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Limited analytical capacity for cyanotoxins in developing countries may hide serious environmental health problems: Simple and affordable methods may be the answer

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ABSTRACT

In recent years, the international demand for commodities has prompted enormous growth in agriculture in most South American countries. Due to intensive use of fertilizers, cyanobacterial blooms have become a recurrent phenomenon throughout the continent, but their potential health risk remains largely unknown due to the lack of analytical capacity. In this paper we report the main results and conclusions of more than five years of systematic monitoring of cyanobacterial blooms in 20 beaches of Montevideo, Uruguay, on the Rio de la Plata, the fifth largest basin in the world. A locally developed microcystin ELISA was used to establish a sustainable monitoring program that revealed seasonal peaks of extremely high toxicity, more than one-thousand-fold greater than the WHO limit for recreational water. Comparison with cyanobacterial cell counts and chlorophyll-a determination, two commonly used parameters for indirect estimation of toxicity, showed that such indicators can be highly misleading. On the other hand, the accumulated experience led to the definition of a simple criterion for visual classification of blooms, that can be used by trained lifeguards and technicians to take rapid on-site decisions on beach management. The simple and low cost approach is broadly applicable to risk assessment and risk management in developing countries.

1. Introduction

The number and importance of harmful algal blooms, especially of cyanobacteria, are increasing worldwide and represent a significant threat to public health. Many developed countries have implemented strategies to monitor and control algal blooms. However, in many developing countries academic reports show that the problem is also present but there are difficulties in establishing monitoring programs and preventive measures. The case of Lake Atitlan in Guatemala is in many ways typical. For several decades it has received untreated sewage, as well as nitrogen and phosphorus runoff from uncontrolled erosion. A significant bloom of cyanobacteria (*Lyngbya hieronymusii*) appeared for the first time in December 2008. A few months later the bloom returned with more intensity and affected society at all levels (http://www.guatemala-times.com/science-environment/environment/1237-guatemalas-lake-atitlan-disaster-the-explanation.html). Despite the prevailing opinion of the scientific community, there have been attempts to control the algal blooms by introducing grass carp or adding algicides into the lake to kill the cyanobacteria without regard for the potential ecological consequences.

Several species of cyanobacteria produce potent toxins such as the microcystins synthesized by various strains of *Microcystis* and *Anabaena*. In Caruaru, Brazil, water contaminated with microcystins caused the death of 52 patients of a hemodialysis clinic (Jochimsen et al., 1998) bringing the problem to the attention of the general public, which drastically changed the country's attitude toward cyanobacterial blooms (Azevedo et al., 2002; Dorr et al., 2010). As a consequence, in the year 2000 the Brazilian

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regulations for drinking water quality were changed to incorporate cyanobacteria and cyanotoxins as parameters that must be monitored for water quality control.

Reports of systematic studies of cyanobacterial blooms in South America are scarce, but publications document the existence of the phenomenon (Ame et al., 2003; Conti et al., 2005; von Sperling and Souza, 2007; Benintende et al., 2008; Johnson et al., 2008; Tundisi et al., 2008; Sant Anna et al., 2011; Unrein et al., 2010). In Uruguay, cyanobacterial blooms have been increasingly evident with *Microcystis aeruginosa* dominating the bloom community in dams, rivers and the estuarine water of the Rio de la Plata (De Leon and Yunes, 2001; Piccini et al., 2006). The La Plata River estuarine system, fifth largest in the world with a population of approximately 67 million people, transports water from the central part of South America through the Paraná and Uruguay basins to the Atlantic Ocean. One of the main factors affecting water conditions the basin is the construction of more than thirty dams for hydropower (Salto Grande, Itaipú, Yacyretá), followed by increasing urbanization, expansion of agriculture and climate variations. These factors have modified the flow, as well as the quality of the water favoring the proliferation of algal blooms posing possible risks for the multiple uses of these water (fishing, drinking water source, recreation). This concern is more than theoretical, as practically all of the *Microcystis* blooms studied in Montevideo (the capital city) produced hepatotoxic microcystins (De Leon and Yunes, 2001; Brena et al., 2006).

Traditional detection of *Microcystis* by microscopic cell counts are time consuming and are not necessarily an accurate indicator of toxicity. Several *Microcystis* species produce toxins (microcystins) at later stages of the cell cycle, and the toxins, which are cyclic peptides of an unusual structure, may persist for very long times in the environment in either an intracellular form or extracellularly after lysis of the cyanobacterial cells. Traditional analytical methods for the determination of microcystins such as mouse bioassay have low sensitivity and may be considered ethically objectionable. Instrumental analysis by HPLC and/or by mass spectrometry, the reference method, is costly and therefore not suitable for high sample throughput and routine monitoring in developing countries. In addition, the microcystins are a family of more than 80 chemical congeners (van Apeldoorn et al., 2007) and the predominant variants in local blooms have not yet been identified, which adds further complexity to HPLC or mass spectrometry analysis.

Immunoassays are very useful for rapid screening of large numbers of samples directly, without the need for complex sample preparation procedures. Enzyme-Linked ImmunoSorbent Assay (ELISA) is a well established and relatively low cost method for the rapid analysis of large numbers of samples. Therefore, the number of U.S. EPA-certified ELISA methods and the level of acceptance by other regulatory agencies of the use of immunoassays in environmental applications are increasing. Moreover, due to the large number of microcystin variants, the use of HPLC methods is limited by the availability of standards so immunoassays of broad specificity may be more suitable for providing global information about possible toxicity. Indeed, existing ELISA antibodies have a broad specificity for the different microcystin variants, which makes this an excellent method for screening for many of the known microcystins (and for unknown variants that have common structural features) simultaneously in a single assay. The comparative advantages of the ELISA prompted the local development of the assay (Brena et al., 2006).

