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Variability of Amnesic Shellfish Toxin and *Pseudo-nitzschia* occurrence in bivalve molluscs and water samples – analysis of ten years of the official control monitoring programme.

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Abstract

As the official control laboratory for marine biotoxins within Great Britain, the Centre for Environment, Fisheries and Aquaculture Science, in conjunction with the Scottish Association for Marine Science, has amassed a decade’s worth of data regarding the prevalence of the toxins associated with Amnesic Shellfish Poisoning within British waters. This monitoring involves quantitative HPLC-UV analysis of shellfish domoic acid concentration, the causative toxin for Amnesic Shellfish Poisoning, and water monitoring for *Pseudo-nitzschia* spp., the phytoplankton genus that produces domoic acid. The data obtained since 2008 indicate that whilst the occurrence of domoic acid in shellfish was generally below the maximum permitted limit of 20 mg/kg, there were a number of toxic episodes that breached this limit. The data showed an increase in the frequency of both domoic acid occurrence and toxic events, although there was considerable annual variability in intensity and geographical location of toxic episodes. A particularly notable increase in domoic acid occurrence in England was observed during 2014. Comparison of Scottish toxin data and *Pseudo-nitzschia* cell densities during this ten-year period revealed a complex relationship between the two measurements. Whilst the majority of events were associated with blooms, absolute cell densities of *Pseudo-nitzschia* did not correlate with domoic acid concentrations in shellfish tissue. This is believed to be partly due to the presence of a number of different *Pseudo-nitzschia* species in the water that can exhibit variable toxin production. These data highlight the requirement for tissue monitoring as part of an effective monitoring programme to protect the consumer, as well as the benefit of more detailed taxonomic discrimination of the *Pseudo-nitzschia* genus to allow greater accuracy in the prediction of shellfish toxicity.

**Keywords:** Domoic acid, Amnesic Shellfish Poisoning, *Pseudo-nitzschia*, monitoring
1. Introduction

Domoic acid (DA) is a heat stable neuroexcitatory amino acid and is the primary toxin associated with Amnesic Shellfish Poisoning (ASP) (Bates et al., 1989). Typically, it is combined with its isomers epio and iso-DA to give the total DA concentration present in a sample (Alexander et al., 2009a). As with other marine biotoxins such as Paralytic Shellfish Toxins (PST) and Lipophilic Toxins (LT), DA can bioaccumulate in filter-feeding bivalve molluscs such as mussels and poses a risk to human health if consumption of contaminated shellfish occurs (Alexander et al., 2009b). DA intoxication in humans was first reported following consumption of mussels from Prince Edward Island in Canada during 1987, where symptoms of vomiting, abdominal pain, diarrhoea, neurological symptoms, memory loss and, in extreme cases, death were recorded (Bates et al., 1989; Perl et al., 1990). DA intoxication is not limited to humans and there have been a number of episodes of its appearance throughout the marine food chain, including fish (Lefebvre and Robertson, 2010), seabirds (Bejarano et al., 2008) and mammals (Anderson et al., 2016; Brodie et al., 2006). Indeed, in spring 2015, a significant DA outbreak along the North American west coast resulted in widespread mortalities of sea birds and mammals, and closures of shellfish harvesting areas (McCabe et al., 2016). Within the United Kingdom (UK), DA has been detected in the urine and faeces of Scottish harbour seals (Phoca vitulina) via consumption of contaminated fish, with potential damaging impacts on their population dynamics (Hall and Frame, 2010; Jensen et al., 2015).

The production of DA is associated with naturally occurring phytoplankton blooms of the pennate diatom genus Pseudo-nitzschia (Bates et al., 1989; Lelong et al., 2012). Pseudo-nitzschia is a cosmopolitan planktonic oceanic genus, with 52 described species at present, 26 of which are known to have the potential to produce DA (Bates et al., 2018; Lelong et al., 2012; Tan et al., 2015; Teng et al., 2016, 2014; Trainer et al., 2012). Within British waters, a diverse range of Pseudo-nitzschia species has been identified, with 13 species present in Scottish waters and a range of species-specific seasonal bloom dynamics (Bresnan et al., 2015; Fehling et al., 2006; Turrell et al., 2008). Within these species, both P. australis and P. seriata have been identified as having the ability to produce DA (Fehling et al.,
In the UK, ASP toxins were first detected in the Shetland Islands in 1997 and since then have resulted in a number of closures of shellfisheries around the UK (reviewed in Hinder et al., 2011). Regulatory monitoring programmes have been established to protect the consumer from ASP. Within the European Union, European Regulation 853/2004 (Anon, 2004) states that DA must not exceed 20 mg/kg shellfish tissue. In addition to shellfish testing, the legislation also stipulates the requirement for monitoring for the presence of toxin-producing plankton (in this case, *Pseudo-nitzschia*) in production and relaying waters. Water samples are typically assessed, and cells identified and enumerated by light microscopy, a technique that works well for genus level detection of *Pseudo-nitzschia* although it is difficult to identify to species level.

