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Multiplex biotoxin surface plasmon resonance method for marine biotoxins in algal and seawater samples

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Abstract A multiplex surface plasmon resonance (SPR) biosensor method for the detection of paralytic shellfish poisoning (PSP) toxins, okadaic acid (and analogues) and domoic acid was developed. This method was compared to enzyme-linked immunosorbent assay (ELISA) methods. Seawater samples (n=256) from around Europe were collected by the consortia of an EU project MIcroarrays for the Detection of Toxic Algae (MIDTAL) and evaluated using each method. A simple sample preparation procedure was developed which involved lysing and releasing the toxins from the algal cells with glass beads followed by centrifugation and filtering the extract before testing for marine biotoxins by both multi-SPR and ELISA. Method detection limits based on IC_{20} values for PSP, okadaic acid and domoic acid toxins were 0.82, 0.36 and 1.66 ng/ml, respectively, for the prototype multiplex SPR biosensor. Evaluation by SPR for seawater samples has shown that 47, 59 and 61 % of total seawater samples tested positive (result greater than the IC_{20}) for PSP, okadaic acid (and analogues) and domoic acid toxins, respectively. Toxic samples were received mainly from Spain and Ireland. This work has demonstrated the potential of multiplex analysis for marine biotoxins in algal and seawater samples with results available for 24 samples within a 7 h period for three groups of key marine biotoxins. Multiplex immunological methods could therefore be used as early warning monitoring tools for a variety of marine biotoxins in seawater samples.

Keywords Biosensor · Surface plasmon resonance (SPR) · Biotoxins · Multiplexing · Paralytic shellfish poisoning (PSP) toxins · Okadaic acid · Domoic acid · Harmful algal bloom (HAB)

Introduction

There is no doubt that harmful algal blooms (HABs) are occurring in more locations than ever before and new sightings are reported regularly with virtually every coastal country in the world being affected (Hallegraeff 1993; Anderson 2009; Chadsey et al. 2012). In addition, there have been more toxic algal species, algal toxins, affected fisheries and economic losses from HABs than ever before (Anderson 2009; Anderson et al. 2012). HABs are caused by the rapid increase in the population of algae in an aquatic system to the extent that it dominates the local planktonic or benthic community. Most species of algae or phytoplankton are not harmful and serve as a food source at the bottom of the food web for many types of marine fish and animals. However, there are some species of algae that are toxic and these toxins can be transferred throughout the food web where they can kill fish, birds, marine mammals and potentially humans. Some of these toxins can be acutely lethal and no antidote exists to any HAB toxin (Glibert et al. 2005). As these toxins are tasteless, odourless and heat and acid stable, normal food preparation procedures will not prevent intoxication if the shellfish is contaminated (Fleming et al. 2006). It is not clear why some algal species produce toxins, possibly to offer some sort of advantage to the producer

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for example; competition for space, to fight predation or as a defence against the overgrowth of other organisms (Anderson 1994). The causes of HABs are much debated with explanations such as changing climate, moving tectonic plates, global changes or human related through pollution, agriculture (Anderson 2009; Anderson et al. 2012) or transport of algal species via ship ballast water (Hallegraeff and Bolch 1992). Whatever the cause, HABs have increasingly become a greater concern because of their adverse effects on the health of people and marine organisms as well as the health of local and regional economies. Economic impacts include the costs of monitoring programmes for shellfish and phytoplankton, short- and long-term closure of harvestable shellfish and fish stocks, reduction in seafood sales, mortalities of fish and impacts on tourism-related industries (Anderson 2009; Anderson et al. 2012).

A number of different types of shellfish poisoning are associated with HABs that contain toxic algae from several dinoflagellate and diatom species. Shellfish grazing on these algae can accumulate the toxins but have the added advantage that they themselves are rather resistant to the toxin effects. Toxins were originally classified corresponding to the acute symptoms in humans and include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP). The principle toxin responsible for PSP is saxitoxin, from which there are 57 analogues to date produced by dinoflagellates mainly from the *Alexandrium* genus (Wiese et al. 2010). The various PSP toxins differ significantly in toxicity with saxitoxin being the reference compound. PSP toxins are neurotoxins which bind to voltage-dependent sodium channels resulting in the blockage of sodium ions transport leading to paralysis (Kao 1996). Adverse effects of PSP toxins include tingling or numbness of the lips which can spread to the neck and face. Headache, dizziness, nausea, vomiting and diarrhoea are also common. In a progressed state, the motor nerves are affected resulting in respiratory and paralytic effects which in some cases can eventually lead to death. The genera *Dinophysis* and *Prorocentrum* produce the causative toxin okadaic acid and the related dinophysistoxins 1–3 (DTX1–3) compounds involved in episodes of DSP. These toxins are potent protein phosphatase inhibitors, mainly PP2A and PP1 (Honkanen et al. 1994) resulting in a loss of cellular fluids. Adverse effects as the name suggests include diarrhoea, gastrointestinal disorder, abdominal cramps, nausea and vomiting. Domoic acid is produced by several species of diatoms from the *Pseudo-nitzschia* genus and cause ASP. The primary action of domoic acid is on the hippocampus, which is involved in processing memory functions (Adams et al. 2009). Adverse effects include nausea, vomiting, abdominal cramps, diarrhoea as well as headache, dizziness and loss of short term memory.

The increasing concerns about marine biotoxins and food safety have necessitated the need for more rapid, robust and highly sensitive detection methods. No legislation is currently available for the testing of marine biotoxins in algal or seawater samples, testing for these toxins is only required for shellfish. Analytical methods have been developed for the quantitative analysis of marine biotoxins in shellfish with these methods undergoing adaptation for the detection in algal and seawater samples. High-performance liquid chromatography (HPLC) with fluorescent detection using both pre-column oxidation (He et al. 2005; Smith et al. 2011) and post column oxidation (Oshima 1995; Wang et al. 2006; Touzet et al. 2007; Montoya et al. 2010) in addition to liquid chromatography tandem mass spectrometry (LCMS/MS; Halme et al. 2012) have been utilised for the detection of PSP toxins in algal and seawater samples with detection limits of nanograms per milliliter. Similarly, HPLC with fluorescence detection (Bravo et al. 2001) and LCMS/MS methods have been applied for the detection of the DSP toxins in algal and seawater samples (Fux et al. 2009, 2011; Hackett et al. 2009; Vale et al. 2009; Gerssen et al. 2011). Many authors describe the detection of domoic acid at pictogram/nanogram per millilitre using LCMS/MS (Wang et al. 2007; de la Iglesia et al. 2008; Alvarez et al. 2009; Mafra Jr et al. 2009; Blanco et al. 2010; Quijano-Scheggia et al. 2010). Pocklington et al. (1990) describes a method incorporating HPLC with fluorescent detection of domoic acid with others using a modified version (Cusack et al. 2002; Fehling et al. 2004; Besiktepe et al. 2008). Although analytical methods are available, it is acknowledged that these methods require skilled personnel and are labour intensive measuring only one toxin group per method.

