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Review

Marine Toxins: Chemistry, Toxicity, Occurrence and Detection, with Special Reference to the Dutch Situation

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Abstract: Various species of algae can produce marine toxins under certain circumstances. These toxins can then accumulate in shellfish such as mussels, oysters and scallops. When these contaminated shellfish species are consumed severe intoxication can occur. The different types of syndromes that can occur after consumption of contaminated shellfish, the corresponding toxins and relevant legislation are discussed in this review. Amnesic Shellfish Poisoning (ASP), Paralytic Shellfish Poisoning (PSP), Diarrheic Shellfish Poisoning (DSP) and Azaspiracid Shellfish Poisoning (AZP) occur worldwide, Neurologic Shellfish Poisoning (NSP) is mainly limited to the USA and New Zealand while the toxins causing DSP and AZP occur most frequently in Europe. The latter two toxin groups are fat-soluble and can therefore also be classified as lipophilic marine toxins. A detailed overview of the official analytical methods used in the EU (mouse or rat bioassay) and the recently developed alternative methods for the lipophilic marine toxins is given. These alternative methods are based on functional assays, biochemical assays and chemical

methods. From the literature it is clear that chemical methods offer the best potential to replace the animal tests that are still legislated worldwide. Finally, an overview is given of the situation of marine toxins in The Netherlands. The rat bioassay has been used for monitoring DSP and AZP toxins in The Netherlands since the 1970s. Nowadays, a combination of a chemical method and the rat bioassay is often used. In The Netherlands toxic events are mainly caused by DSP toxins, which have been found in Dutch shellfish for the first time in 1961, and have reoccurred at irregular intervals and in varying concentrations. From this review it is clear that considerable effort is being undertaken by various research groups to phase out the animal tests that are still used for the official routine monitoring programs.

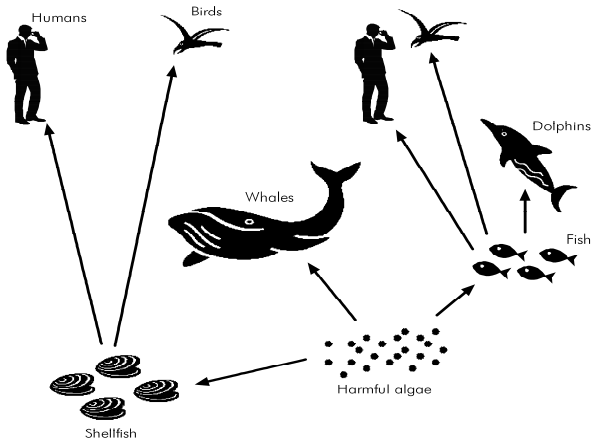
Keywords: lipophilic marine toxins; DSP toxins; alternative methods

1. Introduction

Of the 5,000 phytoplankton species known to date under specific circumstances about 300 of them have a high proliferation rate, resulting in high density algae clouds called blooms. The circumstances for bloom development are not fully understood yet, but specific climatic and hydrographic conditions seem to play a role in the formation of blooms [1–3]. Blooms are sometimes beneficial for aquaculture and marine biology [4]. However, of the 300 phytoplankton species mentioned above, more than 40 species belonging to the classes of dinoflagellates and diatoms are known to produce phycotoxins (marine toxins) [5]. The abundance of these toxic phytoplankton species can vary from thousand until a few million cells per liter. The high abundance blooms of these toxic phytoplankton species are named harmful algae blooms (HABs). It has been suggested that certain phytoplankton species produce toxins to compete for space with other phytoplankton species [6].

Phycotoxins can accumulate in various marine species such as fish, crabs or filter feeding bivalves (shellfish) such as mussels, oysters, scallops and clams. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when substantial amounts of contaminated shellfish are consumed by humans this may cause severe intoxication of the consumer (Figure 1). Throughout the world, toxins produced by algae (including freshwater cyano toxins) are held responsible for approximately 60,000 human intoxications yearly [7]. Shellfish toxins also cause damage to wildlife [8,9] and have a negative economic impact on recreation, tourism and shellfish industry. In Europe an estimated annual loss of 720 M€ for the recreation and tourism industry and 166 M€ for the shellfish industry is due to the occurrence of algae blooms [10,11]. In order to prevent intoxication of the consumer by shellfish toxins, legislation has been developed and monitoring programs have been established worldwide [12,13]. In this review an overview is given of the various types of poisoning syndromes, their corresponding algae and toxins. Furthermore, alternative methods are reviewed that have been developed to replace the animal bioassays that are currently used for the detection of lipophilic marine toxins.

Figure 1. Harmful algae blooms in the food chain and their routes of exposure.



2. Poisoning Syndromes and Corresponding Toxins

Based on their chemical properties marine shellfish toxins can be divided in two different classes: hydrophilic and lipophilic toxins. Toxins associated with the syndromes amnesic shellfish poisoning (ASP) and paralytic shellfish poisoning (PSP) are hydrophilic and have a molecular weight (MW) below 500 Da. Toxins responsible for neurologic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP), azaspiracid shellfish poisoning (AZP) and other toxins such as pectenotoxins, yessotoxins and cyclic imines all have as common denominator a MW above 600 Da (up to 2,000 Da). These toxins have strong lipophilic properties. Therefore, these toxins are generally called lipophilic marine toxins.

2.1. Hydrophilic toxins

2.1.1. Amnesic shellfish poisoning (ASP)

The diatom *Pseudo-nitzschia pungens* is one of the most important species of the more than 10 known producers of domoic acid (Figure 2), the toxin responsible for ASP (Table 1). In addition, a number of toxic DA isomers have been described in the literature [14]. The primary action of DA is on the hippocampus, which is involved in processing memory and visceral functions [15]. DA is a neurotoxin that binds with a high affinity to glutamate receptors. This binding leads to opening of the membrane channels (permeable to sodium). This, in turn, leads to an increased sodium influx and membrane depolarization. The adverse effects reported are gastrointestinal disorders, nausea, vomiting, abdominal cramps and diarrhea. Furthermore, also headache, dizziness and loss of the short-term memory can occur [16,17].