In Great Britain (GB), the Food Standards Agency (FSA) are the central competent authority responsible for official control (OC) monitoring in England and Wales, whilst Food Standards Scotland (FSS) are the central competent authority responsible for Scotland. The Centre for Environment, Fisheries and Aquaculture Science (Cefas) is accredited for the quantitation of DA in bivalve molluscs using High-Pressure Liquid Chromatography with UV detection (HPLC-UV) and has been contracted to conduct testing of shellfish tissue for England, Wales and Scotland, as well as delivery of OC phytoplankton testing for England and Wales, with the Scottish Association for Marine Science (SAMS) conducting OC phytoplankton monitoring for Scotland. Consequently, Cefas and SAMS have completed ten years of combined monitoring for GB, for both the enumeration of phytoplankton in water samples and bivalve testing from designated harvesting locations. This manuscript summarises the results obtained from this monitoring, including concentrations of domoic acid in shellfish samples, temporal and spatial variability, and comparison between water sample results and those for shellfish tissue. Such an assessment will provide a useful additional tool for the determination of DA toxin events, patterns of development and will ultimately contribute towards future risk assessments of relevance to the combined GB biotoxin monitoring programmes.
2. Materials and methods

2.1 Shellfish samples

The samples analysed in this report were collected as part of the official biotoxin monitoring programmes for GB under contract to the FSA and FSS. Samples were obtained over a 10-year period from April 2008 to December 2017 from designated monitoring points in shellfish production and relaying areas within 67 local authority areas. Fifty-one authorities were from England and Wales (7,487 samples) and 16 authorities from Scotland (13,510 samples). Designated OC sampling officers were responsible for the collection, or supervised collection, and preparation of the shellfish for transportation to the testing laboratories. A range of shellfish species were sampled, depending on harvesting activity within each production area: cockles (*Cerastoderma edule*), common mussels (*Mytilus* spp.), Pacific oysters (*Magallana gigas*), native oysters (*Ostrea edulis*), razor clams (*Ensis* spp.), carpet clams (*Ruditapes decussatus*), surf clams (*Spisula solida*), hard clams (*Mercenaria mercenaria*), manila clams (*Ruditapes philippinarum*), queen scallops (*Aequipecten opercularis*) and European otter shells (*Lutraria lutraria*). Shellfish were collected from monitoring points prior to and during periods of active harvesting. The frequency of testing was defined by the competent authorities, with sampling frequencies varying dependent on determined risk, resulting in testing either weekly, fortnightly or once every four weeks. In cases where shellfish monitoring was either fortnightly or four weekly, frequency was increased to weekly if routine results of shellfish tissue or phytoplankton monitoring indicated an increased risk. Shellfish samples were transported live and chilled to the appropriate laboratory using approved and validated cool boxes.

2.2. Analytical methods

2.2.1 Reagents and chemicals

All reagents used throughout the extraction and analysis were >99% purity, HPLC-grade or better. Certified Reference Material (CRM) and certified DA standards were obtained from the Institute for Biotoxin Metrology at the National Research Council Canada (NRCC, Halifax, Nova Scotia, Canada).
2.2.2 Sample preparation

Shellfish samples received were assessed to ensure they were suitable for analysis by the demonstration of organoleptic properties, before being shucked and homogenised to generate a minimum of 100 g of shellfish homogenate, with a minimum number of 10 animals. A 50% methanolic extraction method based on Quilliam et al. (1995) was used to extract the samples.

2.2.3 Liquid chromatography- UV detection

Analysis of filtered methanolic shellfish extracts was performed using LC-UV Agilent 1100/1200 modules (Agilent, Manchester, UK) comprised of quaternary pump, vacuum degasser, autosampler, column oven and UV-diode array detector monitoring at 242 nm. Peaks of DA and epi-DA detected in shellfish samples were summed and quantified against the external calibration standards. The minimum acceptable coefficient correlation ($r^2$) of the calibration curve was taken as 0.999. Toxin concentrations in each sample were calculated as mg DA/kg shellfish tissue.

2.3 Phytoplankton samples

Seawater samples obtained for the Scottish regulatory monitoring programme were collected by OC sampling officers, or under their supervision, as close to the shellfish bed as possible, with the sampling method used being either a PVC sampling tube or a bucket, depending on the depth of water at each site. The sampling tube allowed for the collection of a depth-integrated water sample from 0-10 m. A well-mixed 500 mL sub-sample of this water was then fixed on site with acidic Lugol’s iodine, to obtain a final concentration of approximately 1%. On arrival at the SAMS laboratory, the phytoplankton in a 50 mL sub-sample (detection limit 20 cells/L) was allowed to settle on the base plate of a chamber for a minimum of 20 hours before analysis, following the method described by (Utermöhl, 1958). In Scotland, the sampling frequency was set at weekly for all designated phytoplankton monitoring points between April and September 2006-2012 and reduced at other times of the year. This was extended to include weekly sampling during March from 2013 onwards,
following a risk assessment. Not all the sites that were tested for shellfish toxins were also monitored for phytoplankton.

