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Lipophilic toxins occurrence in non-traditional invertebrate vectors from North Atlantic Waters (Azores, Madeira, and Morocco): Update on geographical tendencies and new challenges for monitoring routines

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ABSTRACT

In the last decades, due to monitoring programs and strict legislation poisoning incidents occurrence provoked by ingestion of naturally contaminated marine organisms has decreased. However, climate change and anthropogenic interference contributed to the expansion and establishment of toxic alien species to more temperate ecosystems. In this work, the coasts of Madeira, São Miguel islands and the northwestern Moroccan coast were surveyed for four groups of lipophilic toxins (yessotoxins, azaspiracids, pectenotoxins, and spirolides), searching for new vectors and geographical tendencies. Twenty-four species benthic organisms were screened using UHPLC-MS/MS technique. We report 19 new vectors for these toxins, six of them with commercial interest (*P. aspera*, *P. ordinaria*, *C. lampas*, *P. pollicipes*, *H. tuberculata* and *P. lividus*). Regarding toxin uptake a south-north gradient was detected. This study contributes to the update of monitoring routines and legislation policies, comprising a wider range of vectors, to better serve consumers and ecosystems preservation.

1. Introduction

Harmful Algal Blooms (HABs), are dense clouds of algae with erratic nature whose forming circumstances are still to disclosure. HABs can vary in species composition (mono or polyspecific), occurrence and frequency (Hallegraeff, 1993; Smayda, 1997). Consequences of global changing together with anthropogenic intervention such as the rising of water temperature and ecosystems eutrophication derived from the anthropogenic intervention are pointed as significative factors for the increase in frequency, persistence, and intensity of these blooms in the past decades (Hallegraeff, 2010; Heisler et al., 2008). So far, about 300 marine phytoplankton species have been reported as bloom-forming, being 40 of them pointed as toxic, noxious or nuisance (Moore et al., 2008; Smayda, 1997). HABs adverse consequences are not measured by cell abundance but by the magnitude of their impact, they can affect an entire ecosystem in different ways by causing high mortalities in wildlife (Conley et al., 2009; Yang and Albright, 1992), affecting economy, namely the fishery and touristic sectors (Hoagland and

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Scatasta, 2006; Morgan et al., 2009), and by inducing human poisoning incidents. Human toxin exposure can occur through via many routes, being the most common by ingestion of contaminated seafood, although there are reports of poisoning incidents provoked by dermal contact and inhalation of aerosolized forms (Backer et al., 2005; Cheng et al., 2005; Fleming et al., 2005; Fleming et al., 2001). Due to market demand, FAO's report on "The State of World Fisheries and Aquaculture", states that global aquaculture had a severe increase since the 1950s, forecasting that this trend will continue with a 3.6% of growth rate every year (Etheridge, 2010). This market globalization, international fish trade increased the need for guidelines, monitoring procedures and the development of reliable detection methods to provide and ensure safety to consumers (Alam et al., 1978; Silva et al., 2013; Takahashi et al., 2001; Yotsu-Yamashita et al., 2001). Since, marine biotoxins are secondary metabolites with phytoplankton and bacterial origin, whose primary goal is to enhance survival strategies to their producers against competitors and predators, yet in this context, human poisoning incidents are an indirect consequence of this surviving strategy (Cembella, 2003; Hallegraeff, 2010). Almost 200 different compounds have been described, divided into 12 different groups (FAO/IOC/WHO, 2004), however, only half of them have a regulatory status: azaspiracids (AZAs), domoic acid group (DA), okadaic acid and dinophysistoxins group (DSPs), saxitoxins (STXs) and recently tetrodotoxin group (TTXs) in the Netherlands (Alexander et al., 2008a, 2009a; Alexander et al., 2009b; Alexander et al., 2008c; Gerssen et al., 2018). Nowadays, in the European Union (EU) and Morocco, the regulated marine toxins are lipophilic toxins, paralytic shellfish toxins and amnesic shellfish toxins. Regarding monitoring programs, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is used in the EU as reference method (European Union Refenrence Laboratory for Marine Biotoxins, 2013; European Union Reference Laboratory for Marine Biotoxins, 2015). The current EU regulatory limit for marine lipophilic toxins in shellfish meat (SM) are for DSPs 160 μg OA equivalents/kg SM; AZAs 160 μg AZA equivalents/kg SM; PTXs 160 μg OA equivalents/kg SM (combination of OA, DTXs and PTXs); and finally, for YTXs 3.75 mg YTX/kg SM (Alam et al., 1978; Silva et al., 2013).

Marine lipophilic toxins are categorized into different groups, which are characterized by different chemical structures and different mechanisms of toxicity. These groups can be produced by the same or by different species of marine microalgae. In this work, we studied the occurrence of these four groups of lipophilic toxins in three different locations (Fig. 1).

