An Evaluation of the risks to food safety and shellfish farming in Great Britain,posed by marine biotoxins from, current and future emerging, marine microalgal species

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An evaluation of the risks to food safety and shellfish farming in Great Britain, posed by marine biotoxins from, current and future emerging, marine microalgal species

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Abstract

Harmful marine microalgae are a global concern, impacting human and ecosystem health as well as having socioeconomic impacts for coastal communities. The changing world climate has an impact on marine organisms including the harmful algal species. These changes will have impacts on species already present in a nations waters whilst also influencing the emergence of novel species. This is assessed here, in part, with regards to Great Britain (GB).

This thesis explores the current extent of a harmful species, *Alexandrium minutum,* globally and in the South of GB. This shows that *A. minutum* occurs widely across the globe with different populations possessing varying toxin profiles. Populations from GB geographically neighbouring areas share similar toxin profiles. Within the South of GB, the current extent of *A. minutum* appears patchy, with evidence gathered by toxin profile analysis but successful germinations of vegetative cells from field samples proving unsuccessful. Experimental work determined a mechanism for the use of chemotaxonomy to differentiate the source of shellfish intoxications, allowing for separation of two key GB saxitoxin producers, *A. minutum* and *Alexandrium catenella.* This technique could enhance routine monitoring data with little additional cost.

Assessment of harmful microalgal taxa considered as non-native species (NNS) to GB suggested that several species could pose a risk of future successful invasion of GB coastal waters, within the next 30 years. This was principally based on the environmental tolerances of NNS. If established the impacts which NNS could impose on GB include similar impacts to native harmful species as well as a higher risk of environmental damage. Experimental work with a high-risk potential invasive species, *Ostreopsis* cf. *ovata,* indicated that this impact could be acute, with rapid mortalities observed in exposed naïve GB mussels.

Taken together this body of work shows the validity of chemotaxonomic assessment of toxin profiles as an additional tool for the tracking of harmful microalgal species as well as proactively assessing the risk and impacts which climate change might have for the future impacts of harmful marine microalgal species around GB.

Contents

List of Figures

List of Tables

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Authors declaration

I Declare that the material contained in this thesis is my own work.

Abbreviations and definitions

- AOAC – Association Of Analytical Chemists
- ASP – Amnesic Shellfish Poisoning
- ASPEC – Automated Solid Phase Extraction Computer
- AZA - Azaspiracid
- AZP – Azaspiracid Shellfish Poisoning
- BHAB- Benthic Harmful Algal Bloom
- C1 - N-sulfocarbamoyl Gonyautoxin 2
- C2 - N-sulfocarbamoyl Gonyautoxin 3
- C3 – N21-sulfocarbamoyl-11α-hydroxysulfate-neosaxitoxin
- C4 - N21-sulfocarbamoyl-11β-hydroxysulfate-neosaxitoxin
- C - Carbon
- CA – California
- CCAP- Culture Collection of Algae and Protozoa
- Cefas – Centre for Environment, Fisheries and Aquaculture Science
- CFP – Ciguatera Fish Poisoning
- dcGTX – Decarbamoyl Gonyautoxin
- dcNeo – Decarbamoyl Neosaxitoxin
- dcSTX – Decarbamoyl Saxitoxin
- DNA – Deoxyribonucleic acid
- doSTX – Deoxydecarbamoyl Saxitoxin
- DSP - Diarrhetic Shellfish Poisoning
- eDNA – Environmental Deoxyribonucleic acid
- EU – European Union
- GB - Great Britain

- GPS – Global Positioning System
- GTX - Gonyautoxin
- H – Hydrogen
- HAB – Harmful Algal Bloom
- HILIC – Hydrophilic Interaction Liquid Chromatography
- HILIC-MS/MS – Hydrophilic Interaction Liquid Chromatography with Tandem Mass Spectrometry
- HPLC – High Performance Liquid Chromatography
- HPLC-FLD - High Performance Liquid Chromatography with Fluorescence Detection
- HPLC-PCOX – High Performance Liquid Chromatography with Post Column Oxidation and Fluorescence Detection
- ICES - International Council for the Exploration of the Seas
- ID - Identification
- LC – Liquid Chromatography
- LC-MS – Liquid Chromatography with Mass Spectrometry
- LC-MS/MS – Liquid Chromatography with Tandem Mass Spectrometry
- LT – Lipophilic Toxins
- M1 – N21-sulfocarbamoyl-11(α/β)-hydroxy-saxitoxin
- M2 – 11(α/β)-hydroxy-saxitoxin
- M3 – N21-sulfocarbamoyl-11,11-dihydroxy-saxitoxin
- M4 – 11,11-dihydroxy-saxitoxin
- M5 – Uncharacterised Saxitoxin Analogue
- M – Molar
- MA – Massachusetts
- MBA – Marine Biological Association

- m:z – Mass to charge ratio
- N - Nitrogen
- Neo - NeoSaxitoxin
- NSP – Neurotoxic Shellfish Poisoning
- NS – Nova Scotia
- NW – Northwest
- O – Oxygen
- Ost - Ostreocin
- OvTX - Ovatoxin
- P - Phosphorous
- PCR – Polymerase Chain Reaction
- PlTX - Palytoxin
- PSP – Paralytic Shellfish Poisoning
- PST – Paralytic Shellfish Toxin
- RCF – Relative Centrifugal Force
- RNA – Ribonucleic acid
- rDNA – Ribosomal Deoxyribonucleic acid
- rRNA – Ribosomal Ribonucleic acid
- RPM – Revolutions per Minute
- SPE – Solid Phase Extraction
- STX – Saxitoxin
- SW - Southwest
- TEF – Toxin Equivalence Factor
- UK – United Kingdom
- USA – United States of America

- YTX - Yessotoxin

Chapter 1 -Harmful algal toxins – global trends, changes and impacts on the UK

1.1 - Harmful algal species

Marine phytoplankton are a diverse group of organisms comprised of many species from a range of unrelated taxonomic groupings. Although they are unrelated, they are often considered together as, at least on a large scale, they often have similar impacts. Crucially they provide primary productivity across a large geographic scale and are considered to provide around half of the photosynthetic primary productivity on Earth (Field et al., 1998). Other important features include the transport of carbon to the deep ocean and subsequent carbon sequestration, the cycling of nutrients and the regulation of the climate (Naselli-Flores and Padisák, 2022).

Within the many groups of microalgal taxa there are a small number which include species known to be harmful. This may be in the form of causing harm to those organisms directly associated with them in the marine environment through to species known to produce compounds which cause toxicity in higher organisms. Of the 5000 recorded species of phytoplankton there are only 200-300 listed as harmful species (Hallegraeff, 2004, 1993; Masó and Garcés, 2006). These predominantly belong to the Dinoflagellates with other harmful species existing in other groups such as the Diatoms and the Raphidophytes (Hallegraeff et al., 2021).

Many species considered harmful are naturally present in the phytoplankton community (Figueiras et al., 2006) and are only known to cause issues when experiencing a bloom. The term ‘Algal Bloom’ is largely undefined, with many different authors using the term to describe vastly different cell proliferations (Smayda, 1997). A bloom is perhaps best described as a rapid atypical proliferation of a given species or in the case of harmful algal bloom (HAB), the term is often used to describe any event causing a deleterious impact, irrespective of cell densities (Masó and Garcés,

2006; Smayda, 1997). This is because ascribing a specific cell density to a bloom event is very difficult, with different species causing issues at significantly different cell concentrations as well as having very different normal cell densities based on life history and competitive characteristics. For example, species in the genus *Dinophysis* (Ehrenberg, 1839) can cause shellfish intoxications at very low cell densities ($10^2 – 10^4$) whereas brown tides species from the genera *Aureococcus* (Hargraves & Sieburth 1988) and *Aureoumbra* (D.A. Stockwell, DeYoe, Hargraves & P.W. Johnson, 1997) can cause damage to ecosystems at much higher cell densities, up to 10^9 cellsL^{-1} (Gobler and Sunda, 2012; Masó and Garcés, 2006). Some species may only become harmful at very high concentrations when water is visibly discoloured, often termed high biomass blooms, whereas others may cause issue when cell concentrations are orders of magnitude lower, such as shellfish intoxications caused by toxin producing microalgal species (Masó and Garcés, 2006).

Other than toxins produced within algal cells, common impacts of harmful blooms include causing localised hypoxia, smothering of or damage to the gills of aquatic organisms, via excessive mucus production or the presence of spines on cell surfaces which can cause mechanical damage (Backer and Miller, 2016; Cullen, 2008; Masó and Garcés, 2006; Sellner et al., 2003). In some cases, even algal species considered to be benign can cause negative impacts through the creation of localised hypoxia during excessive growth or following bloom collapse (Hallegraeff, 2004). These events have been linked with harmful species, and also what is often termed nuisance algae. Mucus production from some species can also have properties not unlike an oil spill, this can foul the feathers of birds and have similar impacts to crude oil exposure on affected individuals (Jones et al., 2017).

1.2 – Harmful algal toxins

Of those species of microalgae considered harmful, around 190 are known producers of toxins which can impact a range of higher organisms (Hallegraeff et al., 2021). Toxins produced by

microalgae are commonly termed phycotoxins. The key toxin groups and their associated impacts are listed in Table 1.1. Some of these compounds have a potent effect in mammalian systems, including humans. The potential exists for these phycotoxins to enter the human food chain through the contamination of marine species, both in the wild and aquaculture. Due to the ability of vector organisms to accumulate high levels of these toxins, monitoring to protect human health is commonplace in many developed nations (De Witte et al., 2022).

Table 1.1: After Turner et al., (2021) showing key human intoxication syndromes with brief comments on causative compounds and symptoms.

Syndrome	Parent toxin	Number of known analogues	Symptoms	Reference
Paralytic Shellfish Poisoning (PSP)	Saxitoxin	57	Paraesthesia, nausea, vomiting, respiratory distress (severe doses: respiratory paralysis and death). Muscular weakness, drowsiness, incoherent speech	(Grattan et al., 2016; Wiese et al., 2010)
Amnesic Shellfish Poisoning (ASP)	Domoic Acid	9	Abdominal cramps, nausea, vomiting, respiratory distress, disorientation, seizures, permanent short- term memory loss, possible neurodevelopmental delay. Coma and death only among the most severe cases	(Grattan et al., 2016; Saeed et al., 2017)
Diarrhetic	Okadaic Acid	11	Gastrointestinal including,	(Dominguez et

Shellfish Poisoning (DSP)			diarrhoea, nausea and vomiting Headache, fever.	al., 2010; Grattan et al., 2016)
Azaspiracid Shellfish Poisoning (AZP)	Azaspiracid	>60	Acute gastrointestinal problems; nausea, vomiting, diarrhoea, and stomach cramps.	(Krock et al., 2019; Twiner et al., 2008)
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxin	>10	Consumption: paraesthesia, abdominal cramps, temperature reversal, diarrhoea, bronchoconstriction, respiratory distress, disorientation. Inhalation: bronchoconstriction, respiratory distress, sneezing, coughing, itchy and watery eye.	(Grattan et al., 2016; Watkins et al., 2008)
Ciguatera Fish Poisoning (CFP)	Ciguatoxin	41	Initial acute gastrointestinal symptoms with multiple neurological issues developing later. Including paraesthesia, numbness and tingling, temperature dysesthesias, loss of coordination and motor control, in extreme cases coma and death. Cases can be prolonged with relapses many years later, recovering patients	(Dickey and Plakas, 2009)

			report depression, fatigue, anxiety and neurosis.	
Palytoxicosis	Palytoxin	20	Consumption: Abdominal cramps, nausea, vomiting, diarrhoea, paraesthesia, bradycardia, rhabdomyolysis renal failure, cyanosis and respiratory distress, in extreme cases fatality. Inhalation: Fever, respiratory distress, bronchoconstriction, mild dyspnea, and wheezes, conjunctivitis Dermal contact: shivering, myalgias, chest pain, weakness, dizziness and speech disturbance.	(Deeds and Schwartz, 2010; Patocka et al., 2018; Ramos and Vasconcelos, 2010)

Monitoring programmes are routinely established to provide information on the level of these phycotoxin contaminants in marine organisms destined for human consumption. Typically, this involves the monitoring of marine bivalve molluscs, which can accumulate several classes of toxins to dangerous levels (De Witte et al., 2022). These phycotoxins are given safe thresholds within food products, with the typical response to breaches of these thresholds being a temporary suspension of harvesting from a given site. Phycotoxins are naturally metabolised and excreted from contaminated organisms but this process takes a varying length of time depending on the toxin group, the contaminated species and the level of contamination (Bricelj and Shumway, 1998; Jauffrais et al., 2012; Mafra et al., 2010; Reguera et al., 2014). Closures of harvesting sites can last for periods of

only a few weeks or prolonged periods of months (Reguera et al., 2014). The result of effective monitoring is a shift of the impact of a seafood contamination from human health issues to economic ones. With closures of shellfish harvesting sites being costly for the producer as well as the ongoing burden of funding the monitoring programme (Anderson, 2009).

Monitoring programmes also regularly screen water samples for potentially toxic microalgal species, to provide an additional layer of protection and act as a broad early warning system. This element is typically carried out via the use of light microscopy with direct assessment and enumeration of species within a small subsample of water collected from sites of interest. Often this is accomplished using the well-established Utermöhl method (Edler and Elbrächter, 2010; Hallegraeff et al., 1995) utilising light microscopy and settlement chambers to evaluate the levels of various algal groups simultaneously (Swan and Davidson, 2011; Touzet and Raine, 2007).

Other toxin groups and their causative organisms are much more difficult to monitor for, this includes toxin groups affecting transient marine organisms (ciguatera in finfish) as well as those emerging toxin groups that have poorly established methods, a lack of analytical reference materials and/or a difficulty of sample collection (e.g. aerosolised toxins from, or cells of, *Ostreopsis* (J. Schmidt, 1901)) (Jauzein et al., 2018; Litaker et al., 2010). In some cases, early warning from cell counts provided by water monitoring can offer a viable alternative trigger for toxin monitoring but in other cases significantly more work is required to provide suitable methods and sampling strategies to enable effective monitoring (Belin et al., 2021; Berdalet et al., 2017).

Of note is that these compounds which are referred to as toxins are not necessarily toxic to the direct predators or competitors of the microalgae which produce them. Although there is evidence of these compounds having a direct impact on certain species of grazers there is also evidence of little to no impact in a host of others with the term grazers covering micro through to mesozooplankton (Turner, 2006). This can be seen in groups such as the copepods, where a range of studies have shown a negative impact of exposure to harmful microalgae, whilst others have not

found evidence of this, either as a result of the species involved in the studies differing or the origin of the exposed population (Turner, 2006). In relation to this last aspect, it has been suggested that those populations of grazers with routine exposure to a harmful microalgal species showed less negative responses than populations of the same grazer without prior exposure to the same harmful algal species (Turner, 2006). As such, the precise ecological role of each suite of compounds has yet to be determined (Cembella and John, 2006). Therefore, whilst toxins will be referred to throughout this thesis it should be noted that in this context this refers to compounds which have a toxic effect on higher organisms, especially humans and is not indicative of toxic effects within the planktonic community, and in some cases, not even in the marine environment.

1.3 – Global distribution and change

Harmful microalgae are widely distributed in the oceans, with reports of harmful species from all of the continents, even the Antarctic and Arctic. As such harmful algae have a global impact, although different regions are variably affected, with different species of microalgae causing the issues or the problems varying in magnitude. Some of the toxin syndromes are currently only known from certain regions, for example, ciguatera fish poisoning (CFP) is almost exclusively associated with waters in the tropics and sub tropics (Chinain et al., 2021; Litaker et al., 2010). Although more recent discoveries of the causative toxins have extended the global range that this syndrome is known to occur within, it still remains confined to warmer waters than those common in temperate regions (Chinain et al., 2021). Other toxins, such as the paralytic shellfish toxins (PST), have been detected across a much wider geographic spread (Etheridge, 2010). Incidents from this toxin have impacted coastlines globally from the Northern coast of Norway to the Southern tip of Argentina (Anderson, 2009). The extent of PST can be attributed to the variety of producers currently known, with several different species, genera and even domains, currently known to be capable of PST synthesis (Etheridge, 2010). This allows for different causative organisms to occupy different niches in

different regions. Conversely, the toxins responsible for CFP are currently only known to be produced by two related genera of dinoflagellate, *Gambierdiscus* (Adachi & Fukuyo, 1979) and *Fukuyoa* (Gomez, Qiu, Lopes & Lin, 2015), limiting the potential geographic spread to those waters able to support their growth (Chinain et al., 2021).

There has long been a suggestion that the incident and extent of harmful algal species are increasing globally (James et al., 2010). With coastal eutrophication and climate change often being cited as key drivers along with human mediated introductions of HAB species, via ballast water and aquaculture species movements (Hallegraeff and Bolch, 1991; James et al., 2010; Smayda, 2007). Recent work under the GlobalHAB initiative however has suggested that this is not the case, with no clear trend emerging globally, with regard to an increasing prevalence of HAB species (Hallegraeff et al., 2021). The increase in monitoring effort and efficacy has been posited as one of the key drivers in the apparent increase of HAB globally, with new records being made of existing issues rather than new issues occurring.

However, it should be noted that regionally there are changes and trends in some areas. Particularly important for this body of work, North Europe emerges as a region where there is evidence of an increasing trend in HAB occurrence and impacts (Hallegraeff et al., 2021).

1.4 - Current impacts in Great Britain

Great Britain (GB) currently experiences annual impacts from harmful algal species, with closures of shellfish harvesting sites being a recurrent issue. There have also been a number of human intoxications within the UK, mostly historic but with some more recent, these are outlined in Table 1.2. There is a well-established monitoring programme for phycotoxins in shellfish in the UK as well as the causative organisms in shellfish growing waters. This monitoring encompasses five regulated groups of shellfish toxins, all of which are included in both UK and EU law (EC 853/2004, 2004; EU 786/2013, 2013). They are the Paralytic (PSP), Amnesic (ASP) and Diarrhetic (DSP) shellfish

poisoning toxins as well as the Azaspiracids (AZP) and Yessotoxins (YTX). The latter three (DSP, AZP & YTX) all possess lipophilic properties and are able to be detected simultaneously using one analytical method, as they can be extracted together owing to their similar properties. In the UK, PSP represents the greatest risk to public health in terms of the severity of the symptoms whilst DSP is the most prevalent toxin group, impacting the most shellfish growing sites for the longest periods on average (Dhanji-rapkova et al., 2018).

Table 1.2: After Bresnan et al., (2021); displaying recorded UK intoxications caused by marine biotoxins of microalgal origin

Intoxication	Year	Number of Human illnesses	Number of human fatalities	Reference
PSP	1827	30	2	Ayres, (1975)
	1858	'Several'	1	Ayres, (1975)
	1888	3	1	Ayres, (1975)
	1904	2	1	Ayres, (1975)
	1909	19	1	Ayres, (1975)
	1958	3	0	Ayres, (1975)
	1968	78	0	Ayres, (1975)
DSP	1997	49	0	Hinder et al., (2011)
	2006	159	0	McDougall and Midgley, (2011)
	2013	70	0	Whyte et al., (2014)
	2019	6	0	Young et al., (2019)

In UK waters the dinoflagellate genus *Alexandrium* (Halim, 1960) is thought to be exclusively responsible for the production of PSP toxins (Brown et al., 2010; Percy, 2006). With three PSP toxin

producing species present in UK waters, *A. minutum* (Halim, 1960)*, A. catenella* ((Whedon & Kofoid) Balech, 1985) and *A. ostenfeldii* ((Paulsen) Balech & Tangen, 1985)*,* the last of which is thought to have a limited distribution and the smallest impact. In Scotland, *A. catenella* is the most commonly implicated producer of PSP toxins and in England and Wales it is thought to be *A. minutum* (See Chapter 2, published as Lewis et al., 2018). The West coast of Scotland and the Southwest of England are the main regions impacted by this toxin group, with few other regions having had recorded instances of PST in shellfish. As such, which PSP toxins have a limited range of impact within GB, with only a few sites being heavily affected although no apparent spatial trend was evident in the paper by Turner et al. (2014), suggesting that recurrence of PSP in shellfish is inconsistent. To date PSP is the only algal biotoxin group which has caused recorded human fatalities within GB.

The most common group of toxins affecting GB shellfish production by enforcing shellfish harvesting site closures are the DSP toxins. This family of toxins are common in shellfish produced on the West coast of Scotland, the Shetland Isles in the North of Scotland as well as in the Southwest of England and have led to prolonged annual closures (Dhanji-rapkova et al., 2018) and the most recent human intoxications from algal biotoxins in the UK (Bresnan et al., 2021). These toxins are produced by members of the dinoflagellate genus *Dinophysis,* with *D. acuta* (Ehrenberg, 1839) and *D. acuminata* (Claparède & Lachmann, 1859) being common causative organisms (Bresnan et al., 2021; Dhanji-rapkova et al., 2018; Tett and Edwards, 2002). Other DSP producers in GB waters are also members of the dinoflagellate group and include *Prorocentrum lima* ((Ehrenberg) F. Stein, 1878) (Tett and Edwards, 2002) and *Phalacroma rotundatum* ((Claparède & Lachmann) Kofoid & J.R. Michener, 1911) (Bresnan et al., 2021).

The Azaspiracids, causing AZP, are also produced by dinoflagellates. These are known to originate in the genera *Azadinium* and *Amphidoma,* with *Azadinium spinosum* (Elbrächter & Tillmann, 2009) being the suspected causative agent of AZP contamination in shellfish from Great Britain. First determined in shellfish originating from Ireland in 1995, this class of toxins has since

been found more widely in Europe (Bresnan et al., 2021). The earliest confirmed instances of AZP contamination, in GB, at levels above regulatory limits occurred in 2011 but have not become a recurrent annual issue. Although there were several more closures through to 2013 there have not been any serious levels of AZP detected in the monitoring programme since (Dhanji-Rapkova et al., 2019). It is possible that there had been elevated levels of AZP prior to 2011 but testing for all lipophilic toxins was carried out via the mouse bioassay at this time, which cannot provide information on the specific category of lipophilic toxins present (Turrell and Stobo, 2007). It was only after the switch, in 2011, to a method utilising liquid chromatography coupled with tandem mass spectrometry that the presence of AZP in shellfish was confirmed (Bresnan et al., 2021).

The final group of the lipophilic toxins is the Yessotoxins, interestingly these compounds appear in the legislation but do not have any known human intoxication syndrome. There is evidence that the yessotoxins cause acute toxicity in mammals following injection but these same toxic affects have not been replicated when the yessotoxins are supplied via oral gavage (Draisci et al., 2000; Tubaro et al., 2003). Presently these compounds remain in the legislation and are still monitored for on a regular basis. The presence of yessotoxins has been detected at a number of sites around Great Britain but have yet to cause a substantial number of harvesting site closures, with only 2 recorded to date (Dhanji-Rapkova et al., 2019). In part this is due to a change in the maximum permitted limit of YTXs from 1mg/kg of shellfish tissue to 3.75mg/kg of shellfish tissue, in September 2013. This change was prompted by the aforementioned lack of human intoxications (EU 786/2013, 2013). If the higher limit had been applied since the YTXs were quantified in GB separately, by LC-MS/MS from 2011 onwards, no sample would have breached the maximum permitted level and the YTXs would have had no recorded impact on human health or socioeconomics within GB. The suspected producer of YTXs in GB is the dinoflagellate *Protoceratium reticulatum,* ((Claparède & Lachmann) Bütschli, 1885) although another producer *Lingulodinium polyedra* ((F. Stein) J.D. Dodge, 1989) is also known to be present in some regions (Dhanji-Rapkova et al., 2019).

The final class of toxins regulated in the UK are the ASP toxins (Rowland-Pilgrim et al., 2019). Unlike those covered above, these toxins are produced by genera of diatom (Bates et al., 2018), rather than dinoflagellate. This means that the ASP toxins and the blooms producing them may occur under different conditions than those that may favour the presence of the other toxin groups. For example as diatoms *Pseudonitzschia* (Yamaguti, 1965) has a requirement for Si which the dinoflagellates do not meaning a lack of Si can limit the growth of *Pseudonitzschia* and other diatoms without negatively effecting the growth of flagellates (Davidson et al., 2012). Within GB the causative organisms of the ASP toxins belong to the genus *Pseudonitzschia* with several toxigenic species having been identified (Tett and Edwards, 2002).

1.5 - Future impacts in Great Britain

It is evident that some regions of GB experience more impact from algal biotoxins than others, with the West coast of Scotland and the Southwest of England being notable for being impacted by multiple toxin classes. In part, this is due to a lack of shellfish production in many parts of the British coastline, resulting in a lack of monitoring for biotoxins but also in a limited scope for impacts to occur. Temperature is thought to be a key driver of algal bloom formation, with increasing temperatures in temperate regions implicated in the elevated risk of Harmful Algal Bloom formation (Gobler, 2020). As such, predicted climatic changes and the warming of temperate waters have been suggested as potential drivers of future HAB events in impacted regions, including around the UK (Baker-austin et al., 2013; Glibert et al., 2014). Furthermore, projected climate change is expected to alter other key factors in driving HAB formation, such as nutrient regimes, freshwater inputs, salinity and stratification (Glibert et al., 2014; Gobler, 2020). This future impact could be an exacerbation of existing annual impacts or could also include the occurrence of new impacts, as microalgal species from other global locations might be able to establish and persist following introduction. Consequently, the introduction of new harmful microalgal species to a region has the

possibility of causing unexpected consequences, as naïve populations of native organisms may behave differently than expected upon exposure to new harmful algal pressures. For example, as previously covered, greater impacts have been observed in naïve populations of copepods exposed to harmful microalgae compared to populations of the same species with a history of exposure (Turner, 2006). This complicates the application of known impacts of a harmful microalgal species to the forecasting of impacts which would be expected if that same species established in a novel location.

1.6 - Thesis Aims and Objectives

The presence of harmful microalgae in British waters constitutes an ongoing threat to both the British shellfish industry and the health of shellfish consumers. The monitoring efforts in place largely guard against the latter but changing global and local conditions mean that the threat posed is not static. This thesis therefore, seeks to explore both current and potential emergent threats to the UK shellfish industry and human populations.

As such, the work presented herein had four key goals:

To assess the status of a currently present toxin producing microalgal group in a global setting and to assess how the global status of this species relates to the situation in GB. The species chosen for this work was the PSP producing species, *Alexandrium minutum*.

Following the initial literature review the next objective was to evaluate both the spread of the producing organism and assess the analysis of the toxins present. Both techniques were undertaken with a view to determining the current spread of *A. minutum* within sites of known occurrence as well as in adjacent areas, not always covered by routine monitoring efforts. The second aspect of tracking the extent of *A. minutum* in GB water was to assess the possibility of whether further analysis of shellfish toxin data could offer a means to identify the causative toxic microalgal species responsible for a shellfish intoxication event. This was made possible due to the complex nature of

the saxitoxin group and the possibility of chemotaxonomic differentiation between microalgal species.

The next aspect of the work undertaken was to look to the future of harmful microalgal presence in GB and to assess future risks rather than current ones. The first aspect therefore was to determine the risk of future successful invasions from a harmful microalgal species not currently known from GB waters as well as to assess the level of harm a future invasion might have, based upon published literature. This provided a means to focus future research and to highlight gaps in existing monitoring efforts or available methods, allowing for time to adjust and make suitable preparations.

Finally, this research aimed to assess the possible impact to GB shellfish and shellfish consumers, via controlled experimental exposure of GB shellfish to a future high risk microalgal invader. This aspect provided ground truthing to the risk assessment undertaken, to assess whether impacts are consistent between published literature from a harmful microalgal species existing range when compared with the impacts in GB shellfish. From the risk assessment undertaken the species chosen for this work was *Ostreopsis* cf. *ovata*, with a known ecological impact as well as the potential to cause human illness.

To address these aims, initial chapters focus on the paralytic shellfish producers in Great Britain, especially *Alexandrium minutum* and its' distribution within England and Wales. A literature review of the current global status of *A. minutum* was first undertaken before a field survey to identify the current range of this harmful species within the South and West of England and Wales. Finally, a controlled feeding experiment, to determine the relationship between the toxin profiles of *Alexandrium* and the toxin profiles of exposed mussels was carried out. These three chapters considered the possibility that future climate change scenarios could result in the northern range extension of *A. minutum,* ultimately leading to a situation where an overlap of the range of both toxic *A. minutum* and toxic *A. catenella* might occur within coastal locations of GB.

The later aspects of the thesis aimed to determine harmful microalgal species which are likely to pose a threat to GB in the future. This included performing a literature-based risk assessment of those harmful species currently considered non-native, assigning risk scores to each harmful species with a predicted capacity to establish within the waters around GB in future decades. The concluding part of the thesis explores the impact of one future high-risk species, on common UK shellfish species, determining the potential food safety implications as well as direct impacts on the shellfish which has implications for food security.

Chapter 2 - A review of the global distribution of *Alexandrium minutum,* dinophyceae with comments on ecology and associated paralytic shellfish toxin profiles, with a focus on Northern Europe

2.i - Preface

The work presented in chapter 2 was compiled for publication and published in the Journal of Phycology as - Lewis, A.M., Coates, L.N., Turner, A.D., Percy, L. and Lewis, J., 2018. A review of the global distribution of *Alexandrium minutum* (Dinophyceae) and comments on ecology and associated paralytic shellfish toxin profiles, with a focus on Northern Europe. *Journal of phycology*, *54*(5), pp.581-598. In terms of work on the manuscript the literature review was designed by myself, A. Turner, L. Percy and J. Lewis. Data collection and manuscript writing was completed by myself. Support with mapping and manuscript review was provided by L. Coates. Support with statistical analysis as well as manuscript review was provided by A. Turner. Manuscript review was also provided by L. Percy and J. Lewis.

2.1 - Introduction

The marine phytoplankton species *Alexandrium minutum* (Halim, 1960), is an armoured dinoflagellate with a global distribution (Hansen et al., 2003; Lilly et al., 2005). It is a known producer of paralytic shellfish toxins (PSTs) the causative agents for the condition in humans known as paralytic shellfish poisoning (PSP). These toxins are a family of neurotoxic alkaloids the parent molecule of which is saxitoxin (STX). To date around 60 saxitoxin analogues have been described, those produced by *A. minutum* fall into the most common groups which are the hydrophilic toxins (Wiese et al., 2010). To date there are very few records of non-toxic *A. minutum*. As a consequence of this scarcity (Touzet et al., 2007a) the predominant impact of *A. minutum* is that caused by the toxic strains. As a result, *A. minutum* is recognised as one of the many harmful algal species which naturally occur around the globe and is known to have had impacts on humans, food security and other marine organisms (Costas and Lopez-Rodas, 1996; Erdner et al., 2010; Ranston et al., 2007;

Santos et al., 2014; Usup et al., 2002). With the continuing expansion of the global human population there is a concomitant rise in the need for safe food sources, the presence of PSTs in seafood poses a risk which is becoming increasingly important (McPartlin et al., 2017), the contribution of *A. minutum* to this risk is therefore an important factor to be assessed.

The first population of *A. minutum* was discovered and described by Halim in 1960 at the entrance to the port of Alexandria in Northern Egypt, from where the genus takes its name. Following many years of discussion around the correct taxonomy for this important group of microalgae the morphological characteristics which define the species were redescribed by Balech in 1989 to provide greater clarity. This identification guide from 1989 provides the basis of many of the identifications which appear within the literature, especially those predating the advent of affordable molecular techniques. In more recent studies a number of molecular techniques, such as PCR (Godhe et al. 2001a, Galluzzi et al. 2004, Touzet et al. 2007a), FISH (Touzet et al., 2009) and microsatellite markers (Casabianca et al., 2012; McCauley et al., 2009), have been utilised, either for the independent or confirmatory identification of *A. minutum*. Work in this field has resulted in a greater understanding of the species complex (Lilly et al., 2005; McCauley et al., 2009) and the reclassification of related species - *A. lusitanicum* (McCauley et al., 2009) and *A. angustibulatum* (Hansen et al., 2003) into *A. minutum* as recommended by Lilly et al. (2005). It has also highlighted variability within some of the morphological traits, such as the presence of a ventral pore, traditionally utilised for microscopic identification (Hansen et al. 2003, Lilly et al. 2005, Touzet et al. 2007a, Touzet et al. 2008, Penna et al. 2008). The occurrence of *A. minutum* has been confirmed by morphological taxonomy at many locations and is still used as a principal tool in species identification for this genus in many areas where molecular tools are either unavailable or have not been applied (Ranston et al. 2007, Satta et al. 2010, Baylón et al. 2015). Often this is because *A. minutum* has not been the primary focus of the research in question (D'Silva et al., 2013). Light microscopy also remains the key methodology in monitoring programmes where molecular tools may not be practicable for regular application (Godhe et al. 2001a).

Details of the distribution of this species have been assessed and reviewed previously (Hansen et al., 2003; Lilly et al., 2005) but this has not been carried out recently. Since the last comprehensive review (Hansen et al., 2003) which detailed global distribution, a number of new populations have been discovered globally, in regions such as central America, which makes the compiling of more recent data a valuable exercise. Further to this, toxin profiles of *A. minutum* populations have been determined from a number of source populations but little work has been conducted to explore the relationship of these. Therefore, the purpose of this brief review is to provide updated details on the current known global distribution of *A. minutum,* as well as the toxin profiles associated with this species globally, alongside information pertaining to the ecology of this important harmful species. Finally, the situation within Northern Europe and the United Kingdom are evaluated in more detail. In this way it is intended that this review offers key information on the current known distribution and toxicity of the species as well as providing an overview of traits which typify areas currently experiencing detected levels of *A. minutum.*

2.2 - Specific ecosystem characteristics associated with *A. minutum*

Geographically *A. minutum* has been detected at sites which are widely dispersed. Although populations may be spatially isolated from one another, the conditions of the local environments where populations are known to occur share similarities. Through a number of laboratory studies and observations of *A. minutum* events in the field it is possible to determine some common characteristics of the conditions which favour the growth and accumulation of *A. minutum* to detectable levels. Predominantly, *A. minutum* is found in sheltered harbours, lagoons, estuaries or embayments, where stratification occurs and hydrographic shear stresses are typically low (Bravo et al., 2008; D'Silva et al., 2013; Delgado et al., 1990; Lassus et al., 2004; Le Bec et al., 2016; Ranston et al., 2007; Touzet et al., 2010b). It is of note that these are normally areas of higher human activity and so also represent areas more likely to be monitored, it is therefore possible that further

populations exist in different habitats which remain to be discovered (Alacid et al., 2017). Those recordings of *A. minutum* from open waters or exposed areas are limited in the literature with only a handful of papers documenting such findings (Godhe et al. 2001a, Yoshida 2002).

The temperature range of *A. minutum* occurrence, as determined from field observations, is broad globally, with cell proliferations occurring from 12 °C (Vila et al. 2005; Touzet et al. 2010a) to 30 °C or more (Glibert et al. 2001, D'Silva et al. 2013). Local populations seem to have a smaller temperature range at which optimal growth occurs, this has been evidenced by variable optimum growth conditions having been determined for different source populations (Grzebyk et al. 2003, Lim et al. 2006, Bravo et al. 2008). The salinity tolerance of *A. minutum* is also broad, ranging from growth in natural samples at 11 (Ranston et al., 2007) to 46 (Abdenadher et al., 2012). Studies on cultures have demonstrated growth at salinities as low as 5, *A. minutum* is therefore considered euryhaline (Lim and Ogata, 2005) and concentrations can develop near to freshwater inputs as well as in lagoons, pools or harbours without such inputs. The optimal levels for both temperature and salinity appear to vary by the geographic location of the source population and potentially represent an acclimation to prevailing conditions (Lim and Ogata, 2005; Van Lenning et al., 2007). This would suggest that *A. minutum* exhibits a degree of local adaptation, presumably altering certain physiological parameters to enable optimum growth conditions to meet those of the local environment. If this is the case *A. minutum* would be able to become competitive in a range of environments if initial introductions are able to survive whilst adaptation takes place.

Nutritionally *A. minutum* is photosynthetic but mixotrophy has been observed (Anderson et al., 2012; Fagerberg et al., 2009). *A. minutum* displays a high affinity for nitrogenous material, displaying relatively low half-saturation constants for both nitrate and ammonium, which is unusual for a dinoflagellate (Maguer et al., 2007). Furthermore *A. minutum* is capable of sequestering both N and P intracellularly to support growth during conditions when nutrients may otherwise be limiting (Labry et al., 2008; Maguer et al., 2007; Touzet et al., 2007b)

Globally *A. minutum* can occur at high abundances, Smayda (1997) suggested that bloom status is species and locality specific, considering a bloom as a rapid increase in cell numbers to levels considerably in excess of background population concentrations allows for a decoupling of bloom status to specific cell densities. Certainly, many authors refer to the proliferation of *A. minutum* within their study areas as a bloom without defining their criteria for what a bloom is, this results in considerable differences in maximum *A. minutum* concentrations within a 'bloom'. Levels of *A. minutum* have been frequently recorded above 10^4 cells per litre (Anglès et al., 2012; Baylón et al., 2015; Bravo et al., 2010a; Chang et al., 1995; Ranston et al., 2007; Touzet et al., 2010b) and maximum densities exceeding 10^7 cells per litre (Chapelle et al., 2015; Hwang et al., 1999; Pitcher et al., 2007) have been reported. A concentration of *A. minutum* above 10^3 cells per litre was used as the criteria to determine a bloom event by Anglès et al. (2012) and above 10^5 in Le Bec et al. (2016). For the purposes of this review, blooms were considered to occur when stated as such in the literature, this follows the criteria applied by Smayda (1997) of bloom status being determined by the conditions considered normal for each individual location.

In many regions experiencing blooms these accumulations tend to occur from spring, into the summer months, March to August in the Northern Hemisphere (Blanco et al., 2009; Guallar et al., 2017; Lassus et al., 2004; Le Bec et al., 2016) and September to February in the Southern Hemisphere (Baylón et al., 2015; Chang et al., 1997; Hwang et al., 1999). There are some cases of winter blooms of this species, with most of these reported in the Northern Mediterranean (Alacid et al., 2017; Bravo et al., 2008). In all cases early onset of blooms coincides with differing conditions depending on the location. Table 2.1 outlines some such bloom initiators where they have been determined.

Table 2.1: A sample of factors attributed to *A. minutum* bloom initiation with the location of occurrence from Lewis *et al.* (2018).

Factor	Specifics	Location	Reference
Competitive advantage	Abnormal conditions suppressed expected bloom of competitor: *Lingulodinium sp.*	Kastela Bay, Croatia	(Marasović et al., 1995)
Nutrient loading	Decreased N:P ratios	Ganzirri Lagoon, Italy	(Giacobbe et al., 1996)
Nutrient loading	Increasing levels of Phosphates	Gulf of Gabes, Tunisia	(Abdenadher et al., 2012)
Salinity & Temperature	Excystment triggered with decreasing salinity and increasing temperature	Sungai Getting, Malaysia	(Lau et al., 2017)
Stratification	Thermal stratification following increasing temperatures	Ganzirri Lagoon, Italy, Baiona Bay, Spain	(Bravo et al., 2010b; Giacobbe et al., 1996)
Temperature	Apparent threshold temperature required for effective cell growth	Bay of Brest, France	(Chapelle et al., 2015)
Tidal event	Rapid increase following spring	Cork Harbour,	(McCoy et al., 2014)

	tide	Ireland	
Tidal event	Bloom maxima attained during times of low tide coefficient, (neap tides)	Bay of Brest, France	(Chapelle et al., 2015)

In several cases the presence of a cyst deposit, alongside a parameter such as those above, has been strongly implied as a primary contributor for a successful population of *A. minutum* developing (Anglès et al., 2012; Bravo et al., 2010b; Cosgrove et al., 2014). The presence of cysts is not enough to guarantee the formation of a bloom, environmental conditions need to be suitable to support cell division following an inoculation from a cyst deposit, the cysts therefore provide for a ready source of vegetative cells which can bloom under favourable conditions. Initiation of a bloom may well be dependent on the specific locality, in all locations there will be several thresholds which need to be met to initiate a bloom. Which of these factors is limiting to bloom formation is likely to vary from site to site based upon prevailing local conditions and the requirements of the local population of *A. minutum.* Consequently, the final trigger condition for bloom formation will vary between sites, this makes it difficult to specify a key parameter which needs to be monitored, instead a range of measurements need to be considered.

Bloom termination is similarly a complex process and is likely to vary between locations and also between years. Many factors have been identified for bloom decline, examples of which are detailed in Table 2.2.

Table 2.2: A sample of factors attributed to *A. minutum* bloom decline with the location of occurrence from Lewis *et al.* (2018).

Factor	Specifics	Location	Reference
Dispersal	Increased wind speed and offshore direction dispersed the bloom	Arenys de Mar, Spain	(Van Lenning et al., 2007)
Encystment	Encystment was the dominant contributor to bloom decline	Penzé estuary, France	(Erard-Le Denn et al., 2000)
Grazing	Microzooplankton are capable of causing substantial reductions in algal numbers	Arenys de Mar, Spain (laboratory study)	(Calbet et al., 2003)
Meteorology	Elevated winds, decreased temperature and irradiance	Cork Harbour, Ireland	(Touzet *et al.* 2010)
Nutrient loading	Decreased availability of phosphates	Ganzirri Lagoon, Italy	(Giacobbe et al., 1996)

Parasitism	High levels of parasitic infection can cause high levels of mortality	Arenys de Mar, Spain; Penzé and Rance Estuaries, France	(Alacid et al., 2017; Blanquart et al., 2016)
Stratification	Decreased water column stability and increasing salinity	Ganzirri Lagoon, Italy	(Giacobbe *et al.* 1996)

Bloom decline is an aspect which needs to be assessed on a case-by-case basis, long term data on the frequency and duration of occurrence becomes a very useful tool in this regard but is normally limited to areas which are well studied or where routine monitoring exists.

Blooms of *A. minutum* are rarely monospecific, *A. minutum* will sometimes form the majority component of the assemblage (Delgado et al., 1990; Maguer et al., 2004) but at other times reaches high densities whilst still being a relatively small proportion of the total algal assemblage (Chang et al., 1997). The proportion of the assemblage represented by *A. minutum* is often not reported as quantifying all species from different phyla can be an extensive task. Consequently, reports are often limited to abundance in relation to other dinoflagellates or simply stating the number of cells present alongside whether it was dominant or not. Where *A. minutum* co-occurs with other potential PST producers or species producing other biotoxins this can increase the complexity of the management strategy required to safeguard human health as well as having a more pronounced impact on the fisheries affected.

2.3 - Life History

The life history of *A. minutum,* which is presented graphically in Figure 2.1, contains both a haploid motile vegetative stage as well as a diploid planozygote formed following sexual fusion, both of these stages are able to encyst leading to a loss of motility and a change in morphology. Two types of cysts are known to feature within the life cycle of *A. minutum*. Of these, one is an asexually produced pellicle cysts which has been found in two forms, the most common of which possesses a thin wall with yellow/brown inclusion bodies, the other retains the theca of the vegetative cell and has rarely been reported. In addition to these asexual cysts, a more resilient resting cyst, possessing a thick double wall can be formed following sexual reproduction. The presence of the thin walled pellicle cyst and the double walled resting cyst has been commonly observed but the thecate cyst has been encountered rarely. One study where all three cyst morphologies were observed in natural samples was conducted by Bravo et al. (2010a), working with sediment traps from the Bay of Baiona, Spain. The different cyst types have also been demonstrated as mechanisms to survive short and long periods of inclement conditions with cyst formation observed in conjunction with deteriorating conditions, such as a reduction in available nutrients (Figueroa et al., 2007). Sexual hypnocysts are also known to form from sexual encounters between cells during normal vegetative cell growth (Figueroa et al., 2015). This has been shown to result in a low percentage of background encystment (Anglès et al., 2012) whenever a population is present in the water column. If these cysts remain in the surface layers of sediments following their mandatory dormancy period, shown to be between one and one and a half months (Figueroa et al. 2007, Bravo et al. 2010b), then they represent a potential rapid inoculum for future *A. minutum* proliferations. Recurrence of *A. minutum* within a location has been shown to result from the formation of 'seed' banks (Cosgrove et al., 2014) composed of resting cysts in surface sediments. Excystment has been demonstrated over a broad range of physical characteristics which affect the vegetative growth of the species and it has been concluded that cyst germination occurs under all conditions where both light and oxygen are available (Blanco et al., 2009; Ní Rathaille and Raine, 2011). Indeed, a study by Anglès et al., (2012)

found a constant low level of background excystment which increased dramatically in periods immediately prior to bloom formation. Consequently, vegetative cells of *A. minutum* are potentially present at very low densities at any sites where cysts have formed but will only escalate to densities likely to cause issue or be detected when conditions within the water column become favourable.

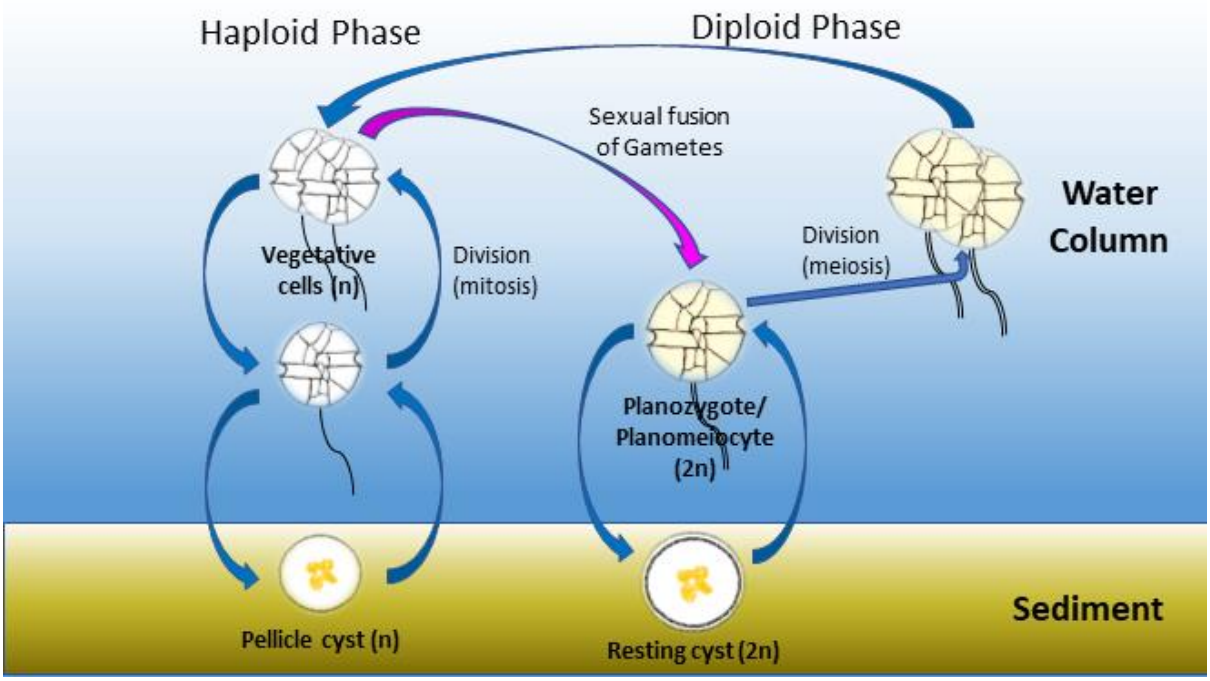


Figure 2.1: Diagram representing the different life cycle stages within *A. minutum* modified after Berdalet *et al.* (2017), from Lewis *et al.* (2018).

Following their formation, *A. minutum* hypnocysts lose their motility and subsequently behave as passive particles with a lower density than clays (Anglès et al., 2010) these then sediment out of the water column and are deposited in the surface layers of the benthos. Where the benthos of a location is dominated by larger particles or the sediment is scoured by strong water movements *A. minutum* cysts are lost from the upper sediment or dispersed (Narale and Anil, 2017) and so are unable to form a future inoculum. Therefore, waterbodies which accumulate and retain fine sediments are required for the successful establishment of long-term cyst deposits (Narale and Anil, 2017). A degree of sediment circulation is required also, to ensure that cysts are not buried too

deeply. Bays and harbours often represent suitable habitats as they typically provide reduced water movement except in proximity to entrances. This allows for the accumulation of the fine sediments required for cyst deposit formation as well as encouraging the development of stable stratified water when temperatures increase towards summer months. The introduction of harmful algal bloom species to new locations through cyst or motile cell transport is an area of concern for food safety reasons as new introductions may bring previously unseen issues with relation to human and ecosystem health. Although the frequency of anthropogenically mediated transfers is debatable (Smayda, 2007) many authors have cited ballast water as a potential vector, although this has only been conclusively demonstrated in relatively few cases (Burkholder et al., 2007), this would suggest that ports and harbours could represent a higher risk environment not only due to favourable conditions for *A. minutum* to persist within them but also potentially as points of introduction where shipping occurs between regions where *A. minutum* is known to occur and new sites.

2.4 - Current global distribution

Since its first discovery at Alexandria port, *A. minutum* has subsequently been isolated from a range of further locations and has now been found to inhabit coastal regions globally, it has now been discovered from the coasts of all continents with the exception of Antarctica. In some areas, including some of those where it has recently been discovered, it is known in only a relatively small range or even a single specific location (Baylón et al., 2015; Pitcher et al., 2007). Several of these areas where occurrence is limited in range represent understudied regions, in some cases comprehensive plankton surveys are being carried out for the first time. In other parts of the world it has been identified at several locations, such as multiple sites around northern Europe (Elbrachter 1998, Nehring 1998, Godhe et al. 2001b, Hansen et al. 2003, Touzet et al. 2007a, Brown et al. 2010, Guallar et al. 2017), which represents a well-studied area both geographically and temporally. The limited known distribution in some areas may be as a result of a lack of suitable conditions or it

could simply be that surrounding areas have not received sufficient attention from research or monitoring groups to determine the presence or absence of *A. minutum*. Figure 2.2 displays the locations of known occurrences of *A. minutum*. No weighting is given to those sites where multiple studies have occurred and in some cases a marker may cover more than one site of occurrence if it is in close proximity to others.