For England and Wales, samples from all sites were obtained fortnightly throughout April-September, and monthly during the rest of the year. Samples were collected by tube or pole sampler and fixed by adding 2 mL Lugol’s solution to 500 mL seawater. The Lugol’s fixed samples were placed into 25 mL Utermöhl chambers and allowed to settle on a level bench at room temperature (detection limit of 40 cells/L). After three hours each sample was given a preliminary examination. If the chamber contained high cell concentrations or too much sediment, which would make identification of cells difficult, then an additional sub-sample was set up in a 10 mL or 5 mL Utermöhl chamber. All samples were allowed to settle for a minimum of 12 hours before being analysed using an Olympus1X71 inverted research microscope (Anon, 2006).

Cells belonging to the genus *Pseudo-nitzschia* were identified and enumerated using inverted light microscopy and cell densities were then calculated to express *Pseudo-nitzschia* spp. concentration in cells/L.

### 2.4 Sample reporting

Shellfish samples with values above a designated “Reporting Limit” (RL) of 1 mg/kg were reported to the competent authorities as “detected”. Samples with concentrations above the Maximum Permitted Limit (MPL) of 20 mg/kg were reported as above “Action Level” (AL). *Pseudo-nitzschia* water samples exceeding 150,000 cells/L for FSA were reported as above trigger level whilst for FSS the trigger level of 50,000 cells/L was used, based on the different risk assessments in England/Wales and Scotland. Samples exceeding the trigger level initiated weekly sampling of shellfish and water until levels fell below regulatory threshold.
2.5 Data assessment

Concentrations of DA were collated from all samples tested since April 2008. Samples which gave values above the reporting limit of 1 mg/kg and above the action levels of 20 mg/kg for the shellfish samples and the relevant *Pseudo-nitzschia* trigger level were used to determine any statistical and/or visual relationships between the groups of data. Data were analysed with Microsoft Excel for Office 365, with statistical analyses performed using R statistical software (R Core Team, 2016) and maps drawn using MapInfo Version 12 (Pitney Bowes Software).

3. Results

3.1 Shellfish samples 2008-2017 - GB

3.1.1 Tissue and species

Domoic acid was detected as a distinct peak at 242 nm, with clear separation from matrix peaks crucial for accurate quantitation (Figure 1A). The annual occurrences of shellfish samples which exceeded RL within GB between the period of April 2008 - December 2017 typically ranged between 2.4 - 4.9% of samples tested (mean = 4.11%) (Figure 2A). The exception to this was 2014 when the percentage was notably higher at 7.7%. The lowest value of 2.4% was observed in 2008 although it is noted that data collection only started from April 2008, which may have impacted the results. Regionally (Figure 2B), Scotland had a larger percentage of samples above RL than England and Wales, with mean values for each region being 5.5 and 1.9%, respectively. The increase in 2014 was observed in both regions, although it was more pronounced in England and Wales, rising almost five-fold to 5.1%, from a mean value of 0.95% during the period 2008-2013, in comparison to Scotland which doubled to 9.1% from an average of 4.7% for the same period. Post 2014, the percentage of samples >RL dropped in both regions, although levels in England and Wales remained higher than previously observed.
Since April 2008, the number of toxic samples that exceeded the MPL of 20 mg/kg has been low, with typically only one sample above this level in each of the years 2008, 2012, 2013 and 2014. These samples were found to contain DA concentrations of 25 (common mussels), 27 (common mussels), 33 (common cockles) and 36 mg/kg (common mussels), respectively (Figure 3A). By contrast, in 2016 four samples were found to exceed the MPL, with DA concentrations occurring chronologically during this period of 49 mg/kg in common mussels, and 24, 29 and 34 mg/kg, all of which occurred in razor clams. The percentage of toxic samples above MPL in GB was low, with toxins >MPL in 0.05% of samples tested in 2008, 2012, 2013 and 2014, although this increased to 0.16% of samples in 2016, all of which were derived from Scottish samples. The majority of the samples analysed since 2008 and containing DA >RL originated in Scotland (Figure 3B), with one mussel sample in 2014 being the first and only sample observed above the MPL in England and Wales (Figure 3C). Overall, linear regression analysis of the data using generalised least squares and autocorrelation structure within years revealed that the increase in the percentage occurrence of DA in shellfish above RL was statistically significant during this period for both England and Wales (p=0.0046) and Scotland (p=0.0135) at the 5% level.