ASP intoxication in humans was first reported in 1987 on Prince Edward Island, Canada [18]. During this toxic episode three people died and more than 100 were admitted to the hospital after consuming blue mussels (*Mytilus edulis)* with high levels of DA [17]. DA occurrence in shellfish is a global issue. In recent years shellfish containing DA have been reported in the USA, Canada, France, United Kingdom (UK), Spain, Ireland and Portugal [18–23]. The European Union (EU) has established a permitted level of 20 mg DA/kg shellfish. In 2009, the European Food Safety Authority

(EFSA) published an opinion on DA [24]. In this opinion the panel recommended that it is safe to consume shellfish which contain less than 4.5 mg DA /kg shellfish in order to not exceed the acute reference dose (ARfD). DG SANCO (responsible for health and consumer protection in the EU) will discuss the EFSA opinion with the different EU member states and this may result in new legislation.

Figure 2. Chemical structure of domoic acid (DA).

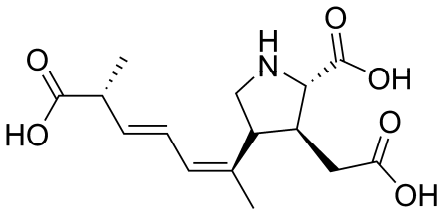


Table 1. Marine toxin groups and their responsible algae.

	Toxin group	Syndrome	Genus	Species	Reference
Hydrophilic toxins	Domoic acid	ASP	Pseudo-nitzschia	australis, calliantha, cuspidata, delicatissima, fraudulenta, galaxiae, multiseries, multistriata, pseudodelicatissima, pungens, seriata, turgidula	[25]
	Saxitoxins	PSP	Alexandrium	angustitabulatum, catenella, fundyense, lusitanicum, minutum, tamarense, tamiyavanichii	[26–28]
			Gymnodinium	catenatum	[26]
			Pyrodinium	bahamense	[26]
Lipophilic toxins	Brevetoxins	NSP	Karenia	brevis, brevisulcata, mikimotoi, selliformis, papilionacea	[8,29] [30]
			Chatonella	cf. verruculosa	[30]
	Okadaic acid and dinophysistoxins and pectenotoxins [1]	DSP	Phalacroma	rotundatum	[31]
			Prorocentrum	arenarium, belizeanum, concavem, lima	[32]
			Dinophysis	acuminata, acuta, arenarium, caudate, fortii, mitra, norvegica, ovum, rotundata, sacculus, tripos	[33–38]
	Yessotoxins		Protoceratium	reticulatum	[29,39]
			Lingulodinium	polyedrum	[29]
			Gonyaulax	polyhedra	[29]
	Azaspiracids	AZP	Azadinium	spinosum	[40]
	Spirolides	–	Alexandrium	ostenfeldii, peruvianum	[41,42]
	Gymnodimines	–	Karenia	selliforme	[43]
			Gymnodium	mikimotoi	[44]

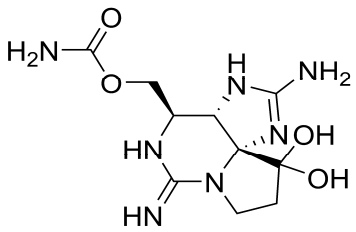
[1] Pectenotoxins do not induce diarrhea but are produced by the same algae as the DSP toxins okadaic acid and dinophysistoxins.

2.1.2. Paralytic shellfish poisoning (PSP)

Dinoflagellates of the *Alexandrium* genus are the producers of saxitoxins (Figure 3), the group of toxins responsible for PSP (Table 1). Within the saxitoxin group around 30 different analogues have been detected [45]. Not every analogue exhibits the same toxicity and nowadays for the most prominent analogues, toxic equivalent factors (TEF) have been established [46]. Saxitoxin causes inhibition of the voltage-gated sodium channel resulting in a reduced action potential [47]. 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 [46,48]. When high levels of saxitoxins are consumed also the motor nerves are affected, resulting in respiratory difficulties and other muscular paralytic effects [49]. Eventually, this may lead to death [50].

First reports of PSP intoxication date to 1920 in California, USA when at least six people died [51]. Until the 1970s PSP toxins were only detected in European, North American and Japanese waters. Nowadays, saxitoxins have been reported in Chile, South-Africa, Australia and other countries as well [52–54]. In most countries monitoring programs have been established to protect the consumer. The EU has established a permitted level of 800 µg saxitoxin 2-HCl equivalents/kg shellfish. Recently (2009) the EFSA published an opinion on the saxitoxin group [46]. In this opinion it is recommended a safe level is as low as 75 µg saxitoxin 2-HCl equivalents /kg in order to avoid exceeding the ARfD [46].

Figure 3. Chemical structure of saxitoxin (STX).



