Review of the currently available field methods for detection of marine biotoxins in shellfish flesh

FS102086

September 2015

Catherine McLeod, Stephen Burrell and Patrick Holland

© Crown Copyright 2014
This report has been produced by Seafood Safety Assessment Ltd. under a contract placed by the Food Standards Agency (the Agency) and Food Standards Scotland (FSS). The views expressed herein are not necessarily those of the Agency or FSS. Seafood Safety Assessment Ltd. warrants that all reasonable skill and care has been used in preparing this report. Notwithstanding this warranty, Seafood Safety Assessment Ltd. shall not be under any liability for loss of profit, business, revenues or any special indirect or consequential damage of any nature whatsoever or loss of anticipated saving or for any increased costs sustained by the client or his or her servants or agents arising in any way whether directly or indirectly as a result of reliance on this report or of any error or defect in this report.

EXECUTIVE SUMMARY

In 2014 the Food Standards Agency in Scotland (FSAS) introduced new guidance for shellfish Food Business Operators (FBOs) on a risk management framework for marine toxins in shellfish. The guidance suggests that end product testing (EPT) of shellfish for marine toxins should be increased when toxins or toxin-producing plankton are detected in samples collected for Official Control (OC) or industry purposes. Many FBOs in the UK are currently implementing the new guidance. Given the increased need for EPT and potential for future increases as more FBOs adopt the guidance, information on appropriate field methods[1] that are available for use by shellfish processing and harvesting businesses is needed. This review was therefore commissioned by the Food Standards Agency to identify kits that can be used by the shellfish industry and to evaluate the kits against internationally agreed method validation guidelines to determine shortcomings.

Nine companies were identified as commercially producing field kits for the detection of amnesic, diarrhetic and paralytic shellfish poisons (ASP, DSP and PSP). No field methods are currently available for azaspiracids, pectenotoxins or yessotoxins (AZA, PTX, YTX), which are also currently regulated in the European Union (EU). The available methods include enzyme linked immunosorbent assays (ELISAs) and lateral flow immunoassays (LFAs) for ASP, DSP and PSP, and the protein phosphatase inhibition assay (PPIA) for DSP. Method performance was evaluated against minimum criteria established by the AOAC (Association of Analytical Communities) and Eurachem for screening methods, and included an assessment of detection capability (including cross reactivity, false negative rates and false positive rates), specificity, and for quantitative methods, precision.

Field methods are a desirable component of the overall risk management framework for marine toxins, as they are relatively inexpensive (i.e. <£15.10 per sample) and enable FBOs to undertake 'real time' testing (<2 hours per sample) of shellfish prior to harvesting or processing, thereby reducing the potential for placing shellfish on the market that could contain excessive levels of marine toxins. In general, the commercially available field methods have appropriate sensitivity, with methods capable of detecting ASP, DSP and PSP at concentrations well below the maximum permissible levels (MPL). The methods were also found to have acceptable specificity, and did not cross react with non-related toxins or other compounds, and in cases in which precision had been investigated, repeatability estimates were also acceptable (i.e. ≤15%). However, the review identifies some technical issues, including:

- For assays to be accepted as alternative methods of analysis in the EU, inter-laboratory studies (ILS) must be conducted in accordance with AOAC and Eurachem guidelines. Only the Biosense ELISA for ASP and the Zeulab PPIA for DSP have met this requirement.
- The ELISAs and LFAs have poor reactivity to the DSP congener DTX-2. DTX-2 has been found to be dominant in ca. 13% of Scottish shellfish, therefore it is important that assays can detect this congener at levels below the MPL to avoid false negatives. It was also noted that DSP assays can give false negative results when high levels of DTX-3 are present and the hydrolysis step is not undertaken to release ester forms.
- PSP ELISAs and LFAs were noted to have poor reactivity to the PSP congeners GTX-1,4, NEO and the decarbamoyl toxins. GTX-1,4 and NEO are of relatively high toxicity to humans and some PSP contaminated shellfish in the UK contain high proportions of these congeners. Therefore, there is a risk of false negative results when using these immunoassays with UK shellfish.

[1]Methods that are portable and can be adapted for use in the field and food-processing environment. They should be applicable in a field laboratory and not require a high level of technical competence or the use of expensive laboratory instrumentation, other than basic equipment such as plate readers and other common electronic devices.

- The LFAs were found to give some false positive results when ASP, DSP and PSP were below the MPL. The false positive rates vary depending on the toxin profile, geographic region, and shellfish species involved. Any positive EPT results can have significant consequences for FBOs due to the closure of harvesting areas or withdrawal of shellfish from the market.
- FBOs using kits in the UK were interviewed regarding their practical experiences with using field kits for EPT. All FBOs currently undertaking EPT use LFAs. The cost of equipment was identified by the FBOs as a significant barrier to implementation of EPT. Many also noted technical difficulties when conducting the tests.

While some limitations of field methods have been identified in this review, the use of such kits by FBOs for EPT of shellfish in the processing and harvesting environment is a positive development, which is likely to lead to improved protection of public health through more intensive monitoring. The findings of the review however, emphasises the importance of field methods being appropriately validated on sample types that are relevant to the UK, and for the kits to have acceptable false negative and false positive rates. In some cases the validation of the field methods may need to be more rigorous, in particular they may require inter-laboratory studies using appropriate sample types, to meet the requirements for alternative methods described in EU law. It is also imperative that there is a clear and efficient process for FBOs to follow when the test kits give a positive response, including the availability of short turn around confirmatory testing facilities and methods.

A series of recommendations have been made (Section 9.2) to address the technical issues and information gaps that have been highlighted in the review, including suggestions regarding potential false negatives, false positives, regulatory acceptance of methods, quality control, enhanced uptake of EPT by industry, and future research. The recommendations are directed to FBOs, Food Standards Scotland, test kit suppliers, and UK Local Authorities for consideration.

GLOSSARY

AOAC	Association of Analytical Communities
ASP	Amnesic shellfish poisons
AZA	Azaspiracid
CCFFP	Codex Committee on Fish and Fishery Products
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
Confirmatory method	Confirmatory method means methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest.
DA	Domoic acid
Detection capability (CCβ)	Detection capability (CCβ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β.
DSP	Diarrhetic shellfish poisons (includes okadaic acid group toxins and pectenotoxins)
Decision limit (CCα)	Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.
DLC	Dioxin-like compound
DL-PCB	Dioxin-like PCB
DTX	Dinophysistoxins
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbant assay
EPT	End product testing
EU	European Union
EU RL	European Reference Laboratory for Marine Biotoxins
False negative	A result which is negative when the true level is ≥ MPL
False positive	For the purposes of this report, two types of false positives are discussed: (a) Samples for which toxin was detected, but no toxin was present (FPa); (b) Samples which have toxin concentrations less than the MPL, which give a positive result (FPb).
FBO	Food business operator
Field method	Methods that are portable, can be adapted for use in the field and food-processing environment. They should be applicable in a field laboratory and not require a high level of technical competence or the use of expensive laboratory instrumentation, other than basic equipment such as plate readers and other common electronic devices.

FSA	Food Standards Agency
FSAS	Food Standards Agency in Scotland
FSS	Food Standards Scotland
GTX	Gonyautoxin
HPLC	High performance liquid chromatography
ILS	Inter-laboratory study
ISSC	Interstate Shellfish Sanitation Committee
IUPAC	International Union of Pure and Applied Chemistry
LA	Local Authority
LC	Liquid chromatographic
LC-FLD	Liquid chromatography fluorescence detection
LC-MS/MS	Liquid chromatography mass spectrometry detection
LFA	Lateral flow immunoassay
LoD	Limit of detection
LoQ	Limit of quantitation
MBA	Mouse bioassay
MPL	Maximum permissible level
MT	Metallothionein
NSSP	National Shellfish Sanitation Programme
OA	Okadaic acid
OC	Official control
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
PDE	Phosphodiesterase
PP	Phosphoprotein phosphatase
PPIA	Protein phosphatase inhibition assay
Precision	Precision means the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result.
PSP	Paralytic shellfish poisons
PTX	Pectenotoxins
QA	Quality assurance
QC	Quality control

Qualitative method	Qualitative method means an analytical method which identifies a substance on the basis of its chemical, biological or physical properties.
Quantitative method	Quantitative method means an analytical method which determines the amount or mass fraction of a substance so that it may be expressed as a numerical value of appropriate units.
RBA	Receptor binding assay
Recovery	Recovery means the percentage of the true concentration of a substance recovered during the analytical procedure. It is determined during validation, if no certified reference material is available.
Repeatability(RSD_r)	Repeatability means precision under repeatability conditions. Repeatability conditions means conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment.
Reproducibility (RSD_R)	Reproducibility means precision under reproducibility conditions. Reproducibility conditions means conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.
RIA	Radioimmunoassay
Ruggedness	Ruggedness means the susceptibility of an analytical method to changes in experimental conditions which can be expressed as a list of the sample materials, analytes, storage conditions, environmental and/or sample preparation conditions under which the method can be applied as presented or with specified minor modifications. For all experimental conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH, temperature) any variations which could affect the analytical result should be indicated.
SAGB	Shellfish Association of Great Britain
Screening method	Screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.
SLV	Single laboratory validation
Specificity (also called ‘selectivity’)	Specificity means the ability of a method to distinguish between the analyte being measured and other substances.
SPR	Surface plasmon resonance
STX	Saxitoxin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

TEF	Toxicity equivalence factor
TEQ	Toxic Equivalency Quotient
Trueness	Trueness means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness is usually expressed as bias.
TTX	Tetrodotoxin
VAM	Validation of analytical methods
YTX	Yessotoxin

TABLE OF CONTENTS

EXECUTIVE SUMMARY 2

GLOSSARY 4

SECTION ONE: INTRODUCTION 10

1.1 OBJECTIVES OF THE REVIEW 10

1.2 SCOPE OF THE REVIEW 11

SECTION TWO: BACKGROUND TO THE REVIEW 12

2.1 THE TOXINS AND TOXIN PRODUCERS 12

2.2 MAIN BIVALVE SPECIES PRODUCED IN THE UK 13

2.3 TOXIN PROFILES OF UK SHELLFISH 14

2.4 OFFICIAL CONTROL PROGRAMME 17

2.5 MANAGEMENT OF TOXIN RISK BY FOOD BUSINESS OPERATORS 18

2.6 EUROPEAN REQUIREMENTS FOR MARINE TOXIN METHODS 20

2.7 METHOD VALIDATION AND QUALITY ASSURANCE 20

SECTION THREE: METHODOLOGY FOR THE REVIEW 24

3.1 LITERATURE REVIEW 24

3.2 EVALUATION OF FIELD TEST METHODS 24

SECTION FOUR: OVERVIEW OF MARINE TOXIN DETECTION METHODS 27

4.1 FUNCTIONAL ASSAYS 27

4.2 IMMUNOLOGICAL ASSAYS 32

4.3 INSTRUMENTAL ANALYTICAL METHODS 33

4.4 BIOSENSORS 34

SECTION FIVE: EVALUATION OF CURRENTLY AVAILABLE FIELD KITS FOR MARINE TOXIN DETECTION 37

5.1 FIELD METHODS CURRENTLY AVAILABLE IN THE UK 37

5.2 EVALUATION OF METHOD PERFORMANCE FOR ASP FIELD METHODS 38

5.3 EVALUATION OF METHOD PERFORMANCE FOR DSP FIELD METHODS 42

5.4 EVALUATION OF METHOD PERFORMANCE FOR PSP FIELD METHODS 48

SECTION SIX: FOOD BUSINESS OPERATORS' VIEWS ON TOXIN FIELD TESTS 57

SECTION SEVEN: STATUS OF FIELD TEST DEVELOPMENT FOR AZASPIRACID, YESSOTOXIN AND PECTENOTOXIN GROUPS 60

7.1 STATUS OF METHODS UNDER DEVELOPMENT FOR AZASPIRACID 60

7.2 STATUS OF METHODS UNDER DEVELOPMENT FOR YESSOTOXIN 60

7.3 STATUS OF METHODS UNDER DEVELOPMENT FOR PECTENOTOXIN 61

SECTION EIGHT: OVERVIEW OF FIELD METHODS FOR CHEMICAL CONTAMINANT DETECTION 62

8.1 SCOPE 62

8.2 Heavy metals 62
8.3 Polyaromatic hydrocarbons 63
8.4 Dioxins and dioxin-like PCBs 64
8.5 Adaptability of methods for shellfish testing 65
SECTION NINE: CONCLUSIONS AND RECOMMENDATIONS 67
9.1 Conclusions 67
9.2 Recommendations 70
ACKNOWLEDGEMENTS 73
APPENDIX ONE: FOOD BUSINESS OPERATOR SURVEY QUESTIONS 74
APPENDIX TWO: FOOD BUSINESS OPERATOR RESPONSES TO SURVEY 75
REFERENCES 77

SECTION ONE: INTRODUCTION

Certain species of marine phytoplankton (primarily dinoflagellates and diatoms) are known to produce toxins. Bivalve shellfish are filter feeders, and when blooms of these toxin-producing plankton occur they can accumulate high levels of the toxins in their digestive tracts and to a lesser degree their muscular tissues. A large number of different toxins are produced by marine phytoplankton, and these are broadly categorized into eight different toxin groups (FAO, 2004; Lawrence et al., 2011). Of these eight groups, the most serious risk to human health are posed by the azaspiracid toxin group (AZA), the domoic acid toxin group (ASP), the okadaic acid and dinophysis toxins group (DSP), and the paralytic shellfish toxin group (PSP).

European Union (EU) law (EC regulations 854/2004, 882/2004, 2074/2005) requires a range of official controls for bivalve molluscan shellfish to ensure that shellfish contaminated with marine toxins at concentrations exceeding maximum permitted levels (MPL) are not placed on the market. Statutory monitoring of shellfish in the UK is undertaken for the most serious toxin groups noted above, as well as for yessotoxins (YTX) and pectenotoxins (PTX), for which the harmful effects in humans are less clear but currently also have EC regulatory levels set. In addition to toxin monitoring, identification and enumeration of potential harmful algal species is also undertaken.

The Food Standards Agency (FSA) and Food Standards Scotland (FSS) manage the Official Control (OC) Programme for shellfish in the UK (FSA in England, Wales and Northern Ireland and FSS in Scotland), including the administration of shellfish testing for marine toxins in accordance with EU law. The OC Programme provides information to support the determination of whether production areas should be open or closed for harvesting depending on the marine toxin status and other factors. The OC Programme does not aim to determine the final marine toxin status of products placed on the market, which is a responsibility of Food Business Operators (FBOs). Food businesses are required under EU law to ensure that shellfish placed on the market are safe for consumption and do not exceed the MPLs stipulated in the EC regulations. Given this need, in April 2014 the FSA in Scotland (FSAS)[2] issued new guidance to provide a framework for FBOs to assess the toxin risk of their products and to implement testing programmes to minimise the risks to consumers. The guidance includes the use of the 'traffic light' tool, which incorporates a series of phytoplankton and flesh triggers for each toxin group. It is recommended that when OC or FBO samples exceed the triggers that FBOs should increase end product testing (EPT) of their products. Many FBOs in Scotland are currently implementing the guidance and are undertaking EPT of their products, particularly at times of increased risk when toxin and plankton levels are elevated.

Given the heightened emphasis on FBOs ensuring that their products are compliant, it is pertinent that up to date information is accessible to stakeholders, regarding suitable test methods that can be used by industry for marine toxins. This review has therefore been commissioned to compile supporting information to assist FBOs to select the most appropriate methods for use in the field.

1.1 Objectives of the review

The objectives of this review are to:

a. Give an overview of the technological basis of methods that have been developed for marine toxin analysis;

[2]Please note as of 1 April 2015, Food Standards Scotland (FSS) has taken on all the functions that were previously exercised in Scotland by the Scottish part of the UK-wide Food Standards Agency: food safety and standards, feed safety and standards, nutrition, food labelling, and meat inspection policy and operational delivery.

b. Identify commercially available field methods for each of the regulated marine toxin groups and critically evaluate the performance of the methods;

c. Identify field methods under development and evaluate the status of the developmental efforts and potential timeframes to commercialisation for toxin groups for which kits are not commercially available (i.e. AZA, YTX and PTX);

d. Develop recommendations on: (a) research that may improve the range of toxin field methods available to industry; (b) validation studies that may facilitate acceptance of field methods by regulators and FBOs; and (c) improved adoption of EPT by industry; and

e. Summarise the field methods currently being used for chemical contaminants in shellfish[3].

1.2 Scope of the review

The review involved a systematic appraisal of the scientific literature and data supplied by commercial test kit manufacturers and other parties to identify and evaluate field detection methods for marine toxins in shellfish that can be used by industry in a field laboratory (i.e. at a bivalve processing or harvesting facility).

For the purposes of this review 'field methods' are defined as methods that are portable, can be adapted for use in the field and food-processing environment. They should be applicable in a field laboratory and not require a high level of technical competence or the use of expensive laboratory instrumentation, other than basic equipment such as plate readers and other common electronic devices. Methods that were only at a research stage of development, or were noted by suppliers to be for 'research use only' were not included in the evaluation.

While the basis of the various detection methods differs, they all require the collection of samples that are representative of the area or shellfish batch, and sample preparation (including shucking) and toxin extraction steps. The quality of data produced by any method is contingent on appropriate sample collection and extraction steps being undertaken, and they also significantly impact on the costs and overall time taken to produce a result. This report focuses on detection methodologies not on sampling and sample preparation methods.

The review focuses on field methods pertaining to the currently regulated toxins in the UK, namely DSP, PSP, ASP, PTX, AZA and YTX. Information on field methods for other 'emerging' toxins (e.g. cyclic imines, palytoxins, brevetoxins etc) that may be regulated in the future are not evaluated in this review. Field methods for some environmental contaminants were also identified to assist in any future deliberations regarding end product testing by FBOs.

The review included evaluation of information obtained from:

- Journal publications
- Government reports
- Conference proceedings
- Information (manuals, protocols, validation reports) and unpublished data obtained from test kit manufacturers
- FBOs.

[3]The initial specification also included identifying field methods for mycotoxins and nutrients in foods. The intention of this part of the specification was to ascertain if methods currently used for other contaminants could be adapted for marine toxin analysis. The scope was narrowed to marine toxins and chemical contaminants in shellfish following discussions with the FSAS regarding the difficulties in adapting methods for other groups of chemicals to suit marine toxin analysis, because a specific method for one analyte e.g. Pb, is unlikely to be applicable to another compound such as saxitoxin.

SECTION TWO: BACKGROUND TO THE REVIEW

This section contains information that was used to support the development of conclusions and recommendations in Section 9 of this review. It includes background information on the regulated toxins and toxin producers, toxin profiles found in UK shellfish, the current risk management system in place in the UK, toxin methods of analysis that are approved in the EU and the method validation requirements if alternative methods such as field kits are to be used.

2.1 The toxins and toxin producers

2.1.1 Amnesic shellfish poisons

The main toxin in the ASP toxin group is domoic acid (DA), which is a water-soluble cyclic amino acid. Several DA isomers have been reported (e.g. epi-domoic acid and iso-domoic acids A-H), but not all have been detected in shellfish (EFSA, 2009b).

Domoic acid is produced by diatoms in the genus *Pseudo-nitzschia* e.g. *Pseudo-nitzschia multiseries* (formerly *Nitzschia pungens f. multiseries*), *P. australis*, *P. seriata*, and *P. pungens*. Eleven species of *Pseudo-nitzschia* have been reported to produce DA, nine species of *Pseudo-nitzschia* have been identified in Scotland. Testing to date confirms that *P. australis* and *P. seriata* in Scotland are DA producers (Swan and Davidson, 2012).

2.1.2 Azaspiracids

Azaspiracids are nitrogen-containing polyether toxins with a unique spiral ring assembly, a cyclic amine and a carboxylic acid. There have been over 30 different congeners identified to date (Kilcoyne et al., 2014; Krock et al., 2014; Rehmann et al., 2008).

The ostensible primary producer of AZA was identified in seawater samples from the North East coast of Scotland in 2008. The organism, *Azadinium spinosum,* is a small (7 – 11 µm) dinoflagellate (Salas et al., 2011; Tillmann et al., 2009) and can be difficult to identify using light microscopy. AZA-1 and AZA-2 are produced by *A. spinosum* (Krock et al., 2009) while some of the other congeners identified are shellfish metabolites. Many more *Azadinium* and related species have been identified, some of which are producers of novel, recently discovered AZAs (Kilcoyne et al., 2014), with their assessment and relevance to public health still to be determined.

2.1.3 Diarrhetic shellfish poisons

The DSP toxins are heat stable lipophilic compounds which include:

- Okadaic acid (OA) and its isomer 19-epi-okadaic acid;
- The OA congeners dinophysistoxin-1 (DTX-1) and dinophysistoxins-2 (DTX-2);
- The 7-acyl derivatives of OA, DTX-1 and DTX-2 that are collectively known as DTX-3 (EFSA, 2008a); and
- Pectenotoxins (PTX).

Pectenotoxins are polyether compounds that frequently co-occur with OA and DTXs. Approximately 15 PTX congeners had been isolated and characterised up until 2009 (EFSA, 2009a). Dinoflagellate species of the genus Dinophysis (*Dinophysis acuta, D. acuminata, D. fortii*) and Prorocentrum (e.g. *Prorocentrum lima, P. hoffmanianum, P. concavum, P. belizeanum, P. rhathymum*) produce OA and DTX group toxins. The IOC-UNESCO list 11 species of *Dinophysis* that are potential DSP producers.

Dinophysis species are planktonic, but *Prorocentrum* species are benthic or epibenthic. Several species of *Dinophysis* have been detected in the UK.

2.1.4 Paralytic shellfish poisons

Paralytic shellfish poisons are a group of toxins composed of related congeners that have been identified in toxic algae and various species of seafood (EFSA, 2009c; van Egmond et al., 2004; Wiese et al., 2010). Saxitoxin consists of a 3,4-propinoperhydropurine tricyclic structure with the molecular formula, $C_{10}H_{17}N_7O_4$. Since the discovery of saxitoxin, 57 different PSP congeners have been identified from various organisms (Wiese et al., 2010).

Paralytic shellfish poisons are produced by some species of marine dinoflagellates in the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium*. Several species of *Alexandrium* have been reported to occur in the UK, including *A. tamarense*, *A. minutum*, *A. ostenfeldii* and *A. tamutum* (Swan and Davidson, 2012) (reviewed in Turner et al. (2014b). *A. tamarense* is commonly reported in the UK and both toxic and nontoxic variants are found (Collins et al. 2009; Touzet et al., 2010). However, *Gymnodinium catenatum*, a PSP producer commonly observed internationally, has not been detected in waters around Great Britain.

2.1.5 Yessotoxins

Yessotoxins are a group of polyether compounds, more than 90 congeners have been described to date (EFSA, 2008c). Yessotoxins are produced by the dinoflagellate *Protoceratium reticulatum; Lingulodinium polyedrum* has also been implicated in YTX production. Both dinoflagellates have been identified in Scottish waters (Swan and Davidson, 2012).

2.2 Main bivalve species produced in the UK

The bivalve shellfish species produced in the UK are shown in Tables 2.1 and 2.2 (aquaculture and fishery products respectively). Mussels (*Mytilus* spp.) are the most significant farmed bivalve species in the UK, followed by Pacific oysters (*C. gigas*) and native oysters (*O. edulis*). Small amounts of King and Queen scallops (*P. maximus* and *A. opercularis* respectively) are also harvested from the in-shore environment, as are manila clams (*Ruditapes philippinarum)*, hard clams (*Mercenaria mercenaria*) and cockles (*C. edule)*.

Regarding wild capture fisheries, scallops represent the most fished bivalve in the UK (not speciated in the fishery statistics), followed by cockles, razors (*Ensis* spp.), mussels, oysters, and surf clams (*Spisula solida*). There is one classified production area for wedge clams *(Donax vittatus)* in Scotland*,* however volumes do not appear in the published fisheries statistics and they are therefore omitted from Tables 2.1 and 2.2.

Table 2.1: Volume (tonnes) of bivalve shellfish produced using aquaculture methods in the United Kingdom (England, Wales, Scotland and Northern Ireland) in 2012 (Munro et al., 2013).

Species	Tonnes
Mussels (*Mytilus spp.)*	26021
Pacific oysters (*Crassostrea gigas*)	1206
Native oysters (*Ostrea edulis)*	111
Hard clams (*Mercenaria mercenaria*)	9
King scallops (*Pecten maximus)*	7
Manila clams (*Ruditapes philippinarum)*	5
Queen scallops (*Aequipecten opercularis)*	0.4
Cockles (*Cerastoderma edule)*	0

Table 2.2: Volume (tonnes) of bivalve shellfish captured in the United Kingdom (England, Wales, Scotland and Northern Ireland) in 2012 (Anonymous, 2013)

Species	Tonnes
Scallops (not speciated)	54,200
Cockles (*Cerastoderma edule)*	2,200
Razor fish (*Ensis* spp.)	900
Mussels (*Mytilus spp.)*	700
Native oysters (*Ostrea edulis)*	211
Surf clams (*Spisula solida*)	6

2.3 Toxin profiles of UK shellfish

This section summarises current knowledge regarding the toxin profile (toxin composition and concentration) of commonly found shellfish species in the UK. The relative proportions of different toxin congeners in shellfish (i.e. the toxin profile) may be expected to vary for several reasons. Firstly, toxin profiles have been demonstrated to differ between algal species and between strains of the same algal species. Toxin composition also has been shown to differ between algal species from different locations, between isolates from the same location, and between isolates maintained under different growth conditions (Anderson et al., 2012; Anderson et al., 1990; Hallegraeff et al., 2012). Secondly, there are large interspecies differences in clearance and filtration rates of bivalve shellfish that inhabit the same environment and these differences have resulted in differential uptake of harmful algae by bivalves and subsequent differences in toxin concentration between species (Li et al., 2001). Thirdly, differences in metabolic transformation of toxins from one congener to another within different species of shellfish results in variations in toxin profiles (Jaime et al., 2007). Finally, differences in the depuration rates of the various toxin congeners in different shellfish species may contribute to variations in toxin profile.

It is important that field kits can detect the toxin congeners of highest concern in the UK. This is a particular concern regarding the PSP and DSP toxin groups, for which there are a variety of different toxin congeners. Data regarding the toxin profiles of PSP contaminated shellfish from England, Wales and Scotland has recently been published (Turner et al., 2014a; Turner et al., 2014b), similar data from Northern Ireland is not available, however data from the Northern Irish monitoring programme indicate PSP toxins are rare in samples from this region. The FSAS also commissioned a report that provides information on the DSP, AZA and YTX profiles of shellfish from Scotland (Turner

et al., 2014a), however similar equivalent information has not yet been published for England, Wales and Northern Ireland. Toxin profile information is not relevant for ASP as DA is the only regulated congener.

While information on the toxin profiles is useful when considering the specificity of field methods, an important caveat is that this historical data is not a certain guide to the future. Many other harmful algal species and strains are known world-wide, each with their own spectrum of toxins, and some of these could bloom in UK waters as potential threats with different risk profiles.

