found between toxin concentration and seafloor composition (percentage of mud, sand or gravel), although a weak but positive correlation was observed between DTX-2 and the percentage of organic matter ($r = 0.64$) and DTX-2 and the percentage of water ($r = 0.56$) (Fig. 4). Similar trend was observed for AZA-2 concentration and organic matter ($r = 0.69$), being more evident in the case of the percentage of water ($r = 0.87$) and depth ($r = 0.64$) (Fig. 4).

Previous studies suggested sediment size could play a role in lipophilic toxin retention by the presence of resting cyst (Mohamed et al., 2011; Pizarro et al., 2018), although this was not obvious in samples from the study area. In most of the stations a high percentage of sands was recorded, not allowing for reliable comparisons. Similar research performed in China did not observe differences between toxin contents

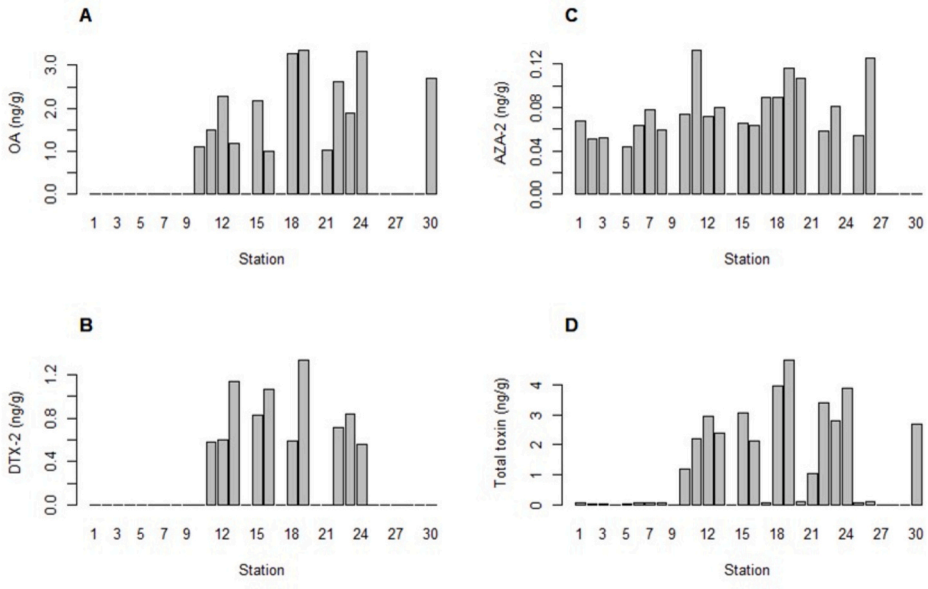

Fig. 3. Concentration (ng/g) of OA, DTX-2 and AZA-2 determined in sediment samples collected in the Arrábida marine protected area, Portugal.

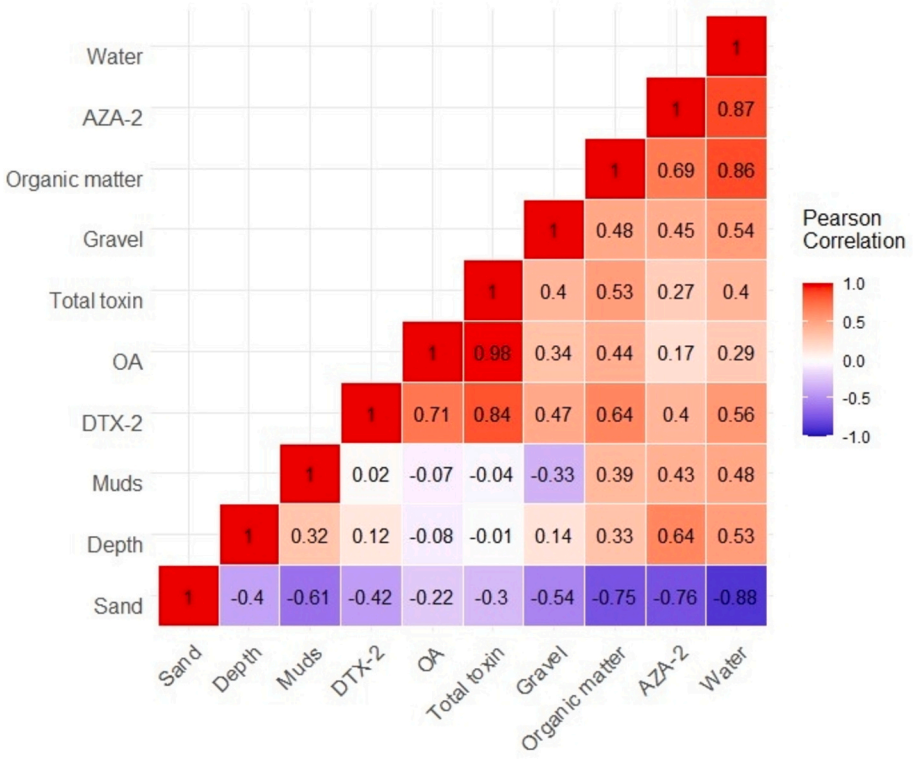

Fig. 4. Correlation matrix showing Pearson correlation between the different variables considered.

and sediment grain average (Liu et al., 2019, 2021). Nonetheless, DTX-2 and AZA-2 concentration may be somehow related to organic matter content, probably due to its higher affinity for this fraction (Li et al., 2022). The percentage of organic matter in samples from this study averaged 3.2 ± 2.3, being in the range of those observed in certain Chinese regions where lipophilic toxins have been also detected (Liu et al., 2019, 2021). Previous studies suggested that some lipophilic toxins may be more stable in detrital organic matter, which agrees with the positive correlation observed (Kuuppo et al., 2006; Li et al., 2022). High correlation between particulate organic matter and DTX-2 and AZA-2 was observed in the study by Li and collaborators (Li et al., 2022). Also, the spatial distribution of lipophilic toxins in sediments from Sishili and Rongcheng Bays (China) showed a trend of high concentrations in areas with land-based inputs likely linked to an elevated organic matter in the bay (Sheng et al., 2023).