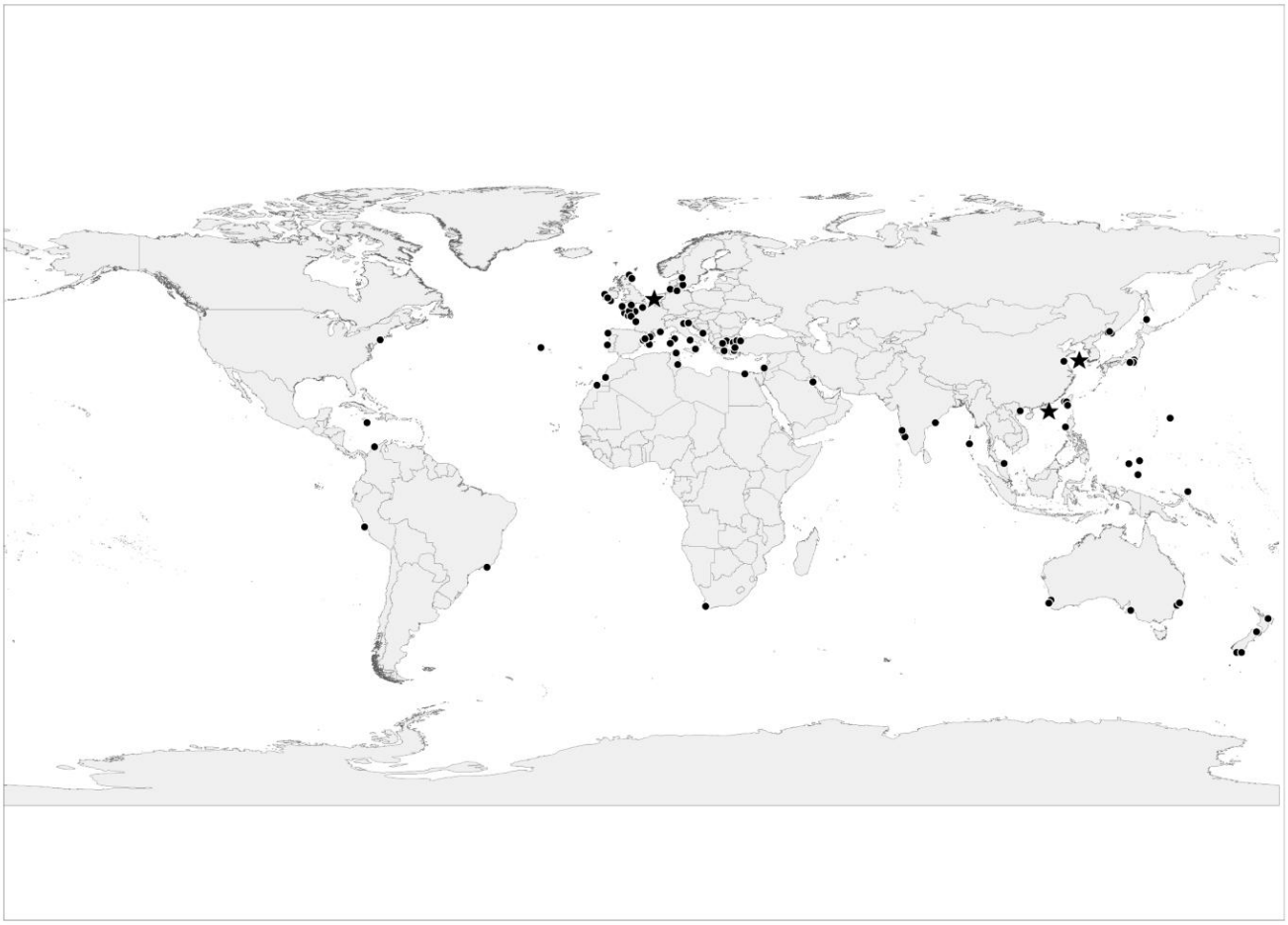


Figure 2.2: Map of the world showing locations from which *A. minutum* has been positively identified. In some areas with higher numbers of identification of occurrence points may overlap. Circles denote confirmed locations. Stars represent regions of occurrence listed in literature or reports but where original literature and definitive location is uncertain, from Lewis *et al.* (2018). Compiled from: (Abdenadher et al., 2012; Abouabdellah et al., 2008; Alkawri and Ramaiah, 2010; An et al., 1992; Aydin et al., 2015; Aydin and Uzar, 2014; Balech, 1995; Başdemir, 2016; Bastianini et al., 2016; Baula et al., 2011; Baylón et al., 2015; Bazzoni et al., 2016; Bouchouicha et al., 2012; Bravo et al., 2010a, 2010b, 2008, 2006; Brown et al., 2010; Chang et al., 1997, 1995; Chapelle et al., 2010; Chen and Chou, 2001; D'Silva et al., 2013; Delgado et al., 1990; Dhib et al., 2013; Dias et al., 2015; Elbrachter, 1998; Eslihan Balkis et al., 2016; Farrell et al., 2013; Franca et al., 1995; Franco et al., 1995; Garcés et al., 2004; Giacobbe et al., 1996; Glibert et al., 2002; Godhe et al., 2001a; Guallar et al., 2017; Guillou et al., 2002; Hallegraeff et al., 1991; Hansen et al., 2003; Harlow et al., 2007; Hii et al., 2016; Hwang and Lu, 2000; Ignatiades, 2012; Klouch et al., 2016b; Klouch et al., 2016a; Lassus et al., 2004; Le Gac et al., 2016; Lim et al., 2011, 2007b; Lim and Ogata, 2005; López-Flores et al., 2006; Lozano-Duque et al., 2011; Maas et al., 2007; MacKenzie and Berkett, 1997; Marasović et al., 1995; Menezes et al., 2007; Montresor et al., 2004; Narale and Anil, 2017; Nascimento et al., 2005; Nehring, 1998; Orlova et al., 2004; Orlova and Morozova, 2009; Percy, 2006; Pitcher et al., 2007; Ranston et al., 2007; Rubino et al., 2017; Santos et al., 2014; Satta et al., 2010; Silva et al., 2011; Tang et al., 2012; Tillmann and John, 2002; Touzet et al., 2008; Touzet et al., 2007a; Touzet and Raine, 2007; Usup et al., 2002; Uzar and Aydin, 2010; Vila et al., 2005; Yahia Kefi et al., 2001; Yang et al., 2010; Yoshida, 2002; Yoshida et al., 2000; Yuki, 1994; Zina et al., 2012)

The study of Lilly et al., (2005) subdivides *A. minutum* into four clades, based upon differences in the D1-D2 regions of the large ribosomal sub-unit DNA. Of these the smallest two clades were comprised of only three strains, with one clade of one and the other containing two, all of which were originally isolated from Japan. The remaining two clades contained the majority of

those strains studied. These two major clades are the Global clade, which, of the strains assessed, includes those populations isolated from areas around Europe, South Africa and Australia and the smaller Pacific clade, which encompasses those populations originating from Asia and New Zealand. Since the work carried out by Lilly et al., (2005) further populations of *A. minutum* have been identified from Cape Town in South of Africa (Pitcher et al., 2007), the Azores (Santos et al., 2014) East Asia (Baula et al., 2011) South America (Menezes et al. 2007, Baylón et al. 2015) and Central America (Ranston et al. 2007, Lozano-Duque et al. 2011).With the exception of the population in South Africa these newly discovered populations have only been identified via the use of morphological traits and as such detailed information on their phylogeny is currently lacking. It is therefore not possible to classify these into either the Global or Pacific clade at this time. This is also the case for less well studied areas or those where culturing of detected populations has not been actively undertaken. As well as lacking phylogenetic information, the toxicity of the populations from Jamaica, Peru and India has yet to be determined. It was noted by Ranston et al., (2007) that there have been no recorded cases of PSP in Jamaica, implying that the *A. minutum* population present there is either not toxic, does not reach abundances sufficient to cause issues in areas where harvested shellfish are also present, or existing management programmes are sufficient to prevent human exposure. As with phylogenetic relationships, where *A. minutum* occurs but cultures have yet to be established it is uncommon for toxicity of a population to have been elucidated. Given the potential impact, on humans, of PSTs from *A. minutum* it should be of paramount importance to determine what if any toxicity is exhibited by a population following discovery.

The more recent identifications of *A. minutum* now increase its known spread to encompass South America, where it was previously unrecorded but also increase its spread in the Southern Hemisphere where it was previously known only from Eastern areas. It is possible that with further studies carried out along the coasts of both Africa and South America the number of detections would increase. From Figure 2.2 it is apparent that under the current taxonomic designation, *A. minutum* is truly a global species. This raises the possibility for human health concerns related to *A.*

minutum across a wide range of geographies. As a species *A. minutum* should be considered by monitoring agencies worldwide, as the detection of further populations seems likely given the ability of *A. minutum* to survive across such a large range of latitudes and longitudes.

2.5 - Current global toxin profile information

Other than morphological and genetic markers toxigenic strains of *A. minutum* also produce determinable PST profiles. The toxin profiles for *A. minutum* populations are presented in Table 2.3 alongside their geographic origin. Where these toxin profiles have been determined in conjunction with complete phylogenies, either within the same study or via separate studies utilising cell cultures sourced from the same location, it is noted as to which of the two clades they belong. The most common method for determination of toxin profiles within the literature has been the use of high-performance liquid chromatography with fluorescence detection and post column oxidation of PSTs. For determination of toxins in algal cultures many studies have utilised high performance liquid chromatography with post column oxidation (HPLC-PCOX) such as the AOAC method (Anon, 2011) or have utilised similar methods developed or refined within their own research group.

From Table 2.3 it is possible to see that the most common toxins produced by *A. minutum* are GTX 1&4 with GTX4 being the most dominant toxin in a range of strains which are widely dispersed geographically. Additionally, both non-toxic and toxic strains belong within the same clade. The exception to this toxic profile appears to occur around Northern Europe with strains identified from Ireland, England and Northern France being dominated by GTX2&3 rather than GTX1&4 or the populations from Denmark and some areas of Northern France containing C1&2 alongside either GTX2&3 or dcGTX2&3. Whether there is an underlying genetic separation between these populations and other members of the global clade which has yet to be determined or if the toxin production in this region is driven by a specific set of environmental parameters or stressors is currently unknown. For the most part other STX analogues are scarce in *A. minutum* strains,

appearing in low or trace levels within most strains analysed. Both STX and Neo occur in higher proportions but only in a limited number of strains analysed, predominantly from New Zealand. This may indicate that these toxins are comparatively scarce or that populations producing these toxins are less well studied.

Table 2.3: Table detailing the toxins present where determined within the literature alongside classification by the clades proposed in Lilly et al., (2005) where this information is available. Toxin abbreviations are as follows: STX – Saxitoxin, GTX – Gonyautoxin, NeoSTX- NeoSaxitoxin C1 – N-sulfocarbamoyl Gonyautoxin 2, C2 - N-sulfocarbamoyl Gonyautoxin 3. From Lewis *et al.* (2018).

Location		**Strain Identifier**	**PST Profile**		**Phylogeny**		**Reference**
			Principle Toxins	**Secondary Toxins**	**Pacific Clade**	**Global Clade**	
Africa	Cape Town Harbour, South Africa	CTCC22	GTX1,4	GTX2,3		X	Pitcher *et al.* 2007
Asia	Do Son, Vietnam		GTX4	GTX1-3, NeoSTX, dcSTX	X		Lim *et al.* 2007
Australasia	Australia	AMAD01, AMAD06, AMAD21	GTX1,4			X	Franco *et al.* 1995

	New Zealand, Bay of Plenty	AMBOPO006	NeoSTX	STX, GTX1-4	X	Chang *et al.* 1997
	New Zealand, Marlborough Sounds	CAWD11, CAWD12, CAWD13	GTX1,2,4, NeoSTX, STX	GTX3	X	MacKenzie and Berkett 1997
Europe	Denmark, Kosor Nor	GHmin04	C1,2	GTX2,3 STX	X	Hansen *et al.* 2003
	England, Fleet Estuary	3.9h	GTX3	GTX2, STX	X	Nascimento *et al.* 2005
	France, Bay of Morlaix	AM89BM	GTX2,3	dcGTX2,3	X	Grzebyk *et al.* 2003
	Ireland, Cork Harbour	CK.A02, CK.A14, CK.A17, CK.A20, CK.A23, CK.D04	GTX3	GTX2, dcGTX2&3GTX2&	X	Touzet *et al.* 2007a, 2007b,

			3		2008
Ireland, Killary Harbour	Kill.A12, Kill.C6, Kill.E4, Kill.G3	Non-toxic	Non-toxic	X	Touzet *et al.* 2007a,
Ireland, Shannon Estuary	SHA.A12, SHA.B11, SHA.B12	Non-toxic	Non-toxic	X	Touzet *et al.* 2007a,
Italy, Gulf of Trieste	AL3T, AL9T	GTX4	GTX3, STX	X	Yang *et al.* 2010
Italy, Gulf of Trieste	AL1T	Non-toxic	Non-toxic	X	Yang *et al.* 2010
Scotland, Orkney Islands	W07/001/01, W07/025/01	Non-toxic	Non-toxic	X	Brown *et al.* 2010
Spain, Ria de Vigo	AL1V, AL2V, AL3V	GTX1,4		X	Franco *et al.* 1995

Spain, Ria de Vigo	AL1V	GTX1,4	GTX2,3	X	Bricelj & Cembella 1995, Yang *et al.* 2011

To determine if any global patterns existed within toxin profile further analysis was undertaken. Where toxin profile was available within the available literature it was collated and normalised to allow for comparison. Additionally, owing to a desire to maximise the data pool but also to reduce variability, allowing data to be classified into a meaningful number of clusters, the epimeric toxin pairs were summed and considered as a single value for further analysis. K-means clustering analysis was performed, in MS Excel, on this toxin profile data for a range of algal strains from different geographic locations. The analysis generated five clusters of PST toxin profiles via iterative generation of minimum distances to each of five centres taken from within the source data. Figure 2.3 graphically represents the geographic spread of the different clusters. The first cluster was predominated by GTX1&4 and covered a broad range of source populations from sites in Europe as well as Australia, Taiwan and Malaysia. The second cluster included those strains producing high levels of GTX2&3 and included most of the strains analysed from Northern Europe as well as some from South Taiwan. The remaining three clusters were smaller in terms of representation in the literature. Cluster 3 contains two strains from New Zealand and one from Brazil, all of which contained a high proportion of Neosaxitoxin. Cluster 4 contains exclusively strains from Northern Europe including those from Denmark and some from Northern France. The defining feature of cluster 4 is the significant amount of C1&2 within the profile, this appears to be unique to this geographic region as of those profiles analysed the C toxins only featured at low levels outside of Northern Europe, in one strain, AL2V, from the Mediterranean. Finally, cluster 5 grouped some strains from the South of England with some from New Zealand, the characteristics of this profile were the presence of GTX2&3 alongside a considerable contribution to overall toxicity by STX, those New Zealand strains in cluster 5 were also found to produce GTX1&4 and in some cases Neo. Data for one strain from Taiwan was also found to fall into cluster 5.

Figure 2.3: Map of the world displaying the geographic distribution of 5 individual toxin profiles as determined by K means clustering analysis. Inlay of NW Europe allows visualisation of the spread of multiple toxin profiles present within this region. From Lewis et al. (2018). Compiled from: (Mascarenhas et al. 1995, Flynn et al. 1995, MacKenzie and Berkett 1997, Chang et al. 1997a, Béchemin et al. 1999, Hwang et al. 1999, 2003, Hwang and Lu 2000, Carreto et al. 2001, Moroño et al. 2001, Chen and Chou 2001, Guisande et al. 2002b, Parker et al. 2002, Guisande et al. 2002a, Hansen et al. 2003, Negri et al. 2003, Grzebyk et al. 2003, Montresor et al. 2004, Chou et al. 2005, Nascimento et al. 2005, Wang et al. 2005, Lim et al. 2006, Percy 2006, Selander et al. 2006, 2008, Lim et al. 2007b, 2011, Menezes et al. 2007, Pitcher et al. 2007, Touzet et al. 2007a, Bergkvist et al. 2008, Touzet et al. 2008, Maas and Brooks 2010, Yang et al. 2010, 2011, Frangópulos et al. 2011, Fabioux et al. 2015; Stüken et al. 2015)

Cluster 1 is the most widely dispersed of the reported profiles encompassing a broad range of different populations globally. Excluding the Taiwanese strains, the remaining clusters are either closely grouped together or represent only a handful of sites. Cluster 2 represents a small distribution in NW Europe, cluster 3 is confined to two locations, cluster 4 like cluster 2 is limited to Northern Europe and cluster 5, whilst occurring at very distant locations is only representative of populations from four locations, two of which are in New Zealand. The data from Taiwan is interesting as cultures from this region were found to exhibit toxin profiles indicative of three of the five clusters generated. Strains from this area occupied clusters 1, 2 and 5. The example of a Taiwanese occurrence in cluster 5 is, additionally, profile information generated within a study where the profile during exponential growth of *A. minutum* fell into cluster 5 whereas the PST profile during the stationary phase was grouped with cluster 1. Work by (Lim et al. 2007a) arrived at a similar conclusion, albeit using a smaller range of toxin profiles during analysis, where a dendrogram was used to display the divergence of profile between strains. It is therefore difficult to generalise regarding the toxin production of *A. minutum* on a global basis, the Global clade contains representatives from three of the four clusters and strains from the Pacific clade fall within two clusters, leading to a number of genetically differentiated strains existing within the same toxin profile cluster. This would imply that the toxicity of a strain is not linked to its clade but more likely to the geographic region from which it is isolated, with specific regions appearing to have characteristic toxin profiles which may be driven by the local environment. Although in both Taiwan and Northern Europe there are areas where two or more profiles exist within a very small geographic range, this may give credence to the notion that toxin profile is genetically driven as these populations will be exposed to very similar environmental conditions but still produce distinct toxin profiles. The similarity in profile between the New Zealand strains included within the analysis and the single strain tested from Brazil is an interesting development. Work to compare these two populations using molecular techniques would be of value in determining what, if any, connectivity

exists between these geographically disparate populations as they represent an otherwise uncommon PST profile within the *A. minutum* species.

With the Exception of Taiwan, where the same region or strain has been assessed repeatedly it is possible to see that the toxin profile remains relatively stable (Flynn et al., 1994; Yang et al., 2011), one strain which has been extensively used within research, AL1V, for example was always found to appear in cluster 1 during our analysis. Some studies have found the PST profile within *A. minutum* and other toxic dinoflagellates to be variable (Hwang and Lu, 2000; Maas et al., 2007) but these have often been studies conducted using cultures exposed to artificial conditions, such as the removal of associated bacteria, and so extrapolating their findings directly to natural populations is not easy (Hansen et al., 2003). It has been demonstrated by other studies that the cellular toxin quota is also modified by environmental conditions, examples of these are detailed in Table 2.4.

Table 2.4: Examples of biotic and abiotic factors and their associated impact on PST quotas in *A. minutum.* From Lewis *et al.* (2018).

Factor	Impact on toxicity	Reference
Associated bacteria	Antibiotic treatment of cultures reduced PST levels by up to 87%	(Maas et al., 2007)
Grazer cues	Cellular toxin quota significantly elevated in the presence of grazer cues	(Selander et al., 2006; Yang et al., 2011)
Growth stage	3 times higher cellular toxicity encountered in exponential than stationary phase	(Mascarenhas et al., 1995)
Nutrient limitation	10 times higher toxicity when exposed to	(Selander et al., 2008)

	grazer cue and limited phosphates	
Nutrient source	4 times higher cellular toxicity when fed with ammonia rather than nitrate	(Lim et al., 2010)
Temperature	Increased temperature resulted in increased toxin production	(Lim *et al.* 2006)

From the analysis of multiple toxin profiles generated globally for *A. minutum* it is possible to determine patterns of toxin profile distribution which could indicate related populations or those which may have diverged. This provides clear regions globally to compare genetically and an indication of where either variance or similarity could be expected. Consequently, the assessment of PST profiles within *A. minutum* populations constitutes another mechanism for assessing global variation within the species.

Studies where the toxicity of *A. minutum* has been determined are quite common but it is unfortunately also common for comprehensive toxin profile data to be omitted in many publications. Instead, the principal toxins and their proportions are listed or only total toxicity given. It is of value, for further research efforts, for the full toxin profile to be released if it is determined, to allow for further exploration of links between genetic structure and toxin production. This would be useful as the toxicity of harmful species is of key importance with regard to the impact which they have on society and health. Holistic studies which have determined morphology, toxicity and phylogeny are more rare still but are also incredibly useful (Hansen et al. 2003; Pitcher et al. 2007; Brown et al. 2010; Touzet et al. 2010b), wherever possible studies of harmful algal species should provide data on all of these aspects and in the case of well-studied populations it is of value to compile this data over time even if it is not possible to determine all of it within a single study.

2.6 - North European distribution and toxin information

There are a number of sites spread across North Europe which are currently known to experience growth of *A. minutum*, for several of these sites incidents of growth are recurrent, happening either annually or sporadically but within the same locations. Figure 2.4 presents a map of Northern Europe highlighting areas where *A. minutum* has been detected within this region as well as denoting whether a specific population was found to be toxic or non-toxic.

Figure 2.4: Map of Northern Europe displaying sites where *A. minutum* has been positively identified. From Lewis *et al.* (2018). Compiled from: (Elbrachter 1998, Nehring 1998, Arzul *et al.* 1999, Guillou *et al.* 2002, Hansen *et al.* 2003, Lassus *et al.* 2004, Nascimento *et al.* 2005, Percy 2006, Touzet *et al.* 2007b, Brown *et al.* 2010, Klouch *et al.* 2016b, Guallar *et al.* 2017)

Of the populations of *A. minutum* in North Europe, some have been extensively researched. For example, sites from the North of Brittany have been well studied, especially the Penzé Estuary. This area has been well characterised with studies detailing *A. minutum* toxicity (Lassus et al., 2004), parasitism (Chambouvet et al., 2008; Erard-Le Denn et al., 2000) and nutrition (Maguer et al., 2004). As a result, a comprehensive knowledge set exists for *A. minutum* in the region and consequently further studies in this area can be well designed using the knowledge already present as a suitable basis. Similarly, the *A. minutum* population originating in Cork Harbour in Ireland has been extensively assessed leading to knowledge of toxicity (Touzet et al., 2007a) bloom dynamics (Touzet et al. 2010a), factors affecting excystment (Ní Rathaille and Raine, 2011) and phylogeny (Touzet et al., 2009). As with the Brittany coast this presents Cork Harbour as a model population to carry out further research with many characteristics being well documented allowing for the exploration of more specific questions. Cultured cell lines from these populations would make an excellent choice if investigating the effect of environment on toxin production, as their PST profile is both well characterised and uncommon globally. This rare toxin profile also makes these populations an important inclusion in any study wishing to investigate genetic divergence between strains globally, the high abundance of GTX2&3 indicating that there may be an underlying genetic distinction between these Northern European populations and others globally. However, conclusions drawn from work conducted on these specific populations may be limited in scope to the local region as *A. minutum* populations exhibit a broad range of tolerance for certain parameters depending upon their origin so that work conducted with one population may not be directly applicable to populations occurring in disparate regions. Work by Hansen et al., (2003) in Denmark was also comprehensive as were the studies in the South of England by Percy (2006) and Nascimento et al., (2005). Although these populations were thoroughly evaluated within these works further work at these sites has either not been conducted or published. Other sites within Northern Europe where *A. minutum* has been identified have been less comprehensively studied. As a result, information is patchy especially relating to longer temporal scales or broader geographic areas than the direct area

of study. It is therefore possible that as yet undiscovered populations exist within this region, without comprehensive surveys the full extent of the species distribution cannot be conclusively stated.

One common feature of *A. minutum* from this region is the production of GTX2&3 or C1&2 in much larger quantities than exhibited elsewhere in the world. Interestingly the production of GTX1&4 which is a common feature of many other *A. minutum* populations is entirely absent from these North European strains. The exact reason for this difference has not yet been determined to our knowledge and further studies assessing this particular aspect would be of interest to improve the understanding of the *A. minutum* species as a whole. It is recognised that work with the genomes of dinoflagellates is complicated by the large size of the total genome, the dynamic nature of the genome evolutionarily and the variability in chromosome number (Casabianca et al., 2017). As a dinoflagellate *A. minutum* exhibits a large genome, where multiple strains of *A. minutum* were assessed they were found to contain a genome size ranging from 22.5-29.6 pgDNA cell (Stüken et al., 2015). Consequently, it may be some time before it is possible to resolve any genetic basis for these global differences or define intraspecific markers which allow for the separation of *A. minutum* populations where differences are known to exist. Recent work by (Casabianca et al., 2012) has successfully resolved genetic differences in multiple strains isolated from within the Mediterranean via the use of microsatellite markers. This is therefore an area warranting further research especially in and between those areas for which an extensive amount of background work has been conducted, such as the populations of Brittany.

2.7 - Current understanding of the *A. minutum* species group in the UK

A. minutum populations are known from both the Northern and Southern extent of the UK. The Scottish algal assemblages have been thoroughly analysed on several occasions and where *A. minutum* has been detected it has been shown to be non-toxic and belonging to the global clade

(Brown et al., 2010), shown as a black X in the North East of Scotland in Figure 2.4. Both populations identified from the south of England, denoted by the two black circles in Southern England in Figure 2.4, have been determined to be toxic with both producing primarily GTX3 alongside GTX2 which appears to be typical of Northern Europe. The population of *A. minutum* from the Fleet Lagoon was also found to be producing STX (Nascimento et al., 2005) in sufficient quantity to cluster it with strains from New Zealand rather than the others in Northern Europe. These two sites in Southern England where toxic *A. minutum* has been identified do not represent the only occurrences of PSP toxicity from southern areas of the UK. Data supplied in Turner et al., (2014) clearly shows that PSP toxicity occurs in shellfish at a number of sites around the South coast with further incidents in Devon, Cornwall and South Wales. The analysis performed within that paper suggested that the toxin profile determined in shellfish via pre-column oxidation and high-performance liquid chromatography in these regions most closely aligned with the profile of *A. minutum* from the Fal, except in the Fowey estuary which showed a profile clustering more closely to that from Scotland possibly due to its STX content. The Fowey profile could be representative of the profile exhibited by *A. minutum* from the Fleet Estuary population, which also contains significant STX. Within our own cluster analysis performed here the populations from the Fal and the Fleet fell into different clusters, the factor affecting this was again the presence of STX in the Fleet population which is absent from the Fal population. Considering the dominance of GTX2&3 in both the Fleet and Fal populations of *A. minutum* it would appear that the PST events in shellfish from the Southwestern regions of the United Kingdom where the algal producer has not been identified are most likely caused by undetermined populations of toxic *A. minutum*. Shellfish toxicity associated with *A. minutum* within the UK has only been demonstrated conclusively in one location with intoxications in the Fal directly linked to the presence of *A. minutum* (Percy, 2006). The only other demonstrably toxic population from the Fleet Lagoon has no known associated shellfish intoxications, although the area does contain shellfish production and is monitored on a regular basis. Therefore, in other UK shellfish harvesting areas which have been affected by PST contamination it can only be inferred from the

evidence supplied by toxin profile that *A. minutum* is the most likely causative algal species and further research within this area could shed more light on the current situation and bring this part of Northern Europe in line with other, comparatively better studied sites within this global region.

Further to this information from within the UK it is worth considering the relationship between the South coasts of England and Wales with other sites containing populations of toxic *A. minutum,* such as the Southeast of Ireland and Northern France. Although dispersal of viable vegetative cells via ocean currents is unlikely across broad geographic scales there is a weak current which runs up the Brittany coast and crosses the mouth of the channel passing Cornwall, this flow then joins the Celtic sea circulation, which runs anti-clockwise to the South coast of Wales and either into the Irish sea or crosses the St George's Channel before flowing South West along the Irish coast (Bailly Du Bois et al., 2002; Brown et al., 2003). The other prevailing currents in the area flow from West to East along the English Channel until joining the North Sea circulation (Bailly Du Bois et al., 2002; Brown et al., 2003). Further to this water from the Celtic Sea flows into and out of the Bristol Channel, along the South coast of Wales due to tidal forcing (Pearce et al., 2012). This has been well described and is shown visually in Figure 2.5, from Bailly Du Bois et al. (2002).

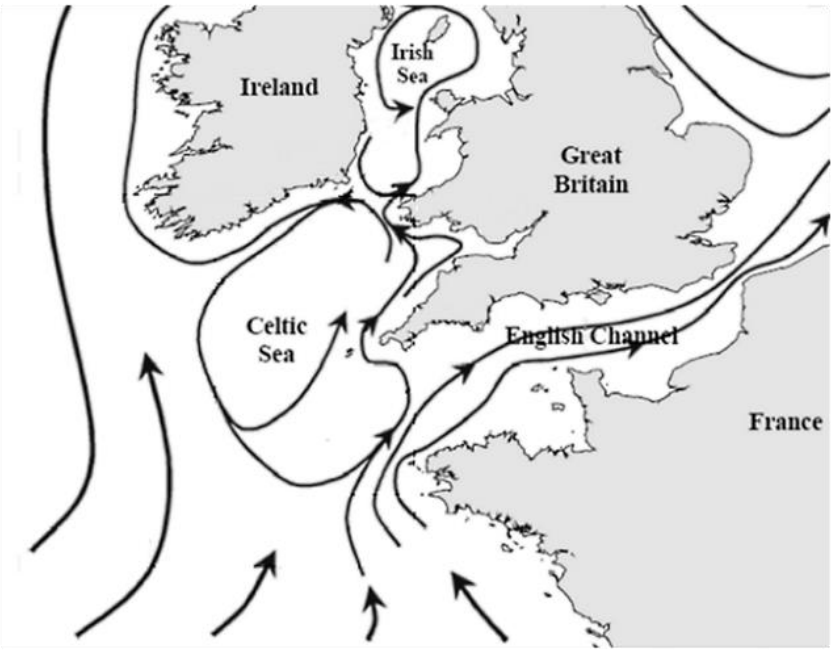


Figure 2.5: Map displaying prevailing currents around Great Britain, Northern France and Ireland, modified from Bailly Du Bois *et al.* (2002). From Lewis *et al.* (2018).

This large-scale water exchange connects the known toxic populations of *A. minutum* in Southern England, Southern Wales, Southern Ireland and Northern France providing the possibility for cells to move between the regions via oceanic transport. These areas are also connected via anthropogenic means. Recreational boating is popular in the area which is noted as being well connected (Pearce et al., 2012) with direct yachting and recreational cruising between Northern France and Southern England as well as Southern Ireland, although this is less common (Tidbury et al., 2016). The South of Cornwall and the region around Cork harbour both scored highly for intensity of pathway activity with regard to the introduction of non-native invasive species in the paper by Tidbury et al. (2016) Earlier work assessing non-native species introductions by Pearce et al. (2012) rated several regions of the South West of England, the South of Ireland and the West of Wales as a medium risk of introduction of planktonic species when considering pathways such as

commercial shipping, in ballast water or biofouling, and the movement of aquaculture organisms. Tidbury et al. (2016) noted also that whilst a lower risk than recreational shipping, commercial shipping posed a risk with live animal movements representing a lower threat to this South-Western region. Nationally, both France and Ireland fall within the top ten in terms of shipping volumes to and from the UK, scoring second and sixth respectively (GOV.UK, 2016). Therefore, whilst shipping into the South-Western region of the UK is relatively light it may still originate from other areas with established populations of *A. minutum.*

Whilst some sites of PST toxicity in shellfish within the Southwest of the UK have been linked to *A. minutum* the remaining areas remain undetermined. In other parts of the world and even within the UK the algal assemblages have been thoroughly analysed and the results published in such a way as to allow more definitive conclusions to be drawn regarding the causative organisms of intoxications both within studied and nearby locations. To provide a greater depth of understanding it would be of benefit for further field surveys to be conducted in the Southwest of the UK to elucidate the distribution of *A. minutum* within the region as well as the potential presence of other noxious algal species. This would allow for a greater understanding of the true extent of *A. minutum* in this region of Europe as well as providing an opportunity to assess relatedness between the populations from this region. In turn this information can be fed into management strategies and decisions to allow for more effective monitoring as well as better planning for any aquaculture expansions in the South of the UK.

2.8 - Conclusion

Globally *A. minutum* has been well studied, with a number of investigations evaluating different features of its biology and ecology. Due to the occurrence of predominantly toxic strains of *A. minutum* it exists as a concern for human health in locations where it occurs alongside the harvest of filter feeding organisms, usually bivalve molluscs. Recent studies highlight that *A. minutum* may well be more widely distributed than is currently realised and given the range of localities it has now

been detected from it should form a species of interest for anyone undertaking a comprehensive phytoplankton survey. Due to the variations in different strains of the species globally, data from areas where limited research has been carried out must be treated cautiously as extrapolation to the *A. minutum* species as whole may not be appropriate. Further to this it is not possible to assume with confidence that these comparatively under studied populations will behave in the same way as those where the literature is more abundant leading to a need to determine certain characteristics for these strains. Conversely well studied areas such as the Ria de Vigo and Penzé Estuary are very useful as they allow for ever more detailed and specific studies to be carried out as groundwork in many areas, such as optimal growth characteristics, has already been completed. In this way studies can be planned more precisely and outcomes can be interpreted with confidence that the results obtained do not arise from the effects of undetermined variables. Due to the potentially harmful nature of *A. minutum* and the expanding knowledge of its distribution it is important that research continues to further our knowledge of the species. The toxin profiles of populations are a key area, especially for any identifications where toxicity has not been tested. Novel statistical analysis of global *A. minutum* toxin profiles has shown that, at present, five distinctive profiles exist globally. Of these profiles, two encompass the majority of strains analysed and both major molecular clades as outlined by Lilly et al. (2005) include representatives of these two most common toxin profiles. Also, further characterisation of populations under the existing clades as well as further work to find genetic markers capable of differentiating between populations would certainly be of value in the comparison of *A. minutum* from different sites and could allow for an understanding of source populations and population divergence within this important global species. Further examination of microsatellite markers may be a promising area for future work in this regard.

Chapter 3 - A shoreline survey of encysting dinoflagellate assemblages in the Southwest of Great Britain, evaluating the spread of *Alexandrium* species

3.1 - Introduction

As introduced in Chapter 1, there are five groups of regulated toxins within GB, of those paralytic shellfish poisoning (PSP) remains the most dangerous to humans. This is due to the potent neurotoxicity of this toxin group, with the key symptoms outlined in Table 1.1 Many PSP intoxication can be mild but higher doses lead to more severe symptoms with paralysis being the most concerning, as paralysis of respiratory muscles can lead to fatalities (Grattan et al., 2016; Wiese et al., 2010). The compounds responsible for causing PSP are collectively known as the saxitoxins, after saxitoxin, the parent molecule within the group and are often referred to as Paralytic Shellfish Toxins (PST). These compounds are produced by species within four genera of marine dinoflagellates, *Alexandrium, Gymnodinium* (F. Stein 1878)*, Pyrodinium* (Plate, 1906) and *Centrodinium* (Kofoid, 1907)*,* as well as some species of cyanobacteria*.* Within tropical Pacific waters *Pyrodinium* is a common occurrence and has been linked to several incidents of PSP especially in the Philippines, where many deaths have been recorded (James et al., 2010; Van Dolah, 2000). In tropical through to warm temperate regions, such as the Atlantic coast of Spain and Portugal, the Mexican Pacific and areas of Australia, the species *Gymondinium catenatum* (H.W. Graham, 1943) has often been associated with PST presence in shellfish (Band-Schmidt et al., 2010; Hallegraeff et al., 2012). Toxic populations of *Alexandrium sp.* can be found globally and in temperate regions, such as northern Europe and Northern America the most common causative organisms of PST are members of this genus (Anderson et al., 2012; Lilly et al., 2007). Saxitoxins have also been found in some freshwater cyanobacteria from genera including *Anabaena* (Bory ex Bornet & Flahault, 1886)*, Cylindrospermopsis* (Seenayya & Subbaraju, 1972)*, Planktothrix* (Anagnostidis & Komárek, 1988) and

Lyngbya (Agardh ex Gomont, 1892) (Lagos et al., 1999; Moustafa et al., 2009; Pearson et al., 2010; Wiese et al., 2010).

As mentioned in chapter 1, the saxitoxins are a group of 57 structurally related tetrahydropurine alkaloids (Turner et al., 2019; Wiese et al., 2010), which are stable under both heat and acidic conditions (Etheridge, 2010). This stability allows them to persist after both common cooking processes and passage through the human gut. Therefore, absorption into the body can occur after consumption even where the contaminated food has been cooked thoroughly, unlike some other contaminants. Within the UK there have been several recorded outbreaks of PSP, the most severe being in 1968 when 78 individuals were hospitalised (Ayres and Cullum, 1978). The other recorded incidents stretch back to 1828 (Ayres and Cullum, 1978; Joint et al., 1997). More recently there were several intoxications of dogs, which resulted in illness in some individuals and unfortunately fatalities in others (Turner et al., 2018). As a result of the 1968 outbreak and the severity of PSP there has been a monitoring programme in place to detect PSP levels in UK shellfish, the typical vector of the toxins into humans, since 1968. Initially this programme was focussed in the Northeast region of England but this has expanded to cover all classified shellfish production areas (Ayres and Cullum, 1978; Franklin, 1991). This monitoring programme has proven to be effective at protecting public health as there have not been recorded human cases of PSP in the UK since the 1968 intoxications (Bresnan et al., 2021; Hinder et al., 2011). The current programme takes a two-pronged approach to the protection of public health. Water monitoring for *Alexandrium sp.* is undertaken to provide an early warning of the presence of potential PSP producers, whilst shellfish from classified production sites are tested for the presence of the toxins themselves (Davidson and Bresnan, 2009; Hinder et al., 2011). Although monitoring has been undertaken for many years the identity of the possible PSP producers is rarely elucidated within this programme. This is a consequence of the methodology employed, with large water volumes screened, typically 25 or 50 mL, to attain high sensitivity. This limits the ability of analysts to manipulate individual cells, making speciation very difficult for many harmful taxa. Finally, a high sample throughput is essential for the

monitoring programme to be cost effective and rapid enough to provide public health protection, this further limits the time analysts have to confirm identity to species level.

The regulations within the UK for the detection of any species of *Alexandrium* require further shellfish samples to be submitted for toxin analysis, as a precaution. The result of this is that the causative organisms of PSP in UK waters have only been conclusively identified through research efforts. This means that although there has been work undertaken in Scotland sufficient to suggest that the causative organism in Scottish waters is *Alexandrium catenella* (Brown et al., 2010), in England and Wales the sites where Alexandrium has been definitively speciated are sparse. In those cases where speciation has been achieved the species found to produce PSP toxins (PST) have been *Alexandrium minutum* and *Alexandrium catenella*. These two species show geographic separation, with those sites in the Northeast of England being affected by *Alexandrium catenella* (Reported as *Gonyaulax tamarensis* (Ayres and Cullum, 1978) and *Alexandrium tamarense* (Joint et al., 1997)) similarly to Scotland and the South of England being affected by *Alexandrium minutum* (Nascimento et al., 2005; Percy, 2006). Although several locations in the South and west of England and Wales have experienced PST contaminations of shellfish the causative organism has only been positively identified twice. The two sites where *Alexandrium minutum* has been conclusively shown to be present are the Fleet Lagoon in South Dorset (Nascimento et al., 2005), and the Fal estuary in South Cornwall (Percy, 2006), both on the south coast, shown as triangles (Figure 3.1).

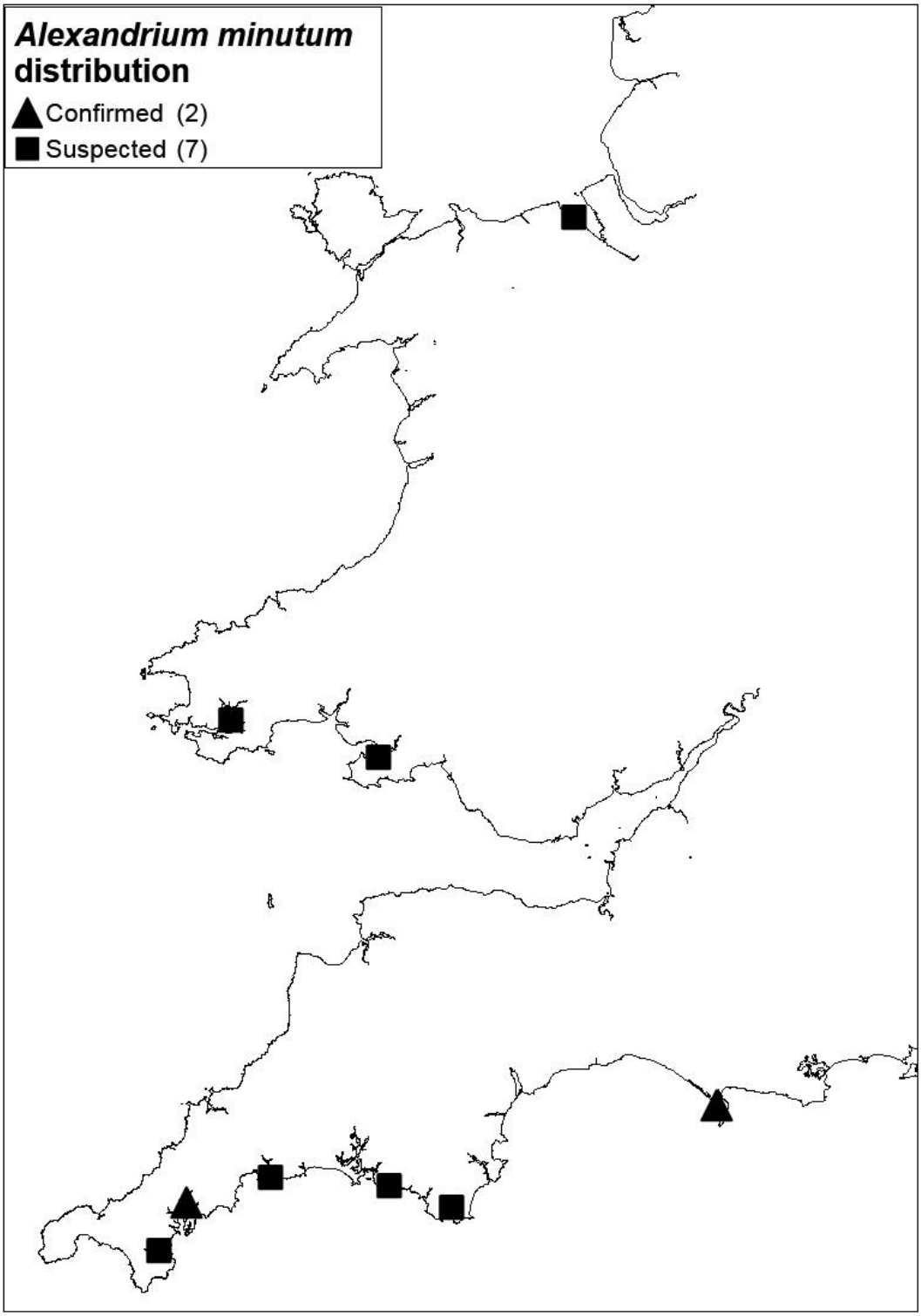


Figure 3.1: Map of the Southwest UK showing sites where *A. minutum* presence has been confirmed by molecular identification, and suspected locations of *A. minutum* based on PST presence in shellfish.

In addition to the two PST producers thought to be most common in UK waters *Alexandrium ostenfeldii* has been identified from the East coast of Scotland in the region around the Firth of Forth (John et al., 2003) as well as the Southwest of England in the Fal (Percy, 2006). This species has been shown to produce saxitoxins (Hakanen et al., 2012; Setälä et al., 2014), with the Scottish strains tested being weakly toxic, producing small amounts of Saxitoxin and Neosaxitoxin (Brown et al., 2010). This species is also commonly linked with the group of compounds called spirolides (Aasen et al., 2005; Ciminiello et al., 2006; Hu et al., 2001; Medhioub et al., 2012). These compounds are the largest group of cyclic imines, a term covering six families of toxins (Cembella and Krock, 2008). The presence of spirolides in shellfish is not yet regulated in Europe (Anonymous, 2010) but there are some concerns around their health impacts in humans. Following injection in mice as part of the lipophilic mouse bioassay it has been demonstrated that they rapidly produce neurological symptoms, convulsions and cramps, often causing death in minutes (Anonymous, 2010; Gill et al., 2003). Alongside the toxin producing species of *Alexandrium* known to be present in the waters around Great Britain, there are also several additional species which do not produce compounds harmful to humans. These include *Alexandrium tamarense* ((Lebour) Balech, 1995) which was split from the *A. tamarense* species complex where it was previously known as *Alexandrium tamarense* Group III (John et al., 2014; Lilly et al., 2007). This ribotype was always found to be non-toxic and is known to be widely distributed in the British Isles. The name also commonly occurs in the literature in place of *A. catenella* as prior to, and even after, the separation of the *A. tamarense* species complex in 2014 (John et al., 2014) references to toxic *A. tamarense* are widespread. Another non-toxic species detected in UK waters is *Alexandrium tamutum* (M. Montresor, A. Beran & U, John, 2004), which has been identified in samples from the East coast of Scotland (Alpermann et al., 2008). Also, non-toxic populations of *A. minutum* have been identified from Scottish waters (Brown et al., 2010), especially around Scottish Islands such as the Shetlands and Orkneys (Bresnan et al., 2008).

A key feature of the *Alexandrium* genus, along with many other dinoflagellates, which makes it a concern for human health over long time scales is the ability to form resting stages (Anderson et

al., 2012; Bravo and Figueroa, 2014; Wyatt and Jenkinson, 1997). These resting stages are normally called cysts and can persist for many years whilst remaining viable (Bravo and Figueroa, 2014), with reports of viability exceeding 100 years (Miyazono et al., 2012). An archetypal life cycle of a cyst forming planktonic dinoflagellate is presented in Chapter 2 section 2.4 as Figure 2.1. This is modelled from the life cycle of *Alexandrium sp.* and is a powerful survival mechanism (Bravo and Figueroa, 2014) for the species in those regions which support the retention of cysts. Being non-motile *Alexandrium* cysts behave as fine silt or clay particles once formed (Anglès et al., 2010; Dale, 1976; Dodge and Harland, 1991), depositing in areas where flow is very low. As such they can most commonly be found in deeper waters and sheltered areas of coastline with low energy, such as embayments, tidal creeks and harbours (See Chapter 2, section 2.3). Typically, dinoflagellate cysts undergo a period of dormancy before entering into a quiescent state where germination can occur (Anderson et al., 2012; Bravo and Figueroa, 2014). Recent work with *Alexandrium catenella* has determined previously unknown cellular control of the dormancy and quiescence cycle. In these studies, from the Gulf of Maine and the Nauset marshes on the East coast of the United States of America, cysts of *A. catenella* have been shown to require a certain amount of chilling, quantified chilling units, to shift between the two states (Brosnahan et al., 2020; Fischer et al., 2018). Cooler periods hasten the onset of quiescence and then after a period of warmer temperatures have been experienced cysts return to a dormant state. This control mechanism of cyst germinability has yet to be demonstrated widely but does appear to occur in *Pyrodinium bahamense* (Plate, 1906) (Lopez et al., 2019). These studies offer an insight into the ability of cysts to avoid excessive germination and so loss, when the prevailing conditions are unfavourable for vegetative cell growth (Brosnahan et al., 2020). This cyclical pattern allows cysts to remain viable across seasonal cycles, avoiding germination in the colder winter months during dormancy before returning to a viable state in time for the optimal conditions in spring and summer (Fischer et al., 2018). If this same cycle is common in temperate dinoflagellates then it also means that the optimum time to collect cysts is during the colder months of autumn and winter, when cyst concentrations are at their highest as active

germination is less likely, preventing loss of cysts to the water column. This was seen in both *A. minutum* and *A. tamarense* from Cork harbour, where those cysts collected in the spring and early summer showed much higher ability to germinate than those from late summer through to late winter (Ní Rathaille and Raine, 2011). Also though, this means that a period of cold storage may be advisable before attempting laboratory germinations, to allow for sufficient chilling to occur for quiescence to begin. This appeared to be the case during the study of Ní Rathaille and Raine (2011) where cold storage eliminated the previously observed seasonality of excystment success, allowing for high levels of successful excystment for all samples.

The cyst forming aspect of the *Alexandrium* life cycle allows for an alternative methodology for the detection of the *Alexandrium* genus at a location, not only can the vegetative cells be observed in water samples but also cysts can be found in sediments where blooms have occurred, either recently or in the past. Due to the many cyst forming species of dinoflagellate, this method can be used to detect many different species as well as being valuable in the detection of diatom species capable of forming auxospores which may also settle from the water column whilst remaining viable.

The cysts of *Alexandrium sp.* have been implicated in bloom initiation in a number of examples. In the Mediterranean, on the Spanish coast, blooms of *A. minutum* have been demonstrated to be initiated by excystment of resting stages in a well-studied harbour (Estrada et al., 2010). The cysts of both *A. minutum* and *A. tamarense* have been thought to originate from cyst banks in the North channel of Cork Harbour, Ireland (Touzet et al., 2010b). In the well-studied region of the Gulf of Maine, on the East coast of the USA, several distinct cyst beds lead to the annual inoculation of vegetative cells required to initiate blooms of *A. catenella* (typically reported from this region as *A. fundyense*) (Anderson et al., 2005; Anderson et al., 2012; Butman et al., 2014).

Within UK waters previous work has shown that the presence of extensive *Alexandrium* cyst beds in the Northeast of England (Joint et al., 1997), these also extend into the Firth of Forth and

offshore into the North Sea. These cyst beds are of *Alexandrium catenella* (reported as *A. tamarense*) and interestingly are not ubiquitous or homogenous in the region (Joint et al., 1997). Further to this *Alexandrium* cyst deposits have been found to extend up the East coast of Scotland, with limited numbers in the Moray Firth but more present in the Orkney Isles and down the complex series of islands and bays of the west coast, at least as far South as the Isle of Rùm (Brown et al., 2010).

Owing to the longevity of cysts their presence in surface sediments suggests the possibility of blooms being seeded in the future, even if a location has not seen problematic proliferations of cells for several years. Being able to detect viable cysts in sediments can be a valuable indicator of the species present and represents a way to determine the extent of a species range, even in the absence of detectable vegetative cell growth. It also has the advantage of allowing for the detection of a species year-round, rather than only during the brief window of active vegetative growth that is common for many of these dinoflagellate species. The presence of cysts from PST producing species of dinoflagellate does not correspond well to current risk from PSP however, although grazing on dinoflagellate cysts has been reported in some groups of organisms (Persson et al., 2008; Persson and Rosenberg, 2003), including bivalves (Persson et al., 2006; Persson and Smith, 2009), this is not widely reported as causing toxicity, with few confirmed examples (Persson, 2000; Persson et al., 2006).

To date, with the causative species of PSP in the southwest of GB only positively identified in two locations, it is difficult to make accurate predictions about PST occurrence in the region. In those cases where the identity of the producer is known this is *Alexandrium minutum* but at other sites, although toxin profiles derived from analysed shellfish are similar (Turner et al., 2014), it is possible that another PSP producing species is present. As such, this shoreline survey was undertaken to sample a wide range of locations in the region, sample cyst forming dinoflagellate species, germinate dinoflagellates and undertake identification and toxin testing of monoclonal isolates established to

determine if other toxic populations of *Alexandrium minutum* or a different PST producer are present in the southwest of Britain. Additionally, other cyst forming dinoflagellate species might be detected using this method and so further information would be provided on the presence of other potential toxigenic species.

3.2 - Methods

3.2.1 - Sample site selection

Sample sites were selected to cover the Southwestern region of Great Britain with the most Northern samples from the West coast of Wales and the furthest East being in Portsmouth. Sites were chosen via satellite imagery, two key criteria were assessed using this technique. Firstly, areas of intertidal coastline or river estuary, which appeared to be composed of muddy sediments were selected. Secondly site selection was refined by choosing areas where a clear access route could be identified, allowing safe access to the proposed sampling locations. Finally, sampling locations were added in areas where commercial shellfish harvesting is undertaken, allowing a degree of consistency between the routine monitoring efforts and the results of this survey. Where PST had been detected in shellfish sampling was undertaken at the shellfish growing area in an attempt to target known areas of PST occurrence. For each sample site, those accessible areas which were most sheltered, allowing for the build-up of fine sediments, were sampled if possible. Initially, this resulted in 30 sample sites (Figure 3.2) being chosen with several sample sites having more than one sample taken for 47 sediment samples collected in total during the winter months from December 2015 to March 2016. Following a PSP event in shellfish from the Swansea Bay docks production area, additional sampling was conducted at this site in January 2017, increasing the sites sampled to 31 (Figure 3.2) and the number of sediment samples taken to 53.