This point is highlighted by events that recently unfolded in Tasmania, Australia, where recurrent blooms of *G. catenatum* take place. The monitoring programme was very focused on managing risk relating to *G. catenatum* and placed an emphasis on phytoplankton monitoring for this microscopically easily recognisable species. However, in 2012 an unprecedented bloom of *A. tamarense* occurred causing high concentrations of PSP in shellfish (Campbell et al., 2013). The recent occurrence of *A. tamarense*, in addition to *G. catenatum*, has re-directed the focus of the management programme towards implementing field methods for the detection of PSP in shellfish. Studies are currently being conducted to ensure that antibody based field methods for PSP are suitable for a variety of different toxin profiles to ensure that the Tasmanian programme is 'future proofed' with respect to the occurrence of a variety of different PSP toxin producers.

2.3.1 Azaspiracid profile

In 2014 the FSAS commissioned CEFAS to appraise the OC results with the objective of evaluating toxin profiles in Scottish shellfish. As part of the review it was determined that 591 samples tested between 2011 and December 2013 (of a total of 7127 samples analysed) contained AZAs at levels exceeding the method LoQ (16 µg/kg). Samples were analysed for the presence of AZA-1, AZA-2 and AZA-3; AZA-1 was found to be the dominant congener and was present in 590 samples. AZA-2 was present in 311 samples and AZA-3 was only present in trace levels in two samples (Turner et al., 2014a).

The authors undertook a cluster analysis and identified two groupings of shellfish:

- Profile 1 group shellfish contained only AZA-1; and
- Profile 2 group shellfish contained a mix of both AZA-1 and AZA-2, and trace levels of AZA-3 in a few samples.

These findings underscore the need for field tests to detect AZA-1 and AZA-2, which appear highly relevant in the Scottish setting. It has been recommended that AZA-6 should also be included in the regulated AZA congeners (Kilcoyne et al., 2014), it is unclear what contribution this makes to the total AZA concentrations of shellfish in the UK, as analysis to date has focused on the regulated congeners AZA-1, -2 and -3 (Turner et al., 2014a).

2.3.2 DSP profile

As part of the CEFAS review of lipophilic toxin data, it was determined that 1524 shellfish samples from Scotland between 2011 and December 2013 had DSP levels above the LoQ (Turner et al., 2014a). On average, OA toxins were dominant over DTX-1 and DTX-2, with most toxicity attributed to OA-esters, rather than free OA. On average, OA esters were the dominant form present in all species, with only low proportions of both free and esterified DTX-1 and DTX-2 present.

The authors also undertook a cluster analysis and shellfish samples were found to fall into three distinct profile groupings:

- Profile group 1 contained OA exclusively with equal proportions of OA and esterified OA;

- Profile group 2 contained OA esters almost exclusively; and
- Profile group 3 contained low free OA, with free DTX-2 dominating and lower near equal proportions of OA and DTX-2 esters.

Overall the authors noted that "there was clear indications of significant differences in OA-group profiles between shellfish samples of different species and in shellfish sampled from different geographical regions" (Turner et al., 2014a).

While OA esters dominated the toxin profiles of most samples of Scottish shellfish, some samples were dominated by free DTX-2 (profile group 3). These findings highlight the need for assays to be able to identify the presence of DTXs in addition to OA, and also the need for incorporation of the hydrolysis step which converts the ester compounds to their parent molecules and enables detection in commonly used methods.

In the review undertaken by CEFAS, PTXs were identified (at levels >16 µg/kg) in only 16 OC samples during the period July 2011 to December 2013 (out of a total of 7127 in shore samples analysed). In each of the samples the only congener detected was PTX-2; PTX-1 and PTX-11 were not detectable. PTX-1 and PTX-2 are currently regulated in shellfish but the relevance of these toxins to human health is under review and they are considered of lower priority for the purposes of this review.

2.3.3 PSP profile

PSPs have been documented to occur in a variety of bivalve molluscan shellfish in the UK, including clams, cockles, mussels, oysters, razors and scallops (McLeod, 2014; Turner et al., 2014b).

A recent review of PSP toxin profiles in UK shellfish noted that there is "some evidence for species related differences" (Turner et al., 2014b) in toxin profiles, specifically:

- Oysters (n=3) were found to be rich in GTX-2,3 in comparison to cockles (n=4);
- Proportions of GTX-1,4, NEO and dcGTX-2,3 in cockles were higher than those in mussels;
- Razor clams showed higher percentages of STX than mean values for mussels;
- Surf clams only contained quantifiable levels of the decarbamoyl toxins dcSTX, dcNEO and dcGTX-2,3.

The authors also undertook a cluster analysis and UK shellfish samples collected over five years were found to fall into four distinct profile groupings:

- Profile 1 group contains GTX-2,3 as the major toxin, with a smaller proportion of STX;
- Profile 2 group contains GTX-1,4 as the major toxin, with smaller proportions of STX, GTX-2,3, C-1,2 and NEO;
- Profile 3 group STX and GTX-2,3; and
- Profile 4 group contained only the decarbamoyl toxins.

The first two groups were found to have profiles very similar to UK strains of *A. minutum* and *A. fundyense* respectively. Profile group 4 was found only in PSP positive surf clams, which are known to produce decarbamoyl toxins through metabolic transformation processes. Profile group 3 was suggested to result from a mix of Profile 1 and Profile 2 inputs. Shellfish samples exhibiting the different toxin profile groupings were localised to different regions of Great Britain:

- PSP positive shellfish with toxin profiles matching group 1 have only been found in south west England and Wales;
- Shellfish with the characteristics of profile 2 (most common profile in GB) have been detected from a range of regions around Scotland and north east England;

- Profile 3 samples have occurred in south west England and west Scotland; and
- Profile 4 is only associated with surf clams from the Forth estuary.

2.3.4 Yessotoxin profile

As part of the review of lipophilic toxin data commissioned by the FSAS in 2014, 224 samples were found to contain YTXs above the method LoQ (0.1 mg/kg); 204 of these samples were mussels with the remainder comprising scallops. All samples were found to contain the parent toxin YTX and 37 samples also contained 45OH-YTX, comprising on average 37 ± 8% of the total toxin loading (Turner et al., 2014a). YTX and 45OH-YTX are currently regulated in shellfish but the relevance of these toxins to human health is under review and they are considered of lower priority for the purposes of this review.

2.4 Official control programme

Regulations (EC) 854/2004 and (EC) 853/2004 specify that shellfish production areas must be periodically monitored to check for the presence of biotoxins in live bivalve molluscs. Table 2.3 shows the MPLs for each regulated toxin group and the toxin congeners that are to be detected in the EU, including the UK.

Table 2.3: Regulated marine toxins and the maximum permissible limit applied in the European Union (current at January 2015)

Marine toxin group	Maximum permissible level	Toxin congeners to be detected
Azaspiracids (AZA)	160 µg/kg	AZA-1, AZA-2, AZA-3
Amnesic shellfish poisons (ASP)	20 mg/kg	Domoic acid
Diarrhetic shellfish poisons (DSP)	160 µg/kg	Okadaic acid, dinophysistoxins (including DTX-1, DTX-2 and DTX-3, including their esters) Pectenotoxins-1 and -2
Paralytic shellfish poisons (PSP)	800 µg/kg	Saxitoxin and any of its analogues for which analytical standards are available
Yessotoxin group (YTX)[4]	3.75 mg/kg	YTX, 45OH-YTX, homo-YTX, and 45OH-homo-YTX

The legislation (Regulation (EC) 854/2004) also requires that EU member states must develop sampling plans for marine biotoxins in shellfish[5] and for toxic phytoplankton and that OC checks are undertaken at regular intervals. Regarding implementing sampling plans, the legislation specifies the following regarding the position of sampling sites and monitoring frequency:

"the geographical distribution of the sampling points and the sampling frequency must ensure that the results of the analysis are as representative as possible for the area considered."

"the sampling frequency for toxin analysis in the molluscs is, as a general rule, to be weekly during the periods at which harvesting is allowed. This frequency may be reduced in specific areas, or for

[4]Regulation (EC) 786/2013 revised the maximum permissible level for yessotoxin from 1 mg/kg to the current limit of 3.75 mg/kg in 2013.

[5]Regulation EC 854/2004 applies to live bivalve molluscs, live tunicates, live echinoderms and live marine gastropods.

specific types of molluscs, if a risk assessment on toxins or phytoplankton occurrence suggests a very low risk of toxic episodes".

The FSS commission risk assessments on a regular basis to support decision-making regarding the monitoring frequency of marine toxins in bivalve shellfish for each production area. Generally during times of heightened toxin risk, the monitoring frequency is weekly. During low risk periods and for toxins that occur rarely, monitoring frequency may be reduced to fortnightly or monthly. Toxin monitoring results generated through the OC programme in the UK are used to support decisions regarding the closure and re-opening of production areas, and elevated results may also instigate further sampling and testing.

The presence of certain phytoplankton in seawater can provide forewarning of toxicity in shellfish, therefore most countries have developed alert levels in seawater (in cells/L) for a series of toxin-producing phytoplankton species. For the UK, the alert levels used for phytoplankton in seawater as part of the OC programme are shown in Table 2.4.

Table 2.4: Phytoplankton alert levels used for Official Control purposes in the United Kingdom. Current at January 2015.

Toxin group	Phytoplankton species	Phytoplankton alert level (cells/litre)		
		England and Wales	Northern Ireland	Scotland
PSP	*Alexandrium* spp.	Presence*	Presence*	40
DSP	*Dinophysis* spp.	100	100	100
	Prorocentrum lima	100	100	100
ASP	*Pseudo-nitzschia* spp.	150,000	150,000	50,000

*Limit of Detection = 40 cells/L

The EU legislation specifies that any changes in the phytoplankton population that could lead to toxin accumulation should result in an increase in mollusc sampling frequency, or precautionary closures of production areas. Consistent with this, in the UK, plankton-monitoring results which exceed the alert levels may be used to increase the frequency of toxin monitoring (particularly for areas in which toxin monitoring is undertaken at a reduced frequency), or trigger precautionary closures or end product testing by industry. Elevated plankton counts can also be used by the FSA and FSS to advise local authorities (LAs) to close areas where toxin monitoring in shellfish flesh has not been undertaken.

2.5 Management of toxin risk by food business operators

Regulation (EC) 853/2004 stipulates that food business operators (FBOs) must ensure that live bivalve molluscs placed on the market for human consumption do not contain marine biotoxins in concentrations that exceed those detailed in Table 2.3. Additionally, Regulation (EC) 852/2004 (Chapter II, Article 5) requires that FBOs implement a HACCP system, which includes the need to establish and implement effective monitoring procedures for major hazards of concern.

While the OC programme involves regular shellfish sampling and testing to support closure and re-opening of shellfish production areas, the programme is not intended to determine the final marine toxin status of products placed on the market (the FSA note in their recent guidance that the OC programme "is not designed to provide confirmation of the health status of the final product placed on the market – this is the legal responsibility of the food business operator").

To assist the industry to meet the requirements of Regulations (EC) 853/2004 and 852/2004, in April

2014 the FSAS issued new guidance on how FBOs can assess biotoxin risk in their products and implement a management strategy to minimise consumer risk. The new guidance can be viewed at: http://www.foodstandards.gov.scot/managing-shellfish-toxin-risks-harvesters-and-processors.

The guidance includes the use of a risk matrix, also known as the 'traffic light' tool, which incorporates a series of phytoplankton and shellfish flesh trigger levels for each of the regulated toxin groups. When toxin or plankton concentrations in OC samples, or in industry-collected samples, exceed the trigger levels a range of different risk management actions are proposed. Two different toxin trigger levels are set for each toxin group: amber and red triggers. Phytoplankton trigger levels are also incorporated into the new guidance. Table 2.5 provides a summary of the toxin and plankton trigger levels.

Table 2.5: Current phytoplankton and toxin levels used to trigger the commencement of end product testing and/or harvesting restrictions, and the associated maximum permitted levels in shellfish flesh

	AZA[a]	ASP	DSP	PSP	YTX[a]
Plankton trigger	None set	150,000 cells/litre	100 cells/litre	40 cells/litre	None set
Toxin trigger (amber)	≥ 80 µg/kg	> Limit of Quantitation	≥ 80 µg/kg	> Reporting Limit	≥ 1.8 mg/kg
Toxin trigger (red)	≥ 160 µg/kg	≥ 10 mg/kg	≥ 160 µg/kg	≥ 400 µg/kg	≥ 3.75 mg/kg
Maximum permissible level	160 µg/kg	20 mg/kg	160 µg/kg	800 µg/kg	3.75 mg/kg

[a] *A. spinosum* (AZA producer) is very difficult to identify in routine analysis of seawater samples due to its small size, therefore there is no trigger level set for this phytoplankton species. For YTX, the OC programme includes monitoring for two YTX producing plankton species, *P. reticulatum* and *L. polyedrum*, however the FSAS note in their guidance that there is 'no recognised trigger level' for these species.

The risk management actions that are suggested if trigger levels are exceeded include heightened EPT and suspension of harvesting. For example:

- If toxin concentrations exceed the amber trigger levels, or plankton samples exceed triggers, it is suggested that harvesting may continue with an increased EPT regime applied to harvested products, or so called 'positive release' which requires batches of shellfish to be tested for compliance.
- If toxin concentrations exceed the red trigger levels it is proposed that harvesting should be suspended.

This review has identified that around 35 FBOs in the UK are currently implementing the FSAS guidance and using plankton and toxin trigger levels to increase the testing undertaken. The use of field methods by FBOs to support decisions on harvesting and marketing of shellfish means that it is very important that the results of such tests are accurate. A false positive test may result in unnecessary destruction of shellfish if products are withdrawn from the market and destroyed based on the result. This can cause large economic losses to the FBOs due to the costs of harvesting and the lost product value. On the other hand, a false negative could result in product containing an excessive amount of marine toxins reaching the market place and being consumed, thus placing consumers at heightened risk of intoxication. Given these implications it is imperative that shellfish testing does not return false negative or false positive results. This has particular implications for field test kits that are generally qualitative or act as screening methods and thus provide less accurate data than quantitative analytical methods used in an accredited laboratory.

2.6 European requirements for marine toxin methods

The EC specifies analytical methods that should be used for marine toxin analysis by competent authorities and where appropriate FBOs. The methods are specified in Regulation (EC) 2074/2005, Regulation (EC) 1664/2006 and Regulation (EU) 15/2011:

- For PSP toxins, the mouse bioassay (MBA) method is the reference method. An instrumental method based on HPLC (AOAC method 2005.06, also known as 'the Lawrence method') may be considered as an alternate method.
- For ASP toxins, an HPLC method is the reference method. The Biosense ELISA (AOAC official method 2006.02) may be considered an alternative method (Regulation (EC) 1244/2007).
- For lipophilic toxins, including OA group toxins, YTX group toxins, PTX group toxins and AZAs, an LC-MS/MS method that was validated by the European Union Reference Laboratory (EU RL) is the reference method. The Zeulab protein phosphatase assay (Okatest) may be considered a supplementary method for the OA group toxins.

The regulations also provide guidance on the use of alternative methodologies to those specified in the legislation. For PSP, the regulation states that "any other internationally recognised method" may be used. For lipophilic toxins, the regulation states that other methods such as immunoassays and functional assays may be used as alternatives provided that:

- They can detect the required congeners (Table 2.3);
- They fulfil the method performance criteria stipulated by the EU RL;
- Intra-lab validation studies have been performed;
- Proficiency studies have been undertaken; and
- That they provide equivalent public health protection.

The EU RL have advised that alternative methods would be expected to be inter-laboratory validated in accordance with IUPAC (International Union of Pure and Applied Chemistry) and AOAC method validation guidelines, and be involved in proficiency testing programmes.

2.7 Method validation and quality assurance

2.7.1 Valid analytical measurement (VAM)

Valid Analytical Measurement (VAM) was a programme of work sponsored by the Department of Trade and Industry as part of the UK's National Measurement System. VAM articulated a set of six principles under which laboratories should operate:

1. Analytical measurements should be made to satisfy an agreed requirement.
2. Analytical measurements should be made using methods and equipment, which have been tested to ensure they are fit for their purpose.
3. Analytical measurements made in one location should be consistent with those elsewhere.
4. Staff making analytical measurements should be both qualified and competent to undertake the task.
5. Organisations making analytical measurements should have well defined quality control and quality assurance procedures.
6. There should be a regular independent assessment of the technical performance of the laboratory.

These principles provide a good basis for assessing not only the available tests that might be suitable for testing of molluscan bivalve shellfish but also the context and laboratory environment in which they might be used.

Principle 1, as defined in the Terms of Reference and the recent EPT guidance issued by FSAS (FSA, 2014), is to assess whether shellfish meet regulatory limits for biotoxins. It is intended that toxin results generated by industry in field laboratories using quantitative or qualitative screening methods may be used to decide whether a given batch of shellfish could be marketed, and also to support decisions on voluntary closure and opening of shellfish growing areas.

Principle 2 covering Fitness for Purpose of test methods forms the main focus of this section. Formal method validations are a key component of assessing new methods. Eurachem (2014) has recently revised their comprehensive guide that expands on the principles recommended by IUPAC e.g. Thompson et al. (2002) and are now broadly accepted internationally (including by the EU RL and Codex Alimentarius). Adequate validation, including some inter-laboratory study, is a prerequisite for accepting a test method as suitable for regulatory use and, for the purposes of this review, would generally include some data from testing of shellfish matrices. In some cases a test may appear very suitable (technical, cost, ease of use) but further validation may be required before it could be recommended for routine use on shellfish samples.

Principles 3 to 6 covering quality control (QC) and quality assurance (QA) are equally important to Item 2 for obtaining valid test results in a laboratory on a consistent, demonstrable basis. Methods with documented high performance, however defined, may not yield satisfactory results for particular samples if there are any issues with items 3 to 6 in the laboratory conducting the testing. To ensure appropriate QA and QC procedures are in place, it is expected that laboratories undertaking OC testing for marine toxins meet the requirements specified in ISO/IEC 17025:2005, and have been accredited by the UK Accreditation Service for this testing. This review is not intended to cover the complex issues surrounding accreditation and QA/QC procedures for analytical laboratories, however some commentary is provided on basic requirements that would be expected to be implemented in a field laboratory (Section 9).

2.7.2 Single laboratory validation (SLV)

A single laboratory validation (SLV) typically follows method development and optimisation as an 'in-house' formal step to assess the accuracy and the repeatability precision of a new method. Information is generally also obtained on calibration (available standards; calibration function), limit of detection (LOD), limit of quantitation (LOQ), specificity and ruggedness. The types of experiments conducted in validation protocols have become relatively standardised e.g. Eurachem (2014) and are applicable across most types of quantitative methods (instrumental; immunoassays; functional assays). However, the details often depend on the specifics of the method and the matrices to which it is applied. Validations of instrumental methods generally put an emphasis on recovery data for fortified blank samples. Validations of commercial ELISA kits may emphasise repeatability and detection limits for dilute standards in buffer, assuming that matrix effects will have been reduced by the extraction/clean up procedure and high levels of dilution. Ruggedness testing is also important for methods that might be used in field laboratories or by less skilled operators e.g. sensitivity of results to changes in volumes of reagents, assay temperature etc. Comparability of results to those from other methods and assessing accuracy through use of certified reference materials are also desirable for SLVs. The EU RL note (Personal Communication, Ana Gago Martinez, December 2014) that SLV is not sufficient for methods that are to be used within the EU to support shellfish safety decisions and that inter-laboratory validation studies are required.

2.7.3 Inter-laboratory study (ILS; Collaborative Study)

The key parameter is the reproducibility precision assessed from the data obtained for sub-samples of the same samples across a number of participating laboratories AOAC (2005); CEN (2013). They also can augment SLV data in other areas such as accuracy, ruggedness and calibration function. These studies provide robust data on the performance to be expected for a method in general use. It is important to recognise that this performance generally will be inferior to that predicted from SLV alone. The precision data from ILS is a key component of the overall uncertainty of measurement for a method (Eurachem/CITAC, 2012). Large ILSs are expensive to conduct but are a requirement for methods that will be widely used for regulatory purposes, particularly internationally. AOAC has formalised protocols for collaborative study of quantitative methods (AOAC, 2005) with the following minimum criteria based on the statistical requirements to obtain robust precision data:

- At least five materials e.g. shellfish samples containing relevant toxins at different levels;
- At least eight laboratories reporting valid data for each material;
- Replication using blind duplicate samples or split levels (Youden pairs).

Very few methods applicable to biotoxins in shellfish matrices have received ILS that meet these AOAC criteria for collaborative study. However, more limited ILS data is always valuable.

2.7.4 Qualitative analyses

The requirements for, and information from, SLV and ILS outlined above are strictly only applicable to methods that provide quantitative data for an analyte, or groups of analytes. Qualitative methods effectively give only a Yes/No answer at a given decision concentration and therefore are used primarily for screening purposes (Eurachem, 2014). The Scotia Rapid (formerly Jellet) test strips, e.g. for PSP, are an important example in the biotoxin field. It is also important to recognise that ELISA tests for PSP, e.g. RidaScreen, are also only screening tests despite their ability to produce a calibration curve for STX and output number values for samples - reasons for this are discussed further in Section 4.2.

Validation of screening methods is best-conducted using samples that have been fully characterised by a suitable quantitative method e.g. the Lawrence LC-FLD method for PSP. Otherwise fortified and blank samples can be used. AOAC have set minimum criteria for collaborative study of qualitative analyses:

- Ten laboratories reporting on two analyte levels per matrix;
- six test samples per level; and
- six negative controls per matrix (AOAC, 2005).

The key parameters for qualitative methods are the rates of false positive and false negative result around a decision concentration or cut-off limit as determined from testing of samples:

- Diagnostic sensitivity = No. true positive by test / Total no. samples above cut-off
- Diagnostic specificity = No. true negative by test / Total no. samples below cut-off

The limit of detection (LOD) for a qualitative method can be determined by testing low levels of fortified blanks until a concentration is reached where replicate samples test say 25% negative, 75% positive. A cut-off limit for that method will be set above the LOD at a concentration where the false negative rate reaches a stated low probability. The selection of the levels of analyte for the SLV and ILS are obviously crucial to obtaining a good estimate of the LOD and establishing a cut-off for the method.

2.7.5 Acceptable performance criteria for screening methods

Screening methods are increasingly used globally for both regulatory and industry based management of marine toxins in shellfish. Due to this increase in usage, in 2012 the Codex Committee on Fish and Fishery Products (CCFFP) developed performance criteria for screening methods that were to be used by competent authorities to select methods that are adequate to support routine toxin monitoring programmes. Unfortunately international agreement on the criteria could not be reached and the committee discontinued their development. The draft criteria include the following requirements:

- Cross reactivity to the toxin congeners should be investigated and well understood;
- Sensitivity to all relevant congeners should be known;
- Blank matrix fortified with other toxins that could possibly be found in samples should be tested to establish negative response;
- Preference given to methods that have undergone ILS;
- False negative rates should be less than 5% at a level equating to half the maximum allowable level, and no false negatives at the maximum level;
- The detection limit should enable detection of biotoxins at half the maximum level.

In addition to these draft criteria, Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results is relevant. This Decision primarily pertains to the presence of residues in products of animal origin, however the principles are also applicable to marine toxins in seafood. The Decision includes the following performance criteria for screening methods:

"Only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant [=false negative] rate of 5% (β-error) at the level of interest shall be used for screening purposes"

Both Commission Decision 2002/657/EC and the draft CCFFP performance criteria stipulate that samples which give positive screen test results should be confirmed by a confirmatory method.

In the absence of specific performance criteria for screening methods for marine toxins in shellfish, the above criteria may be appropriate for the assessment of validity of qualitative and semi-quantitative screening methods.

SECTION THREE: METHODOLOGY FOR THE REVIEW

3.1 Literature review

A literature search was undertaken to provide background information for the review and develop a summary of the types of field methods under development and currently being used in the broader toxin and contaminant food analysis fields. Literature searches were undertaken to collate information on:

- Field methods developed for the regulated marine toxins (DSP, PTX, PSP, ASP, YTX and AZAs); and
- Field methods used for the analysis of chemical contaminants.

Literature searches began with a structured electronic search using the Google Scholar and PubMed search engines. Electronic literature searches commenced with the following key words:

- Shellfish AND Cd, Pb, Hg, PAH, dioxin, PCB AND method;
- Shellfish AND PSP, DSP, ASP, YTX, AZA AND method; and
- Shellfish AND saxitoxin, okadaic acid, domoic acid, pectenotoxin, yessotoxin, azaspiracid AND method.

Papers that were identified through electronic searching were assessed for relevance by initially reviewing the abstracts. Additional papers were accessed using the reference list of reviewed publications, and through searches that used slight variations on the above keywords. Marine toxin experts involved in method development activities provided further publications. Unpublished reports relating to the detection of toxins, contaminants and phytoplankton within Scotland were sourced by enquiry from the FSAS, including a report regarding the toxin profiles of shellfish in the UK (Turner et al., 2014a).

3.2 Evaluation of field test methods

3.2.1 Identification of field methods

An internet search was undertaken in conjunction with the literature review (Section 3.1) to identify field methodologies (see definition in glossary) that can be readily purchased as commercially available 'kits' by FBOs. Only methods that were reasonably portable (all reagents and materials contained within a simple kit[6]) and did not require expensive instrumentation (other than simple plate readers etc) were considered. The assay types identified as field methods through this process included ELISAs and lateral flow immunoassays (LFA) for ASP, DSP and PSP, and the protein phosphatase inhibition assay (PPIA) for DSP (Section 5). Methods in which reagents and materials need to be sourced individually, required some in-house optimisation, and/or non-portable or expensive instrumentation, were not considered to be 'field methods'. Methods, which were noted by the kit suppliers, to be for 'research purposes only' were not included in the evaluation.

3.2.2 Classification of field methods

The validation approach for quantitative, qualitative, confirmation and screening methods differs. Therefore, field methods identified were classified as quantitative, qualitative, confirmatory or

[6] With the exception of reagents that can be readily purchased or sourced, such as water.

screening methods based on the definitions described in Commission Decision 2002/657/EC, as follows:

Quantitative method: a method that determines the amount of a substance so that it may be expressed as a numerical value of appropriate units.

Qualitative method: a method that identifies a substance on the basis of its chemical, biological and physical properties.

Confirmatory method: methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest.

Screening method: methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false negative results.

Using these definitions, the field methods identified were assessed as follows:

1. LFAs were considered to be qualitative screening methods, because they produce a yes/no result for a particular toxin group, and do not unequivocally identify each congener of regulatory relevance (they are immunological assays that provide an overall response to a group of structurally related toxin congeners).
2. ELISAs could be quantitative methods (ASPs) or screening methods (PSP, DSP and PAHs). Although they produce a numerical result, they do not unequivocally measure levels of each congener of regulatory relevance. In the case of ASP, DA is the only relevant toxin and thus ELISAs can be considered quantitative.
3. The PPIA for DSP was considered to be a quantitative method, because it is a functional assay (based on the inhibition of the PP enzyme) that provides a single integrated response to the DSP toxin congeners present with no interferences from other toxins. The assay produces a numerical result that can be directly compared to the regulatory limit for DSP.

3.2.3 Evaluation of field method performance

Commission Decision 2002/657/EC specifies which performance parameters should be assessed for quantitative, qualitative, screening and confirmatory methods. Considering Commission Decision 2002/657/EC, and the minimum validation criteria for screening methods set internationally (AOAC, 2005; Codex, 2012; Eurachem, 2014), the following factors were evaluated (definitions of the factors are contained in the glossary):

1. **Considerations regarding detection capability (CCβ) and decision limit (CCα):**
   a. Is the limit of detection or cut-off value (value at which the test gives a reliably positive response) at or below the MPL?
   b. What is the false negative rate at or above the MPL?
   c. Is the decision limit suitable i.e. is the false positive rate acceptable for samples with toxin levels at half the MPL?
   d. Have false negative and false positive rates been explored using UK shellfish species containing a variety of different toxin mixtures?
2. **Considerations regarding specificity:**
   a. Does the test produce a positive response to each congener of regulatory concern?
   b. Are truly blank shellfish reliably negative?

c. Does the method produce a negative response in the presence of unrelated toxins/compounds that may be expected to commonly occur?

