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High resolution mass spectrometry-based screening reveals lipophilic toxins in multiple trophic levels from the North Sea

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ABSTRACT

Lipophilic marine biotoxins, which are mainly produced by small dinoflagellates, are increasingly detected in coastal waters across the globe. As these producers are consumed by zooplankton and shellfish, the toxins are introduced, bioaccumulated and possibly biomagnified throughout marine food chains. Recent research has demonstrated that ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC–HRMS) is an excellent tool to detect marine toxins in algae and seafood. In this study, UHPLC–HRMS was used to screen lipophilic marine biotoxins in organisms from different trophic levels of the Belgian coastal zone ecosystem. A total of 20 tentatively identified lipophilic compounds was detected. Hereby, the trophic transfer of lipophilic marine biotoxins to the upper trophic level was considered to be rather limited. Furthermore, 36% of the compounds was clearly transferred between different organisms. A significant biotransformation of compounds from the okadaic acid and spirolide toxin groups was observed (64%), mainly in filter feeders. Through a multi-targeted approach, this study showed that marine organisms in the Belgian coastal zone are exposed to a multi-toxin mixture. Further research on both single compound and interactive toxic effects of the frequently detected lipophilic marine toxin ester metabolites throughout the food chain is therefore needed. As a future perspective, confirmatory identification of potential toxins by studying their fragmentation spectra (using new tools such as hybrid quadrupole Q-Exactive™ Orbitrap-MS) is designated.

1. Introduction

The occurrence of marine harmful algae is increasing around the globe (Valdiglesias et al., 2013; Ciminiello et al., 2014; Díaz et al., 2015). Natural dispersal as well as anthropogenic activities (e.g. shellfish translocation, global shipping, and ballast water discharge) have introduced these algae into non-native regions (Hallegraeff, 1998; Miller et al., 2010; Liebich et al., 2012). The discharge of nutrients from domestic, industrial and agricultural waste further contributes to the increased harmful algal bloom (HAB) frequency (Miller et al., 2010). Since filter-feeding bivalves consume algae, the accumulation of one or more lipophilic marine biotoxins from harmful phytoplankton is a well-known food safety threat in the shellfish industry (Marcaillou et al., 2005, 2010; Rundberget et al., 2011; Garcia et al., 2012). As these organisms accumulate marine biotoxins, even more so during HABs, increased concentration levels may be found at higher trophic levels in the food chain (Reguera et al., 2004; Franchini et al., 2010; Costa et al., 2013; Lage and Costa, 2013; Lopes et al., 2013). Lower trophic levels, such as zooplankton that graze on harmful microalgae, may experience considerable adverse effects (Hégaret et al., 2009; Vasconcelos et al., 2010). Moreover, through bioaccumulation, the intoxication of higher trophic level feeders such as fish, marine mammals, seabirds, and humans can occur (Álvarez et al., 2010; Silva et al., 2013; Turner, 2014). Additionally, harmful algae may also directly impact higher trophic levels (e.g. fish) through direct contact or anoxia when large blooms of algae decompose (Hoppenrath et al., 2007; Peperzak and Poelman, 2008; van der Woerd et al., 2011; Turner, 2014).

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Bioaccumulation is a key criterion used in European legislation (Gobas et al., 2009; Schäfer et al., 2015) to assess and manage the safety of chemicals and pollutants in aquatic systems and food webs. Bioaccumulation can be defined as a net accumulation of a chemical by an organism as a result of uptake from all environmental sources (Drexler et al., 2003). Screening estimates of bioaccumulation may include biomagnification, whereby the substance concentration in an organism is greater than in its diet (and the diet being the main exposure pathway), and trophic dilution, whereby the substance concentration in an organism is lower than in its diet. The processes of bioaccumulation of lipophilic marine biotoxins in marine environments are often very dynamic since marine biotoxins are continuously accumulated from the available phytoplankton species and transferred to different organisms through the food chain. Furthermore, bioaccumulation may depend on abiotic factors such as temperature, suspended organic matter and biotic factors such as age, sex and lipid content of an organism. While substantial work has been carried out on lipophilic marine biotoxins production from algal toxin producers and to a lesser extent on the environmental conditions that affect toxin production, very little research has been conducted to investigate trophic transfer of lipophilic marine biotoxins in the marine environment.

The North Sea is a rather shallow semi-enclosed basin of continental shelf water, surrounded by the European continent, the Scandinavian Peninsula and Great Britain (Vanden Eede et al., 2004; Speybroeck et al., 2007). In the past, harmful algae such as dinoflagellates were thought to follow the global trend and increase in the North Sea environment (Edwards et al., 2006; Peperzak, 2003; Hallegraeff, 2010). Recently, however, Hinder et al. (2011) reported a significant decrease in dinoflagellates and increase in diatom abundance, which may indicate an opposing shift in the plankton composition in the North Sea. To date, naturally occurring toxin producers (Klöpper et al., 2003; Krock et al., 2008, 2009; Hinder et al., 2011; Tillmann et al., 2012), shellfish accumulation (James et al., 2002; van der Fels-Klerx et al., 2012), and poisoning reports from around the North Sea have been well documented (Hinder et al., 2011; Whyte et al., 2014). However, research on lipophilic toxin profiles along diverse marine trophic levels is lacking.

As proposed in the Marine Spatial Plan (2014), aquaculture activities are to be developed in the Belgian Part of the North Sea (BPNS) (Maes et al., 2013; MARE, 2015). The 67 km of the near-straight Belgian coastline is characterized by the presence of (sand) beaches, stone groins and concrete dykes (Vanden Eede et al., 2004; Speybroeck et al., 2007). Along this coastline, key edible species can be found such as mussels, oysters and crabs. However, both the broad public and the scientific community are not aware of the occurrence and accumulation of marine biotoxins as no routine HAB monitoring is in place. Therefore, it is important to understand the marine toxin status within the different marine key species of this environment.

The main goal of this study was to investigate the prevalence of various lipophilic toxins in key edible organisms collected at different trophic levels of the BPNS. During the last decade, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was the method of choice to detect *a priori* defined lipophilic marine biotoxins in seafood and marine matrices (Krock et al., 2008; Rodríguez et al., 2015). More recently, high-resolution mass spectrometry (HRMS) has been confirmed as an even better tool to conduct the synchronous detection of targeted and multi-targeted lipophilic marine biotoxins in different matrices because of its highly accurate mass measurements and full-scan properties (Blay et al., 2011; de la Iglesia et al., 2013; García-Altares et al., 2014; Domènech et al., 2014; Orellana et al., 2014, 2015). Here, HRMS analysis was used to study the occurrence and trophic transfer of toxins in marine organisms of the BPNS. Toxin extracts of living marine organisms, sampled both inshore and offshore, were used to study the bioaccumulation of lipophilic marine biotoxins.