Nevertheless, the ability to monitor the detection of marine biotoxins in algal and seawater samples using a sensitive fit for purpose method is increasingly important as it would offer an early warning detection tool authorising important decisions to be taken before the contamination of shellfish. Immunological assays have shown promise as sensitive rapid screening tools. A number ELISA methods exist for the semi-quantitative detection of marine biotoxins with commercial ELISAs (Biosense Laboratories) available for use in seawater (Lefebvre et al. 2008; Del Rio et al. 2010) showing detection limits in the parts per trillion levels for PSP, DSP and ASP toxins. A qualitative lateral flow device is also available for seawater biotoxin detection from Jellet (Jellet Rapid Testing Ltd) with detection limits of 150 and 100–200 ng/ml for ASP and PSP toxins, respectively (Turrell et al. 2008; Brown et al. 2010).

The emergence of biosensor-based immunological assays in the field of marine biotoxin testing has been noted due to the reported added advantages. These include high sensitivity, low limit of detections, portability, specificity and robustness. One of the most promising biosensor methods is based on

SPR; a label-free technique for the sensitive real-time monitoring of molecular interactions. SPR biosensors have proven to be a successful platform for detecting low level contaminants and toxins in food products with many authors describing its applicability in the field of marine biotoxin analysis (Yu et al. 2005; Traynor et al. 2006; Llamas et al. 2007; Campbell et al. 2007, 2009, 2010, 2011a; Fonfria et al. 2007; Marchesini et al. 2009; Rawn et al. 2009; Stewart et al. 2009a, b; Haughey et al. 2011; Van den Top et al. 2011; Yakes et al. 2011). However, all of these methods only describe the analysis of one specific or one group of biotoxins. Multiplexing was achieved using a prototype SPR biosensor from GE Healthcare in a 4×4 layout, with four flow cells each containing four spots, giving a total of 16 analytes for simultaneous detection. Domoic acid, okadaic acid, saxitoxin and neosaxitoxin have all been successfully immobilised on the multi-biosensor chip for the detection of these biotoxins (Campbell et al. 2011b). Within the MIDTAL project, this technology was applied for the multiplex analysis of marine biotoxins in seawater samples. The purpose of the MIDTAL project is to support the common fisheries policy to aid monitoring agencies by providing new rapid tools for the identification of toxic algae and their toxins. The aim of this research was to develop a multiplex SPR biosensor assay for the simultaneous detection of PSP, okadaic acid (and analogues) and domoic acid toxins in seawater samples using a rapid sample preparation procedure and to employ the assay to analyse seawater samples from different sampling sites across Europe.

Materials and methods

Instrumentation

The prototype multiplex SPR biosensor was developed by GE Healthcare (Uppsala, Sweden).

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$^+$ 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). Saxitoxin was purchased from the National Research Council of Canada (Halifax, Canada) and okadaic acid was obtained from LC Laboratories (Massachusetts, USA). Commercial ELISA kits for the analysis of domoic acid (ref. code E.F.1), okadaic acid (ref. code E.F.2) and saxitoxin (ref. code E.F.3) were developed and provided by Centre d'Economie Rurale (CER), Belgium. Nitrocellulose syringe filters (0.45 μm) were obtained from Millipore (Watford, UK) and the 0.5 mm glass beads were obtained from Thistle Scientific Ltd (Glasgow, UK).

Prototype SPR multiplex biosensor methodology

Biosensor chip immobilisation

A series S CM5 sensor chip was docked onto the prototype multiplex SPR biosensor and washed with HBS-EP$^+$ buffer (running buffer). The carboxymethylated surface of the sensor chip was activated using EDC and NHS (1:1, *v*/*v*) and injected over the surface of flow cell 1 for 15 min. Jeffamine 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 allowed to immobilise onto the chip surface overnight at 37 °C on the bench. These steps were repeated separately for okadaic acid (flow cell 2) and domoic acid (flow cell 3). Flow cell 4 was left as a blank control with no toxins immobilised. 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 sensor chip was stored at 4–8 °C until needed. The layout of the chip surface is as illustrated in Fig. 1.

Antibody production

The preparation of immunogens and the production of the saxitoxin antibody (Campbell et al. 2007), okadaic acid antibody (Llamas et al. 2007) and domoic acid antibody

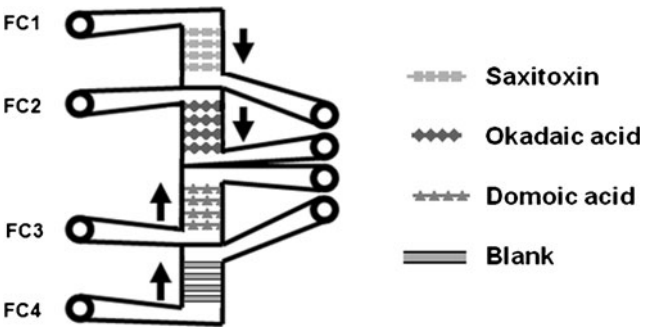

Fig. 1 Layout and spotting of the four flow cells (*FC*) for the prototype multiplex SPR biosensor chip. Four spots of each toxin was spotted in each FC (leaving FC4 as a blank to be used as a control to ensure immobilisation was successful) allowing samples to be analysed in quadruplicate. Arrows show the flow of samples across the individual flow cells and the inlets/outlets for each flow cell being separate from each other

(Traynor et al. 2006) were prepared similar to that published previously.

Biosensor parameters

The multiplex SPR sensor chip was optimised on the prototype multiplex SPR biosensor. The flow rate across the chip surface was 20 μl/min and sample contact time was 480 s. Report points were taken 10 s before injection and 30 s after injection to determine baseline and level of binding on the chip surface. The antibody titre was determined as the response between 300 and 500 resonance units (RU) over 480 s for the 0 ng/ml calibration standard. The saxitoxin, okadaic acid and domoic acid antibodies were therefore diluted 1/750, 1/10,000 and 1/2,000 in HBS-EP$^+$ buffer respectively and mixed 1:1 with samples or standards. The surface of the sensor chip was finally regenerated for 60 s (30 s×2) before analysis of the next sample. The regeneration solution for each toxin was optimised to allow the response to return to baseline after each sample. Regeneration solutions for saxitoxin, okadaic acid and domoic acid were 10 mM HCl/1 % SDS (1:1, *v*/*v*), 250 mM NaOH/acetonitrile (8:2, *v*/*v*) and 100 mM HCl/1 % SDS (1:1, *v*/*v*), respectively. The duration of sample analysis including regeneration of the chip was approximately 13 min for each sample.

