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Evaluation of an ELISA Kit for the Monitoring of Microcystins (Cyanobacterial Toxins) in Water and Algae Environmental Samples

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A recently developed commercial microtiter plate ELISA test kit for microcystins was evaluated for its reproducibility, accuracy, detection limits in real samples, and comparability to results obtained from solid-phase extraction followed by liquid chromatography. Detection limits in the deionized water matrix were 0.05 μg/L, and the overall intra- (two to five replicates) and interkit (three replicates) reproducibility at this level was good (%CV < 10%). Various types of groundwater and surface water samples gave a matrix effect at low concentration levels so that limits of detection were obtained in the range 0.1−0.15 μg/L with the possibility of obtaining a false positive in this range. The limits of quantification were measured at 0.2 μg/L in several types of surface water samples. A fast and simple enrichment step using disposable C18 cartridges allows lower detection limits and is recommended in order to avoid a false positive. No false negative measurements were detected. Reliable correlations between measurements obtained by ELISA and by solid-phase extraction followed by liquid chromatography were obtained in spiked drinking and surface water samples ($n = 8$, $r^2 = 0.989$). In algae samples, the occurrence of several microcystins which may cross-react was shown using mass spectrometry but could not be confirmed due to the lack of commercially available standards. Overall, the assay illustrated the ability to measure concentrations of microcystins-LR and -YR in the range 0.2−4 μg/L in any type of surface water, allowing thus rapid and on-site detection of toxins in cyanobacterial blooms without any pretreatment.

Introduction

Water blooms of toxic cyanobacteria (blue-green algae) are commonly encountered in freshwater and represent an increasing environmental hazard because many strains of cyanobacteria produce toxins, one major group being cyclic heptapeptides named microcystins (MCs) (*1*−*3*). Rapid on-site and reliable analytical methods are required in case of cyanobacterial blooms, since evidence exists for the adverse effects of cyanobacterial toxins for animal and human health (*4*−*9*). Microcystins are a group of cyclic heptapeptides produced by bloom-forming cyanobacteria such as *Microcystis aeruginosa* (*M. aeruginosa*) and over 50 MCs have been isolated so far, with only three being commercially available. One variant of microcystins, microcystin-LR (leucine-arginine) was found to inhibit phosphatase 1 and 2A and to exhibit tumor-promoting activity in rats (*8*). In drinking water, maximal values of 0.1 μg/L for a long-term exposure and 1 μg/L for a short-term exposure have been proposed on the basis of laboratory experiments of toxicity on mice and pigs (*10, 11*).

Current analytical methods for the determination of MCs require an extraction and cleanup procedure followed by measurements using liquid chromatography (LC) (*12*−*19*). While these methods have detection limits as low as 0.1 μg/L in drinking and surface water, they are time-consuming and expensive. Over the past decade, enzyme-linked immunosorbent assays (ELISAs) have been developed for the determination of environmental pollutants. They have been shown to be sensitive, inexpensive, fast, highly specific, portable, simple, easy-to-use, and appropriate for on-site analysis (*20, 21*). Moreover, no sample cleanup is required, and detection limits for many pesticides in surface water are often below the 0.1 μg/L level. ELISAs are particularly well-adapted to environmental monitoring and fate studies, such as aquatic and terrestrial field dissipation, surface water transport, and ground and runoff studies. Validation studies have demonstrated that data generated by ELISAs were comparable to those generated by traditional analytical methods for some targeted pesticides (*20*−*25*). Quality assurance and guidelines for their standardization and validation have been made by several agencies such as the U.S. Environmental Protection Agency (EPA), the AOAC, the U.S. Analytical Environmental Immunochemical Consortium (AEIC), and the German Immunoassay Study Group (*20, 21, 26, 27*).

To date, a few studies have reported the production of antibodies against MCs and their application in laboratory-made ELISAs (*17, 28*−*33*). The first ELISA reported by Chu et al. (*30*) involved coating anti-MC-LR antibodies and showed a working range of 0.5 to 10.0 μg/L, with a minimum detection level of 0.2 μg/L in water. The matrix effect was only studied with tap water spiked at the 1 μg/L level. An and Carmichael (*17*) used anti-MC-LR polyclonal antibodies and obtained an ELISA kit with a working range of 0.5−50 μg/L. The most sensitive was recently described by Nagata et al., with a working range of 0.02−0.5 μg/L using monoclonal anti-MC-LR antibodies (*32*). All these laboratory-made ELISAs have observed high cross-reactivity with MCs other than the MC-LR variant. They have been used for the determination of MCs in surface water where blooms had occurred, and reported concentrations have been in the range 0.1−3000 μg/L with very few confirmation studies (*30, 32*).

Recently, a microtiter plate ELISA kit became commercially available. Its working range between 0.1 and 1.6 μg/L in water makes it appropriate for water monitoring without any sample pretreatment. No report documenting its performance exists in the literature. This work reports here the evaluation of this kit with emphasis on detection limits and matrix effects when applied to various surface water samples and algae samples. Since the calibrator provided with the kit is a nontoxic MC-LR variant, comparison was established between standard curves using the calibrators and the commercial MCs. Comparison is also given with

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results obtained using solid-phase extraction followed by LC.

Procedures

Chemicals. Microcystin-LR and -RR standards were purchased from Sigma (Saint Quentin Fallavier, France); microcystin-YR standard was from Calbiochem Novabiochem (La Jolla, CA). ELISA EnviroGard Microcystins Plate kits were obtained from Rhône Diagnostics Technologies (French distributor of Millipore, Lyon, France). Concentrated standard solutions of each toxin (0.5 mg/L to μg/mL) were prepared in methanol. Phosphate-buffer saline (PBS) was 0.05 M phosphate buffer with 0.10 g/L NaCl at pH = 7.5.

