FINAL REPORT

Research to Support the Development of a Monitoring Programme for New or Emerging Marine Biotoxins in Shellfish in UK Waters

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ABBREVIATIONS

AFBI	Agri-Food and Biosciences Institute
AOAC	Association of Official Analytical Chemists
ARfD	Acute reference dose
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
BTX	Brevetoxin
b.w.	Body weight
CCFFP	Codex Committee for Fish and Fishery Products
Cefas	Centre for Environment, Fisheries and Aquaculture Science
CI	Cyclic imines / confidence interval
CFP	Ciguatera Fish Poisoning
CONTAM Panel	Panel on Contaminants in the Food chain
CRLMB	Community Reference Laboratory for Marine Biotoxins
CTX	Ciguatoxins
DG SANCO	Health and Consumer Protection Directorate General
DSP	Diarrhetic Shellfish Poisoning
DTX	Dinophysistoxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EFSA	European Food Safety Authority
eq.	Equivalent

EU	European Union
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FAO/IOC/WHO	Food and Agriculture Organization of the United Nations/ Intergovernmental Oceanographic Commission of UNESCO/World Health Organization
FITC	Fluorscein isothiocyanate
GYM	Gymnodimines
HAB	Harmful Algal Bloom
HPLC	High-performance liquid chromatography
HPLC-UV	High-performance liquid chromatography-ultraviolet detection
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
i.c.	Intra-cerebroventricular
ICES	International Council for the Exploration of the Sea
IOC	Intergovernmental Oceanographic Commission of UNESCO
i.m.	Intra-muscular
i.p.	Intraperitoneal
i.v.	Intravenous
ISO/IUPAC/AOAC	International Organization for Standardization/ International Union of Pure and Applied Chemistry/Association of Analytical Communities
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LD_{99}	Lethal dose – the dose required to kill 99% of the members of a tested animal population
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
MBA	Mouse bioassay
MLD	Minimum lethal dose
MOE	Margin of exposure
MS	Mass spectrometry
MU	Mouse Unit: the minimum amount needed to cause the death of an 18 to 22 g mouse in 15 minutes
MW	Molecular weight
nAChR	Nicotinic acetyl choline receptor
NSP	Neurological Shellfish Poison
NOAEL	No-observed-adverse-effect level
NRCC	National Research Council Canada
OA	Okadaic acid
OJ	Official Journal of the European Union
PcTX	Prorocentrolides
PFP	Puffer fish Poisoning
PlTX	Palytoxins

PnTX	Pinnatoxin
PSP	Paralytic Shellfish Poisoning
PtTX	Pteriatoxin
PTX	Pectenotoxin
RSD	Relative standard deviation
SAMS	Scottish Association of Marine Science
SPE	Solid Phase Extraction
SPX	Spirolide
SRM	Selected reaction monitoring
SST	Surface sea temperature
TDI	Tolerable daily intake
UB	Upper Bound
UNESCO	United Nations Educational, Scientific and Cultural Organization
US FDA	United States Food and Drug Administration
UV	Ultraviolet
VGSG	Voltgate gated sodium channels
VSP	Venerupin Shellfish Poisoning
WG	Working group
WHO	World Health Organization
YTX	Yessotoxin

EXECUTIVE SUMMARY

The literature survey undertaken by the Centre for Environment, Fisheries and Aquaculture Science (Cefas), the Scottish Association of Marine Science (SAMS) and Agri-Food and Biosciences Institute (AFBI) focused on 3 key areas, these were:

1. To establish a list of potential new and/or emerging harmful algae and toxin threats for UK waters and to consider European Food Safety Authority (EFSA) opinion on new and/or emerging toxins.
2. To assess the suitability of existing and potential methods for the detection of new and/or emerging algae and toxins, and identify knowledge gaps where further research could be focused.
3. To collate information on sampling and testing regimes implemented within other countries, EU and worldwide, for the detection of new and/or emerging harmful algae and toxins.

The aim of this project was to:

1. Identify any suitable existing testing methods or potential new methods that might be investigated to support the development of a risk based monitoring programme for emerging marine biotoxins in UK shellfish harvesting waters, in response to the recent amendment to EU hygiene legislation.
2. Identify knowledge gaps that might be addressed through future research work to support suitable method development.
3. Contribute to the design of a monitoring of emerging marine shellfish biotoxins in the UK.
4. Provide evidence to support the UK's negotiations relating to the monitoring of new and emerging toxins in Europe

The review utilised relevant published peer reviewed scientific literature and reports/ communications from scientists/organisations involved in this field. EFSA opinion and the biotoxin laboratory network were also utilised.

Phytoplankton review

Phytoplankton monitoring methods used in the UK programme were found to be generally fit for purpose and in line with other countries. However, as detailed below, for certain existing and potentially emerging species molecular methods of detection are recommended. Phytoplankton data is also less extensively used to target shellfish sampling than in some other countries. Such an approach may be particularly beneficial outside the peak toxicity season.

The review of potentially new/emerging toxic algae in UK waters through either advection or ballast water introduction highlighted 6 genera/species of concern. The highest risk was associated with *Gymnodinium catenatum,* a Paralytic Shellfish Poisoning (PSP) causative species, which has previously been observed to be capable of translocation and which has been found in ballast water of vessels in UK ports. This species has the potential to survive at UK water temperatures and is therefore of high risk as an invasive species. The current official water monitoring programme is capable of detecting this species using existing light microscopy methods should it be included in the toxic species list used in this programme. It is also recommended to evaluate, in the laboratory, the ability of Spanish strains of this species to reproduce in UK water temperature profiles.

The genus *Karenia* and the species *Alexandrium catenella* were found to have equal risk of establishing viable populations in UK waters if advected/introduced. They could be detected in the official water monitoring programme if included in the toxic species list.

The genera *Ostreopsis* and *Coolia* were found to have similar risk of invasion to *Karenia* and *A. catenella*. However, as these are benthic species, the development of a benthic monitoring capability within the programme would be required for full quantification of these algae.

To aid in the necessary identification of *Karenia* and *Ostreopsis* to species level the application of molecular techniques is recommended.

The final organism recommended for potential inclusion in the toxic species list is *Vulcanodinium* sp. This species was identified in New Zealand as the causative organism of pinnatoxins. These toxins have now been identified in Norway and a risk of this genus becoming established in UK waters therefore exists. While it could potentially be detected by the current light microscopy monitoring programme, this would be dependent on more detailed taxonomic information being made available.

Whilst monitoring at all sites for the above algae is possible, it may be sufficient to enumerate the organisms highlighted at a limited number of selected sentinel sites around the UK. At these sites, full phytoplankton counts would be conducted to identify/enumerate these and any other unexpected harmful species detectable using the monitoring method.

The review highlighted a major concern with the monitoring and detection of *Azadinium* spp. Although this genus has already previously been found in UK waters, and as such is not a new or emerging alga, the current light microscopy based monitoring programme cannot detect this species due to its small size and indistinct morphology (at normal microscope magnification). It is recommended that widespread monitoring for this genus be conducted using molecular Quantitative Polymerase Chain Reaction (Q-PCR) methodology.

The survey returns from EU based monitoring organisations indicated that other organisations felt vigilance was required with regard to the possibility of new/emerging toxic algae. However, no new species other than those already discussed were highlighted.

Toxin review

The review of new/emerging toxins showed the highest overall risks arising from the potential presence of novel AZA and PSP analogs, together with the brevetoxins (BTXs).

The review highlighted new/ emerging risks associated with PSP and lipophilic toxins toxin groups which are currently not monitored within the UK official control programmes. These include most notably the presence of PSP toxins originating

from *Gymnodinium catenatum* and other analogues of the regulated groups of toxins including the large number of azaspiracids (AZAs) identified to date as shellfish metabolic products. For the PSPs *G. catenatum* produces a range of hydrophilic and hydrophobic toxins including gonyautoxin 6 (GTX6). Currently no certified standards are available for GTX6 and other analogues, this leaves a number of options for quantifying these toxins and their associated risks. These include the development of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, the assessment of the Receptor Binding Assay (RBA) technology and toxicity studies to determine risk from identified toxins. For the new analogs of AZAs, the development and validation of the current EURL LC-MS/MS reference method for lipophilic toxins would be required, together with the availability of formally-assessed toxicity equivalence factors from oral toxicity studies, work which is ongoing.

BTX was also shown to provide a high potential risk to consumers in the UK, given the combination of toxin severity and likelihood of occurrence. Although currently not recorded in the UK the potential presence of the causative organisms (*Karenia* spp.) increases the risk. The current shellfish monitoring programme would not detect these toxins and it is recommended that a two stage analysis approach for BTXs be considered utilising a screening assay and confirmatory analysis by LC-MS/MS.

Cyclic imines (CIs) perhaps represent a group of emerging toxins which are already present in UK waters and in shellfish food products. Whilst the likelihood of occurrence may be high, a risk to the consumer is not fully established given the lack of information on human toxicity following oral consumption. The monitoring of these toxins, particularly pinnatoxins and spirolides, is recommended through a screening method and development of analytical methods (LC-MS/MS). However, this may be limited by a lack of analytical standards.

Palytoxins (PlTXs) have been found to be spreading in Europe and are a potential future risk in the UK. Methods for detecting these toxins have not been established and the development of suitable methods is inhibited by the lack of analytical standards for palytoxin group compounds. The production of standards and development of non-animal methods is suggested.

Given the unsuitability of UK water temperatures for the growth of ciguatoxin (CTX) producing organisms, the main risk relates to intoxication from imported products. The recommended monitoring approach is the use of a cytotoxicity screen and LC-MS/MS confirmation. Again, low availability of analytical standards presents a major barrier to the development of both screening and confirmatory methods.

Tetrodotoxins (TTXs) whilst generally present in warmer waters have been linked to a range of bacterial sources which are well known to exist in UK waters and in UK shellfish. There is also the occasional occurrence of the toxins in European waters. However, with an absence of any evidence for intoxication from these highly potent neurotoxins in the UK, it is unlikely that the risk is particularly high in UK cultivated shellfish at present.

Cyanobacterial toxins are perhaps the most difficult to assess in terms of threats to shellfish consumers. The threat has not been demonstrated in the UK to date and the report proposes a review of toxins currently present in UK species of marine cyanobacteria and the development of testing methods to help assess the potential threat of these toxins in the UK.

The majority of the responders to the toxin survey concentrated on toxin groups most commonly perceived as emerging threats within the European context. These were toxins associated with warmer water: ciguatoxins, palytoxins and tetrodotoxins. A lower number highlighted brevetoxins and cyanotoxins. Cyclic imines were thought to be the most likely to be currently present in UK waters.

EFSA review

The EFSA report concluded that there was insufficient data to allow them to characterise the risk associated with exposure to PlTX-group toxins. If the PlTX-group toxins were to be regulated the ability of the current analytical methods to detect the group at any proposed regulatory level would have to be considered. A range of testing methods including cell based assays, immunological based assays

and analytical methods (high performance liquid chromatography [HPLC-FLD] and LC-MS/MS) were considered for development but would require investment.

The EFSA Panel also concluded that whilst the mouse bioassay (MBA) has been used for the detection of cyclic imines, there are ethical reservations concerning its use, and its poor specificity makes it inappropriate as a method of analysis. Receptor assays and LC-MS/MS offer improved methods for the quantification of cyclic imines but further development and validation is required. EFSA found no reports of human illness associated with cyclic imines. Whilst the Panel considered acute reference doses (ARfDs) should be set for the toxin group, there is insufficient data on which to do this.

EFSA recognised that the high toxicity of CTX-group toxins and their emerging occurrence necessitates the development of appropriate strategies to protect human health. The use of *in vitro* assays as screening tests, supported by LC-MS/MS should be developed but EFSA recognised that there is a need for access to certified standards and reference material to allow the development and optimisation of alternative methods to the mouse bioassay.

The MBA has traditionally been the method of analysis for BTX-group toxins but for reasons of animal welfare and poor specificity, it is not considered an appropriate method. EFSA noted that immunoassays and LC-MS/MS are applicable to the detection of the group in shellfish and fish products but further development and validation is required. The Panel indicated that progress in this area would be assisted by the availability of certified reference materials.

Recommendations

The review findings can be utilised within EU negotiations. It is recommended that to comply with Regulation (EC) 854/2004, the FSA should consider extending the current list of phytoplankton monitored in UK waters to include those genera/species highlighted above which are linked with the production of emerging toxins and that have been identified as potentially present in UK waters or with a potential to become present. Given the financial implications of widespread monitoring of these organisms the report recommends, that most are enumerated only at a limited number of sentinel sites (perhaps at a lower frequency than the weekly regulatory

monitoring). However, widespread monitoring with high temporal resolution, of the genus *Azadinium* is recommended.

Phytoplankton monitoring alone will be insufficient, and must be accompanied by the development and application of suitable methods of analyses for the associated toxins in flesh. However the priority given to these toxins as health issues varies throughout Europe. Therefore, careful prioritisation of the methods to be developed is required to avoid unnecessary demand on the limited resources of certified standard material. This is important in some European countries for emerging toxin groups such as PlTX and TTX, with evidence for their presence in shellfish and for CTXs which present a clear recognised risk in finfish. For the UK, it is recommended that if not a full quantitative method at least a screening method should be developed and validated for pinnatoxins. Data on CIs should be recorded as part of the current monitoring programme when possible, to provide the competent authority with occurrence data within UK waters. The report also recommends the development of appropriate screening and confirmatory methods for the detection of BTX-group toxins.

Chapter 1

1. AN OVERVIEW OF THE EFSA OPINIONS ON NEW AND EMERGING TOXINS AND A CONSIDERATION OF THE OPINIONS IN THE LIGHT OF RECENT DEVELOPMENTS IN TESTING METHODS AND TOXICOLOGICAL DATA

1.1 INTRODUCTION

The late 1990s saw a number of food related crises which prompted the setting up of the European Food Safety Authority (EFSA) in January 2002. The Authority was established as an independent source of scientific advice and communication on the risks associated with the food chain. It was seen as a key part of the EU strategy to improve EU food law which aimed to protect the consumer and restore confidence in food production and processing. EFSA provides scientific advice and opinions which underpin European policies and legislation on food safety and aims to ensure member states can take effective risk management decisions. The EFSA's terms of reference cover food and feed safety, nutrition, animal health and welfare, plant protection and plant health. Its primary aim is to provide objective scientific advice based on up-to-date information and knowledge.

Following concerns raised by France and Germany, the European Commission advised EFSA in May 2006 of a possible request related to marine biotoxins. On receipt of the mandate, EFSA requested its Panel on Contaminants in the Food Chain (CONTAM) to address the issue. CONTAM were requested to *"assess the current EU limits with regards to human health, the methods of analysis for marine biotoxins as established in the EU legislation, as well as new emerging toxins".* The Panel were asked to consider international reports and recommendations and any other relevant scientific information.

In response to the request, CONTAM established a Working Group (WG Biotox) which formulated a list of marine biotoxins which should be considered in the opinion and agreed to draft the opinion from a risk assessment point of view.

What followed was a series of opinions on: Okadaic Acid (November 2007), Azaspiracids (June 2008), Yessotoxins (December 2008), Saxitoxins (March 2009), Pectenotoxins (May 2009), Domoic Acid (July 2009) and a summary in August 2009. The Working Group also produced statements on the influence of shellfish processing in March 2009 and on the consumption figure of 400g shellfish meat used in the opinions. The Panel was of the opinion that the current limits for a number of the regulated toxins were not sufficient to protect high consumers against acute toxins. The WG were of the opinion that the MBA for lipophilic toxins was inappropriate due to its high variability and detection capability. It was of the opinion that whilst the bioassay for saxitoxin and its analogues was capable of quantification at the current regulatory limit, it could not be relied on at levels below this.

Having considered the current regulated toxins, the WG went on to consider a number of new/emerging toxins. Opinions were published on Palytoxin (November 2009), Brevetoxin (July 2010), Ciguatoxin (May 2010) and Cyclic Imines (May 2010).

The current UK monitoring programme is aimed at the detection and quantification of the regulated toxins. It is important that the ability of the current testing regime to detect new and emerging toxins is assessed. With this in mind, the EFSA opinions on emerging toxins will be reviewed and recommendations on the modifications required to the current monitoring programme will be discussed based on:

- The risk posed to human health by the toxin
- The risk of occurrence in UK waters
- The methods available for detection and quantification
- The ability of current official control methods to detect the toxins
- The ability of the MBA proposed under EC15/2011 to detect the toxins covered by the EFSA opinions.

1.2 CYCLIC IMINES: SPIROLIDES (SPX); GYMNODIMINES (GYM); PINNATOXINS (PnTX); PTERIATOXINS (PtTX)

1.2.1 Introduction

The cyclic imines (CIs) are a family of marine biotoxins which can be present in shellfish and include spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs).

The main producer of SPX is the dinoflagellate *Alexandrium ostenfeldi.* SPX was first isolated from scallops and mussels in the early 1990s (Hu et al. 1995). EFSA noted that the profile of SPX toxins varies significantly with geographical location (Cembella et al., 2000).

EFSA reported that GYM was first identified in New Zealand oysters in the early 1990s and was linked with the dinoflagellate *Karenia selliformis.*

At the time of submission of the EFSA report, the organism producing PnTXs was yet to be identified. The toxin was first reported in 1995 in Japanese shellfish and a number of analogues have since been identified (PnTX A-G).

PtTXs have not been detected in shellfish from Europe but EFSA have included them in their report because they may be produced in shellfish from PnTXs.

The CIs were discovered through their high toxicity to mice following interperitoneal (*i.p.*) injection. The toxins cause rapid death which leads to interference in the MBA for lipophilic toxins.

In Europe, SPXs have been identified in the causative organism in a number of countries, for example, Scotland (John et al., 2003), Ireland (Touzet et al., 2008a) and Italy (Ciminello et al., 2006). The toxins have also been detected in shellfish in Norway (Aasen et al., 2005), Spain (Villar Gonzalez et al. 2006) and Italy (Pigozzi et al. 2008).

GYMs have not been reported in shellfish from Europe and whilst PnTXs have been reported from Norway, EFSA reported that data from other European countries is limited. There is currently no data linking CIs with incidents of human intoxication.

The members of the CI-toxin group show a high degree of structural similarity and are soluble in organic solvents. The SPXs are the largest group of the CIs and EFSA detailed the structure of the range of SPX analogues. The Panel noted that, like other marine biotoxins, the SPXs can be metabolised in shellfish to fatty acid esters (Aasen et al., 2006; Doucet et al., 2007).

The GYMs are the smallest of the CIs and the chemical structure of GYM-A, GYM-B and GYM-C have been elucidated.

The chemical structure of PnTXs is most closely related to that of the SPXs and a number of analogues have been identified (A-G). It is now thought that PNTX-F and G are the progenitors of all the known PnTxs and PtTXs that are produced through metabolism in shellfish (Selwood et al., 2010). It is thought that PnTX-F is metabolised to PnTX-D and E whilst PnTX-G is metabolised to PnTXs A-C and PtTXs A-C.

The chemical structure of the PtTX analogues has been determined and they are almost structurally identical to PnTxs. The difference is that the cyclohexenyl side chain of the PtTXs ends in a cysteine group.

There are currently no regulatory limits set in Europe or elsewhere in the world for the CI group. The Panel noted that the European Reference Laboratory working group on toxicology proposed a guidance level of 400µg/kg of SPXs/kg of shellfish (CRLMB 2005; Pigozzi et al., 2008).

1.2.2 Occurrence of CI-group toxins

EFSA reported that they were provided with data on the occurrence of CIs by France, Italy, The Netherlands and Spain. GYMs were not detected and evaluation of SPXs only was undertaken. In addition PnTX-G and PnTX-A have been detected in mussels in Norway and Miles et al., (2010) have reported on the occurrence of

SPXs in Norway. The data reviewed by EFSA was all derived from LC-MS/MS analysis. Due to the high LOQ reported by Spain and the lack of quantitative data, the Spanish data was excluded from their calculations.

In analysing the data, occurrence was assessed in whole shellfish and results based on analysis of digestive glands were adjusted by a factor of 5 to convert to whole tissue. From the data supplied by the three European countries considered, concentrations ranged from “not detected” to 105µg SPX/kg. The overall figure for not quantified results was 80%. From occurrence data not included in the original submissions, SPX-C and 20 methyl SPX-G detected in shellfish in Norway had levels of between 4-25µg/kg and 4-20µg/kg, respectively. Miles *et al.* (2010) reported maximum concentrations of 49, 38 and 226µg/kg for SPX-C, 20-methyl SPX G and 13-desmethyl SPX-C, respectively.

The EFSA Panel reported that results from Norway indicated that levels of PnTX A were low whilst PnTX-G was detected generally in the range 5-30µg/kg but levels upto 115µg/kg had been recorded. PtTXs have not been detected in European shellfish.

EFSA considered the distribution of SPXs in shellfish species based on the data submitted from France, Italy and the Netherlands (1801 samples) and determined the 95th percentile levels to be 7µg/kg in clams, 15µg/kg in oysters and 9µg/kg in mussels. SPXs were not detected in scallops. EFSA had no information on the influence on processing on the levels of CIs in shellfish.

1.2.3 Methods of Analysis

Several methods have been described for the detection of CIs. The EFSA report noted that whilst the MBA is considered a simple approach, there are ethical reasons against its use. Functional and LC-MS/MS approaches have been described, but the Panel noted that, to date, none have been formally validated in interlaboratory studies. Whilst certified standards are available for 13-desmethyl SPX-C and GYM-A, the Panel indicated that at the time of writing, there were no other standards available for the CI group.

Mouse Bioassays

SPXs have been detected using the MBA developed for lipophilic toxins (Yasumoto et al., 1978). The toxins caused rapid death and were identified as a source of false positives in the method. The method has also been used to detect GYM-A in clams (Bire et al., 2002) but the symptoms and time to death are not recorded. Based on the work of Mundy (unpublished, 2008) the limit of detection for 13-desmethyl SPX C has been estimated at 5.6µg/kg and for SPX-C a limit of detection (LOD) of 6.4µg/kg. For GYM-A, the LOD has been estimated at about 77µg/kg. Based on studies by Selwood et al., (2010), the LODs for PnTx E, F and G are estimated to be 40, 36 and 13µg/kg. The Panel recognised that the MBA does provide an indication of the overall toxicity of the sample but it does not give information on the individual toxins present. The Panel report that the use of the MBA is highly variable, has not been validated and is not acceptable in many countries for ethical reasons.

Biomolecular methods

Functional assays have been developed on the basis that GYMs and SPXs selectively interact with cholinergic receptors. The inhibition assay developed by Vilarino et al. (2009a) for the detection of GYM-A and 13-desmethyl SPX-C is capable of detecting any of the CIs which interact with the receptor nAChR. The method has been demonstrated to be effective for the detection of GYM-A and 13 desmethyl SPX-C in a number of shellfish species (Fonfria et al. 2010) with LODs reported by the panel as 80 and 85µg/kg respectively. The Panel was of the opinion that the method was rapid and capable of detecting a broad spectrum of CIs but requires a source of receptors and has yet to be validated.

Chemical methods

The structure of CIs makes them particularly suitable for detection by LC-MS/MS and low limits of detection and high specificity can be achieved. It is the current method of choice, with a number of multi-toxin methods reported for the detection of lipophilic toxins (Gerssen et al., 2009a, 2009b; Fux et al., 2007). Extraction is based on aqueous methanol with optional clean up using either solvent partition or solid phase extraction. Both acidic and alkaline liquid chromatography conditions have been

developed to reduce matrix effects. Recoveries of over 90% are reported for SPX-C and GYM-A (Gerssen et al., 2009a) and LODs of 0.8 and 3.7 units respectively have been reported (Gerssen et al., 2009b). For PnTXs, LODs have not been recorded, however, for PnTX-G the lowest level detected by Miles et al., (2010) was 5µg/kg. The Panel report on an interlaboratory study (McNabb et al., 2005) on the determination of lipophilic toxins by LC-MS/MS which included the CIs. In this instance only GYM A was quantified. HORRAT values of 0.8 to 2.0 were reported. The Panel concluded that LC-MS/MS is highly specific and suitable for confirmatory analysis, with LODs lower than other reported methods. However they acknowledge that instruments are expensive and require highly skilled operatives and the lack of certified standards is a significant disadvantage.

HPLC with ultraviolet detection (HPLC-UV) has been described for the detection of GYM-A and B in clam tissue (Marrouchi et al., 2009), with an LOD of 2.4µg/ kg of digestive gland reported. No other methods using HPLC with optical detection are reported by EFSA. The Panel recorded that for general application to the CIs group, the low UV adsorption of the group means confirmatory analysis is required. No interlaboratory validation or proficiency test has been undertaken and the lack of certified standards limits development of the approach. EFSA recorded that LC-MS/MS multi-residue approaches show the most promise for the determination of CIs but validation and the development of standards are required.

1.2.4 Table 1. Summary of methods for CIs

Method	Advantages	Disadvantages
Mouse Bioassay	Official Control method for lipophilic toxins will detect CIs	High Variability Not validated Ethical concerns over use of animals
Receptor Assay	Capable of detecting broad spectrum of CIs	Requires source of receptors May be subject to interference

		Not validated
LC-MS/MS	Number of multi toxin methods available Highly specific Low LODs Validation undertaken for some compounds Current Official Control method can detect CIs	Expensive equipment Skilled operatives required Lack of certified standards for some compounds
HPLC-UV	Method available for GYM Low LOD	Low UV adsorption Confirmatory analysis required No validated methods Lack of certified standards for some compounds

1.2.5 Exposure Assessment

In line with its previous opinions on marine biotoxins, EFSA determined consumption of 400g of shellfish in one meal to represent a large portion size. As CIs are not regulated, there is the potential for all contaminated produce to reach the consumer. EFSA estimated that based on the 95th percentile figure of 8.9µg/kg, consumption of 400g would result in a single exposure of 0.06µg/kg body weight (b.w.) or 3.6µg/ 60kg person. EFSA determined a probabilistic estimate of dietary exposure based on the distribution of both the occurrence data and data on shellfish consumption. The chance of exceeding the exposure estimate of 3.6µg/ 60 kg person corresponding to a portion of 400g containing the 95th percentile concentration (3.4µg / portion) was estimated to be 4%.

1.2.6 Toxicokinetics

EFSA reported that there is no information on the absorption, distribution or excretion of SPXs, GYMs or PnTXs in laboratory animals or humans. Information based on oral administration (Munday et al., 2004; Munday 2008b; Richards et al., 2001) indicate that the compounds are absorbed from the intestinal tract. Information from the EU research project Conffidence (2010) indicates that analogues of SPX-C can be detected in the blood, urine and faeces of mice following oral administration. Rapid recovery following sub-lethal doses of GYM-A or 13-desmethyl SPX-C (Munday et al., 2004) suggests rapid detoxification or excretion.

The EFSA Panel reported that there is no information on the biotransformation of CIs in mammals but it is known that biotransformation and detoxification occurs in molluscs (Hue *et al.*, 1996; Munday, 2008b; Stewart *et al.*, 1997).

1.2.7 Mode of Action

SPXs and GYMs are neurotoxic with a similar mechanism of toxicity. Evidence of their neurotoxic activity is based on inhibition of both muscarinic and nicotinic acetylcholine receptors (nAChR) (Munday, 2008b). PnTXs cause respiratory paralysis in mice and it is thought they also target nAChR (Selwood et al., 2010). There is currently no data on the mode of action of PtTXs. The main difference between GYMs and SPXs is that GYMs show a reversible effect whilst SPXs appear to be irreversible (Molgo et al., 2007, 2008).

1.2.8 Effects in Animals and Relative Toxicity

Toxicity following intraperitoneal injection (i.p.)

SPXs

The most toxic SPXs are 13-desmethyl SPX-C, SPX-C and 20 methyl SPX-G with median lethal dose (LD_{50}) values of 6.9-8.0µg/kg b.w. (Munday, 2008b). Mice receiving lethal doses of SPXs died within 3-20 minutes, with survivors recovering

completely (Munday, 2008b). In mice the neurotoxic symptoms described include, hunched appearance, abdominal breathing, respiratory distress, contractions, tremors and ultimately death (Gill et al., 2003).

GYMs

GYM-A is highly toxic to mice following *i.p*. injection, with an LD_{50} of 80-96µg/kg b.w. The signs of toxicity include hyperactivity, jumping, paralysis and extension of the hind legs. Death occurs within 15 minutes of injection. At sub-lethal doses, prostration and respiratory distress were evident but mice recovered completely within 30 minutes. GYM-A is reported to be 10 times more toxic than GYM-B (Kharrat et al. 2008).

PnTXs

LD_{50} values for PnTXs range between 16-50µg/kg b.w., with the analogues E and F being the most toxic (Rhodes *et al.*, 2010b). The signs associated with toxicity include, hyperactivity followed by a sudden decrease in activity, abdominal breathing, respiratory failure and death occurring within 22-26 minutes. At sub-lethal doses, mice showed abdominal breathing and lethargy but full recovery was evident by 2 hours. The limited data available shows that PtTX B/C mix is 12 times more toxic than PtTX-A (Takada et al., 2001b).

Toxicity following oral administration

SPXs

SPXs are more toxic by the gavage route than when administered on food. By gavage the LD_{50} for the most toxic analogues ranged from 53-176µg/kg b.w. In feeding trials, the LD_{50} values ranged from 500-1005µg/kg b.w. The signs of toxicity observed were similar to those described following *i.p.* injection.

GYMs

The oral LD_{50} for GYM-A by gavage (755µg/kg b.w.) was 8 times higher than *i.p.* injection in mice. No signs of toxicity were observed in mice administered doses of approximately 7500 µg/kg (Munday et al., 2004).

PnTXs

The only data available to EFSA related to PnTXs E+F. The LD_{50} following administration by gavage was 23µg/kg b.w. and 60µg/kg b.w. following

administration in food. The values were estimated from the LD_{50} of algal extract containing 10µg PnTX/mg (Rhodes et al., 2010a). EFSA recorded that these values are the lowest of any of the cyclic imines.

1.2.9 Relative Potency of Analogues

EFSA reported that the CIs appear to act through the blocking of AChR. The evidence suggests that these interactions may differ between the groups, hence why reversible and irreversible effects are seen. The Panel concludes that whilst there is no data on combine exposure, it is reasonable to assume additive toxicity by the different analogues within each group. In Europe, only a limited number of CIs have been detected – the SPXs and the current practice of reporting the concentration as the sum of the analogues, with a factor of one for each analogue is justified.

1.2.10 Observations in Humans

The Panel observed that there have been no reports of human illness linked to CIs. An outbreak of toxicity in Nova Scotia, Canada at a time of high concentrations of SPXs could not be linked to the toxin and clinical signs did not match those observed in mice (Richard et al., 2001). Reports from New Zealand indicated that consumers of shellfish with GYMs present suffered no ill effects (Munday et al., 2004). Similarly reports from the Rangaunu Harbour region of New Zealand, suggest no ill effects among consumers of shellfish with PnTX concentrations up to 200µg/kg of shellfish (McCoubrey, 2009).

1.2.11 Hazard Characterisation

There is no data available on long term studies to establish a tolerable daily intake for the CIs. The Panel noted that oral toxicity varies depending on the method of administration, gavage or in feed. Whilst there is no quantitative data on human toxicity, the Panel were of the opinion that given their acute toxicity, ARfDs should be

established for the different groups. However the Panel indicated that there is insufficient data on which to establish ARfDs.

1.2.12 Risk Characterisation

The Panel reported that data reported on oral toxicity in mice was not appropriate for the establishment of an ARfD. They did however calculate a margin of exposure (MOE) based on the oral LD_{50} and the 95^{th} percentile of exposure (0.06µg/kg b.w.). The MOE ranges from 1000 to 10,000 depending on whether the LD_{50} from gavage or in feed is used. The Panel felt that the LD_{50} from administration in food was the most appropriate. Based on their analysis, they concluded that exposure to SPXs does not raise concern for the consumer. They note however that there is a high degree of uncertainty associated with estimating exposure for the European population due to the lack of occurrence data.

1.2.13 Key Conclusions

Methods of Analysis

- The MBA has traditionally been used for the detection of CIs but it is considered as inappropriate for ethical reasons and its poor specificity.
- Receptor-based methods and LC-MS/MS methods have been developed and would be of value in the quantification of CIs, subject to further development.

Occurrence/Exposure

- SPXs have been identified in a number of European countries and whilst GYMs have not been reported in European shellfish, they have been found in products from outside Europe. PnTxs have been detected in shellfish in Norway
- The Panel estimated a dietary exposure of 3.6 µg sum of SPXs for a 60 kg adult (equivalent to 0.06 µg sum of SPXs/kg body weight (b.w.)).

Hazard Identification and Characterisation

- The toxic signs are consistent with the ability of these compounds to bind and block acetylcholine receptors in the central- and peripheral nervous systems, including neuromuscular junctions.
- No reports of human illness due to SPXs, GYMs, PnTXs or PtTXs have been identified.

Risk Characterisation

- The CONTAM Panel concluded that current estimated exposure to SPXs does not raise concern for the health of the consumer although this conclusion is based on very limited toxicity data.
- Exposure to GYMs, PnTXs and PtTXs could not be estimated from the available data therefore no conclusions could be drawn with respect to any possible risk to consumers.

1.3 BREVETOXIN (BTX)

1.3.1 Introduction

Brevetoxins (BTXs) are lipid-soluble polyether compounds which can be differentiated on the basis of their molecular structure into Type-A (or 1) and Type-B (or 2). The toxins can accumulate in fish and shellfish and are produced by the dinoflagellate *Karenia brevis.* The BTX-group toxins are metabolised in shellfish and fish and several metabolites have been isolated and characterised (Ishida *et al.*, 1995, 1996). Consumers of contaminated product are therefore more likely to be exposed to the metabolites rather than the parent molecules. The toxin causes Neurological Shellfish Poisoning (NSP) which is characterised by neurological and gastrointestinal effects, including nausea, vomiting, diarrhoea, paralysis and coma. Whilst occurrence is limited to the Gulf of Mexico, the east coast of the United States and regions of New Zealand, the discovery of new BTX-producing algae and the

trend in the expansion of bloom distribution suggest the toxin may emerge in other regions. Algal species such as *Chatonella* spp., *Fibrocapsa japonica* and *Hetrosigma akashiwo* have been reported to produce BTX-like toxins (FAO 2004).

Regulatory limits for BTX-toxins have been set in the United States (20 mouse units [MUs] /100g or 0.8mg BTX-2 equiv/kg, US FDA 2001)) and in New Zealand (20MUs / 100g) but the BTX-analogue is not stated (NZFSA 2006, FSANZ 2010).

1.3.2 Occurrence of BTX-group toxins

The EFSA Panel reported that there have been no reports of NSP outbreaks or occurrence of BTX-group toxins in shellfish or fish in Europe. EFSA is ofthe opinion that with the discovery of new BTX producing algae and the expansion of algal bloom distribution, the toxin could emerge in Europe. Levels recorded in shellfish ranged from 880 – 49,000µg BTX-2/kg (based on conversion from MUs). Levels measured by enzyme linked immunoassays (ELISA) in fish in Florida gave levels of 580-600µg BTX-3/kg. The EFSA Panel had no information on the influence of processing on BTX toxins in fish and shellfish and no information on which to estimate exposure for European population.

1.3.3 Methods of Analysis

The determination of BTX-group toxins has been undertaken in algae, shellfish and fish using methods such as MBA, cytotoxicity assays, immunoassays and instrumental methods such as LC-MS/MS. Certified standards for BTX-1 and BTX-2 are commercially available but characterisation of methods for fish and shellfish have been hampered by the lack of certified reference material.

Mouse Bioassays

The classical method of detecting BTX-group toxins is by MBA. Following diethyl ether extraction, the crude lipid-extract is injected *i.p.* into mice and results are reported as Mouse Units (MU) per 100g. It is estimated that one MU is equivalent to 4µg BTX-2 equivalents. Several studies (for example, Dickey *et al.*, 1999) have

indicated that diethyl ether does not extract some of the BTX-group toxins effectively. The MBA does provide a measure of the overall toxicity of the sample and is relatively simple however there are disadvantages. The method gives no information on toxin profiles, there are questions over the extraction efficiency for some of the toxin group, and the bioassay is inherently variable due to specific animal characteristics. In many countries the MBA is considered undesirable for ethical reasons.

Biomolecular methods

Cytotoxicity assays for BTX-group toxins are based on the action of the toxins on voltage-gated sodium channels. Most cytotoxicity assays use neuroblastoma cells and a limit of detection for BTX-1 of 0.25mg/kg (shellfish) has been reported (Manger *et al.*, 1993). However, the assays performance in an interlaboratory trial is reported to have been highly variable (Dickey *et al.*, 2004).

Receptor binding assays based on competitive binding between radio labelled BTX-3 and BTX-group toxins have been reported by a number of workers (for example, Van Dolah *et al.*, 1994; Fire *et al.*,2008). The limit of quantification (LOQ) for receptor assays has been reported as 30µg BTX-3 / kg of oyster (FAO/IOC/WHO, 2004).

Immunoassays based on ELISA and radioimmunoassays (RIA) have been described and the approaches are more specific than the mouse bioassay. The RIA has largely been replaced by ELISA but both approaches are rapid and are applicable to multiple sample analysis.

The EFSA Panels view on cytotoxicity, receptor and immunoassays is that they have the advantage of being applicable to multiple sample analysis but they do not give information on the toxin profile. The cytotoxicity and receptor assays are susceptible to interference from other substances which act on sodium channels whilst the cross-reactivity of the antibody used in immunoassay based methods may require confirmatory analysis of samples identified as positive.

Chemical methods

The application of LC-MS/MS to the detection of BTX-s has been reported widely and the EFSA opinion referenced a number of reports including the application of LC-MS/MS with electrospray ionisation (Dickey *et al.*, 1999) and LC-MS/MS with electrospray ionisation in positive and negative mode using selective ion reaction monitoring (Ishida *et al.*, 2004bc, 2006). The LOQ are reported to be in the range of 0.2 – 2µg/kg for a range of BTX-toxins. The EFSA panel report that an interlaboratory study (Dickey *et al.*, 2004) demonstrated that LC-MS/MS was capable of quantifying samples spiked with BTX-3 at an order of magnitude below the USA regulatory level (0.8µg/kg). EFSA acknowledges that the approach allows the gathering of information on toxin profiles and its high specificity makes it ideal as a confirmatory method. However, they acknowledge that instrumentation is expensive and as yet formal interlaboratory validation studies are lacking.

1.3.4 Table 2. Summary of Methods for Brevetoxin

Method	Advantages	Disadvantages
Mouse Bioassay	Official Control method for lipophilic toxins could be adapted to detect some brevetoxin analogues. Gives an indication of overall toxicity. Relatively simple technology.	Diethyl ether extraction may not extract all Brevetoxin analogues. High Variability. Not validated. Ethical concerns over use of animals.
Cytotoxicity / Receptor Assay	Functional assay which can detect all analogues. Applicable to multiple sample analysis	Highly variable performance in interlaboratory trials. Subject to interference from other substances which act on sodium channels / receptors. No information on toxin profile
Immunoassays	More specific than MBA	Cross reactivity of the

	Rapid tests, applicable to multiple test analysis	antibody may require confirmatory testing of positives No information on toxin profile
LC-MS/MS	Low LOQs / LODs High Specificity Gives information on toxin profile Ideal for confirmatory analysis	Expensive equipment Limited availability of certified standards Interlaboratory validation data is limited.

1.3.5 Toxicokinetics

The EFSA panel reported that information on toxicokinetics is limited to BTX-2 and BTX-3. Peak levels in rats are reported to have been achieved 1 hour after *i.p.* injection, with levels 3 times higher for BTX-2 than BTX-3 (Radwan *et al.*, 2005). BTX-3 administration by intravenous (*i.v.*) injection is cleared from circulation within one minute of administration and eliminated within 24 hours (Poli *et al.*, 1990). Following oral administration, BTX-3 is rapidly absorbed and distributed to all organs with the highest levels found in the liver. Inhalation is considered an important route in the development of NSP and in rats, Benson et al (1999) report that 80% of radiolabelled BTX-3 was absorbed from the lungs to blood and subsequently distributed to all tissues. The majority of BTX-3 was cleared from the major organs within 7 days.

1.3.6 Mode of Action

BTX-group toxins bind with high affinity to the receptor sites of voltage gate sodium channels and can affect mammalian cortical synaptosomes and neuromuscular preparations (Watkins *et al.*, 2008). These effects are associated with significant membrane depolarization. Respiratory effects are also due to the interaction of the

toxins with the voltage gated sodium channels on nerve cell membranes (FAO/IOC/WHO, 2004).

1.3.7 Effects in Animals and Relative Toxicity

The BTX-group toxins cause depression of respiratory and cardiac function, muscular contractions, twitching and leaping, paralysis and death. The EFSA report indicates that there is limited toxicity data available on the BTX group and their metabolites. Signs of intoxication following *i.p.* injection have been described for BTX-B2, BTX-3 and S-deoxy-BTX-B2 with lethal doses inducing immobility after 15 minutes followed by respiratory paralysis and death. At sublethal doses, fast abdominal breathing immediately following injection was noted. Subsequent paralysis occurred with movement regained after 3-5 hours. Following *i.v.* injection, toxic signs and death were immediate. Oral toxicity data for BTX-2 indicates a LD_{50} value of 6600mg/kg b.w. whilst for BTX-3 a figure of 520mg/kg b.w. is reported (Baden and Mende, 1982). The authors report that the difference in values may be due to differences in the absorption rate of the toxin analogues.

Several studies have indicated the potential for BTXs to induce chromosomal damage. BTX-2 induced chromosomal aberrations in hamster ovary cells (Sayer *et al.*, 2006) and DNA damage was found in Jurkat E6-1 cells following exposure to BTX-2, BTX-3 and BTX-6 (Murell and Gibson, 2009).

The EFSA Panel report that *i.p.* toxicity for BTX-2, BTX-3, BTX-B2 and S-deoxy-BTX-B2 are similar but there was insufficient data on which to evaluate the relative potencies of other analogues. It was noted that the oral toxicity of BTX-3 appears to be a factor of 10 higher than that of BTX-2.

1.3.8 Observations in Humans

Symptoms of NSP are apparent within 30 minutes to 3 hours after consumption exposure and may last for several days. Reports of deaths or chronic conditions are not recorded. EFSA report that dermal or inhalation exposure can result in irritant

effects. Most cases of intoxication are recorded in the Gulf of Mexico region and New Zealand. There is no information on the concentration of BTX-toxins in the contaminated shellfish responsible and no observable effect levels in humans have been established. Samples from oysters implicated in an outbreak of NSP contained levels of 35-60 MU whilst oysters from the general region had levels between 48-170MUs. Gessner (2000) estimated a low toxic dose of 42-72MU per person (168-228ug BTX-2).

1.3.9 Hazard Characterisation

The EFSA Panel concluded that there was insufficient data on which to establish a tolerable daily intake. The limited data available suggest that effects could be caused by exposure to 2.4-4.8µg BTX-2/kg b.w. No information was available on no-effect levels and the CONTAM Panel were unable to set an ARfD for the toxin group. The potential carcinogenic effects of this toxin group which have been reported raised concerns over long term exposure to BTXs. The Panel could not comment on the risk of exposure to BTXs in shellfish and fish produce reaching the European consumer due to the limited data available.

1.3.10 Key Conclusions

Methods of Analysis

- The MBA has been used for the detection of BTX-group toxins but due to its poor specificity and animal welfare issues is not considered appropriate.
- In vitro and immunoassays have been developed for the detection of BTX-group toxins. Together with LC-MS/MS they offer the potential for quantification of the toxins but certified standards are required to allow further development and validation.

Occurrence/Exposure

- Up to the time of production of the report, BTX-group toxins had not been reported in shellfish or fish from Europe.
- There is no information on the affect of processing on the levels of BTX-group toxins in fish or shellfish.

Hazard Identification and Characterisation

- The BTX-group toxins cause toxicity through binding to voltage-gated sodium channels.
- There is evidence of potential long-term carcinogenic effects.
- The CONTAM Panel considered that an ARfD should be established for the BTX-group toxins. However, due to the limited quantitative data, this was not possible.

Risk Characterisation

- The discovery of new BTX-producing algae and the apparent expansion of algal bloom distribution, suggest that BTX-group toxins could also emerge in Europe.
- Due to limited data on acute and chronic toxicity the CONTAM Panel could not comment on the risk associated with the BTX-group toxins.

1.4 PALYTOXIN

1.4.1 Introduction

Palytoxin-group toxins (PlTXs) are complex polyhydroxylated compounds with both lipophilic and hydrophilic areas. The toxins are insoluble in nonpolar solvents such as diethyl ether and hexane, sparingly soluble in methanol and ethanol and soluble in pyridine, dimethyl sulphoxide and water.

The toxins occur in soft corals of the genus *Palythoa* and benthic dinoflagellates of the genus *Ostreopsis*. Distribution of *Ostreopsis* is worldwide and has been reported in the Mediterranean Sea since the 1970's with blooms recorded in France, Greece, Italy and Spain. In addition to PlTXs, other analogues of this toxin group are produced by a number of *Ostreopsis* species (e.g ovatoxins, oestreocin). A number of analogues may be produced by the same species, for example, *O. tuberculosa* is known to produce five analogues in addition to PlTXs. EFSA noted that whilst the patient symptoms associated with PlTX are not well defined (muscle pain and weakness, possibly accompanied with fever, nausea and vomiting), it can be fatal (EFSA reported the case of a 49 year old in the Philipines who died within 15 hours following the consumption of contaminated crab). . There are no regulatory limits set for PlTX in Europe or worldwide. However, the report indicates that a provisional limit of 250µg/kg was proposed by the WG on Toxicology of the NRLs for Marine Biotoxins (2005). This compares with a regulatory limit of 800µg/kg for saxitoxin and 160µg/kg for okadaic acid and azaspiracid.

1.4.2 Methods of Analysis

EFSA considered the methods of analysis under bioassays, functional assays and chemical methods. The Panel noted that there was a lack of appropriate certified standards which would have a significant impact on the application of available methods, their validation and the development of new methods.

Mouse Bioassays

EFSA reported that the MBA has been used for the detection of PlTX-group toxins in fish and shellfish tissue. The initial method was not specifically designed for PlTX but as a general method to isolate the causative agent of a food poisoning outbreak in Japan and was based on acetone extraction of whole flesh or hepatopancreas with subsequent evaporation and resuspension in Tween. A modified version (Taniyama, 2002) introduced initial extraction with 75% ethanol followed by partition with diethyl ether after removal of the ethanol. In addition to defattening, this removed potential

interference from okadaic acid and its analogues. EFSA noted that given the partitioning properties of PlTX (Aligizaki et al, 2008), the harmonised EURL method (based on Yasumoto, 1985) would not efficiently extract the PlTX group. The UK harmonised protocol differs in some respects from the EURL protocol but is essentially based on Yasumoto (1985) and the same problem applies. EFSAs assessment of the MBA indicates the advantages of the MBA are that it provides a measure of total toxicity and does not require complex analytical equipment. However, to be quantitative it would have to be calibrated against a certified standard which are scarce. Therefore it was found that the protocol based on Yasumoto (1985) was not suitable and other modifications of this approach are subject to interference from YTX-group toxins and water soluble toxins. The clinical signs in mice for PlTX-group toxins (jerking, stretching, paralysis, convulsions and death within 15 minutes) are severe and pose significant ethical objections to the use of the mouse bioassay. EFSA see the inherent variability and the requirement for specialised handling facilities as practical disadvantages to the use of the mouse bioassay.

Biomolecular methods

Anti-body based methods and those based on cell death have been considered by EFSA under the general heading of biomolecular methods.

Haemolysis Assays

The general principle of haemolysis assays is the ability of the toxin to interact with the Na^+/K^+-ATPase (an enzyme located in the cell membrane which is responsible for maintaining relatively high concentrations of potassium ions and low concentrations of sodium in the cell). The toxin interferes with the function of the enzyme, converting it into a non-specific cation channel which leads to an imbalance in the red blood cell and to delayed haemolysis. The test exploits this characteristic haemolysis and the suppression of this activity in samples pre-treated with Ouabain (an ATPase blocker) to identify the presence of haemolytic compounds in the sample.

EFSA reports on several studies which have used haemolysis neutralisation to quantify the levels of PlTX group toxins in algal, fish and shellfish extracts (Bignami

1993). The advantages of the method are that it is readily adapted to a microtitre plate format, enabling multiple samples to be analysed. It has the potential to detect low levels and can also detect all biologically active members of the toxin group. However, it has low specificity and may detect other haemolytic compounds present in the sample.

Cytotoxicity assays using MCF-7 breast cancer cell lines where cell lysis is measured by the release of lactate dehydrogenase and assays employing neuroblastoma cells were reviewed. Whilst the assays have the advantages of having good detection capabilities and can detect the biologically active PlTX group toxins, they too are prone to interference from other cytotoxic compounds. Using cell cultures also requires specialised facilities for the maintenance and handling of cell cultures to be available.

Immunoassays

Immunoassays are ideal screening tests with low limits of detection capabilities, however, they can be subject to matrix interference. EFSA recorded that whilst a number of ELISA methods have been reported for PlTX, there is little information on their application to detection of PlTX in shellfish tissue. It is noted that there has been little in the way of recent developments in this area and this may reflect that antibodies are not readily available. The effectiveness and accuracy of the methods relies heavily on the performance of the antibody and its ability to detect as wide a range of the PlTX-group as possible. However cross-reactivity may not reflect toxic activity and the accuracy of results obtained would need to be treated with caution. Whilst EFSA in principle see immunoassays as having an application as a screening method, they do indicate that confirmatory testing would be required. Antibody production requires significant amounts of the toxin and EFSA noted there is a lack of standard material which may explain why there have been no recent developments in the area.

Chemical Methods

HPLC-based methods

EFSA reported on one HPLC-FLD method based on pre-column derivatisation and solid phase extraction (Riobo *et al.* 2006). The method has been used to determine

PlTX concentrations in extracts from the dinoflagellate genus *Ostreopsis* and there is no information on its application to shellfish tissue.

HPLC-UV using different mobile phases and column combinations has been frequently applied for the detection of PlTX (Lenoir *et al.*, 2004, Oku *et al.* 2004). However, as indicated above, the application has been largely to algal extracts rather than shellfish tissue. EFSA indicated that limits of detections quoted for the method range from 0.1 to 2µg injected. This compares with 0.75ng injected for HPLC-UV detection. However there is no information on the detection capabilities in shellfish tissue.

In general HPLC methods have the advantages of being fully automated and are relatively inexpensive (although fluorescence detectors are more expensive than UV detectors). It should be noted in the case of HPLC-FLD, its applicability can be reduced by the complexity of sample extraction, clean-up and derivatisation.

LC-MS/MS

A number of methods have been published on the use of LC-MS/MS for the identification and quantification of PlTX group toxins however these have been confined to seawater and phytoplankton extracts. Riobo *et al.* (2006) reported significantly higher LOD and LOQ for LC-MS/MS when compared with FLD when both methods were used for the detection of PlTX in *Ostreopsis*. EFSA suggests that this could have been due to factors associated with the LC-MS/MS system used and that improved detection capability is reported with the use of triple quadrupole (Ciminiello *et al.* 2006). The application of the methodology to the determination of PlTX in shellfish tissue has been reported in France and Italy where it has been used for research and premarket control. Significant differences in the LODs (2 and 36µg/kg, respectively) are thought to be due to differences in the instrumentation used and the matrix tested. EFSA's conclusion was that whilst LC-MS/MS has the potential to provide a sensitive method, capable of detecting and quantifying the PlTX group, instrumentation is expensive and requires highly trained staff. The establishment of LC-MS/MS as the reference method for the lipophilic toxin group (EC Regulation 15/2011) has seen increased use of LC-MS/MS in monitoring laboratories throughout Europe. With the instrumentation and expertise now well

established in many laboratories, the disadvantages noted by EFSA may no longer be an issue.

EFSA noted the application of high performance capillary electrophoresis for the detection of PlTX (Mereish *et al.*, 1991) and that with UV detection, the LODs were significantly improved over HPLC-UV. However they also note that strong matrix effects make the method unsuitable for the analysis of shellfish tissue.

External proficiency tests are a valuable tool in assessing the performance of methods of analysis but this information is not available for the methods highlighted within the EFSA report. The Panel noted that whilst the MBA say has been used to detect the toxin group in a range of fish and shellfish tissue, the method has poor specificity and there are growing concerns over its continued use on animal welfare grounds. Cytotoxicity and immunoassays may be valuable screening tools but issues with crossreactivity and matrix interference will require confirmatory methods to be available. It is EFSA's opinion that the optimisation of HPLC-FLD and LC-MS/MS for use in shellfish tissue is necessary. However they note that for progress to be made, the availability of standard and certified reference material is required.

1.4.3 Table 3. Summary of methods for PlTX

Method	Advantages	Disadvantages
Mouse Bioassay	Widely used for detection of PlTX in fish and shellfish. Provides a measure of total toxicity. No requirement for complex instrumentation. Potential to quantify if calibrated	Potential portioning problems for toxin depending on extraction protocol. Lack of certified standards. No validation. Severe clinical signs in mice.

	against certified standard.	Ethical issues in the use of mice.
Cytotoxicity / Receptor Assay	Readily adapted to a microtitre plate format. Multiple samples can be analysed. Potential to detect low levels. Can detect all biologically active members of the toxin group.	Low specificity – prone to interference from other haemolytic / cytotoxic compounds. Specialised facilities for maintaining and handling cell cultures are required.
Immunoassays	Potential to have low LODs Readily adapted to a microtitre format Potential as a screening method	Little development may reflect lack of antibodies. Effectiveness and accuracy of assays relies heavily on the performance of the antibody. Cross-reactivity may not reflect toxic activity. Confirmatory method required.
HPLC	Number of alternative approaches available using both UV and FLD detection Fully automated and relatively inexpensive	UV less sensitive than FLD. Sample preparation for HPLC-FLD more complex. Application to algal extracts, little information on application to shellfish tissue. Optimisation of HPLC-FLD for use in shellfish tissue required.
LC-MS/MS	A number of methods have been published. Capable of detecting and quantifying the PlTX group.	Optimisation for use in shellfish tissue required. Instrumentation is expensive and requires highly trained staff.

	Approach used in France and Italy for research and premarket control.	Standard and certified reference material is required. No validation

1.4.4 Exposure Assessment

In assessing exposure, EFSA issued a call for data in 2008 on the occurrence of PlTX-group toxins in Europe. In response to the call, only Greece supplied data which was obtained from 11 samples tested by haemolytic neutralisation assay which returned levels significantly above the LOD.

With the identification of *Ostreopsis* species in the Mediterranean, monitoring plans for the species and research projects were in place in a number of countries. Data from these sources were considered by EFSA in assessing exposure. The data was supplied by France and Italy on PlTX group toxin levels in sea urchins and mussels. However the data was obtained from two very different technologies - haemolytic neutralisation assays and LC-MS/MS. EFSA noted further that the data from sea urchins was obtained during a bloom event and during the summer which is outside the normal harvesting time for sea urchins. As a consequence EFSA relied on the data from mussels only to arrive at an illustrative figure for exposure estimate and indicated there was significant uncertainty in establishing the 95^{th} percentile value for PlTX-group toxin. A figure of 185µg of PlTX group toxins per meal was estimated. EFSA also indicated that because of the lack of occurrence data from shellfish intended for consumption, a probabilistic estimate was not possible.

1.4.5 Toxicity

EFSA detailed the complex molecular responses to PlTX group toxins but it is clear that the primary action is on the Na^+/K^+ ATPase in the cell membrane of excitable and non-excitable cells, making skeletal, heart and smooth muscle cells the major targets for the toxin group (Habermann *et al.* 1989 and others). Among the

responses observed in animal models is vasoconstriction causing heart failure and death (Wiles *et al.*, 1974).

The toxin is identified as acutely toxic and the dose required to illicit a response is dependent on the route of administration. Data is available for PlTX and oestreocin-D but none for ovatoxin-A. Work by Wiles *et al.* indicates that there are large species differences following *i/v* administration with rabbits, dog and monkey being more susceptible than mice.

Intraperitoneal administration of PlTX results in LD_{50} values of 0.72µg/kg b.w. Clinical signs associated with toxicity include: stretching of the hind legs, concave curvature of the spine and progressive muscular paralysis. Animals injected with high doses of PlTX died quickly without any obvious morphological signs. Intra tracheal administration at doses of 2µg of PlTX and above resulted in the death of mice within 2 hours of administration. The mice showed paralytic signs and extreme lung pathology (Ito and Yasumoto, 2009). In rats a lethal dose of 5-7.5µg/kg b.w. was established. Sublingual administration in mice (Ito and Yasumoto, 2009) of a single dose of PlTX and oestreocin-D caused inactivity and rapid respiration. Repeated doses of PlTX resulted in scratching and severe pathology but this was not observed for oestreocin-D.

Oral administration of PlTX in mice gave an LD_{50} of 510µg/kg b.w. Sosa *et al.* (2009) reported a LD_{50} of 767µg/kg b.w, with the severity of clinical signs increasing with dose. In rats, an oral 24-hour LD_{50} of >40µg/kg b.w has been reported (Wiles *et al.* 1975).

EFSA assessed PlTX and oestreocin to be equipotent by the oral route and that given they act on the same target and show only minor structural differences, exposure to both was likely to be additive in effect. EFSA had no information on the relative toxicity of ovatoxin-A.

1.4.6 Observations in Humans

PlTX has been associated with human illness for over four decades, however, EFSA recorded that many of the reports are anecdotal and the clinical signs, symptoms

and dose response relationship are not well defined. The EFSA opinion reported on human intoxication following the consumption of fish, crustaceans and following inhalation exposure (Deeds et al. 2010). This report found that algal sources of the toxins have not been generally investigated and no incidents of intoxication linked with bivalve molluscs were detailed.

The most reported signs of PlTX poisoning in humans is the breakdown of muscle fibres leading to myoglobin release into the bloodstream (Rhabdomyolysis), resulting in possible renal failure. Common symptoms are myalgias, generalised weakness, fever, nausea and vomiting. Clinical chemistry shows elevated serum creatine kinase and myoglobinurea. Recovery from PlTX poisoning is reported to take several months. Poisoning can occur through the skin and reports of coughing and fever have been reported from seaside resorts in Italy during periods of *Oestreopsis Ova* blooms (Gallitelli *et al.* 2005 and Ciminiello *et al.* 2006).

Confirmation of PlTX poisoning is by clinical symptoms and confirmation by MBA or other screening assay without quantification. Seafood sources include parrotfish, identified as responsible for an outbreak in Japan (Noguchi *et al.* 1988), hairy crab (*Demania reynaudii*) in the Philippines in 1984 where the content in crab was estimated to be between 200-924µg/kg. However EFSA were of the opinion that this evidence was insufficient for CONTAM to estimate a dose of PlTX and on which to base hazard characterisation.

EFSA reported the association of PlTX with clupeotoxism, a condition associated with the consumption of tropical clupeid fish. Investigations into a poisoning incident associated with sardines by Onuma *et al.* (1999) indicated that PlTX or a similar analogue was present. Whilst *Oestropsis siatnensis* (*Ostreopsis siamensis*) was inferred as the source of the toxin, this could not be confirmed.

1.4.7 Hazard Characterisation

The EFSA report concluded that as no long term toxicity studies had been undertaken, it was not possible to determine a daily tolerable intake. The report cited a number of cases of human intoxication. It also identified that the PlTX group were

toxic by several routes, with intra muscular (*i.m)*, *i.v.* and *i.p.* more potent than administration by intra tracheal. They concluded that oestrocin-D and PlTX were equipotent and therefore dose addition is to be expected given their similar structure and mode of action. There is no reliable quantitative data on acute toxicity in humans but there is good agreement on oral toxicity by gavage and the CONTAM panel agreed on an acute oral dose for PlTX of 200µg/kg b.w. The severe toxicity of internal organs following sublingual administration was noted. This, together with the reduced sensitivity of mice to the toxin, resulted in EFSA applying an additional uncertainty factor of 10, to the factor of 10 for intra and 10 for interspecies variation. This resulted in an estimated oral ARfD of 0.2µg/kg b.w. They concluded that this should apply to the sum of PlTX and ostreocin-D. There is a high risk to humans from aerosol inhalation and the potential for contact through open wounds and EFSA concluded that the ARfD should not apply in these circumstances. The CONTAM panel could not characterise the hazard of ovatoxin-A and other PlTX-group toxins due to the lack of toxicity data.

1.4.8 Risk Characterisation

The CONTAM panel thought it appropriate to use a large portion size (400g) in determining the risk, due to the acute toxic effects of PlTX. Their conclusion was that for a 60kg adult to avoid exceeding the ARfD of 0.2µg/kg b.w. shellfish should not contain more than 30 µg/kg of the sum of PlTX and ostreocin-D.

From the limited data available, the Panel concluded that consumption of shellfish from contaminated areas could result in exposure to PlTX and ovatoxin-A of levels up to 3µg/kg b.w but data on occurrence of PlTX-group toxins in shellfish is limited and a reliable exposure assessment for the European consumers is not possible.

1.4.9 Key Conclusions

Methods of Analysis

- There is no information on the efficiency to extract PlTX group compounds using the extraction protocols currently used for marine biotoxins.
- The MBA has been used but for ethical reasons and its poor specificity, there are growing concerns over its continued use.
- Cell based assays have good sensitivity but are subject to interference and poor specificity and confirmatory analysis would be required.
- HPLC-FLD and LC-MS/MS offer the best way forward but optimisation of these methods and the development of standards and reference materials are required.

Occurrence/Exposure

- There is a lack of occurrence data on PlTX
- The limited data available suggests a dietary exposure limit of 3µg/kg b.w of PlTX and ovatoxin-A.

Hazard Identification and Characterisation

- PlTX and ostreocin-D are acutely toxic and can be assumed to be equipotent.
- Rhabdomyolysis is the most reported sign of PlTX poisoning.
- There are no long term studies on the toxicity of PlTX.
- An oral ARfD of 0.2µg/kg b.w. has been proposed.
- The hazards of ovatoxin-A and other PlTX group toxins could not be assessed due to lack of data.

Risk Characterisation

- To avoid exceeding the established ARfD of 0.2µg/kg b.w, shellfish should not contain more than the sum of 30µg/kg of PlTX and oestreocin-D.

- The most frequently reported PlTX group toxin in the occurrence data is ovatoxin-A. There is no information available on the toxicity of ovatoxin-A and the CONTAM panel could not characterise the risk to European consumers.

1.5 CIGUATOXIN (CTX)

1.5.1 Introduction

Ciguatoxin-group toxins (CTXs) are found mainly in the Pacific, Caribbean and Indian Oceans and are classified as (P), (C) or (I) – CTX-group toxins respectively. The toxin group have also been detected in fish caught in European waters. They occur through the biotransformation of precursor molecules produced by the benthic dinoflagellate *Gambierdiscus toxicus* (Murata *et al.*, 1989, 1990). Ciguatera poisoning is mainly associated with consumption of large predatory fish which have accumulated the toxin from the consumption of smaller contaminated fish. There are no reports of poisoning following consumption of shellfish. The toxin causes the syndrome ciguatera fish poisoning (CFP) characterised by a range of clinical signs, including gastrointestinal, neurological and cardiovascular effects and death can occur from cardiac or respiratory failure. The toxins are lipid soluble polyether compounds with over 20 analogues identified for the Pacific, 10 for the Caribbean and 4 for the Indian variant. Other toxins, such as maitotoxin (MTX) have been isolated from *Gambierdiscus toxicus* but EFSA only considered ciguatoxin in its report.

Whilst no regulatory limits or methods of analysis are set within Europe, EC regulations do require that checks are made to ensure that fishery products do not contain biotoxins such as CTX (EC Reg 854/2004). The USA FDA have proposed guidance levels of <0.1µg/kg for C-CTX-1 and <0.01µg/kg for P-CTX-1. Australia provides guidelines on fish species which may contain the toxin and information on geographical locations where fish may be contaminated. Neither Australia nor New Zealand has set regulatory limits. Japan has a ban on specific fish species such as Barracuda.

1.5.2 Occurrence of CTX-group toxins

The causative organism *Gambierdiscus* spp*.* have been detected as far west as Crete in 2003 (Aligizaki and Nikolaidis, 2008) and as far west as the Canary Islands and Madeira in 2008 (Aligizaki *et al.* 2008). The presence of the toxin producing organisms has been confirmed by the presence of toxin-contaminated fish in Israel (Bentur and Spanier, 2007). Limited occurrence data is available from two recent incidents of human intoxication. An outbreak of human intoxication in the Canary Islands was linked to the consumption of Amberjack (*Seriola Rivoliana*) and the presence of C-CTX-1 was confirmed by *in vitro* assay and LC-MS/MS with levels estimated at 1.0µg/kg. The second incident in Madeira was linked to the consumption of the fish *Seriola dumerili* and *Seriola fasciata*. The presence of a number of ciguatoxin-group toxins were identified and quantified by LC-MS/MS with concentrations of ≥ 1.0µg/kg. EFSA were unable to reach any conclusion on the potential influence of processing on the levels of CTX due to the lack of data.

1.5.3 Methods of Analysis

As with other marine biotoxin groups, development of methods to detect the CTX group are inhibited by the lack of certified reference standards and reference material. A further complication is that P, C and I-variants are all slightly different and methods developed to detect one form may not be applicable to another. Similarly, reference material prepared in one region may not be suitable for development of a method in another area.

Mouse Bioassays

The mostly widely used MBA is that based on the method of Banner *et al.* (1960) and used extensively in the Pacific region. Also described by Yasumoto *et al.* (1971, 1984) it is based on the *i.p.* injection of a diethyl ether extract suspended in Tween. Mice are observed over the first 2 hours and then over 24 hours. The LOQ is approximately 0.56µg/kg and the relationship between dose and time of death is used to quantify the toxin present in the extract. Modified protocols have been

developed (for example Yokoyama *et al.* 1988) to reduce interference from MTXs which are also produced by *Gambierdiscus toxicus* but have a different mode of action. The MBA gives a measure of total toxicity and does not require complex instrumentation. However, it has limitations including that it does not give information on the profile of toxins present, its limit of quantification may not permit detection of regulatory levels of the toxin and the methods have not been validated. In addition there are ethical objections to the use of mouse bioassays.

Biomolecular methods

Anti-body based methods and those based on cell death have been considered by EFSA under the general heading of biomolecular methods. The cytotoxicity assays described are based on the capacity of the toxin to bind to sodium channels, causing them to open at normal cell resting potential. Manger *et al.* (1993, 1994, 1995) have produced an assay which is sensitive at pg/kg levels for the toxin group. A fluorimetric method developed in 2001 for the detection of saxtoxin (Louzao *et al.* 2001) has been adapted for the detection of ciguatoxin-group toxins (Louazo *et al.* 2004).

Manger (1995) reported that results obtained using neuroblastoma cells showed good correlation with the mouse bioassay and Bottein Dechraoui *et al.* (2005a) have reported an LOQ of 0.039µg/kg in fish tissue. This protocol has been adopted by the US FDA and NOAA laboratories for in vitro assay of CTX-group toxins in finfish and is used in parallel with a confirmatory liquid chromatography method.

EFSA recognised the methods are simple, can be automated and are sensitive. However, EFSA questions whether they offer a cost effective method of screening individual fish. The methods do not give information on toxin profiles and have yet to be validated.

Receptor-based assays based on the inhibition of radio-labelled ciguatoxin in the presence of ciguatoxin have been reported (for example, Lombet *et al.*,1987 and Bottein Dechraoui , 1999). The methods have good detection capabilities but require radio-labelled reagents and a source of receptors. These methods have not been

validated. Similarly, methods based on radioimmunoassay have been developed (Hokama *et al.* 1977) but these methods were found to be impractical for routine use.

Radio-labelled approaches have been superseded by ELISA which although improve on previous RIA are still impractical for routine use. The application of monoclonal antibodies led to the development of stick-test procedures (Hokama *et al.* 1989) which were widely used for survey and clinical diagnosis. Membrane immunobead assays with LOD of 32ng C-CTX-1/kg have also been reported and the method of Hokama *et al.* (1998) was subject to an AOAC collaborative study. However, the method was not approved due to a lack of information on the antibody used and difficulties in interpreting the results.

The EFSA panel observed that whilst immunoassays in general are fast and more specific than MBA and are suitable for rapid screening of samples, there are several drawbacks to their use. The antibodies may be specific to region-specific CTX groups, positive results need to be confirmed and there have been no successful international validation studies to date.

