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Variability and profiles of lipophilic toxins in bivalves from Great Britain during five and a half years of monitoring: Azaspiracids and Yessotoxins

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Abstract

Cefas has been responsible for the delivery of official control biotoxin testing of bivalve molluscs from Great Britain for just over a decade. Liquid chromatography tandem mass spectrometric (LC-MS/MS) methodology has been used for the quantitation of lipophilic toxins (LTs) since 2011. The temporal and spatial distribution of Okadaic acid group toxins and profiles in bivalves between 2011 and 2016 have been recently reported. Here we present data on the two other groups of regulated lipophilic toxins, azaspiracids (AZAs) and yessotoxins (YTXs), over the same period. The latter group has also been investigated for a potential link with Protoceratium reticulatum and Lingulodinium polyedra, both previously recognised as YTXs producing phytoplankton. On average, AZAs were quantified in 3.2% of all tested samples but notable inter-annual variation in abundance was observed. The majority of all AZA contaminated samples were found between July 2011 and August 2013 in Scotland, while only two, three-month long, AZA events were observed in 2015 and 2016 in the south-west of England. Maximum concentrations were generally reached in late summer or early autumn. Reasons for AZAs persistence during the 2011/2012 and 2012/2013 winters are discussed. Only one toxin profile was identified, represented by both AZA1 and AZA2 toxins at an approximate ratio of 2:1, suggesting a single microalgal species was the source of AZAs in British bivalves. Although AZA1 was always the most dominant toxin, its proportion varied between mussels, Pacific oysters and surf clams. The YTXs were the least represented group among regulated LTs. YTXs were found almost exclusively on the south-west coast of Scotland, with the exception of 2013, when the majority of contaminated samples originated from the Shetland Islands. The highest levels were recorded in the summer months and followed a spike in Protoceratium reticulatum cell densities. YTX was the most dominant toxin in shellfish, further strengthening the link to P. reticulatum as the YTX source. Neither homo-YTX, nor 45-OH homo-YTX were detected throughout the monitored period. 45-OH YTX, thought to be a shellfish metabolite associated with YTX elimination, contributed on average 26% in mussels. Although the correlation between 45-OH YTX abundance and the speed of YTX depuration could not be confirmed, we noted the half-life of YTX was more than two-times longer in queen scallops, which contained 100% YTX, than in mussels. No other bivalve species were affected by YTXs. This is the first detailed evaluation of AZAs and YTXs occurrences and their profiles in shellfish from Great Britain.
over a period of multiple years.

**Keywords**

Azaspiracids, Yessotoxins, *Protoceratium reticulatum*, LC-MS/MS, Great Britain, Shellfish

**1. Introduction**

Azaspiracids (AZAs) are the most recently discovered group of regulated lipophilic toxins (LTs) produced by marine microalgae, following intoxication events in Europe during the 1990s (McMahon and Silke, 1998, 1996). Although AZAs cause acute symptoms in humans similar to that of classic Diarrhetic Shellfish Poisoning (DSP) toxins, including nausea, vomiting, diarrhoea and stomach cramps (McMahon and Silke, 1996), they are not protein phosphatase inhibitors (Flanagan et al., 2001). Numerous studies report the toxic effects of AZAs at the organ, cellular and molecular level, yet their primary mode of action *in vivo* is still not clear. Interestingly, toxic effects were found to be more severe using crude shellfish extracts compared to purified toxins (Flanagan et al., 2001; Furey et al., 2010). Recently, it has been proved that a combination of AZA and glutaric acid (both found in mussels) is capable of inhibiting the activity of voltage-gated sodium channels (Chevallier et al., 2015).

The chemical structure of AZAs was described as a polyether skeleton with a tri-spiro ring and an azaspiro ring assembly, together with a terminal carboxylic acid function (Nicolaou et al., 2006; Satake et al., 1998). To date, approximately 60 variants have been identified, twenty six of which have been so far confirmed to be produced by four planktonic species of the Dinoflagellate taxon *Amphidomataceae* (*Azadinium spinosum*, *Azadinium poporum*, *Azadinium dexteroporum* and *Amphidoma languida*): AZA1, AZA2, epi-AZA7, AZA11, AZA33-43, AZA50-59 and AZA62 (Kilcoyne et al., 2014b; Kim et al., 2017; Krock et al., 2019, 2012, 2009; Percopo et al., 2013; Rossi et al., 2017; Tillmann et al., 2018, 2017, 2009). There is increasing evidence that the genus *Azadinium* is distributed worldwide (Tillmann et al., 2014). As such, AZAs were reported in shellfish from numerous European countries: Ireland (Furey et al., 2003; Ofuji et al., 1999a), England (James et al., 2002), Norway (Aasen et al., 2004; James et al., 2002), France (Amzil et al., 2008; Braña Magdalena et al., 2003), Spain (Blanco et al., 2017; Braña Magdalena et al., 2003; Tillmann et al., 2017), Portugal (Vale et al., 2008b, 2008a) and Italy (Bacchiocchi et al., 2015). In addition, they were found in shellfish from the North African Atlantic coast (Elgarch et al., 2008; Taleb et al., 2006), China (Yao et al., 2010), North America (Trainer et al., 2013) and South American countries, including Chile (Álvarez et al., 2010; López-Rivera et al., 2010) and Argentina (Turner and Goya, 2015). The majority of reports on AZAs occurrences in bivalves are short-term evaluations and/or from limited geographical areas. The exception is the long-term assessment of AZAs in Ireland, facilitated through two major projects, Astox and Astox II (Hess et al., 2007; Kilcoyne et al., 2014a).

Ladder-shaped poly cyclic ethers yessotoxins (YTXs) were described for the first time three decades ago in Japan (Murata 1987), after inflicting high acute toxicity in the mouse bioassay (MBA). YTXs were originally included in the DSP group, although toxicological studies later revealed that they do not induce diarrhoea and are much less toxic after oral (p.o.) as opposed to intraperitoneal (i.p.) administration (Aune et al., 2002; Tubaro et al., 2003). The effects at
cellular and molecular level have been reported (Alfonso et al., 2003; Callegari et al., 2006; de la Rosa et al., 2001; Korsnes et al., 2011), yet the mechanism of YTX toxicity in animals is not well understood. YTX is the main toxin produced by the dinoflagellate Protoceratium reticulatum (Ciminiello et al., 2003; Eiki et al., 2005; Paz et al., 2007; M Satake et al., 1997), although homo-YTX was also found to be dominant in certain strains (Konishi et al., 2004; Paz et al., 2007; Suzuki et al., 2007) and the toxin profile can be complex (Miles et al., 2006, 2005). Dinoflagellates Lingulodinium polyedra (Draisci et al., 1999; Paz et al., 2004), Gonyaulax spinifera (Rhodes et al., 2006; Riccardi et al., 2009) and Gonyaulax taylorii (Álvarez et al., 2016) have also been linked to production of YTXs. More than 100 YTX analogues have been described so far, some produced by algae and some, mainly hydroxylated and carboxylated derivatives, as products of metabolic transformation in bivalves (Aasen et al., 2005; Ciminiello et al., 2010, 2000, 1999; Finch et al., 2005). Shellfish contaminated by YTXs have been found globally: in Japan (Murata et al., 1987), China (Liu et al., 2017), Norway (Aasen et al., 2005; Dalguji et al., 1998), United Kingdom (Stobo et al., 2008), France (Amzil et al., 2008), Italy (Ciminiello et al., 1997; Masayuki Satake et al., 1997), Morocco (Haddouch et al., 2017), Canada (Finch et al., 2005), Mexico (García-Mendoza et al., 2014), Chile (Yasumoto and Takizawa, 1997), Argentina (Turner and Goya, 2015), and New Zealand (Finch et al., 2005).

AZAs and YTXs were first treated legislatively as a separate group of toxins in 2002 when AZA1, AZA2 and AZA3, and YTX, homo-YTX, 45-OH YTX and 45-OH homo-YTX became regulated within the European Union (EU) (Anon, 2002). Although the reference method at that time was a qualitative and non-specific DSP mouse bioassay (MBA), Maximum Permitted Levels (MPL) were set at 1 mg YTX eq./kg and 160 µg AZA eq./kg of whole shellfish flesh, respectively. For the AZAs MPL, the MBA was capable of detecting AZAs with 95% probability, although the probability fell sharply to only 5% for detecting AZAs concentrations at 0.5 MPL (Hess et al., 2009). The suitability of the DSP bioassay as reference method for AZAs and YTXs detection was questioned not only because of its low sensitivity, but also due to discrepancy in YTXs’ acute toxicities after oral and i.p. administration (Aune et al., 2002; Tubaro et al., 2003). The lack of human intoxications by YTXs prompted an increase of the regulatory limit to 3.75 mg YTX eq./kg in September 2013 (Anon, 2013).