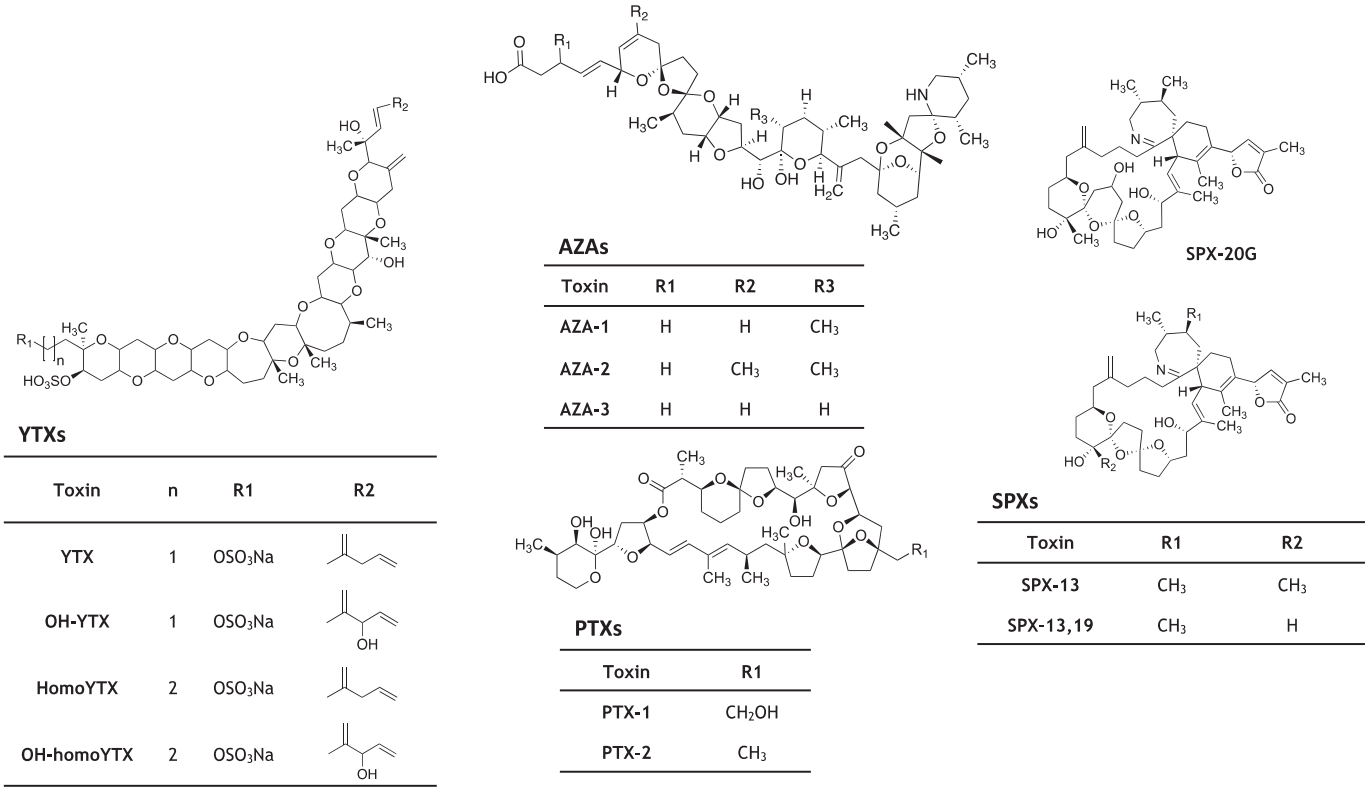
Toxins belonging to the YTX group are cytotoxic polyethers (Fig. 1) first isolated in 1986 in Japan, from the digestive gland of the scallop *Mizuhopecten yessoensis* (Bianchi et al., 2004; Konishi et al., 2004; Murata et al., 1987; Pérez-Gómez et al., 2006). Concerning their origin, dinoflagellate species of *Gonyaulax spinifera*, *Protoceratium reticulatum*, and *Lingulodinium polyedrum* are pointed as main producers (Eiki et al., 2005; Paz et al., 2004; Rhodes et al., 2006). Initially, YTXs were included in the DSP group, due to their lipophilic features. The DSPs characteristic intoxication symptoms are diarrhea through inhibition of phosphatases (Bialojan and Takai, 1988). Since YTXs were not shown to provoke these symptoms, the European Food Safety Authority (EFSA) proposed the guideline for YTXs (Alexander et al., 2008b; Bianchi et al., 2004; Konishi et al., 2004; Ogino et al., 1997; Pérez-Gómez et al., 2006; Tubaro et al., 2011). These heat-stable polyethers were detected mainly in bivalve species (scallops and mussels) from Japan, Italy, Spain, United Kingdom, Norway, Russia, Canada, Chile and New Zealand (Ciminiello et al., 1997; Lee et al., 1988; Murata et al., 1987; Stobo et al., 2003; Vershinin et al., 2006; Yasumoto and Takizawa, 1997). With up to 90 analogs described so far, only 30 were fully characterized, being the most relevant: 45-hydroxyYTX, 45-hydroxy-1a-homoYTX, and 1a-homoYTX (Alexander et al., 2008b).

AZAs are lipophilic phycotoxins with three spiro bonds between rings (Fig. 1). The first AZA isolated was AZA-1 in 1995 following a toxic episode in the Netherlands due to contaminated mussels (McMahon and Silke, 1996). The symptoms of AZA poisoning are nausea, vomiting, diarrhea and stomach cramps. These toxins were detected in several species of filter-feeding mollusks (oysters, mussels, scallops, and clams) (Hess et al., 2003; McMahon and Silke, 1996). Also, one of the main AZA producers are dinoflagellates, such as *Azadinium spinosum* and *Azadinium poporum* (Krock et al., 2008, 2009; Krock et al., 2019; Tillmann et al., 2009). EFSA established the toxic equivalent factors (TEFs) for this group 1 for AZA-1, 1.8 for AZA-2 and 1.4 for AZA-3 (Alexander et al., 2008c).

PTXs are macrocyclic polyethers, unstable in strongly basic conditions and to date 15 different analogs have been isolated (Fig. 1) (Suzuki, 2008). Under EU legislation these toxins were included within the DSP group because they were produced by *Dinophysis* spp. Nowadays, PTXs are considered an independent group since their absence of recorded symptomatology, neither poisoning incidents recorded (European Food Safety Authority, 2009). However, several studies have confirmed that chronical exposure to this group of toxins causes alterations of the actin-based cytoskeleton (Espina et al., 2008). Being the toxicological database of this group is limited and no reports on adverse effects in humans so far, EFSA established provisional TEFs value of 1 for PTX-1, PTX-2, PTX-3, PTX-4, PTX-6 and PTX-11 (European Food Safety Authority, 2009).

Cyclic imines (CIs) are a heterogenic group of macrocyclic compounds having imine and ester groups and spiro unions (Fig. 1) (European Food Safety Authority, 2010; Molgó et al., 2014). The SPXs are neurotoxic and their target is nicotinic and muscarinic acetylcholine receptors (mAChR and nAChR, respectively) in the central and peripheral nervous system and at the neuromuscular junction (Meilert and Brimble, 2006; Molgó et al., 2014). These toxins were discovered in the 90s during a routine monitorization in Canada and New Zealand, but in subsequent years CIs have been also detected in European waters (Amzil et al., 2007; Hu et al., 1995; Seki et al., 1995; Villar Gonzalez et al., 2006). Currently, regulations regarding SPXs in the EU are absent, only a recommended guidance level of 400 μg total SPXs/kg, due to the lack of reports regarding their acute toxicity, though their effects regarding chronical exposure are still to be unravel (European Food Safety Authority, 2010; Otero et al., 2011a). Several detection methods have been developed for this group of compounds like mouse bioassay (MBA), biochemistry assays, LC-MS/MS methods, etc. (Bourne et al., 2010; Gerssen et al., 2009; Marrouchi et al., 2010; Otero et al., 2011b; Yasumoto et al., 1978). Taking into account that these toxins are lipophilic, and they can be extracted with the regulated lipophilic toxins method, they are suited to be included in EU Reference Laboratory for Marine Biotoxins (EU-RL-MB) detection method (European Union Reference Laboratory for Marine Biotoxins, 2015).

In this work, the Portuguese Islands of Madeira and S. Miguel, and the northwestern Moroccan coast were surveyed for four groups of lipophilic toxins. As mentioned above, the rise of water temperature, derived from global change, together with anthropologic inputs, aided the migration and establishment of these phycotoxins from warm ecosystems to more temperate ones. EFSA defined "emergent toxins" as recently discovered nonregulated toxins or reported toxins previously absent (Alexander et al., 2010; Reverté et al., 2014). In this context, these toxins (AZAs, YTXs and PTXs) can be considered emergent toxins in Madeira and Azores despite being regulated they are not common in these areas. On the other hand, SPXs fall into the category of emerging toxins, since they are not regulated or reported in the screened areas monitored in this work. Considering this premises, our primal aim was to search for new non-traditional vectors, unravel geographical tendencies to assess human health risk. In this context, the DSP group was not described in this work because the results obtained for this group were presented in a previous paper (Silva et al., 2015b).



