Importance of strain type to predict the toxicological risk associated with *Microcystis aeruginosa* blooms: comparison of Microtox® analysis and immunoassay

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ABSTRACT

The occurrence of toxic cyanobacterial blooms in aquatic environments, associated with human health problems and animal deaths, has increased the need for rapid, reliable and sensitive methods to determine the toxicity of microcystin produced by cyanobacteria. An *in vitro* Microtox® system and a commercially available microcystin ELISA were used to screen out the potential risk associated with selected *Microcystis aeruginosa* strains (Ma1D–Ma8D). Results showed the existence of three differentiated groups in the selected *M. aeruginosa* strains. Strains Ma7D and Ma6D were determined to be very toxic, strains Ma2D, Ma1D and Ma5D as moderately toxic and strains Ma8D, Ma4D and MA3D as non-toxic. These results agreed with the microcystin concentration values obtained by immunoassay. Although the data obtained by other authors clearly show that Microtox® is not sensitive to microcystins, our results suggested that this bioluminescence assay may prove useful in the preliminary screening of cyanobacterial blooms for microcystin-based toxicity. Additionally, the combination of immunodetection and toxicity-based Microtox® provides a useful addition to the methods already available for detection of cyanobacterial toxins.

Key words | environmental risk, immunoassay, *Microcystis aeruginosa* strains, Microtox®, toxigenicity

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INTRODUCTION

Some species and strains within the freshwater cyanobacterial (blue-green algal) genera *Microcystis*, *Oscillatori*, *Anabaena* and *Nostoc* are known to produce cyclic heptapeptide liver toxins (Carmichael 1997). Microcystins are the most common group of hepatotoxins. To date, over 80 structural variants have been characterized from field samples or isolated strains (Krüger *et al.* 2009). Cyanobacterial toxins can cause illness and death in animals such birds and fish (Codd *et al.* 1989; Carmichael 1992). Consumption of both cyanobacteria and water containing released toxins may cause poisoning. The lethal dose depends on the type of cyanobacterial toxins and species of cyanobacteria, as well as on the age, weight and gender of the exposed organism (Palus *et al.* 2007).

Toxic cyanobacterial blooms in waterbodies used for recreation and drinking constitute hazards to human health by skin contact and ingestion (Falconer 1989). Weather factors, such as temperature (15–30 °C), windless weather and water pH (pH 6–9), also play an important role in the formation of algal blooms (Palus *et al.* 2007). Up to 50% of natural blooms reported in many European countries are related to freshwater cyanobacterial species. Of these, at least 25% were considered toxic (WHO 1999).

One of the first difficulties encountered in confirming a toxic algal bloom is distinguishing it from a non-toxic one. This is because the same species may produce both toxic and non-toxic strains (Volterra *et al.* 2006). Environmental factors may affect microcystin production in *Microcystis* cultures by a factor of 3 to 4 (Sivonen & Jones 1999). However, the capability for microcystin production as such is genetically determined. Strains isolated from the same bloom

sample are constitutively microcystin producing or non-producing (Long *et al.* 2001).

With this premise, Carrillo *et al.* (2003) investigated 26 strains of *Microcystis aeruginosa*, isolated from two water-supply reservoirs in Andalusia (Southern Spain) and three lagoons from Doñana National Park, and their results suggested a significant variation in the production of microcystin among different populations. Rico *et al.* (2006) developed a model to estimate genetic and phenotypic variance in 21 strains of *M. aeruginosa*, and their results suggest that environment may modulate strain mixtures in *Microcystis* populations.

Many tests have been proposed to detect the presence of algal biotoxins. Cyanobacterial toxicity testing has relied on intraperitoneal mouse bioassays; however it is now necessary to develop additional rapid, reliable and sensitive methods. To date, a range of methods have been identified, including a cytotoxicity assay (Chong *et al.* 2000; Huang *et al.* 2009), immunoassay (Fischer *et al.* 2001; Long *et al.* 2009) and high-performance liquid chromatography (HPLC) (Mathys & Surholt 2004; Barco *et al.* 2005). However, the cytotoxicity, immunoassay and HPLC methods all need considerable development, special equipment or skills, and are time consuming.

