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RESEARCH PAPER

Evolving to the optoelectronic mouse for phycotoxin analysis in shellfish

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Abstract Despite ethical and technical concerns, the in vivo method, or more commonly referred to mouse bioassay (MBA), is employed globally as a reference method for phycotoxin analysis in shellfish. This is particularly the case for paralytic shellfish poisoning (PSP) and emerging toxin monitoring. A high-performance liquid chromatography method (HPLC-FLD) has been developed for PSP toxin analysis, but due to difficulties and limitations in the method, this procedure has not been fully implemented as a replacement. Detection of the diarrhetic shellfish poisoning (DSP) toxins has moved towards LC-mass spectrometry (MS) analysis, whereas the analysis of the amnesic shellfish poisoning (ASP) toxin domoic acid is performed by HPLC. Although alternative methods of detection to the MBA have been described, each procedure is specific for a particular toxin and its analogues, with each group of toxins requiring separate analysis utilising different extraction procedures and analytical equipment. In addition, consideration towards the detection of unregulated and emerging toxins on the replacement of the MBA must be given. The ideal scenario for the monitoring of phycotoxins in shellfish and seafood would be to evolve to multiple toxin detection on a single bioanalytical sensing platform, i.e. 'an artificial mouse'. Immunologically based techniques and in particular surface plasmon resonance technology have been shown as a highly promising bioanalytical tool offering rapid, real-time detection requiring minimal quantities of toxin standards. A Biacore Q and a prototype multiplex SPR biosensor have been evaluated for their ability to be fit for purpose for the simultaneous detection of key regulated phycotoxin groups and the emerging toxin palytoxin. Deemed more applicable due to the separate flow channels, the prototype performance for domoic acid, okadaic acid, saxitoxin, and palytoxin calibration curves in shellfish achieved detection limits (IC_{20}) of 4,000, 36, 144 and 46 μg/kg of mussel, respectively. A one-step extraction procedure demonstrated recoveries greater than 80 % for all toxins. For validation of the method at the 95 % confidence limit, the decision limits (CCα) determined from an extracted matrix curve were calculated to be 450, 36 and 24 μg/kg, and the detection capability (CCβ) as a screening method is ≤10 mg/kg, ≤160 μg/kg and ≤400 μg/kg for domoic acid, okadaic acid and saxitoxin, respectively.

Keywords Multiplex · Phycotoxins · Optical biosensor · Shellfish · Antibody · Validation

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Introduction

Phycotoxins are a generic term for groups of relatively low molecular weight compounds produced by phytoplankton in the marine and freshwater environment. These compounds are classified based on their toxic symptomatic properties in humans and physiochemical properties as paralytic, diarrhetic, amnesic or neurotoxic shellfish or ciguateria fish poisoning

toxins [1]. Shellfish have been highlighted in the classification, as this food type is the main vector for poisoning in humans due to bioaccumulation of the toxins following harmful algal blooms. To ensure human safety on the consumption of this food type and to protect the growing aquaculture industry worldwide, legislation and monitoring programmes have been established to monitor both frequency and density of harmful algal blooms and the toxicity of seafood, mainly bivalve mollusks [2–4]. Further legislation [5, 6] laid down and updated the specific methods for testing. Harmful algal blooms are monitored mainly through the use of microscopy techniques for phytoplankton species recognition by cell morphology, and density by cell counts though molecular probe techniques are being considered as superior alternatives in this area [7]. For many years, the toxicity of seafood worldwide was determined using crude biological methods referred to as the mouse bioassays (MBA) [8, 9]. Legislatively, these methods ensured that a representative sample of seafood was safe to eat through two selective extractions for hydrophilic and lipophilic toxins followed by injection of this extract into six mice, three per group. The time of death indicated the toxicity of the sample and hence determined whether the shellfish was safe for human consumption.

European legislation for animal experimentation [10, 11] and global regulatory authorities recognising the ethical issues of these biological methods drove the introduction and implementation of alternative methods [12, 13]. These alternatives focused on a range of functional assays, biochemical methods and various types of analytical methods for marine biotoxin detection [14–16]. Gerssen and coworkers (2010) [4] indicated which methods were being applied in different countries at this time, and the MBA remained the most commonly used. In relation to regulation in Europe, the analytical methods such as high-performance liquid chromatography and mass spectrometry have made the most impact in moving away from the use of the MBA as the toxins can be identified and quantified. Progress in moving away from the MBA was impeded as these methods have drawbacks in that they are expensive to run, identification can only be performed with available standards, and toxicity equivalent factors must be applied, making data analysis laborious [1, 17]. Sample preparation tends to be extensive, for example, for paralytic toxin analysis this HPLC method requires time-consuming oxidation steps [18]. To date, each regulated toxin group—paralytic, diarrhetic and amnesic—requires a different platform and method of analysis so each sample must be analysed by three different recommended methods to cover these groups of marine toxins [19–21]. For this reason, alternative methods are still sought that will allow all toxins to be analysed on a single platform as a single test and permitting the toxins to be identified and measured directly relative to their toxicity factors in a similar manner to that of the MBA.

Although functional assays provide a measure of toxicity in practice, their robustness and transferability have proven difficult, but some success has been achieved with the radiolabelled sodium channel receptor assay for paralytic shellfish poisoning (PSP) toxins [22]. The difficulty in its implementation is the radiolabel. The progression of biosensor-based biochemical assays demonstrating marine biotoxin testing has been substantial [23]. Surface plasmon resonance (SPR) demonstrated the most promise as a rapid real-time detection biosensor platform for marine biotoxins in the combination of sample preparation and analysis time. To date, assays have been developed for the detection of each of the key regulated toxins: domoic acid, okadaic acid and dinophysistoxins and paralytic shellfish poisoning toxins as separate methods [24–28]. However, these marine toxin assays, developed using this technology, has demonstrated their robustness in single laboratory validation [29, 30] and their transferability in inter-laboratory studies [31]. The key to the success of these biosensor methods as antibody-based tests is the correlation of the antibody cross-reactivity to the toxicity of the different analogues within a group or the ability to multiplex using different antibodies [32, 33].

Multiplexing was achieved using a prototype SPR biosensor with a specially designed immobilisation unit in a 4×4 layout, with four flow cells each containing four spots, allowing for the detection of 16 analytes simultaneously [34]. Domoic acid, okadaic acid, saxitoxin and neosaxitoxin have all been successfully immobilised on the multi-biosensor chip for the detection of these biotoxins and the proof of concept shown in buffer for their multiple detection. A modification of this method has been applied to detect these toxins in European seawater samples [35]. Other multiplexing techniques such as chemiluminescence [36], flow cytometry [37] and planar waveguide [38] are also now coming to the fore. However, these devices to date tend to be single channel and there are hurdles for the analysis of toxins from one aliquot of sample extract relative to the varying action levels in shellfish.