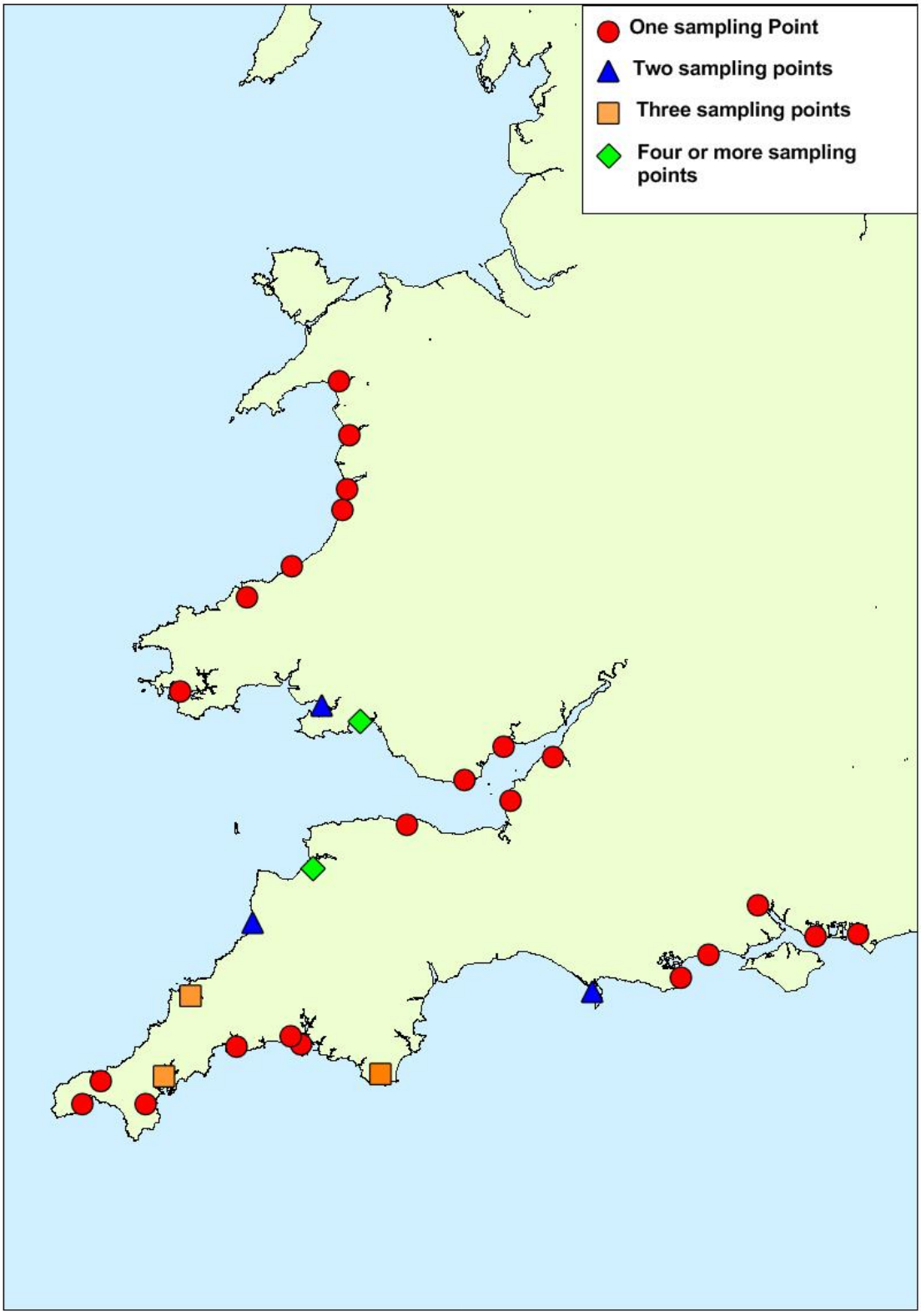


Figure 3.2: Map of the SW of GB displaying sediment sampling sites.

3.2.2 - Field sample planning and equipment

Prior to sample collection sites were grouped by geographic proximity and routes planned to allow for the sampling of as many sites as possible within the available time. This resulted in eight sampling trips in total. The details of each sample trip and the sites surveyed are detailed in table 3.1. Sampling trips were organised around the tide times, allowing for safe collection of samples either on the falling tide or above the incoming tide line. Due to the sampling being undertaken in the Autumn and Winter months special attention was also paid to weather forecasts to ensure that fieldworkers always had access to appropriate clothing and equipment. Additionally, there was the potential for field sampling to be cancelled at short notice if weather conditions were particularly poor, however, the advanced planning and favourable conditions meant that cancellation of a trip or site sampling was not necessary. All field sampling trips were planned with two field work trained researchers being available as well as a further land contact, who was based at Cefas in Weymouth. There was also a communication plan developed and in place for the land contact to cascade notifications to the researchers and other agencies, including the emergency services, in the event of a call for help or an inability to contact fieldworkers at pre-agreed time points.

Table 3.1: Outlining the dates and locations of field sampling trips undertaken to collect sediments from intertidal waters.

Trip dates	Region surveyed	Sites sampled
07/12/2015	North Devon	Chivenor Yelland Appledore
08/12/2015	South Dorset	Fleet Lagoon
10/12/2015	South Devon	Kingsbridge
4-5/02/2016	Cornwall	Bude

		Wadebridge Camel Estuary Padstow Hayle Newlyn Port Navas Point Malpas Fowey
15- 17/02/2016	Wales & East Somerset	Borth-Y-Gest Barmouth Aberystwyth Ynyslas Aberaeron Cardigan Milford Haven Bury Port Barry Harbour St Brides Portishead Weston- super- Mare
09/03/2016	North Devon & South East Cornwall	Porlock Wier St Germans St Johns

		Itchenor Gosport
17/03/2016	South Dorset & Hampshire	Southampton Christchurch Studland
31/01/2017	South Wales	Swansea

In all instances of field sampling a set of standard equipment for sampling as well as for safety were utilised by fieldworkers. Sampling equipment taken from the vehicle to the sampling location was a corer, sample storage containers, film to seal containers and a Global Positioning System (GPS) to record coordinates of sampling. A box grab was taken alongside other sampling equipment in cases where the sample site was inaccessible on foot but could be accessed with the use of a remote grab. In terms of specific safety equipment, fieldworkers utilised auto-inflation life jackets, unless entrapment hazards were present, a depth gauge pole to assess the depth of mud flats, throw ropes, first aid kits and high visibility clothing.

3.2.3 - Sediment sample collection

Upon arriving at a sampling site, researchers assessed local conditions for safety and suitability, expected tide state and weather conditions were confirmed or if they had deviated from forecasts, sampling safety was reassessed. Before undertaking sampling at each site researchers notified the shore contact via mobile telephone and prepared personal protective equipment and sampling equipment whilst in the safe area designated during fieldwork planning. Researchers travelled on foot across the upper shore until reaching the intertidal. At this point local ground conditions were assessed and sampling was targeted for those areas where sediments were composed of mud and fine sediment wherever possible, in practice this meant that sample sites were typically in mud flats, near small creeks or in the lee of large natural or manmade structures.

Normally the ground conditions correlated well with the satellite imagery but in some cases sediments were less suitable than the originally identified location. In these cases, researchers aimed to find a nearby suitable alternative. When a suitable sampling site had been determined sediment cores were taken and the upper 5 cm of the core was removed and stored in an opaque container, made airtight with parafilm. In rare cases the sampling site was inaccessible, either due to a lack of suitable ingress/egress routes or because the sample site was inundated with water, as a result of being within a harbour. When this was the case a box grab was deployed carefully to the substrate, this was then triggered remotely before being recovered. A core was taken from this grab sample and stored in the same manner as for the directly sampled cores. When grab sampling was undertaken within Swansea docks, which was fully submerged, insufficient sediment was captured in the grab and sediments lacked the integrity to take a core. As such sediment was recovered directly from the grab in these cases, these were again stored in the same manner as all other samples. Once stored, samples were transported back to the vehicle and stored in a cool box, containing ice packs. Details of the sample site were recorded and contact was made with the designated land contact to inform them that sampling had been undertaken successfully. At this point all field kit was rinsed with deionised water before being sterilised with Virkon (a virucide and bactericide). Field kit with heavy soiling was scrubbed clean with Virkon solution and deionised water to prevent the carrying of material between sample sites. All field kit was then rinsed with deionised water before being stored, ready for use at the next site.

3.2.4 - Germination and isolation

Upon return to the laboratory all sediment samples were placed into wax coated boxes and stored in the dark at 4 °C. When samples were taken for germination they were removed from cold storage and unsealed. For each sample 0.5 g of sediment was weighed into a 50 mL centrifuge tube and 10mL of sterile seawater (autoclaved and 0.2 µm filtered) was added to resuspend the sediment and form a slurry. Three slurries were produced for each sample, allowing for triplicate analysis of each sample. Samples then underwent 1 minute in a XB6 sonication bath (Grant Instruments,

Cambridge UK) to separate the sediments. Following this samples were gently mixed before being filtered through both an 80 µm and a 20 µm sieve. The sample was rinsed through the 80 µm sieve using sterile seawater, residual material >80 µm was discarded to waste. Sample material collected on the 20 µm sieve was then rinsed with sterile seawater and the particle fraction <20 µm was discarded to waste. The 20 µm sieve was then back rinsed into a 9 cm diameter petri dish with sterile seawater, collecting the 20-80 µm fraction. To this petri dish seawater containing L1 media, at 10% of normal strength, was added until the total volume of rinsed particles and dilute media was approximately 20-25 mL. These plates were then placed in an incubator set at 17 °C with a 14:10 hour light:dark cycle and left for 10-15 days to allow time for dinoflagellate cysts to germinate.

After 10-15 days in the incubator, petri dishes were removed from the incubator and assessed for the presence of motile vegetative dinoflagellate cells using an Olympus SZX7 stereomicroscope. Individual dinoflagellate cells were picked using a drawn glass pipette and were transferred to a watch glass containing sterile seawater. As this survey was intended to investigate the spread of *Alexandrium spp.* only those cells thought to potentially be *Alexandrium spp.* were isolated, with a few exceptions for other potential harmful algal species. From the watch glass dinoflagellate cells were then individually transferred to a well in a 24 well plate, with each well containing 2 mL of sterile seawater containing L1 media at 10% of normal concentrations. Each well was marked and noted with the site number of origin. Once a 24 well plate was filled it was sealed with Parafilm and transferred to the incubator. Once all cells of interest from a sample had been transferred in this way all remaining sample material was discarded to waste and all equipment was sterilised and rinsed using deionised water and 70% ethanol solution.

All wastewater, sediment slurries and plastic ware which was used in germinations or to culture cells was treated with a hypochlorite solution or autoclaved to prevent the discharge of any viable cells, dinoflagellate cysts or other resting stages such as diatom auxospores.

3.2.5 - Algal culture

Well plates were left in the incubator and checked every three to seven days to determine if division of cells had commenced and cultures had established. Once cultures began to grow wells were checked more frequently and when cell numbers began to increase the culture from the well plate was transferred to a small, 5 cm diameter, petri dish containing 7-10 mL of seawater enriched with L1 media at 20% strength. Once transferred to the petri dish, cultures were returned to the incubator and allowed to establish. After the cultures were able to establish, cells were transferred again, to a 50 mL tissue culture flask, with a 0.2 µm vented cap, containing 49 mL of sterile seawater enriched with L1 media. Following transfer to the flasks, cultures were returned to the incubator. Cultures were assessed visually for active growth and when culture density increased, normally after four weeks, cultures were subcultured. Subculture was achieved by transfer of 5 mL of dense culture into a new vented tissue culture flask containing 45 mL of fresh L1 media seawater, at this point two cultures were established for each strain. This process was repeated as necessary to maintain viable cultures. One of these culture lines was used to assess the species identity, the other was utilised for evaluation of culture toxicity.

3.2.6 - Algal identification

Of the two established cultures for each strain, one was utilised for algal species identity checking. To determine the algal species, 3 mL of culture was taken from actively growing cultures and 1ml of dilute neutral Lugol's iodine was added. Of this fixed solution 1 mL was mounted on a glass microscope slide following sample homogenisation by repeated inversion. Identity was assessed using a Nikon Eclipse TE300 inverted light microscope. Observed cells were assessed for gross morphology and measured to determine cell size. Cells were photographed for later assessment.

3.2.7 - Toxin determination

3.2.7.1 Culture toxin extraction

The second of the two culture lines for each strain was taken for Paralytic Shellfish Toxin assessment, this was undertaken for all cultured strains, even those not believed to be *Alexandrium* sp. as there was the possibility of a novel PST producer being identified in the region. Cultures were allowed to reach a high density, where cell aggregations were visible in the culture flasks with the naked eye. This ensured that there was sufficient cellular material for successful toxin content analysis, with a visible cell pellet present during the concentration step. Before toxin concentration assessment a 3 mL sample was taken from each culture, this was fixed as per section 3.2.6 and stored for cell counts, to allow for per cell toxin quotas to be determined. Cell counts were undertaken using a Fuchs-Rosenthal counting chamber and a Nikon Eclipse TE300 microscope (Japan).

Two independent methods were used to assess culture toxicity, a modified version of the routinely employed High Performance Liquid Chromatography with Fluorescence detection (HPLC-FLD) and a recently developed method employing Hydrophilic Interaction Liquid Chromatography (HILIC) with tandem mass spectrometry.

For both methods sample preparation of algal cultures began the same way. Following a sample being taken for cell counts, 50 mL of culture was transferred to a 50 mL centrifuge tube. This tube was then centrifuged at 3400 RCF. At this point, the supernatant was carefully removed so as not to disturb the algal cell pellet. The supernatant was held for disposal following hypochlorite treatment to ensure that no viable cells remained. The pellet was then resuspended in 1.5 mL of 1% acetic acid, this was mixed with the cells using the micro pipettor. After the pellet and acetic acid were homogenised, the cells were lysed via the use of probe sonication. For this process the probe was placed into the centrifuge tube, slightly above the bottom of the tube. Samples were sonicated

for 1.5 minutes at 30% amplitude. After sonication, each sample was once again centrifuged at 3400 RCF for 10 minutes. At this point the residual cell material formed the pellet and the toxins, if present, were in solution with the acid. The supernatant was then transferred to a 5 mL syringe and filtered through a 0.45µm syringe tip filter, into a 4 mL tube. At this point the sample treatment diverged depending on the analysis type to be used, the different methods are detailed in sections 3.2.7.3 and 3.2.7.4.

3.2.7.2 Reagents, chemicals and analytical equipment

The reagents which were used for the toxin extractions and HPLC analysis were of HPLC grade or higher. Chemicals of LC-MS grade were utilised where mass spectrometry was used for analysis. The chemicals used were sourced either from Fisher Scientific (Loughborough, UK) or VWR (Lutterworth, UK). All solid phase extraction (SPE) processes were automated and performed using a Gilson (Dunstable, UK) ASPEC, running Trilution software. Due to two independent methods being utilised for the toxin analysis of cultures, different analytical systems were employed. Where HPLC-FLD analysis was utilised, it was performed using an Agilent 1100/1200 series HPLC with fluorescence detection. For the HILIC-MS/MS analysis two different systems were used, these were a Waters (Milford, MA, USA) Acquity I class UPLC coupled with a Waters Xevo TQ-S triple quadrupole mass spectrometer and an Agilent (Santa Clara, CA, USA) 1290 Infinity II UPLC coupled with an Agilent 6495B triple quadrupole mass spectrometer. Calibration standards for both the HPLC-FLD and the HILIC-MS/MS analytical methods were prepared from certified reference material sourced from the Institute of Biotoxin Metrology, National Research Council Canada (Halifax, NS, Canada). The two methods provide complimentary analysis of several compounds, with the HILIC-MS/MS method able to quantify some additional toxin congeners as well as quantifying epimeric pairs of toxins separately rather than as a sum, as is incorporated within the HPLC-FLD method (Table 3.2).

Table 3.2: Full names for saxitoxin analogues analysed in this study, abbreviations for all saxitoxin analogues. Ability to analyse for each method used (+/-). Where analogue concentrations for epimers are summed under the HPLC-FLD method this is noted. Those toxins marked with a * are only able to be qualified and quantified using the HPLC-FLD method by following additional steps, such as hydrolysis and ion exchange fractionation. As such they were not screened for in this study by this method.

Compound name	Compound abbreviation	HPLC-FLD	HILIC-MS/MS
N21-sulfocarbamoyl-11α-hydroxysulfate-saxitoxin	C1	+ as C1&2	+
N21-sulfocarbamoyl-11 β -hydroxysulfate-saxitoxin	C2	+ as C1&2	+
N21-sulfocarbamoyl-11α-hydroxysulfate-neosaxitoxin	C3	- *	+
N21-sulfocarbamoyl-11β-hydroxysulfate-neosaxitoxin	C4	- *	+
Decarbamoyl-11α-hydroxysulfate-saxitoxin (Decarbamoyl-gonyautoxin 2)	dcGTX2	+ as dcGTX 2&3	+
Decarbamoyl-11β-hydroxysulfate-saxitoxin (Decarbamoyl-gonyautoxin 3)	dcGTX3	+ as dcGTX 2&3	+
Decarbamoyl-11α-hydroxysulfate-neosaxitoxin (Decarbamoyl-gonyautoxin 1)	dcGTX1	-	+
Decarbamoyl-11β-hydroxysulfate-neosaxitoxin (Decarbamoyl-gonyautoxin 4)	dcGTX4	-	+
11α-hydroxysulfate-saxitoxin (Gonyautoxin 2)	GTX2	+ as GTX 2&3	+
11β-hydroxysulfate-saxitoxin (Gonyautoxin 3)	GTX3	+ as GTX 2&3	+
11α-hydroxysulfate-neosaxitoxin (Gonyautoxin 1)	GTX1	+ as GTX 1&4	+
11β-hydroxysulfate-neosaxitoxin (Gonyautoxin 4)	GTX4	+ as GTX 1&4	+
N-sulfocarbamoyl-saxitoxin (Gonyautoxin 5)	GTX5	+	+
N-sulfocarbamoyl-neosaxitoxin (Gonyautoxin 6)	GTX6	- *	+

Deoxydecarbamoyl-saxitoxin	doSTX	-	+
Decarbamoyl-saxitoxin	dcSTX	+	+
Decarbamoyl-neosaxitoxin	dcNEO	+	+
Saxitoxin	STX	+	+
N1-hydroxy-saxitoxin (Neosaxitoxin)	NEO	+	+
N21-sulfocarbamoyl-11(α/β)-hydroxy-saxitoxin	M1	-	+
11(α/β)-hydroxy-saxitoxin	M2	-	+
N21-sulfocarbamoyl-11,11-dihydroxy-saxitoxin	M3	-	+
11,11-dihydroxy-saxitoxin	M4	-	+
Uncharacterized	M5	-	+

3.2.7.3 - Fluorescence detection

To prepare samples for analysis via HPLC-FLD sample material first needed to be oxidised. This was undertaken using a modified periodate oxidising reagent, normally used in the routine monitoring programme to increase the sensitivity of the method for the analysis of scallop species (Turner and Hatfield, 2012). This modification employs a stronger periodate reagent than that employed in the normal method. This is achieved by mixing the reagent with a 5:1:1 mix of 0.03 M periodic acid: 0.3 M ammonium formate: 0.3 M sodium phosphate. Oxidations were performed in the same way as the official 2005.06 method (Anonymous, 2005). Therefore, 100 µL of oyster matrix modifier was added to a glass HPLC vial, this was followed by 100 µL of filtered sample extract. To this mix 500 µL of periodate oxidising reagent was added to the vial, and the mixture was vortex mixed for 5 seconds. The mixture was then left to react for 1 minute before 5 µL of glacial acetic acid was added to the vial and it was vortex mixed for a further 5 seconds to quench the oxidation reaction. At this point the sample was ready for analysis. In addition to the samples a low and a high-

level standard (concentrations shown in Table 3.3) of mix 1, 2 and 3 (mixes shown in Table 3.4) were also oxidised as above. The low level standard of each mix was used to determine instrumental sensitivity levels, with a requirement for each primary peak included in the mix to have a signal:noise of at least 3.0. The high-level standard for each mix was used to define the retention of each relevant toxin peak as well as to draw a rudimentary two-point calibration curve with the low level peak areas from the same mix. In this way the method was semi-quantitative, providing an indicative estimation of toxin concentrations rather than an accurate one.

Following initial issues with the presence and detection of fluorescent co-extractives, which were not found to be any of the saxitoxins (see section 3.4 for further details), an unoxidised extract was also prepared for each sample prior to analysis by HPLC-FLD. This was accomplished by taking 100 µL of matrix modifier and adding it to a glass HPLC vial. To this vial 100 µL of sample was added as above, 500 µL of deionised water was added to the vial, the vial was capped and this mixture was then vortex mixed for 5 seconds. At this point the unoxidised extract was ready for analysis. As such each sample was analysed via two injections, the data from the oxidised and unoxidised vials for each sample constituting one result.

Analysis was undertaken using the modified chromatographic conditions refined at Cefas (Hatfield et al., 2016; Hatfield and Turner, 2012). This makes use of a superficially porous column to reduce run times compared to the official method by half. This allows for more rapid analysis as well as enhanced sensitivity. The HPLC-FLD method used here was a screening method, able to detect relevant compounds but not to quantify them accurately. This was to allow rapid processing of many samples, allowing for toxin screening as it was expected that a number of cultures would not be toxic, as microscopic analysis suggested that they were not from a PSP producing genus. This screen method was qualified with the presence of a high and a low certified standard for each of the toxins present in the assay, shown in Table 3.3.

Table 3.3: Showing the toxin concentrations (µMol) of the two standard levels utilised in the screening of samples via HPLC-FLD.

	Standard concentration (umol)	
Toxin	Low Level	High Level
GTX 1&4	0.049	0.243
dcNeo	0.145	
Neo	0.052	0.258
GTX2&3	0.084	0.422
GTX5	0.264	0.053
STX	0.054	0.269
dcGTX2&3	0.142	0.710
C1&2	0.421	2.103

In order to achieve chromatographic separation of all compounds using this method it was necessary to run some saxitoxin congener standards separately from one another, this was achieved by the use of three standard mixes (Table 3.4). This complexity is necessary to remove the confounding effects of coeluting compounds on interest, which is an issue with this methodology. By separating the compounds in the standards it becomes possible to determine sensitivities for all relevant compounds. When complex mixes are encountered in samples it is necessary to treat the primary peak for each congener as being wholly generated by that compound. As such, although it is suitable as a screen, when this method is interpreted semi-quantitatively it gives rise to inflated toxin concentrations when coelution occurs. This necessitates the additional steps required for the fully quantitative method, to adequately separate analytes in samples as well as standards.

Table 3.4: Standard mix composition for the 3 qualitative mixes analysed. Bold toxin names indicate the primary peak for a given compound and are used to semi-quantitatively compare analytes against.

PEAK NO. & INDICATIVE RETENTION TIME (MINUTES)	STANDARD MIX 1	STANDARD MIX 2	STANDARD MIX 3
1 – 2.00	-	-	**dcGTX2&3**
2 – 2.30	**GTX1&4**	-	dcGTX2&3
3 – 2.80	Neo	-	
4 – 3.00	-	-	C1&2
5 – 4.00	**dcNeo**	dcSTX	-
6 – 4.30	**Neo**	dcSTX	-
7 – 4.80	GTX1&4	**GTX2&3**	-
8 – 5.30	-	**GTX5**	-
9 – 5.80	Neo	**STX**	-

In this analysis the high level (level 5) of each of the standard mixes is used as a retention time marker for each of the toxins present. It also serves as the high-level for semi-quantitative analysis. The low-level standard for each mix (level 1) contains the same compounds but at 20% of the concentration present in the level 5. The low-level standard is used to define a sensitivity limit for each compound as well as forming the low level calibrant for semi-quantitative analysis.

3.2.7.4 - Mass spectrometry detection

As well as analysis by LC-FLD, samples were prepared for HILIC-MS/MS analysis. This method was used to complement the screening by HPLC-FLD but being a recently developed method (Boundy et al., 2015; Turner et al., 2015) was more challenging to run and so was not used exclusively. In part this is due to the complex ionic interactions which occur in the column of the HILIC-MS/MS method, which can be affected by relatively minor changes in the chemical composition and pH of the mobile phases used. As such, the robust routine method was a useful screening technique although fluorescence detection is less specific than mass spectrometry. The protocol used followed the method as presented in Boundy et al., (2015) and validated by Turner et al., (2015). The same sample extract from section 2.2.6.1 underwent further processing before being analysed by the HILIC-MS/MS method.

Prior to mass spectrometry analysis sample extracts needed to have the salts removed, as these interfere with both chromatographic elution and mass spectral response. In order to remove the salts 1 mL of the filtered extract from section 3.2.7.1 was added to a 4 mL TC tube and 5 µL of ammonium hydroxide was added, this was mixed for 5 seconds using a vortex mixer. Each sample was then forwarded for automated carbon clean-up using a Gilson ASPEC XL-4 liquid handler and carbon SPE cartridges (Supelco ENVI-Carb 250 mg/3 mL). Samples were eluted into clean 4 mL TC tubes using 2 mL of 20:80 acetonitrile:water with 0.25% acetic acid. Finally, 100 µL of each desalted extract was diluted with 300 µL acetonitrile in polypropylene UPLC autosampler vials.

Extracts were analysed on one of two triple quadrupole mass spectrometry systems as outlined in 2.6.2. Two column types were also utilised, either a BEH Amide UPLC column (Waters) or a HILIC-Z UPLC column (Agilent) (Turner et al., 2020). In either case the mass spectrometer was run in multiple reaction monitoring (MRM) mode and two transitions for each compound of interest were measured. Each toxin having both a quantitative transition and a qualitative transition to

provide confirmation of analyte identity ensures excellent selectivity and specificity for the method as such there was no need for the analysis of an additional extract analogous to the unoxidised extract needed in the HPLC-FLD screen.

Calibration curves were drawn for each analyte, with seven calibrant standard levels included for each compound analysed. The peak area of the primary, quantitative, transition for each analyte present in a sample was integrated and then taken and compared to the generated calibration curve from the relevant standards, providing a calculated toxin concentration in $nmolL^{-1}$ for each compound of interest individually.

3.3 – Results

3.3.1 - Field sampling

Field sampling was successfully undertaken without a change in the planned site listings in all but one of the planned trips. For the trip needing modification, issues with a hired vehicle reduced the available time for the field sampling trip to Cornwall (4th and 5th of February 2016), where approximately a third of the first day was lost. This resulted in some sites being abandoned and a later finish time for sampling on the first day which in turn affected some of the tide states due to changes in the planned sampling times. Through consultation with the land contact and modifications made as soon as the issue had been identified, much of the planned sampling was able to take place. These modifications to the field plan resulted in changes to the times of some sites but did not affect site access where field sampling was still undertaken. A further field sampling trip was organised to encompass several of the sites which had to be abandoned in the initial Cornwall field sampling trip, as such the coverage of the field sampling programme undertaken was not substantially diminished. Overall, eight sites were abandoned on the initial Cornwall sampling trip, with three of these being sampled during the second trip.

Figure 3.3: image composite of field sampling sites. A – Borth-Y-Gest, Wales. B – Padstow, England. C- Aberystwyth, Wales. D – Studland, England.

No trips needed to be cancelled in their entirety and suitable pre-planning resulted in weather and tides being suitable for all field sampling days, some examples are provided (Figure 3.3). Field workers carried the required PPE and wore appropriate clothing for site visits, resulting in no injuries or ill health. Communication channels were appropriate to ensure the safety of fieldworkers and communication plans resulted in the land contact seeking further clarification or communication when necessary. Although safety equipment for the events of water immersion

(auto-inflate life jackets) and entrapment in mud (throw ropes) were carried where these hazards were present, these items of equipment were not called into use.

3.3.2 - Algal germinations

Following triplicate germination attempts, vegetative dinoflagellate cells were observed from 42 of the 53 samples taken (Table 3.5). Those sites where germinations did not occur fell into two categories. The first category included those sites with muddy sediments but located in the mouth of the Bristol channel and without shelter, affecting three sites: St Brides, Portis Head and Weston-Super-Mare. The second category consisted of sites where sediments contained more coarse material, with sediment samples being mostly sandy, affecting six sites: Bude, Porlock Wier, Studland, Chivenor, Appledore and the Fleet. It should be noted that in the cases of the Chivenor and Appledore sites, where three samples were taken from each, successful germinations were still achieved from the site, but not from all samples. Of the three samples taken at each two samples from Chivenor yielded motile dinoflagellate cells as did two samples from Appledore.

Table 3.5: Site name and ascribed number with germination status from triplicate germination attempts.

Site Number	Site name	Dinoflagellate Germinations Successful +/ Unsuccessful -
1	Bude - Outer harbour	-
2	Bude -Inner harbour	-
3	Wadebridge	+
4	Camel - Petherick Creek	+

5	Padstow	+
6	Hayle	+
7	Newlyn	+
8	Port Navas	+
9	Point	+
10	Fal - Malpas A	+
11	Fal - Malpas B	+
12	Fowey - Cliff	+
13	Borth-Y-Gest	+
14	Barmouth	+
15	Aberystwyth	+
16	Ynyslas	+
17	Aberaeron	+
18	Cardigan	+
19	Milford Haven	+
20	Bury Port (inner)	+
21	Bury Port (outer)	+
22	Barry Harbour	+
23	St Brides	-
24	Portishead	-
25	Porlock Weir	-
26	St Germans	+
27	St johns	+
28	Itchenor	+
29	Gosport	+

30	Southampton	+
31	Christchurch	+
32	Studland	-
33	Weston Super Mare	-
34	Chivenor (Creek)	-
35	Chivenor (Cages)	+
36	Chivenor (Mudflat)	+
37	Yeland (Jetty)	+
38	Yeland (upshore)	+
39	Zeta Birth	+
40	Appledore (Stream)	+
41	Appledore (The pill)	+
42	Appledore (slipway)	-
43	The Fleet (Nearshore)	-
44	The Fleet (Cages)	-
45	Geese Quarry (Cages)	+
46	Geese Quarry (Downstream)	+
47	Geese Quarry (Upstream)	+
48	Swansea - Kings dock	+
49	Swansea - Queens dock Jetty	+
50	Swansea - Dry Dock	+
51	Swansea - Queens dock entrance	+
52	Swansea - River Tawe	+
53	Swansea - River Neath	+

Overall, of 31 sites, 24 provided evidence of the presence of viable dinoflagellate cysts (Figure 3.4). This demonstrates the presence of suitable conditions for cyst deposition and retention at those 24 locations. Those sites lacking successful germinations also tended to be more exposed, with greater exposure to either open water or high currents possible. For those sites where some samples yielded viable cysts and others did not there were some differences in the nature of the sampled material or the site itself. The Chivenor creek sample which did not yield dinoflagellates was taken further up the shoreline than the other two samples from this site. At the Appledore site, the two samples where dinoflagellates germinated were taken from within a small creek as well as from beside a stream, which are inundated at high tide, these sample contained some finer fluvial deposits, compared with the other sample taken from near to the slipway which was more sandy in composition.

Some samples contained high numbers of diatoms alongside the motile dinoflagellates. Additionally, ciliates and even small copepods were also observed in several samples. Where diatoms were prevalent they were consistently chains of small centric diatoms. High numbers of diatoms present necessitated successful washing of isolated dinoflagellates, in those cases where diatoms were inadvertently picked alongside the dinoflagellate the diatoms consistently grew more quickly in the 96 well plates. This resulted in the culture being irretrievably contaminated, normally the dinoflagellate failed to grow. In some cases, where *Scrippsiella sp.* (Balech ex A.R. Loeblich III, 1965) had been isolated, they were able to rapidly grow alongside the diatoms, with both algal species reaching high numbers rapidly. Where wells were contaminated with diatoms, they were not progressed to further culture stages.

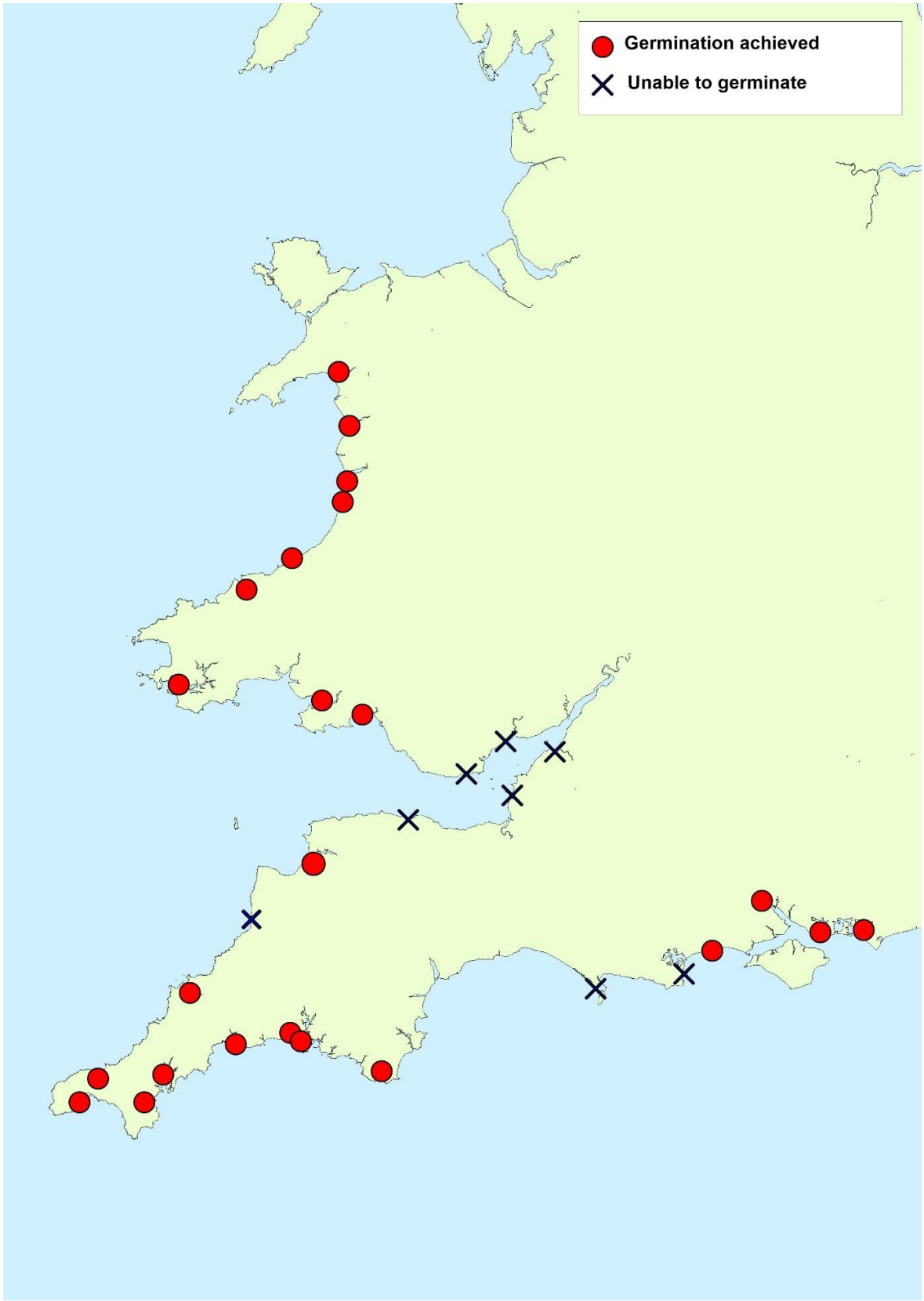


Figure 3.4: Map of the SW of GB displaying results of cyst germination efforts.

From those samples where germination was achieved, cells were successfully isolated and forwarded for continued algal culture. The number of cells successfully transferred to culture from each sample varied, between sites and also between replicates. In total 215 dinoflagellate cultures were established successfully in tissue culture flasks and maintained for further analysis. Unfortunately, an incubator failure occurred before all cultures had been analysed for toxicity or identity, as such data were only collected for 170 of the 215 cultures.

3.3.3 – Algal culture identity

The majority of cultures processed were identified as *Scripsiella sp.* or *Scripsiella* like, with ~88% of all cultures assessed being ascribed to this category. Potential HAB species detected were *Alexandrium sp.* isolated from Bury Harbour in Wales and sites at St Johns, Port Navas and Malpas in Cornwall. Additionally, there were consistent germinations, isolations and culture of *Lingulodinium polyedra* from the West coast of Wales (Figure 3.5). In those samples where *L. polyedra* were observed there were many other smaller dinoflagellates present.

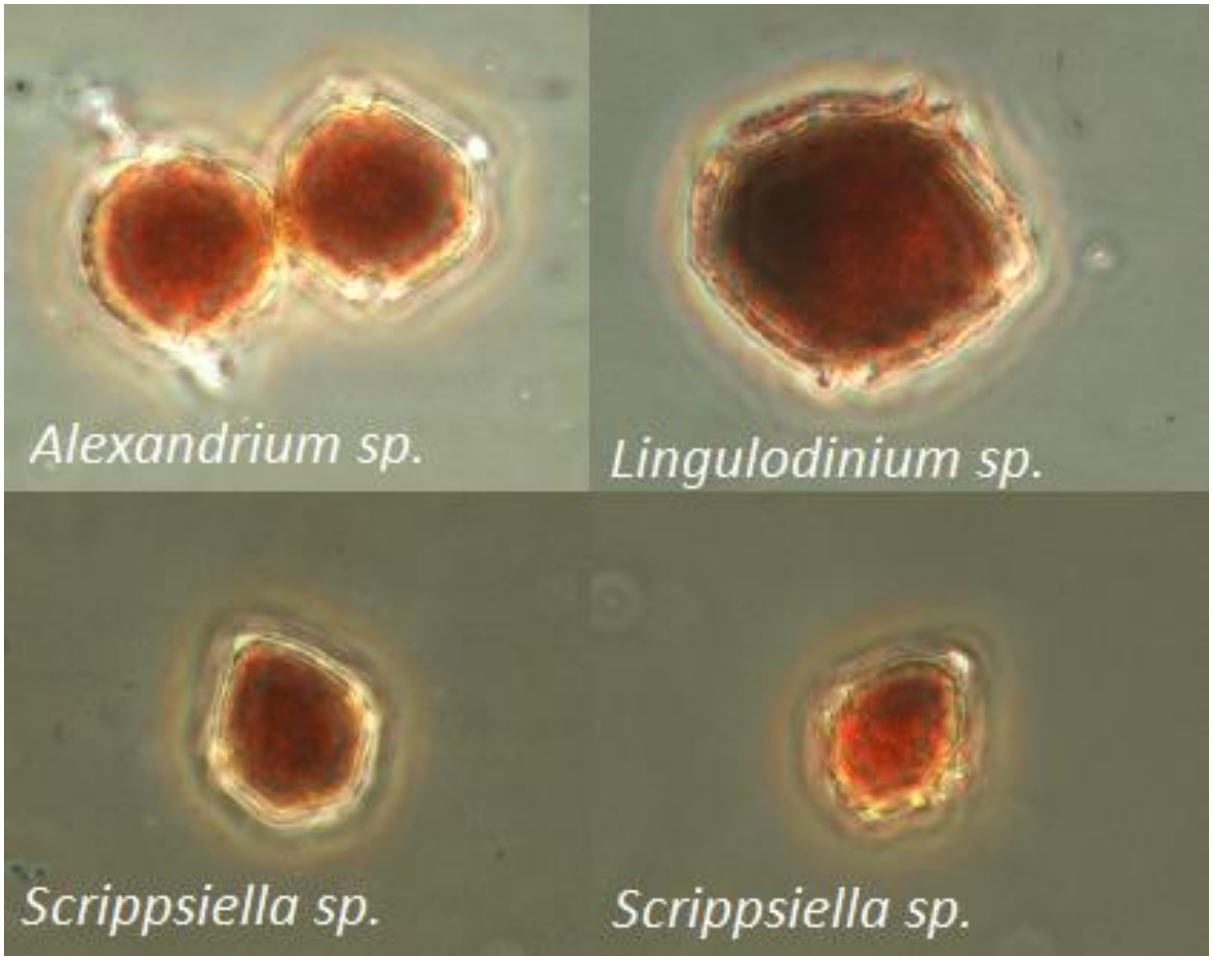


Figure 3.5: composite of images obtained from culture ID screening with assigned genus based upon gross morphological characteristics.

Those cultures established and identified as potential HAB species were few in number and could be determined as belonging to either the *Lingulodinium* (D. Wall, 1967) or *Alexandrium* genera. The region of occurrence for both was the Welsh coastline and the south coast of Cornwall. For *L. polyedra* distribution was most common on the west coast of Wales, although one culture was also established from south Cornwall (Figure 3.6). Conversely, *Alexandrium* was isolated from the south of Wales once as well as repeatedly from the south of Cornwall (Figure 3.6).

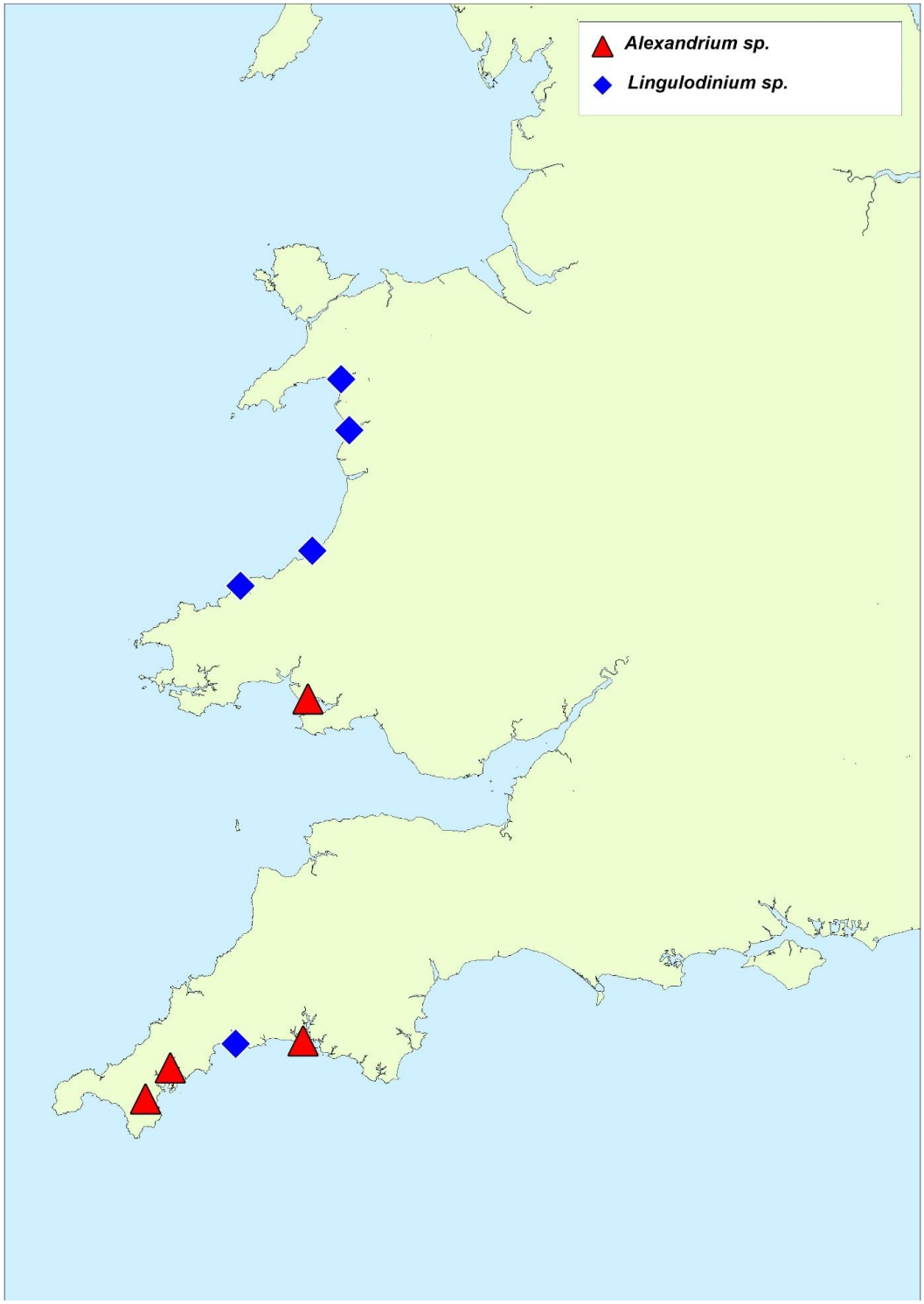
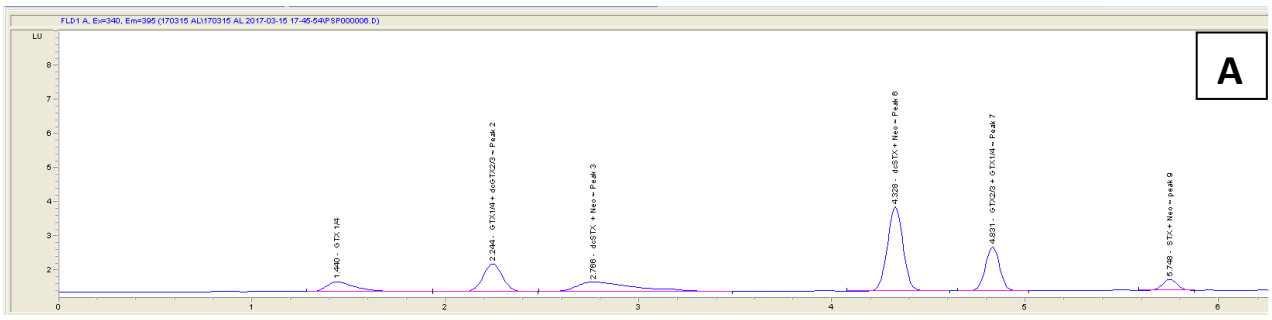


Figure 3.6: Locations in England and Wales which recorded germinations of potentially harmful genera. Sites with germinations of *Alexandrium* are marked as red triangles and sites with *Lingulodinium* are marked as blue diamonds.

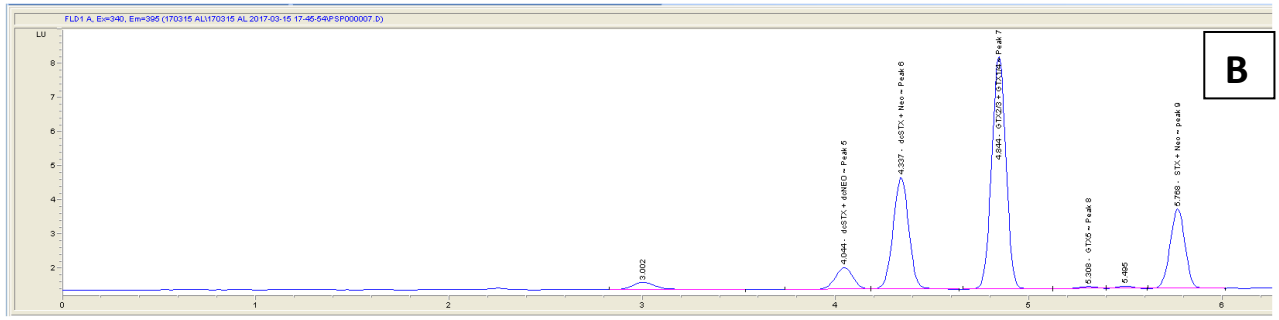
3.3.4 - Algal culture toxicity

Initial results suggested the presence of PSTs in several cultures, with profiles being simplistic, composed of only dcNeo. This was an unusual toxin profile and suggested that there may be an issue with analysis. Subsequently, following the use of unoxidised extracts for toxin confirmation, it was evident that the positive samples were instead analytical artifacts. Due to the high proportion of light absorbing, pigmented compounds present in photosynthetic organisms it is not surprising to see fluorescent compounds as co-extractives in the culture samples. Chromatograms displaying the high-level standards for mix 1 and 2 are shown alongside the results for sample 28iA (Figure 3.7) it is possible to see the peak corresponding to dcNeo is present at the same retention time as that present in the mix 1 standard, this toxin only displays a single peak under this method. However, it is also possible to see that the unoxidised extract contains a peak of almost identical peak area at the same retention time. This evidences that the peak present corresponds not to the toxin but instead to a naturally fluorescent compound which elutes at the same retention time.

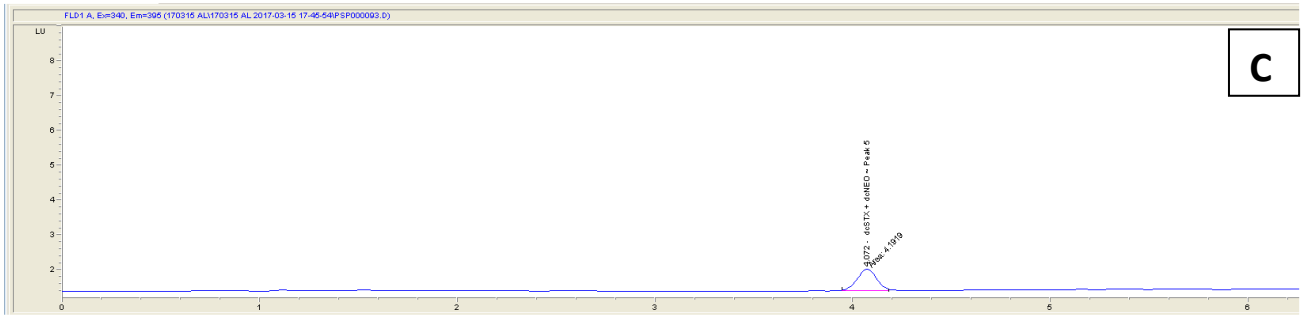
M1L5



M2L5



28iA Oxidised



28iA Unoxidised

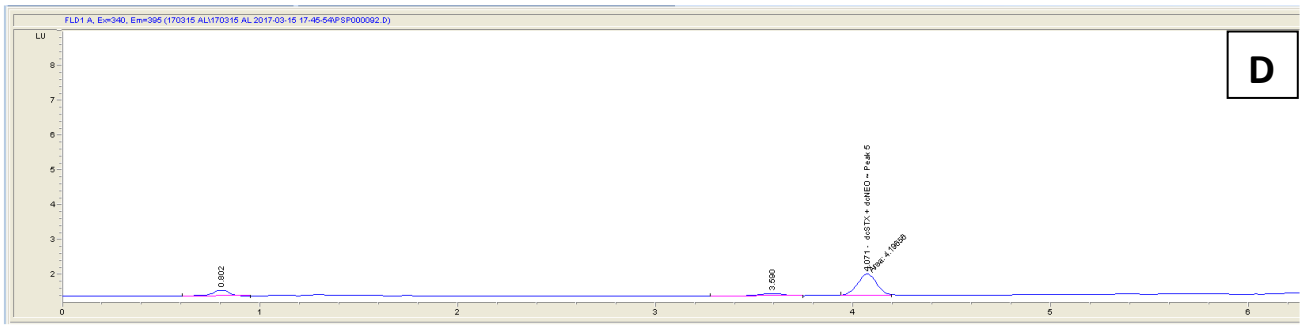


Figure 3.7: Chromatograms generated via HPLC-FLD analysis of analytical standards (A - Mix 1 level 5; B – Mix 2 level 5) and both oxidised and unoxidised sample extracts (C – Oxidised sample; D – Unoxidised sample). Showing the presence of toxin peaks in both oxidised and unoxidised extracts of the same sample at a relevant retention time.

Following the addition of the unoxidised analysis for samples those few cultures exhibiting peaks were found to be non-toxic, with peaks ascribed to naturally fluorescent compounds rather than toxins. This was further confirmed by HILIC-MS/MS, as where cultures were evaluated by both methodologies no toxins were found to be present. This is despite the HILIC-MS/MS method having a broader range of compounds detectable, enhanced sensitivity and a higher degree of specificity over the HPLC-FLD method.

3.4 - Discussion

Field sampling was successfully undertaken at sites covering a wide region of the British coastline. The selection of sites by the use of freely available satellite images proved to be an effective way of targeting viable sites, both in terms of suitable sediment types for collection as well as the ability of researchers to access sites. All sites visited yielded samples with the exception of two in Cornwall, Portreath and Mevagissey. In the case of Portreath this site proved to be inaccessible at the time of sampling, due to being in a small harbour with restricted access. In this case the use of a grab was attempted to gain a sediment sample from an accessible point but due to the presence of large amounts of strand material, mostly large fucoid species, it was not possible to reach the sediment of the harbour bottom. For the planned sample at Mevagissey, the disruption to sampling times (3.3.1) meant that the tide state was inappropriate for sampling as the site was inundated and inclement weather conditions made the deployment of the grab unsafe.

