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Variability and profiles of lipophilic toxins in bivalves from Great Britain during five and a half years of monitoring: Okadaic acid, dinophysis toxins and pectenotoxins

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ABSTRACT

Official control biotoxin testing of bivalve molluscs from Great Britain has been conducted by Cefas for over a decade. Reflecting the changes in legislation, bioassays were gradually replaced by analytical methods, firstly for analysis of Paralytic shellfish toxins, followed by introduction of liquid chromatography tandem mass spectrometric (LC–MS/MS) method for lipophilic toxins (LTs) in 2011. Twelve compounds, representing three main groups of regulated lipophilic toxins, as well as two non-regulated cyclic imines were examined in over 20,500 samples collected between July 2011 and December 2016. The toxins belonging to Okadaic acid (OA) group toxins were the most prevalent and were quantified in 23% of samples, predominantly from Scotland. The temporal pattern of OA group occurrences remained similar each year, peaking in summer months and tailing off during autumn and winter, however their abundance and magnitude varied between years significantly, with concentrations reaching up to 4993 µg OA eq./kg.

Three toxin profiles were identified, reflecting the relative contribution of the two main toxins, OA and dinophysis toxin-2 (DTX2). Dinophysis toxin-1 (DTX1) was less common and was never detected in samples with high proportions of DTX2. Inter-annual changes in profiles were observed within certain regions, with the most notable being an increase of DTX2 occurrences in north-west Scotland and England in the last three years of monitoring. In addition, seasonal changes of profiles were identified when OA, the dominant toxin in early summer, was replaced by higher proportions of DTX2 in late summer and autumn. The profile distribution possibly reflected the availability of individual *Dinophysis* species as a food source for shellfish, however persistence of DTX2 during autumn and winter in mussels might have also been attributed to their physiology. Mussels were the only species with higher average proportions of non-esterified toxins, while Pacific oysters, cockles, surf clams, razors and queen scallops contained almost exclusively ester forms. In addition, a temporal change in proportion of OA and DTX2 free form was observed in mussels.

Pectenotoxin-2 (PTX2) was quantified only on rare occasions.

1. Introduction

Human intoxications caused by consumption of shellfish contaminated with phycotoxins have been reported for decades. Monitoring programmes have been set up worldwide to mitigate the risk and prevent illness and even deaths to the consumer. Within the European Union (EU), shellfish classified production and relaying areas are required to be monitored and tested for a range of possible contaminants, including marine biotoxins (Anon, 2004a). This forms part of a range of control measures aiming at ensuring the safety of bivalve molluscs. Currently there are three main groups of regulated marine biotoxins in the EU: domoic acid, responsible for Amnesic Shellfish Poisoning (ASP), saxitoxin and its derivatives, responsible for Paralytic Shellfish Poisoning (PSP) and the lipophilic toxins (LTs) group which itself represents several sub-groups of polyether compounds which possess similar extractability in organic solvent. Okadaic acid (OA) (Murakami et al., 1982; Tachibana et al., 1981) and related analogues dinophysis toxins (DTXs) (Hu et al., 1992; Murata et al., 1982) were the first LTs identified and linked to Diarrhetic Shellfish Poisoning (DSP) (Yasumoto et al., 1984). The pectenotoxins (PTXs) were originally

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included in the DSP toxin group due to their high acute toxicity in the mouse bioassay (MBA) after *i.p.* administration (Yasumoto et al., 1985). However, toxicology studies indicate that PTXs are much less toxic via the oral route, have a different mode of action and do not induce diarrhoea (Ito et al., 2008; Miles et al., 2004). Consequently, the European Food Safety Authority (EFSA, 2009a) recommended that the toxicity of PTXs should not be expressed as OA equivalents. Nevertheless, due to co-occurrence of PTXs alongside OA and DTXs, PTX1 and PTX2 remain included in the OA-group with a total regulatory limit 160 µg OA eq./kg (Anon, 2004b). Similar limits have been adopted worldwide, although PTXs have been de-regulated in some countries outside the EU (Codex Alimentarius, 2015).

Yessotoxins (YTXs) and Azaspiracids (AZAs) represent another two groups of regulated LTs however their global occurrence in shellfish is less prevalent. OA and DTXs are by far the most abundant and geographically widespread LTs, having been implicated in poisoning outbreaks or harvesting bans in Japan (Suzuki and Watanabe, 2012; Yasumoto et al., 1978), China (Li et al., 2012), around the Mediterranean Sea (Ciminiello et al., 2014; Gladan et al., 2011; Prassopoulou et al., 2009), along the western European coast (Kumagai et al., 1986; Ramstad et al., 2001; Vale et al., 2003; Villar-González et al., 2007), Great Britain (Hinder et al., 2011), Ireland (Carmody et al., 1995), Canada (Taylor et al., 2013), USA (Deeds et al., 2010), Mexico (García-Mendoza et al., 2014), Chile (Lembeye et al., 1993) and Argentina (Gayoso et al., 2002; Turner and Goya, 2015).

The qualitative MBA based on Yasumoto et al. (1978) was the first method utilised in monitoring programmes. Method limitations are well recognised, due to false positives from matrix co-extractives or other non-DSP toxins as well as sensitivity issues and a lack of specificity (Fernández et al., 2003; Suzuki et al., 1996; Takagi et al., 1984). The publication of a chemical detection method using High Performance Liquid Chromatography (HPLC) for quantitation of OA and DTX1 (Lee et al., 1987) facilitated the development of a range of detection methods using either conventional HPLC or HPLC coupled with mass spectrometry (HPLC-MS). Although the chemical methods were often applied to samples from a limited time period and from small geographical areas, they became essential to investigating intoxication incidents (Hossen et al., 2011), characterising new toxin analogues in both shellfish and algae (Draisci et al., 1998; Hu et al., 1992; James et al., 1997), assessing toxin biotransformation in shellfish (Marr et al., 1992; Suzuki and Mitsuya, 2001; Vale and Sampayo, 1999) and understanding the link between toxin accumulation in shellfish and algae source (Bravo et al., 2001; Jørgensen and Andersen, 2007).