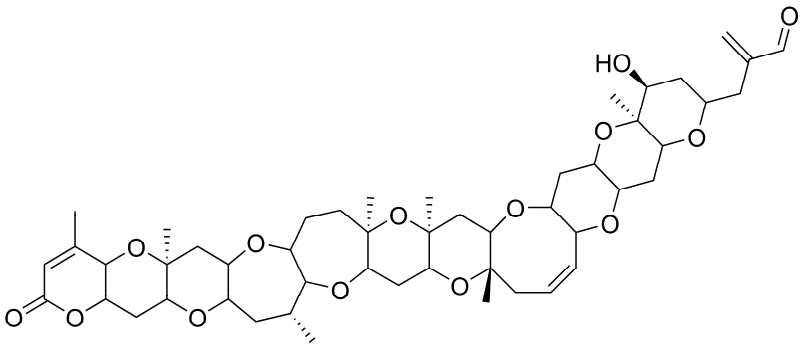
2.2. Lipophilic toxins

2.2.1. Neurologic shellfish poisoning (NSP)

NSP is caused by brevetoxins (Figure 4). These are produced by the algae species *Karenia ssp* (Table 1) [8,30]. Brevetoxins cause opening of the voltage-gated sodium channels, leading to an influx of sodium in the cells and to a complete blockade of the neuronal excitability [55]. Adverse effects observed are diarrhea, vomiting, cramps, rapid reduction of the respiratory rate and cardiac conduction disturbances which can lead to a coma and eventually to death [30]. In addition to consumption of brevetoxin-contaminated shellfish, intoxication can occur due to inhalation of aerosols produced by breaking waves at the shoreline [56,57]. Inhalation of brevetoxin aerosols may result in respiratory problems and eye and nasal membrane irritation. Until now NSP intoxications have been limited to the USA (Gulf of Mexico and Florida) and New Zealand [58,59]. As these toxins have not been found in Europe no legislation has been set for these toxins and no monitoring programs have been established. In the USA, legislation has been set by the Food and Drug Administration (FDA); the current

regulatory limit is 800 µg brevetoxin-2 (PbTx-2) equivalents/kg shellfish [60]. At the time of writing, the EFSA had not published a scientific opinion on NSP-type toxins.

Figure 4. Chemical structure of brevetoxin (PbTx-2).

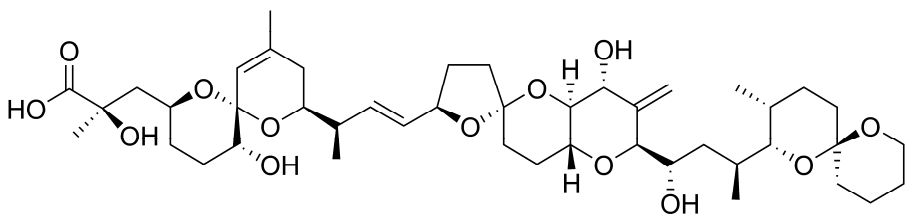


2.2.2. Diarrhetic shellfish poisoning (DSP)

Okadaic acid (Figure 5), dinophysistoxin-1 (DTX1) and -2 (DTX2) as well as the esterified forms of OA, DTX1 and DTX2 are produced by the *Dinophysis* genus (Table 1) [35]. Toxins of the OA group inhibit the serine and threonine phosphatases PP1 and PP2A [61]. This inhibition leads to hyperphosphorylation of proteins involved in the cytoskeletal junctions that regulate the permeability of the cell, resulting in a loss of cellular fluids [62]. Consumption of shellfish contaminated with high levels of OA-type toxins will result in adverse effects such as gastrointestinal disorder, diarrhea, abdominal cramps, nausea and vomiting [63]. Furthermore, OA and DTX1 have been shown to be tumor promoting substances in animal tests [64].

The first documented human intoxication caused by DSP toxins was in The Netherlands in 1961 [65]. Nowadays, high levels of OA group toxins are repeatedly reported in shellfish or algae along the coasts of Europe (UK, Ireland, Denmark, Sweden, Norway, France, Spain, Italy, Portugal, The Netherlands and Belgium), Canada, South America (Chile), Japan, Australia and Africa (Morocco) [63,66,67]. TEF values for OA, DTX1 and DTX2 have been established (Table 2) [68,69]. Within Europe the permitted level for the total amount of OA, DTXs and PTXs in shellfish has been set at 160 µg OA-equivalents /kg shellfish. In 2008, the EFSA panel concluded in their opinion on OA and analogues that OA and DTXs should not exceed 45 µg OA-equivalents/kg shellfish in order to not exceed the ARfD. For PTXs, a separate EFSA opinion has been prepared [70].

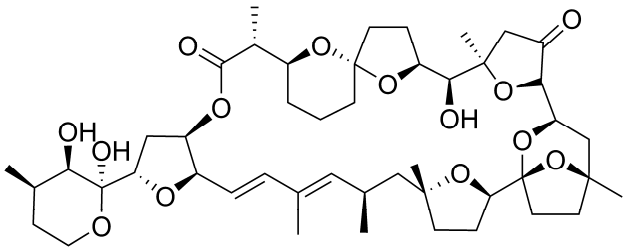
Figure 5. Chemical structure of okadaic acid (OA).



Pectenotoxins (PTXs) (Figure 6) are produced by the same phytoplankton species as toxins of the OA group, the *Dinophysis* genus [33]. Approximately 15 different PTXs have been described to

date [71,72]. Pectenotoxin-2 (PTX2), pectenotoxin-2 seco acid (PTX2sa) and 7-epi pectenotoxin-2 seco acid (7-epi PTX2sa) are the predominant analogues in European shellfish [73]. The toxicity after *i.p.* or oral administration in mice of PTXs is considered to be comparable. After *i.p.* injection of PTX2, liver damage such as the generation of vacuoles and deformation of hepatocytes has been observed [74]. Oral administration of PTX2 resulted in histopathological changes in the liver and stomach of mice but no diarrhea has been observed [75]. No human intoxications by PTXs have been reported yet. As discussed earlier, PTXs are currently included in the European legislation in the OA group but EFSA has recently suggested that the PTXs should be classified individually. The EFSA panel proposed a permitted level of 120 µg/kg PTX2 equivalents (Table 2) [70].

Figure 6. Chemical structure of pectenotoxin-2 (PTX2).



