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Production of a broad specificity antibody for the development and validation of an optical SPR screening method for free and intracellular microcystins and nodularin in cyanobacteria cultures

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ABSTRACT

A highly sensitive broad specificity monoclonal antibody was produced and characterised for microcystin detection through the development of a rapid surface plasmon resonance (SPR) optical biosensor based immunoassay. The antibody displayed the following cross-reactivity: MC-LR 100%; MC-RR 108%; MC-YR 68%; MC-LA 69%; MC-LW 71%; MC-LF 68%; and Nodularin 94%. Microcystin-LR was covalently attached to a CM5 chip and with the monoclonal antibody was employed in a competitive 4 min injection assay to detect total microcystins in water samples below the WHO recommended limit (1 μg/L). A 'total microcystin' level was determined by measuring free and intracellular concentrations in cyanobacterial culture samples as this toxin is an endotoxin. Glass bead beating was used to lyse the cells as a rapid extraction procedure. This method was validated according to European Commission Decision 96/23/EC criteria. The method was proven to measure intracellular microcystin levels, the main source of the toxin, which often goes undetected by other analytical procedures and is advantageous in that it can be used for the monitoring of blooms to provide an early warning of toxicity. It was shown to be repeatable and reproducible, with recoveries from spiked samples ranging from 74 to 123%, and had % CVs below 10% for intra-assay analysis and 15% for inter-assay analysis. The detection capability of the assay was calculated as 0.5 ng/mL for extracellular toxins and 0.05 ng/mL for intracellular microcystins. A comparison of the SPR method with LC–MS/MS was achieved by testing six *Microcystis aeruginosa* cultures and this study yielded a correlation R^2 value of 0.9989.

1. Introduction

Microcystin, a cyanotoxin, was first discovered in *Microcystis* and is also produced by *Oscillatoria*, *Nostoc*, *Anabaenopsis*, *Anabaena* and *Planktothrix* [1,2]. *Microcystis* is the most commonly occurring cyanobacterium found in blooms, worldwide, making the presence of microcystins in freshwater a significant problem. Microcystins are hepatotoxins that act by inhibiting serine/threonine proteases in hepatocytes [3]. Acute toxicity causes the loss of cell function and structure, leading to hepatic haemorrhaging and sometimes death. The worst reported case of microcystin poisonings occurred in a haemodialysis unit in Caruaru, Brazil; where untreated, contaminated reservoir water was used during the dialysis of 126 patients, resulting in the death of 60 [4]. Chronic, low level, exposure has been shown to cause tumour promotion and microcystins are classed as type 2b carcinogens (possibly carcinogenic to humans) [5,6]. Low level exposure has also been shown to have immunotoxic and genotoxic effects as well as causing organelle dysfunction and apoptosis [7–9].

Structurally, microcystins are cyclic non-ribosomal peptides that consist of several uncommon non-proteinogenic amino acids such as dehydroalanine derivatives and the special β-amino acid ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) [10] (Fig. 1). Microcystin-Leucine–Arginine (MC-LR) is one of over 90 known toxic variants and is the most studied by chemists, pharmacologists, biologists and ecologists due to its availability and occurrence [11]. Nodularin, a cyanotoxin structurally similar to microcystin, is produced only by *Nodularia* and is composed of five amino acids [12]. Three of those amino acids are the same as in microcystins: D-MeAsp (3) (D-*erythro*-b-methylaspartic acid), ADDA (5), and D-Glu (6) (D-glutamic acid). In addition to these, nodularin consists of L-Arginine at the *Y* position and Mdhb (2-(methylamino)-2-dehydrobutyric acid) replacing the residues at the *X*, D-alanine and N-methyl-dehydroalanine (Mdha)

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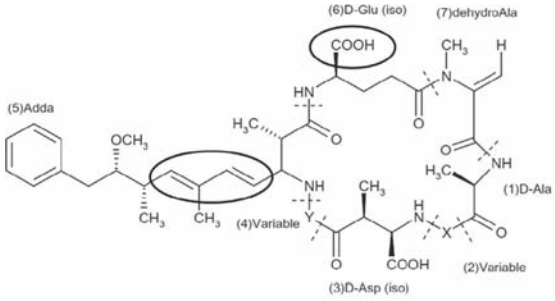

Microcystin variant	LD50 Intraperitoneal mice (μg/kg)	Relative Molecular Weight	X (variable amino acid residue at 2)	Y (variable amino acid residue at 4)
MC-LR	50	995.2	Leucine (Leu, L)	Arginine (Arg, R)
MC-RR	600	1038.2	Arginine (Arg, R)	Arginine (Arg, R)
MC-YR	70	1045.2	Tyrosine (Tyr, Y)	Arginine (Arg, R)
MC-LA	50	910.1	Leucine (Leu, L)	Alanine (Ala, A)
MC-LW	Not Determined	1025.2	Leucine (Leu, L)	Tryptophan (Trp,W)
MC-LF	Not determined	986.2	Leucine (Leu, L)	Phenylalanine (Phe,F)
Nodularin	60	825.0	2-methylamino-2-dehydrobutyric acid (MDhb)*	Arginine (Arg, R)

*MDhb replaces 1,2 and 7 on the parent structure

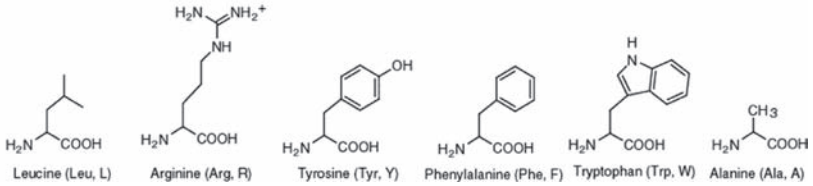

Fig. 1. General structure of microcystins indicating the two key regions required for toxicity and variations from the parent MC-LR.

positions. The uniqueness between both microcystins and nodularins is that they contain the ADDA amino acid component which along with D-Glu are believed to be required for their toxicity.

