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Multihapten Approach Leading to a Sensitive ELISA with Broad Cross-Reactivity to Microcystins and Nodularin

Ingunn A. Samdal,*,† Andreas Ballot,†,§ Kjersti E. Løvberg,† and Christopher O. Miles†,‡

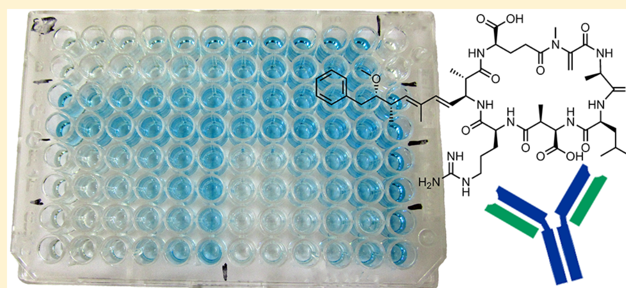
†Norwegian Veterinary Institute, P.O. Box 750, Sentrum, N-0106 Oslo, Norway

§Norwegian Institute for Water Research, Gaustadalléen 21, N-0349 Oslo, Norway

‡Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo, Norway

Supporting Information

ABSTRACT: Microcystins (MCs) are a group of biotoxins (>150) produced by cyanobacteria, with a worldwide distribution. MCs are hepatotoxic, and acute exposure causes severe liver damage in humans and animals. Rapid and cheap methods of analysis are therefore required to protect people and livestock, especially in developing countries. To include as many MCs as possible in a single analysis, we developed a new competitive ELISA. Ovine polyclonal antibodies were raised using an immunogen made by conjugating a mixture of microcystins to cationised bovine serum albumin, and the plate-coating antigen was prepared by conjugating [Asp³]MC-RY to ovalbumin. This strategy was used also to minimize specificity for particular microcystin congeners. Cross-reactivity studies indicate that the ELISA has broad specificity to microcystins and also detects nodularin, providing a sensitive and rapid analytical method for screening large numbers of samples. The limit of quantitation for microcystins in drinking water is 0.04 μg/L, well below the WHO's maximum recommendation of 1 μg/L. The ELISA can be used for quantifying total microcystins in various matrices, including drinking water, cyanobacterial cultures, extracts, and algal blooms, and may be useful in detecting metabolites and conjugates of MCs.



INTRODUCTION

Cyanobacterial toxins can occur in drinking water reservoirs and recreational lakes worldwide, causing gastroenteritis, skin reactions, and liver damage and represent an acute and chronic risk for human and animal health. Lethal poisonings have been recorded in sheep, cattle, horses, pigs, dogs, fish, rodents, amphibians, waterfowl, bats, rhinoceros, wildebeest, and zebras.[1−3] In Kenya and Tanzania, mass mortalities of lesser flamingos and other animals have been attributed to cyanotoxins.[4−6] Acute cyanotoxin poisoning in humans is rare because water containing high concentrations of cyanobacteria has a foul smell and taste. Nevertheless, cyanotoxins are a health risk in developing countries due to chronic exposure to low levels of cyanotoxins in the drinking water. High nutrient enrichments in many South African surface water resources result in eutrophication-related problems and high concentrations of cyanotoxins such as microcystins.[7−9] Only 21% of households in rural South Africa have access to piped indoor water,[8] many water treatment plants in rural areas do not produce water of acceptable quality,[10] and the remaining rural households depend on what is available in lakes, streams, and wells. There is therefore a pressing need for rapid, reliable, rugged, and affordable screening tests for cyanotoxins in such situations.

Microcystins (MC) (Figure 1) are toxic cyclic heptapeptides produced by various cyanobacterial genera, including *Microcystis*, *Anabaena*, *Hapalosiphon*, *Oscillatoria*, *Planktothrix*, and *Nostoc*[11] and are one of the most commonly reported classes of cyanobacterial toxins. The most frequently reported and most toxic microcystin is MC-LR[12] (Figure 1), where L and R represent the amino acids leucine and arginine at the variable 2- and 4-positions, respectively.[13,14] Other variants regularly reported are MC-LA,[15] -YR, and -RR,[13−16] although >150 analogues have been reported.[17,18] Nodularins (NOD) (Figure S1, Supporting Information) are related pentapeptides, produced by cyanobacteria of the genus *Nodularia*[11] with ca. 10 analogues reported.[17,18] The World Health Organization[19] has proposed a provisional guideline value of 1 μg/L of MC-LR (free and cell-bound toxin) in drinking water,[20] stating that "It is important to measure *total* microcystins, which includes microcystins occurring free in water and microcystins bound to or inside cyanobacterial cells and which includes all microcystins, not just microcystin-LR".[19]

The large variety of microcystins, combined with the limited availability of standards, makes such analyses demanding. Available methods include HPLC-UV, LC-MS, enzyme-linked immunosorbent assays (ELISAs), and bioassays. LC-MS

