Handbook of Water Analysis
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Publication details
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Published online on: 29 Jul 2013


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Marine Toxins Analysis

Luis M. Botana, A. Alfonso, M. R. Vyeites, N. Vilariño, A. M. Botana, C. Louzao, and C. Vale

Marine toxins are a very large and complex group of compounds from different origins. They are produced by actinomycetes, cyanobacteria (Leao et al., 2012), sponges (Bondu et al., 2012), bacteria, gastropods (Wang et al., 2006), and so on (Nikapitiya, 2012). Their biological role is intended for defense or predation, but in some cases, their role is unknown or fits in the undefined role of secondary metabolites (Kita et al., 2010, Nikapitiya, 2012, Proksch, 1994). The toxins covered in this chapter, the phycotoxins (produced by algae), affect a specific problem related to food safety, and pose an analytical challenge for health authorities. Phycotoxins are produced by unicellular microalgae, mostly photoautotrophic, heterotrophic, or mixotrophic dinoflagellates (Reguera and Pizarro, 2008) and a few diatoms (Vasconcelos et al., 2010). They are distributed worldwide, and from their ecological environment, they show different patterns of toxin production (Kellmann et al., 2010, Lin, 2011, Litaker et al., 2010, Otero et al., 2010a). Given the extreme diversity of the genes responsible for the production of the toxin skeletons, and the molecular enzymatic machinery for the biosynthesis of the toxins, the polyketide synthases (Fujii, 2009, Monroe and Van Dolah, 2008), the number of marine phycotoxins is very high (several hundreds), although they are classified in few chemical groups (see Table 6.1). These toxins are very relevant to food and health authorities because they accumulate in very high amounts in filter feeding mollusks and gastropods, to levels that may even allow a couple of mussels to reach lethal quantities, or one single conch to be potentially lethal. Even though most of the toxins are not lethal, those few groups that can cause death to humans are especially dangerous. The monitoring and surveillance systems in all producing countries are costly and require international standardization, since shellfish are very important in international trading (Vieites and Cabado, 2008). In addition to the cost of monitoring the presence of toxins in seafood, they are an economical problem for producers, and their presence closes production areas for long periods of time, causing major losses.

Since the European Union (EU) is a major importer and producer of shellfish products, the legislation in the EU becomes a de facto international standard. For many years, EU legislation required the mouse bioassay to be the reference method for the monitoring of most of the marine toxins, and the standard to implement it was achieved by means of regulation 853 (Commission, 2004a) and regulation 2074 (Commission, 2007b). Basically, out of all the regulated marine toxins, only domoic acid was monitored by a chemical method, HPLC with UV detection (International, 2000), while all the other toxin groups were monitored by mouse bioassay. The mouse bioassay was semiquantitative for the saxitoxin group (PSP—paralytic shellfish poison) (AOAC, 1995), and qualitative for the lipophilic group (DSP—diarrheic shellfish poison, azaspiracids, pectenotoxins, and yessotoxins) (Yasumoto et al., 1978).
### TABLE 6.1
Regulated Toxins, Official Detection Methods, and Toxicological Facts