Considerable efforts have been made in the last 20 years in relation to AZAs and YTX detection, resulting in the development of specific, selective and sensitive chemical methods, predominantly based on liquid chromatographic separation followed by tandem mass spectrometric analysis (LC-MS/MS). The first LC-MS/MS method for AZAs analysis was reported by Ofuji et al. (1999b) and for YTXs by Draisci et al. (1998), however advances in technology, together with the increased availability of toxin standards, soon enabled multi-toxin approach analysis. The advantage of simultaneous quantification of several groups of regulated LTs has made LC-MS/MS by far the most suitable candidate to replace bioassays as the reference method. After the multi-toxin LC-MS/MS analysis was proposed by Quilliam et al. (2001), it was further developed and validated using acidic (McNabb et al., 2005), neutral (McCarron et al., 2011; Stobo et al., 2005), moderately alkaline (These et al. 2011, 2009) and alkaline chromatographic conditions (Gerssen et al., 2009). In the European Union (EU), the
LC-MS/MS analysis described by the European Union Reference Laboratory for Marine Biotoxins (EURLMB) was adopted as the reference method for detection and quantitation of marine LTs on 1st July 2011 (Anon, 2011). The use of the DSP mouse and rat bioassays was disallowed within the EU for official control testing purposes of live bivalve molluscs on 31st December 2014.

Cefas has conducted routine official control (OC) biotoxin testing of bivalve molluscs from England and Wales since 2001, and from the whole of Great Britain (England, Wales and Scotland) since 2005. The implementation of the LC-MS/MS method for OC testing on 4th July 2011 enabled the systematic and long-term collection of quantitative and toxin-specific data, over this large geographical area. For the first time in Great Britain, we have been able to evaluate these results from different perspectives, creating a platform for new hypotheses and focused studies. A summary on the prevalence of OA group toxins and Pectenotoxins between 2011 and 2016 was recently published (Dhanji-Rapkova et al., 2018). Here we analyse the abundance, temporal and geographical distribution, and the toxin profiles of the other two groups of regulated LTs: AZAs and YTXs. In addition, potential relationships between YTXs in shellfish and YTXs producing algae, *Protoceratium reticulatum* and *Lingulodinium polyedra*, have been explored in Great Britain for the first time over a period of multiple years.

2. Material and methods

2.1 Shellfish 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. 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. 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 of samples in Scotland was conducted by designated sampling officers or under their supervision, while in England it was conducted by local authority staff. Live samples were 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 December 2016, which constituted 95% of all received OC samples during this period. The remaining 5% was not tested for LTs, either because the analysis was not required according to the routine testing schedule, or the samples originated from production areas already closed for harvesting due to other regulated toxins breaching MPLs. On average, 2,860 samples were analysed each year from Scotland, 630 from England and 130 from Wales. In 2016, samples from England increased by 75% for risk assessment purposes. Ten bivalve molluscs species were collected and their respective proportions in each country are presented in Table 1. The
proportion of samples from England, Wales and Scotland and the proportion of species within each country remained consistent between the years (data not shown). Offshore scallops, received as a part of the Pectinidae monitoring, were not included in the data set.

2.2. Phytoplankton samples

Seawater samples were collected from classified shellfish production areas, the sampling method being either pole sampler, tube or a bucket, depending on the depth of water at each site. The sampling tube allows for the collection of a depth-integrated water sample from 0 to 10 m. A well-mixed 500 mL water sample was fixed on site, to obtain a final concentration of approximately 1% acidic Lugol’s iodine, and then sent to the relevant laboratory for analysis, the Scottish Association for Marine Science (SAMS) for Scottish samples, or Cefas Lowestoft for samples originating from England and Wales. The phytoplankton in either a 50 mL or 25 mL sub-sample was allowed to settle onto the base plate of a chamber for a minimum of 20 h before analysis, following the method described by Utermöhl (1958). Both Protoceratium reticulatum and Lingulodinium polyedra (formerly known as L. polyedrum or Gonyaulax polyedra), were identified and enumerated using inverted light microscopy and concentrations were reported as cells/L, with a detection limit of 20 cells/L or 40 cells/L, depending on the volume of subsample. Genera Azadinium or Amphidoma are not currently monitored by the UK laboratories, due to their small size and difficulty in accurately identifying them to even genus level by light microscopy.

2.3. Analytical methods

2.3.1. Reagents and chemicals

Methanol used for sample extraction was of HPLC grade. Water and acetonitrile used for LC–MS/MS mobile phases and instrument wash reagents were of LC–MS grade. Sodium hydroxide (VWR International Ltd, UK) and hydrochloric acid (Fisher Scientific UK Ltd) were analytical grade. Mobile phase additives, 25–31% ammonium hydroxide and ammonium hydrogencarbonate, were of LC–MS grade (Sigma-Aldrich, Poole, England). Certified reference materials for AZA1, AZA2, AZA3 and YTX were purchased from the Institute of Biotoxin Metrology, National Research Council Canada (NRCC, Halifax, Nova Scotia, Canada). Primary toxin standards were diluted in 100% methanol to form concentrated stock standard solutions prior to further dilution for production of calibration standards in two concentration ranges, depending on the instrumentation used. For the Quattro micro and Xevo TQ the concentration range was 1.6–40 μg/L (AZAs) and 10–250 μg/L (YTX). For the more sensitive Xevo TQ-S, the concentration range was 0.16–4 μg/L (AZAs) and 1–25 μg/L (YTX).

2.3.2. Shellfish sample preparation and extraction

Live shellfish samples were shucked and homogenised to provide a minimum of 100 g of homogenate. Aliquots (2.0 ± 0.01 g) of each sample were weighed into 50 mL polypropylene centrifuge tubes and subjected to an extraction procedure based on the EURLMB protocol (EURLMB, 2015). Methanol (9 mL) was added and vortex mixed for 3 min before centrifuging for 8 min (3,500 rpm). After the supernatants were collected, the remaining pellets were subjected to a second extraction step whereby 9 mL of methanol was added and the content was mixed with Ultra Turrax for 1 min (11,000 rpm) before centrifuging again for
8 min (3,500 rpm). The supernatants from the two extractions were combined in 20 mL volumetric flasks and diluted to 20 mL with methanol prior to filtering with 0.2 μm nylon filters (Phenomenex, Manchester, UK). Prior to analysis, extracts for the Xevo TQ-S were diluted ten-fold with methanol to reduce matrix effects. Despite the dilution, the sensitivity of lipophilic toxins on the Xevo TQ-S instrument remained comparable or better than on the Xevo TQ. Each day, a negative control procedural blank and positive control laboratory reference material (LRM) were co-extracted for quality control assessment.

2.3.3. Liquid chromatography and tandem mass spectrometry

Three different LC–MS/MS instruments were used throughout the study period. An Agilent 1100 High Performance Liquid Chromatographic (HPLC) system (Agilent, Manchester, UK) was coupled to a Quattro Micro triple quadrupole mass spectrometer (MS/MS; Waters Ltd., Manchester, UK). Due to long HPLC run times and limited sample throughput on this instrument, this was utilised only as a backup instrument until February 2013. Two Ultra Performance Liquid Chromatographic (UPLC) systems (Acquity and Acquity I-class) were coupled to a Xevo TQ and Xevo TQ-S triple quadrupole mass spectrometer respectively (Waters Ltd., Manchester, UK). The alkaline (pH 11) LC method described by Gerssen et al. (2009) was adopted with modifications and subjected to single laboratory validation prior to implementation into the routine biotoxin monitoring programmes. Mobile phase A comprised of 2mM ammonium bicarbonate adjusted to pH 11 +/− 0.2 with ammonium hydroxide. Mobile phase B was 2mM ammonium bicarbonate in 90% acetonitrile, also adjusted to pH 11 +/− 0.2 with ammonium hydroxide. Chromatographic conditions for all three LC–MS/MS systems were the same as detailed in (Dhanji-Rapkova et al., 2018). MS conditions for both Waters Xevo instruments are outlined in Supplementary material 1.

2.3.4. LT quantitation

Instrument data were analysed using MassLynx™ v.4.1 (Waters Ltd.). LC–MS/MS performance was checked by applying quality control criteria outlined in internal Standard Operating Procedures (SOPs) and the EURLMB SOP (EURLMB, 2015). External calibrations generated from the analysis of calibrant solutions were used for the quantitation of all target compounds, except for YTXs, where YTX was used for the quantitation of homo-YTX, 45−OH YTX and 45−OH homo-YTX. Individual toxin concentrations were calculated and only results above the designated Reporting Limit (RL) were subjected to further calculations. The RL was defined as either the Limit of Quantitation (LOQ) or the concentration of the lowest calibration standard, whichever was higher. RL for AZA1, AZA2 and AZA3 was set at 16 μg/kg and RL for YTX and analogues was set at 0.1 mg/kg for all tested shellfish matrices. Toxicity Equivalent Factors (TEFs) recommended by the European Food Safety Authority (EFSA, 2009) were applied and toxin concentrations were subsequently summed into representative groups as stipulated by EU legislation (Anon, 2004). The AZA group included AZA1, AZA2 and AZA3 and the combined concentration was expressed in μg AZA eq./kg. The YTX group included YTX, homo YTX, 45−OH YTX and 45−OH homo-YTX and the combined concentration was expressed in mg YTX eq/kg.

2.4. Results reporting and data assessment

Total results for the AZA and YTX groups were reported daily (alongside the OA group) to
the competent authorities, together with a lower and a higher total result calculated from the contributions of validated levels of Measurement of Uncertainty (MU). For the purpose of compliance, enforcement actions were taken on the higher value, but for the purpose of this review, the actual total values were utilised. Individual toxin concentrations were not reported to the competent authorities but were recorded and used in this study.