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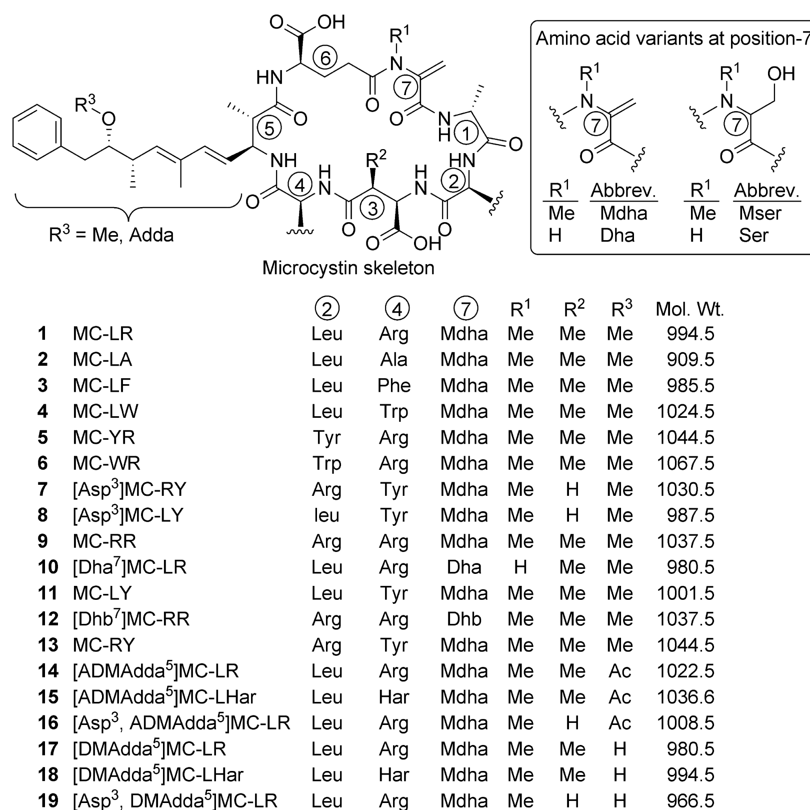


		②	④	⑦	R^1	R^2	R^3	Mol. Wt.
1	MC-LR	Leu	Arg	Mdha	Me	Me	Me	994.5
2	MC-LA	Leu	Ala	Mdha	Me	Me	Me	909.5
3	MC-LF	Leu	Phe	Mdha	Me	Me	Me	985.5
4	MC-LW	Leu	Trp	Mdha	Me	Me	Me	1024.5
5	MC-YR	Tyr	Arg	Mdha	Me	Me	Me	1044.5
6	MC-WR	Trp	Arg	Mdha	Me	Me	Me	1067.5
7	[Asp3]MC-RY	Arg	Tyr	Mdha	Me	H	Me	1030.5
8	[Asp3]MC-LY	leu	Tyr	Mdha	Me	H	Me	987.5
9	MC-RR	Arg	Arg	Mdha	Me	Me	Me	1037.5
10	[Dha7]MC-LR	Leu	Arg	Dha	H	Me	Me	980.5
11	MC-LY	Leu	Tyr	Mdha	Me	Me	Me	1001.5
12	[Dhb7]MC-RR	Arg	Arg	Dhb	Me	Me	Me	1037.5
13	MC-RY	Arg	Tyr	Mdha	Me	Me	Me	1044.5
14	[ADMAdda5]MC-LR	Leu	Arg	Mdha	Me	Me	Ac	1022.5
15	[ADMAdda5]MC-LHar	Leu	Har	Mdha	Me	Me	Ac	1036.6
16	[Asp3, ADMAdda5]MC-LR	Leu	Arg	Mdha	Me	H	Ac	1008.5
17	[DMAdda5]MC-LR	Leu	Arg	Mdha	Me	Me	H	980.5
18	[DMAdda5]MC-LHar	Leu	Har	Mdha	Me	Me	H	994.5
19	[Asp3, DMAdda5]MC-LR	Leu	Arg	Mdha	Me	H	H	966.5

Figure 1. Structures of microcystins discussed in the text.

methods can measure the individual MC congeners present but require high-tech instruments and experienced operators due to the large numbers of MCs with molecular masses/transitions that potentially could be present, including undescribed MC congeners. The analytical challenges this poses is illustrated by a recent LC-MS analysis of water from Hartbeespoort Dam, South Africa, where the sample contained 18 MCs at levels above the LoQ and a further 23 MC congeners at levels below the LoQ.[7] To provide certainty, an analytical method should detect all MCs, regardless of whether analytical standards are commercially available. To measure such low levels of microcystins, and to detect them all, requires a very sensitive analytical method with exceptionally broad specificity.

Immunoassays with a suitable antibody can potentially identify all MCs (and NODs) and are well suited to screening because they can be highly sensitive and cheap, they have high sample throughput, and it is not necessary to possess all MCs as standards provided the assay cross-reactivity is similar toward all analogues. Several ELISAs for MCs are already commercially available.[21−23] However, data on cross-reactivity of the existing assays are relatively limited, cross-reactivities often appear to vary markedly from analogue-to-analogue, and many commercial ELISA kits are too expensive for large surveillance programs in developing countries.

With the exception of the first antibody raised using MC-LA,[24] most MC antibodies and ELISAs have been produced by raising antibodies to MC-LR.[25−39] This approach can lead to a preference for microcystins with arginine in position-4, with some MCs being poorly recognized.[29] MC-RR has also been used for raising antibodies for ELISA development[31,32] but could be expected to be subject to similar limitations. To avoid selection for specific microcystins, a conjugate of the unusual β-amino acid Adda—present at position-5 in most MCs and NODs—was used to raise antibodies with good cross-reactivity to a wide range of MCs.[21,23] However, antibodies directed solely at the Adda moiety should detect free Adda, which can be produced by microbial degradation of MCs and is not toxic[40] and may not adequately recognize congeners containing modified Adda units such as [DMAdda5]- and [ADMAdda5]-MCs. Furthermore, such antibodies should recognize Adda-containing ring-opened "linearized" *seco*-MCs. These are produced during biosynthesis or hydrolysis of MCs but are, however, much less toxic.[41−47]

Alternative strategies for the development of antibodies with broad specificity for whole groups of analytes[48] include immunizing with hapten mixtures.[49] In an attempt to avoid producing antibodies with a preference for MCs containing particular amino acids, we conjugated a carefully chosen mixture of microcystins to cationized bovine serum albumin (cBSA) for immunizing sheep. The resulting polyclonal antisera, together with a plate-coating antigen derived from [Asp3]MC-RY, were combined in an indirect competitive ELISA with exceptional sensitivity and broad specificity. The ELISA gave excellent results with cultures, spiked, and natural samples.

