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Occurrence of domoic acid and cyclic imines in marine biota from Lebanon-Eastern Mediterranean Sea

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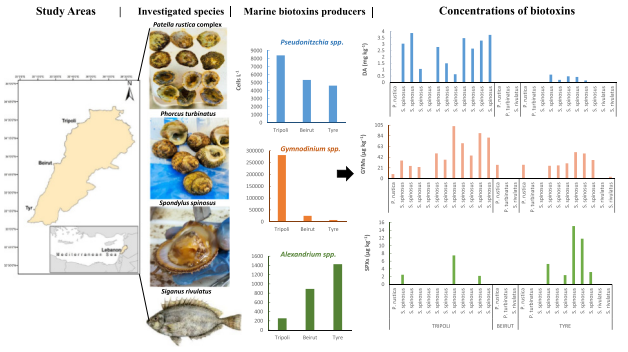
HIGHLIGHTS

- Only DA, GYM-b and SPX exhibited levels above LOD in some species/areas.
- Highest DA, GYMs, and SPXs levels are found in spiny oyster (*S. spinosus*).
- Biotoxins' levels were in harmony with the abundance of their potential producers.
- Repetitive consumption of DA-contaminated seafood could emerge health issues.
- A health risk is unlikely to occur after consumption of food contaminated by CIs.
- More research is needed to fix tolerable daily intake limits for human consumption.
- Spiny oyster *S. spinosus* may be used as a sentinel species in biotoxins' surveys.
- Non-bivalve species can be vectors of toxins and should be monitored as well.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine biotoxins are naturally existing chemicals produced by toxic algae and can accumulate in marine biota. When consumed with seafood, these phycotoxins can cause human intoxication with symptoms varying from barely-noticed illness to death depending on the type of toxin and its concentration. Recently, the occurrence of marine biotoxins has been given special attention in the Mediterranean as it increased in frequency and severity due to anthropogenic pressures and climate change. Up to our knowledge, no previous study reported the presence of lipophilic toxins (LTs) and cyclic imines (CIs) in marine biota in Lebanon. Hence, this study reports LTs and CIs in marine organisms: one gastropod (*Phorcus turbinatus*), two bivalves (*Spondylus spinosus* and *Patella rustica* complex) and one fish species (*Siganus rivulatus*), collected from various Lebanese coastal areas. The results show values below the limit of detection (LOD) for okadaic acid, dinophysistoxin-1 and 2, pectenotoxin-1 and 2, yessotoxins, azaspiracids and saxitoxins. The spiny oyster (*S. spinosus*) showed the highest levels of domoic acid (DA; 3.88 mg kg^{-1}), gymnodimine (GYM-B) and spirolide (SPX) (102.9 and 15.07 μg kg^{-1}, respectively) in congruence with the occurrence of high abundance of *Pseudo-nitzchia* spp., *Gymnodinium* spp., and *Alexandrium* spp. DA levels were below the European Union (EU) regulatory limit, but higher than the Lowest Observed Adverse Effect Level (0.9 μg g^{-1}) for neurotoxicity in humans and lower than the Acute Reference Dose

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(30 μg kg^{-1} bw) both set by the European Food Safety Authority (EFSA, 2009). Based on these findings, it is unlikely that a health risk exists due to the exposure to these toxins through seafood consumption in Lebanon. Despite this fact, the chronic toxicity of DA, GYMs and SPXs remains unclear and the effect of the repetitive consumption of contaminated seafood needs to be more investigated.

1. Introduction

Phytoplankton blooms have been documented since ancient times as their occurrence has been mentioned in some historical documents (Boni, 1992; Zheng and Klemas, 2018). Recently, these blooms are highlighted in newspapers and scientific journals (Boni, 1992; Visciano et al., 2016) as these events occur globally and in increased frequency, and the reporting literature is remarkably growing (Smayda, 1990; Boni, 1992; Vlamis and Katikou, 2015; Vilariño et al., 2018). Phytoplankton cells are critical food for filter-feeding bivalve shellfish (i.e. oysters, mussels, scallops, clams) as well as for the larvae of crustaceans and fish. Among thousands of microalgal species, approximately 300 ones can cause the so-called "red tides" (Hallegraeff, 1995; Lindahl, 1998), and among these species, >100 are producers of persistent natural toxins generating toxic outbreaks known as Harmful Algal Blooms (HABs). HABs can have consequences not only on marine organisms but also on human health (Costa et al., 2017) as well as socio-economic impacts and costs (Visciano et al., 2016). Their direct impact on human health can occur via direct consumption of contaminated seafood, skin contact with contaminated water, and/or inhalation of aerosolized biotoxins (Visciano et al., 2016). These biotoxins can be distinguished in water- and fat-soluble molecules causing various symptoms; toxins soluble in water can cause Paralytic Shellfish Poisoning (PSP) and Amnesic Shellfish Poisoning (ASP), whereas the toxins soluble in fat can cause Diarrhetic Shellfish Poisoning (DSP) and Neurotoxic Shellfish Poisoning (NSP) (FAO/IOC/WHO, 2004; Visciano et al., 2016). Therefore, contamination by marine biotoxins had become a major concern worldwide for public health authorities and aquaculture industry (Liu et al., 2019).

1.1. Lipophilic toxins (LTs) and hydrophilic toxins (ASP and PSP)

Marine lipophilic toxins (LTs) are toxic metabolites grouped in different classes, namely okadaic acid (OA), dinophysistoxins (DTXs) and azaspiracids (AZAs). They are known to cause Diarrhetic Shellfish Poisoning (DSP) incidents (Vale and Sampayo, 2002), and are believed to be tumour promoters (Fujiki and Suganuma, 1993) and/or can induce pathological changes in liver, pancreas, thymus and spleen of mice (Ito et al., 2000). LTs are isolated from various species of bivalves (shellfish) and phytoplankton (dinoflagellates) (Draisci et al., 1996). Pectenotoxins (PTXs) and yessotoxins (YTXs) are not proven to cause diarrhetic symptoms following intoxication (EFSA, 2008, 2009; Vlamis and Katikou, 2015; Ferron et al., 2016), whereas domoic acid (DA) is a known neurotoxin that causes damage in the central nervous system and responsible for Amnesic Shellfish Poisoning (Gago-Martínez and Rodríguez-Vázquez, 2000; Diogène, 2017).

Paralytic Shellfish Poisoning (PSP) toxins make a group of water-soluble neurotoxins produced by dinoflagellates *Alexandrium*, *Pyrodinium*, *Gymnodinium*, but also by the cyanobacteria *Trichodesmium*. These toxins can bioaccumulate in a range of filter-feeding invertebrates (i.e. mollusks, crustaceans, echinoderms) and therefore can cause the poisoning of fish, seabirds, mammals and humans through consumption of these contaminated organisms (Deeds et al., 2008; Ujević et al., 2012; Zamorano et al., 2013; Roje-Busatto and Ujević, 2014). Symptoms can appear within 2 h after eating contaminated shellfish, and start with the lips and tongues' tingling and then the extremities, followed by the weakness of lower and upper limbs, numbness, double vision, dizziness, headache, abdominal pain, vomiting and diarrhea, or more severe symptoms that may culminate with the coma and death (Hurley et al., 2014).

1.2. Cyclic imines (CIs), the emerging toxins

Through the latest research and improved detection methods, toxin groups are updated and new ones are identified as "emerging toxins", such as the cyclic imines (CIs), palytoxin (PlTX) and ciguatoxin (CTX). These toxins show novel appearance in the environment, potentially due to climate change which is affecting the distribution of phytoplankton species (EFSA, 2009, 2010a, 2010b). CIs are lipophilic imine toxins of organic compounds, produced by few species of marine dinoflagellate microorganisms. They can be associated with algal blooms, shellfish contamination and neurotoxicity (Richard et al., 2001). For example, *Karenia selliformis* and *Alexandrium ostenfeldii*/*A. peruvianum* have been correlated with the biosynthesis of gymnodimines (GYM) and spirolides (SPX) (Seki et al., 1995; Cembella et al., 2000; Touzet et al., 2008; Salgado et al., 2015), *Vulcanodinium rugosum* is the producer of pinnatoxins and portimines (Nezan and Chomerat, 2011; McCarthy et al., 2015; Molgó et al., 2017), prorocentrolides have been isolated from *Prorocentrum lima* (Torigoe et al., 1988), and spiro-prorocentrimines are suggested to be produced by *Prorocentrum* species (Lu et al., 2001). These phycotoxins have been found in extracts from contaminated shellfish, natural plankton assemblages, clonal cultures of toxic dinoflagellates, and as resulting products of the shellfish metabolism (fatty acid acyl esters) (Molgó et al., 2007; Guéret and Brimble, 2010; Stivala et al., 2015). The cyclic imines are exemplified by 40 molecules that differ based on its membered rings number, essential components for their bio-activity (Stivala et al., 2015; Molgó et al., 2017). They have been grouped together because of their common imine group as a part of a cyclic ring, which confers the pharmacological and toxicological activity, and due to their similar acute "fast-acting toxicity" in the intraperitoneal mouse bioassay (EFSA, 2010a, 2010b, 2010c; Otero et al., 2011; Reverté et al., 2014; Ujević et al., 2015). These "emerging toxins" accumulate mainly in bivalves and their potent neurotoxicity raises concerns related to seafood safety (Vlamis and Katikou, 2015).