YTXs

Toxin	n	R1	R2
YTX	1	OSO_3Na	
OH-YTX	1	OSO_3Na	
HomoYTX	2	OSO_3Na	
OH-homoYTX	2	OSO_3Na	

AZAs

Toxin	R1	R2	R3
AZA-1	H	H	CH_3
AZA-2	H	CH_3	CH_3
AZA-3	H	H	H

PTXs

Toxin	R1
PTX-1	CH_2OH
PTX-2	CH_3

SPX-20G

SPXs

Toxin	R1	R2
SPX-13	CH_3	CH_3
SPX-13,19	CH_3	H

Fig. 1. Marine lipophilic toxins.

2. Materials and methods

2.1. Chemicals

Acetonitrile, dichloromethane, and methanol were from Panreac (Barcelona, Spain). All solvents were analytical grade and water was obtained from a water purification system (Milli-Q, Millipore, Spain). Formic acid was purchased from Merck (Darmstadt, Germany). Ammonium formate was from Fluka (Sigma-Aldrich, Spain).

Certified reference materials provided by Cifga (Lugo, Spain) were: pectenotoxin-2 (PTX-2, 7.27 ± 0.33 μg/mL), yessotoxin (YTX 7.42 ± 0.49 μg/g), 1a-homoyessotoxin (homoYTX 7.68 ± 0.44 μg/g), azaspiracid-1 (AZA-1 1.36 ± 0.07 μg/g), azaspiracid-2 (AZA-2 1.33 ± 0.11 μg/g) and azaspiracid-3 (AZA-3 1.30 ± 0.09 μg/g).

The quality control standard provided by Cifga (Lugo, Spain) was 20-methyl spirolide G (SPX-20G 7.01 ± 0.61 μg/mL).

2.2. Sampling sites and selected species

In the present study a total of 101 samples were collected, aiming to screen lipophilic toxins in different species of benthic organisms: from echinoderms (*Astropecten aranciacus*, *Echinaster sepositus*, *Marthasterias glacialis*, *Ophidiaster ophidianus*, *Holothuria (Platyperona) sanctori*, *Arbacia lixula*, *Diadema africanum*, *Paracentrotus lividus*, *Sphaerechinus granularis*), to arthropods (*Pollicipes pollicipes*) and mollusks (*Mytilus* spp., *Phorcus lineatus*, *Haliotis tuberculata*, *Onchidella celtica*, *Pattela aspera*, *Patella* spp., *Umbraculum umbraculum*, *Stramonita haemostoma*, *Charonia lampas*, *Cerithium vulgatum*, *Gibbula umbilicalis*, *Aplysia depilans*, *Patella gomesii*, *Patella ordinaria*). Samples were collected in the intertidal zone and by Self-Contained Underwater Breathing Apparatus (SCUBA) diving in three different locations (Fig. 4). Edible and non-edible species were selected due to their inherent economic relevance and by their key position in the food web (Table 1), since phycotoxin bioaccumulation phenomena already been reported in former work (Silva et al., 2015b).

Sample expeditions were performed in three different geographical locations (Azores and Madeira archipelagos (Portugal) and the north-western Moroccan coast), between September 2012 and July 2013 (Fig. 2). Samples of *P. ordinaria* and *P. aspera* were purchased in local markets in Madeira, caught on the northern coast of the island (32°51′17.02″ N; 17°01′54.02″ W). After harvesting, samples were transported to the laboratory in refrigerated containers. If immediate processing was not possible, samples were stored at −20 °C.

2.3. Sample extraction

Samples were extracted following the extraction procedure (Otero et al., 2010; Silva et al., 2015b). The animals were homogenized with a blender (A320R1, 700 W, Moulinex, Lisbon, Portugal), previously the shells were removed when necessary. In some cases, the animals were homogenized in pooled groups in order to obtain 1 g of tissue. In the case of *O. ophidianus*, *P. lividus*, *S. granularis*, *U. umbraculum*, *D. africanum*, *H. sanctori*, *C. lampas*, *M. glacialis*, and *A. depilans* each animal was handled separately since it had enough biomass. Then, 1 g of homogenized tissue was weighed into a centrifuge tube and 3 mL of methanol was added. The sample was homogenized via vortex mixing at maximum speed level. Afterward, the extracts were centrifuged for 10 min at 2932*g* at 4 °C and the supernatant was transferred to a 15 mL volumetric flask. The extraction was repeated twice, the supernatants were combined and concentrated to dryness. Next, residues were re-suspended in 10 mL of Milli-Q water and liquid-liquid extraction with dichloromethane was done. The aqueous layer was extracted again with 10 mL of dichloromethane and the organic layers (20 mL of final volume) were concentrated to dryness and re-suspended in 200 μL of methanol. The determination of PTXs, AZAs, YTXs, and SPXs was performed by ultra-high liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) after filtering a methanolic extract aliquot with 0.22 μm filter (UltraFree-MC centrifugal devices, Millipore).

Table 1
Sampled species description.

Species	Sample type	Trophic level	Sampling site(s)	AN	Ed	MS	Ref
A. aranciacus	Echinodermata asteroidea	2nd level predator	Madeira	2	No	No	(Burla et al., 1972)
A. depilans	Mollusca gastropoda	Grazer	Morocco	1	No	No	(Carefoot, 1987)
A. lixula	Echinodermata echinoidea	Grazer	Madeira/Azores/Morocco	4	No	No	(Bulleri et al., 1999)
C. lampas	Mollusca gastropoda	3rd level predator	Madeira/Morocco	1	Yes	No	(Lin and Hwang, 2001)
C. vulgatum	Mollusca gastropoda	Grazer	Morocco	40	Yes	No	(Nicolaidou and Nott, 1999)
D. africanum	Echinodermata echinoidea	Grazer	Madeira	1	No	No	(Rodríguez et al., 2013)
E. sepositus	Echinodermata asteroidea	2nd level predator	Madeira	3	No	No	(Ferguson, 1969)
G. umbilicalis	Mollusca gastropoda	Grazer	Morocco	100	Yes	No	(Crothers, 2001)
H. sanctori	Echinodermata holothuroidea	Deposit feeder	Morocco	1	Yes	No	(Navarro et al., 2013; Toral-Granda et al., 2008)
M. glacialis	Echinodermata asteroidea	2nd level predator	Madeira/Azores/Morocco	1	No	No	(Knox, 2001)
P. lineatus	Mollusca gastropoda	Grazer	Morocco	86	Yes	No	(Crothers, 2001)
Mytilus spp.	Mollusca bivalvia	Filter feeder	Morocco	30	Yes	Yes	(Buschbaum et al., 2008)
O. celtica	Mollusca gastropoda	Grazer	Morocco	50	No	No	(Dayrat, 2009)
O. ophidianus	Echinodermata asteroidea	Detritivorous	Madeira/Azores	1	No	No	(Ferguson, 1969)
P. aspera	Mollusca gastropoda	Grazer	Madeira	15	Yes	No	(Knox, 2001)
Patella spp.	Mollusca gastropoda	Grazer	Morocco	12	Yes	No	(Knox, 2001)
P. gomesii	Mollusca gastropoda	Grazer	Azores	10	Yes	No	(Knox, 2001)
P. lividus	Echinodermata echinoidea	Grazer	Madeira/Azores/Morocco	1	Yes	No	(Lemée et al., 1995)
P. pollicipes	Arthropoda hexanauplia	Filter feeder	Morocco	35	Yes	No	(Knox, 2001)
S. granularis	Echinodermata echinoidea	Grazer	Azores	1	Yes	No	(Martinez-Pita et al., 2008)
U. umbraculum	Mollusca gastropoda	Grazer	Madeira	1	No	No	(Valdes, 2001)
S. haemostoma	Mollusca gastropoda	2nd level predator	Madeira/Azores/Morocco	15	No	No	(Ramírez et al., 2009)
H. tuberculata	Mollusca gastropoda	Grazer	Azores	1	Yes	No	(Peck, 1989)
P. ordinaria	Mollusca gastropoda	Grazer	Madeira	10	Yes	No	(Faria et al., 2017)