In vivo bioluminescence assays, involving *Vibrio fischeri* have been introduced as a rapid, reproducible and relatively inexpensive means of toxicity assessment. Since its introduction, the Microtox® assay, based upon *V. fischeri* bioluminescence, has been used to assess the toxicity of a wide range of aquatic pollutants (Watanabe 1997; Beg & Ali 2008).

The purpose of this work is to verify the usefulness and sensitivity of Microtox® as a first aid instrument in detecting cyanobacterial blooms that cause toxicological risk in aquatic environments and might interfere in the potabilization process.

MATERIAL AND METHODS

Experimental organisms

Experiments were carried out with eight selected strains of *M. aeruginosa* from the algal culture collection of Veterinary College, Complutense University, Madrid, Spain. These strains had been collected from Doñana National Park. Isolation procedures and culture methods were as described by Carrillo *et al.* (2003). They are named as Ma1D to Ma8D.

Cells were grown axenically in cell-culture flasks with 20 ml of BG-11 medium (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), at 20 °C and a photon irradiance of 60 μmol m^{-2} s^{-1} over the wavelengths 400–700 nm, in a 16:8 h light:dark photoperiod. Cells were maintained in mid-log exponential growth by serial cell transfers to fresh medium. Prior to the experiments, the culture cells were re-cloned (by isolating a single cell) to assure genetic homogeneity in all the cultures.

The cultures for each of the *M. aeruginosa* strains included in the study, with an initial cell density of 10^4 cells ml^{-1}, were kept in 20 ml cell-culture flasks until they reached a concentration sufficient to obtain inocula of 20×10^6 cells ml^{-1}. Four replicates of each culture were included in the study. To standardize the sampling procedure, all strains were cultured at the same time, and the cell inocula were obtained simultaneously. Based on the methodology of Pietsch *et al.* (2001) to obtain cyanobacterial crude extract, and thus study the effect induced by the presence of free toxin in the culture medium, homogenates corresponding to 20×10^6 cells ml^{-1} of each *M. aeruginosa* strain were obtained by freezing (-40 °C) and thawing (three times) and subsequent incubation in a 40 W ultrasonic water bath (Sonicor SC52, New York, USA) for 30 min. Finally, these homogenates were filtered with a 0.2 μ polycarbonate filters, and then each filtrate from each of the cyanobacterial strains (seven strains, four filtrates per strain) was used simultaneously in both assay methods.

Enzyme-linked immunosorbent assay

A commercially available ELISA kit (EnviroGard™ Microcystins Plate Kit, Strategic Diagnostic Inc., Newark DE, USA) was used. At the beginning of the investigation homogenates previously obtained from each strain of *M. aeruginosa* were extracted and assayed using the manufacturer's protocol. Microcystin detection with the kit is based on the action of polyclonal antibodies. The spectrophotometric analysis of microcystins was performed by

measuring the absorbance at 450 nm with a spectrophotometer BOECO S20 (Boeckel Co., Hamburg, Germany). Calculation of results was done manually using a semilogarithmic function by plotting the results of calibration and the negative control.

According to the manufacturer's instructions, the negative control, calibration samples and samples for analysis should be added to the wells and the microcystin-enzyme conjugate added immediately. To improve the ELISA protocol, all samples were added to the wells first and incubated at 25 °C for 30 min. After preincubation, the microcystin-enzyme conjugate was added to the wells and all samples incubated for 30 min. The other steps in the assay were performed as recommended by the manufacturer. Statistical calculations (*t*-test, confidence intervals) were performed using the computer software package GraphPad Prism v4.0 (Graph-Pad Software Inc., USA), and the differences were considered significant at $p < 0.05$.

Microtox® basic test

The Microtox® basic test was carried out using the manufacturer's protocol (Microbics Corporation, Carlsbad, USA). Briefly, a range of culture filtrate dilutions from 45 to 0.56% was made in solvent supplied by the manufacturer. For the reactions, freeze dried *V. fischeri* were reconstituted with 0.01% sodium chloride and 10 ml was mixed with 500 μl of each culture filtrate dilution. A Microtox® Model 500 Analyser (AZUR Environmental, Carlsbad, CA, USA) was used to measure the luminosity from the reconstituted bacteria after 5 and 15 min of exposure to culture filtrate. The luminescence inhibition after a 15 min exposure was taken as the endpoint (Kaiser 1998; Froehner *et al.* 2000). A 2% sodium chloride solution was used for bacterial regeneration, sample dilution and control. The osmotic control was made with 22% sodium chloride osmotic adjusting solution.