All other reagents were of HPLC grade or analytical reagent grade. Methanol was purchased from Prolabo (Fontenay-sous-Bois, France) and acetonitrile from J. T. Baker (Deventer, The Netherlands). LC-quality water was obtained by purifying demineralized water by a Milli-Q filtration system (Millipore, Saint Quentin en Yvelines, France).

Instrumentation. The instrumentation for microLC consisted of two LC-10AS pumps (Shimadzu, Kyoto, Japan) connected to an Accurate 1/70 microflow splitter (LC Packings, Amsterdam, The Netherlands). The outlet was linked to a four-port Valco CIAW valve (VICI, Valco Europe, Switzerland) with an internal loop of 200 nL or 1 μL. UV detection was performed either with a SPD-10A detector (Shimadzu) equipped with a U-shaped microcell with an internal volume of 35 nL (LC Packings) or with a SPD-M10A photodiode array detector (Shimadzu) equipped with a microcell (prototype LC Packings).

The instrumentation for classical LC consisted of a Varian Vista 5500 liquid chromatograph equipped with a UV-200 detector or a 9065 Polychrom diode-array detector (Varian, Palo Alto, CA). The analytical column was connected to a Rheodyne (Cotati, CA) valve. The injection volume was 20 μL.

MicroLC-ES-MS experiments were carried out on a benchtop HP1100 series LC/MSD setup from Hewlett-Packard (Waldbronn, Germany) which was equipped with a dual air-cooled turbomolecular pump vacuum system and incorporates a hinged swing-out spray chamber enabling atmospheric pressure electrospray (ES).

Analytical microLC and LC separations were performed using a 25 cm × 0.32 mm i.d. column packed with 5 μm reversed-phase BioSil C_{18} (Biorad, Hercules, CA) or a 25 cm × 1 mm i.d. and a 25 cm × 4.6 mm i.d. packed with 5 μm reversed-phase Hypersil BDS C_{18} (Life Science, Eragny, France).

Off-line solid-phase extraction was performed on 3 mL Bakerbond SPE C_{18} cartridges packed with 500 mg of octadecyl silica.

The optical density of the ELISA assay was measured using a Ceres 900C BioTek (Osi, Maurepas, France) microplate reader.

Water and Algae Samples. Drinking water 1 (DW1) comes from groundwater and is distributed without treatment except for slight chlorination in order to avoid further contamination in the distribution network. Its mineral composition is, in mg/L, as follows: calcium, 89; magnesium, 4; bicarbonates, 207; sulfates, 27; chlorites, 35; nitrates, 18. Drinking water 2 (DW2) is a drinking water originating from surface water (reservoir A) after conventional chemical treatment. Surface water samples were collected in rivers and reservoirs from various areas of France, to be representative of various compositions of the matrix (limestone area, sandy area, etc.). Their pH stands in the range 7.3−7.9. Surface water 1 (SW1) originated from the Seine river (samples taken in Paris) and surface water 2 (SW2) from the Clain river (samples taken in Poitiers, France). Surface waters 3−5 (SW3, SW4, SW5) came from water reservoirs located in the west of France (respectively reservoirs A−C). All samples were filtered on a Whatman GF/C filter (pore size = 1.2 μm) and stored at 0 °C before analysis.

Cyanobacterial cultures were grown as described in ref *14* and prepared as follows. The cells were separated from the liquid medium by filtration through a GF/C filter. Cellular components were extracted with methanol. Toxins were isolated and concentrated from 200 mL of liquid medium using a solid-phase extraction on a Bakerbond SPE C_{18} cartridge. The activation of the cartridge, the cleanup, and the desorption of the toxins were performed as described above. Samples were analyzed by microLC and then diluted for the ELISA assay to get a toxin concentration in the working range. They were stored at −20 °C before ELISA analysis.

ELISA Procedure. The Envirogard test is a direct competitive enzyme immunoassay. The kit consists of a 96 well microtiter plate coated with anti-microcystin-LR antibodies which are immobilized to the walls of the test wells. It is calibrated with a nontoxic microcystin-LR surrogate at levels equivalent to 0.1, 0.4, and 1.6 μg/L microcystin-LR. Assays of standards or samples were performed following the kit instructions. Briefly, a 100 μL volume of the unknown sample, standard, calibrator, or negative control, is introduced into the well and incubated for 30 min at ambient temperature. A 100 μL aliquot of a microcystin−enzyme conjugate solution is then added and incubated for another 30 min at ambient temperature. The wells are emptied and washed four times with 300 μL of ultrapure water. A 100 μL aliquot of substrate is added to each well and incubated for 30 min at ambient temperature. The substrate is transformed by the enzyme conjugate into a blue compound. A 100 aliquot μL of a 1 N hydrochloric solution is added to stop the reaction, and the solutions turn yellow. The optical density or absorbance A is immediately recorded at 450 nm using the microplate reader. The spectrophotometer was blanked on air.

Standard Curves, Calculation of Microcystin Concentrations, Matrix Effects, and Cross-Reactivy Measurements. The standard curves were established by plotting the percent of the maximum absorbance versus the concentration of the nontoxic calibrator provided in the kit or the concentration of standard microcystin-LR, in log scale. For each run, the negative control and the three calibrators (0.1, 0.4, and 1.6 μg/L equivalent microcystin-LR) were assayed at least in duplicate. Standard calibration curves were drawn using commercial microcystin-LR. Diluted standard solutions (μg/L to μg/mL) were prepared in deionized water, PBS, or various water samples so that the methanol content did not exceed 0.5%. The measurements for the standard calibration curves with microcystin-LR were repeated two, three, or five times.