<table>
<thead>
<tr>
<th>Toxin Representativea</th>
<th>Mode of Actiona</th>
<th>Toxicologyb</th>
<th>Detection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saxitoxin (STX)</td>
<td>Sodium channel</td>
<td>Muscle paralysis and respiratory paralysis (Panel, 2009c)</td>
<td>HPLC (Lawrence et al., 2005)</td>
</tr>
<tr>
<td>30 analogs (PSP group)</td>
<td></td>
<td>ARfD: 0.5 μg/STX equivalent/kg body weight (Panel, 2009a)</td>
<td></td>
</tr>
<tr>
<td>Genus producer:</td>
<td></td>
<td>Legal EU limit: 800 μg PSP/kg shellfish meat</td>
<td></td>
</tr>
<tr>
<td>Alexandrium, Gymnodinium, Pyrodinium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aphanizomenonc, Anabaenac, Lyngbyac</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domoic acid (DA)</td>
<td>Kainate receptor activation</td>
<td>Neuronal damage (Panel, 2009d)</td>
<td>HPLC (Lawrence et al., 1989)</td>
</tr>
<tr>
<td>11 analogs (ASP group)</td>
<td></td>
<td>ARfD: 30 μg/DA/kg body weight (Panel, 2009a)</td>
<td>ELISA (Kleivdal et al., 2007)</td>
</tr>
<tr>
<td>Genus producer:</td>
<td></td>
<td>Legal EU limit: 20 mg DA/kg shellfish meat</td>
<td></td>
</tr>
<tr>
<td>Pseudonitzschia</td>
<td>Phosphatase PP2A inhibition</td>
<td>Diarrhea (Panel, 2008b)</td>
<td>LC-MSd Conditions as in (van Den Top et al., 2011b): 100% methanol extraction hydrolysis for DSP esters MMS C18 (150 × 3, 5 μm) Phase A: water Phase B: acetonitrile/water (90:10, v/v) Both phases with 6.7 mM ammonium hydroxide (pH 11) Negative electrospray ionization MS/MS transitions: OA Q1/Q3:803.5/255.0/113.0 DTX2 Q1/Q3:803.5/255.0/113.0 DTX1 Q1/Q3:817.5/255.0/113.0</td>
</tr>
<tr>
<td>Okadaic acid (OA)</td>
<td></td>
<td>ARfD: 0.3 μg/OA equivalent/kg body weight (Panel, 2009a)</td>
<td></td>
</tr>
<tr>
<td>12 analogs (DSP group, lipophilic toxins group)</td>
<td></td>
<td>Legal EU limit: 160 μg OA equivalent/kg shellfish meat</td>
<td></td>
</tr>
<tr>
<td>Genus producer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinophysis, Protoperidinium Dinophysis, Prorocentrum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yessotoxin (YTX)</td>
<td>Several targets reported</td>
<td>Unknown (Panel, 2008c)</td>
<td>LC-MSe Conditions: same as okadaic acid MS/MS transitions: YTX Q1/Q3:1141.5/1061.7/855.5 HomoYTX Q1/Q3:1155.5/1075.5/869.5 45 OH-YTX Q1/Q3:1157.5/1077.7/871.5 45 OH-HomoYTX Q1/Q3:1171.5/1091.5/869.5</td>
</tr>
<tr>
<td>50 analogs (lipophilic toxins group)</td>
<td></td>
<td>ARfD: 25 μg/YTX equivalent/kg body weight (Panel, 2009a)</td>
<td></td>
</tr>
<tr>
<td>Genus producer:</td>
<td></td>
<td>Legal EU limit: 1000 μg YTX equivalent/kg shellfish meat</td>
<td></td>
</tr>
<tr>
<td>Protoceratium, Lingulodinium, Gonyaulax</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectenotoxin2 (PTX2)</td>
<td>F-Actin Unknown (Panel, 2009e)</td>
<td>ARfD: 0.8 μg/PTX2 equivalent/kg body weight (Panel, 2009a)</td>
<td>LC-MSf Conditions: as same okadaic acid but positive ionization MS/MS transitions: PTX-1 Q1/Q3:892.5/821.5/213.2 PTX-2 Q1/Q3:876.5/823.4/213.2 LC-MSf Conditions: as same pectenotoxin MS/MS transitions: AZA-1 Q1/Q3:842.5/824.5/806.5 AZA-2 Q1/Q3:856.5/838.5/820.5 AZA-3 Q1/Q3:828.5/810.5/792.5</td>
</tr>
<tr>
<td>20 analogs (lipophilic toxins group)</td>
<td></td>
<td>Legal EU limit: 160 μg OA equivalent/kg shellfish meat</td>
<td></td>
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<tr>
<td>Genus producer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinophysis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Azaspiracid1 (AZA1)</td>
<td>Unknown Diarrhea, neurotoxicity (Panel, 2008a)</td>
<td>ARfD: 0.2 μg/AZA1 equivalent/kg body weight (Panel, 2009a)</td>
<td>LC-MSf Conditions: as same pectenotoxin MS/MS transitions: AZA-1 Q1/Q3:842.5/824.5/806.5 AZA-2 Q1/Q3:856.5/838.5/820.5 AZA-3 Q1/Q3:828.5/810.5/792.5</td>
</tr>
<tr>
<td>32 analogs (lipophilic toxins group)</td>
<td></td>
<td>Legal EU limit: 160 μg AZA equivalent/kg shellfish meat</td>
<td></td>
</tr>
<tr>
<td>Genus producer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azadinium</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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*a* Further information is available in reviews by Botana et al. (2009, 2010, 2011).