Total AZA group equivalents (μg AZA eq./kg) and total YTX group equivalents (mg YTX eq./kg) were used for assessment of inter-annual variability in abundance, as well as temporal and spatial distribution. In addition, K-means clustering algorithms were developed in Excel and applied to the relative proportions of AZAs (AZA1, AZA2, AZA3), as well as YTXs (YTX, homo-YTX, 45–OH YTX, 45–OH homo-YTX) in each positive sample. Specific clusters of toxin profiles were identified through generation of minimum distances of each toxin profile to a corresponding cluster centre (Turner et al., 2014). Results utilised for the assessment of toxin profiles were absolute quantified values with no TEFs applied. Statistical analyses were performed using R software (R Core Team, 2018). Maps were created by Cefas, using licenced Mapinfo software.

3. Results and discussion

3.1. Inter-annual variability of azaspiracids

On average, 3.2% of all tested samples contained AZA group toxins above RL (Table 2), however, 94% of those were recorded in the first three years of the study period. AZAs occurrence in shellfish was most prevalent in 2011, with the highest number of samples above MPL (22) and with the highest concentration maximum recorded (518 μg AZA eq./kg). In contrast, AZAs above RL were found only in 39 samples in the last three years of the monitored period (Table 2).

Similar year-to-year differences in abundance were reported from Ireland, where AZAs have been analysed systematically by LC–MS/MS since 2002. While AZAs were not detected or were found only at low levels between 2003 and the first half of 2005, they were prevalent in the subsequent years until 2013 (Salas et al., 2011; Tillmann et al., 2014). In 2014, AZAs were found mainly between January and June, with no new event observed in summer 2014. Only 25 samples from the Irish monitoring programme exceeded the RL between 2015–2017, which was in stark contrast to some previous years (Dave Clarke, Marine Institute, Ireland, personal communication). In Portugal, AZAs were examined in bivalves between 2002 and 2005, but more comprehensively only during 2006, when low levels were reported for the first time, and after implementing a more effective extraction procedure (Vale et al., 2008b, 2008a).

The only peer-reviewed publication on AZAs occurrences in bivalves from Scotland reports a low prevalence between March 2003 and September 2004, when out of 366 samples, 28 (7.7%) were found to be positive (Stobo et al., 2008). However, the abundance was not evaluated for each year separately and no details were offered on AZAs temporal distribution, making comparison with our data challenging. Our data from five and a half years of monitoring may not be sufficient for long-term trend analysis, especially when considering the unpredictable nature of AZAs occurrences in British shellfish.
3.2. Seasonal variability of azaspiracids

AZA group toxins did not follow a regular temporal distribution, even during years when they were prevalent (Fig. 2). AZAs were present from July/August 2011 and peaked in October. Their continuous presence for eight to twelve weeks was observed in as many as 14 RMPs. AZAs persisted through the winter 2011/2012 and were present in low quantities until July 2012. The concentrations started to increase again in late July 2012, reaching maximum levels of 433 μg AZA eq/kg in August. This marked another important AZA event, lasting continuously for >28 weeks at nine RMPs, with various concentrations below MPL recorded until summer 2013. During the two years that followed, until summer 2015, AZAs were quantified only in ten samples. The two, approximately three-month long, AZA events were observed in 2015 (late July – late October) and 2016 (September – December), with the highest concentrations recorded in September.

For a more detailed study of the temporal distribution of AZAs, occurrences in six selected RMPs between 2011 and 2013 were compared (Fig. 3). In the first year monitored, AZAs were present only in northern parts of Scotland, showing a spike in concentrations in early autumn (Fig. 3A, B, C). AZAs were slow to depurate and persisted at low levels throughout winter in the Shetland Islands (Fig. 3A) and Sutherland (Fig. 3B), whereas they were present for only three months in Lewis and Harris (Fig. 3C). The islands of Lewis and Harris are located further to the west than the other two areas, and local conditions may have had an influence on AZAs levels in bivalves. The importance of geographical location was further evidenced by a lack of AZAs in 2011 in other areas situated further south (Fig. 3D, E, F). Despite the absence of a clear spike in concentrations in late summer/early autumn 2012, AZAs appeared in late autumn/winter 2012 and kept fluctuating until late spring 2013.

During the 2012/2013 AZA season along the west coast of Scotland (Fig. 3D, E, F), toxin maxima were recorded in the winter months. In Ireland, the AZA event in 2012 was particularly important, in terms of maximum concentrations reached as well as geographical area affected: AZAs were found mainly between June and November 2012, with some sites affected up to spring 2013, and with maxima recorded in various months, depending on location (Tillmann et al., 2014; Dave Clarke, Marine Institute, Ireland, personal communication). The persistence of AZAs until spring 2013 in certain RMPs was observed in both Ireland and Scotland, suggesting that the 2012/2013 AZA events in these two countries were connected. As such, detailed studies from Killary Harbour in Ireland, exploring the link between A. spinosum and AZAs accumulation in mussels, could provide a possible explanation on fluctuating levels and/or AZAs winter occurrences in Scotland. Azadinium spinosum densities in Killary Harbour, Ireland, oscillated between July and September 2012, which was also reflected in fluctuating toxin levels in shellfish (Tillmann et al., 2014). It was therefore suggested that AZAs in shellfish may be considered a useful indicator for the temporal distribution of toxin-producing Azadinium and consequently shellfish intoxication might not be a result of a single Azadinium bloom, but possibly reflect multiple blooms, advected inshore in pulses (Tillmann et al., 2014). In contrast, an A. spinosum bloom in Killary Harbour showed a different dynamic in July-August 2013, with a sharp increase and decrease in densities, followed by a gradual decline, whilst AZAs in shellfish continued to fluctuate (Kilcoyne et al., 2014a). A rapid increase in toxin levels in mussels was
demonstrated in the Killary Harbour study and also in controlled conditions after being exposed to *A. spinosum* cells (Jauффrais et al., 2012; Salas et al., 2011), although toxin levels may increase even when *A. spinosum* has declined. Since *A. spinosum* was not monitored in Killary Harbour between October 2012 and May 2013, the continuous presence of AZAs during the following winter months in certain Irish sites cannot be explained and extrapolated to the situation in Scotland.

A previous study of *A. spinosum* in culture confirmed its ability to grow in a wide range of temperatures, with the growth significantly slower at 10 °C (Jauффrais et al., 2013b). The toxin quota at 10 °C was more than 20 times higher than at temperatures between 18 °C and 26 °C (Jauффrais et al., 2013b). Nevertheless, blooms were less likely to occur between December and April, when sea surface temperature (SST) in Britain generally fell below 10 °C. However, in the absence of a bloom, shellfish were able to uptake AZAs, once *Azadinium* cells entered senescent phase and toxins were released in the water (Jauффrais et al., 2013a). Indeed, AZA1 increased in colder months (November - February) in passive samplers deployed at Loch Ewe and Scapa in Scotland between 2005 and 2013, whilst the lowest concentrations were usually observed in July (Bresnan et al., 2016). In 2013 however, a series of consecutive monthly positive anomalies were recorded at both sites between January and August 2013 (Bresnan et al., 2016). Such unusually high levels of dissolved AZA1 in water could possibly explain sustained AZAs concentrations in shellfish observed in nearby locations during this period (Fig. 3D, E, F). SST at both Loch Ewe and Scapa remained below 10 °C between January and April in all monitored years (Bresnan et al., 2016). Such conditions were less favourable for *Azadinium* blooms (Jauффrais et al., 2013b), even more so during 2013 with low SST and low salinity anomalies at both sites (Bresnan et al., 2016). Consequently, in the winter months of 2012/2013, AZAs in bivalves were most probably sourced from water, rather than through filter feeding on AZA producers. At some RMPs (Fig. 3B, E, F), where AZAs started to appear as late as Nov/Dec 2012, without a spike in previous months, a physical transport of a senescent bloom or dissolved AZAs from another location may be suggested. Another plausible explanation for winter persistence of AZAs is a decreased ability of bivalves to depurate them during colder months (Tillmann et al., 2014). The potential for the transfer of AZAs through pelagic vector organisms, including larger heterotrophic dinoflagellates and zooplankton feeding on *Azadinium*, has also been discussed (Kilcoyne et al., 2014a; Krock et al., 2009).

Although the 2012/2013 winter occurrence of AZAs in Scotland may be unusual, it is not the first to be reported. In a year-long study between Sept 1999 and Sept 2000, mussels from Bantry Bay, Ireland, were found to contain AZAs between December and February, with some samples exceeding MPL (Furey et al., 2003). Likewise, following significant AZA events in 2012 and 2013, AZAs were found during the winters of 2012/2013 and 2013/2014 in Ireland (Dave Clarke, Marine Institute, Ireland, personal communication). Until more data are gathered, the reasons for winter accumulation of AZAs in bivalves remain unclear.

### 3.3. Spatial distribution of azaspiracids

AZAs did not tend to re-appear annually in the same locations (Fig. 4). The majority of AZA occurrences in the first three years of the monitored period (2011–2013) were reported from Scotland (Table 3). AZAs were found around different regions of mainland Scotland and the
Scottish islands, including Lewis and Harris, the Isle of Skye, the Shetland Islands and the Orkney Islands. We observed a southern shift in AZA occurrences in the first two years of monitoring: while 14 RMPs from the Shetland Islands recorded AZAs above the RL in 2011, including four RMPs above the MPL, only eight did so in 2012. Simultaneously, regions further south showed an increase in AZA occurrences: from only one RMP above the RL in 2011 to five in 2012 within the Argyll and Bute region in south-west Scotland. In 2013, AZAs were no longer found in the Shetland Islands (Fig. 3A), the Orkney Islands and Lewis and Harris (Fig. 3C). In the north-west of mainland Scotland, AZAs were quantified at five RMPs in the first two years but remained only at one RMP in 2013. No further samples above the RL were found in Scotland from January 2014 until December 2016, the exception being only one sample from south-west Scotland, which recorded AZAs just above the RL.