Yessotoxins (YTXs) (Figure 7) are produced by the dinoflagellates *Proceratium reticulatum* and *Lingulodinium polyedrum* [39,76]. Until now up to 90 YTX analogues have been identified [77]. The most abundant toxins found in shellfish are YTX and the metabolites 45-hydroxy-YTX, carboxy-YTX and their corresponding 1a-homologues [78]. Some analogues of YTX have only been found in certain regions such as adriatoxin in the Adriatic sea [79]. When injected *i.p.* the toxicity of YTX is relatively high, with a LD_{50} for mice of 750 µg/kg. In contrast, oral administration of high levels of YTX (7.5 and 10 mg/kg) did only result in some swelling of the heart muscle cells of mice [80]. Until now, no human intoxications caused by consumption of YTX contaminated shellfish have been reported. YTXs levels exceeding the current EU regulatory level (1 mg/kg) have occasionally been found in Italy, Norway and Portugal [78,81,82]. EFSA has suggested that a consumer is protected when shellfish do not exceed a concentration of 3.75 mg YTX-equivalents/kg shellfish [83]. EFSA identified YTX, 1a-homo-YTX, 45-hydroxy-YTX and 45-hydroxy-1a-homo-YTX as the most important YTXs present in shellfish. For these toxins TEFs have been established (Table 2) [83].

Diarrhetic shellfish poisoning is caused by OA and its DTX analogues. YTXs and PTXs are often included in the group of DSP toxins as they often co-occur with OA and DTX analogues although they do not cause diarrhea. Therefore, removal of these toxins from the DSP group should be considered. To our opinion lipophilic marine toxins is a better term to classify the toxins belonging to these groups.

Figure 7. Chemical structure of yessotoxin (YTX).

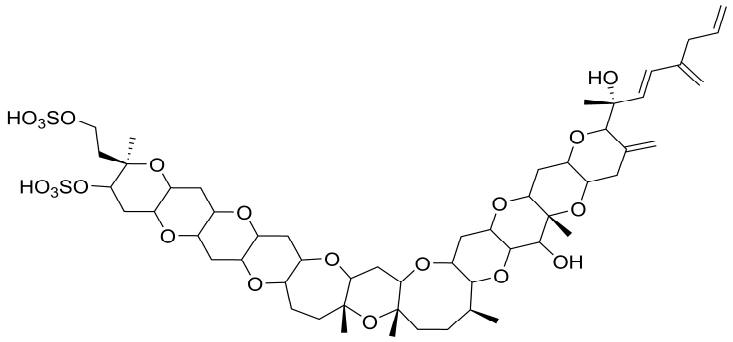


Table 2. Toxic equivalent factors of lipophilic marine toxins.

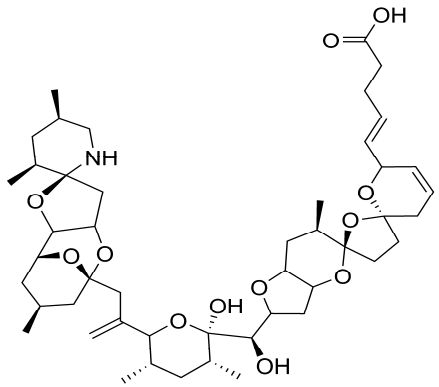
Toxin	TEF	Reference
Okadaic acid	1	[68]
Dinophysistoxin-1	1	
Dinophysistoxin-2	0.6	
Yessotoxin	1	[83]
1a-homo yessotoxin	1	
45-OH yessotoxin	1	
45-OH 1a-homo yessotoxin	0.5	
Azaspiracid-1	1	[84]
Azaspiracid-2	1.8	
Azaspiracid-3	1.4	

2.2.3. Azaspiracid shellfish poisoning (AZP)

For years azaspiracids (Figure 8) were thought to be produced by *Protoperidinium crassipes* [85], although a clear correlation between high algae counts and toxin levels was lacking [86]. Recently, it was discovered that the AZAs are actually produced by a minute dinoflagellate [40,86]. This dinoflagellate, *Azadinium spinosum* , is smaller (12–16 μm) than any of the other toxin-producing dinoflagellates known so far. Until now, 24 different AZAs have been described, with azaspiracid-1 (AZA1), -2 (AZA2), -3 (AZA3) as the predominant ones [87]. The mechanism of action is not yet fully understood, but *in-vitro* experiments in mammalian cell lines showed alterations in the cytoskeletal structure, and an effect on the E-cadherin system, which is responsible for the cell-cell interactions [88–90]. This could explain the toxic effects such as gastrointestinal disorder, diarrhea and abdominal cramps that are observed during AZP intoxication [85,91]. In 1995, the first intoxication due to AZP was reported when at least eight people got ill in The Netherlands after consumption of mussels imported from Ireland. The rat bioassay, normally applied to detect OA type toxins, revealed the presence of diarrhetic toxic activity, where the mouse bioassay lacked detection of these toxins. Since then several AZP outbreaks have occurred in Ireland and by now AZAs have been detected in Ireland, UK, Norway, France, Portugal, Northern Africa (Morocco), South America (Chile) and the USA [67,85,92–97]. According to current EU legislation the total amount of AZAs should not exceed

160 µg/kg AZA1-equivalents [98]. Recently, EFSA reviewed all available toxicity data and suggested that a safe level of AZA toxins in shellfish is below the ARfD of 30 µg AZA-1 equivalents /kg shellfish [84]. Furthermore, EFSA suggested TEFs for three most important AZAs (Table 2) [84].

Figure 8. Chemical structure of azaspiracid-1 (AZA1).

