

EMERGING MARINE TOXINS WITH INCREASING CONCERN IN EUROPE

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Abstract

Marine toxins are produced by some microalgae, accumulate in filter feeding bivalves and through the food chain find their way to humans. Monitoring programs are established to respond to current legislation and focus on regulated marine toxins. However, the ultimate objective is the safety of consumers and the appearance of so called “emerging toxins” have to be considered. These include the discovery of new toxins or new toxin analogues, as well as recent appearance of known toxins in areas where they had not been previously described etc. Aim of the study is recently published data on occurrence, chemical structure, poisoning potential and methods for determination of emerging toxins in European coastal waters to be reviewed. Additionally a comparison of the toxin-producing organisms with their availability in the Black Sea is made.

Key words: *marine toxins, emerging, consumers’ safety, European coastal waters*

1. INTRODUCTION

Marine biotoxins (phycotoxins, algal toxins) are produced by some toxic dinoflagellates and diatoms, accumulate in the filter feeding mollusks and find their way through the food chain to humans causing severe illness.

Marine toxins belong to a variety of chemical families with different mechanisms of action and structures that include relatively simple molecules such as aminoacid derivatives or complex polyketides. Recently, some of these toxins are being described by scientists and agencies with the ambiguous term “emerging toxins” for several reasons. These include the discovery of new toxins (e.g. azaspiracids and pinnatoxins) or new toxin analogues (e.g. acyl ester derivatives of gymnodimines), the recent appearance of known toxins in areas where they had not been previously described or the awareness that not regulated toxins require additional information on their toxicological potential, mechanism of action or importance in the ecosystems (e.g. spirolides). Monitoring programs focusing on marine toxins are established to respond to current legislation (Diogene et al., 2013)

According the European regulations presence and concentration of some of the phycotoxins are determined (Table 1) in order the safety of the consumer to be ensured.

Table 1. Summary of EU reference value, methods for determination and symptoms of poisoning for regulated marine toxins
(Commission Regulation, 2013; Commission Regulation, 2004; Hallengraeff, 2014)

Toxin group		Current EU limits in shellfish meat	Reference methods	Symptoms of poisoning
Hydrophilic toxins	Saxitoxin	800 µg PSP.kg ⁻¹ SM	HPLC – FI	Within 30 min: tingling sensation or numbness around lips, gradually spreading to face and neck; prickly sensation in fingertips and toes; headache, dizziness, nausea, vomiting, diarrhea
	Domoic acid (DA)	20 mg DA.kg ⁻¹ SM	HPLC – UV	After 3–5 hours: nausea, vomiting, diarrhea, abdominal cramps.
Lipophilic toxins	Okadaic acid (OA) Dinophysis toxins(DTXs) Pectenotoxins (PTX)	160 µg OA.kg ⁻¹ SM	LC MS/MS	After 30 min to a few hours (seldom more than 12 hours): diarrhea, nausea, vomiting, abdominal pain
	Yessotoxins (YTX)	3.75 mg YTX .kg ⁻¹ SM		Considered not to pose any health risk
	Azaspiracids (AZAs)	160 µg .kg ⁻¹ SM		Diarrhea

However, scientists and managers that have as an ultimate objective the safety of consumers have to take into considerations “emerging toxins” that could present acute or chronic effects (Diogene et al., 2013). There should be found different ways and mechanisms “emerging toxins” to be studied, since it is important to improve our understanding about them.

This paper aims to summarize and review recently published data on occurrence, chemical structure and poisoning potential of marine toxins, classified by their authors as emerging toxins. In order to evaluate the concern of the cited toxins for the Black Sea researches are filtered only for European waters. Additionally a comparison of the toxin-producing organisms with their availability in the Black Sea is made.

The Black Sea is a nearly enclosed basin connected to the Sea of Marmara and the Sea of Azov by the narrow Bosphorus and Kerch Straits, respectively (Stanev, 2015).

Analyzing the geographic origin of marine alien invasive species in the Black Sea, most of them (43%) are cosmopolite planktonic species, 12% have Atlantic-Mediterranean origin, 27% are North Atlantic species and 18% are Indo Pacific (Skolka et al., 2010). This various nature of the Black Sea species has to be taken in account when discussing the concern of emerging toxins for the Black Sea.

