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# Automated portable array biosensor for multisample microcystin analysis in freshwater samples

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ABSTRACT

An automated array biosensor based on evanescent-wave excitation has been developed for the detection of microcystins (MCs) in freshwater samples. The sensing surface consisted of microcystin-leucine-arginine (MCLR) covalently immobilized onto a planar waveguide (microscope slide). The binding of anti-MCLR monoclonal antibodies, spiked in the sample, to the immobilized MCLR was competitively inhibited by MCLR in solution and the amount of antibody bound to the patterned antigens was revealed using Cy5-labeled rabbit anti-mouse IgG. Surface chemistry has been optimized to improve biosensor performance in terms of sensitivity, regeneration ability and to avoid non specific binding for further application to environmental monitoring. The optimized biosensor assay presents an $IC_{50}$ value of $0.34 \pm 0.01$ μg/L, a detection limit of $16 \pm 3$ ng/L and a dynamic range from 0.06 to 1.5 μg/L MCLR, improving the performance of previously reported devices. Cross-reactivity to other related MCs, such as microcystin-RR (MCRR, 90%), microcystin-RR desmethylated (dm-MCRR, 95%) and microcystin-YR (MCYR, 91%), was also evaluated. The automated microarray can assay up to six different samples in parallel, with a total analysis time of about 60 min. The sensing surface was regenerated with 50 mM NaOH and each chip was reused for, at least, 15 assay-regeneration cycles without significant binding capacity loss. The immunosensor has been successfully applied to the direct analysis of MCs in surface water samples and the results were in close agreement with those provided by LC–MS/MS.



## 1. Introduction

Microcystins (MCs) are a group of dangerous cyclic heptapeptide hepatotoxins produced by several bloom-forming cyanobacteria genera, particularly *Microcystis aeruginosa* spp. Cyanobacteria represent a public health concern because they can grow rapidly in waters rich in organic matter, under warm climate conditions, giving rise to the proliferation of "algae water blooms". More than 80 MCs have been identified up to date (Campo and Ouahid, 2010), and microcystin-leucine-arginine (MCLR), is the most common and potent cyanotoxin. MCs promote the formation of hepatic tumors upon chronic ingestion of contaminated food or drinking water; they can also affect the kidney and the gastrointestinal tract as well as provoke headache, blurred vision, abdominal pain, nausea and vomiting (Bell and Codd, 1994).

MCs are most widely found in freshwaters (e.g. rivers, lakes, reservoirs) where they are released in substantial amounts after bacterial cell lysis or bacterial death (Amé et al., 2010). The World Health Organization (WHO) has set a guideline value of 1 μg/L for total MCLR in drinking water, the major route of human exposure (WHO, 2003). Possible health risks associated with recreational activities have also been investigated (Backer et al., 2008, 2010); therefore, sensitive and reliable analytical methods are required to monitor MC levels in freshwater samples.

MCs are commonly detected and quantified by reversed-phase liquid chromatography (LC) combined either with ultraviolet (UV) (ISO, 2005; Spoof et al., 2010) or with mass spectrometry (MS) detection (Bogialli et al., 2006; Cong et al., 2006; Lawrence and Menard, 2001; Mekebri et al., 2009; Neffling et al., 2009) after a previous sample pre-treatment step. Alternatively, biochemical screening methods, such as enzyme-linked immunosorbent assays (Pyo et al., 2005; Lindner et al., 2004; Mountfort et al., 2005), competitive enzyme immunoassays (Khreich et al., 2009; Long et al., 2009a) or protein phosphatase inhibition assays (Mountfort et al., 2005; Allum et al., 2008) have also been described. These assays usually require long analysis times and may be strongly affected by matrix effects. Recently, several commercial kits (EnviroGard™ Testkit, Abraxis™) and dipsticks (Lawton et al., 2010; Tippkötter et al., 2009) have become available and applied to field testing with minimum sample processing or technical expertise.

