

Biodegradation of Microcystin-LR by Natural Bacterial Populations

Pathmalal M. MANAGE^{1,2}, Chritine EDWARDS² and Linda A. LAWTON²

¹*Department of Zoology, University of Sri Jayewardenepura,
Gangodawila, Nugegoda, Sri Lanka*

²*School of Pharmacy and Life Sciences, The Robert Gordon University,
Aberdeen, U.K. AB25 1HG*

(Received 8 January 2009; accepted 10 March 2009)

Abstract—Microcystin-LR is a potent mammalian toxin which is known to have been responsible for the deaths of domesticated animals, and consequently there is concern as to its environmental fate. An experiment was designed to ascertain the degree of biodegradation of microcystin-LR, by enrichment of natural microbial populations from Loch Rescobie, Forfar Loch and river Carron, Scotland under aerobic conditions. It has been shown that the microcystin-LR degraded at different half-life ($D_{1/2}$) in Lochs Rescobie (6.5 d), Forfar Loch (12.5 d) and river Carron (>14 d). Out of 30 bacterial isolates, 12 strains from Forfar Loch, 9 strains from Loch Rescobie, and 9 strains river Carron were isolated. Three strains from Forfar Loch, 4 strains from Loch Rescobie and 2 strains from river Carron showed strong MC-LR degradability when they were screened by Biolog MT plate using three different concentrations of MCLR ($10 \mu\text{gml}^{-1}$, $1.0 \mu\text{gml}^{-1}$ and $0.1 \mu\text{gml}^{-1}$) as sole carbon source.

Keywords: blue-green algae, algal toxins, microcystin-LR, biodegradation

INTRODUCTION

Microcystin-LR is a toxic cyclic heptapeptide which may be produced by some strains of blue-green algae such as *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc*, and they are extensively associated with toxicoses (Zurawell *et al.*, 2005). The stable cyclic structure of these peptide toxins present many challenges to conventional water treatment facilities which have limited capability for the removal of microcystins (Himberg *et al.*, 1989). Deaths of domestic animals, following the ingestion of toxic blue-green algal scums from algal blooms and human health problems have demonstrated that under certain circumstances, very high concentrations of the toxin are produced that can be lethal. Thus, potential chronic toxicity from microcystins led the WHO to establish a guideline value of $1 \mu\text{g L}^{-1}$ as the maximum concentration of microcystin-LR in drinking water (WHO, 1998). Therefore, information regarding the persistence of this toxin is necessary to make decisions on the use of water bodies for drinking and

recreational purposes and the modality of water treatment following blue-green algal blooms.

Significant advances in water treatment technologies over the last two decades provided solutions for efficient removal of these toxins (Lawton and Robertson, 1999). However, these facilities are expensive to implement and maintain, and their efficiency may decrease under conditions of high carbon load as in highly eutrophic water bodies. Water treatment costs combined with water scarcity and increasing water demand present a huge problem in the developing world where populations are frequently exposed to cyanobacterial toxins amongst other organic and microbial contaminants. Rapala *et al.* (1994) recorded that MC-LR degrade over a 30 day period and more rapidly if the inocula were from locations with history of cyanobacterial blooms. Thus, harnessing microbes seems to be one of the most successful solutions to remove cyanotoxin. One simple, low cost and effective water treatment technology receiving current attention is the use of slow sand filters and biofilms which utilizes the selected biodegrading bacteria to complement the natural microbial flora of the filter for improved removal, providing a low cost solution for the provision of safe potable water (Babica *et al.*, 2005; Bourne *et al.*, 2006; Ho *et al.*, 2006; Tsuji *et al.*, 2006).

This paper presents results from an investigation on the microbial degradation of microcystins LR in three Lochs of Scottish waters where water chemistry and cyanobacteria bloom histories were different. Classical enrichment method was used to isolate bacteria previously shown to contain microbial flora capable of microcystin degradation.

