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Variability of paralytic shellfish toxin occurrence and profiles in bivalve molluscs from Great Britain from official control monitoring as determined by pre-column oxidation liquid chromatography and implications for applying immunochemical tests

Andrew D. Turner *, Ben Stubbs, Lewis Coates, Monika Dhanji-Rapkova, Robert G. Hatfield, Adam M. Lewis, Stephanie Rowland-Pilgrim, Alison O'Neil, Patrycja Stubbs, Stuart Ross, Clothilde Baker, Myriam Algoet

Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, United Kingdom

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ABSTRACT

As the official control monitoring laboratory in Great Britain for the analysis of marine biotoxins in shellfish, Cefas have for the past five years conducted routine monitoring for paralytic shellfish poisoning toxins (PST) using a non-animal alternative method to the mouse bioassay reference method; a refined version of the AOAC 2005.06 pre-column oxidation liquid chromatography method. Application of this instrumental methodology has enabled the generation of data not only on the occurrence and magnitude of PST events, but also the quantitation and assessment of different PST profiles. Since implementation of the method in 2008, results have shown huge variabilities in the occurrence of PSTs, with large spatial and temporal variabilities around the coastline. Mean PST profiles were not found to correlate either with total PST content of the shellfish, the year of sampling or with a few notable exceptions, the shellfish species. Toxin profiles were found to fall into one of four distinct profile types, with one relating solely to the exclusive presence of decarbamoyl toxins in surf clams. The other profile types contained variable proportions of gonyautoxins, N-sulfocarbamoyl toxins, neosaxitoxin and saxitoxin. While some indications of geographical repeatability were noted, this was not observed for all profile types. Consequently, the application of rapid immunochemical testing methods to end product testing would need to be considered carefully given the large differences in PST congener cross-reactivities.

1. Introduction

Phytoplankton are photosynthetic organisms present at the bottom of the food chain in the aquatic environment. Their spatial and temporal variability is affected by a number of factors including climate, water circulation, temperature, nutrients and other localised parameters. Under optimum conditions, they go through periods of active growth, resulting in blooms. Filter feeding bivalve molluscs, such as mussels, oysters, scallops and clams rely on these organisms as a primary source of food. A large number of plankton genera and species are present within marine waters. While the majority of these species are non-toxic, specific organisms under certain conditions are found to produce a range of naturally occurring toxic chemicals. Consequently, harmful toxins and secondary metabolites may accumulate in the flesh of the filter-feeding molluscs, which typically have no adverse effects on the health of the shellfish. Contaminated shellfish may subsequently impact significantly on the health of the human shellfish consumer, with a number of naturally occurring toxins potentially causing very serious illness if enough of the shellfish is consumed and concentrations of toxins have accumulated to high enough levels within the shellfish flesh. A growing number of classes of marine phycotoxins have been identified to date and each contains a diverse range of typically structurally complex bioactive compounds. Groups of toxins known to occur around the waters of Great Britain, include the hydrophilic toxins, specifically the Saxitoxins and Domoic acid, responsible for Paralytic Shellfish Poisoning (PSP) and Amnesic Shellfish Poisoning (ASP) respectively. Also included are a number of lipophilic toxins, including Okadaic acid (OA), Dinophysistoxins (DTXs) and Azaspiracids (AZAs), responsible for Diarrhetic Shellfish Poisoning (DSP). A number of other lipophilic toxins are also detected in GB waters, such as the Pectenotoxins (PTXs), Yessotoxins (YTXs) and cyclic imines where there is little, if any, evidence for acute or chronic

* Corresponding author. Tel.: +44 01305 206636.
E-mail address: Andrew.turner@cefas.co.uk (A.D. Turner).

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toxicological effects following oral exposure. These groups of toxins possess notable structural differences from each other, with each group containing a large range of structurally related toxin analogues, numbers of which are expanding each year through the introduction of more specific and sensitive detection methods.

The PSP-producing Saxitoxins contain a range of more than 30 analogues, which are associated with a number of toxic phytoplankton, including the genera of *Alexandrium* species, together with *Gymnodinium* and *Pyrodinium*. The majority of PSP intoxications from marine origin around the world are thought to relate to the specific species *Gymnodinium Catenatum*, *Alexandrium tamarense*, *Alexandrium catenella*, *Alexandrium fundyense* and *Pyrodinium bahamense* (Shumway, 1990, 1995). Species of *Alexandrium* reported as occurring in the UK are those of *A. minutum*, *A. tamarense*, *A. ostenfeldi* and *A. tamutum* (Higman et al., 2001; Töbe et al., 2001; John et al., 2003; Davidson and Bresnan, 2009; Collins et al., 2009; Brown et al., 2010; Percy, 2006; Percy et al., 2008), whereas *G. catenatum* is currently not detected within the waters around GB (Davidson and Bresnan, 2009). The saxitoxin analogues are of great concern as they are potent neurotoxins which block the voltage-gated sodium channels in excitable cells, suppressing ion permeation. This action can subsequently cause a range of concentration-dependent rapid responses including facial tingling sensations and numbness, nausea, ataxia, dizziness, shortness of breath, paralysis and death through asphyxiation (Schantz, 1969; Halstead, 1978; FAO, 2004).