Even though these devices can detect multiple toxins simultaneously to fully benefit from such a system, a single extraction procedure accounting for the different physiochemical properties of the toxins to be detected and their dilution factors relative to the action level must be devised for shellfish analysis. In previous methods, okadaic acid has been extracted using acetonitrile and PSP toxins in acetate buffer, but PSP toxins cannot be extracted into acetonitrile and similarly okadaic acid by aqueous buffers. Methanol has been used in extractions for all toxins, but for hydrophilic PSP toxins, only 50 % recovery was achieved using 90 % methanol [25]. Each of the toxins has different action levels, and therefore for each toxin, different dilutions of the extract must be evaluated relative to the sensitivity of that test. This can be much more easily accomplished on a multichannel instrument compared to a single-channel array as different dilutions can be added to

each channel and thereby simultaneous detection relative to the different action levels can be achieved.

The aim of the present study was to evaluate two four-channel SPR instruments, a Biacore Q with sequential analysis and a prototype SPR instrument designed for parallel analysis for the simultaneous detection of marine toxins from shellfish.

Materials and methods

Materials

An amine coupling kit (containing series S CM5 sensor chips, 1-ethyl-3(3 dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine) and HBS-EP+ (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) buffer were purchased from GE Healthcare (Buckinghamshire, UK). Jeffamine, ethylenediamine, acetonitrile, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium dodecyl sulphate (SDS) and domoic acid were purchased from Sigma-Aldrich (Dorset, UK). Okadaic acid was obtained from LC Laboratories (Massachusetts, USA). Palytoxin was obtained from Wako. Saxitoxin and PSP toxin analogues, okadaic acid and dinophysistoxins 1 and 2, and domoic acid analytical standards were purchased from the National Research Council of Canada (Halifax, Canada). Shellfish samples were received from National Reference Laboratories within Europe.

Antibody production

Antibodies for the development of the multiplex assay, for the detection of paralytic shellfish toxins, okadaic acid and dinophysistoxins and domoic acid to be representative of each group of the regulated toxins and palytoxin as an emerging toxin, were either produced at Queen's University or sourced through collaboration.

PSP toxins An immunogen of neosaxitoxin conjugated to keyhole limpet haemocyanin (KLH) was prepared via a modification of the Mannich reaction in a similar way as previously described [24]. A New Zealand female white rabbit was immunised monthly with an initial dose of 200 μg of protein followed by 7 monthly doses at 100 μg of protein. The final serum was harvested and stored frozen until use.

DSP toxins The production of the polyclonal antibody for okadaic acid and dinophysistoxins was described in full previously [26], and the final serum was stored frozen until use.

ASP toxins Domoic acid was conjugated to bovine thyroglobulin in a similar manner as described for domoic acid to human serum albumin [28]. A monoclonal antibody using this immunogen was prepared using the fusion procedure [39].

Emerging toxin A monoclonal antibody for palytoxin was sourced externally [40].

Instrumentation

The Biacore Q SPR biosensor and the prototype multiplex SPR biosensor were developed by GE Healthcare (Uppsala, Sweden).

Biosensor chip immobilisation

Each flow cell of a CM5 and a series S CM5 sensor chip was activated in succession on the Biacore Q and prototype multiplex SPR instruments, respectively. The immobilisations of each toxin—saxitoxin, okadaic acid, domoic acid—using the same chemical methodology as previously described [34] were performed but without the use of an immobilisation device. In addition, palytoxin was immobilised onto flow cell 4. In brief, the chip was docked onto the respective instruments and washed with HBS-EP buffer (running buffer). The carboxymethylated surface of the sensor chip was activated using an EDC and NHS (1:1, *v*/*v*) mixture which was injected over the surface of flow cell 1 for 15 min. Jeffamine (0.1 M) in borate buffer (pH 8.5) was injected over flow cell 1 for 30 min to add an amine linker followed by ethanolamine (1 M, pH 8.5) for 15 min to block any non-reacting sites. The surface was washed with HBS-EP+ buffer, and the sensor chip was undocked from the biosensor. Finally, saxitoxin was immobilised onto the chip surface overnight at 37 °C. These steps were repeated in succession for okadaic acid (flow cell 2) and domoic acid (flow cell 3). Slight modifications in the immobilisation protocol for okadaic acid and domoic acid included that the amine linker added was ethylenediamine and the overnight incubation was at 25 °C. The emerging toxin, palytoxin, was additionally immobilised onto flow cell 4 using carbodiimide chemistry, though no linker was required. Each sensor chip was stored at 4–8 °C until needed. The layout of the chip surface for each biosensor and the running plate design for the maximum number of samples in an analytical run are as illustrated in Fig. 1.

Parameters for biosensor evaluation

Biacore Q SPR sensor

The flow rate across the chip surface was set at 20 μL/min, and sample contact time was evaluated at 4 and 8 min. Report points were taken 10 s before injection and 30 s after

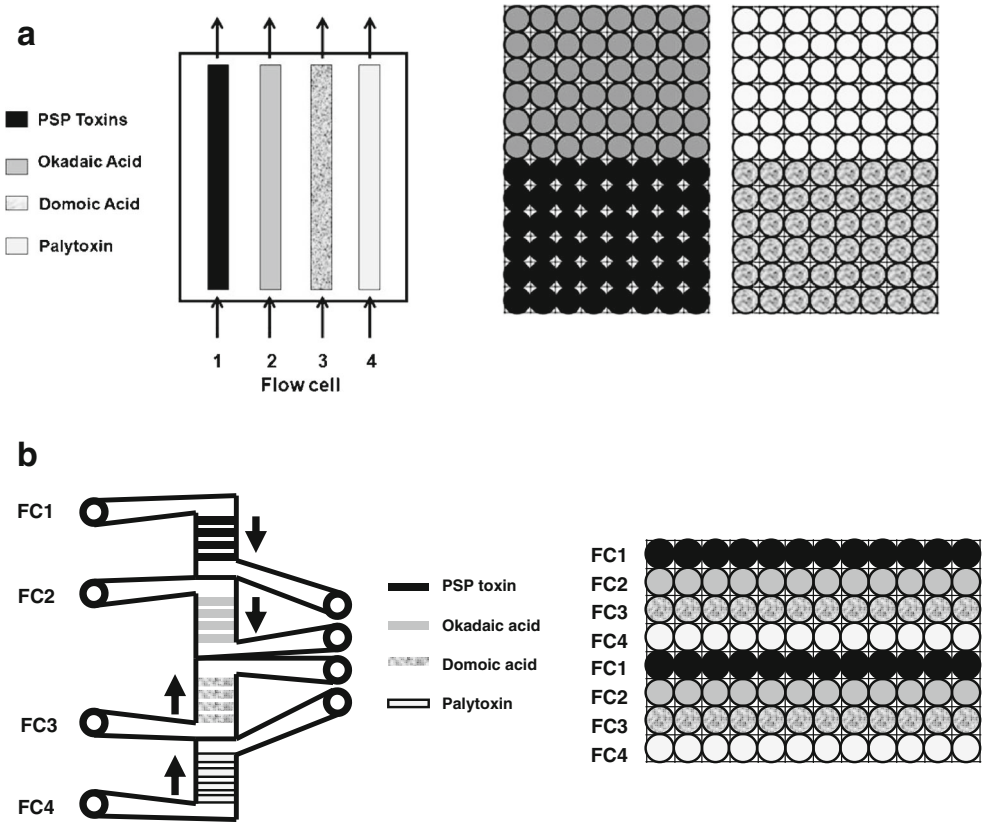

Fig. 1 **a** Biacore Q SPR instrument chip layout and 2×96-well plate design for multiple toxin analysis using a single-needle injection system within a single operation. **b** Biacore multichannel prototype instrument chip layout and 96-well plate design for multiple toxin analysis using the four-needle injection system (one needle per flow cell) within a single operation