Among the 5000 species of extant marine phytoplankton, some 300 species can at times occur in such high numbers that they obviously discolor the surface of the sea. Among them approximately 80 species have the capacity to produce potent toxins (Hallegraeff, 2004).

In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. They are relatively stable and do not degrade or reduce significantly in amount when cooked. In addition, they do not alter the taste of the meat. Detection of contaminated seafood is not straight forward, and neither anglers nor consumers can usually determine whether seafood products are safe for consumption (Ferrante et al., 2013).

Poor chemical information exists on the toxin profile of the Black Sea organisms from the Bulgarian coast. Recent study showed that some toxins (PSP and ASP) have values below the reference limit. However, these results are not reliable because of the quality of the sample tests because farms provide samples for toxicity only once per year. Furthermore, this could lead to potential shellfish intoxications by ASP and PSP during the remaining period of the year. There is no data in the literature on analysis of lipophilic toxins from the Bulgarian coast including DSP toxins because of lack of experts, expertise and equipment (Kalinova et al., 2015; Peneva et al., 2011).

Due to their chemical structure, emerging toxins are classified as lipophilic substances. Traditionally, LC-MS/MS methods with acidic chromatographic conditions are used for their determination (Fux et al., 2007; Quilliam et al., 2001). Hereby various techniques for determination of emerging toxins are presented.

2. CHEMICAL STRUCTURE AND OCCURRENCE OF EMERGING TOXINS

The emerging group of marine biotoxins has been principally monitored in shellfish. Countries as France, Italy, Spain and Norway report mostly palytoxins (PITX, ostreocin-D, ovatoxin-A, homopalytoxin, bishomopalytoxin, neopalytoxin, deopalytoxin and 42-hydroxypalytoxin) and cyclic imines [spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs)]. Toxin levels of some cyclic imines vary from 2–585 ng/g (Amzil et al., 2007) to 11–7950 ng/g (Ciminiello et al., 2010) in shellfish from France and Italy, respectively, whereas reported values for Spanish mussels were in the range of 13–20 ng/g (González et al., 2006). Putative palytoxin was for the first time detected in Italian waters at 1,350 ng for plankton pellet and 1,950 ng for butanol extract. It was therefore suggested that this toxin was the causative agent responsible for the Genoa 2005 outbreak of respiratory illness in people exposed to marine aerosols. Therefore some of these toxins are not only a concern of consumers but also may be seen as a public health problem related to environment (Andjelkovic, 2012).

2.1. Azaspiracids (AZA)

Azaspiracids are a group of lipophilic polyether toxins (Figure 1) at first associated with the diarrhetic shellfish poisoning (DSP) syndrome, based upon provisional epidemiological and etiological characteristics.

Azaspiracid poisoning caused by AZA- contaminated seafood consumption is primarily manifested by diarrhea in humans. Furthermore AZA-2 has been related to arrhythmia appearance in rats, suggesting potential heart toxicity. Azaspiracids might have chronic cardiotoxicity related to hERG channel trafficking and they should not be overlooked when evaluating the threat to human health (Ferreiro et al., 2014). To protect human health, AZA-1, AZA-2 and AZA-3 content in seafood has been regulated by food safety authorities in many countries.

AZA-43 and AZA-2 first time cases respectively in Huelva coast (2009) and North-Central Adriatic (2012-2014) are described below.

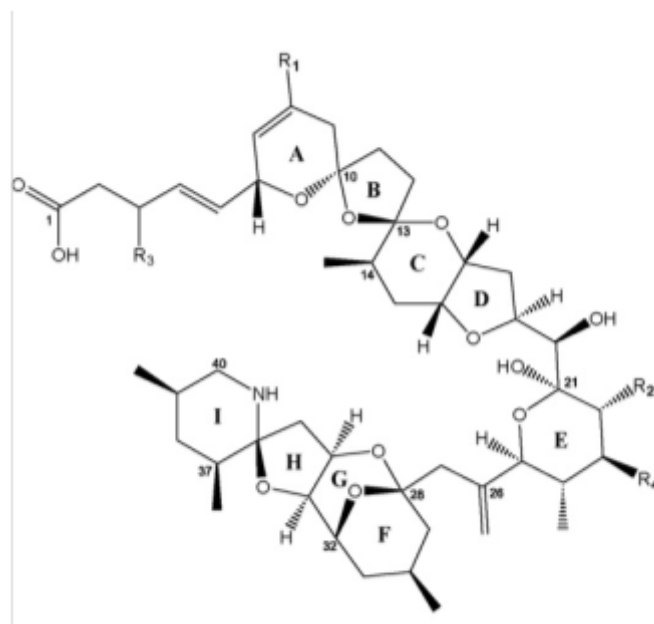


Figure 1. General structure of AZA. R1-R4 vary in -H, -CH₃, -OH in different combinations (Krock, 2009)