MATERIAL AND METHODS

Samples with a range of microcystin exposure histories were collected in September 2006 for MC-LR degradation experiment. Surface water samples from Loch Rescobie, Forfar Loch and River Carron were collected in 1 L sterilized glass bottles and transferred within six hours to the laboratory and stored at 4°C overnight until analysis. Each water sample was passed through a metal sieve (150 µm mesh; Endecotts Ltd., London, UK) to remove zooplankton and vegetation. Samples were processed and analyzed by HPLC-diode array to determine the presence of naturally occurring microcystins (Lawton *et al.*, 1994). MC-LR was extracted from *M. aeruginosa* PCC 7820 (Pasteur Institute, Paris, France) and purified by semi-preparative HPLC as previously described (Lawton *et al.*, 1995). MC-LR was re-suspended in Milli-Q water (0.1 mg mL⁻¹) and sterilized by passage through a 0.2 µm filter (Dynaguard, Fisher, UK).

Biodegradation of MC-LR

Water samples from Loch Rescobie, Forfar Loch and River Carron, north east Scotland were prepared individually by placing 50 ml of freshly collected water in 100 ml sterile Erlenmeyer flasks closed with cotton wool bungs. One µg mL⁻¹ of sterile MC-LR was added to each flask aseptically. 50 ml of sterile controls and test samples were autoclaved in 100 ml Erlenmeyer at 121°C for 15

minutes. Triplicate samples were prepared for all samples and controls. Flasks were incubated at constant temperature (29°C) and shaken at 100 rpm during the experiment period. Aliquots (1 ml) were removed from each flask under sterile conditions at 2 d intervals and frozen immediately (-20°C). The frozen samples were freeze-dried, reconstituted in 80% aqueous methanol (200 µL) and centrifuged at 15,000 × g for ten minutes. The supernatant was removed and analyzed by HPLC (Christne *et al.*, 2008). Half life was calculated as the duration for removal of 50% of the toxin(s) from the start of each experiment.

Isolation of microcystin-degrading bacteria

After 14 d enrichment experiment, one ml of water sample was taken aseptically from each flask from Loch Rescobie (R), Forfar Loch (F) and the river Carron (C). Pour plate method was employed to isolate bacteria (LB agar media; -9.1 g l⁻¹ Tryptone, 4.6 g l⁻¹ Yeast extract, 4.6 g l⁻¹ NaCl, 13.7 g l⁻¹ Agar). Ringer's solution (Oxoid Ltd, UK) was used to make serial dilutions and 1 ml of tenfold serially diluted samples (up to 10⁻⁵) were mixed with 20–25 ml of 1.5% molten LB agar and poured onto sterilized Petri dishes and incubated in the dark at 25°C for 5 days, after which colonies of differing morphologies were re-suspended in liquid LB medium and a pure culture was obtained by repeated streaking (Yamamoto and Suzuki, 1990) onto LB agar (1.5%) plates. The cultures were then subjected to a Gram stain and the bacterial isolates were sub cultured and kept in LB agar slants for further degradation studies and characterization.

Screening of the microcystin-degrading bacteria by Biolog MT2 Plates

The Biolog MT2 plate was used to screen the ability of the isolated bacteria to metabolize MC-LR. This technique is more commonly used for identification and community analysis where specific carbon sources are provided and active metabolism results in the production of a colored product. Each bacterial isolate was tested against three different concentrations (10 µg l⁻¹, 1 µg l⁻¹ and 0.1 µg l⁻¹) of MC-LR in triplicate. These concentrations were chosen because they are in the range between the low, WHO recommended concentrations of microcystin-LR and high environmental levels in natural waters following blue-green algal blooms. A loop of each bacterial strain was transferred to 5 ml liquid LB medium and incubated in dark over night at 25°C. The exponentially growing cultures were then washed twice by centrifugation at 1000 × g for 15 min with re-suspension of bacterial pellets in sterile 0.01M PBS. Then the cultures were incubated at 25°C for 24 hours to let out residual carbon content. Turbidity of all cell suspensions was equalized (A₅₉₀ = 0.35) using spectrophotometer (Pharmacia biotech Nova Spec II). Adjustment was made by addition of bacterial suspension incubated overnight or saline solution.