■ MATERIALS AND METHODS

Materials. MC-LR, -LA, -LF, -LW, -YR, and -WR (**1**−**6**) were from Enzo Life Sciences Inc. (Farmingdale, NY). [Asp3]MC-RY (**7**) was fractionated from an HP-20 extract of *Microcystis aeruginosa* culture NIVA-CYA 548.[50] 2-Hydroxythiepan (2-HTP) was from Toronto Research Chemicals (North York, ON, Canada). Sodium cyanoborohydride, bovine serum albumin (BSA), ovalbumin (OVA), and Freund's incomplete adjuvant (FIA) were from Sigma−Aldrich (St. Louis, MO). Cationized BSA (cBSA) was prepared by standard

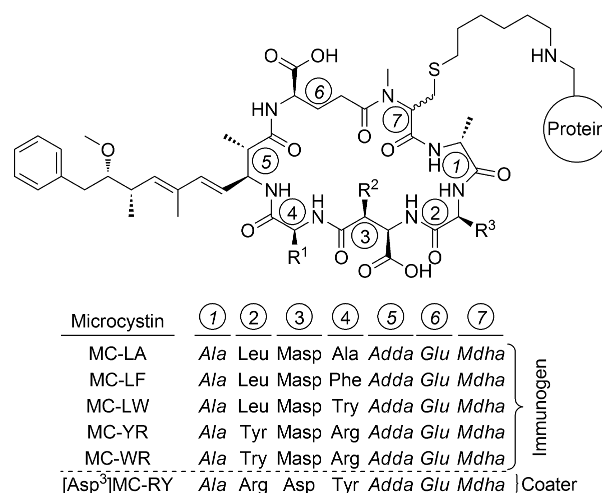
methods.[51] Maxisorp immunoplates (96 flat-bottom wells) were from Nunc (Roskilde, Denmark), poly(vinylpyrrolidone) 25 (PVP) was from Serva Electrophoresis (Heidelberg, Germany), rabbit antisheep−horseradish peroxidase conjugate (antisheep−HRP) from Zymed (now Invitrogen, Paisley, U.K.), and HRP-substrate K-blue Aq. from Neogen (Lexington, KY). *Nostoc* 152 culture,[52] from K. Sivonen (University of Helsinki, Finland), was extracted to provide crude **14**−**16**, and a subsample of the extract was hydrolyzed to give crude **17**−**19**.[7] Certified reference materials (CRMs) of MC-LR, MC-RR (**9**), NOD-R, and [Dha[7]]MC-LR (**10**) were from the National Research Council of Canada (NRC) (Halifax, NS, Canada). Noncertified standards were obtained as follows: MC-LR, MC-RR, MC-YR, MC-LA, MC-LF, MC-LY, MC-LW, and NOD-R (Enzo Life Sciences, Farmingdale, NY); [Dhb[7]]MC-RR (Cyano Biotech, Berlin, Germany); MC-LR (Abraxis-ELISA-kit, Enzo Life Sciences); and [Dha[7]]MC-LR (AgResearch, Hamilton, New Zealand). A reference material (RM) (quantitated by LC-CLND) of MC-LA and nonquantitated standards of MC-LF and MC-YR were from NRC. Inorganic chemicals and organic solvents were reagent grade or better. Plate-coating buffer was carbonate buffer (50 mM, pH 9.6). Phosphate-buffered saline (PBS) contained NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8 mM), and KH_2PO_4 (1.5 mM), pH 7.4. ELISA washing buffer (PBST) was 0.05% Tween 20 in PBS. Sample buffer was 10% methanol (v/v) in PBST, and antibody buffer was 1% PVP (w/v) in PBST.

Cultures of *M. aeruginosa* were isolated from Hartbeespoort Dam (−25.7475, 27.866944) and from the breeder dam at Le Croc crocodile farm (−25.492467, 27.680450), North West Province, South Africa. These produced a range of microcystin variants (Tables S1−S6, Supporting Information) and were used to test ELISA performance.

[Asp[3]]MC-RY (7). A culture (3 L) of NIVA-CYA 548 was extracted with HP-20 resin and then eluted.[50] The eluate was evaporated to dryness, dissolved in MeOH−water (1:4, 5 mL), and chromatographed (SPE cartridge, Hypersep C18, 500 mg, 3 mL; Thermo Scientific) with a stepwise gradient of MeOH (30, 40, 45, 50, 55, 60, 70, 80, 90, and 100%) in water (5 mL per step). LC-MS revealed 98% of **7** in the 40 and 45% fractions (together with 31% of **8**), which were combined. This fraction was further purified by SPE, with a gradient of MeOH (25, 30, 35, 40, 45, 50, and 90%) in water (5 mL per step). The 40% fraction contained 67% of the [Asp[3]]MC-RY (**7**), essentially free of **8** or other peptides by LC-MS, and an aliquot containing 100 μg of **7** was evaporated to dryness under a stream of nitrogen.

Immunogen. MC-LA (**2**), -LF (**3**), -LW (**4**), -YR (**5**), and -WR (**6**) (100 μg each) were dissolved together in MeOH (2 mL), added to a suspension of 2-HTP (550 μg) in MeOH−MeCN (1:1, 600 μL) plus tetrahydrofuran (THF) (200 μL), and held at 40 °C. After 1 h, carbonate buffer (pH 9.2, 50 μL, 0.2 M) was added and held at 40 °C for 2 h and then overnight at ca. 20 °C. Most of the organic solvent was removed under a stream of nitrogen, PBS was added (1 mL), and the residual organic solvent removed under a stream of nitrogen. The ice-cold solution was carefully washed with diethyl ether (4 × 2 mL) to remove unreacted 2-HTP and briefly held under a stream of nitrogen to remove residual ether.