Bivalve and water samples were collected from different areas along the Huelva coast. LC–MS used is a liquid chromatograph coupled to triple quadrupole mass spectrometer and equipped with an atmospheric pressure electrospray ionization (ESI) interface. Separations of lipophilic toxins were performed by reverse-phase chromatography column with gradient elution using an alkaline (pH 11) mobile phase according to Gerssen et al. (2009). Detection was carried out in the negative selected reaction monitoring (SRM) mode for the determination of okadaic acid (OA), dinophysistoxins-(DTX) and yessotoxins (YTX) and in the positive mode for pectenotoxins (PTX), AZA, 13-desmethyln spirolide C (SPX-1) and gymnodimine A (GYM). That was case for AZA-2, -3, PTX-1 and DTX- 2, that were quantified using the reference solutions of AZA-1, PTX- 2 and OA. Toxicities were calculated for each toxin by multiplying the estimated toxin concentration by its corresponding toxicity equivalence factor (TEF) suggested by the European Food Safety Agency (EFSA, 2009). Retroactively, AZA-43 levels in a few selected mollusc samples were estimated via calibration with an AZA-1 standard and expressed as AZA-1 mass equivalents (Tillmann et al., 2017).

The authors pay attention on the consideration of AZA-43 and *Am. languida* as new target toxin and the need for species monitoring systems of the area which should include both analyzing the toxins in shellfish and the presence of the organisms in the water.

Nevertheless, it is well known that the occurrence and development of plankton, and especially of particular plankton species, can be sporadic, intermittent, and generally difficult to predict, especially in light of the continuously ongoing environmental and climate change, which may lead to unexpected interannual variations and exceptional bloom events.

2-year LC-MS/MS study (2012–2014) of the lipophilic toxins profile in mussels from the North-central Adriatic Sea in the context of the presence of toxic phytoplankton concentrations in seawater is made. Traces of Azaspiracid 2 (AZA-2) were observed often in mussels during the study period, confirming for the first time the presence of this biotoxin in Mediterranean seafood (Bacchiocchi et al., 2015).

2.2. Cyclic imines

Cyclic imines (CIs) constitute a quite recently discovered group of marine biotoxins that act on neural receptors and that bioaccumulate in edible tissues of filter-feeding organisms. CIs are grouped together due to the imino group functioning as their common pharmacore, responsible for acute neurotoxicity in mice. Cyclic Imines (CIs) include spirolides (SPXs) (Figure 2) and gymnodimines (GYMs) (Figure 3) produced by algal species from the genus *Alexandrium* and *Karenia*. *Vulcanodinium rugosum* is suggested as pinnatoxin (Figure 4) producing dinoflagellate (Rhodes et al., 2011, Nezan et al., 2011).

Cyclic imines have not been linked yet to human poisoning and are not regulated in Europe, although the European Food Safety Authority (EFSA) requires more data to perform conclusive risk assessment for consumers. Pinnatoxins are chemically stable compounds (Rundberget et al., 2011) and can persist in the water column accumulating in shellfish even when the causative species are absent. A recent study by Hess et al. (2013) has suggested that these toxins may be a significant threat to shellfish consumers.

Emerging CIs are found on Irish coast (2015) and in a multiple European research.

