SEAFOOD TOMORROW

Nutritious, safe and sustainable seafood for consumers of tomorrow

Grant agreement no: 773400

Deliverable 1.5

Validated prototypes for xenobiotics and marine toxins detection in seafood

Due date of deliverable: 30/04/2021

Actual submission date: 21/05/2021

Start date of the project: 01/11/2017 **Duration: 42 months**

Organisation name of lead contractor: IRTA, BFD, AZTI, QUB

Revision: v1

Project co-funded by the European Commission within the H2020 Programme	
Dissemination Level	
PU Public	X
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

Table of Contents

1. Summary

This task focused on prototyping three new fast screening methods targeting two important groups of contaminants: xenobiotics and marine toxins.

(1) Enzymatic inhibition assay for xenobiotics screening in seafood samples:

In this task, AZTI adapted and optimised a colorimetric CYP-based assay validated at lab-scale for the detection of xenobiotics: polycyclic aromatic hydrocarbons (PAH4 which comprises benzo[a]anthracene (BaA), chrysene (Chry), benzo[b]fluoranthene (BbF) and benzo[a]pyrene (BaP)). The assay was applied to the analysis of samples of salmon and seabass.

(2) Immunoassay for the detection of tetrodotoxins (TTXs) in seafood samples:

In this task, QUB supplied the anti-TTX antibody, and IRTA adapted and optimised a colorimetric immunoassay for the extraction and analysis of sample types mussels, razor clams and oysters.

(3) Optical biochip for multiplex detection of regulated marine toxins in seafood samples:

In this task, QUB adapted and optimised an optical biochip previously developed at lab scale for the simultaneous detection of regulated toxins, i.e. okadaic acid (OA), azaspiracids (AZAs), domoic acid (DA), and saxitoxin (STX) and analogues.

Biorex Food Diagnostics (BFD) supported each developer and contributed to getting each innovative test solution to a stage suitable for commercialisation.

2. Objective

To develop three improved fast screening methods for chemical contaminants, tetrodotoxins (TTXs) and multiple regulated marine toxins.

3. Background

Xenobiotics kits

Xenobiotics, chemical substances such as polycyclic aromatic hydrocarbons (PAHs), perfluorinated compounds (PFCs) and brominated flame retardants (BFRs), are environmental contaminants largely produced during industrial processing or synthetised for use in consumer goods. These compounds can accumulate in fish and seafood and pose a risk to consumers and the environment. Although the EU has established legislations regarding the levels and reference methods for some xenobiotics (e.g. PAHs, metals, dioxins and PCBs), several others (e.g. BFRs and PFCs) have not been addressed yet or only recommendations exist for them.

Tetrodotoxin kit

Tetrodotoxin (TTX) is a potent neurotoxin responsible for food poisoning incidents, mainly related with the consumption of some species pufferfish in tropical or subtropical regions of Asia and the Pacific

Grant agreement: 773400

Islands. Since 2007, TTX has been found in some shellfish from European countries such as the United Kingdom, Greece, the Netherlands, Spain, Italy and France, though concentrations have been low so far. Currently TTX presence in shellfish is not regulated in Europe. There is no maximum permissible level, but the European Food Safety Authority (EFSA) affirms that concentrations below 44 μg of TTX equivalents/kg shellfish meat, based on a large portion size of 400 g, do not result in adverse effects in humans.

Optical biochip for regulated marine toxins

Marine biotoxins, saxitoxin (STX), okadaic acid (OA), domoic acid (DA), azaspiracid (AZA) and their toxin analogues/families, are potent toxins naturally produced by algae that can accumulate in fish and shellfish and pose a serious human health risk if consumed. Under EU-Commission regulation 853/2004, maximum permitted levels of these toxins in shellfish intended for consumption and reference analysis techniques have been established and are implemented by national programmes.

4. Methodology

Each developed innovative test solution was evaluated with a view to commercialisation. Market studies were initially performed (Annexes 1, 2 and 3). Each kit developer (AZTI, IRTA and QUB) provided BFD with specific information for the evaluation of costing, kit advantages/features, equipment and availability of raw materials along with IP restrictions and limitations were all assessed. All data was analysed considering manufacturing processes at BFD and comparing with existing information collected during the market studies. Instructions for use (IFUs) were formulated. Dissemination material (flyers and videos) was prepared:

IFU PAHs:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433991747/D1.5_Instructions%20For%20Use-PAH.pdf

Flyer PAHs: https://seafoodtomorrow.eu/wp-content/uploads/2021/04/PAH-Test-Solution-FlyerV.7.pdf

Video PAHs: https://vimeo.com/537197799

IFU TTXs:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433991748/D1.5_Instructions%20For%20Use-TTX.pdf

Flyer TTXs: https://seafoodtomorrow.eu/wp-content/uploads/2021/04/Tetrodotoxins-Flyer_V3.pdf

Video TTXs: https://vimeo.com/537220370

IFU marine biotoxins:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433991749/D1.5_Instructions%20For%20Use-MMB.pdf

Flyer marine biotoxins: https://seafoodtomorrow.eu/wp-content/uploads/2021/04/Marine-Biotoxins-Flyer_07_04_21.pdf

Grant agreement: 773400

Video marine biotoxins:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/434017262/D1.5_Marine%20Biotoxins%20video.mp4

5. Results and Discussion

5.1. Performance of the fast screening methods

Enzymatic inhibition assay for xenobiotics screening in seafood samples

New methods have been developed to quickly screen the presence of PAHs, PFCs and BRFs in fish. The methods include a simple extraction protocol, where the xenobiotic is extracted from the fish sample, and a novel fluorescence enzyme inhibition assay. Two different extraction protocols have been optimised for polar (PFCs) and apolar (BFRs and PAHs) substances. The assays, which can be performed in 60 minutes, allow the detection of xenobiotics to concentrations of less than 10 µg/kg of PAHs, meeting current regulatory limits, and between 90-500 µg/kg of BFRs and PFCs, with proven precision and accuracy (less than 5% false negatives), repeatability and reproducibility. The methods have been tested and validated for salmon and seabass samples. IFU, flyer and video are in the links above.

Protocols are in the report of milestone 4:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433392188/SEAFOODtomorrow_MS4_v1.pdf

Validation data are in the report of milestone 3:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433392187/SEAFOODtomorrow_MS3_v1.pdf

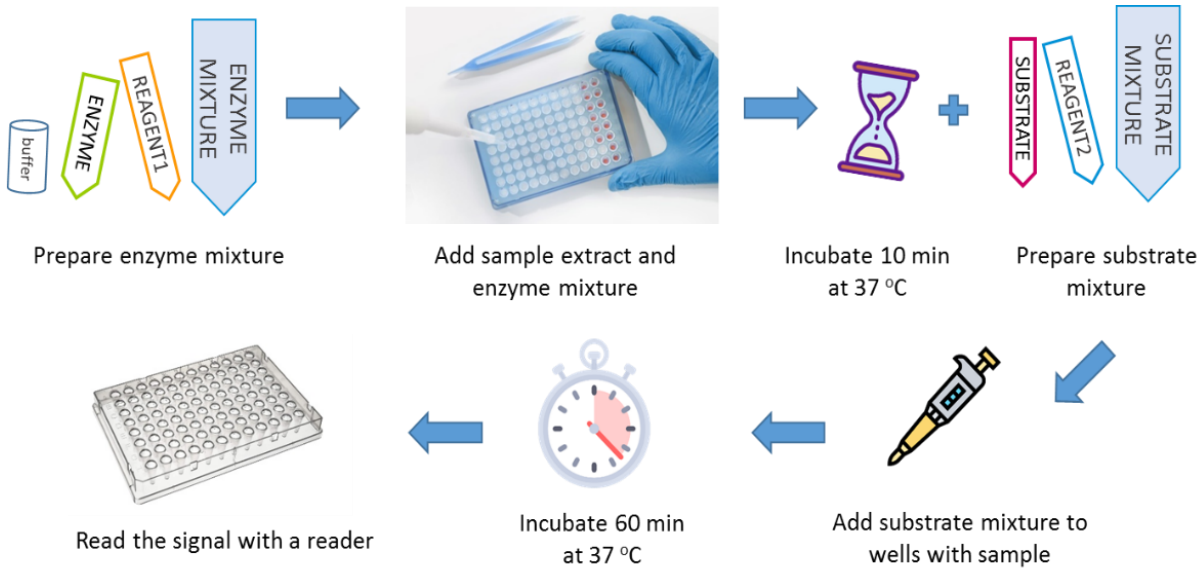

Figure 1. Enzyme inhibition assay for the detection of xenobiotics.

Grant agreement: 773400

Immunoassay for the detection of tetrodotoxins (TTXs) in seafood samples

A new method has been developed to quickly screen the presence of TTXs in shellfish. The method includes a simple and easy-to-implement extraction protocol, where TTX is extracted from a seafood sample, and a novel immunoassay process. This novel immunoassay uses magnetic beads as immobilisation supports and an antibody that specifically recognises TTX and several of its analogues in a competition format. The method allows the detection of TTX at concentrations as low as 1 μg/kg in oysters and razor clams, and 3.3 μg/kg in mussels (levels well below the EFSA guidance threshold). The analysis, which can be performed in 70 minutes, has been validated for three shellfish species (mussels, razor clams and oysters) with proven inter and intra assay precision. This method provides a novel method to guarantee shellfish safety and protect human health. IFU, flyer and video are in the links above.

