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EU REGULATED MARINE BIOTOXINS IN SEA FOOD: ORIGIN, CLASSIFICATION, CHEMICAL STRUCTURE AND INTOXICATION EFFECTS

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Abstract

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Sea food, especially marine shells (mussles, rapana, scallops) are considered as healthy food due to their high value of unsaturated fatty acids and high protein intake. Global production of mussels has serious economic impact. In last years 25% was from European aquaculture. Despite the high nutritional value of shells, they have bad reputation due to the possible toxins which could be present in their meat. Marine biotoxins can be found in all trophic levels of marine food web but are produced by certain phytoplankton species. Classification of current EU regulated marine biotoxins includes two main groups – hydrophilic (PSP and ASP) and lipophilic toxins (DSP and AZP). This review presents their structure, classification and symptoms of intoxication. In addition, EU regulatory limits for marine toxins are discussed.

Key words: marine biotoxins, sea food, harmful marine microalgae, symptoms of intoxication
Abbreviations: HABs – harmful algal blooms; EU – European Union; PSP – paralytic shellfish poisoning; ASP – amnesic shellfish poisoning; DSP – diarrheic shellfish poisoning; AZP – azaspiracid shellfish poisoning; CFP – ciguatera fish poisoning; NSP – neurologic shellfish poisoning; SSP – spiroimine shellfish poisoning; STXs – saxitoxins; DA – domoic acid; OA – okadaic acid; DTXs – dinophysistoxins; YTXs – yessotoxins; PTXs – pectenotoxins; AZAs – azaspiracid group toxin; SM- shellfish meat; neoSTX (NEO) – Neosaxitoxins; GNTX (GTX) – Gonyautoxin; dc – decarbamoyl; do – deoxydecarbamoyl; FAO – Food and Agriculture Organization of the United Nations; IOC – International Olympic Committee; WHO – World Health Organization; EFSA – European Food Safety Authority; i.p. – Intraperitoneal; LD – lethal dose; LTs – lipophilic toxins; KT3 – Killary Toxin-3

Introduction

Marine shells as mussles, rapana, scallops are considered as healthy food due to their nutritional characteristics, namely the high value of unsaturated fatty acids and high protein content (Dobreva et al., 2015). Nevetheless, marine shells could cause sickness explained by the presence of toxins in their meat.

Marine biotoxins (also called phycotoxins or algal toxins) are an acute world wide sea food safety concern. They are produced by certain phytoplankton species (diatoms and dinoflagellates) and accumulate in various marine species such as fish, crabs or filter feeding bivalves (shellfish) such as mussels, oysters, scallops and clams. Marine toxins find their way to humans through the food chain (Figure 1).

The microscopic planktonic algae of the world's oceans are the most important food source for filter feeding bivalve shellfish as well as larvae of commercially important crustaceans and finfish. Therefore, the proliferation of plank-

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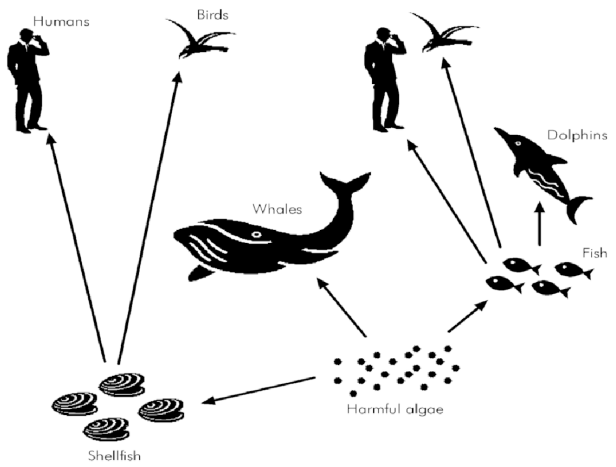


Fig. 1. Harmful algae blooms in the food chain and their routes of exposure (Gerssen et al., 2010)

tonic algae (so-called 'algal blooms', up to millions of cells per liter) in most cases is beneficial for shellfish growth in aquaculture and wild stocks. However, in some cases algal blooms can have negative effects (harmful algal blooms – HABs), causing severe economic losses in aquaculture, fisheries and tourism and can have major environmental and human health impacts. 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 fishermen nor consumers can usually determine whether seafood products are safe for consumption (Ferrante et al., 2013).