Evaluation of seawater matrix effects

Natural seawater (salinity 33–34 ppt) for the evaluation of seawater matrix effects was obtained from Strangford Lough (Co. Down, Northern Ireland, UK). The seawater sample (1 L) was extracted using the method as described for the seawater sample preparation ("Seawater sample preparation" section). The matrix-extracted seawater calibration curve was diluted 1:1 with HBS-EP$^+$ and the final extract was spiked at different calibration concentrations for saxitoxin, okadaic acid and domoic acid (0–50 ng/ml). This matrix extracted seawater curve was compared to a HBS-EP$^+$ buffer/deionised water (1:1) calibration curve and tested on the prototype multiplex SPR biosensor.

Limit of detection

The limit of detection is defined as the lowest concentration of toxin in a sample that can be detected. The theoretical limit of detection for this research was defined as the IC_{20} of the assay. The IC_{20} is defined as the inhibitory concentration of toxin required to reduce the response by 20 % compared to the response when no toxin is present (100 % binding). This was calculated from an average of all calibration curves analysed during this research (n=33). Additionally, 17 extracted negative matrix cultures were assessed by the multiplex SPR biosensor.

Biosensor chip stability

Stability studies were carried out using the series S CM5 multiplex sensor chip on the prototype multiplex SPR biosensor in order to assess the stability of the immobilised biotoxins. This was determined using a 0 ng/ml calibration standard and measured in RU over time. Fresh working antibody solutions were prepared on each occasion and the sensor chip was stored at 4–8 °C prior to use. The sensor chip was assessed at day 0 and then routinely over an 8-week period.

ELISA methodology

The reagents were prepared and the methods were followed as described in the commercial ELISAs manufacturer's protocol (CER, Belgium) and previous publication (Dubois et al. 2010) with the exception that the calibration standards were made up in ELISA dilution buffer/deionised water (1:1). Standards or samples (50 μl) were applied to the pre-coated purified sheep anti-rabbit IgG microtitre plates. Diluted peroxidase conjugate (100 μl) and reconstituted antibody (100 μl) were applied to all wells and incubated at +4 °C for 2 h (okadaic acid and domoic acid ELISA) or overnight (saxitoxin ELISA). The wells were emptied and washed three times (okadaic acid and domoic acid ELISA) or five times (saxitoxin ELISA) with washing buffer. Peroxide/TMB (150 μl) was added to each well and incubated for 30 min in the dark at room temperature. Finally, 1.8 M sulphuric acid (50 μl) was added to each well to stop the reaction and the absorbance was read at 450 nm within 30 min using a Tecan plate reader (Tecan UK Ltd, Reading, UK).

Sample collection

Seawater samples (n=256) were collected during 2009 and 2011 from a number of sites across Europe by the consortia (Table 1) of the MIDTAL EU project. The seawater samples were filtered through a 0.45 μm nitrocellulose filter until the filter clogged (approximately 0.5–2 L). The filter was frozen in a 1.5–2 ml Eppendorf tube at −20 °C and shipped to Queen's University, Belfast for further extraction and analysis for PSP, okadaic acid (and analogues) and domoic acid toxins.

Seawater sample preparation

A number of cell lysis methods were examined including both chemical and physical methods. Optimum cell lysis was observed using the following protocol. The frozen filter was allowed to defrost and was carefully removed from the Eppendorf tube, gently opened and transferred to a 20 ml

Table 1 PSP, okadaic acid and domoic acid biotoxins results for seawater samples (n=256) collected from different sampling sites across Europe during the period 2009 – 2011 as part of the MIDTAL project. Samples were analysed by both testing platforms, the prototype multiplex SPR biosensor and commercial CER ELISA

Partner	Sampling site	Sampling period 2009								Sampling period 2010								Sampling period 2011							
		Months	Total No.	PSP		Okadaic acid and analogues		Domoic acid		Months	Total No.	PSP		Okadaic acid and analogues		Domoic acid		Months	Total No.	PSP		Okadaic acid and analogues		Domoic acid	
				SPR	ELISA	SPR	ELISA	SPR	ELISA			SPR	ELISA	SPR	ELISA	SPR	ELISA			SPR	ELISA	SPR	ELISA	SPR	ELISA
Marine Biological Association, Plymouth, UK (MBA)	Arcachon Bay, France	N/A	0	0	0	0	0	0	0	Jul–Sep	4	0	4	0	0	1	0	Jul–Oct	5	1	5	0	4	0	2
Stazione Zoologica ‘A. Dohrn’ di Napoli, Naples, Italy (SZN)	Gulf of Naples, Italy	Sep–Dec	4	1	4	2	1	3	3	Jan–Dec	12	4	12	4	3	11	8	Jan–Aug	7	6	7	2	6	6	7
Linnaeus University, Kalmar, Sweden (LNU)	Skagerrak area, Sweden	Jul–Dec	6	0	4	0	1	0	1	Jan–Dec	15	5	14	5	8	1	5	Jan–Apr	3	1	3	0	1	1	3
Instituto Espanol de Oceanografia, Vigo, Spain (IEO)	Ría of Pontevedra, Spain	Jul–Nov	10	6	9	10	10	10	10	Mar–Sep	18	15	18	16	18	15	18	N/A	0	0	0	0	0	0	0
Martin Ryan Institute, National University of Ireland, Galway, Ireland (NUIG)	Killary, Cork and Bell Harbours, Ireland	Jul–Dec	8	7	7	5	7	4	8	Jan–Oct	15	2	13	14	11	10	14	Mar–Sep	36	30	36	27	20	31	21
University of Oslo, Oslo, Norway (UO)	Oslofjord, Norway	Aug–Dec	5	0	1	0	2	0	2	Jan–Dec	10	2	7	10	5	5	7	Jan–Jun	6	0	5	0	3	1	6
University of Westminster, London, UK (UW)	Orkney Islands, UK	Oct–Dec	2	0	0	0	1	0	1	Feb–Dec	10	1	10	8	4	5	4	Jan–Oct	10	5	10	2	7	7	10
Technological Institute for t he marine environment control of Galicia, Pontevedra, Spain (INT)	Rias of Pontevedra, Arosa, Muros, Ares-Betanzos, and estuary of Bayona	Sep–Nov	12	8	10	10	7	7	12	Jan–Dec	42	18	39	27	23	32	37	Jan–May	16	9	15	9	15	11	14

tube. The Eppendorf tube was rinsed with deionised water (1 ml) and added to the 20 ml tube containing the filter. This step was repeated to give a total of 2 ml deionised water. The sample was vortexed for 20 s and rotated on a head over head mixer for 20 min. The filter was removed from the 20 ml tube being careful to remove as much liquid from the filter as possible and ensuring that any algal cells had been washed off the filter. The supernatant was transferred to a 5 ml tube containing 0.5 mm glass beads (1 g) and allowed to shake for 20 min on a Merris Minimix shaker (Merris Engineering Ltd, Galway, Ireland). Finally, samples were centrifuged at 3,000×*g* for 10 min and the supernatant was filtered using a 0.45 μm syringe filter.