MC-LR was added to Biolog MT2 plates (Technopath, Ireland) in triplicate to give final concentrations of 10, 1 and 0.1 µg ml⁻¹. Control wells contained sterilized saline solution.

All wells were inoculated with prepared suspensions of test bacteria (150

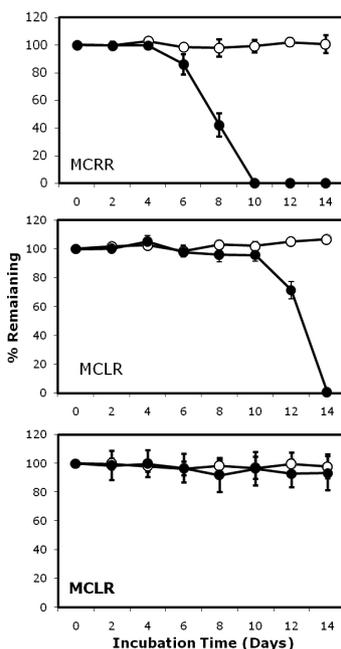


Fig. 1. Percentage of MC-LR in sterile controls (○) and incubated water samples (●) from Loch Rescobie (A), Forfar Loch (B) and the River Carron (C). Standard errors are displayed ($n = 3$).

μl). Plates were incubated at 25°C in plastic containers having moist paper towel to maintain humidity. Absorbance was recorded using microplate reader at 595 nm wavelength (Dynex technologies) immediately after inoculation which was considered as zero hours then at 3, 6, 15, 18, 24 and 48 hours.

RESULTS AND DISCUSSION

No microcystin (extra- or intracellular) was detected in any of the water samples collected in October 2006, confirming that those compounds detected were those added under experimental conditions.

All three water samples were shown to contain microbes capable of degrading the MCLR since no considerable change was detected in the sterile control (Fig. 1). There were significant differences in the rate of degradation; the half-life ($D_{1/2}$) of MC-LR in Lochs Rescobie was 6.5 d (Fig. 1A); Forfar Loch 12.5 d (Fig. 1B) and River Carron was more than 14 d (Fig. 1C). This data is consistent with bloom history of Loch Rescobie that frequently supports microcystin-containing blooms (Richard *et al.*, 1983). Forfar Loch, is eutrophic and has no record of toxic blooms; there have been no reports on blooms in the fast flowing River Carron. Those water bodies with no previous history of microcystin contamination, Forfar and Carron showed a notable lag period before degradation commenced.