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However, they are single use and show detection limits over or close to the provisional guideline value established by WHO. In addition, immunosensors based on different transduction schemes, such as optical (Herranz et al., 2010; Hu et al., 2009; Lindner et al., 2009; Long et al., 2009b), electrochemical (Loyprasert et al., 2008; Yu et al., 2009) and nuclear magnetic resonance (Ma et al., 2009) measurements have been also applied to MCs analysis but they usually have limited applicability for multisample analysis.

Recent improvements in biosensor instrumentation have facilitated the commercialization of fully automated and portable devices, specially suited for field measurements, which can favorably compete with laboratory instrumental techniques (Sapsford et al., 2008). In the present study, a ready portable, low cost and easy-to-use array biosensor has been applied to MC monitoring in environmental waters. The instrument is a licensed version of a laboratory prototype of the US Naval Research Laboratory (NRL) Array Biosensor (Ligler et al., 2007) and enables on-site field applications for simultaneous multisample and/or multi-analyte detection on a single sensing surface.

The assay optimized in the present work is based on the covalent immobilization of MCLR onto the surface of a planar waveguide. Binding of anti-MCLR monoclonal antibodies to immobilized MCLR is competitively inhibited by the hepatotoxin in the sample solution. The amount of antibody bound to the patterned antigens is revealed using Cy5-labeled rabbit anti-mouse IgG, and evanescent wave excitation, and the fluorescent signal is inversely proportional to the concentration of MCLR in the samples. Several parameters affecting immunoassay performance have been optimized, including planar waveguide surface functionalization, anti-MC antibody concentration and nature of the blocking solution and regeneration solutions. The immunosensor has been applied to the simultaneous analysis of MCLR in spiked and naturally contaminated lake and river water samples and the results have been validated by HPLC–MS/MS.

## 2. Experimental

### 2.1. Reagents and materials

MCLR was obtained from AbKem Iberia S.L. (Vigo, Spain). Microcystin-RR (MCRR), microcystin-YR (MCYR), microcystin-RR desmethylated (dm-MCRR) and monoclonal MC10E7 mouse IgG antibodies raised against MCs were provided by Alexis (Läufelfingen, Switzerland). IgG fraction monoclonal mouse anti-biotin and Cy5-conjugated affinity purified rabbit anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

N-hydroxysuccinimide (NHS), 2-(N-Morpholino)ethanesulfonic acid hydrate (MES), 1*H*,1*H*,2*H*,2*H*-perfluorooctyltrichlorosilane (FOTS), gelatine from porcine skin, bovine serum albumin (BSA) and D-biotin (5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-(Trimethoxysilylpropyl)diethylenetriamine (triamino-APMS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were provided by Acros Organics (Geel, Belgium). Borosilicate microscope slides used as planar waveguides were purchased from Thomas Scientific (Swedesboro, USA).

MC stock solutions were prepared in dimethylsulfoxide (DMSO) (1 mg/mL) and stored at −20 °C. MC standard solutions for calibration purposes were prepared daily upon dilution of the stock solutions in phosphate buffered saline (PBS) (20 mM, pH 7.4) containing 0.05% of Tween 20 and 0.3% of powdered non-fat milk (PBSTM, carrier buffer). Water was purified with a Milli-Q system (Millipore, Bedford, MA). All other chemicals used were of analytical reagent grade.