3. **Ruggedness studies:**
   a. Have factors that could influence the results been identified (e.g. different analysts, different storage temperatures for kit reagents, varying room temperatures at time of analysis, rate of heating)?
   b. Have studies been undertaken in which the ruggedness factors identified have been varied?

4. **Precision (mainly for quantitative methods e.g. PPIA and ELISA):**
   a. Has within laboratory repeatability (RSD_r) been determined, if 'yes', what is the RSD_r?
   b. Has between laboratory reproducibility (RSD_R) been determined? If 'yes':
      i. What is the RSD_R?
      ii. Did the inter-laboratory study (ILS) conform with AOAC requirements (five materials, eight laboratories)?

The evaluation criteria noted above were discussed and agreed with the FSS as part of the evaluation process. To ensure inclusion of all available data in the review, suppliers of field test kits were contacted via email to request access to method validation data. Similarly, scientific experts involved in the EU RL network were contacted to seek data regarding method validation of field kits. Some validation data on the use of test kits was obtained through reference to a series of publications.

3.2.4 Food business operators views on field methods

Food business operators in the UK were contacted to gather information regarding their views and real life experiences with the use of toxin field kits. Initially the FSA, FSS and Local Authorities in Scotland were contacted to determine which FBOs perform EPT using field kits. The Shellfish Association of Great Britain (SAGB) was also contacted to determine if FBOs in England and Wales also use field kits for toxin testing. Each FBO identified was then contacted by phone and email and a series of questions regarding the use of the kits were posed (Appendix One). The FBOs included producers, catchers and processors.

SECTION FOUR: OVERVIEW OF MARINE TOXIN DETECTION METHODS

Assays for marine toxins must be designed to detect a variety of toxins, because for each toxin group there may be a range of different congeners present that contribute to the overall toxicity. The most important test methods currently used for marine toxins can be classified into three broad groups: functional assays, immunological assays and instrumental analytical methods. An overview of each method type is provided below, along with examples of specific tests developed, the advantages and disadvantages of each assay type, and their applicability for field monitoring of toxins in shellfish. A summary of the emerging development of biosensors for marine toxins is also provided.

4.1 Functional assays

When humans consume substances such as marine toxins, the toxins interact specifically with cellular assemblies (receptors) that recognise the toxin structure. The interaction of the toxin with such receptors can trigger a cascade of cellular effects, which often culminates in the illness symptoms that are observed when significant amounts of toxin are consumed. Functional assays measure the response of receptors when challenged with toxins, therefore the single response from a functional assay is directly related to the total toxicity of the sample (to the extent that toxin binding to the receptor mimics mammalian toxicity).

A variety of different functional assays have been developed for marine toxins that are based on the molecular toxin targets, some are cell based, others cell free and they use a range of detection strategies including colorimetric, fluorimetric and scintillation methodologies (reviewed in Hess et al. (2006), Botana et al. (2009); Campbell et al. (2011c); Rossini (2005)). Functional assays have been designed for most of the regulated toxin groups, however the mode of action of AZAs is not well understood and thus functional methods have not yet been developed for this toxin group.

4.1.1 Functional assays for ASP toxins

A receptor assay was developed for DA that uses rat glutamate receptors that have been cloned and are expressed in insect cells (DA is an analogue of glutamate). The assay is a competitive binding method in which tritiated (^{3}H) kainic acid competes with DA (present in standards or samples) for binding to the glutamate receptors. Following incubation the microplates are placed in a scintillation counter for quantification. The assay was reported to have an interassay variability of <10% and results agreed well with those obtained from an HPLC method (Van Dolah et al., 1997). The assay doesn’t appear to have been adopted as a regulatory method, possibly due to the ease with which DA can be quantified using instrumental methods (facilitated by the high regulatory limit in shellfish of 20 mg/kg). This method would not be suitable for field use due to the use of radioisotopes and the need for a scintillation counter.

4.1.2 Functional assays for DSP toxins

Okadaic acid has been shown to have an inhibitory effect on serine-threonine phosphoprotein phosphatases (PP), particularly PP1 and PP2A through binding to a site on these enzymes. The inhibition of PP by OA has been suggested to be responsible for the symptoms of DSP in humans, although a recent review notes that there is no *in vivo* evidence to support this (Munday, 2013). Quantitation of OA is based on the decrease of activity of catalytic subunits of PP2A in the presence of OA and incorporates either colorimetric (Honkanen et al., 1996; Ramstad et al., 2001; Simon and Vernoux, 1994; Tubaro et al., 1996) or fluorimetric detection (Mountfort et al., 1999; Mountfort et

al., 2001; Vieytes et al., 1997). The assays are capable of detecting OA, DTX-1 and DTX-2 and, if a hydrolysis step is performed, they can also detect the esters of the parent compounds (DTX-3) (Mountfort et al., 2001). An ILS for the fluorimetric method indicates that the method is robust (González et al., 2002), however it was only limited to three laboratories. Recently, SLV and ILS were also undertaken on the colorimetric based PPIA (Smienk et al., 2013; Smienk et al., 2012), which has been commercialised by Zeulab and is accepted as a supplementary method for OA group toxins in the EU. Further discussion on the technical performance of this method is included in Section 5.3.

A cytotoxicity assay has also been developed for the quantification of DSP toxins, which is based on the disruption of F-actin when neuroblastoma cells are exposed to OA and DTX toxins (Leira et al., 2003). While the assay was rapid and gave similar results to an instrumental method, MBA and the PPIA, the reported LOD of 200 – 1000 µg/kg was higher than the current regulatory limit for DSP (160 µg/kg). Although improvements might be made through refining the extraction procedures this assay has not been commercialised.

4.1.3 Functional assays for PSP toxins

The PSP toxins bind to voltage gated sodium channels, these channels conduct sodium ions through the cell plasma membrane. The binding of PSP toxins to the channels blocks the inward flow of sodium to the cell, ultimately preventing nerve transmission impulses and accounting for the reported paralytic effects of PSPs in humans e.g. muscular paralysis, respiratory distress. The MBA for PSPs was developed in the 1920s in the USA and was first adopted as a reference method in 1959. The MBA (AOAC 959.08) involves using acidic extracts injected parenterally. It is still widely used for shellfish safety testing and is currently the EU reference method.

A receptor binding assay (RBA) was developed in the early 1990s (Vieytes et al., 1993) and further optimised and extensively validated in recent years (Doucette et al., 1997; Van Dolah et al., 2012; Van Dolah et al., 2009). This assay uses sodium channels that are isolated from rat brain membranes and coated on to microtitre plates. Added tritiated saxitoxin (^{3}H-saxitoxin) and PSP toxins contained within sample extracts (or standards) effectively compete for binding to the sodium channels. Following filtration the amount of ^{3}H-saxitoxin not bound to the receptors is measured using an automated scintillation counter (microplate or traditional glass vial). A collaborative ILS demonstrated that the method has acceptable reproducibility (RSD_R) and repeatability (RSD_r) values, which were 33.1% and 25.1% respectively. The assay also performed well in comparison with the MBA (correlation coefficient (r^2) of 0.84) and HPLC (r^2 of 0.92) (Van Dolah et al., 2012). This method has been accepted as an official AOAC method of analysis (Method 2011.27). While the assay offers a robust alternative to the MBA or HPLC methods, and has the advantage of a single response that is proportional to the overall PSP toxicity of the sample, few laboratories have adopted this method for routine use. This may be related to the issues of reliable sourcing for ^{3}H-saxitoxin and managing the use of radioisotopes. Given the need for careful control of radioisotopes, this assay is unsuitable for use in the field or by poorly-trained personal.

Several cytotoxicity assays for PSPs have been developed that measure survival of cells potentiated with ouabain/veratridine[7] (Jellett et al., 1992), or change in the membrane potential of veratridine treated cells (Louzao et al., 2001; Nicholson et al., 2002). In the validation work undertaken, a good correlation was demonstrated between the membrane potential assay and MBA, HPLC and solid phase radioreceptor assays (Louzao et al., 2001; Nicholson et al., 2002). The cell survival and membrane potential assays showed acceptable LOQ, in the range of 0.2 – 20 µg/100 g (compared to

[7]Veratridine transiently opens the sodium channels and ouabain inhibits the sodium pump responsible for removing sodium from inside the cell, this leads to cell swelling and eventual lysis. In the presence of PSP toxins, which block sodium channels, the action of veratridine and ouabain is inhibited and the cells maintain a normal appearance.

the MPL of 80 µg/100 g) and allow for medium to high sample throughput (Jellett et al., 1992; Louzao et al., 2001; Nicholson et al., 2002). An ILS was undertaken for the cell survival assay in 1999 and apparently did not give satisfactory results (reviewed in EFSA (2009c). Neither assay type has been commercialised.

Assays that exploit the effect of PSP toxins on sodium channels are also reported to detect tetrodotoxin (TTX) and its congeners (noted in (Doucette et al., 2000); Llewellyn et al. (1998). TTXs are not structurally related to PSP toxins, however they act in the same fashion and also bind to the sodium channels. The presence of TTXs in bivalves is not currently under regulatory control in the EU, presumably due to a lack of evidence of contamination of bivalves with TTX. However, of interest TTX has recently been detected in bivalve molluscs (oysters and mussels) from the English channel (Turner et al., 2015a).

Another receptor has also been used to develop a functional based assay for PSP toxins. Saxiphilin, which is found in the circulatory fluids of vertebrates and invertebrates, is reported to be specific to PSP toxins and does not bind TTX (Llewellyn et al., 1998). A radioligand binding assay utilising saxiphilin was developed to detect PSP toxins in blue-green algae and bivalves. The assay was shown to be capable of detecting several PSP congeners (Llewellyn et al., 1998). It has not yet been subjected to formal SLV or ILS and still requires the use of radioisotopes which poses a significant barrier to field usage.

4.1.4 Functional assays for Yessotoxins

Several different methods have been developed for the detection of YTX, which may be related to its mode of action (recognising that YTXs have not yet been reported to cause illness in humans). Several assays are based on the interaction of YTX with phosphodiesterases (PDE), which act on cyclic AMP, and other methods that exploit the fragmentation of E-cadherin when cells are exposed to YTX have also been developed.

Alfonso et al. (2004) reported a fast microplate assay involving a fluorescent derivative of cAMP, anthranyloyl-cAMP. YTXs cause an increase in the activity of PDEs, which subsequently hydrolyse anthranyloyl-cAMP, this causes a decrease in fluorescence which is able to be quantified using a plate reader. Pazos et al. (2004) published an optical biosensor method in which a specific PDE was immobilised on the sensor surface, YTX is then added (in samples or standards), and changes in the refractive index associated with the binding and release of YTX to the PDE are then monitored. A surface plasmon resonance sensor has been recently reported that also exploits the interaction between PDEs and YTXs (Fonfria et al., 2008).

Pierotti et al. (2003) reported an assay involving the treatment of MCF-7 cells with YTX and associated observed increases in a 100 kDa fragment of E-cadherin which are measured using immunoblotting detection methods. The authors noted that the assay was time consuming requiring around two working days. A subsequent method was thus developed which involved using two antibodies that recognise E-cadherin and a refined so called ‘slot blot’ procedure, this reportedly shortened analysis time to around seven hours. While the method was reported to be accurate, the authors noted that it was variable with large standard deviations (Pierotti et al., 2007). It has been noted that the E-cadherin method is not specific for YTX and produces a positive response to AZAs (reviewed in EFSA (2008c).

4.1.5 Suitability as field methods

While functional assays have the major advantage of giving a single response that corresponds to the toxicity of the sample, most of the methods rely on the use of cells, radiolabelled isotopes, and/or require the maintenance of receptor preparations. These activities require laboratories with specialised facilities and means that most functional assays are not amenable as field methods or for use by untrained personal. One exception is the Zeulab PPIA for OA-group toxins (the Okatest), which is available in a kit format. While the PPIA is suitable as a field method it is likely that significant training would be required for non-skilled operators. The performance of the PPIA as a field method is evaluated in 5.3.

Table 4.1 provides an overview of the advantages and disadvantages of functional assays. The advantages and disadvantages of functional assays are discussed in further detail in the recent EFSA biotoxin opinions (EFSA, 2008a, 2008c, 2009a, 2009b, 2009c, 2010)

Table 4.1: Summary of the advantages and disadvantages of the major assay types for marine toxin analysis

Assay type	Advantages	Disadvantages	Suitability for field testing
Functional assays	Quick to perform (1-2 hrs)[a]	No information on toxin profile	No
	High throughput	Toxin identification not unequivocal	
	Per sample cost relatively low	Preparation and maintenance of receptors labour intensive	
	Response proportional to toxic potency of toxins	Need for specialised facilities (e.g. radioisotope use)	
	Quantitative results	Need for higher cost equipment	
Immunological assays	Quick to perform (20 mins – 2 hrs)[a]	No information on toxin profile	Yes
	Per sample cost relatively low	Cross reactivity issues may lead to false positives and false negatives	
	Equipment needs are minimal for LFAs	ELISAs require plate readers (expense)	
	QA/QC relatively simple	Concentrations detected may not reflect toxicity for complex toxin groups	
	Simple extraction and detection methods		
	Generally qualitative or semi-quantitative results		
Analytical methods	Quick to perform (5 – 10 mins)[a]	Need toxin standards for each congener of regulatory concern	No
	High throughput	Toxic equivalence factors may be inaccurate for some toxins (i.e. PSP)	
	Can analyse multiple toxin groups in single run	Equipment has high capital cost	
	Methods validated (SLV and ILS)[b] and approved	Requires significant expertise	
	Info on toxin profile provided	Each toxin requires calibration with significant QA/QC	
	Quantitative results	Per sample cost relatively high	

[a] Time estimates are for the detection step only. Shellfish sample preparation (shucking of 10-15 representative specimens and blending of the flesh) is a uniform requirement for all testing. Current methods for marine biotoxins all test a solvent extract of a sub-sample of the flesh homogenate. Although the extraction step may differ somewhat between methods, similar time/costs are involved.

[b] SLV = single laboratory validation; ILS = inter-laboratory study

4.2 Immunological assays

Immunological assays use antibodies to detect the toxin of concern. The antibodies are raised in either animals or cell cultures and can be used in a variety of different test formats i.e. ELISAs, LFAs etc. The detection methods are based on the antibodies ability to recognise and bind the 3D structure of the toxin of interest and 'ignore' other compounds present. Immunological assays are used widely in the food industry and are particularly successful when one chemical is the target of concern.

In the case of marine toxins, most toxin groups comprise a large number of structurally related congeners. Antibodies are raised against particular congeners within a group, and this means the specificity of the antibodies for other congeners in the same group may vary significantly. If the cross reactivity correlates with toxicity then this may not be a major issue i.e. C-toxins have low reactivity in some PSP immunoassays, but are also thought to be of low toxicity. However, if the toxicity of a congener is high but the cross reactivity low (or vice versa) then it is likely that the final result will not reflect the toxicity of the sample. Due to the issues relating to cross reactivity of immunological assays, antibody based tests for most toxin groups can only be considered as screening tests and not as fully quantitative assays (Hess et al., 2006; Humpage et al., 2010; Laycock et al., 2010a). While this is an issue for toxin groups comprising a large number of congeners (i.e. PSP), in circumstances in which only one toxin needs to be detected (i.e. DA) and the antibody has been raised against this toxin, the final concentration will reflect toxicity and the assay can be considered to be quantitative.

There are four main categories of immunoassays that have been developed for toxin analyses: radioimmunoassay (RIA), ELISAs, LFAs and immunosensors (Campbell et al., 2011c; EFSA, 2009c). Campbell et al. (2011c) notes that RIAs have been developed for the PSP and DSP toxins but are no longer used due to issues with the use of radioactivity, thus no further information on these assays is provided in this review. The following provides an overview of the development of ELISAs and LFAs. Immunosensors for marine toxins are discussed in Section 4.4.

4.2.1 ELISAs

Generally ELISAs follow a similar basic procedure: toxin in samples competes with toxin that has been bound to a microtitre plate for antibody that is free in solution. The sample and antibody mix are added to the wells on the microtitre plate and the amount of antibody bound to the toxin on the plate is measured using a colorimetric reaction and a spectrophotometer. The colorimetric reaction is catalysed by an enzyme that is conjugated to the antibody. So the more antibody that is bound to the plate, the higher the signal will be. The signal is therefore inversely proportional to the amount of toxin present in the sample. There are many variations of this basic procedure.

ELISAs have been developed for all the regulated toxin groups, including DSP, PSP, ASP, YTX and AZAs (Briggs et al., 2004; Frederick et al., 2009; Garthwaite et al., 2001; Kleivdal et al., 2007b)(Samdal et al., 2015). Several ELISAs have been subjected to SLV and/or ILS and have been commercialised (Kleivdal et al., 2007a; Kleivdal et al., 2007b; Litaker et al., 2008), including ELISA assays for ASP, PSP and DSP. Section 5 of this report provides an evaluation of current ELISA assays that are commercially available.

Garthwaite et al. (2001) developed a screening format using a single alcohol based extraction and a series of ELISAs to detect ASP, NSP, DSP, YTX and PSP. Similarly, researchers in Belgium developed a single ethanol based extraction following which three ELISAs were performed for the detection of DA, OA and STX (Dubois et al., 2010). Following on from this other researchers have recently been involved in attempts to multiplex immunoassays to enable detection of more than one toxin group in a single detection format, including an assay for simultaneous detection of STX, OA and DA in shellfish (reviewed in Vilarino et al. (2013)). Multiplex assays such as these have not yet been commercialised.

4.2.2 Lateral flow immunoassays (LFA)

These devices generally consist of pre-made strips of carrier material that contains regions where antibodies and toxin have been bound. Several different formats of LFA have been described, but the following format is generally used for toxin analysis. The extract containing the toxin of interest is applied to the strip within the application pad region at one end. The adjacent conjugate pad contains labelled antibodies. A test line further down the strip contains bound toxin. As the sample extract wicks down the strip, any free toxin in the sample competes with bound toxin on the test line for binding sites on the antibody. If no toxin is present in the sample, the labelled antibody is able to bind to the toxin at the test line producing a coloured reaction. If toxin from the sample binds to the antibody it cannot bind to the toxin at the test line and produce colour. Thus the visual response is inversely correlated to the toxin concentration in the sample (more toxin present, weaker colour) (Jawaid et al., 2013b; Laycock et al., 2010b; Posthuma-Trumpie et al., 2009). The LFA are considered a qualitative screening test (positive/negative result). The LFA for toxins also contain stationary control zones, which includes the antibody and thus colour should always form regardless of the level of toxin in a sample. A test result must give an intense colour at the control line to be considered a valid test.

LFAs have been designed for PSP, ASP and DSP toxins, and have been commercialised by Scotia Rapid Testing Ltd (formerly Jellet Rapid Testing Ltd) and more recently by Neogen Europe Ltd. (Anon, 2005; ISSC, 2005; Jawaid et al., 2013b; Laycock et al., 2010b; Laycock et al., 2003). The performance of these tests is discussed in detail in Section 5.

4.2.3 Suitability of immunological assays as field methods

Table 4.1 provides an overview of the advantages and disadvantages of immunological assays. The major drawbacks of immunological assays are: (1) the recognition of toxins by the antibody is not related to the toxin mode of action *in vivo* and therefore does not provide a direct measure of the toxicity of a sample; and (2) they do not provide detailed information on the toxin profile, which can hamper the identification of causative algal organisms.

While these drawbacks hinder quantitation of the total toxin concentrations in samples containing complex mixtures, immunological methods are typically very sensitive, and have relatively simple extraction procedures (Campbell et al., 2011c). The simplicity of the methods means that they are rapid to perform. The LFA are particularly amenable to 'field' analysis, are currently utilised by industry operators in a variety of countries, with rapid results enabling real time decisions on the fate of harvested shellfish products. The performance of various ELISAs and LFAs that have been commercialised is evaluated in Section 5.

4.3 Instrumental analytical methods

Analytical methods for marine toxins generally involve a liquid chromatographic (LC) step that separates mixtures of toxin congeners from each other and the other extractives in shellfish samples using a solvent mixture (mobile phase) flowing through a column packed with a stationary phase. An aliquot of sample extract (or toxin calibration standard) is injected onto the column and the effluent is coupled to a detection system, designed to exploit the spectroscopic properties of the toxins (ultraviolet, fluorometric and/or mass spectrometry). Modern mass spectrometers are highly automated and versatile instruments with very sensitive, specific detection modes for the set of toxins selected for analysis by each method. The identification of a toxin is based on its chromatographic retention time and spectroscopic properties.

For quantitative results the response for each toxin must be individually calibrated against reference standards. The toxic potency (usually the LD_{50}) of each regulated congener has been determined and related to the toxicity of the reference toxin for the group; the ratio of the toxicities is described as the toxicity equivalence factor (TEF) (Botana et al., 2010). To express the total toxin concentration of the sample in 'toxicity equivalents', the concentration of each congener in a group is multiplied by the appropriate TEF and then summed to provide a total adjusted concentration. At the current time the accepted TEFs for the PSP, OA and AZA groups are based on the intraperitoneal LD_{50} of the various congeners (EFSA, 2008a, 2008b, 2009c), although some revision of the TEFs may be required in the future to reflect new information regarding the oral potency of the toxins (Munday et al., 2013).

A variety of analytical methods for marine toxins have been subjected to both SLV and ILS (Lawrence et al., 2005; McNabb et al., 2005; van de Riet et al., 2011; Villar-González et al., 2011). These methods are generally considered to be fully quantitative confirmatory methods.

4.3.1 Suitability of analytical methods for field analysis

Table 4.1 provides an overview of the advantages and disadvantages of analytical methods. The major advantage of analytical methods is that each toxin congener is individually quantified with a high degree of accuracy. This yields valuable information on the concentration and relative proportions of the toxin congeners that are present in a sample. This information can assist in a range of environmental investigations regarding the potential source of toxicity and time at which toxicity occurred. Methods based on LC-MS are increasingly recognised internationally as the reference methods for most toxin groups in seafood (as for mycotoxins, pesticides and other trace contaminants in foods). While these methods are valuable research and regulatory techniques, they require equipment of relatively high capital cost and well-trained technical staff, thus they are not likely to be amenable to use in field labs.

4.4 Biosensors

Biosensors use biological components, such as antibodies or receptors, to recognise and bind the toxin(s) of interest. The degree of binding is then directly detected using one of a variety of technologies such as electrical, thermal or optical signals (Campbell et al., 2011b; Vilarino et al., 2013). A variety of different biosensors have been developed for the detection of marine toxins, but the majority of sensors that have been developed to date use antibodies as the biorecognition component and surface plasmon resonance (SPR) optical biosensor technology as the detection component. The SPR methods involve the adhesion/immobilisation of the toxin of interest onto a chip. The sample is then mixed with the biorecognition component i.e. an antibody or receptor and injected over the surface of the chip. Toxin in the sample effectively competes with the toxin on the chip for binding to the antibody. If the biorecognition component binds to the toxin on the chip this results in an increase in mass on the chip that changes the resonance angle of the light. This change has been found to be inversely proportional to the 'equivalent' total concentration of the toxin(s) present in the sample (Campbell et al., 2011b) and can be calibrated using solutions of the reference toxin. Immunosensors have been developed to detect PSP, DSP, ASP toxins and YTX, in addition to other toxins, which are not currently regulated in shellfish in the EU (e.g. palytoxin, tetrodotoxin, microcystin) (reviewed in Vilarino et al. (2013).

An SPR based immunoassay for detection of PSP in shellfish was developed and subjected to validation studies (Campbell et al., 2010; Campbell et al., 2009; Rawn et al., 2009; van den Top et al., 2011). Single laboratory validation was undertaken on the method in mussels and cockles (Campbell et al., 2010). The detection capability was calculated to be 120 µg/kg for STX-diHCl in mussels. The validation showed low cross reactivity's for the PSP congeners GTX-1,4 (<0.7%) and dcNEO (8.3%).

The LoD of these congeners was 13450 and 1128 µg/kg respectively. Following the SLV, an ILS was also undertaken involving seven laboratories and 20 shellfish samples, with some spiked with low (240 µg/kg STX-diHCl) and high levels of STX (825 µg/kg STX-diHCl), and other naturally contaminated samples (van den Top et al., 2011). HorRat values obtained were <1, indicating that the method precision was acceptable. The RSD_r ranged from 1.8 – 9.6% and RSD_R ranged from 2.9 – 18.3%. Recoveries were also acceptable, with mean values of 94.6 ± 16.8% and 98.6 ± 5.6% for the low and high spiked samples respectively.

Recently SPR methods have been developed for the detection of multiple toxin groups (ASP, DSP and PSP) simultaneously in one shellfish sample 'the optomouse method' (Campbell et al., 2011a; Campbell et al., 2014). An SLV was conducted on the shellfish method and showed promise with the detection capability as a screening method of ≤10 mg/kg, ≤160 µg/kg, and ≤400 µg/kg for DA, OA and STX respectively. It was noted that for OA further work is required to improve the sensitivity because the LoD is currently around the regulatory limit (Campbell et al., 2014). The main advantage of this approach is that three toxin groups can be detected in a single test.

In addition to optical biosensors, electrochemical immunosensors have also been developed for the detection of OA and DA in shellfish tissues using antibody detection systems (Campas et al., 2008; Kreuzer et al., 2002; Micheli et al., 2004) and for DSP toxins by immobilising the PP2A enzyme on to electrodes and electrochemically measuring the cleavage of the substrate catechyl monophosphate (Campas and Marty, 2007). To our knowledge however, electrochemical sensors (whether immune or functional-based) have not been subjected to SLV or ILS or incorporated into shellfish monitoring programmes as yet. The use of electrochemical sensing for toxins in shellfish was recently reviewed by Vilarino et al. (2013), who comments that "although these technologies are very promising, further development and validation are needed".

While most biosensors developed for shellfish toxins utilise antibodies as the binding component, some sensors have also been developed for marine toxins that use alternate 'binders', including:

- Functional receptors (including sodium channels for PSP toxins, and PP2A for DSP toxins) (Campas and Marty, 2007; Campbell et al., 2007);
- Chemosensors (molecules that bind to the toxin through non-covalent interactions and produce changes in light or fluorescence) (Gawley et al., 2007; Gawley et al., 2005);
- Molecular imprinted polymers (MIP) (polymers moulded around molecules of the target of interest or a structural template) (reviewed in Campas et al. (2007).

Further information on alternate binding substrates for PSP toxins, including their advantages and disadvantages, can be found in a recent review by Campbell et al. (2011b). While alternate binders have been investigated for marine toxins, limited studies have been undertaken on the application of these assays to contaminated shellfish. Aptamers (oligonucleotide or peptide molecules designed to specifically bind a target molecule with high affinity) are also noted as a promising avenue for future research.