Those samples where sediments were more coarse consistently showed no germinations of dinoflagellates. This is most likely due to the retention abilities of the sediment for the dinoflagellate cysts, with cysts either being lost in the interstitial spaces and becoming deeply buried or washed away due to higher energy conditions where coarser sediments are present. Typically, those sediments which favour cyst accumulation are those composed of fine particles with a similar behaviour to the dinoflagellate cysts, these tend to accumulate in those areas with low energy whilst sediments of a coarser nature are indicative of higher energy areas and contain fewer dinoflagellate cysts (Brown et al., 2013). High energy areas tend to be well mixed and so as well as being likely to remove cysts also do not favour the development and stability of stratified water column conditions. As such, they are not favourable for cyst accumulation or for the vegetative growth of the dinoflagellate haploid stages (Wyatt and Jenkinson, 1997). The other sites showing no successful germinations were those in the Bristol channel. This area has a very high level of sediment loading in the water, resulting in very high turbidity. There were numerous areas with sediments with clay or silt like properties, seemingly ideal for the accumulation of dinoflagellate resting stages but the turbidity of the water represents a significant barrier to light penetration. As such the conditions are particularly unfavourable for the growth of the motile photosynthetic stages of the dinoflagellates of interest.

The lack of PST being identified in any of the cultures suggests that none of the cultures of suspected *Alexandrium sp.* were toxic strains of *Alexandrium minutum.* Initial results from the toxicity screen undertaken by HPLC-FLD appeared to be promising in terms of identifying a population within the survey area producing PST. Following the addition of the unoxidised analysis of samples it became apparent that those cultures initially thought to be producing PSTs were instead an analytical artifact. This was confirmed by the use of HILIC-MS/MS. Due to the specificity of the mass spectrometry analysis it is not possible for compounds with similar retention properties on column to produce the same fragmentation patterns within the collision cell, it is also unlikely that unrelated compounds exhibit the same mass to charge ratios as found in the ions of interest.

Therefore, even if naturally fluorescent compounds are present they will be excluded from analysis due to structural differences in the compounds, as filtering by *m:z* is a far more reliable discriminator than by the property of fluorescence and retention characteristics alone. As such the HILIC-MS/MS provided a more comprehensive breakdown of the toxins present in an algal sample, along with the ability to avoid the interference peaks that were present in the samples analysed via HPLC-FLD. Furthermore, although it was a less robust method, it was more efficient with respect to the time taken for analysis as the main reason for utilising the screening HPLC-FLD method over the fully quantitative variant, was the complex and lengthy additional steps needed to separate and fully quantify the included PST analogues using the HPLC-FLD technique. It is therefore suggested that the HILIC-MS/MS offers an effective alternative for the analysis of PST in algal cultures, especially in a research context where the pressure of rapid delivery of results often required of monitoring programmes, can be reduced.

All cultures of *Alexandrium sp.* tested were free of toxicity, this alongside morphological characteristics suggests populations of *Alexandrium tamarense* which is known to occur in the south of England.

The discovery of *Lingulodinium polyedra* along the west coast of Wales indicates that there could be issues associated with yessotoxins (YTX) in this region, as *L. polyedra* is a confirmed YTX producer in some parts of the world (reported as *Gonyaulax poleydra* in Draisci et al., 1999). Unfortunately, an incubator failure resulted in the collapse of the *L. polyedra* cultures before testing for the presence of YTX could be undertaken. As such it has not been possible to establish if this Welsh population of *L. polyedra* represents a confirmed source of YTX. As explained in Chapter 1, although the YTX have been shown to be highly toxic by intraperitoneal injection into mice this same toxicity does not present when the toxins are introduced by the oral route (Tubaro et al., 2003). As such there are questions around the regulation of the YTX toxin group (Pitcher et al., 2019) but currently they remain regulated within the EU and the UK at a level of 3.75 mg/kg of shellfish tissue

(increased from 1 mg/kg of shellfish tissue in 2013) (Bresnan et al., 2021; EU 786/2013, 2013) and could therefore be the cause of an enforced shellfish site closure. To date there have been two closure events triggered by YTX in the UK, both in Scotland (Dhanji-Rapkova et al., 2019). Comparing historic data to the revised level of YTX now regulated, there has not yet been a breach above the 3.75 mg/kg maximum permitted level in the UK (Dhanji-Rapkova et al., 2019). As such the risk posed by YTX to food safety appears to be relatively low. There have been implications of ecological damage however, in South Africa a mixed bloom of *Gonyaulax spinifera* ((Claparède & Lachmann) Diesing, 1866) and *Lingulodinium polyedra* caused massive die off of farmed abalone. The bloom was found to be producing YTX but the *L. polyedra* strain isolated from it was not, suggesting *G. spinifera* as the source of the YTX, with YTX being postulated as the ultimate cause of the mortalities in the abalone (Pitcher et al., 2019). As such it is valuable to know that the range of *L. polyedra* in the UK encompasses the west coast of Wales.

Failing to successfully isolate and culture any strains of *Alexandrium minutum* from the study area, including those sites known to have previously experienced *A. minutum* proliferations, is interesting. It is possible that there has been a range retraction, or even a total loss of the species in this region. It is also possible that this reflects a reduction in the quantity of cysts present in the sediments in these areas, making them scarce and so less likely to be detected. During this study, 0.5 g sediment samples were used, theoretically providing a detection limit as low as 2 cysts g^{-1}, in practice however it was not guaranteed that all cysts present would germinate, nor would all of those which did be guaranteed to be isolated and successfully cultured during the process. Consequently, although the quantity of sediment used and the triplicate sediment slurries prepared provided a low potential detection limit this would likely be higher in practice than in theory. Still, if samples were taken from an extensive cyst bed, with a high density of cysts present, it would be highly likely that successful germinations and detections would be made if *A. minutum* were present. It is also possible that the failure to germinate and subsequently culture this species was linked to the methodology employed, rather than reflecting the absence of the species from the sample sites.

Any of the sampling, germination or isolation techniques could be at fault for failing to provide adequate detection of *A. minutum.* In the case of the germination and isolation, this seems less likely as many dinoflagellates were successfully processed and maintained in culture, including several non-toxic *Alexandrium sp.* strains. The conditions utilised for both of these methodological stages are known to be suitable for the growth of this species from this region, as has been shown for culture MBA F5, a toxic variant of *Alexandrium minutum* utilised in later work in this thesis (see Chapter 4 section 4.2.2 for appropriate culture conditions). As such it seems likely that the point where the method is most likely to have been unsuitable is in the sampling strategy. The intention of this study was to cover as broad a range of sites as possible in order to establish the maximum range extent of *A. minutum* which is currently only confirmed in two sites. This meant that whilst it was possible to study some sites fairly intensively, with three or more samples collected within a small area (Figure 3.2) at others it was only possible to take one or two sediment samples. Consequently, some sites were surveyed in a higher resolution than others. This suggests that of those sites where multiple samples were taken the absence of toxic *A. minutum* is likely to be more reflective of the situation than at those sites where it was only possible to take a single sample. It can also be noted that some sites represent a larger area, such as an estuary and tributary network when compared with some sites which were small, enclosed areas, such as small harbours. As such a single sample from a small site may be more representative than two or three samples taken from an expansive site. Unfortunately, due to both time and budgetary constraints it was not feasible to sample each site in exacting detail, as has been achieved in some previous studies (Percy, 2006). Therefore, with more intensive sampling at the sites visited during this study it may be the case that successful germinations of a toxic strain of *A. minutum* could be achieved. Indeed, several sites across England and Wales continue to experience PST contamination of shellfish, albeit at lower levels in recent years than has been reported previously (Bresnan et al., 2021).

Another possibility to improve the sensitivity of this survey would be to employ an alternative form of detection for *Alexandrium.* One possibility would be the use of molecular tools, as there

have been a series of investigations into a variety of techniques. For example, there have been studies into the use of molecular techniques for the identification of the entire *Alexandrium* genus with a view to improving the reliability of speciation and in some cases provide either confirmatory assays or replacements for the currently employed microscope-based techniques (Touzet et al., 2009). Anderson *et al.* (2012) suggested that the use of genetic probes and primers, such as those targeting rRNA, offered the most promise for the use in both research and monitoring of HABs. The use of these has been applied to the identification and quantification of *Alexandrium* genus using a variety of techniques such as real-time PCR (Galluzzi et al., 2004) quantitative PCR (Erdner et al., 2010) RAPD (Touzet *et al.* 2007a) and in the format of a microarray (McCoy et al., 2014). It was noted however those common regions used for identification, the ITS regions and more conserved 5.8 S gene, were able to speciate *A. minutum* but not distinguish amongst toxic and non-toxic forms (Touzet *et al.* 2007, 2008, Penna *et al.* 2008), which would be a desired ability to enhance the understanding of the species but is still currently beyond those techniques utilising these regions. The decreasing cost of next generation sequencing is likely to lead to a more comprehensive understanding of the genomes of a range of HAB species, this may well enhance the ability to design primer and probe sets to allow for increasingly specific identification. With higher throughput sequencing techniques it is also becoming possible to assess environmental DNA, detecting the presence of organisms in water or sediments via detection of exogenous DNA or RNA. The first use of one such technique, Nanopore technology, was applied to harmful microalgal taxa in this way (Hatfield et al., 2020), being able to provide information on the species present in complex mixes of different taxa.

These molecular tools could also offer the possibility to enhance the existing monitoring efforts. However, they come with additional steps and costs and so would need to be supported via increased investment, at least in initial deployment phases when new methods were established. As such, they have yet to become prevalent in monitoring operations for harmful algae, with one of the few examples existing in the Irish monitoring programme (Clarke et al., 2018). This demonstrates the

possibility of a molecular monitoring system but, as of yet, efforts relying solely on the widely employed microscope methods remain prevalent.

Chapter 4 – The value of toxin profiles in the chemotaxonomic analysis of paralytic shellfish toxins in determining the relationship between British *Alexandrium sp.* and experimentally contaminated *Mytilus sp.*

4.i – Preface

The work presented in chapter 3 was compiled for publication and published in Harmful Algae as – Lewis, A.M., Dean, K.J., Hartnell, D.M., Percy, L., Turner, A.D. and Lewis, J.M., 2022. The value of toxin profiles in the chemotaxonomic analysis of paralytic shellfish toxins in determining the relationship between British Alexandrium spp. and experimentally contaminated Mytilus sp. Harmful Algae, 111, p.102131. In terms of contribution to the manuscript, I led on the study design, practical work, data collection and analysis as well as the writing of the manuscript. Both K Dean and D Hartnell contributed to the practical work during the shellfish exposures as well as reviewing the manuscript. Assistance in the analysis of shellfish utilising mass spectrometry as well as study design and review of the manuscript was provided by A. Turner. Finally, both L. Percy and J Lewis provided input for study design and manuscript review.

4.1 – Introduction

As covered in the previous chapters, Paralytic shellfish poisoning (PSP) is a human condition which results from the consumption of seafood, typically bivalve molluscs, contaminated with saxitoxins. These compounds are structurally related neurotoxic molecules (structure and key

analogues shown in Figure 4.1 and Table 4.1). They are produced, primarily, by marine microalgae from the Dinoflagellate phylum with key genera being *Pyrodinium* (Usup et al., 2012)*, Gymnodinium* (Hallegraeff et al., 2012) and *Alexandrium* (Anderson et al., 2012). Due to the differences in the ecological niches of these genera, PST causes problems globally, with different areas being affected due to different algal species (Anderson, 2009). The effects in humans are detailed in earlier chapters (Table 1.1) but critically can be fatal. Due to the severity of PSP there are stringent monitoring programmes in place in many countries where aquaculture can be affected by PSP (Anderson, 2009; Berdalet et al., 2016). Within Great Britain (GB), toxic cell proliferations or shellfish toxicity events have been caused by species of *Alexandrium, A. catenella* (previously reported from northern areas as *A. tamarense* (John et al. 2014, Fraga et al. 2015, accepted in Prud'homme van Reine 2017)) from the coast of Scotland (Brown et al., 2010) and *A. minutum* in Southern parts of England (Percy, 2006). Several sites in GB experience or have experienced, annually recurrent blooms, including several sea lochs on the West coast of Scotland (Swan and Davidson, 2011) and estuaries in South England (Bresnan et al., 2021). These recurrent populations are driven by the ability of many members of this genus to form resilient resting stages as a part of their life cycle, with cysts remaining in sediments for a prolonged period of time before initiating new vegetative populations when local seasonal and hydrographic drivers become conducive (Anderson et al., 2012; Blanco and Lewis, 2014; Bravo et al., 2008; Estrada et al., 2010; Joint et al., 1997). Although not all events in GB are caused by *A. minutum* the life cycle presented in Figure 2.1 is applicable to other member of the genus, including *A. catenella.*

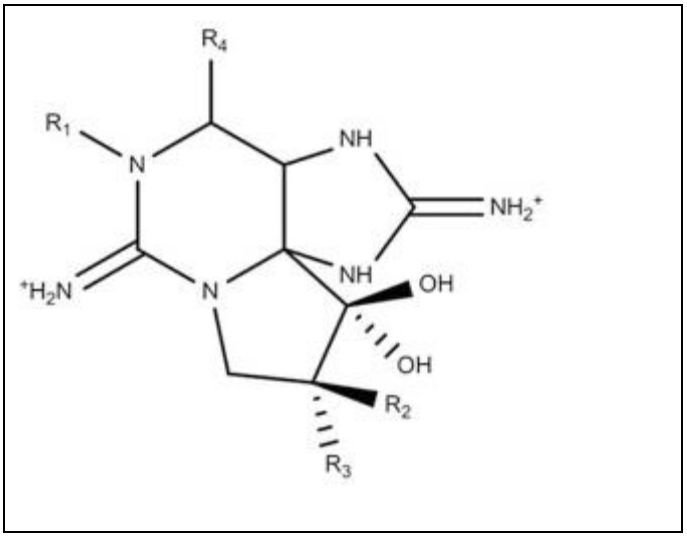


Figure 4.1. The general structure of PST analogs. From Lewis *et al.* (2022).

Table 4.1. Chemical structures, TEFs, and calibrants applied for quantitation and semi quantitation of PST analogs, for full names of congeners see Table 3.4. From Lewis *et al.* (2022).

Group (Charge state)	**Analog**	**R1**	**R2**	**R3**	**R4**	**TEF**	**Calibrant**
C toxins (0)	C1	H	H	OSO	OCONHS	0.01	C1
	C2	H	OSO	H	OCONHS	0.1	C2
	C3	OH	H	OSO	OCONHS	0.02	C1
	C4	OH	OSO	H	OSO_3^-	0.1	C2
GTXs (1)	dcGTX3	H	H	OSO	OH	0.2	dcGTX3
	dcGTX2	H	OSO	H	OH	0.4	dcGTX2
	dcGTX1	OH	H	OSO	OH	0.5[a]	dcGTX2
	dcGTX4	OH	OSO	H	OH	0.5[a]	dcGTX3
	GTX2	H	H	OSO	$OCONH_2$	0.4	GTX2
	GTX3	H	OSO	H	$OCONH_2$	0.6	GTX3
	GTX1	OH	H	OSO	$OCONH_2$	1	GTX1
	GTX4	OH	OSO	H	$OCONH_2$	0.7	GTX4
	GTX5	H	H	H	OCONHS	0.1	GTX5
	GTX6	OH	H	H	OCONHS	0.1	GTX6
STXs (2)	doSTX	H	H	H	H	0.05	doSTX
	dcSTX	H	H	H	OH	1	dcSTX
	dcNEO	OH	H	H	OH	0.4	dcNEO
	STX	H	H	H	$OCONH_2$	1	STX
	NEO	OH	H	H	$OCONH_2$	1	NEO

Monitoring in Europe uses internationally validated methods, for the detection of both potentially harmful phytoplankton groups and the levels of shellfish PST. Identification of harmful taxa is undertaken via the Utermöhl method, utilising light microscopy and settlement chambers to evaluate the levels of various algal groups simultaneously (Swan and Davidson, 2011; Touzet and Raine, 2007). Unfortunately, whilst speciation of *Alexandrium* is possible via light microscopy, when used in a monitoring capacity typically used light microscopy methodologies do not allow for reliable speciation of the *Alexandrium* genus within the timeframe required for the monitoring programme. As such when *Alexandrium* is detected in the UK monitoring programme it is typically recorded only to genus level and there is no scope to determine the toxicity of populations observed (Swan and Davidson, 2011). Toxin monitoring is conducted using a variety of methods and modern chemical techniques can determine a wide range of analogues (Table 4.1). Within the EU the pre-column oxidation (Pre-cox) high-performance liquid chromatography method with fluorescence detection (HPLC-FLD) (Anonymous, 2005), has recently replaced the mouse bioassay (Anonymous, 1959) as the official reference method for the detection of PST in shellfish (Turner et al., 2019). The Pre-cox method has been implemented and run routinely in several countries for many years following collaborative validation. The UK is one such country, having used the technique to monitor PST qualitatively in several shellfish species since 2006 with adoption of the fully quantitative method in 2008 (Turner et al., 2009). This method provides quantitation of the total concentration of saxitoxins by calculating the concentrations of a range of congeners, separately or paired, and summing these and applying toxicity equivalence factors (TEFs). The current EU regulatory limit, for total PST in shellfish, is 800 µg of saxitoxin equivalence per kilogram (STX eq./kg) of shellfish tissue (Alexander et al., 2009; Etheridge, 2010). Upon exceeding these levels harvesting sites are closed temporarily, leading to significant socioeconomic impact (Shumway, 1990). The low levels of quantitation

achievable with modern chemical analytical techniques allow for detailed toxin profile information to be determined in the GB monitoring programme well before dangerous levels of PST are accumulated.

As the PST profile within shellfish and algae can be analysed in detail within a monitoring framework the possibility exists of comparing shellfish toxin profiles and algal toxin profiles. The toxin profile of an algal species is known to vary by strain or population. Also, the toxin profiles of individual algal strains have been shown to be variable within controlled laboratory conditions and experiments subjecting cultures to abnormal or sub optimal culturing conditions (Hwang and Lu, 2000; Maas et al., 2007). Conversely, however, there is evidence to show that the toxin profile of a given strain is relatively stable under normal conditions (Anderson et al., 2012). The toxin profile of the ingested algal population forms the basis for the profiles quantified in contaminated shellfish. Some shellfish species are known to perform rapid interconversions of PST following consumption (Bricelj and Cembella, 1995; Jaime et al., 2007), in GB a good example is the surf clam, *Spisula solida* (Linnaeus, 1758) (Turner et al., 2013), where the rapidity of transformation is mediated via enzymatic conversion and is therefore very efficient (Fast et al., 2006; Lin et al., 2004). These conversions can lead to either modified or entirely novel toxin profiles within the shellfish. Other shellfish species such as Pacific oysters (*Magallana gigas* (Thunberg, 1793)), however, show substantially less interconversion of the toxins and in these cases the link between source algal population and contaminated shellfish can be more easily evaluated (Turner et al., 2012). Differences in accumulation rates between shellfish species have also been observed. The mussel, *Mytilus spp.* (Linnaeus, 1758) is commonly grown in GB for commercial harvesting, both *M. edulis* (Linnaeus, 1758) and *M. galloprovincialis* (Lamarck, 1819) are known to occur in the Southwest of England, as well as hybrids of these two species (Bignell et al., 2011). Members of this genus are known to be both a rapid PST accumulator and depurator (Bricelj and Shumway, 1998).

PST profiles, as determined via the GB monitoring programme, of toxic shellfish were previously evaluated by Turner et al. (2014). That study determined four toxin profiles nationally, with one exclusively appearing in *S. solida* and the other three being derived from the geographic origin of samples. The three clusters were tentatively linked to the source algal species and populations.

The work presented here seeks to elucidate the relationship between the toxin profiles of source algae and contaminated shellfish, under controlled conditions. Using statistical comparisons of toxin profiles in shellfish and algae we aim to determine if an effective diagnosis of the original algal source of those toxins can be made. If practical, identification of source algal populations from a chemotaxonomic analysis of PST profiles in shellfish would add value to the existing monitoring programme without incurring additional costs. The key algal species of interest in this regard were *A. catenella* and *A. minutum*, owing to their presence in GB waters and the possibility of their ranges expanding. To evaluate the value of chemotaxonomic markers in GB two feeding studies were undertaken. To assess the toxin profiles in *Mytilus sp.,* resulting from contamination by different *Alexandrium* species, feeding was undertaken with toxic strains of both species of *Alexandrium* in isolation, as well as when mixed. Additionally, it has been noted that *A. catenella* associated toxin events in Scotland occur earlier in the year than those triggered by *A. minutum* in England and Wales (Lewis et al., 2016). Therefore, feeding was also undertaken with these algal species sequentially, as a geographic overlap of these two species would not necessarily also lead to a temporal one. Work was undertaken with a commercially important bivalve species, *Mytilus sp.,* known to uptake toxins rapidly but also presenting little conversion.

4.2 – Methodologies

4.2.1 - Reagents, chemicals and analytical equipment

All reagents used for toxin extractions and HPLC analysis were of HPLC grade or higher. For the mass spectrometry analysis chemicals of LC-MS grade were utilised. Chemicals were sourced from either Fisher Scientific (Loughborough, UK) or VWR (Lutterworth, UK). All solid phase extraction (SPE) processes were automated and performed using a Gilson (Dunstable, UK) ASPEC, running Trilution software. Toxin analysis was carried out on multiple systems, for HILIC-MS/MS this was performed using a Waters (Milford, MA, USA) Acquity I class UPLC coupled with a Waters Xevo TQ-S triple quadrupole mass spectrometer and an Agilent (Santa Clara, CA, USA) 1290 Infinity II UPLC coupled with an Agilent 6495B triple quadrupole mass spectrometer. For HPLC-FLD analysis an Agilent 1100/1200 series HPLC with fluorescence detection was used. Calibration standards were prepared from certified reference material sourced from the Institute of Biotoxin Metrology, National Research Council Canada (Halifax, NS, Canada).

4.2.2 - Algal culture

Algal species and strains were chosen to represent those species and strains of current importance within GB. Algal cultures were sourced from the Culture Collection of Algae and Protozoa (CCAP, Scottish Association of Marine Science, Oban, Scotland) or the Marine Biological Association (MBA, Plymouth, England). The toxic strains used were *A. catenella* CCAP 1119/27 originally isolated from the West coast of Scotland, *A. minutum* MBA F5 isolated from Southwest England. Additionally, non-toxic strains were used in the second study as control organisms, these were *A. minutum* CCAP 1119/48 which was isolated from the east coast of Scotland and *A. tamarense* CCAP 1119/31 isolated from the Orkney Islands, Scotland. All *Alexandrium* strains were cultured in natural seawater, autoclaved and 0.2 µm filtered for initial flask culture and 0.2 µm

filtered only for large scale batch culture, supplemented with L1 media, with silica. These cultures were held at 17 °C with a 14:10 light:dark cycle. Cultures were initially grown in tissue culture flasks with 0.2 µm vented caps before transfer to large polypropylene bag culture of between 25 L and 35 L in a hanging bag photobioreactor. Bag cultures were gently aerated after an undisturbed growth period to allow cultures to establish. Additionally, the non-toxic haptophyte *Diacronema lutheri* ((Droop) Bendif & Véron, 2011) (CCAP 931/1 – isolated from Millport, Scotland) was cultured to act as a feed supplement within algal exposure work. The required volumes of *D. lutheri* were grown under the same conditions as the strains of *Alexandrium* used but were instead grown in aerated 20 L carboys (Nalgene). Culture cell densities were determined prior to use in feeding, sub samples of algal culture were fixed with neutral Lugol's iodine before being enumerated with a haemocytometer on a Nikon Eclipse TE300 (Japan) inverted light microscope.

4.2.3 - Mussel exposure

Experimental contamination of mussels with two PST producing *Alexandrium* species of relevance to GB was undertaken in closed systems under controlled conditions. Methodologies were based on the core set-up detailed in Higman and Turner (2010). For this study two separate exposures were undertaken. In both studies, adult mussels, *Mytilus sp.,* were sourced from the Swansea Docks production area in the South of Wales, UK.

4.2.4 - Toxin Profile determination study

The first exposure evaluated the toxin profiles in *Mytilus sp.* derived from feeding on toxic *A. catenella* (CCAP 1119/27) and toxic *A. minutum* (MBA F5). This exposure study contained four treatments, one control with no toxic dinoflagellates present and three where toxic dinoflagellates were included. The three toxin containing treatments were *A. catenella* as the sole toxin source, *A. minutum* as the sole toxin source and a third where the two toxic *Alexandrium* species were added

as a mix, providing the mussels with two simultaneous toxin sources. A tank set up of twelve individual 35 L aquaria maintained at 17 °C was established, with 73 mussels allocated to each tank. These tanks represented triplicates of each of the four treatments, with each aquarium a closed system with separate aeration system. Feeding was standardised by algal cellular toxin quota with the mixed feed concentrations determined using an equal toxin concentration contribution to the treatment by both *A. minutum* (MBA F5) and *A. catenella* (CCAP 1119/27). All treatments began with a seven day exposure period followed by a subsequent 14 day period for depuration of toxins. The non-toxic alga *Diacronema lutheri* (CCAP 931/1) was added at a concentration of $2.9x10^7$ $cellsL^{-1}day^{-1}$ to all treatments throughout the 21 days of study to provide a dietary supplement and to stimulate feeding and therefore potential toxin accumulation (Bricelj and Shumway, 1998).

Table 4.2: Showing algal cellular concentrations introduced during the first feeding study. Cell numbers were standardised by cellular toxin quota, with A. catenella being significantly more toxic, leading to the disparity between the quantities of A. minutum and A. catenella. From Lewis *et al.* (2022).

Treatment	*A. minutum* cells/mussel/ day	*A. catenella* cells/mussel/ day	*D. lutheri* cells/mussel/ day
Control	0	0	1.39E+07
A. minutum exposed	4.08E+07	0	1.39E+07
A. catenella exposed	0	3.02E+06	1.39E+07
Mixed exposure	2.84E+05	2.10E+04	1.39E+07

The cell numbers involved in each of the four treatments from the first exposure are shown in Table 4.2. Cell numbers were chosen to provide detectable levels of toxins across the study whilst providing total toxin concentrations low enough to evaluate the toxin profiles of contaminated shellfish at levels relevant to early detection within the monitoring programme. Due to the wide range of cell concentrations observed in natural populations no attempt was made to simulate a specific event. Algal cells were introduced to each system twice daily, at mid-morning and mid-afternoon, with an interval of four to six hours between feeds. A complete water change was undertaken each morning prior to any feeding, in order to remove all faecal material as well as any un-ingested algal cells.

Sampling for toxin analysis consisted of removing ten individuals, immediately before the daily water change, from each replicate at days 1, 3, 5, 7, 9, 11, 15 and 21. These ten individuals were then shucked, with tissues within each replicate being pooled and homogenised. By removing ten individuals this way the impact of variability in toxin accumulation between individual mussels was mitigated and this also kept the sampling in line with the minimum requirements for representativeness as defined in the GB monitoring programme (Pers. Obs.). Prior to feeding commencing, a day 0 sample was taken by pooling individuals from all treatments to result in triplicate samples each consisting of ten individuals. In order to eliminate changes in algal concentrations due to the changes in the number of study organisms, tank volumes and algal feed volumes were modified in line with the reducing mussel population in each study replicate. As samples were taken so the total system volume of each replicate, initially 35 L, was reduced commensurately as were the introduced algal feed volumes. This kept algal concentrations fixed per unit volume and per shellfish (see Table 4.2) for the duration of the study.

4.2.5 - Sequential toxin profile determination study

The second exposure study consisted of two treatments, a non-toxic condition and one with PST containing algal strains. For this study six individual 60 L closed systems, each housing 130 mussels were established at 17 °C with individual aeration, the six tanks were divided into three replicates each of the two treatments. The transition from *A. catenella/tamarense* to *A. minutum* is displayed in Table 4.3 and Figure 4.2, this transition was undertaken with *A. catenella* (CCAP 1119/27) and a toxic strain of *A. minutum* (MBA F5) in one treatment and with non-toxic *A. tamarense* (CCAP 1119/31) and a non-toxic strain of *A. minutum* (CCAP 1119/48) in the control treatment. Non-toxic *Diacronema lutheri* (CCAP 93/1) was also introduced in both treatments at a constant cell concentration of $2.9x10^7$ cells$L^{-1}day^{-1}$ for the 19 days. As with the first study, complete water changes were carried out daily prior to the morning feed. System water volumes and algal feed volumes were modified in line with sampling to ensure consistent levels of exposure during all time points of the study. As in the previous study samples from each replicate consisted of ten individual mussels pooled at each sampling point. The sampling points in the second study were at two-day intervals from day 0 to 17, samples were collected immediately before the daily water change.

Table 4.3: Displaying the algal strains used within the second feeding study and their allocation to different treatments within the study design. From Lewis *et al.* (2022).

	Exposure	**Transition**	**Exposure**
	Day 0-5	**Day 6-12**	**Day 13-16**
Toxic	*A. catenella* CCAP 1119/27. Max concentration. *D. lutheri* CCAP 93/1 consistent	Reducing 1119/27, escalating MBA F5. *D. lutheri* CCAP 93/1 consistent	*A. minutum* MBA F5. Max concentration. *D. lutheri* CCAP 93/1 consistent
Non-Toxic	*A. tamarense* CCAP 1119/31. Max concentration. *D. lutheri* CCAP 93/1 consistent	Reducing 1119/31, escalating 1119/48. *D. lutheri* CCAP 93/1 consistent	*A. minutum* 1119/48. Max concentration. *D. lutheri* CCAP 93/1 consistent

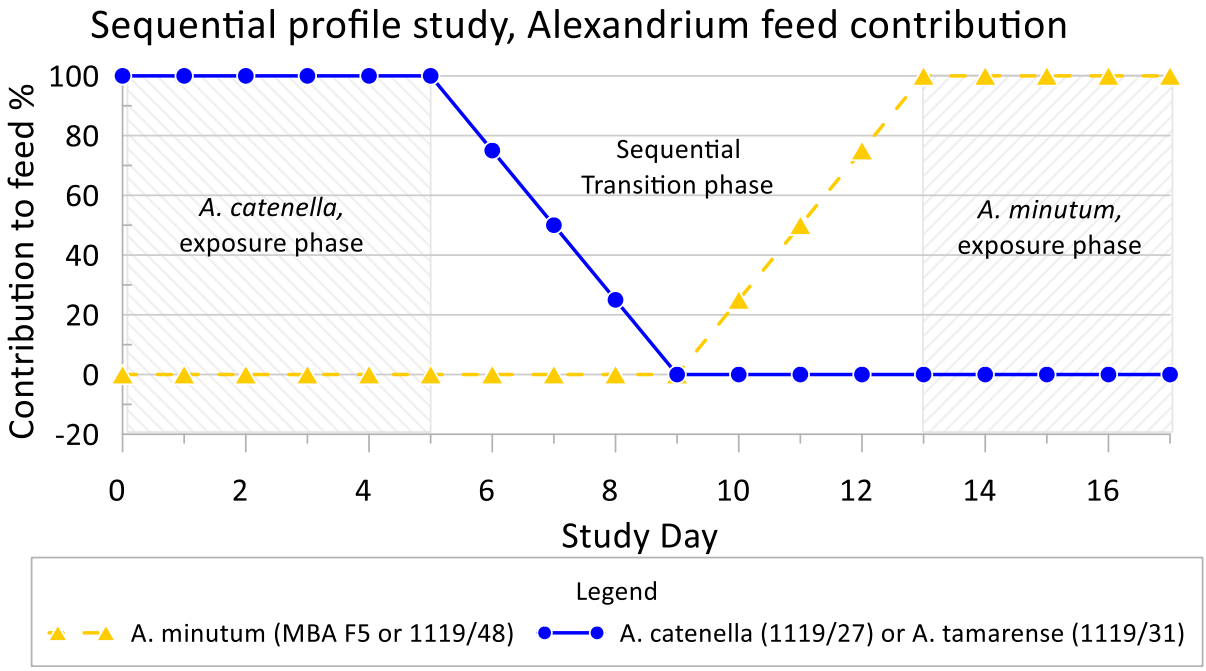


Figure 4.2: Showing the sequential feeding design of the second exposure study, graphically displaying the feeding transition from *A. catenella* (toxic exposure) or *A. tamarense* (non-toxic exposure) to *A. minutum* (toxic or non-toxic strains). From Lewis *et al.* (2022).

4.2.6 - Toxin extraction and analysis

4.2.6.1 - Toxin extraction, algal strains

Triplicate samples were taken from cultures to determine algal toxin profiles and cellular toxin quotas. From each culture 45 mL samples were taken for toxin extraction and 3 mL samples were taken and fixed with neutral Lugol's iodine solution for cell counts. The 45 mL sample underwent centrifugation at 3400 RCF for ten minutes. Following this the supernatant was discarded and the pellet resuspended in 1.5 mL of 1% acetic acid. This concentrated cellular material was then disrupted via the use of probe sonication for 90 seconds. To remove particulates the extract was centrifuged again at 3400 RCF for ten minutes and the supernatant containing the toxins carefully

removed. At this stage 1 mL of the crude extract was taken and 5 µL of ammonium hydroxide solution added, this was mixed and forwarded for automated carbon SPE clean-up. Finally, 100 µL of cleaned extract was diluted using 300 µL of acetonitrile in autosampler vials. These were then forwarded for analysis by HILIC-MS/MS as detailed below.

4.2.6.2 - Toxin extraction, mussels

Two different toxin extraction methods were used to extract toxins from shellfish, both are detailed elsewhere in full. Mussel samples for toxin analysis were composed of the homogenate of ten individuals taken at each sampling point from each replicate, for three samples at each time point per treatment. In all cases 5.0 ± 0.1 g aliquots were weighed into 50 mL centrifuge tubes for each sample.

The first extraction technique was used for all study materials generated during the course of both feeding studies and followed that presented in Turner et al. (2015). This was a single step dispersive extraction and provided extracts suitable for analysis using the HILIC-MS/MS method. This method began with the addition of 5 mL of 1% acetic to each sample. Samples were then mixed using a multi-tube vortex mixer for 90 seconds at 2500 RPM. Mixed samples were then transferred to a boiling water bath for 300 seconds before being cooled in running water for 300 seconds. Samples were then separated via centrifugation at 3400 RCF for 10 minutes. The supernatant was then filtered into a clean centrifuge tube and the samples underwent an automated cleanup step. As covered in Chapter 3.2.7.4 and above in 4.2.6.1 this involved 1 mL of the filtered being added to a 4 mL TC tube and 5 µL of ammonium hydroxide added to this, samples were then mixed for 5 seconds using a vortex mixer. Each sample was then forwarded for automated carbon clean-up using a Gilson ASPEC XL-4 liquid handler and carbon SPE cartridges (Supelco ENVI-Carb 250 mg/3 mL). Samples were eluted into clean 4 mL TC tubes using 2 mL of 20:80 acetonitrile:water with 0.25% acetic acid.

Finally, 100 µL of each desalted extract was diluted with 300 µL acetonitrile in polypropylene UPLC autosampler vials at which point they were ready for analysis via mass spectrometry.

The second extraction technique was performed on selected samples taken from the two feeding studies and was done to ensure direct comparability of results generated in this study with those generated as part of the GB official control monitoring programme allowing for conclusions to be drawn without potentially confounding effects originating from different methods being used. The second extraction was undertaken following that presented for the AOAC 2005.06 method, used in routine testing (Anonymous, 2005). This second method is a two-step exhaustive procedure and was undertaken for those shellfish samples analysed using HPLC-FLD. This extraction process began with the addition of 3 mL of 1% acetic acid to the 5 g of shellfish homogenate. This was then mixed in a multi-tube vortex mixer at 2500 RPM for 90 seconds. Following this samples were placed into a boiling water bath for 300 seconds before being cooled under running water for a further 300 seconds. Once samples were cooled they were mixed for a further 90 seconds using the same 2500 RPM multi-tube vortex mixer. After this all samples were placed in a centrifuge for 10 minutes at 3400 RCF. Supernatants for each sample were then decanted into a 15 mL graduated centrifuge tube. At this stage a further 3 mL of 1% acetic acid was added to the homogenate pellet in the 50 mL centrifuge tube, for each sample. The pellet was then dislodged and the sample mixed for 90 seconds at 2500 RPM using the multi-tube vortex mixer. Samples then underwent a final centrifugation step before the supernatant from the second extraction step was combined with the first for each sample. Sample volumes were then made up to 10 mL via the addition of deionised water.

All samples then underwent automated solid phase extraction using a C18 cartridge, in order to remove residual elements of the shellfish matrix. As with the graphitised carbon cleanup described above this was accomplished using a Gilson ASPEC XL-4 liquid handler but instead made use of C18 cartridges (Strata® C18-T (55 µm,140 A). During the process 1 mL of sample was loaded

onto a cartridge before being eluted into clean, 4 mL graduated TC tubes using deionised water Samples were then adjusted to between pH 6.0 and 7.0 using either 1M sodium hydroxide or 0.1M acetic acid. Finally, sample volumes were adjusted to 4 mL using deionised water. Samples were then further processed via ion exchange fractionation, in order to separate the coeluting toxins and allow for full quantitative analysis. This was also achieved using the automated Gilson system stated above but made use of weak cation exchange cartridges (Strata™-X-CW 33 µm Polymeric Weak Cation 200 mg / 3 mL) and fractionation was achieved by washing cartridges loaded with sample with an increasing salt gradient, 0.3M sodium chloride solution was used for elution into fraction 2 and 2M sodium chloride was used for elution into fraction 3.

To enable detection via fluorescence saxitoxin analogues needed to be derivatised prior to analysis, as this was to be a fully quantitative analysis each sample was analysed four times in order to provide separation of each toxin.

Firstly, the C18 cleaned extract was oxidised using a hydrogen peroxide oxidising agent. 25 µL of 10% hydrogen peroxide was added to a glass HPLC vial, to this 250 µL of 1M sodium hydroxide was added. To initiate the reaction 100 µL of the pH adjusted C18 extract was pipetted into the vial, the vial was capped and mixed for 5 seconds using a vortex mixer. This reaction mixture was allowed to stand for 2 minutes before the addition of 20 µL of glacial acetic acid and a further 5 seconds of vortex mixing. This peroxide oxidised sample was then ready for analysis.

Fractions 2 and 3 were oxidised separately, in the same manner as Chapter 3.2.7.3 this was achieved using a periodate oxidising reagent, although here the standard periodate reagent was used. As such the oxidising periodate mixture was prepared with a 1:1:1 mix of 0.03 M periodic acid: 0.3 M ammonium formate: 0.3 M sodium phosphate. Oxidations were then performed as described in the official 2005.06 method (Anonymous, 2005) on fractions 2 and 3 separately. This comprised of, 100 µL of oyster matrix modifier being added to a glass HPLC vial, this was followed by 100 µL of fractionated sample extract. To this mix 500 µL of periodate oxidising reagent was added to the vial,

and the mixture was vortex mixed for 5 seconds. The mixture was then left to react for 1 minute before 5 µL of glacial acetic acid was added to the vial and it was vortex mixed for a further 5 seconds to quench the oxidation reaction. At this point the samples were ready for analysis via HPLC-FLD.

Lastly, an unoxidised vial was prepared for each sample. This consisted of 100 µL of oyster matrix modifier with 100 µL of the pH adjusted C18 extract being added in a glass HPLC vial. To this, 500 µL of deionised water was added before the vial was capped and the mixture mixed via vortex mixer for 5 seconds. At this stage, the unoxidised aliquot was ready for analysis.

4.2.6.3 - Toxin analysis

For algal strains and all mussel samples, from both exposure studies, toxin analysis was carried out using a HILIC-MS/MS method and followed recently developed (Boundy et al., 2015) and validated methods (Turner et al., 2020, 2015). Calibration standards were prepared from purified certified reference material of toxins sourced from the Institute of Biotoxin Metrology, National Research Council Canada, with six calibrant levels prepared and used for quantitation. This was undertaken using the same analytical technique as described in Chapter 3

To compare mussel samples from this study with data generated in the routine monitoring programme those extracts prepared in accordance with AOAC 2005.06 (Anonymous, 2005) were also analysed following this method. This analysis was undertaken following methods originally described in Anonymous (2005) but making use of the superficially porous column, reduced run time and gradient elution as presented in Hatfield and Turner (2012). Analytical standards were prepared in three mixes of six levels each and a further three level mix to allow for complete separation and quantitation of all PST detected using the HPLC-FLD method.

4.2.7 - Toxin profile cluster analysis

In order to statistically compare complex toxin profiles, results were analysed using K-means clustering via Microsoft excel with the Solver addon. The clustering methods used herein followed those from Turner et al. (2014) with a detailed description being provided in Aravind et al. (2010). In order to compare toxin profiles from samples containing differing quantities of toxins, toxin content data were normalised as percentage contributions to the total toxin concentrations. Normalised data were used throughout the cluster analysis procedure in all cases. Clustering of toxin profiles was performed twice, both internal to this study and between study data and routine monitoring data. Firstly, clustering was undertaken using the data generated within this study via analysis by HILIC-MS/MS. This allowed the assessment of similarity between source algal species and intoxicated shellfish.

Additionally, data generated from this study using HPLC-FLD analysis of select samples was clustered against historic monitoring data. This latter analysis did not include data generated by HILIC-MS/MS as the aim was to assess the clustering performance of the routine monitoring method. Not all samples from all time points were analysed using HPLC-FLD due to the relative inefficiencies of that method when compared to the more recent HILIC-MS/MS method, both in terms of instrumental and extraction methodologies. As such a range of triplicate samples were chosen from across the time points sampled and forwarded for analysis using routine monitoring methods, only this data was included in the study and monitoring cluster analysis.

4.3 - Results

4.3.1 - Algal toxin concentrations

The two toxic *Alexandrium* strains utilised throughout yielded differing toxin profiles. Table 4.4 shows detail of the toxin profiles as well as giving the calculated total toxin concentrations per cell for each strain.

Table 4.4: Showing PST concentrations in terms of pgSTXeqcell^{-1} by compound for both toxic algal strains utilised in this study as determined by HILIC-MS/MS. Principal toxins (>0.1 pgcell^{-1}) are shown in bold. Trace toxins are any detected but quantified as less than 0.1 pgSTXeqcell^{-1}. From Lewis *et al.* (2022).

	pg STXeqcell^{-1}	
	***A. minutum* MBA**	***A. catenella* CCAP**
PST congener	**F5**	**1119/27**
N-sulfocabamoyl toxin 1 (C1)	nd	Trace
C2	nd	0.7
C4	nd	Trace
Decarbamolygonyautoxin 2 (dcGTX2)	nd	nd
dcGTX3	nd	Trace
Gonyautoxin 2 (GTX2)	0.5	0.4
GTX3	0.6	0.6
GTX1	nd	1.9
GTX4	nd	2.1
GTX5	nd	Trace
vDeoxydecarbamoylsaxitoxin (doSTX)	nd	Trace

Decarbamoylsaxitoxin (dcSTX)	nd	Trace
Decarbamoylneosaxitoxin (dcNEO)	nd	Trace
Saxitoxin (STX)	0.1	3.2
Neosaxitoxin (NEO)	Trace	7.5
Tetrodotoxin (TTX)	nd	Trace
Total PST	1.2	16.5

As is expected, *A. catenella* had a higher cellular toxin quota than did *A. minutum.* These toxin quotas are broadly in keeping with some of those reported previously for *A. catenella* and *A. minutum* (Bougrier et al., 2003; Lassus et al., 2004; MacKenzie and Berkett, 1997; McCoy et al., 2014; Touzet et al., 2008; Touzet et al., 2007a). The toxin profiles show substantial differences between the species used, most importantly for this study the *A. minutum* strain produces only trace levels of Neo and no GTX1, GTX4 or C toxins. This allows for a clear differentiation of the strains used here by toxin profile alone. The other *Alexandrium* strains used as non-toxic controls, *A. minutum* 1119/48 and *A. tamarense* CCAP 1119/31, were also analysed but found to contain no toxins.

4.3.2 - Toxin profile determination study

During the first feeding study mussels visibly cleared the algae from the experimental tanks post feeding. There was no outward sign of negative effects in one treatment over another, such as excessive production of pseudo-faeces or extreme mortality. Although various sublethal effects have been reported previously from exposure to *Alexandrium sp.* (Haberkorn et al., 2010; Mello et al., 2013) there was no indication from our observations that these had an impact on feeding behaviour. Mortalities occurred in all of the treatments, although these were occasional and did not occur consistently in one treatment over the others.

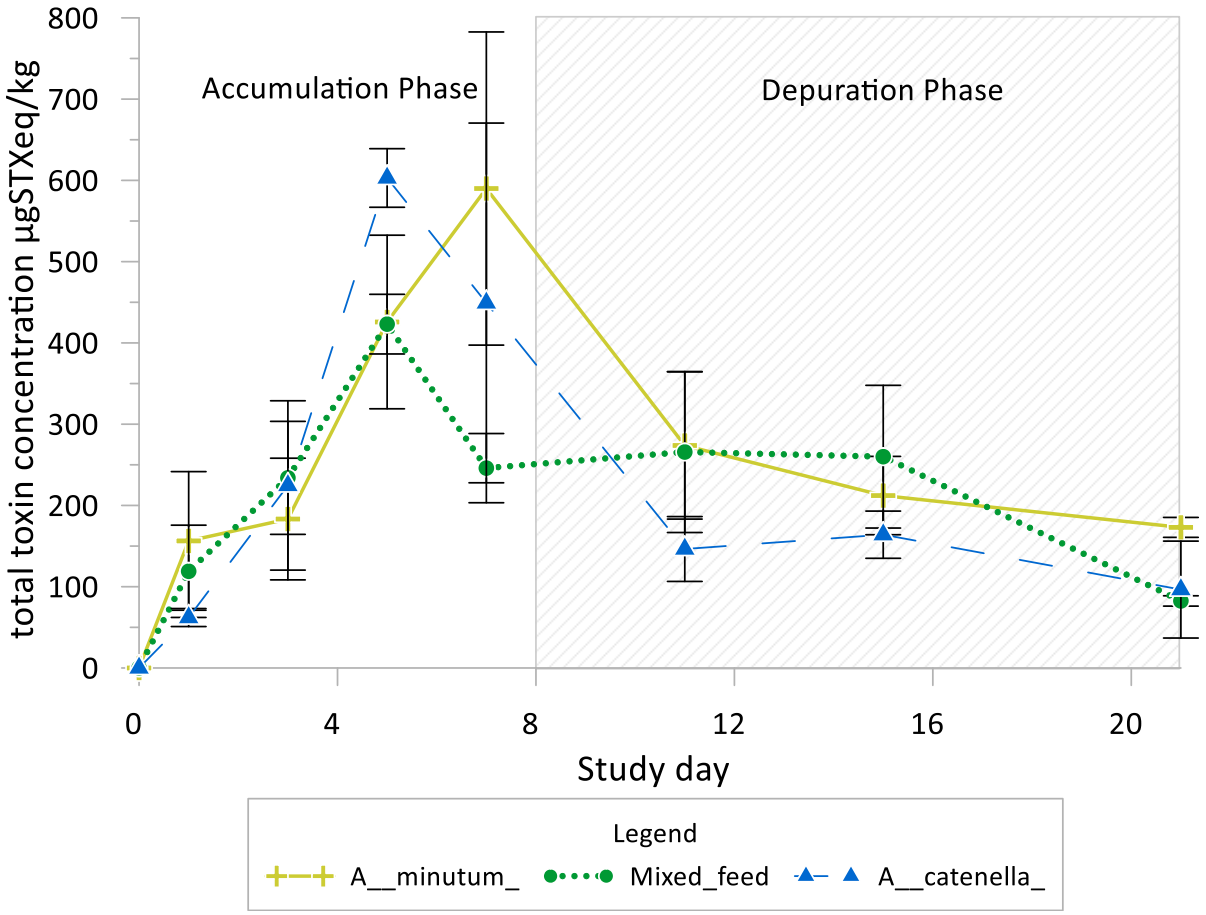


Figure 4.3: Showing the change in total toxin concentrations of the three toxic treatments from the initial feeding study over the course of the 21-day experiment. Toxic cells were introduced for the first 7 days, followed by a period of 14 days for depuration (Hashed region). Control samples were found to be negative and so are not included. From Lewis *et al.* (2022).

Toxin levels increased at a similar rate in the three toxin-fed treatments for the first three days. The toxins in mussels exposed to only *A. catenella* increased more rapidly to a maximum level at day five of the study, whilst those in the *A. minutum exposed* and mixed exposure treatments maintained a very similar increase to a near identical point on day 5. The toxin levels in the mixed exposure then peaked at day five of the study, in the same manner as the *A. catenella* exposed shellfish. The mussels fed on only *A. minutum* as a toxin source continued to increase in toxin concentrations until day seven. Both the *A. minutum* and *A. catenella* exposed treatments reached

very similar levels in total, peaking at 590 µgSTXeq/kg and 603 µgSTXeq/kg respectively. Those in the mixed feed treatment, however, showed a lower average peak total toxin concentration of only 423 µgSTXeq/kg, representing only 70% of the total reached in the other two treatments (Figure 4.3).

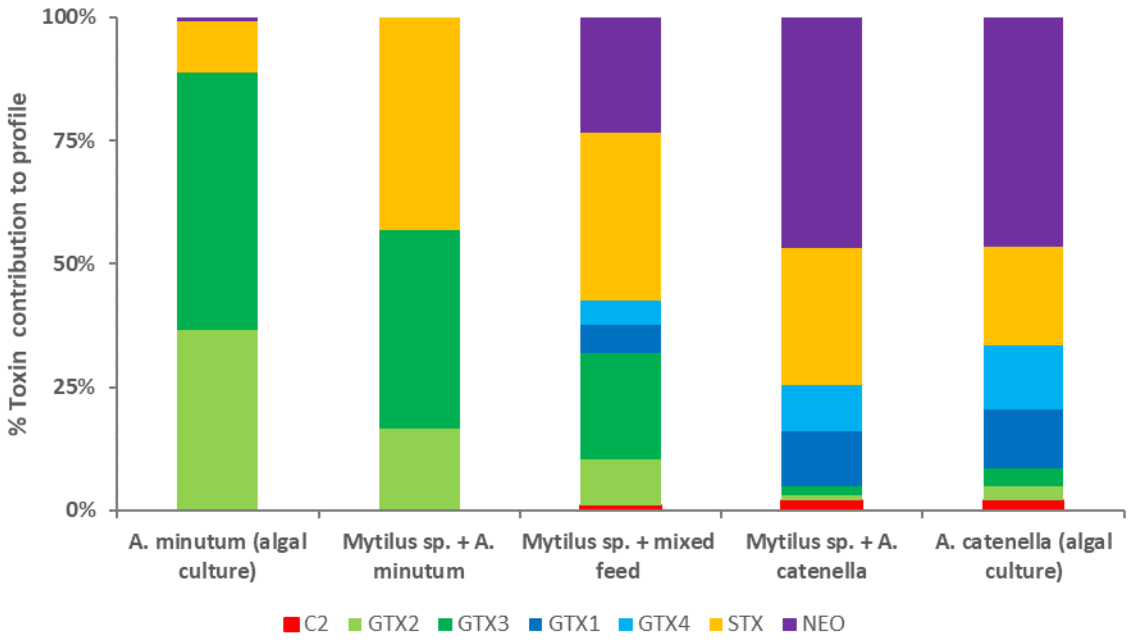


Figure 4.4: Relative toxin profiles generated from the average of all results across the 21 days of the 3 toxic exposure treatments and the average profiles generated from triplicate samples of each toxic microalgal species. All control samples were found to be negative and so are not shown. From Lewis *et al.* (2022).