Chemical methods

HPLC-FLD and LC-MS/MS methods has been described for the detection of the ciguatoxin-group toxins. For HPLC, derivitisation of the CTX-group toxins is required. Yasumoto *et al.* (1993) described a method based on this approach but noted that further work was required to improve the clean-up prior to analysis. Subsequent methods have failed to detect CTX-1 at µg/kg levels and EFSA note that there is a lack of validation and performance characteristics for HPLC-FLD.

The EFSA panel report that the advantage of LC-MS/MS methods is their ability to detect the individual analogues of all the regional variations. Quantification of clinically relevant levels requires an LOD of 0.1µg/kg or less (Lewis, 2001). This has been demonstrated for P-CTX-1, using a concentration step such as solid phase extraction (Lewis *et al.* 2009) and a modification of this approach (Stewart *et al.* 2010) has been established as a reference method for the Public Health Laboratory for Queensland, Australia. EFSA reports a recovery averaging 53% and an LOD of 0.03µg/kg for the method.

LC-MS/MS is highly specific and suitable as a confirmatory method but development of methods, their optimisation and interlaboratory validation is restricted by the lack of certified standards and reference material.

1.5.4 Table 4. Summary of Methods for Ciguatoxin

Method	Advantages	Disadvantages
Mouse Bioassay	Official Control method for lipophilic toxins could be adapted to detect some CTX analogues. Adaptations described to reduce interference Gives an indication of overall toxicity. Relatively simple technology.	May not detect toxins at regulatory limit No information on toxin profile. Not validated. Ethical concerns over use of animals.
Cytotoxicity / Receptor Assay	Simple tests Good sensitivity Can be automated	No information on toxin profiles May not be cost effective for screening fish Not validated
Immunoassays	Rapid More specific than MBA Suitable for screening	Antibodies may be region-specific for CTX Requires confirmatory method Not validated
HPLC-FLD	Method developed for CTX-group toxins	Derivitisation required Poor sensitivity Clean-up requires further improvement Not validated
LC-MS/MS	Can detect individual toxins irrespective of region. Suitable as confirmatory method	May require concentration step to achieve appropriate LODs Further development hampered by lack of certified standards Not validated

1.5.5 Human Consumption and Exposure

The CTX-group of toxins cause acute toxicity and the EFSA Panel considered it appropriate to consider the high percentiles for single meals for exposure assessment. Information on fish consumption was available to the Panel but the methods applied to collecting the data varied significantly. In countries which applied a 1-day (24 hours) recall survey, the 95 percentile consumption ranged from 250-422g per day and the 97.5 percentile range was 300-500g per day. The Panel concluded that data from this group probably represented the distribution of single meals. The data from 2-day surveys indicated a 95 percentile range of 140-178g per day and a 97.5 percentile range of 176-225g per day. Based on this data and data supplied on fish consumption from the UK, EFSA assumed a large portion size of 350g of fish per meal. This compared with the reference figure of 500g quoted in the FAO paper on marine biotoxins (FAO 2004). The lack of data on the occurrence of ciguatoxins in fish prohibited an exposure assessment on the European population.

1.5.6 Toxicokinetics

The EFSA panel could find no studies specifically aimed at the adsorption of CTX-group toxins. However, they note that adsorption through the gastrointestinal route is suggested on the basis of toxicity studies in mice following oral administration (Lehane and Lewis, 2000). Biotransformation has been observed within animals following the administration of C-CTX-1, with both polar and non-polar toxins detected in the blood (Bottein Dechraoui *et al.*, 2005a). Transfer of the toxin group via the placental route (Pearn *et al.*, 1982) and transfer through the mother's milk (Bagnis and Legrand, 1987) have been reported.

1.5.7 Mode of Action

The report identifies the voltage-gated sodium channel as the primary molecular target for the toxins. Binding of the toxins causes the opening of the channel and the influx of sodium to the cell. This leads to membrane depolarisation and functional

impairment of excitable cells. This process, together with increases in the secretion of neurotransmitters, is the underlying cause for the loss of muscle and nerve function which leads to the paralysis observed in animals exposed to the toxin group.

1.5.8 Effects in Animals and Relative Toxicity

It has been established that CTX-group toxins are acutely toxic upon *i.p.* administration. Death times varied from 0.5 to 1 hour and LD_{50} values of 0.25 to 2.3µg/kg b.w. for the toxin analogues which have been reported (Lewis *et al*., 1992). The main signs include hypothermia, piloerection, diarrhoea, lacrymation and terminal convulsions leading to death from respiratory failure. Oral administration of 0.19-0.56µg/kg b.w. produced lethal doses and clinical signs similar to those produced by *i.p*. injection (Ito *et al.,* 1996b). The Panel note that the CTX-group show differing relative receptor affinity which in general correspond to the LD_{50} values established by i.p. administration in mice. The values are recorded in the EFSA report and range from 1 (P-CTX-1) to 0.05 (P-CTX-4B).

1.5.9 Observations in Humans

It is estimated that 10,000 to 50,000 people are affected by CFP each year. However, the Centre for Disease Control and Prevention estimate that only 2-10% of cases are reported. Diagnosis of the disease is based on clinical signs and symptoms which include gastrointestinal, neurological and cardiovascular symptoms. Onset can be as early as 30 minutes or may be delayed to 24-48 hours in milder cases. Death is uncommon and it is suggested that the toxins toxicity to fish may limit the accumulation in live fish (Lewis, 1992). It has been reported that individuals who have previously suffered from the toxin may have a recurrence of symptoms on exposure to levels which do not cause symptoms in others. This may suggest sensitisation or accumulation (De Fouw *et al.*, 2001). The EFSA opinion records that duration and severity of symptoms vary with both ethnic group and sex. It is, however, unclear if this reflects a genetic predisposition or differences in consumption patterns. A few reports record exposure of pregnant women to the toxin

and indicate that the toxin can affect both mother and the unborn child (Pearn *et al.*, 1982 and Fenner *et al.*, 1997).

Despite the large number of cases, there are few in which exposure levels have been estimated. In the Pacific, most cases involved consumption of fish containing 0.1 – 5.0µg P-CTX-1/kg of fish. A review of poisoning incidents in Japan between 1997 and 2006 estimated a concentration of 0.175 - >5.6µg P-CTX-1/kg in fish (Oshiro *et al.*, 2010). C-CTX-1 has been reported to be less toxic than P-CTX-1 (FAO 2004); however cases have been reported with concentrations of 0.6µg C-CTX-1/kg (CDC 2009).

1.5.10 Hazard Characterisation

The Panel observed that there are few oral studies in mice and no long term studies on which to base a tolerable daily intake. EFSA also concluded that there was insufficient data on which to establish an acute reference dose. The Panel concluded that toxicity in the Pacific was associated with levels between 0.1 and 5µg P-CTX-1/kg. Since this range was established using analytical methods which could detect other CTX toxin analogues, an uncertainty figure of 10 should be applied to the lower value to give a concentration of 0.01µg P-CTX-1/kg. The Panel concluded this should cover all CTX-group toxins that could be present and represents the level at which no toxic effects would be expected in sensitive individuals.

The CONTAM panel could not comment on the risk associated with exposure to CTX-group toxins in Europe because of the limited occurrence data associated with fish in Europe. The Panel further concluded that the uncertainties associated with exposure assessment were so large as to make detailed consideration of the potential sources meaningless.

1.5.11 Key Conclusions

Methods of Analysis

- The MBA has poor specificity and detection capability.
- Although widely used, the MBA is not considered appropriate for the detection of CTX-group toxins.
- Cytotoxicity and receptor assays can detect the active analogues but do not provide information on toxin profiles.
- Immunoassays are rapid and simple but tend to be region-specific for the CTX toxins.
- LC-MS/MS can detect all individual toxins but methods require further development.
- None of the methods currently available have been validated.

Occurrence/Exposure

- CTX toxins have been detected in fish in Europe.
- There is insufficient data on which to make an exposure assessment for the European population.

Hazard Identification and Characterisation

- CTX causes acute toxicity by binding to voltage-gated sodium channels
- TEFs should be applied to individual analogues where quantitative methods are used.
- EFSA concluded that a level of 0.01µg P-CTX-1 equivalents/kg should be taken as the concentration not expected to exert an effect in sensitive individuals.

Risk Characterisation

- The Panel could not comment on the risk associated with exposure to CTX-group toxins because of the limited occurrence data available.

1.6 CONCLUSIONS

The EFSA report concluded that there was insufficient data to allow them to characterise the risk associated with exposure to PlTX-group toxins. PlTX has been found in European waters and is known to cause toxicity in humans. If the PlTX-group toxins were to be regulated the ability of the current analytical methods to detect the group at any proposed regulatory level would have to be considered.

Whilst cell-based assays appear to have the lowest LOD for PlTX-group toxins, some assays showed interference with other toxins and any positive results would require confirmation by chemical analysis. The maintenance and management of cell lines would require specialist facilities.

Immunoassay-based methods offer the potential for rapid automated testing for the toxin. However, it was reported that antibodies are not readily available and matrix effects may reduce the sensitivity that can be achieved. The cross-reactivity of the antibody may not reflect the toxicity of the sample, thus accuracy of the result may be questionable and confirmatory analysis would be required.

HPLC-FLD and LC–MS/MS methods have been identified as valuable tools for the determination of the PlTX-group toxins. Most current developments have focused on the application of LC-MS/MS for the detection of the toxin. Lack of certified standards and reference material will inhibit progress and whilst the technologies and expertise are in place, the development of HPLC-FLD and LC-MS/MS based methods for PlTX may require significant investment in time and resources.

The Panel concluded that whilst the MBA has been used for the detection of CIs, there are ethical reservations concerning its use and its poor specificity makes it inappropriate as a method of analysis. Receptor assays and LC-MS/MS offer improved methods for the quantification of CIs but further development and validation is required.

SPXs have been identified in shellfish in a number of countries bordering the Mediterranean, the Atlantic and North Sea. GYMs have not been reported in shellfish from Europe and to date PnTXs have only been reported from Norway, however other countries have not undertaken surveys to detect the toxin. The evidence presented to the Panel suggests that PtTXs are transformed from PnTXs but as yet have not been reported in Europe. No reports of human illness associated with CIs have been recorded and whilst the Panel considered ARfDs should be set for the toxin group, there is insufficient data on which to do this. The EFSA Panel concluded that the current estimated exposure does not raise concern for the health of the consumer but they acknowledge this conclusion is based on very limited data. For the GYMs, PnTXs and PtTXs, EFSA could not draw any conclusions on the risk to consumers, due to the lack of exposure data. The current official control method for the detection of lipophilic toxins (LC-MS/MS) is capable of detecting both GYM and 13-desmethyl spirolide C (CIs for which certified reference standards are commercially available). Effective monitoring could be undertaken by expansion of the current monitoring programme to include the provision of data on cyclic imines to the Competent Authority.

EFSA recognised that the high toxicity of CTX-group toxins and their emerging occurrence necessitates the development of appropriate strategies to protect human health. The use of in vitro assays as screening tests, supported by LC-MS/MS should be developed but EFSA recognises that there is a need for access to certified standards and reference material to allow the development and optimisation of alternative methods to the MBA. Whilst cytotoxicity and receptor assays can provide the levels of detection required, they do not provide information on toxin profiles. Immunoassays are rapid and easy to use but tend to be region-specific for the toxin groups. LC-MS/MS offers the potential for the detection of individual toxin analogues but further development is needed and is hampered by lack of certified materials. The Panel views this lack of certified materials as a block on the optimisation and validation of alternatives to the MBA. To evaluate occurrence and exposure, more information is needed on the occurrence of CTX-group toxins, gambierol and MTXs in fish and other seafood. If it is established that monitoring is necessary for CTX-group toxins in the UK, the most cost effective approach would appear to be the

development of LC-MS/MS methodology, given the limitations of the available antibody and receptor based screening methods which would require the additional development of a confirmatory chemical method. Official control laboratories have the expertise and instrumentation in place but additional resources to support the purchase of reference material, method development and validation would be required. These resources would also be required for the development and validation of a screening test.

The MBA has traditionally been the method of analysis for BTX-group toxins but for reasons of animal welfare and poor specificity, it is not considered an appropriate method. EFSA notes that immunoasssays and LC-MS/MS are applicable to the detection of the group in shellfish and fish products but further development and validation is required. The Panel indicated that progress in this area would be assisted by the availability of certified reference materials.

In characterising the risk, the Panel acknowledged that with the identification of new BTX-producing algae and the trend in expansion of algal bloom distribution, BTX-group toxins could emerge in Europe but due to the lack of data, they could not comment on the current risk of exposure to the toxin group in Europe. The panel recommended the development of immunoassays for screening coupled with the development of LC-MS/MS methods for confirmation. The level of testing required for BTX-group toxins will determine whether it is cost effective to undertake the development of both a screening assay and a confirmatory method. If the confirmatory method can be integrated into existing protocols to provide a multi-toxin approach, it would provide a more cost effective approach to analysis and reduce the demand on expensive equipment time. In any case, the lack of BTX certified standards may limit the development of both screening and confirmatory methods.

Chapter 2

1. POTENTIAL NEW AND EMERGING TOXIN THREATS FOR UK WATERS- PHYTOPLANKTON

1.1 INTRODUCTION

Phytoplankton are free-floating plants found in marine and freshwaters that through their photosynthetic growth form the base of the aquatic food chain. A relatively small subset of these phytoplankton may be harmful to the health of humans. The species that cause harm are now widely referred to as ‘Harmful Algae’. The term “Harmful Algal Bloom” (HAB) is often used to describe their occurrence.

World-wide there are approximately 4000 species of phytoplankton. These are commonly grouped into three functional categories or life-forms: diatoms, dinoflagellates and microflagellates. Approximately 300 of these species of phytoplankton have properties that make them harmful to humans or influence the human use of theaquatic environment.

In terms of human health, the most important consequence is the production of biotoxins by some species of phytoplankton. Approximately 70 harmful species produce such biotoxins, with only a small subset of these currently existing in UK waters.

Typically, biotoxin producing phytoplankton species exist at relatively low densities (ca. few hundred or thousands of cells per litre) and hence do not bloom in the sense of dominating the phytoplankton biomass. This behaviour makes the prediction of their distribution particularly problematic as it is difficult to quantify factors, such as food web interactions, that may govern their abundance.

Biotoxins become concentrated in the flesh of grazing organisms (particularly bivalve molluscs) that filter feed on phytoplankton. In most cases, there are no adverse

effects to these primary consumers, but this concentrating mechanism creates a risk to health if the shellfish are consumed by humans.

The majority of harmful phytoplankton are pelagic, although a number such as *Prorocentrum lima*, *Ostreopsis* spp. and *Gambierdiscus* spp. are semi benthic, living in the sediments or growing epiphytically on seaweed, and are only sporadically present in the water column.

In marine environments, most harmful species are dinoflagellates. Of the dinoflagellate group, some important species are known to produce resting cysts that are a key component of their life cycle. One diatom genus (*Pseudo-nitzschia*) dominates the toxic diatoms. Harmful marine cyanobacteria (sometimes called 'blue-green algae') can be important in some low salinity environments.

This chapter is a review of new and/or emerging toxic phytoplankton threats to UK waters. Including a review of published literature on this area, communication with agencies, research establishments and universities to gather reports and communications listing the occurrence of new/emerging toxin producing species (both within the UK, and European countries including France and Northern Spain). This will also include examination of ballast water research reports for new/emerging species in ballast water and finally the production of a risk based assessment of new/emerging toxin producing species which are present or may become present in UK waters.

1.2 CURRENT SHELLFISH TOXIN PRODUCING SPECIES IN THE UK

In UK waters we are fortunate that the numbers of toxin-producing phytoplankton are limited, with seven species or genera currently being monitored on a routine basis to safeguard human health (Table 1.). Of these, species of dinoflagellates belonging to the genera *Alexandrium* and *Dinophysis* and diatoms belonging to the genus *Pseudo-nitzschia* are the most important in terms of their toxin production.

Small flagellates of the genus *Azadinium* have been recently shown to produce azaspiracids (Tillman *et al.*, 2009), but this organism is not routinely monitored

because of the difficulty (due to its small size) in discriminating between this genus and other benign organisms by light microscopy.

Table 1. Species or genera of potentially biotoxin producing phytoplankton monitored in UK waters to ensure shellfish safety.

Organism	**Toxin : Syndrome**
Alexandrium spp.	Saxotoxin & derivatives: PSP
Pseudo-nitzschia spp.	Domoic acid : ASP
Dinophysis spp.	Okadaic acid & dinophysistoxins: DSP Pectenotoxins PTXs
Prorocentrum lima	Okadaic acid & dinophysistoxins: DSP
Prorocentrum minimum	Venerupin : VSP
Lingulodinium polyedrum	Yessotoxin : YTX
Protoceratium reticulatum	Yessotoxin : YTX
*Azadinium spp.**	Azaspiracids : AZA

*Not currently monitored

1.3 HISTORICAL PERSPECTIVE, CURRENT STATUS, AND RECENT CHANGES

1.3.1 Historical perspective

Species that are harmful to human health have been a natural part of the phytoplankton in UK coastal waters for at least the last 100 years, and hence it is important to note that HABs are not a new phenomenon. Examples of early observations of important UK HAB species are presented below.

Paralytic Shellfish Poisoning (PSP) causative species

Alexandrium: Alexandrium tamarense (previously known as *Gonyaulax tamarensis*), was originally described based on cells collected from the Tamar Estuary (Lebour, 1925).

Amnesic Shellfish Poisoning (ASP) causative species

Pseudo-nitzschia: The presence of *Pseudo-nitzschia seriata* (then named *Nitzschia seriata*) was first recorded in Scottish waters in Loch Hourn in July 1909; in the Firth of Lorne in 1909 and 1910; and both *N. seriata* and *Nitzschia delicatissima* (in Loch Torridon in July 1911 (Herdman and Riddell, 1911, 1912).

Diarrhetic Shellfish Poisoning (DSP) causative species

Dinophysis: Cleve (1900) observed *Dinophysis acuta* in the northern North Sea off Scotland and in the Irish Sea. Herdman and Riddell (1911, 1912) noted the presence of *Dinophysis sp*. in the Scottish west coast sea Lochs Hourn (in July 1908 and 1909) and Torridon (in July 1911), and in the Firth of Lorne in 1909. In addition, Lebour (1917) recorded the presence of *Dinophysis acuminata* in the English Channel (off Plymouth) during a study in 1915 and 1916.

1.3.2 Current status of biotoxin producing phytoplankton species present in UK waters

Paralytic Shellfish Poisoning (PSP) causative species

In UK waters the biotoxin producing genus of most concern, due to its production of highly potent PSP toxins, is *Alexandrium*. Three toxin producing species are known to be present in UK waters: *A. tamarense, A. minutum* and *A. ostenfeldii* (Bresnan *et al.* in press). Other, non-toxic species have also been identified such as *A. tamutum* (Brown *et al.*, 2010; Swan and Davidson, 2012a).

A range of PSP events have occurred in the UK. Ayres (1975) suggested that between 1827 and 1968 there were ten UK PSP incidents with approximately 14 fatalities. The most well documented case being in 1968 in the North East of

England when 78 people showed clinical symptoms of PSP toxicity after consuming mussels (Ayres and Cullem, 1978) contaminated by *A. tamarense* (Robinson, 1968). In recent years monitoring has prevented (known) human health incidents related to PSP intoxication. Worldwide, the Intergovernmental Oceanographic Commission of the United Nations Education, Scientific and Cultural Organisation (IOC-UNESCO) harmful algal bloom programme indicates that there are about 2000 cases of PSP annually with 15% mortality, although not all of these are a result of *Alexandrium*.

A. minutum: this low toxicity species has been identified as a PSP-producer in England, Ireland (Percy, 2008; Touzet *et al.* 2010b) and occasionally in Scotland (Töbe *et al.*, 2001). Its role as a PSP-causing organism is variable and cell densities rarely reach sufficient levels for PSP to cause major problems in the UK. However, significant localised problems occur in Cork Harbour in Southern Ireland (Touzet *et al.*, 2010b).

A. ostenfeldii: This organism has been identified in both Scottish and English waters and has been observed to produce trace amounts of PSP, as well as spirolides (Töbe *et al.*, 2001; John *et al.*, 2003).

A. tamarense: This is the organism of greatest concern in UK waters. It is important to recognise that *A. tamarense* often constitute a very minor component of the phytoplankton assemblage but, through the very high per-cell toxicity of the toxic strains, it remains capable of generating shellfish toxicity even at very low cell densities of only a few hundred cells per litre.

Globally this species exists as five different ribotypes (strains), Groups I-V, (Lilly *et al.*, 2007). Different *A. tamarense* ribotypes have different levels of toxicity. The highly toxic group I (North American) ribotype has long been known to exist in Scottish waters, and has historically been viewed as a potent PSP-producer in this region, with particular hotspots including the Orkney and Shetland Islands.

The non-toxic group III (Western European ribotype) is known to be present in England (and the Irish Republic). However, recently non-toxic forms of *A. tamarense* (group III) have also been identified in Scottish waters (Collins *et al.* 2009), with their

co-occurrence in the water column with groups I cells now having been demonstrated (Touzet *et al.* 2010a).

Amnesic Shellfish Poisoning (ASP) causative species

ASP is primarily caused by the diatom genus *Pseudo-nitzschia*. In UK waters, thirteen *Pseudo-nitzschia* species are known to be present (Fehling *et al.*, 2006; Bresnan *et al.*, 2010). Of these, only three are confirmed toxin producers, *P. australis* and *P. seriata* in Scottish waters, along with *P. multiseries* in English waters (Davidson *et al.*, 2011).

Prior to 1997, there are no records of Domoic acid (DA) in UK waters. However, in 1999, a 49 000 km^2 area in western Scottish waters was closed to shellfish harvesting as a result of high levels of DA in king scallops. Scallop harvesting was first prohibited in June of that year, and the ban remained in force until April 2000. This is the largest fishery closure recorded worldwide (Campbell *et al.*, 2001; Fehling *et al.*, 2004a). Both offshore and inshore waters were affected, demonstrating the potential for significant HAB-generated toxicity incidents in the UK. Frequent fishery closures occurred in subsequent years in the region due to elevated toxicity in scallops.

The finding that the bulk of the toxin (typically >99% of DA) is located in the scallop hepatopancreas has led to a system of shucking and end-product testing of the edible gonad and muscle. This safeguards human health while allowing the offshore scallop fishing industry to be sustained. While the end-product testing of scallops has markedly reduced the human ASP health risk in UK waters, a risk still exists from the potential human toxification from mussels in particular, as DA accumulates (and depurates) rapidly in mussel flesh (Krogstad *et al.*, 2009).

Diarrhetic Shellfish Poisoning (DSP) causative species

The principal cause of DSP in UK waters is the dinoflagellate genus *Dinophysis*. Most commonly observed are *D. acuminata* and *D. acuta*, but with considerable inter-annual variation in their absolute and relative abundances. For example, *D. acuta* dominated in Scottish waters in 2001 but numbers have subsequently declined

as *D. acuminata* has become abundant at those sites monitored by Marine Scotland Science (E. Bresnan, personal comm.).

DSP was first reliably recorded in the UK in 1997 (Dubrow, 1999). Hinder *et al.* (2011) detail 19 subsequent incidents from 1999-2009. While the number of reported events seems very few, this may be due to under reporting.

A second potential causative organism of DSP in UK waters is *Prorocentrum lima*. The semi benthic and epiphytic behaviour of this organism means that it may be under represented in sampling programmes that better sample those species that are pelagic in nature. Scientific studies on its ecology are relatively few in UK waters, although the production of okadaic acid (OA) and dinophysistoxin-1 (DTX-1) from *P. lima* strains isolated from the Fleet Lagoon in Dorset has been studied. This area had previously experienced DSP in shellfish without any observable *Dinophysis* in the water column suggesting that the causative organism could be *P. lima* (Foden *et al.*, 2005; Nascimento *et al.*, 2005).

Azaspiracid producing species

Azaspiracid shellfish poisoning (AZP) was first identified from mussel from Killary Harbour Ireland in 1995 (Ito *et al.*, 2000). Azaspiracids (AZAs) have been identified in UK mussels (James *et al.*, 2002). Initially it was thought that the heterotrophic dinoflagellates *Protoperidinium crasipes* was responsible for AZA production. However, it is most likely that this species is simply a vector for the toxin and recently it has been demonstrated that these toxins are produced by some species of the genus *Azadinium* (Tillman *et al.*, 2009; Tillman *et al.*, 2011). While the AZA producer *Azadinium spinosum* is known to exist in, and was first isolated from, UK waters (Tillmann *et al.*, 2009) it is not monitored as it cannot be routinely identified by light microscopy. Hence, little is known about its spatial or temporal distribution.

Yessotoxin producing organism

Yessotoxins (YTXs) are produced by the marine dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* that are present in UK water. However, a further species *Gonyaulax spinifera* (Rhodes *et al.*, 2006a) has also been implicated in their production. This organism is present in the British Isles (Pybus *et al.*, 2006;

Parke and Dixon, 1976) and is currently not monitored. However, given the lack of evidence of YTX toxicity to humans, and relative infrequency of such toxicity in shellfish samples, the imperative to include *G. spinifera* in any monitoring programme seems slight.

Venerupin producing organisms

Prorocentrum minimum (which is currently regarded as a taxonomic synonym of *Prorocentrum cordatum*) is implicated in the production of hepatoxic or neurotoxic compounds. There is some debate over the toxicity of this organism, with some early reports of *P. minimum* toxicity being erroneous due to the co-occurrence of e.g. toxic *Dinophysis* cells (Kat, 1985). More recently, Grzebyk *et al.* (1997) demonstrated the existence of toxic as well as non-toxic clones of *P. minimum* arguing that this may account for the different observations concerning the toxicity or non-toxicity of blooms of this species. This species is frequently present in UK waters with, for example, its presence being observed in over 50% of regulatory monitoring samples from Scottish waters in 2011.

1.4 FACTORS THAT MAY INFLUENCE THE ABUNDANCE OR TOXICITY OF HAB SPECIES IN UK WATERS

1.4.1 Ballast Water Transfer

The concept of transfer of HAB species in the ballast tanks of merchant shipping was first highlighted by Hallegraeff and Bolch (1991) who investigated the presence of dinoflagellate cysts in the sediment of cargo vessel ballast tanks entering Australian ports.

Smayda (2002, 2005) argue that, while occurrences of ballast water transport mediated introduction do occur, there is relatively little species-specific evidence in support of this theory. However, Smayda (2005) goes on to state “Clearly, Scottish coastal waters are "open" to ballast water and sediment introductions of toxic and benign species of phytoplankton, both immigrant and indigenous species. This

exposure is of concern, given the significant increase in harmful and novel species blooms recorded in European waters”

Hallegraeff and Bolch (1991) found that, of the sediment samples collected from 80 vessels, 40% contained viable dinoflagellate cysts of non-toxic species and 6% contained the cysts of the toxin-producing species, *A. catenella* and *A. tamarense*. A further study surveyed 343 vessels entering Australian ports and found that more than 200 of the vessels contained sediment in the bottom of their ballast tanks and of these 50% contained dinoflagellate cysts (Hallegraeff and Bolch, 1992).

Surveys of 127 vessels arriving at Scottish ports and 76 vessels at English and Welsh ports (MacDonald and Davidson, 1998; Hamer *et al.*, 2001) have been undertaken. Twenty nine phytoplankton species were recorded in the Scottish vessels and 48 for the English and Welsh vessels. Of the vessels entering Scottish ports, motile dinoflagellate cells were found in the ballast water of 76% of these, and cysts were found in 61% of 92 sediment samples collected.

These studies clearly show that motile cells and resting cysts can be transported in ballast water and sediment. Of particular concern is that Hallegraeff and Bolch (1991) concluded that the evidence pointed to the ‘distinct possibility’ that *Gymnodinium catenatum* (a species that is of concern as a potential future invasive organism in UK waters) had been introduced to coastal waters around Hobart in Tasmania (Australia). This organism was recorded in the Scottish ballast waters survey.

Other harmful species were found in the surveys UK ballast waters. These included *Dinophysis*, *Pseudo-nitzschia* and *Alexandrium* species. Perhaps of particular concern is that cysts from the *A. tamarense / catenella* species complex were common in the UK ballast water surveys. While *A. tamarense* is present in UK waters, *A. catenella* is not. These species cannot be distinguished based on cyst morphology. Smayda (2005) noted the recent appearance of *A. catenella* in the Mediterranean Sea, where it is spreading along the north-west coast and producing annually recurrent blooms at ca. 20 °C (Vila *et al.*, 2001). Smayda (2005) therefore finds that, the potential de-ballasting of *A. catenella* cysts into Scottish coastal waters cannot be discounted, given its Mediterranean Sea occurrences.

Smayda (2005) noted concern whether current International Maritime Organization (IMO) guidelines are adequate to protect against harmful species introductions into UK waters. This concern is compounded by a recent study by Roy *et al.* (2012) who demonstrated that, even with transoceanic ballast water exchange that is intended to prevent algal translocations, ballast water remains a potential vector for HAB dispersal.

This problem of ballast water transport will not be easy to solve as it is not yet compulsory to report ship ballast water operations in any ports of the UK, with a recent study that tried to develop a methodology for the estimation of ballast water operations in UK ports identifying numerous ports with imported ballast water of foreign origin (Enshaei and Mesbahi, 2011).

Since ship's ballast water transport between different biological/ environmental/ geographical regions presents a great threat in terms of the possible introduction of invasive species to new areas, studies are on-going to test various ballast water treatments: physical separation, mechanical and chemical methods (Tsolaki and Diamadopoulos, 2010). One of the most controversial options to address this problem is the use of biocides (La Carbona *et al.*, 2010). Other treatments include the use of UV treatments. These have been found to be efficient for numerous phytoplankton species e.g. the nuisance flagellate *Phaeocystis globosa.* This species was unable to recover after UV-C exposure. However, other non-harmful species have been noted to recover including *Chlorella autotrophica* and *Chaetoceros calcitrans* (Martinez *et al.*, 2012). It has also been found that some species, such as *Pseudo-nitzschia,* both toxic and non-toxic are able to re-grow after an initial decline in cell numbers when exposed to UV-C (Liebich *et al.*, 2012).

A recent study by Carney *et al.* (2011) compared the survival of tropical (Goa) and temperate (UK) phytoplankton following incubation for 28 days in darkness and subsequent re-exposure to light. They found population growth observed during the re-exposure period in the tropical population was comparable to that of the temperate population. While this does not necessarily imply that long term survival will occur, it does suggest some degree of risk from long distance ballast transfer to UK waters.

An International Council for the Exploration of the Sea (ICES) workshop on “harmful phytoplankton that could potentially be transported or introduced by ballast waters” was held in October 2012 (ICES, 2010). The report from the workshop concludes that “there was not much information available regarding which species of phytoplankton are capable of surviving in ballast tanks and the associated sediments”. Although there have been several ballast water sampling studies that have resulted in lists of species, in many cases it has not been possible to identify the species reliably beyond genera. Also, it is often assumed that a species has some form of resting stage or cyst but this is not known definitively and it is therefore not possible to know whether a species is capable of surviving for any length of time in a dark ballast tank. For many species there is no detailed information on the temperature and salinity tolerances and in many cases this is not known for certain, particularly for species that are difficult to identify. The report did, however, generate a list of harmful phytoplankton species that could potentially be transported in ballast water that included representatives of the genera *Pseudo-nitzschia, Dinophysis, Alexandrium, Prorocentrum, Azadinium, Karenia, Coolia, Ostreopsis, Gambierdiscus, Gonyaulax* and *Gymnodinium* all of which have the potential to generate human health problems.

1.4.2 Anthropogenic Nutrients (Inorganic)

In temperate-latitude seas such as those that surround the UK, the onset and duration of the phytoplankton production season is controlled by light availability (Svedrup, 1953; Smetacek and Passow, 1990). This results in pronounced seasonality that is broadly determined by the solar cycle. As the light climate improves in late winter/early spring, nutrients that have been mixed into surface waters during winter fuel the spring growth of phytoplankton (the “spring bloom”). During the subsequent production season, it remains that the supply of mineral nutrients largely determines how much phytoplankton growth occurs.

Given this relationship between nutrients and phytoplankton, anthropogenic nutrient enrichment of coastal waters is considered to be one reason for the putative worldwide increase in the occurrence of HABs, and a number of high profile

publications exist that link HABs to anthropogenic nutrients (Glibert *et al.*, 2005; Heisler *et al.*, 2008; Anderson *et al.*, 2008).

Whether or not anthropogenic nutrient enrichment of coastal waters influences the dynamics of HAB species is a complex matter. Key issues relating to this topic have recently been critically reviewed by Gowen *et al.* (2012). This study found that there is no consensus regarding the role of anthropogenic nutrients in stimulating the occurrence of HABs exists.

Attempts to relate trends in HABs to nutrient enrichment are made particularly difficult by the lack of long term time series and a lack of associated environmental monitoring where time series do exist. It is also difficult to decouple the effects of nutrient from those of: increased monitoring and reporting; variability in large-scale meteorological forcing; and the influence of climate change.

For large-biomass HABs, Gowen *et al.* (2012) found the hypothesis that nutrient enrichment can cause HABs to be supported in some water bodies but not in others. However, they also found the global evidence that enrichment brings about an increase in low-biomass HABs of the toxin-producing species that are of particular interest with respect to shellfish toxicity in UK waters to be equivocal.

1.4.3 Nutrient Ratios

The potential for changing nutrient ratios to influence the growth of HAB species are based on the nutrient ratio hypotheses (Officer and Ryther, 1980; Tilman, 1977). These hypotheses suggest that a perturbation in the nutrient supply ratio will result in the environmental selection of particular species, potentially favouring harmful organism (Smayda, 1990; Heisler *et al.*, 2008). As human activity is thought to have increased inorganic nitrogen (N) and phosphorus (P) loads to coastal waters by different proportions (Falkowski, 2000; Conley *et al.*, 2009), resultant changes in the ratio of dissolved inorganic N to P (or in the case of diatoms the ratio of these nutrients to silicon) provide a possible mechanism for an anthropogenically mediated increase in HABs.

The role of nutrient ratios in promoting HAB species was recently reviewed by Davidson *et al.* (2012). These authors found that, at limiting concentrations, evidence that alteration of N:P ratios has stimulated HABs is limited, and primarily based on hypothesised relationships in relatively few locations outside UK waters (in particular: Tolo Harbour Hong Kong and Dutch Coastal Waters), and for harmful/nuisance but non-biotoxin producing species. Even in these cases, an unequivocal causal link between an increase in HABs (in terms of frequency, magnitude or duration) and change in nutrient ratio was difficult to establish.

The role of silicon (Si) in controlling the switch from diatom to dinoflagellate dominated communities was generally supported by experimental evidence (Davidson *et al.*, 2012). But little evidence exists that high N:Si ratios preferentially promote harmful dinoflagellates over benign species.

Hence, given that lack of evidence of a link between HABs and anthropogenic nutrients in UK waters and that the EU and other regulation is likely to further limit the discharge of nutrients to coastal waters, it seems unlikely that any change in shellfish biotoxins will be promoted as a result of inorganic anthropogenic nutrient additions and/or resultant changes in their ratios.

1.4.4 Anthropogenic Nutrients (Organic)

The bulk of the scientific investigation on the role of nutrients in promoting HAB species has related to inorganic nutrients. However studies of the role of dissolved and particulate organic nutrients in the growth of HAB species, while limited, demonstrate the potential for organic nutrients (especially organic N) to support the growth of a range of HAB species. Organic nutrients may also be introduced anthropogenically to coastal waters. This is most clearly evident from changes in farming practices in some parts of the world that have led to a marked increase in the use of urea as a fertiliser, and its resultant run off into coastal waters (Glibert *et al.*, 2006).

The work of Glibert *et al.* (2006) has generated much debate on the role of urea in stimulating HABs. Solomon *et al.* (2010) summarised many of the laboratory studies

of urea utilisation by HAB species, finding that urea supported both higher and lower growth rates compared to rates with nitrate (NO_3) or ammonium (NH_4) as the substrate. As many HAB species are now being identified as being mixotrophic (i.e. being capable of both autotrophic and heterotrophic growth), there is a clear need for better understanding of the role of organic nutrients and fertiliser based urea. In particular, studies of the role of how organic nutrients influence HAB and non-HAB species in competition for environmentally realistic concentrations of organic nutrients are required.

The British Survey of fertiliser practice 2011 (Holmes, 2012) indicates that 358,000 tonnes of urea were used as fertiliser in that year, 7.4% of the total fertiliser used, with urea ammonium nitrate contributing another 7.2%. Most fertilisers are applied in the months of March, April and May, which is before the main season for dinoflagellate growth. However, a more complete understanding of the role of this form of N remains necessary to allow predictions of how changes in terrestrial farming practice may influence coastal HABs.

1.4.5 Factors that may influence cell toxicity

Toxin production by phytoplankton is not necessarily continuous or of a constant amount per cell. This makes understanding the factors governing toxicity important. Most commonly such changes are related to the availability of the mineral nutrients that the cells require for their growth and for toxin synthesis. Davidson *et al.* (2012) reviewed this literature with respect to the two genera, *Pseudo-nitzschia* and *Alexandrium* that have received the most detailed investigation, and which are of particular concern in UK waters. They found that laboratory studies generally demonstrate that nutrient ratios can influence toxin production. For example a lack of Si, P or a range of micro nutrients such as iron (Fe) or copper (Cu) could lead to enhanced domoic acid production by *Pseudo-nitzschia* (Fehling *et al.*, 2004; Wells *et al.*, 2005), but genus and species specific differences and environmental control make extrapolation of these data to the field difficult.

Factors governing the toxicity of *Alexandrium* cells are complex. PSP toxins are nitrogenous compounds and N is required for their synthesis. This suggests that N

stress (i.e. a low N:P ratio) would limit toxin synthesis and a number of laboratory studies are consistent with this having demonstrated enhanced PSP toxin production under P stress (Davidson *et al.*, 2012). However, such observations are typically based on laboratory culture studies, with insufficient evidence being available to draw firm conclusions in the UK or elsewhere of what, if any, factors are influencing cellular toxicity in the field.

1.4.6 Fishing

Another potential mechanism for the modification of planktonic food web is overfishing. This will remove top predators, stimulating small fish stocks, which graze zooplankton, thus relieving phytoplankton grazing pressure (Hallegraeff, 2010). This relief of ‘‘top-down control’’ of the marine food web (Turner and Graneli, 2006) may have as yet uncertain impacts on planktonic community.

1.4.7 Climate Change

The role of climate change in governing changes in the distribution of HAB species has been considered in a number of recent reviews, with Hallegraeff (2010) looking at this relationship on a global basis and Bresnan *et al.* (2010) and Bresnan *et al.* (in press) evaluating knowledge on a UK (and Ireland) specific basis.

The effects of climate change are likely to be many. Some of these, for example increased water temperatures and increased severe weather, may have opposing effects on phytoplankton growth, with little current understanding on how such pressures will interact (as discussed more fully below). There remains a great difficulty in confirming one way or another how the effects of climate change are likely to influence HABs in the UK (or indeed elsewhere). This is further compounded by our lack of understanding of whether the purported increase in HABs in recent decades is real and, if it is, what has caused it.

There is very little doubt that our planet is getting warmer and will continue to warm, because of the consequences of increased atmospheric greenhouse gases. Warmer

weather in temperature latitudes, on average will result in wetter weather because the warmer air carries more water vapour. However, it remains difficult to predict how much warmer and wetter the weather will be in the future in the UK, and how will this influence conditions in the shelf seas within which phytoplankton live. This view is consistent with the extensive review of the potential impact of climate change on harmful phytoplankton (Hallegraeff, 2010) that found “Prediction of the impact of global climate change on algal blooms is fraught with uncertainties”.

Climate change is expected to generate a range of effects in UK shelf seas as detailed below. These include:

1. Increases in sea surface temperature
2. Increase in strength and duration of water column stability through thermal stratification
3. Possible increase in storm events with increased duration of high wind speed events may also be expected
4. Increased carbon dioxide (CO_2) in the atmosphere is expected to lead to ocean acidification and a decrease in the pH of surface waters
5. Potential changes in the patterns of water mass circulation

All of the above have the potential to influence phytoplankton populations, with possible impact on HAB species discussed in section 1.5 below.

1.5 CLIMATE CHANGE SCENARIOS AND THEIR POSSIBLE IMPACT

1.5.1 Increased water temperature

In broad terms, we might expect a change in the balance of diatoms and dinoflagellates in UK waters. Diatoms typically dominate in cool, nutrient-rich, turbulent waters. In contrast, dinoflagellates are adapted to warm and stratified seas. Thus, in these terms, we might expect the pelagic balance to shift from diatoms

towards dinoflagellates as the world warms, giving greater likelihood of HABs. Some evidence for this is provided by studies of sediment records, e.g. the PSP-causative dinoflagellate, *G. catenatum*, is characteristic of warm waters, causing problems, for example, in the Rias of northern Spain (Figueiras and Pazos, 1991) but not at higher latitudes. However, studies of sediment cores from Scandinavian waters indicate that this organism was once present in coastal waters as far north as Bergen in Norway during warm periods in the last two millennia (Thorsen and Dale, 1998). These results support the theory that warm-water species, including HAB organisms are capable of range expansion.

Other sediment record evidence (Marret *et al.*, 2004) suggests that shifts in the location of dinoflagellates growth zones may be related to postglacial changes in sea-level that have altered tidal mixing regimes. Changes in freshwater discharge can also alter tidal mixing regimes. However, as most of our time-series data for phytoplankton are limited to a few decades (at best), definitive proof of these relationships is difficult to establish.

Each phytoplankton species is typically adapted to grow over a range of temperatures that are characteristic of their normal habitat. Growth rates are usually higher at higher temperature, but considerably lower beyond an optimal temperature (Eppley, 1972; Hallegraeff, 2010).

Natural populations of phytoplankton often occur at temperatures suboptimal for photosynthesis, and it is believed that this distribution is designed to avoid risking abrupt declines in growth associated with the abrupt incidence of warmer temperatures (Li, 1985).

Temperature effects on phytoplankton growth and composition are more important in shallow coastal waters, which experience larger temperature fluctuations than oceanic waters. Predicted increasing sea surface temperatures of 2 - 4 ^{o}C may shift the community composition toward species adapted to warmer temperatures as observed in the temperate North Atlantic (Edwards and Richardson, 2004).

Estimates of phytoplankton biomass (using a phytoplankton colour index) indicated an increase in its quantity over the last decade in certain regions of the North and the

East Atlantic and North Sea particularly in winter (Bresnan *et al.* 2013). The increase may be linked to increased water temperature and decreased turbidity which may allow normally light-limited phytoplankton to utilise available nutrients earlier in the year (McQuatters-Gollop, 2007).

1.5.2 Increased water column stratification

Increasing water temperature is linked to increased strength and duration of the seasonal stratification of shelf seas. Again, this is likely to favour flagellate species rather than diatoms, and may have a particular influence on those organisms that have been observed to exist in thin layers, such as *Dinophysis* (Farrell *et al.*, 2012) and *Alexandrium* (Touzet *et al.*, 2010).

An increase in sea surface temperature may therefore facilitate range expansion of more southerly species. Alternatively, it might support the survival of introduced species. Species that have the greatest potential for expansion into UK waters are *G. catenatum* and *Ostreopsis* sp., and these will be discussed in more detail below. Temperature also plays a role in the bloom dynamics of the cyst-forming PSP dinoflagellates such as *G. catenatum* (Bravo and Anderson, 1994).

Enhanced surface stratification, may also lead to more rapid depletion of surface nutrients, as the stratification will acts to prevent nutrient replenishment from deep nutrient-rich waters. This may lead to changes in phytoplankton community composition with smaller nano- and pico- plankton cells, that exhibit the higher surface area: volume ratios and are better able to cope with low nutrient levels favoured over larger cells.

1.5.3 Changes in wind speed and duration

Storm event may act to offset increased thermal stratification and hence promote diatom rather than dinoflagellate species.

Heavy precipitation coincides with storm events and flash floods. Episodic storm events may therefore affect the timing of freshwater flow, residence time, and

magnitude and time of nutrient pulses. This may be particularly prevalent for the restricted exchange environments of Scottish sea lochs.

1.5.4 Ocean acidification

Ocean acidfication is most likely to have an adverse impact on calcifying phytoplankton organisms. While these phytoplankters are not harmful species, it is unclear if reductions in their abundance will open an ecological niche for other, as yet unknown, species to exploit.

An algal bloom generates an increase in pH that is detrimental to further algal growth and may influence species succession (Hansen, 2002). If, through ocean acidification, the pre-bloom pH is lower, then succession events may be altered (Calloway *et al.*, 2012), again with unknown consequences for HABs and benign species.

However studies of harmful species are few, Kremp *et al.* (2012) examined the effects of the changes in environmental factors, such as increased temperature and CO_2 availability, as predicted consequences of global climate change. They cultivated eight strains of *A. ostenfeldii* from the Baltic Sea acclimating isolates for 10 generations. Their findings concluded that increased temperatures caused general increase in growth of the species and their toxin composition was also consistently altered by the increased temperature and the increased CO_2 supply, resulting in the overall promotion of saxitoxin production.

1.5.5 Changing hydrodynamics

Physical conditions are frequently highlighted by some authors as potentially important or over-riding factors in the control of HAB appearance/magnitude, e.g. *Phaeocystis* in the Southern North Sea and German Bight, PSP in Puget Sound, red tides in Tolo Harbour (Gieskes *et al.*, 2007; Breton *et al.*, 2006; Hickel, 1998; Xu *et al.*, 2010). Such physical, and in particular hydrodynamic factors, may also be key to the geographical inconsistency on the influence of HABs, as rates of lateral

exchange, mixing, or dispersion within and between water bodies differ (Gowen *et al.*, 2012).

Hydrodynamics may act in a range of ways. Factors such as the strength of vertical mixing and its consequences for the illumination experienced by phytoplankton are important. Spatio-temporal patterns in stratification also influence phytoplankton species succession. Solar warming of the sea surface or the input of freshwater create surface layers of lower density water. Hence, while nutrient inputs to such layers (either natural or anthropogenic) may stimulate blooms, biomass can be removed through dispersion by currents or the consumption by planktonic and benthic animals. Strong vertical mixing, due to wind, tidal currents, or surface cooling, can carry phytoplankton away from the surface light. This can also suspend large quantities of light-obscuring sediment from the seabed in shallow areas.

Differences in the characteristic hydrodynamic features of water bodies in small regions of restricted exchange and regional seas provide evidence for why HABs occur in some waters, but are less frequent or absent in other areas. For example, the nutrient enriched waters of Tolo Harbour in Hong Kong (Xu *et al.*, 2010), Loch Striven on the Scottish west coast (Tett *et al.*, 1986), and the Seto Inland Sea of Japan (Imai *et al.*, 2006), show increased frequencies of high biomass blooms. Sometimes these waters included blooms with harmful consequences. In contrast, the enriched waters of Victoria Harbour in Hong Kong (Xu *et al.*, 2010), Carlingford Lough on the border between Northern Ireland and the Republic of Ireland (Capuzzo, 2011) and the eastern Irish Sea (Gowen *et al.*, 2008) do not exhibit the symptoms of eutrophication (which may include HABs). This is because their hydrodynamic characteristics (i.e. rapid flushing in Victoria Harbour, and tidal stirring in Carlingford Lough and the Eastern Irish Sea) counteract nutrient enrichment which reduces the potential for development of high biomass blooms.

Offshore, water mass transport processes that are potentially climate sensitive may also influence the transport of advective HAB species. For example, Farrel *et al.* (2012) demonstrated the long distance transfer of *Dinophysis* by costal jets in Irish coastal waters with a speed of ~ 7km day^{-1}, and Fehling *et al.* (2012) suggested

cross shelf transport is important for populations of *Pseudo-nitzschia* in Scottish waters.

The prediction of the changes in large scale water mass circulation or local hydrodynamics is extremely difficult and to extrapolate how these changes will influence HAB species would require water body specific investigation.

1.5.6 Synergistic effects

As discussed by Hallegraeff (2010), the disturbance to an ecosystem by climate change may make it more susceptible to invasive species. However, given the range of different effects of climate change, it is likely that some of these may act in contradictory ways in terms of promoting the growth of phytoplankton. For example, increased stratification may promote dinoflagellates, but increased storminess will counteract that process. Prediction of the result of such interactions remains particularly problematic.

Species such as *G. catenatum* have well-defined seasonal temperature windows that may overlap with UK waters temperature. However, even if survival on the basis of temperature alone was possible, it is not possible to know how the interaction with other factors such as nutrients, turbulence, and grazing would influence the dynamics of the species and its ability to become established in UK waters.

1.6 CHANGES IN PHYTOPLANKTON COMPOSITION IN UK WATERS

Regardless of the difficulties in interpreting how changes in coastal waters will influence HAB populations, it is clear that change is occurring. This is most readily identified in measurements of water temperature. Bresnan *et al.* (2013) review data from a number of sources (Hughes *et al.*, 2009; Frost *et al.*, 2012) that all indicate that, while considerable inter-annual variability exists, an increase in NE Atlantic waters temperature is evident in recent decades. Lowe et al. (2009) also predicted a

(maximum) increase of 2.5 – 4 ^{o}C in the next century in UK waters. Other changes include those in wave height, wind speed, salinity and ocean acidification (Frost *et al.*, 2012) all of which have the potential to influence phytoplankton communities.

Again, the lack of long term records make it difficult to identify how the above changes have influenced the abundance or distribution of HAB species in UK waters. Given the natural inter-annual variability in phytoplankton, it is therefore not surprising that Bresnan *et al.* (2010, 2013) found that while there is considerable inter-annual variability in the occurrence of HAB species in UK waters, no changes can be directly attributed to climate change.

Smayda (2005) noted differences between the Scottish HAB community to those in similar regions and questioned whether these reflect the presence of ecophysiological barriers, or unique selective factors within Scottish coastal water habitats. Given that the current distribution of HAB species cannot be explained, determining how this may change in the future is an even greater task.

Gowen *et al.* (2010) studied the occurrence of HABs and HAB species abundance in relation to anthropogenic nutrient additions to UK and Irish coastal waters. Data sets on nutrients and the growing season (April – September) abundance of HAB species were compiled and analysed statistically. The results showed that of the 168 relationships between HAB species abundance, nutrient loadings, and winter nutrient concentrations examined, only 24 were significant. However, all but 3 of the significant regressions were negative. These results therefore show that in general HAB species abundance in UK and Irish waters was not influenced by enrichment with nitrogen and phosphorus. In fact, the significant negative regressions imply that HAB species were more abundant in un-enriched waters. This does not mean that nutrients suppress the growth of harmful algae. A more likely explanation is that the relevant algae are naturally more abundant in waters to the west and north of our islands, in which there is least anthropogenic nutrient enrichment.

Notwithstanding the lack of a clear driving force, there are a number of examples of possible change in the composition of the HAB community in UK waters. Again it should be noted that the lack of multi decadal time series make it difficult to establish

if these are real changes or simply inter-annual fluctuations. However, with this caveat, they are outlined below.

1.6.1 Changes in PSP toxicity

Although, considerable variability exists, with for example 2006, being a year of extensive PSP toxicity in Shetland, a decrease in PSP toxicity in Scottish shellfish has been suggested over the period since monitoring began in 1990 (Bresnan *et al.*, 2008; Bresnan *et al.*, 2010).

The length of the available time series is still too short to determine if this decrease in toxicity is an on-going trend or a short term fluctuation. However, changes are likely to be governed by one (or more) of three factors:

1. a decrease in the abundance of *Alexandrium* cells
2. a decrease in the toxicity of these cells
3. a shift in the composition of the *Alexandrium* community to include more non-toxic cells.

Establishing which of the above factors, individually or in combination could be responsible for such a change is difficult, as prior to 2006 the collection of phytoplankton samples for the enumeration of HAB species lacked sufficient structure to allow statistical analysis.

Subsequently, the sampling has been more consistent, although the relationship with toxicity is still difficult to establish due to the cessation of biotoxin sampling when a region is closed, potentially as a result of a different toxin.

Recently Swan and Davidson (2012a) analysed the number of samples that exceeded regulatory thresholds for the three major biotoxin species in Scottish waters as a function of year and month between 2006 and 2011 over the months of April to September.

The results of Swan and Davidson (2012a) indicated that while *Alexandrium* (dominated by *A. tamarense*) shows statistically significant differences in abundance

across different months, no statistical difference in the abundance was evident between years over the period 2006-2011 (2 way ANOVA p = 0.576). Such results seem contradictory to those of Bresnan *et al*. (2010). However, a possible reconciliation between these two findings may come from the recent observation of the presence of a non-toxic strain of *A. tamarense* in Scottish waters (Collins *et al.*, 2009; Touzet *et al.*, 2010). A change in the balance of the *A. tamarense* population to include relatively more non-toxic cells would generate less toxicity with the same cell densities. This might suggest a northerly migration of the non-toxic group III *A. tamarense*, or a change in conditions that make it better able to compete with toxic strains. However, insufficient evidence exists to draw any definitive conclusions.

Smayda (2005) notes that *A. minutum* appears to be spreading within European coastal waters and changes of its distribution or abundance in the UK cannot be discounted. However, Smayda (2005) also notes that it exhibits a predilection to bloom in nutrient enriched water, perhaps inhibiting its appearance in the low nutrient regions where the Scottish shellfish industry is typically based.

Ballast transfer is also possible. Hallegraeff (1988) believed that the putative regional and global expansions of *A. minutum* have resulted from bio-invasions facilitated by ballast water conveyance, and specifically into Australian waters. Smayda (2005) notes that more recent molecular evidence suggests that the Australian and New Zealand populations (ribotypes) are indigenous. However, partial genetic and toxicological similarities with European strains of *A. minutum* suggest that one or more introductions from Europe (Mediterranean) of this species into Australian waters from Europe may have occurred and introduced another ribotype (de Salas *et al.*, 2001).

Based on the limited, available evidence, Smayda (2005) therefore found that *A. minutum* does not appear to pose a significant threat to cultured and natural fish and shellfish stocks in Scottish coastal waters.

1.6.2 Change in *Dinophysis* community composition

Bresnan *et al.* (unpublished) suggest that the composition of the *Dinophysis* community in Scottish waters changed over the period 1996-2005 with *D. acuta* dominating from 2000 – 2002 and *D. acuminata* being most abundant at other times.

Such changes may be of some importance as *D. acuta* is thought to produce (maximal) okadaic acid concentrations that are four times greater than those generated by *D. acuminata*. This may result in higher shellfish toxicity and slower depuration. (Anderson *et al.*, 1996)

Dinophysis is currently only monitored to genus level in UK waters. However, should it be monitored to species level at some sentinel sites this may allow a better understanding of the toxicity generated by this organism.

Using data from the Scottish regulatory monitoring programme a statistical analysis of cell counts that were greather than the threshold for biotoxin producing phytoplankton set by the UK NRL network was conducted. Of the three major biotoxin producing species monitored, *Dinophysis* was the only one to show a statistically significant difference in over threshold of 100 cells/l events between years (2006-2011). Tukey's multiple comparisons show that for the summer months (June, July and August) there was a statistical difference between the years 2006 and 2008 for above-threshold *Dinophysis* blooms. An investigation of weather patterns over the whole of Scotland between 2006 and 2011 showed that 2006 was characterized by a cold spring (March-April) followed by a relatively "good" summer, with higher than average temperatures and lower rainfall. The summer of 2008 might be regarded as fairly typical with few anomalies from the 30-year average, although May 2008 was exceptionally warm and sunny with low rainfall.

Raine *et al.* (2010) indicate that *Dinophysis* is typically found in stratified waters, often in high density thin layers within horizontally retentive structures such as gyres or coastal jets (Farrell *et al.*, 2012). These structures are transported to the costs where they dissipate and cause DSP events. Climate change driven changes in water mass circulation could therefore have an influence on HAB transport.

1.6.3 Changes in *Pseudo-nitzschia* community composition

The unprecedented appearance of the ASP toxin DA in UK (Shetland) waters in 1997 (EU NRL 1998) and subsequent major shellfishery closures are possibly indicative of a major shift in the distribution of this species (or in its toxicity). Given that this genus has been present in UK waters since at least 1909 (see section 1.3.1) the lack of historical toxicity is surprising. It is unclear if this is due to poor monitoring, a change in species composition within the genus in UK waters, or a change in physical or chemical conditions in the water column in recent years that is promoting toxicity.

The appearance of *Pseudo-nitzschia* species in UK waters may be linked to periods of bad weather (Fehling *et al.*, 2006; 2012) and hence storm events may promote its appearance (Hinder *et al.*, 2012).

The continuous plankton recorder (CPR), operated by the Sir Alistair Hardy foundation for Ocean Science (SAFOS) using ships of opportunity, provides a long term record of changes in plankton in offshore waters. Hinder *et al.* (2012) used CPR data to analyse the long term changes in six phytoplankton taxa in the northeast Atlantic and North Sea. They propose a fundamental shift in the relative abundances of diatoms and dinoflagellates with a marked decline in dinoflagellates in recent years. They found that increasing wind speed and sea surface temperature resulted in a decrease in some dinoflagellates genera. In contrast these conditions favoured some diatoms, including the potentially toxin producing *Pseudo-nitzschia seriata* group, pointing to a greater risk of high abundances of this organism should climate change trends of increased water temperature and storm events continue. However, it is worth noting that Swan and Davidson (2012b) found no statistical difference in the inter-annual number of *Pseudo-nitzschia* samples from the Scottish monitoring programme that exceeded regulatory threshold.

While *Pseudo-nitzschia* is already present in UK waters, the genus is comprised of a large number of species (Lelong *et al.*, 2012). As the organism is typically enumerated only to genus (or at best to the *delicatissima* and *seriata* “group”) the distribution of the different species within the genus is unknown. Moreover, the

toxicity of these different species is thought to vary and, for toxic species, be influenced by nutrient availability (Fehling *et al.* 2004). Given the demonstration by Hinder *et al.* (2011) of changes in the abundance of this genus with temperature and wind speed and the observation by Fehling *et al.* (2012) of its cross shelf transport (which will presumably be influenced by climate change driven changes in water column physics), it is possible that further changes in the abundance and toxicity of this organism will occur.

1.6.4 Changes in *Azadinium* distribution

As noted above, while *Azadinium* is known to be present in UK waters and azaspiracids have been found in shellfish flesh, the inability to monitor this organism by light microscopy means that there are no data from which to assess if and how its distribution might be changing.

The taxonomy of this genus is rapidly evolving with new toxic and non-toxic species being identified in a range of locations worldwide. Until the organism is better characterised morphologically and spatially, discussion of changes in its distribution would be no more than speculation.

1.6.5 Other changes in the phytoplankton community

HAB data from CPR tows indicates a change in the distribution of HAB species since the 1960s with a general decrease in abundance along the east coasts of the UK (Edwards *et al.*, 2006; Bresnan *et al.*, in press). The above changes in the North Sea have been accompanied by a northward shift of warm-water phytoplankton (Edwards and Richardson, 2004; Richardson and Schoeman, 2004). For example, *Ceratium trichoceros*, previously found only south of the British Isles, has expanded its geographic range to the west coast of Scotland and the North Sea, and the subtropical species *Ceratium hexacanthum* has moved 1,000 km northward in 40 years (Hays *et al.*, 2005). At the same time the *Prorocentrum, Ceratium furca*, and *Dinophysis* have increased along the Norwegian coast, and *Noctiluca* has increased in the southern North Sea. While climate warming may be partly responsible it is

difficult to untangle the role of anthropogenic nutrient additions for some of these species patterns (Bresnan *et al.*, 2013).

1.7 "NEW" SPECIES THAT HAVE POTENTIAL TO IMPACT UK SHELLFISH WATERS

Bresnan *et al.* (2013) state that no harmful species have been observed to enter UK waters as range expansions. Reviewing the minutes of the ICES Working Group for Harmful Algal Bloom Dynamics (ICES 2006-2012) at which new findings of HAB species in UK (or other ICES countries) waters would be reported, confirms this conclusion.

However, a range of other biotoxin producing species from elsewhere in the world may have the potential to "invade" UK waters. Below we evaluate the potential for key organisms to become established in UK waters as a result of climate change and/or ballast transfer.

Anderson (1996) indicated that ~ 75 species of phytoplankton were capable of producing biotoxins. A range of databases include the current list of harmful species and their distribution. These are: the IOC-UNESCO taxonomic reference list of harmful micro algae (http://www.marinespecies.org/hab/index.php), the harmful algae event database (HAEDAT; http://haedat.iode.org//), and the AlgaeBASE database of benign and harmful algae from terrestrial, marine and freshwater habitats (http://www.algaebase.org/). Similar information is also held on other web sites such as the world register of marine species (WoRMS; (http://www.marinespecies.org). Fortunately, only a relatively few of these species have the potential to migrate to UK waters, or survive in UK waters should they be introduced. Hence, below we discuss only those species of potential relevance to the UK.

1.7.1 Cyclic imine toxin producing organisms

Cyclic imines are a family of marine biotoxins including spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs). Although SPXs, GYMs, PnTXs and PtTXs are now known to occur in microalgae and/or shellfish in several parts of the world, no information has been reported linking these toxin groups to poisoning events in humans.

SPXs and GYMs are produced by the dinoflagellates *A. ostenfeldii* and *Karenia selliformis*, respectively.

A.ostenfeldii is known to be present in UK waters. The uncertainties in the distribution of *Alexandrium* species has been discussed above.

GYMs have not been found in Europe, with AlgaeBase indicating that the causative species, *Karenia selliformis*, (Haywood *et al.*, 2004) is restricted to New Zealand and Australia. However, *K. selliformis* has been found close to Europe in Tunisia (Bire et al. 2002, Medhioub *et al*. 2010)

PnTXs and PtTxs were first identified by Japanese researchers in 1995. PtTXs are suggested to be bio-transformed from PnTXs in shellfish, with one organism producing PnTXs now thought to be the dinoflagellate *Vulcanodinium rugosum* dinoflagllates (Rhodes *et al.*, 2011a,b). Recently PnTXs were identified for the first time in shellfish in Europe in Norway (Rundberget *et al.*, 2011) and have been idenitified in UK shellfish (Section 2.5).

1.7.2 Diarrhetic shellfish poisoning producing organisms

This is most frequently related to species of the genus *Dinophysis* which is present in UK waters. As with *Pseudo-nitzschia* a range of different species can cause toxicity but insufficient information exists to evaluate the potential for invasion of new toxic species.

1.7.3 Neurotoxic Shellfish Poisoning producing organisms

Neurotoxin Shellfish Poisoning (NSP) is related to species within the genus *Karenia. Karenia mikimotoi* is common in UK waters and has been responsible for the mortality of farmed fish (Davidson *et al.*, 2007). This organism is not thought to be harmful to humans.

The species *Karenia brevis* is capable of causing NSP through its production of brevetoxins (BTXs) (Brand *et al.*, 2012). Human exposure to the effects of NSP toxins is via two pathways, ingestion of contaminated seafood or via a toxic aerosol generated by wave action. Breathing this aerosol can lead to respiratory asthma-like symptoms in humans and consumption of contaminated foodstuffs leads to Neurologic Shellfish Poisoning (NSP). *K. brevis* was considered to be restricted to the Gulf of Mexico and the east coast of Florida, where it is endemic. However, human cases of NSP were unexpectedly reported in the summer of 1992-1993 on the NE coast of New Zealand. These were associated with a previously unknown dinoflagellate named *K. cf brevis.* This organism is similar to other *Karenia* species (*K. mikimotoi* and *K. brevis*) in morphology, and produced 'brevetoxin-like' lipid soluble toxins (MacKenzie *et al.*, 1995). Summer sea surface temperatures in this region of New Zealand are typically ~ 21^{o}C and hence exceed those of the UK.

Subsequently, a species named *Karenia brevisulcata* was found in New Zealand waters. In 1998 it caused a severe HAB incident in the central and southern east coast of the North Island which devastated all marine life in Wellington Harbour (Chang, 1999; Chang *et al.*, 2001). Over 500 cases of human respiratory distress were reported during this event, although no food poisoning associated with the event was recorded. Symptoms included dry cough, severe sore throat, rhinorrhoea, skin and eye irritations, severe headaches and facial sunburn sensations. The respiratory distress was attributed to exposure to seawater and aerosols and resembled that caused by aerosolised BTXs (Holland *et al.*, 2012). A range of novel toxins were identified in bulk cultures of algae, although no brevetoxins were identified. No blooms of *K. brevisulcata* have since been reported, so it is difficult to speculate on the cause of this outbreak. While it was noted that the water was unusually warm and stratified at the time of the bloom, given that water temperatures

near Wellington (~18 ^{o}C in in summer) are closer to those in the UK, blooms of novel *Karenia* species that are potentially harmful to humans cannot be discounted in UK waters.

1.7.4 Paralytic Shellfish Poisoning producing organisms

Gymnodinium catenatum

Perhaps the organism of the greatest concern in terms of invading UK waters is the PSP causative chain-forming dinoflagellate *G. catenatum*. The species produces cysts that survive prolonged darkness and nutrient-poor conditions and can travel considerable distances in ships´ ballast water (Halegraeff, 1998). It is frequently observed in Northern Spanish (Galician) waters where it was first reported in 1976 after a PSP event (Estrada *et al.*, 1984). Wyatt (1992) hypothesised that the taxon was introduced by the Galician fishing fleet, which during the 70s operated in Argentinian waters, where this species is common.

The species has then apparently expanded into the Mediterranean Sea appearing in the NW Alborán Sea (Delgado, 1990; Bravo *et al.*, 1990), probably transported by currents from the Atlantic Ocean according to the circulation through the Strait of Gibraltar (Gómez, 2003a) and then has expanded along the southern basins of the Western Mediterranean Sea (Gomez, 2003b).

The harmful algal event database (HAEDAT) confirms that *G. catenatum* events are common in the Galician Rias of North West Spain and in Portugal. However, no events were reported for France. One event was reported from the German Bight in 1993, although this may be anomalous.

Water temperatures in Galicia are similar to those in Japanese waters where this species is also common. Such temperatures exceed those typical in UK waters. For example, the seasonal cycle of surface sea temperature (SST) at station L4 off the Devon coast operated by Plymouth Marine Laboratory suggest a winter minimum of 9±1 °C in March and a summer maximum of 17±1.5 °C in August (http://www.st.nmfs.noaa.gov/nauplius/media/time-series/site northsea-plymouth-l4/).

G. catenatum is also present in Australian waters, where it thought to have been translocated by ballast transfer from Japan to Tasmania (Hallegraeff and Bolch, 1992). The Australian Integrated Marine Observing System (http://imos.org.au/) indicates that water temperatures typically range from 12 – 18 ^{o}C, similar, at least in summer, to the data from station L4. Hallegraeff *et al.* (1995) notes *G. catenatum* to bloom in well-defined seasonal temperature windows when water temperature exceeds 14 ^{o}C at the time of bloom initiation, suggesting summer UK blooms are possible.

Further evidence for the ability of *G. catenatum* to survive in waters of temperature representative of that in the UK comes from Band-Schmidt *et al.* (2004). The authors studied the temperature tolerance of a strain of *G. catenatum* from the Gulf of Mexico and found positive net growth to occur between 11.5 and 30 ^{o}C, with optimal exponential growth rates of 0.14 – 0.21 day^{-1} between 15 and 29 ^{o}C. Similarly, Bravo and Anderson (1994) found optimal growth of a different strain of *G. catenatum* from Rio de Vigo Spain, to occur between 22 and 28 ^{o}C, but with net positive growth down to temperatures as low as 11 ^{o}C. While Bravo and Anderson (1994) also demonstrated cyst germination success to be greatest at temperatures 22 - 28 ^{o}C, they also observed some germination at relatively low temperatures, with ~ 50% at a temperature as low as 13 ^{o}C.

This data therefore suggest that the summer water temperature in UK waters, while non-optimal for *G. catenatum,* could potentially sustain its growth. However, winter temperatures seem to be lower than those where it is established elsewhere (with the potential exception of Tasmania). This may limit the development of the species, should it be trans-located to UK waters. However, the species is known to form resting cysts (McMinn *et al.*, 1997) and therefore may be able to overwinter in the sediments should water temperatures become too low for it vegetative growth.

A study based on cyst records of microreticulate species in dated sediment cores along the West Iberian shelf covering the past ~150 years indicated that *G. catenatum* is new to the NE Atlantic and expanding northwards along the West Iberian coast (Ribeiro *et al.*, 2012). Using recent toxic bloom records, an invasive

pathway of this species have been reconstructed, which points toward the species' natural range expansion, but human-mediated introduction cannot be discounted.