In this paper we report the main results and conclusions of a long term cyanobacterial monitoring program, including cyanobacterial species identification, cell count and chlorophyll-a determination. We also describe our approach to the standardization and long-term use of our locally developed ELISA, the results from this systematic monitoring program for microcystins over a 6-year period, and its impact upon public health protection in Montevideo.

2. Materials

Microcystin-LR (MC-LR, purity ≥98.0%) and all other microcystin variants used in this study were purchased from Alexis Biochemicals (San Diego, CA, USA). Goat anti-rabbit IgG-Horseradish Peroxidase (HRP) conjugate was from Pierce Biotechnology (Rockford, IL, USA); bovine serum albumin (BSA, fraction V, purity 98%) and ovalbumin (OVA) were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Purified water was prepared with a Milli-Q plus 185 (Millipore).

Mercury ((o-carboxyphenyl)thio)ethyl sodium salt (Thimerosal) was obtained from BDH Chemicals Ltd., Poole, England. 3,3′,5,5′-tetramethylbenzidine TMB, ethylenediamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), and Minipax absorbent packets (0.5 g) were from Sigma–Aldrich. MC standards were from Alexis Biochemicals.

3. Methods

3.1. Study area and monitoring strategy

The study zone is situated on the Uruguayan coast at Montevideo (the northern coast of the Río de la Plata) between the Santa Lucia River in the west of the city and the Carrasco River to the East (Fig. 1). Hydrological records show an increase in the discharge of the Uruguay River since 1953, with a very sharp growth after 1971, and there have been frequent El Niño events that correlate with severe floods, and La Niña that are associated with droughts (Nagy et al., 1998).

During the summer season (mid-November to the end of March) the beach monitoring program for water quality of the City of Montevideo samples 22 sandy beaches every second day for the analysis of fecal coliform contamination. At each sampling site careful visual observation is performed in order to establish the presence or absence of cyanobacterial blooms. When cyanobacterial colonies are not observed, sentinel samples at six sites are taken once a week for routine vigilance. These samples are analyzed for salinity, temperature and chlorophyll-a. When cyanobacterial colonies are detected, technicians are trained to classify the magnitude of the phenomenon into two well-defined categories: 1. Dispersed cyanobacterial colonies (the colonies are evenly dispersed and can be seen only from a short distance or are observed in the water when the sample is taken) and 2. “Scum” (“bloom”) is when colonies are accumulated and produce a typical deep green color in the water that can be observed from several meters distance. Experiments with the use of more categories proved to be difficult in practice so the distinction into only these two categories proved feasible. If a cyanobacterial bloom is detected, samples are taken for cyanobacterial species identification, cell count, chlorophyll-a determination and microcystin analysis.

Samples were collected in 1-L dark bottles 20 cm below the water surface and were carefully subdivided into three aliquots for determination of chlorophyll-a, cell counts, and ELISA. Aliquots for chlorophyll-a and cell counting were stored refrigerated at 4 °C until used (maximum preservation time 24 h). Aliquots for MC analysis were frozen at −20 °C and stored until analyzed. Cell counting was performed using Box’s methodology (Box et al., 1981), with disintegration of colonies by alkaline hydrolysis (80–90 °C for 10 min, followed by intensive mixing). The isolated cells were counted using a counting chamber (Sedgewick–Rafter or a hemocytometer) with a 400× microscope. In cases of low cell density, Utermhöl methodology (Sournia, 1978) was used. The taxonomic