The timing of DA occurrence varied on an annual basis, with DA presence in shellfish scattered throughout the year. Maximum DA concentration generally occurred during the late-spring to summer period, although there was considerable variation from year to year. Occurrence of DA typically started in early spring and ended during October, with maximum concentration peaking during the summer months, but being recorded as early as April in 2009 and 2015 and as late as September in 2008. Domoic acid events usually occurred earlier in England and Wales than those observed in Scotland. The results therefore indicate large fluctuations in the timing and intensity of DA occurrence in bivalve shellfish throughout GB through the 10-year study period with higher levels typically appearing in England and Wales around May, and Scottish events more variable in timing and intensity but tending to reach maximum levels later, towards the summer to early autumn period.
Table 1 shows that DA was quantified in a wide range of shellfish species with the majority of samples above RL found in common mussels, although this may reflect the fact that this is the highest percentage of species tested (average 65% over the study period). Toxin levels above the MPL were recorded in mussels, cockles, oysters, and razor clams. During 2014 an increase in the percentage of some species found to be above RL was recorded, notably in cockles, mussels, razors and surf clams. An increase in DA occurrence was observed in surf clams with 52.8% of samples tested above RL in 2016, up from 10% in 2009. All queen scallops tested in 2016 were found to be above the MPL.

3.1.3 Spatial Variability of DA occurrence

Figure 4 shows the distribution throughout GB of samples above both RL and MPL during the study period in each of the 10 years monitored. Clear differences were observed between years. The majority of samples with DA above both RL and MPL occurred on the west coast of Scotland, the Outer Hebrides and the Shetland Islands. The frequency of occurrences in the Shetland Islands was seen to fluctuate significantly, with only one event >RL observed in 2013, in comparison to other years such as 2009 and 2010 where widespread toxicity throughout the islands was observed. It is acknowledged that this may be an artefact of ASP testing frequency in the monitoring programme, as sites were closed for the presence of other toxin groups and therefore not tested for DA, despite the presence of *Pseudo-nitzschia* blooms. The number of ASP tests performed monthly each year is detailed in supplementary tables 1 and 2 and highlights the decrease in sampling frequency from 2012 onwards. The most notable change throughout this ten-year period was an increase in the occurrence of DA in shellfish samples from England and Wales, particularly on the south coast of England. This was particularly noticeable from 2014 onwards, following detection of the only toxic sample with DA above MPL in England and Wales since 2008. A DA concentration of 36 mg/kg was recorded in a mussel sample obtained from a site on the south coast of England during May 2014. During this ten-year period of monitoring, the highest toxicity event occurred in 2016 on the west coast of Scotland, with a concentration of 49 mg/kg detected in a mussel sample collected in May.
3.2 Comparison of Pseudo-nitzschia cell counts to shellfish tissue results

Under light microscopy *Pseudo-nitzschia* spp. is identified by a characteristic rod-like structure and is frequently found in chains of overlapping stepped cells (Fryxell and Hasle, 2003) (Figure 1B). The trigger level for *Pseudo-nitzschia* cell counts differs between the two regions with a limit of 50,000 cells/L in Scotland, and a higher limit of 150,000 cells/L in England and Wales. Figure 5 summarises the occurrences of water samples containing *Pseudo-nitzschia* spp. determined above the relevant regional trigger level for each of the ten years of monitoring in GB between April 2008 and December 2017. In England and Wales, the percentage of samples above trigger level was generally low, with an average of 1.6% of the samples during this period being above the trigger level (Figure 5A). The maximum occurrence of 2.7% was in 2013, with the lowest occurrence being 0.5%, observed in 2009. In Scotland, due to the lower trigger levels, the percentage of samples exceeding this threshold was consequently higher, with an average of 11% during this time (Figure 5B). There was a noticeable variation in bloom frequency in Scottish waters, with a maximum occurrence of 17.2% in 2011, decreasing to 6.5% in 2013. When the lower Scottish trigger level was applied to samples from England and Wales, *Pseudo-nitzschia* bloom occurrence was still less frequent than that observed in Scotland, with a mean of 3.7%, a maximum value of 6.8% in 2013, and a minimum value of 1.7% in 2009 (data not shown). Linear regression analysis using generalised least squares and autocorrelation structure within years revealed that there was no statistical trend for both England and Wales, (p=0.184) and Scotland (p=0.086) at the 5% limit.