While both ballast water translocation and range expansion are possible vectors for the transfer to *G. catenatum* to UK waters, the latter is perhaps the more likely. Experiments to investigate the adaptation to and survival of Spanish strains of *G. catenatum* in conditions representative of UK coastal waters are therefore recommended.

Pyrodinium bahamase var. compressum

The armoured chain-forming dinoflagellate, *Pyrodinium bahamense var. compressum*, is included here because of its significant PSP implications. The species was responsible for the greatest number (41%) of global PSP events between 1989 and 1999. This organism is of importance in several countries in the tropical pacific, with the harmful implications of the organism being first recognised in Papua New Guinea. Blooms are often related to monsoon periods with winddriven upwelling being implicated in their formation, potentially through re-suspension of cysts into the water column where excystment and vegetative cell division results in population growth (Davidson *et al.*, 2011). As no records exist outside of tropical waters, its potential for translocation to, and survival in, UK is very slight.

1.7.5 Palytoxin producing organisms

Ostreopsis

Ostreopsis is a benthic dinoflagellate, some species of which produce palytoxin-like toxins that are powerful vasoconstrictors in mammals (Usami *et al.*, 1995). It has been associated with skin irritation for swimmers (Deeds and Schwartz 2010) and respiratory problems for beach users (Ciminiello *et al.*, 2008). Extreme cases following consumption of contaminated seafood have led to vomiting, kidney problems and even death. While such cases are linked to fish and crabs, the possibility of PlTX exposure through shellfish consumption is still unknown (Deeds and Schwartz, 2010) and hence must be considered a risk.

New toxins are being discovered as our detection techniques develop. An example is the identification of a new palytoxin congener ovatoxin-f from fish samples collected in the North Western Adriatic (Caillaud. *et al.*, 2010). The toxin profile was both quantitatively and qualitatively different from any previously reported *O. ovata* toxins.

The first description of benthic dinoflagellates of the genus *Ostreopsis* is from the Gulf of Siam (Thailand) (Schmidt, 1902). *O. ovata* was described much later as an epiphyte of macroalgae in French Polynesia and New Caledonia (Fukuyo, 1981). The GEOHAB, HABs in Benthic Ecosystems report (GEOHAB, 2012) discusses the range expansion of this genus to temperate regions including the Mediterranean Sea, with a number of records of *Ostreopsis* recently having been noted for the French Mediterranean (Sechet *et al.*, 2012; Guidi-Guilvard *et al.*, 2012; Blanfune *et al.*, 2012).

Rhodes (2011) surveyed the global distribution of *Ostreopsis*. No blooms have been reported in the cool temperate waters of Northern Europe with European instances currently restricted to the Mediterranean and Portuguese Atlantic. HAEDAT and AlgaeBase confirm that European events are restricted to southerly latitudes.

While Rhodes (2011) suggests that climate does not appear to have a particular impact on bloom formation or toxin production, Selina and Orlova (2010) report *Ostreopsis* in the Russian waters of the Japan Sea. These waters reached temperatures greater than 20 ^{o}C in summer, but are approximately 0 ^{o}C in winter. Therefore it remains unclear whether this genus could survive in UK shelf sea waters.

Given the apparent rapid global expansion of this organism, the possibility of it becoming established in UK waters cannot be discounted and research on this organism is recommended.

Coolia sp.

Coolia is an armoured, marine, benthic and toxic dinoflagellate species with world-wide distribution. *C. monotis* was considered toxic by Nakajima *et al.* (1981) producing cooliatoxin, a neurotoxic analog to yessotoxin (Holmes *et al.*, 1995; Rhodes and Thomas, 1997). While the IOC list of harmful algae indicated that this

was thought to be due to mis-identification with the toxin producing organism being *C. tropicalis,* recent publications such as Rhodes *et al.* (2010a) are still indicating *C. monotis* is toxic.

If toxicity is restricted to *C. tropicalis* then it is warm water habitat means that it is unlikely to impact UK waters. In contrast, *C. monotis* is a neritic species that is quite common world-wide in temperate to tropical waters (Steidinger and Tangen 1996). Populations have been observed from plankton samples collected from oyster beds, brackish habitats and tidal pools, as well as mangrove environments. While this species is most common in warm shallow waters of the Caribbean and Mediterranean Seas, and the Pacific Ocean (Faust 1992) it was listed by Parke and Dixon (1976) as being present in UK waters and has been identified in the Fleet Lagoon in Dorset (Professor Duncan Purdie, University of Southampton, personal communication).

1.7.6 Cyanotoxin producing organisms

There are approximately 2000 species of cyanobacteria, of which ~ 40 have been identified as toxigenic, with toxicity to hepato-, neuro-, gastro-intestinal and dermatic systems with embryo-lethal, teratogenic, gonadotoxic, mutagenic and tumour-promoting activities. Cyanobacteria and their toxins are of greatest concern in freshwater environments. But they have relevance to low salinity marine waters. In Europe, cyanobacteria are most prominent in the Baltic Sea, but may need to be considered in some estuarine environments in the UK.

Cyanotoxins can be accumulated in shellfish (Duy *et al.*, 2000) and therefore are of concern for shellfish harvesting regulation. As discussed by Ibelings and Chorus (2007) information on this topic remains relatively scarce and mostly related to freshwater. The Scottish Government (2012) assessed the risk to public health from cyanobacteria in inland and inshore waters, noting that some concern exists from the accumulation of the toxins in shellfish, including freshwater and brackish-water mussels and in fish. However, no cases of intoxication from such sources have been reported to date in Scotland.

However, in the US, studies have found evidence of microcystin-contaminated freshwater reaching the marine ecosystem and reported the deaths of marine mammals due to cyanotoxins and confirmed the existence of hepatotoxic shellfish poisoning (HSP) in the Pacific coastal environment (Miller *et al.*, 2010). These findings therefore suggest that we need to consider that humans and animals might be at risk from microcystin poisoning when consuming shellfish harvested at the land-sea interface (Paerl, 2008).

Also important among the marine cyanobacteria is the benthic species *Lyngbya majuscula* that can be found in tropical regions growing in fine strands attached to seaweed and rocks. Mats can rise to the surface to form large floating aggregations that may reach land. *L. majuscula* contain a number of toxic compounds of which debromoaplysiatoxin (DAT), aplysiatoxin (AT) and the lyngbyatoxins A, B and C are the most important. It is responsible for cyanobacterial dermatitis, commonly referred to as ‘swimmers’ itch’ or ‘seaweed dermatitis’. This is a severe contact dermatitis that may occur after swimming in water. The symptoms are itching and burning within a few minutes to a few hours after swimming. Visible dermatitis, blisters and deep desquamation may follow, with eye and respiratory irritation also possible.

Toxins from the genera *Schizothrix* and *Oscillatoria* have also been linked to dermatitis and tumour formation. A further important toxic species is *Nodularia spumigena*, which is of particular concern for human and animal health in the Baltic Sea (Sellner *et al.*, 1997). This species produces nodularin, a monocyclic penta-peptide (closely related to the freshwater microcystins) that acts as a hepatotoxin, by inhibiting protein phosphatase activity. Low-level exposure to these toxins may promote the development of cancer in the liver and other chronic disorders of the gastrointestinal tract. Allergic or irritative dermal reactions have also been reported following recreational exposure.

Given the lack of reports of harmful marine cyanobacterial blooms in UK marine waters and the generally high salinity at most UK shellfish harvesting sites, the widespread risk to UK consumers from shellfish vectored cyanotoxins would seem to be slight. However, locations where runoff from cyanobacterial contaminated fresh water bodies meet marine waters may be of some concern.

1.7.7 Ciguatera Fish Poisoning organisms

The benthic dinoflagellate genus *Gambierdiscus* is responsible for the food-poisoning syndrome ciguatera. Humans are affected by this syndrome through ingestion of tropical fish that have accumulated the ciguatoxins (CTXs) produced by this organism. It is well known in coral reef areas in the Caribbean, Australia, and especially French Polynesia.

In 2004, an outbreak of CFP was reported in the Canary Islands which was followed by two additional occurrences in 2008-2009, with Caribbean ciguatoxin -1 (C-CTX-1) confirmed in fish caught in nearby waters (Boada *et al.*, 2010). This report suggested the possible northward migration of *Gambierdiscus*.

Kibber *et al.* (2012) investigated the growth rate of six *Gambierdiscus* species at a range of water temperatures. They found growth to peak at temperatures typically ~ 30 ^{o}C with the lowest peak being 25 ^{o}C. Little to no growth was found below 20 ^{o}C. Therefore, notwithstanding the possible northwards migration of *Gambierdiscus*, it seems unlikely that this organism could survive in the conditions characteristic of UK shelf seas and is hence of low risk to UK waters.

In addition, Rhodes *et al.* (2010a) noted that extracts of mass culture of the dinoflagellate *Amphidinium cartarae* caused respiratory paralysis in mice at high doses and given the co-occurrence of this organism with *Gambierdiscus australes,* its role in human poisoning attributed to ciguatoxins may warrant further study.

1.8 FEEDBACK FROM UK AGENCIES, RESEARCH ESTABLISHMENTS AND UNIVERSITIES ON THE OCCURRENCE OF NEW/EMERGING TOXIN PRODUCING SPECIES

A survey was circulated to 14 relevant UK and Irish universities/institutes

Eight of the fourteen recipients provided a reply. The questions and replies received are detailed in Appendix 1, with a summary of responses presented here.

A tentative identification of *Karenia papilionacea* was made by Marine Scotland Science (MSS). This organism may produce BTXs (Haywood *et al*. 2004). It has previously been identified in New Zealand, but Haywood (2004) noted that *K. papilionacea* like species have been described from Spain (Fraga and Sanches, 1985). Also a species described as *K. brevis* from Japan in Fukuyo et al. (1990) is more similar to *K. papilionacea* than to *K. brevis*. The observation highlights the possibility of new *Karenia* species appearing in the UK with the potential for introduction of brevetoxins into the food chain and subsequent health implications for the shellfish consumer.

Data from MSS also confirms the (known) presence of a number of different species and strains of *Alexandrium* with different levels of toxicity.

A range of different *Pseudo-nitzschia* species were also observed. As this organism is rarely studied to species it is difficult to determine if these species are new. Isolates would be required to confirm their toxicity.

Returns also suggest some variety in *Dinophysis* species.

In summary, the survey returns indicate the possibility for invasive HAB species and that vigilance is required but, as yet, no new species other than those discussed elsewhere in this report (e.g. *Azadinium*) have clearly recently become established. Although the tentative detection of *K. papilionacea* (Brevetoxin) and *C. monotis* could be a future concern if further studies found these species were becoming established.

Table 2. UK phytoplankton survey recipients

Institution	Contact	Reply received
Marine Scotland Science	Eileen Bresnan	Yes
SEPA	Claire Scanlan	Yes
CEFAS	Steve Milligan	Yes
Environment Agency	Mike Best	No

AFBI	Richard Gowen	No
PML	Claire Widdicome	Yes
SAHFOS	Rowena Stern	Yes
Millport Marine Station	Fiona Hannah	Yes
Napier University	Linda Gilpin	Yes
NUI Galway	Robin Raine	No
Sherkin Island Marine Station	'sherkinmarine@eircom.net'	No
University of Liverpool	Jonathon Sharples	No
University of Southampton/NOC	Duncan Purdie*	Yes
University of Exeter/ ECEHH	Clare Redshaw*	No

*also received the biotoxin survey questions

1.9 FEEDBACK FROM EU AND OTHER COUNTRIES AGENCIES AND RESEARCH ESTABLISHMENTS ON THE OCCURRENCE OF NEW/EMERGING TOXIN PRODUCING SPECIES THAT MIGHT BE ADVECTIVELY TRANSPORTED

An extended survey that also included questions relating to monitoring methodology was sent to a number of international laboratories. The recipients of this survey included a range of other European countries and (due to its similarity to the British Isles) New Zealand.

The list of non-UK organisations is given in Table 3 with the responses received summarised in Appendix 2.

Four of the eight EU recipients provided a reply. No response was received from New Zealand.

Returns indicated, that in common with the UK, there are relatively few different organisms that can be confirmed as new invasive harmful species.

Those new species that are highlighted are *Azadinium* and *Ostreopis* both of which are discussed elsewhere in this report, the former also recently becoming evident in UK waters. It is interesting that *Ostreopis* has not been detected in N. Spain.

The Spanish return also highlights *Prorocentrum rhathymum* and *Karlodinium micrum.* The IOC-UNESCO HAB database indicates the former produces haemolytic toxins not toxic to mice, with the water soluble acetone precipitate being toxic to mice (IOC-UNESCO HAB database), but the database notes that the latter is not accepted as a harmful species.

In summary, the EU survey returns indicate the possibility for invasive HAB species and that vigilance is required but, as yet, no new species other than those discussed elsewhere in this report (e.g. *Azadinium* and *Ostreopsis*) have clearly recently become established.

Table 3. List of EU and non-EU monitoring laboratories surveyed

Country	Institution	Contact	Reply received
Spain	INTECMAR	Yolanda Pazos	Yes
Italy	Stazione Zoologica Naples	Adriana Zingone	Yes
Portugal	IPMAR	Teresa Moita	No
France	IFREMER/REPHY	Catehrine Belin	No
Ireland	Marine Institute	Joe Silke	Yes
Denmark	Orbicon	Per Andesson	No
Netherland	WUR	Ainhoa Blanco	No
New Zealand	Cawthron	Lincoln Mackenzie	No
Norway	IMR	Lars Naustvoll	Yes

1.10 CONCLUSIONS

Alexandrium spp. are known to be present in UK waters presenting a high risk of shellfish toxicity. The distribution of the different species and strains within the genus is poorly understood with previously assumed distributions no longer being thought

to hold true. This may be due to climate change or other factors and indicates that spatial/temporal risk remains high. Invasion of *Alexandrium* spp. such as *A. catenella* is possible, but may currently be limited by temperature. However, sentinel site monitoring of *Alexandrium* would help to clarify the geographical risk of different species.

Pseudo-nitzschia spp. remains a threat in UK waters due to its potential for high toxicity. As for *Alexandrium* spp. the lack of information at the level of species makes temporal/geographical risk assessment difficult and again sentinel site monitoring to species would be of benefit. Similar monitoring would also benefit our understanding of *Dinophysis*. While this genus produces less potent toxins that *Alexandrium* or *Pseudo-nitzschia*, production is still thought to be species specific and hence poorly quantified on the basis on current genus based monitoring.

Azadinium spp are known to be present in UK waters but, due to the lack of monitoring, its temporal/spatial distribution is poorly understood. Monitoring through molecular based methods will be important for this genus.

Of the possible invasive species *Gymnodimium catenatum* presents the highest likelihood of invasion and risk. With climate driven warming of surface coastal waters occurring, it will be important to be vigilant for this PSP producing species.

Other invasive species have the potential to become established in UK waters. Given the examples of unexpected *Karenia* events in New Zealand and *Vulcanodinium* in Norway broader surveillance is required in the UK. For benthic species this will require development of new methods of quantitative cell collection.

1.11 RISK BASED ASSESSMENT OF NEW AND EMERGING TOXIN PRODUCING SPECIES WHICH MAY/ARE PRESENT IN UK WATERS

Based on the literature review and the responses from UK and EU laboratories a risk based assessment relating to biotoxin producing phytoplankton has been produced. Table 4 refers to organisms that are known to be present in UK coastal waters and summarises possible changes and the implications of these. Table 5 details the non native, potentially invasive species, that are most likely to become present in UK waters (although there is no clear evidence that any of these will become present).

The level of risk is based on both the likelihood of toxins occurring and the severity of the hazard. Both aspects are assigned a mark out of five, with 5 representing a high risk and zero representing no risk. The total risk, ranging from zero to twenty-five, is calculated from multiplying the two risk factors. It is noted that any determination of risk relating to the presence of new or emerging toxins where either no UK monitoring has taken place or where the toxicity is currently unknown is subjective. A key describing likelihood and severity ratings is provided with the tables.

Risk Based Assessment

Key:

Likelihood of occurrence (either now or in the future)	**Score**	**Severity**	**Score**
No risk of occurring	0	No toxicity effects	0
Unlikely to occur	1	No acute toxicity, chronic toxicity unknown	1
Possiblity of occurrence	2	Evidence for some toxicity, although actual effects unclear	2
Good potential for occurrence although no evidence currently exists	3	Evidence for toxicity with clear threat to human consumer safety in contaminated products, but no potential for fatality or long term sickness	3
Highly likely to exist or likely to become established	4	High toxicity but with no potential for serious long term illness or fatality even at high concentrations	4
Evidence for presence currently or in recent years within UK waters	5	Extreme toxicity risk with high potential for fatality when present at high enough concentrations	5

Likelihood and Severity factors are scored independently. Total risk is product of the two factors.

Table 4. Current UK HAB species

Organism	Issue	Outcome	Situation	Likelihood (0-5)	Severity (0-5)	Risk level (0-25)
Alexandrium tamarense	High toxicity group I *A. tamarense* now co-exists with non toxic group III.	Difficult to predict toxicity on the basis of cell counts alone	Detected with other *Alexandrium* in current official monitoring programme. Identify composition of rapidly developing or large blooms to species level using molecular methods recommended.	5	5	25
Alexandrium minutum	Unknown distribution	Changes in PSP toxicity	Detected with other *Alexandrium* in current official monitoring programme. Recommended to monitor to species at sentinel sites.	5	5	25
Alexandrium ostenfeldii	Unknown distribution	Changes in PSP toxicity	Detected with other *Alexandrium* in current official monitoring programme. Recommended to monitor to species at sentinel sites.	5	5	25
Pseudo-nitzschia spp.	Potential climate driven increase in abundance (Hinder *et al.* 2012). Factors governing toxic/non	Changes in ASP toxicity	Detected in current official monitoring programme. Monitoring to species (molecular methods)	5	5	25

	toxic species composition poorly understood. Factors governing toxicity poorly understood.		at sentinel sites recommended.			
Dinophysis spp.	Abundance likely related to oceanographic features that may be climate modified. Factors that influence the relative abundance of *D. acuta, D. acuminata* and other *Dinophysis* species poorly understood.	Variable toxicity	Detected in current official monitoring programme. Conduct monitoring to species level (morphology) at sentinel sites recommended.	5	4	20
Azadinium spp.	Unable to monitor by light microscopy. Toxic and non toxic species now identified.	No early warning of AZP toxicity	**Not detected in current official monitoring programme.** Conduct widespread monitoring of this organism using Q-PCR analysis of Lugol's fixed samples recommended as a high priority.	5	4	20
Prorocentrum lima	Current method of sample collection of this (and other) semi benthic species ineffective.	Extent to which this organisms influences DSP toxicity not well understood	Detected in current official monitoring programme if cells present in the water column but **benthic cells which may be available to shellfish not detected in this programme.**	5	4	20

			Recommendation that evaluation of *P. lima* abundance in benthos at sites of unexpectedly high DSP toxins: this first requires the development of a robust sampling methodology.			
Cyanobacteria	Freshwater species can survive in brackish water	Cyanotoxins in marine shellfish	**Not detected in current official monitoring programme** Risk assessment of sites that may be at risk of washout from fresh water species.	2	4	8
Prorocentrum minimum	Present in UK waters	Potentially linked to VSP	Detected in current official monitoring programme.	5	1	5
Gonyaulax spinifera	Present in UK waters	Yessotoxin producer	Not currently monitored	4	1	4

Table 5. Possible invasive HAB species to UK waters

While, as discussed above, the "invasion" of a range of species is possible, those listed below are seen as being most likely to become present in UK waters.

Organism	Issue	Outcome	Situation	Likelihood (0-5)	Severity (0-5)	Risk level (0-25)
Gymnodinium catenatum	Has previously trans-located Has been observed in ballast water of vessels at UK ports Potentially capable of survival in UK water temperatures	PSP toxicity of shellfish	Currently not included in species list monitored within the official monitoring programme but could be detected if included. Recommendation to include in list of species monitored within the UK monitoring programme. Laboratory based experiments on Spanish strains recommended to better evaluate their potential to survive in UK	4	5	20

			waters.			
***Ostreopsis spp*.**	Perceived northwards migration Methodologies for its monitoring not well developed	Aerosol and potential for shellfish toxicity	Currently not included in the list of species monitored within the official monitoring programme. However, could be detected (potentially by light microscopy but more reliably by molecular methods) if present in the water column by this programme. However, as this specied is primarily benthic unless mixed into the water column it would not be detected within the monitoring programme. Recommendation to include in species list of monitoring programme and develop methodology for monitoring this benthic	2	4	10

			species.			
Alexandrium catenella	Has recently invaded Mediterranean Cysts may be present in vessels at UK ports	PSP toxicity of shellfish	Detected with other *Alexandrium* in current official monitoring programme. Recommended to monitor to species at sentinel sites	2	5	10
***Karenia spp*.**	Unexpected blooms of non native biotoxin producing *Karenia* species in New Zealand. Such waters of similar temperature to UK. *Karenia papilionacea* noted in UK waters from survey responses Species toxic to humans difficult to discriminate by	NSP toxicity (aerosol and shellfish)	*Karenia* included to the genus level in the current monitoring programme. Recommended molecular based monitoring at sentinel site(s) to the species level undertaken to identify potentially toxic species.	2	5	10

	microscopy from fish killing species that are currently present					
Vulcanodinium spp.	Pinnatoxins noted in Norway from Survey responses	Pinnatoxins (PnTXs)	Currently not included in list of species. Ppotentially could be monitored within the official monitoring programme (dependant on more detailed taxonomic details becoming available). Recommendation to include in list of species monitored for within the UK monitoring programme.	3	2	6
Coolia spp.	Observation in Fleet Lagoon	Palytoxin toxicity Uncertainty over the	Currently not included in list of species monitored within the official monitoring programme. However, could be detected if present in the water column by this	2	4	8

		toxicity of *C. monotis*	programme but this genus is primarily **benthic and hence cells which, though sporadic mixing, may be available to shellfish would not necessarily be detected**. Should toxicity be clearly establised, it is recommendation to include this genus in the list of organisms monitored in the programme and develop methodology for monitoring this benthic species.			

2. POTENTIAL NEW AND EMERGING TOXIN THREATS FOR UK WATERS- SHELLFISH TOXINS

2.1 INTRODUCTION

Marine biotoxins are accumulated and metabolised in the flesh of grazing organisms such as bivalve molluscs whilst feeding on toxic strains of phytoplankton. Usually the shellfish are unaffected by the toxins, consequently creating a risk to human consumers of contaminated products. In the UK, official control monitoring is conducted on shellfish tissue homogenates for the detection and quantitation of marine biotoxins of algal origin. Toxins reported to the Food Standards Agency (a competent authority) are those from the three main toxin classes regulated under European Union (EU) law and which have been subjected to appropriate validation in relation to the shellfish species of interest.

Paralytic Shellfish Poisoning (PSP) toxins

PSP toxins are highly potent neurotoxins present in a number of different marine algae (Llewellyn, 2006). With accumulation of toxins in feeding molluscs, human consumption of shellfish products can result in a serious risk to the consumer (Etheridge, 2010). Symptoms range from mild tingling, numbness at low levels, to muscular paralysis, respiratory distress and death at higher levels of intoxication.

Amnesic Shellfish Poisoning (ASP) toxins

Amnesic Shellfish Poisoning is caused following consumption of seafood products containing domoic acid and potentially associated isomers (Nikkar and Mijjar, 2000). The toxins are found most notably in scallops, although very little is generally found in the edible parts of the flesh. However, other shellfish including mussels are also known to accumulate domoic acid.

Since implementation of the high performance liquid chromatography with UV detection (HPLC-UV) method more than ten years ago it has been used to detect and quantify ASP in shellfish around the UK. To date domoic acid and its naturally-occurring isomer epi-domoic acid has been identified in cockles, mussels, king scallops, Pacific oysters, razors, surf clams and otter shell clams. Depending on the chromatographic method applied, the method will either separate domoic and epi-domoic acid on column or the two will elute as one chromatographic peak. Total results obtained following either approach are reported as the sum of both isomers. These isomers of domoic acid are well recognised and as such do not constitute new or emerging toxin threats.

Lipophilic Toxins (LTs) including Diarrhetic Shellfish Poisoning (DSP)

Lipophilic marine biotoxins, include those traditionally termed the diarrhetic shellfish poisoning (DSP) toxins. They are a hugely diverse collection of groups of naturally-occurring toxins present in marine phytoplankton and found to accumulate in the fatty tissues of shellfish through natural feeding. Toxins found in shellfish include both the precursor toxins present in the algae and metabolic products. The polyethers okadaic acid (OA) together with the dinophysis toxins (DTXs) are the main contributors to DSP. The principal toxins OA, DTX1 and DTX2 have been identified both in algae and as the causative agents in intoxications (Yasumoto *et al.*, 1978; Kumagai *et al.*, 1986; Hu *et al.*, 1992). Other OA-group toxins identified in shellfish include a mixture of OA/DTX1&2 fatty acid ester metabolic products, termed DTX3, which in some instances can represent large proportions of the total toxin-content (e.g. Villar-Gonzalez, 2008) plus a range of other ester derivatives. Consumption of shellfish contaminated with these metabolic products can trigger hydrolysis to the toxic parent forms. Symptoms of DSP include vomiting and diarrhoea, with full recovery after 3-4 days and no long term health implications proven to date.

Other polyethers included in the suite of lipophilic toxins are the pectenotoxins (PTXs), comprising the precursor PTX2 and a group of more than 14 congeners formed during shellfish metabolism (Draisci *et al.*, 2000).
Another toxin belonging to lipophilic toxin group is yessotoxin (YTX), which together with its huge range of analogues (currently >90) is structurally similar to the brevetoxins and ciguatoxins. Whilst occurrences of both PTX and YTX compounds are known to be widespread, the health effects of both groups remain unclear (Ogino *et al.*, 1997).

Azaspiracid poisoning (AZP) is a more recent syndrome, with symptoms following consumption of contaminated shellfish similar to those exhibited by the classical DSP toxins. AZA toxins are nitrogen-containing polyethers with a large number (>20) of AZA analogues identified to date in shellfish (Rehmann *et al.*, 2008). AZA1, 2 and 3 are most commonly encountered and are identified by EFSA as the highest importance in relation to intoxication (EFSA, 2008).

Lipophilic marine toxins are a hugely diverse range of naturally-occurring toxins with a marine phytoplankton source. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) results from the analysis of UK shellfish over the past few years have indicated periodic occurrences of toxins from all the regulated lipophilic toxin groups, including OA/DTXs, PTXs, YTXs and AZAs. OA-group toxins have been particularly prevalent, with OA, DTX2 and OA-group fatty acid esters all detected and quantified in particular, although DTX1 has been detected at low levels. . Whilst some authors report OA and its methylated analogue dinophysistoxin-1 (DTX-1) as the primary contributors to toxic outbreaks (e.g. van Egmond *et al.*, 1993), other analogues such as DTX2 and the fatty acid ester metabolites (termed DTX3) are also recognised as significant contributors (Quilliam, 2003).

Other regulated lipophilic toxins identified in shellfish include YTX, 45-hydroxy homo YTX, and AZA1-3 with AZA3 generally lower in abundance than the other two. PTX1 has not been detected and although only small concentrations of PTX2 have been detected they were below the limit of quantitation.

Table 6. Biotoxin groups monitored in UK shellfish to ensure shellfish safety.

Toxin class	Toxins
Paralytic Shellfish Poisoning (PSP) toxins	Saxitoxins (STXs)
Amnesic Shellfish Poisoning (ASP) toxins	Domoic (DA) and epi-domoic acid
Lipophilic toxins (LTs) including Diarrhetic Shellfish Poisoning (DSP) toxins	Okadaic acid (OA) & dinophysistoxins (DTXs) Pectenotoxins (PTXs) Yessotoxins (YTXs) Azaspiracids (AZAs)

2.1.1 Current status of biotoxin monitoring in UK shellfish

To ensure protection of the shellfish consumer, the EU reference method for PSP is the Mouse Bioassay (MBA) (Anon, 2005a, 2006). This provides a quantitative determination of PSP toxicity calculated from the time of death observed in replicate mice. Given both ethical and performance issues with the MBA (Turner *et al.*, 2012), there is a continued global trend to move away from continuing reliance on the live animal assay. Alternative methods based on liquid chromatography with fluorescence detection (LC-FLD) have been validated in a number of laboratories and by collaborative study. One of these, known as the “Lawrence method” a pre-column oxidation LC-FLD method (Lawrence and Menard, 1991; Lawrence and Niedzwiadek, 2001; Lawrence *et al.*, 2005) was established as an AOAC International Official Method (OMA 2005.06; Anon, 2005b) and became officially recognised as an alternative method for official control testing of live bivalve molluscs by the EU (Anon, 2006). This and other LC methods provide qualitative information on the presence of individual toxins separated either as toxin oxidation products or in their natural toxin state. With the availability of certified toxin standards, instrumental calibrations enable the quantitation of PSP toxins, which can consequently be used to estimate sample toxicity through calculation of total

saxitoxin equivalents. This requires accurately calculated Toxicity Equivalence Factors (TEFs) which describe the relative potency of individual PSP toxins. As well as the pre-column oxidation method, a post-column oxidation (PCOX) LC-FLD has recently been validated through collaborative study and accepted by the AOAC as an official method (OMA 2011.02; Anon, 2011). More recently still a Receptor Binding Assay (RBA) has been validated and published as AOAC OMA 2011.27. Whilst other methods have been extensively investigated and published, including a number of biomolecular methods, screening tests and mass spectrometric methods, these remain research tools until thoroughly and formally validated.

In the UK, AOAC 2005.06 LC-FLD has been refined, semi-automated, extended to additional toxins and validated for the twelve shellfish species of relevance to the official control monitoring programme. This method involves the pre-analysis oxidation of cleaned-up acidic shellfish extracts prior to chromatographic analysis. Performance characteristics of the method have been demonstrated previously through a number of validation studies, with the sensitivity, linearity, recovery, precision and ruggedness shown to be fit for purpose (Turner *et al.*, 2009, 2010, 2011; Turner and Hatfield, 2012). To date validation has been conducted only for those PSTs available commercially as certified reference standards from the NRC in Canada. The method has been implemented in various forms into the UK official control monitoring programmes since 2006, with full replacement of the PSP MBA occurring in 2011.

Monitoring shellfish for the presence of ASP is a relatively simple process, requiring the application of either isocratic or gradient elution reverse-phase liquid chromatography (LC) prior to ultra-violet (UV) detection. LC-UV methods are used worldwide and are typically based on the method of Quilliam *et al.*, 1989, with or without sample extract clean-up prior to analysis. Validation, optimisation and collaborative trial (Lawrence *et al.*, 1989, 1991) facilitated the interlaboratory assessment of the method performance characteristics and the method was subsequently adopted by the AOAC as an official method for the quantitation of domoic acid in mussels (OMA 991.26; AOAC, 1991). An optimised method was reported by Quilliam *et al.*, 1995 describing the use of a 50% methanol extraction

step and showing excellent toxin recoveries (domoic acid recovery > 95%). The method was subsequently validated in a number of other laboratories (Hess *et al.*, 2001) forming the basis of harmonised standards as published by CEN (CEN, 2008) and the EURL-MB (EURLMB, 2008). The method has been refined and validated at many laboratories worldwide including official control monitoring laboratories within the UK, where it is applied on a routine basis for all species of relevance to the monitoring programmes.

For years a number of qualitative animal (mouse and rat) bioassays were listed in the EU regulations as reference methods for the determination of lipophilic toxins in shellfish. The MBA method of Yasumoto *et al.*, 1978 was most commonly used throughout Europe and the rest of the world, although there are known issues with its performance (Fernandez *et al.*, 2003; Suzuki *et al.*, 2005). These remained as the reference method until 2011, when the legislation changed to stipulate the use of new methods utilising LC-MS/MS. Whilst other methods may be applied given suitable evidence for applicability to all regulated toxins and acceptable performance characteristics, the global move in recent years has been to test, validate and implement LC-MS/MS as the sole monitoring tool for official control testing of bivalves. In the UK LC-MS/MS methods have been validated for all species of relevance to the monitoring programmes. The implementation started in 2011 and since 2012 LC-MS has become the sole monitoring tool for regulated lipophilic toxins, resulting in the complete replacement of the DSP MBA for shellfish control.

The aim of this review is to establish which new/emerging toxins are present or could potentially become present in UK shellfish in addition to those toxins tested for within the monitoring programme. This information will then be used to produce a risk based assessment and matrix allowing the evaluation of potential hazard to shellfish consumers from these new/emerging toxins. The findings will also be used to highlight toxins which present the most serious risk to UK consumers and recommendations for further work in relation to these risks.

2.2 BREVETOXINS

2.2.1 Toxicity risks of brevetoxins (BTXs) to human health

The brevetoxins (BTXs or PbTxs) are a large family of stable lipid-soluble cyclic polyether compounds primarily produced by the dinoflagellate genus *Karenia*, most notably the species *K. brevis* (also known as *Gymnodinium breve* or *Ptychodiscus breve*). The toxins produced by such algae are responsible for toxicity in fish, marine mammals, birds and humans.

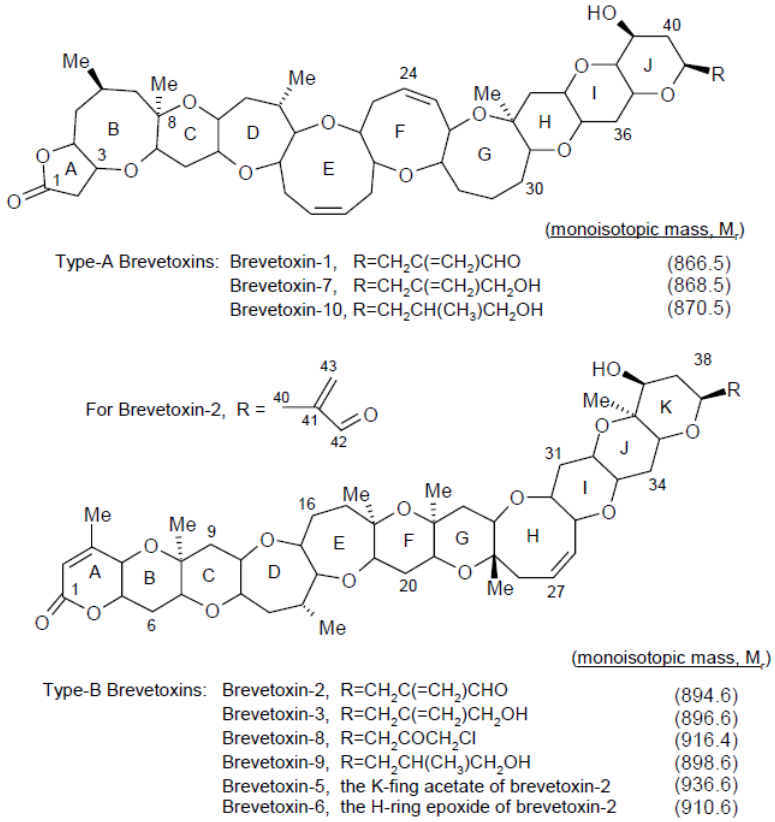


Figure 1. Brevetoxin chemical structures (from Wang, 1997)

Blooms of the causative algae result in both food safety and environmental damage, being responsible for mass mortalities of fish and other marine organisms. BTXs are

neurotoxins which bind to voltage-gated sodium channels. The syndrome is therefore similar to PSP, but less severe. Humans may be affected from BTX exposure through consumption of contaminated shellfish, termed neurotoxic or neurologic shellfish poisoning (NSP), or from exposure to aerosols on or near to marine waters where algal blooms have developed. BTXs are thought to be more toxic to humans following inhalation than following shellfish consumption (Stedinger et al.,2008). In addition to the parent toxins and related products present in the algae, an antagonist, brevenal has also been discovered, a compound which inhibits the toxicity of BTXs through competitive replacement of BTXs at the sodium channel binding site (Bourdelais et al., 2005; Pierce and Henry, 2008). Aerosols are formed as the dinoflagellate cells are broken down, releasing the toxins into the water and subsequently into the atmosphere under appropriate atmospheric conditions. Inhalation can cause irritation in the eyes, nose and throats and may cause respiratory distress (Pierce *et al.*, 1995) particular in asthma sufferers (Fleming *et al.*, 2009). Symptoms appear to be reversible when subjects are removed from the area of exposure, although there is recent evidence for hyper-responsiveness and lung inflammation following repeated exposure to aerosols of PbTx-3 (Zaias *et al.*, 2011).

2.2.2 Shellfish accumulation and depuration

Work conducted to date has shown some evidence for large differences in accumulation and reduction in concentration of toxins and metabolites. In some studies oysters have been shown to exhibit rapid accumulation and reduction, thought to relate to their high filtration rates, where as other species such as clams show longer depuration times (Pierce and Henry, 2008). There is great difference in toxin depuration rates between the different BTXs and related metabolic products, given large differences between the toxin in terms of polarity and hydrophobicity (Otero *et al.,* 2012). In some cases shellfish such as oysters have been found to remain toxic more than 70 days after the dissipation of the source algal bloom (Dickey *et al.* 1999) and there are reports of depuration taking up to nearly one year post bloom (Watkins *et al.*, 2008). In other studies, clearance rates were determined for four different species of bivalves, with results showing significant differences

between scallops, clams and oysters (Leverone *et al.*, 2007) and in four species of invertebrates including a sponge, a tunicate and a clam (Echevarria *et al.*, 2012).

2.2.3 Prevalence of brevetoxins

To date, NSP has been confined to the Gulf of Mexico, the Atlantic coast of the United States of America (U.S.A) and to New Zealand, with no reported occurrence in the UK or Europe (Pierce and Henry, 2008; Paredes *et al.*, 2011). The metabolism of *K. brevis* has been determined in cockles (*Austrovenus stutchburyi*), mussels (*Perna canaliculus*), Pacific oysters (*Crassostrea gigas*), Eastern oysters (*Crassostrea Virginia*) and clams (*Mercenaria sp*.).

To date, NSP has been associated mainly with oysters, clams, whelks, cockles and mussels (Landsberg, 2002, Plakas *et al.*, 2008; Watkins *et al.*, 2008, Abraham *et al.*, 2012). Symptoms of NSP include both gastrointestinal and neurological, typically occurring within a few hours and persisting for no more than a few days (Plakas and Dickey, 2010). A range of symptoms including breathing difficulties and coma have occurred, although no fatal cases of NSP have been reported in Italy (Tubaro *et al.*, 2012). Large documented outbreaks of NSP have occurred in New Zealand during 1992-1993 and periodically along the east coast of the USA (Watkins *et al.,* 2008).

As part of this study a consultation with a wide range of UK and European organisations, including universities, research institutions, monitoring laboratories and other agencies was undertaken with a view to identify whether BTXs was found in the European waters. No reports have been made available describing the occurrence of BTXs in UK or European waters.

2.2.4 Potential for BTXs becoming established in UK waters

Whilst no BTXs have been identified to date in UK waters, with the further global expansion of *K. brevis* blooms and the potentially favourable conditions in UK waters to support the causative organism (Section 1.7.7), these toxins could potential

become established in the UK. Overall, with no reports of clinical cases or toxin occurrence in Europe, but with some trends in expansion of algal producers, the risk of these toxins appearing in European waters is at best moderate (Carmen Louzao *et al.*, 2012). However, given the significant effects resulting from blooms of NSP-producing algae in New Zealand where water temperatures are not too much higher than those in parts of the UK, NSP is a risk that should be assessed.

2.3 PALYTOXINS

2.3.1 Toxicity risks of palytoxins (PlTXs) to human health

Palytoxin (PlTX) (Figure 1) and its analogues are some of the largest, non-polymeric natural compounds currently known with molecular weights in excess of 2500 Da. A non-crystalline white solid, PlTX was originally discovered, isolated and purified from Hawaiian zoanthids (soft corals) belonging to the genus Palythoa (Moore, 1971). Although the origin of PlTX and PlTX-like compounds has not yet been fully elucidated, *Ostreopsis* spp. have been identified as some of potentially several producers (Usami, 1995; Ukena, 2001; Taniyama, 2003; Ciminiello, 2008).

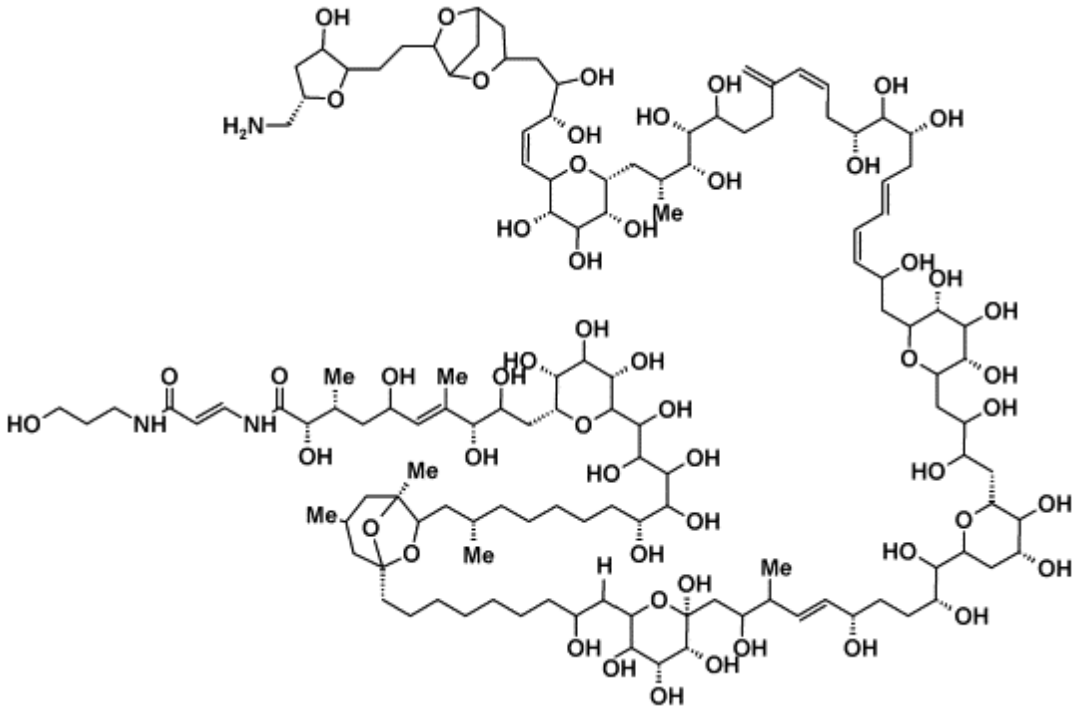


Figure 2. Palytoxin (PlTX)

For some of the human intoxications, the involvement of PlTXs remains unconfirmed and it is sometimes unclear whether incidents can solely be attributed to PlTXs.

In cases where adverse effects on human health have been attributed to PlTX or PlTX-group compounds, the toxin identification and/or quantitation are often incomplete or missing. The toxicological potential of these compounds have yet to be fully evaluated (Tubaro, 2011b).

Traditionally, PlTX and PlTX-like compounds have been linked to incidents of fish or crustacean poisoning, potentially including clupeotoxism, in tropical waters (Noguchi, 1988; Alcala, 1988; Kodama, 1989; Onuma, 1999) sometimes leading to fatalities. Evidence of low levels of a PlTX-like compound in Pacific oysters extracts from New Zealand has been reported (Rhodes, 2002). More recently extensive blooms of *Ostreopsis* sp. along with the presence of PlTX-group compounds have been reported in southern Europe where high levels of PlTX-like activity were recorded in mussels, clams and sea urchins (Aligizaki, 2008; Gorbi, 2012; Amzil, 2012). So far, PlTX poisoning has not been confirmed from the consumption of contaminated shellfish.

PlTXs cause palytoxicosis in humans. In several cases (Alcala, 1988; Onuma, 1999), intoxicated people report stopping eating the contaminated food after detecting a bitter metallic taste. Although the clinical data associated with PlTX poisoning in human is scarce, rhabdomyolysis seems to be the most commonly reported complication (Kodama, 1989; Okano, 1998; Taniyama, 2002). Other symptoms linked with PlTX poisoning are hypersalivation, abdominal cramps, nausea, vomiting and diarrhea sometimes accompanied by mild to acute lethargy.

In addition to the risk of intoxication through consumption of seafood, exposure by inhalation is believed to be an issue. PlTX-group compounds are thought to have been responsible in 2005 and 2006 for skin irritation, rhinorrhea, cough, fever, bronchoconstriction (with or without mild dyspnea) and possible conjunctivitis in population who had spent time on or near a stretch of coastline in Italy (Ciminiello, 2006; Brescianini, 2006; Durando, 2007).

Although not directly related to food safety, a case of human poisoning by PlTXs after contact with zoanthid corals from an aquarium through skin injury has also been reported (Hoffmann, 2008).

The concentration of PlTX-group compounds in shellfish is not regulated in the European Union or in other parts of the world.

2.3.2 Shellfish accumulation and depuration

The uptake of *O. siamensis* by Greenshell^{TM} mussels, Pacific oysters and scallops was studied in New Zealand using *O. siamensis* cells containing PlTX (Rhodes, 2002). Two feeding experiments were carried out for each species; one over 27 hours and the other over 84 hours. Using the haemolysis neutralisation assay (HNA), the mussels were negative for PlTX in both experiments. When fed over the shorter period of time, the oysters and scallops were negative. In the extended feeding experiment, a low concentration (below 750 pg/g) of a PlTX-like material was detected in oysters and the hepatopancreas of scallops contained 1000 pg PlTX equivalent/g. PlTX-like activity was not detected in scallop muscle and roe.

Shellfish samples of mussels (*Mytilus galloprovincialis & Modiolus barbatus*) and clams (*Venus verrucosa*) were collected in 2004, 2005 and 2006 in different locations in Greece and at different times of the year in order to compare the *Ostreopsis* sp. bloom season with periods when *Ostreopsis* sp. are not present in the water. Their toxicity was assessed using the haemolysis neutralization assay (HNA) and the mouse bioassay (MBA) with samples extracted by the two different protocols published (Yasumoto, 1978; Taniyama, 2002) for the latter. The estimated total levels in the shellfish samples determined by HNA ranged from 33.3 – 97.0 µg PlTX eq./kg and the qualitative MBA test was in general agreement (Aligizaki, 2008). These concentrations are higher than the concentrations reported following a feeding experiment involving *O. siamensis*, bivalve molluscs and sea urchins over 3 days in laboratory conditions (Rhodes, 2006b). A proposed explanation for the variation is the different conditions and exposure time since the Greek samples were collected in a natural environment (i*n-situ*) in an area with high abundance of *Ostreopsis* sp. for

3-4 months at a time (Aligizaki, 2008). In the same work, Aligizaki *et al.* (2008) report that toxicity in the shellfish samples would usually be detected 1 or 2 weeks after the start of the *Ostreopsis sp*. bloom and remain until 2-3 weeks after the bloom had receeded.

In summer 2009, bioaccumulation monitoring work was also carried out *in situ* in France in two different locations using mussels and sea urchins. If present, PlTX-group compounds were concentrated in the digestive tract of sea urchins and in the digestive glands of mussels. For both species, the toxicity peaks were in line with the cell concentrations in the water. The PITX-group compounds concentrations in sea urchins (maximum 360 µg PlTX eq./kg) were generally higher than in mussels (maximum 217 µg PlTX eq./kg). Since 2009, trigger levels have been introduced for *Ostreopsis sp*. in sea water (Amzil, 2012).

The values recorded in shellfish in Greece and France exceeded the ARfD of 30 µg PlTX/kg shellfish flesh value proposed in the European Food Safety Authority (EFSA) opinion on PlTX (EFSA, 2009).

2.3.3 Prevalence of palytoxins

Previously confined to tropical and sub-tropical waters, reports of PlTX and PlTX-like compounds have now spread to southern European waters (Aligizaki, 2008; Ciminiello, 2006, 2008 & 2013; Amzil, 2012). To date, PlTXs have been recorded in latitudes approximately between 43°N and 15°S (Aligizaki, 2011).

The extensive survey carried out as part of this review which included a wide range of UK and European organisations, including universities, research establishments, monitoring laboratories and other agencies revealed that PlTXs have not been the object of analysis in the UK and subsequently the presence of PlTX or PlTX-like compounds has not been reported. However, in European waters the presence of *Ostreopsis* sp. known to produce these substances has been reported. More importantly in the context of this study, instances of contaminated shellfish including

bivalves have been reported in Greece, Italy and France (Aligizaki, 2008; Bellocci, 2008; Amzil, 2012).

2.3.4 Potential for palytoxins becoming established in UK waters

Although the origin of PlTX and PlTX-like compounds has not currently been unequivocally determined, a number of hypotheses have been brought forward including biosynthesis by symbiotic microorganisms (Uemura, 1985) and a bacterial origin (Moore, 1982; Carballeira, 1998; Seemann, 2009) supported by the ability of bacterial genera *Aeromonas* and *Vibrio* to produce compounds antigenically related to PlTX (Frolova, 2000). *Ostreopsis* sp. have also been identified as PlTX-group toxins producers (Usami, 1995; Ukena, 2001; Taniyama, 2003; Ciminiello, 2008) but cannot currently be considered as the only source.

The recent widening of *Ostreopsis sp*. distribution to the southern European waters potentially increases the likelihood of these species becoming established in the UK should the environmental conditions (temperature, barometric pressure, hydrodynamics and salinity) be conducive.

In the majority of the European countries and worldwide, PlTXs are not the object of regular monitoring so any record of PlTX-group toxins are patchy and rely on punctual scientific contributions. It is worth noting however, that according to the responses we have had to the survey undertaken, the Italian Regional Agencies for Environmental Protection have performed a regular monitoring programme for the detection of *O. ovata* since 2006. This monitoring is performed along the Italian coasts and shellfish as well as sea urchins are collected sporadically during blooms of the algae for biotoxin analysis. The Campania region of Italy finances a regular monitoring programme on marine organisms and although total PlTXs levels of up to 625 µg/kg were reported, no human intoxication through ingestion have been reported. France has also introduced a specific monitoring programme in the French Mediterranean sea since 2007 (Amzil, 2012) leading to report of PlTX and ovatoxin-a in mussels and sea urchins. The literature includes reports of PlTX-group toxins in

the Adriatic and Mediterranean seas. The survey carried out as part of this study also highlighted that PlTX and ovatoxin-a have been detected in dinoflagellates from Portugal, although these results have not yet been published.

Considering these recent reports, the possibility of PlTX-group toxins becoming established in UK waters and becoming a food safety issue in seafood cannot be completely ruled out.

2.4 CYCLIC IMINES

2.4.1 Introduction

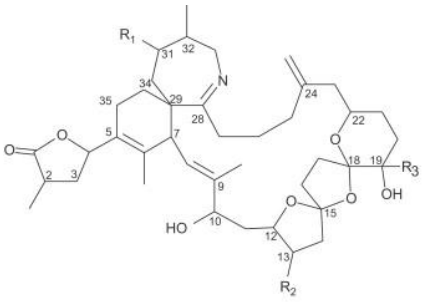
Cyclic imines (CIs) are a group of natural products sharing common macrocyclic features and an active imine moiety (Cembella, 2008). They were discovered in the early 1990s and include: spirolides (SPXs) (Hu, 1995 & 1996a), gymnodimines (GYM) (Seki, 1995), pinnatoxins (PnTXs) (Uemura, 1995), pteriatoxins (PtTXs) (Takada, 2001), prorocentrolides (PcTXs) (Torigoe, 1988; Hu, 1996b) and spiro-prorocentrimine (Lu, 2001). CIs are sometimes referred to as “fast-acting toxins” as they are characterised by acute toxicity in mice following intraperitoneal (i.p.) injection and rapid onset of neurological symptoms potentially leading to death if the compounds are present at elevated concentrations. However, they have not been unequivocally linked to human intoxication.

The concentration of CIs in shellfish is not regulated in the European Union or in other parts of the world.

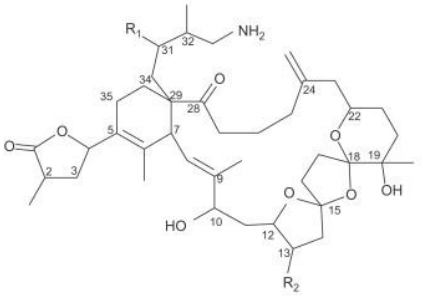
Spirolides

The SPX group is one of the best characterized CIs sub-group and its compounds have been detected in European and North American waters. Six major congeners, namely SPX A, B, C, D, E and F, were initially isolated from lipophilic extracts of the digestive gland of mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*) harvested from aquaculture sites in Nova Scotia, Canada (Hu, 1995 & 1996a)

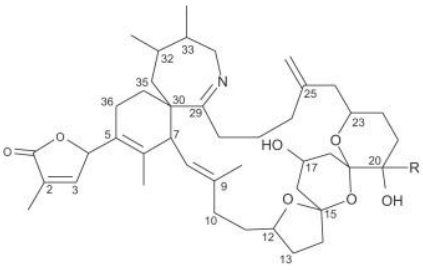
(Figure 3). More recently, SPX H and I have been proposed as additional SPXs (Roach, 2009). SPX derivatives have been isolated and characterized (Hu, 2001; MacKinnon, 2004; Aasen, 2005; Ciminiello, 2007 & 2010). The marine dinoflagellate *Alexandrium ostenfeldii* was identified as the causative organism of spirolide shellfish toxins (Cembella, 2000a). More recently, *Alexandrium peruvianum* has also been identified as a SPXs producer (Touzet, 2008a).



Spirolide	R_1	R_2	R_3	$\Delta^{2,3}$	MW
A	H	CH_3	CH_3	+	691.5
B	H	CH_3	CH_3	-	693.5
C	CH_3	CH_3	CH_3	+	705.5
D	CH_3	CH_3	CH_3	-	707.5
13-desMe-C	CH_3	H	CH_3	+	691.5
13, 19-didesMe-C	CH_3	H	H	+	677.5



Spirolide	R_1	R_2	$\Delta^{2,3}$	MW
E	H	CH_3	+	709.5
F	H	CH_3	-	711.5



Spirolide	R	MW
G	H	691.5
20-Me-G	CH_3	705.5

Figure 3. Structure of some of the known spirolides (Christian, 2008)

Gymnodimines

In New Zealand in 1994, oysters analysed by the MBA for the detection of lipid-soluble marine biotoxins, displayed unusual signs of neurotoxic shellfish poisoning (NSP) which prompted further investigations. As a result of this research, GYM was isolated from dredged oysters (*Tiostrea chilensis* = *Ostrea chilensis*) from the Foveaux Strait, South Island of New Zealand and from a concurrent bloom of what was thought to be *Gymnodinium cf. mikimotoi* (Seki, 1995) (Figure 4). GYM B and

GYM C were also isolated from phytoplankton cultures established from this bloom (Miles, 2000 & 2003). The phytoplanktonic source was later renamed as *Karenia selliformis* (Haywood, 2004).

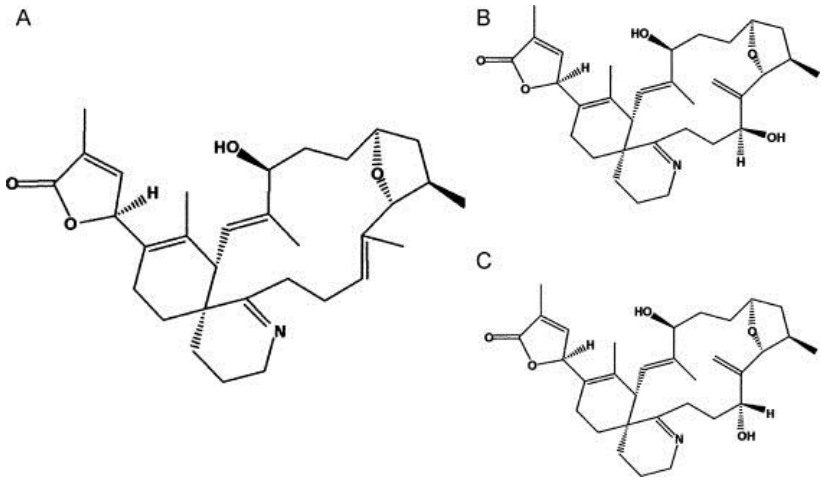


Figure 4. Chemical structure of GYM, GYM B and C (taken from Ben Naila, 2012)

Pinnatoxins

PnTXs are compounds accumulated in shellfish of the genus *Pinna*. PnTX A and B were the first PnTXs isolated from the viscera of *Pinna muricata* from Japan (Uemura, 1995) (Figure 5). Subsequently, PnTX D and C were isolated from the same organism (Chou, 1996; Takada, 2001a). PnTX E, F and G were originally identified from the digestive gland of Pacific oysters (*Crassostrea gigas*) from South Australia (Selwood, 2010). The causative organism of PnTXs in Australia, New Zealand and Japan was recently identified as the dinoflagellate *Vulcanodinium rugosum* (Rhodes, 2011).

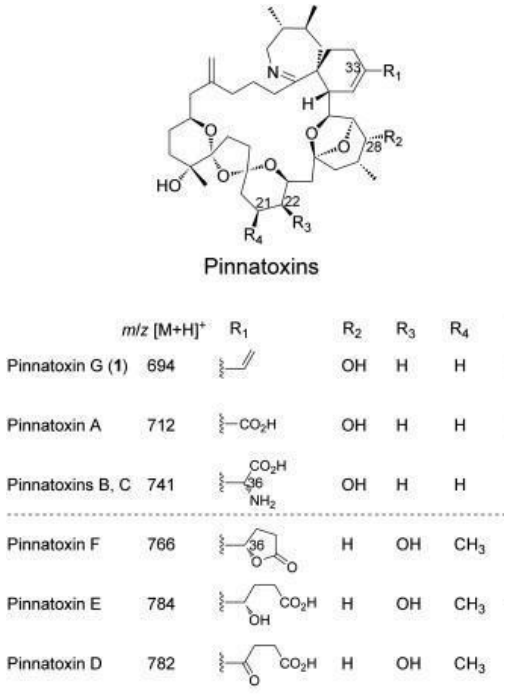


Figure 5. Chemical structure of known pinnatoxins (Rundberget, 2011)

Pteriatoxins

PtTXs were isolated from the Okinawan bivalve *Pteria penguin* (Takada, 2001b) (Figure 6). The origin of PtTXs has not been fully determined but PnTX G may be the precursor of PtTXs A-C via metabolic and hydrolytic transformation in shellfish (Selwood, 2010).

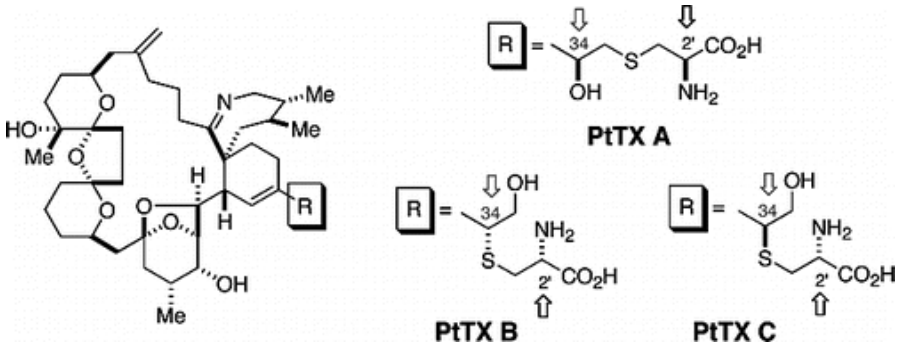
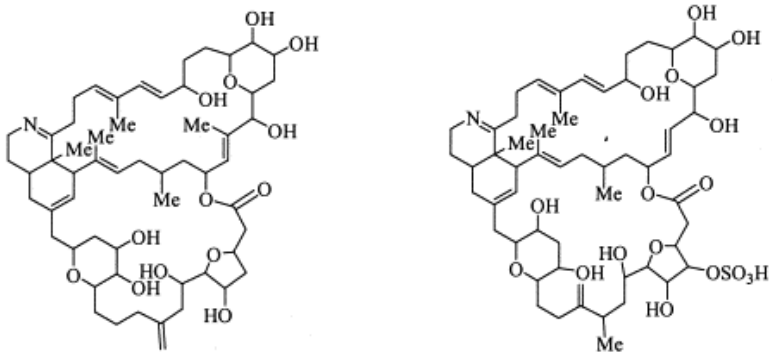


Figure 6. Chemical structure of known pteriatoxins (taken from Hao, 2006)

Prorocentrolides

PcTX A was first isolated from *Prorocentrum lima* (Torigoe, 1988) (Figure 7). Another PcTX, PcTX B, was later isolated from *Prorocentrum maculosum* (Hu, 1996b).



Prorocentrolide A

Prorocentrolide B

Figure 7. Planar structure of the known prorocentrolides

Spiro-prorocentrimine

Spiro-prorocentrimine was isolated from a culture *Prorocentrum sp*. of Taiwan (Lu, 2001).

2.4.2 Toxicity risks of these toxins to human health

Although some seafood poisonings in China and Japan were initially attributed to PnTXs, they were later shown to be caused by *Vibrio* species (Toyofuku, 2006) so no human intoxications have unequivocally been linked to CIs so far and, consequently, the toxicity of CIs to human through ingestion of contaminated seafood is debated.

2.4.3 Shellfish accumulation and depuration

For some of the CIs sub-group, no report related to the shellfish accumulation and depuration has been found. For the other group, the information is detailed in the individual sub sections below.

Spirolides

The uptake of SPXs in paddle crab (*Ovalipes catharus*) has been studied using laboratory feeding trials and Greenshell^{TM} mussels as a vector. The toxin uptake in crab was limited to the visceral tissue and the concentrations were low (Jester, 2009). SPX uptake and detoxification was also investigated in oysters (*Crassostrea gigas*) through laboratory feeding experiments. Four different SPX analogues were detected (13,19-didesmethyl C, 13-desmethyl C, 13desmethyl D and traces of SPX D). After 4 days of exposure, the digestive gland of the oysters contained 83% of the total SPX concentration. SPX seemed to have a toxic effect on the digestive tubules of the oysters but 7 days after exposure, the SPX concentration and the toxic effects had almost completely gone (Medhioub, 2012).

Gymnodimines

LC-MS/MS analysis of clams collected in Tunisia have shown that GYM seems to accumulate preferentially in the digestive gland with a substantial proportion of the total amount also present in the remaining shellfish parts. It is worth noting that the ratio between the amount in the digestive gland and the rest of the shellfish varied from sample to sample (Biré, 2002). In a separate study carried out on Greenshell mussels (*Perna canaliculus*) collected in New Zealand, GYM was found to be concentrated mainly in tissues outside of the digestive gland and, despite the apparent absence of producing organism, the concentration did not decrease over the 5-month study (MacKenzie, 2002).

In a kinetics depuration study carried out over 1 month on clams (*Ruditapes dessicatus*) from the coastline of Tunisia, an exponential discharge of 75% of the total GYM-A content during the first 12 days was observed followed by a slow

depuration for the subsequent days (Marrouchi, 2010). In a different study carried out on the same species of clams from Tunisia, faster detoxification rates were observed in the digestive gland when, following exposure, clams were fed on a non-toxic algae than when they were starved. The detoxification rate was high initially but then decreased and less than 5% remained after 7-8 days (Medhioub, 2010).

Pinnatoxins

LC-MS analysis of Pacific oysters (*Crassostrea gigas*) and razor fish (*Pinna bicolor*) collected from Franklin Harbour in South Australia revealed that both species were contaminated with PnTXs and that the concentrations were higher in the razor fish than in the oysters (Selwood, 2010).

2.4.4 Prevalence of CIs

Spirolides

The presence of SPXs in *Alexandrium sp.* and/or in water sample has been reported in Nova Scotia, Canada (Hu, 1995, 1996a & 2001; Sleno, 2004a & b; Roach, 2009), in Scottish coastal waters (John, 2003), in Denmark (MacKinnon, 2004), in the Gulf of Main, USA (Gribble, 2005), in Norway (Aasen, 2005), in Italy (Ciminiello, 2006) and in Ireland (Touzet, 2008a). The SPX toxin profile produced by *A. ostenfeldii* has been found to vary widely with water depth, location and seasonality (Cembella, 1997; Cembella, 2000b; Cembella, 2001; Ruehl, 2001; MacKinnon, 2002; Gribble, 2005).

SPXs in shellfish seem to be widely distributed. They have been reported in mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*) harvested from aquaculture sites in Nova Scotia, Canada (Hu, 1995, 1996a & 2001), in mussels (*Mytilus edulis*) from Norway (Aasen, 2005), in mussels (*Mytilus galloprovincialis*) and in razor clams (*Enis arcuatus*) from Spain (Villar Gonzáles, 2006), in clams, mussels and oysters from France (Amzil, 2007), in mussels from Italy (Ciminiello, 2010) and in macha (*Mesodesma donacium*) and clams (*Mulinia edulis*) from Chile (Álvarez, 2010).

In addition, fatty acid acyl esters of SPXs have been detected in Norwegian mussels (Aasen, 2006). These compounds have not been detected in phytoplankton so far indicating that the SPXs might be esterified within the shellfish.

SPXs have been detected in water and/or phytoplankton in Denmark (MacKinnon, 2004), Italy (Ciminiello, 2006) and in Ireland (Touzet, 2008a). Following consultation with a wide range of UK and European organisations, including universities, research insitutions, monitoring laboratories and other agencies, reports of shellfish contaminated with CIs can be extended to 13-desmethyl SPX C in Sweden.

Gymnodimines

In a review considering the LC-MS analysis of 217 samples collected in New Zealand between 1993 and 1999 and covering eight different shellfish species, GYM was detected in 155 samples with a maximum concentration as high as 23 400 µg/kg. Samples of Greenshell mussel^{TM} (*Perna canaliculus*), blue mussels (*Mytilus galloprovinvialis*), dredge oysters (*Tiostrea chilensis*), scallops (*Pecten novaezelandiae*), pipi surf clam (*Paphies australis*), paua New Zealand abalone (*Haliotis iris*) were found to be contaminated with GYM demonstrating a wide geographic spread in New Zealand and the potential for a number of shellfish species to be affected (Stirling, 2001).

The first report of GYM outside of New Zealand was in clams (*Ruditapes decussatus*) harvested in Tunisia in December 2000 and analysed using LC-MS/MS. The analysis was carried out on the whole edible meat, on the digestive gland alone and on the remaining edible meat. GYM B and GYM C were not detected in any of the four samples analysed but GYM was detected in all of them (Biré, 2002). GYM was also detected using HPLC-UV in clams (*Ruditapes decussatus*) from the coastline of Tunisia that previously tested positive (i.e fast lethal toxic effects following *i.p.* injection) when assayed by the MBA (Marrouchi, 2010) and by LC-MS/MS (Ben Naila, 2012) who also reported the presence of GYM B and C.

Following LC-MS/MS analysis, GYM has been detected at low concentrations in pipis (*Donax deltoides*), mussels (*Modiolus proclivis*) and in oysters (*Saccostrea*

glomerata) in Australia (Takahashi, 2007) and at low but consistent levels in oyster (*Crassostrea gigas*) samples from the west coast of South Africa (Krock, 2009). The presence of GYM in Canadian waters has also been reported (Munday, 2008b citing Annual report 2003-2004 from the Defense Research and Development Canada). The presence of GYM on the west coast of Canada has also been confirmed in the extensive survey carried out for the purpose of this review.

In addition, fatty acid acyl esters of GYMs have been detected in shellfish samples from Tunisia (De la Iglesia, 2013). These compounds have not been detected in phytoplankton so far indicating that the GYMs might be esterified within the shellfish.

Following consultation with a wide range of UK and European organisations, including universities, research insitutions, monitoring laboratories and other agencies, GYM has not been detected in European waters to date.

Pinnatoxins

PnTXs reports had been limited to the species of the genus *Pinna* in Japan but they have now expanded to Pacific oysters (*Crassostrea gigas*) and razor fish (*Pinna bicolor*) from South Australia (Selwood, 2010), Pacific oysters from Rangaunu harbour in Northland New Zealand (Selwood, 2010; McNabb, 2012c), mussels from Norway (Miles, 2010 Rundberget, 2011), mussels from Canada (McCarron, 2012) and also mussels and clams from France (Hess *et al.* 2013). In addition, data was presented at the symposium on emerging toxins organised by the EURLMB in 2012 reporting the presence of PnTXs in mussels in one production area in France and this was confirmed in the responses to the extensive survey carried out for this review.

In addition, fatty acid acyl esters of PnTXs have been detected in shellfish including Canadian mussels (McCarron, 2012). These compounds have not been detected in phytoplankton so far indicating that the PnTXs might be esterified within the shellfish.