Seawater sample analysis

Seawater samples (n=256) where analysed using the two testing platforms. Depending on the method of analysis, extracts were diluted 1:1 with corresponding buffers; HBS-EP$^+$ buffer for the prototype multiplex SPR biosensor (analysed in quadruplicate) or ELISA dilution buffer for the ELISA (analysed in duplicate).

Results

Biosensor chip immobilisation

Saxitoxin, okadaic acid and domoic acid were successfully immobilised onto the multiplex sensor chip (Fig. 1) based on antibody response and comparison with the blank control flow cell.

Evaluation of seawater matrix effects

Figure 2 shows a comparison of a matrix-extracted seawater/HBS-EP$^+$ buffer (1:1) curve and a deionised water/HBS-EP$^+$ buffer (1:1) curve for saxitoxin (Fig. 2a), okadaic acid (Fig. 2b) and domoic acid (Fig. 2c). It can be seen that both curves are very similar for all three biotoxins with less than 10 % difference observed at each calibration standard for all toxins. Validation and analysis of seawater samples was therefore carried out with a deionised water/HBS-EP$^+$ buffer (1:1) curve.

Limit of detection

The limit of detection for each toxin is based on the detection ability of the prototype SPR instrument and no calculations are incorporated for the concentration step of the sample preparation. The theoretical limit of detection for these assays was defined as the IC_{20} derived from the calibration curves for each toxin. Figure 3 shows calibration curves for both the prototype multiplex SPR biosensor (Fig. 3a) and ELISA (Fig. 3b). The limits of detection (Table 2) for saxitoxin, okadaic acid and domoic acid were 0.82, 0.36 and 1.66 ng/ml, respectively, for the prototype multiplex SPR biosensor (33 runs). Additionally, when comparing 17 extracted negative matrix cultures, the limit of detection incorporating matrix effects (calculated concentration at the mean response minus three times standard deviation) was calculated as 0.93, 0.26 and 2.41 ng/ml for saxitoxin, okadaic acid and domoic acid, respectively, when analysed by multi-SPR. At or below these levels, there is a 99 % chance that the sample is negative. From the analysis using ELISA (13 runs) limit of detections were calculated as 0.01, 0.41 and 0.34 ng/ml for the saxitoxin, okadaic acid and domoic acid ELISAs respectively.

Biosensor chip stability

To determine the stability of the multiplex sensor chip over time, the change in biosensor response measured in RU was monitored over an 8-week period. If the immobilised biotoxins on the sensor chip had have been unstable, there would have been a steady decrease in response over time at each time point. However, from Fig. 4, it can be seen that response did not drop off significantly for any biotoxin, although fluctuations did occur there was ±20 % deviation from day0 observed at the end of the 8-week period for all three biotoxins.

Seawater sample preparation

A number of different cell lysis methods were examined including both chemical and physical methods to obtain a method of cell lysis that would be optimum for different types of toxin-producing algae. These methods included glass bead beating, sonication, freeze–thawing, SDS, copper sulphate, penicillin and lysozyme. Optimum cell lysis was obtained when a freeze–thaw step was combined with glass bead beating. Algal cultures examined included *Alexandrium fundyense*, *Pseudo-nitzschia multiseries* and *Prorocentrum lima* which give 100, 100 and 77 % cell lysis respectively when 16 sub-samples of each algal culture was assessed with optimum cell lysis conditions (data not shown). The optimum cell lysis method was therefore used for the extraction of toxins from seawater samples.

Seawater sample analysis

Analysis of PSP, okadaic acid (and analogues) and domoic acid toxins were performed using both the prototype multiplex SPR biosensor and ELISA for a comparative evaluation. Filtered seawater samples (n=256) were received from eight of the MIDTAL partners for biotoxin analysis. Seawater