Due to their toxic nature the World Health Organisation (WHO) has recommended that a level of 1 μg/L, based on MC-LR, is not exceeded in drinking water. This limit is a consequence of the tolerable daily intake (TDI) of MC-LR being 0.04 μg/L and the assumptions that an average person has a body mass of 60 kg and drinks 2 L of water per day; of total microcystin uptake with 80% attributed to water intake. Although, this WHO limit addresses microcystin contamination it does not address the different analogues or the toxicity of these analogues whereby half the lethal dose (LD_{50}) of known analogues can vary 10-fold on intraperitoneal injection in mice (Fig. 1). There is also no toxicity data available for many analogues due to their unavailability from commercial sources as analytical grade standards.

The analytical methods for cyanotoxins have recently been summarised in a state of the art review [13]. Nonetheless the early methods employed for microcystin detection used mouse/brine shrimp biological assays which measured total toxicity, which were replaced by, non-animal, enzyme based protein phosphatase inhibition assays (PPIAs) [11,14,15]. Both these assays detected toxicity in a sample, but were not specific for microcystins. Analytical techniques, such as high performance liquid chromatography (HPLC) and mass spectrometry (MS), allow for the characterisation and quantification of individual microcystin variants for which analytical standards are available. However with over 90 variants for both microcystins and nodularins, of which not all are available as analytical standards, and with lengthy laborious sample preparation, these methods are regularly used to detect only key microcystins, mainly MC-LR in lake and river samples [16,17]. As more variants are becoming available as standards and with increasing interest in microcystin composition, under different climatic conditions in various contaminated regions globally, other variants are being illustrated as highly prevalent in their occurrence [18]. The unethical, unspecific biological assays and highly skilled, expensive and lengthy characterisation by the physiochemical techniques allowed opportunities for alternative methods for rapid and sensitive total microcystin detection. This led to the development of bioanalytical methods such as enzyme linked immunoassays (ELISAs), using antibodies with specific or generic affinity for total microcystin detection. Several polyclonal and some monoclonal antibodies have been generated against microcystins [19]. Most of the research groups used microcystin-LR as a hapten for immunisation, one used microcystin-LA and one used ADDA [19–21]. In more recent years novel biosensor approaches have been trialled for the detection of microcystin due in part to proofs of concept with the emerging technologies, but as this toxin family is gaining increasing awareness as an emerging threat to human health, the environment and the aquaculture industry. This is due to its heightened occurrence either as a consequence of the increasing frequency of algal blooms or through increased monitoring prompted by scientific discussions on its toxic potency and enhanced focus on climate change and possible effects. Optical based surface plasmon resonance (SPR) detection, although no longer in its infancy as a new technology, has been proven as a screening platform for toxins that offers the capability for rapid, robust real time detection that can be validated to meet European standards as a bioanalytical screening method and assays can be fully transferable in kit format to other laboratories [22–24]. SPR methods have been achieved previously for measuring microcystins in drinking water [2] and in Blue-Green Algae (BGA) supplements [25]. However, significant advancements in these SPR methods in relation to toxin detection and analysis time could be achieved through a more efficient sample preparation procedure and utilisation of an antibody with improved sensitivity and specificity to the toxin family.

To date most biosensor methods have measured the free toxin in water samples not taking into consideration the cyanobacteria cells present. This is an important factor as microcystin is an endotoxin that is mostly released during bloom senescence or cell lysis. Consequently, a screening test measuring only free toxin in the water, and excluding intracellular toxin underestimates the total toxin content within the sample. It is therefore necessary to design a rapid assay that detects the total level of microcystin toxins present in a water sample or bloom to offer a true measure of protection for recreational and drinking water. The detection of the intracellular toxin levels provides an additional early-warning phase of the potential toxin release that will inevitably occur as a bloom starts to expire. Rapid methods often do not consider this factor or the microcystin variants despite claims of being early-warning methods [26]. The current study outlines the production of a highly sensitive broad specificity antibody for the detection of MC-LR, microcystin analogues and nodularin for SPR analysis. The focus of the work moves to the implementation of this antibody into the development and validation of a rapid, sensitive SPR assay

for the detection of total microcystin content, by the separation and quantification of free and intracellular toxin in cyanobacterial cultures.