the injection was completed to determine the baseline and the level of binding on the chip surface. The antibody titre was determined as the dilution required to provide a response between 300 and 500 resonance units (RU) for the 0 ng/mL calibration standard. For the 4-min injection, the saxitoxin, okadaic acid, domoic acid and palytoxin antibodies were therefore diluted at 1/500, 1/10,000, 1/500 and 1/100 in HBS-EP buffer, and for the 8-min injection, at 1/2,000, 1/20,000, 1/1,000 and 1/250 in HBS-EP buffer, respectively. The antibody dilutions were mixed 1:1 with samples or standards manually on a 96-well microtitre plate with calibrants and samples for each toxin analysed in succession for saxitoxin, okadaic acid, domoic acid and palytoxin on each of the four flow cells due to a single needle on the instrument (Fig. 1a). The surface of the sensor chip was regenerated for 60 s (30 s×2) before analysis of the next sample. The regeneration solutions for each flow cell were optimised for each toxin interaction to allow the response to return to baseline after each sample effectively. The regeneration solution for saxitoxin, domoic acid and palytoxin was the same using 100 mM HCl/1 % SDS (1:1, *v*/*v*), and for okadaic acid, it was 250 mM NaOH/acetonitrile (8:2, *v*/*v*). Each regeneration solution was placed in a container which only administered that particular regeneration solution to its dedicated flow cell. The duration of sample analysis for each toxin including regeneration of the chip was approximately 10 min for the 4-min injection and 14 min for the 8-min injection.

Prototype multichannel multiplex SPR biosensor

The operation of the prototype Biacore which is operated through simple to operate software in a similar manner to the Biacore Q was discussed in detail previously [34]. The biosensor parameters for the prototype SPR sensor chip were optimised in a similar way to the Biacore Q. The antibody titre was determined as the dilution required to provide a response between 300 and 500 RU over 8 min for the 0-ng/mL calibration standard. The saxitoxin, okadaic acid, domoic acid and palytoxin antibodies were therefore diluted at 1/1,000, 1/10,000, 1/2,000 and 1/1,000 in HBS-EP+ buffer, respectively. The flow rate across the chip surface was 20 μL/min, and sample contact time was 8 min. Report points were taken 10 s before injection and, 30 s after the injection, was completed to determine the baseline and the level of binding on the chip surface. The antibody was mixed 1:1 with samples, matrix standards or buffer standards manually on a 96-well microtitre plate with lanes 1 and 5 designated for flow cell 1; 2 and 6 for flow cell 2; 3 and 7 for flow cell 3; and 4 and 8 for flow cell 4 (Fig. 1b) due to the four-needle injection system of the instrument and one needle per flow cell. The surface of the sensor chip was regenerated for 60 s (30 s×2) before analysis of the next sample by using the same reagents for each toxin as for the Biacore Q instrument. The duration of sample analysis for all four toxins including regeneration of the chip was approximately 12 min.

Preparation of the assay calibration curves

Calibrants for each of the toxins were prepared in pH 7.4 HBS-EP buffer. The initial calibrant range for saxitoxin was 0–50 ng/mL; for okadaic acid, 0 to 50 ng/mL; for domoic acid, 0 to 250 ng/mL; and for palytoxin, 0 to 200 ng/mL. Working standards were used to produce a toxin calibration curve on the relative flow cell for each toxin based on dose response on the biosensor.

Extraction procedure

The toxin extraction from homogenised shellfish (1 g) was performed using 50 % aqueous methanol (4 mL) at room temperature. A 50 % methanol solution was employed as the extraction solvent to allow for a compromise in recoveries between the lipophilic and hydrophilic toxins. The sample was vortex mixed for 5 s and roller mixed for 30 min. Following centrifugation at 3,500*g* for 10 min, the supernatant was collected. For PSP toxins, domoic acid, okadaic acid and palytoxin, the supernatant dilution was adjusted to 1 in 80, 1 in 500, 1 in 20, and 1 in 2 in HBS-EP+ buffer, respectively. These dilutions in HBS-EP buffer were to match the sensitivity of the antibody in relation to the regulatory limit as outlined in Table 1. Mussels were initially evaluated as the model shellfish matrix for analysis, but toxin analysis was performed for a range of other contaminated shellfish species.

Validation

The method was validated where possible considering the guidelines required by IUPAC [41], AOAC [42] and the Commission Decision 2002/657/EC [43] concerning the performance of analytical methods.

Sensitivity and selectivity

The sensitivity (IC_{50}) and dynamic range (IC_{20}–IC_{80}) of the assay in buffer calibration curves and mussel matrix curves were calculated using Biaevaluation Software V4.1 and analysed to compare matrix effects in the procedure and monitor recovery of the extraction to establish the applicability of the method. Homogenised known negative mussel samples (8×1 g) fortified with each toxin at different concentrations, comparable to the buffer calibration curve, were then extracted and compared to a negative mussel sample that had been extracted and fortified after extraction to achieve 100 % recovery of the toxin at each concentration.

The selectivity is the ability of the method to distinguish between the target toxins and the presence of other potentially interfering analytes. This was assessed for each antibody through the analysis of working standards of a range of shellfish toxins. The toxins assessed with each antibody

Table 1 Summary of the sensitivity (IC_{50}) of the assay for each Biacore instrument in buffer and for the multichannel prototype instrument in mussel matrix spiked before and following extraction

Toxin	Regulatory limit (μg/kg)	Antibody	Biacore Q single channel		Prototype multichannel Biacore				
			IC_{50} buffer curve (ng/mL) 4 min	IC_{50} buffer curve (ng/mL) 8 min	IC_{50} buffer curve (ng/mL) 8 min	Matrix dilution	IC_{50} spiked matrix curve (μg/kg)	IC_{50} extracted matrix curve (μg/kg)	Dynamic range (IC_{20} to IC_{80}) extracted curve (μg/kg)
Saxitoxin	800 STXeq	QUB 7	1.36	0.21	1.44	1 in 40	382	371	144–874
Okadaic acid	160 OAeq	R739	0.81	0.44	0.3	1 in 8	13.9	88.3	36–192
Domoic acid	20 000	12D21D12F5	8.74	9.3	10.0	1 in 500	17,800	20,900	4,000–36,500
Palytoxin	30 (EFSA) 256 (NZ)	PLTX MAB	263.6	90.7	12	1 in 2	215	193	45–500

included those currently covered by EU legislation and which could co-extract with the target toxin. The percent cross-reactivity of each antibody with each potential cross-reactant was determined.