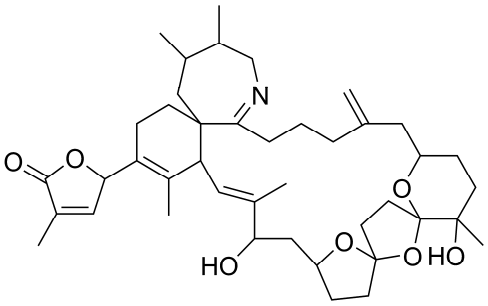


2.2.4. Spirolides and gymnodimines (cyclic imines)

Spirolides (SPXs) (Figure 9) and gymnodimines are toxins belonging to the cyclic imine group. SPXs are produced by *Alexandrium ostenfeldii* (Table 1) [41,99]. Approximately 10–15 different SPXs (including esters) have been found in either algae or shellfish [100–102].

The mechanism of action is not yet completely understood, but *i.p.* injection of shellfish extracts containing SPXs or gymnodimines is causing death of the test animal within minutes [103]. For this reason these toxins have been classified as fast-acting toxins. Intoxications of humans with cyclic imines have not been reported yet. SPXs have been found, however, in algae and shellfish from Norway, Canada, Denmark, Spain and Chile [95,100,104], while gymnodimines thus far has only been detected in algae and shellfish from New-Zealand [44]. Currently, there is no EU-legislation for the cyclic imines. This toxin group is currently being reviewed by the EFSA, who will publish an opinion in 2010.

Figure 9. Chemical structure of 13-desmethyl spirolide C (SPX1).

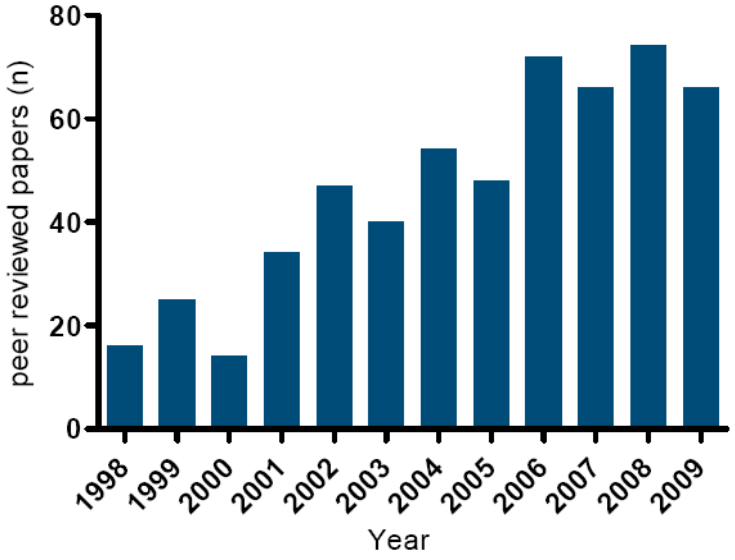


3. Methods of Analysis

For the determination of marine toxins various biological (*in-vivo* and *in-vitro*), biochemical and chemical methods have been described in the literature. However, for lipophilic marine toxins chemical methods for long were not available. In this paragraph, an outline will be given on the official methods stated in European legislation and alternative methods developed in recent years.

The last decade has seen a strong increase in peer-reviewed papers on lipophilic marine toxins (Figure 10). In general, method development and method validation for lipophilic marine toxins was hampered for many years, by the lack of (certified) standards and (certified) reference materials. As shown in Figures 4–9 the chemical structures of the toxins are complex and, consequently, it is too difficult and expensive to synthesize them [105]. Therefore, standards need to be isolated from either contaminated shellfish or algae [106,107]. In recent years considerable efforts have been made to expand the number of available toxins. In 2005, only small amounts of reliable reference standards were available for OA and PTX2. In 2007 YTX, AZA1 and SPX1 became available. Since then, of all important lipophilic marine toxin groups at least one certified standard is available (OA, PTX2, YTX, AZA1 and SPX1). It is expected that other important reference standards such as DTX1, DTX2, AZA2 and AZA3 will become available in the course of 2010.

Figure 10. Number of peer reviewed publications on lipophilic marine toxins in the last decade.



3.1. Current official methods described in legislation and their limitations

EU legislation prescribes a biological test for the determination of OA, DTXs, YTXs, PTXs and AZAs in shellfish. This biological test can be a mouse (MBA) or a rat bioassay (RBA). The MBA was developed in Japan and the RBA in The Netherlands in the 1970s [65,108]. Various laboratories have adjusted the MBA which has resulted in different protocols [109,110]. In Europe a detailed procedure has been described by the Community Reference Laboratory on marine toxins (CRL-MB, Vigo, Spain) in order to standardize the protocol for the MBA [111]. Shellfish extracts are prepared by acetone extraction followed by liquid-liquid partitioning with dichloromethane or diethylether. After evaporation the extract is reconstituted in 1% polysorbate 20 solution. These extracts are injected *i.p.* into three male mice with a body weight of 20 g. Preferably the hepatopancreas of the shellfish should be used, as most toxins tend to concentrate in that part, only about AZAs there can be a discussion if these toxins diffuse into the shellfish flesh [91,112]. If at least two out of the three mice die within 24 hours after injection, the sample is considered positive for lipophilic marine toxins [13]. Unfortunately, low levels of SPXs can also cause mouse death, even within minutes [103]. This

indicates that the MBA lacks specificity. A strong point of the assay is that it can signal the presence of possible new emerging marine toxins. The RBA, an official EU method that is only applied in The Netherlands, is based on consumption of shellfish (see also section "Occurrence of toxic events in The Netherlands"). Starved (24 h) female rats are fed with 10 g of hepatopancreas of the shellfish. After 16 h the consistency (softening) of the faeces is investigated. Severe diarrhea corresponds with toxin levels around the current EU legislation (160 µg/kg OA-equivalents or 160 µg/kg AZA1-equivalents) [68]. A major drawback of the RBA is that YTXs and PTXs are not detected at the regulatory limit because they do not induce diarrhea. Furthermore, the analyst needs to build up experience for a precise interpretation of the test results (texture of faeces). More in general, the limitations of the MBA and RBA are lack of specificity and sensitivity, no elucidation of the toxin profile is possible, and the frequent generation of false positive results. For these reasons, within Europe many countries now use a combination of an animal test and a chemical test (Table 3). Furthermore, the MBA in particular is becoming increasingly unacceptable for ethical reasons and this provides a strong impetus to out phase and replace the MBA.