In contrast to Scotland, AZAs in England were scarce in the first three years of the study (Table 3). The continuous presence of AZAs between April 2012 and May 2013 was observed in only one English RMP situated on the north-east coast, close to the Scottish RMPs affected by the same AZA event. The majority of AZA positive samples from England was detected in 2015 and 2016, all originating from Cornwall, south-west England (Fig. 4).

The first detection of AZAs in UK shellfish was reported by James et al. (2002). The hepatopancreas of a mussel sample, collected in 1998 from Craster, north-east England, was found to contain AZAs (AZA1+ AZA2+AZA3) at 0.13 μg/g. Since this finding, no further data on AZA occurrence in the UK have been published, with the exception of an assessment of Scottish shellfish from 2003 and 2004. In these two years combined, AZAs were found along the Scottish coast, including the Shetland Islands, the Orkney Islands and The Hebrides (Stobo et al., 2008). More data were gathered by Cefas prior to LC–MS/MS implementation into the OC monitoring, when the method was tested on ∼450 OC samples collected from more than 90 Scottish RMPs during 2010, whilst targeting areas affected by LTs. AZAs were detected in 122 samples, with concentrations exceeding the MPL at 16 RMPs spread along the western Scottish coast, the Shetland Islands, The Hebrides and the Isle of Skye, where the highest concentration of 663 μg AZA eq./kg was recorded (unpublished data). The results obtained in 2003, 2004 (Stobo et al., 2008) and 2010, together with our results from the OC programme between 2011 and 2016, suggest that spatial distribution of AZAs varies between years. Similar observations were made in Ireland, where RMPs with AZAs above the MPL were recorded in each year between 2002 and 2013 (Kilcoyne et al., 2014a; Tillmann et al., 2014). While the south of Ireland suffered longer and more intensive AZA events in most years, other areas were affected only in some years. As suggested earlier, the AZAs occurrences in Ireland and Scotland in 2012/2013 were likely to be linked, spreading predominantly along the western coasts of both countries. This does not appear to be the case in 2011 when no AZAs were detected in the north of Ireland above 55°latitude (Kilcoyne et al., 2014a; Tillmann et al., 2014), while in Scotland, the MPL was breached only in northern parts of Scotland, the Shetland Islands and The Hebrides (Fig. 4A).

The first continuous presence of AZAs, lasting approximately three months, was observed in the south-west of England in 2015 and 2016. This seemed to be a localised event, affecting only three and four RMPs in each year, respectively. Even though the presence of AZAs in southern England may have been unusual in the context of the UK, AZAs have been
previously reported at concentrations above the MPL further south off the British Isles: in northern Brittany, France (Amzil et al., 2008; Braña Magdalena et al., 2003), Galicia, Spain (Braña Magdalena et al., 2003) and Andalusia, Spain (Tillmann et al., 2017). Traces of AZAs were found during a two-year long study (2012–2014) in the Adriatic sea, north-west Italy, (Bacchiocchi et al., 2015), in 2016–2017 in the north of Spain (Blanco et al., 2017), during a threear year long study (2003–2006) in Portugal (Vale et al., 2008b, 2008a) and in Morocco (Elgarch et al., 2008).

3.4. AZA profiles

Out of three regulated AZA toxins, AZA1 and AZA2 are known to be phycotoxins. In our study, the proportions of these two toxins were evaluated in order to investigate a possible link with the source phytoplankton. All samples above the RL were considered and on average, AZA1 was the most dominant (81 ± 19%), followed by AZA2 (19 ± 19%). K-mean cluster analysis initially revealed two profiles, Profile 1 with 100% AZA1 and Profile 2 with AZA1: AZA2 ratio of 64% : 36%. It was noticed however, that AZA concentrations in all Profile 1 samples did not exceed 53 μg/kg (no TEFs applied). Coincidently, AZA concentrations in all but one Profile 2 sample did not fall below 25 μg/ kg. Therefore, based on data set with total AZA concentrations above 53 μg/kg, the existence of only one profile in British shellfish could be suggested, with both AZA1 and AZA2 present at a ratio of 66 ± 5% : 34 ± 5%. AZA2 could be quantified in samples with total AZA concentrations above ~30 μg/kg, considering the relative proportion of AZA2 at 34 ± 5% applied.

If AZA3 is found in bivalves, it generally represents a small fraction, however its levels are known to rise after heat treatment (McCarron et al., 2009). Having been identified as a shellfish metabolite, AZA3 was evaluated in our study separately. AZA3 was found above the RL only in two samples and constituted 6% and 4% of the total AZAs, respectively. Furthermore, 11 samples (all mussels) were examined, containing AZA3 above the method LOQ (6 μg/kg for mussels), and the mean AZA3 proportion was 4±1.3%. The shellfish samples utilised in OC biotoxin monitoring were not heat-treated and as such AZA3 was not expected to be frequently recorded. Indeed, AZA3 was rare and, if present, constituted on average only 4% of total AZAs, which is consistent with previous reports on raw shellfish samples exhibiting an A. spinosum profile (Fux et al., 2009). Interestingly, Furey et al. (2003) noted that although AZA3 was frequently found in low proportions in mussels, it was rarely detected in other bivalve species. All 11 samples with AZA3 above LOQ in our study were also mussels. In a laboratory experiment, mussels were able to convert AZA1 to AZA17 and subsequently to AZA3 over time without heat treatment, though the AZA3 proportions were reported to be only 1.7% after four days of exposure to dissolved, semipurified AZA1 (Jauffrais et al., 2013a).

Prevalence of AZA1 with lower proportions of AZA2 is a typical toxin profile associated with A. spinosum. This species was isolated for the first time from the north-east coast of Scotland in 2007, and it was also the first species identified as an AZA producer (Krock et al., 2009; Tillmann et al., 2009). Since its discovery, A. spinosum (strain SHETF6) was also isolated off the coast of the Shetland Islands, with an AZA1: AZA2 ratio of 2.3 : 1 (Tillmann et al., 2012b), and in Ireland, where a similar ratio (2.9 : 1) was reported from strain 3D9 (Jauffrais et al., 2012). In addition to AZA1, A. spinosum was found to also produce AZA33.
and AZA34 (Kilcoyne et al., 2014b), but some recently isolated strains from Norwegian coast showed a different profile. Following a morphological, phylogenetical and chemical examination, two clades within *A. spinosum* species were discovered: Ribotype A strains producing AZA1, AZA2 and AZA33, and Ribotype B strains producing mainly AZA11 and AZA51 (Tillmann et al., 2018).

In the UK, assessment of *Azadinium* spp. is not a requirement of the phytoplankton monitoring programme. Consequently, the link between the toxin profile in shellfish and source algae could not be explored fully. Considering the acquired knowledge on the geographical distribution of *A. spinosum*, its reported incidence and profile off the Scottish coast (Krock et al., 2009; Tillmann et al., 2012b), and several laboratory studies detailing the uptake of AZAs in mussels fed on *A. spinosum* (Jauffrais et al., 2013b, 2012; Salas et al., 2011), it can be suggested that shellfish found contaminated by AZAs in Scotland during the monitored period were likely to be exposed to *A. spinosum* blooms. Further evidence comes from analysis of passive samplers deployed at two sites in Scotland (Loch Ewe and Scapa) between 2005 and 2013, where the predominant toxin was AZA1 with occasional low quantities of AZA2 (Bresnan et al., 2016). Considering the existence of two clades within *A. spinosum* and their distinct toxin profiles (Tillmann et al., 2018), we can also suggest the *A. spinosum* from Ribotype A strains were most likely to be responsible for AZA contamination in British shellfish. However, none of the AZA analogues produced by Ribotype B M. Dhanji-Rapkova, et al. Harmful Algae 87 (2019) 101629 and, with exception of AZA2, none of the analogues produced by other species from Ampidomataceae family (Krock et al., 2019), have been routinely analysed OC shellfish samples. Consequently, the potential contribution of all four AZAs producing species, including those already reported in the north-east Atlantic (*A. spinosum*, *A. poporum* and *Amphidoma languida*), could not be elucidated through the biotoxin monitoring programme.

While the toxin profile characterised by dominance of AZA1 has been prevalent in the northeast Atlantic, there have been reports of AZA2 being the most dominant analogue further south, specifically in some Italian (Bacchiocchi et al., 2015), Portuguese (Vale et al., 2008a), Spanish (Blanco et al., 2017) and Moroccan shellfish (Elgarch et al., 2008; Taleb et al., 2006). Recently, the high proportion of AZA2 found in shellfish from Andalusia, southern Spain, was linked to *Amphidoma languida* (Tillmann et al., 2017), a species phylogenetically close to *Azadinium* (Tillmann et al., 2012a). Although a north-east Atlantic strain of *A. languida* was found to produce AZA38 and AZA39 (Krock et al., 2012; Tillmann et al., 2015), the toxin profile from the Spanish strain was different. In addition to AZA2, it also produced AZA43, a compound with the same mass and similar chromatographic retention as AZA3 (Tillmann et al., 2017). AZA3 is known to be a shellfish metabolite transformed after heat treatment from AZA17, which was previously converted from AZA1 (Kilcoyne et al., 2015; McCarron et al., 2009). Consequently, its presence in some planktonic samples (James et al., 2003) or in shellfish in the absence of AZA1 (Taleb et al., 2006) was intriguing. Considering the similarities between AZA3 and AZA43, Tillmann et al. (2017) suggested that in those cases AZA3 may have been misidentified.