Phycotoxins 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.

Recent study showed that some toxins (PSP and ASP) had values below the limit of quantification in the Black sea coast of Bulgaria. However, these results are not reliable 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 including DSP toxins due to the lack of experts, expertise and equipment (Kalinova, 2015).

Thereafter, this review aims to present the types, structure and poisoning symptoms of regulated by EU marine toxins. Moreover, based on this review our purpose is to initiate a study for the implementation of EU legislative requirements regarding marine biotoxins (initially EU Directive 91/492/EEC and currently EU Regulations 853/2004 and 2074/2005) in Bulgaria.

Materials and Methods

Classification of phycotoxins

Phycotoxins can be divided in two groups hydrophilic and lipophilic. Hydrophilic marine toxins include paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP). Lipophilic toxins include diarrheic shellfish poisoning (DSP), azaspiracid shellfish poisoning (AZP), ciguatera fish poisoning (CFP), neurologic shellfish poisoning (NSP) and spiroimine shellfish poisoning (SSP).

The current EU legislation on marine toxins include PSP toxins (saxitoxin-group (STXs)), ASP toxins (domoic acid (DA), DSP toxins (okadaic acid (OA) and dinophysistoxins DTXs), yessotoxins (YTXs), pectenotoxins (PTXs) and azaspiracid-group toxins (AZAs)). Table 1 presents the current EU limit values for regulated marine toxins.

Table 1

Current EU limits for regulated marine toxins (European Regulation, 2004; Commission Regulation, 2013)

Toxin group	Current EU limits in shellfish meat (A)
STX	800 μg PSP.kg^{-1} SM
DA	20 mg DA.kg^{-1} SM
OA , DTXs and PTX	160 μg OA.kg^{-1} SM
YTX	3.75 μg YTX .kg^{-1} SM
AZAs	160 μg .kg^{-1} SM

Paralytic shellfish poisoning (PSP)

Paralytic shellfish poison (PSP) is produced by numerous microalgae species, mainly toxic marine dinoflagellates species of the genera *Alexandrium*, *Gymnodinium,* and *Pyro-*

dinium and by certain freshwater cyanobacteria such as *Anabaena circinarlis* and *Aphanizomenon flosaquae* (Asakawa et al., 2015)

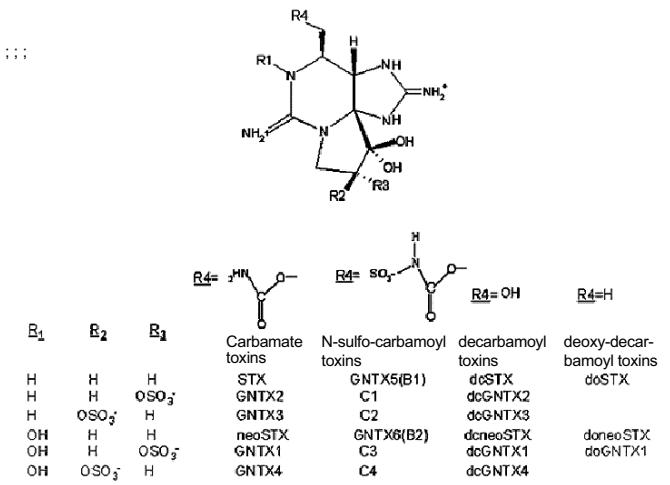
The PSP toxins form a group of closely related tetrahydropurine compounds that make up four subgroups (Figure 2):

carbamate (saxitoxin – STX, neoSTX and gonyautoxins (GNTX1-4);

N-sulfo-carbamoyl (GNTX5-6, C1-4);

decarbamoyl (dc-) (dcSTX, dcneoSTX, dcGNTX1-4);

deoxydecarbamoyl (do-) (doSTX, doneoSTX and doGNTX1) components.