4.4.1 Suitability of biosensors for field analysis

The biosensor methods that have been developed to date generally have the advantages of high speed, sensitivity and accuracy. However, the following limitations are noted:

- Most biosensor methods are immunological and thus suffer issues highlighted previously relating to poor cross reactivity of some toxin congeners, particularly for the PSP toxin group;
- Very few have been subjected to validation studies, with only one SPR based method for PSP having a ILS conducted to our knowledge (van den Top et al., 2011);

- Instrumentation cost can be high; and
- The instrumentation is generally not portable and requires a dedicated laboratory.

The high cost and lack of portability of the instrumentation prohibit the use of biosensors as field methods at this time, however some advances to overcome these limitations have been made. A portable SPR biosensor for the detection of DA in shellfish has been described (Stevens et al., 2007) and recently, portable planar waveguide devices (MBio Diagnostics Inc.) have been developed for the detection of some marine toxins in water and cultures (McNamee et al., 2014; Meneely et al., 2013). The devices exploit fluorescently labelled antibodies as the biorecognition component and toxin in samples compete with toxin on a plastic slide for antibody binding sites. The plastic slide is analysed using a hand held reader and results are displayed on a laptop computer. Several publications suggest that the device can achieve low LoDs for toxins in seawater (McNamee et al., 2014; Meneely et al., 2013) and that they may be able to be adapted for field analysis of shellfish tissues in the future.

SECTION FIVE: EVALUATION OF CURRENTLY AVAILABLE FIELD KITS FOR MARINE TOXIN DETECTION

5.1 Field methods currently available in the UK

An internet search was conducted to identify marine toxin testing kits that are currently commercially distributed in the UK. Section 3.2 outlines the approach taken to identify the field methods. Only methods that are amenable to use in field laboratories were considered i.e. simple to use, produce results in a relatively short timeframe, and do not require expensive instrumentation (other than simple plate readers). Table 5.1 shows companies that currently (March 2015) distribute kits for testing the regulated marine toxins. Only kits for ASP, DSP and PSP toxins were identified. No kits for YTXs, AZAs or PTXs were available. The methods identified were ELISAs and LFAs for ASP, DSP and PSP toxins, and the PPIA for DSP toxins. Section 4 describes the technical basis of these methods.

Table 5.1: Marine toxin test kit suppliers (at March 2015)[1], types of tests distributed, and website details.

Company	DSP toxins	ASP toxins	PSP toxins	Website
Abraxis	PPIA[2] ELISA[3]	ELISA[3]	ELISA[3]	http://www.abraxiskits.com/
Beacon	ELISA	ELISA	ELISA	http://www.beaconkits.com
Bioo Scientific	ELISA	ELISA	ELISA	http://www.biooscientific.com/
Biosense Laboratories	ELISA[3]	ELISA[3]	ELISA[3]	http://www.biosense.com/
EuroProxima	ELISA	ELISA[4]	ELISA[4]	http://europroxima.com/
Neogen Europe Ltd	LFA	LFA	LFA	http://www.neogeneurope.com/index.html
R-biopharm			ELISA	http://www.r-biopharm.com/
Scotia Rapid Testing[5]	LFA	LFA	LFA	http://www.scotiarapidtesting.ca
Zeulab	PPIA[2]	ELISA[4]	ELISA[4]	http://www.zeulab.com/

[1]Mercury Science Inc. currently produces a field kit for ASP and ELISAs for ASP and PSP toxins. ERFA Biotech currently distribute an ELISA for DSP toxins. Both companies note that these products are for 'research purposes only', therefore these kits have not been included in this evaluation. Sceti (Japan) produced a PPIA kit for DSP toxins, which is no longer manufactured and is also therefore not included in the evaluation.

[2]Some components of the Zeulab and Abraxis PPIA kits are identical.

[3]Biosense manufacture the ASP ELISA, Abraxis manufacture the DSP and PSP ELISAs. Biosense distribute all three ELISAs in Europe, while Abraxis distributes all three ELISAs in the USA.

[4]Zeulab distribute the EuroProxima ASP and PSP ELISAs in Spain.

[5]The Scotia Rapid Tests were formerly known as the 'Jellett Tests' (www.jellett.ca) and 'Mist Alert' tests.

To determine if the methods identified are 'fit for purpose' as per Principle 2 of VAM, method validation data was sourced from the suppliers and through the published literature and key performance characteristics were evaluated following the process outlined in Section 3.2.3. Briefly, the reported detection limits, specificity, ruggedness and precision of each method were evaluated in accordance with the acceptable criteria for screening methods established internationally (AOAC, 2005; Codex, 2012; Eurachem, 2014), these parameters are summarised below in a series of tables, and issues identified through this process are noted.

5.2 Evaluation of method performance for ASP field methods

5.2.1 Summary of ASP methods

Six kits are commercially available that are noted to be for use by industry and regulatory agencies for monitoring ASP in shellfish; of these kits, four are ELISAs and two are LFAs. Table 5.2 provides an overview of the ASP kits available, analysis time, sample throughput, cost, validation studies undertaken and method status. All the tests available have been subjected to an in-house SLV, but only two suppliers (Neogen and Biosense) have published the results in peer-reviewed journals (Jawaid et al., 2013b; Kleivdal et al., 2007a). The Biosense ELISA has been subjected to a full collaborative study (ILS), is approved as an official method by the AOAC (AOAC method 2006.02) (Kleivdal et al., 2007b), and is accepted as an alternative method for ASP in the EU. The Neogen LFA has been subjected to a small inter-laboratory study and was approved for use by the United States Interstate Shellfish Sanitation Committee (ISSC) in 2013 as a limited use method[8] to support the US National Shellfish Sanitation Programme (NSSP). Scotia Rapid Testing is in the process of submitting required documentation to the ISSC to seek approval for the ASP LFA.

5.2.2 ASP method performance

Table 5.3 provides an overview of the performance characteristics of the methods.

Detection limit

The LoD of all the methods was below the MPL of 20 mg/kg (Table 5.3). The reported cut off values for the qualitative LFAs were 17.5 and 10 mg/kg (Neogen and Scotia respectively).

False negatives

False negative rates were not reported for the ELISAs because they are considered quantitative assays. The LFAs were reported to give no false negatives at the MPL (Table 5.3); the Neogen kit was used to test 25 naturally contaminated samples (mussels and oysters) containing ≥ 17.6 mg/kg and all samples were positive; and the Scotia LFA was used to test 14 shellfish samples spiked with DA at levels between 7 and 24.3 mg/kg and all samples were positive (testing undertaken by the New South Wales Health Pathology lab). This suggests that the LFA methods meet the requirements of Commission Decision 2002/657/EC which states that the false negative rate should be 5% at the level of interest for screening methods.

Specificity

All the methods are immunoassays and the antibodies used were shown to specifically detect DA. Five of the six suppliers (with the exception of Scotia Rapid Testing) provided the results of studies in which the response of the assays to unrelated toxins/compounds was investigated; none of the assays was noted to have significant cross reactivity with unrelated compounds (cross reactivity studies generally included glutamic acid, glutamine, saxitoxin and other related PSP congeners, and okadaic acid) (Table 5.3). Cross reactivity to structurally related congeners of DA are not reported due to a lack of reference materials for these compounds.

[8]A 'limited use method' allows the use of the method to support the NSSP only in particular circumstances such as opening or closure of production areas, classification activities etc. An HPLC method is also approved as a limited use method.

False positives

For the purposes of this report, two types of false positives are discussed:

a. Samples for which toxin was detected, but no toxin was present (FPa);

b. Samples that have toxin concentrations less than the MPL, which give a positive result (FPb).

Four suppliers (Biosense, Europroxima, Neogen and Scotia) provided evidence (data) that truly blank shellfish give a negative response in the assays, however two suppliers (Beacon and Bioo Scientific) did not provide data on the potential for false positives (FPa) to occur (Table 5.3). With respect to the LFAs, the Neogen assay was found to be reliably negative with DA levels at or below 12.6 mg/kg, but some 'false positives' (FPb) are noted for shellfish with DA levels between 12.6 and 17.5 mg/kg (two mussel samples containing 16 and 16.8 mg/kg DA were positive in 3/10 LFA tests) (Jawaid et al, 2013b). Similarly, the Scotia LFA also gives some false positives (FPb) for shellfish containing low levels of DA, with 28, 44 and 4% of sample batches containing 6 mg/kg DA (the lowest DA level for which data was supplied) giving positive results.

Precision

Precision data for qualitative screening methods (i.e. the LFAs) is not required, as specified in Commission Decision 2002/657/EC. For the quantitative ELISA methods, data regarding the repeatability and reproducibility of the methods is appropriate. Intra-laboratory repeatability data was supplied by Beacon, Biosense and Europroxima and RSD_r values were acceptable (15% and 4.5% respectively), however only Biosense reported between laboratory reproducibility data, with an RSD_R of 23%.

Table 5.2: Overview of commercially available kits for ASP analysis of shellfish tissues

Field test for ASP toxins	Beacon	Bioo Scientific	Biosense (Abraxis)	Europroxima (Zeulab)	Neogen	Scotia
Principle	ELISA	ELISA	ELISA	ELISA	LFA	LFA
Method format	96-well plate	96-well plate	96-well plate	96-well plate	Single sample	Single sample
Qualitative or Quantitative	Quantitative	Quantitative	Quantitative	Quantitative	Qualitative	Qualitative
Method duration[a]	1 hr	1.5 hr	2 hr	45 mins	20 mins	35 mins
Throughput (samples per batch)	High (42)	High (42)	High (36)	High (40)	High[c]	High[b]
Cost per sample	£4.90	£9.52	Biosense: £7.03 Abraxis: £9.36	Europroxima: £7.71 Zeulab: £8.06	£11.04	£14.77
Set up cost[c,d]	High	High	High	High	Medium	Low
Single laboratory validation	Yes	Yes	Yes (published)	Yes (partially published)	Yes (published)	Yes
Inter-laboratory study	No	No	Yes (published)	No	Limited (published)	No
AOAC approved	No	No	Yes	No	No	No
ISSC approved	No	No	No	No	Yes	No
EU approved	No	No	Yes[e]	No	No	No

[a] Analysis times as reported by manufacturers and generally do not include sample preparation time.

[b] Scotia report that 56 samples can be assessed in 2 hours (not including extraction time). Neogen report that 48 samples can be analysed in 1.5 hours.

[c] Low = <£500, Medium = £500 – £2000, High = >£2000

[d] ELISAs require a variety of equipment including plate readers, the latter vary in price from around £2,000 to £12,000 at time of writing. Set up costs of the Neogen LFA is around £1700, which includes the strip reader. The Scotia LFA is generally manually read (set up costs around £100), but has an optional Skannex reader, which costs around £1000.

[e] Accepted as an alternative method in the EU.

Table 5.3: Performance characteristics of commercially available kits for ASP analysis of shellfish tissues

Performance characteristics	Beacon	Bioo Scientific	Biosense (Abraxis)	Europroxima (Zeulab)	Neogen	Scotia
Type	ELISA	ELISA	ELISA	ELISA	LFA	LFA
Limit of detection (or 'cut off value')	0.025 mg/kg	0.03 mg/kg	0.003 mg/kg	0.02 mg/kg	17.5 mg/kg	~10 mg/kg[g]
Limit of quantitation	0.0125 mg/kg	0.1 mg/kg	0.011 mg/kg	0.12 mg/kg	NA	NA
False negatives at MPL	NR	NR	NR	NR[c]	0%[f]	0%[h]
Do blank shellfish reliably produce a negative response (FPa)?	Yes[a]	NR	Yes[b]	Yes[d]	Yes[f]	Yes
Is a negative response given in presence of unrelated toxins?	Yes	Yes	Yes	Yes	Yes	NR
Detection in spiked samples?	Yes (m, cl)[a]	Yes (m)	Yes (o, m, s)	Yes (o, m, s)	Yes (o, m, s, cl, c)	Yes (m)
Comparative data with analytical methods?	Yes	NR	Yes	Yes[e]	Yes	Yes
Detection in naturally contaminated shellfish?	Yes	NR	Yes (s)	Yes[e]	Yes (m, s)	Yes
Detection in UK shellfish?	Yes[a]	NR	NR	NR	Yes (m, s)	NR[i]
Ruggedness studies?	NR	Yes (20%)	Yes (<15%)	NR	Yes	NR
Repeatability (RSD_r)	<15%	NR	15%	5.5%	NA	NA
Reproducibility (RSD_R)	NR	NR	23%	NR	NA	NA
References/data source	Data supplied by company	Data supplied by company	Kleivdal et al. (2007a); Kleivdal et al. (2007b)	Data supplied by company; Dubois et al. (2010)	Jawaid et al. (2013b)	Data supplied by company

[a] Internal data, not supplied (30 blank samples were negative). [b] Established for mussels, oysters and scallops (raw and cooked). [c] Comparisons with LC-MS/MS suggest false negatives unlikely (Dubois et al., 2010). [d] Established for oysters, scallops and mussels (n=20 each). [e] 110 samples (scallops, mussels and oysters) were collected in Belgium and analysed using the same antibodies, ELISA positive samples were analysed by LC-MS/MS and results were noted to correlate well (Dubois et al., 2010). [f] False negative and false positive rates were studied in naturally contaminated mussels and oyster samples. Samples containing ≥ 17.6 mg/kg DA (n=25) were all positive, samples containing at or below 12.6 mg/kg (n=70) were all negative. [g] For shellfish containing 10 mg/kg DA, between 4 and 40% of samples gave negative results (varied by batch). [h] 14 samples spiked at levels greater than 7 mg/kg were all positive. [i] Detection in UK shellfish was reported using a previous ASP assay by Jellet, but this utilised a different antibody type.

NR = not reported.

NA = not applicable. m = mussels, o = oysters, s = scallops, cl = clams, c = cockles.

5.3 Evaluation of method performance for DSP field methods

5.3.1 Summary of DSP methods

Seven different DSP kits are commercially available for monitoring shellfish. One method is a functional assay based on protein phosphatase inhibition by DSP toxins (PPIA), four methods are ELISAs and two are LFAs. Most test suppliers (with the exception of Europroxima and Beacon) note the need to analyse the esters of the OA group toxins and thus include provision in the kits for the toxin extract to be hydrolysed to convert the esters to the parent toxins and incorporate them in the final result. Table 5.4 provides an overview of the DSP kits available, analysis time, sample throughput, cost, validation studies undertaken and method status.

All the tests available have been subjected to an in-house SLV study, however only the SLV for the PPIA method (the OKATEST) has been published in a peer reviewed journal (Smienk et al., 2012). A collaborative ILS on the PPIA method was also undertaken and published (Smienk et al., 2013). The OKATEST validation was also sent to the EU RL for review and the method was subjected to proficiency testing. On this basis the EU RL note that the method has been accepted in the EU as a supplementary method for OA group toxins (but cannot be considered as an alternative to the LC-MS/MS reference method as it does not cover other lipophilic toxins).

Table 5.4: Overview of commercially available kits for DSP analysis of shellfish tissues

Field test for DSP toxins	Zeulab/Abraxis	Beacon	Bioo Scientific	Biosense/Abraxis	Europroxima	Neogen	Scotia
Principle	PPIA	ELISA	ELISA	ELISA	ELISA	LFA	LFA
Method format	96-well plate	96-well plate	96-well plate	96-well plate	96-well plate	Single sample	Single sample
Qualitative or Quantitative	Quantitative	Quantitative	Quantitative	Quantitative	Quantitative	Qualitative	Qualitative
Method duration (excluding 40 min hydrolysis step)[a]	1 hr	1 hrs	1.5 hrs	2 hrs	45 mins	30 mins	35 mins
Throughput (samples per batch)	High: (43)	High (42)	High (42)	High (40)	High (40)	High[b]	High[b]
Cost per sample	Abraxis: £10.13 Zeulab: £11.27	£4.90	£9.52	Biosense: £8.96 Abraxis: £8.42	£7.71	£11.83	£14.77
Set up cost[c,d]	High	High	High	High	High	Medium	Low
Single laboratory validation	Yes (published)	Yes	Yes	Yes	Yes (partially published)	Yes	Yes
Inter-laboratory study	Yes (published)	No	No	No	No	No	No
AOAC approved	No	No	No	No	No	No	No
ISSC approved	No (submitted in 2013)	No	No	No	No	No (submitted in 2013)	No
EU approved	Yes[e]	No	No	No	No	No	No

[a] Analysis times as reported by manufacturers and generally do not include sample preparation time.

[b] Scotia report that 56 samples can be assessed in 2 hours (not including extraction time). Neogen report that 48 samples can be analysed in 1.5 hours.

[c] Low = <£500, Medium = £500 – £2000, High = >£2000

[d] ELISAs require a variety of equipment including plate readers, the latter vary in price from around £2,000 to £12,000 at time of writing. Set up costs of the Neogen LFA is around £1700, which includes the strip reader and hot plate. The Scotia LFA is manually read (set up costs around £100).

[e] Approved as a supplementary method, other tests are required for the detection of the other lipophilic toxins such as pectenotoxins, yessotoxins and azaspiracids.

5.3.2 DSP method performance

Table 5.6 gives an overview of the performance characteristics of the methods.

Detection capability

All suppliers report that the LoD (or cut off value) of their tests is below the MPL (Table 5.6). Table 5.5 shows the cross reactivity of the tests to each regulated DSP congener (OA, DTX-1 and DTX-2). Notably, the reactivity to DTX-2 is lower than the other congeners. In particular, the cross reactivity of the Europroxima assay to DTX-2 was very low (2.6%) (Dubois et al., 2010) in comparison to other assays on the market (20-50%). This assay may not be suitable for UK shellfish, particularly those that are high in DTX-2[9].

Table 5.5: Cross reactivity of the commercially available field kits to the DSP congeners of regulatory concern

DSP toxin	Zeulab (Abraxis)[a]	Beacon	Bioo Scientific	Biosense /Abraxis	Europroxima	Neogen	Scotia	Relative specific toxicity[b]
OA	1.2nM	100%	100%	100%	100%	100%	100%	100%
DTX-1	1.6nM	120%	40%	50%	78%	89%	85-95%	100%
DTX-2	1.2nM	20%	46%	50%	2.6%	47%	30-40%	60%

[a] The method is based on capability of DSP toxins to inhibit protein phosphatase enzymes. Therefore, the inhibitory capacity of the different congeners was evaluated instead of cross reactivity. These values thus represent the IC50 values, the concentration of toxin able to inhibit 50% of the maximum enzyme activity.

[b] Based on EFSA toxicity equivalence factors (EFSA, 2008a)

NR = Not reported.

False negatives

False negative results have been reported for several of the DSP field methods identified.

A spiked matrix study (mussels, scallops, oysters and clams) was undertaken by Neogen to investigate the potential for false negative results at critical levels. All samples spiked at ≥140 µg/kg OA and ≥160 µg/kg DTX-1 were positive. However, only 50% of samples gave a positive result for DTX-2 at 267 µg/kg (Jawaid et al., 2013a)[10]. The potential for a negative result when DTX-2 exceeds the MPL is consistent with the low cross reactivity observed for this congener, and may be a concern for all kits on the market (Table 5.5). A study undertaken by Turner et al (2016) indicates no false negatives for the Neogen LFA in 13 samples containing DSP at levels exceeding the MPL, however the proportion of DTX2 present in these samples is not reported.

The PPIA was subjected to an SLV study in which some comparative testing with an LC-MS/MS method was undertaken. For 37 shellfish samples, one sample gave a false negative result (292 µg/kg LC-MS/MS; <LoQ PPIA (Smienk et al., 2012). Similarly, Eberhart et al. (2013) found that the PPIA gave two false negative results out of a total of 23 samples tested; one clam sample (219 µg/kg LC-MS/MS; 128 µg/kg PPIA) and one Pacific oyster sample (377 µg/kg LC-MS/MS; 131 µg/kg PPIA).

[9]Of the samples containing DSP in Scotland between July 2011 and December 2013 (n=1529), 13% belonged to cluster 3, which is dominated by free DTX-2 (Turner et al, 2014a).

[10]DTX-2 has a TEF of 0.6 compared with 1.0 for DTX-1 and OA. Neogen therefore note that the MPL for DTX-2 should be 267 µg/kg.

The Bioo Scientific MaxSignal DSP assay was reported to give one false negative result for a sample containing 193 µg/kg (as determined by LC-MS/MS, DTX2 content not stated), with the reported value of the ELISA being less than half the MPL (Turner et al, 2016).

The Scotia Rapid Test for DSP was compared with LC-MS/MS in a study undertaken in Washington State to investigate the feasibility of field-testing to provide early warning of toxicity in mussels. The Scotia test was reported to give >30% false negative results (n=23); two blue mussel, two manila clam, and two pacific oyster samples gave negative results, but were found to have >219 µg/kg DSP using LC-MS/MS. The toxin profiles were dominated by DTX-1. The authors attributed the false negatives to potential poor extraction of the toxins using a single methanol extraction method (Eberhart et al., 2013). A subsequent unpublished study by Eberhart et al (2014) investigated the efficacy of the Scotia single methanol extraction. False negatives were not observed for the seven samples that contained DSP above the MPL (all samples had DSP at levels >284 µg/kg), with all samples giving positive visual readings when using the standard Scotia single methanol extraction. The authors noted however, that the antibodies are calibrated to give a mid point response for DTX-1 at 323 µg/kg and "therefore will give false negative readings for DTX-1 at levels ranging between 160 to 300 µg/kg" (Eberhart et al, 2014). Scotia note that for DTX-1 the kits are set to give a positive response at around 35-40% of the control line to reduce the possibility of false negatives. Of note, in a recent study by Turner et al (2015), the Scotia Rapid Test gave no false negative results for 13 samples with DSP at levels exceeding the MPL (toxin profile not reported).

The importance of undertaking the hydrolysis step to detect DTX-3 was highlighted in a study involving the analysis of 40 naturally contaminated samples using the Neogen test strip and LC-MS/MS on extracts that were both hydrolysed and non-hydrolysed. Three false negative results were reported when the extracts were not hydrolysed: one scallop and two mussel samples containing 177, 215 and 452 µg/kg total DSP respectively by LC-MS/MS. The proportion of false negatives will obviously depend on the relative importance of ester forms. This is variable and dependent mainly on the shellfish species and the site (see Section 2.3.2). False negative results were not observed in the Neogen study when the hydrolysis step was performed.

Specificity

The kit suppliers (with the exception of Bioo Scientific and Scotia) reported that the assays give a negative response in the presence of unrelated toxins, including PSP toxins, DA, YTXs, PTXs and AZAs.

False positives

Most providers report that the analysis of truly blank shellfish gives negative results (FPa) as expected. With respect to the potential for false positives (FPb) to occur when levels are <MPL using the qualitative LFAs, as expected Neogen and Scotia found that some shellfish samples containing levels of DSP less than the MPL will give a positive result. For the Neogen LFA, 20% of samples spiked at 60 µg/kg OA and 50% of samples spiked with 120 µg/kg DTX-1 gave a positive response. Scotia reported the proportion of positive samples at ½ the MPL (80 µg/kg) for two batches in 2011 and 2009 was 64% and 32% of the samples respectively. Thus the decision limits for the two LFAs appear to be similar.

The Abraxis ELISA has also been noted to give false positive results (i.e. FPb; ELISA>MPL, LC-MS/MS <MPL) in two published comparative studies. A study on blue mussels from Killary Harbour, Ireland using the Abraxis ELISA, Zeulab PPIA and LC-MS/MS on hydrolysed extracts was undertaken. Results suggested that the Abraxis ELISA overestimated DSP in all samples and 23 false positives (FPb) were

noted[11] (Raine et al., 2011). Blue mussels, pacific oysters and manila clams (n=23) from Washington State were analysed using the Abraxis ELISA, the Scotia Rapid Test, the Zeulab PPIA and were compared to LC-MS/MS. The toxins present were mainly free and ester forms of DTX-1. The ELISA gave five false positives (clams and oyster samples) when compared with the LC-MS/MS (FPb; ELISA result >MPL, LC-MS/MS <MPL or <LoQ). The Zeulab PPIA also returned one false positive result (FPb) for a Pacific oyster sample (PPIA = 266 µg/kg; LC-MS/MS 142 µg/kg) (Eberhart et al., 2013).

Precision

Within lab repeatability data is reported for the Abraxis, Beacon and Europroxima ELISAs (RSD_r <15% and 7.1% respectively) and the Zeulab PPIA (RSD_r ≤11.2%). Between laboratory precision data resulting from ILS is only reported for the Zeulab PPIA (RSD_R ≤ 13.2%) (Smienk et al., 2013; Smienk et al., 2012).

[11]The toxin profiles of the shellfish were not noted.

Table 5.6: Performance characteristics of commercially available kits for DSP analysis of shellfish tissues

Performance characteristics	Zeulab/Abraxis	Beacon	Bioo Scientific	Biosense/ Abraxis	Europroxima	Neogen	Scotia
Type	PPIA	ELISA	ELISA	ELISA	ELISA	LFA	LFA
Limit of detection (or 'cut off value')	44 µg/kg	50 µg/kg	30 µg/kg	100 µg/kg	49.6 µg/kg (mussels) 48.2 µg/kg (oysters)	OA: 120 µg/kg eqs DTX-1: 140 µg/kg eqs DTX-2: 160 µg/kg eqs[i]	75 – 100 µg/kg
Limit of quantitation	56 µg/kg	100 µg/kg	100 µg/kg	NR	NR	NA	NA
False negatives at MPL	2-9%[a]	NR	7.7%[e]	NR	NR[g]	OA: 0% DTX-1: 0% DTX-2: 50%[i]	30%[j]
Do blank shellfish reliably produce a negative response (FPa)?	Yes[b]	Yes[d]	NR	No[c]	Yes[h]	Yes	Yes
Is a negative response given in presence of unrelated toxins?	Yes	Yes	NR	Yes	Yes	Yes	NR
Is a positive response given to each regulated congener?	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Detection in spiked samples?	Yes (m, s)	Yes (m, cl) [d]	Yes (m)	NR	Yes (m, o, s)	Yes (m, s, o, cl)	Yes (m)
Comparative data with analytical methods?	Yes	NR	NR	Yes	Yes	Yes	Yes
Detection in naturally contaminated shellfish?	Yes (m)	NR	NR	Yes (m)	Yes	Yes (m, s, o)	Yes
Detection in UK shellfish?	Yes(m)	No	NR	Yes[cf]	NR	Yes	NR
Ruggedness studies?	Yes	NR	Yes (<20%)	NR	NR	Yes	NR
Repeatability (RSD_r)	≤11.2%	<15%	NR	<15%	7.1%	NA	NA
Reproducibility (RSD_R)	≤13.2	NR	NR	NR	NR	NA	NA
References/data source	Smienk et al. (2013); Smienk et al. (2012)	Company data	Company data	Company data; Raine et al. (2011); Eberhart et al. (2013)	Company data; Dubois et al. (2010)	Company data; submission to US ISSC (proposal 13-113)	Company data; Eberhart et al. (2013); Eberhart et al. (2014)

[a]SLV study, 1 false negative (n=37), 2 false positive results by PPIA (Smienk et al., 2012). Eberhart et al (2013), 2 false negative results (n=23). [b]ILS, 1 lab reported values for 2 of the blank samples, issue considered to be related to lab practices (Smienk et al., 2013). [c]Some false positives (FPb) noted (Eberhart et al., 2013; Raine et al., 2011). [d]Internal data, not supplied (10 blank samples were all negative). [e]1 false negative out of 13 samples >MPL (Turner et al., 2015). [f]Abraxis ELISA suggested to give mixed results on Scottish samples (personal communication Jean-Pierre Lacaze, November 2014). [g]Comparative analysis of positive mussel, oyster and mussels samples (n=110) using LC-MS/MS and ELISA suggests false negatives are unlikely, however only 1 DSP positive sample was reported. [h]20 blank samples of mussels, scallops and oysters gave negative results (Dubois et al., 2010). [i]Cut off limits specified by Neogen in ISSC submission June 2013, 50% of samples containing 267 µg/kg DTX-2 (160 µg/kg OA eqs) produced a negative response. [j]Eberhart et al. (2013) report a 30% false negative rate; two subsequent studies suggest a lower false negative rate is possible, with one study having a false negative rate of 0% for 7 samples with DSP >MPL (Eberhart, 2014). NR = not reported. NA = not applicable. m = mussels, o = oysters, s = scallops, cl = clams

5.4 Evaluation of method performance for PSP field methods

5.4.1 Summary of PSP methods

There are currently eight different immunological based test kits available for purchase for monitoring PSP in shellfish. Six of the methods are ELISAs, and two are LFAs. Table 5.7 gives a summary of the field methods available for PSP analysis in shellfish tissues, the validation studies undertaken, cost of the assays, method duration and the method approval status.

