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Development of a Planar Waveguide Microarray for the Monitoring and Early Detection of Five Harmful Algal Toxins in Water and Cultures

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ABSTRACT: A novel multiplex microarray has been developed for the detection of five groups of harmful algal and cyanobacterial toxins found in marine, brackish, and freshwater environments including domoic acid (DA), okadaic acid (OA, and analogues), saxitoxin (STX, and analogues), cylindrospermopsin (CYN) and microcystins (MC, and analogues). The sensitivity and specificity were determined and feasibility to be used as a screening tool investigated. Results for algal/cyanobacterial cultures (n = 12) and seawater samples (n = 33) were compared to conventional analytical methods, such as high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Detection limits for the 15 min assay were 0.37, 0.44, 0.05, 0.08, and 0.40 ng/mL for DA, OA, STX, CYN, and MC, respectively. The correlation of data obtained from the microarray compared to conventional analysis for the 12 cultures was r^2 = 0.83. Analysis of seawater samples showed that 82, 82, 70, 82, and 12% of samples were positive (>IC_{20}) compared to 67, 55, 36, 0, and 0% for DA, OA, STX, CYN, and MC, respectively, for conventional analytical methods. The discrepancies in results can be attributed to the enhanced sensitivity and cross-reactivity profiles of the antibodies in the MBio microarray. The feasibility of the microarray as a rapid, easy to use, and highly sensitive screening tool has been illustrated for the five-plex detection of biotoxins. The research demonstrates an early warning screening assay to support national monitoring agencies by providing a faster and more accurate means of identifying and quantifying harmful toxins in water samples.

1. INTRODUCTION

During recent decades, there has been an increase in the occurrences of harmful algal blooms (HAB) in many marine and freshwater systems.[1] HABs are caused by the production of biotoxins belonging to three taxa groups; diatoms, dinoflagellates, and cyanobacteria. There is also an increasing number of toxic algal species and algal toxins identified with the economic losses from HABs greater than before.[2] Several algal toxins are classified corresponding to their acute symptoms in humans and include amnesic shellfish poisoning (ASP) toxins, diarrheic shellfish poisoning (DSP) toxins, and paralytic shellfish poisoning (PSP) toxins. Cyanotoxins such as microcystins (MC) and cylindrospermopsin (CYN) initially found in freshwater environments are presenting in brackish and marine environments[3] and have been observed to be spreading from tropical to temperate environments.[4]

Current methods of detection for marine biotoxins are primarily aimed for shellfish where regulations are in place within the EU (Regulation (EC) No. 853/2004) and worldwide (Codex, STAN 292-2008). No regulations are currently in place for the detection of marine biotoxins in aquatic samples. MCs are the only toxin with a guideline value of 1 μg/L in drinking water recommended by the World Health Organization (WHO). Therefore, many of the current methods do not have the required sensitivity to act as an early warning screening method for water quality monitoring.

For marine biotoxins analytical methods such as HPLC and LC-MS/MS were originally developed for their detection in shellfish as an alternative to the mouse bioassay but have been adapted for detection in algal and seawater samples for PSP, OA and DA toxins.[4−12] Analytical tools such as HPLC with UV[13] or MS[14,15] detection have been developed for MC and LC-MS/MS for CYN detection.[4,16,17] Although analytical methods are available, it is acknowledged that these methods require skilled personnel, are labor intensive, can be hindered by the lack of available analytical standards and tend to be specific to a single toxin group. Immunological assays such as ELISA[18−23] and lateral flow devices[24−28] have shown promise as sensitive rapid commercially available screening tools (e.g., Abraxis, Jellet Rapid Testing Ltd., Neogen). The emergence of biosensor based immunological assays have shown many

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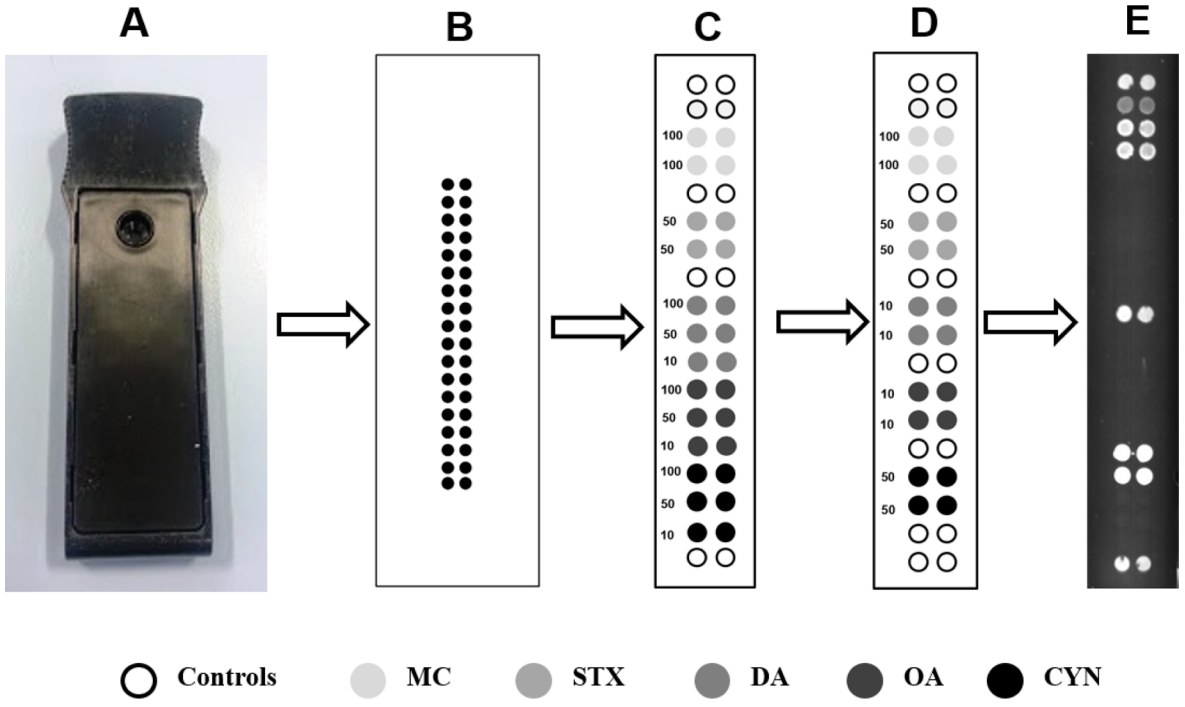