R1	R2	R3	Carbamate toxins	N-sulfo-carbamoyl toxins	decarbamoyl toxins	deoxy-decarbamoyl toxins
H	H	H	STX	GNTX5(B1)	dcSTX	doSTX
H	H	OSO_3^-	GNTX2	C1	dcGNTX2	
H	OSO_3^-	H	GNTX3	C2	dcGNTX3	
OH	H	H	neoSTX	GNTX6(B2)	dcneoSTX	doneoSTX
OH	H	OSO_3^-	GNTX1	C3	dcGNTX1	doGNTX1
OH	OSO_3^-	H	GNTX4	C4	dcGNTX4	

Fig. 2. Chemical structure of PSP toxins (Mons et al., 1998; Quilliam, 2001)

At least 21 PSP toxins, mainly from marine dinoflagellates and shellfish that feed on toxic algae, have been identified (FAO). These groups present different toxicities, with the carbamoyl analogues being the most toxic, followed by decarbamoyl analogues with intermediate toxicity, and *N*-sulfocarbamoyl analogues the least toxic (Vale et al., 2008a; Vale, 2008b).

PSP toxins specifically block the excitation current in-nerve and muscle cells by means of site one of the sodium-channel (Messner et al., 1986). Adverse effects of intoxication with saxitoxins start with tingling or numbness around the lips. These effects spread to the neck and face. In a progressed state, prickly sensation of fingertips, headache, dizziness,nausea, vomiting and diarrhea can occur. Even temporary blindness has been reported (IOC/FAO/WHO, 2004; Alexander et al., 2009a;) When high levels of saxitoxins are consumed the motor nerves are affected, resulting in respiratory difficulties and other muscular paralytic effects (de Carvalho et al., 1998). Eventually, this may lead to death (Azanza, 2006).

In 2005, the Lawrence method was adopted as the official method to detect PSP toxins and then approved by the EU for monitoring these toxins (AOAC, 2005; EC, 2006). It is based on the pre-column oxidation of PSP toxins with hydrogen peroxide and sodium periodate followed by fluorimetric detection. It was validated for the determination of STX, neoSTX, GTX2,3, GTX1,4, dcSTX, GNTX5(B1), C1,2 and C3,4 in molluscs (mussels, clams, oysters and scallops).

According Regulation (EC) No 853/2004 (Commission Regulation, 2004) live bivalve molluscs placed on the market for human consumption must not contain paralytic shellfish poison (PSP) exceeding 800 micrograms per kilogram shellfish meat (Table 1).

***Amnesic shellfish poisoning* (*ASP*)**

Amnesic shellish poisoning has been linked to the diatom *Pseudo-nitzschia* spp. (Bates et al., 1989). Two *Nitzschia* spp. i.e., *Nitzschia navis-varingica* (in tropical to temperate water) (Kotaki et al., 2000) and *Nitzschia bizertensis* (in Tunisia) (Smida et al., 2014) have been recently reported to have ASP productivity comparable to that of toxic *Pseudo-nitzschia* species. In addition, some macroalgae produce ASP- *Chondria armata* (Takemotoand Diago, 1958*), Chondria baileyana* (Kotaki et al., 2000)*, Alsidium corallinum* (Impellizzeri et al., 1975).

Domoic acid (DA) is the main toxin found in a variety of shellfish species. Other minor analogues, about 10 isomers of DA (isodomoic acids A – H and DA 5′ diasteriomer) have been identified in marine samples (Wright et al., 1990; Zaman et al., 1997). The chemical group is called kainates. DA is a crystalline water-soluble acidic amino acid. The structure of DA is presented on Figure 3.

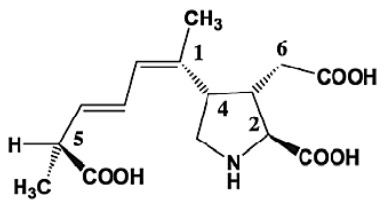


Fig. 3. Structure of domoic acid (Botana et al., 2013)

This neurotoxin acts by blocking some glutamate receptors in the central nervous system which results in depolarization of neurons (Berman and Murray, 1997; Hampson and Manalo, 1998). In humans, symptoms include nausea, gastroenteritis and vomiting, followed by neurological signs such as confusion, lethargy, disorientation, paresthesia, short-term memory loss and, in extreme cases, death (Pulido, 2008).