In the Microtox® test, the inhibition of light emission was measured in relative units of luminescence. The data were used to calculate the EC_{50}, which is the mean sample concentration that causes a 50% reduction in bacteria bioluminescence (Chen & Que Hee 1995; Guzzella *et al.* 1996). The behavior of the bacteria was tested with reference toxins $ZnSO_4.7H_2O$ and phenol, according to

normative AFNOR T90-320 (AFNOR 1991). Toxicity values are the average of four replicates of each filtrate sample, expressed as EC_{50}-15 min with 95% confidence limits.

RESULTS

Microcystin values obtained by ELISA and the 15 min-median inhibitory concentration ($IC_{50(15)}$) values, based on Microtox® assay procedure for all *M. aeruginosa* strains, are shown in Table 1. Microtox® results are shown on an effective concentration scale based on a 50% response, where 1% will be the most toxic value and ≥100% will be the least toxic value. Analysis of these results indicates the existence of three differentiated groups, according to the toxic potential exhibited by the cell homogenates from the *M. aeruginosa* strains.

According to the Microtox® effective concentration levels of toxicity proposed by Bennett & Cubbage (1992), cell homogenates of Ma7D and Ma6D strains with $IC_{50(15)}$ values of 19.98 and 26.65% respectively, could be considered as very toxic; cell homogenates of Ma2D, Ma1D and Ma5D strains with $IC_{50(15)}$ values of 48.11, 55.46 and 63.71% respectively, could be considered as toxic–moderately toxic; and cell homogenates of Ma8D, Ma4D and MA3D strains with $IC_{50(15)}$ values greater than the concentration tested in each sample are considered as non-toxic.

Table 1 | Comparison of microcystin values, expressed in μg/L, and 15 min-median inhibitory concentration ($IC_{50(15)}$) values with associated 95% confidence limits (CL), expressed in % v:v, of homogenates corresponding to 20×10^6 cells/ml from selected *Microcystis aeruginosa* strains

| Strains	No.	EnviroGard® Microcystin (μg/L)	Microtox® $IC_{50(15)}$ CL (95%). (% v:v)
Ma7D	4	46.21 ± 3.666	19.98 (16.41–23.54)
Ma6D	4	35.08 ± 2.746	26.65 (24.69–28.46)
Ma2D	4	3.00 ± 0.005[a,b]	48.11[a,b] (40.68–56.48)
Ma1D	4	2.00 ± 0.001[a,b]	55.46[a,b] (44.89–71.84)
Ma5D	4	2.00 ± 0.001[a,b]	63.71[a,b] (61.87–64.01)
Ma8D	4	0.02 ± 0.003[a,b,c,d,e]	≥100
Ma4D	4	0.01 ± 0.001[a,b,c,d,e]	≥100
Ma3D	4	–	≥100

[a,b,c,d,e]Significant differences ($p < 0.05$) from microcystin concentration and median inhibitory concentrations for cell homogenates of Ma7D, Ma6D, Ma2D, Ma1D, and Ma5D strains, respectively.

The concentration–response relationships obtained for each of the *M. aeruginosa* strains are shown in Figure 1. One-way ANOVA analysis showed that there are significant differences among the three strain groups obtained by the Microtox® assay, but there are no significant differences among the strains within each group.

A comparison between the results obtained by the Microtox® test and ELISA (EnvirogGard®) indicate that there was correlation between the two assays. The highest microcystin concentration values were obtained in cell homogenates of strains Ma7D ($46.21 \pm 3.66\,\mu g\,L^{-1}$) and Ma6D ($35.08 \pm 2.74\,\mu g\,L^{-1}$), which were considered as very toxic in Microtox® tests; moderate microcystin concentration values were detected in cell homogenates of strains Ma2D ($3.00 \pm 0.005\,\mu g\,L^{-1}$), Ma1D ($2.00 \pm 0.001\,\mu g\,L^{-1}$) and Ma5D ($2.00 \pm 0.001\,\mu g\,L^{-1}$), which were considered as moderately toxic in Microtox® tests; the lowest microcystin concentration values were obtained in cell homogenates of strians Ma8D ($0.02 \pm 0.003\,\mu g\,L^{-1}$), Ma4D ($0.01 \pm 0.001\,\mu g\,L^{-1}$) and Ma3D ($0.00\,\mu g\,L^{-1}$), which were considered as non-toxic in Microtox® tests.