Analysis of uncontaminated mussels for interference effects (decision limit (CCα))

This is the limit at and above which it can be concluded with an error probability of α that a sample contains each of the toxins. The determination of CCα, whilst not compulsory as part of 2002/657/EC guidelines, is essential in order to calculate the β error for the detection capability. The CCα for each toxin was obtained by the duplicate analysis of ten individual blank mussel samples when calibrated to a buffer and a matrix-matched curve. The decision limit was calculated as the concentration correlating to the mean response−(2.33×the standard deviation). This provides a 95 % confidence level that a sample with a concentration determined as above this decision limit concentration does contain toxin.

Detection capability (CCβ)

The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. As the regulatory limits are currently set within EC legislation, the permitted levels of 20,000, 160 and 800 μg/kg for domoic acid, okadaic acid and saxitoxin, respectively, were utilised for the purposes of assessing the performance of the method. Uncontaminated mussel samples (n=10) were analysed in duplicate. The same non-contaminated samples were then fortified with each toxin at each regulatory limit and half the regulatory limits and analysed against a buffer and matrix-matched curve.

Recovery

To measure the recovery of each parent toxin (saxitoxin, okadaic and domoic acid) under the assay conditions using this extraction procedure, known negative homogenised mussel samples, aliquots 10×1 g, were fortified with each parent toxin at half the regulatory limit and at the regulatory limit and analysed against both a buffer and matrix-matched curve.

Repeatability

Repeatability of the instrument was assessed by the analysis of mussel samples (n=10). Each replicate was extracted and analysed in duplicate within a run. The repeatability study was performed at each level of toxin fortification, and the repeatability variations (%CV) of the SPR response values were calculated.

Analysis of samples

Shellfish samples (n=48) consisting of mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*), oysters (*Crassostrea Gigas*) and scallops (*Pecten maximus*) were collected from different European locations and supplied for the study as frozen homogenate through national reference laboratories. The samples were analysed using the prototype multiple SPR method and confirmatory information determined using either the AOAC mouse bioassay, AOAC HPLC methods of analysis for PSP toxins or domoic acid or mass spectrometry methods for okadaic acid was provided as available. Different species of bivalve mollusks were analysed and compared for toxin content.

Results and discussion

The focus of the research was to design a multi-bioanalytical SPR method for the identification and detection of four marine biotoxin groups as a single screening test. Two different Biacore SPR instruments were evaluated for their feasibility for this purpose and applicability to marine biotoxin monitoring programmes.

Biacore Q biosensor

The Biacore Q biosensor was designed for food analysis as a single-needle injection instrument with the capability to analyse the biomolecular interaction occurring on a single flow cell at any one time. However, the sensor chip for this instrument was composed of four interchangeable isolated flow cells that could be applied in succession. Using this instrument, a biosensor chip was produced with the four toxins identified one immobilised per flow cell. The buffer calibration curves obtained with a 4- and 8-min injection are shown in Fig. 2, and the sensitivity (IC_{50}) achieved under these conditions for each toxin is illustrated in Table 1. The sensitivity for each regulated toxin was extremely good at low nanogram per millilitre and improved with the further dilutions of the antibody, increasing the competitive binding over the longer 8-min injection for saxitoxin, okadaic acid and palytoxin. The contact times were selected to improve, within a reasonable sample analysis time, the sensitivity of the assay towards palytoxin. Although the feasibility of using this biosensor for the detection of four toxins in a sample was demonstrated, it was through the sequential analysis of each sample over each flow cell with a separate injection per flow cell l. The analysis therefore of the four toxin classes per sample takes 40 and 56 min (depending on injection time). For the duplicate analysis of a six-point calibration curve for each toxin, the duration of the calibration for all toxins alone takes

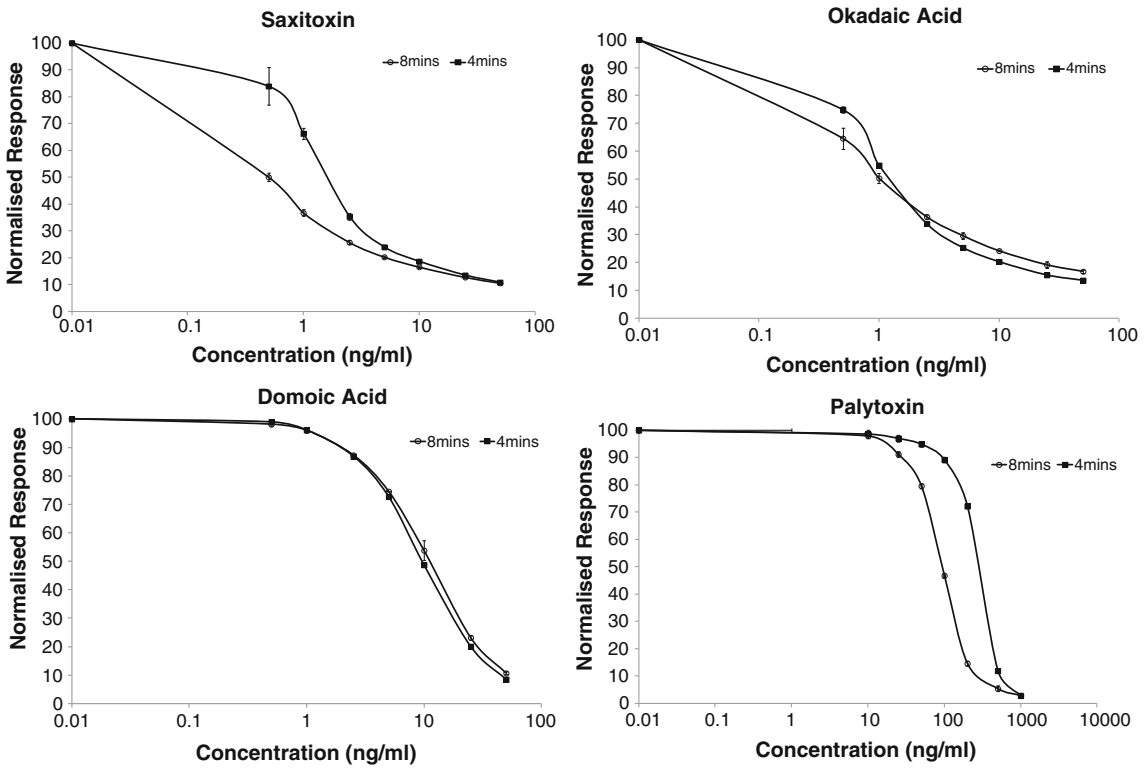

Fig. 2 Calibration curves for saxitoxin, domoic acid, okadaic acid and palytoxin in HBS-EP buffer using 4-min (*filled square*) and 8-min (*empty circle*) injection times to ascertain the surface chemistry and assay viability using a Biacore Q single channel instrument

8 and 11 h for each contact time, respectively. Although 42 samples can be analysed for each toxin, the length of duration of a full analytical run due to the sequential analysis would be too long to offer a practical turnaround time for a marine toxin monitoring programme. Nonetheless, through the manipulation of the chip surface and analysis software, it was demonstrated that the Biacore Q food analysis biosensor could, in principle, be utilised for the multiple analysis of marine toxin analysis using independent channels. However, no further evaluation was undertaken due to the time issues, and the focus of achieving the detection of each toxin class simultaneously was pursued.