Figure 1. Multiplex MBio cartridge (A), spotting layout of slide (2 × 18 spots) (B), version 1 spotting arrangement of slide with biotoxins at three spotting concentrations (C), version 2 spotting arrangement of slide with biotoxins at optimized spotting concentrations (D) and fluorescently imaged cartridge (E). Spotting arrangement of slide including positions of controls (fluorescently labeled bovine serum albumin, buffer, GaM, and GaR antibody controls) and toxins (MC, STX, DA, OA, and CYN).

advantages including high sensitivity, low detection limits, portability, specificity, and robustness. Two of the most promising biosensor methods are based on surface plasmon resonance (SPR)[23,29−39] and planar waveguide[14,40] but as single toxin analysis. Multi toxin analysis using SPR, chemiluminescence, flow cytometry, and microfluidic detection platforms have been described but are currently restricted for portability as are laboratory based tools.[41−46] The MBio biosensor is an innovative technique based on planar waveguide with fluorescence detection showing promise for portable multiplex detection of biotoxins based on its application in the clinical setting for HIV and coinfections.[47] The cartridge is composed of a plastic slide on which a microarray of toxin conjugates are spotted (Figure 1). Light directed at the bottom of the cartridge via the reader allows excitation of the fluorophores of the labeled antibody, enabling the sensitive detection of binding events. When no toxin is present, the antibody binding to the surface is optimal and a high fluorescent signal is generated. When toxin is present in the sample, the antibody binds to the toxin, thereby diminishing the fluorescent signal. Previous authors have described assays utilizing the MBio biosensor for PSP toxins and MC but in a single assay format.[14,48]

The aim of this research was to develop a multiplex assay for the simultaneous detection of five groups of harmful algal toxins. The method was compared with conventional analytical methods and evaluated using seawater samples collected in Europe, in addition to algal/cyanobacterial cultures.

2. MATERIALS AND METHODS

2.1. Instrumentation. The SnapEsi LS system was supplied by MBio Diagnostics Inc. (Boulder, Colorado, U.S.A.). An Acquity UPLC system coupled to a Quattro premier XE mass spectrometer was used for analysis of marine biotoxins and an Acquity UPLC I-Class system coupled to a Xevo TQ-MS mass spectrometer was used for analysis of cyanobacterial toxins (Waters, Ireland). A Waters alliance 2695 separation module HPLC system equipped with a Waters 2475 fluorescence detector (Waters, Ireland) was employed for PSP analysis.

2.2. Reagents. Phosphate buffered saline (PBS) tablets, Tween 20, bovine serum albumin (BSA) and Guillards medium were purchased from Sigma-Aldrich (Dorset, U.K.). Alexa Flour 647 goat antirabbit IgG (GaR) and Alexa Flour 647 goat antimouse IgG (GaM) antibodies were purchased from Invitrogen Ltd. (Paisley, Scotland). Saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), neosaxitoxin (NEO), decarbamoyl neosaxitoxin (dcNEO), gonyautoxins (GTX1/4, GTX2/3, GTX 5), decarbamoyl gonyautoxins (dcGTX2/3), N-sulfocarbamoylgonyautoxin-2 and -3 (C 1/2), okadaic acid (OA), dinophysistoxin 1 (DTX1), dinophysistoxin 2 (DTX2) and domoic acid (DA) were purchased from the National Research Council of Canada (Halifax, Canada). Microcystin leucine-arginine (MC-LR) was purchased from Enzo Life Sciences (Exeter, U.K.). Cylindrospermopsin (CYN) was purchased from n'Tox (France).

BG11, Jaworski's, L1 media and *Alexandrium tamarense* 1119/20 (nontoxic culture) were all purchased from the Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland), *Microcystis aeruginosa* 91342 and 91094 were received from the Laboratory of Ecotoxicology, Genomics and Evolution (LEGE) at the Centre of Marine and Environmental Research (CIIMAR, Porto, Portugal). *Anabaena* spp., *Microcystis aeruginosa* 84/1, *Planktothrix rubescens* 9316, 9743, 9707, and *Planktothrix argardhii* 126 were kindly donated by the University of Portsmouth (UK). *Cylindrospermopsis raciborskii* AWT205, *Alexandrium fundyense* GTCA28, and *Prorocentrum lima* PL2V were kindly donated from Centre

National de la Recherche Scientifique (CNRS, France), University of Maine (U.S.A.) and the Instituto Español de Oceanografia (Spain) respectively.