AN: average number of organisms to compose a sample; Ed: edible; MS: monitoring status; Ref: references.

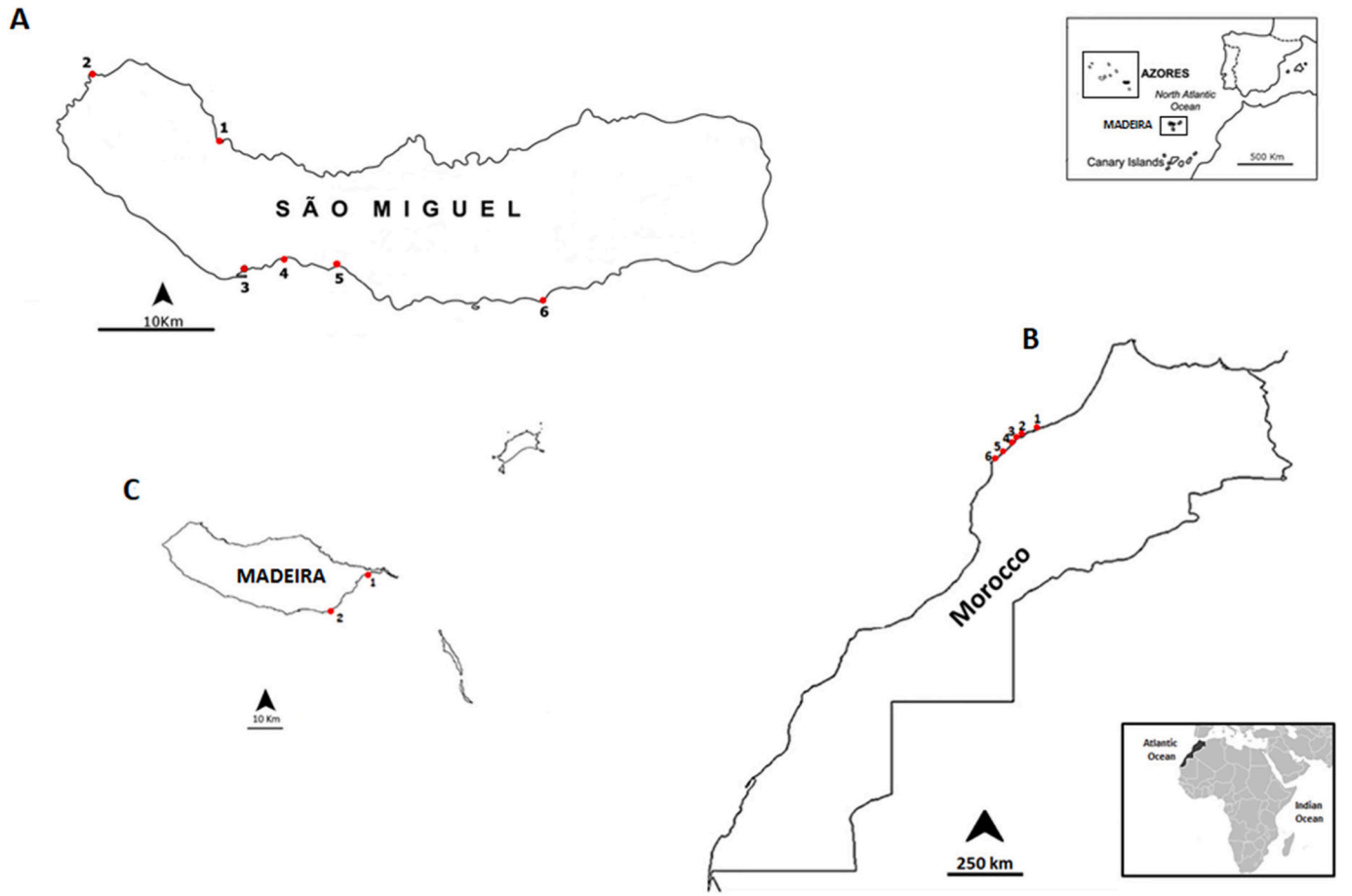


Fig. 2. Location of the sampling sites: (A) São Miguel island coast, Azores archipelago: 1, Cruzeiro; 2, Mosteiros; 3, Étar; 4, São Roque; 5, Lagoa; 6, Caloura. (B) Northwestern Moroccan coast: 1, Casablanca Corniche; 2, El Jadida Haras; 3, El Jadida Sâada; 4, Sidi Bouzid; 5, Mrizika; 6, Oualidia (C) Madeira island coast: 1, Reis Magos and 2, Caniçal.

2.4. UHPLC-MS/MS method

The UHPLC-MS/MS analysis was performed by a 1290 Infinity ultra-high-performance liquid chromatography system coupled to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). The nitrogen generator was a Nitrocraft NCLC/MC from Air Liquid (Spain).

The toxins were separated using a column ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 μm, Waters) at 40 °C. Mobile phase A was composed of 100% water and B by acetonitrile-water (95:5), both containing 50 mM formic acid and 2 mM ammonium formate. The gradient elution (6.5 min) was: starting with 30% B, then 30–70% B for 3 min, then, 70% B was held for 1.5 min and reducing afterward to 30% B over 0.1 min and hold for 1.99 min until the next run. The samples in the autosampler were cooled to 4 °C and injection volume was 5 μL.