3.5. AZA profiles in different bivalve species

AZAs above the RL were quantified in 659 samples over the time period examined, of which
307 (47%) were Pacific oysters and 286 (43%) were mussels. Other species included surf clams, cockles and razors. The mean AZA1 and AZA2 proportions were calculated in samples with total AZAs above 53 μg/kg to enable more accurate assessment of the toxin profiles. AZA1 was the most dominant toxin in all AZAs positive samples regardless of the species. The distribution of AZA1 proportion varied however between mussels, Pacific oysters and surf clams (Fig. 5). In addition, a statistically significant difference in the proportion of AZA1 was found between Scottish and English mussels at the 5% level of confidence (p=0.007, t-test). A much smaller mussel data set for England (n=14) when compared to Scotland (n=88), together with the time span between AZA findings in each country, could have biased the analysis. More data are required to investigate this further for mussel as well as other bivalve species. Total AZA group levels in cockles and razors from our study were too low to enable the assessment of a toxin profile. The differences in AZA1 : AZA2 ratios between bivalve species were also reported by Furey et al. (2003). However, AZA1 proportions in four non-mussel species, ranging from 56% to 82%, were based on a single sample per species and may not be fully representative.

After the first AZA intoxication cases were linked to Irish mussels (McMahon and Silke, 1996; Ofuji et al., 1999a), it soon became apparent that AZAs can accumulate in other shellfish species, including oysters, cockles, clams and scallops (Amzil et al., 2008; Braña Magdalena et al., 2003; Furey et al., 2003; Salas et al., 2011; Tillmann et al., 2017). In our study, despite Pacific oysters constituting only 18% of all tested species, 47% of all AZA positive samples were Pacific oysters, while 42% were mussels. It seems, that unlike OA group toxins, which are accumulated by Pacific oysters to a much lesser degree than by mussels (Lindegarth et al., 2009), AZAs can be efficiently accumulated by both. Furey et al. (2003) reported that mussels and Pacific oysters cultivated at the same location are similarly susceptible to contamination by AZAs. There is a lack of comparative studies on AZAs accumulation by different bivalve species, and we were also unable to conduct a similar evaluation, as OC monitoring focuses on a single representative species in each RMP.

Variability in toxin profile among shellfish species, believed to be contaminated by the same AZA producing algae, may be caused by differences in their biotransformation capabilities. Indeed, the AZA metabolic pathways in mussels have been shown to be complex. With advances in analytical methods, more than 50 AZA analogues have been identified (Kilcoyne et al., 2017; Krock et al., 2019), many of them being shellfish metabolites or a result of apparently abiotic processes such as 22-decarboxylation, 21,22-dehydration or 37-epimerization (Kilcoyne et al., 2017). Laboratory studies revealed the AZA1 and AZA2 taken up by mussels fed by A. spinosum quickly converted to their carboxylated analogues AZA17 and AZA19, respectively (Salas et al., 2011). The biotransformation was so rapid that 25% of total AZAs were metabolites after the first 6 h of feeding, which increased up to 50% in the following two days (Jauffrais et al., 2012). AZA17 does not convert readily to AZA3, likewise AZA19 to AZA6, unless mussels are cooked, or the method incorporates a heating step in the extraction process (Kilcoyne et al., 2015; McCarron et al., 2009). The analysis of AZA analogues other than AZA1, 2 and 3 is not currently a legislative requirement, which has raised a concern that the current OC methods may underestimate total AZA content and total toxicity, by omitting AZA phycotoxins and their shellfish metabolites which are
potentially toxic to humans.

Data obtained through OC monitoring in Great Britain have been invaluable for inter-annual or seasonal assessment of AZAs occurrences, while toxin profile analysis indicated A. spinosum was likely to be responsible for AZAs contamination. However, it has been recognised that more detailed toxin profile analysis is needed to address food safety questions. As such, a separate study will specifically evaluate proportions of AZA analogues, which are not currently included in the OC analysis, in British shellfish.

3.6. Inter-annual and seasonal variability of yessotoxins

Compared to other regulated lipophilic toxins, YTX group toxins have been the least represented in British bivalves. On average, only 2% of all samples tested between July 2011 and December 2016 contained YTXs above the RL (0.1 mg/kg) but their distribution between years varied considerably. Whilst over 3% of samples were found to be YTX positive (above the RL) in 2011 and 2014, there were only 29 positive samples in 2015 and 2016, representing 0.8% and 0.7%, respectively (Table 4). The highest number of samples above the MPL was recorded in 2012. It is important to note that the MPL of 1 mg YTX eq/kg was raised to 3.75 mg YTX eq./kg in September 2013 following change in the legislation (Anon, 2013). Had the previous MPL of 1 mg YTX eq/kg been applied for the entire monitored period, an additional three samples from 2014 would have exceeded it.

A pattern in temporal distribution of the YTX group was not evident, however the concentration maxima were generally reached in British summer months, either in June or July (Fig. 6). Rather than showing a steep increase and decline, YTXs persisted over several weeks, and in some instances for months. Low concentrations were noted as early as spring for most years, although they were often related to localised events. Specifically, all five positive samples in April and May of 2013 originated from a single monitoring point, indicating focused contamination rather than a widespread spring bloom of YTX producers. Occurrence during winter months was not unusual and needs to be again understood in the context of spatial distribution. For example, YTXs were observed in January 2014 at concentrations higher than expected for winter months and were related to a YTX event in the Shetland Islands that had started in the previous summer. During 2014, the most significant YTX season in the time period examined, YTXs were seen continuously between March and December 2014, with concentrations above 0.5 mg YTX eq/kg recorded between April and August.

Overall, the results indicate inter-annual differences in intensity of YTX events. The seasonal distribution of YTXs seemed to be more orderly than for AZAs, as YTXs generally appeared in higher quantities during British summer months (June and July) and slowly tailed off in the late autumn.

There are limited historical records on YTX occurrences in Great Britain. The only report on YTX occurrence details it as being present in 18% of the Scottish samples tested during 2003 and 2004 (Stobo et al., 2008), much higher than we have observed in any of the monitored years. This discrepancy was largely caused by assessing average abundance between March 2003 and September 2004, rather than per calendar year. In addition, Stobo et al. (2008) considered bivalve samples as positive if YTXs were above the Limit of Detection (LOD),
which was at lower level than the RL utilised in our study. While the YTX concentrations in Scotland peaked during the summer months in shellfish (Stobo et al., 2008) and in the passive samplers (Bresnan et al., 2016), studies in other countries reported the highest YTX concentrations in varied times of the year: late spring in Norway (Aasen et al., 2005; Ramstad et al., 2001), during the summer months in Mexico and China (García-Mendoza et al., 2014; Liu et al., 2017), or in the autumn/winter period in the Mediterranean (Bacchiocchi et al., 2015). As such, the temporal distribution of YTX in shellfish should be understood in the context of the temporal distribution of a particular, YTX-producing, dinoflagellate species.

3.7. Spatial variability of yessotoxins

YTXs were almost exclusively present in Scotland, clustered in the south-west region for most of the years monitored. In 2011, their geographical location was limited to RMPs in Argyll and Bute and North Ayrshire, exception being one sample from the single RMP in the Scottish Highlands (Fig. 7A). In the following year, YTXs expanded further into the Highlands and the Shetland Islands (Fig. 7B). Despite their wider geographical spread, the presence of YTXs in these regions was sporadic and unlikely to be a part of a more prolonged YTX event. The highest proportion of positive samples (91%) in 2012 were again found in south-west Scotland, and just above 50% of these originated from a single RMP within the North Ayrshire region.

2013 was the only year when the majority of all YTX positive samples were not concentrated in south-west Scotland. Instead, only four RMPs located along the west coast of mainland Scotland and two RMPs in the Outer Hebrides were affected. For the first time, the majority (65%) of all YTX positive samples originated from the Shetland Islands (Fig. 7C). Here, the YTX event lasted longer compared to other regions, with first occurrences detected at the end of July 2013 and persisting during winter 2013/2014. Low YTX levels were observed continuously in a single RMP in the Shetland Islands until July 2014 (Fig. 8C). The unusual appearance of YTXs in the Shetland Islands during 2013/2014 coincided with an exceptional OA group toxin event (Dhanji-Rapkova et al., 2018).

Between 2014 and Dec 2016, YTXs were concentrated in south-west Scotland (Fig. 7D, E, F). The single YTX positive shellfish sample (above the RL) outside of Scotland was collected in 2014 from south-west England. The only previous assessment on geographical distribution of YTX in Great Britain reported relatively widespread occurrences during 2003 and 2004 around the Scottish coast (Stobo et al., 2008).

3.8. Yessotoxins and YTX producing algae

*Protoceratium reticulatum* and *Lingulodinium polyedra*, the two dinoflagellate species previously linked to YTX production, have been targeted in phytoplankton monitoring, which runs in parallel with the OC biotoxin monitoring programme. In Scotland, *P. reticulatum* was identified in nearly 5% of phytoplankton samples collected between 2011 and 2016. More than three quarters of those samples had low cell densities (< 100 cells/L), appearing sporadically, rather than in developed blooms. *L. polyedra* was even rarer, present in only 0.6% of phytoplankton samples. Consequently, there were limited opportunities to explore the potential relationship between YTXs and YTXs producers. Among the few RMPs where *P. reticulatum* blooms were present in higher densities and/or in a sustained bloom, two
RMPs were identified in south-west Scotland and one in the Shetland Islands (Fig. 8A, B, C). An increase in *P. reticulatum* densities coincided with or was followed by YTXs accumulation in shellfish, while the prolonged persistence of YTXs, after the bloom had ceased, indicated a slow depuration process.