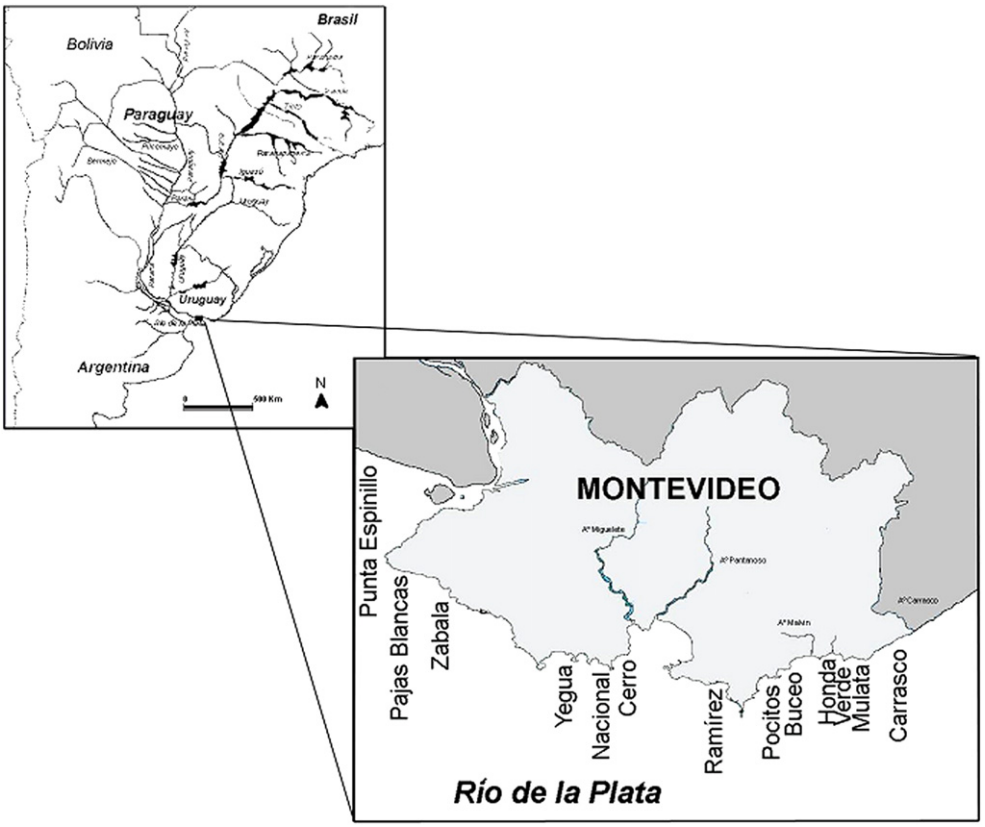

Fig. 1. A map of the Rio de la Plata basin; the sampling area of this study and of the city of Montevideo is shown. The beaches mentioned in the text and tables are shown in their relative locations to each other. The direction of flow of water from the Uruguay River is northwest to southeast in the Rio de la Plata.

observations were performed with a Leitz optic microscope. Phytoplankton analysis in water samples was done collaboratively with the National Direction of Aquatic Resources (DINARA, Montevideo, Uruguay).

Chlorophyll-a was determined spectrophotometrically using cells filtered through glass fiber filters, then extracted in acetone:water 75:25, following APHA Method 10200h (Association, 1998).

In this study we report on blooms occurred over 9 years during the summers from 2000 to 2001 to 2008–2009, with detailed ELISA results for microcystins from six summer seasons (2003–04 to 2008–2009) and isolated historical data starting from 1981.

3.2. Indirect competitive ELISA assay (kit format)

3.2.1. Preparation of plates and reagents

The ELISA assay was based on a rabbit polyclonal antibody raised by using cationized and thiolated ovalbumin (OVA-MC-LR conjugate) as hapten conjugate (McElhiney et al., 2002). Briefly ovalbumin was cationized with ethylenediamine using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, subsequently thiolated with SPDP and then coupled to MC (ratio MC-LR: carrier 15:1).

For immunization, one hundred micrograms of OVA–MC-LR conjugate were dissolved in 250 μL of Phosphate-Buffered Saline (PBS) and vigorously mixed with 250 μL of Freund's Complete Adjuvant (Pierce, Rockford, IL, USA) to form a thick emulsion. This emulsion was then injected subcutaneously into several points on the back of New Zealand white rabbits. After 4 and 8 weeks the animals were immunized intramuscularly with additional doses of hapten conjugates emulsified in Freund's Incomplete Adjuvant. Ten days after the final booster the animals were bled, and after clotting and centrifugation aliquots of the sera were prepared and kept at −20 °C until used.

The coating antigen (BSA-MC-LR) was prepared by coupling MC-LR to thiol groups introduced in cationized and thiolated bovine serum as described for ovalbumin (McElhiney et al., 2002). ELISA plates were coated with the BSA-MC-LR conjugate (100 μL per well containing 70 ng/mL of conjugate in phosphate buffered saline, PBS); then blocked with 200 μL 1% BSA (m/v) in PBS, for 1 h, thoroughly washed four times with PBS-Tween 0.05%, once with water and dried in a chamber at 23 °C ± 2 at 40–50% humidity for 1 h. The plates were preserved in the refrigerator at 4 °C in sealed aluminum foil bags containing desiccant.

Due to the lack of certified standard materials for individual MCs we used MC-LR standard of declared purity, and checked the concentration of the standard by using an extinction coefficient at 238 nm of 39,800 (Blom et al., 2001). Microcystin standards (0.2; 0.6; 1.0; 1.5 and 2.5 μg/L) were prepared in PBS containing 0.1% BSA and 0.5 g/L sodium azide (calibrator buffer). The anti-MC polyclonal serum was diluted 1/15,000 in calibrator buffer and the anti-rabbit-HRP conjugate solution was prepared by diluting the antiserum 1/5000 in PBS containing 0.5% BSA, 0.01% Thimerosal and 0.1 mM TMB. Antibody solutions and MC standards were filtered with 0.2 m cellulose acetate membrane filters (Avantec MFS). Solutions were preserved in the refrigerator at 4 °C in dark glass bottles. Before use, 6 mL of diluted anti-MC polyclonal serum were mixed with 1.34 mL of 1 M Tris buffer, pH 7.5 containing 100 g/L disodium EDTA, 16 g/L NaCl and 1% BSA.

3.2.2. ELISA assay procedure

The mixture containing microcystin standards or adequately diluted samples in calibrator buffer (50 μL per well) and anti-microcystin antibody (60 μL per well) was incubated for 50 min at room temperature with constant agitation (alternatively with manual mixing for 20 s every 10–15-min). After thorough washing,

100 μL of the second antibody (anti-rabbit-HRP conjugate solution) was added to each well and the plate was incubated for 40 min at room temperature. The plates were washed and then 100 μL of the peroxidase substrate (0.4 mL of 6 mg/mL dimethylsulfoxide (DMSO) solution of 3,3′,5,5′-tetramethylbenzidine, plus 0.1 mL of 1% H_2O_2 in water, prepared in a total volume of 25 mL of 0.1 M sodium acetate buffer pH 5.5) was dispensed into each well. The enzyme reaction was stopped after 15 min by the addition of 50 μL of 2 N H_2SO_4. The absorbance was read at 450 nm in a FLUOSTAR optima BMG plate reader using a reference wavelength of 620 nm. Inhibition values were fitted to a linear semi-log plot using Excel, by the equation:

$$\%\,\text{Inhibition} = m \ln(\text{MC concentration}) + b \quad (1)$$

Samples were thawed, filtered, and measured using serial dilutions. Quality controls included the use of at least one control standard (1 μg/L) and one spiked sample per plate. Samples and standards were determined in triplicate.