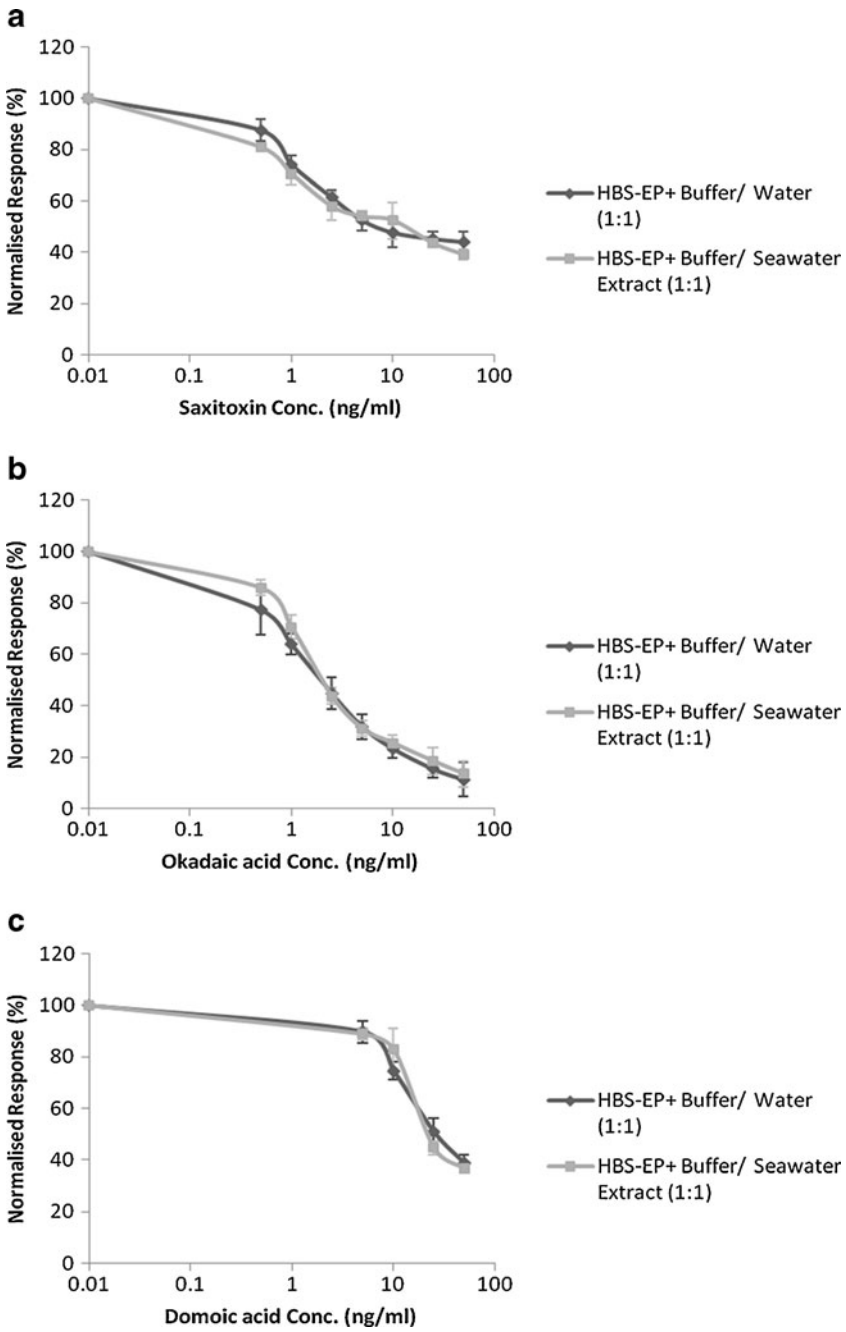

Fig. 2 Comparison of matrix extracted seawater and water curves using the prototype multiplex SPR biosensor in the range 0–50 ng/ml (n=3) for saxitoxin (**a**), okadaic acid (**b**) and domoic acid (**c**) representing flow cell 1, 2 and 3, respectively. Flow cell 4 (blank control) showed a constant response when analysing HBS-EP$^+$ buffer. *Error bars* show standard deviation of response across three separate runs

samples were collected as indicated in Table 1 from various regions across Europe in triplicate (Gulf of Naples, Italy; Skagerrak area, Sweden; Ría of Pontevedra, Spain; Oslofjord, Norway; Orkney Islands, UK), duplicate (Killary, Cork and Bell Harbours, Ireland) or singly (Arcachon Bay, France; Rias of Pontevedra, Arosa, Muros, Ares-Betanzos, and estuary of Bayona, Spain).

PSP toxins were detected in 121 (47 %) and 233 (91 %) of all seawater samples analysed by the prototype SPR multiplex biosensor and ELISA, respectively, during the period 2009–2011 (Table 1). The highest levels of PSP toxins were detected in Ría of Pontevedra, Spain with levels of greater than 100 ng/filter examined between August and November 2009. Significant levels of PSP toxins were also detected by Rias of Arosa, Muros, Ares-Betanzos, and estuary of Bayona, Spain and in the Killary, Cork and Bell Harbours in Ireland. These areas of Spain showed high PSP levels between October and November 2009 and again between June and October 2011. Ireland also showed high levels during May–June 2011.

Okadaic acid and analogues were detected in 151 (59 %) and 157 (61 %) of all seawater samples analysed by the prototype SPR multiplex biosensor and ELISA, respectively, during the period 2009–2011 (Table 1). However when

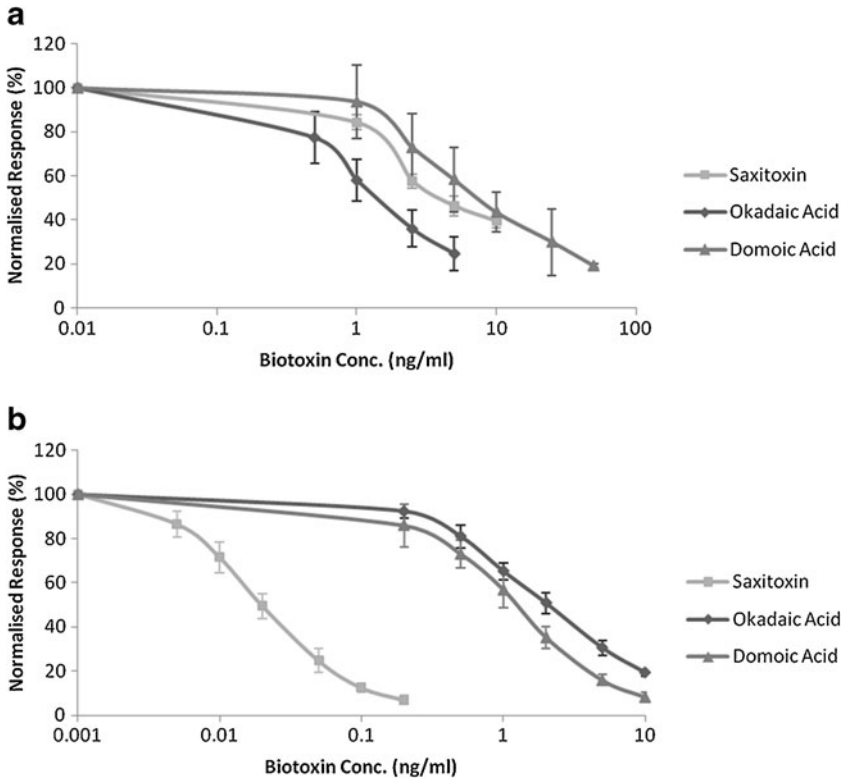

Fig. 3 Calibration curves for both the prototype multiplex SPR biosensor (**a**) and the commerical ELISA from CER (**b**). Calibration curves for saxitoxin, okadaic acid and domoic acid in HBS-EP$^+$/deionised water (1:1) using the protoype multiplex SPR biosensor (n=33). Calibration curves for saxitoxin, okadaic acid and domoic acid in ELISA dilution buffer/deionised water (1:1) using the commercial CER ELISAs (n=13). *Error bars* show standard deviation of response across 33 runs for the multiplex SPR biosensor and 13 runs for the ELISA

examining Table 1, it can be seen that there are slight differences between results for the prototype multiplex SPR biosensor and ELISA with some partners showing a greater number of positive samples for ELISA and others for SPR. Okadaic acid toxins appeared to be more prevalent in Spain with both Spanish regions detecting significant levels over the 2-year sampling period. At Ría of Pontevedra, Spain, levels were high in July 2009 remaining positive throughout the rest of 2009 but increasing again in May 2010 and staying high (>20 ng/filter) until the end of their sampling period (September 2010). Rias of Arosa, Muros, Ares-Betanzos and estuary of Bayona, Spain also showed levels of okadaic acid toxins throughout their sampling period with highest levels detected in July to the start of September 2010. Okadaic acid toxins increased again for these areas at the end of June 2011 and remained until the end of the sampling period (October 2011).