Prototype multichannel biosensor

The prototype biosensor was originally configured to measure the biomolecular interactions on four spots within each of the four flow cells as outlined in Fig. 1b. A specially designed microfluidic immobilisation device which was developed offered the ability for 16 targets to be immobilised onto the chip surface off-line from the machine which would align with the detectors once docked inside the instrument [34]. This device originally designed for the immobilisation of peptides or proteins displayed limitations in stability for the immobilisation of small molecular weight compounds requiring longer reaction times or abrasive chemicals in the reaction. The strategy to activate each flow cell of the chip on the instrument and perform an overnight immobilisation outside of the instrument was evaluated initially using the Biacore Q instrument, and this method of preparing the prototype chip improved the stability and robustness of the chip surface for marine toxin analysis. However, this method of immobilisation reduced the number of different molecular interactions that could be measured on the chip to 4, one target per flow cell. To minimise interference and cross-linking of the toxins on flow cells the order in which the toxins were immobilised was important in the procedure. Using this assay described herein, this biosensor could analyse all four toxins in a single sample in 12 min and could perform a six-point calibration curve in duplicate for all four toxins in 2.4 h followed by a maximum of 18 samples per plate. The simultaneous analysis of the toxins in the sample greatly improved the speed of analysis and thereby the viability of the assay to achieve the goal for suitability in a routine monitoring program. The assay can also be designed as a qualitative screening test as a single replicate test to significantly shorten the run time further.

Sensitivity and specificity

The sensitivity (IC_{50}) of the prototype assay was denoted as the concentration at which 50 % inhibition is achieved in the toxin dose-response calibration curves. The dose-response curves (Fig. 3) and the IC_{50} of the assay for each toxin in buffer, in known negative mussel tissue spiked with toxin and

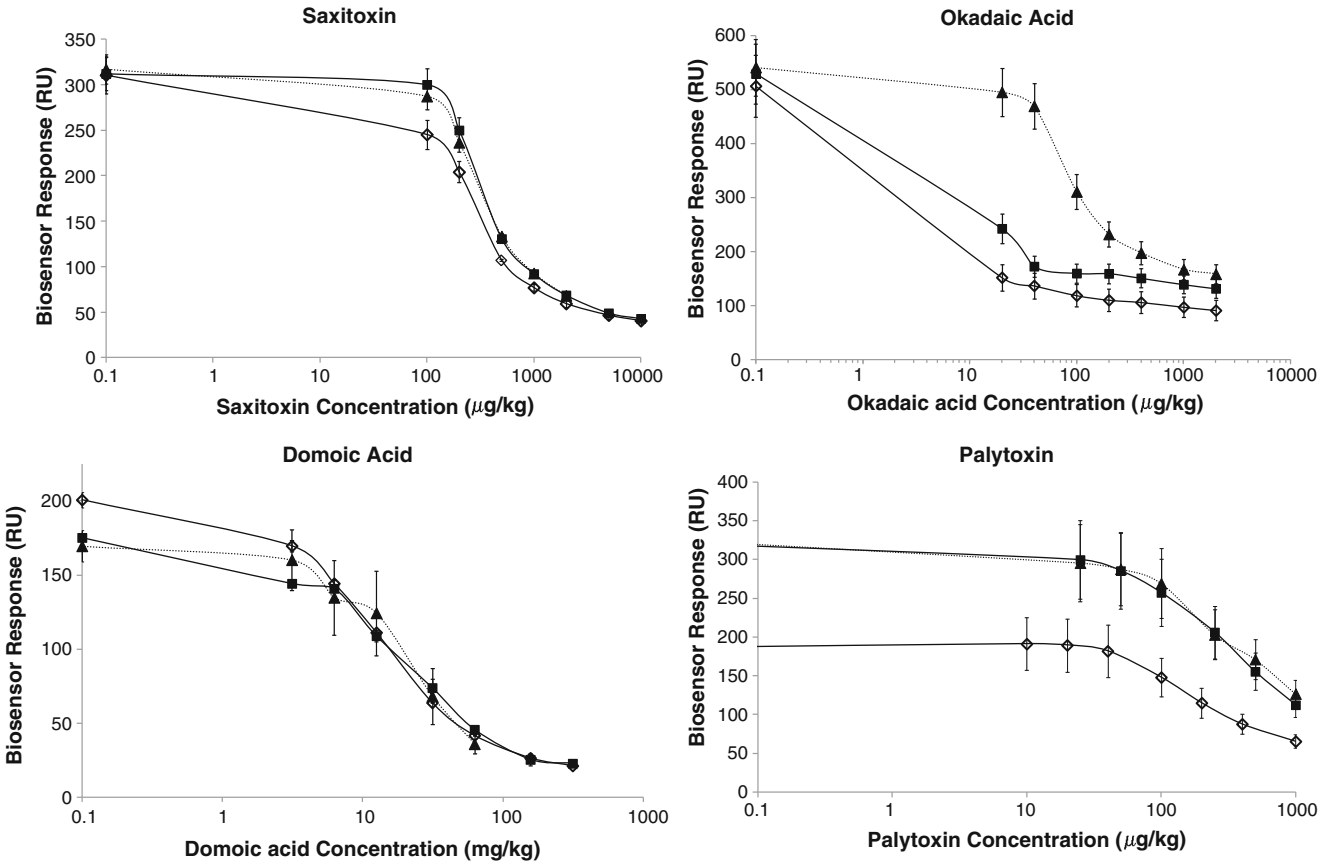

Fig. 3 Calibration curves for saxitoxin, domoic acid, okadaic acid and palytoxin in buffer (*empty rhombus*) and homogenised mussel samples spiked before (*filled triangle*) and after extraction (*filled square*) using the Biacore prototype multichannel instrument

extracted and in negative mussel extract spiked following extraction, are shown in Table 1. The initial matrix evaluation in mussels for this multiplex prototype biosensor demonstrated that the assay was capable of detecting the PSP toxins, okadaic acid and domoic acid at or below the regulatory levels.

Following this preliminary study, the final dilutions of extract in buffer were amended for the PSP toxins to 1 in 80 and for okadaic acid to 1 in 20 to adjust the midpoint of the assay to correlate to the regulatory limits for these toxins and to minimise matrix effects as much as possible through dilution for validation. A difficulty in the palytoxin research throughout was that a limited amount of antibody and toxin was available for the optimization studies. Therefore, the validation of the assay was performed for the regulated toxins only. For both the PSP toxins and domoic acid, the extraction procedure exhibited limited matrix effects on the assay and the recovery of the toxins at the concentrations in the calibration curve was efficient. However, due to the lower dilution of sample extract in buffer, this 50 % methanolic extraction caused enhanced matrix effects and displayed a low recovery for okadaic acid. Similarly for palytoxin at a 1 in 2 dilution, there was a substantial matrix effect but the recovery of the toxin in the extract was still efficient. For these reasons, the study utilised and compared throughout both a buffer curve and an extracted matrix matched curve to determine the format most suitable in the assay. The difficulty with using an extracted matrix matched curve is that known negative material must be available and more toxin is required to spike the sample prior to extraction. However, this is common to most forms of phycotoxin analysis, even when using mass spectrometric techniques. Other options can involve the inclusion of matrix and recovery factors that can be incorporated in the calculations for the final determination of the concentration of toxin in the sample allowing the application of a buffer calibration curve.