3.2.3. Statistical analysis of data

Data are expressed as mean values + SEM. Significant differences between groups was defined as a p value less than 0.05, and was determined by linear regression analysis or by ANOVA followed by t-tests between groups with Tukey's correction for multiple comparisons, as appropriate.

4. Results

4.1. Cyanobacterial blooms in the Rio de la Plata

Cyanobacterial blooms in the Rio de la Plata usually appear during late spring and summer (late November to March–April), producing clearly visible green discoloration of the water. Cyanobacterial scums are most frequently observed in the calm water next to rocky points, and appear occasionally in the middle of the beaches. The most relevant historical records of cyanobacterial scums in the Rio de la Plata since 1981 including some of the results of the systematic monitoring program of the beaches of Montevideo between 2003 and 2008 are summarized in Table 1. The dominant species found was *M. aeruginosa*, followed by other species of *Microcystis*, and less frequently by *Anabaena*. The first data set including cyanobacterial count, species identification and concentration of microcystins, was obtained in January 2001. In 2001 the determination of microcystins was performed in Brazil [Laboratory of Dr. Sandra Azevedo, Universidade Federal do Rio de Janeiro] since there was no analytical capacity available at that time in Uruguay. The high cyanobacterial cell densities and microcystin concentrations among the highest reported values worldwide (Chorus and Bartram, 1999; Rinta-Kanto et al., 2005; Lehman, 2007; Xu et al., 2008) led to a joint Government-University planning process for Uruguay to become self-sufficient in performing microcystin assays to enable characterization and understanding of the problem.

Cyanobacterial blooms along the Montevideo coast are a complex phenomenon influenced by several factors which are not fully understood (Feola et al., 2009; Sienra and Ferrari, 2006). When the discharge of the Uruguay River is very high (close to 10,000 m^3/s), the salinity of the Rio de la Plata is decreased and scums are found on the beaches along the entire coast of the Rio de La Plata. We can hypothesize that cyanobacterial accumulations are formed mostly in the favorable conditions existing in the numerous dams of the basin and are transported by heavy rainfall, thereby reaching the water of the Rio de la Plata. Conversely, during conditions of decreased rainfall we would anticipate decreased transport of scums from the Uruguay River basin and fewer blooms in the Rio de

Table 1
Reports of cyanobacterial scums in the Río de la Plata.

Date	Site	μg MC/L	LD50 mg kg^{-1} (mouse bioassay)	μg MC g^{-1} dry mass	Species	Cyano-bacteria (cell ml^{-1})	Reference
1981	Río de la Plata	–	–	–	Ma	–	Carp-Shin-Sohma (1990)[a]
03/16/92	Montevideo	–	–	–	Ma	–	(Ferrari and Méndez, 2000)
01/27/94	Montevideo – P. del Este	–	–	–	Ma	–	(Ferrari and Méndez, 2000)
01/30/97	Colonia	125	233.3	–	Ma	–	(Ferrari and Méndez, 2000)
03/29/97	P. del Este	–	203.1	–	–	–	(Ferrari and Méndez, 2000) (Méndez and Ferrari, 2002)
02/02/99	Colonia	–	–	100–1000	Ma	–	(De Leon and Yunes, 2001)
01/05/01	Ramírez	–	–	–	Ma, As, Ac	161	Ferrari (2001)[b]
	Pocitos	–	–	–	Ma, Ac	6040	
	Carrasco	–	–	–	Ma, As, Ac	15,300	
01/11//01	Ramírez	–	–	–	Ma, Mw, Mfa	6.5×10^5	Ferrari (2001)[b]
01/12//01	Trouville	250	–	132	Mfa, Ma	2×10^9	(De Leon and Yunes, 2001)[c]
	Ramírez	97	–	194	Mfa, Ma	4.5×10^6	
	P. Carretas	1150	–	48	Mfa, Ma	1.6×10^{10}	
01/19//01	Ramírez	–	–	–	Mfa	2.5×10^7	Ferrari (2001)[b]
01/30//01	Ramírez	–	–	–	Mfa, Ma, Mn, Mw, Ac, As	–	Ferrari (2001)[b]
01/03//02	Buceo (port)	290	–	619	Mfa, Mp	1×10^9	De León and Vidal (2002)[c]
02/19/03	Ramírez	26	–	–	Msp	–	De León (2003)[c]
	Pocitos	12	–	–	–	–	Ferrari (2003)[b]
01/27/04	Pocitos	–	–	–	Ma, Mfa, As	1.3×10^8	Sienra and Piaggio (2004)[d]
02/04//04	Ramírez	2440	–	–	Msp	–	Sienra and Ferrari (2004)[d]
11/19/04	Buceo	40	–	–	Ma	4×10^5	(Ferrari and Méndez, 2004)
01/09/06	Ramírez	1900	–	–	Ma	1.2×10^8	(Feola et al., 2009; Ferrari and Vidal 2006)
12/22//06	Pocitos	20,000	–	–	Ma	1.2×10^7	(Feola et al., 2009; Ferrari and Vidal, 2006)
03/06/08	Pocitos	30,000	–	–	Ma	4.4×10^6	(Feola et al., 2009, Ferrari)[b]

Abbreviations: Msp – *Microcystis* sp., Ma – *M. aeruginosa*, Mfa – *M. flos-aquae*, Mn – *M. novacekii*, Mp – *M. panniformis*, Mw – *M. wessenbergii*, As – *Anabaena spiroides*, Ac – *Anabaena circinalis*, Av –*Anabaena vigueri*.