MS detection was performed using an Agilent G6460C triple quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). Source conditions were optimized to achieve the best sensitivity for all compounds. A drying gas temperature of 350 °C and a flow of 8 L/min. A nebulizer gas pressure of 45 psi. A sheath gas temperature of 400 °C and a flow of 11 l/min. The capillary voltage was set to 4000 V in negative mode with a nozzle voltage of 0 V and 3500 V in positive mode with a nozzle voltage of 500 V. The analysis was carried out using electrospray ionization (ESI) and multiple reaction monitoring (MRM) acquisition in positive and negative mode. The collision energy, cell accelerator voltage, and fragmentor were optimized using MassHunter Optimizer software. Two product ions were analyzed per compound, one for quantification and another for confirmation (Table 2).

2.5. Statistical analyses

Patterns of toxin distribution across geographical regions and organisms were explored with the multivariate technique Principal Components Analysis (PCA). For this analysis, data on less frequent toxins in the data set, with ~1 positive sample were removed, and analogous molecules were grouped into a single class for YTXs and AZAs. PCA was performed with *PCA* function from the FactoMineR *R* package (Lê et al., 2008).

3. Results

In this study, a total of 24 species were collected, from three different locations along the North Atlantic in August and September 2012, and June and July 2013.

In Madeira Island a total of 25 samples were harvested, 37 samples in São Miguel Island (Azores) and 39 samples along the north-western coast of Morocco, for the identify and quantify lipophilic toxins using UHPLC-MS/MS technique in MRM mode.

Each toxin group was quantified by means of several certified reference materials: AZA-1, 2 and 3; YTX and homoYTX; SPX-20G; and PTX-2 (Fig. 3).

Nine-point calibration curves ranging between (20 μg/kg and 0.08 μg/kg) were used for the quantification of AZA-1-3; SPX-20G; and PTX-2. To quantify the YTX group, calibration curves ranged between (50 μg/kg and 0.2 μg/kg). Limit of detection (LOD) and the limit of quantification (LOQ) were determined for each toxin (Table 3).

3.1. Madeira Island

A total of 25 samples were collected in August and September 2012. Lipophilic toxins were detected in 24 samples, 7 mollusks and 17 echinoderms and the average concentrations detected were below the legal limit in the EU (Table 3). PXT-2 was detected only in one sample of *E. sepositus* (353) among the total screened samples. For AZAs, a total of 24 from 25 analyzed samples were positive in AZA-2 and the highest concentration sample for this toxin was *O. ophidianus* (341#1), 53.16 μg/kg. In the case of AZA-1 and AZA-3, a total of 64% and 60% of the samples presented these toxins, respectively, from a total of 25 samples. For AZA-1, the detected concentrations ranged from 0.41 μg/kg in *P. ordinaria* (336) to 6.15 μg/kg in *O. ophidianus* (341#1) and 87% of positive samples were echinoderms from a total of 16 positive samples. Finally, for AZA-3, concentration ranged from 0.31 μg/kg in *P. lividus* (339#1 and 339#2) to 2.08 μg/kg in *O. ophidianus* (341#1) and in this case echinoderms represented 93% of positive samples. Regarding CIs, for SPX-13, a total of 80% of samples contained this toxin, but only two were above the LOQ; *O. ophidianus* (341#1) and *E. sepositus* (353) with 2.49 μg/kg and 0.24 μg/kg, respectively.

Table 2
The main characteristic of UHPLC-MS/MS method in MRM mode for lipophilic toxins.

Compound	Precursor ion	Product ions	CE	CAV	Frag	Polarity	LOD	LOQ
45-OH-homoYTX	1171.5	869.5	88	250	4	Negative		
		1091.5	40					
45-OH-YTX	1157.5	871.5	86	240	4	Negative		
		1077.5	38					
homoYTX	1155.5	869.4	88	250	4	Negative	0.23	0.78
		1075.5	40					
YTX	1141.5	855.4	86	240	4	Negative	0.23	0.78
		1061.5	38					
PTX-1	892.5	213.2	44	175	2	Positive		
		821.5	28					
PTX-2	876.5	213.2	44	175	2	Positive	0.09	0.31
		823.5	28					
AZA-1	842.5	806.5	44	206	4	Positive	0.02	0.08
		824.5	32					
AZA-2	856.5	820.5	44	213	2	Positive	0.02	0.08
		838.5	36					
AZA-3	828.5	792.5	44	216	4	Positive	0.02	0.08
		810.5	32					
SPX-13	692.5	164.2	60	75	4	Positive		
		674.3	36					
SPX-13,19	678.5	164.2	60	260	4	Positive		
		660.2	36					
SPX-20G	706.5	164.2	56	233	4	Positive	0.02	0.08
		688.2	32					

CE: collision energy (V); CAV: cell accelerator voltage (V); Frag: fragmentor; LOD: limit of detection (μg/kg); LOQ: limit of quantification (μg/kg).