2. Material and methods

2.1. Chemicals and analytical standards

Certified calibration solutions for okadaic acid (Certified Reference Material (CRM)-OA-c $14.3 \pm 1.5\ \mu g\ mL^{-1}$), dinophysistoxin-1 (CRM-DTX-1 $15.1 \pm 1.1\ \mu g\ mL^{-1}$), pectenotoxin-2 (CRM-PTX-2 $8.6 \pm 0.3\ \mu g\ mL^{-1}$), azaspiracid-1 (CRM-AZA-1 $1.24 \pm 0.07\ \mu g\ mL^{-1}$), spirolid-1 (CRM-SPX-1 $7.0 \pm 0.4\ \mu g\ mL^{-1}$), and yessotoxin (CRM-YTX $5.6 \pm 0.3\ \mu g\ mL^{-1}$) were obtained from the National Research Council, Institute for Marine Bioscience (Halifax, Canada). Reference material, i.e. shellfish tissue containing OA, DTX-1, AZA-1, AZA-2 and AZA-3, were kindly donated by Dr. M. Andjelkovic. Analytical grade solvents were used for extraction purposes while LC–MS grade solvents were used for UHPLC–MS applications. They were obtained from Fisher Scientific (Loughborough, UK). Ultrapure water was obtained using a purified-water system (Sartorius AG, Goettingen, Germany). Millex-GV syringe filters (PVDF 0.22 μm) were obtained from Millipore (Darmstadt, Germany) and glass beads of 0.5 mm were purchased from Thistle Scientific Ltd. (Glasgow, UK).

2.2. Study area and sample collection

Multiple locations within the BPNS were sampled between July and September 2014. An overview of the study area and the sampling stations is provided in Fig. 1. Six sites were chosen around the Ostend harbor and the adjacent sluice dock, i.e. an artificial seawater basin of 85 ha (station 1- station 6). Another six coastal (open water) sites (Fig. 1, 130, 330, 230, 700, 710, 780) were sampled using the research vessel Simon Stevin. The sampled area is characterized by natural sand banks, with water depths varying between 10 and 24 m. Water samples were taken by Go-Flow® bottles and a CTD (Conductivity, Temperature, Depth) carrousel. Phytoplankton and zooplankton were then isolated by filtering the seawater through a plankton net with a mesh size of 15 μm or 80 μm, respectively. A minimum of 50 L of seawater was filtered at each station. The concentrates were stored into 1-L flasks at 4 °C for further analysis of phytoplankton and zooplankton composition, and toxin screening. The phytoplankton and zooplankton community compositions were determined using a stereo and/or an inverted microscope. Organisms were identified to the lowest taxonomic level possible. Different marine organisms were sampled if present in the environment. Specimens (i.e. shrimp (*Crangon crangon*), shellfish (*Mytilus edulis*, *Crassostrea gigas* and *Patella* sp.), shore crab (*Carcinus maenas*) and fish (*Trachurus trachurus* L. Carangidae)) were either sampled by hand (harbor and sluice dock stations) or using a beam trawl operated from the research vessel. Each sample was stored in a 2-L zipper bag and transported to the laboratory for subsequent analysis.

2.3. Instrumentation

UHPLC analysis was carried out using an Accela UHPLC pumping system coupled to an Accela Autosampler and Degasser (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation of compounds was achieved on a Nucleodur C18 Gravity column according to Orellana et al. (2014, 2015).

Mass spectrometric analysis was carried out on an Exactive™ benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), equipped with a heated electrospray ionization probe (HESI-II) that operated in switching polarity mode. The mass

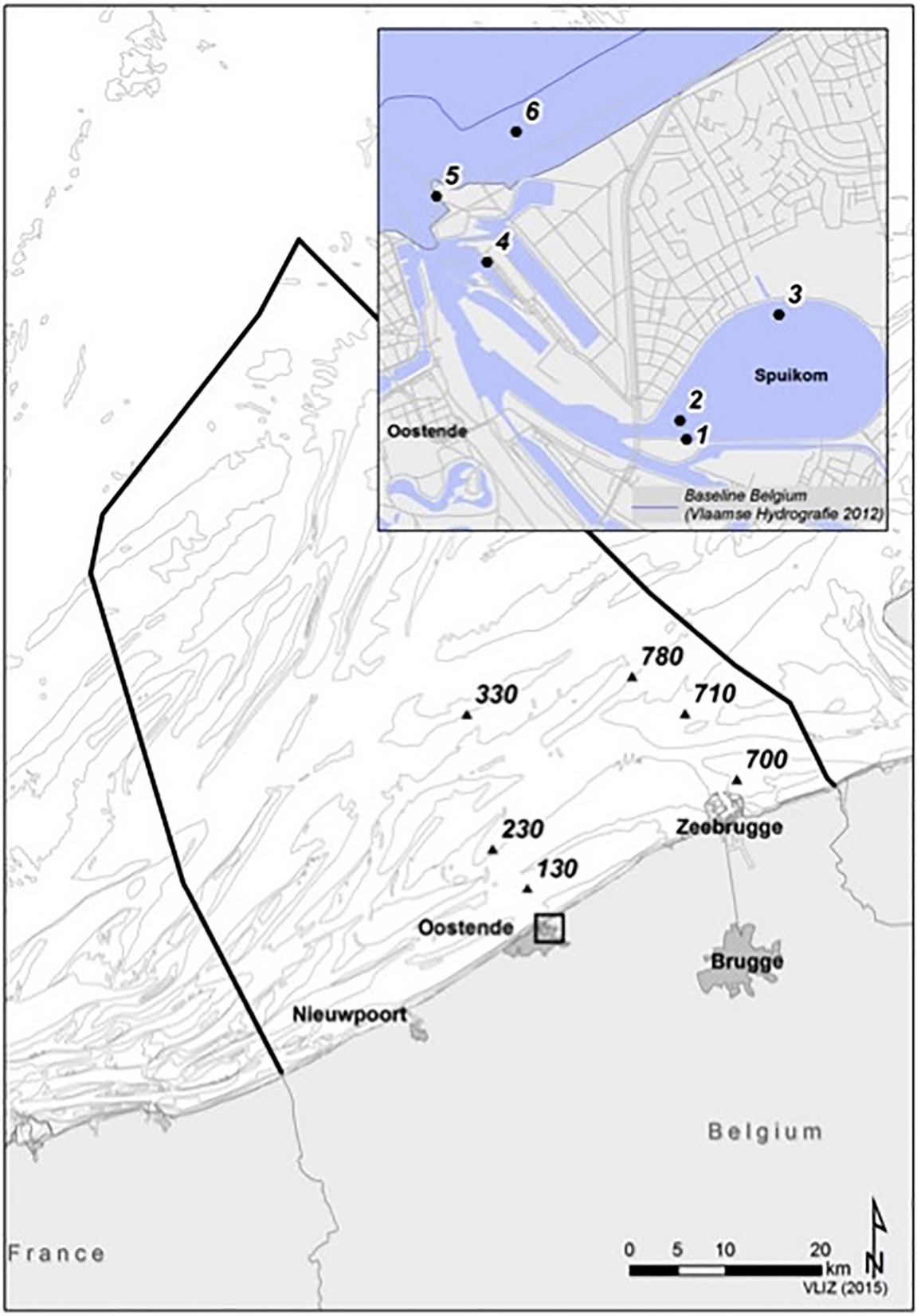

Fig. 1. Map of the Belgian Part of the North Sea, coastal zone and harbor, indicating the locations of the sampling stations. Each number corresponds to a particular station.

resolution was set at 50,000 full width at half maximum for m/z 200 Da. Ionization source working parameters were according to Orellana et al. (2014, 2015). Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San Jose, CA, USA).