1.3. Background of the study

A worldwide increase in the frequency and the geographic distribution of intoxication outbreaks has been noted (Anderson, 1989; Quilliam et al., 1993; Anderson, 1994, 1997; Okaichi, 2004) with some cases described for the first time in new locations (Molgó et al., 2017). Marine biotoxins are heat-stable, largely unaffected by cooking, not detectable by sight or smell, and the seafood in which they are found appears normal (Sobel and Painter, 2005) which explains the high number of people intoxicated (an average of 60,000 cases per year) by these toxins around the globe (Quilliam and Wright, 1993; Lopez-Rivera et al., 2009; Grattan et al., 2016a, 2016b). Seafood poisoning, however, poses serious threats not only to humans but also to marine mammals, seabirds, fish, and many other organisms that ingest the toxin (Reyes-Prieto et al., 2009). It is largely impacting local economies due to its negative effects on tourism, recreation, aquaculture industries (Morgan et al., 2009; García et al., 2016), and on both recreational and commercial fisheries causing about $82 million in economic losses to

the seafood, restaurants and tourism industries (NOAA, 2017). In the Mediterranean, a series of HABs have occurred in various areas during the last 50 years (Totti et al., 2010; Ferrante et al., 2013; Bacchiocchi et al., 2015). Many PSP-related cases were reported in different Mediterranean areas such as France, Italy, Morocco, Spain, Tunisia (Fonda, 1996; Tahri Joutei, 1998; Romdhane et al., 1998; Taleb et al., 2001; Lilly et al., 2002; EU-NRL, 2002). Moreover, mild human ASP intoxications have occurred in Spain, France, Greece and Italy (Friedman et al., 2008). Although DSP toxins were found in harvested mussels in Croatia, no health problems due to consumption of intoxicated seafood were registered there (Orhanovic et al., 1996). However, serious outbreaks affected several thousands of people in other Mediterranean countries (Belin, 1993; Van Egmond et al., 1993; Durborow, 1999; EU-NRL, 2001; EU-NRL, 2002; FAO, 2004; Ferrante et al., 2013; Costa et al., 2017).

The disequilibrium of the marine environment through anthropogenic pressures leads to the occurrence of HABs; a phenomenon that was noted several times in Lebanese coastal waters (Abboud-Abi Saab et al., 2006, 2008a, 2008b; Abboud-Abi Saab and Hassoun, 2017). However, no investigation has been implemented to date in order to quantify biotoxins in marine species neither in Lebanon nor in the Levantine Sea in general. Also, marine biotoxins emerged not only in bivalves, but in other marine species including crustaceans and fish (Costa et al., 2017). Thus, the present paper aims to assess for the first time the biotoxins profile of various marine species, not only in bivalves, from the Lebanese coastal area-Eastern Mediterranean Sea.

2. Study area and methodology

2.1. Study area and investigated species

Marine species were sampled during winter season, between December 2019 and February 2020 from the coastal areas of three Lebanese cities: Beirut (the capital and largest city), Tripoli (the second largest city, located in the North of the country), and Tyre (located in the South of the country and less affected by anthropogenic pressures compared to Beirut and Tripoli; Fig. 1).

Four different species, including one gastropod (*Phorcus turbinatus*), two bivalves (*Patella rustica complex* and *Spondylus spinosus*) and one fish species (*Siganus rivulatus*), were collected either directly from the sea or purchased freshly from the seafood market (Fig. 2). Overall 82 individuals were analyzed and details about species, dates of sampling and locations are mentioned in the Table 1.

2.2. Methods of analyses

2.2.1. Physico-chemical parameters

Surface temperature, phosphate, nitrate, nitrite and chlorophyll-*a* concentrations were measured monthly during the winter season from the vicinity of the study areas in the context of the national monitoring program of the Lebanese coastline conducted by the CNRS-L. Temperature (T) was measured via an ordinary thermometer. Salinity (S) was not measured during the study period (winter season of 2019), therefore it was averaged for winter season (December–February) for the period 2016–2019 ($n = 3, 4, 5$ for Tyre, Tripoli and Beirut, respectively).

Orthophosphates (P-PO_4) were analyzed according to the method described by Murphy and Riley (1962), nitrites (N-NO_2) based on the method described by Bendschneider and Robinson (1952) and nitrates (N-NO_3) following the method of Strickland and Parsons (1968) with a small modification consisting on the use of the ammonium chloride as an activator (Grasshoff, 1961). Samples intended to measure the total chlorophyll-*a* (Chl-*a*) were filtered through a filter paper (Whatmann GF/C) at a low pressure. Pigments were then extracted in 90% acetone in cold and dark conditions for 24 h. The concentration was determined by a spectrophotometer according to the monochromatic method of Lorenzen (1967). The biomass is expressed in quantity of Chl-*a* over the volume of sea water (μg L^{-1}; Abboud-Abi Saab and Hassoun, 2017).

2.2.2. Phytoplankton analysis

Phytoplankton samples were collected few days after the sampling of marine biota (on 02.03.2020 and 03.04.2020) and immediately preserved with Lugol iodine solution at a final concentration of 0.5% for species determination. Phytoplankton cells were counted using the Utermöhl sedimentation method (Utermöhl, 1985). A homogenous sample of 100 ml has been let to settle for 48 h in a 25 mm diameter sedimentation chamber. The base of the chamber was examined with a Wild M 40 phase-contrast inverted microscope. Counting of the phytoplankton species was performed at x40 magnification for better identification of dinoflagellates. Predominant cells were identified to the species level, while taxonomic groups such as small pennate diatoms, flagellates and naked dinoflagellates were counted in groups.

2.2.3. Biotoxins analyses

Lipophilic toxins (okadaic acid (OA), dinophysistoxin-1 and 2 (DTX-1,2), pectenotoxin-1 and 2 (PTX-1,2), yessotoxins (YTXs), azaspiracids (AZAs), gymnodimine (GYM-b), spirolide (SPX) and domoic acid (DA)) were analyzed in homogenized soft tissues of investigated species. An aliquot of 2.00 ± 0.05 g of whole-body shellfish tissue (for gastropod and bivalves) and muscle tissue (for the fish) was extracted (EURLMB-Harmonised SOP, 2015) with 9.0 ml methanol (100%). The sample was vortex-mixed for 3 min, and subsequently centrifuged at 2000*g* or higher for 10 min. at approximately 20 °C. After two centrifugations, the supernatants of the two resulting extracts were combined to make up one extract of 20 ml with methanol (100%). Afterwards, the extract was filtered through a dry methanol-compatible 0.45 μm syringe filter.

In order to detect and quantify the total content of toxins such as OA and DTX, an alkaline hydrolysis was required before LC-MS/MS analysis, to transform the acylated toxins present in the sample into the parent toxins (i.e. OA, DTX-1, and DTX-2) (Mountfort et al., 2001). LC-MS/MS method positive ion mode was applied for detection of AZA1, AZA2, AZA3, PTX1, PTX2, SPX, GYM and DA, while negative mode was used for YTX, homoYTX, 45 OH-YTX, 45 OH-homoYTX, OA, DTX1, DTX2, as well as acylated esters of OA and DTXs detection LC-MS/MS analysis was performed using a triple quadrupole mass spectrometer (Agilent Technologies 6410) equipped with an electrospray ionization source. Chromatographic separation was performed using a 5 μm Poroshell C18, 50 × 2.1 mm Agilent column kept at 30 °C. Chromatography was based on acidic conditions as 2 mM ammonium formate and 50 mM formic acid mobile phases in water (mobile phase A) and 95% acetonitrile (mobile phase B), respectively, were used. All chemicals for biotoxin extractions and instrumental analyses were of LCMS grade. The hydrolysis procedure as proposed by EURLMB (Harmonised SOP, 2015) was applied to investigate total content of OA and DTXs toxins. Method optimization delivers two MRM (MS/MS) transitions per analyte (toxin) that give the highest selectivity and the best sensitivity for identification and quantification (see Table S2 in Supplementary information), along with other parameters of method validation.

Standard certified solutions for calibration were purchased from NRC Halifax Canada and used to prepare multi-toxin standard calibration solutions in six concentrations for analyte determination in positive mode: 2, 4, 6, 12, 20 and 30 ng mL^{-1} for AZA1, AZA2, AZA3, PTX2, GYM and SPX and 30, 60, 90, 180, 300 and 450 ng $mL^{-1}$for DA; and for analyte determination in negative mode: 5, 10, 15, 30, 50 and 75 ng mL^{-1} for YTX, homo-YTX, OA, DTX1, DTX2.