All the tests have been subjected to an in-house SLV study, with four of the methods (Abraxis 'shipboard' ELISA[12], Europroxima ELISA, Scotia LFA, and Neogen LFA) having publications that describe some aspects of the validation (DeGrasse et al., 2014; Dubois et al., 2010; Jawaid et al., 2015; Laycock et al., 2010b; Laycock et al., 2003; Turner et al., 2015b). The Neogen LFA was also subjected to a limited ILS (five laboratories, six oyster samples). Two of the methods (Abraxis 'shipboard' ELISA[12] and Scotia Rapid Test) have been approved as limited use methods by the US ISSC. None of the methods has been accepted as an alternative method in the EU.

[12]Abraxis converted their standard ELISA into a 'shipboard' version which includes a simplified extraction protocol (based on the Scotia rapid extraction method), handheld photometric plate reader and excel spreadsheet and laptop for calculations. The same antibodies are used for the shipboard and standard versions of the ELISA.

Table 5.7: Overview of commercially available kits for PSP analysis of shellfish tissues

Field test for PSP toxins	Abraxis/ Biosense[a]	Abraxis	Beacon	Bioo Scientific	R-biopharm	Europroxima/ Zeulab[b]	Neogen	Scotia
Principle	ELISA	'Shipboard' ELISA[c]	ELISA	ELISA	ELISA	ELISA	LFA	LFA
Method format	96-well plate	96-well plate	96-well plate	96-well plate	96-well plate	96-well plate	Single sample	Single sample
Qualitative or Quantitative	Quantitative	Quantitative	Quantitative	Quantitative	Quantitative	Quantitative	Qualitative	Qualitative
Method duration[c]	1 hr	2 hrs	1 hrs	1.5 hrs	2 hrs	45 mins	15 mins	35 mins
Throughput (samples per batch)	High (42)	High (40)	High (42)	High (42)	High (48)	High (40)	High[d]	High[d]
Cost per sample	Abraxis: £9.24 Biosense: £8.52	£15.10	£5.29	£9.52	£4.81	Europroxima: £7.71 Zeulab: £8.26	£11.04	£14.77
Set up cost[e,f]	High	High	High	High	High	High	Medium	Low
Single laboratory validation	Yes	Yes (partially published)	Yes	Yes	Yes	Yes (partially published)	Yes (published)	Yes (partially published)
Inter-laboratory study	NR	NR	NR	NR	NR	NR	Yes[g]	No
AOAC approved	No	No	No	No	No	No	No	No
ISSC approved	No	Yes	No[h]	No	No	No	No	Yes
EU approved	No	No	No	No	No	No	No	No

[a]Biosense distribute the Abraxis ELISA in Europe. Abraxis converted their standard ELISA into a 'shipboard' version which includes a simplified extraction protocol (based on the Scotia extraction method), handheld photometric plate reader and excel spreadsheet and laptop for calculations. The same antibodies are used for the shipboard and standard versions of the ELISA.

[b]The Europroxima ELISA is distributed by Zeulab in Spain.

[c]Analysis times as reported by manufacturers and generally do not include sample preparation time.

[d]Scotia report that 56 samples can be assessed in 2 hours (not including extraction time). Neogen report that 48 samples can be analysed in 1.5 hours.

[e]Low = <£500, Medium = £500 – £2000, High = >£2000

[f]ELISAs require a variety of equipment including plate readers, the latter vary in price from around £2,000 to £12,000 at time of writing. Set up costs of the Neogen LFA is around £1700, which includes the automated strip reader. The Scotia LFA is generally manually read (set up costs around £100), but has an optional Skannex reader which costs around £1000.

[g]Inter-laboratory study involved five laboratories and six oyster samples.

[h]The method has been submitted to the ISSC for consideration.

5.4.2 PSP method performance

Table 5.11 provides an overview of the performance characteristics of the methods.

Detection capability

All the field tests identified in this report are immunological and the antibodies used in each kit show varying cross reactivities to the PSP congeners (Table 5.8). All kits show very low reactivity to GTX-1,4 and most (with the exception of Neogen) show poor reactivity to NEO.

Some toxin profiles in shellfish can be relatively simple, with one or two congeners dominating, others may be complex with most congeners present at differing concentrations. The overall LoD of an immunological assay is therefore variable, depending on the amounts of each congener present and the cross reactivity in the assay (Laycock et al., 2010b).

All suppliers report that the LOD (or cut off value) of the test for STX is below the MPL (Table 5.11). The cut off values for STX for the Neogen and Scotia LFA tests are 688 and 188 µg/kg respectively. STX is generally the most reactive congener with the other toxin analogues having lower cross reactivities. This suggests that the LODs for the other relevant congeners would be higher than those reported for STX in Table 5.11. Consistent with this, spiking studies undertaken by Neogen and Scotia show that the average concentration of each congener that is required to produce a positive result (the cut-off level) is frequently above the MPL (Table 5.9) (internal data supplied by Scotia; Jawaid et al. (2015)).

A desktop exercise was undertaken as part of this review to determine the response the kits would give for four hypothetical samples, which contained toxin mixtures typical of the four profile groups found in UK shellfish (Section 2.3.3) and with total concentrations of 800 µg STX eq/kg (as would be determined by HPLC). The predicted concentration of each congener when using the kits was calculated using the following equation:

$$\mathbf{conc_{ia} = conc_{HPLC} \times CR/TEF}$$

Where,

$Conc_{ia}$	= Predicted concentration (µg STX-eq/kg) of the congener of interest using an immunoassay
$Conc_{HPLC}$	= µg STX-eq/kg of the congener of interest (as determined using an HPLC method)
TEF	= Toxicity equivalence factor (EFSA)[13]
CR	= Cross reactivity of the congener reported by the immunoassay supplier

The total predicted concentration (µg STX-eq/kg) for each of the four samples was then calculated by summing the predicted concentration of each congener for each kit.

Table 5.10 shows the predicted response of each kit for the four samples. ELISAs were generally predicted to give results below the MPL and the Neogen LFA was predicted to produce negative results, for each of the four samples contaminated at the MPL. The Scotia LFA was predicted to produce positive results for each hypothetical sample. Samples rich in GTX-2,3 and STX (e.g. samples 1 and 3) were predicted to give higher results than those rich in GTX-1,4 or the decarbamoyl toxins (samples 2 and 4 respectively), reflecting the low cross reactivity of most assays to the latter toxins.

[13]The concentration of PSP congeners (as determined by HPLC) in the four hypothetical samples are reported as µg STX equivalents per kg shellfish (because this is how the typical toxin profiles are reported in Turner et al 2014). Therefore, the HPLC concentrations are divided by the TEFs to give the un-weighted concentrations of each congener (i.e. no adjustment to account for toxicity). In cases in which kit suppliers report cross reactivity on a % weight basis, the TEF was converted to a weight ratio using the molecular weight of saxitoxin and the toxin concerned.

Table 5.8: Cross reactivity (%) of the commercially available field kits to the PSP congeners of regulatory concern. Cross reactivity is expressed as reported by kit suppliers as % weight (with the exception of Scotia who report % molar).

PSP toxin		Abraxis (Biosense)[a]	Beacon	Bioo Scientific	R-biopharm	Europroxima (Zeulab)	Neogen	Scotia[b]	Relative Specific Toxicity[c]
Saxitoxin	STX	100	100	100	100	100	100	100	100
Decarbamoyl saxitoxin	dcSTX	29	18	100	20	19.2	56	71	51
Gonyautoxin-2, 3	GTX-2,3	23	12	43	70	5.6	23	79.8	50
Gonyautoxin-5	GTX-5 (B-1)	23	25.6	61	NR	26.2	23	57.2	6
N-sulphocarbamoyl toxin-1, -2	C-1,2	2	1.4	NR	NR	0.2	3	10	5
Decarbamoyl GTX-2, -3	dcGTX-2,3	1.4	0.4	10	NR	0.2	8	15.6	27
Neosaxitoxin	NEO	1.3	0.8	20	12	1.4	129	25.8	92
Decarbamoyl Neo STX	dcNEO	0.6	0.7	4	NR	0.5	28	NR	NR
Gonyautoxin-1, -4	GTX-1,4	<0.2	<0.1	2	NR	<0.1	6	2.56[d]	86

NR = not reported.

[a] Standard format ELISA and 'shipboard' version.

[b] Average cross reactivity's from 5 PSP batches between 2009 and 2014.

[c] Relative specific toxicity was calculated using the specific toxicity values published by Oshima (1995) and expressing the toxicities as a percentage of the specific toxicity given for STX.

[d] Scotia have recently introduced an additional step into the assay which enables the conversion of GTX-1/4 to NEO (and also converts GTX-2/3 to STX). NEO reacts with higher affinity to the antibody than GTX-1,4 and this therefore is considered to improve detection limits for samples which are high in GTX-1,4.

Table 5.9: The mean concentration (µg/kg) of toxins required to generate a positive result using commercially produced lateral flow assays

PSP toxin		Scotia[a]	Neogen[b]
Saxitoxin	STX	220	680
Neosaxitoxin	NEO	850	580
Gonyautoxin-1, -4	GTX-1,4	13000[c]	8055
Gonyautoxin-2, -3	GTX-2,3	350	2830
Decarbamoyl saxitoxin	dcSTX	280	1375
Decarbamoyl GTX-2, -3	dcGTX-2,3	1620	7450
Gonyautoxin-5	GTX-5 (B-1)	500	2400
N-sulphocarbamoyl toxin-1, -2	C-1,2	NR	10855

[a] Internal data provided by Scotia Rapid Testing on the LoDs determined for a batch of kits.
[b] Reported in Jawaid et al. (2015).
[c] The mean concentration of GTX-1,4 to generate a positive result following the new hydrolysis step introduced by Scotia Rapid Testing is 1160 µg/kg.
NR = not reported

Table 5.10: Predicted concentrations (µg/kg) of paralytic shellfish poisons in four hypothetical shellfish samples using commercially available immunoassays. The toxin composition and concentration (µg STX-eq/kg) of the hypothetical samples (n = 4) is shown in the top part of the table (Part A). Each sample has a toxin composition that reflects one of the four profile groups commonly found in shellfish from the UK (estimated from Turner et al, 2014), and a total concentration of 800 µg STX-eq/kg (as determined by HPLC). Numbers in brackets () are the % of each congener in the sample. The bottom part of the table (Part B) shows the predicted response of each commercially available field kit, for each hypothetical sample. Predicted concentrations for each field kit were calculated using the cross reactivity reported for each congener as specified by kit suppliers.

Part A	Sample 1	Sample 2	Sample 3	Sample 4
STX	80 (10)	160 (20)	376 (47)	0
dcSTX	0	0	8 (1)	296 (37)
GTX-2,3	680 (85)	100 (12.5)	296 (37)	0
GTX-5 (B1)	0	0	8 (1)	0
C-1,2	40 (5)	20 (2.5)	20 (2.5)	0
dcGTX-2,3	0	0	28 (3.5)	480 (60)
NEO	0	40 (5)	28 (3.5)	0
dcNEO	0	0	8 (1)	24 (3)
GTX-1,4	0	480 (60)	28 (3.5)	0
TOTAL µg STX-eq/kg (HPLC)	**800**	**800**	**800**	**800**
Part B	**Predicted total PSP concentration (µg/kg)**			
Neogen[a]	443 (neg)	314 (neg)	615 (neg)	270 (neg)
Scotia[a,b]	1253 (pos)	482 (pos)	966 (pos)	402 (pos)
Abraxis	437	219	559	94
Beacon	349	232	527	52
Bioo Scientific	724	264	739	395
Europroxima	165	174	442	52
R-biopharm	1128	319	837	51

[a] Positive and negative results are noted in brackets using the reported cut off values of 680 µg/kg and 188 µg/kg for the Neogen and Scotia kits respectively. [b] Assumes the hydrolysis step has been performed which converts GTX1,4 to NEO and GTX2,3 to STX.

False negatives

The Scotia Rapid Test was used to test a large number of naturally contaminated shellfish from a variety of different countries (including many from the UK). Laycock et al. (2010b) compiled data from the various parallel studies and found for 3492 samples tested, 417 were positive using the MBA, and 1039 were positive using the Scotia Rapid Test. In all, only one false negative was reported to occur (<0.03%), in which the Rapid Test was negative and the MBA gave a level of 450 µg/kg (i.e. less than the MPL) (Laycock et al., 2010b). This is consistent with the predicted response of the Scotia assay to the four hypothetical samples containing typical toxin profiles found in the UK (Table 5.10), in which the Scotia test was predicted to be positive for each sample contaminated at the MPL. PSPs in two mussel samples were not detected using the LFA when spiked levels were just over the MPL: (1) one sample contained 95% GTX-1,4 and 5% STX; and (2) the second sample contained NEO in isolation, which was considered to be representative of a mussel sample that was tested during a PSP incident in 1990 in the UK (Anon, 2007). While the false negative rate for the Scotia assay appears to be very low, they have recently introduced a step which converts GTX-1,4 to NEO prior to detection using the LFA device. NEO has a 10 fold higher cross reactivity in the Scotia assay than GTX-1,4 (26% compared 2.6%). The introduction of this step is reported to improve sensitivity of the assay for shellfish containing high proportions of GTX-1,4 and reduce the possibility of false negatives (Turner et al., 2015b).

Table 5.10 indicates the potential for false negatives to occur when PSP levels are around the MPL when using the Neogen LFA, with all four hypothetical samples predicted to give a negative result. In contrast to the hypothetical analysis, actual validation studies to date have not indicated the occurrence of false negatives for naturally contaminated samples. The Neogen SLV study involved the analysis of 23 naturally contaminated samples using the LFA and LC-FLD. They report no false negatives using the LFA in the study (Jawaid et al, 2015). However, only seven of the samples were above the MPL, these contained very high levels of PSP (six samples contained PSP at concentrations >3 times the MPL), and none of the samples were high in GTX1,4 (to which the assay has a low reactivity). Similarly, an external lab analysed 33 naturally contaminated samples, of which 13 contained PSPs above the MPL, again no false negatives using the LFA were noted (Jawaid et al, 2015). However, it is noted that 9 of the 13 samples contained very high levels of PSP (at least 2.5 fold the MPL). Consistent with the findings of Neogens validation studies, when the performance of the kit was considered for hypothetical samples containing PSP at levels two fold higher than the MPL, the occurrence of false negatives was reduced, with two of the four sample types in Table 5.10 now predicted to give positive responses using the Neogen kit. Levels three fold higher than the MPL resulted in positive responses for all four hypothetical samples. In the limited ILS undertaken by Neogen (five labs analysed six oyster samples in triplicate), samples which contained PSP toxins at concentrations of 0%, 20% or 100% MPL were analysed. One false negative (3%) was reported for a sample, which contained PSP at 100% MPL (no toxin profile information provided). Given the potential for false negatives at the MPL (Table 5.10), further focus on validation studies of samples contaminated at levels close to the MPL is advisable.

Few validation studies have been performed to investigate the potential for false negatives using the ELISAs, however some antibodies have been tested on real shellfish samples. For example, the Europroxima antibodies were used to analyse 110 shellfish samples, however none of the samples contained PSP above the MPL (Dubois et al., 2010). The Abraxis Shipboard ELISA was used to detect PSP toxins in a large number of surfclam samples (DeGrasse et al., 2014), but no samples were above the regulatory limit, and the toxin profile comprised largely STX (82%) to which the antibodies have a high affinity for.

Specificity

Four suppliers report that their assays gave a negative response to the presence of unrelated toxins such as DSP and ASP toxins. The Abraxis ELISA was noted to cross-react with lyngbyatoxin (13%); this does not pose a significant concern, as lyngbyatoxin is unlikely to occur in commercial shellfish. Beacon, Bioo Scientific, R-biopharm and Scotia do not provide information on the specificity of their tests for non-related toxins.

False positives

Limited data is available regarding the response of some of the assays (particularly the ELISAs) to either blank shellfish (FPa), or shellfish containing levels of toxins that are below the MPL (FPb) as determined by MBA or LC-FLD (Table 5.10).

The Abraxis shipboard ELISA had a false positive rate (FPb) of <1% when compared with the MBA for the analysis of surfclams from Georges Bank off the coast of north-eastern USA. The ELISA correlated well with MBA and LC-FLD in this instance, however the kit is calibrated specifically for STX using STX standards, and the profile of surfclams in the Georges Bank is dominated by STX. Thus the performance of the kit on other more complicated toxin profiles is not clear from the available data. The SLV undertaken on the Europroxima ELISA involved the analysis of 20 blank mussel, scallop and oyster samples for which no response was noted (FPa), as expected. However, the accuracy of the assay for shellfish samples that contain PSP levels below the MPL does not appear to have been investigated. No information on the potential occurrence of false positives was provided for the Beacon, Bioo Scientific or R-biopharm ELISAs.

Neogen undertook two separate investigations on naturally contaminated shellfish samples to investigate the occurrence of false positives using their LFA. They found that six samples out of 23 (26%), and one sample out of 33 (3%), gave false positive results when using the LFA compared to LC-FLD and MBA (i.e. FPb, samples for which the true results were <MPL).

False positive results (FPb) have been widely reported for the Scotia Rapid Test and are noted to vary with geographic area and over time:

- 32.4% of sample extracts produced false positive results from samples (n=233) from the state of California (MBA results were between 320 and 400 µg/kg).
- 12.7% of samples tested from New Zealand (n=154) gave false positive results.
- 14.3% of sample extracts from the state of Maine (n=61) gave false positive results.
- 9% of samples (n=69) gave false positive results from shellfish collected in Ireland in 2004 (Anon, 2005) (Clarke, 2004).

Examples of false positive rates using the Scotia Rapid Test for UK shellfish include:

- A comparative analysis of 961 samples (mussels, oysters, scallops, razors, cockles) from the UK was undertaken using the Scotia Rapid Test and MBA; 36 samples (3.7%) were positive using the Scotia test and negative by MBA (Anon, 2007).
- King scallops, mussels, native oyster, pacific oyster, razors, queen scallops and cockles (n=547) from Scotland were tested using the Scotia rapid test and the MBA. 97 samples were positive using the Scotia test and negative by MBA (18%) (Mackintosh and Smith, 2002).
- Samples collected as part of the UK national programme in 2000 were tested using the Scotia test and MBA. A false positive rate of 25% was observed (n=256) when comparing Scotia with MBA. Confirmatory HPLC analysis confirmed that many of these samples contained PSP toxins below the LoD of the MBA (Mackintosh et al., 2002).

Scotia note that false positive rates can be reduced in specific areas by incorporating extra dilution steps.

Precision

Abraxis, Beacon, R-biopharm and Europroxima report intra-laboratory variability (RSDr) of <15% (Table 5.10). None of the ELISA methods for PSP has been subjected to a formal collaborative ILS in which between-laboratory reproducibility was determined. The Neogen LFA method was subjected to a limited ILS involving five laboratories and six samples in which results were generally noted to be acceptable (with the exception of one false negative) (Jawaid et al., 2015).

Table 5.11: Performance characteristics of commercially available kits for PSP analysis of shellfish tissues

Performance characteristics	Abraxis[a] (Biosense)	Beacon	Bioo Scientific	Europroxima (Zeulab)	R-biopharm	Neogen	Scotia
Type	ELISA	ELISA	ELISA	ELISA	ELISA	LFA	LFA
Limit of detection (or 'cut off value') for saxitoxin	0.015 ng/ml	25 µg/kg	9 µg/kg	10.5 µg/kg (muss) 4.9 µg/kg (oys)	50 µg/kg	680 µg/kg	188 µg/kg[l]
Limit of quantitation	200 µg/kg	50 µg/kg	30 µg/kg	13.04 µg/kg (muss) 6.06 µg/kg (oys)	NR	NA	NA
False negatives at MPL	NR	0%[f]	NR	NR	NR	3%[j]	0.03%[m]
Do blank shellfish reliably produce a negative response (FPa)?	Yes[b]	Yes	NR	Yes[g]	NR	Yes	Yes
Do shellfish with PSP at <MPL give a negative response (Lateral flow assays) (FPb)?	NA	NA	NA	NA	NA	No[k]	No[n]
Is a negative response given in presence of unrelated toxins?	Yes[c]	Yes	NR	Yes[h]	NR	Yes	NR
Detection in spiked samples?	Yes[d]	Yes	Yes (m)	Yes (m, o, s)	Yes (m)	Yes	Yes
Comparative data with analytical methods?	Yes[e]	Yes	NR	NR	NR	Yes	Yes
Detection in naturally contaminated shellfish?	Yes[e]	Yes	NR	Yes[i]	NR	Yes	Yes
Detection in UK shellfish?	NR	Yes[f]	NR	NR	NR	Yes	Yes
Ruggedness studies?	NR	NR	Yes (<20%)	NR	NR	Yes	NR
Repeatability (RSD_r)	<15%	<15%	NR	≤12.2%	12% (CV)	NA	NA
Reproducibility (RSD_R)	NR	NR	NR	NR	NR	NA	NA
References/data source	Company data; DeGrasse et al. (2014);	Company data	Company data	Company data ; Dubois et al. (2010)	Company data	Jawaid et al. (2015)	Company data; Laycock et al. (2010b); Laycock et al. (2003); (ISSC, 2005)

[a]Performance characteristics are for both the standard and "shipboard" ELISA. [b]False positive rate noted to be <1% (abstract provided by Abraxis). [c]Cross reactivities with other classes of algal toxins not generally observed, cross reacts (13%) with Lyngbyatoxin. [d]shellfish species not noted. [e]Surfclams with low levels of STX were analysed by MBA, LC-FD and ELISA. [f]Internal data, not supplied (60 samples > MPL, all positive). [g]20 blank mussel, scallop and oyster samples analysed. [h]Cross reactivity to OA, DA, DTX-1, DTX-2 <0.1%. [i]110 samples from Belgium (mussel, oyster, scallops) tested, 1 sample was positive. [j]One false negative (1/30) was returned for a sample with PSP at the MPL in the ILS (Jawaid et al 2015). [k]SLV shows 6/23 naturally contaminated samples gave false positive results when toxin levels were <MPL by LC-FLD or MBA (Jawaid et al 2015). [l]LoD determined from 5 different batches between 2009 and 2014. [m]Scotia report 1 false negative from 3492 samples. [n]false positives extensively documented, rates vary depending on shellfish species, toxin profile, location.

NR = not reported. NA = not applicable. m = mussels, o = oysters, s = scallops, cl = clams.

SECTION SIX: FOOD BUSINESS OPERATORS' VIEWS ON TOXIN FIELD TESTS

Shellfish industry operators utilising field test kits for biotoxins in shellfish were identified using information provided by the FSA/FSS which was sourced from Local Authority enforcement officers. The 35 operators that were identified included producers, catchers and processors (as well as combinations of these activities). The FBOs were contacted by phone and e-mail, to elicit views and real life experience with the use of the kits, with responses recorded from 18 contacts (16 'full' responses, two partial contributions). The objective was to identify kit manufacturers and toxin tests actually in use in the UK, and the practical difficulties (if any) associated with their use along with the costs involved. Industry views on suppliers and kits and comments on how test results influence production and despatch behaviour, were sought. Finally, views on the usefulness of the FSAS guidance material ('End Product Testing for Shellfish Toxins' and 'Managing shellfish toxin risks - Guidance for harvesters and processors') were also queried. Appendix One shows the questions posed to each FBO.

A breakdown of the main findings for each question can be viewed in Appendix Two, the main themes arising are summarised below. Although there are numerous field methods on the market, based on the responses received in this study, the UK market appears to be dominated by Neogen for ASP (9 FBOs), DSP (15 FBOs) and PSP (12 FBOs), with only one respondent using an alternative, the Scotia Rapid Test for ASP.

Selection of Neogen and their kits reflected a number of reasons, with the main ones being convenience, personal service, ease of use and their Scottish location. The company is perceived as having a 'presence', established through advertising, sponsoring training Workshops and by having a stand at industry gatherings (e.g. the ASSG Conference, Aquaculture UK) and as a result was cited as 'the only one we could find'. Other reasons for selection of the kit and the company included 'reputation', recommendation, collaboration during development of the kit, and the complications associated with an ELISA kit previously used (expense, repeat tests and time for testing cited as problematic).

The Scotia user cited the expense of the Neogen equipment (reader and hot plate) combined with the ease of use of the Scotia kit in a 'domestic' environment as reasons for selection. A potential cost concern was raised about apparently random imposition of customs duty on imported test strips from Canada[14].

Comments from users were generally favourable towards Neogen and its operations, along the lines of 'good back up', 'good personal service', 'queries always quickly answered' and 'provided technical support when required'. However, some issues were raised by several respondents, in particular the temperature sensitive nature of storage (18 – 30°C) and the temperature range for using the kit (>18°C). Despite their importance, these issues were felt not to be adequately highlighted by Neogen ('only mentioned in the manual', 'not mentioned in training'), with an observation from one producer that the ambient temperature constraint 'prevents use on a boat or in a shed and therefore this is not a *field* kit in their opinion. (Indeed, every relevant respondent claimed the use of kits was in a dedicated area of their shore base or processing facility, with tests carried out by trained company personnel).

Other concerns included difficulties in 'getting them to work', 'easy to make mistakes', the purchase and delivery of methanol to remote rural locations, imperfect kits (under filled bottles of buffer

[14]Information provided to Scotia Rapid Testing from the UK government suggests that there should be no additional fees for customs and duties on the Scotia tests as the commodity code for the kits are zero rated.

supplies, damaged strips, incorrect results – control test with tap water, control line failure) and incidence of false positives. These issues have led to a degree of loss of confidence in the kits, however Neogen are acknowledged to have recognised the problems and generally reacted positively to consumer concerns.

Despite the issues noted above, the majority of respondents agreed with the statement that the kits were sufficiently user friendly for purpose, that the suppliers provided adequate advice and support, that they experienced no difficulties in interpretation and that they were satisfied with direct liaison with the kit provider. The use of the Neogen plate reader appears to have reduced any difficulties or subjectivity in assessing test results, with 85% claiming no difficulties.

Respondents were almost evenly split between applying tests pre-harvest or on landed batches, however the latter included processors who don't really have the choice and therefore producers favour testing before harvest. This can vary from 'real time' i.e. immediately before harvest (samples returned to base by fast boat or car), to taking a sample the evening before harvesting the following day.