From Table 1, it can be observed that domoic acid toxins were detected in 156 (61 %) and 181 (71 %) of all seawater samples analysed by the prototype SPR multiplex biosensor and ELISA, respectively, during the period 2009–2011. Most regions examined showed some level of domoic acid toxins during the sampling period. The greatest levels of these toxins were detected in Spain and Ireland with high levels also detected in Italy. Ría of Pontevedra, Spain showed the highest levels during June 2010 until the end of their sampling period (September 2010). Rias of Arosa, Muros, Ares-Betanzos, and estuary of Bayona, Spain showed highest levels of domoic acid in May to August 2010 and again in April to October 2011. Killary, Cork and Bell Harbours in Ireland showed seawater samples with domoic acid toxins throughout their sampling period with the highest levels in July and August 2009, May and June 2010 and between May and August 2011. The Gulf of Naples, Italy appeared to show domoic acid toxins more than the other toxins (Fig. 5) with the highest levels of toxins detected in September to November 2010 and again towards the end of their sampling period (April–August 2011).

Discussion

Biosensor chip immobilisation

Multiplexing was achieved using a prototype SPR biosensor in a 4×4 layout, with four flow cells each containing four spots, giving a total of 16 analytes for simultaneous detection. Saxitoxin, okadaic acid and domoic acid were successfully immobilised onto all four spots on flow cell 1, 2 and 3, respectively, as shown in Fig. 1. In this research, each biotoxin was immobilised on all four spots of one flow cell allowing for analysis of samples in quadruplicate. Spotting of biotoxins this way allowed for the prolonged shelf life of the biosensor chip, if one of the spots became unstable, there would still be results from the other three spots remaining. Activation of the chip surface was carried out initially on the biosensor as a simpler step rather than using the

Table 2 Comparison of testing platforms, the prototype multiplex SPR biosensor and commercial CER ELISA showing main characteristics, benefits and limitations for each. Data for sensitivity is based on 33 runs (prototype multiplex SPR biosensor) and 13 runs (ELISA)

Parameter	Prototype multiplex SPR Biosensor				CER commercial ELISA			
Detection capability	PSP, okadaic acid (and analogues) and domoic acid toxins detected simultaneously.				PSP, okadaic acid (and analogues) and domoic acid toxins detected by three separate ELISAs.			
Sensitivity		Mid – point of curve (ng/ml)	Dynamic range of curve (ng/ml)			Mid – point of curve (ng/ml)	Dynamic range of curve (ng/ml)	
		IC_{50}	IC_{20}	IC_{80}		IC_{50}	IC_{20}	IC_{80}
	Saxitoxin	1.71 ± 0.29	0.82 ±0.25	3.57 ± 0.62	Saxitoxin	0.02 ± 0.00	0.01 ± 0.00	0.05 ± 0.01
	Okadaic acid	1.00 ± 0.23	0.36 ± 0.18	2.75 ± 0.53	Okadaic acid	1.37 ± 0.18	0.41 ± 0.10	3.80 ± 0.58
	Domoic acid	5.30 ± 1.97	1.66 ± 1.71	17.60 ± 3.89	Domoic acid	1.06 ± 0.27	0.34 ± 0.13	2.97 ± 0.52
Specifity			Cross Reactivity				Cross Reactivity	
	PSP		Saxitoxin 100%, Neosaxitoxin 113%, Decarbamoyl neosaxitoxin 100%, Decarbamoyl saxitoxin 75%, GTX5 59%, GTX1/4 21%, GTX2/3 6.4%, Decarbamoyl GTX2/3 1%, C1/2 1.4%		PSP		Saxitoxin 100%, Neosaxitoxin 1.4%, Decarbamoyl neosaxitoxin 0.5%, Decarbamoyl saxitoxin 19.2%, GTX5 26.2%, GTX1/4 <0.1%, GTX2/3 5.6%, Decarbamoyl GTX2/3 0.2%, C1/2 0.2%
	Okadaic acid		Okadaic acid 100%, DTX1 68%, DTX2 65%		Okadaic acid		Okadaic acid 100%, DTX1 78%, DTX2 2.6%	
	Domoic acid		Domoic acid 100%		Domoic acid		Domoic acid 100%	
Ease of use	Method is relatively easy but will require initial training				Method is relatively easy but will require initial training			
Speed of analysis (For 24 samples)	Extraction: 2 hr Analysis: 5 hr				Extraction: 2 hr Analysis: 3 hr (Domoic acid and Okadaic acid) or overnight (Saxitoxin)			
Portability	Not possible				Not possible			
Benefits	Relatively fast result, highly sensitive, highly specific, cost effective, high throughput screening, real time results.				Highly sensitive, highly specific, cost effective, high throughput screening.			
Limitations	Supply of biotoxins required for routine analysis				Time consuming, interpretation and analysis of results is required			
Maximum sample analysis per run	24				48 (per ELISA)			
Analysis time per sample	13 min				Dependent on ELISA incubations therefore 3 hr (Domoic acid and Okadaic acid) or overnight (Saxitoxin) independent of number of samples			

immobilisation unit device as described by Campbell et al. (2011b). The immobilisation of toxins on the chip surface overnight provided enhanced stability of the surface compared to the immobilisation unit.

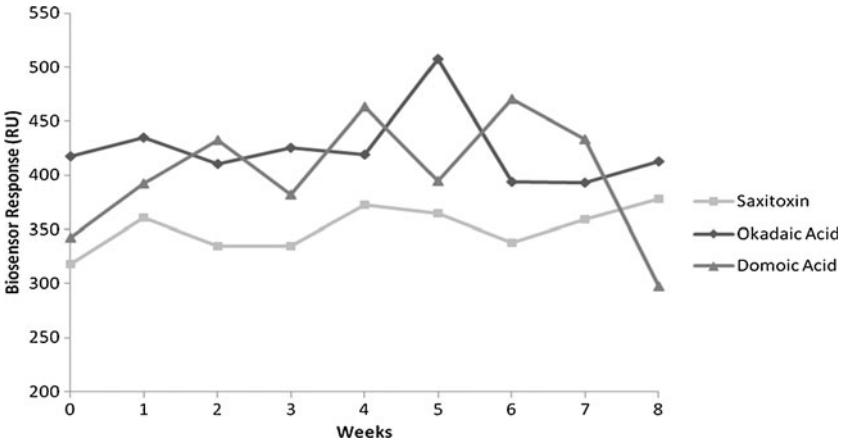

Fig. 4 Multiplex biosensor chip stability assessed over 8 weeks for the prototype multiplex SPR biosensor chip using the 0 ng/ml calibration standard. Stability is based on the biosensor response measured in resonance units (RU) over time

Evaluation of seawater matrix effects

Matrix effects were not observed when comparing seawater and deionised water calibration curves. Less than 10 % difference was observed for all calibration standards for each toxin. This allowed samples to be analysed with a deionised water curve and meant that the method was much easier, quicker and did not require vast volumes of seawater to complete the analysis. Seawater samples were extracted in water so that samples could be analysed by all detection methods by simply requiring a 1:1 dilution with the corresponding detection buffer.