The lack of YTXs in English and Welsh shellfish could be explained by isolated appearances of potential YTX producers in water. Between 2011 and 2016, *P. reticulatum* was found in low densities (up to 360 cells/L) in 16 water samples in total. The single YTX positive shellfish sample (above the RL) in England was collected in 2014 and was preceded by a small *P. reticulatum* bloom, when a density of 100 cells/L was recorded in two consecutive weeks.

*L. polyedra* tends to bloom irregularly in Scottish waters, usually observed on the west coast, including Loch Creran (Fig. 8D). In other parts of the world, blooms of *L. polyedra* can lead to intense red tides and have been associated with both shellfish and finfish mortalities. Although such high-density blooms have not been observed in Loch Creran, occasional increase in cell abundance has been noted, and the highest concentration of 23,680 cells/L was recorded in September 2009 (unpublished data). Due to a lack of *L. polyedra* blooms we were unable to fully examine the potential relationship with YTXs occurrences in bivalves. The only two *L. polyedra* blooms with cell densities just above 2,000 cells/L, observed in Scotland (Fig. 8D) and in southern England, did not result in the accumulation of YTXs in Pacific oysters. Low concentrations of YTX were found in mussels collected briefly in Loch Creran between November 2011 and March 2012 (Fig. 8D). This might have been a residue of a toxin event following the *L. polyedra* bloom in Aug/Sept 2011, although as mussels were not collected in parallel with the bloom, the link between this dinoflagellate species and YTXs in shellfish could not be confirmed. Even though the production of YTXs by *L. polyedra* has been reported (Paz et al., 2004), some strains were found to be non-toxigenic (Howard et al., 2008). To explain the occurrences of high density *L. polyedra* blooms, which did not cause YTX contamination in shellfish in Italy in some years, Pistocchi et al. (2012) suggested high intra-species variability in the toxicity or, alternatively, that this species becomes toxigenic only under certain environmental conditions. There is limited information on toxin production of *L. polyedra* around the British coast. The 2003 study analysing 16 cultures from an UW31 isolate (Loch Creran, Scotland), reported low YTX quantities (0.02 pg/cell) only in one encysted culture (Stobo et al., 2003). So far, the contribution of *L. polyedra* to YTX accumulation in British shellfish is yet to be confirmed.

Our results suggest *P. reticulatum* might have been a source of YTXs in British shellfish. *P. reticulatum* was confirmed as a YTX producer for the first time by Satake et al. (1997a, 1997b). Since then, more evidence has been provided on YTX production by *P. reticulatum*, either in field samples and/or in cultures from various locations worldwide, confirming its global distribution (Aasen et al., 2005; Akselman et al., 2015; Ciminiello et al., 2003; Eiki et al., 2005; Finch et al., 2005; Koike et al., 2006; Liu et al., 2017; Miles et al., 2005; Paz et al., 2004, 2013, 2007; Sala-Pérez et al., 2016; Samdal et al., 2004; Stobo et al., 2003). The concentration of YTXs in shellfish are generally found to peak between one and two weeks following *P. reticulatum* cell density maxima, supported by our own observations (Fig. 8A, B, C) and by other studies (Aasen et al., 2005; Eiki et al., 2005; Koike et al., 2006). Even
brief, relatively low-density blooms of up to 2,200 cells/L were sufficient to cause YTXs contamination in shellfish above 1 mg/kg (Aasen et al., 2005). Blooms may not always exhibit a sharp increase and decline in cell abundancy, and a cumulative effect of a persistent, low-density presence is also likely to impact on toxin accumulation in shellfish, as indicated in Fig. 8A (2014). Indeed, the relationship between P. reticulatum and the accumulation of YTXs in shellfish is complicated by different toxin cell quotas between distant populations of the same species or even isolates from a single population (Aasen et al., 2005; Álvarez et al., 2011; Eiki et al., 2005; Paz et al., 2013, 2007; Sala-Pérez et al., 2016; Stobo et al., 2003; Suzuki et al., 2007). In addition, YTXs production was reported to be influenced by temperature, salinity, light, growth phase, and nutritional conditions (Guerrini et al., 2007; Koike et al., 2006; Mitrovic et al., 2005; Paz et al., 2006; Röder et al., 2012). Some of these factors are influenced by the hydrography of the specific geographical location and are likely to impact on P. reticulatum occurrence and subsequent YTXs accumulation in shellfish (Koike et al., 2006; Ramstad et al., 2001). P. reticulatum has been found to be resilient in a range of conditions (Guerrini et al., 2007), which could at least allow for low-density, infrequent appearances. However, for the bloom to develop and result in YTXs accumulation in shellfish, a combination of factors has to fall into an “ideal” range. Perhaps, these limitations explain why P. reticulatum blooms in Great Britain were often localised (Figs. 7 and 8). Our study did not involve assessing YTXs production by this dinoflagellate, although the production of YTXs has been previously confirmed in an isolate (UW351) of P. reticulatum from the North Sea (Stobo et al., 2003). The presence of YTX, and more occasionally also 45–OH YTX, were confirmed in the water column off the Scottish coast and coincided with low numbers of P. reticulatum cells (Bresnan et al., 2016). This could provide further evidence that this species is the likely source of YTX in Scotland.

3.9. YTX profiles

The mean profiles between four YTX analogues were evaluated and show a high proportion of YTX (95 ± 12%) and a low proportion of 45–OH YTX (5 ± 12%). No homo-YTX or 45–OH homo-YTX were detected in shellfish samples throughout the monitored period. High variability associated with the mean ratio suggested at least two different profiles, depending on the relative proportions of YTX and 45–OH YTX. Cluster analysis confirmed the existence of Profile 1 with 100% YTX, and Profile 2 with 71 ± 8% YTX and 29 ± 8% 45–OH YTX. Toxins, such as 45–OH YTX, that contribute a smaller proportion are likely to go undetected once the total YTX concentration drops below a certain level, as detailed below. The relationship between toxin profile and total YTX concentration was therefore examined in mussels, which constituted 95% of all YTX positive samples (Fig. 9). Only Profile 1 was found in mussels with total YTX concentrations up to 0.23 mg/kg. Both profiles were present in a transition concentration band 0.23–0.5 mg/kg, depending on whether 45–OH YTX fell below or above the RL. We can hypothesise that there is only one true profile for mussels, confirmed in samples above 0.5 mg/kg, consisting of YTX at 74 ± 6% and 45–OH YTX at 26 ± 6%. However, five mussel samples fell into Profile 1 category, despite their concentrations being above 0.5 mg/kg (Figs. 9 and 10A). Further investigation revealed they were collected in 2014 from a single RMP in south-west Scotland and two of these samples with the highest total YTXs (0.943 mg/kg and 1.32 mg/kg), also contained 45–OH YTX at 11% and 9%
respectively (Figs. 9 and 10A). The other 30 YTX-positive samples from the same RMP and collected in the last 5 years, contained 100% YTX at concentrations 0.1–0.4 mg/kg, too low to enable quantification of 45–OH YTX above the RL.

YTX toxin profiles in both shellfish and YTX-producing dinoflagellates have been reported and discussed in multiple studies. Despite considerable variation in the profiles between *P. reticulatum* cultures, YTX has been the prevalent toxin (> 95%) in strains from different regions, including Italy (Ciminiello et al., 2003), Spain (Paz et al., 2013, 2007), Norway (Samdal et al., 2004), UK (Stobo et al., 2003), Germany (Röder et al., 2012), Argentina (Akselman et al., 2015), Chile (Álvarez et al., 2011), Japan (Eiki et al., 2005), Canada (Stobo et al., 2003) and even the Arctic (Sala-Pérez et al., 2016). Many other YTX analogues have been found in low proportions. However, some exceptions have been reported when the dominant toxin was not YTX, but homo-YTXs (Konishi et al., 2004; Paz et al., 2007; Suzuki et al., 2007). In addition, Suzuki et al. (2007) also identified three culture isolates, in which YTX was present but other analogues amounted to 50%. Miles et al. (2005) suggested that the toxin profile in *P. reticulatum* from New Zealand (CAWD40 isolate) is far more complex, containing more than 90 YTX analogues, with YTX representing approximately one third of the total concentration. Many of the YTX analogues were found predominantly in the aqueous fraction, indicating that they are more hydrophilic than YTX and therefore unlikely to be sufficiently extracted by an organic solvent (Miles et al., 2005).

YTX toxin profiles in shellfish have been reviewed by Suzuki (2014). YTX or homo-YTXs were found to be always present, either as the most or the second most dominant toxin. In some instances, both YTX and homo-YTX and related analogues were detected simultaneously (Bacchiocchi et al., 2015; Draisci et al., 1999). We have not identified homo-YTX and homo-derivatives in any of the samples, suggesting the absence of a dinoflagellate species (or a strain of a certain species) producing YTXs of homo structure around the British coast. YTX was always the most dominant toxin, and when both YTX and 45–OH YTX were present, their ratio was 3 : 1. While some YTX analogues like 45–OH YTX and carboxy-YTX were found in small proportions in *P. reticulatum* (Ciminiello et al., 2003), they were present in high abundance in shellfish, indicating they are mainly bivalve metabolites (Aasen et al., 2005; Ciminiello et al., 2010). The conversion of YTX to 45–OH YTX was confirmed ex-vivo in scallops by Suzuki et al. (2005), while rapid oxidation of YTX to 45–OH YTX and more slowly to carboxy- YTX in field mussels was reported by Aasen et al. (2005).