2.3. Algal Culturing. All glassware and media were sterilized prior to use at 121 °C for 30 min. Culturing of algae and cyanobacteria was performed in a UV3 HEPA PCR cabinet to maintain a sterile environment. All cultures were grown at 20 °C (±5 °C) with a 12:12 h light: dark cycle with a light intensity of 170 μmol/m^2/s (*Alexandrium* and *Prorocentrum*), 116 μmol/m^2/s (*Microcystis)* or 5 μmol/m^2/s (*Cylindrospermopsis, Anabaena* and *Planktothrix*).

2.4. Seawater Sample Collection. Seawater samples were collected during 2009−2011 from a number of sites across Europe as part of the MIDTAL (Microarrays for the detection of toxic algae) EU project. The seawater samples were filtered through a 0.45 μm nitrocellulose filter until the filter clogged (approximately 0.5−2 L) depending on level of phytoplankton in the water. The filter was frozen in an eppendorf tube at −20 °C and shipped to Queen's University, Belfast for further extraction and analysis for biotoxins.

2.5. Sample Preparation. Cell lysis was thoroughly examined in previous research.[43] Algal/cyanobacterial (toxic, nontoxic and unknown toxicity) cultures (n = 12) as well as seawater samples (n = 33) were prepared as described previously using the optimized cell lysis method.[43] In brief, cultures (10 mL) and seawater samples (0.5−2 L) were filtered through 0.45 filters and frozen at −20 °C until use. The filter was thawed and toxins extracted using 2 mL deionized water (with the exception that MIDTAL partner NUIG extracted in 5 mL) by vortexing for 20 s, mixing end over end for 20 min, bead beating with 0.5 mm glass beads (1 g) for 20 min on a merris minimix shaker (Merris Engineering Ltd., Ireland) followed by centrifugation at 3000g for 10 min. Finally, the supernatant was filtered using a 0.45 μm syringe filter. Samples were analyzed using the MBio biosensor and analytical methods using the same extract.

2.6. MBio Methodology. *2.6.1. Preparation of Immunological Reagents.* The production of MC-LR transferrin (MC-LR-TR),[14] neosaxitoxin-ovalbumin (NEO-OVA),[48] and cylindrospermopsin-ovalbumin[23] were previously described. Domoic acid-ovalbumin[29] and okadaic acid-ovalbumin[30] were prepared in a similar manner to that described previously for the production of antibodies to these toxins. The preparation of immunogens and the production of the STX antibody,[31] OA antibody,[30] DA antibody,[29] MC antibody,[39] and CYN antibody[23] were previously published.

2.6.2. Cartridge Spotting. Microarrays were printed using a Bio-Dot AD3200 robotic arrayer equipped with a Bio-Jet print head capable of dispensing 20 nL droplets with spot diameters approximately 0.5 mm. Toxin conjugates for DA, OA, and CYN were spotted (version 1 microarray) at three concentrations (10, 50, and 100 μg/mL) in replicates of two (Figure 1C). Following optimization one concentration of toxin was selected (version 2 microarray, Figure 1D) and spotted in replicates of four. Fluorescently labeled protein conjugates and antispecies antibodies were also spotted as controls (Figure 1D). After printing, the waveguide arrays were rinsed with a protein-based blocking agent and dried by centrifugation.

2.6.3. Assay Protocol. Assays were carried out on a specially designed cartridge rack, angled for optimum flow rate enhancing fluid flow. Flow rate was calculated as approximately 60 μL/min. Two assay protocols were designed comprising of a 45 and 15 min assays. Each protocol is described; 45 min assay: The cartridge was preconditioned with MBio assay buffer (150 μL) for 10 min followed by antibody: sample mix (150 μL, 1:1) for 15 min. The cartridge was washed with MBio assay buffer (2 × 150 μL, 5 min), and the secondary labeled antibodies (Alexa Flour 647 goat antirabbit IgG and Alexa Flour 647 goat antimouse IgG) were added (150 μL, 1/200 in MBio assay buffer) for 10 min. The cartridge was washed again with MBio assay buffer (2 × 150 μL, 5 min).

Fifteen min assay: Antibody: sample mix (150 μL, 1:1) was applied to the cartridge for 7.5 min followed by the secondary labeled antibodies (150 μL, 1/200 in MBio assay buffer) for 7.5 min.

For each assay, the cartridge was read immediately on the MBio SnapEsi reader. A series of exposures to gain dynamic range were collected and the final numerical output was scaled that it corresponded to 100 ms exposure.

2.6.4. Evaluation of Seawater Matrix Effects. Calibration curves were prepared in MBio assay buffer and extracted seawater at concentrations across the full dynamic range of each curve for a full evaluation of matrix effects. Final concentrations of calibrants were as follows; DA (0.5−20 ng/mL), OA (0.5−4 ng/mL), STX (0.02−0.5 ng/mL), MC (0.2−10 ng/mL), and CYN (0.5−5 ng/mL). Fresh, natural seawater (salinity 33−34 ppt) was obtained from Strangford Lough (Co. Down, NI). Seawater (1 L) was extracted using the method as described previously for sample preparation.

2.6.5. Limit of Detection. The limit of detection (LOD) is defined as the lowest concentration of toxin in a sample that can be detected. The theoretical LOD for this research was defined as the 20% inhibitory concentration (IC_{20}), which is the 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 calibration curves analyzed during this research (n = 3).

2.7. Comparison with Conventional Analytical Methodology. Four individual analytical methods were employed as follows:

2.7.1. LC-MS/MS: OA (DTX1 and DTX2). Samples were analyzed for OA, DTX1, and DTX2 by LC-MS/MS following the standard operating procedure by the European Union reference laboratory for marine biotoxins (EU-RL-MB, Version 4, Jul 2011).