Phytoplankton blooms can be itself a source of organic matter through direct cell sedimentation or other consumers' faecal material that accumulate on the bottom (Kuuppo et al., 2006). Occasionally, dinoflagellate blooms occur at the mouth of the neighbouring Sado estuary (Santos et al., 2022). The particular location of Arrábida marine

area, facing South and protected from predominant winds by cliffs together with the absence of bottom-disturbing activities, could at some extent favour the sedimentation processes. The high correlation between AZA-2 and percentage of sediment's water could be indirectly related with the organic matter content in the sample. On the other hand, the analyses of sediment porewater in samples from a Chinese estuary revealed levels of lipophilic toxins, even greater than in the bottom boundary layer, but AZA-2 was only detected in the sediment solid phase (Sheng et al., 2023).

The occurrence of lipophilic toxins in sediments reveals that different routes of biotoxin exposure are possible in benthic environments. Biotoxins may be accumulated on the seafloor through sedimentation in particulate matter or dissolved in interstitial water and the risk of biotoxin accumulation in benthic biota may depend on their stability in these phases (Blanco et al., 2018; Jackson et al., 2012).

Particle-mediated transport of biotoxins has been demonstrated in previous studies. Lipophilic toxins were detected in particulate matter in China (i.e. PTX-2, PTX-2 SA, 7-epi-PTX-2 SA, OA, SPX-1, DTX-1-iso) (Chen et al., 2017; Li et al., 2022; Sheng et al., 2023; Wang et al., 2016), and the Baltic sea (i.e. PTX-2, DTX-1) (Kuuppo et al., 2006). Also, DA was detected in suspended particles in California (Sekula-Wood et al., 2011; Umhau et al., 2018). Suspended matter can exhibit important adsorption properties and may have a crucial role in pollutant and biotoxin fate, particularly the most hydrophobic ones. Sedimentation rates to the seafloor were estimated in a few studies. In the Baltic Sea these estimations suggested this sedimentation was very low for PTX-2 (0.01 % of the concentration in suspended matter in 6 weeks), increasing for DTX-1 (1 % of the concentration in suspended matter in 6 weeks), after the peak of *Dinophysis* (Kuuppo et al., 2006). Direct sedimentation after a bloom may contribute to the accumulation of toxins in the bottom, but the low sedimentation rates suggest that most of the toxins are decomposed before getting to the deepest layers (Kuuppo et al., 2006). On the other hand, copepods feeding on toxin-producing microalgae probably contribute to some extent to the transfer of toxins from pelagic to bottom environments through their excretion in faecal pellets (Frangoulis et al., 2022; Jansen et al., 2006; Kuuppo et al., 2006; Wexels Riser et al., 2008).

The partition fraction (Φ) and partition coefficients (kp) were estimated in different Chinese estuaries (Li et al., 2022; Sheng et al., 2023). Lipophilic toxins appeared to be more prone to be present in particulate organic matter and declined in seawater and sediments, although AZA-2 and DTX-2 fractions were still elevated in the sediment-water fraction. Furthermore, the kp increased with organic carbon fraction, especially in pelagic systems, pointing out that particulate organic matter may play an important role in transporting lipophilic toxins among marine compartments, which would be modulated by particular toxin affinities and environmental factors (Li et al., 2022). On the other hand, in the bottom boundary layer the distribution of OA between the particulate and dissolved phases was more balanced (Sheng et al., 2023), consistently with the high stability that this toxin exhibits in both dissolved or adsorbed forms (Blanco et al., 2018).

The present and previous studies confirm, that although sedimentation can be slow and the majority of toxins are likely to be degraded or consumed in the water column, the accumulation and stability of OA, DTX-2 and AZA-2 in sediments may be noticeable. A range of sedimentivorous invertebrates are prey of benthic organisms, such as flatfish, crabs and gastropods, and the gateway for toxin upgrading towards food-webs (Corriere et al., 2021; Mafra et al., 2019; Moreira-González et al., 2022; Sipiä et al., 2000; Zamorano et al., 2013). Furthermore, an experimental test has proved that filter feeders, such as marine bivalves can consume and accumulate biotoxins through particulate matter filtration (A. Li et al., 2018), thus bottom disturbance and resuspension of deposited material are additional possible sources of seafood contamination.

In this study, sediments from the Portuguese coast of Arrábida marine protected area were analysed for the first time, showing quantifiable levels of OA, DTX-2 and AZA-2. The levels found in this study exceed the concentrations reported in other seas and suggest that contamination of marine organisms with marine biotoxins through sediment ingestion is plausible in this region. Arrábida is a little disturbed area where most bottom-impacting fishing activities are forbidden, which could impede the resuspension of toxins in the water column and favour its presence in the bottom layer. On the other hand, considering the proximity of intensive bivalve harvesting zones, a better assessment of marine toxin occurrence in different sea compartments, including sediments, calls for attention. Despite the limited number of samples and periods evaluated, this study fills a gap of knowledge on toxin distribution besides microalgae and filter-feeding organisms and opens a new field on toxin fate and accumulation along sedimentary systems.

CRediT authorship contribution statement

Lucía Soliño: Writing – original draft, Software, Methodology, Conceptualization. **Ana Catarina Braga:** Writing – original draft, Methodology. **Jorge Lobo-Arteaga:** Writing – review & editing, Resources, Funding acquisition. **Pedro Reis Costa:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.117096.

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