Protocols are in the report of milestone 4:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433392188/SEAFOODtomorrow_MS4_v1.pdf

Validation data are in the report of milestone 3:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433392187/SEAFOODtomorrow_MS3_v1.pdf

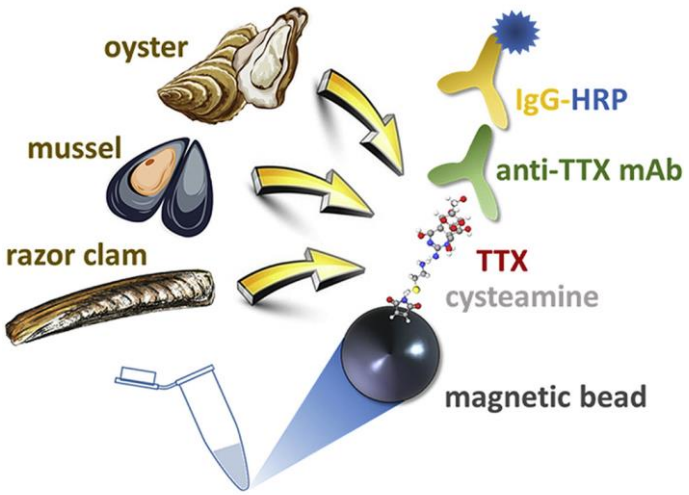

Figure 2. Magnetic bead-based immunoassay for the detection of tetrodotoxins.

Optical biochip for multiplex detection of regulated marine toxins in seafood samples

A new method has been developed to quickly screen the presence of regulated marine biotoxins and TTX (not currently regulated) in shellfish. The method includes simple and easy-to-implement extraction protocols and a novel optical biochip, manufactured using nanoprinting technology, and based on planar waveguide detection of antibody/antigen interactions (immunological competition inhibition assay). Single detection methods have been established for the regulated biotoxins of most concern and TTXs.

Grant agreement: 773400

Users can prepare the shellfish sample extract using low-level technical laboratory equipment, apply the extract to the test cartridge, and read the response on a low cost LightDeck Diagnostics reader which gives a high/low fluorescent signal depending on if the target toxin is present or not. The analysis, which can be performed in 20 minutes, allows for the detection of toxins to concentrations of less that 1ng/mL, meeting all global regulatory requirements, and has been validated as single assays for several shellfish species (mussels and oysters) with proven repeatability and reproducibility. The multiplex format has not been developed, but the technology for single detection has been evaluated and proven fit for purpose for validation requirements. There were substantive delays in this part of the task 1.4 in combining the singleplex assays into a multiplex assay, primarily due to the illness of Katrina Campbell, PI at Queen's University from October 2019 to April 2020. On her return to work, Northern Ireland had entered COVID lockdown, and the university was closed for entry until July 2020. When the university re-opened there were issues with the nanoplotter requiring an engineer to fix the instrument for printing the arrays on the chips. Travel restrictions prevented the engineer / company representative for the equipment from travelling from Germany to Northern Ireland until September 2020. The nanoplotter was deemed broken beyond repair and an order for a new instrument was placed. The instrument arrived and installation was completed in February 2021 towards the end of the extension period of the project. Nonetheless singleplex assays for toxin detection were completed in shellfish matrices. IFU, flyer and video are in the links above.

Protocols are in the report of milestone 4:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433392188/SEAFOODtomorrow_MS4_v1.pdf

Validation data are in the report of milestone 3:
https://asset1.basecamp.com/3890656/projects/18060039/attachments/433392187/SEAFOODtomorrow_MS3_v1.pdf

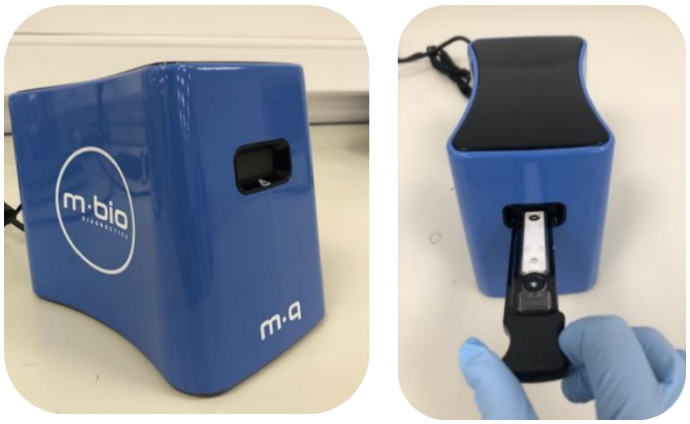

Figure 3. Optical biochip and cartridge for the detection of marine toxins.

Grant agreement: 773400

5.2. Examination of product commerialisation

- Market reports were drafted to investigate the current status of each applicable test solution. Competitors currently present in the market along with regulations to be adhered to were examined in detail. This information was then used to compare the output of each developed test solution to determine the kit advantages, features and limitations (see Annexes 1-3).
- A manufacturing costing was completed that could be used as a guide to determine end user pricing and took into consideration the cost of all raw materials, equipment required, labout cost, packaging, dispense and QC of product. Cost for each test kit was as follows: for xenobiotics (PAH) 117€/kit (48 tests/kit), for tetrodotoxins (TTX) 153€/kit (42 tests/kit), and for marine biotoxins 286€/kit (20 tests/kit) (see deliverable D4.4 - https://asset1.basecamp.com/3890656/projects/18060039/attachments/434041922/SEAFOODtomorrow_D4.4_v1.pdf - for further details).
- To communicate and disseminate the output from this task, protocol videos were produced along with flyers that were used in workshop demostrations as well as in the final event (see deliverable D6.2 - https://asset1.basecamp.com/3890656/projects/18060039/attachments/433991750/SEAFOODtomorrow_D6.2_DEP_v5.pdf - for further details).
- Formulation work instructions were constructed for the Tetrodotoxin Immunoassay and will be available for internal BFD use if commercialisation was to be considered after the end of the project.
- IP, licensing and availability of all raw materials used in the 3 developed test solutions were evaluated and discussed with each test developer:
  - ✓ The antibodies used in the development of the TTX immunoassay and Optical biochip are the propery of QUB and their partners. If antibodies are to be sourced from QUB, the provision would have to be confirmed as available, agreed for use and be economical for kit manufacture.
  - ✓ The optical biochip reader is also required for use at a cost of $4500 and only available from one manufacturer. Appropiate discussions would be required with this manufacturer to discuss if a collaboration is of interest; prior to a partner commencing commercialisation of this developed test solution.
  - ✓ AZTI holds the patent for the enzymatic inhibition assay. If this kit was to be commercialised a discussion between the interested partner and AZTI would have to take place.
- Each test solution was evaluated and limitations of each documented on a factsheet (see deliverable D6.2 - https://asset1.basecamp.com/3890656/projects/18060039/attachments/433991750/SEAFOODtomorrow_D6.2_DEP_v5.pdf - for further details). These were evaluated as part of the commercialisation stage.

Grant agreement: 773400

6. Conclusions

This task has focused on prototyping three new fast screening methods targeting two important groups of contaminants: xenobiotics and marine toxins.

Market reports have been performed. Cost has been evaluated. Kit advantages/features have been analysed. Equipment and availability of raw materials have been assessed. Restrictions and limitations have been identified. Formulations of work instructions and dissemination material (video and flyer) have been prepared.

Grant agreement: 773400

Annex 1 - Market Report for Enzyme Inhibition Assay for Screening of Contaminants in Seafood Samples

Grant agreement: 773400

Helping Create Nutritious, Safe and Sustainable Seafood for Consumers of Tomorrow

Task 1.4: Enzyme Inhibition Assay for Screening of Contaminants in Seafood Samples

Table of Contents

1. Introduction

Polycyclic aromatic hydrocarbons (PAH), a type of persistent organic pollutant, are ubiquitous environmental contaminants. They occur naturally in crude oil, coal and from volcanoes or forest fires, or can be produced through anthropogenic activities such as industrial power generation, vehicle emissions and the incomplete combustion of materials including coal, oil, wood and gas. There are more than 500 known PAHs, and approximately 16 are universally found (Abdel-Shafy and Mansour, 2016). Historically, the confirmation of benzo(a)pyrene present in a food item or substance, was an indicator of PAH contamination. Benzo[a]pyrene is described by the IARC (International Agency on Research for Cancer) as a substance which is carcinogenic to humans (group 1), tracking the presence within food is of the utmost importance (Lerda, 2011). The EU Regulation Commission concluded in 2011 that measurement of one PAH was not a reliable enough marker for presence of all PAHs in food, and so a system of measurement of four PAH compounds was developed. In addition to the maximum level (ML) of benzo[a]pyrene (still measured separately to allow for comparison to older data), a sum ML exists for the following: benzo[a]pyrene, benz[a]anthracene, benzo[b] fluoranthene, and chrysene (PAH4) which is now the official marker of PAH contamination in food samples. The European Food Safety Authority also concluded that the use of more substances in a system (e.g. the proposed PAH8) would not provide much added value when compared to the system PAH4 (European Commission, 2011).