Following consultation with a wide range of UK and European organisations, including universities, research institutions, monitoring laboratories and other agencies further reports of PnTXs in shellfish were received from Ireland and France.

Pteriatoxins, prorocentrolides, spiro-prorocentrimine

PtTXs were first reported in the bivalve *Pteria penguin* (Takada, 2001b) and have not been reported in any other shellfish since. Prorocentrolides and spiro-prorocentrimine were isolated form phytoplankton species and report of their presence in shellfish has not been found.

2.4.5 Potential for cyclic imines becoming established in the UK

Although until recently the majority of the CIs appeared to have been confined to a few locations, the distribution of a number of them now seems to have spread.

To date, LC-MS/MS analysis demonstrated the presence of SPXs (pers. obs.) as well as PnTXs (Cefas unpublished data) in UK shellfish. From the extensive survey carried out encompassing a wide range of UK and European organisations, including universities, research establishments, monitoring laboratories and other agencies, no other CIs compounds have been detected in UK waters.

2.5 TETRODOTOXINS

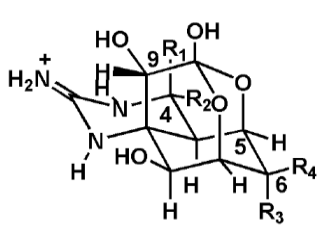
2.5.1 Toxicity risks of tetrodotoxins (TTXs) to human health

Tetrodotoxin (TTX) poisoning occurs in humans following consumption of fish, shellfish, gastropods or other marine animals which are contaminated with TTXs. Many hundreds of intoxications have been reported throughout the affected areas following consumption of fish as well as crabs and scavenging gastropods (e.g. Arakawa *et al.*, 2010).

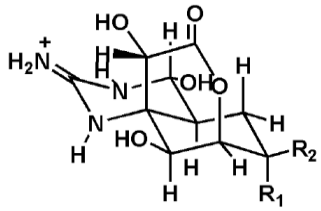
TTX poisoning is the most commonly-occurring lethal marine poisoning in the world (Isbister and Kiernan, 2005), with the toxin being found in the organs of fish from the *Tetraodontidae* family. This includes most notoriously the Puffer fish. This fish is a gastronomic delicacy, known as *fugu* in Japan. Consumption of food products from this fish remains the major cause of fatal food poisoning in Japan. The toxins are recognised by many authors as being exogenous, primarily produced by a range of bacteria (Yasumoto *et al.*, 1988; Wu *et al.*, 2005; Wang *et al.*, 2008; Arakawa *et al.*,

2010) which subsequently accumulate through the food chain and enter the fish as well as in molluscs, gastropods, crustaceans, amphibians and octopus (Arakawa *et al.*, 2010). Therefore these are the only family of marine toxins discussed in this review which do not originate from a marine dinoflagellate or diatom source. However, the link may still be present with some of the primary bacteria being isolated from species of algae (Nagouchi and Arakawa, 2008) and there is still some dispute regarding TTX biosynthesis by bacteria (Chau *et al.*, 2011).

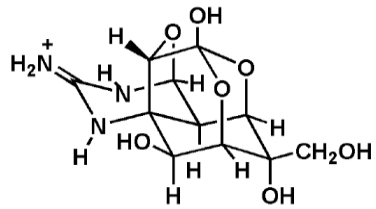
TTX is an extremely potent neurotoxin, with activity similar to that of the saxitoxins (PSP), acting to block sodium conductance at receptor site 1 of sodium channels. The toxins themselves and associated analogues are heat-stable, water-soluble and relatively low molecular weight heterocyclic compounds. Produced by micro-organisms, the first identified as *Shewanella alga* (Yasumoto, 2000) with the list later extended to others (Yu *et al.*, 2004; Wu *et al.*, 2005). A number of analogues have been identified and characterised in fish, gastropod, crab and amphibian species, including four deoxy TTXs (Figure 8).



	R_1	R_2	R_3	R_4	MH^+ (*m/z*)
TTX	H	OH	OH	CH_2OH	320
4-*epi*TTX	OH	H	OH	CH_2OH	320
11-deoxyTTX	H	OH	OH	CH_3	304
6,11-dideoxyTTX	H	OH	H	CH_3	288



	R_1	R_2	MH^+ (*m/z*)
5-deoxyTTX	OH	CH_2OH	304
5,6,11-trideoxyTTX	H	CH_3	272



4,9-anhydroTTX MH^+ *m/z* 302

Figure 8. Chemical structures of Tetrodotoxin and analogs (from Jang *et al.*, 2010)

2.5.2 Prevalence of TTX

The main occurrences of Pufferfish poisoning (PFP) from TTXs are in warm water regions, most notably in the Indian and Pacific Oceans, where the main TTX-containing fish, crabs, gastropods and other marine species predominate (e.g. Chulanetra *et al.*, 2011; Noguchi and Arakawa, 2008; Noguchi *et al.*, 2011; Jang *et al.*, 2010; Leung *et al.*, 2011; Jen *et al.*, 2007, 2008; Sato *et al.*, 1998). Food poisoning incidents in these regions have arisen following ingestion of contaminated fish, gastropods and crabs. In these regions the toxicity of the fish and shellfish are greatly affected by changes to the marine environment, given the exogenous nature of the toxins (Arakawa *et al.*, 2010). It is also noted that in areas where pufferfish is prohibited, TTX poisoning still occurs commonly following consumption of large, medium and small gastropods (Hwang and Noguchi, 2007; Hwang *et al.*, 2007a,b, Noguchi *et al.*, 2011). TTX has been recently identified in a species of carnivorous sea slug present on beaches in New Zealand (McNabb *et al.*, 2010, Oglivie *et al.*, 2012, Wood *et al.*, 2012) subsequently found to be the cause of dog poisonings in the area. TTX was also found in other marine species including *Paphies australis* (McNabb *et al.*, 2013), Pacific oyster (*Crassostrea gigas*) and rock oysters (*Saccostrea commercialis*), thus showing evidence for accumulation of TTXs in bivalve molluscs in waters similar to those encountered in parts of Europe.

In recent years there has been evidence of TTX being present in fish living within European waters, with the occurrence of a migrant puffer fish *Lagocephalus sceleratus* in the waters around Greece (Bentur *et al.*, 2008; Katikou *et al.*, 2009). This migration is known to occur from the Red Sea to the Mediterranean through the Suez Canal and poses a great threat to the region (Nader *et al.*, 2012). Cases of PFP have been widely reported from parts of the Northern coast of Eqypt, the Aegean Sea as well as the Mediterranean between 2005 and 2010, with a number of deaths attributed to TTX poisoning (El Masry and Fawzi, 2011). Other recent

evidence of further migration towards the central Mediterranean in Tunisia has also been reported (Jribi and Nejmeddine Bradai, 2012) suggesting successful adaptation of the species and a trend towards habitat expansion. The toxicity of Pufferfish collected in the Aegean Sea was confirmed as being in some instances higher than the levels required causing human fatalities. Some authors noted that whilst models relating to climate change temperature increases are simplistic at best, there is the potential for increasing temperatures to alter the prevalence and growth rates of TTX-producing organisms such as *Vibrio*, consequently establishing TTX in the more temperate waters of the Atlantic (Silva *et al.*, 2012).

TTX in Europe is not restricted to fish species, with reports of TTX identification in a Trumpet Shell (a marine gastropod) in Portugal (Rodriguez *et al.*, 2008). The case described related to the severe poisoning of a single person following consumption of a *Charonia sauliae* purchased from a fish market in Malaga (Fernandez-Ortega *et al.*, 2010). This highlights the potential risks from gastropod consumption, given that these species are not routinely monitored for TTXs or any other marine toxins. More recently, an extensive sampling study of a range of marine species including gastropods, bivalve molluscs and echinoderms was conducted along the Portuguese Atlantic coast between 2009 and 2010. Results indicated the presence of low concentrations of TTX analogues in a number of gastropod species, which could provide a risk to humans as a result of subsequent biomagnification in the food chain (Silva *et al.*, 2012). There are also reports of TTXs occurring in cultures of marine algae, including *Alexandrium tamarense* a well known PSP-producer which is known to be present in UK waters (Kodoma *et al.*, 1996), although the source of the toxin in these cells may be endocellular bacteria within the algae.

Following consultation with a wide range of UK and European organisations, including universities, research establishments, monitoring laboratories and other agencies, no additional reports of TTX in Eurpean waters have been received in addition to those studies already published in peer-reviewed journals.

Given the high potency of the toxins, high mortality rates and ease of accidental intake of toxic parts, careful monitoring is of high importance. Furthermore the toxins

appear of increasing interest to European monitoring agencies (Hess, 2012a), particularly following suggestions of diversification and habitat expansion (Noguchi *et al.*, 2011).

2.5.3 Potential for tetrodotoxins becoming established in UK waters

To date there are no reports of TTX found in the UK. Given the most likely continued absence of pufferfish or related TTX-containing fish in UK waters, the risk of exposure to the toxins from local catches appears low. Consequently, the main threat from fish intoxication would be the importation of contaminated fish from abroad. Sale of these species of fish is not permitted by EU legislation, so these are not available for purchase at markets or in restaurants. However, there have been reports of private dinner clubs offering *fugu* on the menu, so the risk may not be completely eradicated (e.g. Peck, 2011). In addition there are the dangers from consumption of mislabelled fish products, which have caused TTX poisoning previously (Cohen *et al.*, 2009).

The risk from other marine species is seemingly dependent on the presence of primary producers in UK marine waters. The list of TTX-producing bacterial species isolated from animals is wide although not all the biological sources of TTX are yet accounted for (Chau *et al.*, 2011). The species of TTX-producing bacteria shown to be present in marine organisms to date include *Vibrio, Pseudomonas, Bacillus, Alteromonas, Aeromonas, Pseudoalteromonas, Seratia marcescens* and *Shewanella putrefaciens* (Chau *et al.*, 2011) with many more species identified in marine and deep sea sediments. Several of these bacterial groups are already present in UK waters, especially during the summer months. There is certainly the potential for these bacteria to grow, and especially in the case of *vibrios*, *pseudomonads*, and *aeromonads* the warming projected in the most up to date climate models suggests much of the UK coastlines will warm at least 2-3 °C by the end of the century, making conditions more favourable for these bacteria (summary at: http://ukclimateprojections.defra.gov.uk/22641). One of the most common bacteria that are believed to produce TTX is *Vibrio alginolyticus,* a bacterium which is found

regularly during the summer, especially in bivalve shellfish. However, the debate as to whether these bacteria are the actual source of TTX remains to be resolved.

Following consultation with a wide range of UK and European organisations, including universities, research establishments, monitoring laboratories and other agencies, no reports have been made available describing the occurrence of tetrodotoxins in the UK.

Overall, whilst there is no evidence for TTX being present currently in UK marine life, the risk of primary producers of TTXs occurring in UK waters either now or in the future cannot be completely discounted.

2.6 OTHER TOXINS PRESENT IN UK WATERS

2.6.1 Potential threats for UK waters associated with other toxins

Cyanotoxins

A large number of toxins have been identified which are present in a diverse range of cyanobacteria, also known as blue-green algae. Numerous blooms occur which contain a range of powerful hepatotoxic, neurotoxic or cytotoxic toxins and mass occurrences of toxic cyanobacteria in freshwaters are well documented globally. Some are also known to harm or even kill animals, including fish, following ingestion of contaminated water. Cyanotoxins producing illnesses in humans include the most commonly encountered microcystins, cylindrospermopsin, anatoxin, β-methylaminoalanine (BMAA) and the PSP-producing saxitoxins. According to the Scottish Environmental Protection Agency (SEPA) episodes of cyanobacterial-contaminated drinking water supplies occur periodically with notable recent examples in Scotland requiring water-treatment and/or water-use bans. Large blooms are recorded annually throughout Scotland, England and Wales, with the greatest risks associated with recreational use (e.g. swimmers and boaters). However, there is also the potential for toxins to become bioaccumulated in bivalve

filter feeders. Whilst the risks of intoxication through this exposure route will be lower than those associated with direct exposure to the source bacteria, there is developing evidence around the world for cyanotoxins accumulating and potentially causing sickness following consumption of shellfish or other food products. This has prompted researchers to investigate the dynamics of cyanotoxin accumulation in bivalve molluscs (e.g. Amorim and Vasconcelos, 1999) resulting in strong evidence for bioaccumulation in various organisms including zooplankton, bivalves, gastropods, crustaceans, fish and waterfowl (Chen *et al.*, 2009; Martins and Vasconcelos, 2009). Reviews are published describing cyanotoxins determined in human foods including a wide range of fish species, mussels, crayfish and shrimps (Ibelings and Chorus, 2007). This risk has recently been highlighted by a cyanobacteria working group from the Scottish Government who in their 2012 report on the assessment of risks to public health from cyanobacteria stated: “Another potential source of intoxication for both animals and humans is via bioaccumulation of cyanotoxins in the food chain. The principal concern here would be the accumulation of the toxins in shellfish including freshwater and brackish-water mussels and in fish” (Scottish Government, 2012). Occurrence of freshwater toxins and potential exposure to filter-feeding shellfish has also been recently highlighted by the Food Standards Agency in Wales who in August 2012 expressed concern at the presence of cyanobacteria (*Oscillatoria tenuis*) in a lake system which drains directly into major shellfish production areas. Similarly during 2012 in France, blooms of *Planktothrix* were found in close proximity to oyster production areas, also containing high populations of fish potentially destined for human consumption. Whilst evidence is low in this country, internationally there have been many examples of cyanobacterial toxins of freshwater origin reaching the estuarine environment.

In the US, evidence for the transfer of the cyanotoxins from freshwater habitats to produce secondary impacts in the marine environment has been confirmed with deaths of marine mammals from microcystins intoxication (Miller *et al.*, 2010). Related research confirmed that both wild and farmed bivalves (clams, mussels and oysters) were found to exhibit large biomagnifications (>100 times water concentrations) and slow depuration rates. There is therefore some evidence for the

potential risk of microcystin poisoning when consuming shellfish harvested in estuarine environments.

In some countries such as France and the US, the hazards and associated risks have prompted toxin monitoring laboratories to develop analytical methodologies for monitoring microcystins and other cyanotoxins. Methods have been developed employing the highly specific LC-MS/MS detection as well as other functional assays for the detection and quantitation of specific toxins in a range of fish and shellfish species. The issues are therefore well noted throughout regions of Europe as well as other parts of the world, notably Australia. Awareness of the emerging risk from cyanobacteria is growing, with the upcoming conference on molluscan shellfish safety containing two separate sessions on this issue (ICMSS, 2013).

Toxicity Risks

With a substantial freshwater toxin input required for the creation of a health risk in marine or estuarine shellfish, the overall risk is thought to be slight, but potentially significant. Locations of particular concern as deemed by local knowledge relating to the prevalence of cyanobacterial blooms should be ideally assessed in terms of their location in relation to shellfish harvesting beds. In areas of highest risk, proof of concept studies could be advisable for the determination of cyanotoxins in shellfish. In parallel with this would be the requirement to develop suitable methods of analysis which are applicable to the matrices of interest. With the current global move to the use of both screening (e.g. ELISA) and chemical (LC and LC-MS/MS) methods, both these approaches could be investigated to aid any such preliminary investigations.

Venerupin

Toxic strains of *Prorocentrum minimum* have been implicated in rare reports of venerupin shellfish poisoning (VSP). In Japan, the syndrome caused over a hundred deaths in the 1940s following the consumption of oysters and clams. Symptoms were distinguishable from other known shellfish poisonings, including hemorrhagic diathesis, centrilobular necrosis, and fatty degeneration of the liver, frenzy, unconsciousness, and coma (Akiba and Hattori, 1949). Deaths occurred in about 33% of cases. In Norway, poisoning following consumption of mussels was attributed to the same species, with similar yet less dramatic symptoms (Tangen, 1980, 1983).

Several strains of the organism have been isolated in the UK including sites in the English Channel, although not all of these have been found to exhibit toxicity. Methanolic extracts of some of the cultures from the Mediterranean were found to kill mice rapidly (Grzebyk *et al.*, 1997) whereas the cultures from the English Channel did not. However, the water-soluble venerupin itself has not been chemically characterized (Landsberg, 2002) and there was no conclusive proof that the poisoning was associated exclusively with *Prorocentrum,* although HPLC analysis revealed VSP-toxic cultures did not contain DSP or PSP toxins (Grzebyk *et al.*, 1997).

There are also some arguments that VSP may be associated with microcystins rather than *P. minimum* (Williams *et al.*, 1997).

Toxicity Risks

The risk of *P. minimum* is not confirmed, although the organism is present in UK waters. Currently therefore no information is available relating to the potential impact of this species on the toxicity of shellfish in the UK. The impact of this species is, however, well recognized in relation to animal health and environment stability (Landsberg, 2002; Tango *et al.*, 2005). Whilst some authors do not consider these blooms to be of risk to the human consumer, there is no data describing how this toxin is passed through the food chain. The potential risk to the shellfish consumer therefore needs to be considered (Grzebyk *et al.* 1997). However, with the lack of toxin characterization (the only tested approach to date would be the application of an animal bioassay to assess the toxicity of cultures or shellfish), further research relating to the occurrence of VSP would be required to enable any further developments relating to the detection of this rare syndrome.

2.7 ANALOGUES OF PSP, ASP AND LIPOPHILIC TOXINS

Whilst various factors may influence cell toxicity, potential increases or decreases in average toxicity levels in future years are not perceived as providing a threat to maintaining an effective flesh-monitoring programme. With the use of quantitative analytical instrumentation methodologies for each of the three toxin classes, samples

of greater toxicity will not cause issues with the quantitative approaches employed. In instances of extremely high toxicity, dilutions of extracts prior to analysis may be required to ensure the toxin concentrations are within the range of linearity of the method. Other than the potential need for repeat analysis in these circumstances, no other risks are envisaged. Consequently the next sections address the potential for new toxins analogues from the current regulated groups of toxins to be detected or become established in the future.

2.7.1 Emerging PSP toxin analogues

Toxicity risk of PSP toxin analogues to human health

The toxicity of the PSP analogues (Figure 9) has been debated for some time, with those for the major hydrophilic analogues used internationally for toxicity calculations described by Oshima, 1995 and more recently updated by EFSA opinion. However, the toxicities of the newer analogues described below including the well characterised shellfish metabolites are currently unknown. Modifications to the R4 substituent on the saxitoxin (STX) molecule has very significant effects on the potency of the toxic effect, so assumptions regarding the toxic equivalence of the newer analogues are difficult to make accurately. Llewellyn *et al.*, 2004 have reported that the toxicity of the hydroxybenzoate analogues designated GC1-GC3 may be ten times lower than STX, resulting in some authors proposing the toxicity of these newer analogues should not be particularly high (Vale, 2010). The potency of the deoxodecarbamoyl PSPs has not yet been fully assessed (Etheridge, 2010) but are thought to have low toxicity or no toxicity at all (Riobbo *et al.*, 2011). 13 nor-decarbamoylsaxitoxin (13-nor-dcSTX) found in samples of Argentinean off-shore scallops was proposed to have a high toxicity, given a good correlation between an LC-FLD method and the MBA, when the toxic equivalence of the compound was assumed to be the same as STX (Gibbs *et al.*, 2009). This therefore still highlights the need for reference materials and further toxicity studies to enable the successful detection of these PSPs and determine their effect on public health.

Potential for new PSP toxin analogues becoming established in UK waters

As discussed previously, one of the potential impacts of increased water temperature, ballast water transportation and natural expansion patters of algal blooms may be the impact of new organisms producing PSP toxins in UK waters. For PSP-toxin producers the one species which would result in significantly different toxin profiles would be *Gymnodinium catenatum*. This organism, encountered frequently in Spain and Portugal, contains a number of PSP toxin analogues not encountered in UK samples to date, including decarbamoylneosaxitoxin (dcNEO), decarbamoylgonyautoxins 2 and 3 (dcGTX2&3), decarbamoylsaxitoxin (dcSTX) and gonyautoxin 6 (GTX6) (Escobedo-Lozano, *et al.*, 2012).

G. catenatum is also known to produce a range of other PSP toxin analogues, the deoxydecarbamoyl toxins (doSTX, doneoSTX and doGTX1; Mons *et al.*, 1998). Other structural groups of PSP toxin analogues have been discovered in toxic fractions of *G. catenatum* including the hydroxybenzoate analogues (GC1-GC3; Negri *et al.* 2003) plus related N1-hydroxy GC toxins (GC4-GC6) and di-hydroxybenzoate variants (GC1a-GC6a) (Vale, 2008a, 2011). These groups of toxins have been found to contribute a large proportion of the total toxicity in Australian strains of the algae (12-63 mol%) as well as in strains from other European countries (Negri *et al.*, 2007), but are only known to date to be produced by *G. catenatum*. In addition to these, other metabolites formed in Canadian mussels with *Alexandrium tamarense* source algae were identified (M1 – M5; Dell'Aversano *et al.*, 2005, 2008) and later identified in shellfish contaminated also with *G. catenatum* (Vale, 2010b). Another unusual analogue, 13-nor-dcSTX has been identified in samples of offshore scallops (Gibbs *et al.*, 2009) and its structure elucidated. Whilst its toxicity is currently unknown, taking the assumption that toxicity was equivalent to STX resulted in samples being quantified with total saxitoxin equivalences similar to the toxicities determined by MBA.

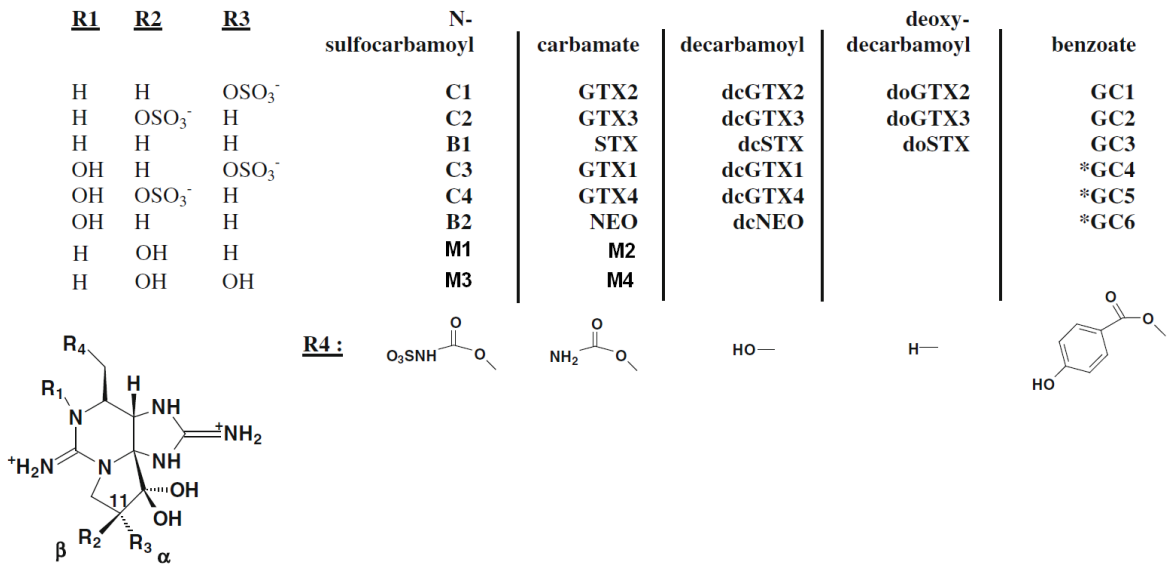


Figure 9. PSP analogue structure (Modified from Vale, 2010)

2.7.2 Emerging ASP toxin analogues

Toxicity risk of ASP toxin analogues to human health

The toxicity of domoic acid has been known for some time, most notably following the widely-reported intoxications in Canada following consumption of contaminated mussels (Quilliam and Wright 1989). The ASP toxins have been identified as potent neurotoxic amino acids which act as agonists to glutamate, a neurotransmitter in the central nervous system. The isomers of domoic acid are also neurotoxins but are known to be less potent than the parent toxin (Wright *et al.*, 1990; Maeda *et al.*, 1986). With lower toxicity and lower prevalence in shellfish in relation to domoic acid, there appears to be little additional risk from any future changes to ASP toxin "profiles". This situation is re-enforced given that the current monitoring methodologies are capable of detecting the majority of the isomers of domoic acid without any significant change to method parameters.

Potential for new ASP toxin analogues becoming established in UK waters

The principal toxin, domoic acid, is found to gradually isomerise to other structures, most notably epi-domoic acid. These isomers are low in relative proportion to domoic acid, but have been found in a number of laboratory cultures and toxic mussel samples (Quilliam *et al.*, 1991). Analysis conducted to date has shown low levels of

some of these compounds in contaminated tissues, although many of these are likely to elute closely, if not remain unresolved, from the major domoic/epi-domoic acid chromatographic peaks. The certified reference standard for domoic acid supplied commercially by the National Research Council (NRC) of Canada and utilised in the UK for calibrating instrumentation for ASP analysis is known to contain epi-domoic acid in addition to isodomoic acid A, D and E (Figure 10) (NRC, DA-e certificate). Matrix reference materials prepared for ASP toxins also contain these additional isomers (e.g. certified reference material CRM-ASP-MUS-C; NRC Certificate of analysis). Consequently, with suitable chromatographic resolution, the presence of these toxins in shellfish tissues could be identified, given their similar relative molar response factors with LC-UV methods. However, use of simpler isocratic methods as currently employed can result in a lack of resolution of these isomers, enabling the reporting of total domoic acid and associated isomers. This is a useful practical approach to take given the known isomerisation which occurs both naturally and under certain treatment conditions. Consequently, there are no perceived risks relating to any potential increases in new isomers of domoic acid, as a result of potential future changes to the abundance and toxicity of the source organisms.

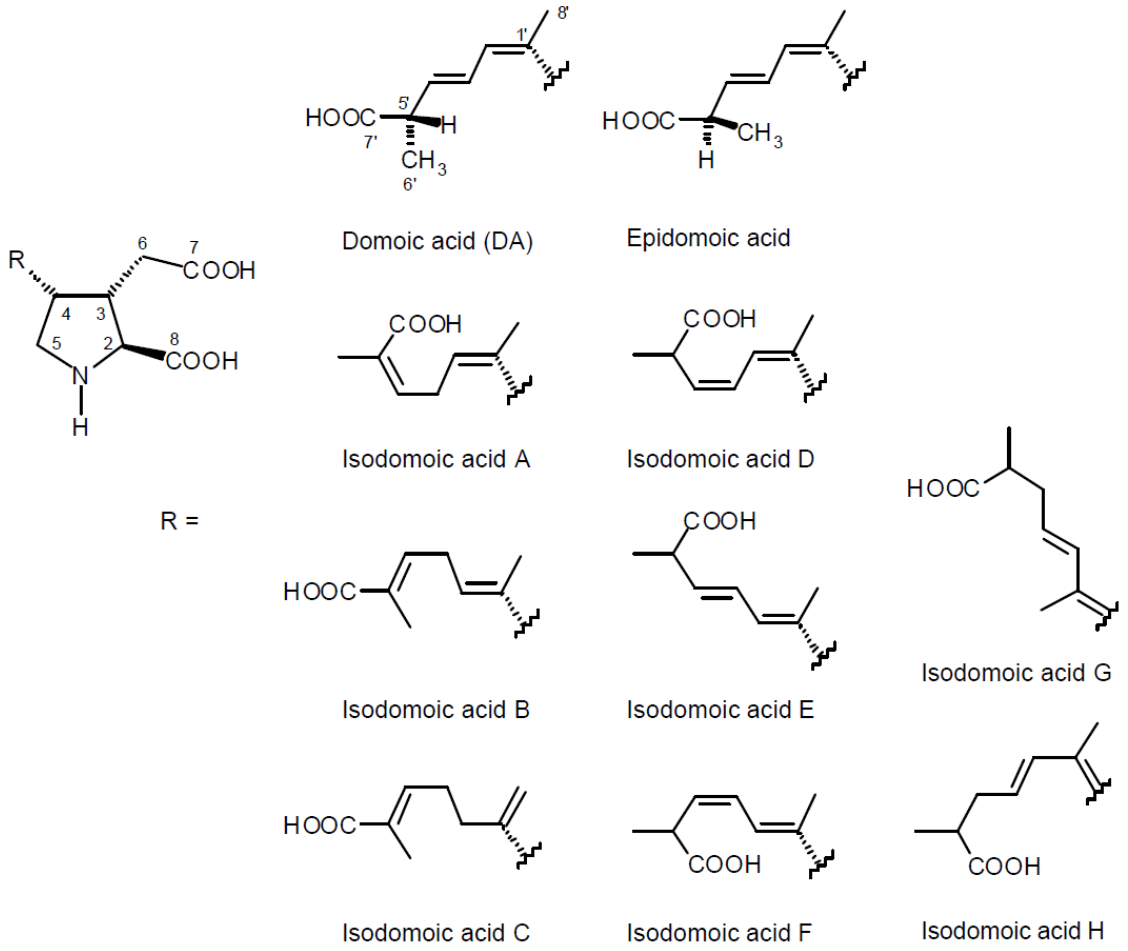


Figure 10. Structure of domoic acid and associated isomers (taken from NRC certificate for domoic acid)

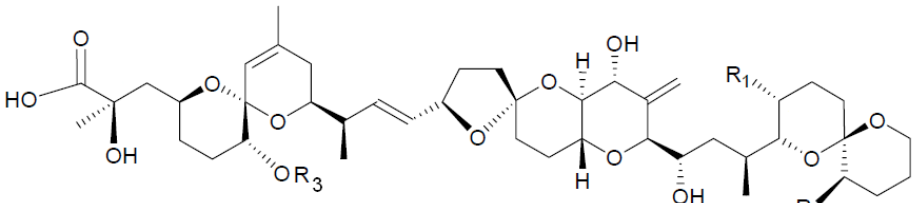
2.7.3 Emerging lipophilic toxins analogues

In recent years, various papers have been published highlighting the detection, quantitation and/or structural elucidation of a large number of analogues related to the parent regulated lipophilic toxins in both algal and shellfish samples. These include members of OA-group toxins, AZAs, PTXs and YTXs.

Emerging analogues of OA-group

Various phytoplankton species are known which can cause the accumulation of DSP toxins in shellfish. Changes to the toxin profiles may occur through the future presence of new strains with different profiles or levels of toxicity. A number of diol-ester derivatives have been reported including those designated DTX4 and DTX5

(Hu *et al.*, 1992; Villar-Gonzalez *et al.*, 2008). Other than the large range of fatty acid substituents in DTX3, no other OA group toxins are recognised, with all of the OA-group esters being identifiable when following the pre-analysis hydrolysis step (11).



R_1	R_2	R_3	
CH_3	H	H	Okadaic acid (OA)
CH_3	CH_3	H	Dinophysistoxin-1 (DTX1)
H	CH_3	H	Dinophysistoxin-2 (DTX2)
H	CH_3	acyl	Dinophysistoxin-3 (DTX3)

Figure 11. Structures of main OA-group toxins

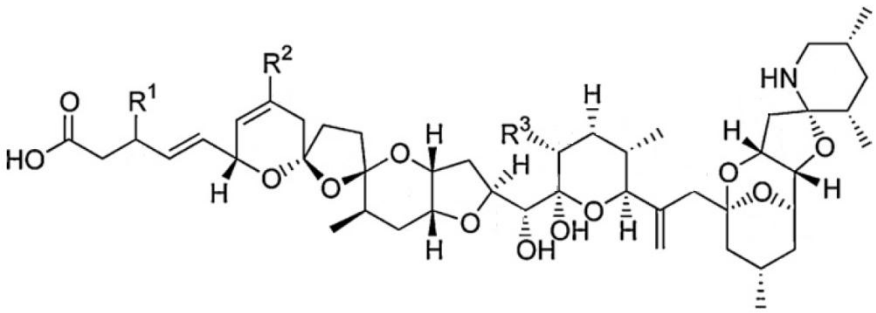
Toxicity risk of OA-group analogues to human health

In recent years an extensive number of papers have been published highlighting the detection, quantitation and/or structural elucidation of a large number of lipophilic toxin analogues in both algal and shellfish samples. The toxicological effects of OA-group toxins are well recognised, providing a significant risk to human consumers if contaminated shellfish is eaten. Even with a growing number of identified OA-esters which appear to have low toxic activity, such compounds are known to hydrolyse during digestion and convert to the toxic parent forms causing intoxication even when esters are present almost exclusively (e.g. Torgersen *et al.*, 2005).

Emerging AZA metabolites

Azaspiracid poisoning (AZP) is a shellfish intoxication syndrome first recorded more recently than the other lipophilic toxin poisonings, following intoxication in consumers of mussels from Ireland (McMahon and Silke, 1996). *Azadinium spinosum* is one of the known producers of the toxins currently present in UK waters (Tillmann *et al.*, 2009). New toxic species of AZA-producing phytoplankton are being identified, but little is currently known about the likelihood of changes to AZA profiles in shellfish in future years. To date AZA1-3 (Figure 12) are the most commonly encountered analogues in shellfish tissue samples (e.g. Ciminiello *et al.*, 2010), although many

more have been identified (e.g. Rehmann *et al.*, 2008; Lehane *et al.*, 2004). The toxins are nitrogen-containing polyether compounds (Figure 12) which are stable at the elevated temperatures associated with cooking. They have been found in mussels, oysters, cockles and clams throughout the UK, Ireland, many other parts of Europe (e.g. Hess *et al.*, 2003; Alfonso *et al.*, 2008; Amzil *et al.*, 2008) and the rest of the world (e.g. Lopez-Rivera *et al.*, 2009). Alfonso *et al.*, 2008 reported the purification of AZA1-3 together with AZA4 and AZA5 (hydroxyl analogues of AZA3; Ofuji *et al.*, 2001) from contaminated mussels collected in Ireland. The occurrence of other AZAs has been reported (AZA6-11) in shellfish extracts (James *et al.*, 2003) rather than cell extracts, suggesting these are metabolic products rather than precursor algal toxins. Other studies have demonstrated significant transformation reactions occurring within heated shellfish tissue, most notably the rapid decarboxylation of AZA17 to AZA3 (McCarron *et al.*, 2009). Proportions of AZA7-11 were found to be very low (<5% of total AZAs) in comparison to the other analogues (James *et al.*, 2003). A total of 32 AZAs have been listed, although this includes 4 which were storage artefacts (AZA29-32) and seven others which have yet to be experimentally determined (AZA18, 20, 22, 24, 26-28; EFSA, 2008b). More recently, other analogues are now being reported such as 3-hydroxy-8-methyl-AZA-1 in newly identified strains of *Azadinium* (e.g. Krock *et al.*, 2012; Gu *et al.*, 2013), providing increasing evidence for the diversity of AZAs even amongst precursor algal strains. Other recent work feeding mass cultured *Azadinium spinosum* to mussels experimentally confirmed the biotransformation of precursor AZA1 and AZA2 to AZA3, 6, 17 and 19 (Salas *et al.*, 2011) as well as AZA-12, 21 and 23 (Jauffrais *et al.*, 2012) . AZA17 and AZA19 were in particular identified as the major metabolites with AZA17 present at levels similar to AZA1 (Jauffrais *et al.*, 2012) or even higher (Salas *et al.*, 2011).



R_1	R_2	R_3	
H	H	CH_3	Azaspiracid-1 (AZA1)
H	CH_3	CH_3	Azaspiracid-2 (AZA2)
H	H	H	Azaspiracid-3 (AZA3)

Figure 12. Structures of regulated AZAs (AZA1-3)

Toxicity risk of AZA metabolites to human health

Whilst mice exposed to AZA exhibit symptoms notably different to those exposed to OA-group toxins (Furey *et al.*, 2010), AZP symptoms in humans, encountered periodically throughout Europe to date, are similar to those encountered following DSP, including nausea, stomach cramps, diarrhoea and vomiting. AZA1-3 have for a number of years been recognised as both the most prevalent in shellfish and the most important in terms of toxicity. EFSA, 2008b reported that the toxicological database is scarce, with only a limited amount of studies are published describing lethality in mice following i.p injection, and with just one intoxication incident being used for determination of an ARfD. This data resulted in the EFSA recommendation for shellfish not to contain more than 30 µg AZA1 eq/kg of shellfish meat, over five times lower than the current regulatory limit of 160 µg AZA1 eq./kg. Repeat-dose toxicity effects including lung tumours were observed only at levels causing severe toxicity so were not considered further (EFSA, 2008b).

Recent studies examining the possible increased toxicity risk following consumption of shellfish containing AZA in combination with OA did not reveal any additive or synergistic effects which would give cause for alarm regarding current regulatory approaches (Aune *et al.*, 2012). Furey *et al.*, 2010 reported that with the AZAs causing serious injury and prevalent in shellfish worldwide, AZA is one of the most serious classes of shellfish toxins. Regulatory control of AZA-exposure is therefore especially important.

Emerging analogues of PTXs and YTXs

The PTXs and YTXs are two other groups of LTs which have yet to have their health threats proven (Ogino *et al.*, 1997). PTXs are a group of macrolactones with at least 14 different analogues (Figure 13). PTX2 is the precursor toxin present in the dinoflagellate, which gives rise to other PTXs through shellfish metabolism (Draisci *et al.*, 2000). Whilst only low levels of PTX2 have been identified in UK shellfish in the last few years, commonly detected PTX-group toxins in other regions of Europe include 7-epi PTX2 seco acid and PTX2 seco acid (Vale *et al.*, 2009) as well as PTX1,4,11 (Ciminiello *et al.*, 2010b). The presence of a range of three series of fatty acid esters of PTXs have also been described (Wilkins *et al.*, 2006). As these toxins are produced by *Dinophysis* species and with these analogues noted in European waters, there is certainly the potential, although no certainty, that these may have appeared in UK shellfish.

Yessotoxin (YTX) has an even more extensive family of analogues (Figure 14). Currently more than 90 have been revealed, but substantially fewer fully identified to date. The most important YTXs identified in shellfish are those regulated in EU legislation, specifically YTX, homo YTX, 45 OH YTX and 45 OH homo YTX (EFSA, 2008). Other structures have been described and identified (Hess and Aasen, 2007). Occurrence of these toxins is known to be widespread globally, with reported contamination throughout Europe and the rest of the world. It is highly likely that some of these analogues are already present in UK shellfish and that profiles may change as patterns of phytoplankton growth and/or shellfish feeding habits change.

Toxicity risk of PTX and YTX analogues to human health

The large and ever-growing number of analogues of PTX and YTX toxins is of interest, but there is limited data available on the chronic effects of YTX in animals and no record of any toxic effects following consumption of YTX in humans (EFSA, 2008a). Mechanisms of toxicity are not determined and data has indicated no clinical signs in mice following oral administration. With a lack of data available for the most important YTXs, there is clearly very little evidence for any toxicity risks from the other YTX analogues which may potentially exist in shellfish. The lack of

documented cases of intoxication with YTX is noted as being consistent with very low acute oral toxicity in animals (Tubaro *et al.*, 2003, 2004, 2008; Munday *et al.*, 2008b). Furthermore there is no evidence for an enhancement in toxicity given the presence of YTX with other toxins, specifically AZA1, especially important given the recorded instances of co-occurrence (Aasen *et al.*, 2011). It is noted that there are moves for these toxins to be deregulated or maximum permitted levels significantly increased, highlighting the general opinion that these toxins should not be regulated.

PTX-group toxicity data is also limited, mostly consisting of acute toxicity studies in mice. Although toxicity in mice following i.p injection is high, there is no evidence for adverse effects in humans following consumption of PTXs (EFSA, 2009). Whilst toxicity equivalence has not been established for PTX analogues as a consequence of this, EFSA did propose provisional TEFs for PTX1, 2, 3, 4, 6 and 11 of 1, until more data was generated. However, PTX7, 8, 9 and 7-epi-PTX2 seco acid were deemed much less toxic and not assigned TEFs (EFSA, 2009). PTX-2 seco acid has been found in small concentrations and is also known to be of low toxicity (Miles *et al.*, 2004). EFSA concluded that shellfish should not contain more than 120 µg PTX2 eq/kg flesh to avoid exceeding the acute reference dose.

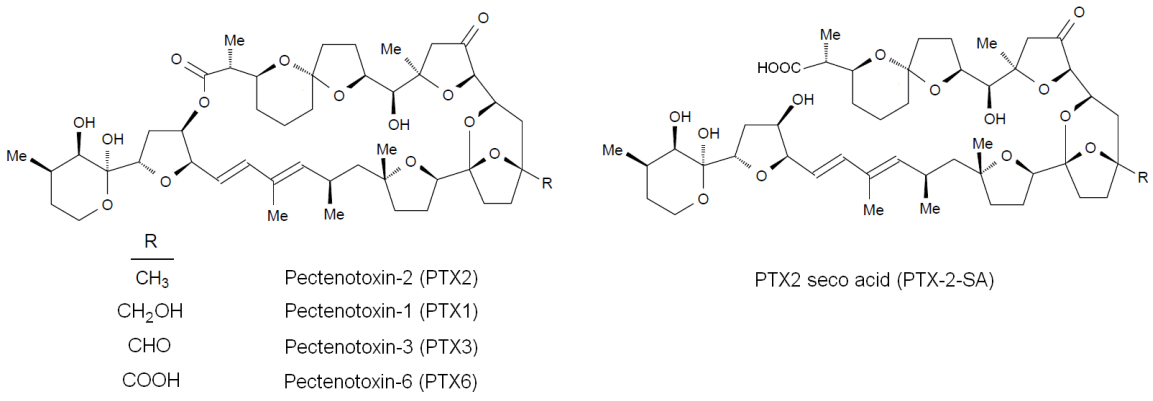
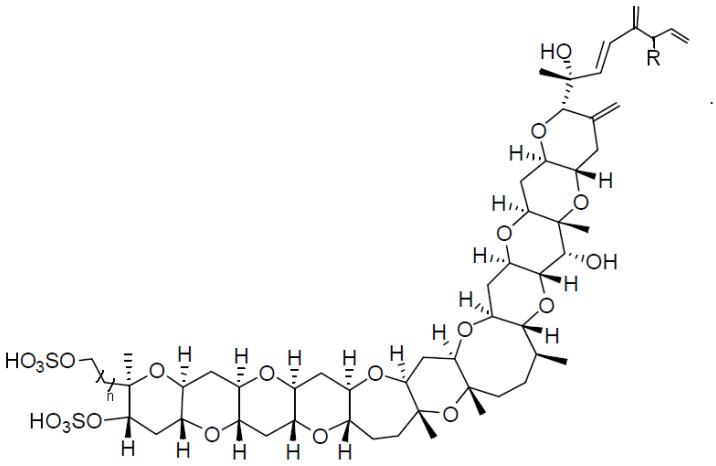


Figure 13. Structures of PTXs



R	n	
H	1	Yessotoxin (YTX)
OH	1	45-Hydroxy-YTX (45-OH-YTX)
H	2	1a-homo-yessotoxin (homo-YTX)
OH	2	45-Hydroxy-homo-YTX (45-OH-homo-YTX)

Figure 14. Structures of YTXs currently regulated under EU legislation

2.8 CIGUATOXINS

2.8.1 Toxicity risks of ciguatoxins (CTXs) to human health

Toxicity

Ciguatera fish poisoning (CFP) occurs in humans following consumption of finfish contaminated with CTXs. The CTXs and gambiertoxins present in the algae are initially eaten by herbivorous fish which remain unharmed. Being lipid soluble, toxins and their more polar metabolites are concentrated primarily in the viscera, liver and gonads resulting in toxin accumulation up the marine food chain in more than 400 species of fish (Lehane and Lewis, 2000) and up to the larger predatory fish which also remain unharmed. Notably, unlike many other marine toxins, significant concentrations of toxins are found in muscle tissue, although the prevalence for concentration in the organs probably explains the low percentage of fatalities in cases of intoxication. It is also apparent that with slow excretion rates, fish can

remain toxic for many years (De Fouw *et al.*, 2001) and individual tropical fish can carry sufficient CTX to poison several humans (Lehane and Lewis, 2000). The toxins are heat stable so cooking of the fish flesh does not remove the risk on intoxication. The toxins are potent voltage-gated sodium channel (VGSC) activator toxins as with the brevetoxins (Lombet *et al.*, 1987), specifically targeting binding site 5 on the α-subunit of neuronal sodium channels. They possess a trans-fused cyclic 13-14-ring structure (Lewis *et al.*, 1998, Murata *et al.*, 1989 and 1990; Figure 15). A number of structurally-distinct groups of toxins and their metabolites have been identified and characterised from different geographical sources, including the Pacific (P-CTX), Caribbean (C-CTX) and Indian (I-CTX) Oceans (Cailaud *et al.*, 2010). Each family of ciguatoxins consists of a range of different toxins, varying in the nature of their structural features and in the potency of the activity. The P-CTXs dominating the profiles of Pacific finfish are P-CTX1-3, with P-CTX-1 the most potent, posing risk to human health at concentrations above 0.1 ppb (Lewis *et al.*, 1999). The dominant toxin in Caribbean fish is C-CTX-1 which accounts for approximately half the toxicity in toxic fish (Vernoux and Lewis, 1997; Lewis *et al.*, 1999), although a great number of potent minor toxins are also known possibly forming as metabolic products of the source algal toxins (Hamilton *et al.*, 2010). I-CTX-1 and 2 are the dominant and equally potent toxins present in the Indian Ocean (Hamilton *et al.*, 2000a and 2000b).

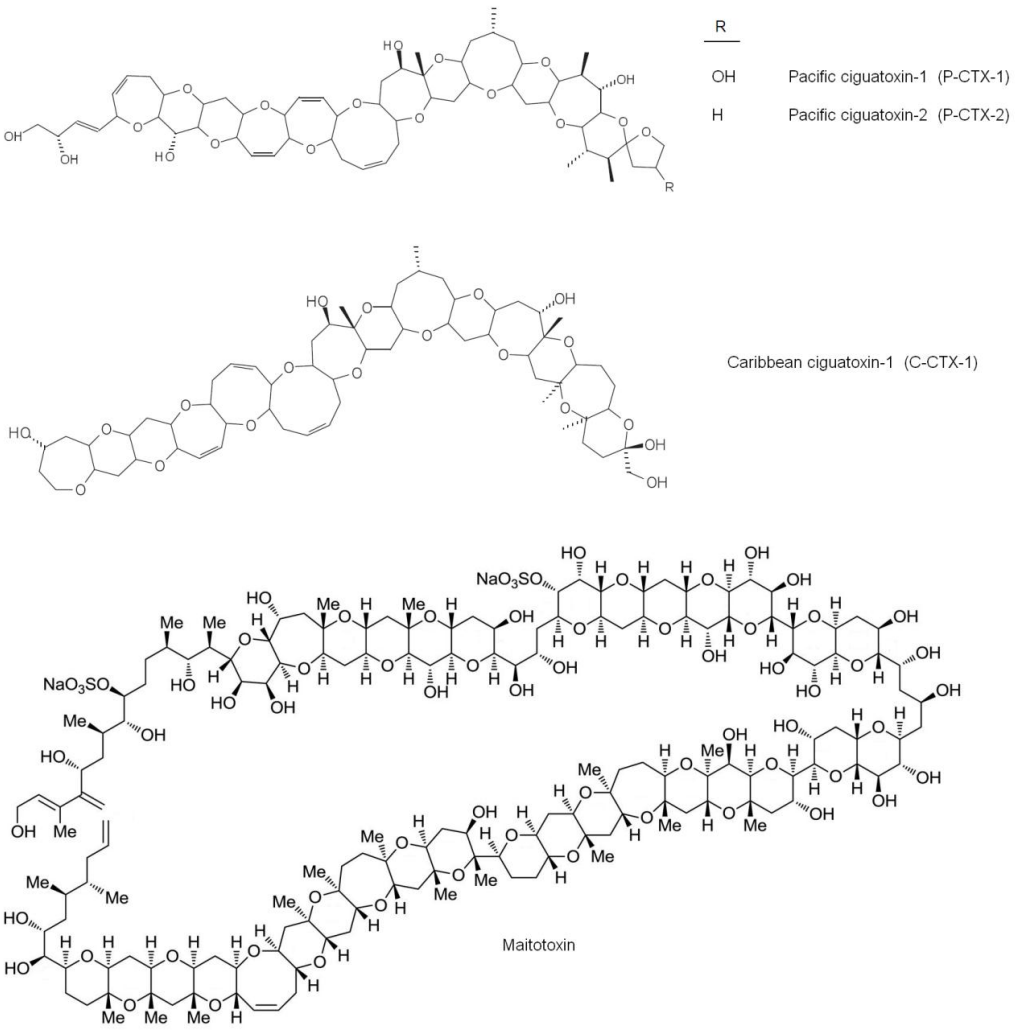


Figure 15. Ciguatoxin and maitotoxin chemical structures

The toxins are known to be highly potent from experimental toxicity studies, with the most toxic P-CTX-1 (or CTX-1B) recording LD_{50} levels of 0.25 µg/kg. P-CTX-3 and C-CTX-1 have shown LD_{50} levels of 3.7 µg/kg, approximately 10 times lower than P-CTX-1 and similar to the potency of the less polar gambiertoxin CTX-4B, or GTX-4B (2.0 µg/kg LD_{50}) (Hamilton *et al.*, 2010). As expected from the highly lipophilic nature of toxins, oral absorption is almost total, as experimentally indicated from similar toxic responses following both *i.p.* or oral routes of administration in mice. Toxic responses have also been determined following repeated administration experiments (Tubaro *et al.*, 2012). The toxicity of a range of chemically defined ciguatoxins including metabolic oxidation products have been published (e.g. Lehane and Lewis,

2000). Recently one of these metabolites, 54-deoxyCTX, was described as more potent than P-CTX-1 (Caillaud *et al.*, 2010a).

In addition to the variations in toxin profile, toxicity in tissues from different geographical regions has also been found to vary significantly. Whilst gastrointestinal, neurological and less commonly cardiovascular symptoms have been recorded, the signs associated with CFP in different regions of the globe have been found to differ (Lewis *et al.*, 1988; Wang, 2008). The symptoms beginning within 1-6 hours of ingesting contaminated fish, the effects can last days, months or even years (Baumann *et al.*, 2010). Whilst many thousands of people are affected annually, the poisoning is rarely fatal (0.1%) (Tubaro *et al.*, 2012), although it is thought that the proportion of CFP cases reported is low (<10%; CDC, 2006). The types of symptoms experienced also depends on the region, with Caribbean cases showing a dominance of gastrointestinal symptoms appearing within 2-12h of ingestions whereas Pacific occurrences appear to show greater proportions of neurological symptoms including sensory disturbances. Diagnosis can be further complicated through very different clinical presentations, even with consumers of the same fish (Tubaro *et al.*, 2012). This may potentially be complicated by accumulation of toxins in the human body following repeated low level exposure or from the combined effects experienced following ingestion of more than one type of toxin (Lehane and Lewis, 2000). Longer-term effects termed chronic ciguatera (CC) are generally rarer but include symptoms such as headaches, chronic fatigue, immune response abnormalities and less frequent cardiovascular symptoms (Tubaro *et al.*, 2012). The large differences in symptoms experienced across different regions of the globe are thought to relate to the differences in chemical structures of the ciguatoxins and subsequent toxicity actions. CTX-1B in particular imparts a risk to health at very low concentrations of around 0.1 pg/g (Pearn, 2001) to 0.175 pg/g (Oshiro *et al.*, 2010). Lehane and Lewis (2000) also summarised the nature and magnitude of the risk of CFP. Many factors are known to affect the probability of contracting the poisoning, including the fish species, location of capture and the sizes of the fish and the portion consumed. Clearly the country of origin is a major factor, with risks quantified by some other authors and shown to vary widely even in regions with known problems.

Maitotoxin (MTX) is a high molecular weight water-soluble toxin also produced by *Gamberdiscus* known to be the most toxic non-proteinaceous natural compound. It is a calcium channel activator, causing an increase in cytosolic calcium levels, stimulating a variety of calcium-dependent processes including cell death (Tubaro *et al.*, 2012). The LD_{50} of MTX following i.p injection in mice has been shown to be 0.5µg/kg (Yasumoto, 2000) or even less than 0.2µg/kg (Wang, 2008). With high water-solubility and subsequent insignificant accumulation in fish flesh, it is thought that this toxin along with another water-soluble compound gambieric acid, are not involved in CFP occurrences (Lehane and Lewis, 2000). However, whilst the role of MTX toxicity in humans is not completely understood, further work would be required to confirm this assumption (Hamilton *et al.*, 2010). In particular, with noted accumulation of MTX in fish liver and viscera, there is significant risk potentially relating to the consumption of eating small fish whole. To date, three forms of MTX have been identified, the high molecular weight MTX-1 and MTX-2 plus the smaller MTX-3, each found to be present in *G. toxicus* (Holmes *et al.*, 1990; Holmes and Lewis, 1994).

Carchatoxins which were found in relation to poisoning following consumption of shark meat resulted in high mortality rates. The toxicological properties were significantly different to those of ciguatoxins (Boisier *et al.*, 1995). The causative toxins carchatoxins A and B were found to have different chromatographic retention characteristics and dose-survival times as compared with the ciguatoxins (Yasumoto, 1998).

Reports from UK and European establishments identifying ciguatoxins in UK and European waters

Following consultation with a wide range of UK and European organisations, including universities, research establishments, monitoring laboratories and other agencies, no reports have been made available describing the occurrence of ciguatoxins in fish products originating from UK waters. Reports of CFP occurring in

Europe predominantly relate to travel to endemic countries bordering known hot spots for the syndrome where causative algae are well known to bloom on a regular basis (FAO, 2004). Outside of the UK, ciguatera has been identified in various regions of Europe with published literature describing recent events in Madeira (Otero *et al.*, 2010), the Canary Islands (Perez-Arellano *et al.*, 2005; Boada *et al.*, 2010, Nunez *et al.*, 2012) and parts of the Mediterranean Sea (Bentur and Spanier, 2007; Raikhlin-Eisenkraft *et al.*, 1988, El Masry and Fawzi, 2011). In the Canary Islands, nine outbreaks have been recorded over three different islands, affecting 68 people. In three of these outbreaks, CTX presence in food consumed was confirmed (Nunez *et al.*, 2012). However, there are still many knowledge gaps relating to the presence of *Gambierdiscus* species and the resulting accumulation in finfish.

2.9 RISK BASED ASSESSMENT ALL TOXINS

Based on the review above, a risk based assessment relating to the presence and potential hazards of biotoxins to shellfish consumers has been produced. Table 7 summarises the toxins that are either known to be present in UK coastal waters and those potentially present or appearing in UK waters. The level of risk is based on both the likelihood of toxins occurring and the severity of the hazard. Both aspects were determined independently and assigned a mark out of five, with 5 representing the highest risk and zero representing no risk. The total risk, ranging from zero to twenty-five, is calculated from the product of the two independent risk factors. It is noted that any determination of risk relating to the presence of new or emerging toxins where either no UK monitoring has taken place or where the toxicity is currently unknown is subjective.

Key:

Likelihood of occurrence (either now or in the future)	Score	Severity	Score
No risk of occurring	0	No toxicity effects	0
Unlikely to occur	1	No acute toxicity, chronic toxicity unknown	1
Possiblity of occurrence	2	Evidence for some toxicity, although actual effects unclear	2
Good potential for occurrence although no evidence currently exists	3	Evidence for toxicity with clear threat to human consumer safety in contaminated products, but no potential for fatality or long term sickness	3
Highly likely to exist or likely to become established	4	Evidence for acute toxicity with clear threat to human consumer safety but no risk of fatality. Known or potential for chronic toxicity.	4
Evidence for presence currently or in recent years within UK waters	5	Extreme toxicity risk with high potential for fatality when present at high enough concentrations	5

Likelihood and Severity factors are scored independently. Total risk is product of the two factors.

Table 7. Current, new and emerging toxin risks

Toxins	Linked to	Situation	Likelihood (0-5)	Severity (0-5)	Risk level (0-25)
PSP toxins from Alexandrium species	*Alexandrium tamarense* *Alexandrium minutum* *Alexandrium ostenfeldi* *Alexandrium catenella*	Detected routinely in official control monitoring.	5	5	25
PSP toxins from Gymnodinium species	*Gymnodinium catenatum*	No PSP toxins specific to *Gymnodinium* detected yet in UK waters.	2	5	10
Other PSP analogues	Shellfish metabolites of PSPs from *Alexandrium*	Unknown presence as not detected by current methods. Present in Canada and Argentina, actual toxicities not yet determined but risk exists given saxitoxin analogues.	3	5	15
ASP toxins	*Pseudo-nitzschia sp.*	Detected routinely in official control monitoring.	5	5	25
OA-group toxins	*Dinophysis sp* *Prorocentrum lima*	Detected routinely in official control monitoring	5	4	20
AZA1-3	*Azadinium sp.*	AZAs detected routinely (AZA1-3 monitored).	5	4	20
Other AZAs	Shellfish metabolites of AZAs from *Azadinium*	Other metabolites noted although not monitored in the UK. Toxicity may be high for some of these although remains undetermined for all analogs.	4	4	16
YTX and PTX toxins	*Dinophysis sp*	YTXs and PTXs found to date in UK, although toxicity risk disputed.	5	2	10
VSP toxins	*Prorocentrum minimum*	Organism present but syndrome rare and	1	3	3

		risk not confirmed.			
Cyanobacterial toxins *(Microcystins, Nodularin, cylindrospermopsin, BMAA, anatoxin, saxitoxins)*	Cyanobacteria	Cyanotoxins not reported in UK in marine shellfish although not monitored. Evidence from other parts of the world suggests the risk exists. With blooms well noted in UK, risk is significant.	2	5	10
Palytoxins	*Ostreopsis sp*.	No detection to date in UK waters, although no flesh testing conducted. Presence in Europe and noted spread of incidents heightens risk.	2	5	10
Brevetoxins	*Karenia sp*.	No detection of brevetoxins to date, although not monitored. Potential presence of causative organisms increases risk.	3	5	15
Cyclic imines	*Vulcanodinium sp. Others*	Recorded in Europe including preliminary evidence for UK presence.	5	2	10
Ciguatoxins, maitotoxins	*Gambierdiscus toxicus*	Little evidence for likely presence in UK waters, although detected in warmer regions of Europe.	1	5	5
Tetrodotoxins	Bacterial source (not proven conclusively), linked most notably to fish, but other marine species including	No TTX recorded in UK, but found in Europe including the Atlantic. Links to commonly-occurring bacteria potentially	2	5	10

	shellfish.	increases risk.			

2.10 CONCLUSION

The regulated toxins PSP, ASP and lipophilic toxins are clearly of high risk to the shellfish consumer if present in contaminated food products. New or emerging risks associated with these toxin groups relate to the potential presence of analogues which are currently not monitored within the UK control programmes. These include most notably the presence of PSP toxins originating from *Gymnodinium catenatum* such as GTX6 and a range of hydrophobic toxins, plus a number of PSP metabolites detected in mussels and scallops by LC-MS/MS analysis. Whilst the toxicity of GTX6 is relatively low compared to STX, high relative concentrations can still result in significant toxicity within highly contaminated samples. Similarly, whilst the toxicity of the shellfish metabolites and hydrophobic analogues remains unclear, high proportions of these toxins could result in a significant risk to the consumer. Other analogues of the regulated groups of toxins include the large number of AZAs identified to date as shellfish metabolic products. Some of these are thought to potentially provide significant risk in terms of toxicity to the shellfish consumer. The OA-group toxins encompassing DTXs and the OA-group esters are well recognised as high risk toxins if consumed in high enough quantities, although these are currently monitored successfully through the LC-MS/MS testing. Whilst only a low number of the YTX and PTX analogues are analysed routinely the risk from these two groups of toxins is thought to be slight.

Cyclic imines represent a group of emerging toxins with some compounds already present in UK waters and in shellfish food products. However whilst some of the are known to occur, the risk to the consumer is not as obvious given the general lack of information on toxicity following oral consumption. Tetrodotoxins whilst generally present in warmer waters have been linked to a range of bacterial sources which are well known to exist in UK waters and in UK shellfish. There is also the occasional occurrence of the toxins in European waters. However, with an absence of any evidence for intoxication from these highly potent neurotoxins in the UK, it is unlikely that the risk is particularly high in UK-cultivated shellfish at present. The other toxin groups such as palytoxins, brevetoxins and ciguatoxins/maitotoxins are all associated generally with warmer marine water sources, with no evidence to date for

presence in UK shellfish. However, these toxins are not monitored in the current control testing, so an absence of data does not guarantee an absence of risk. The brevetoxins in particular may be likely candidates for emerging threats given their presence in the waters of New Zealand, whilst the palytoxins and ciguatoxins are still only found in warmer waters around the Mediterranean and Atlantic islands. Overall the risk is present, but low in comparison to the other toxins listed. Poisoning from venerupin is reported rarely and there is little specific evidence for any high degree of risk. Cyanotoxins are perhaps the most difficult to assess in terms of threats to shellfish consumers. The causative organisms are vast in their number and are certainly prevalent at times within UK water systems. Studies from Europe and further afield have described the accumulation of a wide range of freshwater toxins in marine and brackish water species, including those destined for consumption. So whilst the threat has not been demonstrated in the UK to date, the risk is potentially significant.

Chapter 3

1. ASSESSEMENT OF THE SUITABILITY OF EXISTING AND POTENTIAL METHODS FOR THE DETECTION OF NEW AND/OR EMERGING TOXINS AND IDENTIFICATION KNOWLEDGE GAPS WHERE FURTHER RESEARCH COULD BE FOCUSSED: PHYTOPLANKTON

1.1 INTRODUCTION

The UK phytoplankton monitoring programme is based on the collection of water samples from classified shellish harvesting areas. Samples are collected using a variety of techniques. Most frequently a tube sampler is used, but sometimes with a pole sampler or bucket when water depth is shallow. Samples are collected with a varying frequency, of up to weekly in the high risk summer months. These are fixed with the preservative Lugol's iodine on site and transported (typically by post, but sometimes by hand) to the laboratory for analysis. Sample are then "settled" prior to counting by (inverted) light microscopy (the Utermöhl method). Eight species or genera of phytoplankton (*Pseudo-nitzschia* spp., *Alexandrium* spp., *Dinophysis* spp., *Prorocentrum lima, Prorocentrum minimum, Lingulodinium polyedrum* and *Protoceratium reticulatum*) are enumerated on a routine basis.

Relatively few publications critically assess the methodologies used to collect and analyse samples for the identification and enumeration of biotoxin producing phytoplankton. Those that do include:

- The reviews by ICES (1992) and Anderson (1996) that summarise methods used in regulatory frameworks in a number of countries.
- The manual of Karslon *et al.* (2010) that detail the methodology and the advantages/disadvantages involved in a range of methods using both conventional and molecular methods to enumerate phytoplankton.

- The conference publication of Hess *et al.* (2012b) that reviews the evolution of phytoplankton monitoring technologies.
- Returns of the survey distributed to HAB monitoring organisations in Europe.

It is also expected that the ICES working group for Harmful Algal Bloom Dynamics will produce an update of the 1992 Cooperative Research Report in the next year. Unfortunately, this document was not available for consultation during the preparation of this report. When available, reference to this document for recent developments is recommended.

1.2 COMPARISON OF THE PHYTOPLANKTON MONITORING PROGRAMMES IN THE UK AND OTHER EU COUNTIRES

HAB species enumerated vary from country to country. But all European countries surveyed enumerate the key HAB general of *Alexandrium, Pseudo-nitzschia, and Dinophysis*.

The UK is relatively unusual in enumerating only potential HAB species, with 64% of the countries responding to the survey of Anderson (1996), including most of the European nations, enumerating the whole phytoplankton community. Apart from the countries highlighted by Anderson (1996) some of the countries, for example Ireland (see: http://www.marine.ie/home /publicationsdata /data/ Habs+Search+Database/), also conduct full phytoplankton counts, at some sentinel sites. The surveys returned (Appendix 2) also indicated Spain conducts full phytoplankton counts on all samples.

Such detailed enumeration is obviously much more time demanding that simply monitoring the known HAB species. However, it provides a much greater likelihood of identifying new harmful species should they become present in the water column.

1.2.1 Restrictions implemented on the basis of phytoplankton counts

It is unclear how the threshold concentration for the different HAB species of interest in UK waters was established. The current thresholds in the UK are: for *Alexandrium* spp. presence of cells, *Dinophysis* and *P.lima* 100cells/L and *Pseudo-nitzschia* spp. 150,000 cells/L (50,000 cells/L in Scotland)

Anderson (1996) lists cell threshold concentrations for a range of species and countries. These are very variable and may reflect the differences in toxicity between species and strains in different countries. Little evidence can therefore be gained from other countries to inform the phytoplankton thresholds implemented in the UK.

For the potentially invasive species, Anderson (1996) notes a threshold concentration of “presence” in the UK. This is consistent with the fact that the observation of *G. catenatum* in a UK regulatory monitoring sample would certainly promote some interest. However, in the future if it became necessary to set a more appropriate threshold value there is limited evidence on which to base this. Anderson (1996) found only 2 countries listing threshold concentrations: 2×10^3 cells L^{-1} in Portugal and 500 cells L^{-1} in Spain-Andalucia.

Anderson (1996) also finds the concentration limits to be very variable between countries, with the eventual restrictions implemented in many cases not being clear.

1.2.2 Application of phytoplankton data

While the publication of phytoplankton counts on the FSA web site is an excellent resource for shellfish farmers to plan their harvesting operations, in comparison to some other countries, the UK makes relatively little use of this information for regulatory purposes.

Hess (2012a) note that, in 2007, eight of the European member states that monitor biotoxin producing phytoplankton use this information to trigger shellfish flesh testing. They highlight the example of France when weekly phytoplankton and flesh texting occurs in the high risk period, but in the low risk period flesh testing is only triggered

on the basis of phytoplankton counts. Given the relatively high rate of false negative samples generated by the MBA, Hess *et al.* (2012) suggest that the move to chemical testing will result in an increase in the duration of the low risk period and a still greater role for phytoplankton, particularly as survey responses indicate that, in contrast to the UK, most European countries use phytoplankton data to target shellfish flesh analysis.

1.2.3 Cysts

The ICES CRR (1992) recommends that when harmful species which produce cysts or dormant cells are known to occur, it is useful to monitor during the winter period, the distribution and abundance of cysts in the upper layer of sediment. However, the need for complementary measurements of parameters such as dissolved oxygen and temperature near the bottom that are suggested to be necessary to assess the germination of such seed populations may make this approach preventatively expensive in UK waters.

1.3 CURRENTLY AVAILABLE METHODOLOGY

Pelagic organisms

1.3.1 Sample Collection

Phytoplankton samples are typically collected at shellfish harvesting sites and, following fixation returned to the monitoring laboratory for analysis, typically following settlement, by light microscopy. Results are then reported to the regulator. The various approaches to the different stages of this monitoring protocol are discussed below.

Anderson (1996) and ICES (1996) outlines four possible methods for sample collection:

- Plankton net tow.

This approach is perhaps the simplest, but may miss smaller organisms should they pass through the net mesh (for example *Azadinium* is ~ 10µm in diameter, smaller than the typical 20µm mesh of a plankton net). Also, should the sample have a high biomass of benign species, the concentrating effect of the net tow may make it difficult to identify low abundance HAB species in the resultant sample.

- Water bottle: deployed to multiple depths.

  If a high enough resolution is used, this approach allows for identification of thin layers of harmful species. However, it is more time consuming that other methods of collection and generated multiple samples for analysis.

- Integrated water sample by pump.

  Integrated samples provide an “average” phytoplankton density over the top few meters of the water column (to whatever depth of tubing is used). While pump harvesting of this water is possible, it may result in the breakage of fragile cells and hence offers no obvious advantages of non-pumped collection as detailed below.

- Integrated water Hose / Lund tube sampler.

  The hose system (see Lindhal 1986) is the most common method of sample collection and the method used in the UK when the water is of sufficient depth. The hose is dropped to a set depth and then a tap at the top closed, the bottom end is then retrieved by means of a rope, capturing an integrated water sample. Following gentle mixing in a bucket a subsample of this water can be used for analysis of the average phytoplankton concentration over the depth sampled.

- Bucket / pole sampler.
  In locations where water depth is insufficient to deploy a hose sampler, a bucket or a pole sampler is deployed. While somewhat less quantitative, these are pragmatic and fit for purpose solutions to collection of water in the location it is required.

Replication

The process that introduces the greatest uncertainty in the reported number of HAB species is the collection of a single sample with no replication. While a representative sample is (most likely) collected this lack of replication influences both accuracy and precision. Whilst this is common practice across monitoring organisations (possibly due to financial constraints), it suggests the time and effort required by UKAS accreditation to ensuring the accuracy of the count from this single sample may be disproportionate.