The specificity of each antibody to each of the regulated target toxins was the following: for PSP toxins: saxitoxin dihydrochloride (100 %), neosaxitoxin (113 %), decarbamoyl saxitoxin (75 %), decarbamoyl neosaxitoxin (100 %), gonyautoxin 5 (59 %), gonyautoxin 2/3 (6.4), decarbamoyl gonyautoxin 2/3 (1.1 %), C1/C2 (1.4 %) and gonyautoxin 1/4 (21 %); DSP Toxins: Okadaic acid (100 %), dinophysistoxin 1 (68 %), dinophysistoxin 3 (65 %) and domoic acid (100 %). Each antibody raised for saxitoxin and analogues, for okadaic acid and analogues, or for domoic acid displayed no other measurable cross-reactivity to the other marine biotoxin groups at a concentration of 200 ng/mL.

Analysis of uncontaminated mussels for interference effects (decision limit (CCα))

At the 95 % confidence limit of the mean response of the unfortified samples, the decision limits determined from a buffer calibration curve were calculated to be >0.01, 18 and 70 compared to determination from an extracted matrix curve which were calculated to be 24, 36 and 450 μg/kg for saxitoxin, okadaic acid and domoic acid, respectively. This was the background noise level of the assay, and it can be concluded with a 95 % confidence level that any sample which gives a response correlating to a concentration above these levels for each toxin actually contains that toxin. The CCα value represents possible effects of interfering compounds found in the non-contaminated mussel samples.

Detection capability (CCβ)

The ten unfortified samples produced responses ranging in concentration, but it can be seen from these results for saxitoxin and domoic acid that none of the responses from the unfortified samples overlap with any of the responses from the fortified samples (Fig. 4). Therefore, it can be concluded that the detection capability (CCβ) of saxitoxin and domoic acid as a screening method is ≤400 μg/kg and ≤10 mg/kg, respectively, half the action level for each toxin, when interpolated against a buffer or matrix curve with minimal change in response. Due to the dynamic range of the calibration curves, it is difficult to distinguish between half the action level and the action level for domoic and okadaic acid. However, for the detection of okadaic acid, the CCβ is only truly effective for the action level of ≤160 μg/kg when samples are interpolated against a matrix-matched extracted curve due to both the low recovery of the extraction and inherent matrix effects from the dilution used to achieve the sensitivity required (Fig. 3). Nonetheless, the single laboratory validation of this multiple method shows that it can be performed to a level designed for the adequate sensitivity for the EU regulatory limits set for these compounds. The method can be employed as a semi-quantitative screening test as the 95 % confidence levels can be applied for which decisions can be made on a sample at the three detection points of uncontaminated, half the action level and the action level for each toxin (Fig. 4). It can be stated that below these normalised responses as indicated (Fig. 4), there is a 95 % certainty that the sample contains toxin at or above that level of fortification. For saxitoxin and domoic acid, there was a small variability in these confidence limits when a buffer calibration curve is used compared to a matrix calibration curve. However, for okadaic acid, the difference was significant and a matrix calibration curve is recommended for the assay for the determination of this toxin.

Recovery

The recovery at the two levels of toxin fortification interpolated against a buffer curve and matrix-matched curve are shown in Table 2. The recovery of the assays for saxitoxin and domoic acid was both suitable when determined from both buffer and extracted matrix calibration curves at half the action level and the action level. Due to the polarity of these two compounds, they are extremely soluble in the extraction solvent and due to the dilutions applied in the assay, matrix effects observed were minimal. For okadaic acid, the recovery of the assay at the action and half the action level measured against a buffer calibration curve was approximately 50 %. When using an extracted matrix curve, this increased on average to 130 %.

Repeatability

The repeatability of the SPR responses between runs and between matrix samples is outlined in Table 3. The relative standard deviation (% CV) of the SPR responses was acceptable for each assay. However, with small changes in SPR response, there was greater variability in the toxin concentration determined due to extrapolation from the concentration calibration curves and the dilution factors applied. This was noted in the deviation of the measured recovery for the ten samples at each level (Table 2) and was most significant for okadaic acid due to the narrower dynamic range for this toxin compared to that for the other toxins.

Analysis of samples

On examination of the 48 naturally uncontaminated and contaminated samples with marine toxins, the correlation between the concentrations relative to the action limits determined by the multiple SPR method and the available confirmatory data was extremely good, clearly demonstrating the feasibility of multiplex testing using multichannel instrumentation. Table 4 highlights the results obtained and summarises the data as to the number of samples tested for each toxin above the regulatory limit and the number that presented as a false positive or negative. For both saxitoxin and domoic acid, there were no false negatives or positives determined at each action level of 800 μg/kg or 20 mg/kg, respectively. Although, for okadaic acid when the assay was calibrated to a buffer curve, there were two false-negative results albeit presenting close to the action level and there were levels of okadaic acid detected above the regulatory limit of 160 μg/kg when the mouse bioassay was deemed negative. The mouse bioassay recording a negative indicates that the sample was below the action limit only and not okadaic acid free. Of those four false positives, all but one contained okadaic acid at measurable lower concentrations. The correlation was less decisive for okadaic acid