### 2.2. Instrumentation

Fluorescence imaging was carried out using the Leopard Array Biosensor (Hanson Technologies, USA), a commercial version of the NRL Array Biosensor prototype (Ligler et al., 2007). The instrument is equipped with a 635 nm diode laser source (LAS-635-15, Lasermax). The excitation beam is focused into the edge of the microscope slide waveguide. The fluorescence array intensity developed on the slide is filtered using a long-pass (665 nm) and a band-pass filter (700 ± 35 nm) and measured using a CCD camera (Retiga 1300, Q-Imaging). The system is fully automated and uses two six-chamber reservoir modules, for the samples and tracers, respectively. The slide is mounted vertically and pressed against a six-channel gasket molded in poly(dimethylsiloxane) (PDMS, NuSil Silicone Technology), forming the six-assay flow channels. Each channel is connected on one end to a peristaltic pump and on the other to a 2-way valve that switches between the sample and the tracer that flow through the channels (Ligler et al., 2007). The acquired image is analyzed using a proprietary software control interface that also controls the microfluidic system. The locations and intensities of the fluorescent spots allow identifying and quantifying MC concentration in the samples (Taitt et al., 2008).

### 2.3. Antigen immobilization

Prior to functionalization, the microscope slides were cleaned by immersion in a 10% KOH methanolic solution for 1 h, washed with deionized water and air-dried. Dried slides were treated under argon with 2% triamino-APMS in anhydrous toluene for 2 h, rinsed three times with toluene and stored in anhydrous toluene at 4 °C, until use. A 15-channel PDMS patterning gasket was placed over the silanized surface of the slide, and a 30 μg/mL MCLR solution, containing 100 mM EDC and 50 mM NHS in MES (0.05 M, pH 6.0) was injected into each measuring channel. MES buffer and a solution containing 30 μg/mL biotin, 100 mM EDC and 50 mM NHS were also patterned as negative and positive controls, respectively. The slides were incubated overnight at 4 °C and rinsed with 0.5 mL of PBS (20 mM, pH 7.4). After removal of the patterning PDMS template, the slides were incubated 2 h, at room temperature, in 3% non fat milk in PBS containing 0.5% Tween 20, rinsed with deionized water, dried under argon and either used immediately or stored at 4 °C.

### 2.4. Assay protocol

The patterned slides were placed in the flow chamber of the Array Biosensor as described previously (Section 2.2). All solutions were prepared in PBSTM. MCLR standard solutions (380 μL, 0–100 μg/mL) were mixed with 20 μL of a solution containing anti-MCLR (4 μg/mL) and anti-biotin antibodies (4 μg/mL), loaded into the sample reservoirs, pumped through the channels and allowed to sit static (without flow) for 20 min. Next, the channels were rinsed twice with 0.8 mL PBSTM (1 mL/min) and the flow cells were evacuated. To reveal the anti-MCLR IgG bound to the patterned antigens, a solution (400 μL) containing 2.5 μg/mL Cy5-labeled anti-mouse IgG in PBSTM was loaded into the tracer reservoirs pumped through the channels and allowed to sit static for 20 min. Finally, the slide was rinsed four times with 0.8 mL PBSTM (1 mL/min), the flow cells were evacuated and the slide was imaged and analyzed. A solution of NaOH 50 mM (0.8 mL, 1 mL/min) was flowed for surface regeneration before a new analysis cycle. Total analysis time, including slide regeneration was, approximately, 60 min.

Fluorescent signals were extracted from the CCD images as pixel intensity values ($I$) using the proprietary software provided by Hanson Technologies. The program creates a mask consisting of data

squares (enclosing the areas were MC is patterned) and background rectangles at both sides. The net fluorescence intensity ($S$) for each data square was calculated by subtracting the average background values ($B$) from the average data square ($I$) according to Eq. (1):

$$S = I - B \quad (1)$$

The normalized response was calculated according to the following expression (Eq. (2)):

$$\text{Normalized response} = \frac{S/S_{pc} - S_\infty/S_{pc,\infty}}{S_0/S_{pc,0} - S_\infty/S_{pc,\infty}} \quad (2)$$

where $S_\infty$ is the background signal measured in the presence of an excess of MCLR, $S_0$ is the signal in the absence of MCLR and $S_{pc}$ is the signal measured for the positive control. The normalized response was plotted against the concentration of MCLR (in logarithmic scale), and the experimental data was fitted to a four-parameter logistic equation (sigmoidal) (Eq. (3)):