For an unknown solution, the concentration was directly estimated from standard curves drawn either with nontoxic calibrators, or with the standard solutions in deionized water, or in the matrix sample when specified in the text. The matrix effect was studied by constructing standard curves for drinking water, groundwater, and surface water samples and comparing with the standard calibration curves prepared for deionized water or for the calibrator solutions given with the kit to determine their parallelism. The recovery was determined by analyzing real samples before and after the addition of microcystin-LR and then subtracting the concentration of microcystin-LR present in the sample prior to spiking. Cross-reactivity values were determined by spiking surface water samples with known amounts of cross-reactants and calculated by the ratio of the spiked values and that read on the standard calibration curves.

Method for the Estimation of Reproducibility and Accuracy. To allow direct comparison of several standard curves, the ratio $B/B_o \times 100$, where B is the sample absorbance and B_o the absorbance of the negative control, was calculated and reported against the microcystin concentration on a

semilogarithmic scale. The %CV (coefficient of variation) was calculated as 100-fold of the ratio of the standard deviation to the mean. The reproducibility of the ELISA kit was measured by analyzing replicate samples ($n = 2-5$) with deionized and groundwater samples using the same kit. The %CV corresponding to the negative control was measured using optical density values. Comparison of the reproducibility between kits was made by comparing the $\%B/B_o$ values.

Statistical analysis of the absorbance results was performed using the software JMP (SAS Institute). A Barlett test or a Cochran test is employed to compare the relative standard deviations of several runs. The comparison of the mean data values is carried out using a Student test. The significance level for the Students t test was taken at 5%.

The accuracy of the ELISA kit was tested using various types of water and deionized water as test matrices containing different microcystin-LR concentrations in the working range.

Solid-Phase Extraction and ELISAs. Samples containing less than 0.1 μg/L microcystin-LR were concentrated using disposable Bakerbond SPE C_{18} cartridges. The adsorbent was activated with 5 mL of methanol and then washed with 10 mL of deionized water. A sample of 10 mL was preconcentrated on the cartridge, which was then cleaned with 5 mL of an aqueous solution containing 20% methanol (v/v), and the toxins were desorbed with 5 mL of methanol. After evaporation of the solvent, the residue was dissolved in 1 mL of deionized water containing 0.5% methanol.

LC Analysis. Off-line preconcentration was carried out with 500 mL samples using a C_{18} solid-phase extraction cartridge. The adsorbent was activated with 5 mL of methanol and then washed with 10 mL of deionized water before sample percolation. The cartridge was then cleaned with 5 mL of an aqueous solution containing 30% methanol (v/v), and the toxins were desorbed with 3 mL of methanol acidified with 1% (v/v) trifluoroacetic acid. The residue was evaporated to dryness at 45 °C under reduced pressure and dissolved in 50 or 100 μL of acetonitrile−phosphate buffer (20:80 (v/v)) acidified to pH 2 by addition of concentrated perchloric acid.

The classical LC system was employed for the analysis of groundwater and surface water using the Hypersil C_{18} column of 25 cm × 0.46 cm i.d. The mobile phase consisted of a mixture of solvent A (acetonitrile−phosphate buffer at pH 2.5 (25:75 (v/v)) and solvent B acetonitrile as follows: for groundwater, 0% B at 0 min, 17% B at 35 min, 30% B at 45 min, and 100% B at 50 min; for surface water, 0% B at 0 min, 12% B from 20 to 40 min, 20% B at 50 min, and 100% at 60 min.

MicroLC was used for the analysis in culture media and cellular extracts using a 25 cm × 0.32 cm i.d. C18 column with a mobile phase consisting of acetonitrile−5 mM phosphate buffer acidified to pH 2. The elution gradient employed solvent A made of acetonitrile−phosphate buffer (10:90 (v/v)) and solvent B made of acetonitrile−phosphate buffer (90:10 (v/v)) as follows: 10% B from 0 to 10 min, 28% B at 40 min, 33% B at 60 min, 45% B at 80 min, and 100% B at 90 min.

MicroLC-ES-MS experiments were performed using a 25 cm × 0.1 cm i.d. C18 column. The mobile phase was composed of a linear gradient from 30 to 60% acetonitrile in 40 min. The effluent at a 50 μL/min flow rate was introduced without any split in the ES setup. The ES-MS was used in the positive polarity mode.

Results and Discussion

Dose−Response Curves. Dose−response data with calibrators provided with the kit were collected from three calibrations performed during this study. The data have been reported in Figure 1 (as squares) using the nontoxic microcystin surrogate calibrators. The error bar (+1 SD) at each standard point (five replicates) corresponds to a coefficient of variation lower than 7%, showing thus a good reproducibility. The intra- and interassay reproducibilities were measured using three different commercial kits. Coefficients of variation were in the range 0.2−8.7%. The interassay reproducibility, calculated from $\%B/B_o$ values, was lower than 10%. The overall coefficient of variation corresponding to intra- and interassay dispersion was a little higher with a maximum of 10.9%. The 0.1 μg/L sample gave a 75 ± 5% B/B_o value which was shown to be significantly different from the zero analyte concentration, indicating thus a sensitivity at least equal to 0.1 μg/L when the standard curve is drawn with the calibrators. The concentration corresponding to 50% inhibition (IC_{50}) is 0.30 ± 0.05 μg/L. The values provided by Figure 1 are in agreement with those provided by the manufacturer (0.1 and 1.6 μg/L correspond to 78% B/B_o and 16% B/B_o values, respectively, with a 50% B/B_o value of 0.31 μg/L).