Figure 6 shows the distribution throughout GB of water samples containing *Pseudo-nitzschia* above the FSA and FSS trigger limits during the study period in each of the 10 years monitored. As can be seen, *Pseudo-nitzschia* typically occurred in the Shetland Isles, West coast of Scotland and the SW and SE coast of England. As expected, these sites showed considerable resemblance to those sites with DA in shellfish tissue (Fig. 4).
Due to the fact that Scotland observes a greater number of DA events, and that water sampling occurs on a weekly basis, rather than England and Wales where the water sampling is less frequent, Scottish data from 2008 - 2017 were used to investigate the relationship between *Pseudo-nitzschia* cell counts and domoic acid concentrations in the shellfish. Because of the differing frequencies of water monitoring, matching phytoplankton and shellfish testing data were not available for all samples, which unfortunately coincided with a number of samples above MPL. To examine the possibility of a delay between the bloom maxima and the detection of DA in mussel tissue, the occurrence of DA events exceeding 1 mg/kg was compared with the onset of *Pseudo-nitzschia* cell densities that breached the trigger levels. As shown in Figure 7A, 59% of Scottish DA events were found to be associated with levels of *Pseudo-nitzschia* above the trigger level in the same week. A further 7.5% were associated with a bloom occurring the preceding week, with another 1.5% associated with a bloom two weeks prior to the DA event. When analysed this way, the data show that only 17.6% of DA events were not associated with a significant cell density greater than 0.5 trigger level. Figure 7B illustrates the shellfish toxin results > 1 mg/kg in comparison to the *Pseudo-nitzschia* cell counts enumerated in water samples in the same week. In order to reduce any confounding species variability effects, only data obtained from common mussels were used. No correlation was found between the two variables (n = 265, $r^2 = 0.02$). When the data were analysed seasonally from either January-June, or July-August no correlation was found. Whilst there was only one sample in which DA concentrations exceeded MPL with a value of 25 mg/kg, this had a comparatively low cell density of 70,300 cells/L. Conversely, the sample with one of the highest cell densities of 3,415,080 cells/L had an associated concentration of 4.0 mg/kg. While a relationship between elevated *Pseudo-nitzschia* abundance and DA concentration generally exists, this demonstrates that cell density alone does not predict the concentration of DA measured in shellfish tissue.

To further elucidate the relationship between DA concentration in shellfish tissue and *Pseudo-nitzschia* abundance, selected example events of DA occurrence and/or elevated *Pseudo-nitzschia* abundance were analysed in greater detail. As shown in Figure 8A, a bloom which occurred in the
Shetland Islands during the summer of 2012 exhibited high cell densities, up to 1.3 million cells/L, but shellfish toxicity was low, with a maximum DA concentration of 1.9 mg/kg in mussels occurring two weeks after the bloom maxima. This suggests both the occurrence of a dense *Pseudo-nitzschia* bloom with low DA production and also an increase in toxin production as the bloom diminished. Conversely, there are instances of significantly less dense blooms with maximum densities of only 365,040 cells/L coinciding with high toxicities of 35 mg/kg (Figure 8B), and blooms with cell counts less than the FSS trigger level but still resulting in DA accumulation in shellfish (Figure 8C). There have also been instances where a *Pseudo-nitzschia* bloom above the trigger limit does not result in any detectable DA in shellfish (Figure 8D). Overall, no evidence was found from site-specific studies of any repeatable correlation between cell density and shellfish DA concentration.

4.0 Discussion

While an increase in *Pseudo-nitzschia* bloom densities above regulatory threshold was generally coincident with enhanced DA concentration in shellfish, the abundance of this toxic diatom did not correlate directly with the toxin concentrations quantified in the shellfish samples. This suggests that the relationship between the two is complex and dependent on many factors. On some occasions low cell concentrations were found to produce greater toxin concentration in shellfish samples while in others higher cell counts resulted in tissue toxin concentrations below RL. The occurrence of samples containing *Pseudo-nitzschia* above trigger level has reduced or stayed constant over this time period, whilst the percentage of tissue samples above RL has increased. For example, the elevated toxicity occurrences observed in 2014 and 2016 do not appear to be reflected in observed cell counts. It is noted that due to the sampling frequencies and protocols of the monitoring programme, there is the possibility that shellfish samples may not be tested for DA due to closures for other toxin events, meaning that a dense, potentially toxic bloom may not result in a corresponding toxicity result.