$$\text{Normalized signal} = \frac{A_{max} - A_{min}}{1 + ([\text{MCLR}]/\text{IC}_{50})^b} + A_{min} \quad (3)$$

where $A_{max}$ is the asymptotic maximum (maximum signal in the absence of MCLR), $b$ represents the curve slope at the inflection point, $IC_{50}$ is the concentration of MCLR at the inflection point (concentration giving 50% inhibition of $A_{max}$), and $A_{min}$ is the asymptotic minimum. The detection limit (LOD) was calculated as the MCLR concentration for which antibody binding to the immobilized MCLR was inhibited by 10%, and the dynamic range (DR) of the method was evaluated as the MCLR concentration that produced a normalized signal between 20 and 80% of $B_0$ (normalized signal in the absence of MCLR).

### 2.5. Selectivity studies

Cross-reactivity (CR) studies were carried out by measuring the competitive inhibition curves for other MC variants and non-related water pollutants, under the optimized conditions. CR was calculated according to the following equation (Eq. (4)):

$$\text{CR} = \frac{\text{IC}_{50}(\text{MCLR})}{\text{IC}_{50}(\text{cross-reacting compound})} \times 100 \quad (4)$$

### 2.6. Water sample analysis

Water sampling and MCs HPLC–MS/MS analysis were carried out by the agreement between the Spanish Ministry of Environment and Marine and Rural affairs (MARM) and the "Water Quality Laboratory" (WQL) at CEH-CEDEX on "Technical assistance, research and technological development in matters of concern of the General Directorate of Water of the MARM" (2007–2011).

Water reservoirs at four different locations in Spain ((1) León, (2) Asturias, (3) Toledo and (4) Badajoz) were sampled during the summer of 2010 (period of maximum bloom formation). Samples were collected in Pyrex borosilicate glass bottles and stored in the dark at 4 °C.

Before analysis with the array biosensor, water samples' pH and ionic strength were adjusted by the addition of PBS to a final buffer concentration of 20 mM. The samples were then filtered through a 0.45 μm nylon membrane (Whatman, Maidstone, UK) to remove suspended matter and analyzed in triplicate. Additionally, water samples free of MCs were spiked with increasing concentrations of MCLR to evaluate the matrix effect on the developed immunoassay.

For validation purposes, 10 mL subsamples were spiked with nodularin as internal standard. The samples were filtered using a 0.2 μm membrane and direct analysis of 1 mL aliquots was performed by on-line SPE LC/ESI-MS/MS at the WQL-CEH-CEDEX (Madrid, Spain). The analyses were carried out with an E-Quan System from Thermo Fisher Scientific (San Jose, USA). This system was equipped with a high-flow Surveyor LC and a low-flow Accela LC pump; a HTC PAL autosampler (2.5 mL injections), a six-port column switching valve and a Thermo Fisher Scientific TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, USA). A Hypersil Gold C18 column (20 mm × 2.1 mm, 12 μm) and a Hypersil Gold C18 column (50 mm × 2.1 mm, 3 μm) (Thermo Fischer Scientific, San Jose, CA, USA) were used for preconcentration and analytical separation, respectively. The sample was loaded onto the preconcentration column (mobile phase water:acetonitrile, 97% (v/v), 1 mL/min) and after 1.3 min, the retained MCs were eluted by back-flush elution and transferred to the analytical column. The chromatographic separation was performed using gradient elution, combining solvent A (water) and solvent B (acetonitrile), both containing 0.08% (v/v) formic acid, at a flow rate of 300 μL/min, as follows: 3–30% B: 0.3 min, 30–35% B: 6 min, 35–95% B: 3 min. Finally, the columns were re-equilibrated at the initial conditions. The ESI source was operated in positive ionization mode; spray voltage 3 kV; ion transfer tube temperature 250 °C; sheath gas and auxiliary gas at flow rates of 30 and 5 a.u. (arbitrary units), respectively. Data acquisition was performed in Selected Reaction Mode (SRM) (Table 1S).