Table 3. Methods used for the official control of lipophilic marine toxins.

Country	OA and DTXs	AZAs	PTXs	YTXs	Reference
Norway	MBA Chemical	Chemical	Chemical	MBA Chemical	[113]
Sweden [1]	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[113]
Finland [2]					[113]
Denmark	MBA Chemical	Chemical	Chemical	Chemical	[113]
Ireland	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[113]
United Kingdom	MBA	MBA	MBA	MBA	[113]
Germany	Chemical	Chemical	Chemical	Chemical	[113]
The Netherlands	RBA Chemical	RBA			[113]
Belgium	MBA	MBA	MBA	MBA	[113]
France	MBA	MBA	MBA	MBA	[113]
Austria	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[113]
Portugal	MBA Chemical Biochemical	Chemical	Chemical	MBA	[113]
Spain	MBA	MBA	MBA	MBA	[113]
Italy	MBA Chemical	MBA	MBA	MBA Chemical	[113]

Table 3. *Cont.*

Country	OA and DTXs	AZAs	PTXs	YTXs	Reference
Greece	MBA Chemical	MBA			[113]
Turkey	MBA	MBA	MBA	MBA	[114]
Canada	MBA	MBA	MBA	MBA	[114]
United States [3]					[114]
Venezuela	MBA	MBA	MBA	MBA	[114]
Brazil [3]					[114]
Chili	MBA	MBA	MBA	MBA	[114]
Uruguay	MBA	MBA	MBA	MBA	[114]
Republic of Korea	MBA Chemical	MBA Chemical	MBA Chemical	MBA Chemical	[114]
Japan	MBA	MBA	MBA	MBA	[114]
Thailand	MBA	MBA	MBA	MBA	[114]
New Zealand	Chemical	Chemical	Chemical	Chemical	[113]

MBA = mouse bioassay, RBA = rat bioassay. Chemical = high performance liquid chromatography (HPLC), LC fluometric detection (LC-FLD), LC mass spectrometry (LC-MS), LC tandem MS (LC-MS/MS). Biochemical = enzyme-linked immunosorbent assay (ELISA). [1] Samples for the MBA are analysed in Norway. [2] MBA test for DSP toxins is prohibited. [3] No monitoring established.

From a worldwide perspective, the regulation of the lipophilic marine toxins differs widely. These differences are related to the presence or absence of the toxins in specific regions and on the methodology applied. In the USA the FDA has only installed OA and DTX1 legislation, while no routine monitoring programs for these toxins have been established yet (Table 4) [60,114]. Canadian guidelines only mention maximum levels for OA and DTX1 in digestive glands (Table 4) [115]. In Japan, the level has been expressed in mouse units (MU) which is a common way to express the regulatory limit when the MBA is applied (Table 4) [114]. In Australia and New Zealand a regulatory limit has been established for OA and DTX1, DTX2 and DTX3 (Table 4) [116]. In Europe most types of lipophilic marine toxins can be found in shellfish and as a result EU legislation covers OA, DTXs, PTXs, YTXs and AZAs (Table 4).

Table 4. Permitted levels for lipophilic marine toxins.

Country or Continent	OA, DTXs (µg/kg)	PTXs (µg/kg)	AZAs (µg/kg)	YTXs (µg/kg)	MBA (MU/kg)	Reference
EU	160 WF	Included in OA	160	1000		[12]
United States	200	NR	NR	NR		[60]
Canada	1000 DG	NR	NR	NR		[115]
Japan					50 (~200 µg/kg OA-eq)	[114]
Australia and New Zealand	200 WF	NR	NR	NR		[116]

WF = Whole shellfish flesh, DG = digestive glands, NR = not regulated, MU = mouse unit.

3.2. Development of alternative methods

3.2.1. *In-vitro* assays

Functional assays are currently being developed as alternatives to the bioassays. Functional assays are based on the toxicological mode of action of a group of toxins in a biological process. Advantages of functional assays are their potential for high-throughput screening, detection of new toxins, while there is no need for applying TEF values. Still, false positives or negatives can occur due to matrix substances present in the extract or due to metabolic activation. It is extremely difficult to develop a functional assay that will comprise all lipophilic marine toxins in a single assay. Until now, functional assays have been developed for the OA group toxins, YTXs, PTXs and SPXs. Toxins of the OA group can be determined by protein phosphatase 2A (PP2A) inhibitor assays using fluorometric detection. Several of these assays have been published in recent years [117–119]. A good correlation between the MBA and the PP2A fluorometric assay has been obtained in several laboratories [117,120]. Furthermore, for the OA group toxins and PTXs a cytotoxicity assay based on actin fillament depolymerization in a BE(2)-M17 neuroblastoma cell line has been developed [121]. For the OA group toxins and YTXs an assay was developed based on the reduction of cell-cell adhesion in MCF-7 and Caco-2 cells leading to an accumulation of E-cadherin [122,123]. Also AZA1 showed an effect on the cell-cell adhesion and E-cadherin influx, but these results have not resulted in a functional assay format yet [88]. Unfortunately, with respect to OA and YTX the reproducibility of the assay was rather poor. Therefore the assay should be made more robust prior to routine application. Recently, a fluorescence polarization inhibition assay has been developed for SPXs. The assay uses nicotinic acetylcholine receptor-enriched membranes of the marbled electric ray (*Torpedo marmorata)* and is capable to analyse contaminated mussels with SPX concentrations in the range of 70–700 µg/kg [124]. Of the functional assays developed thus far, most promising results have been obtained with the PP2A assay for the OA group toxins and the nicotinic acetylcholine receptor assay for SPXs. However, successful validation (single- and inter-lab) of these methods is still lacking.