The discrepancy observed between *Pseudo-nitzschia* abundance in water samples and DA in shellfish tissue could be partially attributed to the variability of toxicity between the different species of
*Pseudo-nitzschia*, with some species such as *P. australis* and *P. seriata* producing high levels of DA (Skov, J., N. Lundholm, 1999; Smayda, 2006 and references within) whilst others are not toxin producers. Therefore, a dense bloom with cell counts above the *Pseudo-nitzschia* trigger level may exist in the water column for as long as a plentiful supply of nutrients are available, but the species composition present in the water at the time will dictate the toxicity of the event. At sites within Scotland, *Pseudo-nitzschia* is known to be present throughout the year, indicating the potential for DA events to occur year-round if environmental conditions promoted a bloom of toxin-producing species (Bresnan et al., 2017). It is widely appreciated that light microscopy, the methodology used for routine monitoring of *Pseudo-nitzschia*, cannot accurately discriminate between the different species of *Pseudo-nitzschia*, and those that are not toxin producers, or less likely to be toxic, are also included in the overall cell counts. Nevertheless, it is possible to partially discriminate the *Pseudo-nitzschia* genus into the *P. delicatissima* or *P. seriata* groups by light microscopy through measurement of the valve width of individual cells (*P. delicatissima* group < 3 µm and *P. seriata* group > 3 µm) (Fehling et al., 2004b). This is of value in discriminating those that are likely to have a higher toxin content per cell (Fryxell and Hasle, 2003; Skov et al., 1999). It was noted that instances of high toxicity with relatively low cell densities as shown in Figures 8B and 8C, were associated with blooms recorded as predominantly consisting of *P. seriata* group cells, which have a higher toxin content per cell. Conversely, instances of higher cell densities and no toxicity as shown in Figure 8D, were more associated with blooms dominated by species belonging to the *P. delicatissima* group. A potentially more accurate way to discriminate between the species would be the use of electron microscopy to examine the cell morphology, although quantification of cell abundance and slow sample turnaround time mean it is not practical for routine analysis. Molecular based technologies may also provide an interesting option to potentially allow species-specific identification of *Pseudo-nitzschia* and thus help predict potential toxic events (Andree et al., 2011; Fitzpatrick et al., 2010; Pugliese et al., 2017). Species level identification of *Pseudo-nitzschia* by qPCR is used by the Irish monitoring programme (Brennan and Lyons, 2009; Silke and Gilmartin, 2009). Whilst the current UK monitoring programmes
only detail *Pseudo-nitzschia* levels to the genus level, discrimination into separate sub-groups based on valve width, with differing trigger levels, as utilised in the French monitoring programme may provide further information on the prediction of toxic events (https://envlit-alerte.ifremer.fr/accueil).

The lack of correlation between *Pseudo-nitzschia* cell densities and shellfish toxin concentrations has been observed previously (Bresnan et al 2017; Hinder et al 2011) further indicating that genus level identification of *Pseudo-nitzschia* is not a good predictor of toxicity in shellfish tissue. However, species level identification of *Pseudo-nitzschia* revealed that in the Western English Channel, DA was significantly correlated with the presence of the *Pseudo-nitzschia seriata* group and the *Pseudo-nitzschia pungens/multiseriata* group, although it is noted that this was particulate DA concentration rather than shellfish concentration (Downes-Tettmar et al., 2013).

In addition to the variability in toxicity observed between species, it is known that toxin production may also vary between strains within a single species, which can be dependent on regional variations and other factors (Bates et al., 1998). The presence of both toxigenic and non-toxigenic strains of the same species has been documented for several *Pseudo-nitzschia* species, with a range of environmental factors impacting upon DA production (Almandoz et al., 2017; Sahraoui et al., 2012; Thessen et al., 2009; Villac et al., 1993). The mechanisms behind toxin production are not fully understood, but it is believed that stress conditions could be an initial trigger for *Pseudo-nitzschia* (Hinder et al., 2011). Thus, potentially toxic cells may not continually produce toxins and a higher bloom density does not necessarily equate to increased DA levels. The production of DA in some species of *Pseudo-nitzschia* is thought to be a defence mechanism, linked to the presence of herbivorous grazers, specifically *Calanus* copepods (Lundholm et al., 2018). Other environmental conditions such as irradiance (Cusack et al., 2002; Fehling et al., 2005) nutrient levels, especially silica and nitrate, have been linked to cell growth and DA concentration (Downes-Tettmar et al., 2013; Fehling et al., 2004a). Analysis of a range of environmental factors has suggested that Si(OH)₄ limitation may promote DA production by *P. australis*, with the beginning of autumn suggested to be a potential risk period for *P. australis* driven DA events, as this was the only time of year that this
species was observed in the Eastern English Channel (Klein et al., 2010). Changing trace metal conditions in coastal waters may also have a profound effect upon intracellular DA concentrations and thereby influence the toxic effect of these harmful bloom events (Maldonado et al., 2002), with the off shelf to on shelf transport of phytoplankton including *Pseudo-nitzschia* having been demonstrated on the Scottish west coast (Fehling et al., 2012; Siemering et al., 2016). Oceanic strains of *Pseudo-nitzschia* spp. have also been shown to produce significantly less DA than their coastal equivalents, which may be attributable to either differing environmental or physiological conditions between the two environments, or differing strains (Marchetti et al., 2008). The delayed onset of DA production in *Pseudo-nitzschia* could therefore lead to a scenario whereby a bloom may peak in terms of cell density, but only lead to the toxic contamination of shellfish once the bloom begins to decline. In addition, due to the frequency of the testing regime, or the site being closed due to the presence of other toxins (PST or LT), there may be no shellfish testing during the same week of the bloom peak. Therefore, if a bloom develops suddenly and shellfish testing occurs the following week (often when the bloom has dispersed) and the results are positive, this can appear to be a delayed reaction.