## 3. Results and discussion

### 3.1. Surface derivatization

Covalent coupling of MCLR to the glass slide was carried out using triamino-APM, following activation of carboxylic groups with EDC and NHS (Hermanson, 2008). The selection of this coupling agent was based on previous reports (Smith and Chen, 2008), demonstrating the higher hydrolytic stability of aminosilanes with long alkyl chain linkers for further application of the derivatized surfaces in aqueous solutions.

The use of silane concentrations in the range of 2–4% (v/v) did not significantly affect assay sensitivity (Fig. 1S, in the supplementary material). However, increasing the silanization time from 1 to 2 h led to higher sensitivities and wider DRs ($IC_{50}$ and LOD of 0.75 and 0.032 μg/L vs 0.95 and 0.44 μg/L for 2 and 1 h, respectively) (see Fig. 1S). Longer silanization times (16 h) significantly decreased assay sensitivity and precision, which has been attributed to the formation of aminosilane multilayers on the glass slide (Smith and Chen, 2008). For further experiments the slides were silanized using 2% (v/v) triamino-APM in toluene for 2 h and stored for no longer than 10 days.

### 3.2. MCLR immobilization.

Several parameters affecting the efficiency of the immobilization of MCLR on the silanized surface have been studied, namely: nature and pH of the carrier solution, EDC concentration, preincubation of MCLR with the EDC/NHS mixture before injecting the solution into the patterning channels and MCLR concentration (Fig. 2S). The evaluated ranges and optimum values are collected in Table 1.

### 3.3. Bioassay optimization

Several blocking methods have been evaluated to avoid the non-specific binding of bioreagents on the sensing surface: wet blocking with protein based solutions and vapor-phase blocking methods.

In the first approach, the patterned slides were blocked with solutions of gelatine, BSA or powdered non-fat milk (10 mg/mL, PBS 20 mM, pH 7.4) for 1 h at room temperature. Alternatively, vapor-phase deposition of FOTS (vp-FOTS), a highly fluorinated

**Table 1**
Optimized conditions for MCLR immobilization.

| Parameter | Evaluated range | Optimum value |
|---|---|---|
| Carrier solution | | |
| Nature | PBS and MES buffer | MES buffer 50 mM, pH 6 |
| pH | 5.5–6.5 | |
| [NaCl] (M) | 0–0.5 | |
| EDC concentration (mM) | 0–200 | 100 |
| Pre-incubation of MCLR with EDC/NHS (h) | 0–4 | 0 |
| MCLR concentration (μg/mL) | 10–160 | 30 |

organosilane, was also checked. This method has been reported to be rapid and more effective than wet procedures (Hsieh et al., 2009), resulting in lower background signals and higher antibody recognition efficiencies. Thus, the sensor surface was exposed to the silane vapor, in Ar atmosphere, for 20 min, rinsed twice with toluene and dried with Ar. Blocking efficiency was determined by measuring the background signal intensity between the patterned areas, along the assay channels.

A high non-specific binding was observed without blocking (Fig. 1) and the lowest background signal was measured when using vp-FOTS, although assay sensitivity was also strongly decreased ($B_0$ signal reduced to 5% of the value observed in the absence of blocking). This finding has been attributed to the interactions between FOTS and the guanidine residue of the immobilized MCLR, responsible of antibody recognition (Zeck et al., 2001).