2. Materials and methods

2.1. Materials

Freshwater cyanobacterial cultures, *Microcystis aeruginosa* strains 1450/3 (negative producer) and 1450/6 were obtained from Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland and *M. aeruginosa* strains LEGE 91093 (IZANCYA1), LEGE 91094 (IZANCYA2), LEGE 91095 (IZANCYA25) and LEGE 91096 (IZANCYA33) were supplied by the Laboratory of Ecotoxicology, Genomics and Evolution (LEGE), University of Porto, Portugal (CIIMAR). Jaworski′s and BG11 media were purchased from CCAP, with CCAP cultures grown in Jaworski′s Medium and LEGE cultures in BG11 Medium. Microcystin toxins were obtained from Alexis (now part of Enzo Life Sciences). Keyhole Limpet Haemocyanin (KLH) was purchased from Merck Chemicals Ltd and Bovine Serum Albumin (BSA) from Sigma. CM5 chips, amine coupling kits (containing N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), and Ethanolamine–HCl) and HBS-EP buffer were purchased from GE Healthcare, for use on a Biacore Q biosensor. Cetyltrimethylammonium bromide (CTAB) was obtained from BDH and cell culture media and supplements were obtained from Invitrogen. BIAevaluation software, version 4.1 was used to analyse results.

2.2. Antibody production

2.2.1. Preparation of MC-LR protein conjugates

Both conjugates were prepared using a modified version of the active ester method [27]. *MC-LR-BSA* (*immunogen*): MC-LR (0.5 mg; 63 μL 25% ethanol) was added to BSA (2 mg), and EDC (10 mg: 200 μL deionised water) was added dropwise. The reaction was protected from light and whilst stirring was incubated at room temperature for 2 h. Then EDC (10 mg) was added and the mixture was incubated for a further 2 h. The product was dialysed against 10 mM phosphate buffered saline, pH 7.4.

MC-LR-KLH (*coating antigen*): The KLH conjugate was prepared by adding 0.75 mg EDC (in 100 μL Dimethyl sulfoxide (DMSO)) and 0.75 mg NHS (in 50 μL DMSO) to 0.5 mg MC-LR (in 150 μL 100% ethanol). The reaction mixture was stirred at room temperature, protected from light for 3 h. The active ester solution was added to 2 mg KLH (in 2 mL Carbonate–Bicarbonate Buffer, pH 9.6) and stirred gently, protected from light, for 2 h at room temperature, then overnight at 4 °C. The product was also dialyzed against 10 mM PBS, pH 7.4.

2.2.2. Immunisation procedure

Two BALB/c mice were immunised, on four separate occasions, at 1-month intervals, with 25 μg MC-LR-BSA per injection. For the first two immunisations, immunogen was mixed with the adjuvant Quil-A in sterile saline and administered subcutaneously. The following two subsequent immunisations were delivered by the same route using the adjuvant Pam_3Cys–Ser–$(Lys)_4$ (PCSL). A blood sample (~0.1 mL) was collected ten days after each immunisation to determine the presence of antibodies. The mouse with the strongest serum response, as measured by MC-LR binding on MC-LR-KLH coated ELISA, was selected for the fusion. A final immunisation was performed intraperitoneally without adjuvant. Three days later the mouse was sacrificed and the spleen removed for fusion with an SP2 murine myeloma cell line to produce antibody producing hybridomas using a modified method of Kohler and Milstein [28]. A final mouse bleed was used to optimise ELISA and SPR assays for hybridoma screening and as a positive control in the optimised assays.

All protocols carried out using mice were performed in accordance with a licence issued by the Department of Health, Social Services and Public Safety in the UK under The animals (scientific procedures) Act 1986.

2.2.3. Monoclonal antibody screening by ELISA

Wells of a microtitre plate were coated at 1 μg per well (100 μL) with MC-LR-KLH or KLH (diluted in carbonate–bicarbonate buffer, pH 9.6). Plates were incubated overnight at 4 °C before blocking each well (200 μL per well) with a 1% w/v gelatine solution in 10 mM PBS pH 7.4 and incubated at 37 °C for 1 h. Excess solution was tapped out of the plate and test hybridomas and controls (100 μL) were applied per appropriate well and incubated at 37 for 1 h. To test each hybridoma, supernatant was diluted 1:3 with PBS pH 7.4. For test wells this was applied to 2 × MC-LR-KLH coated wells and for control wells to 2 × KLH coated wells. Positive and negative controls were also included to ensure the functionality of the ELISA; positive controls were the final mouse bleed diluted in PBS pH 7.4 to 1:20,000; negative controls were HAT (Hypoxanthine, Aminopterin and Thymidine) cell culture media diluted 1:3 with PBS. Following incubation, the wells were washed 6 times with ELISA wash buffer (0.9% sodium chloride solution containing 0.0125% (v/v) Tween 20). Goat anti-Mouse IgG labelled with HRP (100 μL at 1:2000) was employed as the detection antibody and incubated at 37 °C for 1 h. Substrate solution, 3,3′,5,5′-Tetramethylbenzidine (TMB), (100 μL) was added to each well and the plate incubated at room temperature (protected from light) for 2 min before stopping the reaction on the addition of 2.5 M Sulphuric Acid (H_2SO_4). Absorbances were read at 450 nm.

A positive response was recorded when the hybridoma media tested displayed a response similar to that of the final heart bleed positive control for the MC-LR-KLH coated wells, and no response for the KLH coated wells. Those with high responses for both MC-LR-KLH and KLH coated wells were deemed false positives, due to non-specific binding. Any positives identified were further screened by inhibition testing. Supernatants were diluted in PBS at 1:2 and 1:10 and analysed when mixed 1:1 with PBS pH 7.4 buffer or MC-LR toxin (100 ng/mL) in PBS pH 7.4. Non-specific binders were those that showed no inhibition of signal in the presence of MC-LR.