Since 1987 HPLC-UV is the regulatory method for determination of domoic acid in shellfish in laboratories (AOAC International, 2000). According Regulation (EC) No 853/2004 (Commission Regulation, 2004) live bivalve molluscs placed on the market for human consumption must not contain more than 20 milligrams of domoic acid per kilogram shellfish meat (Table 1).

Diarrhetic shellfish poisoning (DSP)

Phytoplankton responsible for DSP include *Prorocentrum lima*, and a range of *Dinophysis* species (Yasumoto et al., 1980; Morton et al., 2009; Reguera et al., 2014)

Three groups of polyether toxins – okadaic acid group toxins, yessotoxins (YTXs) and pectenotoxins (PTXs) – with different molecular structures were initially included in the Diarrhetic Shellfish Poisoning (DSP) toxin complex. They often co-occur in natural microplankton assemblages and in filter-feeding molluscan shellfish species exposed to them. It is now well established that the three groups of toxins have different biological effects and that only OA and its congeners are diarrhoegenic (Aune et al., 2002; Miles et al., 2004a; FAO/IOC/WHO, 2014)

Diarrhetic Shellfish Poisoning (DSP) is a human intoxication caused by the consumption of shellfish that contains okadaic acid (OA) and its analogues, the dinophysistoxins (DTX1, DTX2), their diol ester precursors (DTX4 and DTX5 groups), and their acyl derivatives (DTX3 group) (okadaates, OAs herein) (Yasumoto et al., 1985; Domínguez et al., 2010). Okadates are heat-stable polyether compounds and can be found in various species of shellfish, mainly bivalve molluscs. OA and DTX2 only differ in the position of one methyl group in the molecule. DTX1 has one additional methyl group. DTX3 (group) includes a wide range of derivatives of OA, DTX1, and DTX2, esterified with saturated and unsaturated fatty acids. They are products of metabolic transformations that occur in the shellfish (Figure 4) (Suzuki et al., 2001a; Suzuki et al., 2001b).

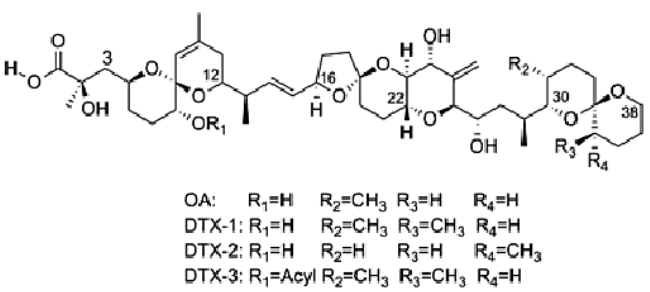


Fig. 4. Chemical structure of OA and regulated DTX

The acute effects of DSP-poisoning are less severe than the effects from other marine biotoxin poisoning syndromes such as paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP), with no known fatalities resulting from intoxication following ingestion of any of the regulated lipophilictoxins (Blanco, 2005). However, DSP poisoning can be widespread and highly unpleasant, with symptoms including severe abdominal pain, nausea, vomiting and diarrhoea (EFSA, 2008). Inhibition of serine/threonine phosphoprotein phosphatases is assumed to constitute the mode of action of okadaates (Cohen et al., 1990). These compounds are also involved in tumor promotion (Fujiki and Suganuma, 1999).

Pectenotoxins (PTXs) are produced by the same phytoplankton species as toxins of the OA group, the *Dinophysis* genus (Draisci et al., 1996).