2.4. Toxin screening

For targeted and multi-targeted toxin screening, the full-scan HRMS data were assessed using an extensive in-house database, comprising information on the molecular formula, $^{13}C/^{12}C$ isotopic ion ratio, and retention time of 142 marine biotoxins (Orellana et al., 2015). A match was based on the presence of the $[M+H]^+$, $[M\text{-}H]^-$, $[M+Na]^+$, $[M+NH]^+$ or $[M+NH_4]^+$ adduct and the corresponding ^{13}C isotopic ion. For each compound, the observed masses were compared to the theoretical masses, whereby mass deviations, expressed in parts per million (ppm), had to be below 5 ppm. Isotope ratios had to be compliant with CD 2002/657/EC (2002).

2.5. Sample preparation

2.5.1. Sample pre-treatment

The concentrated phytoplankton samples were filtered according to Orellana et al. (2015). The same procedure was applied for zooplankton, although the mesh size was adapted to 80 μm to retain zooplankton and remove salts and small phytoplankton. Shellfish samples were pre-treated according to Orellana et al. (2014) and EURLMB SOP (2015). Shrimps and crabs were dissected after storage, whereby the carapax was removed and the tissue was rinsed with ultrapure water to remove any foreign material. After that, 100 g of pooled tissue was homogenized in a blender, with the subsequent steps being similar to the shellfish extraction. Fish were dissected to separate and extract the intestine, stomach, and liver tissue. Intestine and stomach content were stored for posterior analysis.

2.5.2. Toxin extraction

Generic extraction of lipophilic marine biotoxins was applied to explore the occurrence of these compounds within the different organisms of the BPNS. Toxin extraction from phytoplankton was carried out according to Orellana et al. (2015). To extract the toxins from zooplankton, a homogenization step (2 min in the Ultra-Turrax®) was executed at the start of the phytoplankton protocol (Orellana et al., 2015). Toxin extraction from shellfish was modified from EURLMB SOP (2015) and Orellana et al. (2014). Tissues of fish (stomach, intestine and liver) and crab (hepatopancreas and reproductive organs) were dissected and extracted according to the shellfish toxin protocol.

2.5.3. Preparation of matrix matched standards

Matrix matched standards (MMS) were used for quantification purposes. To this end, extracts from 1 wet g of control algae (*Prorocentrum micans*) were spiked with different multi-toxin volumes (0, 25, 50, 75, 100, 150, 200 μL) corresponding to final concentrations ranging from 0 to 8 ng mL^{-1} for all compounds. These calibrations curves were used to quantify toxins in phytoplankton and zooplankton. Secondly, a homogenate of blank mussel tissue was spiked with two different multi-toxin standard solutions to quantify the target toxins in shellfish, shrimp and fish. Concentrations of the first solution were according to Orellana et al. (2014). For the second stock standard solution, mussel extracts were spiked with multi-toxin volumes (0, 15, 30, 45, 60, 75, 90, 120 μL) corresponding to 0, 5, 10, 15, 20, 25, 30, and 40 μg kg^{-1}.

2.6. Bioaccumulation assessment

To estimate trophic transfer of lipophilic marine biotoxins, biomagnification factors (BMFs) were determined. Briefly, BMFs were applied in a single trophic relationship (i.e. phytoplankton-zooplankton; phytoplankton-shellfish; zooplankton-shrimp and zooplankton-fish) since the marine organisms sampled are not part of a multi-trophic relationship at the different sampling stations. The BMF is defined as the quotient of the contaminant concentration at trophic level n (C_n) by that at the next lowest trophic level (C_{n-1}) (Laskowski, 1991; Newman, 2009):

$$BMF = C_n/C_{n-1}$$

where C_n is the wet weight concentration of a compound in the predator, expressed in μg kg^{-1} wet weight, whereas C_{n-1} is the wet weight concentration of the same compound in the prey or diet. If the BMF is less than 1, trophic dilution is suggested (Newman, 2009). To normalize concentrations in organisms, values were corrected by recalculating whole-body concentrations for large organisms like fish.

2.7. Quality assurance

Prior to sample analysis, a standard mixture containing the parent toxins (i.e. OA, DTX-1, PTX-2, AZA-1, YTX and SPX-1) was injected to check the operational conditions of the UHPLC–HRMS instrument.In addition, reference material, i.e. shellfish tissue containing OA, DTX-1, AZA-1, AZA-2 and AZA-3 was used for identification purposes. Identification of these known marine biotoxins was based on the accurate mass (m/z-value) and retention time, as determined by the respective certified standard solution. Additionally, confirmatory identification of the compounds was performed based on the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002). A compound's concentration level was calculated by fitting the peak area into a seven-point calibration curve, constructed by fortifying blank algae or mussels with the mixture of certified standard solutions, as previously described in Section 2.5.3. Each target toxin was quantified using the calibration curves in the matrices described above. Marine biotoxins, for which no analytical standard was at hand, were (semi)quantified using the HRMS response ratio. This is a justifiable strategy as excellent selectivity was observed for the parent toxins, the newly detected metabolites were well separated from other matrix compounds, and there was a strong resemblance of these metabolites with the parent ions in terms of chemical structure, suggesting rather similar ionisation behaviour (Torgersen et al., 2008).

3. Results

3.1. Environmental influence

During sampling, the water column was vertically mixed, which was confirmed by CTD analysis at all sampling stations at sea. The pH ranged from 7.8 to 8.3 in Ostend harbor and from 7.6 to 7.9 offshore. Salinity and temperature values (Table 1) are the average of all measured values (n = 6), registered during the course of the study. Similar environmental conditions in the BPNS have been described by Muylaert et al. (2006) and Van Ginderdeuren et al. (2013). The harbour of Ostend, including the sluice dock Spuikom, is affected by the channel Bruges-Ostend, resulting in lower salinities and higher nutrient and suspended matter concentrations.

Table 1
General description of the sampling sites with description of the marine organisms found (n = 10).

Area	Station	Latitude	Longitude	Mean salinity (psu)	Depth of sampling (m)	Mean T (°C)	Distance to the shore (km)	Sample type
Ostend	1	51° 22′ N	2° 29′ E	30.1	Surface	21.0	<1	Ph, Zoo, Sh, Cr
	2	51° 22′ N	2° 94′ E	30.4	Surface	22.7	<1	Ph, Zoo, Sh, Cr
	3	51° 23′ N	2° 95′ E	30.2	Surface	23.5	<1	Ph, Zoo, Sh, Cr, Li
	4	51° 23′ N	2° 92′ E	31.4	Surface	21.1	<1	Ph, Zoo, Sh, Cr, Oy
	5	51° 24′ N	2° 92′ E	31.1	Surface	20.5	<1	Ph, Zoo, Sh, Cr, Mu
	6	51° 25′ N	2° 93′ E	31.2	Surface	20.1	<1	Ph, Zoo, Cr, Mu
Sea	130	51° 16′ N	2° 54′ E	31.8	1 to 5	15.7	5	Ph, Zoo, Sh
	230	51° 18′ N	2° 51′ E	31.7	1 to 10	15.2	10	Ph, Zoo, Sh
	330	51° 26′ N	2° 48′ E	33.4	1 to 15	16.1	25	Ph, Zoo, Sh, Fi
	700	51° 22′ N	2° 13′ E	30.2	1 to 5	15.3	5	Ph, Zoo, Sh
	710	51° 26′ N	2° 81′ E	31.9	1 to 10	15.2	15	Ph, Zoo, Sh, Fi
	780	51° 28′ N	3° 35′ E	33.3	1 to 15	15.3	20	Ph, Zoo, Sh, Cr

Ph = phytoplankton, Zoo = zooplankton, Sh = shrimp, Cr = crab, Li = Limpet, Mu = mussels, Oy = oyster, Fi = fish.