2.2.4. Monoclonal antibody screening by SPR

2.2.4.1. Preparation of MC-LR coated CM5 chips for SPR. The method developed by Vinogradova and co-workers [25] to immobilise MC-LR to CM5 sensor surfaces was used. Equal volumes of EDC and NHS (0.4 M and 0.1 M in dH_2O, respectively) were mixed and 50 μL applied immediately to a CM5 sensor chip, covering the gold surface completely. After incubating for 30 min at room temperature, protected from light, equal volumes of 5 mg/mL MC-LR (in 25% ethanol) and 0.6 mM CTAB in 10 mM HEPES, pH 7.4 were mixed and 50 μL applied to the chip surface. The chip was incubated overnight, at room temperature protected from light, in a humid environment to prevent drying out of the dextran surface. Following this, 50 μL of 1 M Ethanolamine–HCl, pH 8.5, was added and incubated at room temperature for 20 min. The chip was washed with deionised water, dried under nitrogen and stored at 4 °C.

2.2.4.2. SPR parameters for hybridoma screening. Equal volumes of hybridoma supernatant and HBS-EP pH 7.4 were mixed and tested for binding to MC-LR immobilised on the chip surface. For a positive control, the final mouse bleed, at 1:300 in HAT media

was diluted 1:1 with HBS-EP pH 7.4. A negative control was prepared by mixing HAT media, 1:1, with HBS-EP buffer. Samples were injected over the chip surface at a flow rate of 20 μL/min for 1 min on a Biacore Q. The chip surface was regenerated after each sample injection with 50 mM sodium hydroxide at a flow rate of 20 μL/min for 1 min. Controls were included every 14 samples, alternating between negative and positive. A positive was deemed as such for any sample that gave a signal of approximately 100 RU or above, as lower would be interpreted as non-specific binding. Positive binders were checked for specificity of binding by competition in the assay with and without MC-LR (250 ng/mL). As with ELISA inhibition screening, MC-LR specific binders were characterised by a reduction of signal when MC-LR was present.

2.2.4.3. Monoclonal antibody

On selection of the best monoclonal antibody, the cell line was grown and antibody was concentrated in cell culture media using a CELLine 1000 bioreactor (IBS Integra Biosciences), followed by purification via affinity chromatography using a protein G-sepharose gel column (MAbTrap Kit). Dialysis of the antibody over 24 h in 0.15 M saline (3 × 4 L) was performed. The protein concentration and isotyping of each antibody was determined at $A_{280\ nm}$ and using a mouse monoclonal antibody isotyping kit (Roche Diagnostics), respectively. The antibody was stored frozen at −20 °C until required for use.

2.3. SPR assay development for microcystin detection

2.3.1. Evaluation of assay parameters

Initially, various parameters were investigated in the development of the Biacore Q biosensor assay including: binder dilution, ratio of binder to standard, injection volume, and contact time.

2.3.2. Preparation of assay calibration curve

The sensitivity of the monoclonal antibody to MC-LR was assessed using the Biacore Q biosensor. A stock solution of MC-LR (1 mg/mL) in 100% methanol was prepared and used to prepare an intermediate stock solution of 1 μg/mL in HBS-EP. From this MC-LR calibrants ranging in concentration from 0, 0.5, 1, 2.5, 5.0 and 10 ng/mL were prepared in pH 7.4 HBS-EP buffer. These working standards were used to produce calibration curves based on dose–response on the biosensor.

2.3.3. Sample preparation

The extra and intra-cellular extracts of the *M. aeruginosa* cultures were prepared by conducting cyanobacterial culturing procedures and the development of a rapid sample lysis protocol to release the toxin.

Cyanobacterial culturing: Two cultures from CCAP (1450/3 and 1450/6) were maintained in Jaworski's medium and four from CIIMAR (LEGE 91093, LEGE 91094, LEGE 91095 and LEGE 91096) were grown in BG11 Medium. The, non-axenic, cultures were maintained at 20 °C with a 12:12 h light:dark cycle and a light intensity of 116 μmol/m^2 s. Cultures were maintained in glass Erlenmeyer flasks, with cotton wool plugs, and capped with foil. All consumables were autoclaved at 121 °C, for 15 min, then equilibrated to 20 °C prior to use, with culturing taking place in a UV3 HEPA PCR cabinet, pre-sterilised by UV light. Culture media (200 mL) was seeded with 40 mL of a dense culture, which was approaching stationary phase. Generally cultures were not allowed to drop out of log phase, as they may produce different toxin profiles or levels during the different phases of their life cycle.

Sample lysis: *M. aeruginosa* cultures were lysed to release intracellular microcystins for testing. A sample (50 mL) was centrifuged at 3000*g*; the supernatant was removed and retained for testing the extra-cellular microcystin content and only requiring 0.45 μm filtration prior to use. The pellet was re-suspended in 5 mL HBS-EP buffer and then the cyanobacterial cells were mechanically lysed using a modified method as described by Devlin and co-workers [29]. In brief, 2 mL was transferred to a 5 mL tube containing 1 g of 0.5 mm glass beads and the sample was mixed in an Elcometer 7951 Minimix Paint Mixer for 10 min. The tube was transferred to a centrifuge and spun for 10 min at 3000*g* to remove large particulate matter, with the supernatant then filtered (0.45 μm) to remove the smaller debris. The filtrate was retained and used to measure the intra-cellular microcystin content.