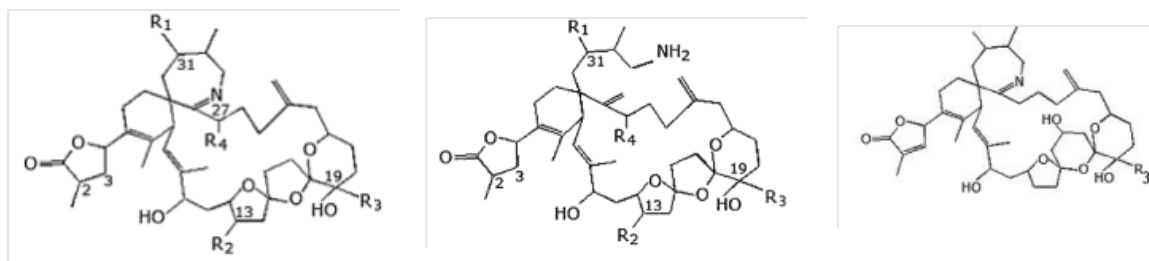


Figure 2. Chemical structures of spirolides (SPXs) (Otero et al., 2010; EFSA, 2010). R1-R4 vary in – H and –CH₃ in different combinations

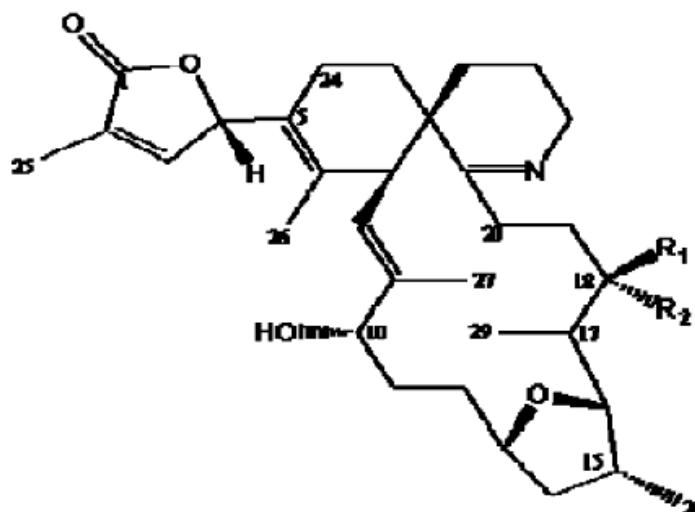


Figure 3. Chemical structure of gymnodimines (GYMs) (Cembella, 2008) R1 and R2 vary in –H and – OH in different combinations

Several commercial shellfish (96 samples) from eight different countries (Italy, Portugal, Slovenia, Spain, Ireland, Norway, Netherlands and Denmark) were analysed for CIs. CIs concentrations in all the samples were analysed on a LC-3200QTRAP and LC-HRMS QExactive mass spectrometer.

In summary, two CIs, pinnatoxin G (PnTX-G) and 13-desmethylspirolide C (SPX-1) were found at low concentrations (0.1 to 12 $\mu\text{g/kg}$ PnTX-G and 26 to 66 $\mu\text{g/kg}$ SPX-1), detected in 9.4% and 4.2% of samples, respectively, at concentrations higher than the LOQ, and in 7.3% and 30% of the samples at concentrations lower than the LOQ (25 $\mu\text{g/kg}$ for SPX-1 and 3 $\mu\text{g/kg}$ for PnTX-G), respectively (Rambla-Alegre et al., 2017).

The first reported incidence of the cyclic imine pinnatoxin G in Irish waters is in 2015. LC-MS analysis of samples obtained using both active and passive sampling methods was carried out on LTQ-Orbitrap Discovery Fourier transform mass spectrometer equipped with heated electrospray ionization (HESI) source.

Vigilance in monitoring coastal waters and shellfish species is paramount, to ensure that newly emerging toxic algal species with serious human health implications are detected prior to any negative impacts on shellfish consumers (McCarthy et al., 2015).

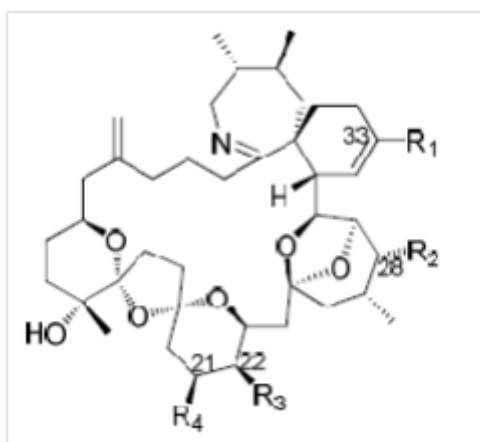


Figure 4. Chemical structure of pinnatoxins (PnTXs) (Miles et al., 2010; Selwood et al., 2010) R1- R4 vary in different combinations

2.3. Palytoxin

Palytoxin (Palytx) is a large, very complex molecule with a long polyhydroxylated and partially unsaturated aliphatic backbone, containing 64 chiral centers (Figure 5) (Kan et al., 2001).