3.2. Sample identification

Phytoplankton species were identified using an inverted microscope, whereas the zooplankton community was analyzed by stereomicroscopy. All organisms were identified to the lowest taxonomic level possible according to literature (Cattrijsse et al., 1997; Vale and Sampayo, 2002; Muylaert et al., 2006; Van Ginderdeuren et al., 2013, 2014; Vansteenbrugge et al., 2015). Microscopic analyses of the samples collected at the 11 sampling stations revealed a dominance of diatoms and *Phaeocystis* within the phytoplankton community. Diatoms were the most abundant group in all samples, characterized by *Chaetoceros* spp., *Rhaphoneis amphiceros, Odontella aurita, Leptocylindricus danicus*, *Actinoptychus senarius* and *Rhizosolenia spp*. The highest numbers of *Phaeocystis* cells (10^2 cells L^{-1}) were found at stations 700 and 710. Dinoflagellates (*P. micans*) were also found but in relatively low quantities (<10 cells L^{-1}). In this study, no other dinoflagellates or toxin producers were found. Zooplankton species showed a rather similar abundance along all samples. Calanoid copepods were most abundant in all stations with an average of 26 ind m^{-3}. Decapoda sp. such as *Brachyura zoea* were also abundant (10 ind m^{-3}) and oyster larvae were found at high concentrations (67 ind m^{-3}) in one inshore station (station 4).

Brown shrimp (*Crangon crangon*) and shore crab (*Carcinus maenas*) were found close to the coast. Mollusks (*Mytilus edulis*, *Crassostrea gigas* and *Patella* sp.) were also sampled along the shoreline. The horse mackerel (*Trachurus trachurus*), a migratory fish species, was found at the offshore stations (330 and 780) (n = 12). Stomach content analysis revealed a diet with a remarkable high amount of small fish and zooplankton species. However, due to advanced decomposition of stomach content it was impossible to identify the diet composition in more detail. Each of the sampled organisms, i.e. crabs, shrimp, mussels, oysters, limpets and fish, were identified *in situ* (Murta, 2000; Van Mol et al., 2007; Folmer et al., 2014).

3.3. Analysis of lipophilic marine biotoxins in different trophic levels

In this study, for the first time in the BPNS, the screening of lipophilic toxins across multiple trophic levels is presented. Moreover, potential emerging toxins like SPXs (Fig. 2) were tentatively identified in different marine organisms

A total of 470 samples were analyzed. Except for shore crabs, at least one or more lipophilic toxins were fully or tentatively detected in all organisms analyzed. Concentrated phytoplankton samples showed very low amounts of lipophilic marine biotoxins in all stations sampled. Certainly, the most common toxin groups found in this study were OA/DTX-2 and SPXs (Supplementary material, Fig. 1)

A high estimated concentration of the tentatively identified fatty ester metabolites from the OA/DTX-2 group, including 16:0, 18:4 and 20:5, was observed in almost all mussels (90%). Parent compounds such as OA/DTX-2 and YTX, however, were less frequently observed (5%) in the sampled biota. The highest concentrations of OA/DTX-2 and YTX found in oyster and mussels were 39.15 μg kg^{-1} and 178.01 μg kg^{-1}, respectively. Additionally, esters from the SPX group were tentatively identified in limpet with concentrations up to 1.35 μg kg^{-1} of SPX D.

High estimated concentrations of diverse tentatively identified fatty ester metabolites of OA/DTX-2 were observed in the stomach content of mackerel. In these fish, the 16:0 OA/DTX-2 fatty ester exhibited the highest estimated concentration found in this study, varying from 658.11 to 711.05 μg kg^{-1} in the stomach content (Table 2).

3.4. Bioaccumulation assessment

In this study, we assumed that the bioavailability of toxins in the water (i.e. extracellular toxins released from toxin producers) are insignificant since dinoflagellates, the main producers of lipophilic marine toxins, were not retrieved (Supplementary material, Table 1). However, influx of toxins (excreted by marine organisms) into the environment and the uptake by some filter feeders such as bivalves and mollusks is possible.

Quantification of marine biotoxins in biota is often an analytical challenge because of the potential for biotransformation of parent compounds and the lack of certified standards. As only six certified standard solutions were at hand, the concentrations of tentatively identified metabolites for each group of lipophilic marine biotoxins were estimated assuming an equal molar and peak response of structurally similar toxins (i.e. the parent compounds) on a molar basis (John et al., 2003; Torgersen et al., 2008; Costa et al., 2015). With respect to the dynamic range, good linearity (R^2) and CVs at the lowest concentrations were obtained for the six targeted compounds, indicating reliable quantification. Quantification of BMFs was performed only if both trophic levels were present at the same time and place during sampling. Furthermore, concentrations of lipophilic marine biotoxins were subjected to the quantification of BMFs only if they were present in both prey and predator (Fig. 3).

3.4.1. Bioaccumulation in zooplankton

Concentrations of OA/DTX-2 in zooplankton slightly exceeded those observed in their diet according to the BMFs ranging from 1.1 to 2.8 for the OA/DTX-2 group (Table 3). Zooplankton also displayed accumulation/transformation of esters from the OA/DTX-2 group (Table 2). A similar BMF was estimated for 16:2 OA/DTX-2.