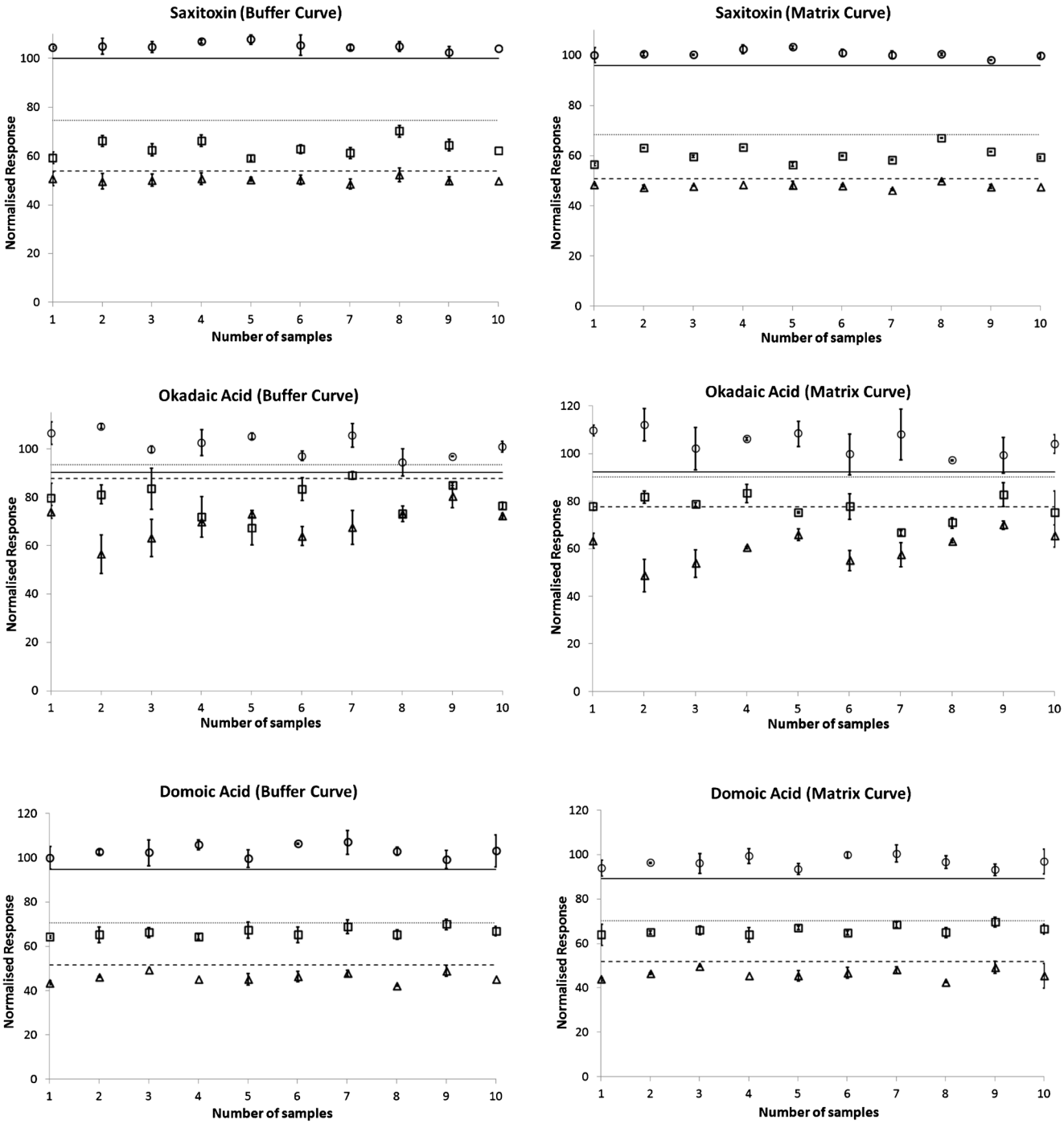

Fig. 4 Distribution of normalised response values on the analysis of samples unfortified (*circle*), fortified at half the action level (*square*) and fortified at the action level (*triangle*). The normalised response representative of the 95 % confidence level for each toxin for the unfortified (*line*), half the action level (*dotted line*) and the action level (*dashed line*) are presented whereby if the normalised response is lower than these levels, there is a 95 % certainty that the toxin concentration is above that level when calibrated either against a buffer or matrix-matched calibration curve

and the DTXs due to the lower recovery in the extraction and matrix effects particularly between species. When the sample results were determined from a mussel matrix curve, no false negatives were recorded but the same four false positives remained. As a screening test for these key regulated toxins, the method has been shown to be fit for purpose for determining samples at the action limit in shellfish meat. Ideally for immunological methods, the goal would be to implement the assay utilising a buffer prepared calibration curve for the determination of toxin content in samples. This would

Table 2 Level of recovery determined from buffer and matrix curves (n=10)

Toxin	Level of fortification (μg/kg)	Level determined from buffer curve (μg/kg)	%CV	% Recovery	Level determined from matrix curve (μg/kg)	%CV	% Recovery
Saxitoxin	400	394±62	15.7	99±16	381±41	10.8	95±10
Saxitoxin	800	748±41	5.5	94±5	837±104	12.4	105±13
Okadaic acid	80	38±10	26.3	48±13	97±21	21.1	121±26
Okadaic acid	160	82±30	36.6	51±19	220±61	27.7	138±38
Domoic acid	10,000	9,980±780	7.8	100±8	7,240±530	7.3	72±5
Domoic acid	20,000	28,750±2,640	9.2	144±13	17,560±1,830	10.4	88±9

eliminate the need of having to source negative shellfish samples for calibration. However, for okadaic acid, this was not achievable based on the extraction efficiency. An extracted calibration curve for each toxin to determine toxin content would minimise extraction efficiency and matrix effects. However, this would utilise a large supply of toxin standards, thereby it may be more applicable to implement correction factors within a calculation to allow for the use of spiked extracts of standards or buffer calibration curves to preserve toxin supplies. A full validation of the assay would need to be completed to achieve this.

Conclusion

The feasibility of the two instruments for the multiplex analysis of shellfish toxins was illustrated, though for time constraints important in turnaround time of analysis, the Biacore Q method would not be as fit for purpose. For saxitoxin and domoic acid, the innovative multiplex SPR method was proven to be sufficiently sensitive to determine the presence at half the regulatory level and above compared to non-contaminated samples. For okadaic acid, some further work on extraction procedures and stability of the chip surface is required to achieve an improved sensitivity. The advantage of this prototype SPR method is that it has the capability to be used as a frontline screening test for the simultaneous detection of the three toxin groups with a single, simple sample preparation procedure. Samples screened at half the action level or above will be confirmed using the appropriate regulatory reference method. Although this methodology has been shown to be fit for purpose, the limitations would be the relatively high cost of Biacore instrumentation for immunodiagnostics and laboratory as the place of use. However, the multichannel system was demonstrated to be a key feature not only in the time of the analysis but also for the detection of these toxins to varying action limits. This allowed for a single extraction and whereby multiple dilutions could be applied to achieve the sensitivity required and allow for the toxin family determination. With the miniaturisation of SPR devices [44], the next stage of evolution of this biotechnology is to take the analysis from the laboratory to multiple channel analysis on-site or remote testing locations to support the aquaculture industry to develop enhanced management systems for harvesting sites and allow improved end-product testing [45]. To date, all end-product tests for shellfish for marine biotoxins can only detect a single group of toxins. Advancements in nanotechnology for miniaturisation and materials for sensor fabrication linked to GPS chips may offer futuristic tools for remote analysis, though sample preparation and design will remain a key component.