2.7.2. LC-MS/MS: DA. Samples were analyzed for DA by LC-MS/MS following the standard operating procedure by the European Union reference laboratory for marine biotoxins (EU-RL-MB, Version 1, Feb 2010).

2.7.3. LC-MS/MS: MC and CYN. An Acquity UPLC HSS T3 column (100 × 2.1 mm^2 id, 1.8 μm particle size) at a column temperature of 45 °C and flow rate of 0.45 mL/min was used. The mobile phases were water (0.1% formic acid) and acetonitrile. The gradient used was based on that published[49] with some modifications to achieve optimal separation. The acetonitrile was held initially at 2% for 1 min, followed by an increase to 70% over 9 min, then washed for 1 min at 85% before returning to 2% for 1 min re-equilibration time.

2.7.4. HPLC: STX (and Analogues). Samples were analyzed for STX and analogues (C1/2, GTX2/3, STX, GTX1/4, dcNEO, NEO, dcGTX2/3, dcSTX, GTX5) by HPLC-FLD following a modification of the precolumn oxidation Lawrence AOAC official method.[50] Modifications to this method included that the toxins were extracted from the seawater and not shellfish samples and as such the C18 cleanup stage was not required.

Table 1. Comparison of the Midpoint of the Curve (IC_{50}) and Dynamic Range (IC_{20}–IC_{80}) of Each Biotoxin When Examining Single and Multi Assays, 45 and 15 min Assay Times and Seawater Matrix Effect for the MBio Biosensor[a]

biotoxin	system	matrix	assay time (min)	mid-point IC_{50} (ng/mL)	dynamic range IC_{20}–IC_{80} (ng/mL)
DA	single	MBio assay buffer	45	0.13	0.03–1.07
	multi	MBio assay buffer	45	1.93	0.56–5.72
	multi	MBio assay buffer	15	1.83	0.63–5.04
	multi	Seawater	15	1.37	0.37–4.43
	LC/MS-MS	Seawater	3	N/A	2.5[b]
OA (and analogues)	single	MBio assay buffer	45	0.24	0.13–0.66
	multi	MBio assay buffer	45	0.42	0.22–0.78
	multi	MBio assay buffer	15	1.06	0.52–2.00
	multi	seawater	15	0.96	0.44–1.94
	LC/MS-MS	seawater	8.5	N/A	2.5[b]
STX (and analogues)	single	MBio assay buffer	45	0.04	0.02–0.06
	multi	MBio assay buffer	45	0.06	0.02–0.15
	multi	MBio assay buffer	15	0.06	0.02–0.18
	multi	seawater	15	0.09	0.05–0.15
	HPLC	seawater	14	N/A	2.5[b]
CYN	single	MBio assay buffer	45	0.23	0.07–0.56
	multi	MBio assay buffer	45	0.19	0.08–0.52
	multi	MBio assay buffer	15	0.39	0.20–0.76
	multi	seawater	15	0.26	0.08–0.72
	LC/MS-MS	seawater	12	N/A	10[c]
MC	single	MBio assay buffer	45	1.48	0.50–3.73
	multi	MBio assay buffer	45	1.25	0.40–3.41
	multi	MBio assay buffer	15	1.46	0.48–3.78
	multi	seawater	15	1.31	0.40–3.35
	LC/MS-MS	seawater	12	N/A	10[c]

[a]Analytical methods used included both LC-MS/MS and HPLC. [b]Denotes the sensitivity of these methods are based on the lowest quantifiable peak observed. [c]Denotes the lowest standard analyzed in the experiment (no peaks in samples observed less than this).

3. RESULTS AND DISCUSSION

The present study outlines for the first time the use of this technology to detect biotoxins in a multiplex format. The increasing concerns due to biotoxins in relation to both food and water quality safety has necessitated the need for rapid, sensitive, portable, high throughput, and multiplex detection systems.

3.1. Assay Protocol. Following the work of Lochhead et al. (2011)[47] and Meneely et al. (2013)[48] the initial optimization for the MBio assay was performed as a single 45 min assay for each toxin group. This allowed the determination of optimal spotting concentration of toxin conjugates, antibody dilutions and to evaluate interference between antibodies and toxins immobilized onto the cartridge. Antibodies were optimized to give a response 300–500 fluorescence units and inhibition was assessed. The spotting and optimization of the microarray for STX and MC were discussed in full in Meneely et al. (2013)[48] and Devlin et al. (2013) to give optimized concentrations of 50 μg/mL using NEO-OVA and 100 μg/mL using MC-LR-TR. For DA, OA, and CYN, three spotting concentrations were assessed at 10, 50, and 100 μg/mL on version 1 of the microarray (Figure 1C). Inhibition and sensitivity were optimum for DA and OA at a spotting concentration of 10 μg/mL while 50 μg/mL was necessary for CYN. This was because the CYN antibody had to be diluted to 1/6000 to show inhibition, as such the response was below the 300–500 fluorescence units required at 10 μg/mL. The final optimum spotting layout of all five toxins can be seen in Figure 1(D).

Each antibody was optimized at dilutions of 1/6000 for DA, OA, STX, and CYN and 1/40 000 for MC giving a response between 300 and 500 fluorescence units as five single assays. It was ascertained that nonspecific binding was not occurring between the binding proteins. All five toxins were then incorporated into a multi assay at 45 min. Finally, the assay was reduced to a 15 min multi assay to be more applicable for use in the field. Antibody dilutions were reoptimized (1/2000 for OA and CYN, 1/5000 for DA, 1/8000 for STX and 1/10 000 for MC) in addition to standards for performance and sensitivity. The midpoint and dynamic range were determined for each assay using BIAevaluation software (Table 1). Cartridges can be batch processed, with up to 32 run in parallel showing stability up to 60 min with average coefficient of variation (CVs) no greater than 1.5% on reading immediately and 60 min later.