Individuals can become exposed to PAHs through various routes, such as:

- breathing in air contaminated with vehicle exhaust fumes, cigarette smoke or smoke from burning wood or coal
- dermal contact with polluted water from farm run-off, sewage and breakdown of plastics/micro-plastics
- ingestion of contaminated water
- ingestion contaminated food which has been sourced from or stored in a polluted place or food which has been cooked/prepared in a polluted manner
  (Kim, Jahan, Kabir and Brown, 2013)

1.1. PAHs in food

PAHs can be formed during processing and domestic food preparation, e.g. through drying, smoking, roasting, baking, frying or grilling. Vegetables can be contaminated through deposition of airborne particles or through being grown in polluted soils (Zelinkova and Wenzl, 2015). Fresh meat, fish, poultry and eggs will not usually contain high levels of PAHs naturally due to metabolism in the species, however marine organisms such as mussels, oyster (filter-feeding bivalves) and lobsters will absorb and

accumulate PAHs from bodies of water (which may have previously been polluted by oil spills or sewage/faming run-off) (European Commission, 2002).

Cooking methods which expose meat to an open flame or smoke will contribute to the presence of PAHs as the pyrolysis of the fats within the meat will generate PAHs and therefore become deposited on the meat. PAHs will become harmful to human health and capable of damage to DNA through bio-activation during the metabolism of the molecules. (Hamidi, Hajeb and Selamat, 2016)

2. PAH in Human Health

The primary concern of exposure to PAHs has been repeatedly described as cancer, foetal development and cardiovascular diseases. Cancer in particular has been linked to skin, lung, bladder, stomach amongst other forms and has been well established in numerous animal studies. (Koene, Prizment, Blaes and Konety, 2016)

Observations as early as 1770's by a surgeon in London, described the incidence of scrotal cancer was becoming significantly more associated within the chimney sweep profession, suggesting the exposure to the environmental toxins such as soot was causing the condition. Later in the 1830's a German surgeon made similar observations in skin cancer incidence and its correlation with workers of coal factories; consequently, scientists began to reproduce cancers by topical application of coal and environmental toxins on rabbits.

The study of these aromatic hydrocarbons was augmented due to the continuous association with human health implications, leading to current research, highlighting the importance of monitoring their presence in any aspect of life where direct human contact or consumption would occur.

2.1. Bio-activation

PAHs can induce toxicity/carcinogenesis when activated by xenobiotic-metabolising enzymes (e.g. Cytochrome P450, epoxide hydrolase and aldoketoreductase). P450 (1A1 and 1B1) catalyses the oxidation of PAHs into toxic and carcinogenic products. (Shimada, 2009)

These enzymes (Cytochrome P450, epoxide hydrolase and aldoketoreductase) mainly participate in the conversion of PAHs to more polar/water-soluble metabolites and these metabolites are readily excreted from the body. However during the metabolism process, a variety of unstable and reactive intermediate products are formed which are capable of attack on DNA causing cell toxicity and/or transformation. P450s and epoxide hydrolase convert PAHs into "the ultimate" carcinogenic metabolites, PAH diol epoxides (highly mutagenic metabolite), and aldoketoreductase converts into reactive PAH o-quinones (produce significant amounts of ROS and interacts with DNA to form stable and depurinating DNA

adducts – a segment of DNA bound to a cancer causing chemical). PAHs are also activated by P450 and peroxidases into reactive radical cations which bind covalently to DNA. (Shimada, 2006)

2.2. Requirement for PAH testing

Many PAHs have been previously defined as human carcinogens by the IARC – specifically benzo[a]pyrene, which is a group 1 carcinogen to humans.

However, the measurement of one PAH may not be sustainable as it is possible for samples to be negative for benzo[a]pyrene and positive for other PAHs.

The ability to detect more than one PAH is desirable, giving better insight of presence of PAHs using PAH4, PAH8 or PAH12. (Public Health England, 2018)

3. Current market and competitors

This assay will be targeted at businesses within the food safety and environment, responsible for the enforcement of legislation regarding the prevalence of PAHs in food items. Other potential end users would find these assays valuable, agencies such as water treatment facilities, environmental protection agencies and others that would encounter PAH toxin within their field of contact.

Whilst there are various kits available and offer detection of PAHs in various mediums, including tissue, there is little offered in the form of testing food products (Table 1). A key focus of the current available kits is the emphasis on Benzo[a]prene, which was predominately pre 2011. After changes to legislation through the European Commission, the sum of 4 compounds has been recommended, known as PAH4 (benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, and benzo[*a*]pyrene). Currently there are no enzymatic based assays offering quantitative assessment in food stuffs, with the industry standard validated method stemming from GC-MS or HPLC with fluorescent detection. (Zelinkova and Wenzl, 2015)

Table 1

Company	Kit Name/Product Code	Sample Types	Run Time/min	Size	LOD (ppb)	Cross Reactivity	Price (USD)
LifeSpan	PAH ELISA KIT LS-F7855	Cel Culture Supernatants, Cell Lysates	270	96T	0.54	Other polycyclic aromatic hydrocarbons	990
Biomatik	Phenylalanine Hydroxylase EKU06579	Serum, plasma, tissue homogenates	180	96T	0.54	No	735
Creative Diagnostics	Benzo(a)pyrene ELISA Kit	Water	135	96T	0.6	No	909
Reagen	Chicken PAH ELISA Kit	Serum, Plasma, Biological Fluids	195	96T	2.34	No cross reactivity claimed	555
SEAFOOD TOMORROW TEST SOLUTION (AZTI)	PAH Enzymatic inhibition reaction	Food Homogenates	60	96T	2	Other polycyclic aromatic hydrocarbons	TBD

4. Legislation

The European Commission has set maximum levels for PAHs (specifically benzo[a]pyrene) in foodstuffs, including 10µg/kg (wet weight) in bivalve molluscs, 5µg/kg (wet weight) in muscle meat of smoked fish and other smoked meats, 2µg/kg (wet weight) in oils and fats which are intended for direct human consumption or as an ingredient in cooking, and 1µg/kg (wet weight) in foods for infants, infant formulae and baby foods. (Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs, 2011)

These allowances are different when considering PAH4 (the previously discussed combination of PAHs). The table below (Table 2) highlights the changes in the measurements from Benzo(a)pyrene to PAH4 alone as of 2014/15 (when the latest changes were implemented in the concentration limits allowed in food stuffs). (European Commission, 2011)

Table 2

Food Stuffs	Benzo(a)pyrene (ug/kg)	PAH4 (ug/kg)
Oils and fats (excluding cocoa butter and coconut oil)	5.0	10
Cocoa beans and derived products	2.0	30.0
Coconut oil	2.0	20.0
Smoked meat and smoked meat products	2.0	12.0
Muscle meat of smoked fish and smoked fishery product	2.0	12.0
Smoked sprats and canned smoked sprats bivalve molluscs heat treated meat	5.0	30.0
Bivalve molluscs	6.0	35.0
Processed cereal-based foods and baby foods for infants and young children	1.0	1.0
Infant formulae and follow-on formulae, including infant milk and follow-on milk	1.0	1.0
Dietary foods for special medical purposes	1.0	1.0

5. Advantages of proposed assay

This assay offers a significant advance in the access of qualitative evaluation of PAHs in the field of food and other mediums that may be in contact with humans. The method used to detect and quantify PAHs was created using HPLC and GC-MS, two highly specialised methods, requiring advanced technical training coupled with expensive equipment and instruments. This assay can be carried out in any setting, requiring the user to have minimal technical experience, with no additional requirements of complex laboratory apparatus, meaning onsite industrial and field use can be performed with ease.
As this bench top assay does not require the previously highlighted complex equipment and advanced training, it is cost effective and users should obtain relatively accurate results well within the legislative guidelines discussed in section 4 of this report.
The sensitivity of the issue has been observed at 2ug/kg determining benzo(a)pyrene and 10ug/kg when determining PAH4, these values indicate the kit would be appropriate for measuring the majority of the food stuffs as the lowest amount permitted in each classification are 2ug/kg (benzo(a)pyrene) and 10ug/kg (PAH4), thus, the kits cut off can detect the lower levels of each group.

6. Limitations

One of the more pressing issues surrounding this assay is the storage and shelf live. In more common areas where enzymatic assays are routinely used as screening methods for toxins, biomarkers and antibodies, the kits can be stored at 2-8°C for up to 18 months. However, due to the nature of this assay, its components and their corresponding requirements for performance, these kits will need to be stored at -20°C for a limited 3 months, or 2-8°C for 1 week, resulting in production limitations as storing after manufacture could result in used/unsold kits.
Whilst stated previous, the kit has the ability to qualitatively detect PAHs in salmon and sea bass that necessitating testing, further research and development would be required to validate the assay to accommodate a larger range of food stuffs. Further research would be required to determine the most commercially viable food products that would merit validation through market demand.

7. Bibliography

Abdel-Shafy, H. and Mansour, M., 2016. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egyptian Journal of Petroleum*, 25(1), pp.107-123.

Eur-lex.europa.eu. 2011. *Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs*. Online. [Accessed 1 February 2021].

Kim, K., Jahan, S., Kabir, E. and Brown, R., 2013. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. *Environment International*, 60, pp.71-80.