HPLC based methods marked a significant progress compared to bioassays but Liquid Chromatographic tandem Mass Spectrometric (LC−MS/MS) separation was the only technique capable of the specific, sensitive and simultaneous quantitation of all regulated LTs. Advances in technology, specifically the introduction of ultra-high performance liquid chromatography (UHPLC) and rapid polarity switching on modern MS instruments, together with the increased availability of toxin standards significantly improved the potential of LC−MS/MS to replace bioassays as the reference method, providing its performance was validated following international guidelines (Anon, 2005). The first single laboratory validation studies for LT LC−MS/MS were conducted by McNabb et al. (2005) and Stobo et al. (2005), followed by collaborative studies (EURLMB, 2011; These et al., 2011; Van den Top et al., 2011). In the EU the LC−MS/MS method described by the European Union Reference Laboratory for Marine Biotoxins (EURLMB) was finally adopted as the reference method for detection and quantitation of marine LTs on 1st July 2011 (Anon, 2011). The LT method involves the two-step methanolic extraction of homogenised shellfish tissue, prior to filtration and alkaline hydrolysis to liberate esterified OA-group toxins. LC−MS/MS analysis of filtered crude extracts and hydrolysed extracts enables the determination of both freely occurring LTs and total OA-group toxins (free plus esterified). The use of the DSP mouse and rat bioassays were disallowed within the EU for official control testing purposes of live bivalve molluscs on 31st December 2014. In some European countries, LC−MS/MS had complemented the MBA for a number of years prior to the change in legislation. Comprehensive data on OA group profiles in shellfish were reported from Ireland (Fux et al., 2009; Hess et al., 2003), Spain (Villar-González et al., 2007) and Portugal (Vale et al., 2008). A detailed and systematic analysis of LT data in bivalves from Great Britain (GB) in recent years has yet to be published. LTs, and OA group toxins in particular are detected in GB shellfish every year and have triggered numerous, and in some instances lengthy harvesting bans of shellfish production areas. The financial impact of toxic events on the shellfish industry is not the only concern as LTs have been responsible for several human intoxication incidents in GB in the last 20 years (COT, 2006; FSA, 2013; Scoging and Bahl, 1998).

Cefas has conducted routine official control (OC) biotoxin testing of bivalve molluscs from England and Wales since 2001, and from Scotland since 2005. Shellfish are tested using methods specified in legislation and accredited to ISO17025 standard by the United Kingdom Accreditation Service (UKAS). Standard Operating Procedures (SOPs) are in place to cover every aspect of the sample treatment and analysis. Cefas implemented the LC−MS/MS method for OC testing on 4th July 2011. Consequently, here we present data on the abundance, concentrations, toxin profiles, temporal and geographical distribution of LTs in shellfish from Great Britain collected between July 2011 and December 2016, focusing on the OA group toxins in this paper, specifically OA, DTX1, DTX2, as well as Pectenotoxins PTX1 and PTX2. The remaining two groups of regulated LTs (AZAs and YTXs), which are known to be produced by different *Dinophyceae* genera and have different toxicological activity compared to OA group toxins, will be presented separately.

2. Material and methods

2.1. Samples

Shellfish samples were collected from representative monitoring points (RMPs), prior to and during periods of active harvesting within classified shellfish production or relaying areas (FSA classification listing; FSS classification listing). On average, 171 ± 9 monitoring points were active each year, 81 ± 3 in Scotland, 75 ± 8 in England and 15 ± 4 in Wales (Fig. 1). Although the number of RMPs in Scotland and England was similar, 77.1% of all samples tested for LTs originated from Scotland with only 19.4% from England and 3.5% from Wales. The differences reflect risk-based sampling frequencies (either weekly, fortnightly or once every four weeks), as defined by the competent authorities (FSA toxin pages; FSS toxin pages). In cases where monitoring was either fortnightly or four-weekly, the frequency was increased to weekly if results of shellfish or phytoplankton monitoring indicated an increased level of risk. Collection was conducted by designated sampling officers with live shellfish transported chilled to Cefas using approved and validated cool-boxes.

In total 20,516 samples were tested for LTs by LC–MS/MS between July 2011 and Dec 2016, which constituted 95% of all received OC samples during this period. On average, 2860 samples were analysed each year from Scotland, 630 from England and 130 from Wales. In 2016, samples from England increased by 75% following a revised risk assessment.

Seven major bivalve molluscs species were collected (Fig. 2), including common mussels (*Mytilus* spp.) (67%), Pacific oysters (*Magallana gigas,* formerly *Crassostrea gigas*) (18%), common cockles (*Cerastoderma edule*) (5.9%), razor clams (*Ensis* spp.) (4.9%), native oysters (*Ostrea edulis*) (1.7%), surf clams (*Spisula solida*) (1.1%) and hard clams (*Mercenaria mercenaria*) (0.85%). The remaining 0.55% of samples received were comprised of manila clams (*Ruditapes philippinarum*), carpet clams (*Ruditapes decussatus*) and queen scallops (*Aequipecten opercularis*). King scallops, received as a part of the *Pectinidae*