Preparation of matrix calibration curve: The matrix curve was prepared using the negative culture (CCAP 1450/3) as described in the sample preparation. Aliquots (1 mL) of filtrate were fortified with MC-LR calibrants (50 μL) to prepare a calibration curve matching in concentration to the calibration curve prepared in buffer only.

2.4. SPR analysis: instrumental parameters

The MC-LR antibody (5C4; 0.88 mg/mL) was diluted 1:1000 with HBS-EP buffer. The working antibody solution was mixed at a ratio of 30:70 respectively of either the calibrant solution or sample and was injected over the chip surface at a flow rate of 20 μL/min for 4 min. Report points were recorded before (10 s) and after each injection (30 s) and the relative response units determined. The chip surface was regenerated with a mixture 75 mM NaOH and 10% acetonitrile at a flow rate of 20 μL/min for 1 min. A typical analytical cycle was completed in approximately 10 min. Calibrants and samples were analysed in duplicate.

2.5. Validation

The method was validated considering the European Commission Decision 96/23/EC criteria concerning the performance of analytical methods and the interpretation of results [30].

2.5.1. Antibody sensitivity and specificity

The sensitivity (IC_{50}) and dynamic range (IC_{20}–IC_{80}) of the assay, calibration curves in buffer only and prepared in known negative culture extract were analysed using the Biacore Q SPR assay to compare matrix effects to establish the method applicability.

Selectivity is the ability of the method to distinguish between the target analyte (MC-LR) and the presence of other potentially interfering analytes [30]. Six of the more common and toxic microcystin variants: MC-RR, MC-YR, MC-LA, MC-LW, MC-LF and nodularin were used to determine the selectivity of the monoclonal antibody to MC-LR. As the monoclonal was raised against an MC-LR conjugate, all responses were normalised against the MC-LR calibration curve. For each curve, the midpoint (the point at which 50% inhibition is achieved; IC_{50}) was calculated and the % cross-reactivity calculated.

2.5.2. Recovery

To measure the recovery of the MC-LR under assay conditions from known negative culture samples in log phase using this procedure, aliquots of extract were fortified with MC-LR at 0.5, 1 and 2 ng/mL and analysed. This level of fortification of the culture pellet corresponds to 0.05, 0.1 and 0.2 ng/mL in the starting sample aliquot of 50 mL.

Analysis of uncontaminated culture 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 MC-LR [30]. 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 was obtained by the duplicate analysis of 20 individual blank cyanobacterial culture samples. The decision limit was calculated as the mean of the calculated concentration + (2.33 × the standard deviation). This provides a 99% confidence level that a sample producing a result above this decision limit does contain MC-LR.

2.5.3. Detection capability

For the optimised assay the detection capability (CCβ), is the smallest content of the substance that can be detected, identified and/or quantified. Twenty negative samples (i.e. toxin free) were analysed, as were 20 negative samples fortified at 0.5 ng/mL MC-LR. All samples were assayed in duplicate and the average concentration calculated.

2.5.4. Repeatability and reproducibility of the assay

To examine the repeatability of the assay, three fortification levels of toxin were used: the WHO recommended limit at 1 ng/mL; twice the recommended limit at 2 ng/mL; and half the recommended limit at 0.5 ng/mL. Ten replicates ($n=10$) were assayed in duplicate for each fortification level used. Samples were fortified after the 50 mL sample was centrifuged and re-suspended in 5 mL HBS-EP, prior to lysis.

To examine the reproducibility of the assay between days this study on repeatability was conducted over 3 days, preparing samples and calibrants fresh each day.

2.6. Comparison of cyanobacterial culture analysis with Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS)

Microcystins from six *M. aeruginosa* culture samples were analysed using the SPR method and sample preparation as described herein and by LC–MS/MS detection using a combination of modified methods [16,31–33]. Culture samples (50 mL) were frozen at −80 °C, lyophilised and then re-suspended in 5 mL of methanol (75% v/v). The samples were vortexed for 1 min and then centrifuged at 3000*g* for 10 min to remove the cell debris. The supernatant was decanted and diluted with deionised water to 15% methanol v/v for solid phase extraction (SPE) using OASIS HLB cartridges, 6 cc, 500 mg (Waters, Ireland). The SPE cartridges were conditioned with methanol and the samples loaded in 15% methanol (25 mL). Cartridges were washed with water, followed by 20% methanol and the microcystin congeners eluted using 6 mL of methanol acidified with 0.1% trifluoroacetic acid. The eluents were dried under a stream of nitrogen at 55 °C and reconstituted in 200 μL of 80% methanol.

Samples were analysed by LC–MS/MS using a Waters Acquity UPLC and a Quattro Premier XE Mass Spectrometer, run in electrospray positive mode (ESI) whereby the microcystin variants were analysed by MRM (multiple reaction monitoring). The samples were calibrated using Quanlynx against a 5-point calibration curve for each congener in a non toxin producing culture 1450/03 extracted in the same manner and fortified with standards.