Koene, R., Prizment, A., Blaes, A. and Konety, S., 2016. Shared Risk Factors in Cardiovascular Disease and Cancer. *Circulation*, 133(11), pp.1104-1114.

Lerda, Donata. 2011. *Polycyclic Aromatic Hydrocardons (PAHs) Factsheet*. Geel: JRC Technical Notes. European Commission

European Commission, 2011. *Polycyclic Aromatic Hydrocardons (PAHs) Factsheet*. Geel: JRC Technical Notes.

Public Health England, 2018. *Polycyclic aromatic hydrocarbons (Benzo[a]pyrene)*. United Kingdom: Public Health England.

Zelinkova, Z. and Wenzl, T., 2015. The Occurrence of 16 EPA PAHs in Food – A Review. *Polycyclic Aromatic Compounds*, 35(2-4), pp.248-284

.

Annex 2 - Market Report for Immunoassay for the detection of Tetrodotoxins (TTXs) in seafood samples

Grant agreement: 773400

SEAFOOD TOMORROW

Helping Create Nutritious, Safe and Sustainable Seafood for Consumers of Tomorrow

Task 1.4: Immunoassay for the detection of tetrodotoxins (TTXs) in seafood samples

Table of Contents

Biorex food diagnostics

IRTA RESEARCH & TECHNOLOGY FOOD & AGRICULTURE

QUEEN'S UNIVERSITY BELFAST

1. Introduction

Foodborne diseases have affected societies and cultures from the beginning of humanity, and arguably before. As time has moved on, humans have evolved from tribal communities to vast cities densely populated and continuing to grow, placing a huge demand on the food supply change across the globe. The diverse changes throughout this development have observed many types of foodborne pathogens, ranging in severity and the impact they have on human health and communities.

One of the challenges posed by global food safety is that very few of those that fall ill due to contaminated food or drink will actively seek medical attention, resulting in under reporting to the public health authorities. In addition, chronic diseases such as kidney or liver failure and some forms of cancers are caused by food pathogens, however, these take a long time to manifest and it is almost impossible to form the link to the causative agent ingested several years previous.

In 2006 the World Health Organisation (WHO) formed international partnerships and a team known as the Foodborne Disease Burden Epidemiology Reference Group (FERG) to lead the initiative (World Health Organization, 2007).

Tetrodotoxin (TTX) is a potent marine neurotoxin found that acts by inhibiting the sodium channels of the neuron cell membrane. Tetrodotoxin binds to site 1 of these channels, blocking the passage of sodium ions through the cell membrane and preventing muscular nervous stimulation (Figure 1). Commonly found in the liver and other organs of creatures such as the puffer fish (see section 1.1), human poisoning occurs when the flesh and/or organs of contaminated species are consumed. It is produced by infecting symbiotic bacteria most commonly from the genus *Vibrio*, *Pseudomonas* and *Bacillus*. Analogues of TTX exist making it more difficult to identify the toxin within a patient who has consumed it (Lago et al., 2015).

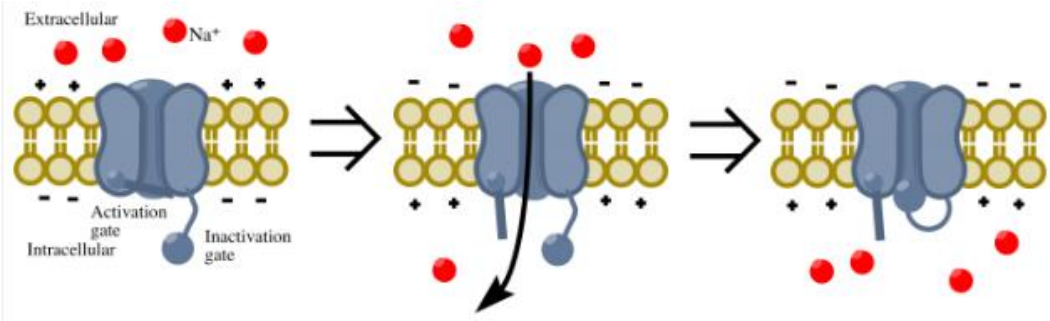

Figure 1. Voltage-gated sodium channel function (Tufts.edu/tetrodotoxin).

The importance of food safety in aquaculture is already crucial with a growing population and greater demand for food; however, failure to detect TTXs within food results in paralysis, mental impairment and lethal respiratory failure of which there is no known antidote (FAO Fisheries Department, 2021). Tetrodotoxin along with its analogues is heat-stable and hydrophilic, meaning cooking preparations cannot denature or remove the toxin. Currently, several detection methods exist to detect TTX in food. Many studies outline limitations, such as that the methods are not sensitive or specific enough to effectively identify or quantify TTXs (detection methods listed on Table 1). Tetrodotoxins oral median lethal dose (LD_{50}) for mice is 334 µg/kg, to show how lethal this can be, the oral LD_{50} of potassium cyanide for mice is 8.5 mg/kg, showing that orally, TTX is significantly more toxic than cyanide. Regarding puffer fish, the regulatory limit set by food regulatory services in Japan is 2 mg TTX equivalents/kg. Regarding shellfish, the European Food Safety Authority (EFSA) has concluded that concentrations below 44 µg of TTX equiv./kg shellfish meat, based on a large portion size of 400 g, do not result in adverse effects in humans (EFSA, 2017), and this value is used as a guideline. Therefore, because of the emergent TTX risk in European shellfish, a rapid, sensitive, specific and portable method of analysis is needed to detect the presence of TTX in seafood.

1.1. Incidence of pufferfish poisoning and other TTX-bearing organisms

The pufferfish has long been a delicacy in China, Japan and other Southeast Asian countries. Known as *fugu*, the prized delicacy is prepared by skilled chefs, trained for many years under strict guidance. The licence to prepare and serve f*ugu* is a government-issued license and requires the qualified individual to fillet, cook and serve the pufferfish. This training often takes between 6-10 years and is in lieu of several written exams and observations of the preparation process. This stringent process is due to the dangers of the toxin as briefly discussed in section 1.

As previously mentioned, TTX is heat-stable and cannot be nullified through the process of cooking, like many other toxins found in foods throughout the world. There are over 30 naturally occurring TTX analogues with no effective antidote currently available.

Pufferfish being a popular and highly sought-after delicacy, TTX is not limited to this species and is abundant in other creatures. Occurring naturally in a variety of vertebrates and invertebrates such as newts, toads, octopus, molluscs, gastropods, snails, starfish, crabs and worms, TTX is spread across distantly related taxa. Recent reports from the EFSA have indicated an incidence of TTXs in bivalve molluscs across Europe. In 2018, the Netherlands reported on a study conducted between 2015 and 2017 whereby 1063 samples were investigated. In oysters, TTX concentrations peaked at 253 µg/kg and 101 µg/kg in mussels, this is beyond the guideline value for TTX recommended by the EFSA. A study published by euro surveillance (Turner et al., 2015) documented the presence of TTXs in the south coast of England in concentrations up to 120 µg/kg, it is speculated that these numbers will rise over the coming decade with countries such as Greece, Holland, Spain, Italy and France reporting TTX in various shellfish species throughout the last 5 years (Vlamis et al., 2015; Gerssen et al., 2018; Leão et al., 2018; Dell'Aversano et al., 2019; Hort et al., 2020).

2. Legislation

In Europe, the European Commission (EC) has implemented specific laws in food hygiene through regulation 853/2044. Chapter 5 of the regulation highlights specific legislation surrounding the laws of live bivalve molluscs entering the market for human consumption and the marine toxin limits allowed before legal sale. Food business operators must ensure that live bivalve molluscs placed on the market for human consumption must not containing marine biotoxins in total quantities that exceed the following limits:

(a) For Paralytic Shellfish Poison (PSP), 800 micrograms per kilogram;

(b) For Amnesic Shellfish Poison (ASP), 20 milligrams of domoic acid per kilogram;

(c) For okadaic acid, dinophysistoxins and pectenotoxins together, 160 micrograms of okadaic acid equivalents per kilogram;

(d) For yessotoxins, 1 milligram of yessotoxin equivalent per kilogram; and

(e) For azaspiracids, 160 micrograms of azaspiracid equivalents per kilogram.
(Magarlamov, Melnikova and Chernyshev, 2017)

However, there is no specific legislation for TTXs due to the low incidence of TTX poisoning in Europe. Food products with risk of TTX poisoning are imported under strict regulatory conditions (European Food Safety Authority. 2021. *Nutrition Applications: Overview And Procedure*). This regulation does state that fishery products derived from poisonous fish of the families Tetraodontidae, Molidae, Diodontidae and Canthigas-teridae should not be marketed.

The Japanese Ministry of Health and Welfare states that within pufferfish products for consumption, a maximum residue limit (MRL) of 2 mg/kg of fish tissue (2000ppb). Japan also prohibits sales or preparation of pufferfish without appropriate qualifications. In America, the FDA does not specify a toxin limit however requires that the importation of pufferfish is restricted and inspected by Japanese Ministry of Health and Welfare officials to ensure certified safety and non-adulterated food products. Because of the potential presence of toxin, pufferfish in the U.S.A. can be considered adulterated as part of their Federal Food, Drug and Cosmetics Act. When importing, states can detain, without physical examination, on all shipments of Puffer Fish, Globe Fish, Swell Fish, Fugu, or other members of the Tetraodontidae family. Regarding shellfish, the EFSA has concluded that concentrations below 44 µg of TTX equiv./kg shellfish meat, based on a large portion size of 400 g, do not result in adverse effects in humans (EFSA, 2017).