Consequently, the toxin profiles in mussels changed dramatically over time: YTX was dominant only at the start of the bloom, 45–OH YTX was the most dominant (twice the YTX proportion) during a *P. reticulatum* bloom, while carboxy YTX was the most dominant analogue after one to six months from initial contamination (Aasen et al., 2005). High relative abundance of 45–OH YTX and carboxy-YTX and its variation over time was also noted by Ciminiello et al. (2010). In our study, however, the low standard deviation associated with 45–OH YTX proportion suggests the ratio between YTX and 45–OH YTX seems to be stable. This could possibly be explained by assessing the proportion of 45–OH YTX in a limited timeframe, either during and just after the bloom when total YTX concentrations were high (> 0.5 mg/kg). During this time window, 45–OH YTX proportions fluctuated generally between 35 and 20% (Fig. 10B), which is comparable to several mussel samples from New
Zealand, Canada and Norway (Finch et al., 2005). Although Aasen et al. (2005) found 45–OH YTX in higher abundance, he noted that, during and up to one month after the bloom, 45–OH YTX contribution followed nearly the same pattern as YTX.

YTX was found in British isolates of *P. reticulatum* (UW351), while 45–OH YTX was not detected in any of the tested cultures (Stobo et al., 2003). Toxin profiles of more recent isolates, coinciding with our monitored period, have not been reported. Nevertheless, based on evidence from other studies, we can suggest that the presence of 45–OH YTX in British shellfish is likely to be a result of metabolic transformation of YTX in bivalves rather than a result of direct accumulation from the phytoplankton source. However, due to a lack of data on other YTX shellfish metabolites (e.g. carboxy-YTX or 45-hydroxycarboxy-YTX), a complete assessment on YTX transformation in British bivalves and seasonal changes in toxin profiles has not been possible.

### 3.10. YTX profiles in different bivalve species

YTXs were found predominantly in mussels where YTX constituted on average 74%, with 45–OH YTX making up the remainder. The only other YTX-positive species was queen scallops, where 20 samples contained only YTX. Interestingly, seven of those queen scallop samples were collected between 4th July and 15th August 2011 from Loch Fyne, North Bay alongside mussels (Fig. 11). This uncommon, simultaneous collection of two bivalve species from the same RMP enabled comparison of YTXs profiles as well their concentrations. Two *P. reticulatum* blooms were recorded at this RMP in March and May, exceeding 2,000 cells/L and 1,000 cells/L, respectively. By the time the LT LC–MS/MS was implemented (4th July 2011), no *P. reticulatum* cells were found in the water and shellfish had already started depurating YTXs. This process was three-times faster in mussels than in queen scallops (Table 5). Different depuration speeds may have been associated with different 45–OH YTX proportions in these two species. To further explore this potential relationship, the depuration of YTX was also compared between mussels from Loch Striven (Fig. 10A) and Arran–Lamlash Bay (Fig. 10B), which showed different 45–OH YTX proportions (10% and 25% respectively). The half-life of YTX in mussels from both areas was 26 days, based on the first 42 days after the bloom has ceased, suggesting the 45–OH YTX contribution does not determine the speed of YTX elimination (Table 5). As such, the inter-species difference in YTX depuration might be caused by a different mechanism. In our study, the comparison between mussels and queen scallops, and between mussels from different areas, was also complicated by several factors. Firstly, there was a limited number of samples with high YTXs concentrations, which in turn meant a limited number of samples with 45–OH YTX quantified above the RL. Secondly, only YTX and three analogues were routinely looked for, while other YTX analogues involved in YTX elimination could have been missed. Thirdly, the samples from Loch Fyne could be evaluated only since LT LC–MS/MS was implemented (4th July 2011), seven weeks after the bloom had ceased. Nevertheless, the half-life of YTX in mussels ranged between 17 and 26 days, which was comparable to 20 days reported by Aasen et al. (2005). The author also reported that the depuration rate remained consistent over a three-month period. Toxin profile comparison with other studies is complicated due to lack of reference compounds for YTX analogues. Consequently, the efficiency of multiple extraction approaches utilised over time may not be well known for all analogues, which is an
important aspect if YTXs with a different degree of lipophilicity are considered (Miles et al., 2005).

4. Conclusions

Despite relatively low occurrences in British shellfish, AZAs pose a threat to the shellfish industry and potentially to the consumer, not only because of their toxicity, but also due to their unpredictability. Large inter-annual differences in abundance were found in our study; the first years with widespread and long-lasting contamination were followed by years with rare and localised AZA events. The toxin profile, comprising of more dominant AZA1 and followed by AZA2, was indicative of *Azadinium spinosum* as the likely source of AZAs. The conventional light microscopy utilised in the phytoplankton monitoring programme is not suitable for detection of small (< 20 μm) AZAs producers of the genera *Azadinium* and *Amphidoma*, and as such there is the absence of an early warning signal for AZAs contamination in shellfish. Molecular techniques can provide a solution however, due to cost related reasons, they have not been implemented in the UK OC monitoring programmes. The understanding of the toxin dynamics within the environment is further complicated by the apparent ability of bivalves to accumulate dissolved AZAs from water and their ability to further metabolise planktonic AZAs into unregulated and potentially more toxic products. Mapping the complexity of AZA profiles in bivalves and the implications for the food safety will be addressed in our future studies.

In comparison, the occurrences of YTXs plankton producers, *Protoceratium reticulatum* and *Lingulodinium polyedra* are regularly monitored. Although *P. reticulatum* was the more abundant out of these two species, it was mainly detected intermittently in low densities, while the developed blooms were rare and fairly localised. *P. reticulatum* was the likely source of the majority, if not all, of the YTX events in GB, evidenced through the spike in YTX concentrations following the bloom and the dominance of YTX in shellfish. While no homo-YTX and 45−OH homo-YTX were detected, 45−OH YTX constituted on average 26% in mussels.

Even though our analysis was not exhaustive in mapping AZA or YTXs producers, or in assessing all known AZA or YTX analogues in shellfish, we have provided valuable information that could facilitate further research in this area.

Acknowledgement

All OC samples analysed in the period between July 2011 and December 2016 were acquired and tested as a part of the FSA and FSS funded OC monitoring programmes. Writing of this manuscript was supported by Cefas funding scheme. In addition to the Cefas colleagues listed as co-authors, we would like to thank Karl Dean, Karsan Dhanji, Diane Partridge and Patrycja Stubbs for their technical assistance, and Myriam Algoet for reviewing our manuscript and providing valuable comments. We would like to thank Dave Clarke from the Marine Institute in Ireland for his helpful assistance regarding AZAs data from the Irish monitoring programme.[CG]
Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2019.101629.

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https://doi.org/10.1016/j.hal.2005.03.005.


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Figure 1. Map of Great Britain showing shellfish representative monitoring points (RMPs) from classified production areas active during the study period (2011 – 2016).
Figure 2. Temporal variability of AZA group toxin equivalents (µg AZA eq./kg) in shellfish collected between July 2011 and December 2016 (n = 659).

--- MPL (160 µg AZA eq./kg)
Figure 3. Temporal variability of AZA group toxin equivalents (μg AZA eq./kg) in six monitoring points from: A) Shetland Islands, B) Highland – Sutherland, C) Lewis and Harris, D) Skye and Lochalsh, E) Highland – Lochaber and F) Argyll and Bute. No AZAs were found in these monitoring points between December 2013 and December 2016. Data from Loch Ewe and Scapa (Bresnan et al., 2016) were included in the discussion.
Figure 4. Spatial distribution of AZA group toxins in (a) 2011, (b) 2012, (c) 2013, (d) 2014, (e) 2015 and (f) 2016. Yellow dots highlight representative monitoring points (RMPs) with AZA group toxins above Reporting Limit (RL) and red dots with AZA group toxins above Maximum Permitted Level (MPL; 160 μg AZA eq/kg)
Figure 5. Box and whisker plot for AZA1 proportions in mussels (n = 102), Pacific oysters (n = 128) and surf clams (n = 9). All samples collected between July 2011 and December 2016 and containing total AZA1 + AZA2 > 53 μg/kg were considered (no TEFs applied). Normal distribution in each category was confirmed by Shapiro-wilk test of normality. Homogeneity of variance was assessed by Levene’s Test. Each category was compared using Welch t-test with “Holm” adjustment of p-value. *** means significance (p < 0.001) * means significance (p < 0.05).
Figure 6. Temporal variability of YTX group toxin equivalents (mg YTX eq./kg) in shellfish collected between July 2011 and December 2016 (n = 402).
Figure 7. Spatial distribution of YTX group toxins in (a) 2011, (b) 2012, (c) 2013, (d) 2014, (e) 2015 and (f) 2016. Yellow dots highlight representative monitoring points (RMPs) with YTX group toxins above Reporting Limit (RL) and red dots with YTX group toxins above Maximum Permitted Level (MPL = 1 mg YTX eq./kg; 3.75 mg YTX eq./kg after September 2013).
Figure 8. Temporal variability of *Protoceratium reticulatum* (cells/L) and YTX group toxin equivalents (mg YTX eq./kg) in four monitoring points: A) Arran – Lamlash Bay (mussels), B) Loch Melfort (mussels), C) Browland Voe (mussels), D) Loch Creran (mussels between November 2011 – March 2012 only, otherwise Pacific oysters). Monitoring in Arran (A) discontinued after 2015.
Figure 9. Relationship between YTX proportion in mussels and corresponding YTXs concentration (no TEFs applied).
Figure 10. YTX group toxin equivalents (mg YTX eq./kg), *Protoceratium reticulatum* (cells/L) and relative YTX proportions of total YTX (YTX + 45-OH YTX) in two monitoring points from south-west Scotland: A) Loch Striven (year 2014) and B) Arran - Lamlash Bay (year 2012)
Figure 11. Temporal change in relative YTX proportions of total YTX (YTX + 45-OH YTX) and YTX group toxin equivalents (mg YTX eq./kg) in mussels and queen scallops collected in 2011 from Loch Fyne.
Table 1. Proportion of shellfish species tested for LTs between July 2011 and December 2016 in Great Britain and in each country within Great Britain. “Other” species include manila clam (*Ruditapes philippinarum*), carpet shell clam (*Ruditapes decussatus*) and queen scallops (*Aequipecten opercularis*).  