[a] Unpublished report, Comission of the Rio de la Plata (Carp-Shin-Sohma).
[b] Unpublished report, National Direction of Aquatic Resources (DINARA), Ministerio de Ganadería y Agricultura, Uruguay.
[c] Unpublished report, Limnology Section, Faculty of Science Universidad de la República, Uruguay.
[d] Unpublished report, Laboratory of Environmental Hygiene, Municipality of Montevideo, Uruguay.

la Plata. Thus, in the summer season of 2008–2009, the most severe drought in the last 20 years, no blooms were observed in Montevideo (Fig. 2).

4.2. Development of local capacity for microcystin analysis in Uruguay

4.2.1. Selection of assay methodology

In addition to benefits of cost and simplicity, ELISA offers major advantages such as high sensitivity and broad cross-reactivity with different microcystin congeners. Indeed, with over 80 known chemical congeners of the microcystins, but detailed toxicological studies only available for the LR variant (which is the most toxic form known), an analytical technique that lumps (rather than splits) the total microcystin content is convenient for a conservative risk assessment. As shown in Table 2, the antibody used in our assay exhibits a broad spectrum of cross reactivity with microcystin congeners.

4.2.2. Assay standardization

To facilitate calculations, instead of the classical ELISA 4-parameter sigmoidal curve, a semi-log linear equation was tested. In the range of 0.2–2.5 μg/L of microcystin, the semi-log linear regression showed a very good fit ($R^2 \geq 0.99$). The assay range is comparable to that obtained with several commercial assays and it is adequate for analyzing drinking water (WHO guideline value 1 μg/L microcystin LR (Chorus and Bartram, 1999) as well as recreational water (Table 3)).

The successful use of ELISA for monitoring water quality requires the standardization of reagents and conditions to ensure reproducible results. The stability of kit reagents (antibodies and standards) and of assay plates were evaluated. To characterize our ELISA methodology we performed a statistical analysis of 43 independently run calibration curves performed over the period of one year with fresh kit reagents. Average data for each standard and for the equation parameters are presented in Table 4. During our detailed analysis of these results we observed an apparent higher slope for the data collected during the summer months of the year tested than for the other times tested. This observation suggested the possibility of a determinant error in these specific assays, possibly related to batch-to-batch variation in the assay reagents used. This effect, though statistically significant, was small (*F*-statistic: 3.586 on 2 and 40 DF, *p*-value: 0.03696). These data were included in our analyzes of the stability of the slope and the intercept (Table 4), and increase the reported standard deviation in the assay. Additional analyzes designed to look for periodicities and autocorrelations in the data did not find any such potential sources of error. We also determined that the assay kit reagents were stable when stored in the refrigerator for one year. The observed curve parameters after 1 year of reagent storage at 4 °C were: $m = -19.41$, $b = 32.82$ are within one standard deviation of the average parameters reported in Table 4, so the use of the ELISA plates and reagents give results that are reproducible over time and the reagents are stable for at least 1 year of refrigerator storage.

During routine use control charts were used to track assay performance and evaluate trends that might be occurring over time. Acceptability limits for the curve parameters *m* and *b* were defined as the mean plus/minus two standard deviations. In each plate at least one control standard of 1 μg/l was determined in triplicate and the acceptability criterion for the average result of this control was in the range of 0.8–1.2 μg/L.

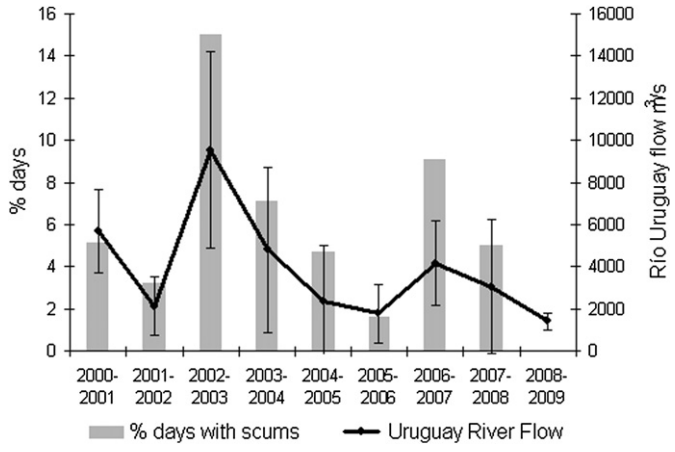

Fig. 2. Frequency of cyanobacterial scums in Montevideo (Rio de la Plata) *vs.* Uruguay River flow (average of each summer season).

Table 2
Cross reactivity of the ELISA assay for available microcystin variants.