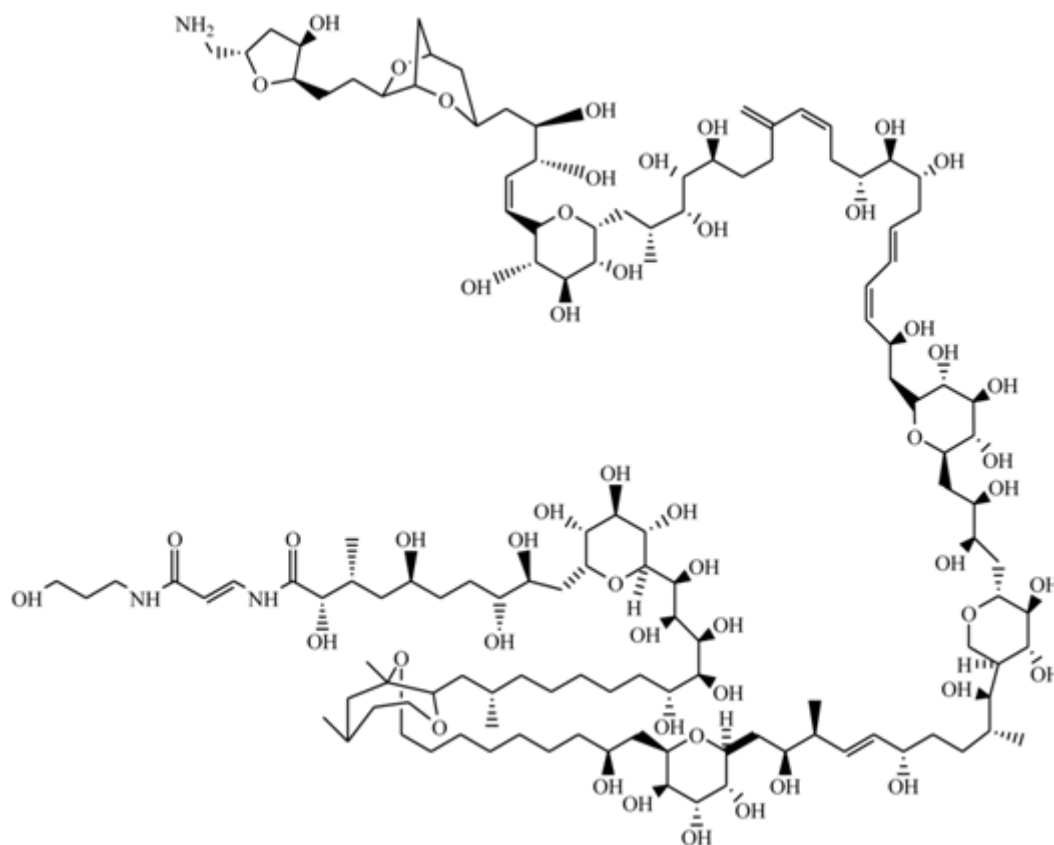


Figure 5. Structure of palytoxin (Ramos et al., 2010)

Ostreopsis spp. present some concern to human health due to their ability to produce congeners of palytoxin, one of the most potent marine toxins so far known (Ciminiello, 2008).

In the temperate region of the Mediterranean Sea, *Ostreopsis* spp. caused relevant negative impacts on human health through skin contact (Tichadou et al., 2010), and contaminated seafood (Amzil et al., 2012). Symptoms associated with these various poisoning events included a bitter/metallic taste, vomiting, diarrhea, muscle cramps, abdominal pain, numbness of the extremities, bradycardia, difficulty breathing, and renal failure (Alcala et al., 1988; Gonzales et al., 1977).

Recent incidences of Palytx are on Italian coast and French Mediterranean coast (2009) registered.

Mediterranean *Ostreopsis* spp. isolates were collected from seawater samples at Taormina, Italy where *Ostreopsis* spp. toxic blooms recur every summer. This site is a semiclosed, shallow bay carpeted by seaweeds. *Ostreopsis* assemblages can be found both in water column and macroalgae, as planktonic and epiphytic microflora.