When queried about a greater likelihood of repeating a test on the same batch of shellfish when there was an initial positive result, 40% agreed; however several producers, including those both agreeing and others who disagreed, claimed that the decision would depend upon other relevant information, including phytoplankton samples, previous results (own and others) and environmental observations. The bias towards repeating a positive result test appears at least partly linked to the degree of confidence in the kits and perhaps partly (for processors) to the potential financial impact of a positive test (i.e. the dumping of shellfish).

Users were questioned on their response in the event of a positive kit result, with a variety of options offered. For scallops, the responses were all identical (almost all were for harvested product), if the whole animal test was positive, the shells were all sent for shucking/processing (and that EPT of the shucked product 'always/inevitably/100%' produced negative results). In the case of other shellfish, the majority of respondents would suspend harvesting, indicating belief in kit results, and the balance would suspend harvesting and repeat the test or suspend harvesting and send a sample to a laboratory for confirmation (particularly if 'concerned' about a particular positive result).

A majority of respondents accessed laboratory based methods in addition to the field tests, mainly Neogen Ltd. but also Cefas and one user of the Agri-Food and Biosciences Institute (AFBI), which is the national reference laboratory for marine toxins in the UK. A number of kit users have access to lab results, as their sites are OC sampling points. There are only a small number of producers who reject the use of lab methods, and this is mainly on the grounds of time ('too slow') rather than cost. Indeed one scallop fisher has switched to using laboratory tests rather than kits allegedly on the grounds of cost (four samples for the three toxin groups per year submitted to Neogen Ltd. for lab based testing).

Costs were noted to be a significant issue for industry. Set up costs for the current Neogen system were reported at:

- £1,500 for the reader;
- £700 for the hot plate for hydrolysis (for DSP); and
- An estimated £300-£500 for miscellaneous pieces of equipment (blender, pipettes, balance, etc).

Reported on-going charges for the Neogen test 'strips' supplied in packs of 24 allegedly varied from £240 to £265 (with outlier reports – one each - of £225 and £300). It's noted that the costs quoted by operators may vary due to possibly poor recollection. This reflects a common perception that the cost of the strips was 'around £10 each'. The consensus 'All Up' cost per test was in the range of £15 - £25, however this – and two higher estimates of £40 and £50 - reflect judgements on costs, labour

charges, allocation of costs to the testing 'space', etc. For the Scotia kits, the user reported a Scotia charge of £400/pack of 24. This price would equal £16.67/strip in contrast to the £11.04/strip for Neogen, however there is no capital expense for the plate reader.

The expense of the start-up equipment for the Neogen kits has led to examples of collaboration between producers:

- In Lochaber, four growers share a plate reader;
- In Shetland all the producers apart from the largest company use the services of the Seafood Shetland Quality Control laboratory;
- In the Western Isles the Council and HIE are assisting the industry establish an End Product Testing regime (using Neogen kits) by funding the purchase and installation of an additional three plate readers to add to the current reader, four hot plates for DSP hydrolysis and the associated equipment and consumables. The equipment will be located across the islands, in Lochs (the current reader), Lochmaddy, Kilbride and Barra;
- In addition there appears to be a number of informal arrangements, whereby growers/fishermen purchase packs of strips and hand them over to a more proficient individual to carry out the test.

With regard to the FSAS publications (and the Appendices relating to field test kits), 75% of respondents found the 2011 leaflet useful, rising to 83% for the 2014 document. Specifically, the usefulness of the Appendices relating to field test kits rose from below 30% for the 2011 leaflet to 50% for the 2014 document. On a further positive note, over 70% of respondents stated they would welcome greater advice from the FSA and FSS on test kits, although this was largely a request for endorsement of specific suppliers and kits.

Further operator observations of interest included:

- One operator uses kits pre- and post-depuration, as they cite that toxins may be present in the water supply;
- An FBO uses kits following the first post-winter algal bloom only;
- Some operators preferred the previous colour card assessment (five shades against control line) used by Neogen to the Accuscan Pro reader (perhaps reflecting a science background);
- One operator noted that the toxin management was 'All a bit muddled – some harvesters test, some don't';
- Several operators commented that it was too expensive and that they hope for cheaper kits;
- It was suggested that information on kits should be distributed to all operators of Approved Establishments, as many fisheries (clams, razors, etc.) are 'not aware';
- One operator noted that they are looking for a kit for AZA; also a Workshop focusing on research and kit developments would be helpful.

SECTION SEVEN: STATUS OF FIELD TEST DEVELOPMENT FOR AZASPIRACID, YESSOTOXIN AND PECTENOTOXIN GROUPS

7.1 Status of methods under development for azaspiracid

The development of field methods for AZAs has been slow mainly due to limited access to purified toxins. Research to date has mainly focused on the production of antibodies where both monoclonal and polyclonal antibodies against AZA have been successfully raised by different groups (Forsyth et al., 2006; Frederick et al., 2009). The polyclonal ovine antibodies were raised against a synthetic hapten, which represented the C28-C40 domain, which is invariant among the AZAs. These antibodies were tested against a partially purified naturally contaminated mussel sample, shown by LC-MS/MS to contain the congeners AZA-1, -2, -3 and -6 and the degree of inhibition of antibody binding was consistent with its total AZA content, rather than by AZA-1 alone. The results suggest that the antibodies raised had similar affinity for AZA-2, -3 and -6 as they did for AZA-1, which was further corroborated through immunoaffinity chromatography (Forsyth et al., 2006). This work is currently being progressed by Samdal and co-workers (Norwegian Veterinary Institute) who have made further refinements to the antibody generation. They have recently published results on the development of an ELISA for quantitating AZA in shellfish (Samdal et al., 2015). It is reported to have an LoQ of 57 µg/kg (well below the MPL), and have cross reactivity to AZA-1-10, AZA-33, AZA-34 and AZA-37-*epi*-1. The method was shown to correlate well with LC-MS/MS on both spiked and naturally contaminated shellfish.

The successful generation of a panel of monoclonal antibodies to synthetic AZA was reported in 2009 (Frederick et al., 2009). These monoclonal antibodies were used by the Frederick/Nicolaou/Botana groups who reported in 2014 on a microsphere-based immunoassay for the detection of AZAs using a Luminex analyser (Rodríguez et al., 2014). The Luminex analysers ease of use and low reagent cost make it applicable for use in a field lab but the overall cost of the analyser (>€100,000) may be prohibitive, although there are cheaper alternatives produced by different companies. The immunoassay is capable of detecting AZAs at concentrations below the EU regulatory limit although no commercialisation routes for this technology are envisaged.

Research carried out by O'Kennedy and co-workers (Dublin City University, Dublin) and Elliot and co-workers (Queens University, Belfast) in the late 2000s sought to generate a recombinant antibody to AZA for incorporation into a specific and sensitive immunoassay-based screening test. However, immunisation of mice with their produced synthetic AZA-KLH conjugate resulted in the immediate death of the animals (Stack, 2011).

Campbell and co-workers (Queens University, Belfast) are currently developing a field test method and they have successfully raised antibodies against AZA. This work has not been published to date.

7.2 Status of methods under development for yessotoxin

There have been several biosensor-based techniques developed with surface plasmon resonance and resonant mirror biosensors based on their interaction with phosphodiesterase enzymes (Fonfria et al., 2008; Mouri et al., 2009; Pazos et al., 2005; Pazos et al., 2004). This interaction produced low detection limits but their specificity was not as good as immune-based biosensors with interactions of the phosphodiesterase and other polyethers and a brevetoxin reported (Mouri et al., 2009). These biosensor technologies, although applicable for use in a field lab due to their ease of use and low cost, have not been made commercially available due to insufficient interest from commercial providers. A microplate assay that is based on the activation of phosphodiesterase enzymatic activity by YTXs (Alfonso et al., 2004) and a direct assay that detects the interaction of the toxins with phosphodiesterase by fluorescence polarisation (Alfonso et al., 2005) have also been developed but

these technologies have not been made commercially available either. Section 4 provides details on the basis of the functional assays described.

AgResearch (New Zealand) in collaboration with the National Veterinary Institute (Norway) developed the first competitive ELISA detection method for the quantification of YTX and its congeners in the early 2000s (Briggs et al., 2004). Polyclonal antibodies were successfully raised which had broad specificity for a large number of YTX congeners, both EU regulated and non-regulated. A pilot product based on this technology was produced by Biosense (Bergen, Norway) but this line was discontinued due to insufficient market interest.

While several assays for YTX have been reported, none has been subjected to SLV or ILS to our knowledge, or is utilised in regulatory monitoring programmes to date. The apparent lack of impetus to validate and implement alternate field methods for YTX may relate to the apparent lack of human illness attributed to YTX.

7.3 Status of methods under development for pectenotoxin

There are very few detection methods for PTXs, particularly those that incorporate a biological component. An ELISA method for PTX was developed in Japan in the early 1990s for which monoclonal antibodies were successfully raised (Sasaki, 1993) but this was never made into a commercial product. Briggs and co-workers (AgResearch, New Zealand) progressed research in this area in the early 2000s by successfully raising ovine polyclonal antibodies, although lack of purified toxin for all the PTX congeners prevented cross reactivity studies to be completed (per. comm. Dr. Christopher Miles, Norwegian Veterinary Institute). Although the kit had very good sensitivities it was never made commercially available. Functional, multi-toxin detection assays have also been developed based on cytotoxicity in different cell models (Cañete and Diogène, 2008) and on the induction in hepatocytes of apoptosis or cytotoxicity by different toxins (Fladmark et al., 1998).

SECTION EIGHT: OVERVIEW OF FIELD METHODS FOR CHEMICAL CONTAMINANT DETECTION

8.1 Scope

This section involves a review of field methods that are currently available for the detection of various contaminants in shellfish tissues. The contaminants that are currently regulated in shellfish in the UK and have been identified as relevant for the purposes of this review include heavy metals (lead, cadmium and mercury), polyaromatic hydrocarbons (PAHs) and polychlorinated dioxins plus dioxin-like polychlorinated biphenyls (PCBs). The maximum permitted levels in shellfish are given in Table 8.1.

Table 8.1: Maximum permitted level for chemical contaminants in shellfish flesh[a]

Contaminant	Maximum level (wet weight)
Lead	1.5 mg/kg
Cadmium	1.0 mg/kg
Mercury	0.5 mg/kg
PAHs	5 µg/kg for benzo(a)pyrene 30 µg/kg for sum of benzo(a)pyrene, benzanthracene, benzo(b)fluoranthene and chrysene
Dioxins and PCBs	3.5 pg/g for sum of dioxins 6.5 pg/g for sum of dioxins and dioxin-like PCBs

[a] Levels should not exceed those laid out in EC Regulation 1881/2006, as amended: (http://www.foodstandards.gov.scot/food-safety-standards/advice-business-and-industry/shellfish)

Similar to marine toxins, instrumental techniques in qualified laboratories, highly specialised for dioxins, are the basis of standard methods for determining chemical contaminants in environmental samples including shellfish and other seafood. In addition to representative samples, there is a need for precautions against adventitious contamination, especially during sample storage and preparation for testing. Field methods that may be used for the detection of the contaminants identified in Table 8.1 are discussed in Sections 8.2 – 8.4, along with commentary on the potential to transfer and adapt the technology for shellfish testing (Section 8.5).

8.2 Heavy metals

Field test kits based on chromogenic reagents are available for some of the heavy metals that can be used in preliminary measurements e.g. on water and sediments from estuaries (US-EPA, 2006). There are a range of suppliers who offer reagents, kits and programmable photometers for testing a wide range of analytes, mainly in water samples. These types of kits have the advantage of being designed for relatively unsophisticated laboratory use and are generally backed by rigorous systems of advice and quality assurance. For example Merck Gmbh offer their Spectroquant kits for lead, cadmium and mercury with LODs in clean water of 10, 2 and 25 µg/L respectively. Provided suitable sample preparation methods are validated for shellfish flesh, these kits could potentially reach the FSA/FSS maximum levels for heavy metals (dilution factors could be as high as 100 for lead and cadmium). A review of testing for lead in blood by WHO (2011) is relevant to shellfish but only instrumental methods are listed as suitable.

Electrochemical sensors for heavy metals have been developed based on glassy carbon electrodes treated with a variety of films to help achieve adequate sensitivity and selectivity. For example, a

portable laboratory monitor is available commercially that can determine a full range of heavy metals in water to µg/L levels (Modern Water, UK). The LODs in water for lead, cadmium and mercury are 0.5, 0.5 and 0.1 µg/L respectively. Applications to biota such as shellfish flesh have not been reported but these low LODs imply extracts could have high dilution factors to enable the monitor to detect metals above the FSA maximum levels.

Heavy metals are also amenable to immunoassay techniques. The route to raising antibodies to heavy metals is following chelation e.g. with EDTA or glutathione. Although ELISA methods based on these principles have been published for cadmium, lead and mercury, only the latter method has been commercialised as the BiMelyze Field Screening Assay (BioNebraska Inc). It has been validated against instrumental methods and accepted as part of method 4500 for screening of soils and solid wastes for mercury (US-EPA, 1998). This kit has not been adapted to biological samples such as seafood and may no longer be commercially available.

Methyl mercury, an important derivative accumulated in seafood, is an added complication. It requires specialised extraction/digestion methods and is prone to volatilisation losses. The mercury must be oxidised to inorganic mercuric ion for all these non-instrumental tests.

Metallothioneins (MTs), involving cysteine complexes, are synthesised by higher organisms in response to challenges from toxic metals. They serve as exposure biomarkers and ELISA kits are available for their analysis, including in fish tissues. However, the level of MTs is only indirectly related to the levels of heavy metals and there is no metal speciation. Therefore their application to regulatory testing of metals in shellfish may be minimal.

8.3 Polyaromatic hydrocarbons

Polyaromatic hydrocarbons (PAHs) are persistent, lipophilic hydrocarbon components of crude oil, petroleum products and their combustion by-products. As such they can accumulate from polluted marine waters into sediments and bivalve molluscs and are markers of incidents such as oil spills. There is a very wide range of PAH congeners. Regulation is generally confined to a subset of the more common, carcinogenic congeners including benzo(a)pyrene, the most toxic. Their testing by instrumental procedures is well established and LC-FL screening for PAHs in water, sediments or foods is relatively fast, effective and inexpensive e.g. US-EPA Method 8310; ISO 17993:2002; ISO 22959:2009. Depending on the end-use of the results, more sensitive and specific methods based on GC-MS or LC-MS may be required.

Several enzyme immunoassay (ELISA) kits for PAHs in water or soil are available. The RaPID test (Modern Water Plc., SDI Inc.) uses tubes in a direct competitive format and achieves high sensitivities for some carcinogenic PAHs through use of selected polyclonal antibodies bound to magnetic particles. This kit uses filtered crude extracts (10 g dry soil plus 20 mL 100% methanol) and has been validated as EPA SW-846 method 4035. Standard ELISAs for PAHs in a direct competitive, multiwell plate format are available e.g. Abnova. They utilise monoclonal benz[a]pyrene antibodies but have broad cross-reactivity to other PAHs. These kits are marketed for research only and specified for water samples. A dilution factor of two can be applied to the LOQs in water provided that the RaPID extraction procedure for soils is suitable for use of these ELISAs with shellfish. Table 8.2 summarises some LOQs for the methods.

Table 8.2: Limit of quantitation of LC-FLD and ELISA methods for PAHs

	LOQ µg per kg			
Method	**benzo(a)pyrene**	**1,2-benzanthracene**	**benzo(b)fluoranthene**	**chrysene**
LC-FLD (fish)	5	5	10	10
RaPID ELISA (soil)	20	6	16	8
Abnova ELISA (soil, est.)	20	25	23	42

In the case of the LC-FLD method, or other chromatographic methods, a wide range of individual PAHs can be determined and the LOQ for the sum of the PAHs is approximately the sum of the LOQs for the individual analytes i.e. ca 30 µg/kg for the four carcinogenic PAHs regulated in shellfish. In the case of the ELISA methods, a range of PAHs can compete for binding with efficiencies depending on their structure and the specificity of the antibodies. Therefore the results from the assay are an approximation of the total PAHs, depending on the congeners present and their cross-reactivities. As there will be a wider range of PAHs present in field samples than just the four regulated PAHs, an ELISA result is likely to be an overestimate of the regulated sum. However, the two ELISAs in the table appear to be adequate for rapid screening of soil samples to flag those exceeding ca 30 µg/kg total PAH. The literature on ELISA performance for PAHs in soil indicates correlations with total PAHs by chromatographic methods are adequate for screening and false negatives are uncommon (Kramer, 1998; Nording et al., 2006; Fillmann et al., 2007).

An ELISA method adapted to shellfish matrices requires an efficient extraction step that provides suitable extracts (solvent compatibility; lack of interferences). The QuEChERS extraction/clean-up protocol (AOAC 2007-01), originally developed and validated for pesticide residue determination in foods, has been shown to be effective for PAH determination in soil and fish by HLPC-FL (Pule et al., 2012a & b). The use of acetonitrile as an extracting solvent in a salting-out condition gave high extraction yields and clean extracts for PAHs. The method does not require co-solvents or evaporations and acetonitrile is compatible with ELISA after dilution to <20% v/v. Commercial kits are available containing extraction tubes, salts, adsorbent and filters. Therefore the QuEChERs procedure should be suitable for use in field laboratories with ELISA tests for PAHs and other low-medium molecular weight contaminants in shellfish.

Immunological techniques are the basis for a range of biosensors for PAHs reported in the scientific literature. These include immuno-sensors utilising surface plasmon resonance (SPR), piezo-electric or amperometric signal transduction. Although detection limits for PAHs in some cases reached levels similar to those for the best ELISAs, no performance data or validations have been reported for real-world samples other than water.

8.4 Dioxins and dioxin-like PCBs

Polychlorinated dibenzodioxins, dibenzofurans and coplanar biphenyls are amongst the most toxic and persistent compounds known, especially 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). There are a large number of congeners of these dioxin-like compounds (DLCs) but many are either minor components or of lower toxicity. They act through the aryl hydrogen receptor (AhR) which is activated by exposure to extremely low concentrations of DLCs. The extremely high lipophilicity of these compounds has implications both for their extraction and clean-up from complex matrices and for their detection by biochemical methods, which rely on aqueous based substrates. The status of immunoanalysis methods for DLCs has been reviewed recently (Tian et al., 2012). A variety of

methods have been reported but only the CALUX assay and an ELISA are of sufficient sensitivity and adequate development stage to approach the validated performance of the reference instrumental method (HRGC-HRMS, EPA method 8295). The CALUX assay is based on recombinant cell lines, stably transfected with dioxin responsive firefly luciferase reporter genes. When dioxins activate AhR, expression of the luciferase reporter gene is induced. Luciferase is easily assayed in a luminometer after addition of luciferin substrate. Validations of CALUX for DLCs in fish (Tsutsumi et al., 2003; Hoogenboom et al., 2006) and fish oils (Hasegawa et al., 2007) have generally demonstrated good correlations with those for the reference method, taking into account the uncertainties and interpretations in the TEQ factors for the range of PCDD, PCDF and DL-PCB congeners present. Although the CALUX assay is sensitive (LOQ 2-5 ng TEQ/kg) and of adequate precision, it requires maintenance of mammalian cell lines, usually rat liver, and is therefore less suited to field laboratories. The related Procept Rapid Dioxin assay is based on the AhR reporter gene activating production of a DNA fragment that is sensitively detected by PCR amplification. This assay has been commercialised (Eichrom Technologies Inc) and evaluated for testing of soil and sediment samples by US-EPA as Method 4430 (Billets, 2011). The biochemical reagents are all supplied *in vitro* in kits including the Ah-Receptor, the aryl hydrocarbon nuclear translocator protein (ARNT) and a small DNA response element (DRE). Therefore the assay does not require cell cultures. However, it has not been validated for matrices other than soils and sediments. Both CALUX and Procept assays require clean extracts and therefore the sample extraction/clean-up procedures are no less complex than those for the reference method.

US-EPA has established an official immunoassay (Method 4025) currently based on a commercially available ELISA kit (CAPE Technolgies LLC, 2009a) that uses monoclonal antibodies raised against TCDD. Reported limits of quantitation for soil are ca. 2 ng TEQ/kg, a sensitivity adequate for regulatory testing for TCDD and other DLCs. The kits provide results that are well correlated to the Toxic Equivalency Quotients (TEQs) determined by the GC-MS reference method. One study showed a statistically non-significant 12% over-estimation by ELISA of DLCs in soils and sediments at 10-10,000 ng TEQ/kg levels (Van Emon et al. 2008). However, in another study on soils containing background levels of DLCs (0.5 to 2 ng TEQ/kg), the ELISA overestimated by a factor of 10 (Deng etal. 2015). ELISA for DLCs has been mainly applied to environmental investigations e.g. Harrison et al. (2010); Deng et al. (2015). Sample extraction/clean-up kits and simplified protocols have enabled testing to be conducted in field facilities with high sample throughputs (40 samples per day) and low cost (US$125/sample; ca. 10% of the reference method). This has enabled intensive sampling of soils or sediments to provide statistically valid environmental screening of large areas for DLCs.

ELISAs for DLCs have been incorporated into validated methods for some biota (Shan et al., 2001) and human milk (Sugawara et al., 2002). Achieving low LOQs required relatively complex sample extraction /clean-up protocols to provide clean, highly concentrated extracts suitable for ELISA. In a related study on dioxin-like PCBs in retail fish, Tsutsumi et al (2006) prepared tissue samples by alkali digestion, solvent extraction and multi-layer column chromatography clean-up. The results for fish extracts obtained using an ELISA based on monoclonal antibodies to PCB-118 correlated well with TEQ concentrations for dioxin-like PCBs obtained by HRGC/HRMS ($r = 0.92$, $n = 26$). Cape Technologies (2009b) have broadly outlined extraction conditions for various foods and feeds to prepare extracts for use with their DLC ELISA but there are no validated protocols for testing shellfish.

8.5 Adaptability of methods for shellfish testing

There are chromogenic, electrochemical and immunoassay kits available, which can detect heavy metals at low concentrations, in particular lead, cadmium and mercury. Some of these tests are packaged with monitoring units that could provide convenience, flexibility (other metals) and some

quality control aspects for small field laboratories e.g. the electrochemistry-based Modern Water PDV6000ultra. It is probable that fully quantitative tests based on this technology could be developed for testing for metals in shellfish. However, there would need to be some method validation before these tests could be recommended. In addition, the inclusion of methyl mercury in the test protocol for mercury would need to be covered.

There are several ELISA kits that may be suitable for screening of PAHs in shellfish e.g. RaPID cPAH but again there is little validation data for foods and none for shellfish. It is likely that suitable extraction/clean-up protocols could be developed e.g. QuEChERS. But the tests could only ever provide screening results for total PAHs due to the complex and varying patterns of PAHs, depending on source, and the varying selectivity of the antibodies for PAH congeners. A suitable decision point below the regulatory limit would need to be established for sending shellfish batches for confirmation of individual congener levels by instrumental methods. The relatively low cost and more rapid screening ability of ELISA could become very relevant following a major environmental pollution event that might contaminate shellfish beds over a wide area e.g. an oil spill.

Testing of foods for dioxin-like compounds (DLCs) is dominated by complex instrumental methods. These speciate the wide range of toxic congeners that are relevant. To achieve low detection limits requires sample extracts that have been subjected to high degrees of clean-up and are highly concentrated. The standard protocols involve large volumes of solvents and evaporation steps. The detection methods also need to be highly sensitive and specific and these attributes have become broadly comparable for DLCs in soil/sediments using instrumental methods and some methods based on ELISA or CALUX. The latter methods still require relatively complex extraction/clean-up protocols and handling of toxic DLC standards. These protocols will not be appropriate for a field laboratory without specialist equipment and training. However, there are no methods based on ELISA or CALUX that have been fully validated for dioxins in shellfish. As with PAHs, these techniques can only ever provide a screening result and confirmation of levels by an instrumental method will be required for samples with TEQ levels exceeding a decision point. Less expensive screening results for DLCs in soil, sediments or fish have proved useful for broad environmental surveys involving relatively large numbers of samples. This role could also be applicable to shellfish that are sentinel species.

The methods described above are based on intrinsic chemical or biological properties of the contaminants. These properties are not shared with marine toxins meaning that direct adaptation of these methods to detection of marine toxins is not possible. For example, the chromogenic assays are based on specific reactions of the metals with various compounds to form coloured complexes, and as such the technology base of these methods cannot be directly transferred for the detection of marine toxins in shellfish. Similarly, ELISAs (and other immunoassays) used for metal, PAH and DLC detection utilise antibodies that are specific to the contaminant of concern and do not cross-react with marine toxins.

SECTION NINE: CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

Field methods are a desirable component of the overall risk management framework for marine toxins, as they are relatively inexpensive tests (i.e. <£15.10 per sample) which enable FBOs to undertake 'real time' testing of shellfish (in <2 hours per sample) prior to harvesting or processing, thereby reducing the potential for placing shellfish on the market that could contain excessive levels of marine toxins.

A major objective of this review was to critically evaluate the performance of commercially available field methods. Key validation characteristics were evaluated in accordance with the acceptable criteria for screening methods established internationally (AOAC, 2005; Codex, 2012; Eurachem, 2014).

In general, the commercially available field methods have appropriate sensitivity, with methods capable of detecting ASP, DSP and PSP at concentrations well below the MPLs. The methods were also found to have acceptable specificity, and did not cross react with non-related toxins or other compounds, and in cases in which precision has been investigated, repeatability estimates are also acceptable (i.e. ≤15%). However, some technical performance issues and knowledge gaps were identified for field methods for each toxin group; these primarily relate to the potential occurrence of false negatives, false positives and the need for inter-laboratory studies.

Issues identified through consultation with the FBOs that utilise field methods relating to the broader operation of field laboratories and use of field methods are also discussed. Recommendations to overcome the technical issues and information gaps identified are presented in Section 9.2.

9.1.1 Potential for false negatives

Data supplied on the use of the ASP field kits suggests that false negative results at or above the MPL are unlikely. The DSP ELISAs and LFAs have a low cross reactivity to DTX-2 raising the possibility that false negative results could occur when analysing shellfish samples that are rich in DTX-2, but have low levels of OA and DTX-1. Approximately 13% of Scottish shellfish contaminated with DSP are dominated by DTX-2 (Section 2.3.2), highlighting the importance of detecting this congener. There is evidence that false negatives occur when the hydrolysis step is not performed to enable the detection of DTX-3. DTX-3 is the most common congener found in Scottish shellfish, emphasising the need to include it in the analysis. Two studies note an incidence of ca. 5-10% false negative results when using the PPIA.

A potential issue was identified regarding the occurrence of false negatives using the Scotia rapid test for DSP, which was attributed to poor extraction of the toxins (Eberhart et al., 2013). Extraction protocols developed for use in conjunction with field methods may have issues with:

1. Too few shellfish and/or insufficient homogenisation to obtain a representative sub-sample of flesh for testing; and
2. Solvent being insufficiently strong, or the solvent/sample ratio too low, to ensure that a high percentage of toxins are extracted; or
3. Extraction conditions too strong such that shellfish co-extractives may interfere with the test.

Extraction conditions for all toxins must be optimised and result in sufficient toxin recovery such that false negative results do not occur at critical levels.