The principal toxins and their normalised contribution to toxin profiles as determined by HILIC-MS/MS analysis are displayed in Figure 4.4. In addition, trace levels (<1.4 µgSTXeq/kg) of C1, dcGTX1, GTX5, doSTX, dcSTX and 11-OH-dcSTX were detected inconsistently. It is likely that some of these results were a consequence of the high sensitivity of the HILIC-MS/MS method and an artefact of very low signals being quantified. No M toxins (Dell'Aversano et al., 2008) were detected, although the method allows for this. The major toxin groups of the *A. minutum* culture and mussels exposed to it were found to be GTX2, GTX3 and STX and the *A. catenella* cultures and mussels

exposed to it were dominated by Neo with other important contributions made up of STX, GTX1, GTX4 and a smaller contribution by C2 and GTX2 and GTX3. The mixed profile includes all of these toxins but at proportions which sit halfway between the other two toxin-fed treatments.

K-means clustering analysis confirmed the relationship between source algal profile and contaminated shellfish whilst supporting a separation between the different treatments. Analysis resulted in four distinct clusters, as shown in Table 4.5.

Table 4.5: Table showing the toxin profiles represented by each cluster centre following the K-means clustering algorithm solution being applied to reduce the sum of squared distances of samples to centres. From Lewis *et al.* (2022).

	C1&2	**GTX1&4**	**GTX2&3**	**Neo**	**dcGTX2&3**	**STX**
Cluster centre 1	0%	0%	65%	0%	0%	35%
Cluster centre 2	0%	0%	49%	0%	0%	51%
Cluster centre 3	1%	10%	34%	25%	0%	30%
Cluster centre 4	2%	22%	3%	49%	0%	23%

Of particular note is that the shellfish from both the mixed and the *A. catenella* exposed treatments formed distinct clusters throughout the 21-day study period but in the shellfish from the *A. minutum* exposed treatment, a divergent profile emerged during the depuration period. This split results in one cluster defining the source algae and those mussels actively feeding on the algae, and a second cluster which contains those mussels in the process of depurating the toxins accumulated from *A. minutum.*

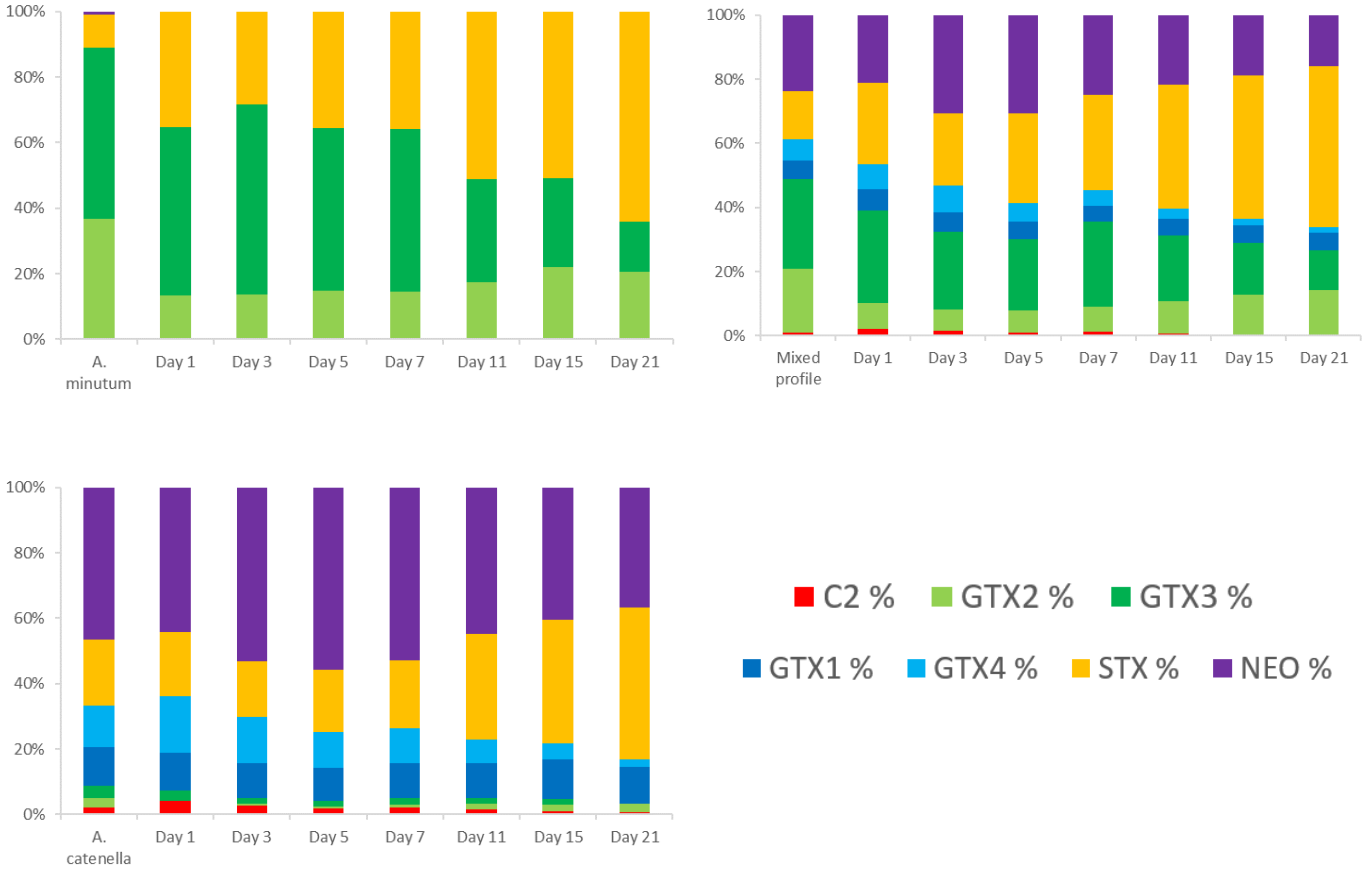
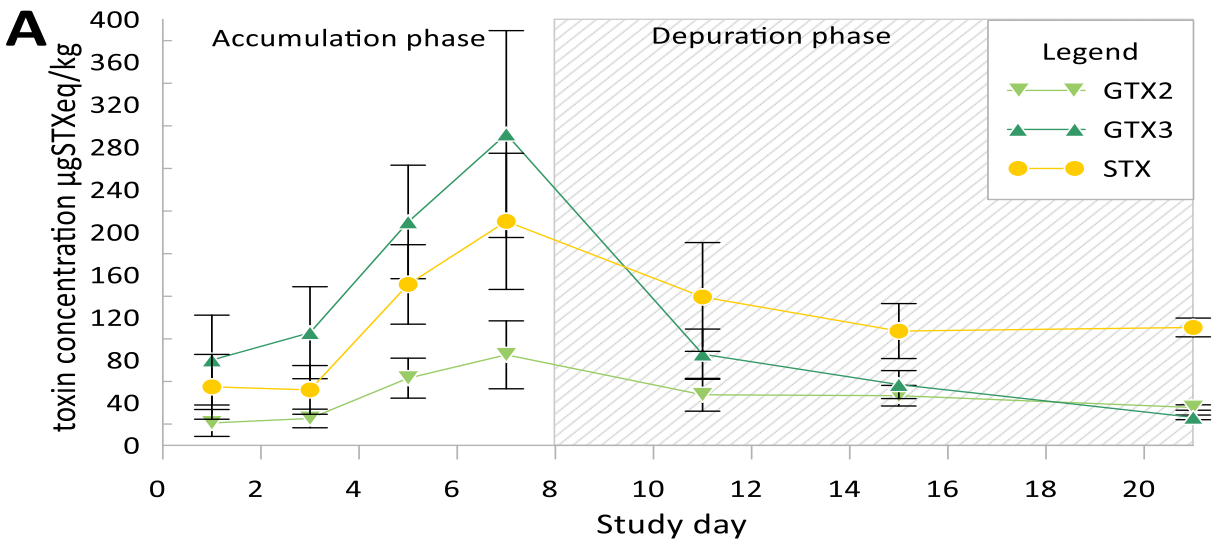
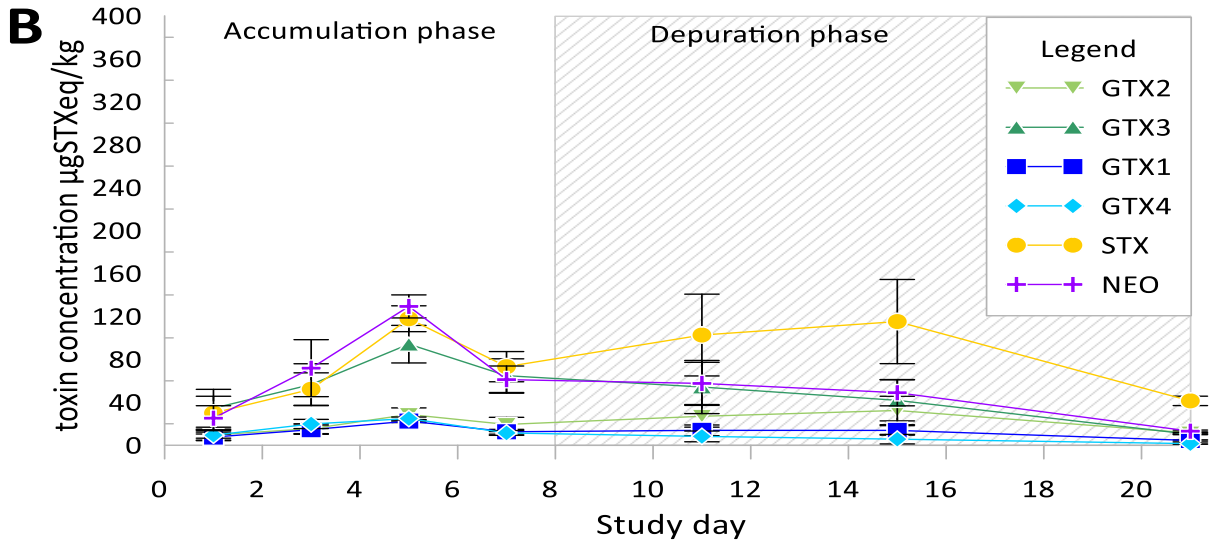


Figure 4.5: Composite Figure showing the chronological toxin profile for the three toxin-fed treatments. **A –** *A. minutum* exposure, **B** – Mixed feed exposure, **C** – *A. catenella* exposure. Each time point is an average result of the experimental triplicates. The left most column represents the toxin profile of the algal source within the treatment, in the case of the mixed algal feed profile this is calculated rather than determined experimentally by assuming an equal contribution from both toxic *Alexandrium* species. From Lewis *et al.* (2022).

The toxin profile within the contaminated mussels changed during the course of the study, with changes measured during all three toxin treatments shown in Figure 4.5. In all treatments there was a change within the relative proportion of the epimeric pairs, with a shift from the β-epimers to their related α-epimer (GTX3→GTX2, GTX4→GTX1). This shift became more apparent when toxic cells were no longer being added to the system, after day seven. Further to this the contribution of Neo to the toxin profile reduced substantially during the depuration phases in the mixed and *A. catenella* fed treatments, falling from 30% to 15% and 55% to 35% respectively as depuration progressed. In all treatments the proportion of STX in the profile increased during the depuration phase. This increase in STX proportion resulted in the divergent toxin profile identified in the cluster analysis for the mussels depurating toxins where *A. minutum* was the feed organism.


A
Accumulation phase
Depuration phase
Legend
GTX2
GTX3
STX
toxin concentration µgSTXeq/kg
Study day


B
Accumulation phase
Depuration phase
Legend
GTX2
GTX3
GTX1
GTX4
STX
NEO
toxin concentration µgSTXeq/kg
Study day

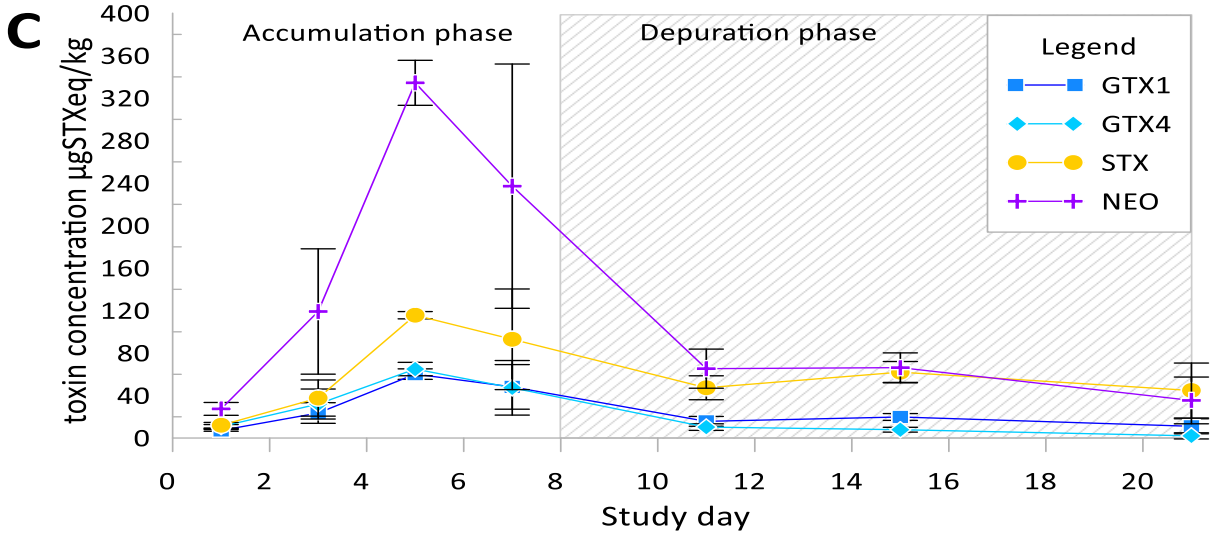

C
Accumulation phase
Depuration phase
Legend
GTX1
GTX4
STX
NEO
toxin concentration µgSTXeq/kg
Study day

Figure 4.6: Composite chart displaying mean (± standard deviation) PST concentrations over time between different treatments. Toxic cells were introduced for the first 7 days, followed by 14 days for depuration (Hashed region). **A** – *A. minutum* exposure, **B** – Mixed exposure, **C** – *A. catenella* exposure. From Lewis *et al.* (2022).

The normalised profiles demonstrate a change in relative toxin proportions but they do not show the changes in overall total toxin concentrations over time. The changes in toxin concentrations of each principal congener following each toxic treatment are shown in Figure 4.6. From this it is possible to see that the increasing proportion of STX in all treatments is a result of the slower rate of depuration of this toxin, where the concentrations of this toxin plateaued during the depuration phase. Additionally, in those treatments where Neo was also present in the profile an increase in the mean STX concentration in the samples was observed within the first seven days of the fourteen day depuration period, although the variability between triplicate samples suggests this increase was not significant. The α and β epimers show a transition with the β epimer contributing more to the total toxin load of the mussels during the uptake and early depuration phase, this is more pronounced in the pair GTX2 and GTX3 than GTX1 and GTX4. In all cases the β epimer began as the majority contributor but this had changed to the α being the more abundant by day 21 of the study.

4.3.3 - Sequential toxin profile determination study

The second study analysed the effects of sequential feeding of the same toxic *Alexandrium* species as those used in the profile determination study. This was carried out with first *A. catenella* (CCAP 1119/27) then *A. minutum* (MBA F5). Increasing levels of PST were accumulated across both exposure phases of the toxic treatment. The control treatment, with non-toxic *A. tamarense* (CCAP 1119/31) and non-toxic *A. minutum* (CCAP 1119/48) remained negative throughout.

As the transition between the toxic algal species occurred and cell concentrations of *A. catenella* introduced into the systems reduced there was a notable decline in the toxin levels of the mussels sampled, this decline continued while the concentration of *A. minutum* being added escalated until the sample taken on day 13 where *A. minutum* was being introduced at the maximum concentration. During the transition period the total concentrations of toxins of the mussels in the toxic treatment fell by 16%. As levels of toxic *A. minutum* reached the maximum input level the toxin concentrations within the shellfish began to rise once more. Similar levels were accumulated during each exposure phase with *A. catenella* exposure reaching a measured high of 724 µgSTXeq/kg and 653 µgSTXeq/kg being accumulated within the *A. minutum* exposure. The *A. minutum* exposure period was slightly shorter than that of *A. catenella* with five days at full concentration rather than seven, this was due to insufficient quantities of *A. minutum* culture to continue to feed for the planned seven days. This was due to contamination occurring within one of the polypropylene bags (4.2.2) housing the *A. minutum* culture. Suspected contamination was initially identified as the culture visually appeared similar to the *A. catenella* culture as cell densities increased. In order to ascertain whether the culture had been contaminated a sample was taken and examined via light microscopy. This presented a dichotomy in cell size indicative of both *A. catenella* and *A. minutum* being present. With the source of both cultures being clonal monocultures the capacity for sexual fusion and the formation of planozygotes (2.3) was ruled out. A further toxin analysis was conducted on a sample from the suspected coculture, this was undertaken using the extraction process from section 4.2.6.1 and the screening HPLC-FLD analysis methodology (3.2.7.3). This revealed a toxin profile similar to that derived mathematically and presented in Figure 4.5, confirming the presence of both species in coculture. Consequently, this bag was not utilised for the final study but there was insufficient time to produce sufficient replacement culture, rather than amending cell concentrations added and consequently toxin dosage, it was decided to instead curtail the *A. minutum* phase of the sequential study.

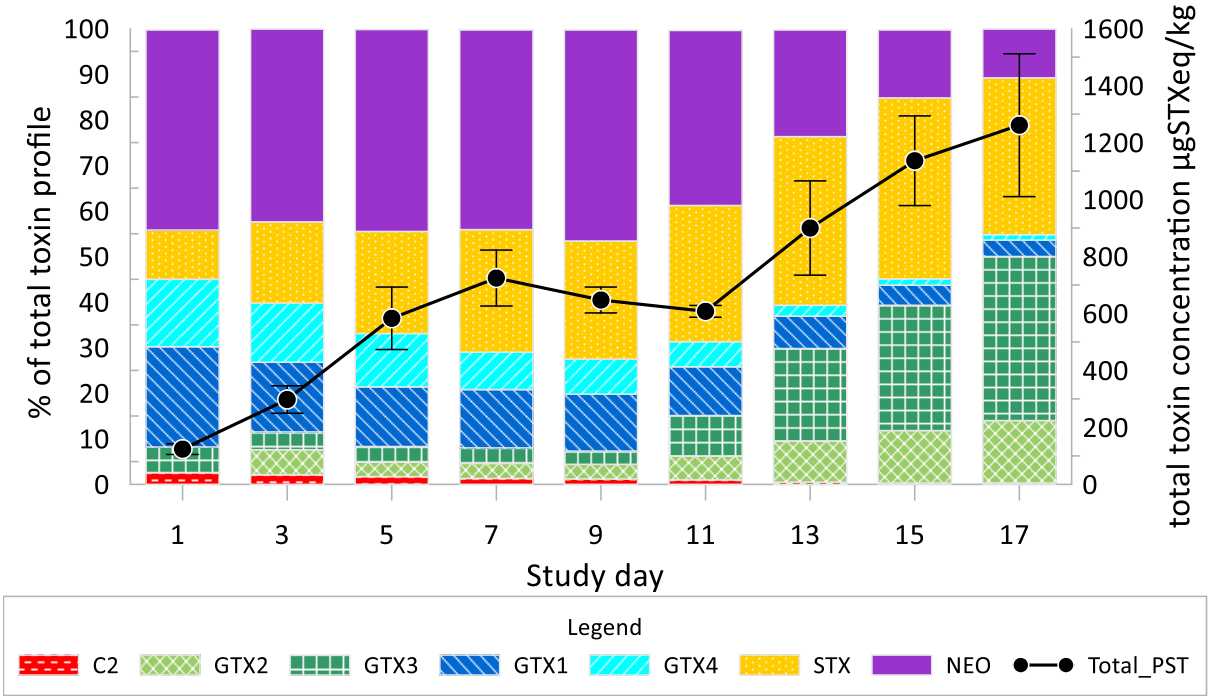


Figure 4.7: Chart displaying the chronological toxin profile and total PST concentrations determined in *Mytilus* sp. by HILIC-MS/MS for the toxin-fed sequential exposure. The three phases of the study are presented in Figure 4.2. From Lewis *et al.* (2022).

The toxin profile derived from exposure to the toxic *A. catenella* strain in the sequential feeding study appeared similar to that in the first, this was confirmed via cluster analysis where early samples from this study clustered with those in the *A. catenella* treatment from the toxin profile determination study as well as the profile of *A. catenella* culture 1119/27. Figure 4.7 displays the change in this profile throughout the study, highlighting the proportional increase in STX over time which was also noted in the treatments from the first study. As *A. minutum* was introduced to the treatment so the profile underwent a rapid shift in its composition. K-means cluster analysis of this data revealed that the profile only continued to cluster with the *A. catenella* results from the first study until T9 whereupon it shifted to the mixed profile at time points 11, 13 and 15 and then to the *A. minutum* toxin profile from the first study at the final sampling point. Day 11 was the first mussel sample collected after the addition of *A. minutum* to the experimental system had commenced,

meaning that the shift in toxin profile from the *A. catenella* cluster to the mixed profile occurred within 24 hours of the introduction of *A. minutum* cells to the system. The cluster defining the depuration phase of the *A. minutum* treatment did not fit any of the sampling points in this second study, which is not unexpected as toxic cells were being introduced on all days of the study with the exception of day 9, so mussels in the toxin exposed treatment were actively feeding on toxic algal cells throughout.

4.3.4 - Cluster analysis comparing experimental results with historic routine monitoring data.

The final aspect of analysis was to take selected samples from the two feeding studies presented here and incorporate them into the cluster analysis performed in Turner et al. (2014) to see whether the experimental data conformed to natural toxin profile information. In order to allow for comparability between study and monitoring data the same extraction and analysis methods used in the British monitoring programme (section 2.6.2) were applied to selected samples from the experiments described herein. A sum of epimers from the HILIC-MS/MS analysis could have been used but this may have introduced additional variability due to differences in method performance and sample processing. Following generation of toxin concentration results in the shellfish, the data were taken and added to the original comparison as performed in Turner et al (2014). The K means analysis was re-solved to allow for changes in each centroid following the addition of new data. This did not result in a shift away from clusters previously determined, suggesting that the additional data points generated in this study were not sufficiently divergent from those found in naturally contaminated samples to force a change.

During the re-assessment, data from this experimental study clustered alongside two of the four pre-existing clusters determined within the GB monitoring programme from 2006-2012. The cluster centres generated are displayed as inserts in Figure 4.8, this Figure also shows the geographic

distribution of the toxins determined via the routine monitoring. Those shellfish experimentally exposed to *A. catenella* clustered alongside monitoring samples originating from Scottish and Northeastern English waters, in cluster 2.

Data from shellfish experimentally exposed to *A. minutum,* clustered with the data from those monitoring samples collected from sites in the South of England and Wales, more specifically with several samples from the Fowey estuary, England and Milford Haven, Wales, in cluster 3.

Like the samples experimentally contaminated with solely *A. minutum,* the samples from the mixed exposure treatment were also clustered with those of cluster 3 except for the first time point which clustered with study samples experimentally exposed to *A. catenella* as a sole toxin source, in cluster 2.

Samples from the toxic treatment in the second feeding study, with the sequential exposure of toxic species, initially clustered in cluster 2 with routine data from Scotland and the NE of England as well as those samples from the toxin profile feeding study contaminated by *A. catenella* only. All samples analysed up to day 9 sat within cluster 2. For the later samples from the sequential feeding study the cluster analysis then classified the remaining days analysed, 13 and 17, with those samples in cluster 3, representing samples from the SW of England, one area on the W coast of Scotland and samples from the *A. minutum* exposure in the toxin profile determination study.

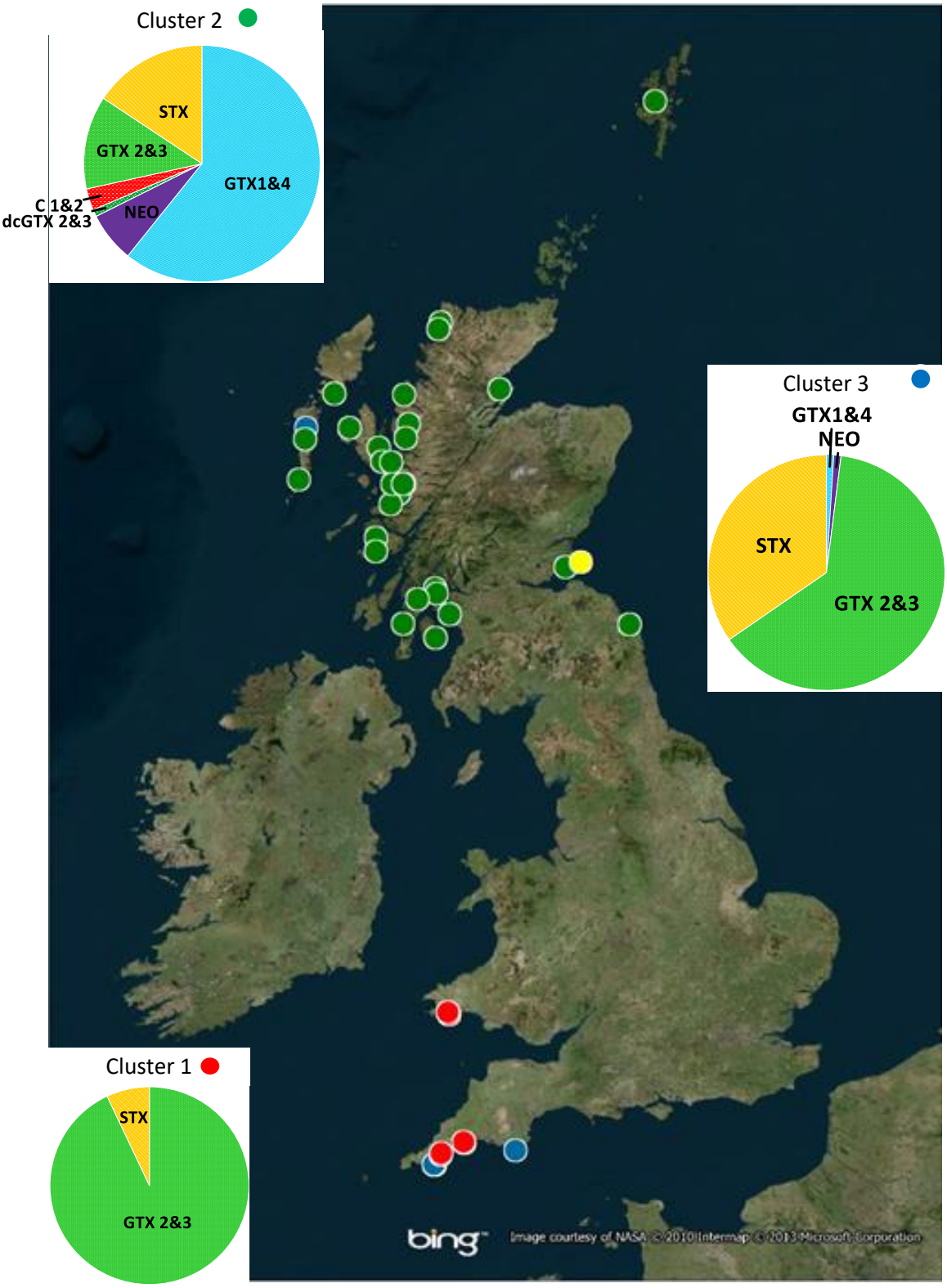

Cluster 2
STX
GTX 2&3
GTX1&4
C 1&2
dcGTX 2&3
NEO
Cluster 3
GTX1&4
NEO
STX
GTX 2&3
Cluster 1
STX
GTX 2&3
bing
Image courtesy of NASA © 2010 Intermap © 2013 Microsoft Corporation

Figure 4.8: Map modified from Turner et al. 2014 showing the geographic distribution of the clusters determined within that paper. Inset pie charts display the toxin composition of each of the 3 cluster centres which sample values from this study were compared against. Data from this study clustered in clusters 2 and 3 when run within the existing data set. The centre for cluster 4 is not shown as this was unique to *S. solida* during the 2014 study and was not represented in this study. From Lewis *et al.* (2022).

4.4 - Discussion

4.4.1 - Feeding studies

The mussels in this study were found to accumulate toxins rapidly, displaying detectable levels of multiple toxin analogues within 24 hours of exposure to toxic algal cells, irrespective of the toxin source. All toxin profiles measured in incurred mussels showed a similarity to those determined in the algal source, as there were no clear or consistent toxins present which could not be derived from the source algae. Although the HILIC-MS/MS method includes a wide range of analytes not typically screened for during routine monitoring by Precox HPLC-FLD, none of these were detected, indicating that the HPLC method currently in use would be suitable for detecting threats posed by the strains of *Alexandrium* used in this study. The more recently determined M toxins (Dell'Aversano et al., 2008), which appear to most commonly be shellfish metabolites of other saxitoxins were not detected during either feeding experiment. This indicates their absence from the organisms used here (both shellfish and dinoflagellates) where they have also been shown to be produced (Qiu et al., 2018). Of key importance for the use of chemotaxonomy in determining the algal species responsible for toxin contaminations, the profiles of the intoxicated mussels from different treatments bear a closer resemblance to their source algal feed than they do to each other.

This is shown in Figure 4.4 and this observation was supported by the outcome of K-means clustering.

Although the maximum toxin concentration attained in both of the treatments with a sole algal source was similar, this average toxin concentrations observed in the mixed feed treatment was considerably lower. The reason for this is uncertain but does not appear to be a preferential feeding upon one species resulting in the exclusion of the other, as the toxin profile generated (Figure 4.4 and Figure 4.5), falls midway between the toxin profiles of the *A. minutum* and *A. catenella* exposed shellfish for all congeners.

The increasing proportion of STX over time in all of the treatments (Figure 4.6), sufficient to result in the generation of a divergent profile in the *A. minutum* treatment, comes largely from the differing depuration rates of the different toxin analogues within *Mytilus sp..* STX is eliminated more slowly than other toxins such that the relative proportion of this congener increases over time. It also appears that STX may be being converted from Neo reductively, as has been shown previously (Bricelj and Shumway, 1998), as STX levels were potentially found to increase during the latter stages of the depuration phase in both the mixed and the *A. catenella* exposed treatments but not in the *A. minutum* exposed treatment, where Neo was not found to be present in the shellfish. Another change within the profile comes from the known conversion in shellfish of β-epimers, predominantly produced by algal strains, to their more stable α-variants (Oikawa et al., 2004; Oshima, 1995). It should also be noted that this conversion of the β-epimers to their α equivalents appears to be occurring for the gonyautoxins with a noticeably higher initial level of GTX3 and GTX4 and a subsequent faster rate of decay of these epimers when compared to GTX2 and GTX1 respectively. In all three toxic exposure treatments the α-epimer became the dominant epimer following the 14-day depuration period (Figure 4.6). As has been highlighted before (Bricelj and Shumway, 1998), an understanding of this conversion rate can be useful for the determination of the time since toxin sources have been removed. The epimeric interconversion did not seem to influence the cluster

analysis however as changes within the ratios did not alter the clustering for either the mixed or *A. catenella* exposed groups.

The sequential feeding study resulted in similar toxin profiles to the initial toxin profile determination study. When exposed to *A. catenella* only at the commencement of the study, *Mytilus sp.* displayed the same toxin profile from the first study. Due to the rapid uptake and the lack of preferential feeding shown *Mytilus sp.* there was then a rapid shift through the mixed toxin profile from the initial study into the *A. minutum* derived profile. The mixed profile appeared during the feeding transition from *A. catenella* to *A. minutum* and persisted until the final sampling point, representing six days of exposure to solely *A. minutum* and seven days since the last exposure to *A. catenella.* This shows that the mixed profile can represent both simultaneous or sequential exposure to two PST producing algal species. The switch to the profile associated with *A. minutum* is a result of the considerably diminished contribution of Neo from 45% during the *A. catenella* exposure down to 11% after several days of *A. minutum* being the toxin source. As such, the transition between profiles in natural populations would largely depend on the dominant species and magnitude (both cell concentration and toxin quota) of the blooms in a sequence as well as their duration. If the mussels had accumulated much higher levels of toxins it is likely that the transition would have been masked or taken longer unless the following exposure was of similar magnitude. Conversely if the initial bloom was smaller than the subsequent one then the transition would occur much more rapidly, potentially skipping the mixed profile within as short a time as 24 hours due to the rapidity at which *Mytilus sp.* can accumulate toxins. Further to this, as the transition between the toxic algal species occurred and cell concentrations of *Alexandrium* introduced into the systems was reduced there was a notable decline in the total toxin levels reached in the mussels sampled, this is in keeping with the premise that *Mytilus edulis* is a rapid detoxifier (Bricelj and Shumway, 1998). Here we presented a seven-day transition window, with toxic cells being introduced on all but one day, albeit at lower levels than during the rest of the study. With a longer window between blooms it is feasible that *Mytilus sp.* would be able to depurate toxin concentrations to levels where a PST

contamination from a subsequent bloom would immediately replace the toxin profile in mussels with one derived from the new source algal population.

4.4.2 - National clustering

The data from our feeding studies did not cluster in an unforeseen manner, with samples known to have been contaminated by a specific producer clustering with those monitoring samples thought to have derived toxins from the same source. Mussels experimentally contaminated with *A. catenella* clustered alongside monitoring samples from Scotland and NE England. This is consistent with the known distribution of toxic *A. catenella* in GB, and the links that this species has with shellfish toxicity in this region (Brown et al., 2010). Similarly, the toxin profile in shellfish, from this study, contaminated with *A. minutum,* clustered alongside profiles of contaminated bivalves originating from two locations in the Southwest. From previous work it is believed that *A. minutum* is the causative organism in this region (See Chapter 2, published as Lewis et al., 2018; Percy, 2006; Turner et al., 2014), as well as the isolation location for the strain (MBA F5) used in this study. These data help to validate the approach of assessing causative algal species by chemotaxonomy, showing that the laboratory derived data from our feeding studies are comparable to data generated within the official control monitoring conducted on field samples.

It should be noted that another cluster, cluster 1, representing what is believed to be *A. minutum* exposed shellfish is derived from the monitoring data, this cluster contains sample data primarily from estuaries in the Southwest of England, as well as samples originating from the Southwest of Wales. One estuary in Southwest England which had samples in cluster 2, also saw some samples display a profile from cluster 1 but none of the samples contaminated during this study were represented by cluster 1.

From this it can be seen that the national clustering approach originally used in 2014 masks the presence of both the mixed exposure treatment and the disparate depuration profile witnessed within the solely *A. minutum* exposed treatment from the first feeding study. It might be of value

therefore, with the additional knowledge gathered from this study, to undertake clustering with at least four cluster centres and to exclude all species other than mussels as the original 2014 study sought to analyse profile similarities for all shellfish species analysed within the monitoring programme within Great Britain. As we have noted, targeting a shellfish species known to undertake limited toxin conversion is a more reliable way to assess causative algal species using toxin profile analysis. The reduced level of cluster separation between the study treatments when also including monitoring data indicates that resolution is potentially lost when analysing large data sets. As such, there may be a need for restrictions to be placed on the data included in K-means clustering to reduce the number of variables influencing the shellfish toxin profile as this will help to highlight the profile divergence inherent to the source algae.

Of note is the finding that when feeding shellfish with relatively stable toxin kinetics with a simultaneous feed of two different toxin profiles or if feeding this sequentially, a novel toxin profile is derived. This is to be expected, especially in the case here where feeding was planned to provide an equivalent dose of toxins from each profile but the rapidity of shift from a profile characteristic of the source algal population to a novel one is of interest. The high accumulation rate of *M. edulis* (Bricelj and Shumway, 1998) is likely to have a key role in this, allowing this species to acquire toxins from the dominant toxic algal species rapidly. This highlights one of the potential weaknesses of attempting to determine the source toxin producing algal species from toxin profile alone. It would be possible to search for an algal strain producing a profile which fits that observed when in reality the analyst would be observing a chimeric profile. Blooms of *A. catenella* in GB typically occur from the Spring into early Summer whilst *A. minutum* begins its growth in the early summer and persists later into the season. If these two were to overlap a scenario similar to our sequential study, feeding study two, would theoretically be the most likely. The chimeric profile might then be confounding if analysing toxin profile data, especially if a single data point were analysed in relative isolation. This finding also highlights the value of clustering profiles statistically rather than solely by analyst's interpretation, in the case highlighted here the range of toxins in the mixed toxin profile bears more

surface level similarities to that of *A. catenella* than the altogether more simple profile of *A. minutum.*

Via the use of statistical clustering it may be possible to determine the source algal populations, tentatively, from the toxin profiles discovered in contaminated shellfish. In order to carry out such comparative work, the toxin profiles of likely algal producers need to be known and well characterised. Whilst it would be possible to utilise profiles ascertained from algal populations from other geographic regions it should be noted that there may well be an overlap of toxin profiles between different strains or species. For example, whilst the *A. minutum* strain used within this study produces no Neo there are other populations globally which have been shown to, such as some strains isolated from New Zealand and Brazil (See Chapter 2, section 2.5 and Table 2.3). Neo is a good discriminatory feature of the toxin profiles of *A. catenella* and *A. minutum* from Great Britain but in other areas this will not be the case. Therefore, if a source algal population has its identity ascribed from the use of toxin profiling as a chemotaxonomic marker there should always be recognition that a recent introduction or range expansion from a previously absent population could give rise to similar results. The use of toxin profiling to infer species is therefore of most use within monitoring programmes to enhance the value of data which is already being gathered and to minimise some of the limitations of the techniques of algal identification and enumeration commonly employed.

The results from the two mussel feeding trials conducted confirmed the PST profiles in shellfish tissue closely resembled the toxin profile associated with the algal strain which produced them. It should, however, be recognised that this relates to a shellfish species where interconversion of toxins to other analogues is not a prevalent feature. If high degrees of interconversion are known to occur, then the interpretation of toxin profiles derived from shellfish is a more difficult process and it becomes very difficult to reliably relate the toxin profile witnessed with a definitive producer. The consistency of the clustering of experimentally contaminated shellfish between studies as well

as when compared to historic monitoring data, shows that the K-means clustering technique produces valid results from which to interpret the PST producing algal species, within GB.

4.5 – Conclusion

Overall, our results have confirmed the suggestion that *Mytilus* sp. exhibits low levels of conversion of toxins and would therefore be an appropriate candidate for use as a sentinel taxon for monitoring of toxin profiles in microalgae. They rapidly accumulate toxins and maintain a relatively stable toxin profile once intoxicated. We have shown that the toxin profile within shellfish may be stable during an intoxication phase but may be subject to change as a result of depuration. Also, a rapid onset of change can be seen if the source population providing the toxins changes. Toxin profiles must therefore not be analysed in isolation if the intention is to gain some indication of algal species involved. Close reference must be made to total toxin loading, changes in accumulation or depuration and linkage to phytoplankton monitoring if it is available.

Countries or regions with well characterised PST producing phytoplankton which make use of chemical analytical techniques, especially within a consistent monitoring framework, could make use of chemotaxonomy to provide source algal tracking. If this is combined with phytoplankton monitoring, then a better understanding of bloom dynamics and shellfish harvesting closures can be gained.

By combining traditional identification, even to the genus level, with inferred species it should be possible to provide a more detailed picture of harmful algal bloom events, without the need for costly additional sampling or testing techniques. The main advantages of having at least a tentative identification of causative organisms during events are the ability to better predict bloom behaviours, inform models and detect novel occurrences of species. This may be used to more

accurately assess the risks to a harvesting site, including potential windows of closures as details of an algal species can be applied rather than a broader genus or phyla related perspective. Within a monitoring framework this technique can be used as an additional layer of quality checking, with unusual toxin profiles triggering investigations into the possible causes, possibly highlighting sampling or processing errors. Also of value, is the ability to identify unexpected occurrences and target further investigative work. By interrogation of toxin profile information from new or atypical intoxications it may be possible to infer a species and so refine questions, hypotheses and sampling strategies to enhance the ability of researchers to plan experiments.

Chapter 5 - Assessing the risk to Great Britain of the emergence of new, non-native, Harmful Algal species

5.1 - Introduction

5.1.1 - Global climate status and its effects on the UK

A critical concern globally is the projected future change in the world's climate. This is an intensely studied field which has provided evidence and forecasts on the changes which have already occurred as well as those which are anticipated to occur in the future. The consensus within the scientific community is that global temperatures are experiencing an increase and that the chief cause is anthropogenic in nature (Betts and Brown, 2021; Defra, 2009; Yoro and Daramola, 2020). However, climate change runs deeper than simply changes in the global temperature, there are regional and local impacts in temperatures which differ in magnitude from the global mean changes. Additionally, the change in temperatures and climate lead to forecasts for the United Kingdom (UK) of more intense weather events (Betts and Brown, 2021), with increased winter rainfall (Betts and Brown, 2021; Defra, 2009) which increases surface runoff and in turn affects nutrient, salinity and stratification regimes in coastal waters but an average of decreased summer rainfall (Betts and Brown, 2021). Further wide scale impacts include an increase in sea levels, and shifts in global prevailing winds, meteorology and water circulation patterns. Furthermore, the increase in the so called "greenhouse gasses" has direct ramifications, with increased atmospheric CO_2 levels leading to greater levels of absorbance into seawater resulting in ocean acidification (Findlay and Turley, 2021). The greater availability of CO_2 suggests that the potential for increased primary production, both in terrestrial and ocean environments, does exist (Hallegraeff, 2010; Johnson et al., 2013).

The predicted rise in average global temperature is currently forecast to be between two and six degrees Celsius by the year 2100, with this largely thought to depend on the actions of global governments in limiting the continued inputs of critical emissions into the atmosphere (Betts and

Brown, 2021; Defra, 2009). There is evidence that it is possible for such a policy switch to be enacted, with the ban on ozone depleting substances resulting in the reduction of damage and the apparent and projected repair of the ozone layer damage which was a key environmental issue of the 1980s through to the early 2000s (Albrecht and Parker, 2019; Karpechko, 2020). Unfortunately, however, the production and emission of greenhouse gases is intrinsically linked with global industries and power output. Consequently, those nations still undergoing extensive industrialisation and with particularly large populations, such as India and China, contribute a significant proportion to global emissions (Ahmad and Zhang, 2020; Yoro and Daramola, 2020). Whilst renewable energy solutions have become increasingly utilised in some nations, where there are also drivers to extend the proportion of power generated by these means further, they still make up a minority of global power output (Ahmad and Zhang, 2020).

It is prudent then to assume that the global projections of increased temperatures are, at the least, likely to hit the lower end of the estimates provided within the most recent International Panel on Climate Change (IPCC) report. This then will have knock-on effects in European waters with projections showing that under a business as usual scenario of warming, a sea surface temperature (SST) rise of 0.2°C per decade is likely (Alexander et al., 2018). It has also been noted that the marine system may be disproportionately affected by climatic changes, with suggestions that the oceans have accumulated ~85% of the excess heat generated from the 1970s through to the early 2000s (Cazenave and Llovel, 2010; Findlay and Turley, 2021). There is also forecast to be an increase in the frequency of high temperature anomalies, so called marine heatwaves, as climate change continues to progress (Alexander et al., 2018). In relation to the UK, the South of the country is forecast to be the most strongly affected region with respect to temperature increases (Defra, 2009).

With the Ocean absorbing a significant quantity of excess heat, UK SST averages for the period 2010 – 2020 have risen by around 0.6-0.7 °C when compared to the period 1961-1990, this (Betts and Brown, 2021; Defra, 2009). Further rises are anticipated, with a number of future climate

scenarios having been modelled, each projecting a different future global emission scenario. These include the Representative Concentration Pathway (RCP) 2.6 and the RCP 8.5, representing an optimistic assessment based on successful limitation of CO_2 production in the first instance and an unmitigated "business as usual" approach in the latter (Pörtner et al., 2019). The temperature increases expected globally by 2100 are divergent between these two scenarios, with the first expecting only 0.8 °C compared with 3 °C under a scenario without controls (Pörtner et al., 2019). In the shorter term, by 2050, however, the two projections are more closely aligned, anticipating between 0.7 °C and 1.3 °C of increase (Pörtner et al., 2019). In UK waters an increase of between 1-2 °C is anticipated by the year 2050 and a total increase of between 1-4 °C by 2100 (Palmer et al., 2018). This is potentially a more significant increase than the global average, with the ranges presented here representing those falling between the climate models of RCP2.6 and RCP8.5, as above. A further scenario, RCP4.5 represents limited success in controlling emissions, in this instance the projected temperature increase for UK surface waters by 2050 is around 1.2 °C, with a forecast total increase of around 2 °C by the end of the century. A rise in the temperature of UK coastal waters of at least 1 °C is therefore a likely future scenario for the year 2050. Further to this, some coastal environments such as estuaries are likely to be more severely impacted, as river waters have also increased in temperature and are more acutely affected by prevailing air temperatures than are oceanic waters (Robins et al., 2016) and as such may be warmer still than surrounding coastal waters.

A further impact, linked directly to both ocean and global temperatures, is that of sea level rise. Sea level rise has been observed already, since the early 1900s and has shown an increase of 1.8 mm per year on average through to 2001 (Cazenave and Llovel, 2010). Continued increases are expected due to both the thermal expansion of sea water, as well as extensive water input from landlocked ice caps (Cazenave and Remy, 2011). These ice caps are primarily situated in Greenland and Antarctica and their continued melting is contributing to the increase current global sea levels. There are variable projections of impact but values of increase from 2005-2100 range from 15 cm to

greater than 50 cm by 2100 (Bosello et al., 2012; Cazenave and Remy, 2011; Nerem et al., 2018) with some projections considering increases of up to 120 cm (Cazenave and Llovel, 2010). The rapidity of ice cap retreat and freshwater input to the oceans is also increasing as the planet warms. The pace of sea level rise has been increasing and has been well monitored since the early 1990s when satellite altimetry has allowed for high frequency monitoring of global sea levels (Cazenave and Llovel, 2010; Nerem et al., 2018). As such sea levels are now increasing at a rate of around 3 mm per year (Cazenave and Llovel, 2010; Cazenave and Remy, 2011) and are expected to continue to rise until at least the year 2300 (Palmer et al., 2018). Within the UK the total increase by the year 2100 is expected to be slightly lower than the global mean increase, although there is a projected variation within the UK with the South experiencing a greater increase than the North (Palmer et al., 2018). Under both the RCP4.5 and RCP8.5 climate projection scenarios, the rate of increase around the UK is also expected to rise, whereas under RCP2.6 the rate of increase will remain more steady (Palmer et al., 2018). With the increase across the 20th century reported as around 1 mm per year (Defra, 2009), the new global rate of 3 mm per year as currently recorded would mean problems associated with sea level rise will materialise more quickly. Whilst sea level rise is likely to have extensive physical impacts on coastal regions (Shennan, 1993), especially in low lying countries and small Island nations (Bosello et al., 2012; Cazenave and Remy, 2011; Pörtner et al., 2019), it is unlikely to impact harmful microalgae on a wide scale in the near future. This is due to the long periods over which substantial sea level rise is predicted to occur but in the short term within the UK it will create more pressure on processes such as coastal erosion, storm surges and the creation of localised flooding (Bosello et al., 2012; Callaway et al., 2012; Defra, 2009). The resultant impacts on harmful microalgal species are therefore likely to be highly variable and have both positive and negative influences on the proliferation of harmful marine microalgae. Sea level rise itself is not likely to influence the proliferation of harmful microalgae bloom formation and as such is not considered as a driver in the following assessment. In the longer term, however, the impacts of sea level rise may

have a synergistic effect with harmful microalgal proliferations, causing multiple pressures on the socioeconomics of coastal communities.

As well as absorbing significant quantities of heat from the atmosphere, the oceans also act as a sink for excess atmospheric carbon, with between a quarter and a third of CO_2 released from anthropogenic sources being absorbed into the ocean (Findlay and Turley, 2021). Interestingly, the synergistic impacts of other climate change, such as temperature, have been found to be insignificant in relation to ocean acidification, where change is directly linked to atmospheric CO_2 levels (Mcneil and Matear, 2017), as such ocean acidification stands as a separate but uncoupled impact of elevated atmospheric CO_2. Historically the absorption of CO_2 has been buffered by the presence of bases in seawater predominantly carbonates, maintaining oceanic pH levels. In more recent times however, the rate of absorption has exceeded the buffering capacity, resulting in a small decrease in the pH of surface waters (Findlay and Turley, 2021). Coastal and more poleward waters are also more prone to shifts in the levels of dissolved inorganic carbon and so consequently pH levels (Raven et al., 2020). There have been suggestions that some microalgal groups, including some harmful species, are favoured by the increase in dissolved CO_2 (Hallegraeff, 2010; Johnson et al., 2013) due to the presence of efficient carbon concentrating mechanisms. These species may synergistically or alternatively form blooms when waters become slightly acidified, with timing following the alkalinisation associated with the spring diatom bloom (Raven et al., 2020). Even where results suggest that the direct impacts of ocean acidification may be relatively weak, such as on microphytobenthos, the impacts which it can have across the marine communities which also influence the microalgae can be much more pronounced (Alsterberg et al., 2013). In these cases therefore, the indirect implications of ocean acidification can still have ramifications at several trophic levels (Alsterberg et al., 2013). There is evidence that elevated CO_2 levels in the ocean not only affect harmful microalgal species through an enhancement of growth but also through the increase in the cellular toxicity of certain marine harmful microalgal species (Hattenrath-Lehmann et al., 2015; Tatters et al., 2013, 2012). In *Alexandrium,* an increase in more potent congeners was

responsible for the increase in cellular toxicity (Hattenrath-Lehmann et al., 2015) whilst a reduction in the availability of silicates in the presence of increasing CO2 levels led to an increase in domoic acid production in *Pseudonitzschia* (Tatters et al., 2012), providing evidence of higher CO2 increasing toxin concentrations in both dinoflagellate and diatom toxin producers. This may suggest that future increased dissolved CO_2 levels might have synergistic effects on the magnitude of the impact of some microalgal species. The long-term implications of elevated CO_2 for microalgae are rarely studied but have shown mixed results in some limited cases, where reduced pH can offset the growth benefits of elevated pH for some species but not others (Bautista-Chamizo et al., 2018).

Changing climate conditions are also expected to alter regional and local weather patterns. One such change expected in GB is an increase in winter rainfall, in turn increasing surface runoff into rivers, although the degree of the effect is catchment specific (Robins et al., 2016). Data from the last 250 years within the Uk indicate that winter rainfall has already started to increase with a further trend towards more heavy downpours in the winter months (Defra, 2009; Watts et al., 2015). This runoff caries essential nutrients downstream into coastal areas and estuaries and has been linked with the eutrophication of coastal areas (Maier et al., 2009; Painting et al., 2013). Increased riverine input additionally alters the salinity of coastal waters and can lead to saline stratification forming. Conversely, there is projected to be a decrease in summer precipitation, although the variability within this is high (Robins et al., 2016) the SW of the UK especially is expected to see a substantial reduction (Defra, 2009). Overall, within the UK, river discharge is expected to continue to increase in winter whilst decreasing in summer (Defra, 2009; Painting et al., 2013; Robins et al., 2016), altering the nutrient inputs into coastal systems across the seasons but with significant variability between river catchments introducing substantial variability to projections. Estuaries can be particularly affected being semi-enclosed, with significant influence from freshwater inputs resulting in less dilution of nutrient inputs than open coastal systems and projected sea level rise, combined with decreased summer river discharge could lead to the trapping of nutrients in these systems (Robins et al., 2016). Another facet of eutrophication is the skewing of

natural nutrient ratios, especially as nations such as the UK have made advances in reducing inputs of nitrogen and phosphates entering the water system but with greater success reducing phosphate contributions (Maier et al., 2009; Painting et al., 2013). As such, nutrient ratios are being skewed towards increasing nitrogen to phosphate ratios in some areas (O'Boyle et al., 2016; Westphal et al., 2020). This altered nutrient ratio has been demonstrated to favour the growth of several mixotrophic microalgal species, including harmful taxa, as well as increasing the toxicity of certain toxic species (Glibert, 2020). Conversely, decreased N:P ratios have also been shown to positively impact some harmful taxa (Heisler et al., 2008). This variability in the effect of changing nutrient ratios highlights the presence of species specific responses to changing conditions and consequently the difficulty in making general statements relating to the impacts of changing environmental conditions. Alongside precipitation, surface wind speeds are predicted to increase across northern Europe (Nazari-sharabian et al., 2018). This would be expected to increase mixing within the water column, concentrate algal growth if directed onshore and increase the resuspension of sediments and benthic nutrients (Nazari-sharabian et al., 2018). With the effect of wind being dependent on many factors, including direction, speed and the local topography it is difficult to infer site specific impacts but it is another important meteorological factor likely to influence the future growth of harmful microalgae in GB coastal waters.