Pectenotoxins (PTXs) are non-diarrhoegenic cyclic polyether lactones, which differ structurally from each other (Figure 5, 6, Table 2, 3, 4) mainly due to:

- the different degrees of oxidation at C43, which is attached to C18, from methyl to carboxylic acid;
- the arrangement or epimerisation of the spiroketal ring system in two of the rings;
- the opening of the large lactone ring in C1–C33 (Burgess and Shaw, 2001; Quilliam, 2003c)

Approximately 15 different PTXs have been described to date (Miles et al., 2004a; Miles et al., 2006). Pectenotoxin-2 (PTX2), pectenotoxin-2 seco acid (PTX2sa) and

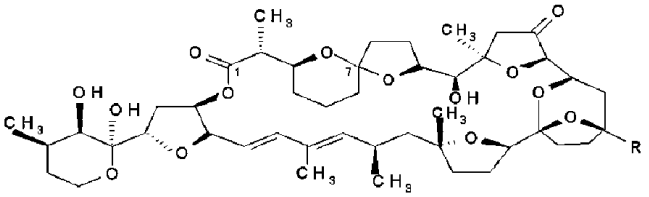


Fig. 5. Structure of pectenotoxins, PTX1- PTX7

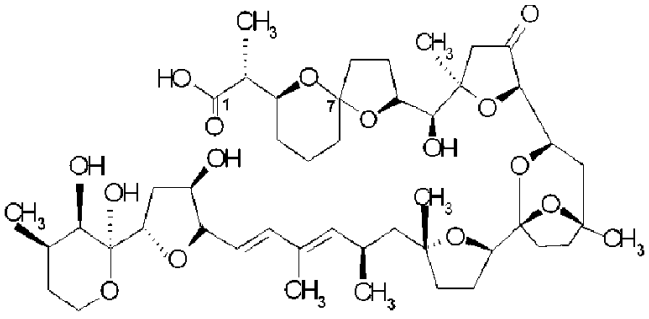


Fig. 6. Structure of pectenotoxins, PTX2SA and 7-epi-PTX2SA

Table 2

Structure of PTX1-7

Name	Abbreviation	R	C7
pectenotoxin-1	PTX1	CH_2OH	R
pectenotoxin-2	PTX2	CH_3	R
pectenotoxin-3	PTX3	CHO	R
pectenotoxin-4	PTX4	CH_2OH	S
pectenotoxin-5	unidentified		
pectenotoxin-6	PTX6	COOH	R
pectenotoxin-7	PTX7	COOH	S

Table 3

Structure of pectenotoxin-2 seco acid and 7-epi-PTX2SA (Yasumoto, et al., 2001)

pectenotoxin-2 seco acid	PTX2SA	CH_3	R
7-epi-PTX2SA	-	CH_3	S

7-epi pectenotoxin-2seco acid (7-epi PTX2sa) are the predominant analogues in European shellfish (Vale and Sampayo, 2002). The toxicity after i.p. or oral administration in mice of PTXs is considered to be comparable. After injection of PTX2, liver damage (generation of vacuoles and deformation of hepatocytes) has been observed (Espina and Rubiolo, 2008). Oral administration of PTX2 resulted in histopathological changes in the liver and stomach of mice but no diarrhea has been observed (Miles et al., 2004b). No human intoxications by PTXs have been reported yet. The European Food Safety Authority (EFSA) panel proposed a permitted level of 120 μg.kg^{-1} PTX2 equivalents (Alexander et al., 2009b).

Yessotoxins (YTXs) are produced by the dinoflagellates *Proceratium reticulatum* and *Lingulodinium polyedrum* (Bowden, 2006; Loader et al., 2007).

Yessotoxins are a group of lipophilic, sulfur bearing polyethertoxins (Figure 7, Table 4).

Until now up to 90 YTX analogues have been identified (Miles et al., 2005). Most abundant toxins found in shellfish

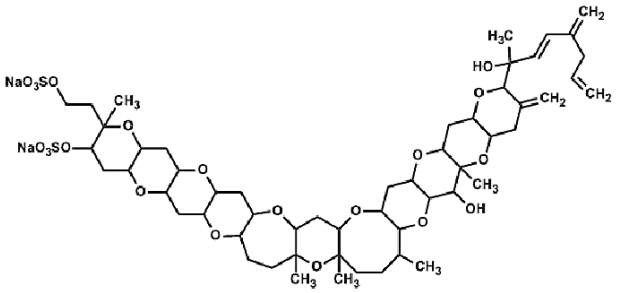


Fig. 7. Structure of yessotoxin (Botana et al., 2013)

are YTX and the metabolites 45-hydroxy-YTX, carboxy-YTXand their corresponding 1a-homologues (Aasen et al., 2005). Some analogues of YTX have only been found in certain regions such as adriatoxin in the Adriatic sea (Ciminiello et al., 1998).