Antibody specificity was assessed in previous publications for the MC, CYN, OA, and STX antibodies.[14,23,33,48] Cross-reactivity data was determined by calculating the midpoint concentration (IC_{50}) of each toxin curve as a percentage relative to the main standard. For PSP toxins these were STX 100%, dcSTX 49%, GTX5 33%, dcGTX2/3 28%, C1/2 25%, dcNEO 8%, GTX2/3 7%, NEO 4%, and GTX1/4 <0.1%. For the MC analogues, these were MC-LR 100%, microcystin arginine-arginine (MC-RR) 108%, microcystin leucine-tryptophan (MC-LW) 71%, microcystin leucine-alanine (MC-LA) 69%, microcystin tyrosine-arginine (MC-YR) 68%, microcystin leucine-phenylalanine (MC-LF) 68% and Nodularin 94%. For CYN, these were CYN 100% and for deoxycylindrospermopsin 5%. Finally for OA toxins these were OA 100%, DTX1 100% and DTX2 60%.

3.2. Evaluation of Seawater Matrix Effects. Comparison of extracted seawater and MBio assay buffer for DA, OA, STX, CYN, and MC showed limited change to the midpoint and

Table 2. Toxin Concentration of 12 Algal and Cyanobacterial Cultures Expressed as ng/mL When Analysed by the MBio Biosensor and Conventional Analytical Methods[a]

algal culture	strain	cell count (cells/mL)	biotoxin present	MBio (ng/mL)	conventional analytical method (ng/mL)	
					total	analogue
Alexandrium tamarense	1119/20	31 700	nd	nd	nd	nd
Alexandrium fundyense	GTCA28	17 000	PSP	70.1	730.0	C1/2: 198.0; GTX2/3: 158.8; STX: 47.5; GTX1/4: 284.5; NEO: 39.2; GTX5:2.0
Prorocentrum lima	PL2V	19 800	OA	108.0	139.7	OA: 112.5; DTX1:27.2
Microcystis aeruginosa	91 342	4 900 000	PSP	0.04	nd	nd
Microcystis aeruginosa	91 094	5 800 000	MC	223.6	248.0	MC-LR: 228.0; MC-LA: 20
Microcystis aeruginosa	84/1	3 950 000	MC	6.9	4.9	MC-LR: 4.9
Cylindrospermopsis raciborskii	AWT205	355 000	CYN	>1250	1283.2	CYN: 1283.2
Anabaena spp.	ANA	400 000	nd	nd	nd	nd
Planktothrix argardhii	126	200 000	MC	35.0	2.6	MC-YR: 2.6
Planktothrix rubescens	9316	145 000	MC	261.2	11.4	MC-LR: 1.4; MC-YR: 10
Planktothrix rubescens	9707	62 500	MC	16.8	nd	nd
Planktothrix rubescens	9743	245 000	MC	189.9	8.3	MC-YR: 8.3

[a]Samples were analysed for all toxins but results show only toxins detected. nd denotes not detected.

dynamic range for each toxin in the 15 min assay protocol (Table 1). Nonetheless, the analysis of seawater samples was carried out with a matrix matched seawater calibration curve to ensure accurate determination of toxin content and to maintain minimal matrix effects.

3.3. Limit of Detection. The theoretical LOD for these assays was defined as the IC_{20}. The LOD (Table 1) for all toxins have been established for each system, assay time, and matrix. The LOD when analyzing single toxin as a 45 min assay (MBio assay buffer) were 0.03, 0.13, 0.02, 0.07, and 0.50 ng/mL for DA, OA, STX, CYN, and MC, respectively. When comparing these LODs but in a multi system (45 min), the LOD for both STX and CYN remained similar, doubled for OA and decreased slightly for MC. DA was the only toxin where a substantial change in the IC_{20} was observed increasing to 0.56 ng/mL compared with 0.03 ng/mL (single assay). When the assay time was shortened to 15 min, the LOD remained relatively similar for DA, STX, and MC while OA and CYN doubled. Finally, when using matrix extracted seawater in the LOD remained similar for MC (0.40 ng/mL), decreased for DA (0.37 ng/mL), CYN (0.08 ng/mL), and OA (0.44 ng/mL) but increased for STX (0.05 ng/mL). MC is the only toxin with a regulatory limit (1 μg/L = 1000 ng/mL) in place for water. The LOD for MC using the MBio biosensor is approximately half this at 0.4 ng/mL therefore sensitivity of all toxins are low enough allowing use as an early warning detection tool.