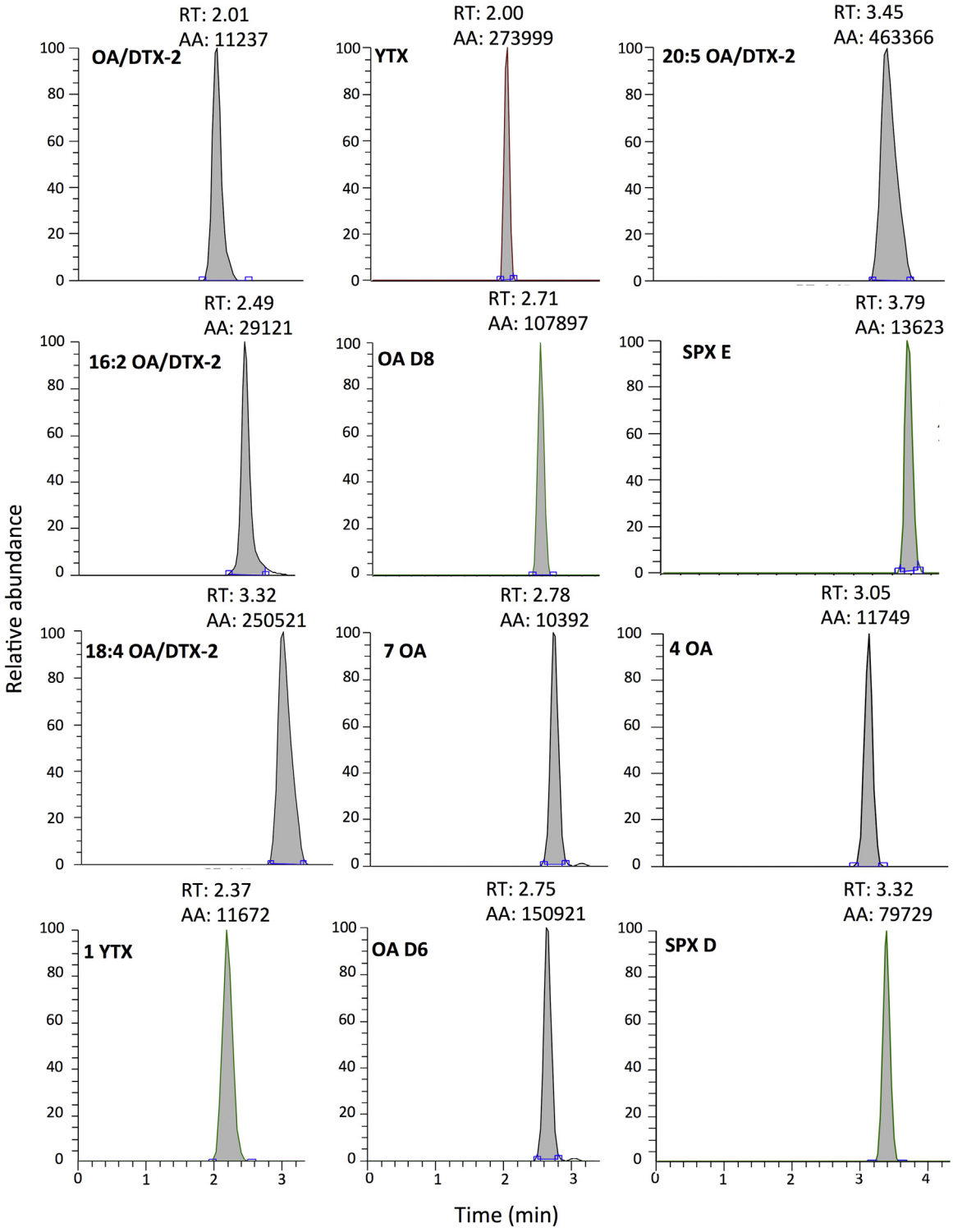

Fig. 2. Chromatograms of OA/DTX-2, YTX and 16:2 OA/DTX-2 for phytoplankton. Furthermore, 1 YTX, 18:4 OA/DTX-2, 20:5 OA/DTX-2, 4 OA, 7 OA, OA D4, OA D8, SPX D and SPX E were observed in many organisms such as shellfish and fish. The mass extraction window was set at 5 ppm.

3.4.2. Bioaccumulation in crustaceans

To the best of our knowledge, the present study is the first to analyze lipophilic marine biotoxins in brown shrimp (*Crangon crangon*). Shrimp BMFs ranged from 1.0 to 5.2 for OA/DTX-2 (Table 3). No other toxins were found in these organisms. For this calculation, it was assumed that shrimps eat small zooplankton species such as calanoid copepods (Boddeke, 1996; Ansell et al., 1999) and macrobenthic species (Oh et al., 2001). Also shore crab (hepatopancreas and reproductive organs) was analyzed for toxins, but no compounds were detected.

3.4.3. Bioaccumulation in mollusks

From the 11 OA/DTX-2 esters that were tentatively identified in this study, only three were found in phytoplankton samples (Table 2). Therefore, BMFs of the OA/DTX-2 group were determined only for the 16:2 OA/DTX-2 fatty ester metabolite in mussels

Table 2
Most prominent lipophilic marine biotoxins and their tentatively identified metabolites detected in different marine organisms. Results were obtained by the software program ToxID (minimum peak intensity of 1000 and maximum mass deviation of 5 ppm), thereby taking into account the $^{13}C/^{12}C$ isotopic ion ratio (CD 2002/657/EC, 2002).