LC–HRMS experiments were carried out on a LC binary system coupled to a hybrid linear ion trap LTQ Orbitrap XLTM Fourier Transform MS (FTMS) equipped with an ESI ION MAXTM source. Sub-fg levels of palytoxin on a per cell basis were detected (Ciminiello et al., 2013).

Four sites located in Nice and Villefranche-sur-Mer, on the French Mediterranean coast, were monitored during the summer of 2009 for the presence of epiphytic and planktonic *Ostreopsis* cf. *ovata*, and that of palytoxin (Palytx) and 2 of its analogues (ovatoxin-a (OVTX-a) and ostreocin-D (OST-D)) in different marine organisms - fish, echinoderms, gastropods, crustaceans and cephalopods. The contamination levels varied geographically and between species, with the herbivorous species generally having higher toxin levels than carnivorous ones. The highest toxin level ever recorded over the course of the study was of 392.2 µg for the sum of OVTX-a and PITX per kg of digestive tube of the flathead mullet *Mugil cephalus* (Bire et al., 2013).

2.4. Tetrodotoxin

Tetrodotoxin (TTX) (Figure 6) is the causative agent responsible for pufferfish poisoning, found mostly in the organs of fish from the Tetradonotidae family. Clinical effects are similar to those of Paralytic Shellfish Poisoning, with symptoms including nausea, diarrhea and vomiting, cardiac arrhythmias and at higher levels respiratory failure, leading to death.

Although pufferfish are thought to use TTX as a defense against predators (Itoi et al., 2014; Williams, 2010) and as pheromone (Williams 2010; Matsumura, 1995), there is little supporting evidence. Larvae of *Takifugu* pufferfish were protected by maternal TTX which had accumulated in the eggs during their development in the ovary. Female and male *Takifugu niphobles* deposited TTX differently during the spawning period (Itoi, 2012). Seasonal changes in the whole body content and tissue distribution of TTX in the pufferfish *T. niphobles*, and intrageneric differences in the sexual maturation of various *Takifugu* species are discussed, the amount of the toxin TTX in the whole body of the pufferfish *T. niphobles* was significantly higher in the maturation/spawning period than in the ordinary period in both sexes. This seasonal increase was achieved by proportionally greater deposition of the toxin in the ovaries and liver in females, and in the skin and liver in males. *T. niphobles* might use the localization of TTX in the skin not only as a biological defense but also, to a lesser degree, as aggregation pheromone (Itoi et al, 2016).

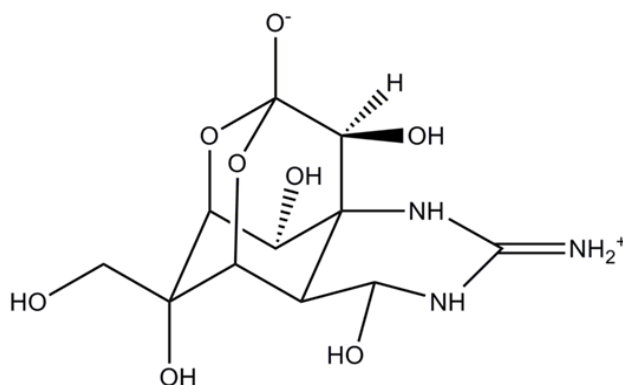


Figure 6. Chemical structure of tetrodotoxin (TTX) (Lago et al., 2015)

Although TTX-bearers are typical of warm waters and it was regarded as a problem confined to Asian countries, recent studies report the possible migration of these toxic species from the Red Sea to the Mediterranean Sea through the Suez Canal (Katikou et al., 2009; Bentur et al., 2008; Zaki et al., 2005).

Hereby cases of tetrodotoxin in 2013-2016 on English, in 2012 on Greek and in 2012-2013 on Mediterranean Turkish coast are cited.

In 2013, however, the first findings of tetrodotoxins in bivalve molluscs anywhere in Europe, with evidence for TTX in mussels and oysters from the South of England was reported. These appeared to be associated with the presence of *Vibrio* spp., in turn linked in the literature to direct production of TTX. Shellfish samples were with hydrophilic interaction liquid chromatography with tandem mass spectrometry was used for detection of TTX and six related TTX congeners. The results from this initial study, together with follow up investigations to assess the prevalence of TTXs in bivalve molluscs from around the inshore waters of the UK confirmed the continued presence of TTX in bivalves showing localised concentrations of the toxins during the warmer months in geographically specific areas of the country (Turner, 2017).