Microcystin variant	IC 50[a] μg/L	% Cross reactivity
LR	0.32 ± 0.03	100
RR	0.45 ± 0.05	68
YR	0.45 ± 0.03	68
LW	0.60 ± 0.02	52
LF	0.42 ± 0.03	77

[a] IC50: Concentration at the midpoint of the inhibition ELISA curve for each variant (experimental details in Methods Section).

4.2.3. ELISA performance in water of the Rio de la Plata

In order to check method performance, seven representative beach water samples taken from different locations in the absence of blooms, with salinities in the range 1–27 (Practical Salinity Scale) were evaluated. Samples were treated as described in the Methods section and spiked with MC-LR at three levels: 0.5, 1, and 2 μg/L (low, medium and high range of the calibration curve). Thirty replicate samples were then measured in ELISAs performed over three consecutive days. The inter-plate reproducibility was better than 30%, and accuracy (calculated from the recovery) was between 83 and 95% over the range of concentrations studied. The limit of detection, calculated as three standard deviations of the lowest standard tested (0.5 μg/L), was determined to be 0.3 μg/L.

4.3. Monitoring results from the Rio de la Plata in Montevideo, 2003–2009

We classified the occurrence of cyanobacteria in three categories, "Absent", "dispersed" and "scums", as described before. The great majority of the samples of the first category have undetectable levels of microcystin (<0.3 μg/L, the limit of detection for the assay) with occasional (less than 20% of the total) samples at the detection limit of the assay. No samples in the category of "cyanobacteria absent" were observed with significantly higher levels of microcystin than the detection limit of the ELISA (0.3 μg/L).

In the second category, identified as "dispersed", the observed values of microcystin ranged from non detected to 65 μg/L in Pocitos (3/6/08) and 58 μg/L in Zabala (3/20/04), but the vast

Table 3
WHO guideline values for recreational water.[a]

Probability of adverse health effects	MC (μg/L)	Chlorophyll a (μg/L)	Cell count (cells/mL)
Relatively low	2–4	<10	2.0 E + 04
Moderate	5–20	10–50	<1.0 E + 05
High	>20	>50	>1.0 E + 05

[a] Adapted from: Chorus and Bartram, 1999.

Table 4
Average parameters from 43 ELISA calibration curves performed within one year.

						$y = m\,(\ln x) + b$	
Concentration of MC (μg/L)	0.2	0.6	1.0	1.5	2.5	*m*	*b*
% Ao	70.8	46.3	35.7	29.4	22.2	−19.54	37.92
SD	5.5	5.3	5.1	4.6	4.73	1.59	4.80
% (RSD)	7.8	11.5	14.2	15.7	21.3	8.1	12.7

Abbreviations used: SD, standard deviation; RSD, relative standard deviation; MC, microcystin. % Ao, percentage of absorbance of the zero concentration (Ao).

majority of microcystin values (94.4%) were in the range of non detected to < 20 μg/L leaving only a minor fraction above the moderate risk limit recommended by WHO (Table 3). The microcystins: chlorophyll-a ratio was highly variable, with a maximum value of 7.6 observed on a single sample on February 2007. The minimum toxicity rates were found in the period December–January 2008, when no microcystins were detected despite the relatively high values of chlorophyll-a.

Finally, in the category of cyanobacterial blooms called "scums" (Table 1, Supplementary material) we observed microcystin values that ranged from 17 to 20,000–30,000 μg/L. The majority of microcystin values found were in the range of 100 to 4000 μg/L. Thus, the presence of cyanobacterial scums was strongly associated with very high concentrations of microcystins in the water. While we will report below that there was a reasonably good correlation between cells per mL or chlorophyll-a measurements and microcystin content on average in these experiments, if we look more closely at individual samples in the "scum" category we can see that the correlations are not consistent at the individual sample level. The variability of toxicity per biomass unit is easily observed when the microcystin:chlorophyll-a ratio is evaluated (Table 1, Supplementary material); this ratio ranged from 0.01 (very low toxicity rate) in November–December 2004 to a value of 11 in December 2006 and March 2008. There was also high variability between cyanobacterial cell numbers and microcystin content at the individual sample level in these scums.

4.4. Aggregated correlations between various measurements

With grouped chlorophyll-a and microcystins data over a 6-year period (Table 5) there are no significantly different increases in chlorophyll-a content and microcystin content as we go from water in which cyanobacteria were not detected visually to water in which disperse colonies were observed ($p > 0.05$, ANOVA, Tukey's correction). These results also indicate that chlorophyll-a would not be a good indicator of the presence of cyanobacteria in the intermediate category (disperse colonies). When scums were observed, there were very large (and significant, $p < 0.001$) increases in both chlorophyll-a and microcystin content, but there was also a very large standard error observed in these values.

When we evaluate the individual samples using all the data, which is dominated by the results when there were dispersed cyanobacterial colonies present, a third of the variance (R^2 value) of the log of the chlorophyll content is explained by the log of the microcystin concentration, and 60% of the variance of the log of the chlorophyll content is explained by the log of the microcystin concentration. If we focus on evaluating only the individual samples during scums, we do not find a linear relationship between either the cyanobacterial cell content or the chlorophyll-a content and the microcystin concentration. On a log–log scale, a linear model shows that only 18% of the variance (R^2 value) in the microcystin concentration can be explained by variations in the chlorophyll content, and only 30% of the variance in the microcystin concentration can be explained by variations in the cyanobacterial cell content. Thus, the correlations become progressively weaker as we preferentially examine the samples with higher concentrations of microcystin present. This observation is biologically reasonable in that the highest concentrations of microcystin tend to be observed in water samples during and subsequent to lysis of large numbers of cells and because microcystins are very stable chemically and are broken down very slowly in the environment (Chorus and Bartram, 1999).