Toxin name	t_R (min)	Elemental composition	Measured accurate mass (m/z)	Mass deviation (ppm)	Ion. mode	Theoretical isotopic ion ratio (%)	Observed isotopic ion ratio (%)	Tropic level	Mean concentration ($\mu g\,kg^{-1}$ wet)
OA/DTX-2	2.08	$C_{44}H_{68}O_{13}$	803.45984	1.39	–	47.59	49.22	Phytoplankton	0.32[a]
OA/DTX-2	2.01	$C_{44}H_{68}O_{13}$	803.45947	0.93	–	47.59	48.15	Zooplankton	0.51[a]
OA/DTX-2	2.05	$C_{44}H_{68}O_{13}$	803.45966	1.16	–	47.59	49.02	Oyster	20.87[a]
OA/DTX-2	2.10	$C_{44}H_{68}O_{13}$	803.45972	1.24	–	47.59	48.82	Shrimp	1.56[a]
OA/DTX-2	2.07	$C_{44}H_{68}O_{13}$	803.45954	1.01	–	47.59	49.05	Fish (liver)	28.79[a]
16:0 OA/DTX-2	3.74	$C_{60}H_{98}O_{14}$	1041.68433	−1.00	–	64.89	59.67	Zooplankton	0.76[b]
16:0 OA/DTX-2	3.68	$C_{60}H_{98}O_{14}$	1041.68351	−1.79	–	64.89	62.78	Mussels	22.30[b]
16:0 OA/DTX-2	3.71	$C_{60}H_{98}O_{14}$	1041.68441	−0.90	–	64.89	58.26	Fish (stomach)	684.80[b]
16:0 OA/DTX-2	3.78	$C_{60}H_{98}O_{14}$	1041.68499	−0.30	–	64.89	63.45	Fish (intestine)	29.70[b]
16:2 OA/DTX-2	2.48	$C_{60}H_{94}O_{14}$	1037.65869	1.55	–	64.89	61.54	Phytoplankton	0.31[b]
16:2 OA/DTX-2	2.53	$C_{60}H_{94}O_{14}$	1037.65873	1.59	–	64.89	60.12	Mussels	23.30[b]
16:2 OA/DTX-2	2.48	$C_{60}H_{94}O_{14}$	1037.65779	0.68	–	64.89	63.88	Fish (stomach)	73.20[b]
18:2 OA/DTX-2	3.72	$C_{62}H_{98}O_{14}$	1065.68604	−2.10	–	67.06	61.46	Zooplankton	0.72[b]
18:2 OA/DTX-2	3.72	$C_{62}H_{98}O_{14}$	1065.68739	−0.90	–	67.06	62.25	Fish (liver)	34.66[b]
18:2 OA/DTX-2	3.72	$C_{62}H_{98}O_{14}$	1065.68676	−1.50	–	67.06	62.29	Fish (stomach)	41.96[b]
18:4 OA/DTX-2	3.35	$C_{62}H_{94}O_{14}$	1061.65613	−0.80	–	67.06	63.21	Zooplankton	5.47[b]
18:4 OA/DTX-2	3.37	$C_{62}H_{94}O_{14}$	1061.65712	0.03	–	67.06	66.25	Mussel	23.31[b]
18:4 OA/DTX-2	3.32	$C_{62}H_{94}O_{14}$	1061.65629	−0.7	–	67.06	64.78	Oyster	36.54[b]
20:5 OA/DTX-2	3.45	$C_{64}H_{96}O_{14}$	1087.67334	0.56	–	69.22	62.76	Zooplankton	5.61[b]
20:5 OA/DTX-2	3.42	$C_{64}H_{96}O_{14}$	1087.67312	0.35	–	69.22	64.98	Mussel	15.08[b]
20:5 OA/DTX-2	3.44	$C_{64}H_{96}O_{14}$	1087.67397	1.14	–	69.22	66.13	Oyster	19.21[b]
20:5 OA/DTX-2	3.39	$C_{64}H_{96}O_{14}$	1087.67284	0.10	–	69.22	62.87	Fish (liver)	27.60[b]
20:5 OA/DTX-2	3.45	$C_{64}H_{96}O_{14}$	1087.67302	0.26	–	69.22	65.13	Fish (stomach)	55.29[b]
OA D6	2.75	$C_{54}H_{82}O_{14}$	953.56287	0.32	–	58.4	53.08	Zooplankton	3.71[b]
4 OA	3.05	$C_{43}H_{66}O_{11}$	757.45557	3.08	–	46.51	43.65	Phytoplankton	0.18[b]
4 OA	3.05	$C_{43}H_{66}O_{11}$	757.45543	2.90	–	46.51	44.86	Fish (stomach)	9.22[b]
7 OA	2.78	$C_{53}H_{82}O_{14}$	941.56305	0.13	–	57.32	54.22	Zooplankton	0.21[b]
7 OA	2.78	$C_{53}H_{82}O_{14}$	941.56356	0.40	–	57.32	52.07	Fish (liver)	17.70[b]
9 OA	4.04	$C_{53}H_{82}O_{15}$	957.55475	−3.40	–	57.32	53.12	Phytoplankton	1.91[b]
9 OA	4.04	$C_{53}H_{82}O_{15}$	957.55603	−2.15	–	57.32	55.12	Fish (liver)	14.18[b]
OA D8 diol ester	2.79	$C_{52}H_{80}O_{14}$	927.54810	0.61	–	56.24	54.88	Zooplankton	0.22[b]
OA D8 diol ester	2.75	$C_{52}H_{80}O_{14}$	927.54839	0.92	–	57.24	55.21	Mussel	9.51[b]
OA D8 diol ester	2.69	$C_{52}H_{80}O_{14}$	927.54805	0.56	–	58.24	58.67	Fish (liver)	40.16[b]
OA D8 diol ester	2.77	$C_{52}H_{80}O_{14}$	927.54774	0.22	–	59.24	57.53	Fish (stomach)	59.13[b]
27-O-Acetyl DTX1 methyl ester	3.88	$C_{48}H_{75}O_{14}$	874.51001	1.84	–	51.92	50.21	Fish (liver)	15.55[b]
27-O-Acetyl DTX1 methyl ester	3.88	$C_{48}H_{75}O_{14}$	874.50813	1.84	–	51.92	49.78	Fish (stomach)	29.71[b]
16:0 PTX-1sa	3.45	$C_{63}H_{102}O_{16}$	1115.72498	0.79	+	68.74	64.18	Fish (stomach)	6.76[b]
YTX	2.02	$C_{55}H_{82}O_{21}S_2$	570.23289	1.17	–	17.37	16.45	Phytoplankton	0.87[a]
YTX	2.01	$C_{55}H_{82}O_{21}S_2$	570.23243	0.36	–	17.37	17.03	Mussels	169.22[a]
1 YTX	2.37	$C_{41}H_{64}O_{21}S_2$	957.34802	2.71	+	44.34	43.89	Phytoplankton	0.01[b]
9 YTX	2.56	$C_{44}H_{68}O_{22}S_2$	1013.37451	2.81	+	47.59	43.54	Phytoplankton	0.06[b]
SPX D	3.35	$C_{43}H_{65}NO_7$	708.48608	3.81	+	46.51	42.51	Mussel	0.97[b]
SPX D	3.32	$C_{43}H_{65}NO_7$	708.48688	4.91	+	46.51	42.51	Oyster	1.13[b]
SPX D	3.32	$C_{43}H_{65}NO_7$	708.48596	3.64	+	46.51	48.59	Limpet	0.73[b]
SPX D	3.32	$C_{43}H_{65}NO_7$	708.48651	4.41	+	46.51	45.59	Fish (stomach)	3.22[b]
SPX E	3.83	$C_{42}H_{63}NO_8$	710.46362	1.37	+	45.43	54.06	Mussel	1.32[b]
SPX E	3.79	$C_{42}H_{63}NO_8$	710.46350	1.21	+	45.43	43.74	Fish (liver)	2.07[b]
SPX E	3.84	$C_{42}H_{63}NO_8$	710.46027	−3.33	+	45.43	44.43	Fish (stomach)	1.05[b]
SPX E	3.83	$C_{42}H_{63}NO_8$	710.45996	−3.77	+	45.43	43.21	Fish (intestine)	1.48[b]
SPX H	4.09	$C_{40}H_{59}NO_6$	672.42413	0.99	+	43.26	41.12	Fish (liver)	1.55[b]
SPX H	4.01	$C_{40}H_{59}NO_6$	650.43878	−4.39	+	43.26	45.79	Fish (stomach)	1.05[b]
SPX H	3.97	$C_{40}H_{59}NO_6$	650.43866	−4.21	+	43.26	38.02	Fish (intestine)	7.91[b]
SPX I	3.77	$C_{40}H_{61}NO_6$	652.45618	−1.51	+	43.26	49.59	Oyster	0.91[b]

*Concentration in ng mL^{-1} or ng g^{-1}
[a] Absolute quantification.
[b] Semi-quantification.

because this compound was detected in both shellfish and phytoplankton (i.e. its food). The remaining of OA/DTX-2 esters was considered to be the result of biotransformation or metabolization processes in mussels. DTXs and PTXs groups were absent in mussels. However, the highest toxin concentration found in mussels in this study was for YTX. The BMFs for YTX in mussels ranged from 142 to 196 but no further YTX metabolites were found in shellfish. These high concentrations were found in one particular sampling station in Ostend harbor and mussels were probably feeding on this specific YTX producer (station 5 in Fig. 1).

3.4.4. Bioaccumulation in fish

In this study, lipophilic marine biotoxins' estimated concentrations were especially high in the stomach and liver of the analyzed mackerel. The 16:0 OA/DTX-2 fatty acid acyl ester concentration was highest, i.e. 684.80 $\mu g\,kg^{-1}$, in the stomach