Shellfish toxicity levels are dependent on both uptake and depuration rates of shellfish, which can vary between species. Bresnan et al. (2017) reported a marked difference in DA uptake and depuration between scallops and mussels, with scallops displaying a significantly reduced depuration rate. Differences have also been observed between oysters and mussels with mussels exhibiting both faster uptake and elimination rates when compared to oysters in a laboratory study, which also linked depuration rates to animal size (Mafra et al., 2010). Bogan and colleagues demonstrated that smaller scallops exhibited a more rapid uptake of DA (Bogan et al., 2007). Location of animals within the water column has also been suggested to be linked to toxicity. Scallops located on the sea floor were found to depurate slower than those suspended from mussel rafts (Blanco et al., 2006). There have also been instances of lags between toxin blooms and resultant toxicity as shown in Figure 8B which has been documented by other authors (Bresnan et al., 2017). It is possible that in those instances where toxic events occurred with no apparent cause could be due to bloom patchiness, inadequate sampling or
rapidly changing environmental conditions. This suggests that in addition to the choice of species for an indicator, location and position within the water column may be critical to best reflect the shellfish harvesting area.

The results presented here indicate that within this period there were large fluctuations in the timing and intensity of DA in bivalve shellfish throughout GB during the 10-year study period, with higher levels appearing in England and Wales in the spring to early summer and with Scotland peaking towards the summer to early autumn. These events are consistent with other studies in Scotland, whereby the later season blooms were associated with DA events, which has been attributed to differing species composition (Bresnan et al., 2017). Spring populations are typically associated with *P. delicatissima* which are not usually associated with DA production in Scottish coastal waters, whereas later blooms typically consist of *P. australis* and *P. seriata* (Bresnan et al., 2017). This is also consistent with studies from the Argentine sea where DA concentration was found to be significantly correlated with the presence of *P. australis* (Almandoz et al., 2017).

Whilst the levels of DA in shellfish is low throughout the UK, the data shown here indicate a significant increase in both DA and toxicity occurrence throughout this period, particularly in England and Wales, although longer time-course analysis is required to determine if this trend will continue. This apparent increase in shellfish DA concentrations appears to be occurring with no rise in *Pseudo-nitzschia* cell concentrations or bloom frequency. This would suggest either a change in the phytoplankton community, and/or a change in environmental factors favouring toxin production. Environmental factors such as increases in nutrient availability from elevated pollution of coastal waters had also been proposed as possible causes of toxic blooms of *Pseudo-nitzschia* spp. (Mos, 2001). Analysis of DA contamination in king scallops in the English channel has revealed that since 2000, toxic events have become more frequent, which is consistent with the data observed here (Husson et al., 2016). Furthermore, the authors concluded that speciation of *Pseudo-nitzschia* blooms is required to allow an accurate link to toxicity events and that major climatic events can trigger toxicity. Climate data for
the UK show an increase in the UK annual average temperature, increases in winter precipitation levels and resultant increases in winter run-offs (UK, 2016). It is also noted that the winter of 2013/2014 was an extremely wet winter and suffered a succession of winter storms which were particularly prevalent in southern England and resulted in elevated precipitation levels (Schaller et al., 2016), with heavy rainfall having been suggested to limit levels of key nutrients such as $\text{Si(OH)}_4$ and $\text{NO}_3$ which the authors suggest may have promoted the production of domoic acid in the Eastern English Channel (Klein et al., 2010).

Hinder and colleagues performed an analysis of 50-year data from the continuous plankton survey which has shown an increase in *Pseudo-nitzschia* spp. abundance during this time, driven by increases in sea surface temperatures and elevated wind (Hinder et al., 2012). Recently, a DA risk assessment model for the US West Coast has shown that elevated levels of DA in razor clams are linked to warm water phases of the northeast Pacific as a results of climate change (McKibben et al., 2017). The elevated water temperatures are suggested to affect *Pseudo-nitzschia* spp. abundance and toxicity in a variety of ways, ranging from altered cellular and metabolic responses to hydrodynamic alterations. Elevated water temperatures have also been linked to elevated toxicity due to the competitive success of certain toxic *Pseudo-nitzschia* species isolated from Californian waters (Zhu et al., 2017). In Denmark, increased water temperatures have been suggested to be linked to a shift in the species composition of the *Pseudo-nitzschia* genus, with a shift towards dominance by the toxigenic *P. pungens*, and an increasing relative abundance of *P. seriata* and *P. americana* over the last 100 years (Lundholm et al., 2010). A recent high-resolution climate projection for the north-west European shelf has suggested that the habitat of most species will be altered by the mid-end of the century, including a southward habitat suitability shift for *P. australis* (Townhill et al., 2018). An understanding of how climate change will impact upon phytoplankton communities (Dees et al., 2017; Gobler et al., 2017) and how this in turn will affect toxicity events is essential to help manage safe and sustainable shellfish production in the future (Wells et al., 2015).
Overall, it can be concluded that whilst levels of DA within GB shellfish are low, these levels may be increasing, particularly in England and Wales, without an increase in \textit{Pseudo-nitzschia} bloom frequencies. It is also apparent that these events and their location can be fairly unpredictable from year to year, making a risk assessment unreliable based on \textit{Pseudo-nitzschia} abundance alone. This highlights the requirement for an effective tissue-testing programme to avoid any potential food safety issues. Whilst phytoplankton monitoring often predicts shellfish toxicity, due to the complex and diverse nature of the \textit{Pseudo-nitzschia} genus, further information on species composition, aided by modern technologies that enable the detection of toxic species/strains, will be of great benefit to the monitoring programmes.