Table 5
Chlorophyll-a and microcystin levels in water samples from the Rio de la Plata.

	Not detected	Dispersed	Scum
Chlorophyll a (2003–2009)			
N	332	172	25
Average	8.8	18.5	3500
Standard error	20.0	28.8	1900
Microcystins (2003–2009)			
N	94	107	28
Average	nd	3.9	3300
Standard error	–	9.7	1900

N: number of samples.

4.5. Risk management approach

In a monitoring program for algal blooms in recreational water, it is critical to rapidly classify the potential risk of the blooms. To this end, beaches and the surrounding water were visually monitored throughout the summer seasons for detecting the presence of either dispersed cyanobacterial colonies ("dispersed") or accumulated colonies ("scums"). Recreational use of the beaches in the summer is a major attractant for tourism so there is a great interest to maintain the beaches open and accessible. When no cyanobacterial colonies were observed the beaches were kept open, and as reported above, microcystin levels in all of the samples tested were consistent with this being a sound decision from a public health perspective (microcystin levels <0.3 μg/L). On the other hand, when scums were observed, microcystin concentrations were in the range of "high probability of adverse health effects" in 27/28 (96.4%) of the samples tested and in these cases people are warned not to swim. These same samples would also have been scored as "high probability of adverse health effects" by their chlorophyll-a content or cell counts.

The samples classified as "dispersed" are the most critical for decision making. Fig. 3 shows the microcystin and chlorophyll-

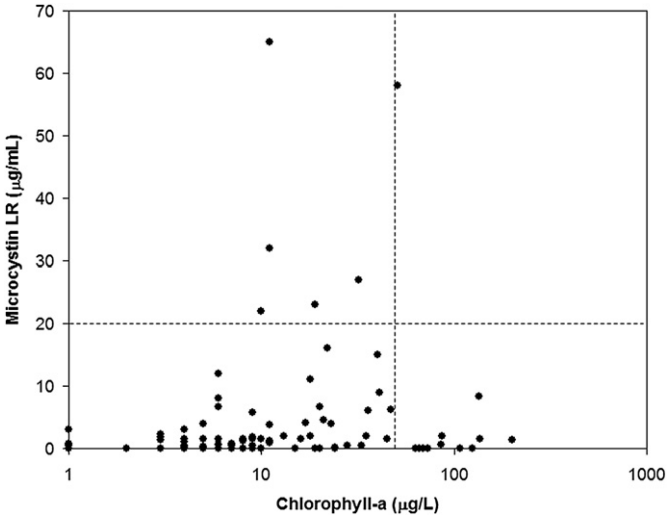

Fig. 3. Microcystins *vs.* chlorophyll-a in the samples of the "dispersed" category. The WHO guideline risk limits for microcystin-LR (20 μg/L) and chlorophyll-a (50 μg/L) are shown as dashed lines.

a content of the individual samples in this category. As mentioned before, following the WHO guideline for moderate risk (<20 μg/L of microcystin-LR equivalents), only 6 out of the 107 samples (5.6%) will indicate that the water is not suitable for recreational use, and so availability of the microcystins results in a timely fashion helped guide decisions as to when to close beaches for swimming and bathing in order to protect human health. On the other hand, using the chlorophyll-a WHO guideline value for high risk (50 μg/L) only one of these 6 samples would have been correctly classified, while the rest would have been wrongly considered harmless. In addition, 11 of the "dispersed" category samples (10%) presented high chlorophyll-a results (>50 μg/L) that would have wrongly led to closure of the beaches.

4.5.1. Chlorophyll a and cyanobacterial count as risk indicators

We classified samples in which at least two of the three parameters—total cells, chlorophyll-a, and microcystin concentration—were measured, as containing low, medium or high levels of each component evaluated according to the WHO guideline values. When we examined the 21 samples where both total cyanobacterial cells and microcystin concentration were measured, we observed agreement between 19/21 of the samples. The two samples that did not agree were both cases of medium cells, low microcystins, suggesting that assessment of risk by quantification of total cell number alone would cause the beaches to be closed more often than is necessary. For the 23 samples where we had data for both total cell number and chlorophyll content, the values were in agreement 20 out of 23 times. For the three samples where these measurements did not agree, there were 2 times where chlorophyll values were high and total cells were medium, and once chlorophyll was medium and cells were low suggesting that chlorophyll a could overestimate the cyanobacterial cell count present. However, these conclusions are preliminary due to the low number of cell counts available in the intermediate category.

When we examined 220 samples where both total chlorophyll and microcystin concentration were measured, we observed agreement between 155/220 of the samples. In the intermediate category of risk (15 samples with microcystin in the range 4–20 μg/L) the chlorophyll values underestimated the true microcystin content in 30% of the cases. To summarize the main conclusions, in Table 6 we compare the predictive values, specificity and sensitivity of chlorophyll determination with visual detection of scums, as indicators of samples with high probability of adverse health effects (microcystin concentration higher than 20 μg/L). Both tests show similar sensitivity (about 80%) and negative predictive values (97%). Interestingly, the visual detection test shows much higher positive predictive values (96.0 *vs.* 67.6% for chlorophyll) and higher specificity (99.5 *vs.* 93.7% for chlorophyll).