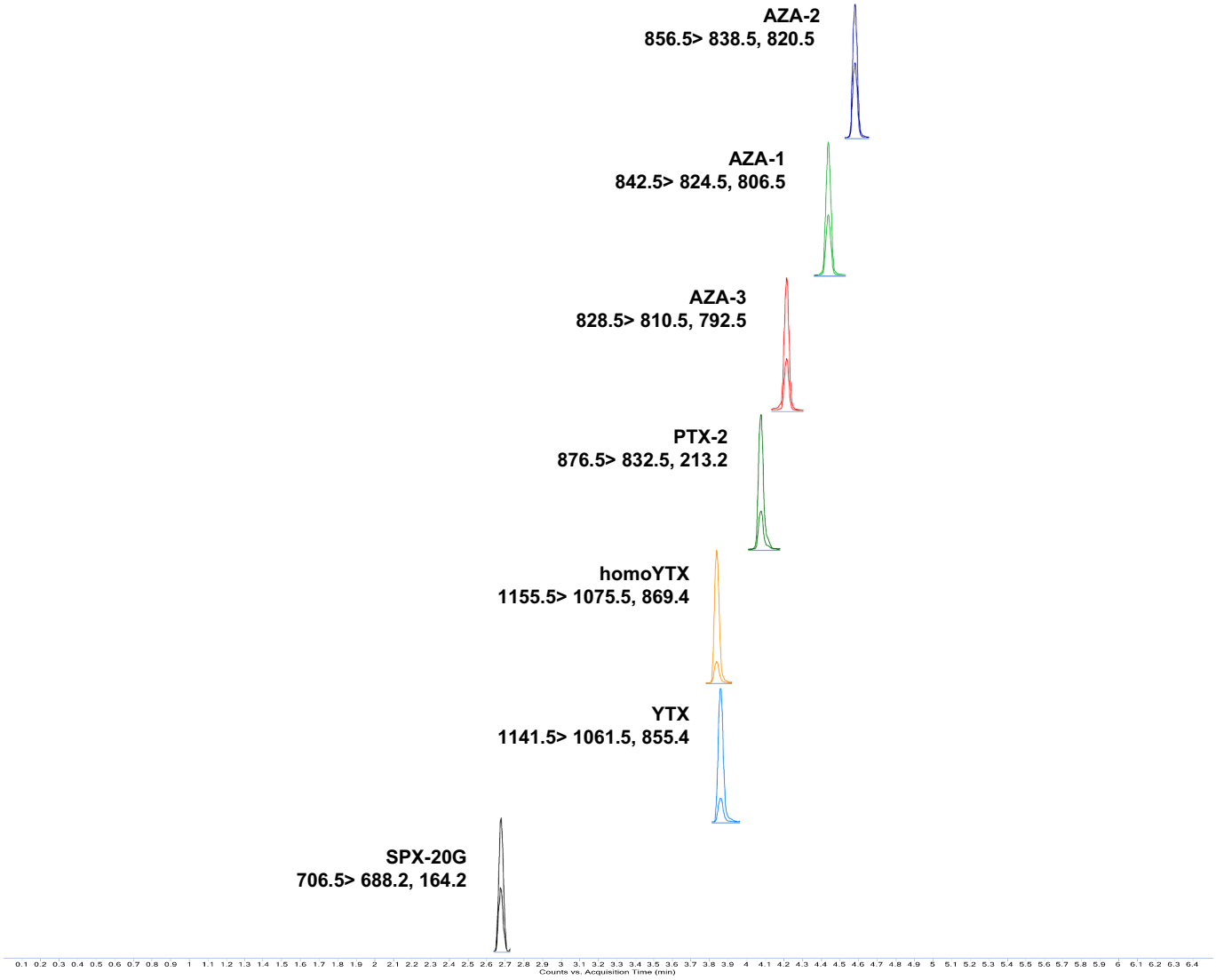


Fig. 3. MRM chromatograms of reference materials obtained by UHPLC-MS/MS.

3.2. São Miguel Island (Azores Archipelago)

From the 37 samples analyzed 31 samples were positive for lipophilic toxins. Three positive samples for YTXs were detected, two echinoderms and one mollusca. One of them, *S. granularis* (425#1), contained 45-OH-homoYTX and the other, *M. glacialis* (411#3), presented YTX and all of them under the EU legal limit for human consumption (Table 4). The echinoderm *O. ophidianus* (435) was the only sample containing SPX-13 below the LOQ. In this location, 31 samples were positive for one, two or three different AZAs, 81% were echinoderms. The phycotoxin AZA-1 was present in 41% Azores samples. The 15 positive samples for AZA-1 (73% echinoderms) contained concentrations ranging from 0.28 μg/kg in a sea-urchin *S. granularis* (425#1) to 18.75 μg/kg in *O. ophidianus* (424). Again, sample 424 had the highest concentration of AZA-2, 124.91 μg/kg. In São Miguel island the echinoderms represent 76% of the collected samples and 81% of all these samples were positive for AZA-2 all of them under the legal limit. Seven echinoderms were positive in AZA-3, containing the starfish *O. ophidianus* (424) the largest quantity, 19.38 μg/kg, and *S. granularis* (425#1) the lowest, 0.61 μg/kg.

3.3. Moroccan coast

At this location, a total of 39 samples were collected in July 2013 and 26 were positive for lipophilic toxins (Table 5). The YTX group was detected in a total of ten samples, nine mollusks, and one echinoderm, with detected concentrations ranging from 1.16 and 29.9 μg YTX/kg SM. It is noteworthy to mention that two samples showed 4 YTXs analogs, the bivalve *Mytilus* spp. (477) and the gastropod *S. haemostoma* (447). SPX-13 was only present in one sample, *M. glacialis* (473), below the LOQ. In Morocco, the incidence of AZAs was lower compared with the other two locations. For instance, in Madeira 96% of samples were positive and 84% in the Azores while in Morocco only 75% were positive samples for AZAs from total sample of each location. The Moroccan samples containing AZAs were 67% mollusks and 27% echinoderms all of them lower concentration than 3 μg AZA/kg SM.

4. Discussion

The main aims of this work were to search for new potential vectors for lipophilic toxins in order to better assess public health threats and unravel potential geographical patterns regarding lipophilic toxin routes. In this study, about 24 species (101 samples) of edible, with commercial interest, and non-edible benthic organisms. Our results show positive hits above 80% of the screened samples.

We report 19 new vectors for these lipophilic phycotoxins (Fig. 4A), belonging to three different phyla: mollusks (*P. ordinaria*, *P. aspera*, *A. depilans*, *S. haemostoma*, *U. umbraculum*, *H. tuberculata*, *P. lineatus*, *G. umbilicalis*, *C. vulgatum*, *C. lampas*), arthropods (*P. pollicipes*), and echinoderms (*P.*

Table 3
Sample information from Madeira Island. Lipophilic toxins analyzed by LC-MS/MS in MRM mode.

Sample type	Species	Code	PTX-2 (μg/kg)	AZA-1 (μg/kg)	AZA-2 (μg/kg)	AZA-3 (μg/kg)	SPX-13 (μg/kg)
Mollusca gastropoda	*P. ordinaria*	336		0.41	1.23		< LOQ
	P. aspera	337			0.16		< LOQ
	P. aspera	344			0.33		< LOQ
	S. haemastoma	346		0.85	2.35		< LOQ
	P. aspera	350			1.44		< LOQ
	U. umbraculum	351			1.25		< LOQ
	C. lampas	354		1.62	14.75	1.04	
Echinodermata echonoidea	*P. lividus*	339#2			1.25	0.31	< LOQ
	P. lividus	339#3			1.21	0.31	< LOQ
	P. lividus	339#4		1.53	7.85	1.35	< LOQ
	A. lixula	345			0.40	0.33	< LOQ
	A. lixula	347		0.81	3.67	0.37	
	P. lividus	348#1		0.90	5.03	0.76	< LOQ
	P. lividus	348#2		0.98	5.54	0.89	< LOQ
	P. lividus	348#4		0.88	4.92	0.77	< LOQ
	S. granularis	349		1.18	5.89	0.97	
	D. africanum	355#1		0.97	0.87		< LOQ
	D. africanum	355#2			0.20		
Echinodermata asteroidea	*A. aranciacus*	340		1.67	7.80	1.08	< LOQ
	O. ophidianus	341#1		6.15	53.16	2.08	2.49
	O. ophidianus	341#2		2.31	22.98	0.98	< LOQ
	O. ophidianus	341#3		2.04	20.13	1.39	< LOQ
	M. glacialis	342		0.78	1.51		< LOQ
	E. sepositus	353	5.91	1.05	6.64	1.11	0.24

lividus, *A. aranciacus*, *O. ophidianus*, *M. glacialis*, *A. lixula*, *S. granularis*, *E. sepositus*, *D. africanum*). Fig. 4B, represents the percentage of each toxin per location. The AZA-2 was presented a high amount in São Miguel and Madeira islands considering the other lipophilic toxins analyzed in these samples being Madeira much more accused. While in Morocco the amount of homo-YTX current in eight samples corresponds to almost 50% of total toxin amount. In this location, YTXs are the most important group since the four YTX analogs were detected in two samples in significant quantities.