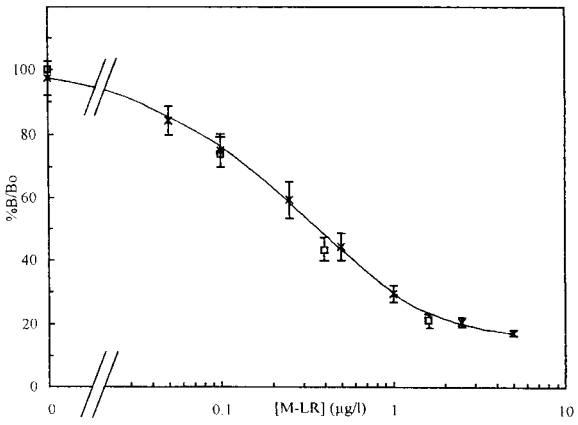

FIGURE 1. Comparison between the ELISA calibration curve obtained using deionized water spiked with the commercial MC-LR (×) and values (□) obtained with the three standard solutions provided with the kit (mean value from five replicates). The error bars represent ±1 standard deviation from the mean.

To study the matrix effect, which requires that the samples be spiked with known amounts of microcystin-LR, the dose−response curve obtained by spiking deionized water with microcystin-LR in the range of 0.05−5 μg/L has also been reported in Figure 1, showing thus a good agreement between the two curves. The value of 85 ± 5% B/B_o obtained for the 0.05 μg/L level is significantly different from the zero control so that, in deionized water, the detection limit is 0.05 μg/L. The working range is confirmed from 0.1 to 1.5 μg/L. Therefore, the dose−response curve drawn with the calibrators can be used for the determination of samples when spiking is achieved using the commercial microcystin-LR.

Reproducibility. Within kit and between kits repeatability was calculated on the results corresponding to the negative control and the three calibrator concentrations. The precision of the assay in standard conditions was estimated by the relative standard deviation using the A/A_o results. Two replicates per assay were run in the first and the second kits, and five in the third kit. At a fixed concentration level, according to the Bartlett test the dispersion of the absorbance data was shown to be similar for the three kits. The average dispersion (within kit RSD) ranged from 5 to 6.4%. The comparison of the within kit RSD values obtained for each concentration level, using the Cochran test, did not show any dependence of the within kit RSD with the concentration, and an average RSD of 5.5% could be calculated. For the concentration levels of 0 and 1.6 μg/L, the means do not differ significantly between the three kits, whereas, for the 0.1 and 0.4 μg/L levels, a dispersion between kits of 8 and 10% was found. Finally, reproducibility was in the range of 5−11%. The average total standard deviation was calculated

as 6.9%, which represents the error margin on one measurement performed with any kit, at any concentration level.

Accuracy and Matrix Effect for Various Aqueous Samples. To examine potential matrix interference, in addition to PBS, various matrices were studied which include several types of drinking and surface water. Matrix effects can be expected because, in the data sheet provided with the kit, it is recommended to always use a calibrator which has a matrix comparable to that of the sample. Spiked concentrations, the mean of the concentration obtained using calibration curves, and the corresponding standard and relative standard deviations, as well as recoveries calculated by the ratio of the spiked values and concentration given by ELISA measurements, have been reported in Table 1. Prior to ELISA analysis, each natural sample was analyzed by LC in order to verify that it did not contain any standard microcystins. Therefore, results of Table 1 can be assessed for accuracy of the ELISA measurements.

First, these values indicate the occurrence of interferences from the sample matrix at spiked concentrations lower than 0.25 μg/L for all samples including spiked solutions of PBS, as shown by corresponding recoveries higher than 130% for most of the samples. False positive values are obtained in drinking and surface water samples, since nonspiked samples give ELISA measurements in the range of 0.05−0.12 μg/L. However, it is worthwhile to note that no false negatives have been observed with these samples. For samples spiked in the range of 0.05−0.10 μg/L, if we exclude results obtained from spiked PBS, concentrations given by ELISA measurements range from 0.1 to 0.14 μg/L. In the range of 0.25−2.5 μg/L, the accuracy is good, taking into account the standard deviation, because the concentration is plotted on a logarithmic scale. Surprisingly, a strong matrix effect due to PBS is observed, which was verified for two different kits and which could be due to the high concentration of PBS that we used.

One important result was that the matrix effect obtained in surface water was not very different from that observed with drinking water samples. Figure 2 shows the calibration curves constructed with four surface water samples, and the solid line represents the calibration curve in deionized water. The absorbance deviation varies within the range of 1−15% according to the sample. The comparison of the RSD shows that they can be considered as homogeneous within each water sample and between the samples. A mean deviation of 7.5% was obtained. Owing to the log scale, the values obtained for reservoir B water seem to be very different from the standard curves, but spiked values join those of the standard values as soon as the spiked level is 0.2 μg/L. This figure clearly shows that the limit of quantification in surface water is around 0.15−0.20 μg/L, with a working range of 0.2−1.5 μg/L and a 50% B/B_0 concentration of 0.35 ± 0.08 μg/L. Reliable measurements can be performed between 0.2 and 1.5 μg/L, using the standard curve with recoveries in the range of 80−110%. As expected, higher concentration levels are subject to a larger uncertainty and recoveries for samples spiked at 2.5 μg/L are in the range 70−140%. But this uncertainty can be easily overcome by sample dilution. Since different types of surface waters coming from various areas of France have been selected, our results suggest that for concentration higher than 0.2 μg/L, it is not necessary to use a calibrator which has a matrix comparable to that of the sample. For river Seine samples, calibration curves were established on two different kits with Seine river water collected at two different periods and were shown to be similar. However, for the monitoring of water reservoirs or rivers likely to contain microcystins chronically, it can be suggested that a calibration curve be used, established in this matrix at a time when no cyanobacteria are present. But one has to be aware that values below 0.15−0.20 μg/L should be considered very carefully, even if this precaution is taken. In such a case, a preconcentration step is strongly recommended. Bovine serum albumin (BSA) water collected at two different periods was shown to be similar. However, for the monitoring of water reservoirs or rivers likely to chronically contain microcystins, it can be suggested to use a calibration curve established in this matrix at a time when no cyanobacteria are present. But one has to be aware that values below 0.15−0.20 μg/L should be considered very carefully, even if this precaution is taken. In such a case, a preconcentration step is strongly recommended.