3.2.2. Biochemical methods

In immunochemical methods antibodies are used that show affinity with specific structural parts of a toxin. Analogues of these toxins can often also be detected by cross-reactivity, but no information is gained about differences in toxicity. Therefore, methods such as enzyme-linked immunosorbent assay (ELISA) can only be used for screening of shellfish samples. For some of the lipophilic marine toxin groups immunochemical methods have been developed. For the OA group an ELISA has been converted to a lateral flow immunochromatographic (LFI) format. The test strips allow the analysis of toxins on site without the use of lab facilities [125]. In principle, this would enable shellfish industry to carry out these tests themselves. A recent study on these test trips showed that a relative high number of samples (45%) were misidentified as positive [126]. Further research is needed to make this LFI suitable for routine monitoring purposes. Other biochemical methods that are currently under development for the OA group make use of amperometric immunosensors and immunobiosensors using surface plasmon resonance (SPR) [127,128]. A sensitive ELISA for YTX has been developed with good correlation to a chemical method based on liquid chromatography/mass spectrometric

detection. Its working range would make this ELISA suitable for routine monitoring [129,130]. The advantage of this YTX ELISA is the cross-reactivity towards many YTX analogues [129], although it is unclear whether these analogues are toxic. Other promising biochemical methods for YTXs are SPR based biosensors, a resonance mirror bioassay and fluorescence polarization [131–133]. For the PTXs, AZAs and SPXs no biochemical methods are available yet. Most promising results have been obtained with the OA and the YTX group ELISA. Provided proper validation is carried out, these rapid screening biochemical methods can be used for high sample throughput analysis of shellfish toxins.

3.2.3. Chemical methods

In the 1980s, the first chemical detection methods developed for the OA group toxins were based on liquid chromatography (LC) coupled to fluorometric detection (LC-FLD). As most lipophilic marine toxins lack chromophores, a derivatisation step was required. For toxins of the OA group 9-anthryldiazomethane (ADAM) [134] and for PTXs and YTXs 4-[2-(6,7–dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) have been used as derivatisation reagents [135,136]. A major drawback of LC-FLD is its limited selectivity for the OA group toxins as well as for the PTXs and YTXs. The derivatisation step is rather laborious and can be critical. For AZAs and SPXs no LC-FLD methods have been developed. This is probably due to the fact that these toxins were only discovered in the mid 1990s when LC (tandem) mass spectrometry [LC-(MS)/MS] became increasingly popular.

In recent years much effort has been put in the development of LC-MS/MS methods that are dedicated to either detecting the specific classes of lipophilic marine toxins or detecting as many as possible different lipophilic marine toxins in a multi-toxin method. Many of the methods developed for specific classes of lipophilic marine toxins focused on either structure elucidation or on discovery of new lipophilic marine toxins. For example for the OA group toxins LC-MS/MS techniques have been used to identify new DTXs [137–140]. By now up to 40 different toxins belonging to the OA toxin group have been identified using LC-MS/MS [140,141]. Several LC-MS/MS methods have been developed to detect new toxins (YTXs and PTXs) in either algae or shellfish [71,77,142–145]. Furthermore, LC-MS/MS has been used to investigate the transformation of toxins into metabolites. The conversion of YTX to 45-OH-YTX and 45-COOH-YTX and the conversion of PTX2 to PTX2sa have been studied by LC-MS/MS [75,78,146]. Another LC-MS/MS method was developed to determine up to 24 different AZAs in a single analysis [87]. Some dedicated methods were used to study the metabolic processes taking place when AZA contaminated mussels are heat-treated [147]. Also, with the help of LC-MS/MS new SPX analogues have been identified that are either produced in algae or in shellfish [101,102].

Most of the methods described above were used for research purposes and were not intended for the monitoring programs. Nowadays, several LC-MS/MS methods are available to determine most or all toxin classes belonging to the lipophilic marine toxins. The first two multi-toxin LC-MS/MS methods for lipophilic marine toxins were developed in 2001 [148,149]. Unfortunately, one method did not include the YTXs [148] while the other one used a laborious sample clean up procedure based on liquid-liquid extraction and various solid phase extraction procedures [149]. Therefore, these methods were not suitable for routine monitoring programs. In 2005 two new multi-toxin methods were

developed that included toxins from all regulated lipophilic marine toxin classes in the EU [150,151]. These methods were in-house validated and good performance characteristics were obtained. Drawbacks were the exclusion of spirolides in one method [151] and poor chromatography for some compounds in the other one [150]. In 2007 a very high pressure liquid chromatography (VHPLC)-MS/MS method was developed. With this method it was possible to analyse 21 marine lipophilic toxins in only 6.6 minutes [152]. It should be mentioned that the separation and detection could only be accomplished by the newest generation LC and MS equipment. This VHPLC-MS/MS method has not been validated yet. The latest developed multi-toxin method was published in 2009. By a different choice of chromatographic conditions, all chromatography problems have been solved and the method has been in-house validated [153]. All prominent lipophilic marine toxins were included in this method. Currently, for this method a full collaborative validation study according to international guidelines is in preparation.