3. Results and discussion

3.1. Monoclonal antibody production

For the monoclonal antibody production 449 hybridomas were screened by ELISA and SPR analysis but only three hybridomas produced antibodies that provided a specific response to MC-LR, of which only one demonstrated suitable sensitivity and specificity as a broad specificity antibody to microcystin congeners. Following purification the monoclonal antibody was isotyped as IgG 1, κ light chain with a protein concentration of 0.88 mg/mL at A_{280}.

3.2. Assay development

To allow for the detection of small analytes on a Biacore Q instrument the most frequently selected formats are antibody based competitive inhibition assays [18,19]. Due to the small size of the microcystin molecule (~1 kDa) this approach was also adopted in the present study. In the development of the assay various parameters such as antibody dilution, contact time, injection type and % ratio of antibody to sample were investigated to determine the conditions for the optimum sensitivity of the assay (IC_{50}) and the maximum binding capacity (R_{max}) of the assay under these conditions. A summary of the data generated is presented in Table 1.

It was observed that by increasing the antibody dilution thereby decreasing the protein concentration, for each constant % ratio and contact time, improved the sensitivity of the assay by decreasing the IC_{50} concentration for microcystin-LR. However, with antibody dilutions increasing by 2-fold the R_{max} decreased by

Table 1
Evaluation of SPR assay parameters on the sensitivity of the assay for MC-LR as defined by the IC_{50}.

Antibody dilution	Contact time (s)	Injection type	30% Antibody ratio IC_{50} (ng/ml)	40% Antibody ratio IC_{50} (ng/ml)	50% Antibody ratio IC_{50} (ng/ml)	30% Antibody ratio R_{max} (RU)	40% Antibody ratio R_{max} (RU)	50% Antibody ratio R_{max} (RU)
250	60	Normal	6.171	9.052	14.492	314.6	427.8	550.3
250	60	Quick	6.563	9.414	14.273	329.8	435.0	545.8
250	120	Normal	5.654	8.612	13.320	674.2	873.5	1113.4
250	120	Quick	6.081	9.005	12.926	671.9	896.0	1083.4
250	180	Normal	5.812	8.350	12.130	1003.8	1326.6	1628.4
250	180	Quick	5.819	8.502	12.806	1007.3	1330.4	1624.4
250	240	Normal	5.033	7.556	11.645	1286.5	1675.8	1981
250	240	Quick	5.321	7.809	11.901	1273.8	1658.2	1969.7
500	60	Normal		5.411	7.546		218.4	280.4
500	60	Quick		5.568	7.838		213.4	272.0
500	120	Normal	3.355	4.951	7.160	332.9	438.0	548.0
500	120	Quick	3.362			329.8		
500	180	Quick	3.259	4.646	7.305	490.5	663.7	778.8
500	240	Quick			6.431			1098.1
1000	120	Quick	1.894	2.671	3.992	142.1	188.5	233.6
1000	180	Quick	1.807	2.573	3.529	212.7	281.3	356.0
1000	240	Quick	1.764	2.418	3.431	283.1	380.1	473.2
2000	120	Quick	1.667	2.154	2.890	79.5	104.7	130.6
2000	180	Quick	1.577	2.103	2.799	116.8	155.0	195.0
2000	240	Quick	1.534	2.000	2.686	155.3	206.7	257.5

2-fold and with dilutions greater than 1 in 2000 the R_{max} achieved was not suitable for assay development in the 4 min timeframe to preserve the assay as a rapid test. Increasing the % ratio of antibody to standard on a volume to volume basis decreased the sensitivity of the assay even though the R_{max} increased. By increasing the % antibody in this manner the final concentration of MC-LR in the antibody standard mix was reduced which reduced the overall assay sensitivity. However, increasing the contact time where other factors remained constant had a minimal effect on the sensitivity. By increasing the contact time and hence the volume allowed a suitable R_{max} to be achieved at increased antibody dilutions for assay development. The difference between a normal injection and a quick injection was the least significant but the slightly slower normal injection provided marginally better sensitivity compared to when the quick injection was applied. The final parameters selected were an antibody dilution of 1 in 1000 mixed in a 30% ratio with a quick 4 min injection time as described in the assay parameters.

For the assay, matrix matched curves were used, as the comparison between the buffer and matrix curves in the assay development illustrated that although there was a suitable overlay, at either ends there was some degree of separation, which would result in less accurate quantification of toxin content in samples at these regions (Fig. 2). For this reason, the lysis procedure was used to prepare lysate of the CCAP 1450/03, a non toxin-producer, which was used for matrix matched calibrants, negative and fortified samples. Under these optimised conditions the sensitivity (IC_{50}) of the SPR assay was 1.76 ng/mL with a dynamic range (IC_{20}–IC_{80}) of 0.69–4.24 ng/mL.

3.3. Assay validation

3.3.1. Specificity

The cross-reactivity was tested for its ability to bind to a range of commonly occurring toxic variants. MC-RR and MC-YR, along with MC-LR are the most common [14], and most toxic variants, followed by MC-LA, MC-LW and MC-LF [20]. As the monoclonal antibody was raised against MC-LR all cross-reactivities were normalised against this toxin′s binding as expressed as an IC_{50}, i.e. the concentration of MC-LR needed to cause 50% inhibition of binding signal. The figure calculated for MC-LR was set as the 100% value and the IC_{50} concentrations for the other toxins measured against this.