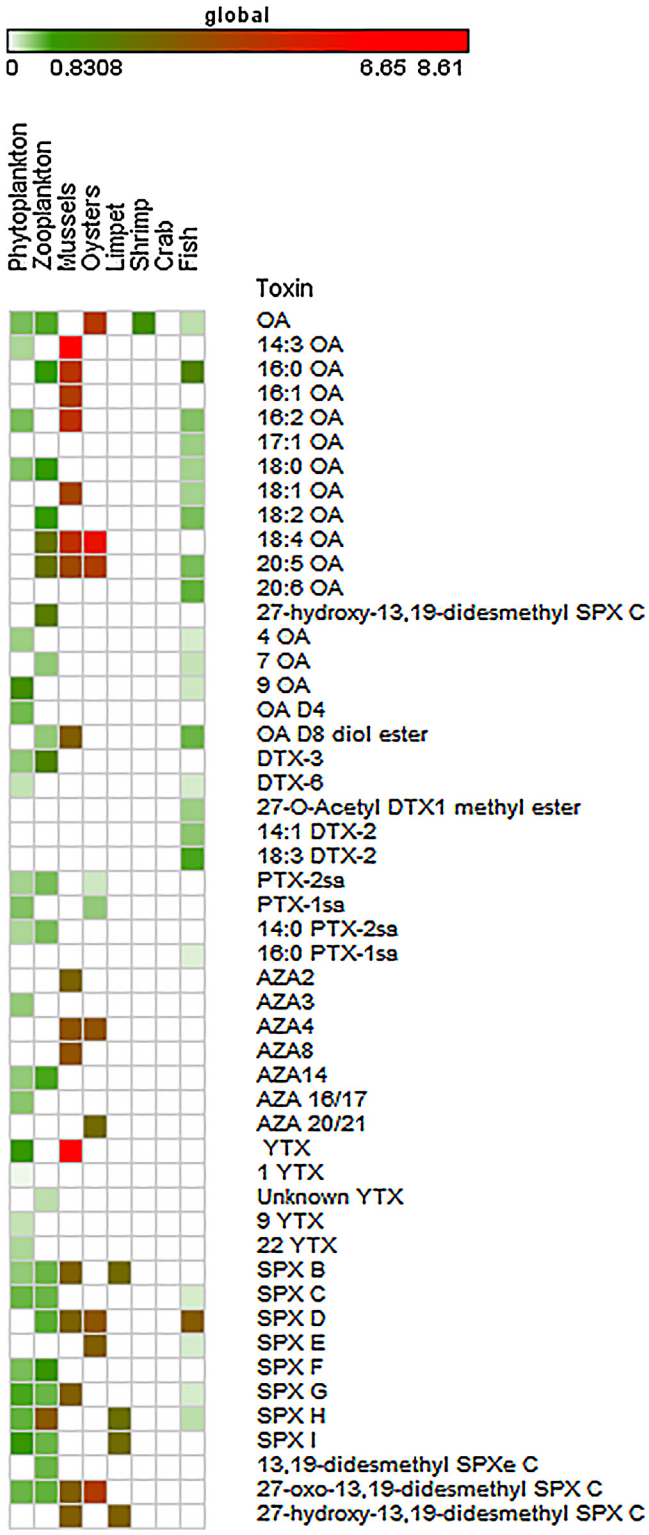

Fig. 3. Heat map depicting the average concentration of the LMBT accumulated in different marine organisms sampled in this study. Toxins that significantly decreased are displayed in light green, while toxins that significantly increased are displayed in red. Mussels and oysters showed a remarkable concentration of mainly two groups of toxins i.e. okadaic acid and spirolides and its esters metabolites in the BPNS.

content (Table 2). However, the concentrations within these organs may not be compared directly to those found in a whole organism (phytoplankton, zooplankton, shellfish). Instead, they should be recalculated to estimate the whole-body concentration of the fish. The highest BMF factor was 0.7 for OA/DTX-2 in fish (Table 3).

4. Discussion

This study yielded new findings on the occurrence, screening and accumulation of fully and tentatively identified lipophilic marine biotoxins in marine organisms in the southeast area of the North Sea. Despite that there were no toxin producers identified, significant estimated concentrations of lipophilic marine toxins were found in key edible organisms for the first time in the BPNS. OA/DTX-2, SPXs and their metabolites were the lipophilic marine biotoxin groups that were most abundantly accumulated and transferred between organisms. Both groups of toxins have been commonly reported in phytoplankton samples collected around the North Sea (Rundberget et al., 2011; Gerssen et al., 2011; van der Fels-Klerx et al., 2012; Whyte et al., 2014) and in farmed shellfish (Klöpper et al., 2003; Stobo et al., 2008; Gerssen et al., 2010; Rundberget et al., 2011). Gerssen et al. (2010) also reported OA in low concentrations in different shellfish collected in the Dutch coastal zone (cockles and clams), while Stobo et al. (2008) reported the presence of OA, DTXs, PTXs, YTXs and AZAs in farmed shellfish (cockles, king scallops, queen scallops) in Scottish waters. However, studies on the occurrence and distribution of lipophilic marine biotoxins in other marine organisms such as fish, shrimp and limpets have not been conducted for the North Sea.

Reports of HABs such as dinoflagellates are uncommon in the BPNS. Moreover, there are no reports or cases of poisoning events during the last decades. The lack of such reports can be attributed to limited shellfish production in this zone and the absence of monitoring programs of HABs and shellfish species. On the other hand, spring algal blooms of non-toxic species such as *Noctiluca scintillans* and *Phaeocystis globosa* (Van Mol et al., 2007; Van Ginderdeuren et al., 2014) have often been observed and they are considered harmful species due to their significant indirect effects on local ecosystems via their high biomass and oxygen depletion upon degradation (Peperzak, 2003). In this study, diatoms from the group of *Chaetoceros* spp. were observed in most of the stations sampled. However, no algal toxin producers were found in any station sampled. Nonetheless, Orellana et al. (2013) described a toxin producing strain of *Dinophysis acuminata* isolated from the sluice dock in Belgium. The toxin profile of this strain consisted of OA/DTX-2 and PTX-2. These compounds were identified and confirmed by HRMS. In addition, Wołoszynska and Conrad (1939) described a mussel-poisoning episode in Belgian waters, related to the dinoflagellate *Pyrodinium phoneus.* Nowadays, *P. phoneus* is considered synonymous with *A. ostenfeldii* (Balech, 1995), but it is not entirely clear whether this dinoflagellate really represents *A. ostenfeldii* or not (Balech and Tangen, 1985; Hakanen et al., 2012). Lipophilic marine biotoxin producers in the BPNS such as *Dinophysis* sp., *Prorocentrum sp.* and *Alexandrium* sp. probably grow in low concentrations (<10 cells L^{-1}) since algal blooms have not yet been reported in this part of the North Sea. Although low concentrations of several SPX metabolites were found in this study, the occurrence of SPXs in shellfish tissue in the BPNS poses a high threat because A. ostenfeldii (being the top producer of SPXs (Kremp et al., 2014)) has been reported as a paralytic toxin producer in neighboring waters of the BPNS as well (Van de Waal et al., 2015).

Numerous studies have focused on the bioaccumulation of toxins or chemicals such as persistent organic pollutants (Martin et al., 2004; Kelly et al., 2008; Lepom et al., 2012), cyanotoxins (Ibelings et al., 2005; Ferrão-Filho and Kozlowsky-Suzuki, 2011) and pesticides (Muir et al., 2003; Moraleda-Cibrián et al., 2015) in marine organisms. In this context, HABs are known as an entry point of toxins for benthic communities (Landsberg et al., 2009;

Table 3
Lipophilic marine toxin groups found in different marine organisms with the estimated concentrations ($\mu g\,kg^{-1}$ wet) and lowest and highest estimated biomagnification factors.