Best blocking efficiencies were obtained using non-fat milk that also provided the lowest background signals (3% vs 11% and 17% for gelatine and BSA, respectively). BSA and gelatine, rendered an important decrease of assay sensitivity ($B_0$ decreased by about 20% and 10% respectively, with respect to that of a non-blocked surface) that could be explained due to the small size of the toxin compared to that of proteins that may hinder antibody recognition by the immobilized MCLR. A correlation was observed between

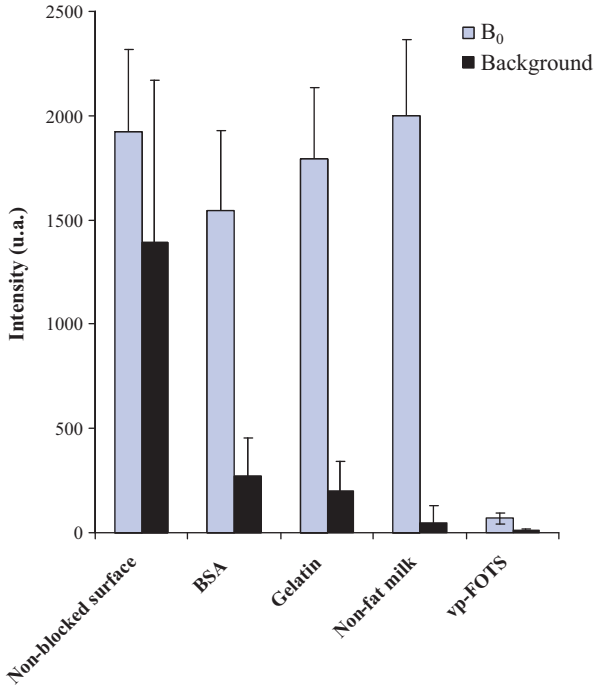


**Fig. 1.** Comparison of the efficiency of different blocking solutions: BSA, gelatine, non-fat milk, vp-FOTS. The silanized slide was patterned with MCLR (30 μg/mL), biotin (30 μg/mL) as positive control, and MES buffer as negative control. Tracer solution: 2.5 μg/mL Cy5-labeled anti-mouse IgG.

**Table 2**
Effect of the anti-MC antibody concentration.

| [Anti-MC Ab] (μg/mL) | LOD (μg/L)[a] | $IC_{50}$ (μg/L)[a] | Dynamic range (μg/L) |
|---|---|---|---|
| 0.2 | 0.016 ± 0.003 | 0.34 ± 0.01 | 0.06–1.5 |
| 0.4 | 0.32 ± 0.02 | 1.12 ± 0.03 | 0.5–2.2 |
| 0.6 | 1.8 ± 0.2 | 3.89 ± 0.08 | 2.2–6.4 |
| 0.8 | 2.1 ± 0.2 | 4.15 ± 0.06 | 2.7–6.7 |

[a] $ts/\sqrt{n}$, $t_{95\%}$.

the molecular weight of the protein in the blocking solution and the decrease in the $B_0$ signal, being lower in gelatine (~50 kDa) or non-fat milk solutions (mainly caseins, 23–37 kDa) than in BSA (~66 kDa) (Golden and Sapsford, 2008). Increasing non-fat milk concentration from 10 to 30 mg/mL led to more effective blocking and thus all the slides were incubated with 30 mg/mL non-fat milk for 90 min prior to use.

Immunoassays were carried out using PBS containing 0.3% non-fat milk and 0.05% Tween 20 (PBSMT) as carrier buffer (Herranz et al., 2010). Different sample incubation times were tested for optimal sensitivity. Increasing the incubation time from 10 to 20 min allowed a decrease of more than 50% in the $IC_{50}$ value (0.78 μg/L and 0.34 μg/L, respectively) but longer times did not significantly improve assay performance. Finally, an incubation time of 20 min with the Cy5-tracer antibody was also selected for assay development, as shorter times lead to both, lower signals and poorer precision.

Anti-MC antibody concentrations were evaluated in the range of 0.2–0.8 μg/mL for optimal dose–response for MCLR samples (toxin concentration from 0 to 1 μg/mL) (see Table 2), as well as best LODs. Decreasing antibody concentration yields lower $IC_{50}$ values as well as lower LODs and narrower DRs, as expected; however, antibody concentrations lower than 0.1 μg/mL provided a very low precision. Thus, a concentration of 0.2 μg/mL was selected for further experiments.