3.4. Sample Analysis and Comparison with Analytical Methods. *3.4.1. Algae and Cyanobacteria Sample Analysis.* Algal/cyanobacterial cultures (n = 12; marine = 3 and freshwater = 9) were analyzed using the MBio biosensor (Table 2). The known toxic cultures (GTCA28, PL2V, 91094, AWT205) were all shown to be producing toxins while the nontoxic culture (1119/20) was negative. The toxic *Alexandrium fundyense* culture GTCA28[48] showed 70.1 ng/mL of PSP toxins by the MBio biosensor compared to 730.0 ng/mL (457 STXeqs/ml) by HPLC-FLD. Differences detected were due to the cross-reactivity profile of the antibody, but the sensitivity offered by the microarray allows for lower levels of STX to be determined compared to the HPLC method. The HPLC data confirmed that this culture was producing mostly GTX1/4 (284.5 ng/mL), C1/2 (198.0 ng/mL), and GTX2/3 (158.8 ng/mL) as well as lower concentrations of STX, NEO, and GTX5 (47.5, 39.2, and 2.0 ng/mL respectively). *Prorocentrum lima* PL2V is a known toxin producer of OA and DTX1,[8,51,52] which was confirmed by both the MBio biosensor (108.0 ng/mL) and LC-MS/MS (139.7 ng/mL) with the LC-MS/MS detecting OA (112.5 ng/mL) and DTX1 (27.2 ng/mL). Both the MBio biosensor (>1250 ng/mL) and LC-MS/MS (1283.2 ng/mL) detected *Cylindrospermopsis raciborskii* AWT205 as positive for CYN production. The *Planktothrix* cultures and *Microcystis* 84/1 are producing MC as measured by the MBio biosensor. These results were confirmed by LC-MS/MS with the only discrepancy being for *Planktothrix rubescens* 9707, positive for MC by the MBio biosensor but negative by LC-MS/MS. This may be because of the cross-reactivity profile of the MC antibody, which may be detecting a MC variant that is not included in the LC-MS/MS profile. The *Anabaena* spp. was negative for all toxins on the MBio assay and LC-MS/MS.

3.4.2. Seawater samples. Seawater samples (n = 33) were analyzed by the MBio biosensor and analytical methods (Table 3).

DA: There were five samples (MBA 5, SZN 5C, NUIG 39, NUIG 42, and INT 28) showing DA detection by the MBio biosensor (result greater than IC_{20}) with no DA present by LC-MS/MS. For samples MBA 5 and SZN 5C this was due to sensitivity with the MBio biosensor showing much lower sensitivity. Sample INT 28 showed a possible peak for DA by LC-MS/MS but too much background noise meant the sample could not be accurately quantified. For DA only one toxin analogue is available as an analytical standard but there are various isoforms that occur of DA which would potentially be detected by the antibody. The biosensor assay will cumulatively detect all toxins whereby the LC-MS/MS will detect and identify only DA. At this level of detection, the MBio is more sensitive and whereas more than one analogue could be present below the detection level of the LC-MS/MS, giving a cumulative response greater than the detection level by MBio. This explains why no DA was detected in samples NUIG 39 and 42 by LC-MS/MS when the MBio showed approximately 4 ng/filter. Where DA was detected by LC-MS/MS, the MBio results were positive (+), but in general it appears that the MBio may offer enhanced sensitivity for detection.

Table 3. Results and Comparison of MIDTAL Seawater Samples (n = 33) When Analyzed by the MBio Biosensor and Compared with Analytical Methods (HPLC or LC-MS/MS)[a]

			DA			OA (and analogues)			STX (and analogues)			CYN			MC		
			MBio		LC-MS/MS	MBio			MBio			MBio		LC-MS/MS	MBio		LC-MS/MS
partner	sampling site	sample no.	(ng/filter)	+/−	(ng/filter)	(ng/filter)	+/−	LC-MS/MS (ng/filter)	(ng/filter)	+/−	HPLC (ng/filter)	(ng/filter)	+/−	(ng/filter)	(ng/filter)	+/−	(ng/filter)
Marine Biological Association (MBA), Plymouth, U.K.	Arcachon Bay, France	5	3.1	+	nd	2.0	+	nd	<IC20	−	nd	0.9	+	nd	nd	−	nd
Stazione Zoologica "A. Dohrn" di Napoli (SZN), Naples, Italy	Gulf of Naples, Italy	5C	1.6	+	nd	1.7	+	nd	0.2	+	nd	0.6	+	nd	nd	−	nd
		19C	>20	+	29.8	1.9	+	nd	>0.4	+	nd	0.5	+	nd	nd	−	nd
		22B	11.7	+	6.8	1.9	+	nd	0.1	+	nd	0.4	+	nd	nd	−	nd
Linnaeus University (LNU), Kalmar, Sweden	Skagerrak area, Sweden	10C	>20	+	104.0	0.9	+	OA: 3.52	0.1	+	nd	nd	−	nd	<IC20	−	nd
		14C	nd	−	nd	2.2	+	OA: 4.62	>0.4	+	GTX2/3: 1.3 STX: 0.5	nd	−	nd	nd	−	nd
		17A	<IC20	−	nd	nd	−	nd	nd	−	nd	<IC20	−	nd	<IC20	−	nd
Instituto Español de Oceanografia (IEO), Vigo, Spain	Ría of Pontevedra, Spain	6B	7.9	+	7.7	>6	+	OA: 283	>0.4	+	C1/2: 242.5 NEO: 13.8 dcGTX2/3: 28.6 dcSTX: 18.3 GTX5:75.2 C1/2: 15.2	0.6	+	nd	nd	−	nd
		10B	>20	+	208.5	>6	+	OA: 134.9 DTX2:42.0	>0.4	+	GTX1/4: 17.8 dcNEO: 2.4 GTX5:3.0	0.7	+	nd	nd	−	nd
		16B	>20	+	1845.0	>6	+	OA: 97.35	<IC20	−	nd	0.6	+	nd	nd	−	nd
		18C	>20	+	1182.3	>6	+	OA: 289.08	0.1	+	nd	0.3	+	nd	nd	−	nd
		20B	>20	+	23.1	>6	+	OA: 84.36	nd	−	nd	0.6	+	nd	nd	−	nd
		23C	>20	+	7.0	>6	+	OA: 1460.07	0.1	+	STX: 0.4 GTX1/4: 25.9 dcNEO: 5.3	0.8	+	nd	1.8	+	nd
Martin Ryan Institute, National University of Ireland (NUIG), Galway, Ireland[b]	Killary, Cork and Bell Harbours, Ireland	2A	>50	+	13.0	>15	+	OA: 25.3 DTX2:14.2	0.4	+	nd	2.7	+	nd	22.9	+	nd
		16A	>50	+	53.3	4.3	+	nd	0.1	+	nd	1.9	+	nd	4.0	+	nd
		23A	<IC20	−	nd	nd	−	nd	<IC20	−	nd	1.4	+	nd	nd	−	nd
		39	3.9	+	nd	4.8	+	nd	>1	+	GTX2, 3:55.5; dcGTX2,3:12.4	1.8	+	nd	<IC20	−	nd
		42	4.7	+	nd	2.8	+	nd	>1	+	GTX2, 3:297.3; dcGTX2,3:18.9	0.7	+	nd	nd	−	nd
University of Oslo (UO), Oslo, Norway	Oslofjord, Norway	7A	1.0	+	2.4	4.2	+	OA: 6.87	0.1	+	nd	nd	−	nd	nd	−	nd
		8B	7.0	+	6.0	nd	−	nd	0.2	+	nd	0.2	+	nd	nd	−	nd
		11B	nd	−	nd	nd	−	nd	nd	−	nd	nd	−	nd	nd	−	nd
University of Westminster (UW), London, U.K.	Orkney Islands, U.K.	6B	>20	+	54.4	2.0	+	OA: 3.63	0.2	+	C1, 2:0.7	0.5	+	nd	nd	−	nd
		12C	<IC20	−	nd	nd	−	nd	0.1	+	nd	0.4	+	nd	nd	−	nd
		18B	>20	+	287.5	1.6	+	nd	0.3	+	nd	0.7	+	nd	1.8	+	nd
		21B	>20	+	184.9	2.7	+	nd	>0.4	+	GTX2,3:0.8;	0.6	+	nd	nd	−	nd