Limit of detection

The sensitivity of each antibody to the corresponding biotoxin on each flow cell was assessed and optimised. The antibody titre was optimised from the antibody dilution that gives a response between 300 and 500 RU over the contact time of 480 s. Regeneration solutions were optimised for each flow cell and hence biotoxin. The fluidics of the prototype multiplex SPR biosensor allows separate regeneration solutions to be used for each of the flow cells; however, the same regeneration solution must be used for the four spots within a flow cell. This is an important consideration if different biotoxins are to be immobilised on each spot of one flow cell. Toxins with similar regeneration conditions would need to be spotted on the same flow cell to maintain the baseline of the flow cell on the sensor chip.

At the optimised antibody titres, calibration curves for each toxin were obtained (Fig. 3). The mid-point (IC_{50}) and dynamic range (IC_{20}–IC_{80}) for each biotoxin was assessed and compared to those of the commercial CER ELISA (Table 2). The development, optimisation and validation of the ELISAs for the three marine biotoxins have been discussed in previous research (Dubois et al. 2010) in relation to shellfish extracts. In this research, the ELISAs were used for the analysis of seawater samples. These ELISAs have not previously been used for the analysis of biotoxins from algal or seawater samples. A comparison of sensitivities for the prototype multiplex SPR biosensor and the ELISA methods are shown (Table 2). Analysis with the prototype multiplex SPR biosensor gives IC_{50}s of 5.3 ng/ml or less compared to 1.37 ng/ml or less for the ELISAs. Limit of detections (based on IC_{20}s) for saxitoxin, okadaic acid and domoic acid were 0.82, 0.36 and 1.66 ng/ml, respectively, for the prototype multiplex SPR biosensor (33 runs) compared with 0.01, 0.41 and 0.34 ng/ml for the saxitoxin, okadaic acid and domoic acid ELISAs (13 runs),

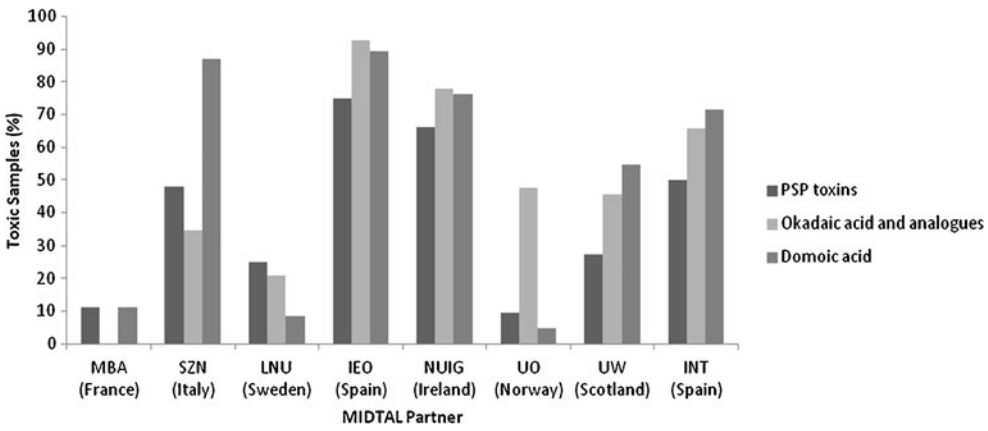

Fig. 5 Comparison of levels of toxic seawater samples detected across Europe as part of the MIDTAL project when analysed on the prototype multiplex SPR biosensor

respectively. Additionally, the analysis of 17 extracted negative matrix cultures by multiplex SPR displayed 99 % certainty limits of 0.93, 0.26 and 2.41 ng/ml which are very comparable to the IC_{20}s with variations due to matrix effects. The saxitoxin and domoic acid ELISAs showed greater sensitivity than the prototype multiplex SPR biosensor although similar sensitivities were achieved for okadaic acid between the two detection systems. The saxitoxin ELISA proved to be very sensitive being able to detect to the picograms levels, with less than 0.5 ng/ml detection limits achieved for the other two biotoxins. A major advantage of the ELISA is their ability to analyse a large number of samples with results for 46 seawater samples available after extraction and analysis within approximately 9 h for okadaic acid and domoic acid and the following day for saxitoxin (overnight incubation required). However, three ELISAs would need to be completed compared to the prototype multiplex SPR biosensor which is capable of the simultaneous detection and analysis of all three biotoxins in one single analysis (24 seawater sample results for three key marine biotoxins in 7 h). A comparison of the prototype multiplex SPR biosensor and commercial ELISAs showing benefits, limitations and main characteristics for each testing platform is presented (Table 2).

Biosensor chip stability

The biosensor chip proved to be stable over the 8-week period examined. The biosensor chip would have shown deterioration if a steady decrease in response was observed; however, this was not the case for any biotoxin on the sensor chip (Fig. 4). Although variation can be seen in response over the 8-week period, this is more likely to be due to fresh working antibody solutions being made up fresh every day. This could be improved by making up a working strength antibody solution and using this across all experiments; however, it was decided that stability of the antibody would be compromised and this would need to be examined in a separate study after we were sure the biosensor chip was stable. The domoic acid immobilisation appears to be the most problematic for the biosensor chip with variation sometimes observed; however, this is seen for all calibration standards and so becomes relative when analysing samples, as a calibration curve will be run with every batch of samples.