*b* Further information is available in EFSA opinions (Panel, 2008a,b,c, 2009a,b,c,d,e, 2010).

*c* Blue green algae (cyanobacteria).

*d* Bioassay until 2014 (Regulation, 2011).


ARfD (acute reference dose) is the amount of toxin in food that can be ingested in 24 h without risk to consumer.
In the past, the use of animals for the monitoring of marine toxins has been a major source of controversy, more so after the publication of a directive (86/609) for the elimination of animal tests that would require replacing the use of animals by alternative tests (Communities, 1986), and optimizing their use, whenever possible, following the three R criteria (reduction, refinement, replacement) (Hess et al., 2006). As a consequence, progress was made in terms of international validation of alternatives to monitor marine toxins. Regulation 1244 (Commission, 2007a) allowed the use of ELISA as an alternative to HPLC for the detection of domoic acid and analogs, and regulation 1664 (Commission, 2006) allowed the substitution of the mouse bioassay for AOAC method 2005.06 (AOAC, 2005), a liquid chromatography method with precolumn derivatization and fluorescence detection. Regulation 1664 states that AOAC method 2005.06 would replace the mouse bioassay only for those compounds for which the method was validated, as the scope of the validation of this method was limited to a few compounds: saxitoxin, neosaxitoxin, gonyautoxins 2 and 3, gonyautoxins 1 and 4, decarbamoyl saxitoxin, gonyautoxin 5, C1, C2, C3, and C4 (Lawrence et al., 1995). This chromatographic method was proven not to be fit for purpose (Ben-Gigirey et al., 2007); hence, an extension of the method was reported for decarbamoyl NeoSaxitoxin and decarbamoyl gonyautoxins 2 and 3 (Turner et al., 2009), and the method was refined for several matrices such as oysters, cockles, and clams (Turner et al., 2010).

The complexity of this HPLC method compared to the fluorescent postcolumn oxidation method developed by Oshima et al. (1984), as reported by several groups (DeGrasse et al., 2011, Rodriguez et al., 2010, Turner et al., 2011), and the limited scope of this method increase the need for better methods for routine monitoring. There is now an ongoing multilaboratory validation that continues a successful single-laboratory validation study that was conducted for the LC postcolumn oxidation analysis of saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins (GTX) 1-5, decarbamoyl gonyautoxins (dcGTX) 2 and 3, decarbamoyl saxitoxin (dcSTX), and N-sulfocarbamoyl-gonyautoxin-2 and 3 (C1 and C2) in mussels (Mytilus edulis), soft shell clams (Mya arenaria), scallops (Placopecten magellanicus), and oysters (Crassostrea virginica) (Van de Riet et al., 2009).

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### 6.1 Lipophilic Detection by LC-MS

Upon request of the EU, a working group was created by the European Food Safety Agency (EFSA) to evaluate the risk to humans and the methodology that was being used for marine toxin analysis to date. The main conclusions drawn by this working group cover several topics, as summarized in Table 6.1. In general, it highlighted the great need for validation of methods, the unavailability of toxicity data for most of the toxins (both acute and long term), the lack of pharmacokinetic information, and the lack of consumption and epidemiological information. Following directive 86/609 to replace animal use (Communities, 1986), one of the main consequences of the working group conclusions was that the mouse bioassay should be replaced. As a consequence, the EU issued in January 2011 a new regulation (Regulation, 2011), that would fundamentally change the current scenario for marine control and monitoring. Basically, this regulation requires that lipophilic toxins are monitored by either mouse bioassay or liquid chromatography coupled to mass spectrometric detection (LC-MS), but the reference method becomes mass spectrometry after July 2011, although both methods may coexist for 3 years. As a consequence, international validation efforts took place to provide a reliable validated LC-MS method. Three simultaneous exercises were organized, with coordinators based in laboratories in Germany (These et al., 2011), Spain (EU-RL, 2011), and Holland (van Den Top et al., 2011b) (see Table 6.1). The results from by these studies have prompted the EU to implement the use of LC-MS as the reference method.