This mixed-MC-hapten solution was added to 10 mg of cBSA in 3.0 mL of PBS, and 100 μL of sodium cyanoborohydride (50 mg/mL in water) was added to form the immunogen (Figure 2). After 2 h, another 50 μL of



Microcystin	1	2	3	4	5	6	7	
MC-LA	*Ala*	Leu	Masp	Ala	*Adda*	*Glu*	*Mdha*	Immunogen
MC-LF	*Ala*	Leu	Masp	Phe	*Adda*	*Glu*	*Mdha*	
MC-LW	*Ala*	Leu	Masp	Try	*Adda*	*Glu*	*Mdha*	
MC-YR	*Ala*	Tyr	Masp	Arg	*Adda*	*Glu*	*Mdha*	
MC-WR	*Ala*	Try	Masp	Arg	*Adda*	*Glu*	*Mdha*	
[Asp[3]]MC-RY	*Ala*	Arg	Asp	Tyr	*Adda*	*Glu*	*Mdha*	Coater

Figure 2. Structures of the immunogen and plate-coating antigen. The immunogen consisted of an equal mix of MC-LA, MC-LF, MC-LW, MC-YR, and MC-WR conjugated to cBSA, whereas the plate-coating antigen consisted of [Asp[3]]MC-RY conjugated to OVA, both conjugated through a thiol linker at position-7 (see Scheme S1 in the Supproting Information). For each analogue, the amino acids (standard 3- and 4-letter codes) at each of the seven positions are shown below the structure. Amino acids common to all the immunogen components, and to the plate coating antigen, are shown in italic text.

cyanoborohydride solution was added and the reaction mixture stored overnight at 4 °C. The immunogen was washed through multiple centrifugations with PBS in a Vivaspin 2.0 mL concentrator with 10 000 MW cutoff (Sartorius Stedim Biotech GmbH, Goettingen, Germany) to remove unbound microcystins and excess reagents. The immunogen (MC-mix−cBSA) was aliquotted, lyophilized, and stored at −20 °C.

Plate-Coating Antigen. [Asp[3]]MC-RY (**7**) (100 μg) in MeOH (400 μL) was added to carbonate buffer (pH 9.7, 100 μL, 0.2 M), followed by a suspension of 2-HTP (91 μg) in THF−MeOH (10:1), and the mixture was allowed to react at ambient temperature. After 16 h, carbonate buffer (200 μL) was added and the reaction held at 40 °C, and then a further 200 μL of buffer was added 24 h after that. The hapten was separated from excess 2-HTP and unreacted **7** by SPE (Hypersep C18, 500 mg, 3 mL; Thermo Scientific) with a step-gradient of MeOH in water (10, 20, 30, 40, 50, 60, and 80%, 5 mL per step). LC-MS showed the hapten in the 40% MeOH fraction, which was evaporated under a stream of nitrogen, dissolved in MeOH−water (1:1, 100 μL), and PBS (100 μL) was added. This solution was mixed with 10 mg of OVA in 800 μL of PBS. $NaBH_3CN$ (5 mg in 100 μL water) was added to form the plate-coating antigen (OVA−[Asp[3]]MC-RY, Figure 2), which was washed, aliquotted, lyophilized and stored as for the immunogen.

LC-MS. LC-MS^2 analysis was as described previously.[50] Quantitation was performed in scan mode (*m*/*z* 900−1200) relative to the most appropriate standard (e.g., MC-LR for microcystins containing a single Arg residue, etc.) using the $[M + H]^+$ ions. Confirmation of the identities of the microcystins and their 2-HTP-derivatives was obtained by examination of the compounds' MS^2 spectra as described elsewhere.[50]

Polyclonal Antibodies. Three sheep (Nor X breed) were immunized with MC-mix−cBSA. Immunogen was prepared as an oil-in-water emulsion by mixing antigen (200 μg in 0.6 mL)

and FIA (0.4 mL) in a sterile syringe and administered subcutaneously near the supramammary lymph node at 4-weekly intervals. Test bleeds (10 mL) were taken from the jugular vein 2 weeks after the second immunization. After the fourth and subsequent immunizations, bulk bleeds of up to 450 mL were collected in blood bags (Fenwal, Lake Zürich, IL) 2 weeks after immunization. The antisera were separated by centrifugation at 3000 rcf for 20 min (Jouan Br4i Centrifuge with S40 rotor, Jouan, Saint-Herblain, France), aliquotted and stored at −20 °C. Animal experiments were in accordance with the three R's, laws, and regulations and approved by The Norwegian Animal Research Authority.

ELISA. Maxisorp immunoplates were coated with plate-coating antigen (Figure 2) (in coating buffer, 100 μL/well) overnight at room temperature. After incubation, excess was removed by aspiration and the plates were washed four times with PBST and blocked for 1 h with 1% PVP in PBST (200 μL/well) and then washed twice with PBST.

Noncompetitive assays were used to estimate titers of the antisera giving maximal absorbance of 1.0. This was done by combining equal volumes (50 μL) of sample buffer (10% MeOH−PBST) and a dilution series of antisera in antibody buffer (1% PVP−PBST) in the wells and incubating for 1 h. After washing four times with PBST, bound antibody was detected by adding antisheep−HRP conjugate in antibody buffer (100 μL at 1:3000) for 2 h and then washing four times before addition of the HRP substrate (100 μL/well). After 15 min, the reaction was stopped by adding 50 μL of 10% H_2SO_4, and absorbances were measured at 450 nm using a plate reader (Wallac 1420 Victor^2 *multilabel counter*, Wallac, Turku, Finland). All incubations were performed at ca. 20 °C.