Still here is only limited TTX data for pufferfish in the Mediterranean Sea (Katikou et al., 2009; Rodríguez et al., 2012). Thus, determination of TTX levels in pufferfish is necessary to determine if there is a human health threat. There might also be an economic benefit if the pufferfish could be processed and sold for human consumption, when the TTX level is less than the regulatory limit.

There have been a few reports suggesting that TTX may have pharmacological effects which may provide an alternative use for these fish (Yu, 2008).

A triple quadrupole mass spectrometer coupled to a HPLC system operating with the ESI (electrospray ionization) interface in positive ion mode was used for determination of changes in the levels of TTX in the gonads, livers, intestines, skins and muscles as a function of season and sex. The authors conclude it can be dangerous to consume pufferfish, including the edible muscle, from the Eastern Mediterranean Sea (Kosker et al., 2016).

During official shellfish control for the presence of marine biotoxins in Greece in year 2012, a series of unexplained positive mouse bioassays (MBA) for lipophilic toxins with nervous symptomatology prior to mice death was observed in mussels from Vistonikos Bay–Lagos, Rodopi. This atypical toxicity coincided with the absence or presence in trace levels of all known lipophilic, PSP and ASP group toxins in mussels, as well as with the presence of *P. Minimum* (syn. = *P. cordatum*, as the currently taxonomically accepted name) at levels up to 1.89×10^3 cells/L in the area's seawater.

LC-MS methods in compliance to the requirements of EU Decision 2002/657/EEC were applied. An acquity UPLC system coupled to a TQD mass spectrometer equipped with a Z-Spray ESI source was used for sample analysis. Results showed the presence of TTXs. The sum of TTXs concentrations (TTX, 4-epiTTX and 4,9-anhydroTTX) exceeded the level of 200 µg/kg in all four consecutive samples.

The TTXs levels were substantially higher than those recently reported by Turner *et al.* (2015) in bivalve mollusks (blue mussels and pacific oysters) harvested in 2013–2014 from the south coast of England, along the Channel. This is expectable, taking into account that TTX is traditionally occurring in warmer tropical or subtropical waters and that Greece is located more southern compared to England, with a substantially warmer climate (Vlamiš et al., 2015).

2.5. Goniodomin A (GDA)

Goniodomin A (GDA) a highly toxic macrolide polyether (Figure 7) is produced by two other dinoflagellate species *Alexandrium monilatum* (Hsia et al., 2006) and *Alexandrium hiranoi* (erroneously identified as *A. pseudogonyaulax* in Murakami et al., 1988) in American and Japanese waters, respectively. This biologically active toxin has been associated over decades with fish mortality.

Toxicity of *A. pseudogonyaulax* strain isolated from Bizerte Lagoon (Mediterranean waters, Tunisia) was investigated. Cellular toxin production was determined using high pressure liquid chromatography coupled to mass spectrometry (HPLC/MS). Results showed for the first time that *A. pseudogonyaulax* contains goniodomin A (Zmerli Triki et al., 2016).

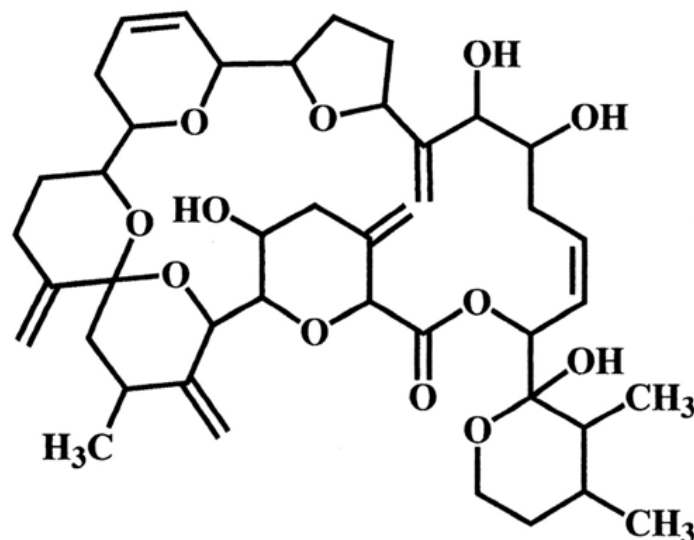


Figure 7. Chemical structure of goniodomin A (GDA) (Matsunaga et al., 1999)

The distribution of the recently described (2009-2016) emerging toxins is shown on figure 8. Obvious is the increasing concern and the appearance in the Black Sea might be expected.