3. Current methods of detection and market trends

The initial methods for detecting TTX were bioassay platforms including both mouse and cell culture. The mouse bioassay has proven to be an unfavoured method, carrying ethical issues around the pain and suffering of animals and the selectively and capability to detect and quantify analogues of TTX. Recognising this, scientists began developing alternative methods for screening TTX, in the forms of immunoassays and instrumental analysis.

Instrumental analysis methods, such as Liquid Chromatography coupled to Mass Spectrometry (LC-MS) or fluorescence detection, provide good limits of detection (LODs) but are complex, expensive and require skilled personnel (Turner, Higgins, Higman and Hungerford, 2015). Commercialisation of these methods is not feasible as they require specialised training to operate, significantly expense to

maintain and run, large space required to house equipment and time consuming. Table 1 outlines the current testing methods with their effectiveness and limitations.

Method	Advantages	Disadvantages
Mouse bioassay	1) Estimates combined effect 2) Allows (semi) quantification, similar to STX	1) Requires the use of animals 2) Does not discriminate between TTX and STX 3) Does not provide information on the toxin profile 4) Not interlaboratory validated
Cell-based assays methods	1) Estimate combined effect as they use the mechanism of action of the toxin group 2) Allow high-throughput screening	1) Do not provide information of the toxin profile. 2) Do not discriminate STXs from TTXs 3) Not interlaboratory validated
Antibody-based methods (SPR, ELISA)	1) Allow estimation of the concentration within antibody cross reactivity 2) Allow high-throughput screening	1) Do not provide information of the toxin profile 2) Not interlaboratory validated 3) Do not estimate overall toxicity 4) Due to point 3, may overestimate largely the amount of equivalent TTX if high amounts of low active compounds are present and detected 5) Only detect the presence of the toxins that the antibody cross-reactivity allows
Chemical-analytical methods	1) Provide information on toxin profile 2) Allow quantification of TTX and each analogue	1) Do not provide information about toxicity 2) Highly dependent on available TEF 3) Highly dependent on available standards 4) Not interlaboratory validated

Table 1: A table comparing different analytical techniques in detecting Tetrodotoxin, highlighting both advantages and disadvantages. (Magarlamov, Melnikova and Chernyshev, 2017)

3.1. Relevant competitor products

Immunoassays currently offer the best method to detect TTX presence as they are both quantitative and qualitative in analysis, providing a measurement that is informative of the total TTX analogues present in a sample. They are also more sensitive than physicochemical methods, giving more confidence to both the customer and the producer in the quality of

product in food safety analysis. Current immunoassays available for TTX testing are ELISA kits presented on Table 2.

Company	Kit Name/Product Code	Sample Types	Run Time (min)	Size	LOD (µg/kg)	Cross Reactivity	Price (USD)
Creative Dianostics	Tetrodotoxin (TTX) ELISA Test Kit/ DEIANJ48	Tissue, Liver, Fish	75	96T	8	No	1480
r-Biopharm	EuroProxima Tetrodotoxin (TTX) ELISA/ 5191TTX	Fish, Shellfish	90	96T	20	No	Not Provided
Unibiotest Products	TETRODOTOXIN (TTX) ELISA TEST KIT/ BA-UBT-UN011	Liver, Fish Tissues	75	96T	8	No	900
Reagen	Tetrodotoxin (TTX) ELISA Test Kit/ RNA97011	Fish, Water	90	96T	Not Provided	Not Provided	830

Table 2: Table highlighting immunoassays available from diagnostic suppliers currently offering ELISA kits and associated samples accommodated, run time, detection limitations, cross-reactivity and current consumer cost.

4. Current market & target consumers

The target user and businesses for this product will predominately fall within the food safety and environment agencies monitoring the prevalence of TTXs in shellfish around Europe and Eastern Asia. However, there is speculated use for food safety diagnostic agencies in regions where puffer fish or shellfish with high risk are produced or imported. The Japanese Ministry of Health and Welfare regulates the processing of pufferfish with qualified professionals to remove the contaminated components. Companies such as QIMA which provide shellfish product testing

services in Asia and Centre for Environment Fisheries and Aquaculture Science (CEFAS) in the UK which monitors and regulates aquatic life and produce for contamination and changes.

4.1. Consumer statistics for potential TTX-containing food

Within the UK, shellfish are expected to generate 2.2% (Business Gateway, 2018, Market Report – Shellfish Production) of overall aquaculture industry revenue in 2019. Most of the shellfish farmed in the UK are mussels, although small quantities of oysters and scallops are also farmed. The proportion of revenue derived from shellfish farming has increased over the past five years. According to the Scottish Salmon Producers' Organisation, the value and production of shellfish increased by 6% and 31% respectively in 2016 (IBISWorld, Aquaculture in the UK, July 2018). 85% of adults eat fish or shellfish, showing that seafood has an ingrained role in British diets, 51% state that shellfish is a part of their diet (Mintel – Fish and Shellfish, UK, December 2017). Exports are expected to account for over 60% industry revenue in the current year, so operators that have a strong presence in export markets are likely to do particularly well (IBISWorld, Aquaculture in the UK, July 2018). In Scotland, mussel production increased by 6% in 2017 to 8,232 tonnes. The greatest contribution in regional mussel production was from Shetland, accounting for 6,647 tonnes or 81% of Scotland's total. There was a 69% increase in the production of mussels for on-growing in 2017. Pacific oyster production increased by 42% from 2016. The Strathclyde region produced 61% of Scotland's farmed Pacific oysters. Queen scallop production increased by 76% since 2016 and the production of farmed scallops increased by 34%, both these sectors continue to target small niche markets (Marine Scotland, Scottish Shellfish Farm Production Survey 2017, May 2018). These figures for the UK alone give evidence for the increase in consumer demands for shellfish and so the predicted increase in demand for food safety diagnostics.

5. Proposed assay

The proposed assay developed in the SEAFOOD^{TOMORROW} project is a magnetic bead-based colorimetric immunoassay, which has been applied in the detection of the TTXs presence in shellfish (oysters, mussels and razor clams). The use of magnetic beads as TTX immobilisation allowed detection of TTX at levels as low as 3.3 µg/kg in mussels and 1 µg/kg in oysters and razor clams.

This assay would allow rapid screening of the TTXs contents in shellfish at significantly lower levels than the European Food Safety Authority guideline value (44 µg/kg), permitting the use of screening

capability on site at harvesting locations within 90 min, thus, equipping facilities to assess potentially compromised livestock before moving into the next stages of the food consumption line.

5.1. Advantages of proposed assay

Whilst there are TTXs detection methods available, they exhibit various disadvantages, supporting the incentive for the development of an assay that would improve the field of TTX detection, leading to a more accessible detection assay for a wider range of users.

The central framework of the project was aimed at reducing or equalling assay time relative to currently available platforms, decreasing the LOD in addition to manufacturing the kit at a lower cost than market competitors.

When testing various species of shellfish (oysters, mussels and razor clams), the assay exhibits a significantly lower LOD than available competitor kits. Furthermore, a reduction in assay duration was achieved through refinement of the method, with a completion time of 70 min. Together, reducing the total assay time and achieving a lower LOD, creating a faster assay with improved sensitivity, the assay offers superiority in the market.

5.2. Limitations of proposed assay

A critical aspect of the assay is that the validation has been performed only with razor clams, oysters and mussels, and the market share would require a more extensive range and robust sample variation. Prospective candidates would include more species of shellfish. The assay could also be applied to puffer fish, but an extensive validation study should be performed.

6. Bibliography

Al-Jawdah, A., Nabok, A., Jarrah, R., Holloway, A., Tsargorodska, A., Takacs, E. and Szekacs, A. (2018). Mycotoxin Biosensor Based on Optical Planar Waveguide. Toxins, 10(7), p.272.

Bane, V., Hutchinson, S., Sheehan, A., Brosnan, B., Barnes, P., Lehane, M. and Furey, A. (2016). LC-MS/MS method for the determination of tetrodotoxin (TTX) on a triple quadruple mass spectrometer. Food Additives & Contaminants: Part A, 33(11), pp.1728-1740.

Campbell, K., Rawn, D., Niedzwiadek, B. and Elliott, C., 2011. Paralytic shellfish poisoning (PSP) toxin binders for optical biosensor technology: problems and possibilities for the future: a review. Food Additives & Contaminants: Part A, 28(6), pp.711-725.

Dell'Aversano, C. et al. 2019. First detection of tetrodotoxin and high levels of paralytic shellfish poisoning toxins in shellfish from Sicily (Italy) by three different analytical methods. Chemosphere 215, pp. 881–892

European Food Safety Authority. 2021. Nutrition Applications: Overview And Procedure. [online] Available at: <https://www.efsa.europa.eu/en/applications/nutrition> [Accessed 15 January 2021].