Concerning the screened species, we report 79% of positive hits for new vectors, in Fig. 5 is displayed toxin incidence distribution pattern multivariate analysis. Curiously, the detected latitudinal pattern of lipophilic toxins uptake is reversed in comparison to previous work for the hydrophilic group of STXs (Silva et al., 2018). In the present work, the percentage of positive results follows a south-north gradient: being the presence of screened toxins less expressive in the Moroccan coast (67%) in comparison with the Azores and Madeira archipelagos (88%

Table 4
Sample information from Azores archipelago. Lipophilic toxins analyzed by LC-MS/MS in MRM mode.

Sample	Species	Code	45-OH-homoYTX (μg/kg)	YTX (μg/kg)	AZA-1 (μg/kg)	AZA-2 (μg/kg)	AZA-3 (μg/kg)	SPX-13 (μg/kg)
Echinodermata echinoidea	*S. granularis*	409#1				0.85		
	S. granularis	409#2				0.77		
	S. granularis	409#3				0.97		
	S. granularis	409#4			0.6	0.76		
	S. granularis	409#5				0.98		
	S. granularis	425#1	12.96		0.28	1.15	0.61	
	S. granularis	425#2				1.11		
	S. granularis	425#3				1.12		
	A. lixula	423			0.56	0.67		
	A. lixula	429				0.61		
	A. lixula	432				0.56		
	A. lixula	442				0.11		
Echinodermata asteroidea	*M. glacialis*	411#1				1.13		
	M. glacialis	411#2			0.29	0.12		
	M. glacialis	411#3		3.51		0.18		
	O. ophidianus	412			13.97	66.98	17.15	
	O. ophidianus	424			18.75	124.91	19.38	
	M. glacialis	426#1				0.95	0.75	
	M. glacialis	426#2				1.33	0.78	
	M. glacialis	428				0.30		
	M. glacialis	433#1			0.38	1.25		
	M. glacialis	433#2			0.59	0.87		
	O. ophidianus	435			9.79	36.90	7.68	< LOQ
	O. ophidianus	440			13.54	42.65	11.75	
	M. glacialis	441			0.25	0.69		
Mollusca gastropoda	*S. haemostoma*	413				1.26		
	C. lampas	414		28.63	0.98	2.08		
	S. haemostoma	431			1.07	0.86		
	S. haemostoma	434			0.35	1.34		
	H. tuberculata	437				0.29		
	S. haemostoma	443			0.77	1.32		

Table 5
Sample information from Moroccan coast. Lipophilic toxins analyzed by LC-MS/MS in MRM mode.

Sample	Species	Code	45-OH-YTX (μg/kg)	45-OH-homo YTX (μg/kg)	YTX (μg/kg)	homo-YTX (μg/kg)	AZA-1 (μg/kg)	AZA-2 (μg/kg)	AZA-3 (μg/kg)	SPX-13 (μg/kg)
Mollusca bivalvia	*Mytilus* spp.	447	9.45	28.72	5.11	29.93		1.06		
	Mytilus spp.	453		2.93			0.83	0.97		
	Mytilus spp.	465	2.88	8.48		12.32		0.08		
	Mytilus spp.	468				3.06	0.29	1.36		
	Mytilus spp.	485				1.41		0.11		
Mollusca grastropoda	*P. lineatus*	448				6.00		0.91		
	P. lineatus	449						0.95		
	A. depilans	451						0.50		
	C. vulgatum	454						0.45		
	P. lineatus	455			1.16		0.65	0.14		
	G. umbilicalis	460						0.82		
	G. umbilicalis	469						0.19		
	P. lineatus	470				8.19		0.22		
	C. lampas	475					0.33	1.63	0.88	
	A. depilans	476						1.15		
	S. haemostoma	477	1.07	2.25	1.09	6.27		0.73		
	P. lineatus	482					0.93	0.79		
	G. umbilicalis	483						0.14		
Echinodermata echinoidea	*P. lividus*	452						0.36		
	P. lividus	457#1						0.14		
	P. lividus	457#2						0.10		
	P. lividus	472						0.82	0.24	
	P. lividus	479						0.98	0.28	
Echinodermata asteroidea	*M. glacialis*	463						1.17		
	M. glacialis	473	2.20		2.73	9.15	0.34	2.78	0.66	< LOQ
Arthropoda hexanauplia	*P. pollicipes*	464						0.83		

and 84% respectively). This could be due to the distribution of the main producers along the screened coasts, though more studies are needed to clarify this matter.

Moreover, the two principal components accounted for 75% of data variance, being the first component strongly positively correlated with the presence of AZAs and SPX-13, a signature of most samples from Azores and Madeira archipelagos. In fact, Kaufmann et al. (2015) reported the presence of *Azadinium* cf. *dexteropor* in the Madeira Island, corroborating our data in this sampling ground (Kaufmann et al., 2015). The second principal component is positively correlated with the presence of YTXs, which is mainly associated with samples from Morocco. In summary, the geographical patterns are the main explanation for the variance observed in toxin distribution and are defined by a differential toxin profile. Among organisms, there are no significant differences except for *O. ophidianus*, and to a lesser extent *Mytilus* spp. which tends to accumulate significantly more toxins than others. The reason for that outcome probably is due to the feeding habits of each organism and their trophic level, more studies are needed to clarify this matter.

Regarding consumer's preferences, the commercialization and demand for crustaceans, gastropods, and echinoderms have increased in the last years (FAO, 2015). Therefore, it is pertinent to adjust monitoring strategies and update legislation regarding limit values. Nevertheless, reference methods have been developed and optimized for bivalves, and it is proofed that monitoring based only on this group of

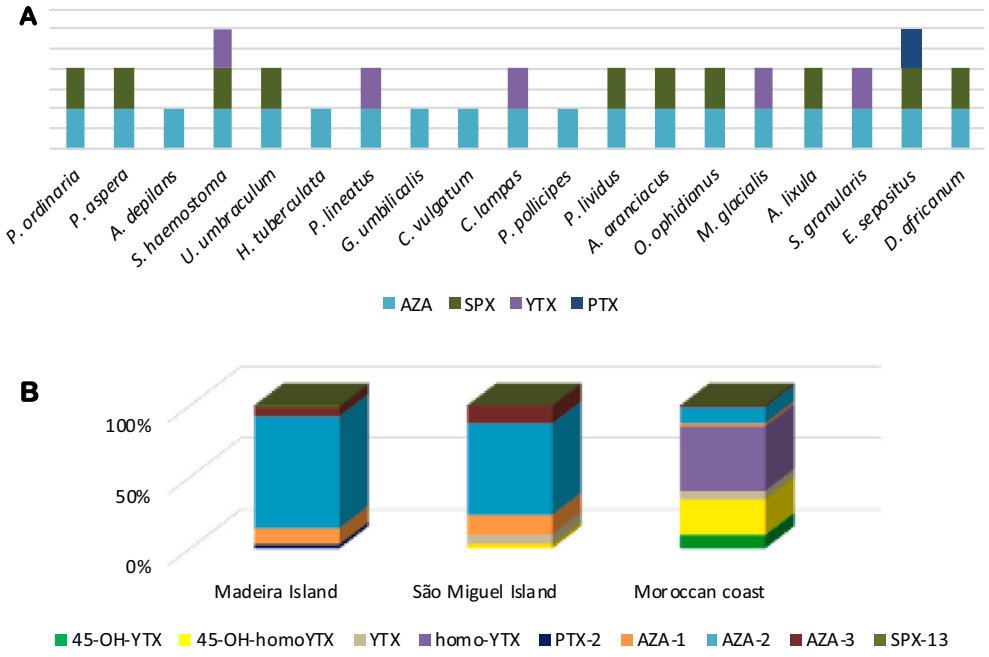


Fig. 4. A. Species identified as new vectors for each group of screened toxins. B. Percentage of each lipophilic toxin considering total toxin amount.