Safety

Marine biotoxins are responsible for incidents of paralytic, amnesic and diarrhetic shellfish poisoning. Therefore, when using standard solutions, special care should be taken. Gloves and eye protection should be worn at all times. Appropriate disposal methods should also be utilised by immersing toxin-contaminated materials in sodium hypochlorite solution. The

Table 3 Repeatability of the SPR responses between analysis and between samples (n=10)

Toxin	% CV on SPR responses between duplicate analysis			% CV on SPR response between samples		
	Negative	Half action level	Action level	Negative	Half action level	Action level
Saxitoxin	1.3	0.5	1.1	1.4	10.8	8.1
Okadaic acid	4.3	7.2	4.9	3.7	8.8	10.7
Domoic acid	1.7	1.4	2.7	2.2	3.0	3.3

Table 4 Results for Samples 1 to 48 for each method of analysis

Sample number	Sample species	PSP toxins				Okadaic acid+DTXs				Domoic acid		
		Multiplex SPR (μg/kg)		AOAC HPLC method (STX eqs μg/kg)	Mouse bioassay (STX eqs μg/kg)	Multiplex SPR (μg/kg)		LC-MS (okadaic eqs μg/kg)	Mouse bioassay (okadaic eqs/kg)	Multiplex SPR (mg/kg)		HPLC (mg/kg)
		Buffer curve	Mussel curve			Buffer curve	Mussel curve			Buffer curve	Mussel curve	
S1	Mussel	*1,100*	*1,300*	*2,000*	*858*	19	32	ND	NEG	5.6	1.9	NEG
S2	Mussel	431	384	658	440	82	120	ND	NEG	0	0	NEG
S3	Mussel	*1,200*	*1,341*	*2,630*	*1,154*	29	63	ND	NEG	1.4	0	NEG
S4	Mussel	*1,274*	*1,255*	*838*	*810*	71	110	ND	NEG	0	0	NEG
S5	Mussel	87	250	ND	NEG	144	*178*	*189*	*POS*	3.5	0	NEG
S6	Mussel	310	340	ND	NEG	36	75	151	NEG	1.7	0	NEG
S7	Mussel	13	90	ND	NEG	43	84	0	NEG	1.1	0	NEG
S8	Mussel	16	79	ND	NEG	*170*	*200*	*181*	*POS*	2.7	0	NEG
S9	Mussel	14	96	ND	NEG	47	87	0	NEG	3.1	0	NEG
S10	Mussel	66	98	ND	NEG	*187*	*230*	*176*	*POS*	2.9	0	NEG
S11	Mussel	12	79	ND	NEG	*239*	*307*	*176*	*POS*	0	0	NEG
S12	Mussel	4	0	ND	NEG	*195*	*240*	130	NEG	1.5	0	NEG
S13	Scallop	33	0	ND	NEG	71	30	ND	NEG	*>100*	*>50*	*20*
S14	Mussel	36	0	ND	NEG	56	82	ND	NEG	0.9	0	NEG
S15	Oysters	60	2	ND	NEG	1	8	ND	NEG	2.9	0.5	NEG
S16	Mussel	72	3	ND	NEG	153	*176*	*245*	*POS*	0	0	NEG
S17	Scallop	102	8	ND	NEG	6	28	ND	NEG	*>100*	*>50*	*53*
S18	Scallop	191	45	ND	NEG	*306*	*321*	27	NEG	*>100*	*>50*	*30*
S19	Scallop	166	31	ND	NEG	1	0	ND	NEG	*>100*	*>50*	*57*
S20	Scallop	297	120	ND	NEG	*357*	*368*	48	NEG	*>100*	*>50*	*26*
S21	Mussel	*1,758*	*3,377*	*ND*	*1,443*	1	1	ND	NEG	3.8	1.0	NEG
S22	Mussel	*2,620*	*>4,000*	*ND*	*800*	52	78	ND	NEG	3.2	0.7	NEG
S23	Mussel	*2,118*	*>4,000*	*ND*	*2,228*	2	21	ND	NEG	1.3	0	NEG
S24	Mussel	210	56	0	NEG	*214*	*234*	0	NEG	3.9	1.1	NEG
S25	Mussel	4	0	0	NEG	1	35	0	NEG	1.3	0.6	NEG
S26	Mussel	4	8	ND	500	1	0	ND	NEG	1.1	0.4	NEG
S27	Cockles	4	78	ND	325	1	0	ND	NEG	3.4	2.4	NEG
S28	Mussel	322	392	ND	696	1	19	ND	NEG	0.4	0	NEG
S29	Scallop	4	96	ND	NEG	43	81	ND	NEG	6.2	4.3	3
S30	Scallop	2	109	2	NEG	1	42	ND	NEG	1.2	0.4	NEG

Table 4 (continued)

Sample number	Sample species	PSP toxins				Okadaic acid+DTXs				Domoic acid		
		Multiplex SPR (μg/kg)		AOAC HPLC method (STX eqs μg/kg)	Mouse bioassay (STX eqs μg/kg)	Multiplex SPR (μg/kg)		LC-MS (okadaic eqs μg/kg)	Mouse bioassay (okadaic eqs/kg)	Multiplex SPR (mg/kg)		HPLC (mg/kg)
		Buffer curve	Mussel curve			Buffer curve	Mussel curve			Buffer curve	Mussel curve	
S31	Scallop	60	220	ND	NEG	46	83	ND	NEG	6.8	4.6	3
S32	Scallop	68	229	ND	NEG	45	82	ND	NEG	8.7	5.7	3
S33	Scallop	100	265	ND	NEG	50	85	ND	NEG	*43*	*24*	*20*
S34	Scallop	94	257	ND	NEG	60	91	ND	NEG	*52*	*29*	*20*
S35	Mussel	49	204	ND	NEG	*200*	*175*	*196*	*POS*	0	0.1	NEG
S36	Scallop	93	257	ND	NEG	147	142	18	NEG	*46*	*25*	*20*
S37	Mussels	147	169	ND	NEG	9	90	48	NEG	2.1	1.6	NEG
S38	Mussels	171	203	ND	NEG	5	76	126	NEG	1.2	0.3	NEG
S39	Mussels	4	0	ND	NEG	1	27	ND	NEG	0.3	0	NEG
S40	Scallop	13	0	ND	NEG	19	119	ND	NEG	*50*	*>50*	*106*
S41	Mussel	19	0	0	NEG	17	113	ND	NEG	0.3	0	NEG
S42	Mussels	264	339	ND	575	1	0	ND	NEG	0.6	0	NEG
S43	Mussels	265	340	ND	<200	1	37	ND	NEG	0.3	0	NEG
S44	Cockles	*2,758*	*1,101*	*2,428*	*1,150*	8	87	ND	NEG	0.6	0	NEG
S45	Cockles	*3,993*	*>4,000*	*2,789*	*2,150*	24	132	0	NEG	0.7	0	NEG
S46	Cockles	*4,252*	*>4,000*	*2,003*	*990*	12	102	0	NEG	0.9	0	NEG
S47	Cockles	*3,993*	*>4,000*	*2,238*	*1,250*	31	149	0	NEG	0.8	0	NEG
S48	Cockles	*2,111*	*>4,000*	*1,096*	*960*	28	142	0	NEG	1.0	0	NEG
Number of samples greater than the action level		11	11	11	11	8	10	6	6	9	9	9
Number of false positives relative to the action level		0	0	0	0	2	4	0	0	0	0	0
Number of false negatives relative to the action level		0	0	0	0	4	0	0	0	0	0	0

ND not determined, *NEG* negative below the action level, *POS* positive above the action limit (italicized)

same attention should be paid to contaminated shellfish samples.

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