Competitive ELISAs were performed (as above) by adding appropriate amounts of standard or sample and antiserum to the wells after blocking. Standards in methanol, usually MC-LR (500 ng/mL), were diluted in PBST to give a methanol concentration of 10%, and then in a threefold dilution series in sample buffer, giving standard concentrations of 50, 16.7, 5.56, 0.62, 0.20, 0.069, 0.023, 0.0076, and 0.0025 ng/mL. All sample and standard dilutions were done in duplicate. The remaining ELISA steps were done as above for the noncompetitive ELISA.

Optimal concentrations of plate-coating antigen (2 μg/mL), antiserum 80289 (1:6500), and antisheep−HRP (1:3000) were determined by checkerboard titrations followed by optimization of the standard curve. Assay standard curves were calculated using logistic treatment of the data. The assay working range was defined as the linear region at 20−80% of maximal absorbance (A_{max}).

Cross-Reactivity. The relative sensitivity of the immunoassay toward the available MCs and NOD-R was determined by assaying a dilution series of each compound in sample buffer. The I_{50} values (molar concentration giving 50% inhibition) were expressed as a percent I_{50} relative to the MC-LR CRM.

Matrix Effects. Tap water (Oslo, Norway) and non-microcystin-producing *M. aeruginosa* cultures (NIVA-CYA 841, NIVA-CYA 843, NIVA-CYA 845, NIVA CYA 848, and NIVA-CYA 849) were analyzed in duplicate in dilution series. Initial studies indicated a matrix effect suppressing the signal in the first dilution. This was investigated by running a non-competitive ELISA with dilution series of tap water samples and measuring the effect on A_{max} of addition of MeOH, PBS, and/or Tween 20. Based on this, 10-times concentrated PBST (45 μL) and 50 μL MeOH were added to samples (405 μL) of water and algal cultures, so that the ionic strength, pH, Tween 20, and MeOH concentrations were similar to those in the sample buffer. Consequently, the initial sample dilution is 1.2-fold, which is corrected for during data processing.

Validation. The optimized assay was validated by spiking MC-LR CRM into tap water and a non-MC-producing *M. aeruginosa* culture (NIVA-CYA 845). The tap water and culture samples were freeze−thawed twice, and to aliquots (4.05 mL) of each were added the following: MC-LR (0, 10, 25, 50, or 100 μL; 50 μg/L in MeOH); MeOH (500, 490, 475, 450, or 400 μL); and 10-times concentrated PBST (450 μL). Each spiked sample thus contained 4.05 mL of culture or water, 0.5 mL of MeOH, and 0.45 mL of conc. PBST, giving a total volume 5.0 mL with MC-LR at 0, 0.1, 0.25, 0.5, and 1.0 μg/L. Aliquots of the spiked samples were frozen and stored at −20 °C until analysis. Each sample was assayed in eight replicates per plate. The total number of plates was seven, with the assay performed on five different days for the tap water, and with six plates on four different days for the algal culture. Recoveries were estimated by comparing the ELISA results with the calculated MC-LR concentrations.

Intra-assay variation was determined from variation of the mean MC-LR concentration of each of the eight replicates for the four different concentrations on one plate. The interassay (interplate) variation was determined from variation of the mean MC-LR concentrations on each plate over 5 days for the tap water and 4 days for the spiked cultures.

Analysis of Cultures. Eleven *M. aeruginosa* cultures (NIVA-CYA 840−849 and AB2013 07) were freeze−thawed twice and ultrasonicated (Branson 3200, Soest, Netherlands) for 10 min. Samples were analyzed directly by ELISA (starting with a 1.2-fold dilution with 10-times concentrated PBS followed by a threefold dilution series with sample buffer). Aliquots of the ultrasonicated cultures were also diluted with an equal volume of MeOH and filtered (Spin-X 0.22 μm nylon, Costar, Corning, NY), and the filtrates were analyzed by both ELISA (starting with a 10-fold dilution to adjust for MeOH in the assay) and LC-MS.

RESULTS AND DISCUSSION

Hapten and Conjugation. Deciding which substructures within the analyte should be recognized by the antibodies, and which should not be recognized, is a critical step in designing hapten chemistry to generate an immunoassay with appropriate specificity. Just as important, in the case of microcystins with numerous variants with modifications in many positions, is it to balance the preference of the antibodies for certain groups/amino acids in order to achieve uniform cross-reactivity.

We wished to produce antibodies with broad cross-reactivity to microcystins and, if possible, nodularins, while avoiding a strong preference for particular amino acids, such as Adda. We therefore aimed to produce antibodies recognizing the general cyclic peptide structure of microcystins, but without a high degree of specificity for particular amino acid side-chains present at positions 2−4 and 7, which are the most variable in MCs.

Immunogen. Therefore, we applied a multihapten strategy using an immunogen containing microcystins with a variety of amino acids at position-2 (Leu, Tyr, and Trp) and -4 (Ala, Phe, Trp, and Arg). MC-LA, -LF, -LW, -YR, and -WR were selected for the immunogen (Figure 2), in the hope that the immune response would be directed to the common structural elements shared by these congeners.

Table 1. Molar Cross-Reactivities of Antiserum 80289-5b with a Series of Microcystin Analogues