Tissue samples were also analyzed for the presence of PSP toxins based on the High Performance Liquid Chromatography with fluorescence detection (HPLC-FLD) method (First Action 2005.06 AOAC Official Method) with pre-chromatographic oxidation using a Varian ProSTAR 230 HPLC analytical system coupled with a ProStar 363 fluorescence detector (excitation 340 nm and emission 390 nm) and a

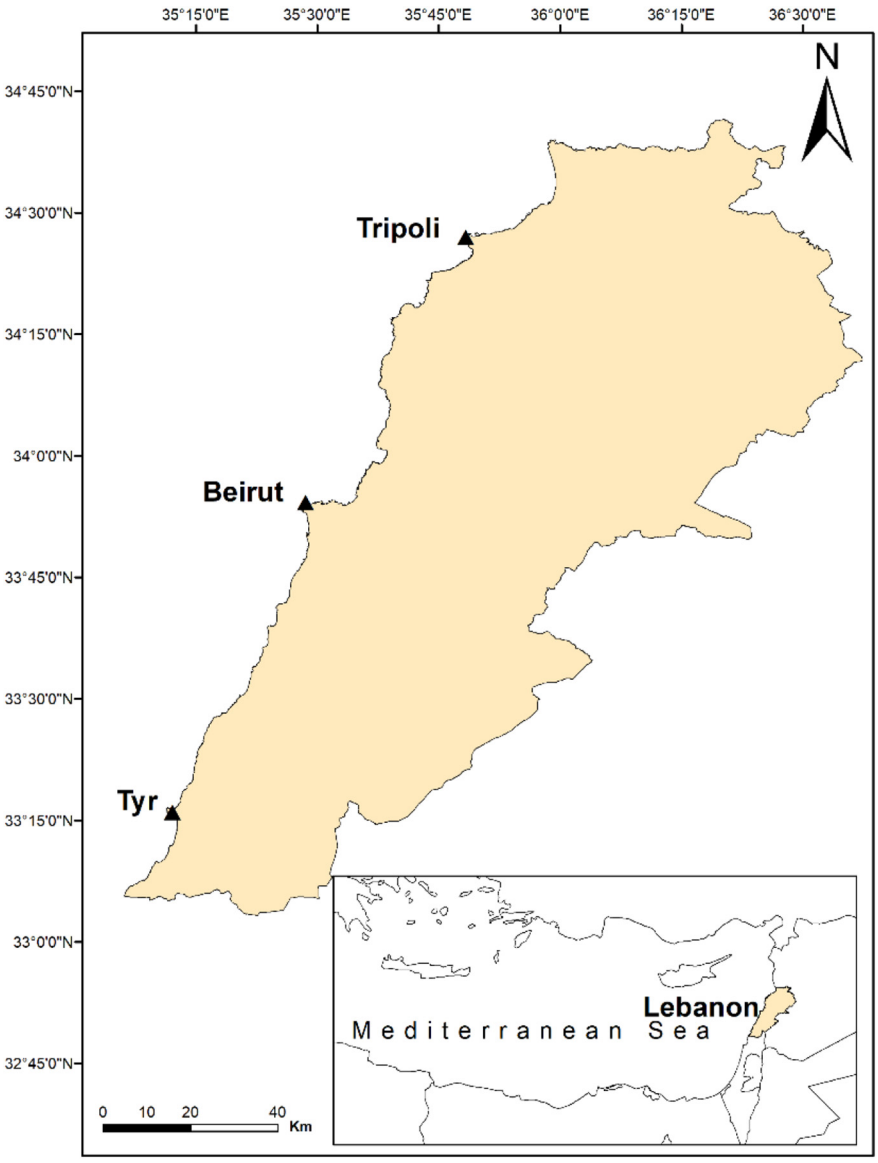

Fig. 1. Map of Lebanon illustrating the sampling stations (black triangles): a) Tripoli, b) Beirut and c) Tyre.

ProStar 410 autosampler. Separation of toxin oxidation products was carried out on reversed-phase C18 column (Supelcosil, 150 × 4.6 mm, 5 μm particle size) protected by a guard cartridge (Supelguard C-18, 20 mm; Supelco, Oakville, Canada) and temperature was kept at 30 °C. Mobile phases consisted of (A) 0.1 M ammonium formate and (B) 0.1 M ammonium formate in 5% acetonitrile solutions, flow rate was set to 1.5 mL min^{-1}, run time to 15.00 min and partial loopfill volumes were set to 25 and 50 mL for peroxide and periodate oxidized samples, respectively. The method was used for determination and quantification of: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NEO), decarbamoylneosaxitoxin (dcNEO), gonyautoxins 1 and 4 (GTX 1,4), gonyautoxins 2 and 3 (GTX 2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX 2,3), gonyautoxin 5 (GTX 5) and N-sulfocarbamoylgonyautoxin 1 and 2 (C 1,2) in samples. LODs have been determined based on a 3:1 signal-to-noise ratio were: 6.81 for GTX-1,4, 0.39 for GTX-5, 4.22 for C-1,2, 2.09 for dcNEO, 1.91 for dcSTX, 1.78 for NEO, 2.14 for GTX-2,3, 1.07 for dcGTX-2,3 and 1.07 μg kg^{-1} for STX.

Homogenized tissue samples were weighed to 5.00 ± 0.10 g and submitted to extraction by adding 3 ml of 1% acetic acid, vortexing, heating in the water bath, remixing and cooling. Subsequently, the sample was centrifuged for 40 min at 3600 ×*g* and 20 °C and the supernatant decanted. The pellet was re-extracted with 3 ml of 1% acidic acid, vortexed and centrifuged under the same conditions. Two supernatants were pooled and diluted to final crude extract volume of 10 mL with deionised water and filtered. Solid phase extraction (SPE) purification procedure was based on Lawrence et al. (2005) where samples were cleaned with Varian (Varian, USA) Bond Elut C-18 (500 mg per 6 ml) solid phase extraction (SPE) cartridges. First, they were conditioned by methanol and deionised water, then the eluate of 1 mL of sample crude extract and 2 mL of deionised water was collected, pH corrected to 6.5 and volume of this C-18 purified extract adjusted to 4 mL with deionised water. As we found no PSP toxins present in the samples, second purification with SPE-COOH cartridges was not carried out. Aliquots of C-18 purified samples were submitted to oxidation with peroxide and periodate oxidants prior to HPLC analyses. In order to eliminate peaks from naturally fluorescent compounds present in the samples, aliquots of the extracts mixed with the matrix modifier (extracts from PSP-free oyster tissue) were analyzed without prior oxidation. Pre-column peroxide and periodate oxidations were performed on aliquots of the SPE-C18 cleaned extracts to detect and quantitate the non-N-hydroxylated and N-hydroxylated toxins, respectively. As there were no PSP toxins found in the samples, periodate oxidation of fractions was not performed.

Fig. 2. Investigated shellfish species: (a) *Patella rustica* complex, (b) *Phorcus turbinatus*, (c) *Spondylus spinosus* during its preparation in the laboratory, and (d-e) *S. spinosus* at the street market in El Mina-Tripoli.

3. Results

3.1. Biotoxins

Levels of lipophilic toxins [OA; DTX-1,2; PTX-1,2; YTXs; AZAs] and hydrophilic PSP toxins were below the LODs and thus not discussed hereafter. Only domoic acid (DA), gymnodimine (GYM-b), and spirolide (SPX) were found above the LODs in some species/areas (Fig. 3) and therefore were presented in the sections below.

➢ DA

All samples of *Phorcus*, *Patella* and *Siganus* showed concentrations below the LOD for Amnesic Shellfish Poisoning toxin, DA (<0.1025 mg kg^{-1}). However, only 18 and 33% of the *S. spinosus* samples collected from Tripoli and Tyre respectively, showed concentrations below the LOD, whereas the other 82 and 87% respectively, demonstrated concentrations above the LOD (Fig. 4a) with an average of 2.60 ± 1.08 mg kg^{-1} for *S. spinosus* samples taken from Tripoli, 6-fold higher than the one from Tyre (0.38 ± 0.17 mg kg^{-1}). DA concentrations in those samples ranged between a minimum of 0.15 mg kg^{-1} in Tyre and a maximum of 3.88 mg kg^{-1} in Tripoli (Fig. 4a). All DA results were below the maximum permitted level for consumption (20 mg kg^{-1}) set by the Regulation 853/2004/EC.

➢ GYMs

The main peak of GYM corresponds to GYM-b. GYMs hereafter refer to both GYM-b and its derivative (the other peak, Fig. 3). GYMs were also detected in our samples (Fig. 4b) with concentrations above the LOD (2.36 μg kg^{-1}) in 66% of the samples. GYMs levels varied between 3.33 μg kg^{-1} in *S. rivulatus* (Tyre) and 102.9 μg kg^{-1} in *S. spinosus* (Tripoli) with levels below the LOD in all gastropod samples (Fig. 4b). The spiny oyster *S. spinosus* is the species that showed the highest GYMs

Table 1
Sampling dates, locations, and number and characteristics of investigated species.

Date	Station	Longitude	Latitude	Species	Number of individuals	Source	Trophic level	Edibility
03-12-2019	Tripoli	35°48′27″	34°26′57″	*P. rustica* complex	12	Collected from the sea	Grazer	Occasional
03-12-2019	Beirut	35°28′32″	33°54′5″	*P. rustica* complex	12	Collected from the sea	Grazer	Occasional
14-01-2020	Tyre	35°12′10″	33°16′2″	*P. rustica* complex	12	Collected from the sea	Grazer	Occasional
14-01-2020	Beirut	35°28′34″	33°54′5″	*P. turbinatus*	12	Collected from the sea	Grazer	Occasional
14-01-2020	Tyre	35°12′10″	33°16′2″	*P. turbinatus*	11	Collected from the sea	Grazer	Occasional
14-01-2020	Beirut	35°28′34″	33°54′5″	*S. rivulatus*	2	Seafood market	2nd level predator	Frequent
14-01-2020	Tyre	35°12′10″	33°16′2″	*S. rivulatus*	1	Seafood market	2nd level predator	Frequent
19-02-2020	Tripoli			*S. spinosus*	11	Seafood market	Filter-feeder	Frequent
19-02-2020	Tyre			*S. spinosus*	9	Collected from the sea	Filter-feeder	Occasional