Frequency of sample collection

Anderson (1996) states that sampling should be carried out at least weekly and that during development of a HAB sampling should be intensified to daily (although this seems impractical in most monitoring situations). While reduced sampling frequency methodologies are being suggested for the Water Framework Directive (Abramic *et al.*, 2012) this is not appropriate for shellfish toxicity related monitoring.

The returns from the survey carried out as part of this study indicate that weekly sampling, at least in the high risk spring/summer/autumn period is the norm in most European countries.

Benthic organisms

It is known that at least one potentially harmful organism (*Prorocentrum lima*) that is present in UK waters has a primarily benthic life cycle. Subsequently, the cells will only be identified during routine weekly monitoring if turbulence or other factors mixes them into the water column at the time of tube sampling. Given that such a mixing event will not necessarily coincide with water column sampling, it is unlikely that current sampling approaches will give a good early warning of the toxification of rope grown shellfish mussels by these benthic organisms.

Given that a number of the potentially invasive species of concern (e.g. *Ostreopsis* sp.) are benthic organisms, some degree of benthic sampling (even if only at a range of sentinel sites) would be prudent in UK waters. In Italy where *Ostreopsis* Spp. are

the major organism of concern, macroalgae samples are collected for analysis of this benthic species (survey response Appendices 1 and 2).

Methods for the collection of benthic algae are not as well standardised as for pelagic organisms. Anderson (1996) details the approach taken by Quod *et al.* (1995) for collecting benthic dinoflagellates responsible for ciguatera fish poisoning from macro-algae on which they live. This includes:

- Collection of macro algae (20g)
- Vigorous shaking in seawater
- Sieving of seawater (150 µm)
- Counting of dinoflagellates in < 150 µm fraction.

A form of this method is also proposed by GEOHAB (2012) as a possible means of collecting benthic algae, although that publication does not detail the exact methodology used to liberate the cells. Moreover, it cautions that normalising cell abundance values is problematic and may vary if results are presented per unit surface area or per unit biomass.

Although there maybe logistical difficulties in obtaining macroalgae (for example tidal state), the sampling methods are relatively simple. However, it would require a detailed validation of the above protocol to ensure that it generated reproducible results.

GEOHAB (2012) noted that alternative protocols exist for the collection of benthic harmful algae. These are:

Vacuum collection: this can usually be accomplished with minimal destruction of the habitat and allows a variety of substrates to be sampled. However, samples tend to be dirty and hard to count.

Use of artificial substrates: sampling of artificial substrates (for example a car windscreen) are proposed as a means of providing more easily standardisable data.

In all cases, access to the benthic environment is potentially problematic and as noted by GEOHAB (2012) none have been systematically evaluated.

1.3.2 Sample fixation

The fixative used within regulatory monitoring programmes is Lugol's iodine (either acidic or neutral). This preserves the cells and generated a brown colouration that makes them easier to visualise by light microscopy. Lugol's iodine has a low toxicity to humans and is used in a diluted state.

Other potential fixatives that are sometimes used in a research environment, such as formaldehyde are toxic (possibly caricinogenic) and should only be used in a fume hood by trained staff. Whilst alcohol free formaldehyde fixed samples of less than 10% are not considered dangerous and not subject to transport regulations,the concentrated solution is still required prior to its dilution in the sample bottles preventing fixation being conducted on site by sampling officers.

In conclusion, whist Lugol's staining may make the identification of some organisms, such as *Alexandrium tamarense* more difficult (as the plate structure is obscured), it remains the most appropriate fixative to use in a regulatory monitoring context.

1.3.3 Sample Enumeration

Standard light microscope methods

Based on Anderson (1996) and the survey returns that were received (Appendix 1 and 2), it is understood that the universal default method of cell enumerations in regulatory monitoring programmes is by light microscopy using the Utermöhl technique. The only exception is that Norway also uses the filtering-semitransparent filter method and Palmer-Malony counting chamber.

The Utermöhl method is clearly fit for purpose for routine enumeration of the majority of the harmful organism that are present in UK waters. However, for a sub set of

organisms in particular the genera *Alexandrium* and *Azadinium*, the method is problematic as outlined below:

Alexandrium

The difficulty with the enumeration of this genus is principally related to the identification of the highly toxic species *Alexandrium tamarense*. This species is indistinct from both other *Alexandrium* species and also from other benign dinoflagellates of similar size. A considerable degree of training and skill is therefore required to identify and enumerate this species.

Visibility of the key morphological features of *A. tamarense* depend on it orientation on the microscope slide, identification therefore frequently requires the removal of the cover slip from the settling chamber and manual manipulation of the cell with a pin. This is a time consuming and skilled process which cannot be performed by automated particle counting methods (see Section 1.3.4).

Finally, while Scottish waters were traditionally understood to contain the toxic (North American) group I ribotype of *A. tamarense*, and English waters the non-toxic (Western European) group III ribotype There is increasing evidence (Collins *et al.*, 2009; Touzet *et al.*, 2010) indicating the presence of non-toxic group III cells in Scottish waters. This means the observation of a bloom of *A. tamarense* does not necessarily mean shellfish toxicity should be expected and therefore for large blooms further corroborative evidence of the strain of *A. tamarense* present should be sought (most likely by molecular methods, see Section 1.3.4).

Azadinium

The genus *Azadinium* has only recently been identified as the organism that produces azaspiracid toxins (Tillman *et al.* 2009). Unfortunately it is a small cell (~10 µm), with no clear morphological characteristics that can be routinely identified within regulatory monitoring programmes using light microscopy at the x20 magnification. The use of higher magnification is not possible within a routine monitoring programme that has to rapidly analyse a large number of samples light microscopy, it is therefore not suitable for the enumeration of this organism.

1.3.4 Alternative enumeration methodologies

The range of alternative methods of phytoplankton cell enumeration are detailed in Karslon *et al.* (2010) and are summarised below

Alternative Light Microscope Methods

Alternative settlement methods:

-Settlement bottle method

This is a modified Utermöhl technique that relies on the observation and enumeration of cells after sedimentation using an inverted microscope. It differs from normal regulatory sample handling protocols in that a preserved water sample is immediately transferred to a plastic tissue culture flask, which is then placed on the microscope stage for enumeration. The advantages of this method are limited to the potential removal of sub sampling errors that may occur when filling the Utermöhl chamber from the sampling bottle. However, given that sample settling is still required this is outweighed by the reduction in optical resolution due to the thickness of the sampling bottle. Enumeration is also less easy using this method, particularly as samples are enclosed and cannot be manipulated (as is necessary for *Alexandrium*).

Alternative counting chambers:

-Haemocytometer

This is a counting slide method for cultures and extremely high concentrations of small cells. It is unsuitable for routine water monitoring because it contains a very small sample volume and statistically robust counts or environmentally relevant concentrations of cells cannot be achieved.

-Palmer Maloney cell

This is an alternative counting slide method for cultures and extremely high concentrations of small cells. While it is sometimes used in Norwegian monitoring, it is unsuitable for routine water monitoring in the UK because it contains a very small sample volume giving a limit of detection of 10,000 cells L^{-1}.

-Sedgewick Rafter Cell

This is a traditional counting slide that can hold 1 mL of sample volume. The chamber has a transparent base that is sub divided into 1000 units. The small volume of the chamber makes it unsuitable for low abundance biotoxin producing species.

Filtering methods

-Calcofluor staining

This method uses Calcofluor White to stain the cellulose thecal plates of dinoflagellates that can then be identified and enumerated by epifluorescent microscopy. The method is limited to thecate dinoflagellates it is not therefore applicable to routine monitoring of a mixed community. However, this method is useful in the identification of species such as dinoflagellates on a non-routine basis.

-Semitransparent filters

Collection of the water samples onto semi-transparent filters provides a method to concentrate enumerated cells by light microscope. The method is rapid but fragile cells may be destroyed during the filtration process. It may also be difficult to identify low abundance cells within the biomass that accumulates on the filter. While sometimes used in Norwegian monitoring it is not suitable for routine monitoring in the UK.

-Filter-transfer-freeze method

The method requires a quick freezing of a microscope slide at the collection sites and is therefore not very practical in a regulatory monitoring context.

Particle counting methods

-Flow cytometery

Flow cytometry is a popular tool for the rapid identification and enumeration of different populations in mixed microbial communities (Sekar *et al.*, 2004). Flow cytometry involves the direction a beam of laser onto a hydro-dynamically focused stream of liquid containing the cells of interests. Multi parameter discrimination and enumeration of cells is then based on the forward angle scatter (FSC) (0.5-5°), side

angle light scatter (SSC) (15-150°) and fluorescence at a range of wavelengths. Detection and discrimination is typically based on bi-plots of light scatter, autofluorescence or laser light excitation of fluorescent stains or probes (Sekar *et al.*, 2004; Kalyuzhnaya *et al.*, 2006).

Flow cytometry is most appropriate for the enumeration of small cells such as pico-plankton or bacteria. Larger phytoplankton, particularly when fixed with Lugol's iodine do not generate a scatter pattern that allows the genus or species level discrimination required for HAB monitoring.

However, the use of flow cytometry to enumeration fluorescently labelled HAB (typically using fluorescence *in situ* hybridisation [FISH]) species may have potential in a monitoring context. With FSA and NERC funding such a method is currently being developed at SAMS for toxic and non-toxic ribotypes of *A.tamarense*. A drawback is that the RNA probes required do not work on Lugol's fixed samples. However, given this caveat, results with relatively high density cells (even within natural communities) are encouraging and tests of lower densities of *A. tamarense* are ongoing. It is hoped that the method will allow the determination of whether developing blooms are toxic or non-toxic.

-Imaging flow cytometry

The most high profile and commercially produced, instrument in this category is the FlowCAM. This instrument is similar to a flow cytometer but also contains an imaging microscope allowing an image of each enumerated particle to be saved. Software allows for "training" of the instrument to assist in analysis and classification (Poulton and Martin, 2010). It has been used in the detection of the high biomass HAB *K. brevis* (Buskey and Hyatt, 2006).

The instrument has the potential to allow more rapid enumeration of cells of interest than can be achieved by a microscopist. However, it may be most appropriate for high abundance organisms or those that are morphologically distinct (e.g. *Dinophysis*), as it does not require the skill of a microscopist to identify difficult organisms such as low abundance *A. tamarense*.

A further disadvantage of the method for routine monitoring of HAB species within mixed populations is that preservation of the sample is not recommended as the loss of fluorescence makes discrimination more difficult. In addition, heavy particle loads can interfere with the image capture. This may require sample dilution, with a resultant increase in the limit of detection.

Again, as with the flow cytometer, the use of fluorescent probes in combination with FlowCAM may provide a method for identification and/or enumeration of strains that are difficult to deal with using standard light microscopy. However, further development is required to have confidence in data relating to low abundance organisms in mixed populations.

While other imaging techniques, for example for the identification of *P. minimum* exist (Gelzinis *et al.*, 2011), these remain developmental and are unlikely to be of use in a monitoring context in the near future.

Electrochemical detection

Electrochemical biosensors combine a biochemical recognition with signal transduction for detection of specific molecules. As part of the EU ALGADEC project, a hand held device was developed using a PalmSens portable potentiostat for the detection of rRNA targeted oligoneucleotide probes. The method remains in prototype with probes existing for only a limited number of organisms. Given that the ALGADEC project ended in 2006, it is unclear if further development of this method is likely.

Molecular methods

-Whole cell hybridisation

Fluorescence *in situ* hybridisation (FISH) has been mentioned above in combination with flow cytometer of FlowCAM methodology. The basis principal of FISH is that target cells are hybridised with fluorescently labelled oligonucleotide probes that bind to target sequences of rRNA. This results in bright labelling of the entire cells when viewed under a fluorescence microscope or a strong fluorescence signal in a (appropriately tuned) detection channel of a flow cytometer or FlowCAM. Further details of the methodology can be found in Töbe *et al.* (2010) and specifically for *A. tamarense* in Touzet *et al.* (2008b, 2010).

Advantages of this method are it is relatively inexpensive and sensitivity and that the cells can still be visually observed. With appropriate probes simultaneous labelling and detection of multiple species is possible. Disadvantages lie in the current lack of available probes and the rigorous specificity testing on local strains required. The RNA specificity of the probe prevents it from being used on Lugol's fixed samples so cells may be lost in (unfixed) transport and during processing.

In New Zealand, FISH assays are used as a supporting tool to microscopy to provide additional information to regulators (Töbe *et al.*, 2010). Such an approach may have benefits in the UK, for example for the discrimination of toxic and non-toxic *A. tamarense* should large blooms of this organism occur.

-Hybridisation and microarray detection

A micro-array consists of DNA sequences that are applied to the surface of a glass slide in an ordered array. Micro-array application for the detection of harmful algae requires micro-array production, sample collection and preparation, hybridisation and data analysis.

A large range of studies have sought to develop micro-array technology with applications to harmful algae including Metfires and Medlin (2004), Ki and Han (2006), and Gescher *et al.* (2007). The EU MIDTAL project has recently sought to further develop these technologies and Taylor *et al.* (2013) conducted a trial application of the MIDTAL microarray in Orkney Island waters finding it able to identify but not quantify target HAB species.

The potential of this method to allow parallel detection of a range of different species makes is appealing for regulatory monitoring. However, Gescher *et al.* (2010) note a number of significant disadvantages that still limit the applicability. These include issues similar to other molecular techniques outlined above in that the methods remain developmental with probes existing for only a few phytoplankton species and the requirement for local validation. However, Gescher *et al.* (2010) note that while both RNA and DNA can be used for identification, the former is preferred as the cell contains rRNA in a higher number that can be more easily extracted using

commercial kits. This will present the same issues with respect to the inability to use Lugol's fixation that were noted above for other molecular methods.

Sample throughput is also relatively slow with a trained person requiring 6 hours to process up to eight samples before analysis. Hence, while more rapid than microscopy, it is not markedly quicker.

The survey response from Spain (appendices 1 and 2) implies that this method is used there with a large number of probes being available locally. However, routine use of this technology to enumerate harmful phytoplankton within a UK regulatory monitoring context would seem to remain a significant distance in the future and not feasible for the use in the routine monitoring. However, similar to the FISH methodology noted above, with some development to target the approach for UK organisms it may be suitable for occasional verification of hard to identify organism when identification to species or strain is required.

-Sandwich hybridisation

Sandwich hybridisation (SHA) employs two DNA probes that target rRNA sequences. A capture probe is attached to a mechanical solid support. This is then submerged in a sample and hybridises with the target molecule. To detect captured molecules a second hybridisation step used a DNA probe conjugated to a signal probe. The resulting "sandwich" is detected using an enzyme colour reaction.

Advantages of this method are its robustness and semi-automated nature. However, probes exist for only a limited number of species and require regional verification. The RNA basis of the method means that it is not suitable for Lugol's fixed samples.

-Tyramide signal amplification with fluorescence in situ hybridisation (TSA-FISH)

TSA-FISH is a method in which enhanced fluorescence signal can be obtained from molecular probes by labelling the probe with the enzyme horseradish peroxidase which catalyses the subsequent deposition of fluorescein isothiocyanate (FITC) labelled tyramide. Detection is by solid phase cytometry.

Orozco and Medlin (2012) review recent advances in this technology. The advantage of this method is its enhanced detection capabilities. Disadvantages include very high set up costs, complex sample processing and the availability of probes for only a few species.

-Quantitative polymerase chain reaction (QPCR)

QPCR (sometimes called “real time PCR”) is a technique used to amplify *in vitro* a target sequence of DNA by heating and cooling an initial reaction mixture (including target DNA, primers, a DNA polymerase enzyme, and buffer containing various salts) in a defined series to temperature steps (Galluzzi and Penna, 2010).

The method is highly sensitive and while molecular biology laboratory skills are required, no taxonomic expertise is necessary. Drawbacks include availability of probes for only a limited number of target species and the need to check their specificity locally. In addition only one species or strain can be analysed at a time unless a more complex multiplex reaction is performed.

The use of QPCR for HAB monitoring is reviwed by Antonella and Luca (2007). Given its specificity and sensitivity QPCR is perhaps the most promising of the alternative enumeration methods and is used in the Irish monitoring programme to ideniify *Azadinium* and to speciate *Pseudo-nitzschia*. Given its specificity for particular organisms exclusive use of QPCR would run the risk of non-detection of new, unexpected, or invasive species. However, used in combination with microscopy it is potentially a powerful tool to enumerate those organisms that are not easily or rapidly identified by microscopy. The survey response indicates that, in Irish waters, QPCR is used to enumerate *Azadinium* and to occasionally identify *Pseudo-nitzschia* to species level to allow determination of potential toxicity of blooms. Similar methods are also used in Italy to identify *Azadinium* and *Ostreopsis*.

1.3.5 Indirect estimates of phytoplankton biomass

The most readily available indirect estimate of phytoplankton abundance is based on fluorescence, either though directly deployed instruments (e.g. tethered to buoys),

satellite remote sensing, or through the collection of water samples that are processed in the laboratory.

Fluorescence simply provides an estimate of the chlorophyll content of the phytoplankton community and hence its biomass. The reliance on total chlorophyll means that the information is most valuable for high biomass organisms, with applications including red tides in Ariake Sound Japan (Ishizaka *et al.*, 2006). Development of algorithms for the specific detection and classification of harmful species based on parameters such as absorption, total backscatter, and water-leaving radiance has had some success in identifying blooms. An example of its use was the work on the dinoflagellate *Karenia mikimotoi* by Davidson *et al.*, (2009). However, further development is required to make such systems operational (Shutler *et al.*, 2012). Davidson et al. (2009) used remote sensing within the monitoring programme for the *Karenia mikimotoi* that operates in Scottish west coast waters on a commercial basis, using a combination of ocean colour and microscopy ground truthing. This system gives sufficient predictive ability that operational decisions such as the routing of the well boats that transport live fish to processing plants are made on the basis of its results.

A similar approach is followed by a HAB forecasting system developed for *K. brevis* blooms in the Gulf of Mexico (Stumpf *et al.*, 2009). HAB forecasts are made twice weekly during bloom events, using a combination of satellite derived image products, wind predictions and a rule-based model derived from previous observations and research. Blooms are detected and defined using ocean colour satellite images, bloom transport is then predicted using hydrographic modelling with passive particle transport. This system is now operational in the region, with the federal government making the twice-weekly forecasts and working closely with state agencies for ground verification.

As discussed by Hess *et al.* (2012b) satellite detection while spatially extensive, has a range of drawbacks making it generally not suitable for biotoxin producing HAB detection. These include:

1. the lack of a guarantee that cloud free images can be obtained
2. satellite images are only relevant for surface populations

3. that only the dominant organisms are detected.

Given that biotoxin producing HAB species rarely dominate the phytoplankton biomass in UK waters. This final shortcoming is perhaps the most relevant as when species information is available it is not possible to determine the fraction of the biomass that may actually be harmful.

Buoy deployed fluorescence detection instruments are equally problematic, sampling at a single location and depth only and requiring relatively frequent and expensive servicing. The detection of fluorescence alone raises the same issues with lack of organism specificity as satellite imagery.

1.3.6 Mathematical modelling approaches

The ability to forecast the appearance and the toxicity of, harmful phytoplankton would be of considerable benefit to both the aquaculture industry and regulatory bodies. Accurate and sufficiently timely prediction would allow the industry to initiate management strategies to minimize economic losses. It would also allow regulators to best focus the resources within monitoring programmes. A range of mathematical model based approaches have been applied to the problem of predicting the appearance and toxicity of harmful algae.

Risk assessment style models

Geographical areas that have well-defined and understood seasonal weather patterns and physical oceanography are potentially more amenable to prediction than areas (such as the UK) which have more variable weather patterns. An early example for the rias of Galicia (NW Spain) was presented by Fraga *et al.* (1988). In that study, the relaxation of upwelling due to a change in wind direction resulted in the transport of established offshore populations of *Gymnodinium catenatum* into the rias where toxicity of shellfish is a frequent occurrence. Refinements have since been presented by Crespo and Figueras (2007).

Similarly Raine *et al.* (2010) developed a simple model that was used operationally to predict blooms of *Dinophysis* and resulting shellfish toxicity in the bays of the

southwest of Ireland. A wind-driven two-layer oscillatory flow exchanges a substantial proportion of the bays' volumes, and harmful algal events arise with the associated transport of harmful populations into them. The model therefore uses meteorological data to determine if wind-driven water exchanges will occur. If the time of year is correct, *Dinophysis* cells are present outside the bays and a toxic event is predicted. The model was trialled in 2005 by estimating wind indices from the five-day weather forecast. Results were published in real time on the web. The simplicity of the approach suggests that it may be of use in other regions where advection is the key process and a clear understanding of the mechanisms of water circulation exists.

Computational mathematical models

A wide range of mathematical models are now being developed to answer research questions related to the growth and toxin production of harmful algae. Many developments are detailed in recent volume of the Journal of Marine Systems that details the proceedings of the GEOHAB modelling workshop held in Galway in June 2009 with, for example, Glibert *et al.* (2010) reviewing issues related to the modelling of HABs and eutrophication. All HAB modelling studies are not reviewed here but a range of modelling methodologies are identified and their strengths and weaknesses discussed through the examples below.

-Ecosystem based models

The ability of coupled physical/biological ecosystem models to simulate HABs was reviewed by Allen *et al.* (2008) with particular reference to high biomass blooms in eutrophic coastal seas using the POLCOMS-ERSEM model. The ecosystem model was found to have some degree of success in predicting blooms in comparison to remotely sensed ocean colour estimates of chlorophyll. This is partly related to the relatively poor ocean colour estimates of chlorophyll in turbid coastal (case II waters). However, it seems clear that computational developments would be required to obtain a robust HAB forecasting model even for high biomass species.

An attempt to produce an operational ecosystem-HAB model is ongoing within the PREVIMER project (http://www.previmer.org/) (Penard, 2009; Hess *et al.*, 2012b).

This aims to provide short-term forecasts along the French coastlines bordering the English Channel, the Atlantic Ocean, and the Mediterranean Sea. PREVIMER had published the results of model forecasts on the web since 2006, but the system remains experimental. While phytoplankton based predictions for most regions are restricted to primary production based on a coupled physical/biological ecosystem models, the model for the Brittany region includes simulation of the appearance of *Pseudo-nitzschia* and the risk of associated toxicity. This is achieved by augmenting the biological model with a simple *Pseudo-nitzschia* specific model derived by Davidson and Fehling (2006).

-Individual based models

An alternative modelling approach combines a physical model with an individual based model (IBM) that considers only the specific organism of interest. These models calculate biological variables while following individual (or meta-) particles in space. Virtual particles are subject to advection and diffusion and their position is tracked around the model domain. Advection and diffusion come from flow fields generated by a hydrodynamic model. The virtual particles may hold biological properties e.g. chlorophyll biomass or number of cells.

An example of an IBM model is that of *Alexadrium fundyense* in the Gulf of Maine. *A. fundyense* blooms are transported in a generally south-western direction along the coast by plume advection with wind-driven force being capable of moving the plume offshore. A coupled physical/biological model is therefore employed in the region to predict the development and transport of the bloom. The model configuration is presented by McGillicuddy *et al.* (2005), Stock *et al.* (2005), and He *et al.* (2008). This model is one of the most successful examples of operational prediction of the dynamics of a harmful phytoplankton species. However, it must be recognised that it was only developed on the basis of considerable background science that provided an understanding of the hydrography of the region and the physiology of the organism. An important feature in the initialisation of the model is a detailed knowledge of the distribution of *A. fundayense* cysts in the region prior to the growth season. This information is gathered annually by ship based cyst surveys, with obvious resource implications.

An IBM model system is being developed in UK waters within the Asimuth project (www.asimuth.eu). The model domain, that is currently restricted to the Scottish west coast in Argyll, employs an unstructured approach (Chen *et al.*, 2003) that allows the fjordic sea lochs within which most shellfish farms are locates to be resolved. Initial development relates to the fish killing species *Karenia mikimotoi*, but in principal the model can be adapted for other advective biotoxic species such as *Dinophysis* or *Pseudo-nitzschia*.

-Statistical models

Statistically based models typically use regression techniques to allow bloom prediction. Examples include the analysis of blooms of *Pseudo-nitzschia*. Anderson *et al.* (2009) used linear hindcasting to determine the environmental conditions associated with *Pseudo-nitzschia* spp. blooms in the Santa Barbara Channel. Subsequently both Lane *et al.* (2009) and Anderson *et al.* (2010) used logistic regression, a generalised linear model based technique, for the prediction of *Pseudo-nitzschia* blooms in Monterey Bay and Chesapeake Bay respectively.

An important factor in the development of statistical models is sufficiently good quality time series of HAB and environmental data from which the relationship between HABs and the environment can be determined. In UK waters it is unusual for sufficiently detailed environmental data to be collected near shellfish farms making it difficult to widely develop similar approaches in UK waters. A preliminary study of such an approach in the Firth of Lorne within the EU project WATER (www.nppwater.com) (Wang *et al.*2011, unpublished) was unable to obtain sufficient predictive power to produce an operational system.

1.4 COMPARISON OF MONITORING METHODOLOGIES AND RECOMMENDATIONS FOR IMPLEMENTATION IN THE UK

Table 1 below summarises the various components of phytoplankton monitoring programmes and compares practice in the UK to other locations (based on evidence from literature and survey responses). The UK methodologies are generally appropriate and in line with international norms. Where UK practices differ from the norm, or where no accepted procedure exists, recommendations for change are made.

Table 1. Summary of monitoring methodologies

Process	Method **(from survey responses and literature review)**	Recommendation
Organisms monitored and enumerated	While some local variations occurs (e.g. *Ostreopsis* in Italy) most countries monitor the same core list of organisms. Majority of European countires carry out full enumeration of phytoplankton species in all site or ina selection of	List of organism enumerated in the UK is appropriate based on knowledge of HAB species currently present (with the caveat that *Azadinium* is not monitored). **Full counts at a number (3-5 UK wide) sentinel sites are recommended to give a better chance of**

	sentinel sites In the UK seems only HAB phytoplankton sepceis are fuly enumerated.	**identifying emerging toxic species.**
Pelagic Sample collection	Typically by Lund Tube Depth of sample collection is variable Some use of Niskin Bottles	Current UK methodology by Lund Tube is in line with international norms. Collection analysis of replicate samples would increase data accuracy and precision.
Benthic sample collection	Not widely undertaken Lack of an accepted standard protocol	The “invasion” of benthic harmful species is a possible threat. **It is therefore recommended that a method of sample collection capable of producing reproducible results is developed and**

		implemented at sentinel sites.
Sample fixation	Almost all monitoring programmes use Lugol's iodine	Current UK methodology (use of 1% Lugol's iodine) is in line with international norms. No clear benefit to the use of an alternative fixative. Heath and safety requirements would make the use of most alternative fixatives problematic for sampling officers and for posting samples.
Sample transport	Varies between post and collection/transport by monitoring laboratory	No evidence to suggest any benefits from changing current UK system of postal transport.
Sample enumeration	Utermöhl method is almost universally used	Current UK methodology of sample settling followed by light microscopy using Utermöhl method is in line with international norms.

		The method is fit for purpose for most species. Other microscope methods are inappropriate for low abundance HAB species. Biomass methods (e.g. satellite and/or fluoremetry) are inappropriate for low abundance HAB species. Cytometry bases counting technologies have promise but are not yet well enough developed for routine use. Most likely these will be used in combination with fluorescent molecular probes. Use of Lugol's fixed samples problematic with these instruments. Identification and enumeration of some organisms require molecular techniques (see below).
Molecular approaches	Used in some countries particularly for *Pseudo-nitzschia* and *Azadinium*	***Azadinium* monitoring is recommended.** These are not currently adopted in the UK although

		development is ongoing. Most appropriate method is QPCR, as currently applied in Ireland (and being developed for operation with FSA funding in the UK by SAMS). QPCR method can be applied on Lugol's fixed cells. **QPCR analysis of *Pseudo-nitzschia* to species at sentinel sites recommended.** **FISH (or single cell PCR) analysis of *Alexandrium tamarense* blooms recommended to determine group I/III composition and hence likely toxicity (method currently in development).** Other molecular methods (e.g. micro-arrays) have promise but still have significant technical challenges to overcome. No clear benefit over QPCR & FISH as

		yet.
Frequency of sample collection	This is almost universally weekly, with a reduction in frequency in winter in some countries.	Current UK methodology of weekly sample collection in summer is broadly in line with international norms, although winter sampling is more frequent in many other countries.
Use of the data	Web publication in some countries (e.g. Ireland, Norway, UK)	Web publication of counts as conducted in UK is not widespread, but occur is a number of other countries. No disadvantages to this can be found.

1.5 DISCUSSION OF IDENTIFIED KNOWLEDGE GAPS IN CURRENT MONITORING METHODS

Table 1 (Chapter 3, section 1.4) summarises UK monitoring practices in comparison to those elsewhere in the EU. In general the system in the UK is thought to be robust and generally in line with those in most other EU countries. However, a number of knowledge gaps exists these are highlighted below along with recommendations on how these might be addressed to reduce risk to the shellfish consumer.

1.5.1 Knowledge gaps:

1) A lack of monitoring of *Azadinuium* species (the causative organism of Azaspiracid poisoning). This is currently being addressed by a FSA funded research programme to develop molecular detection methods.
2) A lack of full phytoplankton counts to species capable of identifying new or invasive species spatially or temporally
3) A poor use of phytoplankton data to target flesh testing
4) A lack of an ability to identify blooms of *Alexandrium* and *Pseudo-nitzschia* to species to provide information on their potential to generate toxicity
5) An inability to adequately monitor harmful benthic species

1.5.2 Recommendations, in priority order:

1) Molecular techniques (QPCR) should be employed to enumerate *Azadinium* on a widespread basis.
2) Full phytoplankton counts to species level should be instigated at a number of sentinel sites UK wide. Approximately 5 sites co-incident with major shellfisheries and/or major ports are recommended. Analysis of these samples would reduce the risk of shellfish toxicity due to an unexpected and hence non-monitoried invasive HAB organism.

3) The use of collected phytoplankton data to better target shellfish toxicity testing (particularly outside traditional high risk periods) is recommended.
4) The use of molecular techniques (FISH or single cell PCR) is recommended to confirm *Alexandrium* and *Pseudo-nitzschia* identification during bloom events. In particular the distinction between blooms of toxic and non toxic ribotypes of *A. tamarense* is recommended.
5) A risk assessment should be conducted of locations that may have significant populations of benthic HAB species. Benthic sample collection should be instigated at these sites. Research into consistent methods for such benthic sample collection and subsequent analysis is required.

2. ASSESSMENT OF THE SUITABILITY OF EXISTING AND POTENTIAL METHODS FOR THE DETECTION OF NEW/EMERGING TOXINS AND IDENTIFICATION KNOWLEDGE GAPS WHERE FURTHER RESEARCH COULD BE FOCUSSED: SHELLFISH TOXINS

2.1 BREVETOXINS

2.1.1 Introduction

Brevetoxins belong to a group of heat-stable ladder-shaped polyethers along with other marine toxins such as ciguatoxin, maitotoxin and yessotoxin. Two distinct structural brevetoxin types (type A and type B) are defined according to their backbone structure (Lin *et al.*, 1981; Simizu *et al.*, 1986). The naturally-occurring PbTx-1 (BTX-1) and PbTx-7 are type A BTXs which possess 10 fused polyether rings whereas the more abundant PbTx-2 (BTX-2) along with PbTx-3 and PbTx-9 are Type B, possessing 11 fused polyether rings (Rossini and Hess, 2010). These parent algal toxins form a multiple of other naturally-occurring analogues and metabolic products have been identified (Abraham *et al.*, 2012; Baden *et al.,* 2005; Colman *et al.,* 2003; Plakas and Dickey, 2010; Otero, *et al.,* 2012). PbTx-1 and PbTx-2 which are found to dominate toxin profiles in the source algae are often not found in the shellfish flesh. Instead their metabolites, including a number of cysteine and taurine conjugates, are common metabolites in brevetoxin-contaminated shellfish (*e.g.* Bottein Dechraoui *et al.*, 2007; Abraham *et al.*, 2012). From data generated to date, it appears as though the Type-B toxins are most prevalent in shellfish, and therefore most important regarding detection and quantitation for food safety control purposes (Ann Abraham and Leanne Flewelling personal communication).

2.1.2 Animal bioassays

Whilst a fish bioassay has been used for assess the toxicity of algae and purified toxin extracts (Viviani, 1992), the mouse bioassay (MBA) remains the primary

bioassay for detection of NSP. The MBA has been used in affected regions for the assessment of shellfish toxicity, but alternative methods have been extensively investigated to address the performance issues of this test which include a lack of specificity and ethical issues (Dickey, *et al.*, 1999; Hokama, 1993; Poli, 2008; Plakas *et al.*, 2010). However, the MBA is still required until there is more detailed knowledge on all the BTXs causing actual intoxication and increased availability of certified standards and reference materials for the development, validation and implementation of non-animal alternatives. A standardised MBA procedure is generally used (based on APHA, 1970) which relates the time of mouse death following *i.p*. injection of diethyl-ether shellfish extracts. In the US shellfish showing toxicity by the MBA are considered unsafe, with a guidance level of 20 MU / 100g used to prohibit shellfish harvest. Disadvantages of this assay relate to its the inability to detect all BTXs, in particular the more polar metabolites, which have been found to contribute to the overall toxicity of both U.S and New Zealand shellfish (Dickey *et al.*, 1999; Nozawa *et al.*, 2003; Murata *et al.*, 1998). Modified protocols include the one used in New Zealand were instigated which incorporated acetone extraction and partitioning with dichloromethane (DCM) to aid in the detection of these polar metabolites. However, interferences in the assay from Gymnodimine resulted in the authorities reverting to the original APHA protocol (Fernandez and Cembella, 1995). The LC-MS/MS analysis of oyster samples from Rangaunu Harbour in New Zeland previously showing NSP MBA positive results showed no detectable concentrations of BTXs (LOD quoted as 10µg/kg).This further highlighted the potential specificity issues with the assay with authors concluding that OA-group compounds may have been responsible for the positive NSP MBA (McNabb *et al*., 2008).

The assay is still considered the primary tool for effective monitoring and will continue to be applied in this manner until replacement methods providing at least an equivalent level of protection have been formally assessed and validated.

2.1.3 Chemical methods

As with any chemical method involving quantitation of specific toxins or toxin groups, methods of analysis require determination alongside certified reference materials. To date the only commercially available reference material standards are for BTX-2 and BTX-3. As a result, and possibly as a consequence of the availability issue, no analytical methods have been validated formally through interlaboratory study.

Extraction

BTX analysis is complicated by both matrix interferences and metabolism (Wang *et al.*, 2004b). Requirements for successful extraction of BTXs prior to analysis are highly dependent on the nature of the samples under investigation and the physico-chemical properties of the BTX toxins present. BTXs produced by algae are lipophilic compounds, but extraction of all BTXs is made more complicated by the presence of hydrophilic shellfish metabolites (Plakas *et al.*, 2004; Wang *et al.*, 2004a) and also polar metabolites from aerosols (Abraham *et al.*, 2006)). PbTx-2 also appears to rapidly and irreversibly bind to or react with shellfish tissue components, resulting in poor recoveries of the toxin from spiked shellfish homogenates (Plakas *et al.*, 2002; Ishida *et al.*, 2004).

Extraction of BTXs in seawater has been achieved through trapping toxins on hydrophobic solid phase extraction sorbents and eluting with methanol (Pierce *et al.,*2003). Toxins in samples of marine aerosols have been collected with glass fibre filters in air sampling devices, with toxins extracted from filters using acetone (Pierce *et al.*, 2005). In addition to the di-ethyl ether extraction method prior to the MBA, both acetone and methanol solutions have been used for extraction of BTXs from shellfish, with the latter deemed more suitable for extraction of the more polar metabolites (Otero, *et al.,* 2012). 80% methanol solutions have been used for successful extraction of BTXs in shellfish implicated in NSP in New Zealand (*e.g.* Nozawa *et al.*, 2003). Other workers have reported the use of additional clean-up steps including the removal of neutral lipids from acetone extracts of oysters with

hexane and C18 SPE clean-up, plus the purification of oyster extracts with normal phase (silica) SPE to improve toxin recoveries (Wang *et al.*, 2005).

Conventional chromatography methods

Conventional chromatographic methods relying on spectroscopic detection or following functional derivatisation are not commonly reported, with HPLC and diode array UV detection mostly undertaken for fractionation of toxic extracts prior to confirmation by LC-MS (Poli et al, 2000). Methods have been developed for the HPLC-UV detection of BTXs on sorbent filters used to concentrate toxins present in aerosols and seawater (Pierce at al., 2003). Dickey *et al.*, 1992 reported the use of diethylaminocoumarin carbamate for the derivatisation of BTXs to facilitate HPLC-FLD detection. Analysis of diethylamino-coumarin carbamate BTX-3 showed two peaks corresponding to the two expected hydroxyl substitutions, with identities confirmed by mass spectral analysis.

Shea (1997) reported the use of Micellar electrokinetic capillary chromatography (MEKC) with laser-induced fluorescence (LIF) detection to measure four BTXs at trace levels. The method required pre-analysis derivatisation of BTXs with a terminal alcohol group to form highly fluorescent derivatives. The approach was applied to the analysis of PbTx-2, 3, 5 and 9 in cell cultures and fish tissue, providing excellent method detection limits of approximately 4pg/g.

LC-MS methods

A major focus for development of analytical instrumentation methods has been on the use of LC-MS methodologies given the high degree of specificity they provide (Baden *et al.,* 2005). To date LC with electrospray mass spectrometric (LC-ES-MS) and tandem mass spectrometric (LC-MS/MS) methods have been used extensively for the identification of BTXs in algae, fish and shellfish (Abraham *et al.*, 2012; Dickey *et al.*, 1999; Hua *et al.*, 1995, 1996; Hua and Cole, 2000; Ishida *et al.*, 2004 and 2006; Nozawa *et al.*, 2003; Plakas *et al.*, 2002 and 2004; Plakas and Dickey, 2010; Poli *et al.*, 2000; Wang, 2007; Wang *et al.*, 2004a; Wang and Cole, 2009).

With the use of LC-MS and LC-MS/MS detection methods, highly specific identification of individual BTX congoners can be successfully conducted, given the availability of appropriate analytical standards. Even without standards, published data on expected diagnostic parent and product ions could potentially be used a screening tool for BTXs in shellfish, although no performance data would be available when taking such an approach.

Wang *et al.* (2004a), Plakas *et al.* (2004) and Abraham *et al.* (2006) all described the use of LC-MS for analysis of BTX metabolites present in oysters using reverse-phase chromatography with selected ion monitoring in positive mode. Dickey *et al.* (2004) reported good performance of an LC-MS method used in a thirteen laboratory collaborative study for the analysis of oysters spiked with PbTX-3 (mean recovery = 78%). Results compared well to those generated by ELISA and the MBA. However, results were not so good for the measurement of naturally contaminated shellfish containing BTX metabolites, resulting in poor between lab variability. Pierce and Henry (2008) also described the use of full scan LC-MS with a single quadrupole detector for the determination of BTXs in water, air and shellfish samples.

Plakas *et al.* (2002) reported the use of both LC-MS/MS and accurate mass measurement for the identification of toxin metabolite structures. Ishida *et al.* (2004) and Nozawa, *et al.* (2003) both described the use of selected reaction monitoring (SRM) LC-MS/MS analysis for a range of specific BTXs. They applied this method to the determination of toxin profiles in cockles, mussels and oysters implicated in an NSP event in New Zealand. Most recently, the technique has been used for the characterisation of BTX metabolites in hard clams exposed to blooms of *Karenia brevis*, confirming the absence of the principal algal toxins (PbTx-1 and PbTx-2) and the presence of a range of metabolites including products of oxidation, reduction, hydrolysis and amino acid/fatty acid conjunction (Abraham *et al.*, 2012). These results returned from these methods have been found to correlate well with those determined by the NSP bioassay and the ELISA method.

Generation of important BTX and BTX metabolite standards is continuing (Selwood *et al.*, 2008) including the availability of six different BTXs and metabolites from Cawthron Institute in purified form (Selwood, personal communication). It is likely that future developments will enable the formal validation of suitable LC-MS methodology for the quantitation of BTXs in shellfish. Work is still ongoing to evaluate the use of BTX biomarkers in further developing LC-MS/MS methods for monitoring BTX exposure and toxicity in shellfish (Abraham *et al.*, 2012). In New Zealand, routine monitoring of BTXs by LC-MS is ongoing and quality control procedures have been developed including recovery and calibration slope controls (McNabb, 2011). In 2012 Cawthron published a single-laboratory validation of a LC-MS/MS method for six BTXs (PbTx-3, BTX-B5, S-desoxy BTX-B2, BTX-B2, PbTx-2 and BTX-B1) in four species of shellfish, including mussels, oysters and clams. Recovery and precision appeared acceptable for the majority of toxins and the method provided good levels of sensitivity (LOD and LOQ) and ruggedness to experimental deviations (McNabb et al., 2012a).A low number (n=4) of natural samples contaminated with BTXs prevented the thorough comparison of LC-MS/MS results against the NSP MBA, although the mean ratios between the test results were used to propose a conservative regulatory action of 0.8mg/kg BTX-2 equivalents.

2.1.4 Biomolecular methods

The development of a wide range of biomolecular detection methods including both functional and biochemical assays has been shown to be applicable to a variety of sample types. Methods applied to BTXs include a cytotoxicity assay, receptor binding assay and immunoassays. Given the modes of action, some of the assays are applicable to the detection of more than one group of marine toxins (e.g. Bottein Dechraoui *et al.*, 2005b).

Cytotoxicity assay

The cytotoxicity or neuroblastoma (N2A) assay is a sensitive and useful screening tool for detecting BTXlike activity (sodium channel enhancers) in extracts of shellfish (Manger *et al.*, 1993). It is based on the actions of BTXs, as well as ciguatoxins, on voltage gated sodium channels. The response of the assay reflects the mixtures of BTXs and has been used for examining cytotoxic fractions of shellfish extracts (Plakas *et al.*, 2002; Wang *et al.*, 2004a; Murata *et al.*, 1998). Whilst some authors describe that neuroblastoma cells are used due to the high numbers of sodium channels in their cell wall (Campas *et al.*, 2007), their advantages may relate more to practicalities and ethics. These are cancer cells, easily grown and maintained as secondary cultures, so there is no ongoing requirement for primary culture nerve cells, use of which requires animal sacrifice (Hungerford, personal communication). Whilst the assay cannot distinguish between individual BTXs, the assay is more sensitive to less polar BTX metabolites (Dickey *et al.*, 1999) and the relative sensitivity of the assay to three separate metabolites has been found to parallel the relative sensitivity determined by the MBA (Bottein Dechraoui *et al.*, 2010). Issues have been noted in the past with use of the assay for shellfish toxicity testing due high variability in an interlaboratory study, albeit involving a low number of laboratories (Dickey *et al.*, 2004). Matrix effects and a poor correlation with the BTX MBA in contaminated oyster samples have also been described (Plakas *et al.*, 2008), with differences in assay performance remaining unexplained. The method requires long incubation times, particularly when needing to achieve high sensitivity of analysis. Given the applicability of the assay to detection of CTX and the higher sensitivity of the assay for CTX as compared to BTXs, potential specificity issues with the assay have been noted (Bottein Dechraoui *et al.*, 2005b). However, in practice this is not an issue given that these two toxin groups are not found in the same organisms i.e. NSP being found mostly in shellfish as opposed to CFP in fish. Flexibility could be an advantage, as with minor modifications the assay can be utilised for testing for both sodium channel blockers (saxitoxin and tetrodotoxin) and activators (BTX and CTX) (Poli, 2008). Overall, whilst the method provides a useful and sensitive tool, it was not deemed an appropriate replacement for official control monitoring in place of the MBA by some authors (Dickey *et al.*, 2004; Campas *et al.*,

2007). However, with further experience in the method and with routine protocols in place for passaging the assay cells there could still be potential for further developments in this area, whilst the assay certainly remains an effective research tool.

Receptor binding assays

This is the simplest of the pharmacology-based assays which measures BTXs in shellfish extracts by competitive displacement of a tritium-labelled BTX (^{3}H-PbTx-3) from sodium channel binding sites in isolated rat brain membranes (Trainer and Poli, 2000) or whole cell preparations (Bottein Dechraoui *et al.*, 2007). The method is a relatively simple, sensitive and rapid tool for BTX analysis. Its performance has been demonstrated through an interlaboratory study on shellfish samples containing multiple BTXs, and it is considered a potential option for replacement of the MBA (Dickey *et al.*, 2004). Whilst the method is also applicable to ciguatoxins, it has been found to be 3-24 times more sensitive to BTX analysis than for ciguatoxins (Bottein Dechraoui *et al.*, 2005b). The binding affinity of BTX metabolites has been investigated and found to be variable depending on the specific toxin (*e.g.* Bottein Dechraoui *et al.*, 2010), with more polar metabolites showing ten times less the affinity for the receptor as compared with PbTx-3. Membrane preparations from animal tissues are required but whilst the use of the radiolabel can be perceived as a disadvantage, the technique is becoming more popular particularly as a similar assay has become an official method of analysis for saxitoxins (AOAC Official Method 2011.27; Anon, 2011). Another noted disadvantage is the presence of matrix effects in the assay (Whitney *et al.*, 1997). An RBA with a high throughput format using microplate scintillation technology has also been described (Van Dolah *et al.*, 1994) which allows parallel assay completion within 3 hours.

More recently still, a competitive fluorescence-based binding assay was reported for study of the inhibition of binding at the BTX receptor in rat brain synaptosomes. The authors reported a rapid assay (< 3 hours), applicability to both Type A and Type B BTXs whilst removing the need for radiolabelled materials and potentially providing another area of research for continued developed of RBAs (McCall *et al.*, 2012).

Immunoassays

Immunoassays are structure-based in vitro methods which are found to be highly specific and sensitive detection methods (Plakas and Dickey, 2010). They are also applicable to field monitoring scenarios and allow high throughput analyses. A competitive radioimmunoassay (RIA) was originally developed to detected PbTX-2 and PbTx-3, with a limit of detection of 1 nM (Trainer and Baden, 1991). A sensitive and specific radioimmunoassay was also developed by Poli and Hewetson, 1992, specific for PbTx-2 toxins with no cross reactivity to type 1 and an improved limit of detection of 0.3 ng/mL (Poli, 2008). However, these are now no longer being developed due to issues relating to disposal of radioactivity (Campbell *et al.*, 2011).

Since this time, work has continued predominantly with the preparation of specific monoclonal antibody enzyme-linked immunosorbent assays (ELISA). These assays bind toxic BTXs and non-toxic derivatives with the same level of activity and work has shown that more than one antibody would be required for detecting the full range of BTXs. Whilst the antibody is highly specific to the B-type BTXs it showed low cross reactivities with A-type BTXs, it also showed low cross reactivity to other marine toxins which may have interfered with the assay (Naar *et al.*, 2002). It is also noted that similar immunoassay responses will be found with all other B-type toxins (Naar *et al.*, 2002: Bottein Dechraoui, *et al.*, 2007). The NSP ELISA developed by the University of North Carolina Wilmington (UNCW) and described by Naar *et al.*, 2002, was found to provide high sensitivity (0.025 mg/kg) analysis of BTXs in shellfish, seawater and clinical specimens. It was also found to have no issues in relation to matrix interferences, even without any form of pre-treatment, dilution of purification. The collaborative study of Dickey *et al.* (2004) showed that this assay performed well for the detection of BTXs in contaminated oysters, with results correlating well with those from the MBA and with LC-MS (Plakas *et al.*, 2008). This ELISA has been validated in-house and proposals have been put in place for AOAC validation by collaborative study and adoption of the method as a Type 1 National Shellfish Sanitation Program (NSSP) analytical method to replace the MBA for NSP monitoring (Proposal 07-104). However, this validation was never realised and the kit remains commercially unavailable as a testing tool.

Maucher *et al.* (2007) described another ELISA developed in New Zealand incorporating anti-PbTx-2 antiserum and demonstrated excellent cross reactivities for B-type toxins in relation to PbTx-3. This was found to have a working range of 90-2100 pg PbTx-3 eq./mL and recoveries for PbTx-3 and two metabolites varying between 58% and 87%. The assay was successfully applied to the analysis of BTXs in dolphin blood. Bottein Dechraoui *et al.* (2010) concluded that the same ELISA provided the most sensitive bioassay for certain BTX metabolites (dihydroBTX-B and BTX-B2) but not for others (N-palmitoyl-BTX-B2).

More recently a faster commercial NSP ELISA assay has been made available (Abraxis) based on the work originally published by Maucher *et al.* (2007) which is a quantitative and sensitive assay applicable to both water and shellfish samples. The relatively simple and cost-effective test is also a direct competitive ELISA based on the recognition of BTX by specific antibodies, with detection of colourimetric changes in plates with a 96 well format. Whilst providing a quantitative result by quantitation against PbTx-3 standard, the manufacturers note this is a screening tool and positive results should ideally be confirmed by a suitable alternative method. The specificity of the test has been demonstrated for to exclude cross reactivity with a wide range of organic and inorganic compounds. Single laboratory validation of the assay has been conducted for acceptance of the method for use in the NSSP. Toxin recovery is quoted as 86% for water and 104% for shellfish samples, with a working linear range of 0.5 to 100ng/g in shellfish extract. The acceptable ruggedness and repeatability of the assay has also been demonstrated. The specificity of the assay is thought to be similar to that of the UNCW ELISA, being high for type-B toxins (83% to 133% relative to PbTx-3) although lower for PbTx-6 and PbTx-1 (13% and 5%) (ISSC, 2009). It is hoped that a multi-laboratory validation will be conducted within the next few years, incorporating this ELISA amongst other confirmatory techniques (Abraham, personal communication), with the target of recognition by the ISSC within the next two years (Rubio, personal communication). A note of caution relates in general to the use of commercial test kits for toxin testing, with the potential for test kits to vary between production batches and/or for the manufacturers to change performance characteristics. Such changes could potentially compromise their use

for reproducible analysis of shellfish for toxin activity. Currently there are no ELISAs available containing an antibody mix that determines both BTX structural types equally well. Overall, these assays are useful as screening tools, but cannot be used for the determination of sample toxicity when unknown profiles of toxins are present. Therefore other confirmatory methods are required for full quantitation of toxicity or for the determination of BTX profiles in shellfish.

Biosensor methods

In 2009, researchers published work describing the application of a Surface Plasmon-Resonance detection method for PbTx-2 together with a range of other ladder-shaped polyethers including YTXs (Mouri *et al.*, 2009). The method uses one specific YTX (desulfated-yessotoxin; dsYTX) which is immobilised on a sensor chip. The technique involves the detection of the ability of analytes to inhibit the binding of phosphodiesterase II to the immobilised dsYTX. Detection was successful for PbTx-2 as well as YTX and dsYTX itself. Dose-response curves were generated for PbTX-2 enabling the confirmation of half inhibitory concentrations in the low µM range. Whilst demonstrating potential, there are clear specificity issues given responses from potentially many different ladder-shaped polyethers, and the technique has not been tested in samples of water, culture of shellfish (Vilarino *et al.*, 2009b). A major advantage with biosensor methods is the inherent sensitivity, enabling the dilution of matrix effects which can be an issue. The equipment itself can be very expensive and probably impractical for high throughput monitoring unless used for a large number of different tests. This is heightened through the need to either buy kits from manufacturers or to develop binders and chips in-house.

Other biosensor methods reported for BTXs and potentially useful as research or screening tools (Campbell *et al.*, 2011) include one consisting of a screen printed electrode system for electrochemical immunosensor detection which enabled detection of PbTx-3 with an LOD of 1 ng/mL (Kreuzer, *et al.*, 2002). Kulagina *et al.* (2006) also reported the use of a neuronal network biosensor (NNB) for the detection of PbTx-3 in both solution and diluted seawater. Although the method was found to provide good sensitivity of detection (0.296 and 0.430 ng/mL in buffer and diluted

seawater respectively), there was a lack of specificity given a twenty-fold increase in method sensitivity for the detection of saxitoxin.

Table 2. Summary of methods applicable to the determination of BTXs in shellfish.

Method	Advantages	Disadvantages
Mouse bioassay (MBA)	• Primary tool for toxicity assessment • History of use and prevention of sickness	• Inability to detect all BTXs • Ethical issues • Variable performance
Cytotoxicity assay	• Sensitive functional assay • Use of cultured vs primary cells	• Matrix effects, high variability • Poor correlation with MBA • Noting limited data on performance characteristics of method • Time consuming
Receptor binding assays (RBA)	• Simple, sensitive, rapid • Good performance in collaborative study • Promising fluorescence-based binding assay	• Variable affinity for BTX metabolites • Requirement for animal tissues and radiolabel • Matrix effects • Limited development to date with fluorescence-based binding assay
ELISA	• Specific for type-B and sensitive • High throughout, fast turnaround and “in the field” • Low matrix effects • Good correlation with MBA and LC-MS • Good single lab validation and multi-lab study anticipated	• Lower cross reactivity for A-type BTXs • Screening tool only – no toxicity or profile data provided • Valuable quantities of toxin required to produce antibodies • Potential issues with commercial kits, with manufacturers changing properties or performance characteristics
Conventional chromatography	• Use of MEKC-LIF, LC-UV and LC-FLD reported • Some degree of specificity	• Very limited data available for determination of low numbers of toxins • Lack of standards and equipment • Proof of concept required for all

		appropriate toxins
LC-MS(MS)	• Highly specific • Sensitive • Single laboratory validation performed (NZ)	• Expensive instrumentation • Lack of all suitable standards
Biosensor methods	• Useful research screening tools • High sensitivity • Matrix effects can be diluted	• Lack of specificity • Expensive instrumentation for biosensors

2.1.5 Suitability of existing and potential methods for BTX testing

A more attractive approach for determination of BTX sample toxicity could potentially be the application of one of the biomolecular methods described. The receptor binding assay is gaining popularity for the determination of PSP in shellfish samples and given its status as an Official Method of Analysis following AOAC validation, the application of the BTX RBA method is a potential way forward. However, with the BTX method not yet shown to be truly effective for the full range of BTX congoners and the systems not currently in place within the UK official control testing regime, this would be a long term developmental process. Similarly the cytotoxicity and biosensor methods whilst showing great potential are not yet in a position to be considered as potential replacement methods given specificity issues and other conclusions drawn by researchers relating to their use as official control monitoring tools. They remain however potentially effective research tools.

The ELISA methods have been shown to provide a sensitive determination of B-type BTXs in particular, with good specificity in terms of a lack of cross reactivity to other marine biotoxins. Whilst the response to Type-A toxins is significantly lower, the response is still there at useful concentrations. Currently there are no ELISAs reported containing an antibody mix responding to both toxin types equally. The methods also appear to have limited effects from matrix components present in shellfish extracts, as well as seawater and clinical samples. With the assay appearing to correlate well for type-B toxins and metabolites with results determined

using LC-MS and the brevetoxin MBA in a variety of shellfish species, this could be a good candidate for potential future methods. Whilst the original ELISA was developed and validated by the University of North Carolina Wilmington (UNCW), the test does not appear to be offered commercially. Consequently the Abraxis ELISA is the one commercial test available which could potentially be tested, validated with suitable shellfish samples and potentially implemented given the determination of appropriate performance in collaborative study at some stage in the future.

Regarding chemical methods, conventional chromatographic detection methods have been reported but there is limited published data on method performance to make these appear viable approaches for further investigations. LC-MS methods, however, provide a more promising alternative approach. The high specificity and sensitivity of the technique together with the increasing availability of some of the important BTX standards, makes this a good choice for future investigations. Given the presence of this technology at both of the official control laboratories within the UK monitoring programme, this could offer a potential applicable and cost-effective solution for future assessment and validation.

Overall it is recommended that both chemical and biomolecular methodologies should be investigated. Based on the information available in the literature at present and from discussions with leading researchers, the ELISA and LC-MS methods appear to be the most applicable for further investigation and potential development for application to official control monitoring in the UK. Depending on the scale of the testing, a first stage screen with a suitable commercial kit which has been validated for the samples of relevance, followed by confirmation by LC-MS/MS currently appears to be the most suitable option. However, two important developments would still be required. Firstly to improve the response of the ELISA methods for type-A brevetoxins and secondly to ensure production of certified brevetoxin reference standards to facilitate the development and validation of accurate quantitation methodologies.

2.1.6 Identification of knowledge gaps with regards to BTXs which might be addressed through further research or method development

The knowledge gaps relating to research requirements for prevalence and detection of BTXs in shellfish are currently wide. These include:

- Identification of additional algal species which produce BTXs, in particular those found to grow well in water conditions relevant to the UK at present or in the future
- Analysis of algal cultures by suitable methods for assessment of presence of BTXs in water samples
- The determination of shellfish species that accumulate BTXs and associated depuration rates
- The determination of BTX metabolites by LC-MS profile studies in relevant bivalve species
- Continued evaluation of MBA-replacement methods, in particular including ELISA and LC-MS
- Develop understanding of BTX and BTX metabolites toxicity in relation to human exposure, including long term assessment of intoxicated people to determine potential long term affects

From the above and in the context of risks to UK waters and shellfish, the first stage would be the identification of whether the blooms with potential for BTX production occur in the UK. The next step would be the development and application of methods for the identification of toxins in harvested phytoplankton. Knowledge regarding the producers and parent toxins will then enable studies to be conducted on the uptake of these toxins into shellfish species of relevance to the UK shellfish industry. This could include both the sampling and analysis of naturally-contaminated shellfish from areas of algal blooms and/or laboratory uptake and depuration studies on shellfish fed with mass cultured phytoplankton species. With suitable contaminated materials, methodologies would need to be assessed and developed in relation to UK shellfish matrices. From the review conducted here, it is proposed that this should consist of the assessment of both ELISA screening tests and LC-

MS/MS confirmation. With suitable methods in place, further confirmatory studies would need to be conducted to assess patterns of metabolism in UK shellfish, thereby determining the most prevalent toxins accumulating in shellfish flesh and the most suitable laboratory protocols for determining and quantifying their presence. Ultimately any such methodologies would need to be formally validated to determine method performance characteristics in relation to UK shellfish species.

2.1.7 Proposed options for routine monitoring of BTXs to meet legal requirements

Currently there are no regulatory limits for BTXs in shellfish or fish in Europe. In other parts of the world including the USA, New Zealand and Australia, maximum permitted levels have in practice been set at 20 mouse units (MUs)/100g shellfish flesh. Expressed in BTX-2 (PbTx-2) equivalents this is equivalent to 0.8 BTX-2 eq./kg. Without regulatory limits set in EU legislation, one potential approach would be to adopt the regulations utilised in those regions currently conducting active monitoring for BTXs. Based on the evidence gathered during this review, an effective approach for routine monitoring is likely to include:

- Screening of shellfish samples using a suitable assay, such as the ELISA
- Application of a suitable quantitative confirmation assay. Removing the MBA as an option due to ethical considerations, the strongest recommendation is for application of a confirmatory LC-MS/MS method for the quantitation of BTXs in samples determined as positive by the screening tools employed

In order to meet legal requirements associated with official control monitoring of bivalve molluscs, each of these assays would need to undergo a series of validation studies to determine full performance characteristics of the method. In addition the performance would need to be demonstrated as being able to provide at least the same level of effectiveness as the MBA. An important part of this would be the refinement of the ELISA screening method to improve the response of the assays towards the type-A brevetoxins. Similarly, successful and fit-for-purpose quantitative LC-MS/MS methods could only be safely implemented following validation of the

methods through use of certified reference standards. Both these developments would be recommended to enable the legal requirements of brevetoxin analysis to be met. With a full collaborative study likely in the near future for both the ELISA and LC-MS/MS methods, a suitable level of interlaboratory validation will hopefully be in place to enable implementation of the method, providing there is evidence for acceptable method performance in UK shellfish demonstrated through additional single laboratory validation studies. Any such developments would need to be reviewed and the stakeholders consulted as deemed appropriate by the UK competent authority.

2.2 PALYTOXINS

2.2.1 Introduction

The initial chemical structure of PlTX was elucidated by two different groups working independently (Uemura, 1981; Moore, 1981). PlTXs are large, heat stable organic molecules made of long, partially unsaturated aliphatic chains interspaced with cyclic ethers, 64 chiral centers, 2 amide groups and 40-42 hydroxyl groups depending on the molecule (Tan, 2000). Despite more than 10^{21} potential stereoisomers for PlTX (Patockaa, 2002), the synthesis of a stable carboxylic form was completed in 1989 (Armstrong, 1989) which later lead to the synthesis of the compound (Suh, 1994).

Different PlTX have been described depending on their origins. A PlTX with a molecular weight of 2659 was isolated from Tahitian *Palythoa sp.* and 2 isomeric hemiketals with a molecular weight of 2677 were isolated from Hawaiian *P. toxica* (Moore, 1981). A compound with the same molecular weight and structure as one of the hemiketal isomers was isolated from Japanese *P. Tuberculosa* (Uemura, 1981). Four minor PlTXs were subsequently isolated from *P. Tuberculosa* (homopalytoxin, bishomopalytoxin, neopalytoxin and deoxypalytoxin) and their structures elucidated (Uemura, 1985). PlTX was also found to be present in *P. vestitus* from Hawaii (Wiles, 1974), in *P. caribaeorum* (C-PlTX) (Béress, 1983), *P. mammilosa* and *Zoanthus sp.* (Gleibs, 1995) from the Caribbean sea and *P. caesia* from the Pacific (Gleibs, 1999). In the same work, Gleibs et al. reported PlTX presence in animals,

including mussels, living near the zoanthid colonies and its spread along the coral reef food chain.

Other reports include the occurence of PlTX in two species of Xanthid crabs from the Philippines (Yasumoto, 1986), in several species of fish (Fukui, 1987; Noguchi, 1988; Kodama, 1989; Onuma, 1999; Taniyama, 2003), in the sea anemone *Radianthus macrodactylus* (Mahnir, 1992) and in *Platypodiella sp.* crustaceans living in close proximity with *Palythoa* colonies (Gleibs, 1995).

Several PlTX-like substances have since been described including a fluorescent structural isomer of PlTX from a xanthid crab living in coral reef beds called Lophozozymus pictor (Lau, 1995). PlTX analogues have also been isolated from dinoflagellates of the genus Ostreopsis and were reported in Ostreopsis siamensis (Usami, 1995; Rhodes, 2002). Ostreocin-D was isolated from O. siamensis (Ukena, 2001), mascarenotoxin-A, and –B from a sample of O. mascarenensis also containing a few cells of O. ovata (Lenoir, 2004), ovatoxin-a from O. ovata (Ciminiello, 2008), 42-hydroxy-palytoxin (42-OH-PlTX) from Palythoa subspecies (Ciminiello, 2009) and mascarenotoxin-C, ovatoxin-b, -c, -d, -e and -f from O. ovata (Rossi, 2010; Ciminiello, 2011a).

2.2.2 Mouse bioassay.

The MBA for PlTX is based on the neurotoxic effect of the extract. The toxicity is determined in relation to the death time of the animal following *i.p.* injection. The MBA symptoms have been described in detail (Riobo, 2008a) and are thought to clearly differ from the symptoms triggered by the more common marine biotoxins in particular within the first 15 minutes of the assay.

The EU harmonised protocol for analysis of DSP toxins in shellfish by MBA does not extract PlTX group toxins efficiently (EFSA, 2009). Different methods have been used for the extraction of PlTXs from shellfish prior to mouse bioassay (Yasumoto, 1978; Teh, 1974; Taniyama, 2002) some of them using hepatopancreas and others

using whole flesh. Following *i.p.* injection, the observation period can be as different as 4 (Fukui, 1987; Tan, 2000), 24 (Alcala, 1988; Onuma, 1999; Riobo, 2008a) and 48 hours (Rhodes, 2002; Taniyama, 2002 & 2003).

In the bioassay proposed by Teh (1974), the toxin amount in mouse units (MU) could be calculated using the equation provided and the death time in minute. This was applied to the detection and quantitation of PlTXs in crabs (Yasumoto, 1986; Alcala, 1988). However, the results of PlTXs analyses in the MBA vary between laboratories depending on the mouse strain, gender and weight of the animals rendering the assay unsuitable for quantitative analysis of the toxin (Vale, 2008). Riobo et al highlight in their study (Riobo, 2008a) a high death time variability and a death time overlap for different concentrations thereby confirming the unsuitability of the assay as a quantitative tool. In the same work, Riobo et al describe the LD_{50} value for PlTX as 294.6 ± 5.384ng/kg and states that it could potentially be used as a semi-quantitative assay although the dynamic concentration range would be restricted to concentrations less than 375ng/kg with a limit of detection (LOD) around 250ng/kg.

The MBA could be used as an investigative tool for the determination of the causative agents responsible for a poisoning outbreak (EFSA, 2009) as it provides a direct measure of toxicity and the symptoms are quite specific to the PlTX group. However, it presents all the drawbacks associated with animal bioassay including possible extraction interferences (toxins and other compounds soluble in the aqueous phase) and ethical issues.

2.2.3 Chemical methods

A number of chemical methods are available for the analysis of PlTX and PlTX-like compounds. High performance capillary electrophoresis (HPCE), HPLC-UV or HPLC-FLD and LC-MS have been developed and used in the various matrices studied over the years. Some of them are more suited to the analysis of shellfish matrices but none of them have been validated.

Extraction

PlTX group toxins are insoluble in chloroform, ether and acetone, sparingly soluble in methanol and ethanol and soluble in pyridine, dimethyl sulfoxide and water (Moore, 1971). In the same work, 70 % aqueous ethanol was used for extraction of PlTX prior to isolation and purification. Ethanol and methanol, potentially mixed with varying proportion of water, have been the main extraction solvents for PlTXs. Following a recent study on extraction recovery in mussel digestive gland (Amzil, 2012), methanol/water (90/10) was shown to be the most effective solvent. Acetone has also been used for the initial extraction. Depending on the matrix and the analysis procedure, partitioning using hexane and butanol may take place potentially followed by SPE.

To date there is a lack of harmonised extraction procedure for PlTX group toxins and no extraction method has been the object of a validation programme mainly because of limiting factors such as the lack of standards and contaminated material.

Chromatography methods

HPCE

A sensitive HPCE method was developed for identification and detection of PlTX (Mereish, 1991). It uses UV detection and the UV spectrum shows the two chromophores at 230 and 263 nm expected for PlTX. The LOD was estimated at 0.5 pg/injection. The work was carried out in PlTX solutions but the methodology is not suitable for the analysis of shellfish due to strong matrix effects (EFSA, 2009)

HPLC-UV

Due to the presence of two chromophores in its molecule, the UV spectrum for PlTX shows two UV-absorption peaks at 233 and 263 nm with a ratio between both peaks of 1.71 (Moore, 1971). This characteristic UV absorption profile can be used to verify the presence of the toxin in samples. HPLC-UV methods have been widely used for the detection of PlTX-group toxins mainly in *Ostreopsis* extracts with different combination of column and mobile phases (Mereish, 1991; Lenoir, 2004; Oku, 2004; Riobo, 2006) although, besides the method used during purification of PlTX from crabs and reported by Yasumoto et al. (1986) there are no reports of PlTX quantitation in shellfish using HPLC-UV (Riobo, 2012). The reported LODs range

from 0.1 to 2 µg injected and are much higher than the LODs reported for other chemical methods.

HPLC-FLD

In an attempt to improve on the sensitivity of the HPLC-UV method, a pre-column derivatization method was developed to detect and quantify PlTX analogues in *dinoflagellates*. In order to eliminate matrix interferences, a clean up step was developed and included (Riobo, 2006). The method LOD for derivatized PlTX was 0.75 ng standard injected.

The method is also suitable for detection of PlTX-group compounds in shellfish (Riobo, 2011). However, the LOD and LOQ of the method in shellfish are currently unavailable.

LC-MS methods

A number of different LC-MS methods have been used for the analysis of PlTX-group compounds in seawater and phytoplankton (Lenoir, 2004; Penna, 2005; Ciminiello, 2006 & 2008; Riobo, 2006; Ciminiello, 2013). More recently, LC-MS analysis work carried out on mussels, sea urchins and anchovies (Ciminiello, 2011b) and on the digestive gland of mussels and wedge clams as well as on whole tissue of Manila clams and sea urchins was reported (Amzil, 2012). The recovery in this body of work was matrix dependent and ranged from 75 to 115%. The LOQs in whole sea urchin tissue was 25 µg/kg and 23 µg/kg in whole mussel tissue (Amzil, 2012). The values obtained in this study did not include the efficiency of the extraction procedure.