Seawater sample preparation

Devlin et al. (2011) examined a number of cell lysis methods and compared these with the standardised extraction method for PSP toxins in phytoplankton (Ravn et al. 1995) using the algal culture *Alexandrium tamarense*. This author found that shaking with a 5 mm steel ball bearing released similar if not better PSP toxin when compared to the method by Ravn et al. (1995). However during this research, it was found that glass bead beating (0.5 mm) showed a higher level of cell lysis than the steel ball bearing when analysing three different types of algal cultures. Slight differences between methods may explain these results as a different species of *Alexandrium* was examined. *A. fundyense* in this research compared to *A. tamarense* by Devlin et al. (2011). Glass bead beating was also carried out using only a vortex by Devlin et al. (2011) compared to the Merris Minimix shaker in this research. It appeared that Devlin et al. (2011) has based the success of cell lysis on toxin concentration detection by analysis with HPLC and not percentage cell lysis of algal cells. This research based cell lysis on percentage cell lysis achieved by taking cell counts before and after the different cell lysis methods. Initial experiments showed that glass bead beating may be an efficient method of lysing cells for *A. fundyense* and *P. multiseries* with 100 % cell lysis obtained for both however it only gave approximately 69 % cell lysis for *P. lima*. A number of cell lysis experiments were therefore further examined using the *P. lima* culture. Alternative cell lysis methods including chemical and physical methods had little/no improvement in cell lysis for this culture. Results indicated that optimum cell lysis was achieved for *P. lima* when algal samples underwent a freeze–thaw step followed by beating with 0.5 mm glass beads (77 %). Diatom cell walls such as those from the *Pseudo-nitzschia* genus are composed of two overlapping sections known as the thecae. They are not of uniform thickness and have arrangements of thicker and thinner areas. The body of dinoflagellate cells are covered by a cell wall made of cellulose, valves or plates. *Alexandrium* are considered armoured dinoflagellates meaning that they have thecal plates of varying thickness and orientation surrounding the cell like armour. The particular size, shape and arrangement on the theca are characteristic of a particular *Alexandrium* species. In comparison, the cell wall of *Prorocentrum* consists of two halves, which are laterally compressed. The differences in the cell wall structure may be a possible reason why the cells of *Prorocentrum* are more difficult to lyse compared to that of *Alexandrium* and *Pseudo-nitzschia*. The glass beads may be able to penetrate these cell walls much easier due to theca arrangement of *Alexandrium* and the thinner cell walls of *Pseudo-nitzschia.*

Seawater sample analysis and comparison

Two hundred fifty-six filtered seawater samples were received from eight of the MIDTAL partners for biotoxin analysis. Extracts were analysed for the presence of toxins (PSP, okadaic acid and domoic acid toxins) by two testing platforms, the prototype multiplex SPR biosensor and ELISAs.

Discrepancies between results for the PSP toxins are due to the ultra-enhanced sensitivity of the ELISA with the

commercial saxitoxin ELISA from CER being 82 times more sensitive than the SPR method. The detection limit for the saxitoxin ELISA is 0.01 ng/ml compared to 0.82 ng/ml for SPR (Table 2).

For okadaic acid results, both platforms show a similar level of sensitivity with 0.36 and 0.41 ng/ml for SPR and ELISA, respectively (Table 2). The cross-reactivity profile of the antibodies (Table 2) used in both methods may help to explain differences observed. ELISA shows 78 % cross-reactivity for DTX1 compared to only 68 % for SPR. In comparison, the ELISA shows very poor cross-reactivity to DTX2 at only 2.6 compared to 65 % for SPR. Examining okadaic acid results in Table 2, during the 2011 sampling period, there were a higher number of samples that tested positive by the ELISA compared to SPR. These samples may have therefore contained a higher level of DTX1. In comparison, in 2010, there was a higher level of samples testing positive by SPR compared to ELISA. In these cases, DTX2 may have been present which was therefore potentially detected by SPR and not ELISA due to ELISA's poor cross-reactivity for DTX2.

For domoic acid toxins, the numbers of positive results are greater for ELISA than the prototype multiplex SPR biosensor. This can be explained by the greater sensitivity of ELISA (0.34 ng/ml) compared to SPR (1.66 ng/ml) therefore showing five times greater sensitivity (Table 2). In conclusion, variations of toxin levels between the two testing platforms are therefore likely due to the enhanced sensitivity of the ELISAs for PSP and domoic acid toxins and the differences between antibody cross-reactivity profiles for okadaic acid toxins. However, all samples that showed significant levels of marine toxins by ELISA also showed significant levels by the multiplex SPR biosensor.

In total 47, 59 and 61 % of seawater samples tested positive (result greater than the IC_{20}) for PSP, okadaic acid and domoic acid toxins, respectively, using the prototype multiplex SPR biosensor. From Fig. 5, it can be observed that toxic samples were received (mainly) from Spain and Ireland with significant levels of all three key toxins detected in these regions. Orkney Islands, Scotland also showed seawater samples positive for all three toxins but to a lesser extent than Spain and Ireland. Gulf of Naples, Italy showed higher levels of domoic acid toxins in their seawater samples compared to the other key toxins while okadaic acid toxins were more prevalent in Oslofjord, Norway although levels were not particularly high (0.5–2 ng/filter). The Skagerrak area of Sweden showed a small number of positive seawater samples for all three key toxins while Arcachon Bay in France appeared to show the least levels of toxic seawater samples.

Finally, when comparing toxin detection results with the species microarray and cell counts, generally in most cases when toxin is present, there is a candidate toxic species present. This work is discussed in the other publications by the MIDTAL consortia.

Conclusions

A multiplex assay has been developed and validated for the semi-quantitative, simultaneous screening of three types of key marine biotoxins in seawater samples. Results for PSP, okadaic acid and domoic acid toxins can be detected in as little as 13 min from one seawater sample (after extraction) in real time. Method detection limits based on IC_{20} values for PSP, okadaic acid and domoic acid toxins were 0.82, 0.36 and 1.66 ng/ml, respectively, for the prototype multiplex SPR biosensor. Using these detection limits, 47, 59 and 61 % of seawater samples tested positive for PSP, okadaic acid and domoic acid toxins, respectively, with toxic seawater samples found mainly from the Rias of Pontevedra, Arosa, Muros, Ares-Betanzos, and estuary of Bayona, Spain and the Killary, Cork and Bell Harbours of Ireland. The simplicity of the assay means it could be used as a first action screening method in a monitoring laboratory for the presence of these biotoxins in seawater samples. Many European countries test and identify phytoplankton in seawater samples in shellfish harvest areas with many setting trigger levels for certain toxic phytoplankton species. In Northern Ireland, these levels are set at >0 cells/L for *Alexandrium* species, >100 cells/L for *Dinophysis*/*Prorocentrum* species and >150,000 cells/L for *Pseudo-nitzschia* species. However, it is often impossible to differentiate between certain species of toxic algae; and if toxic species are present at trigger levels, this would initiate further testing of shellfish whether or not biotoxins are present. A viable alternative would be to monitor seawater samples for the presence of biotoxins. An early warning detection tool would be extremely beneficial to the aquaculture and fisheries industry to allow economic decisions to be made quickly in relation to the harvesting process for shellfish. Although this is a laboratory-based method, it still has many advantages and benefits over the current detection systems in its ability for multiple toxin group analysis. The next step in this research would be to develop a multiplex portable assay for the submersible vehicle. Without comprehensive phytoplankton and shellfish monitoring programmes, there is currently no way to ensure that shellfish are safe for human consumption.

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