A European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis

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This review considers the ethical and technical problems currently associated with employing mouse bioassays for marine-toxin analysis and the challenges and the difficulties that alternative methods must overcome before being deemed applicable for implementation into a regulatory monitoring regime. We discuss proposed alternative methods, classified as functional, immunological and analytical, for well-established European toxins as well as emerging toxins in European waters, highlighting their advantages and disadvantages. We also consider emerging tools and technologies for future toxin analysis.

Even though regulatory bodies have recently recommended analytical methods for a number of toxins, there is still scope for functional and immunological methods in rapid screening and detecting emerging toxins. Future developments foreseen in the analysis of marine biotoxins are multiplex-based analysis, miniaturization and portability for on-site testing. However, the longstanding lack of reference materials and standards continues to pose a severe limitation on progress in development, validation and therefore implementation of any alternative method based on the criteria stipulated by European Union legislation.

Keywords: Analytical method; Antibody; Biosensor; Functional assay; Immunology; Marine toxin; Mouse bioassay; On-site testing; Rapid screening; Receptor

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1. Introduction

Marine biotoxins are naturally-occurring poisonous substances synthesized by microscopic toxin-producing algae or their associated bacteria, though normally in non-harmful quantities. However, a combination of increased temperatures, sunlight and nutrient-rich waters is believed to cause rapid algal reproduction and thereby lead to potentially ''harmful algal blooms''. Worldwide, increasing occurrences of toxic blooms are thought to be linked to climate change, increased ocean eutrophication and commercial shipping [1]. These toxins transfer through the trophic chain into shellfish and fish. Molluscan shellfish are bivalve-filter feeders and ingest the algae, whereupon toxins may increase to levels that are potentially lethal to humans or other consumers (e.g., marine mammals and birds). Hence, as this has major implications for public health, seafood destined for human consumption is routinely monitored by regulatory bodies worldwide and is deemed fit for consumption based on regulatory limits and methods established to prevent acute poisoning [2–7]. The monitoring of marine toxins is vital to the aquaculture industry, as these toxins may cause substantial ecological damage and economic losses through frequent or prolonged contamination and closure of harvesting sites [8].

Marine biotoxins detected worldwide, but particularly in European waters, were originally classified based on their acute symptomatic effect in humans following intoxification. The three main groups monitored in the European Union (EU) are:

- Paralytic Shellfish Poisoning (PSP) toxins;
- Diarrheic Shellfish Poisoning (DSP) toxins; and,
- Amnesic Shellfish Poisoning (ASP).

However, as alternative detection methods are considered, classification is beginning to focus more on chemical structures and properties of the toxins. DSP toxins have in recent times become known as lipophilic toxins incorporating okadaic acid, dinophysistoxins, azaspiracids,

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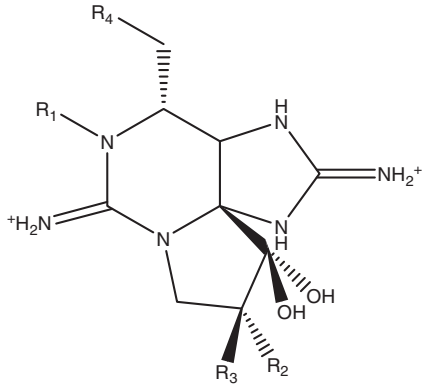
pectenotoxins and yessotoxins with the last two not proved to cause diarrheic symptoms following intoxication. For each of these three main toxin groups and subgroups, the occurrence of the toxins, their chemical characteristics, toxicokinetic evaluations, human-exposure assessments and detailed review of potential methods of analysis have in recent years been published by the European Food Safety Authority (EFSA) as scientific opinions [9–14]. The diversity of the numerous analogues or natural enzymatic metabolites of marine biotoxins has been described [15]. Fig. 1 highlights the structure of the parent or reference toxin within each group and an indication of the number of relative analogues or natural enzymatic metabolites. Table 1 lists the producers of the toxin, mechanism of action and effects in humans in addition to the current European Union (EU) reference methods of analysis and regulatory limits in shellfish meat applied in the monitoring regimes.

Currently, EU regulations stipulate that the reference methods for the detection of marine biotoxins are two distinct animal bioassays based on the hydrophilic [16] and lipophilic [17] solvents used for the extraction procedure. The detection of domoic acid is an exception where the reference method is high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [3,5]. HPLC with fluorimetric detection (HPLC-FLD) for saxitoxin and analogues [4] and an enzyme-linked immunosorbent assay (ELISA) for domoic acid [5] are officially accepted as screening methods but the reference methods for these toxins are the aforementioned.

However, this review also includes prospective emerging toxins to European waters [e.g., cyclic imines, palytoxin, tetrodotoxin, maitotoxin, ciguatoxins and neurotoxin-poisoning brevetoxins (Fig. 2)], as their occurrence could have severe implications with regards to seafood safety [18]. In addition, as the shellfish trade expands globally with increased exports and imports to and from regions of the world where these toxins are prevalent, effective monitoring methods will need to be in place within the EU. Although EFSA has published scientific opinions for emerging toxins (Table 2) [19–22], with the exception of tetrodotoxin, these toxins are not specified by the current EU regulations. At present, their detection is coincidental, as some co-extract with DSP or PSP toxins using the specified sample-preparation protocols for the EU-approved animal bioassays. However, in many other regions of the world, animal bioassays are the method of choice for monitoring these phycotoxins in various seafoods. This review discusses the problems

(a) PSP toxins (> 30 analogues) [85]



			Carbamate toxins	*N*-Sulfocarbamoyl toxins	Decarbamoyl (dc) toxins	Deoxydecarbamoyl (do) toxins
R_1	**R_2**	**R_3**	**R_4:** $OCONH_2$	**R_4:** $OCONHSO_3^-$	**R_4:** OH	**R_4:** H
H	H	H	Saxitoxin (STX)	B1 (GTX 5)	dc-STX	do-STX
H	H	OSO_3^-	Gonyautoxin (GTX) 2	C1	dc-GTX 2	do-GTX 2
H	OSO_3^-	H	GTX 3	C2	dc-GTX 3	do-GTX 3
OH	H	H	Neosaxitoxin (NEO)	B2 (GTX 6)	dc-NEO	
OH	H	OSO_3^-	GTX 1	C3	dc-GTX 1	
OH	OSO_3^-	H	GTX 4	C4	dc-GTX 4	

Figure 1. Chemical structure of the parent/reference toxin(s) for marine biotoxin groups regulated by the European Union.