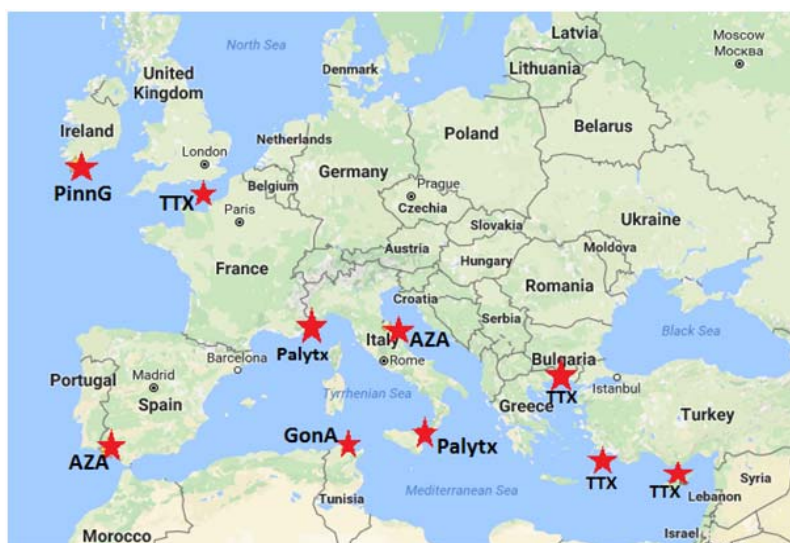


Figure 8. Map of emerging toxins in European coastal waters

On the Bulgarian Black Sea coast are detected various harmful algal species (Table 2). Some of them, e.g. *Alexandrium* spp. produce the regulated PSP, but also as seen above the emerging GDA, SPXs, GYM's etc. As well as *Prorocentrum minimum*, which is known to produce venerupin, hereby its TTXs release is described. To our knowledge there are no studies on the toxicity of these species on the Bulgarian coast.

Table 2. Harmful species detected in the Black Sea (Dzembekova et al., 2014)

Harmful algal species	Known toxin produced
<i>Pseudo-nitzschia</i> sp.	DA
<i>Alexandrium</i> sp.	STXs, GTXs
<i>Dynophysis</i> sp.	OA, DTXs
<i>Gonyaulax spinifera</i>	YTX

The Bosphorus is a typical example of an exchange flow through which the exchange occurs between the denser Sea of Marmara (38 psu) and the Black Sea (18 psu) (Öztürk et al., 2013; Öztürk et al., 2012) and invasive species might be expected on the Bulgarian coast. It is important to consider the combined influence of environmental parameters (e.g. salinity and temperature) on HAB species toxicity as responses may differ when microalgae are faced with changes in only one abiotic factor (Laabir et al., 2013).

3. CONCLUSION

Marine toxins, including emerging toxins, constitute, at present, the most important challenge for shellfish harvesting and marketing. Global production of mussels has serious economic impact. During 2010 it was 1 901 313 tonnes, of which 476 656 tonnes was from European aquaculture (FAO, 2014). However, substantial amounts of contaminated shellfish are consumed by humans, and this may cause severe intoxication. Approximately 60 000 human intoxications yearly with overall mortality of approximately 1.5% are related to toxins produced by algae (including freshwater cyanotoxins) (Kantiani et al., 2010). In case toxic products make it to market consumers need to be recouped if fatalities or illness occur.

The collection of data about emerging toxins is of great importance to refine and complete the risk assessment of these newly appeared toxins and has to be encouraged in order to provide reliable information for setting up a regulatory level that would protect the consumers of edible marine organisms.

3.1. Prospective work

Our ambitious goal is within the project: Determination of marine toxins in Black Sea shells and plankton, first data on emerging toxins and their distribution on Bulgarian coast to be published.

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