Fao.org. 2021. FAO Fisheries Department Review Of The State Of World Aquaculture Safety Of Food Products From Aquaculture. [online] Available at: <http://www.fao.org/3/W7499E/w7499e06.htm>

Food Standards Agency, 2015. MANAGING SHELLFISH TOXIN RISKS. GUIDANCE FOR HARVESTERS AND PROCESSORS. [online] https://www.foodstandards.gov.scot/. Available at: <https://www.foodstandards.gov.scot/> [Accessed 11 January 2021].

Gerssen, A. et al. 2018. First Report on the Occurrence of Tetrodotoxins in Bivalve Mollusks in The Netherlands. Toxins 10(11), p. 450.

Horie, M., Kobayashi, S., Shimizu, N. and Nakazawa, H. (2002). Determination of tetrodotoxin in puffer-fish by liquid chromatography-electrospray ionization mass spectrometry. The Analyst, 127(6), pp.755-759. [Accessed 15 January 2021].

Hort, V. et al. 2020. First Detection of Tetrodotoxin in Bivalves and Gastropods from the French Mainland Coasts. Toxins 12(9), p. 599

Knutsen, H., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Nebbia, C., Oswald, I., Rose,

M., Roudot, A., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H., Arnich, N., Benford, D., Botana, L., Viviani, B., Arcella, D., Binaglia, M., Horvath, Z., Steinkellner, H., van Manen, M. and Petersen, A. (2017). Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA Journal, 15(4).

Lago, J., Rodríguez, L., Blanco, L., Vieites, J. and Cabado, A., 2015. Tetrodotoxin, an Extremely Potent Marine Neurotoxin: Distribution, Toxicity, Origin and Therapeutical Uses. Marine Drugs, 13(10), pp.6384-6406.

Leão, J. et al. 2018. Preliminary Results on the Evaluation of the Occurrence of Tetrodotoxin Associated to Marine Vibrio spp. in Bivalves from the Galician Rias (Northwest of Spain). Marine Drugs 16(3), p. 81

Ligler, F. (2009). Perspective on Optical Biosensors and Integrated Sensor Systems. Analytical Chemistry, 81(2), pp.519-526.

Magarlamov, T., Melnikova, D. and Chernyshev, A., 2017. Tetrodotoxin-Producing Bacteria: Detection, Distribution and Migration of the Toxin in Aquatic Systems. Toxins, 9(5), p.166.

McGrath, T., McClintock, L., Dunn, J., Husar, G., Lochhead, M., Sarver, R., Klein, F., Rice, J., Campbell, K. and Elliott, C. (2015). Development of a rapid multiplexed assay for the direct screening of antimicrobial residues in raw milk. Analytical and Bioanalytical Chemistry, 407(15), pp.4459-4472.

McNamee, S., Elliott, C., Greer, B., Lochhead, M. and 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), pp.13340-13349.

Mukundan, H., Anderson, A., Grace, W., Grace, K., Hartman, N., Martinez, J. and Swanson, B. (2009). Waveguide-Based Biosensors for Pathogen Detection. Sensors, 9(7), pp.5783-5809.

Noguch, T. and Arakawa, O. (2008). Tetrodotoxin – Distribution and Accumulation in Aquatic Organisms, and Cases of Human Intoxication. Marine Drugs, 6(2), pp.220-242.

Stump, E., Ralph, G., Comeros-Raynal, M., Matsuura, K. and Carpenter, K. (2018). Global conservation status of marine pufferfishes (Tetraodontiformes: Tetraodontidae). Global Ecology and Conservation, 14, p.e00388.

Turner, A., Powell, A., Schofield, A., Lees, D. and Baker-Austin, C. (2015). Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance, 20(2), p.21009.

Turner, A., Higgins, C., Higman, W. and Hungerford, J., 2015. Potential Threats Posed by Tetrodotoxins in UK Waters: Examination of Detection Methodology Used in Their Control. Marine Drugs, 13(12), pp.7357-7376.

Vlamis, A. et al. 2015. First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate Prorocentrum minimum. Toxins 7(5), pp. 1779–1807

World Health Organization, 2007. WHO Initiative To Estimate The Global Burden Of Foodborne Diseases. First formal meeting of the Foodborne Disease Burden Epidemiology Reference Group (FERG).

Yakes, B., Deeds, J., White, K. and DeGrasse, S. (2011). Evaluation of Surface Plasmon Resonance Biosensors for Detection of Tetrodotoxin in Food Matrices and Comparison to Analytical Methods. Journal of Agricultural and Food Chemistry, 59(3), pp.839-846.

Annex 3 - Market Report for Optical biochip multiplex detection Marine Biotoxins

Grant agreement: 773400

SEAFOOD TOMORROW

Helping Create Nutritious, Safe and Sustainable Seafood for Consumers of Tomorrow

Task 1.4: Optical biochip multiplex detection marine biotoxins

Biorex food diagnostics

IRTA RESEARCH & TECHNOLOGY FOOD & AGRICULTURE

QUEEN'S UNIVERSITY BELFAST

Contents

1. Introduction

Marine biotoxins are naturally occurring chemicals, caused by certain types of toxic algae, particularly Dinoflagellates, which accumulate in fish and shellfish. When people consume such contaminated seafood, depending on the toxins, the symptoms can be diarrheic, paralytic, amnesic, and neurologic (Visciano et al., 2016). The toxins responsible for most shellfish poisonings are water insoluble, heat and acid-stable, and ordinary cooking methods do not eliminate the toxins making them a threat to the food industry and consumers worldwide. Some groups of toxins are established in the European Union (853/2004) and the monitoring of shellfish is necessary to comply with the regulation before they can be placed on the market:

1.1.Paralytic Shellfish Poisoning (PSP)

The group of shellfish identified in cases of PSP consists mostly of bivalve molluscs. This group includes mussels, clams and, to a lesser extent, oysters, scallops and cockles in temperate zones. The PSP toxins are present in some genera of dinoflagellates and one species of blue-green algae. A commonly referenced PSP toxin is Saxitoxin (STX), STX is a neurotoxin that acts as a selective sodium channel blocker. One of the most potent known natural toxins, it acts on the voltage-gated sodium channels of neurons, preventing normal cellular function and leading to paralysis. In mammals, maximal toxicity is 400µg of STX per gram of shellfish meat or 400000ppb (Cusick and Sayler, 2013).

1.2 Amnesic Shellfish Poisoning (ASP)

ASP is an illness caused by consumption of the marine biotoxin called domoic acid (DA). DA is a kainic acid-type neurotoxin, in the brain, DA especially damages the hippocampus and amygdaloid nucleus. It damages the neurons by activating AMPA (an agonist for a highly regulated receptor which mediates fast synaptic transmission) and kainate receptors, causing an influx of calcium. Although calcium flowing into cells is a normal event, the uncontrolled increase of calcium causes the cell to degenerate (Grant, Burbacher, Faustman and Grattan, 2010).

1.3 Diarrhetic Shellfish Poisoning (DSP)

DSP and its symptoms usually set in within about half an hour of ingesting infected shellfish, this syndrome manifests itself as intense diarrhoea and severe abdominal pains, nausea and vomiting may sometimes occur too. This type of shellfish poisoning is typically caused by okadaic acid (OC), it is produced by several species of dinoflagellates, and is known to accumulate in both marine sponges and shellfish. Okadaic acid is a potent inhibitor of specific protein phosphatases and is known to have a variety of negative effects on cells (Valdiglesias et al., 2013).

1.4 Azaspiracid

This includes azaspiracids along with, brevetoxins, pectonotoxins and yessotoxins and some DSP toxins such as okadaic acid and dinophysistoxins. Much less is known about the impact of these toxins on human health with vague mechanisms of action for most. The grouping of these toxins is relatively ineffective as their respective mechanisms of action are vastly different and require more specific analysis, for example, domoic acid has neurotoxic and potential endocrine effects whereas azaspiracids have cardiotoxic effects in humans (Trainer et al., 2013). First identified in 1995 following several outbreaks in the Netherlands from contaminated shellfish in Killary, Ireland. This Phycotoxin inhibits the hERG voltage gated potassium channel, causing Nausea, vomiting, diarrhoea and cramp.

2. Legislation

The Mouse Bioassay (MBA) protocol for STX-group toxins is able to quantify these toxins at the current EU regulatory limit value, but not below approximately 370 µg STX equivalents/kg shellfish meat, which is far above the concentration compatible with the ARfD for STX-group toxins.

Europe - Commission Regulation 853/2004		
Marine Biotoxin Group	**MRL per kg of Flesh**	**MRL/ppb**
Paralytic Shellfish Toxins	800µg	800
Amnesic Shellfish Toxins	20mg	20000
Okadaic Acid, Dinophysistoxins and Pectenotoxins	160µg	160
Azaspiracids	160µg	160

(Regulation (ec) no 853/2004 of the European parliament and of the council)

3. Methods of detection

- Regulation EC No 2074/2005 stipulates which test methods may be used to detect marine biotoxins.
- Commission Regulation 853/2004 and subsequent regulations lay down the health conditions and methods of analysis of certain marine biotoxins, for the production and placing on the market of live bivalve molluscs.
- As part of the controls to protect public health, Commission Regulation 854/2004 requires the Competent Authority (FSA) to carry out monitoring of relaying and production areas for the presence of toxin-producing phytoplankton in water and biotoxins in shellfish tissue.

(laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation)

3.1. PSP Detection Method

The paralytic shellfish poison content of edible parts of molluscs (the whole body or any part edible separately) must be detected in accordance with the biological testing method or any other internationally recognised method. The mouse bioassay (MBA) for detection of paralytic shellfish toxins has been formally validated in an interlaboratory trial and a standardized AOAC method is available. The test does not provide analogue-specific data but gives a result in equivalents of STX in European Union and United States of America protocols or in equivalents of dc-STX in Japan. The receptor-binding assay (RBA) using tritiated STX is also an assay that gives a sum value for STX-equivalents. This test also has undergone formal inter-laboratory validation and has reached a good level of acceptance in some countries. Analogue-specific methods for analysis of STXs are based on separation by liquid chromatography and fluorescence or mass spectrometric detection, and a number of protocols have been validated. However, these methods require several analytical runs per sample in the case of complex natural toxin profiles (Van Dolah et al., 2012).

3.2. ASP Detection Method

The total content of amnesic shellfish poison of edible parts of molluscs (the entire body or any part edible separately) must be detected using the high-performance liquid chromatography (HPLC) method or any other recognised method. If the results are challenged, the reference method shall be the HPLC method (López-Rivera et al., 2005).

Initial efforts in method validation focused on using the extraction protocol for the PSP MBA, but this method has been superseded by the knowledge that DA is not stable in strongly acidic conditions. Hence, methods using an extraction protocol based on aqueous methanol with HPLC-UV detection are now generally preferred and such methods have undergone collaborative trials for validation. As DA is a relatively simple compound with one major analogue and a single epimer, an ELISA has also been developed and a collaborative trial permitted interlaboratory validation and formal standardization as an AOAC method (Toxicity equivalence factors for marine biotoxins associated with bivalve molluscs, 2016).

3.3. Lipophilic Toxin Detection Method

Mouse bioassay remains the standard method of lipophilic toxin detection whereby extraction of hepatopancreas and purification to test for the amount of toxin being present in mouse using HPLC (Bodero et al., 2018). According to regulation (EC) No 853/2004, three mice shall be used for each test. Where two out of three mice die within 24 hours of inoculation with an extract equivalent to 5g of hepatopancreas or 25g whole body, this shall be considered a positive result for the presence of one or more toxins (Nollet, 2004).

3.4. Alternative detection methods & Requirements for difficult analysis

Regulation (EC) No 853/2004 allows for a series of methods, such as HPLC with fluorimetric detection, liquid chromatography (LC), mass spectrometry (MS), immunoassays and functional assays, such as the phosphatase inhibition assay, shall be used as alternatives or supplementary to the biological testing methods, provided that either alone or combined they can detect analogues and that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection. Whilst these methods can be used and are considered accurate screening and analytical practices in the toxins detection, methods are limited to how they function and if they are applicable at identifying the toxins accurately. AZA and various analogues require detection by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (McLeod, Burrell and Holland, 2015). This analogue-specific methodology has now replaced the lipophilic mouse bioassay (MBA) in some regions, although for practical reasons the MBA continues to be used in many countries. It should be noted, however, that the MBA has never been formally validated for lipophilic marine biotoxins. For the OA-group of toxins, there is an enzyme-based assay, based on the inhibition of phosphoprotein-phosphatase 2a (PP2a). This assay has also been recently validated and is accepted in some countries. This assay will provide a sum of OA-equivalent toxicity present in a sample.

3.5. Current kits available on the market

Table below displays currently available commercial kits that specifically target marine biotoxins. It contains the accepted matrices the kits are capable of testing, run time, LOD and cross reactivity.

Company	Kit Name/Product Code	Accepted Matrices	Analysis Run Time/min	Size	LOD(ppb)	Antibody Cross Reactivity	Price
Saxitoxin ELISA kits							
EuroProxima	Saxitoxin Elisa/5191SAXI	Mussel, Oyster	45	96T	Not Provided	Multiple	Not Provided
creative-diagnostics	Saxitoxin ELISA Kit/DEIA6819	water samples, other contaminated samples	60	96T	0.015 ng/mL	Multiple	1850 USD
creative-diagnostics	Saxitoxin ELISA Kit/DEIA-XY38	shellfish samples (mussel, scallop, oyster)	45	96T	Mussel:10ppb Oyster:5ppb	Multiple	1060 USD
Perkin Elmer	MaxSignal® Saxitoxin (PSP) ELISA Test Kit/1034-02	Mussels	<90	96T	3	Multiple	Not Provided
Eurofins	Saxitoxin Plate Kit/52255B	Drinking water, ground water, and surface water	60	96T	0.015 ng/mL	Multiple	Not Provided
Beacon Analytic Systems	Saxitoxin Plate Kit/20-0174	Mussel, Lobster Tomalley	60	96T	Not Provided	Multiple	Not Provided
Domoic Acid ELISA kits							
Company	**Kit Name/Product Code**	**Accepted Matrices**	**Analysis Run Time/min**	**Size**	**LOD(ppb)**	**Antibody Cross Reactivity**	**Price**
EuroProxima	DOMOIC ACID ELISA/5191DOMO	Scallop, Mussel, Oyster	45	96T	Not Provided	Multiple	Not provided

creative-diagnostics	Domoic Acid (ASP) ELISA Kit/DEIA6821	water samples, shellfish, algal extracts	85	96T	10	None	1,880USD
Bioo-Scientific Laboratories	MaxSignal® Domoic Acid (ASP) ELISA Test Kit/1117-01	Mussels	<90 min	96T	30	Not Provided	Not provided
Biosense Laboratories	ASP ELISA/A 31300401	Bivalve molluscs, algal samples, seawater and body fluids of marine mammals	85	96T	10	None	345 EUR
Abraxis Kits	Domoic Acid (ASP) ELISA/ON0021	Water and shellfish samples	105	96T	2.2	None	Not provided
Bio-Equip	Domoic Acid (ASP) ELISA Test Kit/RNA97007	Mussels, algae and water samples	<90	96T	30	None	Not provided
Okadaic Acid ELISA kits							
Company	**Kit Name/Product Code**	**Accepted Matrices**	**Analysis Run Time/min**	**Size**	**LOD(ppb)**	**Antibody Cross Reactivity**	**Price**
Creative Diagnostics	Okadaic Acid (DSP) ELISA Kit/DEIA6822	Mussels	80-90	96T	100	Multiple	1650USD
Abraxiskits	Okadaic Acid (DSP) ELISA/520021	Water and shellfish samples	80-90	96T	100	Multiple	520EUR
Europroxima	OKADAIC ACID ELISA/5191OKA	Mussels, Oysters	45	96T	40	Multiple	Not Provided
Bioo-Scientific	MaxSignal® Okadaic Acid (DSP) ELISA Test Kit/1110-01	Mussel, Water	<90	96T	6ppb Water Samples 30ppb Mussel Samples	Not Provided	Not Provided

4. Multiplex Technology

Numerous publications related to paralytic shellfish poisoning (PSP) in the last decade demonstrate the movement of single-analyte biosensor technology to multi-analyte devices. As an immune-sensor, this product has the potential for the development of a multiplex Planar wavelength biosensor (PWB) for detection of multiple PSP causing toxins. Multiplex PWB's currently exist in post-research stages of development for multiplex detection of algal toxins including domoic acid (DA), okadaic acid (OA, and analogues), saxitoxin (STX, and analogues), and Azaspiracid (aza and analogues).

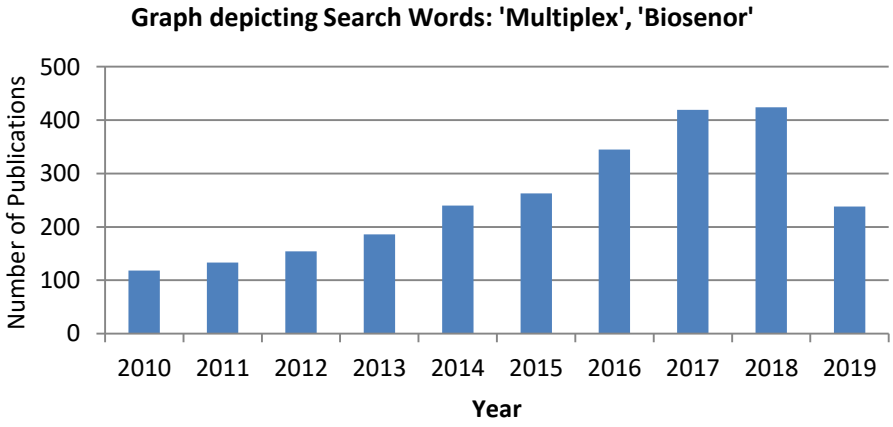

Figure 1: Publications each year from 2010 on NICB database from search words 'Multiplex' and 'Biosensor' (2019 is incomplete at the time of this report)