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Table 3. continued

			DA			OA (and analogues)			STX (and analogues)			CYN			MC		
			MBio		LC-MS/MS	MBio			MBio			MBio		LC-MS/MS	MBio		LC-MS/MS
partner	sampling site	sample no.	(ng/filter)	+/−	(ng/filter)	(ng/filter)	+/−	LC-MS/MS (ng/filter)	(ng/filter)	+/−	HPLC (ng/filter)	(ng/filter)	+/−	(ng/filter)	(ng/filter)	+/−	(ng/filter)
											STX:0.2						
Technological Institute for the marine environment control of Galicia (INT), Pontevedra, Spain	Rias of Pontevedra, Arosa, Muros, Ares-Betanzo s, and estuary of Bayona	28	4.0	+	nd	>6	+	OA: 11.64	>0.4	+	GTX2,3:25.9	0.3	+	nd	nd	−	nd
		30	>20	+	575.6	1.9	+	OA: 4.12	nd	−	nd	nd	−	nd	nd	−	nd
		34	<IC20	−	nd	>6	+	OA: 67.35	<IC20	−	nd	0.2	+	nd	nd	−	nd
		40	>20	+	89.4	>6	+	OA: 151.31	0.2	+	GTX1,4:41.8; dcNEO: 17.0	0.4	+	nd	nd	−	nd
		41	4.5	+	4.2	>6	+	OA: 140.73	nd	−	nd	0.7	+	nd	<IC20	−	nd
		51	>20	+	3267.4	<IC20	−	nd	nd	−	nd	0.4	+	nd	nd	−	nd
		65	>20	+	89.9	>6	+	OA: 43.41	>0.4	+	C1,2:158.2; GTX2,3:7.7; dcGTX2,3:26.3; dcTX: 7.8; GTX5:29.8	0.4	+	nd	<IC20	−	nd
		69	>20	+	384.0	>6	+	OA: 147.54	>0.4	+	C1,2:113.9; GTX2,3:18.3; dcGTX2,3:13.4; dcSTX: 12.2; GTX5:35.4	0.4	+	nd	nd	−	nd
summary	total		33		33	33		33	33		33	33		33	33		33
	+		27		22	27		18	23		21	27		0	4		0
	−		6		11	6		15	10		12	6		33	29		33

[a]Results are expressed as ng/filter and sample dilutions are neat. [b]Denotes samples for NUIG were extracted in 5 mL water compared to 2 mL water for all other samples hence explaining difference in off scale concentration results. nd denotes no toxins detected, + denotes that result is > IC_{20}, − denotes that result is < IC_{20}.

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OA and DTXs: No DTX1 was detected in any sample when analyzed by LC-MS/MS. When OA (and DTX2) are detected by LC-MS/MS the MBio was positive (+) by screening. Seven samples (MBA 5, SZN 5C, SZN 19C, SZN 22B, NUIG 42, UW 18B, and UW 21B) showed OA present (result greater than IC_{20}) when analyzed by the MBio biosensor with no OA present by LC-MS/MS. Samples NUIG 16A and NUIG 39 showed OA by the MBio biosensor at levels that should have been detected by LC-MS/MS but were not. There is a possibility that other OA derivatives are being detected by the antibody in the MBio biosensor assay at low levels which are not detectable or included in this LC-MS/MS method. Sulfated water-soluble analogues of OA such as DTX4 and DTX5 have been reported in *Prorocentrum lima* and *P. maculosum*, respectively, in addition, the so-called diol esters were reported in both *P.lima* and *Dinophysis acuta*.[53] The cumulative detection of toxins will be measured by the MBio biosensor.