5.1.2 - Global status of harmful microalgae

As reported in the introductory chapter, there has been a long-held view by many in the field of Harmful algal blooms (HAB) and bloom impacts, that there is an increasing trend in extent and magnitude of HAB impacts globally (Pörtner et al., 2019). For the first time this has been systemically evaluated as a key aspect of the GlobalHAB project. The key findings from this initiative were that there was no clear global pattern with regards to the impacts or occurrence of harmful marine microalgae but it was determined that certain regions were experiencing a trend towards increasing impacts (Hallegraeff et al., 2021). On the other hand, some regions display either no discernible change or a decline in the impacts of harmful species of microalgae.

One region which was shown to be experiencing an increase in the impacts of Harmful microalgal species is Europe (Hallegraeff et al., 2021). In the assessment by Hallegraeff et al., (2021) this region is broad and encompasses multiple water bodies, with limited interconnection, including the North-eastern portion of the North Atlantic, Baltic, North and Arctic seas. As such, even an increasing trend in this part of the world does not imply that all harmful algal species are experiencing an upward trend in their extent or magnitude, nor does it suggest that the increasing trend in the whole region is being experienced equally or at all in all of the water bodies and coastal regions which provide the principal source of data and site of impacts. However, the finding of an identifiable increase in harmful algal impacts in the region does suggest that prediction of future changes and threats is a valuable exercise in potentially affected locations. It was noted that alongside an increase in impacts due to heightened monitoring and in some areas, expanded aquaculture (Hallegraeff et al., 2021), there have also been incidents of new, emergent harmful microalgal issues, with the first records of endemic ciguatera within European Islands occurring in 2004, with more events in subsequent years (Bresnan et al., 2021).

5.1.3 - Harmful microalgae in a changing world

An important consideration in the expansion of harmful microalgal species are the drivers of bloom formation, or the growth of impactful levels of those species causing issues at low cell densities, and how future projected climate changes will impact these. Temperature is known to be a key driver for the growth of microalgae (Kibler et al., 2015; Kulk et al., 2020; Peperzak, 2003) but it is far from the only factor. As described in Chapter 1, even within one species, *Alexandrium minutum*, bloom drivers share commonalities between affected sites but the critical bloom trigger factor can vary on spatial and temporal scales. This makes accurately assessing the future impact of climatic variation on marine microalgae, including the harmful species, fraught with difficulty. There are indications that certain toxigenic species, namely *Alexandrium catenella* (reported as *A. fundyense*) and *Dinophysis acuminata,* are likely to see an increase in both their growth rates and the window within which impactful cell densities can be sustained, as a result of changing climate

(Gobler et al., 2017). As a general observation of macro-organisms, there has been a poleward shift of marine species in response to increasing global temperatures. In the Northern hemisphere this has resulted in those species reliant on cool water conditions shifting northward with the range of warmer water species expanding to encompass higher latitudes (Mieszkowska et al., 2013).

It is often the case that only a small proportion of factors are considered in studies seeking to model the influence of these changes. Additionally, harmful microalgal species are often not considered, instead the models utilise much higher taxonomic levels, looking at the impact on organisms grouped loosely by life history or niche specialisation. A further consideration is that as climate change has increasing impacts, it is likely that planktonic communities will begin to change also. This changing community will bring with it a host of changes in the interactions of microorganisms, the dissolved chemicals present in the water column, the availability of prey or precursor chemicals and interactions with macro-organisms. For example, it has been hypothesised that future conditions are likely to increase the stress experienced by diatom species in temperate waters, reducing their potential competitive effectiveness (Glibert, 2020), this in turn has the potential to reduce the pressure on dinoflagellate species, including many of the harmful taxa. Furthermore, future climate conditions are forecast to have a negative impact on zooplankton communities, with projected declines across differing future climate scenarios (Pörtner et al., 2019). As these are a key group of grazers of phytoplankton, including harmful species, this may ease a pressure on the proliferation of harmful marine microalgae, in some areas.

5.1.4 - Harmful microalgal impacts

The impacts of harmful microalgae can be broad, of particular note being those impacts for which there is currently no active monitoring or risk mitigation in place. As covered in chapter 1, harmful microalgae can have range of impacts in the marine environment but also further afield with regards to human health impacts as a result of contamination of food products. As a great many

filter feeding organisms are poikilothermic, their metabolic rates are driven by the environmental temperature (Callaway et al., 2012). The result of an increase in coastal water temperature is therefore an increase in metabolic rate (Findlay and Turley, 2021), whilst the organism is within its thermal tolerance limits (Callaway et al., 2012). This results in an increase in feeding activity, potentially leading to an increase in the accumulation of algal biotoxins possible within a given timeframe. This means that the existing impacts of harmful marine microalgae may become more pronounced, if the harmful species is able to bloom or proliferate under future conditions, alongside new impacts becoming established, from current or emergent species.

Molluscs are often a focal group of organisms in the study of toxic marine microalgae. In North Europe the primary impact of toxic harmful microalgae is most often recorded as bivalve mollusc shellfish intoxications which require management actions to ensure seafood safety (Bresnan et al., 2021). There have been a number of incidents where people have been poisoned by the ingestion of contaminated shellfish but owing to the establishment and success of monitoring programmes the frequency and magnitude of outbreaks remain low throughout the region (Bresnan et al., 2021). The primary solution to algal toxin contamination of shellfish in classified growing areas is to issue a temporary closure notice, prohibiting the collection of shellfish from the production area for the duration of the toxin contamination (Anderson, 2009). Testing normally continues throughout the closure period and once two consecutive samples have been found to be below the maximum permitted levels, a site can reopen for commercial harvesting. As such, although closures of shellfish beds are effective for the protection of public health, they are not without cost. The closures vary in length but those which persist for many weeks or months can have substantial socioeconomic impacts (Hoagland et al., 2002). Therefore, incidents of toxin contamination can prove to be costly to coastal communities in affected areas, even when customers are protected and the shellfish themselves suffer no acute affects. Alongside food safety concerns, which whilst costly can be effectively managed, there are several reports of fisheries experiencing significant losses as a result of the impacts of harmful microalgae (Anderson, 2009). This is not dependant only on the

specific type of harmful microalgae present but also the species and situation of the affected organisms. For example, whilst the blue mussel (*Mytilus edulis*) is known to be highly resilient to the sodium channel blocking action of saxitoxins, the surf clam species (*Mesodesma donacium* (Lamarck, 1818)) has been shown to suffer intense mortalities in the presence of high levels of saxitoxin (Álvarez et al., 2019). This is due to differences in the sodium channel morphology between species but also between populations of the same species, for example populations of the surf clam (*Mya arenaria* (Linnaeus, 1758)) saw a significant change in the susceptibility to saxitoxins, with a single mutation in the sodium channel leading to a 1000-fold decrease in affinity to saxitoxin binding between populations of clams exposed to saxitoxin and those with no history of exposure (Bricelj et al., 2010) . These potential ecological impacts have wide ranging implications, if the species impacted is commonly fished or an important aquaculture species then acute impacts carry a food security risk. These two risks may not be mutually exclusive, with a potential presence of both food safety and food security risks under some circumstances.

An important aspect to consider regarding the impact of harmful marine microalgae is that these do not exist in isolation of prevailing environmental conditions. As such, climatic changes may have synergistic effects in the exacerbation of the impacts associated with harmful marine microalgae. For example, in future, organisms (such as some bivalve species) will be pushed to their temperature tolerance extremes within their current range. This will leave them more susceptible to stress from other sources, such as disease or parasites but also from harmful microalgal impacts which might otherwise be sublethal if the organism was in a state of good health. Additionally, the changing conditions may lead to the establishment of new microalgal species or an escalation of small-scale current issues. This has the potential to expose naïve populations to novel harmful microalgae, with a result that populations with no history of exposure may not have developed specific mitigation processes or behaviours that other similar populations in heavily impacted regions possess.

An emerging concept in both assessing and dealing with potential harm to humans is the "One Health" approach. In principle, this is a framework considering that human, animal and environmental health are all intrinsically linked (Gibbs, 2014). As such it can be considered that, a negative impact on animal or environmental health has a negative impact on human health. An obvious and early focus of this principle was the consideration of zoonotic diseases and the control of the spread of these between animal groups and humans (Gibbs, 2014). The One Health approach is intended to foster interdisciplinary working, with multiple expertise required for a comprehensive assessment. From this early origin, One Health has been adopted more widely and the concept has been applied to several health concerns, including harmful algae. These considerations of harmful algae and their impact on One Health have included broad studies (Backer and Miller, 2016; Nwaji et al., 2023) or more focussed evaluations in relation to a specific group of marine organisms (Turner et al., 2021). Considering the severity of an emergent harmful microalgal species under the framework of One Health requires the potential impacts on human health to be considered alongside impacts on animal health as well as possible ecosystem or environmental damage. Microalgal species presenting a risk to the health of aquatic organisms can affect food security or negatively impact marine ecosystems and so these facets are of importance alongside human intoxication risks.

5.1.5 – Invasive potential

Although changing environmental conditions will lead to the creation of new niches this does not in itself, result in the successful invasion of novel harmful species to an area. In order to have recurrent or severe impacts, an invasive species needs to establish in a new area (Smayda, 2002). This is one aspect which is poorly studied and understood in the field of harmful microalgae, with very few examples of species invasion or range extension having been conclusively demonstrated (Smayda, 2007). Furthermore, conditions favouring the growth of a harmful species in isolation does not suggest that the same organism will become dominant in all communities, as interactions between microalgal scales and across trophic levels can be important (Glibert, 2020). It is known that there are various dispersion routes for algal species, from natural vectors such as

oceanic currents (Smayda, 2007) to anthropogenic ones, such as the ballast water of ships (Hallegraeff and Bolch, 1992, 1991) or adherence to marine litter (Katsanevakis and Crocetta, 2014; Masó et al., 2003). Unfortunately, due in part to a lack of suitable baseline data, it is often impossible to determine precisely when a species became established in a given area. Even in the case of harmful microalgal species, where impacts can arise and be tracked, providing evidence of new populations being present, new detections can also arise as new areas are added to monitoring programmes or new techniques provide a greater degree of sensitivity or the ability to detect new groups of organisms or compounds. A limited number of studies have evaluated sediment cores, allowing for the assessment of historic populations of cyst forming dinoflagellates (Klouch et al., 2016a). These studies have been able to gain some insight into the historic presence of harmful species, albeit in a limited geographic region. Consequently, there are few examples available to determine the true extent of species transfer between planktonic communities. The successful invasion of non-native species is considered a high risk in the aquatic environment (Callaway et al., 2012), although microalgal species are often not considered due to a lack of baseline information.

5.1.6 – Aim of this review

The aim of this review is to take a short-term risk assessment approach to harmful microalgal species which are not currently known within British waters. This would allow for the targeting of future work towards the highest risk species considering projected anthropogenic climate change. In order to achieve this the following objectives were determined:

Collate and assess the predicted impact of climate change on future GB conditions, projected to 2050.

Collate a list of harmful algal species, both globally and within GB.

Collect data on the growth requirements of harmful algal species not currently known from GB waters.

Collate data on the impacts of harmful algal species not currently known from GB.

Analyse collected data via a risk funnel approach to allocate risk scores to each species.

From the risk scores generated in this way, it was possible to compare the risk posed by harmful microalgal species not known from GB waters. This allowed for a ranking of the risk posed by the species assessed within this chapter.

5.2 – Methodology

5.2.1 – Species selection

In order to compile a comprehensive list of harmful algal species with the potential to cause future impacts in GB waters, the International Council for the Exploration of the Seas (ICES) list of harmful algae was consulted. The list generated was cross referenced against the World Register for Marine Species to ensure that the correct nomenclature was utilised for each of the harmful species, as of 2020 when the work gathering literature began. Furthermore, the web platform Algaebase was referenced to determine if each of the harmful species was currently known from GB waters. The information of recorded occurrence was supplemented in those cases where literature searched was able to demonstrate the presence of a species of interest in GB waters. In some cases, a species had only been identified in a few or even a single source previously, as this assessment was intended to cover non-native species and future emergence these species were still excluded from further assessment. This meant that some harmful microalgae not currently well known from GB waters do not appear further in this chapter, although it does not exclude them from forming future issues. This might be the case with what can be considered cryptic species, maybe present only in a limited number of sites or at low levels, therefore not yet causing an issue. No method of detection was excluded from providing evidence of presence/absence although in the majority of cases records were from sources using traditional morphological descriptors to assign species status.

To address current presence/absence status consistently across taxa, all microalgal species known from GB waters would have needed to have their presence/absence status rigorously assessed and corroborated which was a task beyond the scope of this assessment. Indeed, reassessing historic records of occurrence in some taxa would be of great value but also a substantial undertaking in its own right, needing a substantial effort dedicated to it. As there will not only be cases of misidentification for some species but others may also have become locally extinct or have receded to cryptic status.

The initial list compiled of non-native harmful microalgal species resulted in over 100 algal species of interest which are known globally but with no recorded presence in GB waters. These species were then taken forward for further assessment as a part of this body of work.

5.2.2 – Search parameters

In order to approach the task of reviewing literature in a consistent manner between species but with many to cover, the search terms of "*Genus species* environment" were used within the web of science platform with genus and species being replaced with each of the harmful taxa on the list, in turn but the term environment remaining consistent. These terms were chosen to yield those papers with information on the environmental requirements and tolerances of the harmful algal species of interest. Only currently accepted taxonomic names were used for the searches. It is recognised that this might limit the availability of literature for certain species.

5.2.3 – Paper selection

All papers which were accessible were included in the study but due to the large body of literature for review, this encompassed the majority of articles listed in the Web of Science search outcomes with access to papers both via subscription and open access, those papers which were not readily accessible were excluded from the literature screen. Papers were then reviewed and were included in the final data compilation if they provided species specific information under one or more of the headings shown in Table 5.1. Where they provided corroborative information which had

already been determined, a note was added to show that more than one source had concluded the same ranges of tolerance. Additionally, it was noted wherever the data were generated via the use of cultured material. This provides a valuable and, in some cases, the only available information source for certain tolerances or requirements of some species. However, where possible data collected from environmental measurements taken from field studies was preferred. Papers which did not contain specific or salient information were not included in the body of literature assessed herein.

5.2.3 – Data mining

Papers were screened for the presence of data which could be linked to a definitive species identification of a harmful taxa of interest. Data were tabulated under the broad headings of invasive potential and impact. The specific data targeted are detailed in Table 5.1. Data generated via the culturing of relevant species were included, this was essential in filling in critical components of the microalgal species environmental tolerances even though data generated in this way does not provide evidence of environmental conditions suitable to stimulate a bloom, as it does not account for synergisms between abiotic factors, or the biotic interactions present in nature. In many cases, certain parameters for species of interest were only able to be ascribed values from the results of laboratory studies on cultured material. Of these parameters, several could be ascribed numeric values which could be directly compared, such as temperature and salinity. Others, including niche and predicted change were more difficult to compare between species, but were intended to provide the potential for an exclusion if certain criteria for growth of a harmful species were not present in GB waters, greatly reducing the invasive potential. An example of this would be if a species needed the presence of shallow, stony coral reefs as a substrate, as presently these structures do not exist in GB waters. A complete list of the papers utilised in the compilation of data related to species is presented in Appendix 1, as not all of these papers have also been cited in the main body of the text some appear in both the thesis bibliography and the appendix whereas those used for data acquisition and not also cited in text appear only in Appendix 1.

Table 5.1: detailing data categories extracted from literature included within this review

Invasive potential	Impact
Temperature tolerances	Compounds produced
Salinity tolerance	Taxa affected
Nutrient requirements	Extent of impacts
pH tolerance	
CO_2 requirements	
Environmental niche	
Possible transport vector	
Predicted change	

During the data mining process it became apparent that some parameters were more widely reported than others. Many papers provided data relating to those parameters with quantitative values assigned, such as salinity and temperature but it was less common to find descriptions of a specific niche. A further parameter which was rarely directly measured were nutrient loads with few studies pushing a microalgal species to the limits of nutrient tolerances. This parameter was also one for which future predictions are vague, with changes discussed in more broad terms and a key focus of change being the changes predicted in precipitation and the influence which this has on surface run off and nutrient delivery from the land. As such, whilst data were collected wherever possible for all criteria listed in Table 5.1, the majority of assessment of invasive potential was made on those more quantitative parameters such as salinity and temperature.

Whilst mining data from collected papers, data relating to organisms which were only identified either to genus level, or without a definitive speciation, such as those labelled *cf.* were not included in the final data table. Mostly this allowed for the linking of data to an individual species, to allow for the risk assessment to consider the impact of each independently. It did limit the gathering

of data in some groups, such as *Ostreopsis* where the taxonomy is still unclear and so the currently preferred system is to identify *Ostreopsis ovata* (Fukuyo, 1981) as *Ostreopsis cf. ovata.* Although this limitation led to the exclusion of some work of interest it ultimately standardised the process of data acquisition between taxonomic groups and many papers evaluated made use of definitive statements of identification, even in those genera or species with less than certain taxonomy.

5.2.4 - Case studies

In order to assess future environmental conditions likely to be present in GB waters, two locations at different latitudes were selected as case study sites. The current average conditions for these were recorded and then amended based upon current climatic projections. As there is very little information with respect to quantifiable changes in many parameters, rainfall for example, in practical terms this resulted in looking at forecast changes to temperatures. Consideration of other changes was much more difficult but as temperature is considered a critical factor in microalgal survival and proliferation this gave a useful metric to ascertain whether the survival or growth of a species was plausible based upon the thresholds published within the searched literature. The southern most site selected was St. Austell bay, Cornwall and the Northern site was chosen as the Shetland Islands. These two sites are characterised by different prevailing environmental conditions being approximately 1,100 km apart. Both case study sites currently have classified shellfish production areas and are therefore indicative of suitable locations for molluscan aquaculture at their respective latitudes.

5.2.4.1 - Cornwall

St Austell bay is a large bay opening into the English Channel. Currently the average summertime sea temperature reaches 17 °C in the month of August with the winter average being 8.9 °C and the lowest values recorded at the end of winter, in March. Adjusted upwards for projected changes by 2050 this would result in an average summer sea surface temperature of 18 °C with winter temperatures averaging around 10 °C.

5.2.4.2 - Shetland

Unlike the St Austell region, the Shetland Islands are a complex series of small Islands, with many bays and inlets. It is home to a successful shellfish growing industry composed of many separate farms. The average temperatures are considerably cooler than those experienced in the South of Cornwall. The average for the summer is 13.1 °C and in Winter this drops to 8.2 °C. Following an adjustment in the same manner as the data from Cornwall this would suggest average summer sea surface temperatures of 14.1 °C and 9.2 °C in the winter.

Consequently, the two case study sites included as part of this risk assessment share similar projected winter sea temperatures, with a little under 1 °C separating the averages, but a more substantial 4 °C difference apparent in the summer averages. The two sites were therefore considered separately at the third stage of the risk assessment process, when the potential for successful invasion and growth of an emergent species was considered.

5.2.5 - Risk assessment

The risk assessment was undertaken on all species not currently known from GB waters. As such the first exclusion occurred for all species currently recorded from GB waters, Additionally, where a GB identification of a species was found in the literature as data mining was undertaken this species was also excluded from further assessment. The principal aspects of the risk assessment process are pictorialised in Figure 5.1.

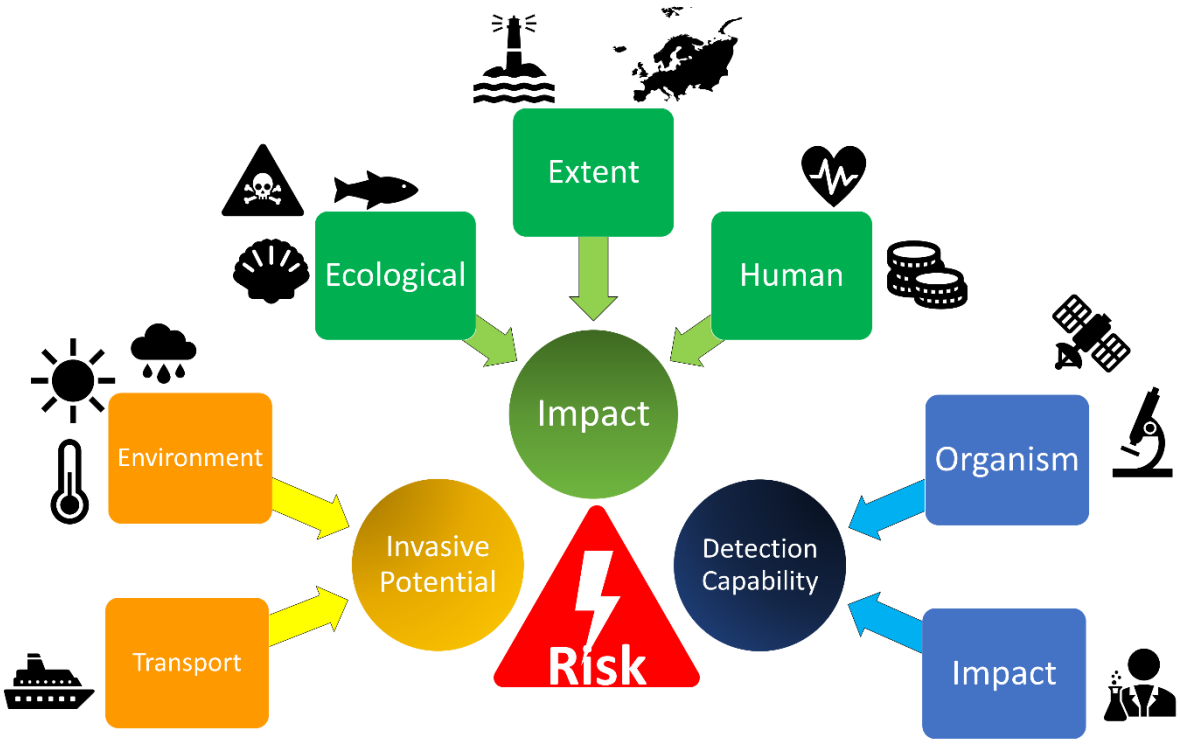


Figure 5.1: graphic showing the inputs into the risk assessment process. Colour coding is carried through to the risk funnel (Figure 5.2) approach utilised for the overall risk assessment of each species. Blue is used to display detection capabilities, Yellow is used for the factors influencing invasive potential, and Green is used to display those factors relating to the impact of the microalgal species.

For the risk assessment process itself, a risk funnel approach was taken. This involved filtering the potentially invasive species through a series of criteria, with the initial criteria being used as exclusion/inclusion parameters before the final criteria were given weighted values and multiplied together to provide a final risk rating for those species not excluded during an earlier stage of the risk funnel process. The risk funnel utilised is shown in Figure 5.2.

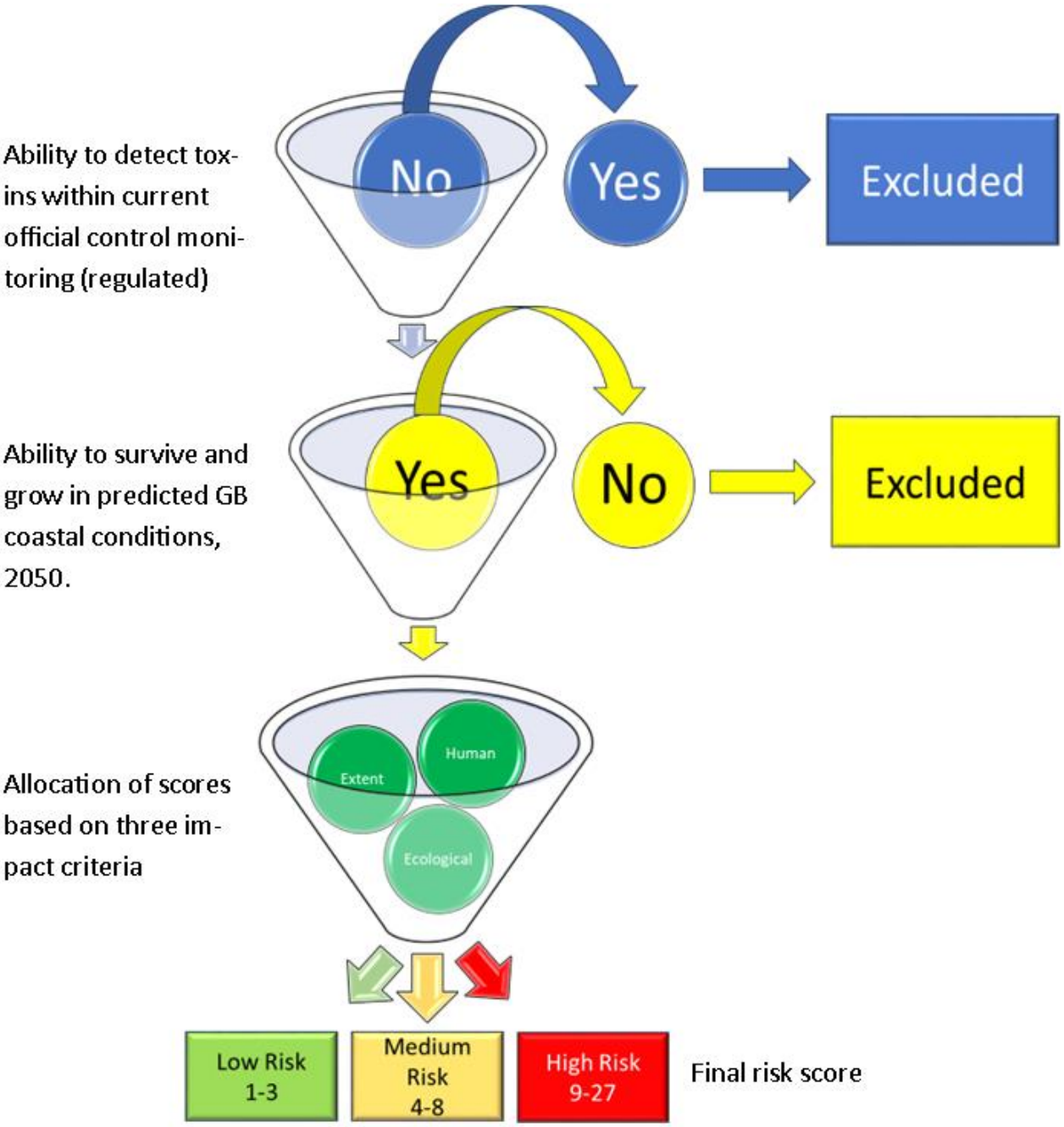

Ability to detect toxins within current official control monitoring (regulated)
No
Yes
Excluded
Ability to survive and grow in predicted GB coastal conditions, 2050.
Yes
No
Excluded
Allocation of scores based on three impact criteria
Extent
Human
Ecological
Low Risk 1-3
Medium Risk 4-8
High Risk 9-27
Final risk score

Figure 5.2: risk funnel utilised in the assessment of risk posed by each toxic species. Colours in each stage are those from Figure 5.1, highlighting the three-step approach to funnelling risk. The first (Blue) involved an exclusion step based upon the capacity to detect the toxins produced by potentially invasive species, with exclusion for those covered by official control monitoring. The second (Yellow) involved exclusion for those species without the required environmental tolerances to grow in GN coastal waters (predicted conditions 2050). The final step (Green) consisted of allocating three factors hazard scores and multiplying them together to attain a risk score between 1 and 27.

A key factor in the risk to the health and wellbeing of seafood consumers is the potential for contamination to go undetected in food produce. As such, a key factor pair in the risk assessment was the ability of assessed algal species to contaminate seafood and the current ability of monitoring programmes to detect that contamination. In cases where there is an established monitoring programme for toxins of concern, producing species of microalgae were excluded from the remainder of the risk assessment process. This meant all species known to produce only paralytic shellfish toxins (PST), amnesic shellfish toxins (AST), diarrhetic shellfish toxins (DST), azaspricaid shellfish toxins (AZT) or yessotoxins (YTX) were excluded at this stage and not assessed further. This was due to the comparatively low risk of impact of these species invading, both in terms of human health but also ecological damage due to the toxins. The chemical analytical techniques in use within the GB monitoring are targeted methodologies, therefore unable to detect any compounds beyond those included in the method. If an invasive toxin producer were to establish and cause contamination of shellfish with targeted toxin groups, these would be detected even if the producer was unknown, therefore the current monitoring would still offer protection of public health. Ecosystem impacts from toxin production could also be assumed to be analogous to the impacts already experienced within the environment from other producers of the same toxin class, which is currently low in UK waters. It was therefore decided that, within this risk assessment framework, effort should be invested in determining the potential impacts of a potentially invasive species producing a toxin class considered novel within GB waters.

The One Health impact of those species passing the first two criteria of having impacts not currently covered under the existing shellfish monitoring framework and considered as being a

potentially invasive species within the next 30 years, were then assessed for the extent and severity of their potential impact. This was undertaken by providing a score of between one and three to each of three separate risk categories aiming to capture impacts on humans, other organisms and the environment. These values were then multiplied together for each species assessed in this manner, resulting in a final score for each of between 3 and 27. Utilising multiplication is common when making use of risk matrices (Venkataraman, 2021), such as that presented in Table 5.2. The higher the value generated, the higher the assessed risk. The criteria utilised for this aspect and the weighting given to each are outlined in Table 5.2.

Table 5.2: Risk factor parameters and scoring system utilised.

	Risk Score		
	1	2	3
Risk factor			
Ecological impact associated with proliferation	No impact or sublethal deleterious effects on fish/shellfish or other species.	Lethality to a small range of relevant species	Severe impact to relevant species and the wider environment and ecology of an area
Health impact associated with proliferation	No toxic effect known in humans	Sublethal, debilitating, reversible impacts on human health	Potential fatalities and/or other irreversible impacts on human health
Environmental impact associated with proliferation	Localised	Regional	Transregional/national

5.3 - Results and discussion

5.3.1 - Risk assessment outcome: general

The first exclusion was performed on those listed harmful microalgal species which have been recorded in GB waters. As these are known to be present but either having no current impact or their impact is already assessed and controlled, via monitoring from either the official control programmes or independent industry led efforts. The outcome of these exclusions is recorded in Table 5.3.

Table 5.3: List of species excluded from further assessment due to a previous identification in GB waters. Compiled from multiple contemporary sources including single instances of detection and older checklists, such as (Dodge, 1981; Parke and Dixon, 1976) with supplemental findings from other papers incorporated where found.

Genus	Species
Halamphora	*coffeaeformis* ((C. Agardh) Levkov, 2009)
Pseudo-nitzschia	*australis* (Frenguelli, 1939)
	calliantha (Lundholm, Moestrup & Hasle, 2003)
	delicatissima ((Cleve) Heiden, 1928)
	fraudulenta ((Cleve) Heiden, 1928)
	multiseries ((hasle) Hasle, 1995)
	pseudodelicatissima ((Hasle) Hasle, 1993)
	pungens ((Grunow ex Cleve) G.R. Hasle, 1993)
	seriata ((Cleve) H. Peragallo, 1899)
	subpacifica ((Hasle) Hasle, 1993)

Phaeocystis	*globosa* (Scherffel, 1899)
	pouchetii ((Hariot) Lagerheim, 1896)
Prymnesium	*parvum* (N. Carter, 1937)
	polylepis ((Manton & Parke) Edvardsen, Wikrem & Probert, 2011
Dinophysis	*acuminata*
	acuta
	caudata (Saville-Kent, 1881)
	fortii (Pavillard, 1924)
	infundibulum (J. Schiller, 1928)
	norvegica (Claparède & Lachmann, 1859)
	tripos (Gourret, 1883)
Phalacroma	*rotundatum*
Alexandrium	*catenella*
	minutum
	ostenfeldii
	tamarense
Gonyaulax	*spinifera*
Lingulodinium	*polyedra*
Protoceratium	*reticulatum*
Pyrodinium	*bahamense* (Plate, 1906)
Amphidinium	*carterae* (Hulbert, 1957)
Karenia	*brevis* ((C.C. Davis) Gert Hansen & Moestrup, 2000)
	mikimotoi ((Miyake & Kominami ex Oda) Gert Hansen & Moestrup, 2000)
Karlodinium	*veneficum* ((D.Ballantine) J.Larsen, 2000)
Prorocentrum	*cordatum* ((Ostenfeld) J.D.Dodge, 1976)

	lima
	rhathymum (Loeblich III, Sherley & Schmidt, 1979)
Azadinium	*spinosum*
Fibrocapsa	*japonica* (S.Toriumi & H.Takano, 1973)
Heterosigma	*akashiwo* ((Y.Hada) Y.Hada ex Y.Hara & M.Chihara, 1987)

The second aspect of the assessment was to exclude those species of microalgae which are not currently known to be present in GB waters but where the toxic compounds they produce are already incorporated into the GB monitoring programme. As covered in chapter 1 this meant that any species recorded as producing toxins currently incorporated into the retained EU legislation (EC 853/2004, 2004; EU 786/2013, 2013) were not progressed to the third stage of the risk assessment process. Table 5.4 outlines which species were excluded at this stage of the process and is grouped by toxin class. From Table 5.4 it can be seen that a substantial number of ASP producing *Pseudo-nitzschia* are not currently found in GB waters but as the genus and the toxins are already covered in the monitoring programme the impact of invasion would be lowered. Similarly, there are several species of *Prorocentrum* (Ehrenberg, 1834) not currently recorded in GB waters but which have been found to be capable of producing some of the DSP toxins, this formed the largest group of excluded species based on the impact being confined to a currently monitored toxin group. Where a species was found to produce known compounds from the literature but also be capable of producing other unknown or untargeted compounds, these species were not excluded from further risk assessment.

Table 5.4: Species excluded from further risk assessment based upon their impact falling within the toxin groups currently included within the official control monitoring programme within GB.

ASP		PSP		DSP		AZA		YTX	
Genus	Species	Genus	Species	Genus	Species	Genus	Species	Genus	Species
Nitzschia	*bizertensis* (B.Smida, N.Lundholm, A.S.Hlaili & H.H.Mabrouk, 2014)	*Alexandrium*	*tamiyavanichii* (Balech, 1994)	*Dinophysis*	*miles* (Cleve, 1900)	*Amphidoma*	*languida* (Tillmann, Salas & Elbrächter, 2012)	*Gonyaulax*	*taylorii* (M.C.Carbonell-Moore, 1996)
	navis-varingica (N.Lundholm & Ø.Moestrup, 2000)		*taylori* (Balech, 1994)		*sacculus* (F.Stein, 1883)	*Azadinium*	*dexteroporum* (Percopo & Zingone, 2013)		
Pseudo-nitzschia	*cuspidata* ((Hasle) Hasle, 1993)	*Gymnodinium*	*catenatum*	*Prorocentrum*	*caipirignum* (Tillmann & Elbrächter, 2011)		*poporum* (Tillmann & Elbrächter, 2011)		
	multistriata ((Takano) Takano, 1995)				*cassubicum* ((Woloszynska) J.D.Dodge, 1975)				
	turgidula ((Hustedt) G.R.Hasle, 1993)				*emarginatum* (Y.Fukuyo, 1981)				
					faustiae (M.C.Carbonell-				

					Moore, 1996) *hoffmannianum* (M.A.Faust, 1990) *maculosum* (M.A.Faust, 1993) *mexicanum* (Osorio-Tafall, 1942) *texanum* (Henrichs, Steidinger, P.S.Scott & L.Campbell, 2013)				

Table 5.5: outcome of the risk assessment matrix applied to those species passing through the earlier stages of the risk funnel. Those species marked with an * lacked detailed tolerance data but did have sufficient data to assign values in the impact assessment. As such they were included so as not to overlook the potential impacts.

Species	Risk Factor			Risk rating
	Ecological impact associated with proliferation	health impact associated with proliferation	Environmental impact associated with proliferation	
Alexandrium pseudogonyaulax ((Biecheler) Horiguchi ex K.Yuki & Y.Fukuyo, 1992)	2	2	1	4
Alexandrium taylori (Balech, 1994)	2	1	1	2
*Coolia santacroce** (Karafas, Tomas & York, 2015)	2	1	1	2
Fukuyoa ruetzleri ((M.A.Faust, Litaker, Vandersea, Kibler, W.C.Holland & P.A.Tester) F.Gómez, D.X.Qiu, R.M.Lopes & Senjie	1	3	1	3

Lin, 2005)				
*Fukuyoa yasumotoi** ((M.J.Holmes) F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin, 2015)	1	3	1	3
Gambierdiscus caribaeus (Vandersea, Litaker, M.A.Faust, Kibler, W.C.Holland & P.A.Tester, 2009)	1	3	1	3
Gambierdiscus carolinianus (Litaker, Vandersea, M.A.Faust, Kibler, W.C.Holland & P.A.Tester, 2009)	1	3	1	3
*Gambierdiscus polynesiensis** (M.Chinain & M.Faust, 1999)	1	3	1	3
*Ostreopsis mascarenensis** (Quod, 1994)	1	3	1	3
Ostreopsis ovata	3	3	1	9
Ostreopsis siamensis (Johs.Schmidt, 1901)	2	3	1	6
*Amphidinium gibbosum** ((L.Maranda & Y.Shimizu) Flø Jørgensen & Shauna Murray, 2004)	2	1	1	2
*Amphidinium operculatum** (Claparède & Lachmann, 1859)	2	1	1	2
*Karenia bicuneiformis** (Botes, Sym & Pitcher, 2003)	2	2	1	4
*Karenia brevisulcata** ((F.H.Chang) G.Hansen & Ø.Moestrup, 2000)	3	2	2	12

*Karenia concordia** (F.H.Chang & K.G.Ryan, 2004)	3	2	1	6
*Karenia cristata** (L.Botes, S.D.Sym & G.C.Pitcher, 2003)	3	2	1	6
*Karenia longicanalis** (L.Botes, S.D.Sym & G.C.Pitcher, 2003)	3	1	1	3
Karenia selliformis (A.J.Haywood, K.A.Steidinger & L.MacKenzie, 2004)	3	2	1	6
*Karlodinium armiger** (Bergholtz, Daugbjerg & Moestrup, 2006)	3	1	1	3
*Karlodinium conicum** (Salas, 2008)	3	1	1	3
Karlodinium gentienii (E.Nézan, N.Chomérat & R.Siano, 2014)	2	1	1	2
Margalefidinium polykrikoides ((Margalef) F.Gómez, Richlen & D.M.Anderson, 2017)	3	1	2	6
Polykrikos hartmannii (W.M.Zimmermann, 1930)	3	1	1	3
Heterocapsa circularisquama (Horiguchi, 1995)	3	1	2	6
Vulcanodinium rugosum (Nézan & Chomérat, 2011)	1	3	1	3
*Chatonella antiqua** ((Y.Hada) C.Ono, 1980)	3	2	2	12

5.3.2 - Risk assessment outcome: Specific (top ranked)

Following the application of the risk assessment matrix to those species not excluded in the earlier steps of the risk funnel, there were 27 species with risk scores attributed to them, as shown in Table 5.5. This was the case for the St Austell case study, where the recorded growth and survival temperatures of assessed species were considered. For the Shetland Islands there were no additional species which passed the exclusion criteria, but of those with comprehensive environmental data only four species exhibited growth or survival at the projected 2050 summertime average temperature of 14.1 °C. These were *Ostreopsis ovata, Karenia selliformis, Heterocapsa circularisquama* and *Vulcanodinium rugosum.* Of these species, both *Ostreopsis ovata* and *Vulcanodinium rugosum* had minimum temperature tolerances of the projected summertime average for the Shetland Islands.

Overall, the majority of assessed harmful species were excluded from the analysis, or the literature assessed offered insufficient data to allow for a comprehensive assessment that passed the methods criteria. Of those species passing through the complete risk funnel more showed invasive potential for the St Austell bay case study site than for the Shetland Islands. This is not unexpected, considering the projected change in temperatures is upward for the waters surrounding GB, meaning that the warmer site is a higher at a higher risk of successful invasion and establishment of species moving Northward from warmer waters. For those four species which had information on temperature tolerances and where those tolerances were found to be at or above the projected summertime average for the Shetland Islands, half were found to have a survival or minimum growth temperature at that average. This suggests that both *Ostreopsis ovata* and *Vulcanodinium rugosum* would likely be unsuccessful invaders, with the fluctuations of temperature in the Shetland Islands during summertime meaning a considerable portion of the time conditions would be ill suited to growth of these species without prior adaptation.

Assessing the risk score against the final scoring presented in the risk funnel in Figure 5.2, it was possible to assign each assessed species a risk category of low, medium and high. In total 16 species were ranked in the low-risk category (risk score 1-3), seven were ranked as medium risk (risk score 4-8) and four species recorded a high risk rank (9-27). The high-risk category was so wide compared to the others due to the multiplication method utilised, meaning that scores above 1 achieved in all categories results in a rapid increase of risk score.

The majority of those species with a risk score were found to be a low risk after a theoretical invasion, with the majority of the risk coming from either the production of ciguatoxins, in the case of those species from the genera *Gambierdiscus* and *Fukuyoa,* or the environmental damage associated with species from *Amphidinium, Karlodinium, Coolia, Alexandrium* and *Polykrikos.* The medium risk category tended towards species with a high environmental impact as well as wide extent of impact, this was the case for some of the *Karenia* species, *Margalefidinium polykrikoides* and *Heterocapsa circularisquama.* In all of these cases, the reported ability of these species to cause widespread blooms and therefore impacts (Cortés-Altamirano et al., 2019; Matsuyama, 2012; Park et al., 2019) was a key factor in their elevated risk upon invasion. Finally, those species which were assessed as being of the highest risk all scored at the highest level in two categories, *Ostreopsis ovata* and *Ostreopsis siamensis,* or had a high score in one category with then a medium score in the remaining two, *Karenia brevisiculata* and *Chatonella antiqua.*

It became apparent as the task progressed that when defining the parameters for the Risk Assessment, it was relatively simple to choose relevant features which may affect microalgal growth as these have been well studied and documented (Masó and Garcés, 2006; Sellner et al., 2003). Conversely, it was very difficult to gather the data on certain parameters for any or all species. Similarly, whilst it was possible to gather data on the current state of case study locations, finding published information on the projections into the future was much more challenging. As such, whilst a great deal of data were gathered for the majority of assessed species only a small proportion of

this was able to be of use when informing the risk assessment. Consequently, the site and risk assessments utilised less of the algal growth factors than originally intended when assessing the suitability of a candidate species to be able to survive and grow in GB waters. Still, the data were of value in excluding a wide range of potentially invasive species whilst capturing the threats of those remaining throughout the process.

As such, a hierarchy of risk could be established with a key emergent feature being that the most likely future invaders would have a broader ecological, and so food security impact, than the current harmful algal species known to be present. Largely, this was due to the current impacts being as a result of the shellfish toxin groups known to cause intoxication of shellfish with compounds harmful to humans and other terrestrial organisms but showing little or no effect in the common aquaculture bivalve species where the monitoring is focussed. On the other hand, those species scoring highly in the risk assessment process here were known to cause a direct sublethal threat to people in areas adjacent to coastal waters but have not been associated with large outbreaks of food poisoning, although it has been found the compounds can accumulate in edible species (Amzil et al., 2012; Echevarria et al., 2012; McFarland et al., 2015; Watkins et al., 2008). Also, these species of *Ostreopsis* and *Karenia* are better known to cause widespread damage within the environment when they reach high concentrations. Other algal groups which cause widespread seafood contamination with compounds falling outside of the scope of the GB monitoring programme are constrained to warmer waters, predominantly being producers of Ciguatera. Although Ciguateric fish poisoning is a common occurrence in those afflicted regions, the species responsible for the production of those toxins were mostly not found to have a survival threshold within the temperature range projected for the southern GB case study site. Only *Gambierdiscus carolinianus, G caribaeus* and *Fukuyoa ruetzleri* had evidence supporting survival at the projected summertime temperature average (Kibler et al., 2012; Leung et al., 2018; Tanimoto et al., 2013). Even in these cases the temperatures required for growth fell above the projected summertime maxima of the sites assessed, suggesting that even if survival was possible during some seasons, this

would be unlikely to equate to active growth, in turn suggesting that there would be little risk of contamination of grazing fish populations. Furthermore, there are some fundamental differences between the community structures of grazing fish between those warm water areas considered as ciguatera endemic and the colder temperate waters of GB (Ebeling and Hixon, 1991). This includes not only the fish present but also the habitats which they inhabit and the trophic structures surrounding them. This could well pose a further barrier to the successful establishment of a ciguatera producing microalgal species, with temperate reefs often colonised by macroalgae forming a canopy above the algal turfs in contrast to tropical systems where there is rarely a macroalgal canopy (Ebeling and Hixon, 1991). Ciguatera toxin producing microalgal species are often associated with algal turfs and so a change in their distribution or light availability might reduce the possibility of successful establishment.

5.3.2.1 - Karenia selliformis and Karenia brevisiculata

Although this species was one of the medium risk species, *Karenia selliformis* was found to possess one of the most favourable growth temperature tolerances of all species assessed. Presently *K. selliformis* is known from the tropics and the southern hemisphere, in both Australia and New Zealand. The temperature tolerances of *K. selliformis* were favourable for growth in GB waters at both of the case study sites evaluated in this assessment, with minimum temperature tolerances of 10 °C and with a very wide upper tolerance of up to 32.5 °C (Feki-Sahnoun et al., 2017; Feki et al., 2013). This was one of the few species showing the potential for growth at both of the case study sites. This species also has a broad salinity tolerance, allowing for growth from estuarine waters through to fully marine and up to hypersaline levels of up to 45 (Feki et al., 2013; Salem et al., 2015). The most credible barrier to the successful invasion of GB waters by this species is the geographic separation from known global locations of occurrence and GB. The impacts associated with *K. selliformis* are most severe in the environment, with reports of mortalities among fin fish and shellfish (Brand Campbell and Bresnan, 2012). This is thought to be attributable to an unidentified ichthyotoxin as well as the presence of gymnodinimines (Tatters et al., 2010; Walid et al., 2010).

There have also been unconfirmed suggestions of the ability of this species to produce brevetoxins, the causative agent of Neurotoxic shellfish poisoning (Brand Campbell and Bresnan, 2012). This last aspect does provide a suggestion that there would be a human health risk associated with outbreaks of the species but as this was unconfirmed it was not considered within the risk score assigned.

Similar issues are ascribed to *K. brevisiculata* as*, K. selliformis* and although it does not have the suggestion of brevetoxin production, sodium channel disruption has been observed (Brand, Campbell and Bresnan, 2012). It has been shown to cause widespread marine mortalities affecting a number of invertebrate taxa, fish and also other algal species in a single bloom, affecting a wide area (Kröger et al., 2006). Unfortunately, in the literature reviewed there was little information on the environmental tolerances of *K. brevisiculata,* instead the average environmental parameters of Wellington Harbour (New Zealand), the area where the reported bloom occurred, are presented. These are analogous to the conditions found in the SW of GB, suggesting that there is a risk of successful growth were *K. brevisiculata* to reach these waters, although it was noted that *K. brevisiculata* bloomed under unusually high water temperatures (Kröger et al., 2006). This might suggest that *K. brevisiculata* exists as a cryptic species in Wellington harbour, able to rise to prominence in the community when the conditions allowed.

5.3.2.2 - Chatonella antiqua

The only Raphidophyte scoring as high risk, *Chatonella antiqua,* is known for causing widespread mortality of finfish (Daranas et al., 2001) and has also been reported as producing brevetoxins (Brand, Campbell and Bresnan, 2012). As such, they have a potentially large-scale ecological impact as well as the ability to negatively impact human health via molluscan vectors. As brevetoxins are the causative agents of Neurotoxic Shellfish Poisoning (NSP) (Watkins et al., 2008), there is a possibility of a bloom of *C. antiqua* intoxicating shellfish from a production area and at present, there is no established monitoring programme for brevetoxins in shellfish in GB. Methodologies and monitoring programmes have been established in high-risk areas, principally

around the Florida coast (Watkins et al., 2008). Although brevetoxins have an effect on nerve cells, they have not been associated with human fatalities (Watkins et al., 2008), symptoms instead are a mix of neurological and gastrointestinal effects (James et al., 2010; Watkins et al., 2008). There have also been incidents of aerosolised toxins causing an impact on human health at beaches in Florida (James et al., 2010), these were, however, associated with blooms of *Karenia brevis* so the possibility of *C. antiqua* causing this nature of impact is speculative rather than demonstrated. As such, the considerations in this risk assessment were centred on the environmental impacts and the possibility of shellfish intoxications. Furthermore, there was a lack of environmental information in the papers reviewed for this assessment, making the invasive potential of this species hard to accurately assess here.

5.3.2.3 - Ostreopsis ovata/siamensis

Both species of *Ostreopsis* which scored highest in this assessment share similar characteristics although the extent of their risks varies. Of the two, far more data were available in the literature accessed for this assessment concerning *O. ovata.* As a result, *O. ovata* was found to have a slightly more broad temperature tolerance range, with survival documented as low as 14 °C (Scalco et al., 2012) and *O. siamensis* at 15 °C (Tanimoto et al., 2013; Zina et al., 2012). Both of these lower temperatures would allow for the growth and survival of these species within the southern case study site. The impact of *O. siamensis* was more limited than that of *O. ovata,* with the production of palytoxin found alongside yessotoxins (Zina et al., 2012) giving rise to potential human intoxications, but the environmental impacts within the literature reviewed here cited sea urchins as the primary marine invertebrate taxa negatively impacted by blooms of *O. siamensis* (Shears and Ross, 2010; Tartaglione et al., 2016), without substantial evidence of wider reaching impacts. On the other hand, *O. ovata* has similarly been shown to produce a wide range of compounds harmful to humans, including palytoxins and ovatoxins (Ciminiello et al., 2014a; Durán-Riveroll et al., 2019; Guerrini et al., 2010) as well as additional reports of hemolytic activity (Granéli et al., 2011). An additional route of human exposure to harmful compounds produced by O. ovata is via the

production of aerosols, which can be carried onshore and directly impact the respiratory functions of beach goers (Vidyarathna and Granéli, 2013). Further to this, this species has been well documented as causing mass mortalities amongst multiple groups of marine organisms (Aligizaki et al., 2008; Mecozzi et al., 2011), suggesting the possibility of widespread ecological damage in areas affected by blooms. Consequently, *O. ovata* scored highly in terms of both human health impacts and environmental damage, lending it the high-risk factor score of 9.