The cross-reactivities were determined as follows: MC-LR 100%; MC-RR 108%; MC-YR 68%; MC-LA 69%; MC-LW 71%; MC-LF 68%; and Nodularin 94%. For total microcystin content this provided an average cross-reactivity of 80.7%.

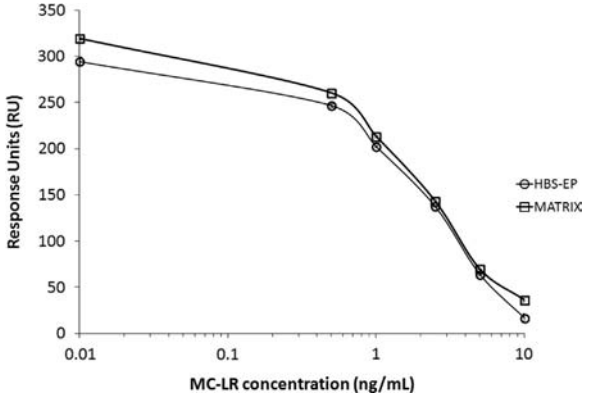

Fig. 2. Overlay of matrix and buffer curves, demonstrating slight disparity at low and high concentrations (which would result in less accurate concentration analysis being achieved in samples falling into these regions of the calibration curve).

3.3.2. Recovery

The ten samples fortified at the 3 levels of MC-LR fortification 0.5, 1 and 2 ng/mL and analysed over 3 days displayed average recoveries of 112.5, 85.4 and 76.3% respectively (Table 2).

3.3.3. Decision limit (CCα)

The effect on the assay of possible interference from non-producing culture samples was determined by the analysis of 20 MC-LR-free samples. Using the calculation stated previously the decision limit (CCα) for the SPR was calculated to be 0.35 ng/mL. This is the noise level of the SPR assay and it can be concluded with a 99% confidence level that any sample which gives a response above 0.35 ng/mL by SPR contains MC-LR. The value represents possible effects of interfering compounds found in the culture. This decision level of the SPR assay correlates directly with the extracellular measurement of the microcystin toxins in the supernatant but for the intracellular toxins prepared from a 50 mL starting sample whereby a 10-fold concentration step has been applied as part of the sample preparation this decision limit then correlates to 0.035 ng/mL for the intracellular toxins which demonstrates an extremely sensitive overall assay.

3.3.4. Detection capability

For the optimised assay the detection capability (CCβ) of the SPR assay is the smallest concentration of the MC-LR that can be detected, identified and/or quantified. The average calculated concentration for the negative population was 0.18 ± 0.07 ng/mL (C.V.=38.9%) and 0.53 ± 0.03 ng/mL (C.V.=5.7%) for the 0.05 ng/mL MC-LR fortified population. As no overlap between the negative and fortified population occurred at this level (Fig. 3) the detection capability was calculated to be 0.5 ng/mL which correlates to that for the extracellular toxins. Similarly, on taking into account the 10-fold concentration step in the sample preparation method utilised this overall assay offers a highly sensitive detection capability of 0.05 ng/mL for intracellular microcystins.

3.3.5. Repeatability (intra-assay) and reproducibility (inter-assay) of the assay

Table 2 shows the calculated concentrations, their standard deviations, % C.V.s and recoveries for each day (intra-assay

Table 2
Recovery, repeatability and reproducibility of the assay at the three fortified levels over the three days of analysis.

Repeatability	Fortification level (ng/mL)	Measured concentration (ng/mL) ± S.D.	C.V. (%)	Mean recovery
Day 1	0.5	0.53 ± 0.03	6.2	104.8
	1	0.74 ± 0.06	8.0	74.6
	2	1.52 ± 0.09	5.9	75.2
Day 2	0.5	0.61 ± 0.03	4.5	122.7
	1	0.99 ± 0.09	9.3	97.5
	2	1.53 ± 0.09	6.1	76.6
Day 3	0.5	0.55 ± 0.03	5.3	109.3
	1	0.84 ± 0.04	4.9	83.1
	2	1.53 ± 0.14	9.1	76.5
Reproducibility (Days 1–3)	0.5	0.56 ± 0.04	7.5	112.5
	1	0.85 ± 0.13	15.0	85.4
	2	1.53 ± 0.01	0.6	76.3

analysis) and across the 3 days (inter-assay analysis), to assess repeatability and reproducibility, respectively. For the intra-assay analysis all % relative standard deviations (C.V.s) were below 10% and recoveries ranged from 75.2% to 122.7%. The inter-assay analysis had % relative standards deviations (C.V.s) below 15% and recoveries ranging from 76.3% to 112.5%. This analysis incorporated the use of two biosensors and two different chips to fully test the robustness of the procedure. The low relative standard deviations for repeatability and reproducibility demonstrated that a robust method was achieved.