compound	origin	quantitation	n	assay range (pg/mL)[a] I_{20}	I_{80}	I_{50}	MW	I_{50} (nM)	% CR[b]
MC-LR[c]	NRC	NMR/CLND	15	40	882	187	994.5	189	100
MC-RR[c]	NRC	NMR/CLND	4	54	1089	243	1037.5	234	80
NOD-R[c]	NRC	NMR/CLND	3	42	1122	216	824.9	261	72
[Dha7]MC-LR[c]	NRC	NMR/CLND	3	44	905	200	980.5	204	92
MC-LA	NRC[d]	CLND	4	56	1141	252	909.5	277	68
MC-LF	NRC[e]	as per label	4	43	811	187	1044.5	189	100
MC-YR	NRC[d]	as per label	3	89	1570	372	985.5	356	53
MC-LR	Enzo	as per label	15	44	899	198	994.5	199	95
MC-RR	Enzo	as per label	3	115	1942	462	1037.5	445	42
NOD-R	Enzo	as per label	4	52	1388	269	824.9	326	58
MC-YR	Enzo	as per label	3	133	1755	476	1044.5	456	41
MC-LA	Enzo	as per label	4	92	1810	404	909.5	444	42
MC-LF	Enzo	as per label	3	78	1172	299	985.5	304	63
MC-LY	Enzo	as per label	3	114	1499	411	1001.5	411	46
MC-LW	Enzo	as per label	3	51	863	209	1024.5	204	93
[Dha7]MC-LR	AgResearch	as per label	4	98	2071	449	980.5	457	41
MC-LR	Abraxis	as per label	2	62	975	239	994.5	240	79
[Dhb7]MC-RR	Cyano	as per label	3	86	1869	396	1023.5	387	49

[a] I_{20}, I_{50}, I_{80} are the concentrations of analogue giving 20, 50 and 80% inhibition, respectively, of binding of antibody to the coating antigen (OVA–[Asp3]MC-RY). [b] % CR = 100 × (I_{50} MC-LR CRM)/(I_{50} analogue). Interassay variation (CV) was 4–24% for I_{50}-values based on 2–15 competition curves. [c] Certified reference material (CRM). [d] Originally from Sigma–Aldrich. [e] Originally from Enzo Life Sciences Inc.

The antibodies' ability to recognize the large variety of microcystins depends on the choices made about the orientation of the hapten on the immunogen and on the plate-coating antigen. There are three approaches for conjugation of microcystins to carrier proteins: (a) through the carboxylic acids in positions 3 and/or 6 using carbodiimides,[24,25,28,30,33,35] (b) through coupling using glutaraldehyde,[34,37] or (c) through the double bond in position-7 using aminoethanethiol.[27,29,31,36,38,39]

We chose thiol derivatization at position-7 with 2-HTP, a cyclic thiohemiacetal. In hydroxylic solvents, 2-HTP exists in equilibrium with its ring-opened thioaldehyde form, and, in weak base, the thiolate reacts readily with activated double bonds via Michael addition (Scheme S1, Supporting Information) to introduce a short side-chain containing an aldehyde.[53] Reactions of 2-HTP with microcystins were monitored by LC-MS analysis and were terminated when the reactions were >95% complete. Aldehydes react efficiently with amino groups on proteins via reductive amination in the presence of sodium cyanoborohydride,[51] and this procedure was used for coupling the 2-HTP-derivatized microcystins to the carrier proteins (Scheme S1, Supporting Information).

Plate-Coating Antigen. For small molecules such as microcystins, ELISA format options are limited to competitive assays, where the antibody can bind either to the microcystin on the plate-coating antigen or to free microcystins from the sample. Because the assay is based on competition between the plate-coating antigen and the analyte for the antibody's binding sites, the plate-coating chemistry is important when developing a competitive immunoassay, especially with polyclonal antisera. To complement our mixed-immunogen approach, we wished to use a plate-coating antigen made from a hapten that shared none of the amino acids present in the "variable" positions 2–4 in the haptens of the mixed immunogen (Figure 2). Few such microcystins are commercially available; however, [Asp3]MC-RY was conveniently isolated from *M. aeruginosa* culture (NIVA-CYA 548)[50] and conjugated to ovalbumin (Scheme S1, Supporting Information) (Figure 2). Thus, antibodies in the antiserum that are capable of binding to the plate-coating antigen would be expected to be those with a strong affinity for microcystin-like cyclic peptides, but without a strong preference for particular amino acid side-chains in positions 2–4. Furthermore, the site of conjugation of all the haptens is position-7, (Figure 2), which is proximal to the carrier protein and therefore unlikely to generate antibodies directed toward it. Thus, the immunogen/plate-coating antigen combination used to develop this assay was expected to lead to an assay recognizing MCs, but without a strong preference for which amino acids are present at position-2, -3, -4, and -7. As these are the four most common sites of variation in the known MCs,[18] it was hoped that the resulting ELISA would possess broad specificity toward MCs and might even detect NODs.

Antibodies. Microcystin-specific antibodies were detectable by indirect ELISA in antisera from all three sheep after the first booster injection. Antiserum titers were determined by noncompetitive ELISA. Antiserum from sheep 80289 gave the highest antibody titer and was usable at 1:6500 dilution after five immunizations. The titer increased with increasing concentrations of plate-coating antigen for sheep 80289 and 80206, but not for 90164. Screening for competition was performed with MC-LR, and 50% inhibition was obtained at 258 and 295 pg/mL for sheep 80289 and 80206, respectively, in the unoptimized assay. Based on standard curves with a range of microcystins, assay development proceeded using serum from the fifth bleed from sheep 80289 (80289-5b).

Unoptimized ELISA analyses showed that antibodies in the antiserum recognized OVA–[Asp3]MC-RY and that binding was inhibited in a concentration-dependent manner by MC-LR (Figure S2, Supporting Information). This indicated that the antibodies produced by immunization with the MC-mixture recognized both [Asp3]MC-RY (on the plate coating antigen) and MC-LR and might have the desired cross-reactivity characteristics.

ELISA Optimization. In a sensitive ELISA, the assay conditions, such as the concentration of reagents, the blocking agent, temperature, etc. need to be optimized. Criteria used to evaluate optimization were maximum absorbance, working range (I_{20}–I_{80}), I_{50}, slope of the curve, and limit of quantification (I_{20} based on a mean of several assays and multiplied by the dilution factor (i.e., 1.2 for water and culture samples and 10 for MeOH-extracts)). Optimal concentrations of assay reagents were determined by checkerboard titrations and standard curves. Based on the means from 13 assays using the MC-LR CRM, the optimized assay had a working range (I_{20}–I_{80}) of 40–882 pg/mL, and the I_{50} was 187 pg/mL.