Bovine serum albumin (BSA) was added to a Seine river sample at a concentration of 1 and 10 mg/mL but without any significant effect to eliminate part of the matrix effect.

TABLE 1. Matrix Effect in Drinking and Surface Water Samples: Agreement between Spiking Levels and Values Given by ELISA Measurements ($n = 2$) and Corresponding Recoveries[a]

water sample	spiked level (μg/L)	level using the std curve (μg/L)	std dev (μg/L)	recovery (%)
DWI	0	0.0	0.01	–
	0.05	0.05	0.01	90
	0.1	0.07	0 0.01	70
	0.25	0.18	0 01	71
	0.5	0.41	0.03	82
	1	0.75	0.04	75
	2.5	2.25	0.07	90
DW2	*0*	*0.05*	*0 0.1*	
	0.05	*0.1*	*0. 02*	*200*
	0.10	*0.14*	*0. 02*	*140*
	0.25	0.32	0.02	128
	0.5	0.55	0.05	110
	1	0.95	0.11	95
PBS solution	*0*	*0.12*	*0.09*	–
	0.1	*0.21*	*0.07*	*205*
	0.25	0.29	0.02	114
	0.5	0.48	0.04	95
	1	1.15	0.28	115
	2.5	2.95	0.07	118
Seine river (1)	0	0	0.01	–
	0.05	0.035	0.02	70
	0.1	0.07	0.02	67
	0.25	0.21	0.02	83
	0.5	0.41	0.11	81
	1	0.87	0.08	87
	2.5	2.28	0.32	91
Seine river (2)	0	0	0.02	–
	0.1	0.1	0.01	95
	0.5	0.44	0.09	88
Clain river	0	0.03	0.02	–
	0.05	0.04	0.03	74
	0.1	0.05	0.03	53
	0.25	0.24	0.04	94
	0.5	0.47	0.12	93
	1	0.84	0.06	84
	2.5	2.5	0.21	100
reservoir A	0	0.02	0.02	–
	0.05	0.06	0.02	119
	0.1	0.11	0.01	113
	0.25	0.24	0.02	94
	0.5	0.54	0.06	109
	1	0.94	0.05	94
	2.5	1.8	0.22	72
reservoir B	*0*	*0.12*		
	0.05	*0.12*	*0.02*	*250*
	0.1	*0.13*	*0.02*	*135*
	0.25	0.26	0.03	106
	0.5	0.50	0.06	100
	1	0.95	0.08	95
	2.5	3.5	0.16	140

[a] Values providing spiking recoveries above 130% have been reported in italics.

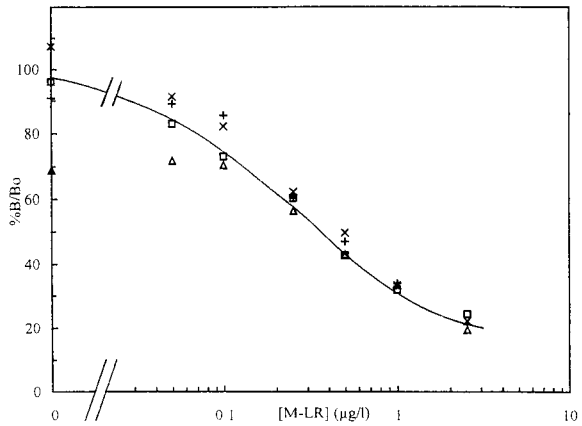

FIGURE 2. Matrix effect for surface water samples spiked with MC-LR: (×) Seine river; (+) Clain River; (□) reservoir A; (△) reservoir B. Mean of two replicates.

Effect of the Addition of Methanol. Since cyanobacterial extracts or environmental samples may contain a small percentage of methanol, the effect of 0.5 and 2.5% (v/v) methanol in nonspiked and spiked deionized and surface water samples was studied. A slight difference between the theoretical level and the experimental level was noticed, but it was not significant.

Cross-Reactivity. Cross-reactivity is expected, owing to the similarity of structures of the microcystins and derivatives which differs primarily in the two L-amino acids plus methylation and demethylation on the two unusual amino acids. The first sensitive ELISA kit made by Chu et al. (*30*) using polyclonal antibodies with high affinity to the microcystin-LR has shown high cross-reactivity with microcystin-RR and microcystin-YR variants, but less reactivity with variants microcystin-LY and microcystin-LA. An and Carmichael tested the cross-reactivity of a laboratory-made kit using anti-microcystin-LR polyclonal antibodies with 18 microcystins and nodularin (monocyclic pentapeptides with similar structure than those of the heptapeptide microcystines) variants (*17*). They have found that the hydrophobic amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) which has the (*E*) form at the C6 double bond was essential for these toxins to express antibody specificity. Strong cross-reactivity was obtained with a nontoxic (monoester of glutalic acid microcystin-LR) and methylated variants of microcystin-LR. Another laboratory-made kit using monoclonal anti-microcystin-LR antibodies was shown to cross-react with microcystin-RR and to a less extent to microcystin-YR (*33*).