For PSP field methods, the cross reactivity of the kits to most congeners other than STX is low, particularly for GTX-1,4, NEO[15] and the decarbamoyl toxins. GTX-1,4 and the decarbamoyl toxins have been found to be dominant congeners in some UK shellfish (Section 2.3.3), ensuring that the assays are reactive to these congeners is thus of high importance. A desktop exercise was undertaken in this review to determine if the commercially available kits would theoretically give a positive result for samples containing PSP at the MPL and with toxin profiles that are typical of UK shellfish. Using the cross reactivities documented by the suppliers, the exercise highlighted the potential for false negative results to occur (Table 5.10). While the desktop exercise highlights the potential for false negatives, limited comparative data from Neogen, and more extensive data from Scotia, indicates that the false negative rates for the LFAs have been low on samples exceeding the MPL by MBA (toxin profiles not determined) and HPLC. Nonetheless, Scotia Rapid Testing have recently introduced a step which converts GTX-1,4 to NEO prior to detection. The introduction of this step is reported to improve sensitivity of the assay (Turner et al., 2015b) and will reduce the possibility for false negatives.

9.1.2 False positives[16]

The Scotia and Neogen LFAs for ASP, DSP and PSP have been shown to produce positive results when toxin levels are below the MPL. This is to be expected because these qualitative assays are designed to avoid false negatives at the MPL, and thus the trigger level for a positive must be set below the MPL. False positive rates have been found to vary depending on the toxin profile involved. For example, Scotia have found false positive rates for PSP of 3.7, 18, and 25 in different studies of UK shellfish, and Neogen report false positive rates for PSP of 26 and 3% in two studies of naturally contaminated shellfish. Few shellfish have been tested in the UK containing high levels of C-toxins to which some tests are unduly sensitive, given their very low mammalian toxicity. Should blooms of *G. catenatum* occur in UK waters in the future, shellfish could be contaminated with high levels of C-toxins and a high rate of false positives may be likely. False positives have also been noted to occur using the Abraxis ELISA and the Zeulab PPIA for DSP toxins, however the lack of comparative testing of naturally contaminated shellfish using ELISAs alongside analytical methods means that the occurrence of false positives is generally not well understood.

A study undertaken by DeGrasse et al. (2014) is instructive regarding the integration of screening tools for fishermen, the authors note: *"While the JRTs [Scotia Rapid Tests] are simple, easy-to-use kits and no false negatives (worst case scenario from a public health perspective) were observed using them, the high incidence of false positives makes it difficult to incorporate them, as currently calibrated, as the onboard screening tool of choice……Harvesting decisions made based on JRT results would be overly conservative and would prevent harvest in locations where shellfish were in fact safe for human consumption, which is not an economically viable option for the fishing industry."* (DeGrasse et al., 2014).

Given the potential economic burden on industry associated with false positive results (prevention of harvest or withdrawal of product from the market), it is imperative to measure and improve the performance of the field methods. It is also important to establish protocols for clearing or release of shellfish that test positive using the kits for screening, but for which the true toxin concentration may be below the MPL. During bloom events, toxin levels can quickly exceed the MPL and then slowly decline, but may hover around the limit for weeks. In situations such as these it is crucial that FBOs and the FSS have precise information on toxin levels in shellfish to inform management decisions, thus field testing needs to be backed up by timely and cost effective laboratory based

[15] with the exception of the Neogen LFA, which has a high reactivity to NEO

[16] A false positive is considered to be a result which is either positive or >MPL when the true level is <MPL (FPb)

confirmatory tests. Commission Decision 2002/657/EC and the draft CCFFP performance criteria for screening methods note that positive screen tests should be confirmed. In relation to laboratory testing to clear implicated shellfish, FBOs noted perceived problems with laboratory-based testing of shellfish in timeframes that would enable harvested products that were subsequently 'cleared' to be sold in a palatable state, or returned 'live' to the marine environment.

9.1.3 Inter-laboratory study

Only the Biosense ASP ELISA and Zeulab PPIA for DSP have been subjected to full collaborative studies that meet the AOAC and Eurachem validation requirements for quantitative methods (minimum of eight laboratories, five test materials). The Neogen ASP, DSP and PSP LFAs were subjected to limited inter-laboratory evaluations (four, three and five laboratories respectively), but these fall short of meeting the AOAC guidelines for the validation of screening methods (ten laboratories and six test samples required). The EC regulations on alternative marine toxin methods state the need to meet the requirements of the EU RL (Section 2.6), which includes ILS, thus to facilitate acceptance of the LFAs collaborative studies which meet the AOAC guidelines for screening methods would be required.

9.1.4 Quality control

The ELISAs and PPIA assays incorporate positive (saxitoxin standards) and negative (blank) controls in the analysis (generally 12 wells per microtitre plate are dedicated to controls). Additionally, most ELISA and PPIA suppliers recommend that samples be run in duplicate. These QC measures provide assurance that the assays perform as expected, with respect to samples that contain toxins above the MPL or are negative.

The LFAs have an in-built 'control' line, which contains antibodies and should form regardless of the toxin level in the sample. The control line provides assurance that the strip is performing as expected, however it does not control for issues that may occur during the sample preparation and extraction. Ruggedness studies undertaken by Neogen on the PSP LFA suggest that small deviations in the preparation of the extract could impact the final result of the assay e.g. lack of mixing resulted in false negative results for samples containing PSP at the MPL, as did a 10% reduction in the volume of the sample extract added to the buffer (Jawaid et al., 2015). Neither the Scotia nor the Neogen LFA kits incorporate external positive or negative control samples to provide assurance that the assays are giving appropriate responses during field use.

9.1.5 Barriers to industry testing

Affordability

Food business operators identified that cost was a significant barrier to wider adoption of EPT within the industry. The costs associated with undertaking a field test for marine toxins includes several parameters:

- Capital cost of the equipment and materials required to undertake the test
- Cost of the test kit and associated reagents
- Costs for the employment of staff and associated overheads
- Costs associated with buildings/infrastructure for the room/facility in which testing is undertaken.

The ELISAs (£4.81 – £15.10) and PPIA (£10.13 – £12.48) are marginally cheaper on a per sample basis than the LFAs (£11.04 – £14.77)[17] (Tables 5.2, 5.4 and 5.7). However, the capital cost of equipment for the ELISAs and PPIA is more expensive than the LFAs; a microtiter plate absorbance reader (for ELISA or PPIA use) costs ca. £5,000 – £15,000 (depending on functionality of the plate reader), whereas the Neogen strip reader currently retails at £1,123. To overcome issues relating to cost, several collaborations between producers in which equipment was jointly purchased and shared were initiated. In some regions, LAs purchased equipment for the communal use of several producers to overcome financial constraints.

Technical expertise

The majority of FBOs who undertake EPT are currently using the Neogen LFAs. FBOs noted some technical issues in using the kits, including 'difficulties getting them to work' and 'easy to make mistakes'. While the Scotia tests are not widely used by FBOs in the UK, issues with test interpretation regarding evaluating the colour change on the test strip have been noted (Mackintosh and Smith, 2002). A recent study evaluated the use of an automated scanner in conjunction with the Scotia PSP assay and found that this removed the subjectivity of the test (Turner et al., 2015b). This automated scanner can now be purchased through Scotia for use with their ASP and PSP assays. The introduction of the automated strip reader by Neogen was generally viewed by FBOs as positive as it removed the need for FBOs to evaluate the strip colour change. Further training opportunities and workshops to assist overcoming technical issues in using the test kits were welcomed by FBOs.

9.2 Recommendations

The following recommendations have been made to address the technical issues and information gaps that have been highlighted in this review. The recommendations made are directed to FBOs, FSS, test kit suppliers, and/or LAs, as indicated in parenthesis following the recommendations.

Recommendations regarding potential for false negatives:

1. Given the low reactivity of DSP field methods to DTX-2 (Table 5.5) and the prevalence of shellfish containing high proportions of DTX-2 in Scotland, further validation studies should be undertaken to verify that UK shellfish with DSP profiles that are high in DTX-2 produce a positive response at the MPL. [Test kit suppliers]
2. DTX-3 is only detected by DSP field methods if an hydrolysis step is performed. Given the dominance of DTX-3 (OA esters) in UK shellfish (Section 2.3.2), it is recommended that end users of the field methods undertake the hydrolysis step. [FBOs]
3. It is recommended that further validation of PSP field methods is undertaken to ensure that the probability of false negatives at the MPL for commonly found toxin profiles in the UK is acceptable. Such studies should evaluate the method's ability to detect PSPs in shellfish with profiles that match the four categories found in the UK (Section 2.3.1), particularly shellfish with high proportions of GTX-1,4 and decarbamoyl toxins, and containing total concentrations around the MPL [Test kit suppliers]
4. Given the low reactivity of field kits to the PSP congener GTX-1,4, the introduction of an hydrolysis step to convert GTX-1,4 to NEO as implemented recently by Scotia Rapid Testing,

[17]The costings for the ELISAs and PPIA are based on the full utilisation of the 96 well plate i.e. ca. 40 samples run in duplicate simultaneously, costs increase considerably if fewer samples are analysed.

should be considered by other test providers to improve the sensitivity of field methods to this toxin. [Test kit suppliers]

5. Extraction protocols for preparing samples for testing vary widely between field kits and even between validated instrumental methods. Although extraction conditions may vary between toxin classes such as for water-soluble PSPs and lipophilic DSPs, there is scope for rationalisation of methods that would simplify sample preparation and benefit all labs. It is recommended that research is commissioned by FSS to: i) Establish the simplest extraction protocols that provide high and reproducible recoveries for each toxin group from the most important shellfish species; ii) Rationalise these protocols as much as possible for multi-class toxin extraction; iii) Initiate validations to ensure the optimised extraction protocols are compatible with field kits. [FSS]

Recommendations regarding false positives[18]:

6. To assist in reducing the economic impact of positive kit results on the business operations of FBOs, It is recommended that positive kit results are subjected to confirmatory laboratory testing to obtain accurate information on toxin concentrations (consistent with Commission Decision 2002/657/EC and the draft CCFFP performance criteria for screening methods). [FBOs]
7. It is recommended that FBOs provide the results from samples that give positive kit responses and subsequent confirmatory lab tests to a single person/agency that is responsible for collating dual testing results such that false positive rates and the associated economic burden can be evaluated in the future. [FSS and FBOs]
8. It is recommended that a protocol (in the form of a flow chart) be developed and agreed to by FBOs, LAs and the FSS regarding the actions that should be taken when a field method gives a positive result. To assist in overcoming issues relating to false positives with field methods, the protocol should consider ways in which shellfish that are implicated might be 'cleared' or 'released' through an efficient process to obtain laboratory based confirmatory data. Key issues to be addressed include: how results will be confirmed, what timeframes should be adhered to, what happens to product in the interim period and following receipt of confirmation results etc. [FSS, LAs and FBOs]

Recommendations on method acceptance

9. It is recommended that toxin field methods used by FBOs to support decisions on the safety of shellfish are subjected to inter-laboratory studies that are in accordance with the AOAC minimum criteria for qualitative analysis (AOAC, 2005). The results of the ILS could be submitted to the European Union Reference Laboratory for Marine Biotoxins for evaluation to ascertain if the method would be considered as an acceptable alternative method in the EU. [Test kit suppliers]

Recommendations on quality control

10. It is recommended that FBOs using field methods (particularly LFAs) should incorporate the use of positive and negative control samples for ASP, DSP and PSP. Such controls should be run periodically to provide further assurance that the LFAs are producing valid results. It is suggested that the FSS and kit suppliers could explore the feasibility of facilitating the development and distribution of matrix controls using naturally contaminated and toxin-free shellfish homogenates (using samples tested through the OC programme). [Test kit suppliers, FBOs, FSS]

---

[18]A false positive is considered to be a result which is either positive or >MPL when the true level is <MPL (FPb)

Recommendations to improve uptake of EPT by industry

11. It is recommended that FBOs and LAs explore further opportunities for collaboration between producers and processors to form 'field testing centres' responsible for testing shellfish from a wider geographical region in which the costs of capital equipment and operations are shared. [FBOs, LAs]
12. Regarding improving industry access to confirmatory testing facilities, laboratory test turn around times and responsiveness to industry needs, it is recommended that a business case be developed to explore the feasibility of establishing a marine toxin-testing laboratory with analytical capability that is located closer to high volume shellfish production areas such as the Shetland Islands. [FBOs]
13. It is suggested that the FSS and LAs consider facilitating regional marine biotoxin workshops for producers and processors. It is recommended that the workshops provide basic background information on harmful algal blooms, marine toxins, methods of analysis and information resources that are available to FBOs (such as the SAMS phytoplankton forecasts etc). Collaboration with kit providers to hold training sessions in conjunction with the workshops would also be beneficial. [FSS, LAs, Test kit suppliers]

Recommendations on research to broaden the range of field methods available

14. There are currently no commercial field kits for the detection of AZAs in shellfish. Although field methods are also lacking for PTXs and YTXs, these toxin groups are of lower priority due to their lower toxicities and this may lead to their deregulation in shellfish. This review identified that the Norwegian Veterinary Institute (Samdal and co-workers) and Queens University (Campbell and co-workers) have both developed antibodies to the AZAs of regulatory concern and are currently developing/validating assays. It is recommended that consideration be given to providing additional resources to assist in expediting the commercialisation of assays for AZAs. [Test kit suppliers, FSS]
15. Consideration could be given by the FSS to supporting research that aims to develop field focused biosensor methods for toxins in shellfish (such as the planar waveguide devices for marine toxins in water and algal samples). [FSS]

ACKNOWLEDGEMENTS

We gratefully thank the following test kit suppliers for providing method validation information and reviewing this report for accuracy prior to finalisation: Abraxis, Beacon, Bioo Scientific, Biosense Laboratories, Europroxima, Neogen Europe, R-biopharm, Scotia Rapid Teting, Zeulab.

Douglas McLeod is thanked for facilitating the industry survey regarding views on the performance of field methods.

Kasia Kazimierczak is thanked for critical review of this report and the provision of data and information to support the review.

Stephen Cameron is thanked for useful discussions and for review of the recommendation section.

APPENDIX ONE: FOOD BUSINESS OPERATOR SURVEY QUESTIONS

The following questions were posed to each food business operator identified as undertaking testing using toxin field methods:

1. Which kits do you use for which toxins? Have you used different types previously?
2. What were the reasons for selecting this particular kit?
3. Have you used/considered using laboratory methods (and gaining validated quantitative results)? If not, is this because of the cost?
4. Are the kits used onshore (e.g. at your shore base) or at the harvest/ fishing location (i.e. on board a vessel/raft)?
5. Are the kits used **before** harvesting/fishing commences? (Or on a harvested/landed batch of shellfish?)
6. If used onshore, are the tests carried out by a (trained) company person or at a third party resource (e.g. a local technical facility)?
7. Are they sufficiently practical/user friendly for use by non-scientific staff?
8. Have you received adequate/sufficient advice from the supplier in the use of the kits 'in the field' (i.e. no temperature control/cold ambient temperature, unstable working conditions on board vessels?
9. Have you encountered any difficulties in their use?
10. Have you experienced any issues in the interpretation of results? are you more likely to query/repeat a positive result compared to a negative result?
11. How long does it take to carry out a test onshore and/or at a harvesting/ fishing site??
12. Can you give a 'round figure' estimate of the set up costs for using the kits (training, purchase of equipment, set aside of testing area, etc)?
13. What is the charge per kit (either for a single test or a pack of tests)? Do you consider this to be a reasonable charge? What was the total cost to you/your company of the purchase of kits during 2014?
14. What do you do in the event of a positive test result? [Suspend harvesting? Suspend harvesting and repeat test? Continue harvesting and repeat test? Suspend/continue harvesting and send sample for laboratory analysis?]
15. Was the FSAS leaflet ('End Product Testing for Shellfish Toxins') published in 2011 useful in explaining the role of End Product Testing in risk management? Was it useful in identifying the various kits available to purchase?
16. Have you read the FSAS document: 'Managing shellfish toxin risks – Guidance for harvesters and processors'? If so, was the updated information in Appendix A, detailing available test kits and laboratory methods, useful?
17. Would greater advice from the FSAS or alternative sources on suitable kits be a positive development? Are you satisfied with direct liaison with suppliers?

APPENDIX TWO: FOOD BUSINESS OPERATOR RESPONSES TO SURVEY

The following provides a summary of the responses to the questions (Appendix One) posed to each industry operator. Note that the actual questions posed varied slightly from those planned in Appendix One. NA = no answer provided.

Summary of responses:	
Number of operators approached	35
Number of non-responses (3 April)	14
Number of partial responses (e.g. no information on number of lab tests undertaken etc	5
Number of full contacts/replies	16
1. Which kit/toxin?	Neogen: PSP 12 Neogen: DSP 15 Neogen: ASP 9 Scotia: ASP 1
2. Why?	**Neogen:** a) Ease of use, convenience, personal service b) Based in Scotland (not much choice!) c) ‘Only one we could find’ d) 'Presence', Workshop e) Recommendation, reputation f) Cost of ELISA kits used previously g) Earlier collaboration **Scotia:** a) Ease of use in domestic environment b) Neogen start up costs
3. Have you previously used a different kit?	Neogen - No: 13; Neogen - Yes: 3 (2 Jellett, 1 Elisa) Jellett - Yes: 1 (Neogen) NA: 1
4. Considered/used lab methods?	Yes: 8 (Neogen: 4; Cefas: 3; AFBI: 1) No: 7 (OC site: 4; Too slow: 2; Cost: 1) NA: 1 (SSQC)
5. Location for use of kits?	Onshore/Base: 14 Offshore: 0 NA: 3
6. Trained company personnel?	Yes: 14 Other: 1 NA: 2
7. Test before harvesting or landed batch?	Before: 7 Landed batch: 9 NA: 2
8. Sufficiently user friendly?	Yes: Neogen - 11; Scotia - 1 No: Neogen - 3 NA: 2

9. Adequate advice/support?	Yes; Neogen - 14; Scotia - 1 No: 0 NA: 2
10. Difficulties/Issues identified (Neogen):	• Storage at 18-30°C • Use at >18°C • Some fishermen have had difficulties • 'Couldn't get them to work' • Easy to make mistakes • Purchase/delivery of methanol • 2 batches incorrect (positives when tested with tap water) • Damaged strips; under filled buffer • False positives (Cefas for confirmation) • Control line failure
11. Difficulty in interpretation?	Yes: Neogen - 2 (incl 1 'difference with OC results') No: Neogen - 12; Jellett - 1 NA: 2
12. More likely to repeat a positive result?	Yes (6) No (9) NA (1)
13. Time for completing tests?	DSP: 20-30 minutes (max of 90 mins, incl hydrolysis step) PSP: 30 - 60 mins ASP: 30 mins
14. Set up costs?	Consensus (Neogen): £2K (Reader@ £1500 + other kit (£700 for Hot Plate)
15. Kit charge?	Consensus Neogen: £240-£265/pack of 24 (11 of 12 responses); £15-£25/test all up Scotia: £400/pack of 24 (plus customs duty when charged)
16. In the event of a positive result?	Suspend harvesting: 6 Suspend & repeat test: 6 Send scallops for shucking: 3 Suspend and lab for confirmation: 2
17. 2011 leaflet useful?	Yes: 12; No: 4; Can't Remember: 2
18. 2011 Appendix re kits useful?	Yes: 5; No: 10; Can't Remember: 3
19. 2014 Guidance useful?	Yes: 15; No: 3
20. 2014 Appendix re kits useful?	Yes: 9; No: 9
21. Welcome greater advice (kits) from FSAS?	Yes: 13 (assess kits and advise, endorse) No: 5 (2: 'not necessary')
22. Satisfied with direct liaison with supplier?	Yes: 15 No: 2 NA: 1

REFERENCES

Abnova Co., Taipei, Taiwan. Benzo(a)pyrene ELISA kit KA1493. Brochure, 7pp. http://www.abnova.com/products

Alfonso, A., Vieytes, M.R., Yasumoto, T., Botana, L.M., 2004. A rapid microplate fluorescence method to detect yessotoxins based on their capacity to activate phosphodiesterases. Analytical biochemistry 326(1), 93-99.

Alfonso, C., Alfonso, A., Vieytes, M.R., Yasumoto, T., Botana, L.M., 2005. Quantification of yessotoxin using the fluorescence polarization technique and study of the adequate extraction procedure. Analytical biochemistry 344(2), 266-274.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The globally distributed genus Alexandrium: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10-35.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., 1990. Toxin composition variations in one isolate of the dinoflagellate Alexandrium fundyense. Toxicon 28(8), 885-893.

Anon, 2005. Summary of data submitted to the laboratory methods committee, Jellet Rapid Testing, Nova Scotia Canada.

Anon, 2007. The use of the Jellet rapid testing kit for PSP toxins detection in the UK statutory monitoring programme for marine biotoxins. CEFAS, p. 31.

Anonymous, 2013. Scottish sea fisheries statistics 2012. Marine Scotland, Edinburgh, Scotland, pp. 1-110.

AOAC, 2005. Guidelines for collaborative study procedures to validate characteristics of a method of analysis. Official methods of analysis, Appendix D. AOAC International.

Billets, S. (2011). Final report on the performance of the Eichrom Technologies Procept Rapid Dioxin assay for soil and sediment samples. EPA/540/R-11/002. 34pp.

Botana, L.M., Alfonso, A., Botana, A., Vieytes, M.R., Vale, C., Vilariño, N., Louzao, C., 2009. Functional assays for marine toxins as an alternative, high-throughput-screening solution to animal tests. TrAC Trends in Analytical Chemistry 28(5), 603-611.

Botana, L.M., Vilariño, N., Alfonso, A., Vale, C., Louzao, C., Elliott, C.T., Campbell, K., Botana, A.M., 2010. The problem of toxicity equivalent factors in developing alternative methods to animal bioassays for marine-toxin detection. TrAC Trends in Analytical Chemistry 29(11), 1316-1325.

Briggs, L.R., Miles, C.O., Fitzgerald, J.M., Ross, K.M., Garthwaite, I., Towers, N.R., 2004. Enzyme-linked immunosorbent assay for the detection of yessotoxin and its analogues. Journal of agricultural and food chemistry 52(19), 5836-5842.

Campas, M., de la Iglesia, P., Le Berre, M., Kane, M., Diogene, J., Marty, J.L., 2008. Enzymatic recycling-based amperometric immunosensor for the ultrasensitive detection of okadaic acid in shellfish. Biosensors & bioelectronics 24(4), 716-722.

Campas, M., Marty, J.L., 2007. Enzyme sensor for the electrochemical detection of the marine toxin okadaic acid. Anal Chim Acta 605(1), 87-93.

Campas, M., Prieto-Simon, B., Marty, J.L., 2007. Biosensors to detect marine toxins: Assessing seafood safety. Talanta 72(3), 884-895.

Campbell, K., Haughey, S.A., van den Top, H.J., van Egmond, H.P., Vilarino, N., Botana, L.M., Elliot, C., 2010. Single laboratory validation of a surface plasmon resonance biosensor screening method for paralytic shellfish poisoning toxins. Analytical Chemistry 82, 2977-2988.

Campbell, K., Huet, A.C., Charlier, C., Higgins, C., Delahaut, P., Elliott, C.T., 2009. Comparison of ELISA and SPR biosensor technology for the detection of paralytic shellfish poisoning toxins. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 877(32), 4079-4089.

Campbell, K., McGrath, T., Sjolander, S., Hanson, T., Tidare, M., Jansson, O., Moberg, A., Mooney, M., Elliott, C., Buijs, J., 2011a. Use of a novel micro-fluidic device to create arrays for multiplex analysis of large and small molecular weight compounds by surface plasmon resonance. Biosensors & bioelectronics 26(6), 3029-3036.

Campbell, K., McNamee, S.E., Huet, A.C., Delahaut, P., Vilarino, N., Botana, L.M., Poli, M., Elliott, C.T., 2014. Evolving to the optoelectronic mouse for phycotoxin analysis in shellfish. Analytical and bioanalytical chemistry 406(27), 6867-6881.

Campbell, K., Rawn, D.F., Niedzwiadek, B., Elliott, C.T., 2011b. Paralytic shellfish poisoning (PSP) toxin binders for optical biosensor technology: problems and possibilities for the future: a review. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 28(6), 711-725.

Campbell, K., Stewart, L.D., Doucette, G.J., Fodey, T.L., Haughey, S.A., Vilarino, N., Kawatsu, K., Elliot, C., 2007. Assessment of specific binding proteins suitable for the detection of paralytic shellfish poisons using optical biosensor technology. Analytical Chemistry 79, 5906-5914.

Campbell, K., Vilariño, N., Botana, L.M., Elliott, C.T., 2011c. A European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis. TrAC Trends in Analytical Chemistry 30(2), 239-253.

Cañete, E., Diogène, J., 2008. Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma× glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. Toxicon 52(4), 541-550.

Cape Technologies LLC, Maine (2009a). Immunoassay analysis of ppt (pg/g) range PCDD/Fs in soil and sediment using rapid extraction and rapid cleanup. Application Note AN-008. 7 pp. http://www.cape-tech.com/

Cape Technologies LLC, Maine (2009b). Technical considerations for TEQ analysis of foods and related samples. Technical Note TN-008. 3pp.

CEN, 2013. Guideline for statistical data treatment of inter-laboratory tests for validation of analytical methods. CEN/TR 10345, Brussels.

Clarke, D., 2004. PSP Bioassay Validation Report. Marine Institute, Ireland, pp. 1 - 34.

Codex, 2012. Report on the electronic working group on the proposed draft performance criteria for screening methods for marine biotoxins in the standard for raw and live bivalve molluscs, CX/FFP 12/32/8, p. 18.

DeGrasse, S., Conrad, S., DiStefano, P., Vanegas, C., Wallace, D., Jensen, P., Hickey, J.M., Cenci, F., Pitt, J., Deardorff, D., Rubio, F., Easy, D., Donovan, M.A., Laycock, M., Rouse, D., Mullen, J., 2014. Onboard screening dockside testing as a new means of managing paralytic shellfish poisoning risks in federally closed waters. Deep Sea Research Part II: Topical Studies in Oceanography 103, 288-300.

Deng, Y.Y., Jia, L.J., Zhang, K. Yin, H.W. (2015). Combinatorial biochemical and chemical analyses of polychlorinated dibenzo-p-dioxins and dibenzofurans in agricultural soils from Chongming Island, Shanghai, China. *Bull. Eviron. Contam. Toxicol*. **94**, 183-187.

Doucette, G.J., Logan, M.M., Ramsdell, J.S., Van Dolah, F., 1997. Development and preliminary validation of a microtiter plate-based receptor binding assay for paralytic shellfish poisoning toxins. Toxicon 35(5), 625-636.

Doucette, G.J., Powell, C.L., Do, E.U., Byon, C.Y., Cleves, F., McClain, S.G., 2000. Evaluation of 11-[3 H]-tetrodotoxin use in a heterologous receptor binding assay for PSP toxins. Toxicon 38(11), 1465-1474.

Dubois, M., Demoulin, L., Charlier, C., Singh, G., Godefroy, S.B., Campbell, K., Elliott, C.T., Delahaut, P., 2010. Development of ELISAs for detecting domoic acid, okadaic acid, and saxitoxin and their applicability for the detection of marine toxins in samples collected in Belgium. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 27(6), 859-868.

Eberhart, B.T., Moore, L.K., Harrington, N., Adams, N.G., Borchert, J., Trainer, V.L., 2013. Screening tests for the rapid detection of diarrhetic shellfish toxins in Washington State. Mar Drugs 11(10), 3718-3734.

Eberhart, B.T., Moore, L. and Trainer, V.L. 2014. TAP Report Prepared for Dorothy Easy, Scotia Rapid Testing. March 2014. 1 - 15.