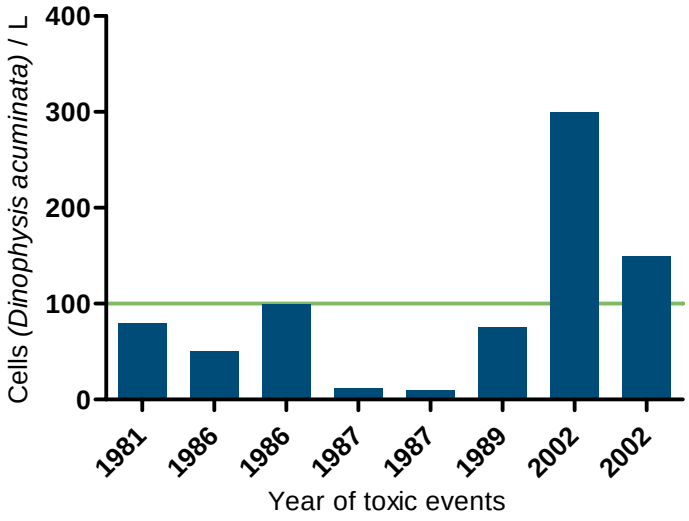
4. Occurrence of Toxic Events in the Netherlands (1960–2009)

In The Netherlands until now only DSP has occurred and the other toxic syndromes (ASP and PSP) have not been reported. Only in 2002 one shellfish sample has been tested positive for domoic acid (unpublished data provided by M. Poelman). Therefore, this historic overview only deals with the DSP syndrome. The first incidences outside The Netherlands were reported in Japan (1976 and 1977) [109]. Japanese researchers found *Dinophysis fortii* the algae producing this toxin. Therefore, the toxin was named Dinophysistoxin and the poisoning syndrome was named Diarrhetic Shellfish Poisoning (DSP) [154]. In 1982 the structure of the causative toxin, dinophysistoxin-1, was finally elucidated [155].

In The Netherlands the first incidences of poisoning associated with consumption of mussels were reported in July and August 1961 [65]. People that had consumed mussels experienced abdominal cramps, vomiting and severe diarrhea. At the same time, in the Eastern Scheldt and the Wadden Sea high concentrations of the dinoflagellates *Prorocentrum micans* , *P. triestinum* , *P. minimum* and *Dinophysis acuminata* were reported. In the following years, these algae were isolated from the gastrointestinal tract (hepatopancreas) of the mussels. Following this episode, human intoxications re-occurred in The Netherlands in 1971 (mussels from the Eastern Scheldt), 1976, 1979 (mussels from Wadden Sea) and 1981 (mussels from the Eastern Scheldt and Wadden Sea) [156–158]. In 1979 a rat bioassay was developed for the detection of these toxins and to prevent human intoxication [65] and this RBA was adopted as the official method of control for the detection of diarrhea causing toxins in The Netherlands. The monitoring program for DSP toxins in the Netherlands includes an early warning system and the pre-market analysis of shellfish on the presence of ASP, PSP and DSP toxins. The early warning system monitors the various potential toxic algae in sea water. The RBA was used to test if *P. micans* and *P. minimum* were responsible for the adverse effects observed in 1961. However, mussels contaminated with cultivated algae were fed to rats, no adverse effects were observed [65]. Therefore, it remained doubtful if these algae were responsible for the toxin production. In 1981 it was demonstrated that in the Netherlands the responsible algae for the toxin production in the Eastern Scheldt and Wadden Sea was *D. acuminata* [159]. In 1986 and 1987 DSP toxins were again detected in the Wadden Sea, but due to the established monitoring program shellfish areas were closed and no human intoxications were reported [160,161]. In October 1989, a minor episode of DSP

toxicity occurred in the Wadden Sea; no incidences of human illness were reported. The production area was closed during the presence of DSP-toxins. In 2002 *D. acuminata* caused the presence of DSP-toxins in mussels from the Wadden Sea. This was followed by a closure of the production area for several weeks (unpublished data provided by M Poelman). By means of an LC-MS method low levels of toxins could be detected in mussels several weeks before the RBA picked up levels above the EU regulatory limit. In this case intoxication of local fishermen was observed, while the RBA detected levels of DSP toxins after closure of the fishing area (unpublished data provided by M. Poelman). In 2005 and 2007 the presence of *D. acuminata* in the Wadden Sea triggered the application of a (delayed) monitoring program using LC-MS/MS. These analyses showed the presence of OA in mussels at levels well below the current regulatory limit, ranging from 18 till 68 µg OA equivalents/kg shellfish. The presence of high numbers of *D. acuminata* triggered analysis of shellfish by LC-MS/MS again in 2009. No detectable amounts of any DSP toxins were found. These results and also those obtained on earlier occasions indicate that there is no clear correlation between the counts of potential toxic algae and toxic events (Figure 11). With respect to the EFSA opinion there are some concentrations found in 2005 and 2007 that are above the ARfD of 45 µg OA equivalents /kg shellfish. Therefore, in case legislation is changed towards the EFSA opinion more positive samples will be found in The Netherlands. Overall, in the last decade in shellfish of Dutch waters only low levels of OA equivalents have been found.

Figure 11. Number of *Dinophysis acuminata* cells per liter of sea waters on the corresponding years of toxin detection. The line indicates an action limit, above 100 cells per liter corrective measures are taken.



5. Conclusions

Blooms of algae responsible for production of lipophilic toxins occur quite frequently within European waters. Finally, almost 50 years after the first occurrence of DSP in The Netherlands chemical methods are now available for the detection of all relevant lipophilic marine toxins in shellfish. These methods can help to decide upon closure of shellfish harvesting areas and to prevent intoxication. Due to changes in climate and algae transport through ballast water future blooms and

toxic episodes cannot be excluded. Therefore, method development should continue and monitoring programs should be maintained. Furthermore, considerable effort is undertaken by various European research groups and the European Union to out phase the animal tests that are still used for the official routine monitoring programs.

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