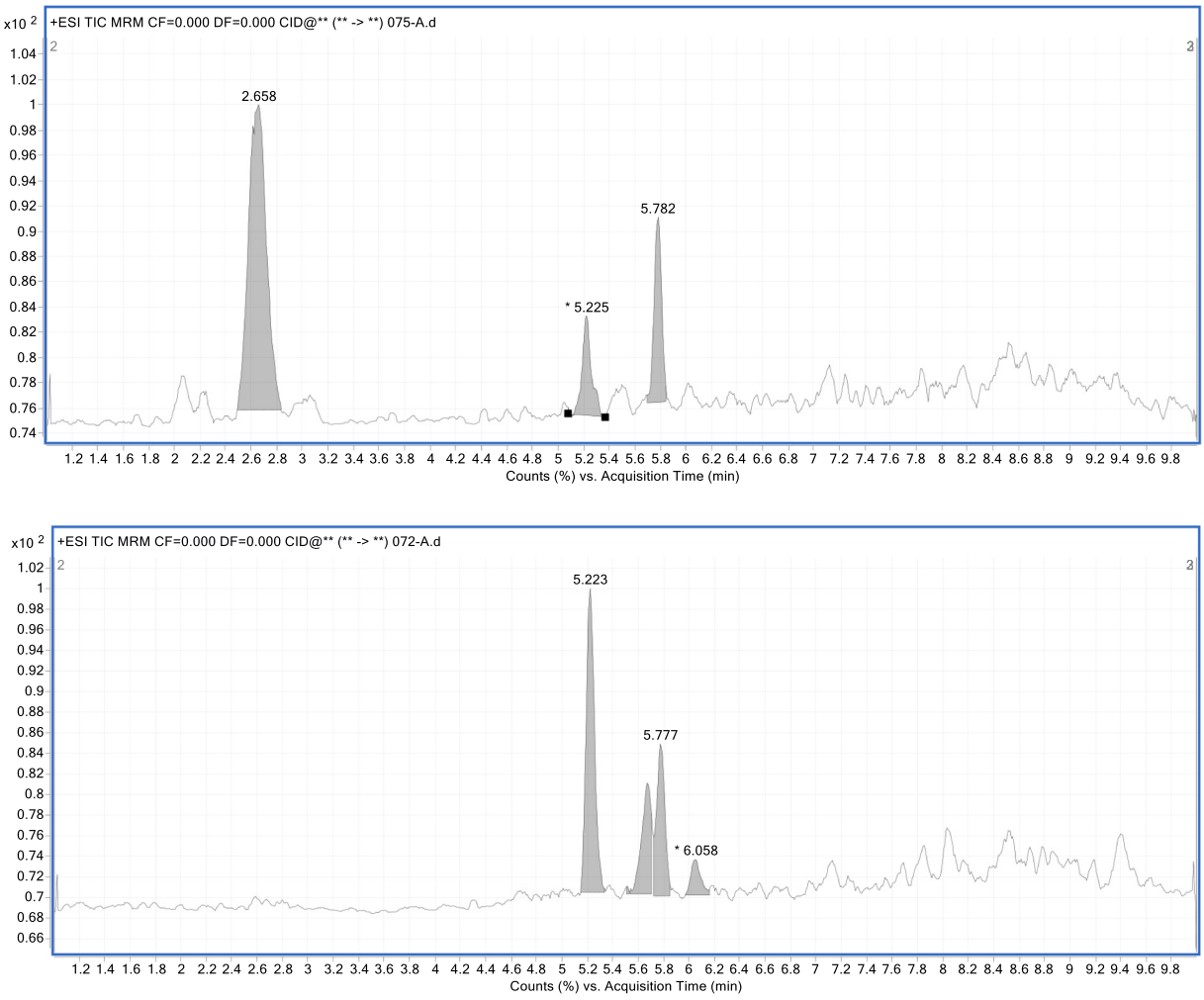

Fig. 3. Multiple reaction monitoring (MRM) chromatogram confirming the presence of: (a) DA (RT = 2.658 min) and GYM (RT = 5.782 min); (b) GYM (RT = 5.777 min) and SPX (RT = 6.058 min); in *Spondylus spinosus* sample from Tripoli (3.88 DA mg kg^{-1} and 24.80 GYM μg kg^{-1}); (a) and Tyre (49.29 GYM μg kg^{-1} and 11.79 SPX μg kg^{-1}); (b).

concentrations with an average of 56 ± 27 μg kg^{-1} from samples of Tripoli, 1.5-fold higher than the average of Tyre's samples (36 ± 11 μg kg^{-1}). The limpet *P. rustica* complex represented the second contaminated species by GYMs with the highest averages from Tyre (26.9 μg kg^{-1}), Beirut (26.8 μg kg^{-1}) and Tripoli (8.7 μg kg^{-1}), respectively. Whereas the lowest GYM concentration was detected in the fish *S. rivulatus* from Tyre (3.33 μg kg^{-1}).

➢ SPX

SPX (13-Desmethyl-spirolide C) was only detected in the spiny oyster *S. spinosus* (Fig. 4c). Concentrations varied between a minimum of 2.18 and a maximum of 15.07 μg kg^{-1} in oysters collected from Tripoli and Tyre, respectively (Fig. 4c), and were mainly found in oysters taken from Tyre (67%) whereas only 27% of *S. spinosus* samples from Tripoli were contaminated by this toxin. The average of SPX concentration in *S. spinosus* taken from Tyre (7.54 ± 5 μg kg^{-1}) is almost 2 times higher than the average of Tripoli's samples (4.05 ± 2 μg kg^{-1}).

3.2. Physico-chemical parameters

During the sampling period (winter season), the highest hydrographic parameters (temperature and salinity) were measured in Beirut. The lowest temperature was recorded in Tyre and comparable salinities were measured in both Tripoli and Tyre (Table 2).

Nutrients' concentrations showed that the ecological quality status of the three study areas can be considered "Good" based on criteria recommended by Karydis (2009) for the Eastern Mediterranean coastal waters. Tripoli showed the highest orthophosphate concentrations (4- and 1.4-fold higher than the levels in Beirut and Tyre, respectively), while the highest nitrate and nitrite concentrations were measured in Tyre. Nitrate concentrations in Tyre were 4- and 2-fold higher than the values of Beirut and Tripoli respectively, whereas nitrite concentrations in Tyre were 2-and 1.7-fold higher than the values in Beirut and Tripoli, respectively (Table 2).

Same as the nutrients, the highest primary production was obtained also in Tyre with the highest chlorophyll-*a* and pheopigment concentrations, 7- and 2.5-fold higher than the Chl-*a* levels in Tripoli and Beirut, respectively (Table 2).

3.3. Phytoplankton populations

Phytoplankton analysis shows the presence of the genesis that produce the biotoxins found in this study, namely *Pseudo-nitzschia* spp., *Gymnodinium* spp., and *Alexandrium* spp. producers of domoic acid, gymnodimines, and both gymnodimines and spirolides, respectively (Fig. 5).

In general, the concentrations of *Pseudo-nitzschia* spp., *Gymnodinium* spp., and *Alexandrium* spp. are low with the highest concentrations detected for *Gymnodinium* spp. *Pseudo-nitzschia* spp. were found in the three studied areas with concentrations always higher than 4500 cell

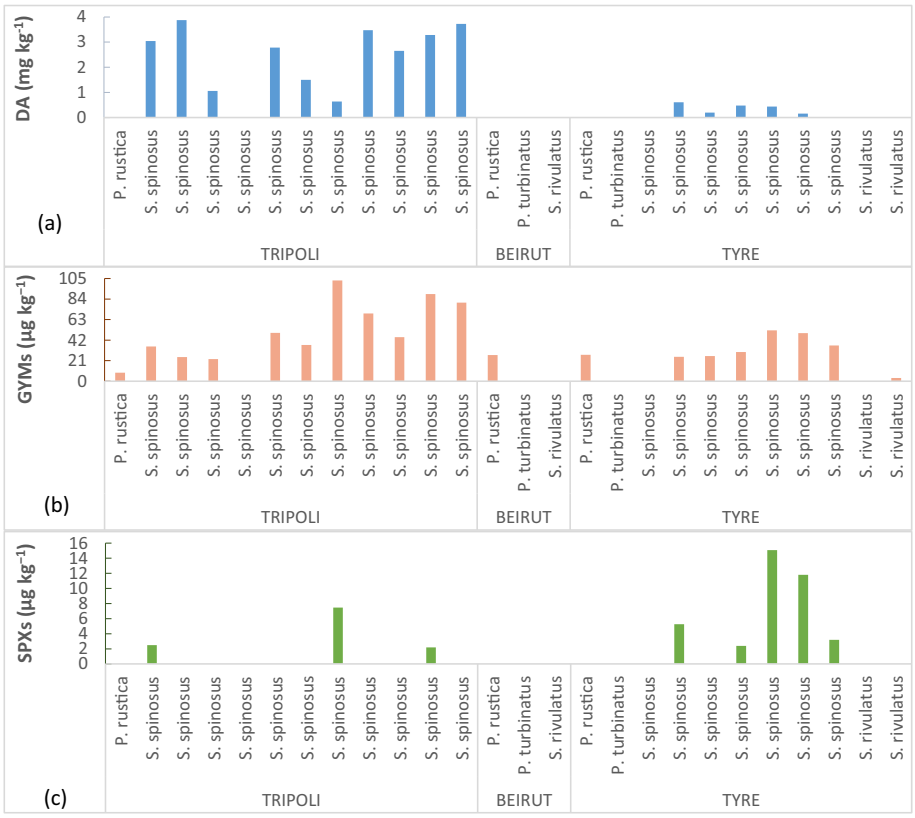

Fig. 4. DA (a), GYMs (b), and SPXs (c) concentrations in the various studied species collected from Tripoli, Beirut and Tyre. Only values above LODs are presented (DA: 0.1025 mg kg^{-1}; GYMs 2.3568 µg kg^{-1}; SPXs: 2.0484 µg kg^{-1}).