2.2.4 Biomolecular methods / Functional assays

Biomolecular assays applied to the PlTX group can be classed into different categories; i.e. the procedures using cell death, those using antibodies and those using the binding site.

Cytotoxicity assay

Potassium release

PlTX has been shown to cause a rapid release of potassium (K^+) from cells. It preceeds the haemolysis and appears to be the primary cytotoxic action (Tan, 2000). The amount of K^+ released is concentration dependent and can be measured by K^+ selective electrode, a flame photometer or an atomic absorption photometer. The sensitivity of the method is approximately 1pM using rat and human erythrocytes (Habermann, 1989; Tan, 2000).

Assays using cell lines

These assays are based on the morphologic changes caused by the toxin, usually allow detection of PlTXs in the picomolar range (Riobo, 2011) and can be used as an alternative to animals. In the presence of toxin, cells are monitored for the characteristic morphological changes and cell damage. Quantitation is achieved through the combined use of dyes and spectrophotometry.

Several types of cells have been used in cytotoxic assays including rat 3Y1 cells (Oku, 2004), MCF-7 breast cancer cell line (Bellocci, 2008; Sala, 2009; Simone, 2011), neuroblastoma cells (Cañete, 2008; Espiña, 2009; Ledreux, 2009).

The assay developed by Bellocci et al (2008) has been tested for PlTX and ostreocin-D on mussels and sea-urchins. Its specificity was demonstrated using other toxins and its LOD for PlTX-group toxins was estimated in the region of 10 ng PlTX/kg shellfish tissue (EFSA, 2009).

The cytotoxicity assays using the neuroblastoma cells include ouabain pre-treatment. The assays developed by Cañete et al. (2008) and Ledreux et al. (2009) both use Neuro-2a cell-based bioassays and the same agent to measure the mitochondrial oxido-reductase activity. The assay developed by Espiña et al. (2009) uses BE(2)-M17 human neuroblastoma cells and add a different agent to monitor the same activity. The estimated LOD of these assays is in the region of 50 µg PlTX/kg shellfish tissue.

Haemolysis Assay

PlTX is a potent but relatively slow haemolysin in mammal erythrocytes. It induces prelytic release of potassium that eventually leads to haemolysin (Habermann, 1981). This property has enabled the development of a haemolysis assay as the amount of haemoglobin released was found to be dependent upon the incubation time and the PlTX concentration. The concentration of the PlTX-like toxin in an unknown sample can be determined by incubating erythrocytes with a number of PlTX standards at different concentration levels and measuring the haemoglobin released from the cells after a fixed period. The haemolysis evolution is monitored by measuring the absorbance at 540-595 nm. The subsequent use of ouabain, a glycoside binding to the sodium pump in the same place as PlTX and therefore a direct inhibitor of PlTX, can demonstrate the specific presence of PlTX-like activity in the sample.

To improve the stability and reproducibility of the response a variant of the original haemolytic assay was developed (Riobo, 2008b) involving sheep erythrocytes, operation at a moderate temperature (25°C) and partial inhibition of the PlTX by ouabain. A 18-24 hours incubation time is recommended and the absorbance should be measured at 405 nm. Although the LOD for this assay would vary with the origin of the erythrocytes, a value in the region of 0.5 pg is thought to be achievable (Riobo, 2011)

Immunoassays

Several antibody-based methods have been used for the detection of PlTX-group toxins. A radioimmunoassay (RIA) was developed and consisted in labelling PlTX with ^{125}I-Bolton-Hunter reagent bound specifically to rabbit anti-PlTX antibody. The extent of binding increased progressively with repeated immunizations. The assay was sensitive since only 0.27 pmoles of unlabelled PlTX was required for 50% inhibition of binding in the ^{125}I-PlTX-anti-PlTX reaction. MTX, teleocidin, okadaic acid, debromoaplysiatoxin and 12-O-tetradecanoylphorbol-13-acetate at concentrations 10–100 times higher than PlTX did not affect binding so the assay appeared to be specific to PlTX (Levine, 1988). The method is unable to distinguish between biologically active and inactive PlTX (Tan, 2000) and its potential for application to other PlTX-group compounds is not known.

Subsequently a sandwich ELISA where five PlTX-specific ELISA for quantitation of PlTX in crude extracts of P. tuberculosa were developed (Bignami, 1992). The detection limit was estimated at 10 pg PlTX for the sandwich ELISA but the authors reported the possible occurrence of matrix interference. Recently, a competitive ELISA method using anti-PlTX phage antibodies was developed by means of phage display technology to rapidly isolate single-chain antibodies directed against PlTX (Garet, 2010). The analysis was applied to samples of mussels and clams and although matrix effects were present, they were minimised by dilution. The apparent recoveries were calculated and were between 64 and 113% (mean 90%) in mussels which seemed acceptable. However, the apparent recoveries in clams were quite high between 84 and 181% (mean 131%). The method detected PlTX specifically with a LOD of 0.5 pg/mL.

With the possible exclusion of the RIA, the immunoassay methods are fast, easy to use and may be applied to enable screening of a large number of samples. However, they do not provide any information on toxin profile, the anti-bodies are not always readily available and the cross-reactivity is not necessarily an indication of toxicity.

Fluorescence polarization

Fluorescence polarization (FP) is a spectroscopic technique based on exciting a fluorescent molecule with plane-polarized light and measuring the polarization degree of the emitting light. The FP detection method for PlTX is based on the interaction between the Na^+, K^+-ATPase and PlTX. The enzyme is labelled with a reactive ester of carboxyfluorescein. As it binds with PlTX, the FP of this resulting protein-dye conjugate decreases when the concentration of the toxin increases and the difference is measured. The performance of the method has been assessed in *dinoflagellate* extract as well as in blank and fortified mussel extracts. A clean up step was added to the method in order to avoid matrix effects. The method is easy and quick, its LOQ was estimated at 10 nM and its LOD at 2 nM (Alfonso, 2012).

Table 3. Summary of methods applicable to the determination of PlTXs in shellfish.

Method	Advantages	Disadvantages
Mouse bioassay (MBA)	• Direct toxicity assessment • History of use and prevention of sickness	• Possible interferences • Ethical issues • Variable performance • No indication of toxin profile
Cytotoxicity assays	• Sensitive functional assay • Direct toxicity assessment • Capable of detecting all PlTX-group compounds with mode of action similar to PlTX	• Maintenance of cell cultures required • No indication of toxin profile
Haemolysis assay	• Direct toxicity assessment • High sensitivity • Capable of detecting all PlTX-group compounds with mode of action similar to PlTX	• Variable performance between laboratories depending on erythrocytes origin • Potential ethical and logistical issues • No indication of toxin profile • Possible interference from other haemolytic compounds
ELISA	• Rapid and easy to use • Might be used as a screen	• No indication of toxin profile • Anti-bodies not always readily available • Cross-reactivity does not always reflect toxicity • Cross reactivity with PlTX-like compounds not assessed
FP	• Good repeatability • Low detection limit • Not expensive	• Applicability to other PlTX-group compound not investigated • No profile information • No indication of toxicity
HPCE-UV	• Can be automated • Some degree of specificity	• Potential matrix effects • Not developed for bivalve molluscs • Lack of availability of standards and equipment
HPLC-UV	• Can be automated • Could provide a toxin profile	• Lack of sensitivity for PlTX • May only be applicable to some PlTX-group compounds

	• Some degree of specificity	• Requires toxicity equivalent factors as there is no direct indication of toxicity
HPLC-FLD	• Can be automated • More specific than HPLC-UV • Sensitive	• Performance characteristics in shellfish tissue not assessed
LC-MS/MS	• Can be automated • Highly specific • Sensitive • Give information on the toxin profile • Can screen and measure PlTX-group compounds individually	• Expensive instrumentation • Lack of availability of all standards • No indication of toxicity

2.2.5 Suitability of existing and potential methods for PlTX testing

PlTXs are not currently regulated in the EU or in the rest of the world. There is at present no recognized official method for PlTX and the scientific groups who have carried out analysis have mainly developed their methodologies or combination of methodologies according to their need and available facilities (e.g: whether the toxin profile and/or the toxicity are required). Although several extraction methods are available, the existing information on their performance is scarce. A PlTX standard isolated from *P. tuberculosa* is available commercially but there are no standards for the growing list of PlTX-group compounds. During the first meeting of the working group on toxicology of the National Reference Laboratories for marine biotoxins (Italy, Oct. 2005), a provisional limit of 250 µg/kg shellfish was proposed by the European Reference Laboratory. Since then, the Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) has advised that shellfish meat should not contain more than 30 µg/kg of the sum of PlTX and ostreocin-D (EFSA, 2009). Reducing the potential limit by a factor of eight has narrowed the list of analysis method potentially applicable.

The testing regime currently applied in the UK is not suitable for the detection of PlTX-group compounds. The LC-MS technique would be the most likely candidate although, the additional compounds could not be easily added to the existing method and a different instrumental method may be required. The HPLC-FLD method has the potential to become a monitoring tool but this will depend on its performance in shellfish and on its applicability and performance for PlTX analogues. The FP method would provide good sensitivity in mussels and might also have potential as a monitoring tool. However, its performance characteristics need to be further assessed and be tested in species other than mussels in order to ensure the clean up procedure developed adequately address the matrix effects from all species since inter-species differences have been shown in other marine toxin analysis.

2.2.6 Identification of knowledge gaps with regards to PlTXs which might be addressed through further research or method development

PlTX has been an issue in tropical and sub-tropical areas for over 40 years but its recent occurrence in European waters has refocused the attention of the scientific community and a number of studies covering issues such as toxicity, structure and analysis have flourished. Although these have provided a number of valuable answers, they have also raised additional questions. In particular, the list of PlTX-like compounds is rapidly expanding and very little is known about them so the knowledge gaps relating to research requirements for prevalence and detection of PlTX-group compounds in shellfish are currently wide. These include:

- Toxicity information relevant to the exposure routes for PlTX and PlTX-like compounds
- Toxicological information for PlTX-group
- Identification of the toxin profile and metabolite relevant to seafood and shellfish especially (i.e. although a number of PlTX-group compounds may be detected in algae, the suite of compounds relevant to food safety may be different)

- Continued evaluation of method for detection and analysis of PlTX-group toxins in seafood and shellfish in particular
- Harmonisation and validation of analysis method for PlTX and PlTX-like compounds enabling direct comparison of results across several laboratories
- Purification of all PlTX analogues in sufficient quantities to produce standards and reach certification
- Unequivocal elucidation of the structure of all PlTX-like compounds to enable better understanding of the molecules and their likely behaviour but also for possible synthesis of standards
- Asserting the *in vivo* mechanism of action of PlTX and other related toxins

2.2.7 Proposed options for routine monitoring of PlTXs to meet legal requirements

Based on the evidence gathered during this review, an effective approach for routine monitoring might include:

- Screening of shellfish samples using an ethical assay directly related to PlTX toxicity at least until toxicity equivalent factor are available for chemical methods
- Application of a suitable confirmatory method for quantitation such as an LC-MS method providing high specificity and toxin profile

In order to meet legal requirements associated with official control monitoring of bivalve molluscs, each of these assays would need to undergo a series of validation studies to determine full performance characteristics of the method. In addition the performance would need to be demonstrated as being able to provide at least the same level of effectiveness as the MBA.

2.3 CYCLIC IMINES

2.3.1 Introduction

Spirolides

SPXs are the largest group of CIs with currently 16 SPX analogues isolated. Until recently, they were characterised by a spiro-linked tricyclic ether ring system and an unusual seven-membered spiro-linked cyclic iminium moiety but a spirolide subclass including two compounds displaying a spiro-linked dicyclic ether ring system has now been proposed (Roach, 2009). SPXs can be split into three types; type A, type G and type H. Amongst other differences, the type A SPXs and their derivatives have a 6,5,5 polyether ring system whereas the type G SPXs present a 6,6,5 polyether ring system and the type H present a 6,5 polyether ring system. All SPXs are soluble in methanol and chloroform and are therefore readily extracted in lipophilic fractions of shellfish prepared for the mouse bioassay.

SPX A-D were initially isolated and the planar structure of SPX B and D was described as two lipid-soluble macrocycles containing a spiro-linked tricyclic ether ring system (Hu, 1995). Following further work, the planar structure of SPX E and F was also described. SPX E and F are keto amines without the characteristic heptacyclic iminium ring. Taking into account the absence of activity in the mouse bioassay by i.p. injection of these compounds, it was proposed that the cyclic imine moiety was the spirolide pharmacore (Hu, 1996a). However, following the inactivity in the mouse bioassay (i.p. injection) of SPX H displaying this function, it was suggested that the cyclic imine moiety is not the only structural requirement for toxicity (Roach, 2009). SPX E and F are thought to be metabolites produced in shellfish as they have so far not been detected in phytoplankton samples from culture or collected in the field. The structural elucidation of SPX A and C was carried out by Hu et al (2001). The differences in chemical structures between SPX C and D, presenting an additional methyl substitution on the imine ring, and SPX A and B may be significant. The first two compounds are resistant to oxalic hydrolysis whereas the other two compounds are converted to the biologically inactive SPX E and F when the same reaction is applied (Hu, 1996a & 2001; Christian, 2008). SPX G was isolated from a culture of *A. ostenfeldii* from Denmark (MacKinnon, 2004). SPX H and I were isolated from *A. ostenfeldii* and their structure determined (Roach,

2009). The identification of SPX derivatives started with the isolation and structure elucidation of 13-desmethyl-C SPX present in shellfish extracts and in cultures of A. ostenfeldii (Hu, 2001). Another SPX derivative was identified as 13,19-didesmethyl SPX C in a culture of A. ostenfeldii isolated from Denmark (MacKinnon, 2004). 20-methyl-SPX G was isolated and its structure elucidated from the digestive gland of steamed Norwegian mussels (Mytilus edulis) (Aasen, 2005). The structure of 27-hydroxy-13,19-didesmethylspirolide C was elucidated from a culture of A. ostenfeldii (Ciminiello, 2007) and the last two SPX derivatives, 27-hydroxy-13-desmethyl spirolide C and 27-oxo-13,19-didesmethyl spirolide C, were isolated from the same culture (Ciminiello, 2010a). The molecular structure of some of the known spirolides is given in.

Gymnodimines

The planar structure of GYM, also sometimes referred to as GYM-A, has been determined (Seki, 1995). The absolute stereochemistry of this compound was elucidated a couple of years later (Stewart, 1997). The structure of GYM B has been identified (Miles, 2000) and the structure of GYM C was elucidated and found to be a stereoisomer of GYM B (Miles, 2003).

GYMs include a spirocyclic imine ring and a 16-membered macrocycle.

Pinnatoxins

PnTXs are macrocyclic compounds composed of a 6,7-spiro ring, a 5,6-bicyclo ring and a 6,5,6-trispiro ketal. The planar structure of PnTX A, was the first one to be elucidated (Uemura, 1995) followed by PnTX D, B and C (Chou, 1996; Takada, 2001a). The structure of PnTXs E-G have also been determined (Selwood, 2010).]The absolute stereochemistry of PnTX A was established by total synthesis (McCauley, 1998) and the total synthesis of the biologically active PnTX A and of PnTX G was achieved by Araoz et al. (2011). The absolute stereochemistry of PnTX B and C was confirmed by total synthesis (Matsuura, 2006). Recently, another potential PnTX compound has been isolated from a Vulcanodinium rugosum culture and its structure is currently being elucidated (Zeng, 2012).

Pteriatoxins

The absolute stereochemistry of PtTXs A, B & C was confirmed by total synthesis (Hao, 2006).

Prorocentrolide***s***

PcTX A is an amorphous solid and the planar structure of this toxic macrocycle has been elucidated.

Spiro-prorocentrimine

Spiro-prorocentrimine is a polar lipid-soluble toxin (Lu, 2001).

2.3.2 Mouse bioassay

SPXs and GYMs were originally discovered and isolated following interferences in the MBA used for the analysis of lipophilic toxins in shellfish since they are readily soluble in polar organic solvents. Their characteristic neurological response in the MBA shortly after *i.p.* injection explains the use of the term “fast acting” toxins and has been described (Munday, 2012a; Otero, 2012). LOD for some SPXs, GYM A and for some PnTXs have been estimated (EFSA, 2010)

The MBA symptoms are quite specific to the CIs group and the analysis would provide a direct measure of toxicity in the mouse. However, the toxicity to human through seafood consumption remains uncertain and it presents all the drawbacks associated with animal bioassay including possible extraction interferences (toxins and other compounds soluble in the aqueous phase) and ethical issues.

2.3.3 Chemical methods

In the process of compound isolation, purification and structural identification, mass spectrometry, nuclear magnetic resonance (NMR) and liquid chromatography have all been used extensively and for the majority of the CIs, LC-MS remains the base of the analysis. Some functional assays (see below) have been developed for

application to some SPXs and/or GYM but no alternative to LC-MS methods have been found for other CIs.

Conventional chromatography methods

Most of the CIs lack chromophores rendering their detection by optical methods quite unspecific. Nevertheless, an HPLC-UV method for the analysis of GYM-A in clams (*Ruditapes decussatus*) from the coastline of Tunisia has been developed. The extraction was carried out on the digestive gland using acetone followed by diethyl ether and dichloromethane. The method recovery exceeded 96% with a LOQ of 8 ng/g of digestive gland (Marrouchi, 2010).

LC-MS methods

LC-MS and LC-MS/MS methods have been widely used for analysis of CIs (Cembella, 1999; Hu, 2001; Stirling, 2001; Takada, 2001a & b; Biré, 2002; MacKenzie, 2002; Aasen, 2005; Ciminiello, 2006; Villar Gonzales, 2006 & 2007; Fux, 2007; Gerssen, 2009; Krock, 2009; Alvarez, 2010; Medhioub, 2010; Miles, 2010; Selwood, 2010; McNabb, 2012b). The methods are based on reversed-phase LC-MS. They are highly specific (presence of the imino function) and sensitive. The shellfish is extracted with methanol or aqueous methanol potentially followed by partitioning with chloroform or a solid phase extraction procedure.

2.3.4 Biomolecular / Functional methods

More recently alternative analysis methods have been developed for GYM-A and 13-desmethyl SPX C based on the knowledge of the toxins' mechanism of action.

Fluorescent polarization (FP)

An FP assay was developed for analysis of 13-desmethyl SPX C and GYM A in shellfish (Vilariño, 2009). The assay has been designed as a competitive inhibition assay where the binding of fluorescent α-bungarotoxin, a toxin from snake venom, to the nAChR enriched membrane of *Torpedo marmorata* is inhibited by GYM and SPXs and is detected by fluorescence polarization. The shellfish (mussels) was

extracted using acetone, the solvent evaporated and a partitioning step using water and hexane was carried out followed by extraction with chloroform. The residue was reconstituted after evaporation and filtered prior to analysis of the resulting extract. The recovery for the extraction method in mussel matrix for GYM A and 13-desmethyl SPX C were estimated at 63.6 % and 87.4 % respectively. Okadaic acid, yessotoxin and BTX-2 were tested for cross-reactivity and did not interfere with the assay. The applicable concentration range in mussels was 50-2000 µg/kg for GYM-A and 70-700 µg/kg for 13-desmethyl SPX C (Vilariño, 2009).

Subsequently, the performance of the assay was further tested on four different shellfish matrices (mussels, clams, cockles and scallops). The average recovery rates were reported as 90.6 % and 89.6 % for GYM and 13-desmethyl SPX C respectively. The quantitation range in all tested species was 80-2000 µg/kg for GYM and 85-700 µg/kg for 13-desmethyl SPX C (Fonfria, 2010a). For mussels and cockles, although the matrix interference was low, the data were statistically different from buffer controls suggesting that for routine analysis a calibration curve prepared in shellfish matrix may be advisable (Fonfria, 2010a). In the same study, the variability for 13-desmethyl SPX C was reported as lower than 14 % however, the variability for GYM was greater than 15 %. In addition, the detection of 13,19-didesmethyl SPX C was studied in mussels using the same fluorescence polarization method (Fonfria, 2010b). The extraction recovery was reported as 77.7 % and the quantitation range was 40-200 µg/kg of shellfish meat.

A direct fluorescence polarization assay, involving the labelling of the nAChR from *Torpedo marmorata* with a derivative of fluorescein, was developed for the direct detection and quantitation of SPXs in mussels samples. The assay is based on the change in fluorescence polarization of the labelled nicotinic receptor when bound by a SPX toxin. Three extraction procedures were tested and the extraction procedure taken forward included extraction of the shellfish with methanol and partitioning with dichloromethane followed by evaporation, reconstitution and filtration. The assay was applied to 13-desmethyl SPX C and 13,19-didesmethyl SPX C but 13-desmethyl SPX C was the only one tested in shellfish matrix (mussels). The usable range was reported as 50-350 µg/kg of shellfish meat and the method recovery was 88%

(Otero, 2011b). The cross reactivity of the method was tested with two other lipophilic toxins and they were found not to interfere with the assay.

Solid-Phase receptor-based assay

Based on the same principle, another receptor-based method was developed using the competition of 13-desmethyl SPX C with biotin-labelled α-bungarotoxin, for binding to nAChRs and the immobilisation of the α-bungarotoxin receptor complex on streptavidin-coated surfaces. The quantitation of the immobilized receptor is achieved using a specific anti-nAChR antibody. Three different detection methods have been tested; chemiluminescent, fluorescent or colorimetric detection (Rodriguez, 2011). The shellfish (cockles only) was extracted using acetone, the solvent evaporated and a partitioning step using water and hexane was carried out followed by extraction with chloroform. The residue was reconstituted after evaporation and filtered prior to analysis of the resulting extract. The recovery of this extraction method was estimated at 67.8 %. The detection range of the assay for 13-desmethyl SPX C in cockles was estimated at 40-1000 µg/Kg. Although the chemiluminescent detection offered the best sensitivity (~40 µg/Kg in shellfish meat), it appeared to suffer from matrix effect whilst the fluorescence and the colorimetric did not appear to be significantly affected. The assay appears capable to detect 13-desmethyl SPX C with higher sensitivity and a wider dynamic range than the fluorescent polarization assay. The cross-reactivity with other regulated toxins was tested and they were not found to interfere with the results of the assay. In the absence of certified standards, cross reactivity with other CIs has not been tested. The method was also applied to GYM but the detection limit of the method for GYM was 10 times higher than for 13-desmethyl SPX C (Rodriguez, 2011). Although this assay is sensitive and specific for the detection of neurotoxins targeting nAChRs, its selectivity is low. In order to address this drawback and provide toxin identification/confirmation, the technique was coupled to mass spectrometry detection (Araoz, 2012). The performance of this assay was tested in four shellfish species; clams (*Glycymeris glycymeris*), oysters (*Ostrea edulis*), mussels (*Mytilus galloprovincialis*) and scallops (*Pecten maximus*) spiked with a mixed standard solution containing GYM-A, 13,19-didesmethyl SPX C, PnTX A, 13-desmethyl SPX C, 20-methyl SPX G and PnTX G. The most potent antagonist was 13,19-

didesmethyl SPX C followed by 13-desmethyl SPX C, PnTX G, 20-methyl SPX G, GYM-A and PnTX-A. Cross-reactivity with five other marine toxins was checked and they were not found to interfere (Araoz, 2012).

Table 4. Summary of methods applicable to the determination of cyclic imines in shellfish.

Method	Advantages	Disadvantages
Mouse bioassay (MBA)	• Direct toxicity assessment in the animal • History of use and prevention of sickness	• Qualitative • Possible interferences • No indication of toxin profile • Ethical issues • Variable performance • Not validated
Fluorescence polarization (competitive)	• Quick • Technically easy • Some toxicity assessment (only for the targeted mechanism of action)	• No indication of toxicity for toxins with a different mode of action • No information on toxic profile • Dependent on availability of receptors from *Torpedo marmorata* electric organ • Validation data limited • Standards required to test performance for other analogues
Fluorescence polarization (direct)	• As competitive FP • More sensitive than the competitive assay • Easier than competitive FP	• As competitive assay • Tested only in one shellfish species for one SPX
Solid-phase receptor-based assays (RBA)	• Simple, sensitive, rapid • Good performance in collaborative study • Promising fluorescence-based binding assay	• Variable affinity for BTX metabolites • Requirement for animal tissues and radiolabel • Matrix effects • Limited development to date with fluorescence-based binding assay
HPLC-UV	• Can be automated • Quantitative method	• Low specificity • Standards required for some CIs • Not validated
LC-MS(MS)	• Can be automated • Highly specific	• Expensive instrumentation • Lack of availability of all relevant

	• Sensitive • Toxin profile information available	standards • No indication of toxicity

2.3.5 Suitability of existing and potential methods CIs analysis

CIs are not currently regulated in the EU or in the rest of the world. Although additional studies have been reported in the past couple of years, the information on the toxicity of CIs is still limited and mainly confined to acute toxicity studies with a distinct lack of information on chronic effects. Progress have been made in relation to the mechanisms of action of some CIs (e.g: SPXs, GYMs) but they have not been entirely elucidated either. Owing to the fact that no human toxicity incident has been unequivocally linked to CIs, their inclusion in the list of regulated toxins is still under debate in the scientific community.

So far, LC-MS has been the detection method of choice for analysis of CIs in shellfish and although the method is suitable, the availability of certified standard commercially is currently limited to 13-desmethyl SPX C, 13,19-didesmethyl SPX C, 20-Me SPX G and GYM-A. 13-desmethyl SPX C and GYM-A are currently the best characterised toxins of the CI group but no inter-laboratory studies have been reported for the validation of these toxins and there is no information related to the validation of an analytical method for the other CIs.

The testing regime currently applied in the UK is not suitable for the detection of CIs. The LC-MS technique would be the most likely candidate since 13-desmethyl SPX C and GYM-A could be added to the current lipophilic toxin method. Other SPXs, SPX derivatives and potentially some of the PnTXs could also be added to the method at least as a qualitative screen.

2.3.6 Identification of knowledge gaps with regards to CIs which might be addressed through further research or method development

Considering the presence of some CIs in UK waters and shellfish, additional knowledge in this area would directly benefit the UK.

- Toxicity information in particular related to chronic effect and potential synergistic effects (e.g: GYM & okadaic acid)
- Toxicological information
- Acyl-esters of CIs have been identified in shellfish so their bio-availability to human through shellfish consumption needs to be investigated
- Purification and availability of certified standards
- Validation of method of analysis
- Development of an ethical screening assay directly related to toxicity
- Occurrence data required

2.3.7 Proposed options for routine monitoring of CIs to meet legal requirements

Based on the evidence gathered during this review, an effective approach for routine monitoring might include:

- Monitoring of the known organism(s) producing CIs
- Screening of shellfish samples using an ethical assay directly related to CIs toxicity
- Application of a suitable confirmatory method for quantitation such as an LC-MS method providing high specificity and toxin profile

In order to meet legal requirements associated with official control monitoring of bivalve molluscs, each of these assays would need to undergo a series of validation studies to determine full performance characteristics of the method. In addition the performance would need to be demonstrated as being able to provide at least the same level of effectiveness as the MBA. It would be recommended that further toxicological assessment of toxins was undertaken to support this.

2.4 TETRODOTOXINS

2.4.1 Introduction

Tetrodotoxin is a low molecular weight neurotoxin, with unique structure determined in 1964 (Tsuda *et al.*, 1964). The toxin is soluble in acidified water, stable in neutral and weakly acidic solution and following heating.

2.4.2 Animal bioassays

The tetrodotoxin MBA has been used for the determination of toxicity for over 35 years (Kawabata, 1978), with a revision to the protocol published by Yasumoto, (1991). The assay is similar to the PSP MBA, with the exception that acetic acid is used in place of hydrochloric acid. An English translation of the protocol was provided by Yotsu-Yamashita and published in the AOAC General Referee Reports of the Journal of the AOAC International in 2006 (Hungerford, 2006). Fish tissue samples are ground and heated with 0.1% acetic acid, prior to filtration or centrifugation and dilution to volume. Aliquots are injected into male mice (of a specific size and strain) and death time, as judged from cessation of respiration, is used to determine toxicity. Dilutions and repeat analysis may be required depending on the time of death, as there is a low dynamic range of the relationship between dose and response. Kawabata *et al.* (1978) described the calculations of toxicity from median death times, expressing the result as a mouse unit (MU), with 1 MU equivalent to 220ng TTX. It is noted that with similar modes of action, positive TTX MBA results cannot be discriminated from samples potentially containing saxitoxins, some of which have been known to be present in certain species of fish (e.g. Sato *et al.*, 2000).

The method has been used for many years to assess the toxicity from TTX in fish tissues and extracts prepared from other marine species such as gastropods (e.g. Wang *et al.*, 2008). Recently the assay has been used to confirm toxicity in fish

species from the Mediterranean Sea (Katikou *et al.*, 2009) and to measure the TTX toxicity in parts of the gastropod which caused a case of intoxication in Malaga in 2007 (Fernandez-Ortega *et al.*, 2010).

Chemical methods

A recent review of instrumental analysis methods for TTXs is provided by Asakawa *et al.* (2012). The requirement for reference materials to enable the testing, refinement and validation of chemical methods is again strong. To date relatively few standards are commercially available, with just two companies (Sigma and Alexis) providing purified tetrodotoxin as commercial products (EURL-MB web-site). As a result, and possibly as a consequence of the availability issue, no analytical methods have been validated formally through interlaboratory study, although chemical methods have been extensively applied to the identification of TTXs in a wide range of poisonous species.

Conventional chromatography methods

Liquid chromatography with post-column fluorescence derivatisation was developed by Yasumoto *et al.* (1982) and Yasumoto and Michishita (1985) for determination of TTX and further reported by Yotsu *et al.* (1989). Extraction was conducted using weak acetic acid, prior to reverse-phase ion-pairing chromatography to separate TTX and the congeners 4-epiTTX and 4,9-anhydroTTX (Lee *et al.*, 1989). Detection was achieved after post-column treatment with sodium hydroxide (NaOH), yielding highly fluorescent amino-quinazoline derivatives. Analytical sensitivity was good (5ng per injection on-column), with good reproducibility (≤5%) and a good correlation observed between the HPLC method and the MBA. The method was also applied to ethanolic extracts of toads in combination with pre-column clean-up where the same 3 TTX analogues were separated and detected (Mebs *et al.*, 1995). A modified procedure involving three extractions with 1% acetic acid in methanol was developed, incorporating additional clean up with chloroform and ultrafiltration for the detection of TTX in gastropods and pufferfish (Chen *et al.*, 1998). Method recovery was excellent (91±5%), with good limits of detection (< 0.2 µg TTX/g) and detector linearity. The method separated the 3 TTX analogues found to coexist in the samples analysed and an excellent correlation with the MBA was observed. The

same method has also been applied to the analysis of TTXs in newts, with results compared against isolated standards (Hanifin *et al.*, 1999; Yotsu-Yamashita *et al.*, 2003; Yotsu-Yamashita *et al.*, 2012). Modified chromatographic and post-column reaction conditions plus an additional pre-column C18-SPE extract clean-up of extracts were used to quantify TTXs in gastropods associated with food poisoning events (Jen *et al.*, 2007, 2008). The method provided a rapid quantitative determination of TTX, 4-epi-TTX and anhydro-TTX, with a 1 µg/mL LOD, TTX recovery of 90% and a linear range of 1-500 µg/mL for TTX. Post-column derivatisation LC-FLD of TTX in urine and serum from patients suspected of TTX ingestion has also been reported. Kawatsu *et al.* (1999) described the use of immunoaffinity chromatography following production of a monoclonal antibody specific to TTX to enable separation of TTX from interfering matrix components. TTX method recoveries were 50-60% with the method sensitive, specific and applicable for determination of TTX in urine. With SPE employed as an alternative clean-up, method precision was acceptable (13-15%) and the linear ranges were 20-300 ng/mL and 5-20 ng/mL for urine and serum analysis respectively. LOQs were 5 and 20 ng/mL for serum and urine respectively, although recoveries were limited (O'Leary *et al.*, 2004).

Yu *et al.* (2010) reported the use of HPLC-UV for the detection of TTX in the urine and plasma of humans intoxicated with pufferfish poisoning. Pre-analysis C18-SPE and weak ion-exchange clean-up steps were employed prior to reverse phase chromatography. Method LODs were reported as 10 ng/mL, with acceptable recovery (>87%) in both matrices.

Other non mass spectrometric analytical methodologies reported previously include the use of gas chromatography for detection of pre-column derivatised TTX applied to autopsy materials following cases of fatal intoxications (Suenaga and Kotoku, 1980) and capillary zone electrophoresis. The latter reported as sensitive, rapid and reliable (Cai *et al.*, 2003). Neither of these approaches have been utilised in more recent investigations of TTX contamination or poisonings.

Overall conventional chromatographic techniques have been well used over the years for TTX detection in a wide range of sample matrices, with fluorescence detection in particular providing a sensitive and fairly specific approach. However it is noted that the specificity will be improved through use of mass spectrometric detection, which would also theoretically improve the detection of certain TTX analogues, most notably 5-deoxyTTX and 11-deoxyTTX) which are difficult to detect by fluorescence (Shoji *et al.*, 2001).

MS detection methods

Mass spectrometry has also been applied to TTX detection, using a variety of less commonly used approaches, including thin-layer chromatography with fast atom bombardment (TLC-FAB-MS) and electrophoresis/FAB-MS (Nagashima *et al.*, 1988), ion-spray MS (Pleasance *et al.*, 1992) and LC with FAB-MS (Hashimoto *et al.*1994)**.** Gas chromatography with MS detection has also been applied to the detection of TTX in human urine and plasma, following a lengthy two-stage clean-up with C18 SPE cartridges and sample derivatisation (Leung *et al.*, 2011).

Various LC-ES-MS methods have been developed for identification of TTX congeners (e.g. Diener *et al.*, 2007; Shoji *et al.*, 2001; Nakagawa *et al.*, 2006; Wang *et al.*, 2008; Rodriguez *et al.*, 2008; Jang *et al.*, 2010). Shoji *et al.*, 2001 originally described in detail the application of LC-MS methodologies for TTXs, reporting both selected ion monitoring (SIM) and tandem mass spectrometry (MS/MS). Their work enabled the comparison of responses and retention times between LC-MS and LC-FLD methods and the confirmation of good MS detector linearity over the range of 50 to 1000 pmol. A standard mixture of TTXs was used to confirm that the SIM ion intensities for each of the analogues was not significantly different to TTX, consequently showing that quantitation of TTX analogues could be performed with a single calibration curve prepared from TTX. This idea was later extended with the quantitation of TTX analogues using TTX as an internal standard (Chen *et al.*, 2011). MS/MS was used to confirm fragment ion spectra for each of the TTXs, which each showing characteristic fragmentation patterns, enabling the generation of a quantitative LC-MS/MS method for TTXs.

The same team later reported the use of Hydrophilic Interaction Liquid Chromatography (HILIC)-ESI-SIM-MS for TTXs after pre-column clean-up with a reverse-phase resin. This enabled separation of TTX isomers without the use of ion-pairing reagents in the mobile phase (Nakagawa *et al.*, 2006).

Wang *et al.* (2008) described the use of LC-MS for the analysis of gastropods for TTX and four TTX analogs (deoxyTTX, anhydroTTX, 4-epiTTX and oxoTTX). Following extraction with 80% methanol solution with 1% acetic acid and liquid-liquid extraction clean-up, analysis was conducted using HILIC with positive mode SIM targeting all TTXs. Diener *et al.* (2007) reported the detection of a number of TTX analogues including TTX, anhydroTTX, 4-epiTTX and 11-deoxyTTX using a HILIC separation with 25 min run time. The approach was later optimised by Yotsu-Yamashita *et al.* (2011).

Utilisation of LC-MS to determine the distribution of TTX analogues in Japanese marine puffer fish *Fugu pardalis* revealed 5,6,11-trideoxyTTX was the major component in all tissues (Jang and Yotsu-Yamashita, 2006). Jang *et al.* (2010) extended this work to examine the distribution of TTXs in a number of other puffer fish species. Quantitation was conducted in SIM mode, as this was found to provide greater sensitivity for TTXs then MRM peaks generated following MS/MS detection, although the latter technique was used for confirmatory purposes.

McNabb *et al.* (2010) confirmed the presence of TTXs in MBA-positive sea slugs, using both full scan MS to identify parent ions, prior to the application of a LC-MS/MS quantitative assay. The method was also applied to various samples of vomit following dog poisoning, consequently confirming the TTX-cause of dog deaths in the area where the sea slugs were found. Subsequently a programme of sampling and analysis was conducted to examine the presence of TTXs in a wide range of marine organisms (>380 samples representing 53 taxa), with LC-MS results confirming the presence of TTX in six of these species (Oglivie *et al.*, 2012). Use of this analytical method has therefore provided a valuable risk assessment tool to facilitate the dissemination of information regarding the potential hazards from TTX to local people. In New Zealand a secondary method has also been developed,

involving alkaline hydrolysis of TTXs to a C9 base (quinazoline). Acetic acid extracts of tissue are boiled in 1M NaOH for 45 min before SPE clean-up and LC-MS. Matrix-matched TTX standards are used to confirm low concentrations of TTX and the differences in concentrations determined between the free TTX and C9 based are used to show the presence of other TTX analogues. When applied to a gastropod containing 0.01 mg/kg TTX, results showed improved detection limits for the C9 base and greater analytical specificity (Selwood *et al.*, 2012). This approach has recently been published by McNabb *et al.* (2013) who report acceptable recoveries for both methods (94 to 120%) and within laboratory reproducibility's (6 to 27%) for both sea slug and bivalve matrices.

Evidence for the presence of TTXs in gastropods from Europe has been demonstrated with the use of both LC-MS/MS (Rodriguez *et al.*, 2008) and UPLC-MS/MS (Silva *et al.*, 2012). MRMs were described for the major transitions and quantitation performed, achieving an LOD of 16 ng/mL and 1.7 ng/mL for the two methods respectively (Silva *et al.*, 2012).

Both LC-SIM-MS and LC-MS/MS approaches have subsequently been utilised for the identification of the cause of poisoning outbreaks in the US, Japan, Thailand and other tropical/sub-tropical areas, enabling confirmation of TTX contamination and/or the clarification regarding relative presence of STX and TTX in neurotoxic fish specimens (Deeds *et al.*, 2008; Cohen *et al.*, 2009; Chulanetra *et al.*, 2011; Jen *et al.*, 2008).

MS detection methods have proved useful when confirming TTX poisoning diagnosis, given the low concentrations of toxins present in clinical samples. A review of the different experimental parameters used for the analysis of TTXs in human samples is provided by Leung *et al.* (2011). Akaki (2006) reported the use of LC-MS/MS for the detection of TTX in fish tissue together with human serum and urine. TTX recoveries were found to range between 79-90% in pufferfish tissue and 93-101% in clinical samples, and the method successfully applied to the determination of TTX in a range of fish and human samples. Confirmation of the presence of TTX in patient's blood was also conducted using LC-MS/MS (Jen *et al.*,

2007, 2008) as well as in the blood and urine from the patient intoxicated from a trumpet shellfish in Europe (Fernandez-Ortega *et al.*, 2010). Rodriguez *et al.* (2008) reported that the LC-MS/MS method was capable of detecting TTX and 5,6,11-trideoxyTTX in both shellfish and in patient body fluids, suggesting that a combination of pre-analysis clean-up of urine and LC-MS quantitation is a very useful technique for diagnosing TTX-intoxication. Jen *et al.* (2008) also highlighted that C18 SPE clean-up was essential for removal of matrix effects from subsequent analysis, resulting in TTX recovery of 90-95%. Others have reported TTX recovery at similar levels with the use of C18 clean up and ultracentrifugation (Tsai *et al.*, 2006). A column-switching method has also been described enabling the on-line clean-up and detection of TTX in serum (Hayashida *et al.*, 2003).

Biomolecular methods

Cytotoxicity assays

Kogure *et al.* (1988) described a tissue culture assay for TTX as well as STXs developed using standards of these toxins. The method worked with the toxins blocking the cellular swelling and death resulting from the veratridine enhancement of sodium influx into the mouse neuroblastoma cell line in the presence of ouabain. The assay enabled the semi-quantitation of TTX based on the percentage of living cells remaining. The authors proposed this as a simple, inexpensive and sensitive technique capable of replacing the MBA, although noted the potential requirement to standardise against either chemical or immunological assays

Hamasaki *et al.* (1996 a,b) subsequently reported an improved method for detection of TTXs using the mouse N2A cell line. Improvements originated from the use of a water-soluble tetrazolium salt to enable automatic measurement with a microplate reader, in place of the time consuming and tedious cell-counting process. This was applied only to the detection of TTX in bacterial cultures.

Receptor binding assays

Functional methods relying on native receptors have been developed for TTX in recent years. These include methods relying on sodium ion channels from rat brain membrane preparations and radio-labelled toxins, either STX (^{3}H-STX) or TTX (^{3}H-

TTX). The signal produced is inversely proportional to the toxicity of the sample extract (Davio *et al.*, 1984; Powell *et al.*, 1999). Davio and Fontelo, 1984 reported a competitive displacement assay for the detection of both STX and TTX using the STX label. The assay was found to have a sensitivity of 0.8 ng/mL and 0.6 ng/mL TTX for buffer and human plasma respectively, with a useful TTX standard curve of 0.8 to 70ng TTX/ml. The mean recovery of the method for TTX-spiked plasma samples was shown to be 108 ± 10% over a range of concentrations.

Doucette *et al.* (2000) described a competitive receptor binding assay (RBA) using radiolabelled TTX, given concerns with the availability of radiolabelled STX. The LOD based on 70% total binding was approximately 2-4 ng STX eq./mL sample extract, similar to that reported when using ^{3}H-STX as the radioligand (Doucette *et al.*, 1997). Furthermore the correlation between toxicities in PSP-positive algae and shellfish determined using both radioligands was shown to be high ($r>0.9$) indicating the two isotopes can be interchanged for the measurement of PSP activity. Therefore there has been some demonstration of the applicability of the method to TTX as well as STX. However, with no further demonstration of the method reported for TTXs, validation studies would be required to demonstrate performance characteristics for TTX analysis in suitable sample matrices. It is likely that the method would perform well, given the applicability and ruggedness of the RBA for STXs.

Given the method of action, the assay is clearly not specific enough to distinguish between TTX and saxitoxin congeners, although with use in combination with HPLC or LC-MS confirmation methods this should not be an issue. However the current requirements for ^{3}H-labelled components can make the method expensive and present practical difficulties. It is likely in some countries that with the availability of LC-FLD and LC-MS instrumentation, the RBA is likely to remain unpopular (Yasumoto personal communication).

Immunoassays

Immunoassays structure-based in vitro methods such as the immunoassays can potentially provide specific and sensitive detection methods. The specificity is of

great interest as a successful assay would enable discrimination from other toxins such as the other toxins relying on sodium ion channels.

As with methods for other emerging toxins, the ELISA is perhaps the most common immunoassay format applied to TTX detection to date. Raybould *et al.* (1992) reported the production of a monoclonal antibody which enabled the development of a direct TTX detection method using alkaline phosphatase-labelled antibody. The assay was reported as showing good sensitivity (IC_{50}) of 6-7 ppb and compared well with HPLC and MBA.

Two ELISA assays were reported by Neagu *et al.* (2006) based on a toxin-alkaline phosphase conjugate, prepared in-house and utilising either spectrophotometric or electrochemical detection. The dynamic ranges of analysis were 4-15 and 2-50 ng/mL and the LODs 2 and 1 ng/mL respectively.

Zhou *et al.*, 2007 described the optimisation of an in-house ELISA preparation using a microtiter plate format. A 0.1% acetic acid extraction with chloroform partition clean-up was applied prior to the assay. Results were reported as showing a range of linearity between 5-5,000 ng/mL with an LOD of 0.05 ng. Recoveries from spiked fish tissue were excellent (97-105%) from both muscle and gonad matrices over a range of concentrations (2-500 ng/mL TTX), with acceptable variability of the results (5-14%) at ≥10 ng/mL. The authors also demonstrated a good correlation with HPLC results, albeit on a limited number of spiked samples. However, all these approaches require time consuming preparation of antibodies and other reagents in-house, so the production of reproducible assay formats is questionable.

A later version of the immunoassay was reported as providing highly specific TTX detection with minor cross-reactivity to anhydro-TTX. For the analysis itself, quantitative determination was achievable within 90 minutes and the assay was shown to be sufficiently sensitive, linear and quantitative results correlating well with the MBA (r=0.987;Kawatsu *et al.*, 1997). Recoveries were also shown to be good over a range of spiking TTX concentrations in three different species of pufferfish (97-103%; over 250-2000 ng/g). Tao *et al.* (2010) also reported an assay based on a

monoclonal antibody which was specific to TTX, with 1% cross reactivity to STX. Similar sensitivity and working range was determined, with an LOD of 5 ng/mL and recoveries from TTX-spiked samples ranging from 80-110%. The assay was also applied to the quantitation of TTX in wild puffer fish tissues.

More recently Stokes *et al.* (2012) reported a 96-well plate modified immunoassay using a commercial monoclonal antibody specific to TTX. The results showed the sensitive, accurate determination of TTX with good apparent repeatability within the laboratory between different plates (n=2). However, the assay offers no specificity to other TTX congeners as it yet to be tested on fish or gastropod tissues. The authors therefore recommended the use of confirmatory analysis for representative samples.

Wang *et al.* (1996) and later Gong *et al.* (2005) developed an ELISA employing a monoclonal antibody for quantitative analysis of TTX, which subsequently resulted in the development of a commercial microplate ELISA test kit (Zhonwei Inc., Beijing, China). Wang *et al.* (2008) utilised this product for confirmation of TTX content in isolated cultured strains of bacteria present in toxic gastropods. Zhong *et al.* (2011) reported performance characteristics of an ELISA including TTX recoveries from spiked muscle samples between 65 and 93%, intra and inter-batch repeatability <8% and a LOD of 1 ng/mL. A commercial ELISA marketed by REAGEN LLC in the US is according to the manufacturers a fast acting sensitive screening tool for TTX in pufferfish and water samples (Reagen, 2012). The performance characteristics reported are a recovery range of 70-120%, with sensitivity of 10 ng/mL and reproducibility of <15% for samples. Whilst this is a promising development, there are no guarantees that commercial products like this will remain available for the long term and/or whether product performance characteristics will remain consistent. Variability of performance and/or removal from production could severely affect any monitoring programmes relying on the assay for regular high throughput testing.

Biosensor methods

In 1998, Cheun *et al.* reported the use of a tissue biosensor (electrophysiological assay) for the determination of both STXs and TTXs. The sensor measured the transfer flow of sodium ions across a frog bladder membrane within a flow cell,

transfer which was sensitive to the presence of TTX. The sensor was found to provide a linear response against TTX concentrations and could detect low levels of TTX in two pufferfish samples and results correlated well with the MBA.

A single laboratory validation of a screening method for TTX detection using a Surface Plasmon Resonance (SPR) Biosensor has been reported, specifically for application to the gastropod species *Charonica lampas lampas* (Barnes *et al.*, 2011). The extraction procedure involved acetic acid and sodium acetate prior to dilution in assay buffer and SPR detection. No significant matrix effects were noted and the Decision Limit (CCα) and Detection Capability (CCβ) were 100 and ≤ 200 µg/kg respectively. The method recovery was good, with 98-99% at 400 and 800 µg/kg and 112% at the lower concentration of 200 µg/kg and associated intra and inter-batch precision acceptable (4-8%). With the assay showing very low cross reactivity with regulated marine toxins including saxitoxins (<0.01%) the method has been proposed as an effective screening method for TTX and thereby potentially applicable to other TTX-containing species including other gastropods and fish. The method has also been reported for the optimised determination of TTX in pufferfish liver, muscle and human urine matrices. TTX concentrations determined in ten fish tissue extracts compared well with those quantified following LC-MS/MS (Taylor *et al.*, 2011).

Other sensor detection methods include surface-enhanced Raman scattering (SERS) with silver nanoparticle arrays (Lin *et al.*, 2009) with which TTX concentrations at 0.9 ppb were reported as being detectable.

Another approach recently published is the use of Fluidic Force Discrimination (FFD) immunoassays, a technique that uses antibody recognition in a flow-based system. The technique was adapted by Yakes *et al.* (2010) for the detection of TTX, showing a proof of concept for a potential assay with a large dynamic range (1 to 100,000 ng/mL). This therefore prevents the need for sample dilution as argued by the authors. However, the accuracy of the method was questioned at the time following evidence for high variability in the responses used to generate standard curves, although work is ongoing to improve this issue (Yakes *et al.*, 2010).

Table 5. Summary of methods applicable to the determination of tetrodotoxins in shellfish.

Method	Advantages	Disadvantages
Mouse bioassay (MBA)	• Standard method accepted worldwide • Applicable to many sample matrices	• Ethics, costs, throughput • Low dynamic range, requiring repeat analyses • Little validation data
Cytotoxicity assay	• Sensitive methods	• Limited development and application to TTXs • Research tools only at present
Receptor binding assay (RBA)	• Sensitive and specific to TTX/STX toxins • Likely to work well for TTXs given success with STX RBA	• No validation for TTX in fish performed to date • Potential issues with ^{3}H-TTX availability • Lack of specificity between STX and TTX
ELISA	• Common application to date • Good recoveries reported from fish tissues • Comparable to MBA • Low cross reactivity to STX • Rapid 96-well plate format • Commercial kit available	• Low cross reactivity to TTX analogues • No guarantee commercial kit will not change performance over time + other availability issues
Conventional chromatography	• Well developed and sensitive LC-FLD methods • Performance characteristics demonstrated as mostly acceptable	• Potential specificity issues • Validation required for species of relevance • Not all congeners determined
LC-MS(MS)	• Highly specific, sensitive and linear methods • Applied successfully to	• Expensive • Standards requirement

	gastropod, fish and clinical samples • Useful confirmatory methods for food and clinical samples • Some performance characteristics demonstrated appear acceptable	
Biosensor methods	• Sensitive assays • SPR perhaps most developed and assessed • Good validation data reported for TTX detection in gastropods	• Expensive instrumentation • More validation required to assess applicability to relevant samples

2.4.3 Suitability of existing and potential methods for TTX testing

The MBA although applicable to samples of relevance, for the UK most likely to include gastropods and potentially imported fish samples, it would not be an appropriate monitoring tool given the UK commitment to the reduction and replacement of animal testing. This is especially true given the number of other methodologies available which could be tested, validated and implemented.

From the review conducted, it is evident that a two-method approach could be applicable, incorporating both a functional screening test such as an ELISA, followed by a more specific confirmatory method. With both conventional LC-UV and LC-FLD methods already validation and implemented into official control monitoring programmes for ASP and PSP toxins, there is potential for the testing and validation of methods using this instrumentation before application to TTXs. If found to be fit for purpose for samples received in the UK, this would provide a more cost-effective approach than use of MS detector methods. However, LC-MS/MS, currently applied to the determination of lipophilic toxins in shellfish, could also be applied in the current regime. The practicalities of incorporating a confirmation MS method for the lipophilic TTXs into the current analysis for the suite of lipophilic toxins would need to

be assessed. It is likely that a separate analysis would be required for TTXs, potentially combining with a general hydrophilic toxin LC-MS/MS method for STXs if required.

The biomolecular methods reviewed appear sensitive techniques, but development is less extensive for TTX in comparison with other emerging toxins such as the ciguatoxins and brevetoxins. Furthermore it is noted that neither the cytotoxicity or receptor binding assay are currently in place within the UK testing laboratories. Both these approaches would require extensive investment in both instrumentation and expertise, so at present would not appear the best options for assessment.

SPR biosensors are also complex and expensive instruments requiring a high level of expertise to run. However, one of the UK official control laboratories (AFBINI) does have access to an SPR instrument which has already been used for the validation of a TTX method for gastropods (Barnes *et al.*, 2011). As such, this method could potentially be utilised for TTX detection on behalf of all the UK biotoxin monitoring programmes.

Overall the UK is at present currently unprepared for the detection of TTXs in shellfish or fish samples. From the review conducted and given the instrumentation and expertise presently in place within the UK monitoring community, the following recommendations are made:

- To accumulate TTX standards to enable the testing and validation of both LC-FLD and LC-MS/MS methods for TTX confirmation.
- To assess the performance of the commercial TTX ELISA and/or any other suitable immunoassay available in kit form for applicability to samples as a screening test.
- To continue the validation of the AFBINI SPR method (developed at Queens University Belfast and validated at AFBINI as part of the Interreg project ATLANTOX) for TTX in all species of relevance.

- To conduct a UK-wide assessment of the above methods on a range of suitable samples and to make subsequent recommendations on performance and applicability
- Where possible to ship suitable samples to international laboratories for the assessment of contaminated samples using other biomolecular techniques

2.4.4 Identification of knowledge gaps with regards to CIs which might be addressed through further research or method development

The knowledge gaps relating to research requirements for prevalence and detection of terodotoxins in marine animals are currently wide. Following identification of TTXs in gastropods and fish species within European waters, there is a clear need for further research including:

- Identification of sources of TTXs in UK waters, both those present now and potentially in the future
- Analysis of bacterial cultures by suitable methods for assessment of presence of tetrodotoxins in water samples
- The determination of marine species of relevance that may accumulate TTXs and associated depuration rates
- The determination of specific TTX profiles studies in relevant species
- Relationship of toxicity to specific fish species and fish size
- Evaluation of MBA-replacement screening methods, in particular the commercial ELISA and SPR biosensor
- Develop understanding of TTX and TTX metabolites toxicity in relation to human exposure, including long term assessment of intoxicated people to determine potential long term affects
- Validation of quantitative confirmatory methods for applicability to samples of relevance to the UK

2.4.5 Proposed options for routine monitoring of TTXs to meet legal requirements

Currently the sale of *fugu* is prohibited in the EU under EU regulations Regulation (EC) 853/2004 and 854/2004 and in other countries such as the US importation is prohibited (Gessner, 2000). In Japan the risk of intoxication is reduced greatly through application of legislation relating to the preparation and marketing the products, although intoxications and fatalities do still occur. Whilst EFSA have not produced an official statement about tetrodotoxins, with the occurrence of the toxins in Europe in both fish and shellfish products, further research, surveillance and risk assessment appear necessary (Silva *et al.*, 2012), with regulation potentially being considered (Paredes *et al.*, 2011). In the US there is also no established regulatory limit for TTX, but with use of the STX MBA for routine monitoring, the presence of TTX would be detected in bivalve products. However, with the UK no longer relying on animal testing for marine biotoxins detection, this option is no longer available.

2.5 ANALOGUES OF PSP, ASP AND LIPOPHILIC TOXINS

2.5.1 Suitability of existing and potential methods for identified analogues of PSP, ASP and lipohpilic toxins and identification of knowledge gaps for further research

PSP toxins

With use of the AOAC 2005.06 pre-column oxidation LC-FLD method, the decarbamoyl toxins dcNEO, dcSTX and dcGTX2&3 are easily detected in a qualitative screening step, and following ion-exchange clean-up may be quantified appropriately, given the availability of these toxins as certified reference standards. Even with the co-occurrence of these toxins some of which form identical oxidation products prior to analysis, the methodology is able to calculate concentrations of toxins. The presence of GTX6 is, however, an issue which must be dealt with in a different way. Currently no certified standards are commercially available for GTX6, which leaves a number of options for quantifying this toxin in contaminated shellfish:

- Firstly, the use of non-certified standards is one option, given that the UK has in store a number of ampoules of well characterised standard solutions of GTX6, supplied by the NRCC. These have been used previously to quantify the concentrations of GTX6 present in proficiency test samples, with acceptable results reported by the proficiency test providers. Availability of such standards would clearly be a potential risk if this toxin was seen frequently in future shellfish samples
- Secondly, a hydrolysis step has been proposed and tested by interlaboratory study for the quantitation of GTX6 (Ben-Gigirey *et al.*, 2012). This involves the acid hydrolysis of GTX6 to NEO in an ion-exchange cleaned fraction, prior to quantitation against NEO calibrants. This subsequently enables the quantitation of the precursor GTX6.
- A third option could involve the quantitation of GTX6 against calibrations prepared for other PSP toxins. Accurate quantitation could be achieved if a toxin was picked which demonstrated a repeatable response which was similar to that of GTX6. The routine running of the current non-certified GTX6 standards would enable the determination of relative response factors for GTX6 in relation to other suitable toxins, making this approach a possibility.

Options 1 and 2 have been utilised in parallel in a previous EURL study for the quantitation of GTX6 in clam samples. The results from both methods compared well with each other and with the assigned results reported by the proficiency test provider. This indicates that these methods are likely to be appropriate for future monitoring. Ideally, the availability of GTX6 standard is preferred given the additional complexity and time required to perform hydrolysis of sample extracts prior to extra analyses. Option 3 would be a last resort in the absence of GTX6 standard, given the assumptions made and the increased contribution of the quantitative method to total measurement uncertainty. It is also noted that other PSP-producers such as *Pyrodinium bahamase var. compressum* is also a producer of GTX6 as well as other PSP toxins (Oshima, 1989; Usup *et al.*, 1994). However there is no evidence for any likely arrival of this species into UK waters.

Given the hydrophobicity of the benzoate PSP analogues, the C18 SPE step employed to clean up acidic extracts of shellfish prior to LC-FLD would remove these components from the samples under analysis. A modified LC method or LC-MS/MS would be required to identify these components in crude extracts (Vale, 2010). However, despite the high proportions of these analogues in the algae, results to date have indicated these toxins are present only at trace levels in bivalve flesh. Confirmation of a suspected carbamoylase activity in live shellfish converting benzoate analogues to decarbamoyl counterparts was confirmed with in vitro incubation experiments with digestive tissues from a variety of species of mussels, cockles, clams and oysters (Vale, 2008b). Therefore, even with high proportions of benzoate type PSP toxins present in phytoplankton, there appears to be little risk from these toxins accumulating in the shellfish.

The M1-M5 toxins identified in Canadian mussels are not responsive to LC-FLD detection, so current fluorescence based monitoring methods will be unable to detect these if present in UK shellfish tissues. LC-MS/MS detection methods were used for identifying these toxins and could be one option for identifying such metabolites if deemed a risk worth mitigation. Whilst these toxins have been detected in significant amounts in a number of European bivalves, their potential formation from GTX5 (and theoretically GTX6; Vale, 2010a) means that these toxins are unlikely to exist in UK shellfish, where GTX5 is rarely identified. LC-MS/MS methods for PSP detection and quantitation are fraught with complexities. Whilst a number of methods have been reported over the years (e.g. Dell'Aversano *et al.*, 2005), the successful application of this technique to quantitative shellfish monitoring requires the availability of suitable calibration standards. With only the 13 major hydrophilic PSP analogues currently available commercially through the NRCC, there are few advantages to be gained at present as compared to the LC-FLD methods available for the quantitation of the major toxins. Detection of the newer analogues resulting from contamination from *G. catenatum* is possible, but quantitation is unlikely to be highly accurate, particularly given the sensitivity issues and significant matrix effects known to effect the ionisation of many of the PSP toxins in the MS/MS detector. To date no successful single laboratory validation has been published for the LC-MS/MS quantitation of PSP toxins in shellfish, making the extension of this technique unlikely

for these additional toxin threats within the next few years, without significant development in standard availability and a robust approach reported for combating matrix effects and sensitivity issues.

13-nor-dcSTX has been successfully analysed using a post-column oxidation LC-FLD method, with confirmation using LC-MS/MS and Nuclear Magnetic Resonance (NMR) spectroscopy. To date, the only shellfish containing these toxins were offshore scallop samples harvested from Argentina (Gibbs *et al.*, 2009). Purified standard or an aliquot of contaminated extract would be required to determine the chromatographic elution of this toxin when using the current UK pre-column oxidation LC-FLD method. It is likely that with successful fluorescence detection described previously, detection of this toxin would be possible with the current UK methods, once elution and detection characteristics have been assessed.

Other non-chemical methods could be utilised for detection of a wider range of PSP analogues, but currently the only method validated for use in shellfish is the including the receptor binding assay (RBA; AOAC, 2011). This is a highly specific functional assay utilising the interaction of PSP toxins with the native voltage-gated sodium channel receptors. It provides a measure of total sample toxicity as a result of the affinity-related toxin binding, previously found to reliably represent total toxicity of individual congeners (Usup *et al.*, 2004). No data is currently known for the affinity of the newer congeners discussed above, so further work would be required to establish the accuracy of the method for assessing sample toxicity within shellfish samples contaminated with *G. catenatum*-related toxins together with the hydrophobic analogues and other toxin metabolites.

More development work still would be required for the potential application of antibody-detection based immunoassays for monitoring newly-emerging PSP toxin congeners. These include both the commercial ELISA kits and lateral flow immuno-chromatography kits, currently available (reviewed by Etheridge, 2010). These products provide a fast and easy to use detection method, but there are known complications amongst other things with cross-reactivity variabilities between the major PSP analogues. There is no known data available for the cross reactivity of

any of these products towards the majority of the emerging PSP toxins described here, so further work would be required to determine these experimentally before the methods could be applied to samples containing high proportions of these congeners. The challenges relating to these antibody-detection methods also apply to some of the reported Surface Plasmon Resonance (SPR) biosensors (e.g. Fonfria *et al.*, 2007) although more recent versions utilising sodium channel receptors and both monoclonal and polyclonal antibodies was shown to provide improved cross reactivities to a larger number of PSPs (Campbell *et al.*, 2007). A single laboratory validation was conducted on a screening SPR biosensor method and applied to the detection of PSP toxins in mussels and cockles (Campbell *et al.*, 2010) as well as a number of naturally contaminated shellfish matrices (Haughey *et al.*, 2011. Cross reactivity was highly variable, including low values for important toxins such as GTX1&4 (<0.7%) and other toxins known to be present in *G. caternatum* (e.g. dcNEO), although the comparative analysis between the assay and HPLC was generally good. Also, to our knowledge there is no data available to date on the cross reactivity of the assay for the emerging PSP analogues described here, so further work would be required to elucidate these. Whilst the method would certainly still be useful as a screening assay given that these newer analogues are likely to be co-existing with the major PSP toxins in shellfish flesh, confirmatory analysis is likely to still be required.

Overall it is noted that the discovery of these additional PSP toxins highlights a need to assess the prevalence, toxicity and suitable detection methods for these compounds. With potentially toxic analogues remaining in shellfish flesh, there could be a need for shellfish monitoring programmes to incorporate these into surveillance where *G. catenatum* in particular acts as a major toxin source for bivalve molluscs (Etheridge, 2010). However, with a lack of data on the presence and toxicity of these compounds, more research would be needed to describe this risk with greater clarity. In the UK, with no evidence to date from *G. catenatum* and with LC-FLD method validation studies showing generally good correlations between the analytical and mouse assays, there appears to be a low current level of risk to the UK shellfish from the presence of significant levels of new PSPs which may result in an under-estimation of sample toxicity if remaining undetected. However ongoing research

would still be recommended, including the development and application of LC-MS/MS methods for the determination of additional PSP toxins.

ASP toxins

The proportions of the additional isomers of domoic acid are generally low in naturally contaminated shellfish samples. They are also noted as being of lower relative potency. With the incorporation of these isomers into the overall determination of total domoic isomers in tissue samples, either through chromatographic resolution and individual isomer quantitation or through a simplified isocratic LC-UV analysis and the determination of all isomers in one chromatographic peak, the toxic contribution from all these compounds should be assimilated into the routine ASP results. Whilst confirmatory LC-MS analysis could potentially be conducted on a range of shellfish samples to assess the relative proportions and toxic contributions from other domoic acid isomers, with the risk to human health not likely to be of major concern, this is not an approach needed urgently. Whilst a number of National Reference Laboratories are looking at incorporating domoic acid into the lipophilic toxin LC-MS/MS methodology, this is focussed more on developing efficiency and potentially removing the need for a separate ASP LC-UV method. However, current recovery issues for domoic acid using the lipophilic toxin extraction method are complicating this development. Overall the current LC-UV method and LC-MS/MS alternatives are fit for the purpose of monitoring the presence of domoic acid and naturally-occurring analogues.

Lipophilic toxins

OA-group toxins

The situation with the presence of OA, DTX1, DTX2 and a range of acyl esters (DTX3) in contaminated shellfish is well known. OA, DTX1&2 are all easily analysed directly in crude shellfish extracts by the current LC-MS/MS reference method, applied throughout the UK official control monitoring laboratories. Whilst the DTX3 esters cannot be determined directly at present, a relatively simple alkaline hydrolysis step enables the conversion to parent toxins and facilitates quantitation of the toxic threat. With this current approach all OA-group toxins can be adequately

identified through the use of current monitoring methodologies. Other approaches have been reported including the direct determination of OA-esters by MS/MS, although this is challenging and complicated by the lack of standards, longer analysis times and a complex set of MRM transitions (Quilliam, *et al.* 2012).

Other methods are available including the use of conventional HPLC with fluorescence detection. These have been extensively reported using a wide variety of pre-analysis derivatisation reagents (e.g. Lee *et al.*, 1987; Quilliam *et al.*, 1998; Louppis *et al.*, 2010; Dickey *et al.*, 1993; Kelly *et al.*, 1996; Ramstad *et al.*, 2001; Akasak *et al.*, 1996; Morton *et al.*, 1996) with some approaches showing evidence for sensitive, selective method performance. Whilst many of these are time consuming and laborious they can potentially provide an alternative option for those laboratories who have no access to the more expensive MS detector technologies.

Other methods reported include an optical biosensor-based immunoassay, which was reported as comparing well with LC-MS/MS detection without the need for alkaline hydrolysis for DTX3 toxins (Stewart at al., 2009). A number of rapid test kits are also available commercially including ELISAs, lateral flow and protein phosphatase inhibition assays (PP2A). Whilst these are potentially useful tools for end-product testing, they are currently not applicable for official control testing of lipophilic toxins given their specificity to only OA-group toxins.

Overall therefore, for the UK official control monitoring programme which already relies on LC-MS/MS detection for quantitation of DSP toxins, there would be no advantage gained from assessing any of these alternative approaches in the context of monitoring new or emerging DSP toxins.

AZAs

With AZA toxin producers being relatively recently identified, there is a more limited selection of methodologies available for the detection of AZAs. One conventional HPLC method has been reported, involving the derivatisation of AZA toxins prior to analysis by HPLC-FLD (McCarron *et al.*, 2011). Although this was developed as a secondary analytical method to aid reference material characterisation, the method

showed good potential for application to shellfish screening, with ≥98% conversion efficiency during derivatisation and good method performance characteristics. However no further work has been reported including any interlaboratory validation and the method has not been applied to any of the minor AZA analogues.

Consequently, LC-MS/MS methods remain the method of choice for the identification and quantitation of AZA toxins. These methods are highly suited for the detection of AZAs, with an increasing amount of literature available describing the application of MS in the elucidation of newer analogues of AZA. The approach is fully capable of detecting individual AZA analogues at concentrations well below the regulatory limit, although the accuracy of quantitation is potential compromised without a larger range of certified standards for instrument calibration purposes. A large number of studies have been conducted investigating the presence of different analogues and also determining method performance characteristics such as extraction efficiency (Hess *et al.*, 2005). Other investigations have been reported describing methods for reducing matrix effects for the determination of AZA and other lipophilic toxins in shellfish flesh (e.g. Kilcoyne and Fux, 2010). Ultra-performance LC (UPLC) has also been utilised as a fast efficient separation method prior to both tandem mass spectrometry (MS/MS) (Fux *et al.*, 2007) and accurate mass detection for the detection of both known and new analogues (Rehmann *et al.*, 2008).

PTXs and YTXs

Other than the MBA which will be removed as a potential regulatory method of analysis by the end of 2014, conventional HPLC methods have been reported for PTXs, which again require sample derivatisation to form fluorescent products. Some of these have been performed using the same reagents as for OA-group derivatisation (e.g. Lee *et al.*, 1989) with others including Yasumoto's group reporting its application to the derivatisation of carboxyl PTXs such as PTX6, PTX7 and the PTX2 seco-acids (Yasumoto *et al.*, 1995, 1989; Daiguji *et al.*, 1998a). Lee *et al.*, 1989 also reported a similar approach for the determination of YTXs in shellfish, with Rawn *et al.* 2005 using the approach to confirm the presence of YTX in mussels from Norway and Japan. Whilst good performance characteristics have been reported by some authors for such methods (e.g. Ramstad *et al.*, 2001) and others

using them to aid identification of new YTX and PTX analogues (Daiguji *et al.*, 1998b), there is a relatively low amount of data available for the performance of these methods with no interlaboratory validation conducted to date.