5.3.3 – Risk assessment process

The risk assessment process utilised in this chapter was able to provide a graded score of risk posed by each harmful microalgal species which passed through the earlier stages of the risk funnel. The process used allowed for the exclusion of harmful microalgal taxa based on available information and allowed the final assessment of only those harmful taxa with theoretical establishment capability and posing novel risks to GB waters. However, a lack of information for several harmful species meant that some microalgal species passed through the filter stages of the risk funnel without adequate assessment, this ensured that they were not overlooked with respect to the risk they could pose but might also mean that they should have been excluded had more data been available within the literature returned during the searches used. This could result in the over estimation of the risk posed by certain species, especially those noted with an * in Table 5.5. Conversely, there was an evident difference in the amount of literature returned under differing search terms, with some harmful taxa returning many more relevant papers for data mining than others. This meant that it was possible to collect far more data for some species than others, providing a more comprehensive data set to later evaluate them with. This suggests that some microalgal species have attracted significantly more research effort than others, which is to be expected, but this also makes it more straight forward to conduct future work with those taxa already well researched, further compounding the issue.

Another possible bias in the final assessment scores were the criteria assigned to each risk factor score. Although it was possible to derive a numeric score for each species, the scoring of the criteria was subjective, with the categories and numbers assigned to each chosen based on the perceived value of their impact. Finally, as remarked above, it was difficult to find specific predictions for many of the environmental criteria for which data were gathered for each of the harmful taxa assessed. This meant that whilst data for many criteria were collected, the value of this was not able to be incorporated into the risk assessment conducted here. With regard to this last element however, it should be noted that predicted shifts in parameters such as salinity or nutritional requirements were considered but not seen to act as a barrier to the potential establishment of the harmful taxa assessed. As the data for these criteria were either very broad in the case of many harmful taxa or the projected future changes to these parameters in GB waters are either nebulous or are unlikely to shift beyond the tolerances found within the species assessed where these data were present.

5.4 - Conclusion

Of the two case study sites assessed, the SW of GB appears to be at higher risk of invasion from a non-native harmful microalgal species than does the North of Scotland. This would then be a suggested region for more intense surveillance with this in mind.

From the assessment conducted here one key element common to those high risk microalgal species was their ability to negatively impact the marine environment. A great many harmful species known from the coastal waters of GB are those responsible for producing the shellfish toxins, not typically associated with environmental impacts but instead a threat to human health if they enter the human food chain. Furthermore, many of the species of harmful marine microalgae globally which are not known in GB coastal waters, also produce these same toxin classes. Owing to the well-established and comprehensive monitoring programme in place within GB for those regulated classes of biotoxins (PSP, ASP, DSP, AZP & YTX), the emergence of further

producers is primarily a threat to the socioeconomics of the regions where shellfish production is important in the local economy. Whilst this impact could be severe for these communities, additional producers of these regulated toxin groups are unlikely to present a serious risk to human or aquatic organism health, unless the producing organism additionally harbours compounds capable of causing damage to marine flora and fauna. Consequently, it seems that the greatest risk from an emergent species would be one where both human and environmental aspects might be observed.

A primary candidate in this regard from this assessment would be *Ostreopsis ovata,* which was shown to produce compounds capable of causing human intoxications as well as negatively impacting a range of marine organisms. This species has also been associated with the release of toxic aerosols, presenting a new risk pathway for human intoxications were it to arrive in GB waters. Finally, *O. ovata* is well researched internationally, meaning that of those high-risk species from this assessment, it was the most well represented in the literature and so had the most comprehensive data set to analyse for invasive potential. This risk assessment therefore posits *O. ovata* as the most likely high risk non-native species for the SW of GB by 2050.

Chapter 6 - Assessing the potential impacts on food safety and food security in the event of an emergent future bloom of *Ostreopsis* cf. *ovata* within the Southwest of Great Britain

6.1 - Introduction

Following the risk assessment undertaken in chapter 5, *Ostreopsis ovata* was identified as a high-risk potential invasive species for the Southwest of Great Britain by the year 2050. As such, this species was selected for experimental assessment, to determine its potential impacts on farmed shellfish from the Southwest region of Great Britain (GB).

The genus *Ostreopsis* (J. Schmidt 1901) contains twelve species of which seven are considered harmful (Akselman and Fraga, 2023). The impacts of this genus are spread from the tropics through to temperate waters as the genus is widely distributed (Penna et al., 2012), with an increasing trend of occurrence in temperate waters (Berdalet et al., 2017). Unlike any other harmful algal species, including those belonging to the genus *Alexandrium,* investigated earlier in this thesis, *Ostreopsis* is a benthic species. Rather than the vegetative cells existing in a planktonic form they instead adhere to solid substrates, such as rocky structures, both natural and artificial, and macrophytes such as macroalgae and seagrasses (Fraga et al., 2012; Parsons and Preskitt, 2007; Totti et al., 2010). Along with *Gambierdiscus, Prorocentrum* (Ehrenberg, 1834) and *Coolia* (Meunier, 1919), *Ostreopsis* is therefore considered as one of the harmful benthic genera (Parsons et al., 2012), with proliferations of these sometimes labelled as benthic harmful algal blooms or BHAB (Berdalet et al., 2017). In order to bind to substrates, *Ostreopsis* produces mucous exudates which bind to the surface of the substrate, the *Ostropsis* cells then inhabit this mucous layer (Giussani et al., 2015). This layer and the cells within may become dislodged from the surface of substrates, as the cells are considered only loosely adhered this can occur with limited water movement, this can also result in *Ostreopsis* appearing in the water column (Accoroni and Totti, 2016; Monserrat et al., 2022; Pistocchi et al., 2011). The cells are motile, able to swim to adhere to substrates as well as to

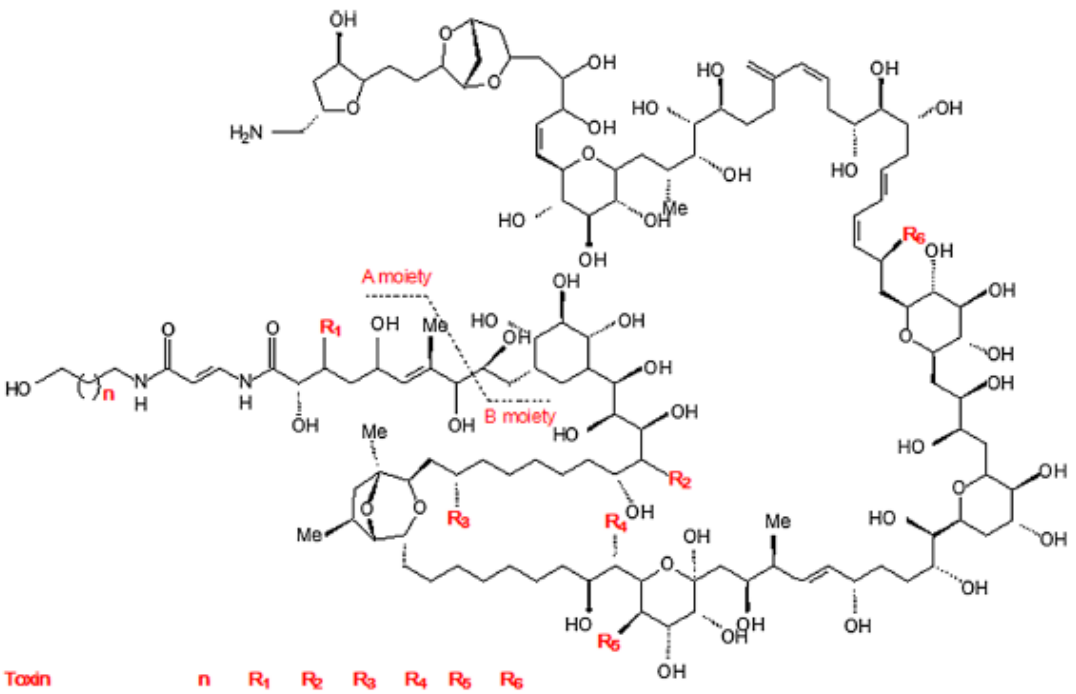
facilitate sexual fusion within populations (Accoroni and Totti, 2016). Although *Ostreopsis* is recognised as an important genus, the taxonomy of this group of microalgae is less clear (Parsons et al., 2012). This stems from a lack of morphological characteristics which can be considered truly diagnostic of a morphospecies, resulting in many descriptions which could apply to more than one species as several characteristics overlap (David et al., 2013; Parsons et al., 2012). This complicates the taxonomy as morphological identification between species of *Ostreopsis* can be challenging, with morphological plasticity observed within the genus (Accoroni and Totti, 2016). Furthermore, although there are genetic distinctions between species there are also substantial genetic differences within species, meaning that the molecular evidence is also yet to be fully refined. As such, two important species, *O. siamensis* and *O. ovata* have been suggested to be better referred to at present as *Ostreopsis.* cf. *siamensis* and *Ostreopsis* cf. *ovata* respectively, until the molecular taxonomy can be fully refined (Parsons et al., 2012; Penna et al., 2010). As such, throughout this chapter hereafter, the term *Ostreopsis* cf. *ovata* or *O.* cf. *ovata* will be used.

Within the *Ostreopsis* genus, *Ostreopsis* cf. *ovata* is a known harmful species. The lifecycle of *O.* cf. *ovata* is similar to many planktonic dinoflagellates regarding its timings and features, although it is predominantly epiphytic, epizoic or epilithic during its lifecycle. Although *Ostreopsis* is primarily considered a benthic genus, there have been reports of species being found free living in the phytoplankton, (Accoroni and Totti, 2016). It is thought that these free-living stages might be a result of dislodgment of previously attached cells and so *Ostreopsis* is considered tychoplanktonic alongside epibenthic (Faust and Gulledge, 2002). Cell division was observed to occur overnight, during hours of darkness (Bravo et al., 2012). Furthermore, both asexual and sexual reproduction have been observed along with the formation of three types of cysts, similar to *Alexandrium minutum* as covered in chapter 2. These cyst types were pellicle, thin-walled and thecate, with the first two types able to germinate within 24 hours of suspension in fresh water and variable rates of germination achieved for the thecate cysts (Bravo et al., 2012). A further study found similar pellicle cysts to be capable of rapid germination, within a matter of days, with double walled cysts seemingly

constituting a longer-term resting stage, showing viability for at least five months (Accoroni et al., 2014). Viable cysts were found embedded in mucous, suggesting a possible protective capacity for the cysts, with double walled cysts remaining viable for several months at least and it was posited that this constituted an overwintering strategy within the species (Accoroni et al., 2014; Bravo et al., 2012). There appeared to be a temperature threshold for successful germination of the double walled resting cysts isolated from culture, of 25 °C, this might be a mechanism by which the timing of germination is coordinated to result in the success of the subsequent vegetative population and has been suggested also for other encysting dinoflagellate species (Accoroni et al., 2014). This is an important mechanism to be present in a potentially invasive species as it suggests a capacity for survival at suboptimal conditions, allowing an encysting microalgal invader to more easily establish a viable population in subsequent years to its initial introduction.

The harmful nature of *O* cf. *ovata* stems from the production of a range of compounds, collectively known as the palytoxins (PlTX). These were initially isolated from the soft coral genus *Palythoa,* from which the toxin family derives its name (Deeds and Schwartz, 2010). The palytoxins are large molecules, with both hydrophilic and hydrophobic properties and has one of the longest carbon chains of any known natural product (Ramos and Vasconcelos, 2010). In a similar manner to other marine biotoxins of algal origin, it is thermally stable and so is not inactivated by boiling and is stable under neutral conditions (Ramos and Vasconcelos, 2010). Palytoxin itself is considered to be one of the most toxic non-proteinaceous compounds currently discovered in nature (Patocka et al., 2018). There are four classes of palytoxin currently described; palytoxins, ovatoxins, ostreocins and mascarenotoxins, with around 20 compounds across the four groups (Patocka et al., 2018). The structures of palytoxin and some of the congeners are shown in Figure 6.1. The biological mechanism for the toxic effects of both palytoxin and ostreocin-D have been determined, with both having the same target and mode of action but with palytoxin being the more potent of the two compounds. The toxins bind to sodium-potassium pumps, which cross cell membranes and render them open cationic channels, leading to a breakdown of the ability of cells to regulate

concentrations of cations (Deeds and Schwartz, 2010; Ramos and Vasconcelos, 2010). Further work has shown that ovatoxin-a has a toxicity between that of palytoxin and ostreocin-d (Pelin et al., 2016). Although the palytoxin compounds differ in toxicity they are considered to be highly potent with the European Food Safety Authority suggesting a safe threshold of total palytoxins in contaminated shellfish of only 30 µg/kg of shellfish tissue (EFSA, 2009). There is further evidence of damage to the cytoskeleton and tumour promotion within cell assays (Patocka et al., 2018; Ramos and Vasconcelos, 2010). Although the palytoxins were first identified in soft coral, members of the *Ostreopsis* genus have subsequently been found to be a producer. For *O.* Cf. *ovata* specifically, a range of congeners have been detected associated with this species. Multiple ovatoxins have been described from Mediterranean *O.* cf. *ovata,* with ovatoxins a, b, c, d, e and f described, as well as putative palytoxin (Ciminiello et al., 2012, 2010). Typically, ovatoxin-a is a primary constituent of the toxin profile but other ovatoxins in *O.* cf. *ovata* can make up a substantial component of the toxin profile (Accoroni et al., 2011; Ciminiello et al., 2010; Guerrini et al., 2010) and ovatoxin-f has been shown to be dominant in at least one strain, with ovatoxin-a the second greatest contributor (Ciminiello et al., 2012). A separate profile within *O.* cf. *ovata*, where ovtx-a is entirely absent and toxicity is instead constituted of ovtx-b and c has also been observed (Tartaglione et al., 2017). Any method employed for the detection of toxins associated with *O.* cf. *ovata* in either algal cellular matrices or shellfish needs to be capable of detecting not only palytoxin but preferably also the ovatoxins to ensure that all likely associated toxins are identified.



Toxin	n	R_1	R_2	R_3	R_4	R_5	R_6
Palytoxin	1	Me	OH	Me	H	OH	OH
42-hydroxypalytoxin	1	Me	OH	Me	OH	OH	OH
Homopalytoxin	2	Me	OH	Me	OH	OH	OH
Bishomopalytoxin	3	Me	OH	Me	OH	OH	OH
Neopalytoxin	1	-	OH	Me	H	OH	OH
Deoxypalytoxin	1	Me	OH	Me	H	OH	H
Ostreocin B	1	H	H	H	OH	OH	OH
Ostreocin D	1	H	H	H	OH	H	OH
Ovatoxin a	1	Me	OH	Me	OH	H	H

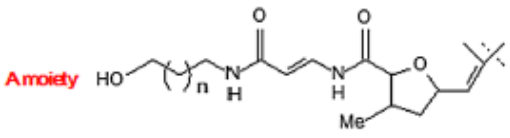


Figure 6.1: Structure of palytoxin and congeners, from Patocka et al., (2018)

It has been found that *O.* cf. *ovata* prefers shallow waters with temperature ranges between 14 °C and 32 °C (Scalco et al., 2012). It also shows resilience in areas experiencing relatively high levels of water movement (Parsons and Preskitt, 2007), growing where wave action suppresses other benthic dinoflagellates. Nutrient limitation is thought to substantially impact the growth of *O.* cf. *ovata,* with considerable drops in cell abundances during culture experiments where nutrients were limited when compared to unlimited controls (Accoroni et al., 2014). With regard to substrate preferences, a preference for hard substrata, such as mollusc shells has been observed (Richlen and Lobel, 2011), as well as for finely branching macrophytes (Accoroni et al., 2011). When cell numbers increase and toxin concentrations, both intracellular and extracellular, in the mucilage are therefore

also elevated (Giussani et al., 2015), wide ranging health impacts can occur. In humans the principal mechanism for exposure is either dermal contact for bathers or inhalation of toxin containing aerosols from coastal users (Accoroni and Totti, 2016). It has also been shown that the *O.* cf. *ovata* cells themselves can be transported in marine aerosols, leading to direct contact with not only toxins but also cells (Ciminiello et al., 2014b). Typical symptoms following these routes of exposure include fever, bronchoconstriction, conjunctivitis, rhinorrhoea mild dyspnoea and skin irritation, with serious cases needing hospital treatment (Accoroni and Totti, 2016; Deeds and Schwartz, 2010; Ramos and Vasconcelos, 2010). There have also been incidences of food poisoning from palytoxins, with fatalities reported, these have been associated with crabs and finfish in the Philippines and Madagascar respectively (Faimali et al., 2012). The condition known as clupeotoxism, a human condition resulting from eating contaminated sardines, herring or anchovies, is also suspected to be caused by those fish being contaminated with toxic *Ostreopsis* (Accoroni and Totti, 2016). As well as affecting humans there are several instances of *O.* cf. *ovata* being associated with ecological damage, negatively impacting a range of marine organisms. Blooms of *O.* cf. *ovata* have been found to cause mortality of sea urchins, including larval stages (Totti et al., 2010; Vidyarathna and Granéli, 2012). Other organisms affected include molluscs, crustacea, holothurians and poriferans (Accoroni and Totti, 2016; Turner et al., 2021), with even macroalgae being found to suffer mortality in association with blooms of *O.* cf. *ovata* (Accoroni and Totti, 2016). A study assessing the impact on mussels from the Mediterranean (*Mytilus galloprovincialis*) observed mortality in study organisms, with complete mortality within 24 hours with a cell density of 1,000,000 cellsL^{-1} and mortality of around 10% after 48 hours of exposure to 300,000 cellsL^{-1} (Carella et al., 2015). In addition to the mortalities, sublethal inflammation and lesions of multiple shellfish tissues was observed after 24 hours of exposure. Recovery after 21 days was found to be incomplete following only 48 hours of exposure, suggesting potential long-term consequences for molluscan health even after sublethal exposure to *O.* cf. *ovata* (Carella et al., 2015). As well as these negative impacts on marine organisms there is evidence of the ability of bivalve shellfish, specifically mussels, to accumulate palytoxins

originally derived from *O.* cf. *ovata* (Accoroni and Totti, 2016). As such, *O.* cf. *ovata* poses a potential threat to human health, directly through inhalation and skin contact and via seafood intoxications, as well as the health of marine ecosystems. This makes it a particular concern for food safety, food security and public health reasons.

The distribution of *O.* cf. *ovata* is broad, covering the Pacific, North Atlantic, South Atlantic, Indian and Mediterranean seas (Durán-Riveroll et al., 2019; Penna et al., 2012). In the Mediterranean, *O.* cf. *ovata* is known to co-occur with two other species of *Ostreopsis, O.* cf. *siamensis* and *O. fattorussoi* (Accoroni et al., 2016). It has been suggested that the genus is increasingly expanding in temperate regions, with a substantial increase in publications relating to *Ostreopsis* detection in temperate waters after 2000 (Shears and Ross, 2009). The extent of *O.* cf. *ovata* in the North Atlantic is of particular concern for GB. Initially in the North Atlantic *O.* cf. *ovata* was described from the Southwestern Portuguese coast (David et al., 2013, 2012). Most recently *O.* cf. *ovata* has been detected along the French Atlantic coast as far as the Basque region (Chomerat et al., 2022) with *O.* cf. *siamensis* being reported even further North (Kevin et al., 2021). This is indicative of a progressive Northern extension of the range of the genus *Ostreopsis* and specifically *O.* cf. *ovata* with this highlighting further the risk of a future invasion of this species, as suggested in chapter 5. With poleward range shifts being predicted to be common (Sorte et al., 2010), offering an explanation for the continued northern discoveries of *Ostreopsis.* There has even been suggestion that blooms of *Ostreopsis* are likely to go undetected even if human intoxications occur unless they are severe enough to require hospitalisation (Pfannkuchen et al., 2012). This would likely be exacerbated in regions where monitoring programmes and appropriate sampling for detection are lacking and there is no history of such events and so a lack of public awareness.

6.1.1 Aims and objectives

This chapter aims to assess the potential impacts of an emergent population of *O.* cf. *ovata* in the Southwest of GB, in the context of both food safety and food security, as both aspects could

be threatened by an emergence of this species. The other risk which an emergent population of *O.* cf. *ovata* could pose within GB would be the threat to public health of aerosolised ovatoxins, which could cause direct intoxication of humans utilising coastal regions. This was beyond the scope of this chapter however, with no facilities to safely test such a possibility. As such the work presented herein is constrained to the impacts which *O.* cf. *ovata* could have on shellfish health and the uptake of ovatoxins into shellfish via direct consumption of *O.* cf. *ovata* cells.

The first aim was to determine the impact *O.* cf. *ovata* could have on the health and uptake of palytoxins within GB shellfish, following direct exposure to live cells of *O.* cf. *ovata.*

To achieve this work was undertaken to expose naïve shellfish from the SW of GB to toxic and non-toxic strains of *O.* cf. *ovata* under controlled conditions. These shellfish would be observed for behavioural changes during exposure. Shellfish would also be analysed for the presence of palytoxins to assess uptake. Additionally, shellfish would be sampled for potential future analysis by the histopathology team at Cefas if there were obvious health impacts on the study animals. This would help to determine the impact of exposure to the two different *O.* cf. *ovata* strains employed within this study.

Additionally, there was a requirement to develop tools to detect the toxins produced by *O.* cf. *ovata* and the experimental work within this chapter provided samples to assess the viability of mass spectrometry-based assays, recently established at the Centre for Environment, Fisheries and Aquaculture Science (Cefas), to provide identification and quantitation of palytoxin analogues. A secondary aim was therefore to apply these new techniques to the experimentally challenged shellfish tissues as well as algal cultures.

This was to be achieved by utilising a recently developed and implemented mass spectrometry methodology for the analysis of palytoxins in GB shellfish as well as strains of *O.* cf. *ovata*, with the intention of providing a range of samples and sample toxicities to analyse. This is

intended to add to a potential toolkit for the monitoring and mitigation of food safety risk from the potential future occurrence of *O.* cf. *ovata* within UK waters.

6.2 - Methodology

6.2.1 - Experimental design

The experiment was designed to allow for the identification of impacts caused by the presence of both an *O. ovata* strain producing palytoxins/ovatoxins, as well as a non-toxin producing strain. The non-toxic strain used in this study was *O.* cf. *ovata* CCMP 3496, originally isolated from Madeira Island, Portugal, the toxic strain was *O.* cf. *ovata* CBA 3322, which was originally isolated from Italian waters in the NW Adriatic Sea. Mussels exposed to the microalgae were observed on a daily basis. Throughout the study mussels were sampled for histological analysis and the remaining parts of the organisms taken for the analysis of toxins. The mussels utilised were sourced from a wild population in the Southwest of England, near to one of the case study regions utilised during the risk assessment in chapter 5 (5.2.4.1). As discussed in chapter 4, both *Mytilus edulis* and *Mytilus galloprovincialis* are present in UK waters, as well as hybrids thereof (4.1). As a result, the precise species of *Mytilus* utilised in this study was not determined and so is more correctly referred to as *Mytlius sp.* throughout.

6.2.2 - Shellfish sourcing from the Southwest GB

6.2.2.1 - Site selection and approval from the Fish Health Inspectorate

As the samples would undergo histological analysis there was a possibility of finding internal parasites or diseases not connected with the exposure study, which would impact upon the future viability of a food business. As this project was a not a sanctioned monitoring or inspection effort it was decided that the needs of businesses should be strongly considered. In discussion with the Fish Health Inspectorate (FHI), the Tamar estuary was suggested as a suitable site for the collection of shellfish. There is currently no commercial shellfish production in this region, as it is already subject

to a disease designation for the protozoa *Marteilia* (Grizel, Comps, Bonami, Cousserans, Duthoit & Le Pennec, 1974) (Figure 6.2). There is also a well monitored area within the estuary, Jupiter Point, which has been used for the collection of marine organisms, including mussels, for research work at the Cefas. Finally, this estuary is within the high-risk area of the Southwest of GB identified in chapter 5, meaning that it is more likely to be within the affected area, of a successful invasion of *O. ovata.*

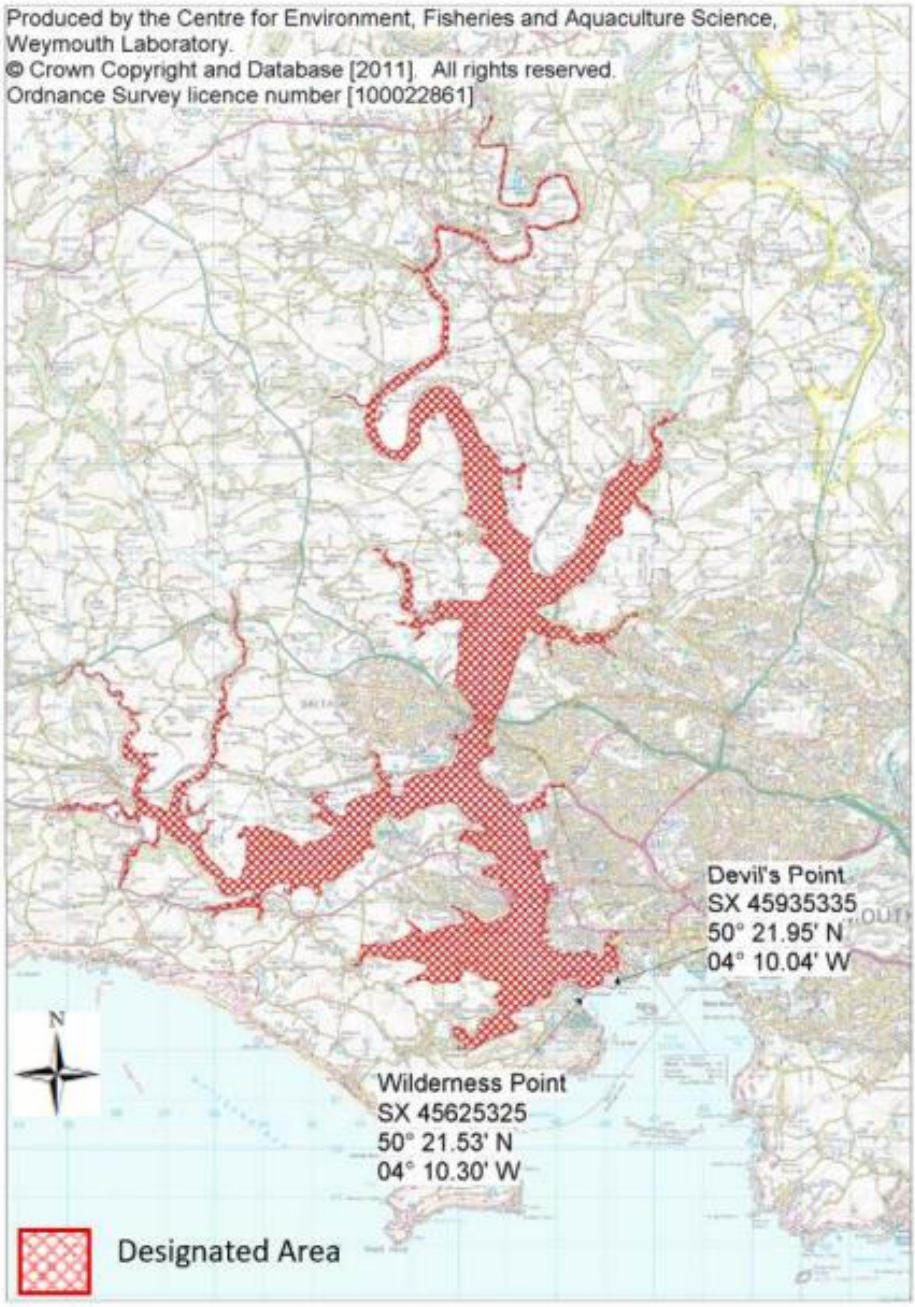


Figure 6.2: Map showing the designated area for control of *Marteilia refringens* (Grizel, Comps, Bonami, Cousserans, Duthoit & Le Pennec, 1974) in the River Tamar

Due to the nature of the site and the restriction on harvesting of shellfish from the area, approval to remove shellfish for the purposes of this study was sought and granted by the Fish Health Inspectorate. Alongside approval, further guidance on biosecurity practices were provided.

6.2.2.2 - Fieldwork

Following on from the attainment of approval to sample shellfish from within the Tamar estuary (Figure 6.2), field trips were planned prior to each tank study. Field trips were undertaken at Jupiter Point (Figure 6.3), which is a small accessible beach in the Lynher River, which feeds into the Tamar. A secondary site, Torpoint, was selected for its close proximity to Jupiter Point, this was intended to be a reserve site which could be visited in the case that insufficient organisms were sourced from the primary site. Each study required the collection of *Mytilus sp.* from wild populations, as there is no commercial production in the area to purchase the required organisms from. . Due to the tides at the sample site and the need for a spring low tide for the exposure of parts of Jupiter Point, field trips needed to be planned around the tidal cycle, limiting the available days for successful field sampling. Consequently, as suitable sampling dates were separated by several weeks, only a single sampling trip could be undertaken per study. During the fieldwork *Mytilus sp.* were collected from the intertidal, washed in local estuary water to remove the worst of the mud and debris before being transferred to polypropylene bags and being placed in a cool box for transport back to the laboratory.

Figure 6.3: Satellite image (from Google Maps) of the *Mytilus sp.* sample site, Jupiter Point, River Lynher, Tamar Estuary, Cornwall UK

Although biosecurity is always an important consideration during fieldwork, the disease designation for the Tamar estuary meant that additional biosecurity measures were added to the previous fieldwork routine from Chapter 3. As with the previous fieldwork, two Cefas staff members were present for each fieldtrip undertaken. To reduce biosecurity risk, no sites other than Jupiter point and Torpoint were visited. Additionally, field gear was sprayed with the Virkon virucide after use before being placed in a sealed box and taken back to the laboratory. Upon arrival at the laboratory all gear which may have been exposed was washed thoroughly with Virkon, ensuring complete coverage with at least 15-minute contact time before leaving it to dry. Shellfish collected were immediately taken into the secure aquaria facility within the Cefas experimental aquatics

facility. All other equipment which could not be cleaned was disposed of as clinical waste, via incineration.

6.2.3 - Ostreopsis cultivation and sourcing of strains

The non-toxic strain of *O. ovata,* CCMP 3496 was sourced from the National Culture Collection of Marine Algae. The toxic strain of *O. ovata,* CBA 3322 was kindly provided by staff from the University of Urbino, Italy. Both algal cultures were grown in sterilised natural seawater enriched with L1 nutrients without Si. Seawater was pumped from Newton's Cove, Weymouth UK, and had a salinity around 34 PSU this was then filtered at 0.2 µm before being autoclaved to ensure sterility. Cultures were grown in incubators maintained at 22 °C with a light to dark ratio of 14:10. Cultures were grown in an escalating size series of vented Falcon tissue culture flasks. Starting at 50 mL, before being transferred to 125 mL and finally 750 mL flasks as culture densities increased. All culture flasks had a 0.2 µM vented cap to allow for gas exchange without allowing for contamination by bacteria.

In order to assess whether the impact of *O. ovata* on naïve shellfish was related to ovatoxins or if it was via a different means, the study was designed to expose mussels to both an ovatoxin producing strain of *O. ovata* and a non-toxic strain of the same species. In this way it would implicate unknown bioactive compound(s) or mechanical damage, induced hypoxia or potentially smothering from mucous, if deleterious effects were seen in the shellfish when exposed to the non-toxic strain. Whereas, if impacts were exclusive to the toxigenic strain it would suggest the ovatoxins themselves as potentially being a key element in the impacts observed.

To provide some reference materials for the verification of mass spectrometry performance when analysing palytoxins for this work, a bulk culture of the toxic *O.* cf. *ovata* CBA 3322 was grown in addition to the culture intended for the mussel exposure. Around 3 L of this material was produced, the cultures were left until a dense layer of mucous and cells were present on the base of the flask. At this time the culture was extracted for palytoxins as per section 6.2.6 with extracts

being combined to form a total volume of 50 mL of cell extract. This material was available to analyse during mass spectrometry analysis, to ensure that relevant compounds were detectable and within the acquisition windows of the method.

6.2.4 - Tank set-up and mussel exposure

6.2.4.1 - System design

Following collection, the sampled mussels were brought to the secure tank room facility at Cefas, Weymouth. Prior to the study commencing the mussels were housed in a flow through system, this consisted of a tank fed by a constant inflow and outflow of filtered seawater, all collected individuals were held in a suspended basket, following cleaning of detritus and biological material from their shells. This flow through system was maintained at 18 °C to allow the mussels to acclimate to the study conditions.

The available system limited each study to just two treatments, so two separate studies were required to accommodate all four treatments (Table 6.1). In the first study, the two treatments were a negative control and a daily dosage of 200,000 cellsL^{-1} of non-toxic *O. ovata* CCMP 3496. In the second, the two treatments consisted of a negative control and a treatment with a daily dosage of 200,000 cellsL^{-1} of toxic *O. ovata* CBA 3322. In all treatments a small quantity of commercial shellfish diet was added as a feed supplement, with Shellfish Diet 1800 (Reed Mariculture, Hallow, UK) was used for this purpose. This is a commercial shellfish feed consisting of a concentrate of five marine microalgal species known to provide a mixed feed for bivalve species. The five species present in the shellfish diet are *Isochrysis* (Parke, 1949)*, Pavlova* (Butcher, 1952), *Tetraselmis* (F.Stein, 1878), *Thalassiosira weissflogii* ((Grunow) G.Fryxell & Hasle, 1977) & *Thalassiosira pseudonana* (Hasle & Heimdal, 1970). This was diluted prior to addition and was added at a rate of 80,000 cellsL^{-1} in the final volume of 500 mL in the experimental container.

Table 6.1: Showing the breakdown of each treatment for each study

	Non-toxic *O.* cf. *ovata* exposure (Study 1)		Toxic *O.* cf. *ovata* exposure (Study 2)		Short term toxic *O.* cf. *ovata* exposure study (following study 2)
Treatment	Treatment 1 (no PlTX)	Treatment 2 (no PlTX)	Treatment 3 (PlTX)	Treatment 4 (no PlTX)	Treatment 3 (PlTX)
Feed source for *Mytilus sp.*	*Ostreopsis* cf. *ovata* CCMP 3496 + Dilute Shellfish diet 1800	Dilute Shellfish diet 1800 only	*Ostreopsis* cf. *ovata* CBA 3322 + Dilute Shellfish diet 1800	Dilute Shellfish diet 1800 only	*Ostreopsis* cf. *ovata* CBA 3322 + Dilute Shellfish diet 1800
Number of *Mytilus sp*. individuals per treatment	30 (6 at T0 followed by 24 exposed)	30 (6 at T0 followed by 24 exposed)	30 (5 at T0 followed by 25 exposed)	30 (5 at T0 followed by 25 exposed)	15
Number of *Mytilus sp.* individuals taken at each sampling point	6	6	5	5	5
Number of sampling points	5 (T0, T1, T2, T3, T4)	5 (T0, T1, T2, T3, T4)	6 (T0, T1, T2, T3, T4, T5)	6 (T0, T1, T2, T3, T4, T5)	3 (T1, T2, T3)

Interval between sampling points	24 hours	24 hours	24 hours	24 hours	2 hours

Prior to each feeding, a 100% water change was undertaken on each container, during the daily water change the containers and mussels were also rinsed to remove any adhered *Ostreopsis* cells. Water from the previous 24 hours was discarded into a container for treatment with sodium hypochlorite at a level of 50 ppm, with at least 18 hours of contact prior to disposal. After the disposal of the water from each vessel clean seawater was added to each container, from a reservoir. The required volume of *Ostreopsis* culture and dilute shellfish diet was then added to each container before it was made up to the 500 mL total volume for the study with clean seawater, as required. In this way, the total volume of each container was kept constant throughout all treatments, with the amount of algal culture altered daily based upon cell counts undertaken prior to each daily introduction of cells. A cross section of the system is provided in Figure 6.4.

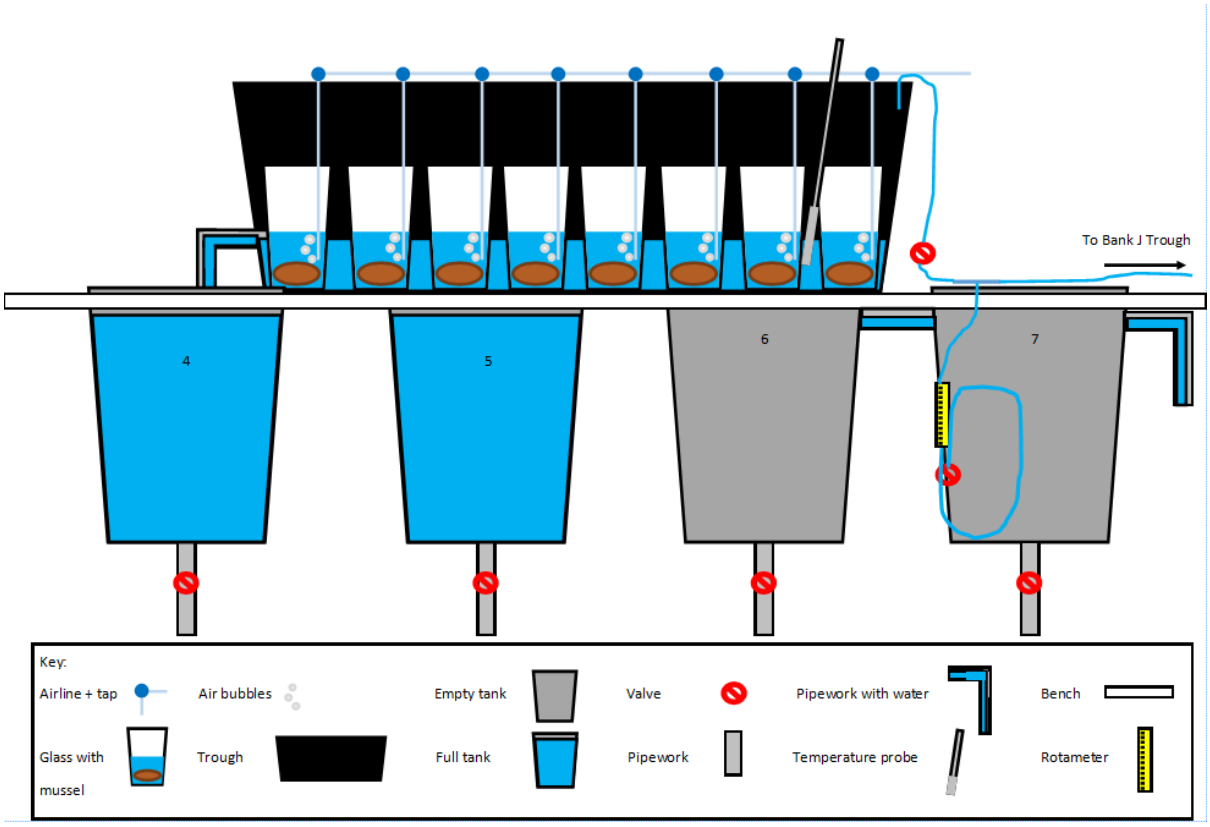


Figure 6.4: Schematic displaying system design cross-section of a single water bath trough with sample containers, drainage and treatment system. In the non-toxic *O.* cf. *ovata* exposure study (study 1 – comprised of treatments 1 & 2) each trough housed 24 containers each with a single study animal and during the toxic *O.* cf. *ovata* exposure study (study 2 – comprised of treatments 3 &4) each trough housed 25 study animals in separate containers.

During the field sampling trips the number of live mussels found was low. As such, fewer individuals than planned were able to be collected during each trip and a different number of individuals was found between the two field sampling efforts. Due to this variability in the number of mussels collected during fieldwork each of the two studies contained a slightly differing number of *Mytilus sp.* individuals. Consequently, the precise numbers used in each study differed, as well as the sampling strategies, details of both studies can be found in the sections below and further in the sampling section (6.2.4.3, 6.2.4.4 and 6.2.4.5)

The system for the first study consisted of 48 individual plastic 1.14 L containers housed in a pair of troughs which were used as water baths, held at 18 °C, with each water bath housing 24 containers. Each individual container had aeration provided via a filtered air supply and was lidded to limit the chance for transfer from one container to another. During the study the total volume of each container was kept constant at 500 mL and in each, a single mussel was housed. The distribution of the treatments for the first study is shown in Figure 6.5.

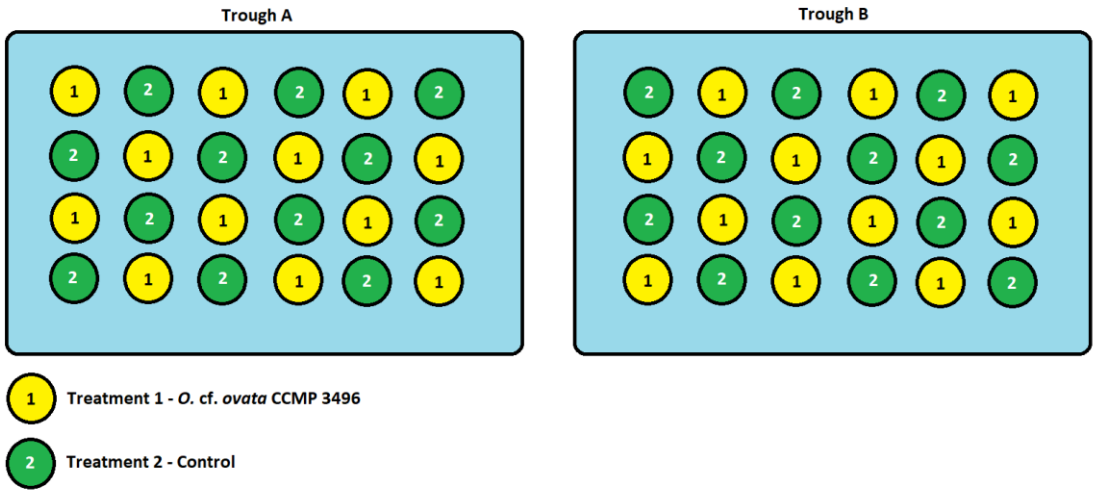


Figure 6.5: Schematic of treatment layout within water bath troughs used in non-toxic *O.* cf *ovata* exposure (study 1), featuring 2 treatments: Treatment 1 – non-toxic *O.* cf. *ovata* exposure and Treatment 2 – dilute shellfish diet only as a control.

The second study consisted of slightly more containers, with 50 vessels being present. All other parameters were the same, with the exception of the strain of *O.* cf. *ovata* used. The second field trip did not provide sufficient individual mussels to fully stock both treatments. As such, to maintain an even number of mussels in both the control and the exposed treatments, 50 mussels, 25 in each treatment were used for the second study rather than the 48 mussels in total, 24 per treatment, used in the first of the studies. The distribution of the two treatments in the water bath

are shown in Figure 6.6. Each water bath housed 25 plastic containers in the second study, rather than 24.

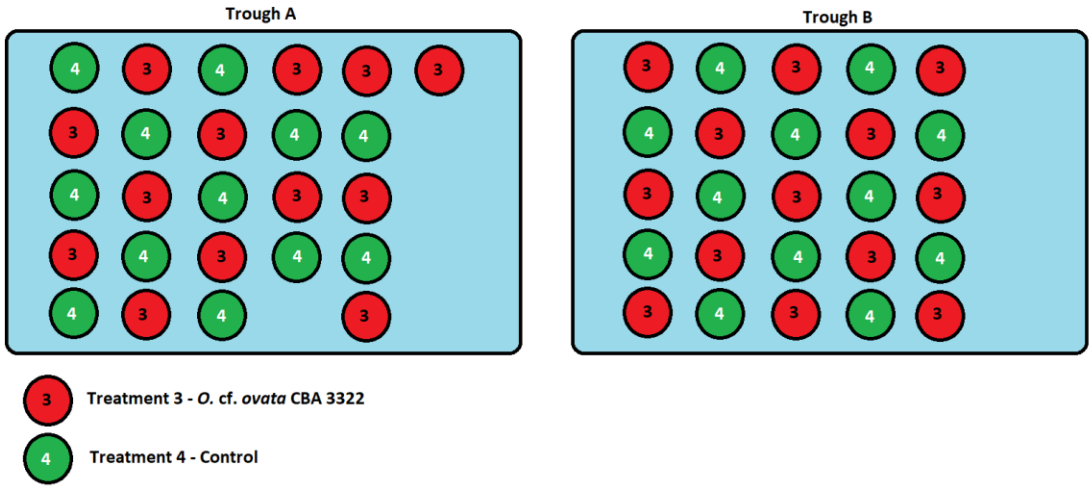


Figure 6.6: Schematic of treatment layout within water bath troughs used in toxic *O.* cf *ovata* exposure (study 2) featuring 2 treatments: Treatment 3 – toxic *O.* cf. *ovata* exposure and Treatment 4 – dilute shellfish diet only acting as a control.

6.2.4.2 - Study design including biosecurity measures

Due to the work involving both toxins and also live cultures of non-native species of microalgae the decision was made to locate the work in the secure experimental facility at Cefas Weymouth. As such, the troughs acting as water baths drained into 80 L tanks which could be isolated from the wastewater flow when cells were introduced to the containers holding the mussels. This additional wastewater capture and treatment step covered the period of highest risk, when the *Ostreopsis* cells were being added to the system. This contained wastewater was treated with hypochlorite (50 ppm) for at least one hour, prior to it being discharged to the secure ozone treatment plant, located within the Cefas secure facility. At all other times water flowing through the troughs was treated using the ozone treatment route only. The secure ozone unit undertakes

between 7 and 21 treatments of wastewater each day with a typical concentration of ozone of 80 g/m³. These two treatment steps acted to neutralize all viable *Ostreopsis* cells as well as ensuring that any *Martelia* released were also destroyed, rendering the remaining wastewater safe to be discharged in accordance with Cefas wastewater discharge license.

6.2.4.3 - Sampling strategy, non-toxic O. cf. ovata exposure (Study 1)

In the first study, six animals were sampled at the commencement of the study to provide a time zero (T0) sample of animals prior to any exposure. The remaining 48 mussels were equally divided between the two treatments, a control with only dilute shellfish diet and an exposed treatment with non-toxic *O.* cf. *ovata.* This allowed for the sampling of six mussels from each treatment following 24, 48, 72 and 96 hours of the study. This resulted in a study lasting four days, with the number of study mussels declining each day as sampling was undertaken. Further to this, all animals sampled were initially processed for histopathology, with a section through the centre of the organism being taken. The remaining tissues were pooled and frozen at -80 °C prior to toxin extraction to assess the presence of palytoxins. At each time point a water sample was taken immediately prior to discarding the contents of each container. This water sample was fixed with neutral Lugol's iodine solution before being assessed via light microscopy, for the presence of residual *Ostreopsis* cells.

6.2.4.4 – Sampling Strategy, toxic O. cf. ovata exposure (Study 2)

In the second study, slightly more animals were sourced, allowing for five days of exposure. This required a reduction in the number of animals sampled at each time point, reduced to five from six. For consistency, five animals were sampled prior to exposure commencing to form a T0 sample

set for the second study. The sampling strategy for the second study was to sample five animals per treatment at 24-hour intervals up to five days of exposure. As with the non-toxic *O.* cf. *ovata* exposure (study 1), all suitable animals were sectioned for histopathology analysis with the remainder being taken for toxin analysis. As there were mortalities in the second study, some individual mussels were not processed for histopathology as their state was too deteriorated prior to sampling to allow for successful analysis. Where possible, mortalities were added to the individuals sampled at each time point, to attempt to diagnose the cause of death. This reduced the number of available animals for the subsequent time points, ultimately resulting in the study completing after 48 hours of exposure.

6.2.4.5 – Study design and sampling strategy, Short term exposure

During the second study, in treatment 3 with exposure to toxic *O.* cf. *ovata* cells, there was a high level of mortality (detailed fully in sections 6.3.2 and 6.4). This left 15 organisms from the control treatment in study 2 but no mussels in the associated exposure treatment. As a result of these mortalities removing an entire treatment after 48 hours the second study ended prematurely. To make best use of the 15 remaining mussels which had been collected an additional study was rapidly planned. Due to the rapidity of the mortalities in the exposed treatment it suggested that there would be value in observing exposed mussels at more frequent intervals. As such, a short-term exposure study was designed to make best use of these organisms. In this final treatment mussels were exposed to the toxic strain of *O.* cf. *ovata* CBA 3322 at the same levels as those mussels in treatment 3 (detailed in Table 6.1). As there were 15 animals left in the control treatment after the close of the second study, this allowed enough mussels for one treatment with three time points to be assessed whilst retaining the ability to sample five animals at each. Prior to the exposure commencing a 100% water change was undertaken, in keeping with all other intervals between exposure for all treatments. Sampling and observations were then undertaken at two-hour intervals,

with the first organisms sampled at two hours post exposure, then subsequent samples taken at four and six hours post exposure. These animals were processed for histopathology and toxin analysis, in the same manner as those from 6.2.4.3 and 6.2.4.4. As these study mussels were from the same stock utilised in the toxic *O.* cf. *ovata* exposure (study 2) it was possible to make use of the same T0 mussels sampled for that study as a bass-line comparison.

6.2.5 - Analytical Reagents, chemicals and equipment

In the same manner as the work presented in chapter 4 (4.2.1) all reagents used for toxin extractions were of HPLC grade or higher. For the mass spectrometry analysis reagents of LC-MS grade were utilised. Chemicals were sourced from either Fisher Scientific (Loughborough, UK) or VWR (Lutterworth, UK). Toxin analysis was carried out using a Waters (Milford, MA, USA) Acquity I class UPLC coupled with a Waters Xevo TQ-S triple quadrupole mass spectrometer. Calibration standards were prepared from certified reference material sourced from the FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

6.2.6 – *Ostreopsis* cell extraction

Cell cultures of *O.* cf. *ovata* were analysed for toxins in a similar manner to *Alexandrium* cultures in chapters 3 and 4. When cell cultures became visually dense triplicate cell toxin samples were taken by decanting 50 mL of cell culture into a 50 mL centrifuge tube. Cultures were homogenised via gentle mixing within the culture flasks prior to sampling. A further 3 mL sample was taken and fixed with 1 mL of neutral Lugol's iodine solution, for cell enumeration within the sampled culture. Enumeration was conducted using a haemocytometer and a Nikon Eclipse TE300 (Japan).

The 50 mL cell cultures then underwent centrifugation at 3400 RCF for 10 minutes. Following this step the supernatant was carefully drawn off and treated with sodium hypochlorite (50 ppm) to eliminate any residual cells which had been dislodged from the pellet. Cell pellets were then resuspended in 1.5 mL of 80:20 methanol:water. Cells were disrupted to release intracellular toxins via the use of a probe sonicator. The sample was placed into an ice bath and sonicated for 1.5 minutes. Following this step, samples were filtered into a glass LCMS/MS vial through a 0.2 µm syringe tip filter. At this stage samples were ready for analysis via the LCMS/MS method detailed below (6.2.8)

6.2.7 - Mussel extraction

Tissue extraction was carried out using a dispersive technique in a similar manner to Selwood et al., (2012) but utilising methanol:water 8:2 rather than 1:1 by volume, as this has been shown to be a more effective extraction solvent for palytoxins in mussels (Ciminiello et al., 2011).

To extract toxins from individual mussels, the remaining portion of mussel tissue following sampling for histology analysis were placed in individual 50 mL centrifuge tubes. The mass of remaining tissue was recorded for each sample. Samples were kept frozen at -80 °C until extraction. Prior to extraction shellfish samples were suspended in extraction solvent (80:20, methanol:water) and homogenised using a stick blender (UltraTurrax) at 12,000 RPM. The ratio of solvent to sample varied depending on the mass of shellfish tissue remaining after the sampling for histology had been undertaken. Typically a solvent:sample ratio of 3:1 was used (volume:weight). For the smallest samples, where remaining tissue mass was less than 1 g a 9:1 ratio was used and for those samples between 1.0 and 1.5 g a 5:1 ratio was used. This ensured sufficient volume in the centrifuge tube for the blender probe to effectively homogenise the tissue.