When injected *i.p.* the toxicity of YTX is relatively high, with a LD 50 for mice of 750 μg.kg^{-1}. In contrast, oral administration of high levels of YTX (7.5 and 10 mg.kg^{-1}) did only result in some swelling of the heart muscle cells of mice (Aune et al., 2002). No human intoxications caused by consumption of YTX contaminated shellfish have been reported until now. YTXs levels exceeding the current EU regulatory level (1 mg.kg^{-1}) have occasionally been found in Italy, Norway and Portugal (Draisci et al., 1999; Aasen et al., 2005; Vale et al., 2008b). EFSA has suggested that a consumer is protected when shellfish do not exceed a concentration of 3.75 mg YTX-equivalents.kg^{-1} shellfish. EFSA identified YTX, 1a-homo-YTX, 45-hydroxy-YTX and 45-hydroxy-1a-homo-YTX as the most important YTXs present in shellfish.

In March 2002, the European Commission laid down the following rules:

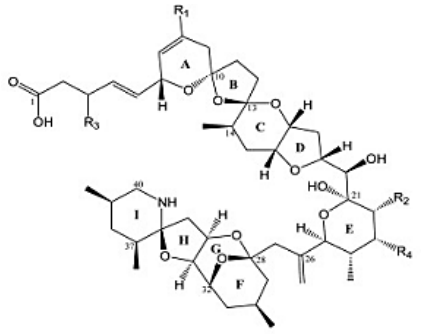
Maximum level of OA, DTXs and PTXs together, in edible tissues (whole body or any part edible separately) of molluscs, echinoderms, tunicates and marine gastropods shall be 160 mg OA equivalents.kg^{-1}.

Table 4

Chemical characteristics of lipophilic marine toxins (Botana et al., 2013)

Toxin groups	Chemical class	Main compounds (Mr)	Formula	Toxin analogues covered by the EU legislation
Okadaic acid (OA)	Polyether, spiro-keto assembly	OA (804)	$C_{44}H68O_{13}$	"OA, DTX-1, DTX-2, DTX-3
Petecnotoxin (PTX)	Polyeter, ester macrocycle	PTX-1 (874.5)	$C_{47}H_{70}O_{14}$	PTX-1, PTX-2
Yessotoxin (YTX)	Ladder-shaped polyether	YTX (1141)	$C_{55}H_{82}O_{21}S_2$	YTX, 45-OH-YTX, homo-YTX, 45-homo-YTX
Azaspiracid (AZA)	Polyether, second amine, 3-spiro ring	AZA-1 (841.5)	$C_{47}H_{71}NO_{12}$	AZA-1, AZA-2, AZA-3

*EU – European Union



Toxin	R_1	R_2	R_3	R_4
AZA-1	H	CH_3	H	H
AZA-2	CH_3	CH_3	H	H
AZA-3	H	H	H	H
AZA-4	H	H	OH	H
AZA-5	H	H	H	OH
AZA-6	CH_3	H	H	H
AZA-7	H	CH_3	OH	H
AZA-8	H	CH_3	H	OH
AZA-9	CH_3	H	OH	H
AZA-10	CH_3	H	H	OH
AZA-11	CH_3	CH_3	OH	H

Fig. 8. Structures of the fully structurally characterized azaspiracids AZA-1 to AZA-11. (Krock et al., 2012)

Maximum levels of YTXs in edible tissues (whole body or any part edible separately) of molluscs, echinoderms, tunicates and marine gastropods shall be 1 mg YTX equivalents.kg^{-1}.

According Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 (Commission Regulation, 2011) as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs the EU-RL LC-MS/MS method shall be the reference method for the detection of marine toxins. This method shall determine at least the following compounds:

okadaic acid group toxins: OA, DTX1, DTX2, DTX3 including their esters,

pectenotoxins group toxins: PTX1 and PTX2,

yessotoxins group toxins: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX;

Current EU limits for regulated DSP toxins are presented in Table 1.

Azaspiracid Shellfish Poisoning (AZP)

Azadinium spinosum (Tillmann et al., 2009), *Azadinium poporum* and *Amphidoma languida* (Krock et al., 2012) are recognized as AZA- producing organisms.

AZAs consist of a six-membered cyclic imine ring and like most polyketides – of a linear carbon chain, which is cyclized at several points in the molecule by ether bridges (Figure 8) (Satake et al., 1998b; Nicolaou et al., 2006).

The current list of AZA analogue (Figure 8) comprises naturally occurring structural variants of AZA-1 (Rehmann et al., 2008). Only AZA-1, AZA-2 and AZA-3 have been found in plankton samples (James et al., 2003a; James et al., 2003b), while all other variants were detected in shellfish and are regarded as shellfish metabolites.

The main symptoms of azaspiracid poisoning (AZP) in humans are nausea, vomiting, diarrhea and abdominal cramps that can persist for several days. In addition, a slowly progressing paralysis was observed in the mouse assay using the mussel extracts. These neurotoxic symptoms were quite different from typical DSP toxicity (Satake et al., 1998a). It was then that azaspiracid (formerly called Killary Toxin-3 or KT3) was identified and the new toxic syndrome was called azaspiracid poisoning (AZP).

Chronic exposure to the toxin in mice led to development of lung tumours (Ito and Satake, 2002). DSP has been shown to be a tumour promoter and AZA is a tumour initiator . Obviously AZA may present a greater risk during times when DSP toxins are co-occurring in shellfish (Ito, 2008) Studies have shown that AZA1 is cytotoxic to a range of cell types, and cytotoxic effects are both time and concentration dependent (Twiner et al., 2005). Other studies suggest that AZA4, unlike any of the other AZA analogues is a novel inhibitor of plasma membrane Ca2þ channels; it inhibits Ca2þ entry by stored operated channels in human T-lymphocytes (Alfonso et al., 2005).

Liquid chromatography hyphenated with tandem mass spectrometry (LC-MS/MS) is perhaps the most effective means of AZA determination in shellfish. AZA represents a group of structurally similar polyethers with different toxicologies and without an analytically discern able chromophore. AZA is present at trace amounts amidst the very complicated matrix of shellfish, often in conjunction with other shellfish toxins, including DSP and spirolides (James et al., 2004; Alvarez et al., 2010).

With this method detected should be at least AZA-1, AZA-2 and AZA-3 (Table 4). Maximum permitted level of regulated AZA analogues is 160 µg.kg^{-1} SM (Table 1).

Traces of Azaspiracid 2 (AZA-2) were observed often in mussels, confirming for the first time the presence of this biotoxin in Mediterranean sea food (Bacchiocchi et al., 2015).The AZA profile of mussels from the North-central Adriatic Sea showed a predominance of AZA-2.This report differs from that generally reported data for shellfish of the North Sea, but resembles with shellfish toxicity from the Atlantic coasts of Morocco and Portugal. This is perhaps due to the presence of AZA producers other than *Azadinium spi-*

nosum. The very low levels of AZAs detected in mussels do not represent a risk to public health, but suggest the need to pursue monitoring of these compounds and to identify their biogenic origin, as well as to consider environmental and climatic changes in progress and their influence on phytoplankton population composition.

Conclusions

Marine shells are extraordinary healthy food and should not be excluded from the menu. Hereof marine biotoxins, which are accumulated in shells, should be studied. Geographical area and the climate conditions have certain influence on the different toxin profiles and still new toxins are being added to the main groups.

Production of Black Sea mussels is increasing in Bulgaria. Still toxin profiling of Black Sea shells and microalgae from Bulgarian Black Sea coast is not sufficient. In addition specific conditions (salinity, temperature etc.) are present and different toxin may emerge. Consequently the toxin presence in Black sea mussels from Bulgarian coast should be studied and revealed.

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