A number of biomolecular methods have been reported for YTXs including biosensors and immunosorbent assay (ELISA), although none of these are specific or have been validated (EFSA, 2008a). Similarly for PTXs a number of antibodies have been developed (Briggs *et al.*, 2005) although not progressed to validation to date (Briggs *et al.*, 2008).

The current reference method (LC-MS/MS) is the most appropriate for detection of YTX and PTX analogues in shellfish. For the quantitation of a wide range of PTXs and YTXs and given the impracticalities associated with the production of certified standards for each of these, a similar molar response for each analogue must be assumed relative to the most similar YTX or PTX compound available as a standard. The literature contains a great deal of information of the determination of these compounds, so there is the potential with modern sensitive and specific mass spectrometric detectors to utilise MRM fragment patters to screen plankton and/or shellfish samples to determine the potential presence of those analogues unavailable as standards. Using this approach, followed by more confirmatory methods should enable data to be generated concerning the prevalence of YTX and PTX analogues in UK waters. However, other approaches have been reported including the determination of acyl ester PTXs by both direct analysis and analysis following enzymatic hydrolysis to their PTX2sa and epi-PTX2sa counterparts (Quilliam *et al.*, 2012). Overall however it is noted that there is unlikely to be any need to progress this, given that there are moves to have these toxins removed from the list of regulated toxins.

2.5.2 Proposed options for routine monitoring of analogues PSP, ASP and lipophilic toxins to meet legal requirements

PSP toxins

Regulation (EC) 2074/2005 specifies that the paralytic shellfish poison (PSP) content of the edible parts of bivalve shellfish must be detected in accordance with the biological testing method (MBA) or any other internationally recognised method. Consequently any “new” PSP analogues should be incorporated into the routine monitoring of PSP to meet the regulatory requirements. However, this is only possible once appropriate analytical standards are available and the method has been validated following international guidance for each of the compounds of interest. Given the high level of validation conducted on the LC-FLD methods to date within the UK biotoxin monitoring laboratories, a cost-effective option would be to update methodologies as and when new toxins are identified in UK shellfish which become available as certified standards. As such it would be important for certified standards to be made available for those toxins most likely to affect UK shellfish given the introduction of *G. catenatum* into the marine environment. Production of other analogues such as the hydrophobic hydroxybenzoate PSPs or the deoxydecarbamoyl PSPs appears less of an urgent matter to address at present.

In parallel with the modification of these confirmatory analytical instrumentation methods, the continued development of screening assays such as the SPR biosensor or Receptor Binding Assay could provide an additional level of safety, although development would be required in the UK network. The RBA in particular has been validated through collaborative study for a wide range of species and toxin profiles, although some further work would be required to assess its performance for the detection of novel PSP analogues. With the SPR technology potentially available to the current UK monitoring network, this option is also worth some consideration. However, the preferred approach given the expertise and equipment in place, is the continued development of pre-column oxidation LC-FLD methods, with parallel development of confirmatory LC-MS/MS and post-column oxidation LC-FLD methodologies for research and development targets.

ASP toxins

Regulation (EC) 2074/2005 states that the total ASP content in the edible part of shellfish must be detected using HPLC, or any other recognised method. If challenged, the reference method will be the HPLC method. With the current LC-UV method successful in its applicability to domoic acid and its associated isomers, any potential increases in the latter would not cause any problems with the current methodologies in place within the UK monitoring programme. As a result no development needs are anticipated to ensure the legal requirements are met for the detection of new profiles of ASP toxins.

Lipophilic toxins

The lipophilic toxin analogues specified by EU Regulation 2074/2005 are okadaic acid (OA) and dinophysistoxins (DTX) including DTX3, pectenotoxins (PTX1-2), yessotoxins (YTX, 45 OH YTX, homo YTX and 45 OH homo YTX) and azaspiracids (AZA1-3). Furthermore this suite of toxins should be supplemented by any new analogues of public health significance as they are discovered and following availability of standards and toxicity equivalence factors.

For YTXs there is no evidence encountered for any additional analogues posing a threat to consumer health, with the general move amongst scientists to deregulate or raise regulatory limits (EFSA 2008a). This suggests that the risk from the potential presence of the considerable number of new YTX analogues is not of a high concern to shellfish consumers.

Given the prevalence of AZP in a wide variety of shellfish species around Europe and the potential severity following intoxication, the AZAs represent the greatest threat to shellfish consumer safety in terms of the potential presence of a wide variety of potentially toxic analogues. It will be important for the UK laboratories to assess the potential presence of AZA analogues which are being shown to exhibit significant toxicity. Any such analogues identified as increasing risk should be monitored using modified LC-MS/MS methodologies. For this to occur successfully,

purified standards would be required to either run standards or at the very least allow the optimisation of instrumental parameters to enable the application of appropriate MS/MS MRM transitions for detection. Quantitation would need to be conducted using response factors generated from the calibrations of the major AZA toxins until calibrants of the other AZA analogues become available.

Overall, with the LC-MS/MS the designated reference method in EU legislation and the technique fully implemented into the UK official control monitoring programmes, the most appropriate option for conducting analysis of potentially new or emerging lipophilic toxins is to maintain this approach. It will be important however for the UK network to maintain awareness of the important developments, particularly where the most serious lipophilic toxins such as the AZA analogues detected in shellfish, are shown to provide a significant risk to the shellfish consumer. With this in mind, it will be important for the UK laboratories to assimilate the information provided by other researchers and/or monitoring laboratories, where possible building up a library of suitable reference materials which would facilitate the development and application of MS/MS detection methods. With the availability of contaminated materials containing the more toxic shellfish metabolites, laboratories should be able to assess the potential presence of these in UK shellfish and develop protocols and methodologies to monitor these appropriately.

2.6 CIGUATOXINS

2.6.1 Introduction

At present a simple, rapid assay providing an accurate assessment of fish tissue toxicity has yet to be fully developed (Dickey and Plakas, 2010). A range of methodologies have been developed to provide biological assays as well as *in-vitro* and chemical alternatives to CTX detection. A detailed review of these including the specifics of extraction and clean-up protocols may be found in Caillaud *et al.*, 2010a. One major factor affecting the ability of laboratories to test, refine, validate and control assays is the availability of reference standards including contaminated

matrix materials. For CTXs, this presents a major problem with currently no known certified or even non-certified reference standards commercially available for such studies. Those laboratories which have stored contaminated materials, have only limited quantities available for their own use, therefore there is limited global availability of contaminated materials. Table 6 summarises the known sources of CTXs which have been made available to research laboratories in the past.

Table 6. Summary of CTX standards utilised to date (none are commercially available)

Source	Standards	Comments
T. Yasumoto, Japan	Pacific CTXs	From contaminated fish, algal cultures, and synthetic material from collaboration with Hirama
M. Hirama, Japan	Pacific CTXs	Synthetic CTXs and fragments thereof
EURL-MB	Unknown	Collaborating to produce a reference material
R. Lewis, Australia	Pacific CTXs	From contaminated fish
R. Dickey, USA	Caribbean CTXs and contaminated materials	From contaminated fish
France NRL	Contaminated materials	
Netherlands NRL	Contaminated materials	
Kam et al, China (Hong Kong)	Pacific CTXs	From contaminated fish

2.6.2 Animal bioassays

A number of animal bioassays have been developed and used for toxicity assessment over the years, including the use of mice, cats, chickens, shrimp and mosquitoes (Lehane and Lewis, 2000; FAO, 2004). The MBA, offering the most

reliable bioassay model, has over many years been most commonly used for detection of CTX (Banner *et al.*, 1960) and MTX (Holmes et all, 1990). The method involving injection of 20 mg ether extracts of fish muscle and observations of duplicate mice for up to 48 hours has been refined by Yasumoto *et al.*, 1984 and is still widely used for CTX detection. Specific clinical signs denote qualitative identification and time of death enables the calculation of total toxicity levels (Yasumoto *et al.*, 1984). The lethality of CTXs in the assay vary depending on the profiles present, with a LD_{50} of 0.25 ug/kg for P-CTX-1 and 3.7 ug/kg for C-CTX-1. Spiked samples have been used to validate the method and have indicated recoveries of 63±14% (Lewis and Sellin, 1993), with similar recoveries following four variations of the ether-water partition clean-up step. In addition to the recognised ethical issues associated with the assay, sensitivity issues result in low-levels of ciguateric fish remaining undetected by the test (Lehane and Lewis, 2000) and some assay interferences are recognised. However, Wong *et al.* (2009) report that use of a modified clean-up involving an additional solid phase extraction (SPE) step may remove lipid-based matrix interferences and shorten death times, consequently potentially improving the limit of detection of the method (Caillaud *et al.*, 2010a). The test has been used extensively for research purposes as well as less frequent monitoring of fish for market (Dickey and Plakas, 2010), with an example of the latter being the study of Wong *et al.* (2005) who describe the use of the assay for monitoring of CTX in fish from markets and import sources.

2.6.3 Chemical methods

As with any chemical method involving quantitation of specific toxins or toxin groups, methods of analysis require determination alongside certified reference materials. To date the reference material standards are **not** commercially available, with analytical development conducted utilising trace amounts of standards received as gifts. As a result, and certainly as a consequence of the availability issue, no analytical methods for CTXs or MTXs have been validated formally through interlaboratory study. Since occurrence of the Madagascan shark poisoning fatalities, no further work has been published in relation in to the detection or structural elucidation of the carchatoxins.

Extraction

Extraction of dissolved CTX and MTX in seawater has been achieved through use of solid-phase adsorption tracking (SPATT) devices. Caillaud *et al.* (2011) reported detection of both groups of toxins following exposure of resins to dissolved toxin solutions and phytoplankton cultures. Extraction recovery was estimated at 85%-91% for CTX-1 depending on the time of SPATT exposure and 66% for MTX. Whilst field experiments are yet to take place, the technique was proposed as a potential monitoring tool in areas of high risk, and also as a method for recovering toxins from culture to facilitate the production of reference standards (Caillaud *et al.*, 2011).

Extraction procedures for toxins in algal cultures are relatively simple**.** Examples include the use of acetone extraction solvent prior to chromatographic purification (Murata *et al.*, 1990) and methanol, 50% methanol and liquid partitioning (Chinanin *et al.*, 2010). It must be emphasized however that the more toxic forms are generally not produced directly by the algae, with the exception of one oxidized form found in an isolate from Japan (Yogi *et al.*, 2011).

Chemical analysis methods for the determination of CTXs in shellfish are complicated by the need for rapid extraction and simple clean-up methods that minimise significant matrix effects. Various options are available in the literature with many based on an initial extraction using acetone extraction prior to a number of liquid-liquid partition steps involving water:di-ethyl ether prior to aqueous methanol and hexane (Yasumoto *et al.*, 1984), dichloromethane:aqueous methanol partition (Pauillac *et al.*, 1995) and water:diethyl ether and cyclohexane:methanol partitioning steps (Legrand *et al.*, 1998). In the latter example, extracts were further purified by chromatography. Variations were also reported by Vernoux and Lewis (1997). Alternative extraction methods have included the use of methanol:hexane (3:1), with use of the methanolic partition for subsequent clean-up and analysis (Lewis *et al.*, 2009). The same authors also used solid phase extraction (SPE) as an enrichment step to improve subsequent method sensitivities (Lewis *et al.*, 2009) and Gel Filtration Chromatography used for purification purposes prior to LC-MS/MS

detection (Pottier *et al.*, 2002a). Yasumoto (2012) reported that extraction of toxins is more efficient from the cooked flesh.

Conventional chromatography methods

CTXs do not possess strong chromophores to enable selective determination of toxins in fish extracts. However, HPLC-UV has been employed for chromatographic purification of toxic fractions (e.g. Vernoux and Lewis, 1997 and reviewed by Dickey and Plakas, 2010).The presence of a primary hydroxyl group in many of the CTX congeners has been exploited for derivatisation to fluorescent esters prior to HPLC with fluorescence detection (HPLC-FLD). One HPLC-FLD method using post-column derivatisation of chromatographically-separated CTXs was developed using alkaline oxidation with peroxide and ammonium hydroxide (Sick *et al.*, 1986). Whilst sensitivity was demonstrated, the authors reported poor precision and accuracy of the method. Yasumoto subsequently investigated the use of the reagent 1-anthroylnitrile, which enabled the characterisation of 10 CTX toxins with good detection linearity over the 1 to 100ng range (Yasumoto *et al.*, 1993). Analysis of CTXs in fish was subsequently conducted using reverse phase HPLC under a range of different isocratic HPLC conditions used for the separation of the primary fluorescent esters (Lee *et al.*, 1989). Yasumoto and co-workers also noted that efficient clean-up protocols would be required to improve the selectivity and sensitivity of methods, especially important given the complexity of fluorescent matrix components and the low concentrations of CTXs causing illness (Lee *et al.*, 1989). Dickey *et al.* (1992) used diethylaminocoumarin carbamate for the derivatisation of P-CTX-1 prior to HPLC-FLD as also reported for brevetoxins, with a detection limit of 0.5 to 1.0ng. The low yield of the labelling reaction highlighted the need for optimised reaction conditions or more appropriate fluorescent reagents to be used. Additionally, analysis of CTX analogues without the primary hydroxyl groups would not be suitable.

Preparative-scale HPLC-UV has been used for isolating two analogues of maitotoxin (MTX) with 15µm C18 columns (5-28cm), a MeOH mobile phase and detection at 210nm (Miller *et al.*, 1989). Holmes *et al.* (1990) reported the use of HPLC for purification of MTX1 and MTX2. Also a method involving capillary zone

electrophoresis with UV detection has been reported for MTX in both standard solutions and purified fractions of algal cultures. Results indicated good sensitivity (50pg), with confirmation using a cytotoxicity test (Bouaicha *et al.*, 1997). The use of solid phase extraction was recommended for extraction of toxins and analysis using this technique when investigating the presence of MTX in fish.

Reverse-phase HPLC has been used to isolate two toxins termed carchatoxin-A and B from contaminated shark meat, although structural elucidation was not possible (Boisier *et al.*, 1995). Analytical-scale HPLC with a 75% MeOH mobile phase and UV detection at 210nm showed different (less polar) chromatographic properties to CTX-1 (Yasumoto, 1998).

LC-MS methods

Given the success of mass spectrometric detection methods for a large number of CTX and GTX analogues, a large number of published methods focus on this technique.

Ionspray mass spectrometry has been applied to the determination of a wide range of polyether toxins, including CTX (P-CTX-1) and MTXs (MTX-2 and MTX-3). CTX-1 was detected at 1ng, although lipid-based matrix effects in crude extracts of fish flesh affected this determination significantly (Lewis *et al.*, 1994). Structural elucidation of CTXs has also been demonstrated using Fast-Atom Bombardment tandem mass spectrometry (FAB-MS/MS; LeGrand *et al.*, 1998; Yasumoto *et al.*, 2000).

Reverse phase LC-MS has been used for the characterisation of CTXs in fish, allowing for example the identification of P-CTXs in moray eels (Lewis and Jones, 1997). C-CTX and I-CTXs have also been identified and characterised using LC-MS in a number of different species of fish and purified extracts (Vernoux and Lewis, 1997; Lewis *et al.*, 1998; Hamilton *et al.*, 2002a,b; Pottier *et al.*, 2002a,b, 2003). Accurate mass analysis has also been employed to compare CTXs from different sources (Hamilton *et al.*, 2002b). LC-MS analysis of a range of contaminated fish tissues from Japan demonstrated specificity issues with the presence of multiple

interfering peaks, therefore requiring two clean-up stages prior to analysis (Oshiro *et al.*, 2010). Some discrepancies between the LC-MS and MBA toxicity data were in that study attributed to the existence of CTX congeners which were not detected by LC-MS due to the absence of standards. Otero *et al.* (2010) recently reported the use of Ultra-Performance LC with MS (UPLC-MS) utilising both full scan and selected ion monitoring (SIM) for the determination of CTXs in fish species from Madeira. Interestingly the quantitative results compared favourably with those determined using an electrophysiological assay.

In particular, LC-MS/MS has proven itself to be a useful and highly specific tool for determining CTX profiles in algal cultures and contaminated tissues, enabling the determination of individual analogues of each CTX family. Lewis *et al.* (1999) originally reported the use of reverse-phase LC with positive mode electrospray MS/MS for the determination of sub-ppb concentrations of both P-CTX and C-CTXs in fish extracts. Detection limits of 0.04 and 0.1 ppb were determined for P-CTX-1 and C-CTX-1 respectively, with acceptable detector linearity. Matrix effects were noted resulting in response suppression for both CTXs in spiked fish extracts, thought to relate to the presence of lipid-based co-extractives, although the accuracy was improved through use of CTX internal standards. Evidence for reliable applicability to a range of Caribbean fish extracts was reported with a good correlation between results returned by the method and the MBA (Lewis *et al.*, 1999). LC-MS/MS has also been used for confirmation of CTX presence in seals from Hawaii, providing evidence for trophic transfer of CTXs to marine mammals (Bottein Dechraoui *et al.*, 2011) and for confirmation of profiles in source algae (e.g. Roeder *et al.*, 2010).

Lewis *et al.*, 2009 reported a rapid extraction LC-MS/MS method (termed CREM-LC-MS/MS) for the determination of CTXs in fish tissues. A simple methanol/hexane extraction was proceeded by a two-step SPE clean-up (reverse-phase then normal phase) before analysis using reverse-phase chromatography and MS/MS detection in positive mode. The LC gradient utilised was reported as effectively separating P-CTX-1 from lipids potentially causing matrix suppression, whilst conducting each analysis with fast cycle time of 12 min. Studies revealed good linearity between 6 to

600pg with an estimated P-CTX-1 recovery of approximately 90% (Lewis *et al.*, 2009). The approach therefore appears applicable to the detection of P-CTX-1 at clinically relevant levels (Lewis *et al.*, 2009). This method was subsequently refined and used to establish a referee analysis method for P-CTXs in fish in Queensland, Australia (Stewart *et al.*, 2010). The extraction was conducted on cooked then re-frozen fish tissues, prior to homogenisation/extraction in 3:1 methanol/hexane, a modified clean-up without the second normal phase SPE step, prior to LC-MS/MS. Results obtained from positively contaminated fish samples allowed the determination of a P-CTX-1 reporting limit of 0.07µg/kg, therefore below the 0.1 µg/kg concentration thought to initiate CFP (Lehane and Lewis, 2000). The analysis of negative controls showed good method specificity, although spiked sample analysis showed variable performance with excellent precision but some poor recoveries (varying between 27% and 75%). Hamilton *et al.* (2010) have also reported the examination of forensic samples associated with a fatal intoxication from P-CTXs, using both LC-MS and LC-MS/MS in combination with a receptor binding assay. More recently detailed LC-MS/MS studies have been conducted facilitated by the use of 14 different reference toxins for CTXs. This enabled the assessment of CTX-1B and CTX-3C type toxins in a range of fish and source algae from the Pacific, with the authors concluding the method provided a rapid, specific and accurate confirmation (Yogi, *et al.*, 2011). The technique has also been applied recently to C-CTXs implicated in poisonings, with the use of SRM LC-MS/MS for the determination of C-CTX-1 and a range of other minor analogues in cytotoxic fractions of uncooked and cooked fish products (Abraham *et al.*, 2012). The study confirmed C-CTX-1 to be dominant biomarker in the fish species analysed, with very little difference in results obtained between uncooked and cooked tissue. LC-MS/MS has also been used to report the emerging risk relating to CTXs in the Canary Islands (Boada *et al.*, 2010), with results highlighting the presence of ciguateric fish in the temperate waters of the Eastern Atlantic. It has also been applied recently to the monitoring of dissolved CTX and maitotoxin in algal cultures using solid-phase adsorption tracking devices (Caillaud *et al.*, 2013).

Whilst authors have in the past discounted analytical methods as cost-effective tools for routine screening (Lewis, 2001), these methods have proved extremely useful for

identification of specific toxins and characterisation of CTX profiles in a wide variety of sample matrices, with reports of good correlation with the MBA (Yasumoto, 2012). With the continued development and production of standards, such confirmatory methods are likely to provide a useful, practical and fit-for-purpose tool for screening fish for the most prevalent and toxic CTXs. As with the brevetoxins and potentially other emerging toxin threats, one approach could involve the application of both biomolecular screening tools in tandem with LC-MS/MS confirmatory tests (Stewart *et al.*, 2010). Use of faster chromatography methods as reported for CTXs by Caillaud *et al.* (2011) will also benefit the application of the technique to high throughput routine monitoring.

Biomolecular methods

In vitro methods applied to CTX include cytotoxicity assays, receptor binding assays, and immunoassays. An electrophysiological assay has also been reported (Otero *et al.*, 2010). Some of these assays are particularly useful for monitoring the activity of extracts or purified fractions, particularly when new analogues are discovered or toxin standards are unavailable. They are generally highly sensitive and without the use of live animals present a more ethical solution to the need for toxicity assessment screening for CTXs in fish tissues.

Cytotoxicity assay

Cytotoxicity assays using cultured cells provide a more ethical approach to monitoring quantitative toxicity in samples. Whilst a range of cell lines have been utilised for CTX identification, the main focus has been on the use of neuroblastoma (N2A) cells due to high sensitivity to CTXs and practicalities of cell line handling (Caillaud *et al.*, 2010a). Caillaud *et al.* (2010a) provide a detailed review of cytotoxicity methodologies available for CTX detection. In the US, outbreaks of ciguatera are investigated using a highly sensitive sodium channel specific cytotoxicity screening method employing N2A cells (Dickey and Plakas, 2010), as also applied for the detection of brevetoxins (Manger *et al.*, 1993). Positive samples are subsequently confirmed with use of LC-MS, with the whole process taking three days (Robertson, 2012). Use in parallel with other biomolecular methods such as the

receptor binding assay, has enabled the detection of both ciguatoxins and brevetoxins (Bottein Dechraoui *et al.*, 2005a) in a wide range of water (Caillaud *et al.*, 2011), culture (e.g. Fraga *et al.*, 2011), fish tissue (e.g. Caillaud *et al.*, 2012), uncooked and cooked meal remnants (Abraham *et al.*, 2012) and blood samples (e.g. O'Toole *et al.*, 2012) as well as from the blood and organs of marine mammals (Bottein Dechraoui *et al.*, 2011).

As originally described by Manger *et al.* (1994, 1995) delineation of Voltage-gated sodium channels (VGSC) specific activity in the 3-(4,5-dimethylthia/ol-2-yl)-2,5-diphi-iiyltclrazoliumbromide (MTT) cell bioassay is achieved through control experiments, by withholding the auxiliary toxins ouabaine and veratridine (-O/V) to define a flat reference baseline against which VGSC activity is measured. In this way VGSC active toxins like the CTXs are clearly evident versus less specific toxins that could cause a response in the –O/V controls. Activators like CTXs give different response curves from blockers such as saxitoxins since the latter cause cell rescue and the former, cell death. Some authors have questioned the cost-effectiveness of applying this assay to routine screening of fish (FAO, 2004). The sensitivity of the assay as applied to fish extracts has been demonstrated (Manger, 1995) and reconfirmed as fit for purpose in relation to toxicity levels causing consumer poisonings (Caillaud *et al.*, 2012), with LOQs approximately 0.01 CTX-1B eq./g.

Other assays have been developed with the use of neuroblastoma cells treated with voltage-sensitive dyes (Louzao *et al.*, 2004). Results indicated rapid detection of purified toxins in the nanomolar range but successful analysis of actual fish extracts was not reported. In the use of voltage sensitive dyes to develop rapid cell assays, flow cytometry imparts improved signal to noise ratios by electronically selecting the most responsive cells. This approach has been applied for determination of toxicity in fish extracts, with a resulting highly sensitive, rapid assay (Manger *et al.*, 2013). To achieve routine use, automation, additional calibration, and validation are needed.

The cytotoxicity of MTX has been investigated through the use of mammalian fibroblast cell lines. Cell viability and morphological alterations were measured using

Neutral red uptake (NRU) allowing quantitation of toxicity of MTX as well as okadaic acid (OA), with qualitative observations being used to increase specificity. The technique has so far been applied to extracts of phytoplankton (Fessard *et al.*, 1994; Fraga *et al.*, 2011). Caillaud *et al.*, 2010b have also reported the detection and quantitation of MTX-like compounds using the N2A assay, reporting its usefulness as a rapid screen for detection of toxicity in cultures.

Receptor binding assays

The sodium channel receptor binding assays (RBA) or radiolabelled ligand binding assay (RLB) using ^{3}H-PbTx-3 for determination of CTXs as well as brevetoxins, are potentially useful screening tools for the assessment of tissues implicated in intoxications. Brevetoxin is used due to the low availability of CTX standards. The method is based on binding competition between CTX present in a sample with ^{3}H-PbTx-3 for the voltage dependent sodium channel in rat brain synaptosomes. The assay was initially conducted in a test-tube format, although Van Dolah *et al.* (1994) later described the use of a high throughput microplate RBA for CTXs and brevetoxins, which rapidly reduces the time of analysis to less than three hours and avoids extensive manipulation of individual samples. The assay is rapid and capable of analysing samples in parallel and the preliminary data was reported as comparing well with the MBA (Van Dolah *et al.*, 1994). The RBA also provides a quantitative assessment of total sample toxicity, with the relative binding of different analogues mirroring their individual toxic equivalence. Detection limits are reported as being in the pmol range (Van Dolah and Ramsdell, 2001), so fit for purpose in terms of quantifying toxicity in fish tissues. The method has been used for determination of CTXs in cultures (e.g. Chinain *et al.*, 2010) and fish tissues for risk assessment purposes (e.g. Darius *et al.*, 2007) and alongside a cytotoxicity and/or LC-MS assay for identification of CFP in fish tissues including outbreak samples from the Caribbean (Poli *et al.*, 1997), the Indian Ocean (Hamilton *et al.*, 2002a,b, 2003) and Australia (Hamilton *et al.*, 2010). Bottein Dechraoui *et al.* (2005b) have described its use alongside a cytotoxicity assay to discriminate CTXs from brevetoxins in fish from Florida.

The assay has been applied successfully for the determination of relative toxicities of CTX congeners. Advantages therefore relate to the ability of the assay to identify toxic tissues and in combination with LC-MS confirmatory methods enable the elucidation of a growing number of toxic CTXs (e.g. Hamilton *et al.*, 2002a,b, 2003). Disadvantages described relate mainly to the perceived method complexities and the requirement for radiolabelled materials.

For the determination of MTX, a high throughput microplate ^{45}Ca flux assay has been described (van Dolah *et al.*, 1994), similar to a format reported by the same authors for brevetoxins and CTXs.

Immunoassays

Immunoassays have been pursued with the aim of producing simple, highly sensitive and specific methods of detection for CTXs, although these efforts have been hampered until very recently due to the extreme scarcity of purified CTXs. Preparation of CTX-protein conjugates required for raising antibodies is not feasible under these constraints, although attempts were made over the years using partially purified materials. This shortage of purified toxins also made well controlled, statistically meaningful characterization and evaluation of immunoassays difficult. The absence of any official guidance levels further complicated the problem since no target limits of quantitation were agreed on. One unfortunate outcome of these problems is that no commercial test kit for CTXs has ever proven accurate enough to pass validation.

A radioimmunoassay (RIA) was developed as early as 1977 for CTX detection in fish (Hokama *et al.*, 1977). The authors proposed this as a practical, specific and simple assay, using it to distinguish positive outbreak samples from non-toxic fishes and indicating a fair correlation with a mongoose assay. Later Hokama *et al.* (1984) developed an enzyme immunoassay which showed similar performance to the RIA but without the cost and practicality issues. Although the assay is thought to be compromised significantly by poor specificity issues (Dickey and Plakas, 2010) including cross reactivity issues with other toxins such as okadaic acid, brevetoxin

and maitotoxin (van Dolah and Ramsdell, 2001). The approach was developed and simplified further with the use of a stick test (Hokama, 1985) and a solid phase immunobead assays (S-PIA) with a rapid visual colour test (Hokama, 1990). According to these authors, the assessment of these stick tests with the ELISA and the MBA showed good comparison and was applied to the examination of clinically implicated fishes. Finally an assay was commercialised (Ciguatect, Hawaii Chemtect International, Pasadena, California, 91109), the analytical precision assessed in naturally contaminated fish tissues following a mini-collaborative study (Park *et al.*, 1992) and plans were in place to evaluate method performance through a more extensive interlaboratory study (Park, 1994, 1995). The test also detects the presence of okadaic acid and other polyether compounds, so specificity was potentially an issue. However, Dickey *et al.*, 1994 reported unfavourable comparison with MBA results, with significant proportions of both false negatives and false positives.

Another commercially available test kit “Cigua-check” based on a membrane immunobead assay (Hokama *et al.*, 1998) has been evaluated for potential application to the determination of CTXs in fish (Garcia Camacho *et al.*, 2007). Wong *et al.* (2005) found poor agreement between the Cigua-check and MBA tests and describing the confirmatory determination as being hard to achieve. Biefang *et al.* (2011) also concluded from their study that with a low level of agreement in results determined by multiple analysts and a lack of agreement with the cytotoxicity assay the Cigua-check was not reliable. However, these conclusions have been refuted by the test kit providers who questioned the validity of the comparison with the cell bioassay (Ebesu and Campora, 2012).

Perhaps more important to potential users, an attempt to validate the commercially available immunobead assay was made, but was unsuccessful, with the final manuscript ultimately rejected following AOAC Int. review (Hungerford, 2009). Although many references can be found in the literature to use of this kit, it has been proven to be inaccurate, producing false negative results following the testing of ciguatera outbreak-implicated samples as well as false positives (Wong *et al*. 2005). These results figured prominently in the decision to not give AOAC Int. approval as

an official method (Hungerford, personal communication). At the present time, this kit is no longer commercially available.

Antibodies specific to synthetic fragments of CTX were reported as providing an accurate and reliable semi-quantitative screening tool when applied to fish from Hawaii (Campora *et al.*, 2008a,b). The 96-well format method, reported as taking 4 hours to complete when applied to a large number of two different fish species, was found to compare well with the neuroblastoma cell assay. Method performance characteristics including recovery, precision, sensitivity and linear range were also reported as acceptable. However, the method utilized the same antibody as the immunobead Ciguacheck assay so is subject to the same issues (Hungerford, personal communication).

More recent efforts pursuing immunoassays are yielding promising results using a sandwich format to produce direct ELISAs. A key factor in producing useful antibodies for the sandwich ELISAs has been the total synthesis of CTXs now achieved in Japan (Hirama *et al.*, 2001) and further use of synthetic fragments to raise monoclonal antibodies to each end of the CTX 3C molecules Oguri *et al.* (2003), which enabled the production of an ELISA detecting CTX-3C at low ppb concentrations without cross reactivity to other polyether toxins. Tsumuraya *et al*. (2006, 2010, 2012) subsequently reported the generation of antibodies against P-CTX, CTX-1B and 51-OH-CTX-3C, also without specificity issues against BTXs, OA and maitotoxin. Due to structural similarities among the toxins it is possible to detect several toxins using four different antibodies. In the sandwich format one antibody is used to capture the toxin molecule at one end while the second antibody allows detection via a conjugated enzyme. Use of this direct sandwich approach does not require 2 unique antibodies per toxin, due to structural similarities among the CTXs (Tsumuraya, *et al.* 2013). The high specificity and “tunable” nature of this approach is important since, as pointed out by Lehane and Lewis (2000), false positives can result from low specificity or conversely, there is the risk of false negatives if the antibodies do not detect important toxic analogues. These approaches are thought by some authors to provide the best potential for fast, simple and accurate screening of fish samples (Dickey and Plakas, 2010). Due to the importance of developing a fit-

for-purpose test kit, work has recently started with European funding (CIGUATOOLS project) to develop a new rapid test kit and supporting reference standards capable of detecting CTXs in both European and global waters. With the project completion due towards the end of 2014, there is the potential for further developments in this area of CTX testing.

For MTX, developments have been less extensive. However, Bignami *et al.*, 1996 described the first production of MTX-specific antibodies in mice and the subsequent preparation of a competitive immunoassay. It was found to detect purified MTX standard at 45ng/mL with no specificity issues in relation to other polyether marine toxins, including CTX, gambierol, yessotoxin, BTX, OA and PlTX (Bignami *et al*, 1996).

Table 7. Summary of methods applicable to the determination of ciguatoxins in shellfish.

Method	Advantages	Disadvantages
Mouse bioassay (MBA)	• Well known and well used • Good recovery of toxins	• Lack of sensitivity • Lack of specificity • Ethical issues
Cytotoxicity assay	• Useful screening for sample or fraction toxicity • Parallel format • Highest Sensitivity (0.01 ppb) • Applicable to wide range of sample matrices • Small amount of toxin used	• Cell culture techniques required (aseptic technique, cells must be maintained) • Operator skill in plating cells • Incubation several hrs to overnight
Receptor binding assay, RBA	• Useful screening for sample or fraction toxicity • Parallel format • Good comparison with MBA • Applicable to wide range of	• Requirement for radiolabelled materials • Operator skill in preparing membranes

	sample types	on filters
Flow cytometry with Voltage Sensitive Dyes	• Response in minutes – 1 hr • Sensitive (sub ppb) • Auto sampling option • Single standard required	• Still in development • Somewhat expensive • Cell culture techniques required (aseptic technique, cells must be maintained)
Direct (sandwich) ELISA	• Sensitive (sub ppb) • Most rapid assay for CTXs • High specificity • Parallel format	• Still in development • Not commercially available • Requires sample cleanup
Pre-column labeling LC	• Less expensive than MS detection	• Unsuitable for analysis of CTX analogues without the primary OH groups. • Interference from lipids
LC-MS(MS)	• Highly specific • Quantitative	• Consumes large quantities of scarce standards • Multiple standards required • Expensive equipment

2.6.4 Suitability of existing and potential methods for CTX testing

Published methods for the testing of shellfish for CTXs are summarised above in Table 7. As discussed above, the MBA is a commonly-used method for the

determination of CTX toxicity in fish tissues. Whilst method performance characteristics have been identified, issues are noted regarding specificity and sensitivity in addition to the ethical problems associated with live mammal assays. In the EU regulations there is currently provision to undertake analysis of emerging toxins using the current MBA for lipophilic toxins. However, it is not clear whether the current extraction method would be suitable for the determination of CTXs. In addition, with a clear move away from reliance on animal assays for marine biotoxin testing, the UK would not currently be in a position to validate new versions of the assay applicable to CTXs, given the complexities associated with animal licences and the likely high numbers of mice required to routinely screen for CTXs in fish. Given the MBA performance issues, laboratories in regions most affected by the toxins have conducted a great deal of development to assess alternative biological and chemical-based assays.

In practice to date, many laboratories actively involved in the identification of CTXs in fish tissues, cultures and other matrices have utilised a combination of one or more of the aforementioned assays. As early as 1997, Poli *et al.*, reported the identification of C-CTXs as the cause of poisoning events through the combined use of receptor binding assay and LC-MS. Many other studies have employed the use of LC-MS in combination with the cytotoxicity assay (e.g. Abraham *et al.*, 2012) and/or an immunoassay (e.g. Wong *et al.*, 2005) or multiple combinations thereof (e.g. Boada *et al.*, 2010). From the evidence gathered in this review, in particular from those regions experiencing CFP as a real threat from the local marine environment, it is clear that at present a dual assay approach is the safest option to take. The availability of toxin standards of the important precursors and metabolites is still low, with the majority of purified standards made available to researchers as gifts resulting in very few being available commercially. Consequently, the ability of any given laboratory to obtain enough standards in order to conduct appropriate method validation following IUPAC/AOAC guidance for quantitative LC-MS/MS determination will be doubtful. However, with the UK testing regime already set up to conduct LC-MS/MS analysis routinely for other marine toxins, this approach is certainly one of high interest and provides some practical advantages, relating to the instrumentation required already being in-house.

The biomolecular methods such as the cytotoxicity assay and the receptor binding assay both appear very useful and applicable techniques for screening fish tissues and for the assessment of sample toxicity. These have been highly beneficial to laboratories conducting CFP analysis and there is plenty of evidence to suggest these are fit for purpose assays, well suited for screening fish samples for food safety purposes. However, neither of these assays are currently in use with the UK biotoxin monitoring programmes. For the cell assay the type of expertise and instrumentation required are those associated with microbiology or cell culture in virology, such as observing aseptic technique, using laminar flow hoods, CO_2 incubators, etc. In some UK laboratories these may already be available, which could have a major impact on cost. Since the cells must be maintained it is best to have personnel specifically tasked with setting up, validating, and implementing such assays. It is recognized, however, that it is less costly and also less laborious to maintain cells than a mouse colony (Hungerford, personal communication). The receptor binding assay, although less challenging in some ways and more easily within reach of a variety of laboratory personnel, as currently practiced uses radionuclides and so would require appropriate licensing, safety and disposal procedures. These would again be available at some UK laboratories if required and development costs could be met. For highest efficiency a 96-well scintillation reader is also required for the RBA. In requiring much less in quantity and variety of CTX standards than LC-MS(MS) either RBA or cell assay may potentially represent a suitable starting point for developing CTX capabilities in the UK.

A reliable, cheap and fast test kit which is amenable to all CTXs and is accurate and reproducible would be a highly beneficial tool to both industry and the analytical scientist. Whilst great progress seems to have been made over the years, there is no evidence to date that such a product exists that can be relied upon for accurate and safe assessments of food safety. There is also the risk of reliance upon one single commercial product which can over the years be changed by the manufacturers, potentially affecting method performance and applicability to control testing.

Overall it is clear that currently the testing regime in the UK, as with most other regions in the EU and the rest of the world, is currently unprepared for the safe detection of CTXs in fish. From the work conducted to date and given the equipment and expertise currently in position within the UK biotoxin monitoring programmes, the recommended approach would be as follows:

- To accumulate CTX standards facilitating the development of LC-MS and LC-MS/MS methods for CFP confirmation. With availability of suitable standards, these methods could be applied initially as research tools to enable the assessment of CFP toxin profiles in suspect fish samples
- To assess the most appropriate screening assay for samples of relevance, ideally involving the collaboration with ciguatera experts already utilising these techniques. Once assessed to determine the method most appropriate to the UK programme, given available technology and expertise. Unless significant advances are made in reliable and accurate rapid test kits, this is likely to involve either the cytotoxicity or receptor binding assay. Whilst not yet reported, with availability of a suitable antibody and standard material, development of an SPR method might also be an option, with the technique also available within UK monitoring laboratories. The decision is also likely to be affected by the potential use of these assays for the determination of other toxin groups, with application of either assay potentially applicable to a large number of shellfish and fish poisoning syndromes.

2.6.5 Identification of knowledge gaps with regards to CTXs which might be addressed through further research or method development

The knowledge gaps relating to research requirements for prevalence and detection of CTXs in seawater and finfish are extensive. Key knowledge gaps include the need for:

- UK-wide surveillance initiatives to collect data on suspected cases of CFP, as conducted in the Canary Islands.
- Identification of additional algal species which produce CTX precursors**.**

- Identification of algal species to determine the presence of toxins in areas of relevance.
- Analysis of algal cultures by suitable methods for assessment of presence of CTX precursors in water samples.
- Determination of the factors which stimulate dinoflagellate growth, in particular the production of toxins. Given that many wild populations of *G. toxicus* do not produce CTX precursors**,** monitoring for algal blooms will not necessarily allow prediction of toxic events.
- The determination of fish species that accumulate CTXs and the development and dissemination of risk management protocols to minimise the impact of ciguatera.
- The determination of CTXs by LC-MS profile studies in relevant fish species and development in the production of appropriate reference standards.
- Continued evaluation of MBA-replacement methods, in particular including a cheap, reliable screen for application to fish and clinical samples and the provision of fit for purpose and validated confirmatory techniques involving either bioassay and/or LC-MS techniques.
- Develop further understanding of CTX toxicity in relation to human exposure, including long term assessment of intoxicated people to determine potential long term effects.

Overall a large amount of further research, method development and risk assessment is required before the UK monitoring programme could benefit from the potential application of CTX determination in fish sourced within or imported to the UK.

2.6.6 Propose options for routine monitoring of CTXs to meet legal requirements

There are no current limits for CTX-group toxins in the EU, although EC Regulations 853/2004 and 843/2004 dictate that seafood products must not be placed on the market that contain CTXs or other toxins dangerous to human health. In addition to

the absence of designated European regulatory limits, there are currently no specified reference methods of analysis for CTXs in fishery products.

Regulations or risk management guidelines are present in other parts of the world, including the US where guidance levels are stated for both C-CTX and P-CTX equivalents based on outbreak studies and with concurrence of Australian and Japanese experts. In the US, Japan, Australia and New Zealand, practical measures are required for minimising the risk of fish products containing CTXs, including controls on the species of fish or areas of harvest. This approach is necessitated by both the absence of rapids tests and also the variation in toxins levels between individual fish. Within the EU, regulations are incorporated into French legislation, subsequently applicable to import products sourced from outside the EU (FAO, 2004). For French overseas territories, a list of fish species potentially contaminated with cigutoxins is available which are not allowed to be marketed. Additionally in some areas random sampling of fish is conducted for analysis of cigatoxins. In other parts of the world bans on selling products have also been instigated to protect public health (Paredes *et al.*, 2011). Proposals for cost-effective management of the risk in currently high risk areas still require the successful development, validation and application of appropriate methods of analysis as well as the development in expertise in fish identification by local enforcement officers and the commitment of retailers (Clua *et al.*, 2011).

DELIVERABLE 2 – ACTION PLAN

Table 8. Knowledge gaps, associated risks and potential research and development areas applicable for assessing and control the risk to consumers from new/emerging toxin threats.

This section summarises the current status with biotoxin threats as described in detail in the above sections. Table 8 summarises the gaps in knowledge identified from the literature review and from knowledge of the current approaches taken in the UK official control monitoring program. The table includes information on all the toxins discussed in this review, including those currently regulated, new analogues of regulated toxins and the new/emerging toxin groups. The main risks identified through this review are also summarised in Table 8, along with the developments which may be required to develop appropriate controls to reduce the risks. The controls are the assessments and/or method developments described throughout this review which may be utilised for the determination of toxin threats and include either or any combination of the application of current techniques, the modification of current approaches to tailor methods to new toxins or the development and application of new methodologies. The table also listed the risks perceived after these developments have been instigated; in particular highlighting any further work or developments required that may currently be impossible or impractical. Finally, the table summarises the priorities for these controls to be implemented. A rank system was employed, with the values derived from the various factors highlighted during this review, including the likelihood of the risk occurring, the severity of the risk and the situation currently in place regarding monitoring.

Toxins	Knowledge gaps	Risks	Controls (Research and development proposals)	Risk after controls	Priority (rank)*
"New" AZAs	No information on presence of other AZAs in UK shellfish Unknown toxicities of analogues potentially present	Potential high toxicity from other analogues which may be present.	To utilise published MS/MS methods to screen samples for the presence of AZA metabolites in UK shellfish. To further develop toxicity studies on prevalent analogues facilitating the development of risk knowledge.	With developed methods and knowledge of toxicity, screening could be implemented, reducing risk.	1
PSP toxins from Gymnodinium species	Method performance for GTX6 toxin, GTX6 standards availability	Lack of GTX6 standards hindering quantitation. Other toxins are monitored routinely.	Assessment of relative response factors for GTX6. Validation of hydrolysis method Provision and validation of current method in UK species for GTX6.	Risk significantly reduced if standards available and method performance validated.	2
Other PSP analogues	No knowledge of PSP metabolite presence in UK shellfish Toxicity of analogues unknown	Unknown presence as not detected by current methods. Present in Canada and Argentina, toxicity unknown so potential risk exists HPLC validation to date shows no evidence for under-estimation of PSP in comparison with MBA, so no evidence for major threat from unknown PSP toxins	Development of LC-MS/MS method for PSP toxins. Assessment of shellfish samples for other analogues using published MS/MS data. Toxicity studies to determine risk from identified toxins. Assessment of Receptor Binding Assay (RBA) for application to UK samples where evidence for the presence of other PSP analogues has been obtained. Assessment of RBA performance and applicability to UK samples.	Risk lessened if method shown to be applicable. But no standards available for performance testing – at best a qualitative test. RBA potentially provides an additional option if risk from these other analogues is shown to be significant.	2
Brevetoxins	No UK experience of BTX detection methods. No evidence for	No detection of brevetoxins to date, although not monitored. Potential presence of causative organisms	Development of two-stage analysis approach for BTXs involving a screen analysis and confirmation.	Availability of standards and contaminated materials would facilitate	3

	presence of toxins in UK waters.	increases risk. Absence of monitoring regime for BTXs heightens potential risk for intoxication from contaminated products. No data on UK shellfish species which potential accumulate BTXs	ELISA methods once refined to include type-A toxins appear most suitable for monitoring purposes, in combination with LC-MS/MS confirmation. Both methods need assessment and development on samples of relevance to UK. Standards of relevance and naturally-incurred materials need to be obtained and used to validate methods. Once assessed, methods to be applied to range of shellfish samples in areas of higher risk to screen and confirm the presence of BTXs. Work ideally conducted in collaboration with international collaborators, including participation in upcoming interlaboratory studies. Studies to assess shellfish metabolism of BTXs to determine accumulation and depuration rates, generation of shellfish metabolites and ultimately determine which toxins are present in the greatest proportions and are in need of toxicity assessment.	method development. With methods in place, screening and confirmation could be applied to samples of perceived higher risk or as a general screen of selected harvesting areas. Once assessed, testing methods would drive the reduction of risk to the consumer. Risk remaining would relate to lack of knowledge regarding metabolites of unknown toxicity.	
Cyclic imines	Toxicity information in particular in terms of chronic effects Lack of information on potential synergistic effect (e.g GYM & OA) Toxicity and bio-availability of the acyl-esters identified in shellfish	Recorded in Northern Europe including preliminary evidence for UK presence.	Monitoring for CIs in particular PnTXs and SPXs including acyl-esters. Purification and availability of certified standards. Harmonisation and validation of analysis method. Development of ethical methods for PlTX-group analysis in shellfish to enable direct toxicity measurement.	Method development and monitoring would enable a more informed assessment of the risk.	4

Palytoxins	Limited knowledge of the toxin profile and metabolite relevant to seafood and shellfish especially No harmonised method available for the analysis of PlTX and PlTX-like compounds No evidence of presence or absence of PlTX and PlTX-like compounds in UK waters Unequivocal identification of organisms responsible for production of PlTX-group compounds Acute toxicity data for PlTX analogues and chronic toxicity data for PlTX-group Lack of information on mechanism of action for the PlTX-group	No detection to date in UK waters, although no flesh testing conducted. Presence in Europe and noted spread of incidents heightens risk	Monitoring for presence of the PlTX-group compounds. Purification of the PlTX analogues, giving priority to those relevant to seafood and shellfish in particular, to produce standards and reach certification. Continued evaluation of method for detection and analysis of PlTX-group toxins in seafood and shellfish in particular. Harmonisation and validation of analysis method for PlTX-group compounds. Development of ethical methods for PlTX-group analysis in shellfish to enable direct toxicity measurement.	Method development and monitoring would enable a more informed assessment of the risk.	4
Tetrodotoxins	Validation of biosensor method for TTX conducted, although no knowledge regarding presence in UK waters. No chemical methods applied to TTX detection in UK to date. Exact sources of TTX yet to be determined	No TTX recorded in UK, but found in Europe including the Atlantic. With toxins known to be highly toxic, likelihood of occurrence appears low given no evidence for intoxication to date Links to commonly-occurring bacteria potentially increases risk	Two stage analysis proposed incorporating both a functional screening test such as the ELISA, followed by a specific confirmatory method. Following the sourcing of toxin standards and contaminated tissues, both the screening test and confirmatory methods (conventional HPLC and LC-MS/MS approaches) could be assessed in terms of method performance and applicability to samples of interest. With SPR biosensor technology within	Risks reduced following development of suitable methods of analysis and application to the screening of a range of UK shellfish samples to determine likelihood of presence of TTXs. Understanding of potential	4

			the UK, this technique could also be assessed in parallel with other screening and confirmatory methods. Application of suitable methods for the screening and confimatory assessment of UK shellfish samples for TTXs, generating data for the development of risk knowledge. Application of methods to the analysis of bacterial cultures common in UK waters which are known to produce TTXs elsewhere in the world. Subsequently to determine potential risks from bacterial sources. Following any identification of TTXs in UK samples, to develop understanding of metabolism of toxins in species of interest.	presence of toxins in bacterial food sources would provide further data to facilitate a greater understanding of potential risks.	
Cyanobacterial toxins *(Microcystins, Nodularin, cylindrospermopsin, BMAA, anatoxin, saxitoxins)*	Strong evidence for occurrence of cyanobacterial blooms in UK. Likelihood of accumulation of toxins in estuarine shellfish is lower than the likelihood for uptake of marine algae. No knowledge of potential accumulation in UK shellfish, therefore knowledge gaps concerning risks at present.	Cyanotoxins not reported in UK in marine shellfish although not monitored. Evidence from other parts of the world suggests the risk exists. With blooms well noted in UK, risk is potentially significant, although the size of this risk is difficult to predict on the information available.	Review to determine toxins currently present in UK blooms, enabling the determination of potential risks from local cyanobacterial species. Testing, development and application of both screening and confirmatory methods for detection of cyanobacterial toxins identified as providing the greatest potential threat from accumulation in shellfish or fish species. Application of methods to screen samples in areas of identified risk, to provide data on likely presence or absence of toxins in food products. Following any identification of risk, specific species and toxins of interest to be further studied, generating more specific performance data. Analysis results to be utilised to produce recommendations for future studies.	Number of diversity of toxins is enormous. Substantial amounts of work required to set up methods. Should be done in conjunction with experts working in field. Even with monitoring methods assessed, a large number of unknowns remain. Application of screening and confirmatory methods would provide at best a preliminary indication of the potential threat from consumption of	4

				cyanotoxins in UK fishery products. Ongoing long term development would be required to grow this approach into a robust and fit for purpose monitoring programme if the risks were identified as being significant.	
Ciguatoxins, maitotoxins	No UK experience of CTX detection methods. No evidence for presence of toxins in UK waters.	Whilst likelihood of presence appears very low, the toxicity threat would be significant if CTXs emerged. The risks relate to the absence of any monitoring strategies to cope with this threat.	Combination of screening/toxicity test (e.g. cytotoxicty assay/receptor binding assay plus LC-MS/MS confirmation). Accumulation of CTX standards to facilitate method testing and development. Testing of screening methods for samples of relevance in collaboration with international experts. Identification of most appropriate method. Analysis of algal cultures and contaminated tissues using chosen methods. Surveillance initiatives for data collection and risk assessment, determining in particular the CTX analogues and fish species affecting the UK Further understanding of toxicity of CTXs of relevance to UK	Risk reduced following development of suitable methods of analysis. Risk remaining would relate to lack of knowledge regarding metabolites of unknown toxicity.	5
VSP toxins	Very little known about the syndrome and the causative compounds	The presence of the toxicity-causing compounds is not known or assessed. However, risk seemingly low due to rarity of VSP, with no reports in UK to date.	None possible until VSP event confirmed and contaminated materials made available to monitoring laboratories.	Full assessment of causative toxins required in materials confirmed to be relate to VSP required before controls can be put	6

				in place.	
PSP toxins from Alexandrium species	Detected routinely	Currently covered with monitoring programme	None required – HPLC method in place.	-	-
ASP toxins	Detected routinely	Currently covered by monitoring programmes.	None required – HPLC method in place	-	-
OA-group toxins	Detected routinely	Currently covered by monitoring programmes.	None required – LC-MS/MS method in place.	-	-
AZA1-3	Detected routinely	Currently covered by monitoring programmes.	None required – LC-MS/MS method in place.	-	-
YTX and PTX toxins	Large number of analogues not analysed for	YTXs and PTXs found to date in UK, although toxicity risk disputed – likely that regulations relaxed	With likely increase in maximum permitted levels or removal of toxins from legislation, the risk is not thought to be great, so no R&D measures are proposed.	-	-

Chapter 4

1. REVIEW OF INFORMATION ON TESTING REGIMES IMPLEMENTED WITHIN OTHER COUNTRIES, EU AND WORLDWIDE, FOR THE DETECTION OF NEW/EMERGING HARMFUL ALGAE AND TOXINS : GLOBAL VIEWS FOR SUITABILITY OF TESTING METHODS

(Survey responses in Appendix 3)

1.1 REVIEW OF DATA, GLOBAL VIEW AND REPORTS FROM THE RELEVANT EMERGING TOXIN MEETINGS

1.1.1 Brevetoxins

Throughout the EU there have been very few instances of threats from BTX producing algae highlighted in the literature, at conferences or in working group meetings. At a recent EURL symposium on new or emerging toxin, no references were made or presentations given relating to BTX methods or issues. Similarly at a recent EURL working group on emerging toxins, BTXs were not discussed, with the focus mainly on other threats already identified in EU waters. However there is some opinion that the threat currently experienced in New Zealand, the east cost of the USA and the Gulf of Mexico may expand in future years, potentially affecting the EU.

New Zealand scientists are actively involved in both the production of standards and the development of methodologies for testing of BTXs, amongst other things incorporating BTX standards into their quality-controlled routine LC-MS/MS method for shellfish monitoring. A single laboratory validation has been published (McNabb et al., 2012a) although the toxins have only been seen once in recent years. Work progressing in recent years at the University of Vigo in Spain has shown some recovery issues for PbTx analogues (e.g. PbTx-2), which resulted in the need for solid phase immunoaffinity extraction clean-up prior to reverse-phase LC-MS/MS

quantitation. With this approach a highly reproducible and linear method with good toxin recovery has been demonstrated (Leao-Martins, 2011).

In the US, FDA scientists have maintained a program of work to identify the best options for monitoring BTXs, including both the ELISA and LC-MS confirmatory methods. The work has also been expanded to pursue the identification of BTX exposure biomarkers and toxicity in bivalve shellfish (Plakas, personal communication). Ann Abraham from the FDA is currently leading studies to validate both ELISA and LC-MS/MS methods, with current results supporting ELISA as a screening method and LC-MS as a confirmatory method (Abraham, personal communication). The organisation have not found the limited cross reactivity to Type-A BTXs to be a significant problem, given the dominance of Type-B BTXs in shellfish as determined by LC-MS metabolite profiling studies. The examination of both oyster and clam tissues naturally contaminated with BTXs during *K. brevis* blooms by both methods and showing good correlation between the assays (Plakas *et al.*, 2008; Abraham *et al.*, 2012) supports these decisions.

Overall, it appears that the preferred approach to future monitoring programmes is the application of both ELISA and LC-MS/MS methods for the detection and quantitation of BTXs and BTX biomarkers in shellfish. This may change if any of the other biomolecular research tools become assessed in greater depth and formally validated. At present, there has been no feedback that this is likely to happen in the near future.

1.1.2 Palytoxins

Over the recent years, PlTXs that had historically been confined to tropical and sub-tropical areas have spread to southern European waters. At a recent EURL symposium on new or emerging toxins, a presentation was given on the acute and sub-acute oral toxicity of PlTXs in mice. Another presentation was given on the determination of PlTXs in samples from an *Ostreopsis sp.* outbreak on the Catalan coast. An outbreak of *O. ovata* in the Algarve in 2011 was also reported and

although shellfish samples were taken for PlTX analysis at a later date, the results were not available at the time of the symposium. An oral presentation was also given by the French NRL for marine biotoxins at the AOAC annual meeting in October 2012. The work taking place in France under the “PlTXpêche” project was presented including the occurrence data of PlTXs in marine products from the French eastern Mediterranean coast was presented (Hossen, 2012). At the same conference, a chemical transformation approach as a detection tool applicable to PlTX by LC-MS was presented (McNabb, 2012b). More recently still at the AOAC Toxins Symposium in Baiona (May 2013), work was presented showing the presence of ovatoxin in a number of marine species including sea urchins and the digestive tubes of fish (Brissard, 2013).

Overall and since the reports of PlTX in European waters started, the gathering of scientific data has picked up pace and research groups have focused mainly on toxicity, producing organisms, analogues and structure elucidation as well as detection methods. The potential for application of PlTX detection and quantitation method to shellfish matrices has been given more and more consideration and overall, the LC-MS and the cytotoxicity assays seem to have had most of the attention in this domain.

1.1.3 Cyclic Imines

Since the first report of CIs in the 1990s, numerous studies have taken place in order to increase our understanding of this toxin group. At a recent EURL symposium related to new or emerging toxins, CIs were the subject of several presentations. One of the presentations considered the occurrence of PnTXs and SPXs in Norway and indicated that the occurrence of these CIs is wide spread in Norwegian shellfish with relatively high concentration levels (e.g: up to 226 µg/Kg 13-desmethyl SPX C in mussels in 2009). Another presentation reported on the CIs in France in particular considering the similarities and differences between SPXs and PnTXs whilst the last presentation on CIs considered the coupling of bio-analytical and physicochemical methods for detection of CIs.

Since report of SPXs and PnTXs have taken place in Europe, an increased in scientific studies has taken place in particular, the isolation and structure elucidation of new toxic compounds (e.g: SPX-related compounds) and the development of detection methods in particular the functional assays. However, at the moment, LC-MS remains the preferred method for analysis of these toxins.

1.1.4 Tetrodotoxin

Responders to the survey who identified TTXs as likely to be present now or in the future included those from New Zealand (published work), Spain, Portugal and the Netherlands.

The Greek NRL have presented information on the detection of TTX in pufferfish caught in Greek waters, noting the trend for these fish to be spreading westwards. Attendees at the EURL MB meeting on emerging toxins recognised that emerging toxins was an issue for fish as well as shellfish, noting that options for control were likely to include the management of risk through bans on fish sales from certain areas or from certain specific species. The need to respond to incidents of human intoxication was recognised as essential for developing knowledge relating to toxicity and applicability of detection methods.

Portugal (both CIIMAR and IPIMAR) recognised the risk from TTXs in Portuguese waters and the potential for this risk to increase with global warming and high latitude migration.

None of the remaining responders, including UK, Denmark, Canada, France, Germany, Norway and Sweden noted any risks in relation to intoxication from TTXs. However, some of these organisations did recognise the need for both suitable screening methods and more toxicological data.

1.1.5 Cyanotoxins

Two responders to the survey highlighted cyanotoxins as potential threats. In Portugal, cyanotoxins such as the neurotoxin β-N-methylamino-L-alanine (BMAA) have been detected in estuarine cyanobacteria. Researchers reported the importance of the need to detect and assess the occurrence of cyanotoxins such as microcystins in coastal organisms, the risk from which has been recently identified. The stability of the microcystins was specifically mentioned as well as their highly toxic effects. In Australia, food safety issues have been identified with the presence of Nodularin in lake systems. Both responders recognise the importance of developing methods for assessing these threats and developing expertise in monitoring capabilities.

None of the remaining responders highlighted cyanotoxins as an emerging threat to the food safety of shellfish in the UK or Europe.

1.1.6 Ciguatoxin

Survey responders who identified ciguatoxin as likely to be present currently or likely to appear in the future included researchers, agencies and other institutes from Portugal, France (Canaries and Mediterranean), Italy, New Zealand, USA, Australia and Spain.

In the US, Hawaii and Florida have the majority of cases where there is significant risk to human health. The US FDA uses a two-tier approach for monitoring CFP involving the N2A screening assay to screen for sodium channel activity with subsequent confirmation in positive samples by LC-MS/MS (Robertson, 2012). Hungerford (2012) noted that the success of the assay for CTXs related to its high sensitivity, enabling the detection of low parts per trillion of CTX1B and consequently provides a rugged and specific assay whilst using only trace amounts of CTX standards. Emerging issues noted include the invasion of lion fish, causing big problems to ecosystems in Florida and the general spread of CFP to new regions.

The FDA recognise the strong need for development of reference materials suitable for the control and validation of testing methodologies, plus the identification and validation of reliable and rapid field test methods. James Hungerford, co-chair of the AOAC Marine and Freshwater Toxins Task Force points out that having the cell based assays available has proven to be important in studying Ciguatera many laboratories, particularly in combination with LC-MS. However he emphasizes that the cell assays, and in indeed all of the analytical methodology, still remain essentially research tools until the method performance can be thoroughly evaluated against FDA guidance limits (Hungerford, 2012).

In Japan, huge efforts are ongoing regarding the structural elucidation of CTXs and the development of methodologies. In particular the need for reference material production is recognised and work is ongoing to produce this from contaminated fish (Yasumoto, 2012). However progress over the years has been hampered by funding issues as a consequence of CFP not being viewed as a major issue in the country.

In Spain, the control measures put in place to assess fish products initially involved use of the Cigua-Check test kit in tandem with the MBA, cytotoxicity tests and LC-MS/MS. With the rapid test kit product no longer available, controls mirror those conducted by the FDA in the US, with a cytotoxicity screening method prior to confirmation by LC-MS/MS. The application of the N2A assay appears successful with recognition that the method is very sensitive and applicable to determine CTXs in Amberjacks found to date in the Canary Islands (Boada *et al.*, 2010, Diogene, 2012). It was noted that since controls have been in place, the only people affected were sports fisherman, with no products on the market causing intoxication. The need for effective functional assays to provide an assessment of toxicity was also flagged as a strong requirement (L. Botana, University of Lugo) in addition to the need for simplified or screening LC-MS/MS techniques (J. Blanco, CIMA, Spain). An European Commission FP7 project is also underway comprising consortium members from Spain, focusing on the development of a rapid antibody-based test kit and supporting reference standards for ciguatoxins (Cigua Tools, 2013).

In France, as reported by the NRL in their survey response as well as in published literature and conference presentations, the importance of ciguatoxins given its identification in the Canary Islands. The MBA is currently used for monitoring CTX, but the importance of developing LC-MS/MS and/or cytotoxicity and/or receptor binding assays was recognised. The NRL of Italy also found CTXs represented a significant risk for fish consumers, highlighting that the issue could affect any EU country as a result of contaminated imported fish. The organisation also use an MBA based on a EURL protocol, with the lack of standards, contaminated materials and funding preventing the development of a replacement or confirmatory chemical method. CTXs and MTXs are recognised as "new things" in New Zealand (McNabb, Cawthron, pers com).

At the EURL-MB working group on Emerging Toxins, 22nd May 2012, the EURL-MB in collaboration with the University of Vigo and Professor Yasumoto in Japan presented a UPLC-MS/MS for detection of CTXs in fish, involving two different extraction protocols for C- and P- CTXs. Work was still ongoing to optimise the method, evaluating matrix effects and improving clean-up procedures, but with progress hampered by lack of reference materials. The EURL also noted that for Spain, ciguatoxin would be the priority emerging toxin group, given the occurrences of human intoxication. The collaboration was also focusing on the isolation and purification of standards (A. Gago-Martinez, personal communication). The confirmation of the emerging toxin threat to Spanish and Portuguese waters was also provided by Leao-Martins *et al.*, 2012.

Many of the responders to the survey recognised the practical problems associated with monitoring fish for CFP. Issues flagged included most notably the lack of certified reference standards, as well as the tedious and complex methodologies currently available. There was a consensus that rapid screening methods were essential tools, but that currently no perfect solutions were available. The costs of developing these methods were also recognised as being extremely high, which in many cases has prevented the proactive assessment of potential methodologies to combat any developing risk. The need for proper risk assessment studies was also flagged by Dr Diogene (IRTA, Spain), without which the EU cannot act. He also

emphasised the need to determine what levels of CTXs are being detected, before regulatory limits can be set. In Portugal, the importance of monitoring CTXs was raised, noting both environmental and human impacts in Portuguese waters (Portugal NRL, IPIMAR), also likely to increase due to global warming and migration to higher latitudes (Vasconcelos, CIIMAR, Portugal). Dr Vasconcelos also stipulated the need to increase the study on molecular mechanisms leading to toxin production and to assess geographic and seasonal patterns more thoroughly.

The Netherlands NRL recognised the geographical location was an important factor in determining the risk of CTXs to consumers of fish products. They recognised the importance of the poisoning to the more Mediterranean countries, whereas the issue would be less of a risk in areas around the UK and Northern Europe. They also raised the need for developing screening techniques using high resolution (accurate mass) mass spectrometry, functional assays and other cell screening methodologies. The functional assays were flagged as providing the greatest potential, given their ability to replace the MBA for toxicity determination and detect toxins not covered by specific LC-MS detection methods. They also highlighted the practical problems relating to the development of methods for emerging risks, where usual funding is unavailable prior to a risk being proven, together with the requirement for more toxicological data.

None of the remaining responders from Northern Europe (including UK, Denmark, Sweden, Germany, Ireland and Norway) raised any concerns in relation to the potential occurrence of ciguatoxins.

In summary, common themes determined following a review of current global views therefore include:

- A lack of toxicological data on these toxins
- Strong requirement for reference material standards
- Need for both rapid sensitive screening assays and confirmatory methods
- Still a great deal of work required to properly assess and validate methodologies

- General recognition of issues relating to confirmatory chemical methods when measuring a growing number of compounds
- Need for greater communication between agencies, to ensure incidents are noted and appropriate data is gathered to assist in establishing a Lowest Observable Adverse Effect Level (LOAEL). This can then be used to generate an Acute Reference Dose (ARfD) and aid the establishment of a regulatory limit.
- The importance of ensuring medical authorities are aware of the facts relating to CFP and the need for reporting any incidents is recognised.
- To prioritise the need for developing suitable methods and setting regulatory limits for emerging toxins
- To develop understanding regarding outbreaks and fish species affected, noting the inconsistency in affected species from different regions of the world
- Overall, the prioritisation needs for the EU community is complicated as a result of the relevance of each emerging toxin threat in individual member states
- Overall the EURL working group on emerging toxins concluded that future work should be prioritised and that ciguatoxins appeared to fall into the category of threats which were clearly a problem with wide health and economic impact.

1.2 SUMMARY OF FINDINGS AND CONCLUSIONS RELATING TO WHETHER MONITORING STRATEGIES WOULD MITIGATE RISKS IDENTIFIED

Following the responses from survey participants, a summary of collated information has been produced (Table 1). Brief summary information has been provided by a number of laboratories, which in some instances has provided further information which can be used in conjunction with literature evidence for the determination of appropriate monitoring strategies for new or emerging toxin risks.

On the whole, the majority of responders concentrated primarily on the toxin groups most commonly perceived as emerging threats within the European context, most notably describing the risks from occurrence of the warmer water species – CTXs

and PlTXs, with other also including mention of TTXs. A lower number of responders highlighted the potential risks from BTXs and cyanotoxins. CIs were highlighted as the most likely to be present in the colder waters of the UK.

For analogues of PSP toxins not currently monitored in the UK control programmes, approaches could include the development and application of LC-MS/MS detection methods and/or the use of the Receptor Binding Assay (RBA). In the UK where LC-MS/MS technologies are already implemented for the quantitation of lipophilic toxins in shellfish, this technique could be potentially applied for the assessment of the presence of other PSP analogues for which LC-FLD methods are not applicable. In addition, if risks from novel PSP analogues are determined through LC-MS/MS investigations, a parallel approach involving analysis by RBA could provide an additional confirmatory tool enabling an overall assessment of toxicity risk to the shellfish consumer. For the specific case of GTX6, a major PSP analogue in shellfish contaminated with *Gymnodinium catenatum* toxins, current LC-FLD methods could be modified and tested to assess performance characteristics for this toxin. Throughout the rest of the world, official control monitoring of shellfish for PSP is conducted using either LC-FLD methods or the MBA. Developments are underway to implement the RBA into a number of state health programs within the US, although to date there is no validation data showing the performance of the method for these additional toxin analogs. With the additional absence of a formally validated LC-MS/MS method and the lack of validation for many of the new PST analogs using LC-FLD, there are currently no formally validated approaches which could be applied directly without additional study and validation. In the UK at present the current monitoring approach would identify the presence of GTX6 and the strategy would be to quantify against non-certified well characterised standards which are kept in storage within UK control laboratories. The availability of certified reference standards would enable more accurate quantitation of GTX6 if this was found to emerge as a significant risk in future shellfish control samples.

For the suite of lipophilic toxins, it is clear from the literature that many other analogues exist which may potentially contribute to total shellfish sample toxicity. Given the evidence for low or no toxicity of YTX and PTX toxins, there is little

perceived risk associated with continuing the current approach for the monitoring of these toxins, particulary given the noted moves to deregulate these toxins. Whilst many analogues of OA and DTX toxins are known, including a wide range of acyl esters, the presence of these toxins is quantified through the current analytical approach taken. Therefore no further developments are expected for detection of OA-group toxins. The major risk from novel analogues of regulated toxins is likely to relate to the potential presence of AZAs, formed through shellfish metabolism. The most suitable approach for detection of these toxins would be the expansion of the lipophilic toxin LC-MS/MS monitoring method to incorporate these analogues. Ongoing studies are currently investigating both the presence and toxicity of these AZAs in a range of shellfish tissues, although to date methods have not been validated or implemented in any regions of the world to monitor routinely for these toxins. With the availability of contaminated shellfish samples containing these toxins, the published information on chemical structures and mass spectral characteristics, instrument methods could be optimised to incorporate these into a screening test. With availability of reference standards or knowledge of toxicity equivalence factors, quantitative analysis could be conducted. Without reference standards for calibration purposes, equivalent response factors for each of the new AZAs would have to be assumed until proven otherwise in-house.