L^{-1} (Fig. 5a). The highest concentration of *Pseudo-nitzschia* spp. was identified in Tripoli which is in harmony with the highest DA levels also measured in Tripoli (>8000 cell L^{-1}; Fig. 4a). Although, Beirut showed concentrations of *Pseudo-nitzschia* spp. slightly higher than in Tyre, DA levels were higher in Tyre (Fig. 4). *Gymnodinium* spp. were also found in all three stations with concentrations above $25*10^4$ cell L^{-1} in Tripoli (Fig. 5b) which is in concordance with the highest GYMs levels in the same study area (Fig. 4b). However, the high GYMs levels in Tyre are not in harmony with the lowest *Gymnodinium* spp. concentrations found in Tyre (~7500 cell L^{-1}; Figs. 4b and 5b). Furthermore, *Alexandrium* spp. were found in the three prospected stations with concentrations always below 1500 cell L^{-1} and a maximum detected in Tyre (Fig. 5c) in agreement with SPXs highest concentrations measured also in Tyre. Otherwise, Beirut showed concentrations of *Gymnodinium* spp. higher than the ones found in Tyre and *Alexandrium* spp. higher than the ones found in Tripoli which are not in harmony with the lowest concentrations of GYMs and SPXs both measured in Beirut (Figs. 4 and 5).

The results demonstrate that microphytoplankton species are the dominant groups in the three studied areas (Fig. 7b). Dinoflagellates constitute the dominant microphytoplankton group in both Tripoli and Beirut, where the highest temperature and salinity were measured, while diatoms are the dominant group in Tyre were the lowest T and S and highest nitrate and nitrite ions were measured (Fig. 7a; Table 2). Furthermore, the dominant species were *Gyrodinium* spp. and *Gymnodinium* spp. both had their highest concentrations in Tripoli (Fig. 6).

4. Discussion

Based on the available literature, no biotoxins' measurements have been previously conducted on the same marine species studied in the present paper which open the window for further studies to assess the seasonal and long-term patterns of biotoxins in these species.

The Lebanese coast is highly urbanized. This narrow coastline (~240 km) is subject to heavy anthropogenic pressures mainly from sewage and industrial outputs, therefore, witnessing frequent algal blooms in heavily polluted areas (Abboud-Abi Saab and Hassoun, 2017). Around 70% of the Lebanese population live in coastal areas which has stressed seawater quality and aggravated, together with the growing urbanization and the expansion of industrial activities, the effects of marine organic pollution (MOE/UNDP/ECODIT, 2011). Lebanon's coastal waters receive about 65% of the total sewage via at least 53 major sewage outfalls spread along the Lebanese coastline

Table 2
Average levels of physico-chemical parameters from surface waters (~0.5 m) during winter in the study areas.

Location	Temperature	Salinity[a]	Phosphates	Nitrates	Nitrites	Chl-*a*	Pheopigments
Units	°C		µmol L^{-1}	µmol L^{-1}	µmol L^{-1}	mg m^{-3}	mg m^{-3}
Tripoli ($n = 2$)	20.05 ± 0.95	38.49 ± 0.01	**0.3 ± 0.07**	2.2 ± 0.8	0.164 ± 0.035	0.075 ± 0.05	0.09 ± 0.05
Beirut (n = 2)	**21.25 ± 0.25**	**38.59 ± 0.54**	0.07 ± 0.009	1.18 ± 0.15	0.125 ± 0.016	0.21 ± 0.07	0.22 ± 0.03
Tyre ($n = 1$)	18.5	38.47 ± 0.22	0.22	**5.09**	**0.28**	**0.55**	**1.92**

Bold values refer to the maximum values of each parameter.
[a] Salinity values were averaged for winter season (December–February) of the period 2016–2019 (n = 3, 4, 5 for Tyre, Tripoli, and Beirut respectively).

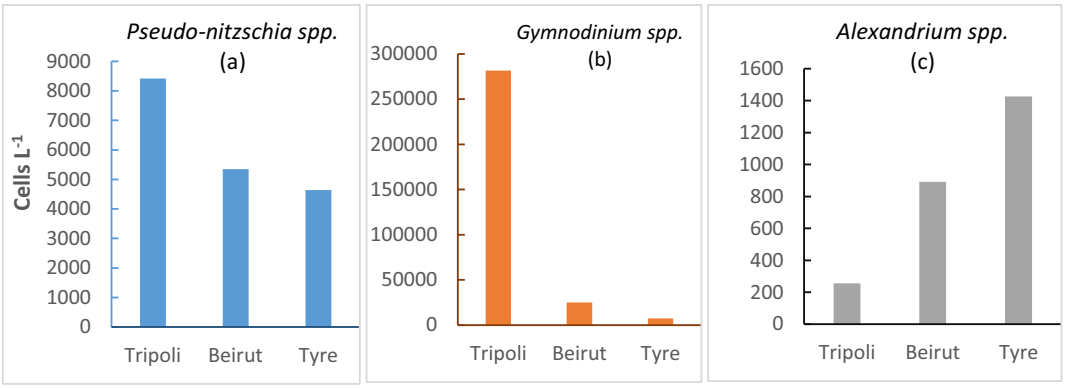

Fig. 5. Number of cells per liter (Cell L^{-1}) for phytoplankton species that produces a) domoic acid: *Pseudonitzchia* spp., b) GYMs: *Gymnodinium* spp., and c) GYMs and SPXs: *Alexandrium* spp. in Tripoli, Beirut and Tyre in 02.03.2020 and 03.04.2020.

(CDR/LACECO, 2000; MOE/UNDP/ECODIT, 2011) which explains the slightly high nutrients levels in Tyre and Tripoli as the collected samples might be influenced by nearby port activities (Table 2). Also, rivers carry pollutants produced by agricultural runoff and sewage directly to the sea (MOE/UNDP/ECODIT, 2011). In addition to the abovementioned facts, only 66% of dwellings and businesses are connected to an improved sewer network in 2007, which explain the Lebanon's rank: 90th among 163 countries, based on the Environment Performance Index (EPI), indicating a lower performance in terms of environmental sustainability (Emerson et al., 2010). These facts explain the occurrence of toxins' producers (Figs. 5 and 6) and/or HABs in many coastal areas, and hence justify the relatively high levels of biotoxins (Fig. 4) accumulated in marine biota. In addition to these anthropogenic pressures, climate change is impacting the phytoplankton structure and diversity through modifications in temperature, salinity, precipitation, nutrients and dissolved oxygen patterns, increased frequency and intensity of extreme weather events, ocean warming and acidification, changes in contaminants' transport pathways, and intrusion of invasive species (Molgó et al., 2014). All these new patterns are affecting HABs that are increasing in frequency, severity and biogeographical level (Molgó et al., 2014), and have been associated to the occurrence of harmful and/or potentially toxic species (Abboud-Abi Saab and Hassoun, 2017).

4.1. Biotoxins in Lebanese waters and the Mediterranean

Although DA concentration was not measured previously in the spiny oyster *S. spinosus*, many studies have been conducted on other marine organisms, mainly harvested mussels and oysters. These studies showed comparable or lower DA levels, such as in the Eastern Adriatic, Morocco, Bizerte Lagoon of Tunisia, Languedoc-Southern France, Spain and Portugal (Table 3). Whereas in Greece, 83 to 95% of all sampled mussels (*M. galloprovincialis*) and venus clams (*Venus verucosa*) contained <1 μg g^{-1} in 2002 and 2003, respectively with a maximum of 14.0 μg g^{-1} in mussels in 2002 and 4.2 μg g^{-1} in mussels and 5.6 μg g^{-1} in venus clams in 2003 (Kaniou-Grigoriadou et al., 2005).

GYMs' range in our samples is higher than the ones obtained in many Mediterranean areas, such as in Greece, Morocco, and Eastern Adriatic (Table 3). While no GYMs were detected after analyzing several raw and processed commercial bivalves in eight European countries (including 4 Mediterranean ones: Italy, Portugal, Slovenia, Spain) over

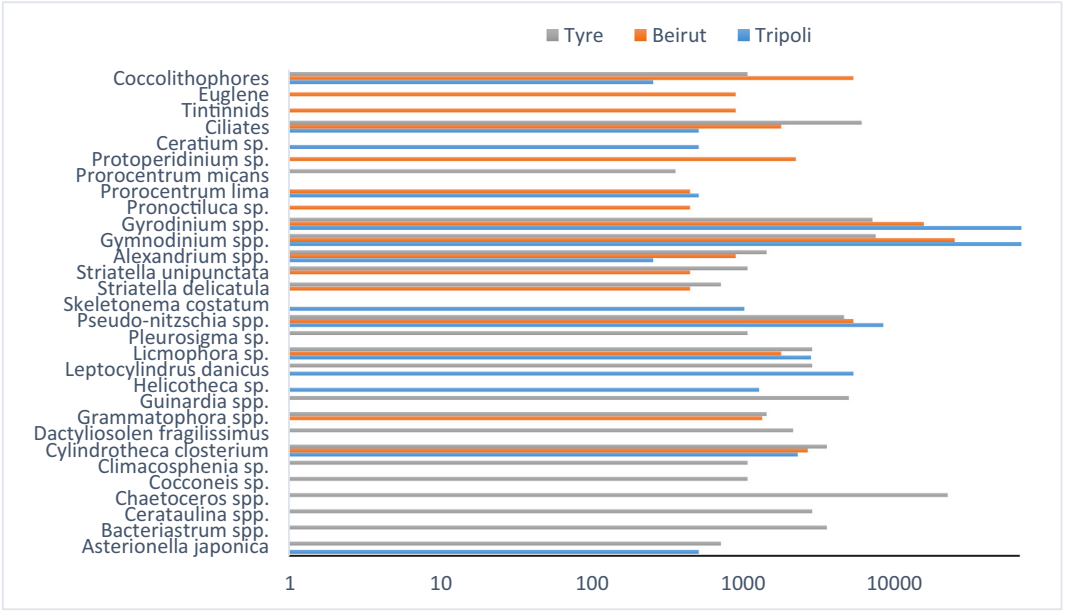

Fig. 6. Concentrations of phytoplankton species found in Tripoli, Beirut and Tyre in 02.03.2020 and 03.04.2020.