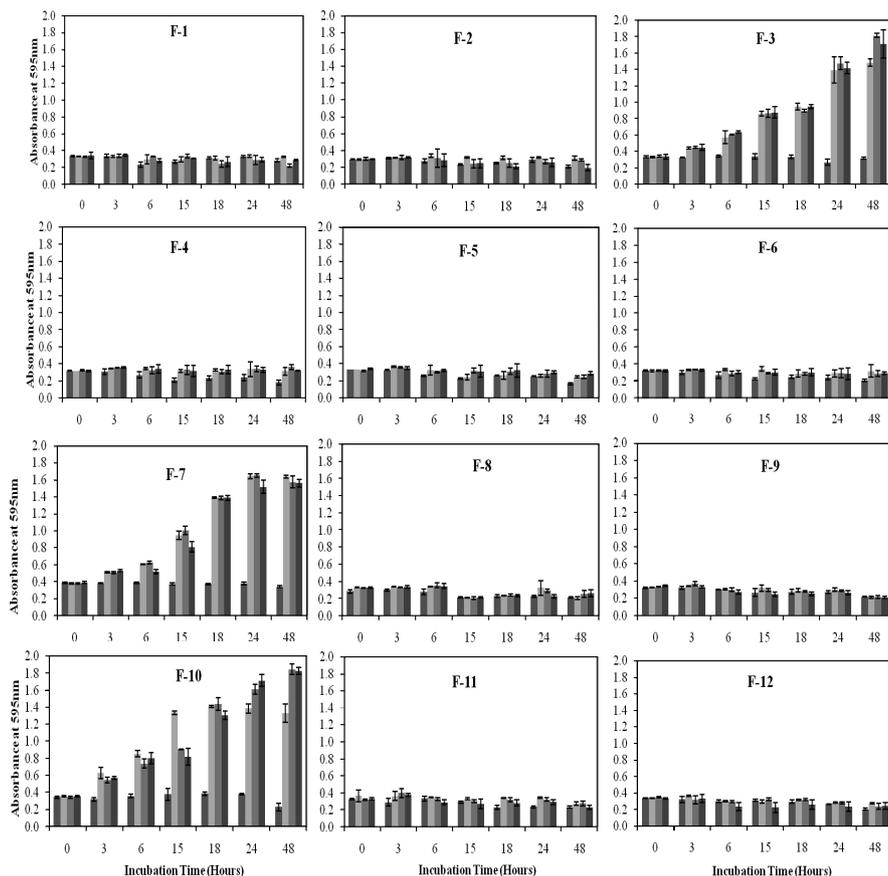


Fig. 2. Biolog screen of MC-LR utilization by bacteria isolated from Forfar Loch during 0 to 48 h incubation. (Control, $0.1 \mu\text{g}/\text{ml}^{-1}$, $1 \mu\text{g}/\text{ml}^{-1}$ and $10 \mu\text{g}/\text{ml}^{-1}$ at each incubation are shown from left to right.) Control has no addition of carbon source. MC-LR was added as the carbon source at concentrations $0.1 \mu\text{g}/\text{ml}^{-1}$, $1 \mu\text{g}/\text{ml}^{-1}$ and $10 \mu\text{g}/\text{ml}^{-1}$. Error bars represent standard deviations from triplicate measurements.

The results presented by Christine *et al.* (2008) on the half-life MC-LR degradation in Loch Rescobie (4 d), Forfar Loch (9 d) and the river Carron (13 d) were different from the present study which may be due to presence of different bacterial communities and water chemistry at the time of sampling. Previous studies have shown that past exposure to microcystins results in considerably faster degradation rates in natural waters (Christoffersen *et al.*, 2002). In contrast, the half-life of MC-LR degradation in River Carron was more than 14 d and it was similar to the slower rates recorded in water from Finnish lakes with no previous occurrence of microcystin-producing blooms (Rapala *et al.*, 1994). Analysis of the sterile controls showed no loss of MC-LR (Fig. 1) but a slight increase of