EFSA, 2008a. Marine biotoxins in shellfish - okadaic acid and analogues. The EFSA Journal 589, 1-62.

EFSA, 2008b. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – azaspiracids. The EFSA Journal 723, 1-52.

EFSA, 2008c. Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish – yessotoxin group. The EFSA Journal 907, 1 - 62.

EFSA, 2009a. Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish – pectenotoxin group. . The EFSA Journal 1109, 1-47.

EFSA, 2009b. Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish – domoic acid. . The EFSA Journal 1181, 1 - 61.

EFSA, 2009c. Scientific opinion of the panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish - saxitoxin group. The EFSA Journal 1019, 1-76.

EFSA, 2010. Scientific opinion on marine biotoxins in shellfish - emerging toxins: the brevetoxin group. The EFSA Journal 8(7), 1677.

Eurachem, 2014. The fitness for purpose of analytical methods – A laboratory guide to method validation and related topics, In: Magnusson, B., Örnemark, U. (Eds.), 2 ed, p. 62.

Eurachem/CITAC, 2012. Quantifying uncertainty in analytical measurement, In: Ellison, S., Williams, A. (Eds.), 3 ed.

FAO, 2004. Marine biotoxins: food and nutrition paper no. 80. United Nations Food and Agricultural Organisation, Rome, p. 295.

Fillmann, G., Bicego, M.C., Zamboni, A., Fileman, T.W., Depledge, M.H., Readman, J.W. (2007). Validation of immunoassy methods to determine hydrocaron contamination of estuarine sediments. *J. Braz. Chem. Soc*., **18**, 774-781.

Fladmark, K.E., Serres, M.H., Larsen, N.L., Yasumoto, T., Aune, T., DØskeland, S.O., 1998. Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. Toxicon 36(8), 1101-1114.

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(1-2), 167-170.

Forsyth, C.J., Xu, J., Nguyen, S.T., Samdal, I.A., Briggs, L.R., Rundberget, T., Sandvik, M., Miles, C.O., 2006. Antibodies with broad specificity to azaspiracids by use of synthetic haptens. Journal of the American Chemical Society 128(47), 15114-15116.

Frederick, M.O., De Lamo Marin, S., Janda, K.D., Nicolaou, K., Dickerson, T.J., 2009. Monoclonal antibodies with orthogonal azaspiracid epitopes. Chembiochem 10(10), 1625-1629.

FSA, 2014. Managing shellfish toxin risks: Guidance for harvesters and processors, In: Agency, F.S. (Ed.), pp. 1-34.

Garthwaite, I., Ross, K.M., Miles, C.O., Briggs, L.R., Towers, N.R., 2001. Integrated enzyme-linked immunosorbent assay screening system for amnesic, neurotoxic, diarrhetic, and paralytic shellfish poisoning toxins found in New Zealand. J AOAC Int 84(5), 1643-1648.

Gawley, R.E., Mao, H., Haque, M., Thorne, J.B., Pharr, J.S., 2007. Visible fluorescence chemosensor for saxitoxin. Journal of Organic Chemistry 72, 2187-2191.

Gawley, R.E., Shanmugasundaram, M., Thorne, J.B., Tarkka, R.M., 2005. Selective detection of saxitoxin over tetrodotoxin using acridinylmethyl crown ether chemosensor. Toxicon 45(6), 783-787.

González, J.C., Leira, F., Fontal, O.I., Vieytes, M.R., Arevalo, F.F., Vieites, J.M., Bermudez de la Puente, M., Muniz, S., Salgado, C., Yasumoto, T., Botana, L.M., 2002. Inter-laboratory validation of the fluorescent protein phosphatase inhibition assay to determine diarrhetic shellfish toxins: intercomparison with liquid chromatography and mouse bioassay. Analytica Chimica Acta 466, 233-246.

Hallegraeff, G.M., Blackburn, S.I., Doblin, M.A., Bolch, C.J.S., 2012. Global toxicology, ecophysiology and population relationships of the chainforming PST dinoflagellate *Gymnodinium catenatum*. Harmful Algae 14, 130-143.

Harrison, R.O., Scotti, D., Clemmey, T., Doncet, S. (2002). Implementation of EPA method 4025 (immunoassay screening) for dioxin site assessment and remediation. CAPE Technologies Inc., Technical Reference TR-006, 28pp.

Hasegawa, J., Guruge, K.S., Seike, N., Shirai, Y., Yamata, T., Nakamura, M., Handa, H., Yamanaka, N., Miyazaki, S. (2007). Determination of PCDD/Fs and dioxin-like PCBs in fish oils for feed ingredients by congener-specific chemical analysis and CALUX bioassay. *Chemosphere* **69**, 1188-94.

Hess, P., Grune, B., Anderson, D.B., Aune, T., Botana, L.M., Paolo, C., van Egmond, H.P., Halder, M., Hall, S., Lawrence, J., Moffat, C.F., Poletti, R., Richmond, J., Rossini, G.P., Seamer, C., Vilageliu, J.S., 2006. Three Rs approaches in marine biotoxin testing. ATLA 34, 193-224.

Hoogenboom, R.,, Bovee, T., Traag, W., Hoogerbrugge, R., Baumann, B., Portier, L., van de Weg, G., de Vries, J. (2006). The use of the DR CALUX bioassay and indicator polychlorinated biphenyls for screening of elevated levels of dioxins and dioxin-like polychlorinated biphenyls in eel. *Mol. Nutr. Food Res*. **50**, 945-57.

Honkanen, R.E., Stapleton, J.D., Bryan, D.E., Abercrombie, J., 1996. Development of a protein phosphatase-based assay for the detection of phosphatase inhibitors in crude whole cell and animal extracts. Toxicon 34(11), 1385-1392.

Humpage, A.R., Magalhaes, V.F., Froscio, S.M., 2010. Comparison of analytical tools and biological assays for detection of paralytic shellfish poisoning toxins. Analytical and bioanalytical chemistry 397(5), 1655-1671.

ISSC, 2005. Proposal for a rapid screening method for PSP to the ISSC (proposal 05-110). Jellet Rapid Testing Ltd, pp. 66 - 73.

Jaime, E., Gerdts, G., Luckas, B., 2007. In vitro transformation of PSP toxins by different shellfish tissues. Harmful Algae 6(3), 308-316.

Jawaid, W., Campbell, K., Melville, K., Holmes, S.J., Rice, J., Elliott, C.T., 2015. Development and validation of a novel lateral flow immunoassay (LFIA) for the rapid screening of paralytic shellfish toxins (PSTs) from shellfish extracts. Analytical chemistry.

Jawaid, W., Hooper, M., Muirhead, P., Klein, F., Rice, J., 2013a. Validation study of the Reveal 2.0 DSP Test for the qualitative detection of the okadaic acid group of toxins in shellfish. Neogen Corporation, Lansing, USA, p. 14.

Jawaid, W., Meneely, J., Campbell, K., Hooper, M., Melville, K., Holmes, S., Rice, J., Elliott, C., 2013b. Development and validation of the first high performance-lateral flow immunoassay (HP-LFIA) for the rapid screening of domoic acid from shellfish extracts. Talanta 116, 663-669.

Jellett, J.F., Marks, L.J., Stewart, J.E., Dorey, M.L., Watson-Wright, W., Lawrence, J.F., 1992. Paralytic shellfish poison (saxitoxin family) bioassays: automated endpoint determination and standardization of the in vitro tissue culture bioassay, and comparison with the standard mouse bioassay. Toxicon 30(10), 1143-1156.

Kilcoyne, J., Jauffrais, T., Twiner, M., Doucette, G., Aasen, J., Sosa, S., Krock, B., Sechet, V., Nulty, C., Salas, R., Clarke, D., Geraghty, J., Duffy, C., Foley, B., John, U., Quilliam, M., McCarron, P., Miles, C., Silke, J., Cembella, A., Tillman, U., Hess, P., 2014. Azaspiracids - toxicological evaluation, test methods and identification of the source organisms (ASTOX 2). . Marine Environment and Health Series.

Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., 2007a. Single-laboratory validation of the biosense direct competitive enzyme-linked immunosorbent assay (ELISA) for determination of domoic acid toxins in shellfish. J AOAC Int 90(4), 1000-1010.

Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., Goksoyr, A., Briggs, L.R., Holland, P., McNabb, P., 2007b. Determination of domoic acid toxins in shellfish by biosense ASP ELISA - A direct competitive enzyme-linked immunosorbent assay: collaborative study. J AOAC Int 90(4), 1011-1027.

Krämer, P.M. (1998). A strategy to validate immunoassay test kits for TNT and PAHs as a field screening method for contaminated sites in Germany. *Anal. Chim. Acta*, **376**, 3-11.

Kreuzer, M.P., Pravda, M., O'Sullivan, C.K., Guilbault, G.G., 2002. Novel electrochemical immunosensors for seafood toxin analysis. Toxicon 40, 1267-1274.

Krock, B., Tillmann, T., Martens, H., Gu, H.F., Nielsen, L.T., Busch, J., Garcia, F., Place, A., Cembella, A.D., 2014. New toxins in the group of emerging toxins: azaspiracids, dinophysis toxins, spirolides and karlotoxins. , International conference on harmful algae, Wellington, New Zealand.

Krock, B., Tillmann, U., John, U., Cembella, A.D., 2009. Characterization of azaspiracids in plankton size-fractions and isolation of an azaspiracid-producing dinoflagellate from the North Sea. Harmful Algae 8(2), 254-263.

Lawrence, J., Loreal, H., Toyofuku, H., Hess, P., Iddya, K., Ababouch, L., 2011. FAO Fisheries and Aquaculture Technical Paper 551, Assessment and Management of Biotoxin Risks in Bivalve Molluscs, Food and Agriculture Organisation of the United Nations. Food and Agriculture Organisation of the United Nations, Rome, Italy.

Lawrence, J., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. Journal of AOAC International 88(6), 1714-1732.

Laycock, M.V., Donovan, M.A., Easy, D.J., 2010a. Sensitivity of lateral flow tests to mixtures of saxitoxins and applications to shellfish and phytoplankton monitoring. Toxicon 55(2), 597-605.

Laycock, M.V., Donovan, M.A., Easy, D.J., 2010b. Sensitivity of lateral flow tests to mixtures of saxitoxins and applications to shellfish and phytoplankton monitoring. Toxicon 55(2-3), 597-605.

Laycock, M.V., Jellett, J.F., Morgan, W.H., 2003. Characteristics and applications of the Jellet rapid tests for PSP and ASP, In: Holland, P., Quilliam, M.A. (Eds.), HABTECH 2003 Workshop, Nelson, New Zealand.

Leira, F., Alvarez, C., Cabado, A.G., Vieites, J.M., Vieytes, M.R., Botana, L.M., 2003. Development of a F actin-based live-cell fluorimetric microplate assay for diarrhetic shellfish toxins. Analytical biochemistry 317(2), 129-135.

Li, S.-C., Wang, W.-X., Hsieh, D., 2001. Feeding and absorption of the toxic dinoflagellate *Alexandrium tamarense* by two marine bivalves from the South China Sea. Marine Biology 139(4), 617-624.

Litaker, R.W., Stewart, T.N., Eberhart, B.L., Wekell, J.C., Trainer, V.L., Kudela, R.M., Miller, P.E., Roberts, A., Hertz, C., Johnson, T.A., Frankfurter, G., Smith, G.J., Schnetzer, A., Schumaker, J., Bastian, J., Odell, A., Gentien, P., Gal, D., Hardison, D., Tester, P.A., 2008. Rapid enzyme-linked immunosorbent assay for detection of the algal toxin domoic acid. Journal of Shellfish Research 27(5), 1301-1310.

Llewellyn, L., Doyle, J., Negri, A., 1998. A high-throughput, microtiter plate assay for paralytic shellfish poisons using the saxitoxin-specific receptor, saxiphilin. Analytical biochemistry 261, 51-56.

Louzao, M.C., Vieytes, M.R., Baptista de Sousa, J.M., Leira, F., Botana, L.M., 2001. A fluorimetric method based on changes in membrane potential for screening paralytic shellfish toxins in mussels. Analytical biochemistry 289(2), 246-250.

Mackintosh, F., Smith, E., 2002. Evaluation of MIST Alert rapid test kits for the detection of paralytic and amnesic shellfish poisoning toxins in shellfish. Journal of Shellfish Research 21(2), 455-460.

Mackintosh, F.H., Gallacher, S., Shanks, A.M., Smith, E.A., 2002. Assessment of MIST Alert™, a commercial qualitative assay for detection of Paralytic Shellfish Poisoning toxins in bivalve molluscs. Journal of AOAC International 85(3), 632-641.

McLeod, C., 2014. A critical review of the current evidence for the use of indicator shellfish species for the purposes of biotoxin and chemical contaminants monitoring in the Scottish shellfish production areas. Seafood Safety Assessment Ltd., Scotland, pp. 1 - 124.

McNabb, P., Selwood, A., Holland, P., 2005. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. Journal of AOAC International 88(3), 761-772.

McNamee, S.E., Elliott, C.T., Greer, B., Lochhead, M., Campbell, K., 2014. Development of a planar waveguide microarray for the monitoring and early detection of five harmful algal toxins in water and cultures. Environmental science & technology 48(22), 13340-13349.

Meneely, J.P., Campbell, K., Greef, C., Lochhead, M.J., Elliott, C.T., 2013. Development and validation of an ultrasensitive fluorescence planar waveguide biosensor for the detection of paralytic shellfish toxins in marine algae. Biosensors & bioelectronics 41, 691-697.

Merck KGaA, Darmstadt, Germany. *Water and Food Analytics*. Brochure, Sept. 2013.

Micheli, L., Radoi, A., Guarrina, R., Massaud, R., Bala, C., Moscone, D., Palleschi, G., 2004. Disposable immunosensor for the determination of domoic acid in shellfish. Biosensors & bioelectronics 20(2), 190-196.

Modern Water Plc, Guilford, UK. *Trace metal monitoring technologies for field, laboratory and industry*. Brochure, August 2013. http://www.modernwater.com

Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M.A., Truman, P., Hannah, D.J., 1999. Evaluation of the fluorometric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. Toxicon 37, 909-922.

Mountfort, D.O., Suzuki, T., Truman, P., 2001. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Toxicon 39, 383-390.

Mouri, R., Oishi, T., Torikai, K., Ujihara, S., Matsumori, N., Murata, M., Oshima, Y., 2009. Surface plasmon resonance-based detection of ladder-shaped polyethers by inhibition detection method. Bioorganic & medicinal chemistry letters 19(10), 2824-2828.

Munday, R., 2013. Is protein phosphatase inhibition responsible for the toxic effects of okadaic Acid in animals? Toxins 5(2), 267-285.

Munday, R., Thomas, K., Gibbs, R., Murphy, C., Quilliam, M.A., 2013. Acute toxicities of saxitoxin, neosaxitoxin, decarbamoyl saxitoxin and gonyautoxins 1&4 and 2&3 to mice by various routes of administration. Toxicon 76, 77-83.

Munro, L., Wallace, I., Mayes, A., 2013. Scottish shellfish farm production survey: 2012 report. Marine Scotland Science, Edinburgh, Scotland, pp. 1-20.

Nicholson, R.A., Li, G.H., Buenaventura, E., Graham, D., 2002. A rapid and sensitive assay for paralytic shellfish poison (PSP) toxins using mouse brain synaptoneurosomes. Toxion 40, 831-838.

Nording, M., Frech, K., Persson, Y., Forsman, M., Haglund, P. (2006). On the semi-quantification of polycyclic aromatic hydrocarbons in contaminated soil by an enzyme-linked immunosorbent assay kit. *Anal. Chim. Acta*, **555**, 107-113.

Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. Journal of AOAC International 78(2), 528-532.

Pazos, M.J., Alfonso, A., Vieytes, M.R., Yasumoto, T., Botana, L.M., 2005. Kinetic analysis of the interaction between yessotoxin and analogues and immobilized phosphodiesterases using a resonant mirror optical biosensor. Chemical research in toxicology 18(7), 1155-1160.

Pazos, M.J., Alfonso, A., Vieytes, M.R., Yasumoto, T., Vieites, J.M., Botana, L.M., 2004. Resonant mirror biosensor detection method based on yessotoxin-phosphodiesterase interactions. Analytical biochemistry 335(1), 112-118.

Pierotti, S., Albano, C., Milandri, A., Callegari, F., Poletti, R., Rossini, G.P., 2007. A slot blot procedure for the measurement of yessotoxins by a functional assay. Toxicon 49(1), 36-45.

Pierotti, S., Malaguti, C., Milandri, A., Poletti, R., Rossini, G.P., 2003. Functional assay to measure yessotoxins in contaminated mussel samples. Analytical biochemistry 312, 208-216.

Posthuma-Trumpie, G.A., Korf, J., van Amerongen, A., 2009. Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. Analytical and bioanalytical chemistry 393, 560-582.

Pule, B.O., Mmualefe, L.C., Torto, N. (2012a). Analysis of PAHs in soils with Agilent Bond Elut HPLC-FL. Application Note 5152, Agilent Technolgies Inc. 8pp.

Pule, B.O., Mmualefe, L.C., Torto, N. (2012b). Analysis of PAHs in fish with Agilent Bond Elut QuEChERS AOAC kit and HPLC-FL. Application Note 5441, Agilent Technolgies Inc. 8pp.

Raine, R., Wilson, A.M., Hermann, G., Lacaze, J.P., 2011. A comparison of assay techniques for the analysis of diarrhetic shellfish poisoning toxins in shellfish, In: Sauve, G. (Ed.), The international conference on molluscan shellfish safety. Springer Science, Prince Edward Island, Canada, pp. 205-213.

Ramstad, H., Shen, J.L., Larsen, S., Aune, T., 2001. The validity of two HPLC methods and a colorimetric PP2A assay related to the mouse bioassay in quantification of diarrhetic toxins in blue mussels (*Mytilus edulis*). Toxicon 39, 1387-1391.

Rawn, D.F., Niedzwiadek, B., Campbell, K., Higgins, H.C., Elliott, C.T., 2009. Evaluation of surface plasmon resonance relative to high pressure liquid chromatography for the determination of paralytic shellfish toxins. Journal of agricultural and food chemistry 57(21), 10022-10031.

Rehmann, N., Hess, P., Quilliam, M.A., 2008. Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (Mytilus edulis) by ultra ‐ performance liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry 22(4), 549-558.

Rodríguez, L.P., Vilariño, N., Louzao, M.C., Dickerson, T.J., Nicolaou, K., Frederick, M.O., Botana, L.M., 2014. Microsphere-based immunoassay for the detection of azaspiracids. Analytical biochemistry 447, 58-63.

Rossini, G.P., 2005. Functional assays in marine biotoxin detection. Toxicology 207(3), 451-462.

Salas, R., Tillmann, U., John, U., Kilcoyne, J., Burson, A., Cantwell, C., Hess, P., Jauffrais, T., Silke, J., 2011. The role of *Azadinium spinosum* (*Dinophyceae*) in the production of azaspiracid shellfish poisoning in mussels. Harmful Algae 10(6), 774-783.

Samdal, I.A., Løvberg, K.E., Briggs, L.R., Kilcoyne, J., Xu, J., Forsyth, C.J., Miles, C.O., 2015. Development of an ELISA for the Detection of Azaspiracids. Journal of agricultural and food chemistry. *In press.*

Sasaki, K., 1993. Structural elicudiation and preparation of useful derivatives of diarrhetic shellfish toxins, Faculty of Agriculture. Tohuku University.

Shan, .G, Leeman, W.R., Gee, S.J., Sanborn, J.R., Jones, A.D., Chang, D.P.Y., Hammock, B.D.(2001). Highly sensitive dioxin immunoassay and its application to soil and biota samples. *Anal Chim Acta*. **444**, 169–178.

Simon, J.F., Vernoux, J.P., 1994. Highly sensitive assay of okadaic acid using protein phosphatase and paranitrophenyl phosphate. Natural toxins 2, 293-301.

Smienk, H., Domínguez, E., Rodríguez-Velasco, M.L., Clarke, D., Kapp, K., Katikou, P., Cabado, A.G., Otero, A., Vieites, J.M., Razquin, P., Mata, L., 2013. Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal of AOAC International 96(1), 77-85.

Smienk, H.G., Calvo, D., Razquin, P., Dominguez, E., Mata, L., 2012. Single laboratory validation of a ready-to-use phosphatase inhibition assay for detection of okadaic acid toxins. Toxins 4(5), 339-352.

Stack, 2011. Studies on the immunosuppressive effects adn detection of naturally occurring toxins, School of biotechnology. Dublin City University, Ireland, p. 327.

Stevens, R.C., Soelberg, S.D., Eberhart, B.-T.L., Spencer, S., Wekell, J.C., Chinowsky, T.M., Trainer, V.L., Furlong, C.E., 2007. Detection of the toxin domoic acid from clam extracts using a portable surface plasmon resonance biosensor. Harmful Algae 6(2), 166-174.

Sugawara, Y., Saito, K., Ogawa, M., Kobayashi, S., Shan, G., Sanborn, J.R., Hammock, B.D., Nakazawa, H., Matsuki, Y. (2002). Development of a dioxin toxicity evaluation method in human milk by enzyme-linked immunosorbent assay - assay validation for human milk. *Chemosphere*. **46**, 1471-6.

Swan, S.C., Davidson, K., 2012. Monitoring programme for the presence of toxin producing plankton in shellfish production areas in Scotland: Reporting period: 01 January 2011 – 31 December 2011. Scottish Association for Marine Science, Oban, p. 70.

Thompson, M., Ellison, S., Wood, R., 2002. Harmonised guidelines for single laboratory validation of methods of analysis. Pure applied chemistry 74, 835-855.

Tian, W., Xie, H. Q., Fu, H., Pei, X., & Zhao, B. (2012). Immunoanalysis Methods for the Detection of Dioxins and Related Chemicals. *Sensors (Basel)*, **12**, 16710–16731.

Tillmann, U., Elbrächter, M., Krock, B., John, U., Cembella, A., 2009. *Azadinium spinosum* gen. et sp. nov. (Dinophyceae) identified as a primary producer of azaspiracid toxins. European Journal of Phycology 44(1), 63-79.

Tillmann, U., Salas, R., Jauffrais, T., Hess, P., Silke, J., 2014. AZA: The producing organisms: biology and trophic transfer, In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 3 ed. CRC Press, Taylor and Francis Group Boca Raton, p. 26.

Tsutsumi, T., Amakura, Y., Nakamura, M., Brown, D.J., Clark. G.C., Sasaki, K., Toyoda, M., Maitani, T. (2003). Validation of the CALUX bioassay for the screening of PCDD/Fs and dioxin-like PCBs in retail fish. *Analyst* **128**, 486-92.

Tsutsumi, T., Amakura, Y., Okuyama, A., Tanioka, Y., Sakata, K., Sasaki, K., Maitani, T. (2006). Application of an ELISA for PCB 118 to the screening of dioxin-like PCBs in retail fish. *Chemosphere* **65**, 467-73.

Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., Yasumoto, T., 1996. A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination of mussels. Toxicon 34(7), 743-752.

Turner, A.D., Hatfield, R.G., Pun, R., Stubbs, B., Algoet, M., 2014a. Review of the PSP and lipophilic toxin profiles observed in Scottish shellfish submitted through the FSAS Official Control Monitoring Programme between the date of implementation of the analytical methods and December 2013. CEFAS, pp. 1-41.

Turner, A.D., Powell, A., Schofield, A., Lees, D., Baker-Austin, C., 2015a. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Euro Surveillance 20(2), 1-7.

Turner, A.D., Stubbs, B., Coates, L., Dhanji-Rapkova, M., Hatfield, R.G., Lewis, A.M., Rowland-Pilgrim, S., O'Neil, A., Stubbs, P., Ross, S., Baker, C., Algoet, M., 2014b. Variability of paralytic shellfish toxin occurrence and profiles in bivalve molluscs from Great Britain from official control monitoring as determined by pre-column oxidation liquid chromatography and implications for applying immunochemical tests. Harmful Algae 31, 87-99.

Turner, A.D., Tarnovius, S., Johnson, S., Higman, W.A., Algoet, M., 2015b. Testing and application of a refined rapid detection method for paralytic shellfish poisoning toxins in UK shellfish. Toxicon 100, 32-41.

Turner, A.D., Goya, A.B., 2015. Comparison of four rapid test kits for the detection of okadaic acid-group toxins in bivalve shellfish from Argentina. Food Control 59, 829-840.

US-EPA (1998). Method 4500: BiMelyze® Soil Extraction Kit and BiMelyze® Mercury Assay Tube Kit. Protocol, 15pp. ftp://ftp.dec.state.ny.us/der/ASP2005-CD/Exhibit%20D/SW-846%20Files/SW-846-IVa%20Draft/4500.pdf

US-EPA, 2006. Contaminants and Toxic Chemicals: Heavy metals, Pesticides, PCBs, and PAHs. Ch. 12 in: Volunteer Estuary Monitoring Manual, 2nd Ed., EPA-842-B-06-003. http://www.epa.gov/owow/estuaries/monitor/

van de Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., MacNeil, J.D., Quilliam, M.A., 2011. Liquid chromatography post-column oxidation (PCOX) method for the determination of paralytic shellfish toxins in mussels, clams, oysters, and scallops: collaborative study. Journal of AOAC International 94(4), 1154-1176.

van den Top, H.J., Elliott, C.T., Haughey, S.A., Vilariño, 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(11), 4206-4213.

Van Dolah, F., Leighfield, T.A., Haynes, B., Hampson, D., Ramsdell, J.S., 1997. A microplate receptor assay for the amnesic shellfish poisoning toxin, domoic acid, utilizing a cloned glutamate receptor. Analytical biochemistry 245, 102-105.

Van Dolah, F.M., Fire, S.E., Leighfield, T.A., Mikulski, C.M., Doucette, G.J., 2012. Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study. Journal of AOAC International 95(3), 795-812.

Van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., Rawn, D.F., 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. Journal of AOAC International 92(6), 1705-1713.

van Egmond, H., van Apeldoorn, M., Speijers, G., 2004. Food and nutrition paper on marine biotoxins. FAO.

Van Emon, J. M., Chuang, J. C., Lordo, R. A., Schrock, M. E., Nichkova, M., Gee, S. J., & Hammock, B. D. (2008). An enzyme-linked immunosorbent assay for the determination of dioxins in contaminated sediment and soil samples. *Chemosphere*, **72**, 95–103.

Vieytes, M.R., Cabado, A.G., Alfonso, A., Louzao, M.C., Botana, A.M., Botana, L.M., 1993. Solid-phase radioreceptor assay for paralytic shellfish toxins. Analytical biochemistry 211, 87-93.

Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M., Botana, L.M., 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Analytical biochemistry 248, 258-264.

Vilarino, N., Louzao, M.C., Fraga, M., Rodriguez, L.P., Botana, L.M., 2013. Innovative detection methods for aquatic algal toxins and their presence in the food chain. Analytical and bioanalytical chemistry 405(24), 7719-7732.

Villar-González, A., Rodríguez-Velasco, M.L., Gago, A., 2011. Determination of Lipophilic Toxins by LC/MS/MS: Single-Laboratory Validation. Journal of AOAC International 94(3), 909-922.

WHO, 2011. Brief guide to analytical methods for measuring lead in blood. IOMC and World Health Organization, Geneva. 14pp.

Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids: saxitoxin and its analogs. Mar Drugs 8(7), 2185-2211.