According to the data sheet included within the kit under evaluation, the concentrations resulting in 50% B/B_0 are given as 0.32 μg/L for the RR variant and 0.38 μg/L for the YR variant, corresponding to CR_{50} values of 97 and 81%, respectively. No significant cross-reactivity with other MC variants is mentioned.

The cross-reactivity has been measured with Seine river water samples spiked with 0.7 μg/L of microcystin-YR or 0.3 μg/L microcystin-RR. At these levels, CR values 93 ± 15% for microcystin-YR and 73 ± 25% for microcystin-RR have been obtained. Therefore, since the values are measured in real samples and are not measured at 50% B/B_0 (but at 80 and 70% B/B_0), our experimental values are in good agreement with those provided by the manufacturer. The effect of these cross-reactants has been studied by spiking drinking and surface water samples with the three microcystins standards.

Gascon et al. have demonstrated that when cross-reactants are present in the sample, the concentration could be calculated as the sum of the products of the cross-reactivities by the concentration (*34*). A sample of Paris drinking water spiked with the three microcystin standards, each at 0.1 μg/L, and a sample of surface water containing the three microcystin standards, each at 0.5 μg/L, were analyzed using HPLC and the ELISA test. The first sample was assayed at 0.35 μg/L equivalent microcystin-LR, with a RSD of 13.4% on the absorbance ($n = 2$). Taking the cross-reactivities into account, the theoretical level is 0.28 μg/L equivalent microcystin-LR. The second sample was diluted three times in order to measure a concentration close to the IC_{50} of the test. The calculated concentration was then 0.48 μg/L equivalent microcystin-LR, and the experiment gave a concentration of 0.65 μg/L equivalent microcystin-LR.

TABLE 2. Effect of an Enrichment Step (Enrichment Factor of 10) Using Solid-Phase Extraction with a C_{18} Silica Disposable Cartridge for Samples Containing Less Than 0.1 μg/L of Microcystin-LR ($n = 2$ or 3)

		SPE-ELISA	
	spiked level (μg/L)	concn after enrichment (μg/L)	recovery (%)
deionized water	0	0 ± 0.02	
	0.05	0.40 + 0.15	80
DW1	0.04	0.36 + 0.02	90
Seine water	0	0.08 ± 0.05	
	0.04	0.60 ± 0.15	150

Sample Pretreatment. To remove the matrix effect and to obtained reliable values, a concentration step can be performed using solid-phase extraction (*35*−*38*). Such an enrichment was applied to samples containing less than 0.1 μg/L microcystin-LR. Deionized water, drinking water, and surface water samples were spiked with 0.04 or 0.05 μg/L microcystin-LR and were concentrated 10 times on a C18 SPE cartridge. Results are reported in Table 2. The dry residue corresponding to deionized water was dissolved in PBS, which explains the higher SD on the data compared to SD obtained with spiked drinking and surface water which were dissolved in deionized water. A blank of extraction was performed with deionized and surface water. The concentrations determined by ELISA were not statistically different from the zero concentration for nonspiked deionized water and gave a concentration of 0.08 ± 0.05 μg/L for the nonspiked river water sample, indicating thus that the matrix effect is not totally eliminated by the enrichment step. However, such a false positive result should correspond to a very low concentration of 0.01 μg/L in the original sample. For samples spiked with 0.04 or 0.05 μg/L spiked samples, the results are correct and recoveries of 80−150% were obtained. It should be noticed that recoveries have been calculated on theoretical values of the enriched extracts and that the enrichment step itself gives recoveries of 85−90%, which reduces the total recovery when it is taken into account. Therefore, such an enrichment procedure enables the detection of toxin in drinking water and in surface water at concentrations as low as 0.05 μg/L.

Comparison between SPE-LC and ELISA. Spiked drinking and surface water samples were analyzed using solid-phase extraction of 500 mL of water and liquid chromatography. An intermediary cleanup step was applied after percolation of samples through the C_{18} cartridge and before the desorption step by washing the packing bed with 5 mL of a water−methanol mixture containing 20% methanol (v/v). Figure 3 shows the chromatogram obtained with drinking water samples spiked with 0.1 μg/L each of the three commercial MCs. Detection limits are lower than the 0.1 μg/L level in drinking water. In surface water, the detection limits are slightly higher but still good, as shown by Figure 4a, which represents the LC chromatogram corresponding to the analysis of an extract of surface water spiked with 0.5 μg/L

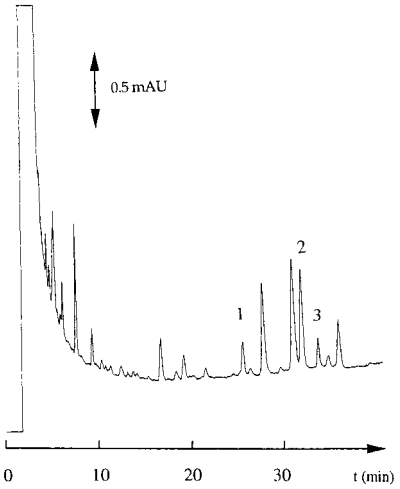

FIGURE 3. Chromatogram corresponding to the analysis of 500 mL of drinking water spiked with 0.1 μg/L of MC-RR (1), MC-YR (2), and MC-LR (3). See experiment discussion for off-line preconcentration and LC analysis.