Specificity. The response of the optimized assay to MC-LR, MC-RR, MC-LA, MC-LF, MC-YR, [Dha^7]MC-LR, MC-LY (**11**), MC-LW, [Dhb^7]MC-RR (**12**) (Figure 1), and NOD-R (Figure S1, Supporting Information) was tested over a range of concentrations to determine cross-reactivity (Table 1 and Figure S2, Supporting Information). All microcystin standards caused concentration-dependent inhibition of antibody binding. Based on I_{50} values, the molar cross-reactivities relative to MC-LR were 68–100% for the RM/CRMs of microcystins and nodularin and 41–100% for all microcystins tested (Table 1). The antibodies in the assay therefore have a similar affinity for these other microcystins and nodularin as they do for MC-LR. Because they were raised against whole MC-congeners, rather than an Adda-containing fragment, we expect that the antibodies would not cross-react strongly with Adda itself but were not able to obtain a standard of Adda to test this. However, the assay responded to [ADMAdda^5]- (**14–16**) and [DMAdda^5]-microcystins (**17–19**) in the *Nostoc* 152 extract and its hydrolysate, indicating cross-reactivity toward MCs containing modified Adda-analogues, although the degree of cross-reactivity could not be measured due to the unavailability of pure quantitative standards. NOD-R differs from MC-LR only in the absence of the Ala^1 and Leu^2 (present in MC-LR) and methyl substitution of the olefinic group at position-7 (Mdhb instead of Mdha). Thus, the minimal reduction in cross-reactivity toward NOD-R (72%), relative to MC-LR, confirms that the antibodies in the assay do not have a strong requirement for the amino acids at positions-1, -2, and -7. The high cross-reactivities of [Dha^7]MC-LR (92%) and MC-LA (68%) also indicate that the *N*-methyl group at position-7 and the amino acid at position-4 have little effect on antibody recognition. That the competitive ELISA format relies on inhibition of binding of antibody to the [Asp^3]MC-RY-containing plate coating antigen, which differs at positions-2, -3, and -4 from both MC-LR and from all the congeners used in the immunogen, suggests the assay will detect MCs with variants at these positions. These results indicate that good cross-reactivity can be expected for MC congeners varying at position-7, -1, -2, -3, or -4, which covers ca. 80% of the MC congeners reported in the literature,[18] with some cross-reactivity (not yet quantified) for congeners varying at position-5. The ELISA therefore appears to be capable of detecting over 90% of the reported MC congeners. It should be noted, however, that although the assay estimates the total content of MCs, this does not necessarily reflect the toxicity of the sample because the congeners' structures affect their toxicities. In addition to free MCs, the ELISA can also be expected to cross-react strongly with conjugated MCs—especially those formed via conjugation of cysteinyl residues to position-7 (e.g., cysteine and glutathione conjugates).

Standards. The availability of reliable standards is important during development and validation of analytical methods. One obstacle in this study was the relatively limited array of MC analogues for cross-reactivity studies. Another problem was the apparent quantitative variation in the commercial standards supplied, relative to the RM/CRMs from NRC. This resulted in measured cross-reactivities (Table 1) that were as little as one-half of those obtained with the CRMs from NRC, presumably due to differences in concentration. This interstandard variation made it difficult to establish true values for cross-reactivities for analogues for which no independently quantitated standards were available. Availability of good standards and reference materials is important for all analytical methods, and the criteria for what constitutes a good standard may vary between analytical methods. For example, standards adequate for LC-MS may contain contaminating analogues which do not interfere with quantitation due to their differences in mass and retention time. However, for antibody- and protein-phosphatase-based detection, contaminating microcystin analogues can affect the response. And, even for highly pure standards, all analytical methods require standards to be quantitatively accurate in order to produce reliable results. Therefore, cross-reactivity data in this and other studies should be regarded only as indicative, except where high-purity quantitatively verified standards have been used.

Matrix Effects. Tap water gave matrix effects when undiluted, seen as a suppressed signal. Suspecting that this was caused by the differences in ionic strength, pH, and Tween content between the sample (fresh water) and the assay buffer, we tested addition of the assay-buffer components (MeOH, PBS, and/or Tween 20) to the sample such that their concentration in the sample was the same as in the assay buffer. This is illustrated in Figure S3 (Supporting Information) where A_{max}-values (relative to sample buffer) from the treatments are shown after a range of dilutions. Addition of PBST and MeOH, such that the concentration of MeOH, PBS, and Tween 20 in the sample was the same as in the sample buffer, completely abolished matrix effects without dilution being required. Thus, to analyze samples of fresh water and algal cultures with minimal dilution, we adjusted for the lack of salt, buffer, and Tween-20 as described in Materials and Methods.

Validation. The optimized ELISA was validated by spiking MC-LR into samples of tap water and the nonmicrocystin-producing *M. aeruginosa* culture NIVA-CYA 845. The samples were spiked with four concentrations of MC-LR (Table 2), with the content of PBS and Tween adjusted as described above. Recoveries were determined by comparing the ELISA results with the calculated MC-LR concentrations.

The measured concentrations of MC in spiked water were 91–120% (Table 2) of the calculated concentrations of MC-LR, with a mean recovery of 105%, while the measured concentrations in the spiked culture samples were 87–106%, with a mean recovery of 99% (Table 2). Both recoveries are well within the acceptable limits for assay recovery (100% ± 20). The mean interassay coefficient of variation was 9.5%, while the mean intraassay coefficient of variation was 15% for the spiked cultures.