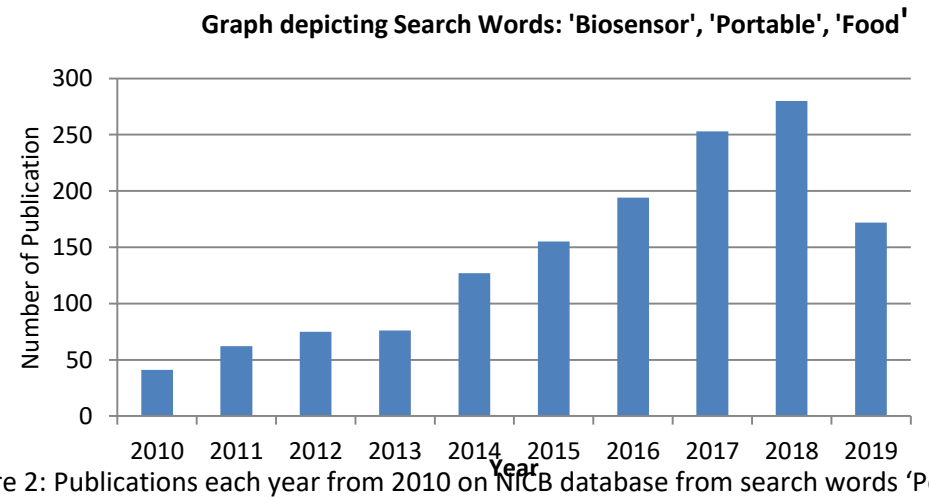

Figure 2: Publications each year from 2010 on NICB database from search words 'Portable', 'Biosensor' and 'Food' (2019 is incomplete at the time of this report)

Within the past decade, the need for easy to use and portable equipment has become more necessary in the growing industry of food diagnostics. Figure 1 & 2 show how the amount of publications with keywords Biosensor, Portable, food and multiple, biosensor have increased since 2010 demonstrating the increased demand from industry for more applicable and portable products, offering multiple analytes tested quickly on a consolidated platform.

The target consumers and businesses for this product will predominantly be food safety and environment agencies monitoring the prevalence of marine biotoxins in shellfish around Europe and Eastern Asia, there is potential as well for food safety diagnostic companies in regions where shellfish with high risk are produced or imported.

5. Geographical regions of outbreaks

The majority of the countries and regions in the world do eat shellfish in some capacity through local fisheries or importing of fresh or frozen products and therefore these marine biotoxins can show up in outbreaks. That being said the vast majority of outbreaks from 1970 – 2010 have been primarily observed in European countries such as Belgium, France, Norway and Sweden. The European outbreaks are predominately stemming from okadaic acid causing DSP. The graph below demonstrates the number of cases and where these cases were detected and the type of contaminate detected.

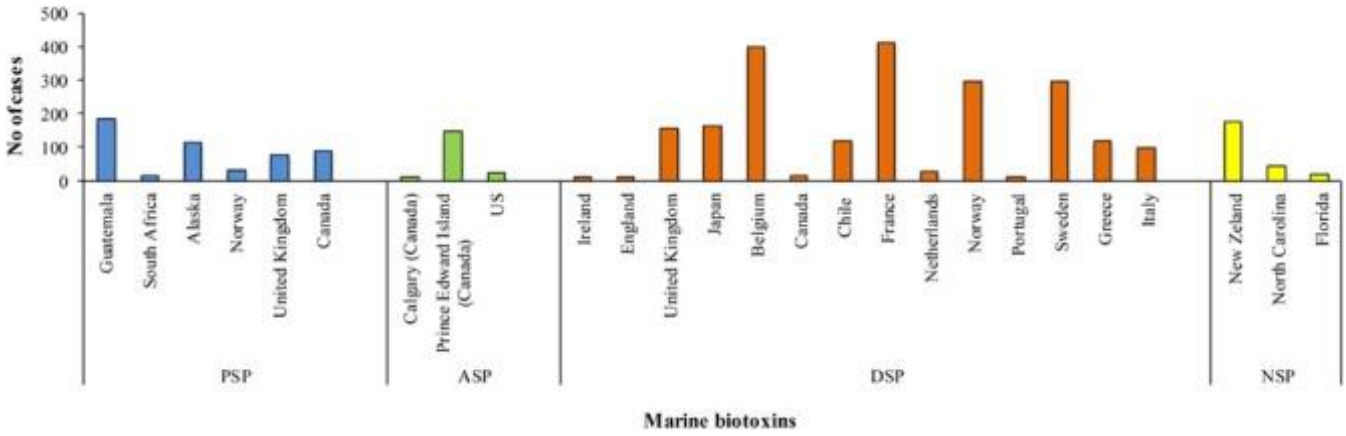

Figure 3: Outbreaks (number of cases) of poisoning due to marine biotoxins occurred from 1970 through 2010. (Visciano et al, 2016)

6. Proposed assay

The proposed assay developed in this project is a multiplex assay, capable of screening multiple problematic marine biotoxins in a rapid and convenient platform that is both user friendly and offers a detection limit of 1ng/ml, one of the lowest on the market. This assay was also developed, targeting two of the more prevalent food stuffs consumed, mussels and oysters, making an exceptionally powerful and accurate screening tool for the prevention of deadly biotoxins reaching market.

6.1. Advantages of proposed assay

Other forms of detection methods exist for the detection of these compounds, and are typically aligned with ELISA, MBA or LC-MS. These methods require specialist training and often, specialised facilities and licencing to perform. The format of this assay, allows minimally trained individuals to conduct the testing of the food stuffs with no specialist training or qualifications required.

In addition the assay offers a LOD of 1ng/ml, this is not only within the required legislative prerequisite for testing but offers one of the most competitive sensitivities commercially available.

Furthermore the sample preparation and assay run time combined, offer an exceedingly rapid assessment of samples, the sample preparation for immunoassay based devices are longer and the assay required several more steps, equipment and incubation times. The same is considered in LC-MS, which requires a more thorough preparation, long run time and additional result analysis.

6.2. Limitations of proposed assay

One of the limitations to consider is oysters and mussels are not the only species to contain the biotoxins being tested for, further expansion to other species and matrices i.e. razor clams and water should be explored and validated.

7. Bibliography

Bodero, M., Gerssen, A., Portier, L., Klijnstra, M., Hoogenboom, R., Guzmán, L., Hendriksen, P. and Bovee, T., 2018. A Strategy to Replace the Mouse Bioassay for Detecting and Identifying Lipophilic Marine Biotoxins by Combining the Neuro-2a Bioassay and LC-MS/MS Analysis. *Marine Drugs*, 16(12), p.501.

Cusick, K. and Sayler, G., 2013. An Overview on the Marine Neurotoxin, Saxitoxin: Genetics, Molecular Targets, Methods of Detection and Ecological Functions. *Marine Drugs*, 11(12), pp.991-1018.

Grant, K., Burbacher, T., Faustman, E. and Grattan, L., 2010. Domoic acid: Neurobehavioral consequences of exposure to a prevalent marine biotoxin. *Neurotoxicology and Teratology*, 32(2), pp.132-141.

López-Rivera, A., Suárez-Isla, B., Eilers, P., Beaudry, C., Hall, S., Fernández Amandi, M., Furey, A. and James, K., 2005. Improved high-performance liquid chromatographic method for the determination of domoic acid and analogues in shellfish: effect of pH. *Analytical and Bioanalytical Chemistry*, 381(8), pp.1540-1545.

McLeod, C., Burrell, S. and Holland, P., 2015. *Review of the currently available field methods for detection of marine biotoxins in shellfish flesh*. Food Standards Agency. method comparison between the Monobind AccuBind® ELISA and Diasorin diagnostic kits was performed. Biological specimens from low, normal, and high Vit D level populations were used; the values ranged from 9.5ng/ml to 200ng/ml. Overall, a total of 83 specimens were evaluated. The least square regression equation and the correlation coefficient were computed for the AccuBind method when compared to the reference method, Diasorin. Monobind requires a high level of correlation and a target value for the correlation coefficient greater than 0.9 when developing products.

Nollet, L., 2004. *Handbook of Food Analysis, Second Edition*. New York: Chapman and Hall/CRC.

Trainer, V., Moore, L., Bill, B., Adams, N., Harrington, N., Borchert, J., da Silva, D. and Eberhart, B., 2013. Diarrhetic Shellfish Toxins and Other Lipophilic Toxins of Human Health Concern in Washington State. *Marine Drugs*, 11(6), pp.1815-1835.

Valdiglesias, V., Prego-Faraldo, M., Pásaro, E., Méndez, J. and Laffon, B., 2013. Okadaic Acid: More than a Diarrheic Toxin. *Marine Drugs*, 11(11), pp.4328-4349.

Van Dolah, F., Fire, S., Leighfield, T., Mikulski, C., Doucette, G., Andersson, Å., Bean, L., Couture, D., DeGrasse, S., DeLeon, A., Dell'Ovo, V., Flewelling, L., Holland, P., Langlois, G., Lewis, R., Masuda, M., McNabb, P., Mikulski, C., Niedzwiadek, B., Porntepkasemsan, B., Rawn, D., Sombrito, E., Srisuksawad, K., Suarez, B., Subsinserm, S. and Tubaro, A., 2012. Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study. *Journal of AOAC INTERNATIONAL*, 95(3), pp.795-812.

Visciano, P., Schirone, M., Berti, M., Milandri, A., Tofalo, R. and Suzzi, G., 2016. Marine Biotoxins: Occurrence, Toxicity, Regulatory Limits and Reference Methods. *Frontiers in Microbiology*, 7.

laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation.

Toxicity equivalence factors for marine biotoxins associated with bivalve molluscs. 2016. Rome: Food And Agriculture Organization Of The United Nations.

Regulation (ec) no 853/2004 of the European Parliament and of the council. VII.