\textbf{Acknowledgments}

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Bibliography


nov. (Bacillariophyceae), a toxigenic species from the strait of Malacca, Malaysia. Harmful Algae 34, 17–28. https://doi.org/10.1016/j.hal.2014.02.005


Table 1. Summary of the percentage of individual species found to contain DA >RL during study period per year. The number in brackets shows the no. of samples >RL. * = samples collected from April-December only

<table>
<thead>
<tr>
<th>Year</th>
<th>Cockles</th>
<th>Mussels</th>
<th>Native oysters</th>
<th>Pacific oysters</th>
<th>Queen scallops</th>
<th>Carpet clams</th>
<th>Hard clams</th>
<th>Manila clams</th>
<th>Otter shells</th>
<th>Razor clams</th>
<th>Surf clams</th>
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<tr>
<td>2008*</td>
<td>0</td>
<td>3.0 (51)</td>
<td>0</td>
<td>1.5 (3)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>6.0 (12)</td>
<td>3.3 (68)</td>
<td>0</td>
<td>0.3 (1)</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>1.6 (1)</td>
<td>10.0 (2)</td>
<td></td>
</tr>
<tr>
<td>2010</td>
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<td>3.7 (71)</td>
<td>0</td>
<td>2.6 (10)</td>
<td>80.0 (12)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>7.7 (1)</td>
<td>1.3 (1)</td>
<td>3.3 (1)</td>
</tr>
<tr>
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<td>2.6 (5)</td>
<td>3.7 (67)</td>
<td>0</td>
<td>4.1 (17)</td>
<td>0</td>
<td>0</td>
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<td>N/A</td>
<td>4.3 (5)</td>
<td>8.8 (3)</td>
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<td>2012</td>
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<td>3.8 (47)</td>
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<td>5.0 (16)</td>
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<td>0</td>
<td>0</td>
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<td>2.5 (28)</td>
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<td>N/A</td>
<td>8.7 (2)</td>
<td>0</td>
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<tr>
<td>2014</td>
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<td>9.3 (14)</td>
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<tr>
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<td>6.1 (31)</td>
<td>100.0 (3)</td>
<td>18.8 (3)</td>
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<td>2017</td>
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<td>4.7 (17)</td>
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<td>3.8 (2)</td>
<td>0</td>
<td>N/A</td>
<td>11.6 (13)</td>
<td>94.4 (17)</td>
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Figure 1. Identification of (A) Domoic acid in a shellfish extract by RP-HPLC with UV detection at 242 nm (m denotes matrix peaks) and (B) Pseudo-nitzschia seriata group (valve width 7.9 µm) by phase contrast light microscopy.
Figure 2. Summary of domoic acid occurrence within Great Britain during the period 2008-2017. The percentage of samples tested which were above the Reporting Limit (RL) of 1 mg/kg were calculated for both GB (A) and when sub-divided into geographical regions (B). The number in brackets is the total number of samples tested for DA.
Figure 3. Summary of domoic acid (mg/kg) in individual shellfish samples from (A) Great Britain combined, (B) Scotland, and (C) England and Wales between April 2008 and December 2017. Dashed line indicates action limit of 20 mg/kg.
Figure 4. Spatial distribution of ASP toxicity events between April 2008 and December 2017. Yellow dots indicate samples with DA concentrations higher than the reporting limit of 1 mg/kg, and red dots highlight samples above the action limit of 20 mg/kg. Black dots indicate all sampling sites.
Figure 5. Summary of the percentage of water samples containing *Pseudo-nitzschia* above the FSA trigger levels in (A) England and Wales or the FSS trigger level in (B) Scotland received between April 2008 and December 2017. The number in brackets is the total number of samples tested for *Pseudo-nitzschia*. 
Figure 6. Relationships between shellfish domoic acid concentration and *Pseudo-nitzschia* levels. A, a scatter plot of corresponding domoic acid concentrations above 1mg/kg in mussels and *Pseudo-nitzschia* measurements obtained from Scotland between April 2008 and December 2017. B, Percentage occurrence of DA events above 1 mg/kg in Scottish mussels when compared to *Pseudo-nitzschia* bloom events.
Figure 7. Selected Scottish episodes of elevated domoic acid concentration in common mussels and Pseudo-nitzschia levels in the water obtained between 2007 and 2017.