Analysis of Cyanobacterial Cultures. To confirm its applicability to real samples, the ELISA used to analyze MCs in 11 cyanobacterial cultures (Figure 3 and Table S7, Supporting Information). These cultures were also analyzed by LC-MS for

Table 2. ELISA Analysis of Tap Water and *Microcystis* Culture NIVA-CYA 845 Spiked with MC-LR

	concentration (μg/L)				
matrix	MC-LR added	MCs by ELISA	%	n^a	CV (%)
tap water	0	0.01	–	7	58[c]
tap water	0.1	0.12	120	7	18
tap water	0.25	0.25	100	7	13
tap water	0.5	0.54	108	7	12
tap water	1[b]	0.91	91	7	15
culture	0	0.01	–	6	73[c]
culture	0.1	0.10	100	6	10
culture	0.25	0.22	87	6	17
culture	0.5	0.51	103	6	5
culture	1[b]	1.06	106	6	6

[a]Number of separate assays run. [b]Maximum level in drinking water as recommended by WHO.[19,20] [c]Measured percentage CVs for microcystin-free samples are high because the concentrations are below the LOQ.

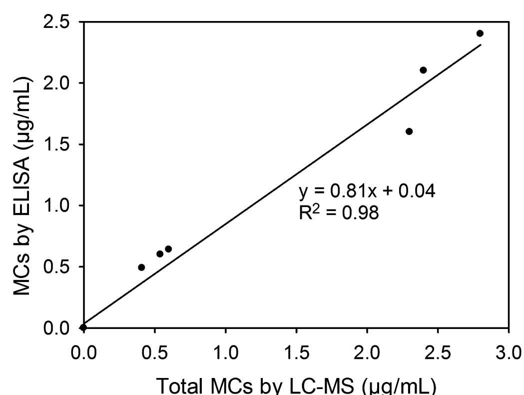


Figure 3. Total microcystins by ELISA and LC-MS for 11 *M. aeruginosa* cultures (ELISA results of cultures compared to LC-MS on culture extracts). Details in Table S7, Supporting Information.

comparison. Cultures of NIVA-CYA 844, 845, 848, and 849 were negative by both methods. Very low levels of microcystins were detected in NIVA-CYA 841, 842, and 843 by ELISA (0.20, 0.36, and 0.07 ng/mL) when analyzed directly, whereas the 50% MeOH extracts (which necessitate starting at a 10-fold dilution for the ELISA due to the MeOH content) of these cultures were negative by both ELISA and LC-MS. Cultures of NIVA-CYA 840, 846, and 847 and of strain AB2013-07 were strongly positive and contained similar amounts of MCs by both methods (Figure 3). By LC-MS, NIVA-CYA 840 was dominated by [Asp^3]MC-LR and [Asp^3,Dha^7]MC-LR, NIVA-CYA 846, and NIVA-CYA 847 by [Asp^3]MC-RY and [Asp^3]MC-LY, and AB2013-07 by MC-LR, MC-YR, and MC-LA. The small differences between the LC-MS and ELISA results may be due to differences in the standards (the LC-MS method did not use the CRMs), cross-reactivities in the ELISA, matrix effects, and variation in interanalogue response factors in the LC-MS. Indeed, the data in Table 1 suggest that some commercial microcystin standards may contain significantly less toxin than stated on the label, and use of such standards in the LC-MS would lead to overestimation of the microcystin content of samples. Nevertheless, the LC-MS and ELISA analyses parallel each other closely (slope 0.8, R^2 0.98; Figure 3), despite major differences in microcystin profiles (Tables S1−S6 of the Supporting Information) in these cultures. This, together with the absence of false positives in the non-microcystin-producing cultures, confirms the broad cross-reactivity of the ELISA and its utility for analysis of naturally contaminated samples.

In summary, we report development of an ELISA with broad specificity for MCs and NODs, so that the total content of analogues and metabolites can be measured. Suitable antibodies were obtained using an immunogen containing a carefully chosen mixture of MCs, and the antiserum, when used with a plate-coating antigen containing a microcystin with complementary structural elements, resulted in an ELISA with exceptional sensitivity and broad specificity. The reagents should be suitable for the development of other immunoassay formats, including biosensors, and the broad specificity of the antibodies makes them suitable for immunoaffinity cleanup and preconcentration prior to HPLC or LC-MS analysis. The multihapten approach used here may be useful for other analyte families where broad, but high specificity is required for a large group of structurally related congeners.

This ELISA provides a sensitive and rapid analytical method that uses inexpensive instrumentation for analyzing MCs in water samples. Although the cross-reactivities for MC congeners tested in the assay were 42−100%, some of this variability appears to be due to the quality of the available standards. It is therefore highly desirable that a wider range of independently quantitated—and, preferably, certified—microcystin standards become available to facilitate validation of chromatographic, immunoassay, and bioassay methods for this important group of cyanobacterial toxins.

■ ASSOCIATED CONTENT

Supporting Information

Structure of NOD-R, ELISA inhibition curves, ELISA matrix effects, scheme showing the conjugation reaction using 2-HTP, table of cyanobacterial cultures tested by ELISA and LC-MS, their MC-profiles established by LC-MS, and detailed instructions for running the ELISA. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*Phone +47 2321 6226; fax +47 2321 6201; e-mail ingunn.samdal@vetinst.no.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

2-HTP	2-hydroxythiepan
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
ADMAdda	9-acetoxyDMAdda
cBSA	cationized bovine serum albumin
CLND	chemiluminescence nitrogen detector
CRM	certified reference material
DMAdda	9-desmethylAdda
ELISA	enzyme-linked immunosorbent assay
FIA	Freund's incomplete adjuvant
HRP	horseradish peroxidase
MC	microcystin
NOD	nodularin
NRC	National Research Council of Canada
OVA	ovalbumin
PBS	phosphate-buffered saline
PBST	PBS with 0.05% Tween 20
PVP	poly(vinylpyrrolidone) 25
RM	reference material
SPE	solid-phase extraction

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