Table 3
Concentrations of DA, GYM and SPX in Marine biota collected from various Mediterranean areas.

Type	Scientific name	Country	Region	DA (μg g^{-1})	GYM (μg kg^{-1})	SPX (μg kg^{-1})	References
Rough cockle (clam)	*Acanthocardia tuberculata*	Croatia		0.17–0.77	2.65–15.77	0.98–5.90	Ujević et al. (2019)
Smooth clam	*Callista chione*	Croatia		0.28	1.17–6.14	0.65–2.14	Ujević et al. (2019)
		Morocco		4.9	–	–	Rijat Leblad et al. (2013)
Palourde clam	*Ruditapes decussatus*	Tunisia	Gulf of Gabes	–	460–1290	–	Marrouchi et al. (2010)
Mussel	*Mytilus galloprovincialis*	Tunisia	Lagoon of Bizerte	0.13–0.86	–	–	Bouchouicha-Smida et al. (2015)
		France	Languedoc	0.8	–	–	Amzil et al. (2001)
		Croatia		0.11-1.65	–	–	Ujević et al. (2019)
			Šibenik Bay	–	BDL[a]	–	Gladan et al. (2011)
			Area of Dubrovnik	–	BDL	–	Čustović et al. (2012)
			Mali Ston Bay	–	BDL	–	Arapov et al. (2015)
		Spain	Ebro delta bays	~5	–	–	Giménez et al. (2013)
			Galicia	–	–	1.2–6.9	Moreiras et al. (2019)
			Catalonia	–	–	2.2–16	García-Altares et al., 2014
		Portugal		~5	–	–	Vale and Sampayo (2001)
		Morocco	Jamâa Ouled Ganem	–	5.6	BDL	Haddouch et al. (2017)
Oyster	*Ostrea edulis*	Tunisia	Lagoon of Bizerte	0.42–1.04	–	–	Bouchouicha-Smida et al. (2015)
Pacific Oyster	*Crassostrea gigas*	Spain	Catalonia	–	–	3.5–6.6	García-Altares et al. (2014)
Spiny oyster	*Spondylus spinosus*	Lebanon	Tripoli	0.64–3.88	22.64–102.9	2.18–7.48	This study
			Tyre	0.15–0.61	25–51.9	2.38–11.79	This study
Scallop	*Flexopecten proteus*	Croatia		0.1117–1.6567	–	–	Ujević et al. (2010)
Scallop	*Pecten jacobaeus*	Croatia		0.1117–1.6567	–	–	Ujević et al. (2010)
			Šibenik Bay	–	BDL	–	Gladan et al. (2011)
Gastropod-Sea snail	*Phorcus turbinatus*	Lebanon		BDL	BDL	BDL	This study
Limpet	*Patella rustica* complex	Lebanon	Tripoli	BDL	8.7	BDL	This study
			Beirut	BDL	26.78	BDL	This study
			Tyre	BDL	26.93	BDL	This study
Fish	*Siganus rivulatus*	Lebanon	Beirut	BDL	BDL	BDL	This study
			Tyre	BDL	3.33	BDL	This study

[a] BDL = below the detection limit.

2 years (Rambla-Alegre et al., 2018). Similarly, no GYM-A contamination was detected in clams and mussels collected in Ganzirri and Faro lakes (connected to each other and to both Ionian and Tyrrhenian Seas; Mattarozzi et al., 2019). Moreover, plankton samples from Ebro Delta (NW Med) contained very low GYM levels. Otherwise, very high GYM concentrations were measured in the South of the Mediterranean, at least 5-fold higher than the values obtained in our study, in the digestive glands of clams from the Tunisian coast (Marrouchi et al., 2010; Table 3), and a maximum of 2136 μg kg^{-1} was recorded in clams from the Gulf of Gabes-Tunisia (Ben Naila et al., 2012).

SPX was measured in 67 and 27% of the spiny oysters (*S. spinosus*) collected from Tyre and Tripoli respectively (Fig. 4c). SPX values are higher than the ones measured in plankton and shellfish samples from the North-Western Mediterranean and the Eastern Adriatic (Table 3). Whereas wider ranges with high SPX concentrations were recorded in mussels and oysters from Catalonia-Spain (Amzil et al., 2007; García-Altares et al., 2014) and in Greek shellfish where concentrations ranged from trace levels up to 118 μg kg^{-1} (Katikou et al., 2012; Vlamis and Katikou, 2015). An interesting study showed that 9.4% of the samples ($n = 47$) collected from raw and processed commercial bivalves from several European countries were contaminated by SPX with concentrations varying between 26 and 66 μg kg^{-1} (Rambla-Alegre et al., 2018). However, below detection levels or no SPXs have been observed in other parts of the Mediterranean such as in Morocco, and Ganzirri and Faro lakes where all clams and mussels were free of SPXs (Mattarozzi et al., 2019).

4.2. Sentinel species for biotoxins survey

All above-cited studies (Table 3) show that DA levels in oysters are higher than the ones measured in other marine species such as mussels. Also, clams can hold DA for up to 1 year in the natural environment, or several years after being processed, canned or frozen (Ferriss et al., 2017). In fact, the measurement of DA concentrations in different marine species, such as in the present study, can help to identify organisms that are more prone to accumulate DA than other ones. These organisms will therefore be considered as early warning tools for potential problem with the toxin, also known as "sentinel" species. However, both oysters and mussels are considered as bio-indicators to address ecosystem contamination and are used as sentinels for potential integration of biotoxins (Turki et al., 2014). Based on our study, the spiny oyster *S. spinosus* is the only species showing DA levels above detection limit (Fig. 4a), relatively high compared to results obtained in the above-mentioned studies (Table 3). These findings are in harmony with

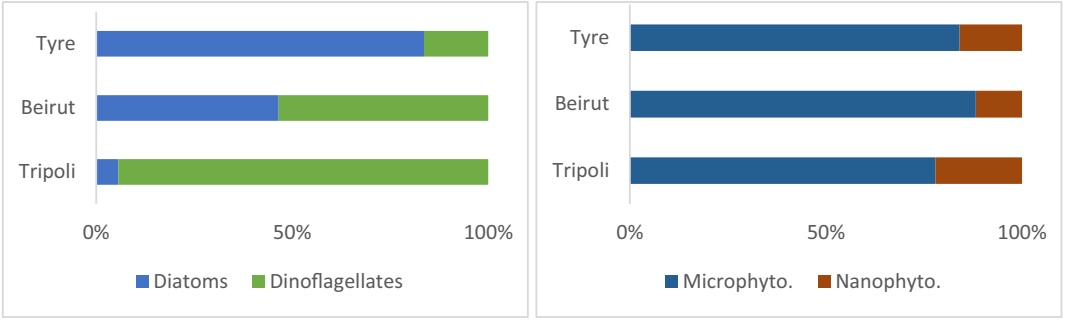

Fig. 7. Percentage of phytoplankton populations (a) diatoms and dinoflagellates, (b) total micro- and nano-phytoplankton in Tripoli, Beirut and Tyre in 02.03.2020 and 03.04.2020.

other studies, for example when mussels and oysters were simultaneously collected in Bizerte Lagoon (Tunisia), oysters showed higher DA levels than mussels, and this difference was attributed to the time needed for depuration (Bouchouicha-Smida et al., 2015). A statement that was also obtained by Blanco et al. (2002) who found that mussels appear to depurate themselves for DA faster than oysters. Although GYMs were found in various species in the present study, the spiny oyster *S. spinosus* showed the highest levels (average 48.4 ± 24 μg kg^{-1}; Fig. 4b). Whereas, SPXs were found only in *S. spinosus* (Fig. 4c). Thus, among the prospected marine organisms in the present study, the spiny oyster *S. spinosus* can serve as a sentinel species that might be monitored regularly to survey DA, GYMs, and SPXs levels.

The same trend was also observed for metals in fishery products collected along the Lebanese coast. The bivalve *S. spinosus* showed the highest concentrations of cadmium (Cd), lead (Pb) and arsenic (As) compared to fish (*Siganus rivulatus*, *Lithognathus mormyrus* and *Etrumeus teres*) and shrimp (*Marsupenaeus japonicus*) (Ghosn et al., 2019). These findings suggest that the spiny oyster can be used as sentinel species to monitor various contaminants in the marine environment, and evaluate any potential human risk poisoning in Lebanon.