Toxin group	Organisms	Mean concentration ($\mu g\,kg^{-1}$ wet)	BMF	
			Lowest	Highest
OA/DTX-2	Phytoplankton	0.8	–	–
OA/DTX-2	Zooplankton	2.1	1.1	2.8
OA/DTX-2	Shrimp	1.5	1.0	5.2
OA/DTX-2	Oyster	25.5	47.2	82.5
OA/DTX-2	Mussels	25.4	70.3	84.9
OA/DTX-2	Whole body Fish	0.4	0	0.7
YTXs	Phytoplankton	0.3	–	–
YTXs	Mussels	169.2	164.2	225.5

Reizopoulou et al., 2010; Bricelj et al., 2012). However, in the present work, low estimated concentrations of tentatively identified lipophilic marine biotoxins were observed in phytoplankton samples, including fatty acid ester metabolites. These results correlate with the poor abundance of dinoflagellates observed in phytoplankton samples and could explain the absence of toxin producers in the phytoplankton analyzed under the microscope. Also in the zooplankton groups, sampled in this study, low estimated concentrations of toxins were observed. Zooplankton groups, like copepods, are significant grazers on harmful algal species and are therefore main entry points for the transfer of biotoxins to pelagic food webs (Turner and Tester, 1997; Calbet and Landry, 2004; Turner, 2014). In this study, copepods were the most abundant zooplankton group in all stations sampled. Similar findings on high copepod abundance have been reported in other studies on the BPNS (Van Regenmortel, 2012; Van Ginderdeuren et al., 2014) demonstrating that they are a key component in the pelagic ecosystem. Regarding the low abundance of dinoflagellates and toxins in the phytoplankton samples from this study, the low estimated concentrations of lipophilic marine biotoxins detected in the zooplankton samples could possibly be explained by the potentially limited availability of prey (i.e. harmful algae) at the sampling stations. Another explanation could be that copepods might not be efficient in the digestion of algae, as was indicated by Jansen et al. (2006). They reported the efficient feeding of copepods (*C. helgolandicus*) from the North Sea on a HAB of *Dinophysis norvegica,* however, a large number of intact cells of *D. norvegica* was found in the faecal pellets of the copepods. Furthermore, worldwide research on the accumulation of lipophilic marine biotoxins in zooplankton species revealed that some copepods are rather inefficient in retaining ingested HAB toxins (Kozlowsky-Suzuki et al., 2006; Turner, 2014).

Although estimated concentrations of toxins were low in the phytoplankton and zooplankton samples in this study, fully and tentatively identified lipophilic marine biotoxin concentrations were remarkably high in the shellfish filter feeders, such as mussels and oysters. Shellfish filter feeders are the most important and most monitored indicators of marine toxins around the world since there are many edible species that are being extensively cultivated and they are able to accumulate high concentrations of marine toxins.

Studies on bioaccumulation and transfer of lipophilic marine biotoxins between marine organisms in the North Sea on the other hand are limited. Recently, Silva et al. (2013) suggested, based on their comparative results between species, that mussels can be a vector for the accumulation and toxin transfer of OA and SPX between different benthic mollusks and echinoderm species. Indeed, the present work demonstrated a remarkable accumulation and transformation of two groups of toxins i.e. OA and its esters and SPXs in shellfish filter feeders (mussels and oysters) (Table 2). The toxins present in mussels differed from that observed in oysters collected at the same time and place (Table 2). Similar results have been reported by amongst others Torgersen et al. (2008), Kacem et al. (2010), and Pitcher et al. (2011), who suggested that this difference is due to the differential rates of accumulation, gut assimilation and/or biotransformation of lipophilic marine biotoxins in mussels and oysters. The highest estimated concentrations of toxins and more particular 16:0 OADTX-2 esters in organs, such as stomach and liver, were found in mackerel. In literature, this toxin was reported as the major fatty acid ester in shellfish such as mussels and clams (Vale, 2006; Torgersen et al., 2008). In this regard, the in this work tentatively identified toxin fatty acid esters constitute the incentive for further research that relates to the identification of the tentatively identified marine toxins and their toxicity. Moreover, accumulation of marine biotoxins from harmful algae related to paralytic shellfish poisoning in fish have been reported in sturgeon (Fire et al., 2012), sardine (Costa et al., 2010), mackerel (Castonguay et al., 1997; Lage and Costa, 2013), and white seabream (Jester et al., 2009; Costa et al., 2012). However, there is a limited knowledge on the accumulation of lipophilic marine biotoxins in North Sea fish. Mackerel does not feed directly on algae, which means that toxin levels may not correlate well with toxigenic algae. In fact, Atlantic horse mackerel is known to prey on copepods and decapod larvae in coastal areas (Cabral and Murta, 2002). Since toxin producers were not found in this study and low estimated concentrations of lipophilic marine biotoxins were quantified in plankton samples and a large amount of prey was found in the stomach content, it is suggested that mackerel must have ingested a high biomass of contaminated prey to accumulate these high levels of toxin fatty acid esters metabolites in the stomach and liver (Castonguay et al., 1997; Lage and Costa, 2013). Because these fish in the BPNS are migratory species and therefore exposed to different environments, the uptake of toxins may be linked to their migration route and the food they consumed elsewhere rather than to the location where the fish were sampled.

In the last decade, crustaceans have been reported as new lipophilic marine biotoxins vectors (Turner, 2014; Costa et al., 2013). In Portugal, for example, Vale and Sampayo (2002) reported OA and domoic acid toxins in shore crab (*Carcinus maenas*). In Norway, Torgersen et al. (2008, 2005) reported AZAs and OA fatty acid esters such as 14:0, 16:1, 16:0 and 18:1 in shore crab (*Cancer pagurus*). Accumulation of lipophilic marine biotoxins in crab was also recently reported by Andjelkovic et al. (2015) after feeding crab with mussels containing high concentrations of OA and AZAs. However, Andjelkovic et al. (2015) noticed a remarkable elimination of the toxins, confirming that crab may release toxins faster than other marine organisms. In this study, shore crab was the only organism that did not show accumulation of toxins in the different organs dissected. This corroborates the observations of Chen and Xie (2005) who suggested that large crustaceans like *Macrobrachium nipponense* are inefficient accumulators of toxic products such as microcystins.

5. Conclusions

Through a multi-targeted screening approach, this study showed that various (edible) species occurring in the BPNS are exposed to a multi-toxin mixture. While both the single compound and the mixture toxic effects of these esters are not known, it is remarkable that they are found across all trophic levels. Not only is it imperative to further evaluate the possible interactive, toxic, accumulative and secondary poisoning effects of these mixtures of natural toxic compounds on the health of these marine organisms, it is also vital to evaluate the potential risk of these seafood products to human health. While the total amount of toxins in the entire body of an organism, such as fish, can be underestimated,

BMFs can be valuable for the evaluation of the potential risk of this seafood for human consumption. Furthermore, marine organisms can also act as bioindicators of lipophilic marine biotoxins and their status in the ecosystem. Monitoring different marine organisms during the early stages of a toxic algal bloom can provide information on toxin profiles necessary to prevent future intoxication or economic loss in marine activities such as aquaculture or tourism. The next challenge will, however, involve the prediction of the trophic transfer and effects of lipophilic marine biotoxin groups in seafood. Furthermore, a new challenge for the BPNS will be to develop a monitoring program for marine biotoxins, including the non-regulated SPX group in phytoplankton and edible marine organisms. This will help to protect public health, fisheries and future aquaculture activities. As a future perspective, confirmatory identification of potential toxins by studying their fragmentation spectra (using new tools such as hybrid quadrupole Q-Exactive™ Orbitrap-MS) is designated.

Declaration of interest

The authors declare no conflict of interest.

Authors contribution

All authors have approved the final article. The following describes the individual contributions of each author:

Experimental design: Lynn Vanhaecke, Colin Janssen, Maarten de Rijcke, and Gabriel Orellana; Drafting and revision of the manuscript: Lynn Vanhaecke, Colin Janssen, Lieven Van Meulebroek, Maarten de Rijcke, and Gabriel Orellana; Sampling, analysis of sampling, and data interpretation: Maarten de Rijcke, Lieven Van Meulebroek, and Gabriel Orellana.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2017.03.005.

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