Finally, to disrupt the interaction between the antibody and the immobilized MCLR and thus regenerate the biosensor surface for further assays, a 50 mM NaOH (0.8 mL, 1 mL/min) solution was used. A 5 min blocking step with non fat milk in PBS (3%, v/v) between consecutive assays, was sufficient to avoid the non-specific interactions when the slide was reused for up to fifteen assays.

### 3.4. Biosensor characterization

#### 3.4.1. Analytical characteristics

Fig. 2 shows a representative CCD image of a patterned waveguide used to test freshwater samples and the competition inhibition curves obtained under the optimized conditions using MCLR standard solutions in the range of 0–1000 μg/L. The fluorescent signals were normalized with respect to positive controls (biotin), as stated in Section 2.4, to account for variability between channels and slides (Kulagina et al., 2007). The $IC_{50}$ value and the LOD were 0.34 ± 0.01 μg/L ($n$ = 9) and 16 ± 3 ng/L ($n$ = 9), respectively. The DR was from 0.06 to 1.5 μg/L. The sensitivity of the developed immunosensor was superior to other reported methods for MCLR analysis (see Table 2S) and the LOD is below the provisional guideline value established by WHO for drinking water.

Reproducibility within slide channels was excellent with RSDs of the normalized signal ranging from 4 to 9%. The slide to slide reproducibility of the competitive immunoassay ($n$ = 3 slides) was also good with RSDs ranging between 4 and 21% depending on the MCLR concentration (mean RSD in the calibration range 13%). The variation in biosensor response was negligible after, at least, 15 assay-regeneration cycles with no significant differences in the

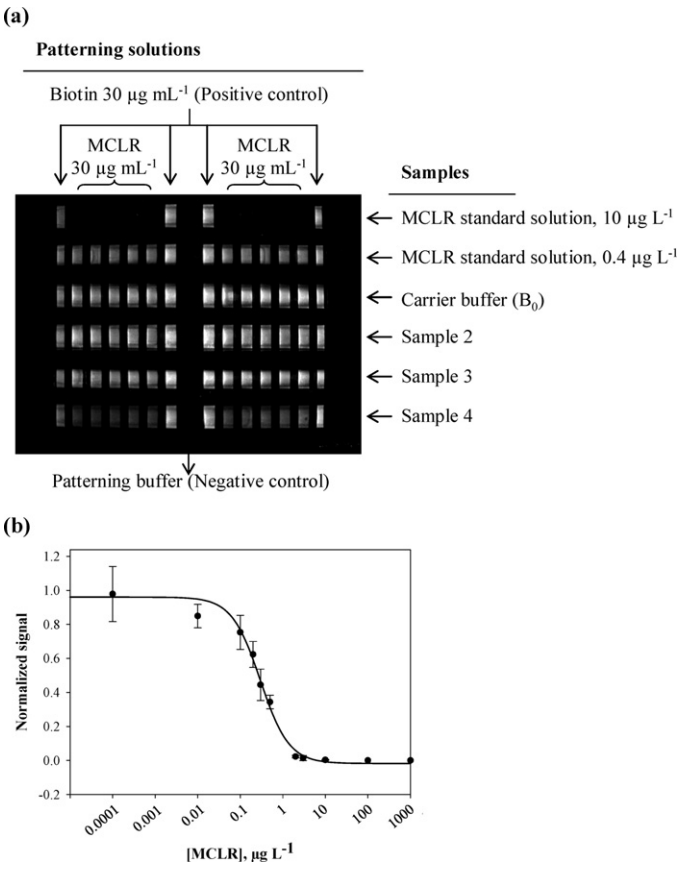


**Fig. 2.** Analysis of MCs using the developed bioarray. The silanized slide was patterned with MCLR (30 μg/mL), biotin (30 μg/mL) as positive control, and MES buffer as negative control. The slide was assayed with MCLR (0, 0.4 and 10 μg/L) and three river water samples (samples 2–4), in the presence of 0.2 μg/mL of anti-MCLR and 0.2 μg/mL of anti-biotin (positive control) antibodies. Tracer solution: 2.5 μg/mL Cy5-labeled anti-mouse IgG. (a) Biochip image. (b) Competitive calibration curve. (Error bars are standard error of the mean for three slides and three spots per slide, $n = 9$.)