DISCUSSION

Bacterial bioassays have been investigated to determine if they can provide simple routine methods for cyanotoxin detection. The one that has received the most attention is the Microtox® bioluminescence assay which indicates toxicity by a reduction in the light emitted by the test
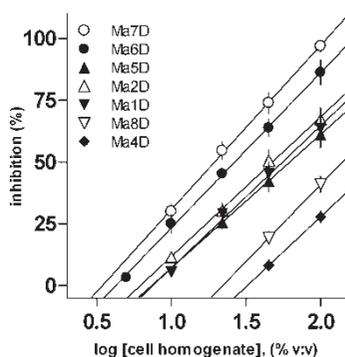
Figure 1 | Concentration–response relationships of cell homogenates corresponding to the selected *Microcystis aeruginosa* Ma7D (○), Ma6D (●), Ma5D (▲), Ma2D (△), Ma1D (▼), Ma8D (▽), and Ma4D (◆) strains, obtained by Microtox® Test. Points represent means ($n = 4$), vertical lines show SD mean.

bacterium (*V. fischeri*). Initial investigations suggested that this system may be suitable for the rapid detection of microcystins in bloom samples (Lawton *et al*. 1990), although more detailed analysis revealed that the assay responded to unknown components of cyanobacterial extracts rather than microcystins (Campbell *et al*. 1994).

Several studies clearly indicate there is no correlation between response in the Microtox® assay and cellular content of the known cyanotoxins (Lawton *et al*. 1994; Vezie *et al*. 1996). In contrast, other authors have found a relationship between Microtox® toxicity and harmful algae proliferation (Bruno *et al*. 1990, 1994; Derby *et al*. 2003). These differences in the results obtained by Microtox® test could be due to the wide variability of toxic substances dissolved in water, but also could be accounted for by their low toxicity for *V. fischeri* or to the chemistry of toxins, because only hydrosoluble toxins were tested by this methodology. In addition, Microtox® toxicity detection may be limited because not all phytoplankton toxins affect the viability of the tested bacteria in the same way (López-Flores *et al*. 2010).

However, when speed in obtaining results is of paramount importance, Microtox® represents the toxicological test of preference. Similarly, previous studies performed in our laboratory demonstrated the possible use of the Microtox® bioassay to obtain preliminary evaluation of toxigenicity of selected isolates of *Aspergillus fumigatus*, because it provides a fast measure of toxic compounds secreted by the microorganism (Alba *et al*. 2009). The results obtained in the present work suggest that this idea may be applied to the identification of blooms of *M. aeruginosa* strains, classifying them according to the potential toxicological risk.

These results are in agreement with those obtained by other authors, who examined toxic and non-toxic strains obtained from cyanobacterial blooms (Tarczynska *et al*. 2001; Nalecz-Jawecki *et al*. 2002) and obtained substantial differences between the two types of strains, with respect to the toxicological risk for microcystins.

The sensitivity and time requirements of the Microtox® bioassay were also compared with those of the immunodetection procedure (EnvirogGard®) in this report. When compared to the toxicity results obtained for each cyanobacterial strain with those obtained with immunodetection

procedure, it seems that the sensitivities and time requirements of the two are similar, and therefore we conclude that the results of the Microtox® bioassay coincide well with those of the EnvirogGard® test. However, the combination of these two detection methods should provide the validation which is required for both determination of the toxicity of an environmental sample and specific identification of the *M. aeruginosa* strain detected.

In summary, for rapid evaluation of the potential risk caused by blooms of specific *M. aeruginosa* strains, the combination of ELISA and toxicity-based Microtox® provides a useful addition to the methods already available for detection of cyanobacterial toxins.

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