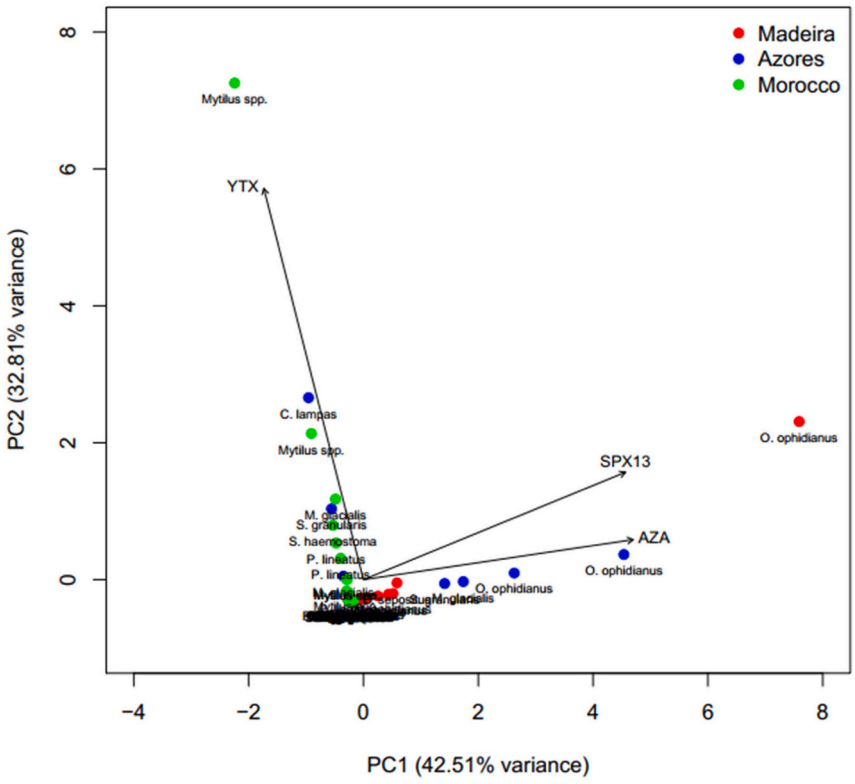


Fig. 5. Principal components plot of individual samples from the three geographical regions surveyed. Individuals are represented by colored circles and variables by arrows. Individuals are labeled with the corresponding species name.

organisms is a simplistic approach and underestimates human health risk (Silva et al., 2015a; Silva et al., 2018; Silva et al., 2015c).

Although acute intoxications have been mitigated by the implementation of monitoring plans, there is still much to unravel regarding the marine biotoxins field, and risk assessment should be re-evaluated. Knowledge on chronical exposure are scarce, and an effort should be done on information gathering to upgrade tolerable daily intake doses as well as regulated levels. Academy and Health sector should be closer in order to promote the collection of epidemiological data, all enhancing the improvement of consumers protection. In the present work, four lipophilic toxin groups were detected in edible and non-edible species, though the threat of edible species is fast-forward to understand, non-edible species pose an indirect but probably higher threat, since marine toxins can be biomagnified along with the food web and transferred to the offspring, contributing to the imbalance of the food chain and consequently the ecosystem (Lin and Hwang, 2001; Roué et al., 2016; Silva et al., 2013; Silva et al., 2015b).

It is noteworthy to mention that some samples displayed the presence of more than one group of toxins, samples #353 corresponding *E. sepositus*, was positive for the four screened groups, #447 corresponding to *S. haemostona* was positive both in AZA and YTX groups, this also emphasizes the need to investigate what impact of toxic combinations in human health, even at sub-lethal dosages.

Also, it is important to highlight that lipophilic toxins were detected in commercial species (*P. aspera*, *P. ordinaria*, *C. lampas*, *P. pollicipes*, *H. tuberculata*, and *P. lividus*), although in concentrations below the recommended values given by EFSA (Alexander et al., 2008b; Alexander et al. 2008c; European Food Safety Authority, 2009, 2010). Despite this, we stress the need for revision and update of present legislation policies, and we hope that our data helps to take the necessary step forward.

5. Conclusions

An sampling effort translated in the harvesting of 101 samples belonging to 24 different benthic organisms in three different locations (Madeira Island, São Miguel Island and northwestern Moroccan coast), during 2012 and 2013, aiming to determine new vectors for four groups of lipophilic toxins (YTX, AZA, SPXs and PTX) using UHPLC-MS/MS technique. With 80% of positive hits, we report a total of 19 new vectors, 53% of them gastropods (*P. ordinaria*, *P. aspera*, *A. depilans*, *S. haemostoma*, *U. umbraculum*, *H. tuberculata*, *P. lineatus*, *G. umbilicalis*, *C. vulgatum*, *C. lampas*), 42% of them echinorderms (*P. lividus*, *A. aranciacus*, *O. ophidianus*, *M. glacialis*, *A. lixula*, *S. granularis*, *E. sepositus*, *D. africanum*) and 5% crustaceans (*P. pollicipes*). All detected values were below the recommended EFSA limits and ranged from 0.08 and 124.91 μg/kg SM. PTX-2 was only detected in a single starfish sample from Madeira, in contrast, the AZA group was the more prevalent in all sampling sites. Geographical tendencies were detected, materializing in a south-north gradient regarding the presence of these phycotoxins. Since consumer's preferences in gastropods, echinoderms and crustaceans is a growing tendency in the last few years, it is a major concern to update monitoring policies and legislations regarding limit uptake values.

We hope our work represents a step forward to better understand the real risks for human and environmental health.

CRediT authorship contribution statement

M.S. and V.V. conceived the idea, M.S. performed the sampling, M.S. and I.R. performed the sample analyses and wrote the paper. A.B. contributed to the experimental design and statistical analyses. M.H. collaborated in sample collection. A.A. contributed with experimental design. V.V. and L.M.B. contributed to funding and to materials and analyses tools. V.V., B.S., A.I.N., and M.K. collaborated in the sample collection and provided sampling and laboratory facilities. All authors participated in proof reading of the manuscript.

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.

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