Following the homogenisation step additional extraction solvent was added to each sample extract to yield a final ratio of 9:1 solvent to sample for every sample. Due to the variable mass of

each sample, differing volumes of extraction solvent needed to be added, the 9:1 ratio ensured that final volumes resulted in a tissue concentration of 0.10 g/mL for the majority of the samples. For the largest samples it was not possible to accommodate this ratio in the centrifuge tubes and so this ratio was reduced to a level where the solvent and sample could be suitably mixed during the extraction step. In all cases of reduced ratios final tissue concentrations were adjusted to either 0.11 g/mL or 0.14 g/mL. These cases of divergence were recorded so that the correct dilution factor could be applied following analysis.

Extraction of homogenised samples was undertaken by mixing in a multi tube vortex mixer for three minutes at 2500 RPM. Following this step all samples were placed in a centrifuge for ten minutes at 3400 RCF. Once separation between the solid tissue and the liquid extraction solvent had been achieved, the supernatant for each sample was decanted into a clean 50 mL tube before being stored at -80 °C prior to analysis.

6.2.8 - Toxin analysis

Principal toxin analysis was undertaken using tandem mass spectrometry. To prepare samples for mass spectrometry analysis, 500 µL of the previously extracted samples were filtered through a 0.2 µm syringe filter, into glass mass spectrometry vials and positioned within the autosampler. The method used was adapted from Murray *et al.* (in prep). In total Palytoxin and ovatoxin analogues were quantitatively assessed in the run, with quantitation against a prepared seven-point calibration curve of pure palytoxin. The analogues included in the methodology and their ionic transitions are included in Table 6.2 and the concentrations of the seven calibrants utilised are detailed in Table 6.3.

Table 6.2: Palytoxin analogues quantified against the palytoxin standard curve as well as their primary quantitative and secondary qualitative transitions, after Murray (in prep).

Compound	Abbreviation	Primary (quantitative) Transition	Secondary (qualitative) Transition
Palytoxin	PlTX	876.1>372.2	876.1>76.1
Palytoxin (Palythoa sp.)	PlTX	870.1>327.2	870.1>76.1
NeoPalytoxin	NeoPlTX	870.1>309.2	870.1>76.1
Deoxy-Palytoxin/Ovatoxin d	Deoxy-PlTX/OvTX d	870.8>327.2	870.8>76.1
HomoPalytoxin	HomoPlTX	880.8>341.2	880.8>76.1
BishomoPalytoxin	BishomoPlTX	885.5>355.2	885.5>76.1
Palytoxin b/Ovatoxin K	PlTX b/OvTX K	890.1>327.2	890.1>76.1
42-OH-Palytoxin	42-OH-PlTX	881.5>327.2	881.5>76.1
Ovatoxin a	OvTX a	865.5>327.2	865.5>76.1
Ovatoxin b	OvTX b	880.1>371.2	880.1>76.1
Ovatoxin b isomer	OvTX b isomer	880.1>327.2	880.1>76.1
Ovatoxin c	OvTX c	885.5>371.2	885.5>76.1
Ovatoxin e	OvTX e	870.8>343.2	870.8>76.1
Ovatoxin f	OvTX f	874.8>327.2	874.8>76.1
Ovatoxin g	OvTX g	860.1>327.2	860.1>76.1
Ovatoxin h	OvTX h	860.8>327.2	860.8>76.1
Ovatoxin i	OvTX i	879.5>327.2	879.5>76.1
Ovatoxin j1/j2	OvTX j1/j2	884.8>327.2	884.8>76.1
Ostreocin A/Ostreocin B	Ost A/Ost B	866.8>313.2	866.8>76.1

Ostreocin D	Ost D	861.4>313.2	861.4>76.1
Ostreocin E1	Ost E1	855.4>313.2	855.4>76.1
Novel compound (O. sp. 3)	-	833.1>230.2	833.1>212.2
Mascarenotoxin A	MscTX A	846.1>327.2	846.1>76.1
Mascarenotoxin B	MscTX B	851.5>327.2	851.5>76.1
Mascarenotoxin C	MscTX C	859.5>327.2	859.5>76.1

Table 6.3: Palytoxin calibration standard concentrations utilised for sample analysis.

Palytoxin standard level	**Palytoxin concentration ugL-1**
1	25.00
2	10.00
3	5.00
4	1.00
5	0.50
6	0.10
7	0.05

For analysis of toxins a Waters xevo TQ-S coupled to an Infinity I-class UHPLC was used. This was set to multiple reaction monitoring mode. Separation of the palytoxin analogues was achieved using a Waters Acquity BEH C18; 50 x 1 mm; 1.8 µm column held at 40 °C, with gradient elution, detailed in Table 6.4. The mobile phases utilised were water with 0.1% formic acid for mobile phase A and acetonitrile with 0.1% formic acid for mobile phase B. Source conditions are detailed in Table 6.5, the collision cell, in quadrupole two, utilised argon as the fragmentation gas. Method run time

was 8.5 minutes in total per sample injection, with an injection volume of 5 µL used for all samples and standards. Calculation of unknown concentrations was undertaken within the mass lynx software, with the target lynx program used for the integration and analysis of chromatographic peaks returned for analysis. In order to check the ongoing performance of the method, a complete set of standards was injected every 30 sample cycles. Additionally, a continuing calibration check of the level 4 standard was inserted into the sequence between each pair of full standards, following the first 15 sample injections between each set of standard brackets.

Table 6.4: displaying the gradient properties for the analysis of PlTX via LC-MS/MS

Time (minutes)	Mobile phase A (%)	Mobile Phase B (%)
0	95	5
1	75	25
6	65	35
6.2	10	90
7	10	90
7.2	95	5
8.5	95	5

Table 6.5: displaying the MS source conditions for the analysis of PlTX

Polarity	ES+
Capillary (kV)	1.00
Source Temperature (°C)	150
Desolvation Temperature (°C)	500
Cone Gas Flow (L/Hr)	150

Desolvation Gas Flow (L/Hr)	1000
Collision Gas Flow (mL/Min)	0.14
Nebuliser Gas Flow (Bar)	7.00

6.3 – Results

6.3.1 - Observed behaviour

Mussels in the three treatments where no toxic *O.* cf. *ovata* was present (treatments 1, 2 and 4) behaved normally, apparently open and feeding during the duration of the study. During the water changes where each mussel underwent significant disturbance, mussels in these three treatments would close until the final additions of cell culture and seawater were added to return each vessel to the 500 mL working volume. Within a few minutes mussels would be open, with normal feeding behaviour seemingly restored. Mussels exposed to toxic *O.* cf. *ovata* (treatment 3 and short exposure) conversely remained shut, with seemingly no return to normal behaviour after the water change. This cessation of feeding was apparent during multiple checks, several hours after the introduction of the toxic *O.* cf. *ovata* cells. Additionally, there was an obvious presence of suspended mucous in experimental vessels where toxic *O.* cf. *ovata* had been added (treatment 3), often adhering in places to the shells of mussels. This mucous was cloudy, with occasional patches of red/brown, presumed to be aggregations of *Ostreopsis* cells. After 24 hours, at sampling, in treatment 3, individuals were either open and dead or moribund, only able to close their shells with assistance. The mucous which was present in vessels containing toxic *O.* cf. *ovata* (treatment 3 and short exposure) was absent in the other treatments. Mussels sampled in treatments 1, 2 and 4 also appeared healthy at sampling, with the adductor muscle needing to be cut in order to allow for the shells to be opened for dissection.

6.3.2 - Mussel mortality

During the non-toxic *O.* cf. *ovata* expsure (study 1) there were no mortalities recorded during the four days of the study from either of the two treatments. However, in the toxic *O.* cf. *ovata* expsoure (study 2) mortalities occurred rapidly in treatment 3, exposed to the toxic strain of *O.* cf. *ovata,* CBA3322. There were no observed mortalities in the control organisms during the two days of the second study before it was closed prematurely following the death of all mussels assigned to the *Ostreopsis* exposed treatment. Mortalities across all four treatments are compared in Figure 6.7, although mortalities were only observed in the treatment with the presence of toxic *O.* cf. *ovata* (treatment 3).

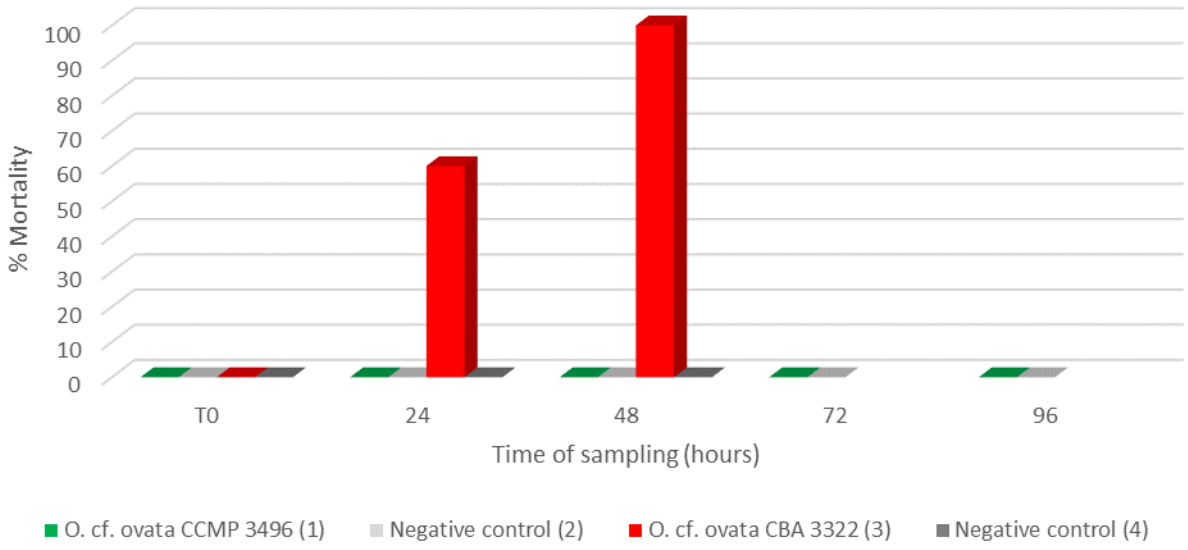


Figure 6.7: Histogram displaying cumulative mortality within all treatments as a % of treatment population. Treatment number is included in brackets after the treatment description.

Within treatment 3 a mortality rate of 60% was observed after 24 hours of exposure, reaching 100% by the 48-hour sampling point. This resulted in 15 animals being sampled at 24 hours with the

remaining 10 animals in treatment 3 sampled at 48 hours. Three animals were unable to processed for histopathology analysis due to their state of deterioration following mortality, these were all sampled after 48 hours of exposure. Although these three individuals showed signs of tissue degradation, they were forwarded for toxin analysis as they had not begun to decompose completely.

During the short exposure study, mussels also remained closed, with no evidence of feeding across the three time points where sampling was undertaken, two, four and six hours after exposure to toxic *O.* cf. *ovata.* Although there were no recorded mortalities in the short exposure study two individual mussels appeared to be moribund at the six-hour sampling time point. In both cases these mussels had started to open in the experimental containers and were not found to close on removal from the water. Neither had begun to gape fully though and still retained some ability to hold their valves closed.

6.3.3 – *Ostreopsis* cf. *ovata* toxicity

Following analysis of the two strains of *O.* cf. *ovata* utilised in this study, strain CCMP3496 was not found to contain any palytoxin analogues whereas CBA3322 produced a range of congeners. Cellular toxicity for strain CBA3322 was found to be 20.8 pg cell^{-1} with the majority of toxin present being ovatoxin-a, followed by ovtx-b. A detailed breakdown of the toxin profile determined within this study is shown in Figure 6.8.

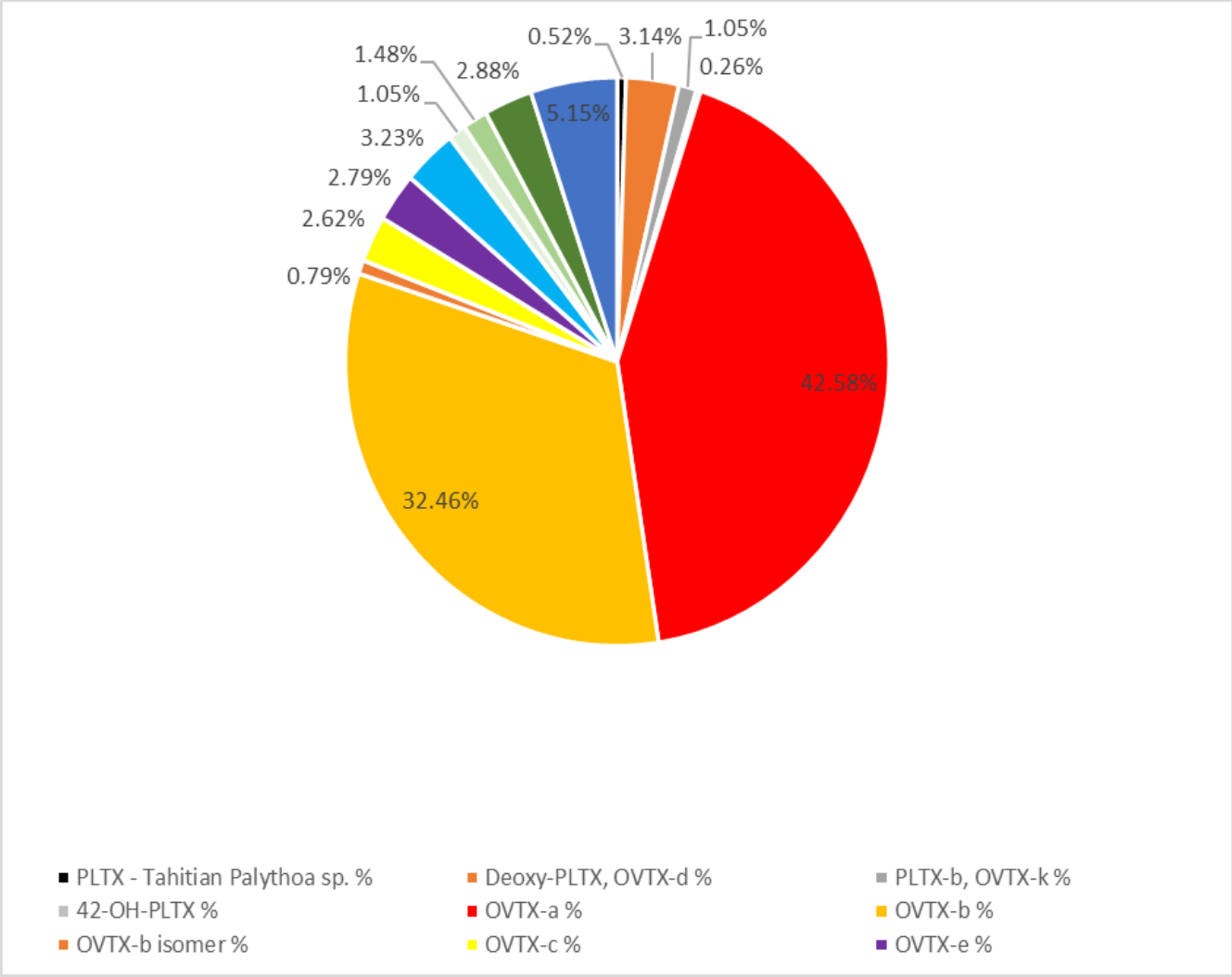


Figure 6.8: Pie chart displaying the average proportion of the detected palytoxin congeners within triplicate analysis of *O.* cf. *ovata* strain CBA3322.

With the exception of ovtx-f all of the ovatoxins included in the mass spectrometry method were detected in the samples analysed. The majority appeared at low levels, with only ovtx-a and ovtx-b being major components of the toxin profile, with 43% and 32% respectively, thereby accounting for 75% of the total toxin concentrations. The next highest contributor to the total toxin content was mascarenotoxin-C and only a trace amount of pltx itself was detected.

6.3.4 - Mussel toxicity

Analysis via LC-MS/MS indicated that the lower level of detection for this method was the level 6 standard, 0.1 µgL-1, the level 7 standard 0.05 µgL-1 returned inconsistent peaks indicating that this level of toxin concentration would not be considered consistently detectable. This also suggested that the limit of quantitation be set at the level 6 standard, with values for toxin concentrations below 0.1 µgL-1 being below the linear dynamic range used. The R^2 value for the calibration curve derived for the palytoxin standards was 0.97, indicating a consistent calibration slope across the 5 batches of calibrants injected. Furthermore, all four level 4 continuing calibration checks showed a divergence of no more than 12.2% of the expected value. These quality control checks compare favourably with the checks implemented within official control monitoring for the statutory monitoring of lipophilic biotoxins within shellfish, via LC-MS/MS.

Following sample analysis it was discovered that only very low levels of toxins appeared in the mussel tissue samples, in the range of nanograms, rather than micrograms, per kilo of shellfish tissue. Due to the low levels returned these would all be considered to fall below the limit of quantitation. The method did show excellent sensitivity in this regard however, with discernible chromatographic peaks being detectable in many of those samples exposed to the toxic strain of *O.* cf. *ovata* (CBA 3322). Although the values fall below the effective LoQ of the method, the calculated concentrations of total palytoxins are displayed in Figures 6.9, 6.10 and 6.11 for the non-toxic exposure, toxic exposure and short exposure studies respectively. Within all analysis conducted this was the dominant toxin identified within the shellfish tissues.

Sample designations were derived from the time point of sampling, followed by the trough housing the sample container, then the column from which the sample was taken and finally the row. Between the first and second study the column designations were changed from letters to roman numerals, to prevent the repetition of letters A and B which were being utilised to refer to

the troughs. Additionally, a T or a C was added as a suffix in the second study to denote whether the sample was a control (C) or exposed to toxic *O.* cf. *ovata* (T). An example sample designation would therefore be T1 B iii 3 T, denoting a sample taken at time point 1 (24 hours) from trough B, column iii row 3 and it was an exposed sample. For the short exposure study, the prefix SE was added, as all samples in this study were exposed to toxic *O.* cf. *ovata* the T suffix was dropped as it was redundant in this case.


T4 AB 3
T4 AB 1
T4 BE 1
T4 AC 4
T4 AE 2
T4 BA 3
T4 BF 3
T4 BB 3
T4 BE4
T4 AB 4
T4 AF 2
T4 BD 3
T3 AF 1
T3 BC 1
T3 BF 4
T3 AE4
T3 AD 1
T3 BC 3
T3 BA 2
T3 BB1
T3 AD 4
T3 BD 1
T3 AA1
T3 AA 3
T2 BB2
T2 BD 2
T2 AD 3
T2 BF 2
T2 AC 4
T2 AA 2
T2 AE 1
T2 BC 4
T2 BA 4
T2 AF 4
T2 AB 2
T2 BE 2
T1 BD 4
T1 AC 2
T1 BA 1
T1 AF 3
T1 AA 4
T1 BB 4
T1 BF 1
T1 BC 2
T1 BE 4
T1 AD 2
T1 AE 3
T1 AC 1
1st T0 F
1st T0 E
1st T0 D
1st T0 C
1st T0 B
1st T0 A
Sample designation
0
1
2
3
4
5
6
7
total pltx ng/kg
72 hour exposure (control)
96 hour exposure (control)

Figure 6.9: Histogram showing trace level concentrations of Pltx detected in shellfish samples from samples in the non-toxic *O.* cf. *ovata* exposure (study 1). Grey represents mussels from the control group sampled after 72 hours. Black represents mussels from the control group sampled after 96 hours.

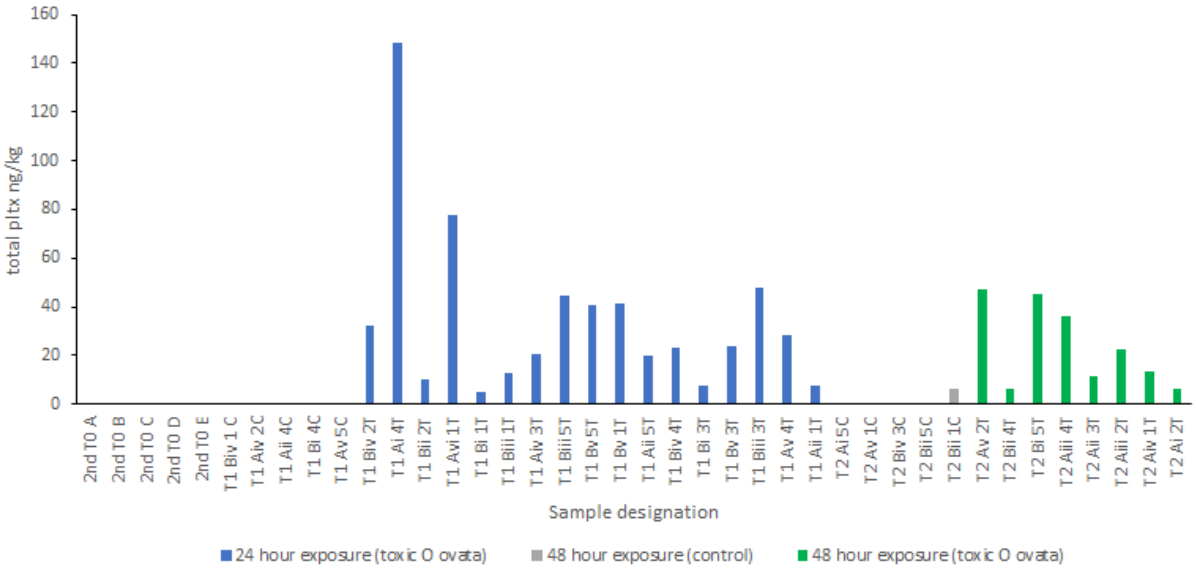


Figure 6.10: Histogram showing trace level concentrations of Pltx detected in shellfish samples from samples in the toxic *O.* cf. *ovata* exposure (study 2). Grey represents mussels from the control group. Blue represents mussels exposed to toxic *O.* cf. *ovata* for 24 hours and green represents mussels exposed to toxic *O.* cf. *ovata* for 48 hours.

All of the samples with trace levels of OvTX a were exposed to the toxic strain of *O.* cf. *ovata* with the exception of samples T4 AB1 and T3 BC 4. Both of these samples were from the first study, where no toxic *O.* cf. *ovata* was used, the samples were T3 BC 3 and T4 AB1 and both were from the control treatment in the first study, with no *O.* cf. *ovata* present at all. In both cases the values present were very low, and it is likely that these are artefacts as opposed to true toxin presence. However, as these are compared against the samples where toxic *O.* cf. *ovata* was present, it was also found that similarly low levels were present in several of these. Within those samples exposed

to toxic *O.* cf. *ovata,* only two had no indication of toxin presence, these were both from the short-term exposure study, SE2 Bv 4 which was exposed for two hours prior to sampling and SE4 Biii 4 which was exposed for four hours prior to sampling. In all other cases the presence of toxic *O.* cf. *ovata* in the treatment resulted in the apparent detection of very low levels of ovatoxin a in the analysed mussel samples. Within all of the mussel samples exhibiting detectable levels of toxins, an average of 91% of the toxins detected were ovatoxin-a.

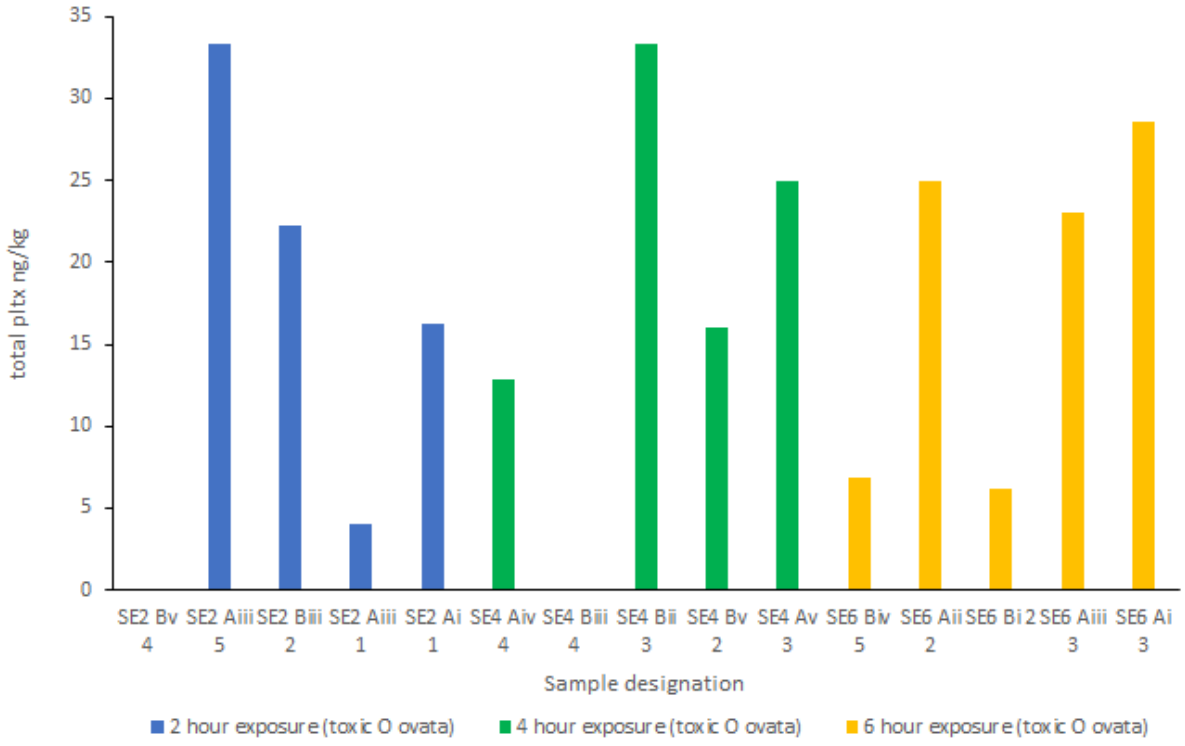


Figure 6.11: Histogram showing trace level concentrations of total Pltx detected in shellfish samples during the short exposure study. Columns in blue were from mussels which had 2 hours of exposure, columns in green were from mussels which had 4 hours of exposure and columns in yellow were from mussels with 6 hours of exposure.

6.4 - Discussion

The key finding of the exposure of GB mussels to both a toxic and non-toxic strain of *O.* cf. *ovata* was the complete mortality of those mussels where toxic *O.* cf. *ovata* was present after 48 hours of exposure. As a result of the initial findings the histological samples taken have now been forwarded for full analysis within the histopathology team.

Of the population exposed to toxic *O.* cf. *ovata* in treatment 3 of study 2 over half were found to have died after 24 hours of exposure. In the follow up short term exposure study, there was some indication that individuals were becoming moribund at the 6-hour time point, although none appeared to have died. This is in keeping with previous reports of mortalities of marine invertebrates, as a result of exposure to *O.* cf. *ovata,* both in the wild and during controlled experiments (Neves et al., 2017; Privitera et al., 2012). When compared to a similar study utilising mussels from within the known current range of *O.* cf. *ovata,* the key difference is the extent and speed of mortality given the cell concentrations employed in this study. The work by Carella et al. (2015) used a cell concentration of 300,000 cells L^{-1} as the low-level exposure in their study, this was 50% higher than those employed here and yet they displayed mortalities of only 10% within the study population as opposed to 100% reported here. It could be the case that this is due to differences between the particular strains of *O.* cf. *ovata* used in these separate studies or it could be indicative of a degree of tolerance inherent within populations of *Mytilus sp.* which naturally come into contact with proliferations of *O.* cf. *ovata.* As the mussels used in this study are believed to be naïve with respect of exposure to *O.* cf. *ovata,* this suggests a susceptibility to the impacts of this microalgal species which may not exist within its current range. It is also possible that the mussels used within this work were in a poor state of health, given that they were taken from an area known to harbour the parasite *Martelia refringens.* This may have meant that the mussels were already in a weakened state, with the introduction of the toxic strain of *O.* cf. *ovata* enough to push

them beyond their physiological tolerances. There has been evidence of damage to digestive tubules and high levels of mortality in the oyster, *Ostrea edulis* (Linnaeus, 1758) as a consequence of infection with *M. refringens* (Carrasco et al., 2015) but has not been found to cause the same high mortality in mussels (Carrasco et al., 2015; Karagiannis and Angelidis, 2007).The damage caused by the proliferation of *M. refrigens* in host *Mytilus* is in the degradation of digestive tubules and epithelium, which can cause infiltration of the parasite into the surrounding tissues (Carrasco et al., 2015; Karagiannis and Angelidis, 2007). The potential impact of infection by *M. refringens* on the study mussels will be elucidated following the completion of the histological analysis of the samples take within this study. This should reveal whether the individuals were infected with *M. refringens* as well as allowing a comparative analysis of *Ostreopsis* exposed individuals to the un-exposed control populations, this should allow for the separation of pathology related to the parasite, if present, and that associated with the exposure to cells of *Ostreopsis.* Furthermore, this study showed no mortalities associated with the introduction of a non-toxic strain of *O.* cf. *ovata,* indicating that either the Palytoxins present in the toxic strain of *O.* cf. *ovata* are responsible for the mortalities or another effect linked to the particular strain used is the key factor, this demonstrates that strain specific impacts exist. As such, the mortalities in those mussels exposed to toxic *O.* cf. *ovata* suggest a potential food security risk in the instance of an emergence of this species of harmful microalgae within GB. In contrast however, the mussels exposed to the non-toxic strain of *O.* cf. *ovata* did not present mortality across the 96 hours of the exposure period. This would suggest that the impact caused by a successful invasion of *O.* cf. *ovata* into GB waters would be strain dependent, with some strains capable of causing significant ecological impacts whilst others might remain undetected with no immediate ramifications being observed.

Toxin uptake within the exposed mussels was only observed in a very limited capacity. Consequently, it seems that mortalities in GB *Mytilus sp.* occur so rapidly as to reduce the risk of harmful levels of toxin accumulation. It is conceivable that in the presence of a lower cell concentration of toxic *O.* cf. *ovata,* mussels might survive long enough to become contaminated and

so present a food safety risk. This would require further experimental work to determine a non-lethal cell concentration for the toxic strain utilised and then to investigate whether, at a lower cell concentration, sufficient toxic cells would be present to allow for toxin accumulation at rates exceeding depuration and excretion rates. Previous cases of PlTX poisoning in humans via ingestion have been with fish and crustaceans as the vector organisms, rather than bivalves (Deeds and Schwartz, 2010), suggesting that the bivalve route is either unreported or unlikely. As sublethal effects on *Mytilus galloprovincialis* and *Crassostrea tulipa* (Lamarck, 1819) (reported as *Crassostrea gasar* (Deshayes, 1830)) have previously been reported when exposed to *O.* cf. *ovata* (Carella et al., 2015; Sales et al., 2021), it would also be important to assess the effects of a longer exposure. It might be the case that mortality would be delayed but still occur prior to dangerous levels of ovatoxins accumulating within the tissues of the mussels.

Whether the behaviour of the mussels would continue to be affected at lower cell concentrations of toxic *O.* cf. *ovata* would also be of importance. The apparent avoidance strategy of keeping the valves closed for a prolonged period would ultimately impact the accumulation of toxins but also interrupt feeding and so, if persistent, impact on growth and survival. If even low-level proliferations occurred in the coastal waters of GB these impacts might have a long-term effect within the environment. Additionally, this particular effect would be difficult to track prior to more serious issues appearing.

The tandem mass spectrometry methodology utilised within this study showed good levels of performance, with consistent performance even with a long run encompassing many samples. The extraction methodology resulted in successful extractions of toxins, both within algal cell pellets and shellfish tissues. In practice, for a monitoring programme, it would be more likely to test shellfish in a similar manner to existing official controls. This would be via the pooling and homogenisation of several individuals from the same location, and the testing for toxin concentrations from this homogenate. This would be conducted then, in a similar manner to the

toxin analysis presented in Chapter 4. Interestingly, although the levels of toxins seen in tissues here were very low, they were variable with a spread of toxin concentrations evident after equivalent exposures. It must be noted though, the variability is likely to be wide with the concentrations presented herein being below the calibration range.

Taken together the low toxin uptake and the high mortality suggest a low chance for a human health impact but a high possibility of a considerable environmental impact. There is also a possibility that other marine species could be impacted, as more taxa than just bivalves have been shown to be affected negatively by *O.* cf. *ovata* in the past (Faimali et al., 2012; Giussani et al., 2015; Neves et al., 2017; Simonini et al., 2011). As such, it can be considered that the One Health impact (Turner et al., 2021) of *O.* cf. *ovata* in GB waters would be more considerable than the limited potential for human health impacts demonstrated in this study suggests.

Further study with exposure of *Mytilus sp.* to lower cell concentrations of the toxic strain of *O.* cf. *ovata* could provide evidence relating to the low-level chronic impacts of this strain on GB mussels. Further, if a low cell concentration did not result in acute mortality, the capacity for toxin uptake in the shellfish may increase. There may also be value in assessing whether the presence of cell extracts of the toxic strain of *O.* cf. *ovata* have an analogous impact on mussels, as the presence of bioactive compounds or the palytoxins themselves may be the direct cause of the mortalities within the mussels. However, previous work has demonstrated limited effects of cell extracts compared with cell suspensions (Faimali et al., 2012; Giussani et al., 2015; Neves et al., 2017). It may therefore be the case that the mucous produced by the toxic strain of *O.* cf. *ovata* utilised in this study has a role in the mortalities induced in the exposed mussels, as this has been suggested as a factor in mortality of *Artemia salina* in previous work (Giussani et al., 2015). The upcoming histological analysis of the organisms from the study detailed here may well provide an indication of the mode of action of the fatal effect which the toxic strain exhibited in this work. It may also highlight some similarities between the effects of the toxic and non-toxic strain, as although the

gross symptoms between the two exposure treatments were significantly different, there might be a less obvious impact of the non-toxic strain. This is further possible, as the exposure to the non-toxic strain was for only 96 hours, which may not have been sufficient for impacts to become fatal.

Consequently, this work provides evidence that the impact of a successful emergence of *O.* cf. *ovata* could be considerable within GB waters. This means that this species should be considered an ongoing threat to GB waters, with ongoing surveillance to enable early detection being of value. The successful use of the LC-MS/MS methodology here for the analysis of a diverse range of palytoxins confirms that this method is suitable for a range of matrices of interest and has a high level of sensitivity. This means that the potential for the protection of human health via seafood intoxication is currently available within GB, at Cefas. However, the rapid mortality, before the accumulation of high levels of toxins, means that the impact of emergence might instead be limited to the health of the shellfish, impacting coastal communities indirectly instead, as HAB impacts on the environment are known to negatively impact on local socioeconomics (Karlson et al., 2021; Turner et al., 2021). If this were the case, the more likely exposure route for humans would be via aerosols, as demonstrated in the Mediterranean (Tichadou et al., 2010). In this instance, the detection of the cells of *O.* cf. *ovata* would be critical in managing this species if it were to appear in GB coastal waters and become successfully established.

Chapter 7 – Thesis conclusion

Within this body of work a current and a range of potential emergent harmful microalgal threats to human and environmental health within Great Britain (GB) have been assessed. Beyond those species assessed in detail within this body of work, there are a number of other harmful microalgal species of interest, both already in GB waters and with the potential for future establishment. As such, there is significant scope for additional work in these areas, highlighting the breadth of the topic and the importance it holds because of the wide-reaching implications of harmful algal proliferations, with new information emerging even in relation to well-studied species. This is evidenced by the novel work included in this thesis, undertaken with *Alexandrium minutum,* despite this genus and species having been a focus of scientific research for decades. Furthermore, there are likely to be further harmful impacts discovered and more compounds found within existing or new toxin classes.

7.1 - Current threat

Early chapters of this thesis delved into the current state of *Alexandrium minutum* revealing that there is a complex distribution of toxin profiles globally, with *A. minutum* being widely distributed. There are likely to be further findings of *A. minutum* in future as more comprehensive monitoring, coastal or sediment surveys are undertaken in areas which have not been well researched to date, or as new species detection methods are utilised. This demonstrates that *A. minutum* continues to be a key paralytic shellfish toxin (PST) producer globally, with toxin profiles often containing some of the most potent saxitoxin congeners, GTX1&4 and STX itself. Contrary to this wide distribution and importance, the sediment survey undertaken in Chapter 3 seemingly highlighted that *A. minutum* has become scarce in sites where it was previously prevalent, in the South of Cornwall (SW England) and the South of Wales. Furthermore, around the South and West

of GB *A. minutum* does not seem to have extended its range to new areas. This highlights that published distributions of species tend to increase over time, as it is common to include occurrence data from historic sources, without the ability to verify the continued presence of a species in a particular region. As such, there is a typical build towards more areas being flagged as within the range of a species, whilst recession of a range is much more difficult to confirm and consequently does not seem to be a common focus of research. The disappearance of macro species can be well recognised and is often a target of considerable interest but microorganisms often go unnoticed as it requires considerable focussed effort to gather data on them. For example, a review of evidence for extinction events in soil biota highlighted the disparity in data sets between an extensive above ground, macro focussed, data set and the scarce below ground data set, where a critical component of the system is microscopic (Veresoglou et al., 2015). This stems from the requirement for specific and active monitoring of the microscopic world, with data collection on macro species often being possible without supplemental equipment in contrast to the microscopic world which requires a bridge to allow humans to observe it, along with assumptions about the robust and widespread nature of microorganisms (Veresoglou et al., 2015).

Further work evaluating the PST producers presented in both Chapter 3 and Chapter 4 demonstrated no evidence of a novel PST producer from the field survey in Chapter 3 but did highlight the value of utilising chemotaxonomy as a low-cost supplement for existing monitoring efforts. Chapter 4 has shown that in regions with multiple PST producers it may be possible to discriminate between them by virtue of differences between the saxitoxin congeners which they produce. As modern instrumental chemical techniques enable the determination of toxin profiles, these data are created as a part of existing monitoring efforts incorporating such tools. Therefore, with preliminary work such as that presented here in Chapter 4, it is possible to enhance the value of this existing data if there is a sufficient difference between the toxin profiles of regional PST producers. Evaluation of current and historic PST profiles in shellfish allows for the provisional speciation of the causative microalgal species, although this comes with the caveat that a shellfish

species used in this manner must not have a substantial capacity to transform PST during shellfish metabolism, as this can lead to substantial divergence between the PST profiles of the producer and the shellfish flesh. By using this chemotaxonomic approach both shellfish monitoring data and phytoplankton monitoring data can be enhanced, as when these data sets are combined it should be possible to determine timings of cell proliferations of relevant harmful species. At present the genus level identification within the phytoplankton monitoring can be updated to include a tentative species ID, at least for toxic species, and the causative species for toxin events in shellfish where no *Alexandrium* cells are detected simultaneously can still have a causative organism ascribed to it. It is also possible to return to historic data which have been generated for toxin profile analysis, which is more feasible than returning to preserved water samples to retrospectively sample individual cells for morphological speciation. The more detailed the toxin profile information the greater the level of resolution able to be achieved, with the mass spectrometry analysis undertaken in Chapter 3 and 4 providing a greater level of information than the pre-column liquid chromatography with fluorescence method also utilised. However, as was seen in Chapter 4, data are more easily comparable when generated using the same methodology. This means that as chemical testing becomes more commonplace chemotaxonomy can become more widespread and valuable but the occasional method changes within regulatory testing frameworks need to be considered when assessing longer term data sets.

Chapters 2 through 4 have provided a global overview of the status of *A. minutum* as well as assessing its current distribution and providing validation of a supplemental technique for the ongoing tracking of this distribution. These aspects both serve to fulfil the first two of the objectives for this body of research laid out in Chapter 1. With Chapter 2 providing a comprehensive, novel, assessment of *A. minutum* globally whilst incorporating elements of chemotaxonomic evaluation which were of importance to the work of Chapters 3 and 4. The two experimental chapters which followed have offered further insight into the local distribution of *A. minutum* in GB whilst also highlighting some of the challenges of targeted field sampling for harmful microalgae in the

intertidal. Finally, the demonstration of the validity of using a chemotaxonomic approach for species separation of shellfish intoxications caused by *A. minutum* and *A. catenella* within GB, has improved the toolkit available within the GB monitoring framework as well as highlighting the potential for the use of a similar technique with other toxin groups or in other nations, with appropriate ground truthing.

7.2 - Emergent threats

In addition to current threats, there are a large number of harmful microalgal species which have not so far been detected in the waters around GB. The data gathered in Chapter 5 show that, from a GB perspective, there appear to be a number of non-native harmful algal species which have tolerances ill-suited to the successful invasion and establishment in GB waters, even when considering the shifts predicted as a result of global climate change. Global changes are expected to favour some microalgal species in temperate waters, with such species expected to migrate poleward as ocean warming continues. Consequently, there are a limited group of harmful species which can be considered as being at a high risk of emerging in GB waters in the next two decades. A key finding from Chapter 5 is that the most likely invaders with a high predicted impact have been shown to be responsible for not only human health risks but also environmental health risks. This feeds into the assessment of threats using a One Health approach, recognising the fundamental link between human health and environmental health. As such, those harmful microalgal species which are not known to produce compounds which can be carried through the trophic web to humans and cause human syndromes after ingestion, may still have an impact on human health, albeit indirectly (Turner et al., 2021). One such example is *Heterocapsa circularisquama,* which has negatively impacted a range of shellfish species in Japan, with many reports of mass mortalities as well as a range of sub-lethal but still negative impacts (Matsuyama, 2012). This species was a candidate in the final stage of the risk funnel presented in Chapter 5, with the mid value score it received as a result

of the reported wide reaching negative impacts on shellfish. There are also species where a threat exists to both the environment and directly to human health. The risk assessment conducted in Chapter 5 highlighted that both *Ostreopsis* cf. *siamensis* and *O.* cf. *ovata* fall into this latter category. This, along with their high-risk scores made them prime candidates for further research.

This risk assessment framework provides a system for the evaluation of future risk as well as a numerical ranking with which to prioritise each species. This contributes to the successful completion of the third goal laid out in Chapter 1. With the work undertaken in Chapter 5 able to be added to or expanded, with either new information as it is published or by the addition of new harmful microalgal species as more continue to be discovered. It would also be plausible to take the risk funnel approach and change the parameters for exclusion/inclusion to assess different criteria from the same data set, such as the assessment of threat to differing case study sites.

The risk funnel and scoring system employed offered a mechanism to streamline the final assessment of species to those of harmful taxa with novel harmful impacts and/or those not known from GB waters. There were some potential biases within this assessment methodology however, detailed in section 5.3.3. The accuracy of the risk assessment could be further improved with more literature searching and a continued expansion of the underpinning data set, as new literature becomes available. Ultimately, there was a tendency towards better assessment for those harmful microalgal taxa where the literature searches yielded the most papers. This made the assessment of the most heavily researched harmful microalgal species the most straight forward and the most comprehensive. This highlights the ongoing need for work within the harmful algae field and the disparity between research efforts between species.

Consequently *O.* cf. *ovata* was selected for a more detailed study on its potential impacts if it were to successfully appear in GB waters. Utilising controlled conditions these impacts were explored by challenging naïve *Mytlius sp.* with cultured cells of two strains of *O.* cf. *ovata.* This study highlighted disparate impacts of the two strains assessed. One of the two strains was found to

produce a range of palytoxin-like toxins called ovatoxins, whilst the other was not. The toxin-containing strain caused rapid, total mortality within exposed shellfish whilst the non-toxic strain caused no outward health degeneration. This highlights that generalisations around the impacts of harmful microalgal species are likely to be challenged by cases which do not fit the generalised pattern. For example, the emergence of *O.* cf. *ovata* in GB waters which was similar to the non-toxic strain (CCMP 3496) might give rise to no human illness and no environmental damage. This would be in contrast to the typical association of *O.* cf. *ovata* proliferations with a discernible negative impact on One Health. The variability inherent in biological systems is key to the challenge of fitting broad statements to harmful microalgal impacts (Cullen, 2008). Nonetheless, the work included here demonstrates the possible damaging impacts of the emergence of a toxic population of *O.* cf. *ovata* with the possibilities of three modes of action, direct impact on humans via aerosolised toxins, contamination of seafood and mortality or ill health within bivalve molluscs in the environment (Accoroni and Totti, 2016; Deeds and Schwartz, 2010). As such *O.* cf. *ovata* can be considered to pose a high risk to GB from a One Health perspective.

The work presented in Chapter 6 provides initial evidence in support of the fourth and final goal from Chapter 1. With initial evidence indicating a potentially serious impact on GB shellfish in the event of a new harmful microalgal species becoming established within coastal GB waters. There are some immediate aspects of continuing work which have now commenced, with the analysis of samples via histopathology anticipated to provide information on the mode of action of the toxic strain of *O.* cf. *ovata* responsible for the high levels of mortalities observed. This will contribute to the ongoing assessment of the impacts of *O.* cf. *ovata.* At this stage the early evidence serves to highlight the possible disparity between impacts within a harmful algal species existing range and those it may have if it expands into new regions. These novel findings provide a suitable basis for further experimentation as well as evidence of the importance for vigilance with regard to the possible future emergence of this species in GB coastal waters.

7.3 - Future work

Work to be considered for the expansion of the studies presented herein would be of value, with the experimental focus on only two toxin classes and three microalgal species leaving others to be explored.

Work to undertake comprehensive baseline surveys around the coast of GB could help to highlight the extent of all existing threats, in terms of both the species present in GB coastal waters and the range which each species occupies. Unlike the more targeted field sampling undertaken as part of this body of work, these would benefit from using untargeted methods for species detection and the sampling of multiple ecosystems, such as plankton sampling, macrophyte/substrate sampling and sediment sampling. In this regard, the employment of modern molecular tools, such as eDNA sequencing (Hatfield et al., 2020), might allow for the detection of not only those species currently known but also elucidation of the prevalence of cryptic species within the waters around the country. Another emerging technology which could be of value for planktonic samples is remote imaging flow cytometry (Luo et al., 2021). Such deployable devices, either on fixed platforms or mobile unmanned vehicles (Griffiths, 2008), can provide near real time imaging and identification of the micro-planktonic community. A further advancement in this field is the increasing use of image recognition software and machine learning (Luo et al., 2021), enabling rapid morphological ID without the need for taxonomic specialists after the training stages of image classifiers and occasional ongoing validity checks. It may even be best practice to combine multiple techniques to provide the most comprehensive data set possible in a study of this kind, with toxin analysis and chemotaxonomy as employed in this body of work being able to further feed into the assessment of presence of harmful strains of a given species as well as the assessment of risk thus associated.

The findings of Chapter 6 make it apparent that the emergence of harmful microalgal species in GB waters could have significant consequences for seafood safety, seafood security, or potentially, both. As such, the development of detection and quantification methods for both the causative microalgae and the toxic compounds produced in many cases, should be a priority. The risk assessment undertaken in Chapter 5 can help to assess the priority of method development, so that those non-native species of highest risk might be addressed first. To date the ichthyotoxic microalgal species have received less research attention than those with a direct impact on human health (Hallegraeff et al., 2017). As such the compounds responsible for their ichthyotoxic properties have not always been determined, indeed as the cause of harm to other marine species is not always the result of a toxin, it may even be the case that there is no such compound to discover (Hallegraeff et al., 2017). Reports of harmful impacts in finfish from a range of high biomass blooms, as a result of not only harmful taxa but also others considered non-harmful, if they reach sufficient cell densities. This can be as a result of reduced oxygen, mechanical damage to gills or production of high levels of compounds such as ammonia. The harmful fish killing microalgal taxa are often associated with gill damage but the specific mechanism is not always known and could include the release of reactive oxygen species, polyunsaturated fatty acids or in some cases phycotoxins (Hallegraeff et al., 2017). Only with further assessment, including under controlled conditions, is it likely that the true extent and cause of these impacts will be discovered. It is also worth mentioning that further experimental work with current harmful microalgal species would be of benefit, although in several cases this is currently constrained by a lack of suitable culturable strains to work with. The ability to work with harmful species in controlled experimental settings remains a key aspect in evaluating the impacts associated with them.

7.4 – Concluding remarks

Overall, this body of work has been able to demonstrate the continued importance of well-studied harmful algal species as well as the capacity to continue to build upon the existing research to further our understanding of these. The value of new data assessment tools has been shown for these well-monitored toxin classes. Furthermore, latter chapters have highlighted that the risks from harmful microalgal species are likely to change as a result of global climate change, creating the possibility for alterations in existing impacts but also the emergence of new ones. To this end, work presented here highlights the potential scope of the emergence of even a single harmful strain of a known toxic species, with the potential for significant impacts in important aquaculture species. The work undertaken and presented within this thesis has successfully addressed the core goals laid out in Chapter 1. With significant contributions across the four goals set out at the opening of the thesis. In this way there has been a novel contribution, including the publication of the contents of two of the chapters, to the understanding of the current and potential future state of harmful microalgal impacts in GB coastal waters.

Appendix 1

This appendix contains a specific bibliography of those papers which were utilised to provide species specific data, collated as part of Chapter 5. As covered in section 5.2.3.

Abadie, E., Chiantella, C., Crottier, A., Rhodes, L., Masseret, E., Berteaux, T., Laabir, M., 2018. What are the main environmental factors driving the development of the neurotoxic dinoflagellate Vulcanodinium rugosum in a Mediterranean ecosystem (Ingril lagoon, France)? Harmful Algae 75, 75–86. https://doi.org/10.1016/j.hal.2018.03.012

Abadie, E., Kaci, L., Berteaux, T., Hess, P., Sechet, V., Masseret, E., Rolland, J.L., Laabir, M., 2015. Effect of nitrate, ammonium and urea on growth and pinnatoxin G production of vulcanodinium rugosum. Mar. Drugs 13, 5642–5656. https://doi.org/10.3390/md13095642

Accoroni, S., Ceci, M., Tartaglione, L., Romagnoli, T., Campanelli, A., Marini, M., Giulietti, S., Dell'Aversano, C., Totti, C., 2018. Role of temperature and nutrients on the growth and toxin production of Prorocentrum hoffmannianum (Dinophyceae) from the Florida Keys. Harmful Algae 80, 140–148. https://doi.org/10.1016/j.hal.2018.11.005

Accoroni, S., Romagnoli, T., Colombo, F., Pennesi, C., di Camillo, C.G., Marini, M., Battocchi, C., Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Tartaglione, L., Penna, A., Totti, C., 2011. Ostreopsis cf. ovata bloom in the northern Adriatic Sea during summer 2009: Ecology, molecular characterization and toxin profile. Mar. Pollut. Bull. 62, 2512–2519. https://doi.org/10.1016/j.marpolbul.2011.08.003

Accoroni, S., Tartaglione, L., Dello, E., Pichierri, S., Marini, M., Campanelli, A., Dell, C., Totti, C., 2017. Influence of environmental factors on the toxin production of Ostreopsis cf . ovata during bloom events. Mar. Pollut. Bull.

Al Muftah, A., Selwood, A.I., Foss, A.J., Al-Jabri, H.M.S.J., Potts, M., Yilmaz, M., 2016. Algal toxins and producers in the marine waters of Qatar, Arabian Gulf. Toxicon 122, 54–66. https://doi.org/10.1016/j.toxicon.2016.09.016

Aligizaki, K., Katikou, P., Nikolaidis, G., Panou, A., 2008. First episode of shellfish contamination by palytoxin-like compounds from Ostreopsis species (Aegean Sea, Greece). Toxicon 51, 418–427. https://doi.org/10.1016/j.toxicon.2007.10.016

Aligizaki, K., Nikolaidis, G., Katikou, P., Baxevanis, A.D., Abatzopoulos, T.J., 2009. Potentially toxic epiphytic Prorocentrum (Dinophyceae) species in Greek coastal waters. Harmful Algae 8, 299–311. https://doi.org/10.1016/j.hal.2008.07.002

Almeda, R., Cosgrove, S., Buskey, E.J., 2018. Oil Spills and Dispersants Can Cause the Initiation of Potentially Harmful Dinoflagellate Blooms ("Red Tides"). Environ. Sci. Technol. 52, 5718–5724. https://doi.org/10.1021/acs.est.8b00335

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