A definite link has not been established between human poisoning and CIs so their risk to human through consumption of seafood and shellfish in particular is currently under debate. Some of the CIs (SPXs and PnTXs) have, however, been detected in European and UK waters and monitoring for these toxins would be useful to inform a risk assessment. From the literature review and the survey responses, LC-MS/MS is the most widely used method for the analysis of CIs in shellfish and whilst certified standards are available for some, they are lacking for other analogues of these sub-groups and for other sub-groups such as PnTXs. The availability of these standards would enable assessment of method performance for each analogue and deployment of a highly tested reliable method.

Whilst VSP has been shown on rare occasion to result in intoxication within shellfish consumers, the syndrome is thought to be extremely rare. Whilst the causative organisms are well noted in UK waters, no evidence of toxicity has been reported. Without any information on the specific toxins of interest, no appropriate monitoring strategies can be recommended for routine monitoring of shellfish flesh. Further data would be required on the likely active compounds before any further work can progress.

BTXs were not highlighted by any of the European responders to the survey, with only New Zealand mentioning the risk to their own shellfish programme. It is certainly clear that these toxins have to date only been detected in a few global areas and there is no evidence currently for these to be expanding into the UK marine waters or even other parts of Europe. Suitable monitoring strategies appear to involve the combination of both a screening assay and confirmatory analysis. In the US, researchers have been involved for some time in the assessment of ELISA methods for BTXs, with the current Abraxis ELISA considered a potentially useful screening tool for BTXs. Confirmatory LC-MS/MS methods have been developed in a number of high profile monitoring laboratories, and with the availability of well characterised standards, these methods could be tested, developed and part-validated to provide useful monitoring tools. As such the recommended approach would be to source relevant standards, to test and validated both the ELISA and LC-MS/MS method for BTXs and important metabolites before applying the two-method approach to a broad screen of samples harvested within UK waters. This would enable the generation of data on the potential presence of BTXs in UK waters and aid the assessment of risk from this particular group of toxins.

CTXs are recognised as a high risk within certain specific areas of Europe, but given the occurrence to date of the causative algae in only warmer marine waters, the overall risk to UK consumers is currently thought to be very low. From the responses received and literature review conducted, a dual method approach is recommended as the most appropriate for the detection and quantitation of CTX toxins in fish products. Currently either the cytotoxicity assay or receptor binding assay are considered most suitable options, although these would represent significant, time-

consuming and expensive deviations from the current testing methods utilised within the UK monitoring laboratories. Confirmation of positive samples would be conducted most appropriately using LC-MS/MS methodologies. However, large amounts of work are still required globally before any suitable methods can be tested, validated and implemented into any routine monitoring. Most notably this requires the generation and supply of reference standards to facilitate the testing and optimisation of methods. These are currently scare and highly expensive, with none currently available commercially. Even with standards available, work would be need to assess the precursor toxins likely to be present in UK waters, and further research would be required to identify metabolic products which may also represent a significant risk to the fish consumer. Once such data has been generated, surveillance initiatives would be required to establish the presence and likely frequency of occurrence of these toxins in UK marine products. However, this would not be a simply process, given the complexities noted elsewhere in the accumulation of CTX toxins in different species and with different toxin profiles.

Taking into account the spread of PlTX-group compounds to European waters, they are becoming more of a potential threat to UK waters and, factoring in the toxicity of PlTX, it is not surprising that several responders (mainly from southern European countries) have put them forward as a risk. Although LC-MS/MS has been used for analysis, the survey highlighted issues with limit of detection and limit of quantitation. Work is currently on-going at the EURL to enable optimisation of a LC-MS/MS method for PlTXs and assessment of its performance characteristics. The ELISA and the fluorescence polarization methods are also showing potential as screening methods at least but further work and assessment of performance is needed before either method can be used for routine monitoring. For performance assessment and reliable application of either of these methods, purified/certified standards are required but are not currently available. In the context of food safety, toxicity data related to the PlTXs analogues relevant to shellfish in particular is also needed in order to inform the risk assessment and potentially develop targeted analysis. Although sources of PlTX-group compounds have been identified in European waters, there currently remains some uncertainty on the involvement of other

organisms. The toxin causative agents need to be unequivocally identified to enable the implementation of an early warning system in the current monitoring programme.

Cyanobacterial toxins are not generally thought of in the same context as new and emerging marine toxins, given their origin from freshwater ecosystems. Consequently there is not a major focus on this threat from the majority of active marine toxin researchers currently engaged in research to determine threats and risks in marine shellfish. Nevertheless there is evidence for potential accumulation of some highly toxic compounds in marine organisms from a freshwater cyanobacterial source. This area of research is an extremely complex one, and there is still a huge amount of work to be done to develop methods suitable for monitoring these toxins in the wide range of marine species potentially affected by these toxins. A large number of different methodologies have been reported to date and some of these have shown some evidence for accumulation of cyanotoxins in potential food products. In the UK there are known occurrences of toxic algal blooms in freshwater systems, but to date no evidence that these have affected shellfish harvesting beds. However, there is the potential for this to occur, and the risk should be assessed. A review of shellfish harvesting areas should be made to assess the potential for influx of cyanotoxins into a harvesting zone. Through this review, proposals could be made concerning which areas may be of highest risk, and these could subsequently be incorporated into any future monitoring programmes. In parallel with this is the need to test, develop and validate suitable methodologies. Application of both screening tests and confirmatory analyses would be appropriate for the assessment of the presence of cyanotoxins. However, performance characteristics of each method would first need to be assessed to ensure the methods were applicable to the species of interest. This would be a complex, time consuming and expensive process given the large number and wide diversity of organisms potentially involved in the production of cyanotoxins. A review of the toxins most likely to be present in UK water systems close to shellfish beds would be appropriate to identify the toxins most likely to result in risk to the consumer.

Table 1. A summary of collated information and survey responses, highlighting best monitoring practises

Algae/Toxins	Possible monitoring strategies	Most appropriate monitoring strategies
PSP toxins from *Alexandrium species*	Currently covered within water and flesh monitoring programmes.	Continue current monitoring approaches.
PSP toxins from *Gymnodinium species*	Lack of GTX6 standards hindering quantitation. Other toxins are monitored routinely. Inclusion of *G. catenatum* in current water monitoring programme.	Assessment of relative response factors for GTX6. Validation of hydrolysis method in conjunction with direct quantitation approach. Provision and validation of current method in UK species for GTX6. Inclusion of *G. catenatum* in current water monitoring programme.
Other PSP analogues	Development of LC-MS/MS methods for PSP with suite of analytes including “new” PSP analogues. Collaboration with organisation with access to RBA , for assessment of samples potentially containing other PSP analogues. Causative organisms currently covered by water monitoring programme to genus level.	Development of LC-MS/MS method for new PSP toxins is likely to be the most appropriate and practical given the inability of LC-FLD to identify some of these analogues. Whilst RBA appears a suitable approach, the technique is currently not used with the UK monitoring programme, unlike LC-MS/MS which is utilised for lipophilic toxin analysis. A validated LC-MS/MS method which has been developed to mitigate against known significant matrix effects could be modified to incorporate the additional toxins of interest. This could be applied for the screening of selected shellfish samples where the presence of novel analogues may be suspected. Methodology could be developed in collaboration with partner organisations who already have access to suitable contaminated materials.
ASP toxins and *Pseudo-nitzschia*	Currently covered within flesh and water monitoring programmes.	Continue current monitoring approaches
OA-group toxins and *Dinophysis* and *P. lima*	Currently covered within flesh and water monitoring programmes	Continue current monitoring approaches.
AZA1-3 and	Toxins currently covered with monitoring programme	Continue current monitoring approaches.

***Azadinium* species**	*Azadinium* spp. not detected by current water monitoring programme.	Initiate monitoring for *Azadinium* spp. by Q-PCR.
Other AZAs	Development of LC-MS/MS methodology to incorporate additional AZA analogues into suite of toxin analytes. No other monitoring strategies are thought to be appropriate for the testing of these analogues.	To obtain suitable contaminated materials, and ideally purified standards where possible, to aid the development of the LC-MS/MS method for additional AZA analogues. To utilise published MS/MS methods to screen samples for the presence of AZA metabolites in UK shellfish. In particular focussing on metabolites identified by other organisations as major components. If other AZAs are found to be present to identify most significant toxins and where possible conduct method performance assessment of the method.
YTX and PTX toxins and causative algae	Major analogues currently covered with monitoring programme Major causative organisms currently covered in water monitoring programme.	With little threat perceived from additional analogues, continue current monitoring approaches.
VSP toxins and *P. minimum*	Monitoring to involve the identification of toxic algae which may be linked to reports of VSP. Contaminated materials known to be linked to cases of intoxication would be required to enable the assessment of potential causative toxins.	Toxicity assessment of UK strains of *Prorocentrum minimum.* Further research could include LC-MS/MS analysis of toxic cultures or contaminated shellfish to determine potential toxins of interest.
Cyanobacterial toxins and cyanobacteria	Monitoring of cyanotoxin blooms in areas of relevance to shellfish growth and harvesting. Application of screening tests such as ELISA for assessment of known cyanotoxins. Application of HPLC and/or LC-MS/MS methods for	Review of shellfish harvesting areas to assess the potential risk from cyanobacteria growing in freshwater systems of close proximity. Cyanobacterial monitoring in areas highlighted as potentially linked to shellfish harvesting areas. Application of validated testing methodologies involving both screening and confirmatory techniques for the analysis of high-risk shellfish

	confirmation and quantitation of toxins in shellfish.	products for cyanotoxins.
Palytoxins and *Ostreopsis* spp.	Recorded in southern Europe. Qualitative monitoring using a suitable screening test although the current methods require further development and/or assessment. *Ostreopsis* could be included to toxin species list in current monitoring programme. However, it is benthic in habitat and may be missed by current sampling practises.	Further study into the suitability of the LC-MS/MS, ELISA and FP method in the context of a routine monitoring programme with a view to use a relatively rapid screen method followed by quantitative confirmation. Validation of the method(s) taken forward for application to the routine monitoring programme. Inclusion of *Ostreopsis* spp. in current water monitoring programme and development of benthic monitoring strategy.
Brevetoxins and *Karenia* spp.	ELISA methods appear most suitable for screening purposes. Survey responders described the use of LC-MS/MS detection for the screening of shellfish for BTXs, with or without sample clean-up. Others recommended the most appropriate monitoring strategy was to include both a screening and a confirmatory approach using LC-MS/MS detection and quantitation. Both methods need assessment and development on samples of relevance to UK. Standards of relevance and naturally-incurred materials to be obtained and used to generate method performance data.	Development and validation of both an ELISA screen and LC-MS/MS quantitative confirmation method. Both methods need assessment and development on samples of relevance to UK. Standards of relevance and naturally-incurred materials to be obtained and used to generate method performance data. Validated methods to be applied to range of shellfish samples in areas of higher risk to screen and confirm the presence of BTXs. Inclusion of *Karenia* spp. in current water monitoring programme to the genus level and implementation of molecular based monitoring to

	Karenia spp. could be included to toxin species list in current monitoring programme at the genus level.	species level.
Cyclic imines and causative organisms	Recorded in Northern Europe including preliminary evidence for UK presence. Qualitative monitoring for the CIs in particular SPXs and PnTXs. Inclusion of causative organisms in current water monitoring programme.	Although lacking direct toxicity information, LC-MS/MS is currently considered the most appropriate method for analysis of CIs. The method performance needs to be assessed for all relevant analytes as well as species and the method will also need to be validated. Inclusion of causative organisms in current water monitoring programme.
Ciguatoxins, maitotoxins and causative algae	Whilst likelihood of presence appears very low, the toxicity threat would be significant if CTXs emerged. The risks relate to the absence of any monitoring strategies to cope with this threat. Causative algae unlikely to establish in temperate waters.	Combination of screening/toxicity test (e.g. cytotoxicty assay/receptor binding assay plus LC-MS/MS confirmation). Accumulation of CTX standards to facilitate method testing and development. Testing of screening methods for samples of relevance in collaboration with international experts. Identification of most appropriate method. Analysis of algal cultures and contaminated tissues using chosen

		methods. Surveillance initiatives for data collection and risk assessment, determining in particular the CTX analogues and fish species affecting the UK. Further understanding of toxicity of CTXs of relevance to UK.
Tetrodotoxins	Survey responders did not mention specific monitoring strategies for TTXs. However, literature reveals that LC-MS/MS provides an appropriate tool for detection and quantitation of the toxins in contaminated shellfish and fish products. Functional screening testing such as the ELISA also identified as potentially useful tools. SPR biosensor technique is also validated for some species (trumpet shell) and potentially applicable to other shellfish or fish species of interest.	For shellfish of higher risk, ELISA functional screening and/or SPR biosensor analysis, followed by confirmatory LC-MS/MS analysis. Surveillance initiatives recommended for determination of prevalence of TTX in UK shellfish, thereby enabling risk assessment to be conducted to inform potential future monitoring regimes.

Chapter 5

1. SUMMARY OF EVIDENCE TO SUPPORT EU NEGOTIATIONS FOR MONITORING APPROACHES WHICH ARE REASONABLE AND RELEVANT

1.1 Introduction

The document provides a comprehensive overview of the likelihood of occurrence or emergence of certain toxic algae and their toxins in UK waters.. Some of the phytoplankton responsible for the production of these toxins have been detected in UK waters. In addition, a number of toxic species have been identified which, although not currently present in UK waters, have the potential to become established. This expansion of the range of species monitored may impact on the current methods of sampling and analysis. Sampling regimes may require to be altered to include benthic species, test methods may have to be adapted or new methods implemented. All have resource implications for sampling officers, testing laboratories and ultimately, the Competent Authority.

Similarly, this primary level of monitoring has to be supported by appropriate analytical methods for the toxins themselves. Where existing methods can already detect the toxins (such as the detection of cyclic imines and gymnodimine by LC-MS/MS) there is minimum resource implications. For other toxin groups, method development and validation is required and this is significantly inhibited by the lack of certified standard material.

The literature review has defined and listed potential new and/or emerging toxin threats for UK waters and assessed the risk of these algae and toxins (Chapter 2, Table 4, 5 and Table 7). Table 1 in this Chapter orders the new and emerging algae and toxins from these assessments in order of highest to lowest risk.

Table 1. New and emerging algae and toxins assessed in order of highest to lowest risk *(based on Chapter 2, Tables 4, 5 and 7).*

Algae	
1	*Azadinium* spp.*
2	*Gymnodinium catenatum*
3	*Karenia s*pp., *Ostreopsis s*pp, *Alexandrium catenella*
4	*Coolia* spp†., Cyanobacteria
5	*Vulcanodinium rugosum*
Toxins	
1	New AZA analogues
2	New PSP analogues and brevetoxins
3	Cyanobacterial toxins, Palytoxin, Cyclic imines,Tetradotoxins, PSP toxins from *Gymnodinium catenatum*
4	Ciguatoxins, Maitotoxins
5	VSP toxins and other lipophilic toxins not covered above

* Although *Azadinium* has been recorded in UK waters previously, it is not currently detected in the UK water monitoring programme and with its associated severity rating is of subsequent high risk.

† Toxicity of this genus not yet clearly established and needs to be confirmed.

1.2 *Azadinium* and Azaspiracid analogues

The genus *Azadinium* is present in UK waters but is too small for detection by the current water monitoring programme (Chapter 2, Section 1.3.1). As such it has been assessed as a high risk due to the inability of current monitoring methods to detect it. EU legislation does not specify the species to be monitored but there is a requirement for member states to monitor for the presence of toxin-producing plankton in production and relaying waters and biotoxins in live bivalve molluscs (854/2004). Any results suggesting an accumulation of toxins in mollusc flesh must be followed by intensive sampling. The inability of the current monitoring programme to detect *Azadinium* means that the current water monitoring programme does not satisfy this EU requirement. To satisfy this legislation it is recommended that the most appropriate methods for section of this organism is widespread monitoring of this organism using Q-PCR analysis of Lugols's fixed samples.

There is currently no information on the presence of AZA analogues other than AZA1-3 in UK shellfish. However, it is thought likely that either there will be changes to AZA shellfish profiles in the future or that other AZA metabolites may already be present in some shellfish samples (Chapter 2, Section 2.6). The recommended and most appropriate method for detection of AZA analogues is the utilisation of LC-MS/MS methods. These methods are highly suited for the detection of AZAs and the approach is fully capable of detecting individual AZA analogues at concentrations well below the regulatory limit, although the accuracy of quantitation is potential compromised without a larger range of certified standards for instrument calibration purposes. In addition there is the importance of determining the relative toxicities of these new analogues. With the potential for some of these to be higher in toxicity than AZA1-3, assuming equivalence to these may result in potential under-estimation in total AZA toxic burden.

1.3 *Alexandrium catenella, Gymnodinium catenatum* and *PSP analogues*

This species has recently invaded the Mediterranean and could potentially become established in UK waters. However, it would be detected within the current monitoring programme to the genus level and produces PSP toxins which could be

detected by the shellfish monitoring programme. No changes are required to the current monitoring regime for this species.

G. catenatum was assessed as the highest risk non established new algae in UK waters. Although not recorded in UK waters it has been detected in ballast waters in UK ports and has the potential to establish (Chapter 2, Section 1.7.6). It is not currently included in the list of species for identification in the water monitoring programme but if included could be detected. The current programme would be appropriate for monitoring of this species.

Regulation (EC) 2074/2005 specifies that the paralytic shellfish poison (PSP) content of the edible parts of bivalve shellfish must be detected in accordance with the biological testing method (MBA) or any other internationally recognised method. Consequently any “new” PSP analogues should be incorporated into the routine monitoring of PSP to meet the regulatory requirements. However, this is only possible once appropriate analytical standards are available and the method has been validated following international guidance for each of the compounds of interest. Given the high level of validation conducted on the LC-FLD methods to date within the UK biotoxin monitoring laboratories, a cost-effective option would be to update methodologies as and when new toxins are identified in UK shellfish which become available as certified standards. If the *G. catenatum* was detected before the standards were available, the MBA could be used if it was felt that there was a danger of the toxicity being under estimated by HPLC. Alternatively, work conducted to date using well characterised but non-certified standards would enable relative response factors to be estimated. This could provide the option of semi-quantifying concentrations of these toxins using HPLC.

The current shellfish monitoring programme should be developed as required and would be an appropriate response to EU requirements. However, development of LC-MS/MS method for new PSP toxins is also recommended as ultimately the most appropriate and practical given the inability of LC-FLD to identify some of these analogues. Whilst RBA appears a suitable approach, the technique is currently not

used with the UK monitoring programme, unlike LC-MS/MS which is utilised for lipophilic toxin analysis. However, consideration of the current monitoring programmes to include this monitoring routinely may lead to resource and capacity problems which would have to be addressed.

1.4 *Karenia* and Brevetoxins

Algal species of the genera *Karenia* have been found in UK waters and toxic species are viewed as being a risk of establishment (Chapter 2, Section 1.7.5). The current water monitoring programme is able to detect these algae to the genus level. However, this would not identify potentially toxic species were they to become present. To allow species to be discerned, the use of molecular based monitoring within the water monitoring programme is viewed as the most appropriate (Chapter 3, Section 1).

BTXs have not been detected in UK shellfish, as a consequence of the historical lack of monitoring for these toxins. The potential presence of the causative organisms in UK waters makes this group one of the higher risk emerging toxins highlighted in this report. Currently there are no regulatory limits for BTXs in shellfish or fish in Europe. Without regulatory limits set in EU legislation, one potential approach by the EU would be to adopt the regulations utilised in those regions currently conducting active monitoring for brevetoxins. Were this approach to be taken based on the evidence gathered during this review, an effective and reasonable approach for routine monitoring is recommended to include (Chapter 3, Section 2.1):

- Screening of shellfish samples using a suitable assay, such as the ELISA
- Application of a suitable quantitative confirmation assay. The MBA is one option for monitoring, noting some issues with performance. However, if the MBA was removed as an option due to ethical considerations, the strongest recommendation is for application of a confirmatory LC-MS/MS method for the quantitation of brevetoxins in samples determined as positive by the screening tools employed.

This combination of water and flesh testing would be the recommended progression for *Gambierdiscus* and NSP monitoring were it required within EU regulations.

1.5 *Vulcanodinium rugosum* and Cyclic Imines

V. rugosum has been detected in Norway and its temperate nature would allow for its potential establishment in UK waters (Chapter 2, Section 1.7.3. It is not currently included in the list of species identified in the UK water. However, providing further more detailed taxonomic information is published or made available, it would potentially be possible to detect this species in the current water monitoring programme.

A number of the CIs group toxins have been found in Europe although CIs are not currently regulated in the EU or in the rest of the world. Owing to the fact that no human toxicity incident has been unequivocally linked to CIs, their inclusion in the list of regulated toxins is still under debate in the scientific community.

The testing regime currently applied in the UK is not suitable for the detection of CIs. To instigate testing for these toxins the LC-MS technique would be the most likely candidate since 13-desmethyl SPX C and GYM-A could be added to the current lipophilic toxin method and other SPXs, SPX derivatives and potentially some of the PnTXs could also be added to the method at least as a qualitative screen (Chapter 3, Section 2.3). Based on this review a reasonable approach for the monitoring of these toxins would be:

- Screening of shellfish samples using a functional assay directly related to CIs toxicity
- Application of a suitable confirmatory method for quantitation such as an LC-MS method providing high specificity and toxin profile

1.6 *Ostreopsis* and Palytoxin

No blooms of the genus *Ostreopsis* have been found in cool temperate waters and it seems restricted to the Mediterranean and Portuguese Atlantic. However, it has been found in cooler waters and it cannot be discounted as a potential emerging species in UK waters (Chapter 2, Section1.7.7). This genus is not currently included in the UK water monitoring programme, although it could be detected. However, as a predominantly benthic species the detection of this species by current monitoring methods would be expected to be poor. The recommendation for monitoring of this species would be to initially include it in the list of species monitored and ideally to develop a benthic monitoring methodology.

PlTXs have not been detected in UK waters although this is a consequence of an absence of monitoring for these toxins (Chapter 3, 2.2). However they are regularly found in other areas of Europe including Italy. There are currently no regulatory limits set for PITXs in Europe or worldwide but an appropriate monitoring approach based on the evidence gathered during this review, would include:

- Screening of shellfish samples using an ethical assay directly related to PlTX toxicity at least until toxicity equivalent factor are available for chemical methods
- Application of a suitable confirmatory method for quantitation such as an LC-MS method providing high specificity and toxin profile

However, the lack of standards for these toxins would limit the development of these methods. There needs to be a coordinated approach to the development of any methods to make best use of the limited resources.

1.7 Tetrodotoxin

To date TTX has not been found in the UK and occurrences of Puffer Fish Poisoning (PFP) are limited to warm water regions. TTX has been reported in European waters

around Greece, Egypt and Tunisia as well as in gastropods harvested from Spain. The risk within UK waters is assessed as being low. Currently the sale of *fugu* is prohibited in the EU under EU regulations Regulation (EC) 853/2004 and 854/2004. Whilst EFSA have not produced an official statement about tetrodotoxins, with the occurrence of the toxins in Europe in both fish and shellfish products, further research, surveillance and risk assessment appear necessary, with regulation potentially being considered. Based on the evidence gathered in the review and given the instrumental and expertise presently in place the following recommendations for the monitoring of TTX are suggested:

- Analysis of bacterial cultures, contaminated marine organisms for the Identification of sources of TTXs in UK waters
- Evaluation of MBA-replacement screening methods, in particular the commercial ELISA and SPR biosensor
- Development and validation of quantitative confirmatory LC-MS/MS methods for applicability to samples of relevance to the UK

These would be proposed as a reasonable approach to the development of monitoring if required within the EU.

1.8 VSP

There have been no reports of VSP in the UK to date. Little is known about this syndrome and the review assessed it as low risk. At present the only method for detection of venerupin would be animal assay.

1.9 Conclusions

Regulation (EC) No 854/2004 states that classified relaying and production areas must be periodically monitored to check for the presence of toxin-producing plankton and biotoxins in live bivalve molluscs. To comply with the regulation, FSA should consider extending the current list of phytoplankton monitored in UK waters to

include those species linked with the production of emerging toxins and identified as present in UK waters.

For those toxic species not yet present in UK waters but with the potential to occur. It could be interpreted that there is no current requirement to monitor. However, some form of infrequent monitoring of these species would provide an early warning of changes in the phytoplankton population. This would provide FSA with a proactive response rather than reactive.

Phytoplankton monitoring alone will be insufficient and must be accompanied by the development and application of suitable methods of analyses for the associated toxins. However the priority given to these toxins as a health issues varies throughout Europe. Therefore, careful prioritisation of the methods to be developed is required to avoid unnecessary demand on the limited resources of certified standard material. This is important for emerging toxin groups such as palytoxin, tetrodotoxin and ciguatoxin which present a recognised risk in some European countries.

The opinion expressed at the Seminar on emerging toxins (Vigo, May 2012) was that DGSANCO was only likely to react to pressure from Competent Authorities and on scientific data. Therefore FSA may require some form of survey data to support any action (or inaction) taken by them. For phytoplankton, the extension of the current range of species monitored could be helpful in providing data. Whilst for toxins, information can be accumulated through the current monitoring programme for some members of the the CIs and GYM groups.

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APPENDIX 1

Responses to phytoplankton questionnaire: UK & Ireland

Within your research/monitoring have you identified any new species within the phytoplankton of UK coastal or shelf waters in recent years (the last ~ 10 years)?
Marine Scotland *Yes* ***SEPA*** *SEPA provided a species list for the last 10 years but did no state if they regarded any of these species to be "new"* ***CEFAS*** *Not as far as we are aware.* ***PML*** *One unidentified diatom* ***SAHFOS*** *Yes* ***Millport*** *Basically we have not recorded any species not already listed as having been found in UK waters.* ***Napier*** *No* ***Southampton*** *New species to a location?* *If so Prorocentrum lima and Coolia monotis from the Fleet Lagoon*

If the answer to the above question is yes, can you provide us with any further details, e.g. species, location, abundance, frequency of observation, possible cause etc.

Please also include any relevant references.

Marine Scotland

Karenia papillionaceae: First suspected observation in Scalloway in Shetland in 2009. Single cell.

Dinophysis tripos: this was exciting for us in Scalloway, Shetland. One occasion, summer 2012.

Other identifications are not identification of species new to Scotland but first time to analyse samples using appropriate microscopy/molecular techniques to identify cells identified as spp. to species level.

Identifications in this category include:

A. tamarense (Group I and Group III), A. ostenfeldii, A. minutum, A. tamutum (Collins et al. 2009, Brown et al., 2011)

Chrysochromulina polylepis: at Stonehaven (unpublished)

Prorocentrum minimum: confirmation using SEM (unpublished)

Phaeocystis scrobiculata: (Bresnan and Collins in prep)

Pseudo-nitzschia: (Huge overlap with Johanna's work here)

P. Americana, P. australis, P. caciantha, P. cuspidata, P. delicatissima, P. decipiens, P.

fraudulenta., P. hemii, P. micropora, P. multiseries , P. pungens, P. pseudo-delicatissima, P. subpacifica, P. seriata (Brown and Bresnan 2008, Bresnan et al in prep)

SEPA

See attached spreadsheet

SAHFOS

Genetic monitoring of English channel water samples in 2011 has identified:

Karlodinium: April lat-lon 4.01, 49.09; -4.11, 49.69

Pseudo-nitzschia: (P. pseudodelicatissima, P.delicatissima), May lat-lon -4.04, 49.69

Chrysochromulina: April lat-lon 4.17, 49.99; May lat-lon -4.04, 49.69

Phaeocystis: March -4.12, 49.80; April lat-lon -4.11, 49.69

Pseudochatonella: Feb lat-lon -4.12, 49.94; April, position lat-lon -4.11, 49.69

Pfiesteria: (although evidence suggest this genus may not be a HAB)

*Karenia**:** Al –Kandari et al. 2011 Harmful Algae, 10, 636-643*

Dinophysis: (different genotypes) July 2003, North sea

Southampton

Occasional sampling in Fleet lagoon and isolates of above (P. lima and C. monotis) produced

We looked for Gambierdiscus and Ostreopsis in the Fleet but did not find them!

APPENDIX 2

EU monitoring laboratory responses are detailed below

Spain: Monitored phytoplankton

What genera or species of potentially biotoxin producing phytoplankton are routinely enumerated in your waters?
All microplankton species present in the samples are identified and quantified at the maximum taxonomic level possible and not only toxic ones.
Have you identified any new biotoxin producing species within the phytoplankton in recent years? **(If so can you provide us with any further details, e.g. species, location, abundance, frequency of observation, and any relevant publications)**
Regarding the emerging toxins, those we have more recently confirmed are *Karlodinium micrum* and *Prorocentrum rathimum*. **For years we know *the Lingulodinium polyedra, Protoceratium reticulatum, and Gonyaulax spinifera, Karenia mikimotoi, Karenia papilonacea, Takayama helix and Alexandrium ostenfeldii*.** **We have not detected until now nor *Gambierdiscus* nor *Ostreopsis* nor *Vulcanodinium rugosum*.**
Which of the monitored organisms are most problematic to human health in your region?
The most lethal is *Gymnodinium catenatum* and second *Alexandrium minutum* (PSP toxins). **The most frecuent is *Dinophysis acuminata*. Also we have, less often, *Dinophysis acuta* and many other *Dinophysis* species (lipophilic toxins).** **Much less often we have *Pseudo-nitzschia australis* (ASP toxins).** **Other species are less dangerous or frequent.**
If new species have been identified, do you know the cause (e.g. ballast transfer, climate induced migration, unknown…)
My opinion is that, in most cases, the cause is an improvement in the scientific

knowledge and/or in the methodology of the monitoring systems.

Spain: Phytoplankton sample collection, analysis and reporting

What method or methods are used to collect samples for the enumeration of biotoxin producing phytoplankton?
A hose sampler, Lindahl 1986 and plankton nets 10 µ mesh. Sometimes oceanographic bottles.
Do you monitor benthic species? If so, how are samples collected?
No
Who collects the samples: industry or scientists or another contracted organisation?
In several ways: **1. - Our own staff, every day, at production areas.** **2. - Samples from an oceanographic ship that we are using on a cooperation agreement with the Spanish Institute of Oceanography in Vigo.**
Are any other parameters measured at the sampling sites (e.g. water temperature, salinity….)
CTD vertical profiles (salinity, temperature, pH, oxygen, fluorescence, transmittance, irradiance, hydrocarbons), spectrofluorimetry Chlorophyll, nutrient salts and organic carbon. **Buoys** **Ferry Box.**
What method is used for phytoplankton preservation prior to analysis?
For each the sample: Formalin, Lugol and in vivo. **For some samples: Calcofluor, DAPI, Glutaraldehide.**
How are the samples transported to the laboratory for analysis? (e.g. by post?)
Our own staff, by car.
Are the samples analysed by microscopy using the Utermöhl method? **If not, what alternative method is used?**
We use the method of Utermöhl, for quantification of samples fixed with Lugol under inverted microscope. **We observe the *in vivo* samples, and formalin-fixed samples, calcofluor, Dapi under direct optical microscope for qualitative analysis and taxonomic**

studies.

Some samples, fixed with glutaraldehyde, are sent to the Vigo and Coruña University for the electron microscope.

Are any molecular methods of analysis used? If so, what methods and for what species?

We have the microarray method from Linda Medlin because we were involved as partners in a European project called MIDTAL.

Species Level Probes: *Alexandrium* NA,WE,TA, species complex, *Alexandrium minutum*, *Alexandrium tamarense* (North America), *Alexandrium tamarense* (North America), *Alexandrium tamarense* (Temperate Asian), *Alexandrium ostenfeldii*, *Alexandrium ostenfeldii, Chrysochromulina polylepis, Prymnesium parvum, Karenia mikimotoi* and *brevis, Karenia mikimotoi* and *brevis, Karenia mikimotoi, Karenia brevis, K. brevis, K. brevis, Karlodinium veneficum, Karlodinium veneficum, Karlodinium veneficum, Karlodinium veneficum, Karlodinium veneficum, Karlodinium veneficum, Prorocentrum lima, Prorocentrum lima, Prorocentrum micans, Prorocentrum minimum, Gymnodinium catenatum,Dinophysis acuminata+ dens+sacculus, Dinophysis acuta+D.fortii, Dinophysis acuminata, Dinophysis acuta, Dinophysis norvegica, Phalacroma rotundatum, Pseudo-nitzschia australis, P. multiseries, P. pungens, P. americana, P. australis & P. multistriala, P. delicatissima, P. fraudulenta and P. subfraudulenta, P. fraudulenta, P. australis & P. seriata, P. australis & P. seriata, P. brasiliana, P. brasiliana, P. brasiliana, P. caciantha, P. caciantha, P. caciantha, P. calliantha, P. calliantha, P. calliantha, P. calliantha, P. calliantha, P. delicatissima, P*. cf. *delicatissima* Clade4,*P*. cf. *delicatissima* Clade4, *P. arenysensis, P. delicatissima, P. galaxiae, P. galaxiae, P. galaxiae, P. hemeii, P. multiseries, P. multiseries, P. multiseries, P. multiseries, P. multistriata, P. multistriata, P. pseudodelicatissima & P. cuspidata, P. pseudodelicatissima & P. cuspidata, P. pungens, P. pungens, P. subpacifica,,P. seriata,P. turgiduloides,P. turgiduloides,P*. cf. *delicatissima* Clade4,*P. hasleana, P. hasleana, Chloromorum toxicum, C. toxicum, C. toxicum, C. toxicum, Gymnodinium catenatum, G. catenatum, G. catenatum, Heterosigma akashiwo, H. akashiwo, H. akashiwo, H. akashiwo, H. akashiwo,

H. akashiwo, H. akashiwo, H. akashiwo, Pseudochattonella farcimen
Within what time frame post receipt are phytoplankton samples analysed?
Samples from the production areas, analyzed for taxonomy and qualitative evaluation, immediately: hours. **The oceanographic samples, for quantitative analysis, the next day: one day.**
Are samples collected for phytoplankton enumeration at all shellfish harvesting sites or just a targeted sub-sample of these? How are the sampling sites chosen?
The samples that are analyzed for enumeration are collected from representative oceanographic stations representing both production areas as external and internal areas and are used for prediction. **A scientific panel of experts designed an initial network of oceanographic stations. Later, more stations were added.**
What frequency are samples collected for enumeration of biotoxin producing phytoplankton? Does this frequency vary with season? If so how?
The samples for enumeration are collected once per week. **No, the frequency does not vary with the seasons.**
Do phytoplankton results influence frequency of shellfish flesh testing location or frequency?
Yes, the results of phytoplankton analysis are included in the Action Plans.
Are the data made public? If so how?
www.intecmar.org **We carry several types of reports:** **Report of toxic cell count in oceanographic stations which is updated on our web and sent by e-mail, each time that we have new results. It closes at the end of the week.** **http://www.intecmar.org/pdfs/zonas_2010.pdf** **Report on the situation of the production areas (Action Plans) which includes information from toxins, assessment of oceanographic conditions and the evolution of toxic phytoplankton. It is updated each time that there are significant results and at least twice a day.** **http://www.intecmar.org/pdfs/zonas_1401.pdf**

Weekly Report which includes oceanographic data for each station, chlorophyll, etc. Species counts and is updated once a week.
http://www.intecmar.org/pdfs/Zonas_2025.pdf
We also participated in publications as yearbooks
http://www.intecmar.org/PDFs/Anuarios/2006/Anuario_ingles.pdf
, books, conferences proceedings etc.

Italy: Monitored phytoplankton

What genera or species of potentially biotoxin producing phytoplankton are routinely enumerated in your waters?
Pseudo-nitzschia, Alexandrium, Dinophysis, Ostreopsis
Have you identified any new biotoxin producing species within the phytoplankton in recent years? **(If so can you provide us with any further details, e.g. species, location, abundance, frequency of observation, and any relevant publications)**
We have just submitted a paper on a new AZA-producing Azadinium from our waters. We have also identified toxin production in some dinoflagellates and diatoms, i.e. A. andersonii, P. multistriata, P. galaxiae, but this information is published
Which of the monitored organisms are most problematic to human health in your region?
Ostreopsis
If new species have been identified, do you know the cause (e.g. ballast transfer, climate induced migration, unknown…)
unknown

Italy: Phytoplankton sample collection, analysis and reporting

What method or methods are used to collect samples for the enumeration of biotoxin producing phytoplankton?
Niskin bottle and net samples
Do you monitor benthic species? If so, how are samples collected?
macroalgae sampling by scuba diving
Who collects the samples: industry or scientists or another contracted organisation?
Both local environmental agency (ARPAC) and scientific institutions (our Institute)
Are any other parameters measured at the sampling sites (e.g. water temperature, salinity….)
T, S, nutrients, chl, zooplankton and the whole phytoplankton assemblage at our LTER sampling site. **T,S, Nutrients, light for benthic microalgae**
What method is used for phytoplankton preservation prior to analysis?
Formol fixation
How are the samples transported to the laboratory for analysis? (e.g. by post?)
We collect them
Are the samples analysed by microscopy using the Utermöhl method? **If not, what alternative method is used?**
Yes
Are any molecular methods of analysis used? If so, what methods and for what species?
Yes, we use molecular analyses for Ostreopsis and Pseudo-nitzschia species

mainly
Within what time frame post receipt are phytoplankton samples analysed?
Net samples (qualitative) the same day of collection, Niskin samples (Utermohl) within sevral months. **Within a week for Ostreopsis**
Are samples collected for phytoplankton enumeration at all shellfish harvesting sites or just a targeted sub-sample of these? How are the sampling sites chosen?
The sampling site for plankton is the LTER-MC station, far from any shellfish harvesting site. For Ostreopsis we sample a few sites known to have the most abundant populations, while ARPAC samples all along the coasts of the Campania region
What frequency are samples collected for enumeration of biotoxin producing phytoplankton? Does this frequency vary with season? If so how?
Weekly for the plankton samples – but the aim is not only to enumerate biotoxins-producing phytoplankton **Weekly for benthic microalgae, but only from June to November**
Do phytoplankton results influence frequency of shellfish flesh testing location or frequency?
No for phytoplankton, yes for Ostreopsis
Are the data made public? If so how?
Yes for Ostreopsis, they go to a Regional database and reports are published on lien after some time.

Ireland: Monitored phytoplankton

What genera or species of potentially biotoxin producing phytoplankton are routinely enumerated in your waters?
Routinely encountered: Dinophysis spp., Alexandrium spp., Pseudonitzschia spp, Small Azadinium like species are recorded and where necessary checked by gene probe
Have you identified any new biotoxin producing species within the phytoplankton in recent years? **(If so can you provide us with any further details, e.g. species, location, abundance, frequency of observation, and any relevant publications)**
Azadiniun spinosum,
Which of the monitored organisms are most problematic to human health in your region?
Dinophysis, Azadinium
If new species have been identified, do you know the cause (e.g. ballast transfer, climate induced migration, unknown…)

Ireland: Phytoplankton sample collection, analysis and reporting

What method or methods are used to collect samples for the enumeration of biotoxin producing phytoplankton?
Lund tube or Surface sampling
Do you monitor benthic species? If so, how are samples collected?
Not at the moment
Who collects the samples: industry or scientists or another contracted organisation?
Industry under the instruction of Competent authority (Sea Fisheries Protection Authority)
Are any other parameters measured at the sampling sites (e.g. water temperature, salinity….)
Shellfish Toxins
What method is used for phytoplankton preservation prior to analysis?
Lugols
How are the samples transported to the laboratory for analysis? (e.g. by post?)
Post
Are the samples analysed by microscopy using the Utermöhl method? If not, what alternative method is used?
Yes
Are any molecular methods of analysis used? If so, what methods and for what species?
Occasional molecular test using qPCR to differentiate Pseudonitzschia and presence of Azadinium

Within what time frame post receipt are phytoplankton samples analysed?
3Days max… 95% are analysed within 2 days
Are samples collected for phytoplankton enumeration at all shellfish harvesting sites or just a targeted sub-sample of these? How are the sampling sites chosen?
All shellfish sites are asked to submit water samples
What frequency are samples collected for enumeration of biotoxin producing phytoplankton? Does this frequency vary with season? If so how?
Weekly Summer and Monthy in Winter
Do phytoplankton results influence frequency of shellfish flesh testing location or frequency?
Phytoplankton is one of the key drivers to determine shellfish testing frequency along with time of year and sub-threshold toxin concentration and trends
Are the data made public? If so how?
All data on www.marine.ie/habs

Norway: Monitored phytoplankton

What genera or species of potentially biotoxin producing phytoplankton are routinely enumerated in your waters?
Mussel Dinophysis spp (all species, D. acuta, D. norvegica, D. acuminata most common) Alexandrium minutum, A. tamaranse, A. ostenfeldeii Azadinium spp (A. spinosum) Protoceratium reticulatum Prorcentrum lima (when in the water column)

Prorocentrum minimum (when in high concentration over longer time periods) Fish Chrysochromulina Prymnesium Pseudochattonella
Have you identified any new biotoxin producing species within the phytoplankton in recent years? **(If so can you provide us with any further details, e.g. species, location, abundance, frequency of observation, and any relevant publications)**
No. The last “new” producer where Azadinium
Which of the monitored organisms are most problematic to human health in your region?
Dinophysis and Alexandrium
If new species have been identified, do you know the cause (e.g. ballast transfer, climate induced migration, unknown…)
Pseudochattonella was the last “new” species. There has not been stated explanation for it

Norway: Phytoplankton sample collection, analysis and reporting

What method or methods are used to collect samples for the enumeration of biotoxin producing phytoplankton?
A hose covering 0-3 meters depth in the routine monitoring program. Monitoring performed by IMR for “Fish – HAB” is collected using NISKIN water sampler.
Do you monitor benthic species? If so, how are samples collected?
NO, only when they observed in the water column
Who collects the samples: industry or scientists or another contracted organisation?
A combination. For some location the industry is collecting samples, other station by staff from the Food safety Authority and other by IMR staff.
Are any other parameters measured at the sampling sites (e.g. water temperature, salinity….)
Only at station run by IMR
What method is used for phytoplankton preservation prior to analysis?
Lugol solution for water samples, formalin for net samples
How are the samples transported to the laboratory for analysis? (e.g. by post?)
By post, express over night
Are the samples analysed by microscopy using the Utermöhl method? If not, what alternative method is used?
Only for some sampling stations. For HAB monitoring the “filtering- semitransparent filter” method is used and Palmer-Malony counting chamber (see IOC manual 55, Karlson et al 2010 (Unesco))

Are any molecular methods of analysis used? If so, what methods and for what species?
No
Within what time frame post receipt are phytoplankton samples analysed?
All HAB samples are sampled on Monday and are reported every Thursday. In most cases the same day as received, or within 2 days.
Are samples collected for phytoplankton enumeration at all shellfish harvesting sites or just a targeted sub-sample of these? How are the sampling sites chosen?
The routine program cover harvesting sites as well as areas without mussel farming.
What frequency are samples collected for enumeration of biotoxin producing phytoplankton? Does this frequency vary with season? If so how?
Within the routine program there is a weekly sampling (Monday). For some stations only during the summer periods, other covers spring and autumn in addition, some few the whole years.
Do phytoplankton results influence frequency of shellfish flesh testing location or frequency?
Yes and no. For the industry there will not be performed any toxin analysis if there are HAB species above regulation levels. There has to be a period with no HAB species before toxins analysis is performed. In the routine monitoring program there is a fixed frequency of toxin samples. For some station there is monthly samples and other (a few) every two weeks.
Are the data made public? If so how?
The Norwegian Food Safety Authority is the owner of the data from the Routine monitoring program, giving weekly advises, giving number of cells and amount of toxins for the sampled stations. Advice given on internet and App.

APPENDIX 3

Shellfish toxin responses to survey

CFIA Quebec

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
Specific to the CFIA Longueuil Lab (Quebec Area) are Domoic Acid and Paralytic Shellfish Toxins
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
N/A
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
N/A
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
N/A
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
a) PSP-PCOX methodology (AOAC 2011.02) **b) N/A** **c) N/A (see CFIA Dartmouth Lab)**
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
N/A
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
N/A
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
N/A

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs.

Portugal

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
We have detected BMAA (β-N-methylamino-L-alanine), spiroloides and tetrodotoxin.
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources).*
Tetrodotoxin, Ciguatoxins, Palytoxin and analogues.
We would appreciate links to or copies of any reports or communications relating to the above if any are available. The following papers will be sent with this document.
Baptista, M. S., Cianca, R. C. C., C. Almeida, M. R., Vasconcelos, V. M. 2011. Determination of the non protein amino acid β-N-methylamino-L-alanine in estuarine cyanobacteria by capillary electrophoresis. Toxicon 58: 410-414 Cianca, R. C. C., Baptista, M. S., Silva, L.P., Lopes, V.R. , Vasconcelos, V. M.. 2012. Reversed-phase HPLC/FD method for the quantitative analysis of the neurotoxin BMAA (β-N-methylamino-L-alanine) in cyanobacteria. Toxicon 59: 373-378. Silva, Marisa; Azevedo, Joana; Rodriguez, Paula; Alfonso, Amparo; Botana, Luis M., Vasconcelos, Vítor. 2012. New Gastropod Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of the Atlantic Ocean. Marine Drugs 10:712-726 Cianca, R. C. C., Baptista, M. S., Lopes, V.R. , Vasconcelos, V. M.. 2012. b-N-methylamino-L-alanine in novel Portuguese cyanobacterial isolates from Minho, Douro and Vouga Rivers. Amino Acids 42:2473-2479 Vitor Vasconcelos, Mafalda Batista, Rosa Cianca, Joana Azevedo, Marisa Silva. 2012. Emergent Marine Toxins in Europe: New Challenges for Scientists and Regulatory Authorities. Toxicon 60:147
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
Tetrodotoxin, Ciguatoxins, Palytoxin and analogues. All of the have caused environmental and human intoxications in the Portuguese costal area and will likely increase due to global warming and migration to higher latitudes.
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
We use HPLC and LC-MS for the above toxins but more sensitive methods should be developed envolving ELISA or enzymatic assays due to the fact that palytoxins and ciguatoxins are not eeasy

to analyse by these chemical methods.
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
Increase the study on new toxin vectors apart from the common studies using bivalves and fish. We have detected high amounts of Okadaic acid in gastropods and equinoderms that are also part of the human food chain. It is necessary to unravel the molecular mechanisms that led to the production of these toxins so as to develop molecular methods to be used as early warning approaches. This has been successfully done with toxins from freshwater cyanobacteria so such as approach in the marine environment is a need. Another important issue is to improved and increase the monitoring of the emerging toxins, study geographical and seasonal patterns so as to have enough evidences for risk assessment and future inclusion in the legislation. Detect the occurrence of freshwater borne toxins such as microcystins produced by cyanobacteria in coastal organisms.
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
No there are no official methods for these toxins so a significant effort has to be made on optimizing methodologies for extraction and analysis so as to perform intercalibration exercises.
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
Some of this toxins and variants are not well studied form a toxicological and ecotoxicological points of view. The routes of toxin transfer are also very important so as are the new vectors that are not covered by regular monitoring plans. Recently some studies also have shown the possibility of occurrence of freshwater borne cyanobacterial toxins in coastal areas and their accumulation by marina organisms. This is especially important for the hepatotoxic microcystins, which are very stable and have acute and chronic effects such as tumour promotion. So I believe that some attention has to be given to this issue as well. Another important aspect is the cumulative effects of different toxins in the organisms. The different toxins may occur at the same time in the same organisms, so mixtures of toxins should be studied to understand whether we can find synergism or antagonism in these occurrences.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Denmark

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
The main toxins found in Danish waters: OA, OA esters, YTX (traces), PSP toxins (rare), ASP toxin (rare); see: Kevin Jørgensen, Per Andersen, Bjarne Ring Thorbjørnsen (2010) Review of toxic episodes and management strategies in relation to Danish mussel production. Proceedings of the 7th International Conference on Molluscan Shellfish Safety, Nantes, France, 14-19th June 2009, page 271-277. We have not looked for new toxins. However, from 1984 until July 1 2011 the DSP and PSP MBA have been used and we have not seen any reactions in MBA that could not be explained by presence of the normal toxins, which have been found and verified by chemical methods.
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Germany

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
We are an institute of the food control in Germany. We didn't analyse water on marine biotoxins
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
No identification
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
-
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
-
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
a) ASP; DSP; PSP b) none; c) ciguatera, NSP
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
-
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
No view
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Ireland – Marine Institute

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
AZAs, OA group including PTXs, YTXs, ASP and PSP. The MI also screens for SPX and PNTs both of which have been detected at low levels in Irish shellfish.
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
Pinnatoxins have been identified in Irish shellfish. Currently, no other new or emerging toxins are being monitored for. The causative organisms of these new and emerging toxins are not routinely monitored for in phytoplankton samples.
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
n/a
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
The AZAs are quite problematic in Ireland with a number of poisoning incidents being reported since they were first detected in 1995. It would not be surprising if they also became problematic for other countries on the Atlantic seaboard in the coming years. Although PNTs have been detected in Irish shellfish the levels are low (quantitation yet to be confirmed) and to date very little toxicological data is available for this group but generally they do not seem to be of major concern at the present time.
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
We currently use the alkaline LCMSMS method (Gerssen et al., 2009) and have traces set up for pinnatoxin and spirolides detection.
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
Screening (phytoplankton/chemical) for other known toxins (ciguatoxins, palytoxins, brevetoxins, tetrodotoxin) More toxicological data required for PNTs and other groups
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
Lack of standards/RMs for many of the new/emerging toxins hinders effective screening/monitoring programs.
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement

for biological assays, need for reference materials, toxicology and validation needs.
As above

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

NRLMB of ITALY

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?

Concerning regulated toxins: we detected **yessotoxins, okadaic acid, pectenotoxins, saxitoxins, domoic acid.**

Concerning emerging toxins: we detected

- since 2003, **spirolides**;
- since 2006, **palytoxin-like compounds (mainly ovatoxin-A).**

Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*

Palytoxin-like compounds could be of significant risk due to the presence of *Ostreopsis ovata* in Mediterranean waters: currently reported in Greece, Croatia, Italy, Spain and France.

We would appreciate links to or copies of any reports or communications relating to the above if any are available.

- Aligizaki K., Katikou P., Milandri A., Diogène J. 2011. Occurrence of palytoxin-group toxins in seafood and future strategies to complement the present state of the art. Toxicon, 57(3): 390-9.
- Ciminiello P., Dell'Aversano C., Fattorusso E., Forino M., Tartaglione L., Boschetti L., Rubini S., Cangini M., Pigozzi S., Poletti R. 2010. Complex toxin profile of *Mytilus galloprovincialis* from the Adriatic sea revealed by LC–MS. Toxicon, 55: 80-88.
- Pistocchi R., Guerrini F., Pezzolesi L., Riccardi M., Vanucci S., Ciminiello P., Dell'Aversano C., Forino M., Fattorusso E., Tartaglione L., Milandri A., Pompei M., Cangini M., Pigozzi S. and Riccardi E. 2012. Review. Toxin Levels and Profiles in Microalgae from the North-Western Adriatic Sea - 15 Years of Studies on Cultured Species. Mar. Drugs, 10: 140-162.

Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).

- **Palytoxins**, see conclusion of the paper: Aligizaki K, Katikou P, Milandri A, Diogène J. 2011. Occurrence of palytoxin-group toxins in seafood and future strategies to complement the present state of the art. Toxicon, 57(3): 390-399.
- **Ciguatera toxins** represent a significant risk for fish consumers. The presence of these toxins in fish has been reported in Canary Islands (Spain) and Madeira Island (Portugal). Other European Countries could also face the problem due to import of fish caught in Caribbean.

Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*

Spirolides and gymnodimines

We included spirolides and gymnodimines transitions in the LC-MS/MS standard operating procedure for lipophilic toxins.

Palytoxins

We currently use a LC-MS/MS internal methodology but the LOQ is not adequate for the limits proposed by EFSA. We also apply the haemolysis neutralization assay developed by Riobò et al. 2006, on the basis of Bignami 1993. The method is sensitive, but it cannot discriminate among

palytoxin analogues. Both methods have pros and cons.

Ciguatoxins

We have been using the mouse bioassay, following a protocol developed by the EURLMB. The lack of standards, positive samples and funds prevented us from the implementation of a chemical method.

Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.

Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.

Palytoxins

Since 2006 the Italian Regional Agencies for Environmental Protection have been carrying out a regular monitoring programme for the detection of *Ostreopsis ovata* along our coasts. Results of these activities are yearly reported to our Ministry for Environment. Only in some cases, during blooms of the microalga, marine organisms (mainly shellfish and sea urchins) have been collected for biotoxin analysis. A regular monitoring programme on marine organisms, financed by the Regional Competent Authority, is carried out only in the area of Naples (Regione Campania). A summary of the results is attached. Although total palytoxins in seafood were up to 625 µg/kg, no cases of human intoxication through ingestion have been reported. Concerning analytical methods, LC-MS/MS seems to be the most promising option, but the limit of detection/quantification must be carefully evaluated.

Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.

We highlight the following needs:

- reference materials for palytoxin-like compounds are needed for method validation;
- purified materials of palytoxin-like compounds (in particular ovatoxin-a) are needed to conduct toxicology studies;
- reference materials for ciguatoxins are needed for method development and validation;
- risk assessment is needed for palytoxin analogues.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

New Zealand

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
OA, DTX-1 (DTX-2 really rare), YTX and hydroxlated metabolites (homo YTX rare), PSP (alexandrium and gymnodinium (c toxins) sources). We're looking at CTX's, MTX and Palytoxin as "new things"
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
Brevetoxins seems to be something not many people expect up your way, but we've had it here, and you are (relatively) much closer to Florida than us.
We would appreciate links to, or copies of any reports or communications relating to the above if any are available.
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
PSP
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
PSP RBA, maybe – we're not 100% happy with HPLC.
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
What's happening with palytoxin?
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
Palytoxin, do this, Selwood, A. I., van Ginkel, R., Harwood, D. T., McNabb, P. S., Rhodes, L. R. & Holland, P. T. 2012 A sensitive assay for palytoxins, ovatoxins and ostreocins using LC-MS/MS analysis of cleavage fragments from micro-scale oxidation. Toxicon 60, 810-20.
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
Reference materials, see above, cheers

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Norway

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
OA-, AZA-, PTX-, YTX-, STX- and DA-group toxins of the regulated. Several spirolides, mostly 13C-desmethyl, spirolide G and 20-methyl- spirolide G. Also several pinnatoxins with pinG as main
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
The above mentioned
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
All published Toxicon, RCMS and CRT
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
Not sure about any of these new will be a significant risk. To little toxicology
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
LC-MS/MS analyses like the EURL sop will cover these
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
Toxicology of Spirolides and Pinnatoxins
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
Need both reference materials/standads and more toxicology befor a proper risk assessment can be made

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Portugal

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
In addition to the "classic" toxins (Lipophilic, ASP and PSP toxins), Tetrodotoxins have also been detected in Portuguese continental coastal waters, and Ciguatoxins in the Madeira Archipelago.
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
Tetrodotoxins, Ciguatoxins and Palytoxins are considered the most eminent emerging toxins in Portuguese waters. Phytoplankton species producers of Ciguatoxins , i.e. Gambierdiscus spp. were observed in Madeira , and producers of Palytoxins, i.e. Ostreopsis spp. , were observed in Madeira, Azores and continental waters.
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
See the following reports/articles: Harmful Algae News 45:12-13 Harmful Algae News 42: 6-7 Harmful Algae News 42: 1-2 Analytical Chemistry 82(14): 6032-6039 Analytical Chemistry 80(14): 5622-5629 Marine Drugs 10(4): 712-726.
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
Regarding to shellfish consumers strictly, we believe that tetrodotoxin may be of higher risk and eventually Palytoxins too. The risk of Ciguatoxins is related with finfish consumers.
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
Sensitive toxicity based assays and chemical methods (LCMSMS, QTOF-LCMSMS) would be of great importance if developed and applied for these groups of toxins. These methodologies currently in use to detect the mentioned emergent toxins are still not developed in our lab as a routine analysis.
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
There is lack of toxin standards, as well as, there is lack of knowledge on the toxicology of many toxins and toxins derivatives.
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
Most other countries use mouse bioassay or chemical methods such as liquid chromatography with mass spectrometry to routinely detect these emerging toxins. Due to the limited expertise on testing methods for those emerging toxins we prefer not to suggest a method.

Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
As these toxins are new for most of the European countries, insufficient knowledge has been noted regarding several topics, such as detection methodologies, risk assessment & management, reference materials, toxicological studies. It would be of great importance the implementation of a cooperative European research program (involving as many countries as possible) to investigate all these gaps and to obtain the necessary data to build and support EU legislation.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Netherlands

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
For research we've investigated the presence of various pinnatoxins and spirolides. For routine monitoring we analyse 13-desmethyl spirolide-C (quantitative) and pinnatoxin-G (qualitative). Where we regularly detect 13-desmethyl spirolide C (at low levels <10µg/kg) and do not detect pinnatoxin-G.
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
Depending on the definition of future, and the EU waters we're talking about. The more Mediterranean countries will probably face an increase of more (sub)tropical algae species and related toxins (i.e. ciguatera, palytoxins and maybe also tetrodotoxins). For Northern Europe temperatures probably remain too low to have an increase of these (sub)tropical emerging toxins but frequencies of known HABs could be increasing. So for UK and northern Europe waters I think the only risk at this moment are the cyclic imines. But toxicity towards humans is unknown for these compounds and this should be investigated before establishing legal limits and/or apply monitoring regimes for these toxins.
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
I think the EFSA opinions deal with most of this issues
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
Difficult to answer as there are quite some knowledge gaps that should be filled first before a descent decision could be made based on the risk assessment.
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
a- We use LC-MS/MS testing methodologies b- Underdevelopment are broadscreening approaches using high resolution mass spectrometry and for detection of 'unknowns' functional assays based on qPCR and other cell screening techniques (embryotic beating hart cells for compounds targeting Ca/K/Na channels) c- Use of antibody based (multi-plex approaches) From this I think that the functional assays need the highest urgency as this can really replace the MBA also for the detection of 'new emerging' or unknown toxins which are not incorporated in the routine programs based on LC-MS.
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
I think that more effort should be given to sampling procedures as detection methods are established or under development. Laboratories produce small errors (<20%) in toxin quantification but collection of the correct sample which represents the whole production area with a high confidence interval is much more difficult resulting in large deviations in toxin results easily >100%.

Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
In most cases there is no additional financing for implementing new/emerging toxin methods before it is proven there is a real risk. This risk is partly proven with occurrence data that is (not) produced in routine programs. So this is a vicious circle. In our case we implemented the cyclic imines within the lipophilic toxin LC-MS/MS method. But to create data on toxins that need a specific sample extraction as well as detection method we do not have the proper financial support in the monitoring plan.
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
In general I think the term new or emerging toxins is not that strong because these compounds are I.P. toxic to mice and are therefore classified as toxin before having any knowledge about bioavailability, oral toxicity and or exposure levels. So more knowledge is definitely needed on the toxicology part but this is largely hampered by the lack of reference materials. Furthermore, personally I do not like the approach of having fixed methods stated in legislation as at the moment they are present in legislation improved methods are available but can't be used for official control. Therefore I' am more in favour of the vet drugs or pesticide approach were performance characteristics are defined and that a method should perform acceptable in PT schemes.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

Spain 1

What toxins have been detected in your waters? **Have you looked for any new or emerging toxins as part of your monitoring or research activities?**
All the lipophilic toxins except palytoxins, saxitoxin and analogs, tetrodotoxin, ciguatoxins and domoic acid and analogs **Yes**
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
All the lipophilic toxins except palytoxins, saxitoxin and analogs, tetrodotoxin, ciguatoxins and domoic acid and analogs. Palytoxins were identified (Ostreopsis, hence ostreocins and analogs) in the South of Portugal.
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
See attatched articles.
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
Functional methods are the only ones that do provide coverage of any analog with a common mechanism of action, hence of toxicity. See list of methods we developped: **(1-14). In my opinion, antibody-based methods are not adequate to the detection of marine toxins given the large number of chemical analogs, for the same reason LC-MS are not fitted to identify such a large number of compounds.**
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
In my opinion, antibody-based methods are not adequate to the detection of marine toxins given the large number of chemical analogs, for the same reason LC-MS are not fitted to identify such a large number of compounds. See article (15)
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate. See article (16)
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs. See article (15)

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs

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***2**. Llamas, N.M., Stewart, L., Fodey, T., Higgins, H.C., Velasco, M.L., Botana, L.M., Elliott, C.T. (2007). Development of a novel immunobiosensor method for the rapid detection of okadaic acid contamination in shellfish extracts. Anal Bioanal Chem, 389, 581-587.*

***3**. Fonfria, E.S., Vilarino, N., Vieytes, M.R., Yasumoto, T., Botana, L.M. (2008). Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin. Anal Chim Acta, 617, 167-170.*

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***7**. Araoz, R., Vilarino, N., Botana, L.M., Molgó, J. (2010). Ligand-binding assays for cyanobacterial neurotoxins targeting cholinergic receptors. Anal Bioanal Chem, 397, 1695-1704.*

***8**. Vilarino, N., Louzao, M.C., Vieytes, M.R., Botana, L.M. (2010). Biological methods for marine toxin detection. Anal Bioanal Chem, 397, 1673-1681.*

***9**. Otero, P., Alfonso, A., Alfonso, C., Araoz, R., Molgó, J., Vieytes, M.R., Botana, L.M. (2011). First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. Analytica chimica acta, 701, 200-208.*

***10**. Perez, S., Vale, C., Alonso, E., Alfonso, C., Rodriguez, P., Otero, P., Alfonso, A., Vale, P., Hirama, M., Vieytes, M.R., Botana, L.M. (2011). A comparative study of the effect of ciguatoxins on voltage-dependent Na+ and K+ channels in cerebellar neurons. Chemical research in toxicology, 24, 587-596.*

11. *Rodriguez, L.P., Vilarino, N., Molgó, J., Araoz, R., Antelo, A., Vieytes, M.R., Botana, L.M. (2011). Solid-phase receptor-based assay for the detection of cyclic imines by chemiluminescence, fluorescence, or colorimetry. Analytical chemistry, 83, 5857-5863.*

12. *van den Top, H.J., Elliott, C.T., Haughey, S.A., Vilarino, N., van Egmond, H.P., Botana, L.M., Campbell, K. (2011). Surface plasmon resonance biosensor screening method for paralytic shellfish poisoning toxins: a pilot interlaboratory study. Analytical chemistry, 83, 4206-4213.*

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14. *Rubiolo, J.A., Lopez-Alonso, H., Alfonso, A., Vega, F.V., Vieytes, M.R., Botana, L.M. (2012). Bioengineered protein phosphatase 2A: Update on need. Bioengineered, 4, Press*

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16. *Otero, P., Alfonso, A., Alfonso, C., Rodriguez, P., Vieytes, M.R., Botana, L.M. (2011). Effect of uncontrolled factors in a validated liquid chromatography-tandem mass spectrometry method question its use as a reference method for marine toxins: major causes for concern. Anal Chem, 83, 5903-5911.*

Spain 2

What toxins have been detected in your waters? Have you looked for any new or emerging toxins as part of your monitoring or research activities?
Okadaic acid, DTX2, DTX1, acyl-(OA and DTX2) and diol-(OA and DTX2), PTX2, Domoic acid and isomers (mainly epi-DA, Iso-D, Iso A), Saxitoxin, NeoSTX,GTX1-6, dcSTX, dcGTX3, C1-C4, 13desmethylSPXC, azaspiracids 1-3 (only 1 sample). Mouse bioassay is being used in order to detect non-analyzed toxins, and a number of toxins are being monitored in mussels and passive samplers in an on-going research project (OA, DTX1, DTX2, AZA1-5, PTX2, palytoxin, brevetoxin (PbTX-2), gymnodimine, SPXA, SPXB, SPXC, SPXC2, 13,19 didesmethyl SPXC, 13 desmethyl SPXC, SPXD, 13-desmethyl SPXD, 20-Methyl SPXG, SPX E, SPXG, Pinnatoxin A-G, Pteriatoxin A-C.
Please identify any specific new/emerging toxins which you believe may be present currently or in likely to appear in the future within UK/EU waters (*Please indicate whether there is any evidence for this, e.g. from phytoplankton identification or from other sources)*
Other spirolides, pinnatoxins, ciguatoxin, palytoxin
We would appreciate links to or copies of any reports or communications relating to the above if any are available.
No results have been published up to date.
Please highlight which of these toxins (and why) you think may be of significant risk in relation to shellfish consumer food safety in UK/EU waters (*based on either published or unpublished data*).
Palytoxin, ciguatoxin (highly toxic, some cases reported) (*Nordt, S. P., Wu, J., Zahller, S., Clark, R. F., & Cantrell, F. L. (2011). Palytoxin Poisoning After Dermal Contact With Zoanthid Coral. Journal of Emergency Medicine, 40(4), 397–399; Deeds, J. R., & Schwartz, M. D. (2010). Human risk associated with palytoxin exposure. Toxicon, 56(2), 150–162.), Sosa, S., Del Favero, G., De Bortoli, M., Vita, F., Soranzo, M. R., Beltramo, D., et al. (2009). Palytoxin toxicity after acute oral administration in mice. Toxicology Letters, 191(2-3), 253–259*.; (*Boada, L. D., Zumbado, M., Luzardo, O. P., Almeida-González, M., Plakas, S. M., Granade, H. R., et al. (2010). Ciguatera fish poisoning on the West Africa Coast: An emerging risk in the Canary Islands (Spain). Toxicon, 56(8), 1516–1519*), some spirolides (*Otero, P., Alfonso, A., Rodriguez, P., Rubiolo, J. A., Cifuentes, J. M., Bermudez, R., et al. (2012*). *Pharmacokinetic and toxicological data of spirolides after oral and intraperitoneal administration. Food and Chemical Toxicology, 50(2), 232–237)*. Pinnatoxins (*Munday, R., Selwood, A. I., & Rhodes, L. (2012). Acute toxicity of pinnatoxins E, F and G to mice. Toxicon, 60(6), 995–999*).
Please highlight any toxin testing methodologies which a) you currently use b) are under development or c) you are aware of, which may be targeted at testing for these new and emerging toxins. *In particular emphasising those tests which you feel should be developed or applied with the greatest urgency.*
A) Mouse bioassay, LC-MS/MS, LC-UV(DAD)
Please describe any knowledge gaps where you feel further research of new/emerging toxins or development of testing methods would benefit UK/EU shellfish monitoring programmes.
Simplifying or screening techniques for LC-MS/MS analysis, functional assays adapted to monitoring dynamics.
Do you have any views on the suitability of toxin testing methods for new/emerging toxins currently implemented in official control monitoring programmes and/or under development in other countries (*published or unpublished*). Please give specific examples where appropriate.
Mouse bioassay continues to perform reasonably well, but with the progressive implementation of LC-MS/MS techniques the number of bioassays carried out are going to be substantially reduced and consequently the probability of detection will be also reduced. For LC_MS, the most important

problem is the increasing number of the compounds to be monitored, that generates both, conceptual and practical uncertainties. Mainly TEFs (how toxicity is estimated from toxin concentrations), limits of the equipment to measure a continuously growing number of compounds to be monitored, impossibility (or near impossibility) to have reference materials for all compounds and to estimate the precise recoveries...
Please highlight any other views you may have relating to the risk of new/emerging toxins, including but not restricted to methodologies, risk assessment and risk management, requirement for biological assays, need for reference materials, toxicology and validation needs.
It is important to fine-tune the estimations of toxicity both, those used to establish the legal limits and those obtained with the techniques of quantification. Good estimates of those toxicities would allow to maintain low or to reduce the uncertainty factor and find an optimum balance between safety and commercial activity. This includes, among other aspects, the availability of reference materials and precise estimates of TEFs. It is also needed a framework to fit the different techniques that can be used (at European or worldwide level) and that are unlikely to give the same results for the different techniques when they are near the allowable limits.

Note all references to "New and emerging toxins" refers to those toxins not currently listed in EU legislation concerning official control testing of bivalve molluscs