Although the validation studies, which compare results from samples carefully exchanged in controlled conditions, provide acceptable results in most of the cases, it is noteworthy that even in these controlled conditions, the mouse bioassay did perform in some cases better than the analytical alternative. A recent article has questioned the validity of the results provided by these interlaboratory exercises (Otero et al., 2011). In essence, the study shows that LC-MS is a rather complex technology where, unless a careful interlaboratory study is done on the equivalence of all variable parameters, the results provided will give gross errors far higher than the variability of the mouse bioassay. One of the conclusions of the EFSA working group was that the mouse bioassay would provide an uncertainty of 40–50% to detect...
okadaic acid, while this study shows that a variation of up to 200% can be observed by LC-MS by just changing the commercial source of the solvents, or slightly modifying the elution pH, without any matrix. If the matrix component is added to the equation, then variation increases. Therefore, the use of LS-MS as a reference method has several drawbacks that do not allow its use with all guaranties, unless all factors are controlled. The last interlaboratory exercise acknowledges the need for more tight control in each of the parameters (van Den Top et al., 2011b).

## 6.2 Standards and Toxic Equivalent Factor

One requirement associated with analytical detection, and recognized by all LC-MS users as a problem, is the need to have standards to quantify the levels of analyte in a sample. The availability of standards has been a historical problem in this field, although just recently there is a supplier of certified reference standards in Europe, CIFGA (www.cifga.com), that adds to the standards available from the NRC in Canada. But even with certified standards, there is a tendency to quantify one toxin with a calibration curve for another compound in the group, that is, quantity dinophysistoxin-1 with a calibration standard of okadaic acid. This has been proven to be a major source of error (Otero et al., 2011) and should be avoided, so that each compound is quantified against its own calibration plot.

Once a compound is quantified, even if the quantification has been done under perfect conditions, the result has to be translated into the toxicity of the reference compound of the group, that is, amount of okadaic acid in the case of phosphatase inhibitors. Therefore, it is critical to understand and know the relative toxicity of each of the compounds of a group with regard to the reference compound. This value, the toxic equivalent factor or TEF, is not understood with the available information for many of the phycotoxin analogs. Therefore, there is always an unavoidable error when reporting toxic levels of a toxin group from results obtained by LC-MS (Botana et al., 2010), although there is a major revision of some TEF values in the literature (Aune et al., 2007, 2008, Otero et al., 2012, Perez et al., 2011, Rubiolo et al., 2011, 2012, Vale et al., 2008).

## 6.3 Regulated Toxins and Food Safety

One of the striking consequences of the partial implementation of the EFSA working group conclusions was that food safety actually decreased. This paradoxical phenomenon was caused by three different factors:

a. The mouse bioassay for lipophilic compounds would detect the sum of toxic activities of each lipophilic compound; hence, the limits would be 1 mg/kg yessotoxin and 160 μg/kg mussel flesh equivalents for okadaic acid, dinophysistoxins, pectenotoxins, or azaspiracids in combination, but LC-MS would report each toxin group independently, so it is possible to market samples with up to 160 μg/kg of each of the toxin groups, since the additive toxic effect of these groups would only be detected in the animal assay. Therefore, a marketed sample may contain 1 mg yessotoxin and 4 times 160 μg/kg of each of the toxin groups, clearly levels far higher than the bioassay would allow.

b. Owing to the nature of the LC-MS, detection and quantification can be only performed against a standard; therefore, a routine monitoring laboratory would seek only regulated toxins. All other toxins, known to be present in the European coasts, but not specifically regulated (tetrodotoxin (Rodriguez et al., 2008), ciguatoxins (Otero et al., 2010b), palytoxins (Aligizaki et al., 2008, Cagide et al., 2009, Ciminiello et al., 2006, 2008), cyclic imines (Gonzalez et al., 2006, Villar-Gonzalez et al., 2007)), will not be detected by LC-MS because they are not seeked, there is no standard (ostreocins, ciguatoxins), or there is no recognized method (ciguatoxins, ostreocins). Although shellfish is not a major contributor to the dietary exposure of chemical contaminants, it is recognized as a risk to certain population groups, such as breast-feeding women or very young children (Gueguen et al., 2011).
c. The legal change in toxin detection was not accompanied by a modification of toxin levels, which was one of the major tasks of the EFSA working group (Panel, 2009b, 2010); as a consequence, only the suggestion to modify the method was taken into account, but not the recommendation to modify the toxic levels or the consumer portion size figure of 400 g for a 95th percentile (Panel, 2010) that should replace the 100 g value currently in place. Recent analysis of food poisoning events (Hossen et al., 2011) indicate that lowest observable adverse effect level (LOAEL) established by the EFSA working group, about 50 μg OA equivalents per person, were accurate (Panel, 2008b).