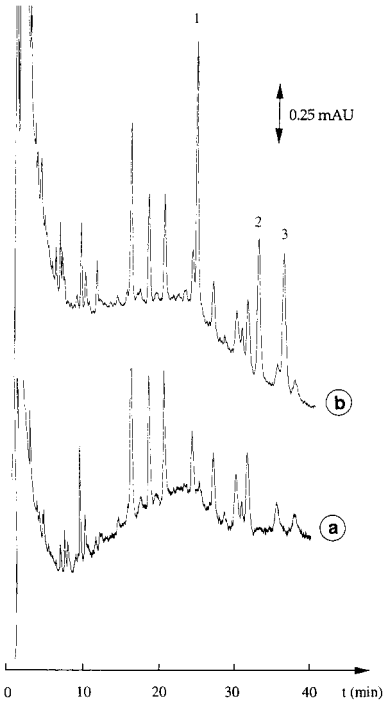

FIGURE 4. Chromatogram corresponding to the analysis of 500 mL of surface water from reservoir B, nonspiked (a) and spiked (b) with 0.5 μg/L of MC-RR (1), MC-YR (2), and MC-LR (3). See experiment discussion for off-line preconcentration and LC analysis.

of each MC. For comparison the chromatogram of the extract of the nonspiked sample is represented in Figure 4b. None of the three MCs was detected in the sample, despite a bloom that had recently occurred in the reservoir.

Several samples were analyzed by SPE-LC, and direct ELISA measurements were used for samples containing more than 0.25 μg/L of MC-LR or SPE-ELISA for samples with lower content. Figure 5 shows the good agreement between the two measurements. However, one has to be aware that, in the case of blooms, water can contain MCs other than MC-LR, as shown below.

Application to Cyanobacterial Samples. Aqueous samples and cellular extracts likely to contain microcystins were assessed by the ELISA test. These samples came from

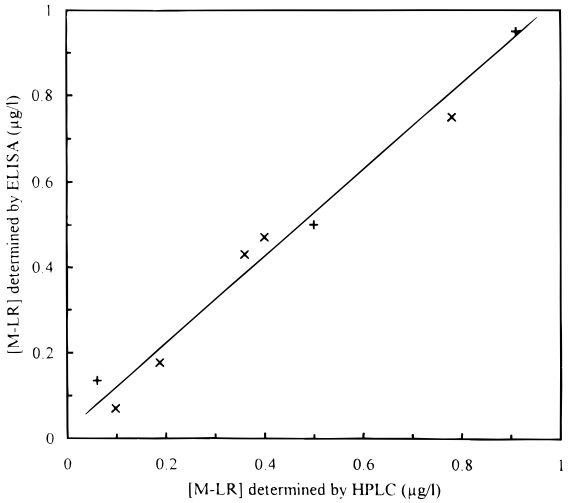

FIGURE 5. Comparison of the results obtained by LC and ELISA for the determination of MC-LR in spiked drinking water (×) and surface water (+) samples.

laboratory-cultured strains as well as samples taken in a reservoir when blooms had occurred. The concentrations of standard microcystins have been determined in each sample.

Aqueous Samples. The analysis of the aqueous culture media of two strains of *Microcystis aeruginosa* PCC7806 and PCC7813 of cyanobacteria has shown that only microcystin-LR on the three standards could be identified by LC. The culture water has been previously concentrated by a factor 500 using solid-phase extraction for LC determinations. Results of Table 3 indicate the concentration given by SPE-LC. ELISA determinations have been made from the same extract, after dilution and without using the raw water of the culture medium. Values given by ELISA are about 5−10 times higher than the concentration of MC-LR determined by SPE-LC. This result can be explained by a strong matrix effect or by the occurrence of cross-reacting microcystin variants. The first reason is unlikely to occur due to the high dilution of the extract. Moreover, the extracts of the aqueous sample PCC7806 have been spiked with known amounts of MC-LR, and the corresponding recoveries are 88 and 125% so that calibration curves are parallel to the standard curve.

The dilution of the extracts from *M. aeruginosa* PCC7813 by a factor of 5 gives a decrease of the same factor with an ELISA value of 0.74 for the extract diluted 2 000 000 and 0.15 when diluted 10 000 000. On the other hand, the effect of the dilution of the extract from *M. aeruginosa* PCC7806 did not provide a decrease in the dilution ratio.

Therefore, the difference between LC and ELISA measurement originates from the occurrence of other MC variants with different degrees of cross-reactivity. The effect of the dilution will depend on the cross-reactivity values. The analysis of the culture media of *M. aeruginosa* PCC7806 is given in Figure 6. The chromatograms obtained by injection of the nonspiked and spiked extracts are given in Figure 6a,b, using microLC coupled to UV DAD. The comparison of the chromatograms shows that only MC-LR (peak 2) on the available standards is present in the extract. The UV spectra of peaks 1−4 are close to those of MC-LR. Figure 6c shows a part of the chromatogram obtained by microLC-ES-MS. Peaks 1 and 2 were not resolved with the 1 mm i.d. column, but the MS spectra confirmed that peaks 1 + 2 corresponded to MC-LR and demethyl MC-LR (named (D-asp3)M-LR). Peaks 3 and 4 have also been identified as MCs with a characteristic mass fragment of 134 (*38*).