initial $B_0$ value (a larger number of cycles were not evaluated). This is a clear advantage over previous developments using the same sensing scheme (Ngundi et al., 2006).

### 3.4.2. Cross-reactivity

CR was studied for the most common MC variants found in freshwater samples: MCRR, dm-MCRR and MCYR (Sivonen and Jones, 1999), obtaining CRs of 90%, 95% and 91%, respectively. Since the antibody recognizes [4-arginine]-MCs (Zeck et al., 2001) with similar sensitivities, the biosensor allows the quantification of the total concentration of the most common MCs in environmental water samples.

Non related compounds that may be present in surface waters, such as pesticides (e.g. atrazine, carbaryl) or antibacterials (e.g. enrofloxacin, chloramphenicol) were not recognized by the antibody (CR < 0.002%), in the studied concentration range (0–1000 μg/L).

### 3.4.3. Analysis of freshwater samples

In order to investigate the presence of matrix effects, samples checked to contain undetectable levels of MCs were spiked with increasing concentrations of MCLR, in the range of 0–100 μg/L and analyzed using the immunosensor. The comparison of the dose-calibration curves obtained in these samples and in Milli Q water showed no statistically significant differences (confidence level 95%).

**Table 3**
Analysis of freshwater samples ($n = 4$).

| Sample | MC concentration (μg/L) | |
|---|---|---|
| | Biosensor | LC–MS/MS |
| 1 | <LOD | nd |
| 2 | <LOD | nd |
| 3 | 0.24 ± 0.02 | 0.23 ± 0.03[b] |
| 4 | 0.55 ± 0.06 | 0.64 ± 0.05[c] |

nd, not detected.
[b] dm-MCRR.
[c] MCRR (0.23 ± 0.01) μg/L + MCLR (0.41 ± 0.05) μg/L.

Freshwater samples, collected from different geographic areas in Spain, were analyzed using the developed immunosensor and by on-line SPE-LC–MS/MS for validation purposes. The results are reported in Table 3. No significant differences were observed (confidence level 95%) in any of the tested samples demonstrating the excellent performance of the biosensor and its suitability for the direct analysis of MCs in surface waters at the low ng/L level, without the need of previous clean-up or preconcentration steps.

## 4. Conclusions

A biosensor consisting of MCLR covalently immobilized onto planar optical waveguides has been developed for the determination of MCLR in freshwater samples. The working principle is based on a competitive fluoroimmunoassay performed within a multi-channel flow cell placed on the waveguide surface. The immunosensor allows detection of MCLR at levels below the provisional guideline value of 1 μg/L proposed by WHO. Moreover, it shows acceptable stability, reusability (at least 15 assay-regeneration cycles with the same chip) and excellent precision. The configuration of the instrument used in this work allows the simultaneous analysis of up to six samples within short analysis times (one complete measurement-regeneration cycle can be accomplished in 60 min), and the system is fully automated, portable, and highly competitive with laboratory techniques. The $IC_{50}$ and the LOD are lower than those reported for other biosensors based on different transduction techniques, including label-free methods such as SPR. The biosensor has shown excellent CR for other MC variants, such as MCRR, dm-MCRR and MCYR, which appear together with MCLR in environmental waters. Finally, the biosensor has been successfully applied to the direct analysis of surface water samples without any clean-up or preconcentration steps, and the results were fully comparable to those obtained by LC–MS/MS as an alternative technique.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.12.016.

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