3.4. Comparison of cyanobacterial culture analysis with Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS)

The microcystin concentration was determined in 6 cyanobacterial cultures by both SPR and LC–MS/MS (Table 3). Five out of the six cultures tested positive and the overall the toxicities were ranked: 91095 > 91093 > 91096 > 91094 > 1450/06 > 1450/03 and this ranking correlated by both SPR and LC–MS/MS methods displaying an R^2 value of 0.9989 (Fig. 4). The relatively small differences between the quantification determined by the two methods might be explained by the differing sample preparation methods. It should be noted that the LC–MS/MS method utilises a time consuming freezing followed by freeze-drying for lysis, followed by an expensive SPE clean-up, whereas the SPR method uses only the rapid and low cost bead beating for lysis. In addition, the SPR method measures the total toxicity relative to the cross-reactivity profile of the antibody and with average cross-reactivity factor of 1.2 applied for total microcystin in unknown samples the correlation of concentration would be improved. Nonetheless, as a screening method, with a set WHO limit of 1 μg/L this SPR assay is a rapid and efficient tool for determining total microcystin presence.

The cultures were investigated for the six microcystins for which the cross-reactivity was determined but only MC-LR and MC-LA were determined in the toxin producing cultures with MC-LR being the dominant toxin.

4. Conclusions

In this study a novel broad specificity monoclonal antibody for microcystins and nodularin was used to develop a rapid and sensitive SPR assay to detect microcystins. The levels of microcystins present were calculated as intracellular and free, to give the total microcystin content displaying the versatility of the assay, whereby the user can test for total or compartmentalised microcystins, as required. For free microcystins the detection capability was determined as 0.5 μg/L and for the intracellular microcystins the detection capability was 10-fold lower at 0.05 μg/L due to the concentration step applied in the sample preparation. A concentration step could also be included for the free microcystins measurement but would require a SPE clean-up step which would make the method slower and more expensive. The WHO recommended limit is 1 μg/L (1 ng/mL) and detection at this concentration was rapidly achieved by the SPR assay. The validation showed good levels of both precision and accuracy, as indicated by low % relative standard deviations and recoveries greater than 75% in the intra and inter-assay study and comparable data to LC–MS/MS analysis.

Thus herein is presented an assay for the rapid testing of samples, using a simple sample preparation, to test at or below the WHO recommended limit of 1 μg/L for total microcystins in water. In comparison to the SPR method of Herranz and co-workers [2] which displayed enhanced sensitivity for MC-LR detection but only in surface water, this approach has a much quicker cycle time,

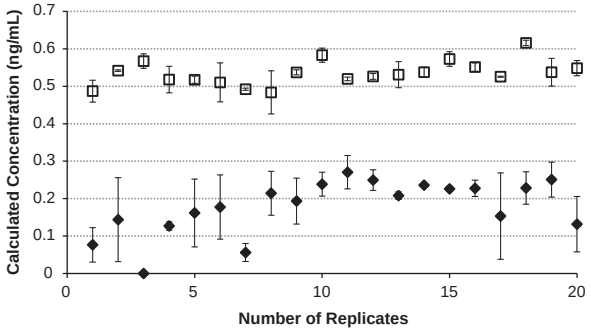

Fig. 3. Lowest detectable limit (CCβ) of the SPR assay was determined at 0.5 ng/mL, as there is no overlap recorded between negative (⬥) and samples fortified at 0.5 ng/mL (□).

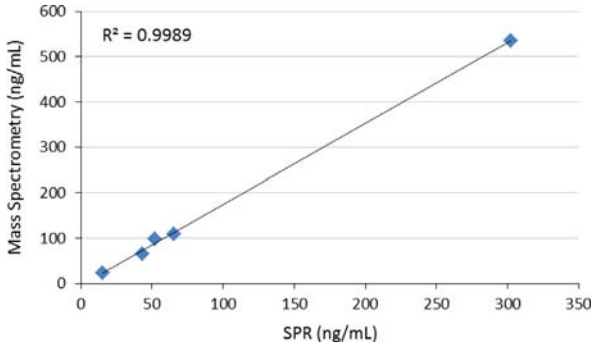

Fig. 4. Comparison of SPR and Mass Spectrometry results for microcystin producing cultures.

Table 3
Microcystin levels of *Microcystis aeruginosa* cultures as determined by SPR analysis with toxin characterisation and quantification by mass spectrometry with differences between results indicated (R^2=0.9989).

Culture	SPR Analysis			Mass Spectrometry Analysis			Difference (x-fold)
	Pellet (Cell) concentration (ng/mL)	Supernatant concentration (ng/mL)	**Total concentration (ng/mL)**	Concentration of MC-LR (ng/mL)	Concentration of MC-LA (ng/mL)	**Total concentration (ng/mL)**	
1450/6	9.6	9.0	**18.5**	23.6	0.0	**23.6**	1.3
91093	65.4	9.6	**75.0**	93.6	14.5	**108.1**	1.4
91094	42.9	8.4	**51.3**	53.2	11.3	**64.5**	1.3
91095	175.0	237	**412**	531.9	4.3	**536.2**	1.3
91096	51.4	7.9	**59.3**	85.7	13.2	**98.9**	1.7
1450/3	< 0.5	< 0.5	< **0.5**	0	0	**0**	N/A

of 8 min, compared to their 60 min cycle. The chip performance of the present method at 800 cycles per flow cell has been shown to be substantially better compared to 40 cycles per chip which further reduces the cost per analysis of the procedure. Thus a validated, rapid, automated and quantitative screening assay is presented as an early warning detection system before natural lysis of a cyanobacterial bloom for both free and intracellular microcystins.

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