5. Discussion

The occurrence of cyanobacterial scums in the Rio de la Plata was first observed more than 30 years ago (1981). *Microcystis*, the dominant genera in the Rio de la Plata produce microcystins, potent liver toxins identified as a threat to human and animal health for more than 100 years (Francis, 1878).

Our first question was whether we could design and implement an ELISA-based monitoring program to quantify microcystin concentrations in the Rio de la Plata during harmful cyanobacterial blooms. The answer to this question is a very strong yes, and this is a strong argument for the utility of this approach for routine monitoring purposes in developing countries based upon the low assay cost per sample and the lack of necessity for sophisticated analytical equipment. We have supplied assay kits and training for technicians in regulatory agencies and/or drinking water purveyors in Uruguay, Paraguay, Perú and Guatemala, and for the binational (Argentina–Uruguay) FrePlata Project (Environmental Protection of the Río de la Plata [http://www.freplata.org]).

Our second question was what concentrations of microcystins were present in the Rio de la Plata beaches of Montevideo during the blooms in the summer months? As shown in Table 5 they are very high during scums, and can occasionally exceed WHO guidelines for recreational water use during the presence of dispersed colonies of cyanobacteria (Fig. 3). Thus, a program for rapidly measuring microcystin levels in these beach water should be an integral part of any strategy for appropriate protection of public health.

Our third question was whether measurements of cyanobacterial cells or of chlorophyll-a can be used as surrogate measurements to estimate the actual amount of the cyanobacterial toxins present. The correlation between cells per mL or chlorophyll-a, and microcystins tends to be best at the lower levels of chlorophyll-a and microcystins, and to break down in the samples with very high concentrations of these components. The lack of correlation between chlorophyll-a and microcystin concentrations has been reported before (Ha et al., 2011). The use of either cyanobacterial count or chlorophyll as risk indicators would cause the beaches to be closed more often than necessary and in the case of chlorophyll

Table 6
Predictive values, specificity and sensitivity of chlorophyll-a determination *vs.* visual detection as indicators of recreational water health risk.

		High probability of adverse health effects (WHO) MC > 20 μg/L (high MC)		
		Positive	Negative	
Chlorophyll test	Positive Chlo >50 μg/L	True Positive High Clo/High MC 25	False Positive High Chlo/MC < 20 12	→ Positive predictive value 67.6%
	Negative Chlo ≤50 μg/L	False Negative Low Chlo/High MC 5	True Negative Low Chlo/MC < 20 178	→ Negative predictive value 97.3%
		↓ Sensitivity = 83.3%	↓ Specificity = 93.7%	
Visual detection test (scum detection)	Positive (scum)	True positive Scum/MC > 20 24	False positive Scum/MC < 20 1	→ Positive predictive value 96.0%
	Negative (no scum)	False Negative No scum/MC > 20 6	True Negative No scum/MC < 20 189	→ Negative predictive value 96.9%
		↓ Sensitivity = 80.0%	↓ Specificity = 99.5%	

Positive predictive value = TP/(TP + FP); negative predictive value = TN/(FN + TN).
Sensitivity = TP/(TP + FN); specificity = TN/(FP + TN).

there is also a high percentage of false negatives so it would not be protective for public health.

It is noteworthy that the visual detection of scums showed much higher positive predictive value and better specificity than chlorophyll–a (Table 6). Therefore, the criterion locally adopted for beach management, that is, a) warn not to swim in scums and, b) keep the beaches open in the presence of dispersed colonies unless microcystin analysis indicates high risk, seems appropriate for achieving a balance between protecting public health and keeping the beaches open for tourism.

Our fourth, and final, question was whether we could identify seasonal or other factors that are responsible for fluctuations in the extent of harmful cyanobacterial blooms and production of microcystins in the Rio de la Plata. Hydrological records show evidence of an increase both in rainfall and runoff in the La Plata Basin after 1970. El Niño events have also had an impact on stream flows in the basin. In the middle section of the Paraná River, the four largest discharges on record followed the four El Niño events of 1905, 1982–1983, 1992, and 1998. In a study to be reported elsewhere (manuscript in preparation), we will present monitoring data from the Rio de la Plata for 2009 and 2010 (La Niña and El Niño years, respectively) that suggest that flow rates of the Uruguay River into the Rio de la Plata, with concurrent impacts on river salinity, nutrient content, and turbulence, are a critical determinant of the occurrence of harmful cyanobacterial blooms on and around the beaches of Montevideo. The actual mechanisms connecting water flow rate and microcystin production in the Rio de la Plata are not well understood. It is known that salinity can affect cyanobacterial growth. In addition, some strains produce as much as 2- to 4-fold more toxins at high phosphorus levels, and non-nitrogen fixing species such as *Microcystis* and *Oscillatoria* produce more toxins under nitrogen-rich conditions. *Microcystis* produced higher concentrations of microcystins when aggregated in a stationary state under conditions of low growth rate (Ha et al., 2011). Further studies will be required to determine which of these factors are critical in the causation of harmful cyanobacterial blooms in the Rio de la Plata.

6. Conclusions

Limited analytical capacity in developing countries may hide serious environmental problems, hampering risk assessment and management as well as the establishment of preventive and remediation measures. Local development of a microcystin ELISA made possible the establishment of a sustainable monitoring program that revealed peaks of extremely high concentrations of toxins, and demonstrated the poor value of commonly used predictive parameters for risk evaluation. Moreover, our results show that a simple and practical criterion, the visual detection of scums, can be used as a front line element for rapid beach management decisions, complemented with easy and fast laboratory methods.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2012.10.052.

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