Table 3 shows data for water taken in reservoir C after cyanobacterial blooms. Sampling 1 corresponded to deep water, whereas sampling 2 corresponded to the water

TABLE 3. Microcystins in Culture Media and Cellular Extracts of Cyanobacteria and in Environmental Samples, As Determined by the HPLC Analysis (Microcystin-LR) and the ELISA Assay (Microcystin-LR Equivalent)[a]

	dilution of SPE extracts	spiked level (μg/L)	concn (μg/L) SPE-LC	concn (μg/L) ELISA		recovey (%) ELISA
Cyanobacterial Strains						
culture media				crude extracts		
M. aeruginosa PCC7806	1 000 000	–	0.11	0.69		
	2 500 000	–	0.045	0.52		
		0.2	0.245	0.77		125%
		0.5	0.545	0.96		88%
	10 000 000	–	<0.03	0.15		
M. aeruginosa PCC7813	2 000 000	–	0.075	0.74		
	10 000 000	–	<0.03	0.15		
cellular extract				crude extract	cleaned on C_{18}	
M. aeruginosa PCC7806	20 000		0.33	nd	1.5	
	100 000		0.065	1.2	0.55	
	500 000		0.013	nd	0.15	
M. aeruginosa PCC7813	10 000		0.16	nd	3.2	
	100 000		0.016	>10	0.5	
	1 000 000		<0.03	>10	0.18	
	5 000 000		<0.03	>10	0.1	
Environmental Water Samples						
reservoir C						
sampling 1	0.2		2.5	0.88		
	2		0.25	0.13		
	5		0.1	0.08		
reservoir C						
sampling 2	5		2.5	>10		
	25		0.5	2.20.45		
	100		0.12	0.45		

[a] nd = not determined.

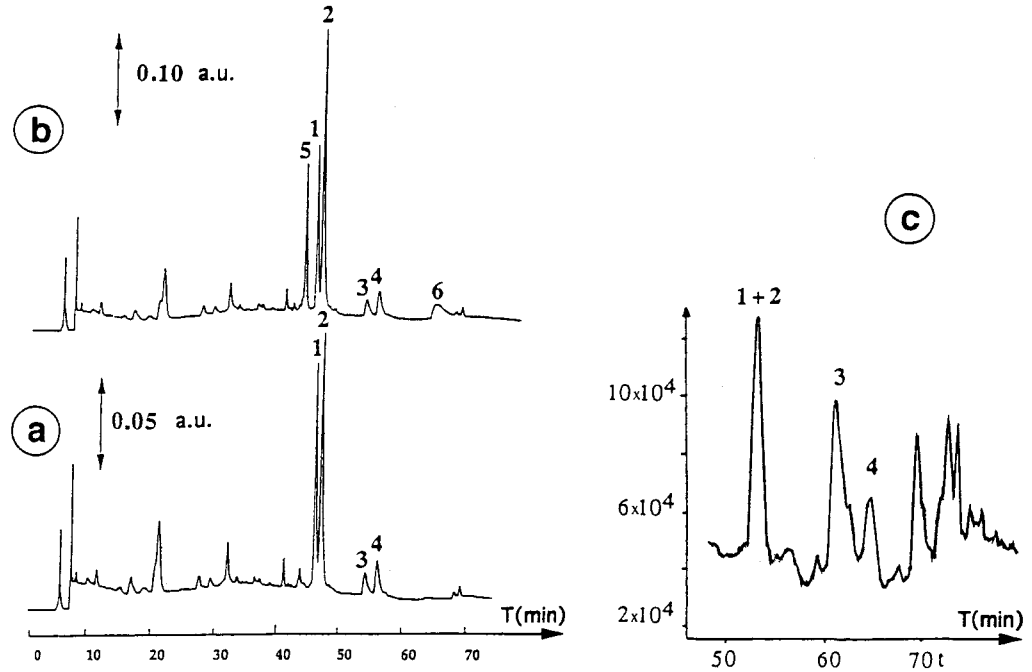

FIGURE 6. Chromatogram corresponding to the analysis of an off-line extract of 130 mL of the aqueous culture media of *Microcystis aeruginosa* PCC7806 obtained (a and b) using microLC with 25 cm × 0.32 mm i.d. column coupled to UV DAD and (c) using microLC with 25 cm × 1 mm i.d. column coupled to ES-MS in full scan and positive ion mode: (a) extract nonspiked and (b) spiked with 20 ng of MC-LR (peak 2), MC-YR (peak 5) and MC-RR (peak 6). See experiment discussion for analytical conditions.

sampled at the surface layer where the bloom occurred. In sampling 1, results obtained by LC and ELISA are low and not much different. In sampling 2, the LC analysis indicated 6.5 μg/L of MC-RR and 12 μg/L of MC-LR together with other potent MCs as indicated by UV spectra, which explains the higher values provided by ELISA.

Since the standards are not available, it is impossible to measure the cross-reactivity and better validate this ELISA kit with natural samples for semiquantitative or quantitative measurements. However, results of Table 3 indicate clearly that this ELISA kit is useful as a screening tool in the case of blooms.

Cyanobacterial Cellular Extracts. ELISA measurements have also been applied to diluted extracts of the cultured algae. In the case of cellular extracts, the dilution did not remove the matrix effect and a cleanup was necessary to eliminate some cross-reactants and reduce the matrix effect, since no measurement could be performed without this step. The ELISA test results in higher microcystin concentrations than the HPLC analysis, as for the corresponding aqueous culture extracts. Toxins other than microcystin-LR have also been identified by LC-MS.

Our results are different from those published by Nagata et al. (*32*), who observed good agreement between the sum of the concentrations of MC-RR, MC-YR, MC-LR, and MC-demethyl-LR determined by LC and the ELISA value in Japanese water extracts of natural cyanobacterial blooms and cultured cyanobacteria. Since high cross-reactivity has been measured by these available MCs, the sum could be correlated to the ELISA value. In France, the occurrence of several blooms has been observed especially in lakes, dams, and reservoirs and samples have been studied by LC-MS, showing the occurrence of MCs other than the standards. Mass cultures of algae taken in sites of blooms are now performed in order to isolate microcystins of interest. A better validation for on-site monitoring using ELISA is therefore expected. The main problem for validation studies remains the lack of standards MCs.

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