6.4 Functional Assays

One of the consequences of the new legal regulation in Europe, and hence in the global shellfish market, is that only a selected, though not complete, set of toxins are controlled in marketed products. As a consequence, there is a growing concern among producers for the possibility of a “toxin leak” to the market, and this is increasing the pressure to have premarketing screening tests that would reproduce a response similar to the mouse bioassay, alerting of possible problems caused by a combination of toxins or specific analogs (Aligizaki et al., 2008, Bire et al., 2002, Villar-Gonzalez et al., 2006, 2007). The presence of palytoxin in Greece (Aligizaki et al., 2008), pinnatoxin in Norway (Rundberget et al., 2011), or gymnodimine in Tunisia (Bire et al., 2002) has been reported, and since these are nonregulated toxins, their presence should be screening before or along official testing. For this reason, functional assays, defined as assays that use receptors or binding transducers of the toxin action, are the best approach, as they can detect any compound that interacts by a given mechanism of action (Alfonso et al., 2009, Araoz et al., 2010, Botana et al., 2009, 2011, Fonfria et al., 2010a,b, Vilarino et al., 2009, 2010). Although antibody-based methods are not included in this category, as antibodies do not interact in a fashion that resembles the activity of the toxin, they might be useful to screening methods, once their cross-reactivity is well defined. There are new technologies that use antibodies, and specially surface plasmon resonance (SPR) has provided several interesting screening options for okadaic acid, saxitoxin, brevetoxin or yessotoxin, and palytoxin analogs (Alfonso et al., 2005, Campbell et al., 2007, Fonfria et al., 2007, 2008, Llamas et al., 2007, Mouri et al., 2009, 2007, Pazos et al., 2004, 2005, Yakes et al., 2011). The cross-reactivity can be designed to even match the TEF of certain toxins, as it was the case for an SPR (surface plasmon resonance) method for okadaic acid and analogs (Stewart et al., 2009a, b). Also, interlaboratory exercises have shown that SPR-antibody-based methods might be suitable for routine screening of saxitoxin and analogs (Campbell et al., 2010, van Den Top et al., 2011a). There are also ELISA methods available for most of the toxins groups known (Empey Campora et al., 2008), of which the domoic acid ELISA has been put in the legislation (see above). Although there is no commercial functional assay for marine toxins, many methods are now available for each of the toxin groups. They include the groups of palytoxin (Alfonso et al., 2012, Espina et al., 2009, Ledreux et al., 2009), cyclic imines (Rodriguez et al., 2011), okadaic acid and dinophysistoxins (Della Loggia et al., 1999, Draisic et al., 1994, González et al., 2002, Ikehara et al., 2010, Leira et al., 2002, 2003, Tubaro et al., 1996, Viedes et al., 1997), ciguatoxins (Darius et al., 2007, Louzao et al., 2003, 2004), brevetoxins (Louzao et al., 2003, 2004, Truman et al., 2002), pectenotoxin (Canete and Diogene, 2008), saxitoxin (Louzao et al., 2001), and yessotoxin (Alfonso et al., 2004, 2005). One of the major drawbacks for the detection of marine toxin using functional assays is the need to have approved interlaboratory validation for each method, so that the results they provide have some legal value. This is not only complex but also slow, and therefore, very few methods will be ready in the future for this purpose. Nevertheless, it is worth mentioning a successful validation exercise to quantify saxitoxin and analogs that uses a radioreceptor assay, which will be used in some American countries for the monitoring of saxitoxin and analogs (Van Dolah et al., 2009).

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