4.3. Biotoxins' producers

DA production is mainly attributed to the presence of *Pseudo-nitzschia* spp. which bloom events preceded high DA concentrations in mussels, as demonstrated in Croatian shores-Eastern Adriatic (Ujević et al., 2010). *Pseudo-nitzschia* genus comprises cosmopolitan species, found worldwide (Hasle, 2002; Trainer et al., 2009; Churro et al., 2009; Loureiro et al., 2009; Lundholm et al., 2010), as well as in various Mediterranean areas (Quiroga, 2006; Congestri et al., 2008; Loureiro et al., 2009; Sahraoui et al., 2011, 2012). In Lebanon, *Pseudo-nitzschia delicatissima* is one of the dominant species in some areas and can account for 7 to 51% of the total microphytoplankton populations (Abboud-Abi Saab and Hassoun, 2017). During winter season (December to February), the percentage of *P. delicatissima* usually varies between 7 and 25% depending on the station and its highest concentration is obtained during the decrease of both temperature and salinity (November–May), mainly in stations influenced by terrestrial inputs such as rivers and sewages (Abboud-Abi Saab and Hassoun, 2017). In our phytoplankton samples collected during winter season, the highest abundance of *Pseudo-nitzschia* spp. was found in Tripoli (Fig. 5a), however, *Pseudo-nitzschia* spp. constitute 1, 2.5 and 3% of the total microphytoplankton community in Tripoli, Beirut, and Tyre respectively where in the latter station, T and S had the lowest values (Table 2). These results show that the high DA levels are not correlated to the occurrence of *Pseudo-nitzschia* spp. bloom during the study period. The same finding was highlighted in other studies stating that the maximum DA concentration does not coincide with *Pseudo-nitzschia* spp. blooms; other unidentified species could be responsible. In fact, high DA levels above the allowed limit were measured in soft tissues of *Pecten maximus* during the continuously low *Pseudo-nitzschia* spp. abundance (James et al., 2005). This was documented in various Mediterranean areas such as Ebro Delta, Arenys de Mar and Vilanova (Busch et al., 2016; Giménez Papiol et al., 2013), as well as in the Eastern Adriatic where potentially dangerous DA concentrations can be accumulated in some shellfish species even when *Pseudo-nitzschia* spp. is found in low abundance (Ujević et al., 2010). Similarly, DA production was primarily attributed to another species: *Nitzschia bizertensis* in Bizerte Lagoon-Tunisia (Bouchouicha-Smida et al., 2015). However, a good correlation between high DA levels and *Pseudo-nitzschia* spp. was observed in the vicinity of river Krka estuary (central Adriatic Sea) where high DA concentrations and high abundance of *Pseudo-nitzschia* spp. ($>1.0 \times 10^6$ cells L^{-1}) correlated with temperature and salinity minima in February (Ujević et al., 2010). The same trend was also found in the Bay of Banyuls-sur-Mer, North-Western Mediterranean Sea during spring (Quiroga, 2006), along the Latium region coast-Middle Tyrrhenian Sea (Congestri et al., 2008), and in the Eastern Adriatic (Ujević et al., 2010).

GYMs production is mainly attributed to the dinoflagellate *Karenia selliformis*, formerly identified as *Gymnodinium selliforme* (McKenzie et al., 2002; Haywood et al., 2004; Kremp et al., 2014). *Gymnodinium* spp. is widely detected in the Mediterranean (Reñé et al., 2011 and references therein), including the Levantine Sea (Siokou-Frangou et al., 1999). The expansion of *K. selliformis* is demonstrated to be supported by temperature increases with spatial development differences near touristic areas (Feki et al., 2013). In Lebanon, *Gymnodinium* spp. is among the dominant phytoplanktonic species in coastal waters ranging from 6 to 29% between October and December, with the highest concentrations recorded in stations influenced by river discharges (Abboud-Abi Saab and Hassoun, 2017). Phytoplankton analysis in the present study, showed that *Gymnodinium* spp. constitute 5, 12 and 42% of the total microphytoplankton populations in Tyre, Beirut and Tripoli, respectively (Figs. 5b and 7). These results explain the high GYMs levels measured in Tripoli (Fig. 5b) and are in harmony with many studies around the Mediterranean where the presence of GYMs was correlated with high abundance of *Gymnodinium* spp. Moreover, in the Eastern Adriatic the presence of GYMs was obtained twelve days after the occurrence of *Gymnodinium* spp. bloom (7.1×10^5 cells L^{-1}) (Gladan et al., 2011). Also, Marrouchi et al. (2010) have noted that the highest GYMs levels were detected in November and December of each year after a bloom of *K. selliformis* generally after a strong sunshine. Here too, the high GYMs levels were detected during the same period in this study. Otherwise, a positive correlation between *K. selliformis* and nitrate and a negative one with total phosphorus was found (Feki et al., 2013). However, in our study, the highest GYMs concentrations were obtained in oysters from Tripoli, although the highest GYMs levels in the bivalve *Patella rustica* complex were measured in samples from Tyre where also the highest concentrations of nitrates, nitrites and Chlorophyll-*a* and lowest phosphates were measured (Fig. 4b; Table 2). A longer dataset is needed to better understand the correlation between the measured concentrations of GYMs and the physico-chemical parameters.

SPXs are known to be the largest CI group (Chatzianastasiou et al., 2011). These toxins are produced by dinoflagellates *Alexandrium ostenfeldii* (Cembella et al., 2000; Touzet et al., 2008), and *A. peruvianum* (Moestrup et al., 2011; EFSA, 2010c). In Lebanese waters, *Alexandrium* spp. was detected almost along the entire coastal area, reaching a maximum of 4752 cells L^{-1} in spring (Abboud-Abi Saab and Hassoun, 2017). Likewise, *Alexandrium* spp. was found in the three studied areas with the highest abundance in Tyre, in accordance with the high SPXs levels measured in oysters from that area (Figs. 4c and 5c). In fact, *Alexandrium* spp. have the ability to colonize multiple habitats and to persist over large regions through time. Thus, this species is widespread globally, present in coastal, shelf and slope waters of the Northern and Southern Hemispheres (Lilly et al., 2007 and references therein), including the Mediterranean Sea where the diversity of *Alexandrium* appears to be higher than elsewhere, reflecting the level of taxonomic scrutiny more than an actual distribution (Anderson et al., 2012). Therefore, this high diversity prevented us to differentiate between *Alexandrium* spp. species in our samples. It is noteworthy to mention that this genus is generally benefiting from climate change effects on land runoff, terrestrial outputs and nutrients variability, water masses circulation and water column stratification (Goffart et al., 2002), such as the case of *Alexandrium minutum* in the Mediterranean Sea (Valbi et al., 2019).

4.3.1. Obstacles in the identification

Pseudonitzschia genus comprises >30 taxa of which 11–12 are potential DA producers (Lundholm, 2011). Many other species need to be further studied in order to evaluate its biology, ecology and toxicity to improve the monitoring and prediction of toxic species' blooms. Improved taxonomic equipment, jointly with molecular tools should be used in future systematic studies to appropriately distinguish between

toxic and non-toxic species in Lebanese waters. Also, *Gymnodinium* genus contains around 234 identifiable species (Thessen et al., 2012), and the light microscope is not enough to well differentiate between *Gymnodiunium* species on a hand, and between *Gymnodinium* and *Karenia* species on the other hand, particularly that they resemble to each other under the light microscope. Besides, other studies demonstrated that GYMs are also produced by *Alexandrium ostenfeldii* (Harju et al., 2016; Van de Waal et al., 2015; Van Wagoner et al., 2011) and *Alexandrium peruvianum* (Van Wagoner et al., 2011), species not easily recognized via a light microscope as well. Furthermore, a lack of coherence between the presence of GYMs and their potential producers was argued in many studies (Medhioub et al., 2009; Harju et al., 2016; Busch et al., 2016) which may be attributed to the presence of undescribed species as alternative sources for the detected toxins and raises concerns about the necessity to upgrade morphological identification tools. *Alexandrium* genus comprises >30 morphologically defined species, at least half are known to be toxic or have otherwise harmful effects (Anderson et al., 2012). Using a light microscope, *Alexandrium* spp. look very comparable, particularly *Alexandrium ostenfeldii* and *A. peruvianum* that are morphologically very similar, but can be separated based on their cell size, on the shape of their platelets, and the right anterior margin of their plates (Balech, 1995). Moreover, in the genus *Alexandrium*, the advent of molecular techniques challenged the classification of species based on morphological characters by showing that: i) a high level of genetic diversity is present within the same morphospecies, and ii) some characters for separation of closely related morphospecies show a broad range of variability and do not match molecular genetic clustering. These features, however, are too hard to be distinguished via light microscope which is the main obstacle to further identify *Alexandrium* spp. in Lebanese waters.

4.4. Toxicity and regulations of the detected biotoxins

For DA, the European Food Safety Authority (EFSA) estimated the Lowest Observed Adverse Effect Level (LOAEL) as 0.9 μg g^{-1} for neurotoxicity in humans and established an Acute Reference Dose (ARfD) of 30 μg kg^{-1} body weight (b.w.) (EFSA, 2009). All *S. spinosus* samples taken from Tyre had values lower than the estimated LOAEL, whereas 82% of *S. spinosus* taken from Tripoli showed DA concentrations higher than the LOAEL but lower than the ARfD (Fig. 4a). These concentrations raise concerns about potential short and long-term neurotoxicity effects on humans. On a short term, DA intoxication may cause gastrointestinal symptoms within 24 h. after the ingestion of contaminated organisms (Vilariño et al., 2018). On a long-term, DA may induce morphological changes in the hippocampus of rats (Pulido, 2008), and a chronic DA toxicosis syndrome in sea lions has also been revealed in their natural habitat (Goldstein et al., 2008). These studies show that chronic effects were more often seen in juvenile animals showing higher susceptibility of the developing brain to DA exposure (Doucette and Tasker, 2016). While on humans, studies on Native Americans, who consumed >15 razor clams per month with < 20 μg DA g^{-1} over 4 years, show that they suffered mild memory decline (Grattan et al., 2016a, 2016b). Also, a recent human health study revealed that high razor clams (with safe levels of DA) consumers would have worse everyday memory than non-consumers or low consumers based on dietary exposure of 10 days and 1 year prior to assessment (Grattan et al., 2018; more about DA symptoms in Vilariño et al., 2018). Therefore, not only short-term but also long-term DA neurotoxicity, due to repetitive consumptions, may be associated with low level, chronic exposures in adults who are heavy consumers of contaminated marine organisms.