STX and analogues: Samples SZN 5C, SZN 22B, LNU 10C, IEO 18C, NUIG 2A, NUIG 16A, UO 7A, UO 8B, UW 12C, and UW 18B all showed very low levels of STX when analyzed by the MBio biosensor but with no detection by HPLC-FLD. This was due to the sensitivity of the MBio biosensor being extremely low at 0.05 ng/mL and approximately 50 times less than what can be detected by HPLC-FLD. Sample SZN 19C showed detection of PSP toxins by the MBio biosensor at levels that should be detected by HPLC but was not and again most likely due to sensitivity and cross-reactivity of the antibody. Low levels of combined toxins could be present undetectable as individuals by the HPLC method or another PSP toxin derivative could be detected by the antibody used in the MBio biosensor assay which was not included as a standard in the HPLC method as currently there are 57 PSP analogues recognized.[54] Samples IEO 23C, UW 6B, and INT 40 all show low levels of STX by the MBio biosensor in comparison to high levels by HPLC. Results for MBio are quantifiably lower for STX due to the antibody cross-reactivity, whereby the antibody was raised to toxicity rather than concentration. However, the antibody also has lower cross-reactivity to hydroxylated toxins such as GTX1/4.[31] In this case, the antibody shows the proof of principle of the assay as a rapid screening tool to determine PSP presence but a cocktail mix of antibodies to PSP[35] could improve the correlation to toxin concentration.

CYN: The MBio biosensor showed 27 samples positive for CYN (result greater than the IC_{20}) with no CYN detected in any sample when analyzed by LC-MS/MS. The LC-MS/MS method only includes analysis for the main CYN and not any CYN analogues such as deoxycylindrospermopsin or 7-epicylindrospermopsin. The MBio assay was extremely sensitive for the detection of CYN with a LOD of 0.08 ng/mL compared to 10 ng/mL (lowest standard) by LC-MS/MS (125 fold more sensitive). Therefore, with this sensitivity achieved, even with a cross-reactivity of 5% as described by Elliott and co-workers (2013)[23] for deoxycylindrospermopsin, the CYN antibody could be detecting other CYN analogues to a measurable extent. No CYN was detected in any of the algal cultures tested (Table 2) other than *Cylindrospermopsis raciborskii* AWT205 and no interference or nonspecific binding was observed from other binding reagents. It may be that the CYN assay requires desensitization when monitoring levels are established for this toxin. However, it may also mean that human exposure to CYN albeit at low levels may be higher than previously realized. This is worthy of further research to confirm, through more sampling and utilizing enhanced monitoring and analytical techniques.

MC: For MC, four samples were positive by the MBio biosensor (result greater than IC_{20}) which do not show detection by LC-MS/MS. The LC-MS/MS method detects seven MC variants including MC-LR, MC-RR, MC-YR, MC-LA, MC-LW, MC-LF, and nodularin. However, there are approximately 90 MC variants[55] so the MC antibody in the MBio assay could be detecting other MC variants especially for the NUIG 2A sample which showed 22.9 ng/filter. Recent data suggests that MC analogues are being produced in the oceans by a number of marine species[3] providing evidence that MC presence in these seawater samples is plausible.

When seawater samples were analyzed by the MBio biosensor, 82, 82, 70, 82, and 12% of samples were positive (result greater than the IC_{20}) for DA, OA, STX, CYN, and MC, respectively. In comparison, only 67, 55, 36, 0, and 0% of those samples showed detection by analytical methods for DA, OA, STX, CYN, and MC, respectively. No false negative results are reported for the MBio biosensor, differences in reported concentrations of samples have been attributed to antibody cross-reactivity profiles and the superior sensitivities of the MBio biosensor. It was therefore highly unlikely to see close agreement between the antibody based tests and analytical methods when comparing the LODs (Table 1). These methods which are generally used as a confirmatory analysis are not as sensitive in comparison to the MBio biosensor. Therefore, the MBio biosensor shows better promise as an early warning screening method for determining the presence of harmful algal toxins in water before they may reach a detrimental toxicity level.

In summary, a highly sensitive multiplex assay has been developed for the semiquantitative, simultaneous screening of five biotoxins. Monitoring programmes for HAB phytoplankton and biotoxins have become a necessity because of the potential dangers to human health and significant economic impacts. Present day monitoring of seawater samples is based on species identification and enumeration using light microscopy which is often insufficient to give definite species identifications and thus assess the potential toxicity of the water. Failure to detect a potentially toxic species is intolerable because of the possible consequences for human health. However, considering all species as potentially toxic is not an option either because of the economic consequences of closing a shellfish industry. A viable alternative would be to monitor water samples for the presence of biotoxins. The simplicity and sensitivity achieved with the MBio biosensor means it could be used as an early warning monitoring tool for the potential presence of harmful algal toxins in water samples. This would allow the aquaculture and shellfish industries to make important economic decisions in relation to HABs and toxins. This technology demonstrates feasibility as a portable, rapid, easy to use, and highly sensitive multitoxin detection and is a major advancement in the field of biotoxin detection. Prior to implementation a full validation and interlaboratory trial of the assay should be conducted following accreditation guidelines (e.g., AOAC or IUPAC). Additionally, the feasibility of the translation of the MBio device for detecting toxins to the regulatory levels in seafood samples as a way forward for total toxicity testing is worthy of further research. The technologies adaptability for a field study on board a boat, to shorelines to test water samples and modified for the analysis of seafood samples to regulatory levels are also areas of interest.

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Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

There was an error in column head 12 of Table 3 in the version of this paper published October 31, 2014. The correct version published November 3, 2014.