<table>
<thead>
<tr>
<th>Species</th>
<th>Great Britain</th>
<th>Scotland</th>
<th>England</th>
<th>Wales</th>
</tr>
</thead>
<tbody>
<tr>
<td>mussels (<em>Mytilus</em> spp.)</td>
<td>13745 (67%)</td>
<td>71%</td>
<td>51%</td>
<td>70%</td>
</tr>
<tr>
<td>Pacific oysters (<em>Crassostrea gigas</em>)</td>
<td>3687 (18%)</td>
<td>17%</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>native oysters (<em>Ostrea edulis</em>)</td>
<td>359 (1.7%)</td>
<td></td>
<td>9.0%</td>
<td></td>
</tr>
<tr>
<td>cockles (<em>Cerastoderma edule</em>)</td>
<td>1207 (5.9%)</td>
<td>4.6%</td>
<td>6.5%</td>
<td>30%</td>
</tr>
<tr>
<td>razor clams (<em>Ensis</em> spp.)</td>
<td>1005 (4.9%)</td>
<td>6.2%</td>
<td>0.60%</td>
<td></td>
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<tr>
<td>surf clams (<em>Spisula solid</em>)</td>
<td>224 (1.1%)</td>
<td>1.1%</td>
<td>1.3%</td>
<td></td>
</tr>
<tr>
<td>hard clams (<em>Mercenaria mercenaria</em>)</td>
<td>175 (0.85%)</td>
<td></td>
<td>4.4%</td>
<td></td>
</tr>
<tr>
<td>other</td>
<td>80 (0.55%)</td>
<td>0.27%</td>
<td>1.8%</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>total samples tested for LT</td>
<td>1887</td>
<td>3610</td>
<td>3543</td>
<td>3676</td>
</tr>
<tr>
<td>total &gt;RL</td>
<td>214</td>
<td>212</td>
<td>194</td>
<td>3</td>
</tr>
<tr>
<td>(11%)</td>
<td>(5.8%)</td>
<td>(5.5%)</td>
<td>(0.1%)</td>
<td>(0.6%)</td>
</tr>
<tr>
<td>&gt;RL &lt;MPL</td>
<td>192</td>
<td>208</td>
<td>193</td>
<td>3</td>
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<tr>
<td>&gt;MPL</td>
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<tr>
<td>maximum concentration</td>
<td>518</td>
<td>433</td>
<td>189</td>
<td>127</td>
</tr>
<tr>
<td>(μg AZA eq./kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RL = Reporting Limit; MPL = Maximum Permitted Level (160 μg AZA eq./kg); *July 2011 to December 2011 only
### Table 3

Number of active representative monitoring points (RMPs), number of RMPs with AZA group toxins above Reporting Limit (RL) and above Maximum Permitted Level (MPL) in each country in each year. Relative proportion to active RMPs is expressed as percentage (%) in brackets.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td><strong>Active RMPs</strong></td>
<td></td>
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</tr>
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<td>Great Britain</td>
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<td>172</td>
<td>183</td>
<td>170</td>
<td>155</td>
</tr>
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<td>77</td>
<td>78</td>
<td>84</td>
<td>85</td>
<td>83</td>
</tr>
<tr>
<td>England</td>
<td>75</td>
<td>79</td>
<td>76</td>
<td>86</td>
<td>74</td>
<td>62</td>
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<tr>
<td>Wales</td>
<td>17</td>
<td>20</td>
<td>18</td>
<td>13</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td><strong>RMPs with AZA group &gt; RL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Great Britain</td>
<td>34 (20%)</td>
<td>36 (21%)</td>
<td>17 (10%)</td>
<td>3 (1.6%)</td>
<td>4 (2.4%)</td>
<td>4 (2.6%)</td>
</tr>
<tr>
<td>Scotland</td>
<td>33</td>
<td>35</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>England</td>
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<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Wales</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>RMPs with AZA group &gt; MPL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Great Britain</td>
<td>7</td>
<td>2</td>
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<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Scotland</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>England</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Wales</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

RMPs = representative monitoring points; RL = Reporting Limit; MPL = Maximum Permitted Level (160 μg AZA eq./kg)
Table 4. Number of OC shellfish samples from Great Britain tested for lipophilic toxins (LT), the total number of samples containing YTX group toxins above Reporting Limit (RL), the number of samples with YTX group toxins below and above the Maximum Permitted Level (MPL) in each year, and associated percentage of all samples analysed each year.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>total samples tested for LT</td>
<td>1887</td>
<td>3610</td>
<td>3543</td>
<td>3676</td>
<td>3734</td>
<td>4065</td>
<td>20515</td>
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<tr>
<td>total &gt;RL</td>
<td>63</td>
<td>54</td>
<td>96</td>
<td>131</td>
<td>29</td>
<td>29</td>
<td>402</td>
</tr>
<tr>
<td>(3.3%)</td>
<td>(1.5%)</td>
<td>(2.7%)</td>
<td>(3.6%)</td>
<td>(0.8%)</td>
<td>(0.7%)</td>
<td>(2%)</td>
<td></td>
</tr>
<tr>
<td>&gt;RL &lt;MPL</td>
<td>62</td>
<td>49</td>
<td>96</td>
<td>131</td>
<td>29</td>
<td>29</td>
<td>396</td>
</tr>
<tr>
<td>&gt;MPL</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>6</td>
</tr>
<tr>
<td>maximum concentration</td>
<td>1.1</td>
<td>1.8</td>
<td>0.8</td>
<td>1.4</td>
<td>0.4</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>(mg YTX eq./kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

RL = Reporting Limit; MPL = Maximum Permitted Level (1 mg YTX eq./kg; 3.75 mg YTX eq./kg since September 2013); *July 2011 to December 2011 only
Table 5. Depuration rates of YTX in mussels and queen scallops based on least square fits of data

1the first 42 days after *P. reticulatum* bloom had ceased (Loch Striven and Arran), 2the first 42 days of simultaneous collection, but 49 days following *P. reticulatum* bloom

<table>
<thead>
<tr>
<th>Species</th>
<th>Area</th>
<th>45-OH YTX</th>
<th>( r^2 )</th>
<th>Half-life (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mussel</td>
<td>Loch Striven (Fig. 10A)</td>
<td>10%</td>
<td>0.799</td>
<td>26(^a)</td>
</tr>
<tr>
<td>mussel</td>
<td>Arran - Lamlash Bay (Fig. 10B)</td>
<td>25%</td>
<td>0.973</td>
<td>26(^a)</td>
</tr>
<tr>
<td>queen scallop</td>
<td>Loch Fyne (Fig. 11)</td>
<td>0%</td>
<td>0.891</td>
<td>53(^b)</td>
</tr>
<tr>
<td>mussel</td>
<td>Loch Fyne (Fig. 11)</td>
<td>26%</td>
<td>0.876</td>
<td>17(^a)</td>
</tr>
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### S1 Waters Xevo TQ and Xevo TQ-S MS/MS acquisition method utilising electrospray ionisation (ESI).

<table>
<thead>
<tr>
<th>Ionisation mode</th>
<th>Compound</th>
<th>Transition</th>
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<th>Xevo TQ-S</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>Cone (V)</td>
<td>Collision (V)</td>
<td>Cone (V)</td>
</tr>
<tr>
<td>Negative</td>
<td>YTX</td>
<td>570.5&gt;467.4</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>570.5&gt;396.2</td>
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<td>40</td>
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<tr>
<td></td>
<td>homo-YTX</td>
<td>577.5&gt;474.2</td>
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<td>577.5&gt;403.2</td>
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<td>40</td>
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<tr>
<td></td>
<td>45-OH YTX</td>
<td>578.5&gt;467.4</td>
<td>38</td>
<td>30</td>
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<td>578.5&gt;396.2</td>
<td>30</td>
<td>40</td>
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<tr>
<td></td>
<td>45-OH homo-YTX</td>
<td>585.5&gt;474.2</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>585.5&gt;403.2</td>
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<td>40</td>
</tr>
<tr>
<td>Positive</td>
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