To comply with European Commission (EC) Regulations and to ensure consumer protection, monitoring of causative organisms in water and PSP toxins in shellfish from within classified harvesting areas is a statutory requirement for member states and for third countries wishing to export live shellfish to the European Union (EU). Water samples are typically monitored by light microscopy a technique that works well for detection of the three toxic genera of PSP-producing phytoplankton. However, while the presence of these algae is a good indicator of risk, this does not necessarily result in the presence of toxins within shellfish flesh. Some species of *Alexandrium* are found not to produce PSP toxins (e.g. SAMS, 2012), while toxin-producing species may in some cases not produce toxins (Touzet et al., 2007). In addition toxins may sometimes not be accumulated in the flesh of some shellfish species for various reasons including the presence of other plankton food sources (Bricelj and Sumway, 1998), the location of the shellfish within the water column or the feeding habits of the shellfish themselves (Sekiguchi et al., 2001). Consequently, there is the need to monitor for the presence of toxins within the shellfish flesh, for which a series of legal controls are imposed (Anon, 2004). EC Regulation 853/2004 describes the statutory limits of toxins in flesh for the three major groups of toxins, ASP, PSP and lipophilic toxins. For PSP, the maximum permitted level (MPL) or regulatory action limit (AL) is 800 μg saxitoxin equivalents (STX eq.) per kg of shellfish flesh (Anon, 2005b). In the United Kingdom (UK) including Great Britain (GB; Scotland, England and Wales) and Northern Ireland, the Food Standards Agency (FSA) is the competent authority responsible for official control monitoring. In Great Britain, the Centre for Environment, Fisheries and Aquaculture Science (Cefas) are contracted to carry out co-ordination of the official control monitoring programme and to deliver all the shellfish flesh testing for all regulated marine biotoxins, including PSP.

The European Union's (EU) reference method for detecting PSP toxins is the mouse bioassay (MBA) (Anon, 2005a and 2006). However, Cefas and the FSA have been committed to moving away from animal assays in the statutory monitoring programme and have in recent years pursued the development and implementation of sensitive and validated alternative methods to ensure the continued safety of the shellfish products while reducing and ultimately replacing the use of animals. An instrumental chemical method involving Liquid Chromatography with Fluorescence Detection (LC-FLD) and commonly referred to as the "Lawrence method" had been developed and gone through single and inter-laboratory validation (Lawrence et al., 2005). In 2005, this method was adopted by the AOAC as an official, first action method (method AOAC 2005.06) (Anon, 2005b) and was subsequently approved by the EU as an alternative to the MBA for those toxins and shellfish species detailed in the published validation reports (Regulation EC 2074/2005 as amended) (Anon, 2006a). While available for use as an allowable alternative method in official controls, the method required further refinement and validation before being approved for application to the UK official control monitoring of shellfish (Turner et al., 2009), as the method had to be shown to deal with complex and variable matrices as well as differentiate toxins from non-toxic compounds and from toxins of other groups (Thompson et al., 2002; Anon, 2006b). The increased commercial availability of analytical PSP standards also provided more tools to enable full and thorough assessment of method performance characteristics. In GB, a programme of method validation funded by FSA and Cefas was undertaken, to validate and quality assure the LC-FLD methods to a suitable level for official control testing. Initially, a qualitative LC-FLD method based on AOAC 2005.06 but using hydrochloric acid extracts prepared following the MBA extraction procedure (Anon, 2005a), was validated and shown to be suitable for the screening of shellfish prior to the quantitation of PSP toxicity using MBA in screen-positive samples only (Cefas, 2007). The full quantitative LC method was subsequently refined to improve performance and practicalities of use within a high throughput testing environment and validated between 2007 and 2010 for the 12 species of shellfish monitored routinely under the scope of the Official Control programme. The validation enabled the determination of LC-FLD method performance characteristics for mussels (*Mytilus edulis*), Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*), common cockles (*Cerastoderma edule*), hard clams (*Mercenaria mercenaria*), razor clams (*Ensis sp*), whole king scallops (*Pecten maximus*), whole queen scallops (*Aequipecten opercularis*), manila clams (*Ruditapes philippinarum*), otter clams (*Lutraria lutraria*), carpet shell clams (*Ruditapes decussatus*), surf clams (*Spisula solida*) and processed king scallops (adductor only and adductor and roe presentations). Validation demonstrated acceptable performance characteristics of the method for each species (Turner et al., 2009, 2010, 2011) while demonstrating method performance issues for scallops, resulting in further refinements and validation of a modified methodology for these species (Turner et al., 2012b). Subsequently the LC-FLD was implemented into the official control monitoring programme of GB. Specifically, in April 2008 the full quantitative AOAC 2005.06 method was implemented for mussel species, followed by implementation for cockles, hard clams and razors clams in June 2010. Following an additional period of investigation into the relative performance of the official methods for oysters (Turner et al., 2012a) the quantitative method was implemented for oyster species in June 2011, before the MBA was removed for all remaining species in August 2011. During the interim periods, the qualitative LC-FLD screen was used to identify the qualitative presence of PSP toxins in cockles, oysters and whole scallops, with remaining species being analysed by MBA alone.

Consequently, Cefas has now completed the fifth season of monitoring GB shellfish samples from designated harvesting locations using the AOAC 2005.06 LC-FLD method for determination of PSP in mussels. In addition, data has also been generated throughout this period for the quantitation of PSP in non-mussel samples by LC-FLD, in any samples shown to be PSP positive by either the LC-FLD screen or by MBA. This report describes the work done and summarises the results obtained from the first 5 years of monitoring. As well as enabling the determination of total saxitoxin