Although an acute toxicity of CIs has been demonstrated through MBA positive samples containing only gymnodimine and spirolide toxins (Ujević et al., 2015), no acute poisoning in humans has been directly related to seafood contamination yet (Marrouchi et al., 2013; Harju et al., 2016; Visciano et al., 2016). In fact, there is limited information regarding CIs absorption, distribution, metabolism and excretion in animals or in humans (Chatzianastasiou et al., 2011). Thus, neither an acute reference dose (ARfD) nor a tolerable daily intake (TDI) have been suggested to prevent acute or chronic toxicities, respectively (Ben Naila et al., 2012). Despite this fact, the chronic toxicity of GYMs remains unclear as its role in the development of neurodegenerative illnesses like Alzheimer's or Parkinson's diseases has been debated (Alonso et al., 2011; Marques et al., 2010, 2014). As a result, the levels of CIs in marine seafood have not yet been regulated in the European Union or elsewhere. Gymnodimine was demonstrated to be highly toxic by intraperitoneal injection (lowest lethal dose: LD50 = 96 μg kg^{-1}), however, when ingested with food (>7500 μg kg^{-1} in mice) GYMs show low toxicity (Munday et al., 2004). In our study, the maximum GYMs level was 102.9 μg kg^{-1} a bit higher than the LD50 (causes acute toxicity via intraperitoneal injection in mice) and much lower than the concentration that shows toxicity when ingested with food in mice (Fig. 4b). These results are in harmony with the above-mentioned studies suggesting that GYMs are of low risk to humans consuming contaminated shellfish. Furthermore, the EFSA has assessed the risk for spirolides as low, taking into account the 95th percentile of the concentration of SPXs of 9, 15 and 7 μg kg^{-1} in shellfish meat for mussels, oysters and clams respectively (Ben Naila et al., 2012), which is in the range of the concentrations found in the spiny oysters in this study (Fig. 4c). Moreover, a margin of exposure (MOE) approach was used for the risk characterization of exposure to these toxins (Rambla-Alegre et al., 2018) via the very limited toxicity data, by dividing the LD50 value by the estimated 95th percentile of exposure from shellfish consumption. Taking into consideration that the LD50 following administration of SPXs in the food is ~500 μg kg^{-1} (b.w.) and that the MOE is estimated to be in the range of 2941–4545 μg kg^{-1} (Rambla-Alegre et al., 2018), it is unlikely that a health risk exists due to the exposure to SPXs through seafood consumption in Lebanon.

4.5. Contamination in non-bivalves species

DA vectors to humans are not restricted to clams, mussels, oysters, and scallops but also squids, sardines, anchovies, crabs, lobsters and other marine organisms may play this role (Lopez-Rivera et al., 2009). Many studies revealed important DA levels in non-bivalve species such as in the cephalopod, common cuttlefish (*Sepia officinalis*) in Portugal (Costa et al., 2005) and Morocco (Ben haddouch et al., 2016). These studies demonstrated that despite having below-EU-regulatory-limit DA concentrations in muscles (0.7 and 16 μg g^{-1} in Portugal and Morocco respectively), high DA levels were measured in digestive glands of cuttlefish (50 to 241.7 μg g^{-1} in Morocco and Portugal respectively). In our study, DA levels were below the detection limit in the muscle of the fish *S. rivulatus* (Fig. 4a), however no measurements were conducted for its digestive glands. This step is very necessary in future studies since in some countries, including Lebanon, whole juvenile pelagic fishes are consumed (i.e. without evisceration) and in this case they might represent a risk to human health since DA can reach harmful levels in their digestive glands. Moreover, DA was also detected in coastal waters of the Sea of Marmara in Turkey (0.96 and 5.25 μg mL^{-1}; Dursun et al., 2016). Such measurements in seawater can be helpful in a systematic monitoring program to prevent the DA bioaccumulation by marine biota. Furthermore, gymnodimines have been detected in many shellfish species, including greenshell mussel, blue mussel, scallop, cockle, clams, oyster and abalone (MacKenzie et al., 2002; Stirling, 2001). However, other organisms such as crustaceans, gastropods and fish have also been reported as vectors (Shumway, 1995; Deeds et al., 2008), including the gastropod *Phorcus turbinatus* and fish *S. rivulatus* as demonstrated in this study.

5. Conclusions and recommendations

This study reports for the first time the occurrence of lipophilic toxins, domoic acid (hydrophilic toxin) and cyclic imines in marine biota from various areas of the Lebanese coast.

- Levels below the detection limits (LOD) were obtained for okadaic acid (OA), dinophysistoxin-1 and 2 (DTX-1,2), pectenotoxin-1 and 2 (PTX-1,2), yessotoxins (YTXs), azaspiracids (AZAs), and hydrophilic toxins (ASP and PSP). Only domoic acid (DA), gymnodimine (GYM-b) and spirolide (SPX) exhibited levels above the detection limits in some species/areas.
- The highest domoic acid and gymnodimine levels (3.88 mg DA kg^{-1} and 102.9 μg GYM kg^{-1}) are found in spiny oysters (*S. spinosus*) collected from Tripoli in accordance with the occurrence of relatively high abundance of genuses that produce these toxins in the same station (*Pseudo-nitzchia* spp. and *Gymnodinium* spp.), whereas highest concentrations of spirolides (15.07 μg SPX kg^{-1}) were obtained also in spiny oysters but from Tyre in harmony with the important abundance of this toxins' producers (*Alexandrium* spp.) in this area.
- DA levels were below the EU regulatory limits, but higher than the Lowest Observed Adverse Effect Level (0.9 μg g^{-1}) for neurotoxicity in humans and lower than the Acute Reference Dose (30 μg kg^{-1} b. w.) both set by the EFSA. These concentrations raise concerns about potential effects that could emerge in humans due to repetitive consumption of DA-contaminated seafood.
- Taking into consideration that the lowest lethal dose (LD50) following administration of GYMs and SPXs in Mouse bioassays, it is unlikely that a health risk exists due to the exposure to both toxins through seafood consumption in Lebanon. Despite this fact, the chronic toxicity of GYMs and SPXs remains unclear and more research is needed to fix tolerable daily intake (TDI) limits for human consumption.
- The results shed the light on the importance of oysters (spiny oyster: *Spondylus spinosus*) as a sentinel species that bio-integrate various toxins. As biotoxins were found in non-bivalve species, it is important to monitor phycotoxins not only in shellfish and commercial species but also in various organisms of the marine trophic chain as they can be vectors of toxins to commercial/edible seafood and ultimately to humans.

Recommendations:

* *Phytoplankton identification tools*: The main obstacle for a better identification of potential biotoxins' producers in Lebanese waters is the difficult taxonomical aspects of phytoplankton species in general. As Taylor (1985) has stated: "Nowhere is the value of taxonomy more readily apparent than in its application to toxic species". Traditional criteria and tools (such as the light microscope) used to identify phytoplankton species cannot be so helpful in toxic species' taxonomy, as the "morphospecies" vary and different criteria besides the morphological alone must be used (Taylor, 1991; Cembella, 2003). Therefore, new identification developments (such as genetic and molecular tools, electronic microscope, flow cytometer, etc.) have reached the stage where they are routinely used in research and some monitoring programs over the last few decades (Anderson et al., 2005). These developments can help better identify the potential producers of biotoxins in Lebanese waters to upgrade their monitoring in this highly populated area.
* *Undescribed species*: Filter-feeding shellfish do not need dense blooms of toxic algae to eventually accumulate amounts of toxin harmful to humans. Many of the most serious algal-related human health hazards are not necessarily associated with dense obvious blooms (Wu et al., 2015). Consequently, as the occurrence of high phycotoxins does not always coincide with a bloom of the potential producer (as stated in Section 4.3), there may be novel and yet undescribed species as alternative sources for the detected toxins and better identification tools are also needed.
* *Type of samples, raw or cooked*? Most official monitoring programs analyze raw shellfish tissue to determine the content of lipophilic and hydrophilic toxins. However, most shellfish are eaten cooked or steamed, so heat-treated tissue seems to be the most relevant form to analyze. Heat treatment can lead to a two-fold concentration of toxins due to water loss. These issues should be recognized in relation to future risk assessments to establish and control permissible toxin levels (Wu et al., 2015).
* *Regulatory limits and toxicity studies*: Although in our study, DA levels were below the regulatory limits and cyclic imine levels are not yet regulated, it may become crucial to set up regulatory limits for marine organisms' consumption. In fact, it has become a matter of concern to assess their risks on human health at short and long-terms, as their repetitive consumption might be dangerous for their consumers. Thus, a consensus is emerging that further studies should be conducted to enhance our understanding of the potential producers, their ecology and distribution, their eco-toxicological behavior as well as their toxicity in marine organisms and humans (i.e. gastrointestinal absorption, tissue disposition, and crossing of the blood–brain and placental barriers, etc.). Substantial progress has been obtained on the characterization of phytoplankton genus producing toxins, but the genes involved in their production, and the pathways leading to the biosynthesis of the various families of toxins still to be explored. Also, the ecological factors favoring HABs need to be better identified and more information is needed on the environmental distribution and risks of chronic exposure to these phycotoxins (Picot et al., 2011; Molgó et al., 2017; Farabegoli et al., 2018).
* *Regular survey*: A regular monitoring program is necessary to start building a reliable, accurate estimates of bloom toxicity and to study their potential impacts on marine species as well as on human health.

CRediT authorship contribution statement

Abed El Rahman Hassoun: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Visualization; Writing - original draft & editing.

Ivana Ujević: Methodology; Data curation; Validation; review & editing.

Céline mahfouz: Writing-review & editing.

Milad Fakhri: Funding acquisition; Resources.

Romana Roje-Busatto: Methodology; Writing - original draft; Writing - review & editing.

Sharif Jemaa: Sampling, Editing.

Nikša Nazlić: Formal analysis.

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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Appendix A. Supplementary data

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