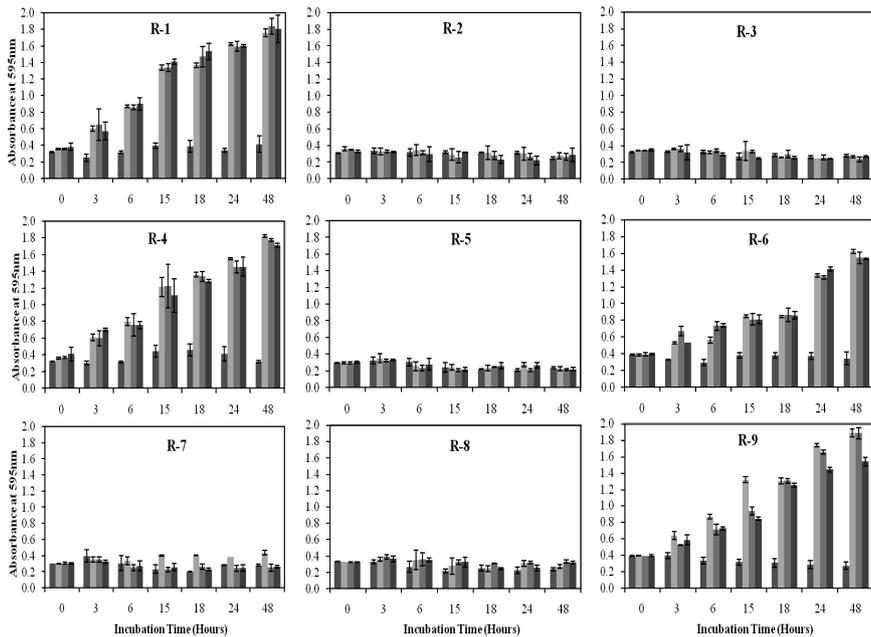


Fig. 3. Biolog screen of MC-LR utilization by bacteria isolated from Loch Rescobie during 0 to 48 h incubation. (Control, $0.1 \mu\text{g/ml}^{-1}$, $1 \mu\text{g/ml}^{-1}$ and $10 \mu\text{g/ml}^{-1}$ at each incubation are shown from left to right.) Control has no addition of carbon source. MC-LR was added as the carbon source at different concentrations $0.1 \mu\text{g/ml}^{-1}$, $1 \mu\text{g/ml}^{-1}$ and $10 \mu\text{g/ml}^{-1}$. Error bars represent standard deviations from triplicate measurements.

MCLR due to evaporation during the experiment confirming that the observed degradation was due to the microbial populations already present in the water samples.

A total of 30 bacterial strains; 9 from Loch Rescobie, 12 from Forfar Loch and 9 from River Carron were isolated two weeks after the enrichment experiment. Different types of colonies were isolated from the Petri dish on the basis of size, color and morphology, and used for subsequent experiments. All bacterial isolates were found to be Gram negative. Full microbial identification was not performed and it was decided to elucidate all isolates for their ability to degrade microcystin-LR before any detailed classification was made. Those bacterial strains and sterile control (milli-Q, Autoclaved 121°C , 15 min) were screened by exposing to three different concentrations of MCLR of 10, 1 and $0.1 \mu\text{g/ml}^{-1}$ using BIOLOG MT plate. Metabolism of the substrate in particular wells results in the formation of formazan, producing color change of the tetrazolium dye. BIOLOGMT2 microplates contain the redox chemicals without any substrate and hence allow the use of specific substrates like MCLR. Three isolates from Forfar Loch (F3, F7 and F10) were active and the isolate F7 and F10 showed very

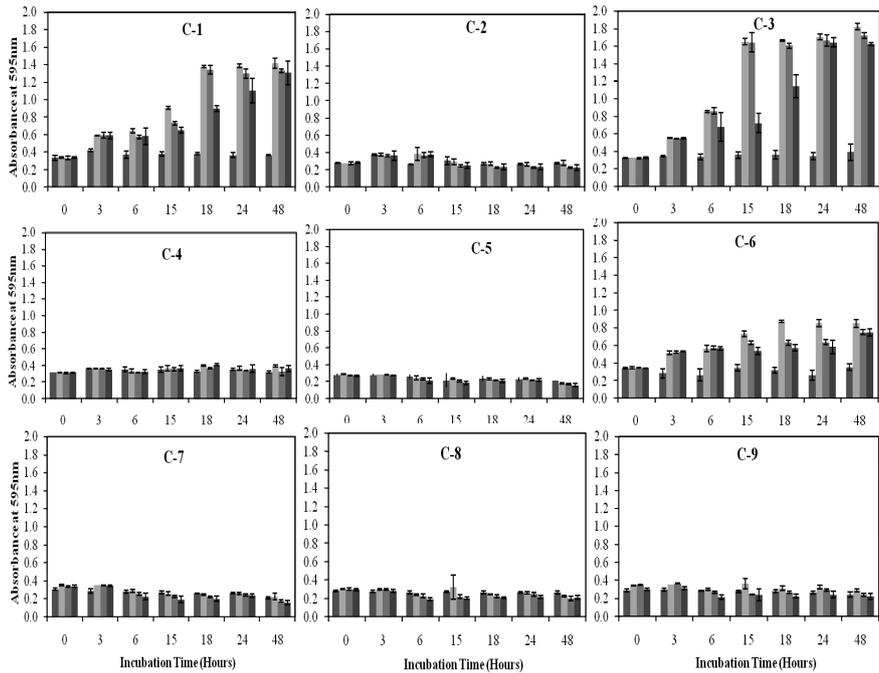


Fig. 4. Biolog screen of MC-LR utilisation by bacteria isolated from the River Carron during 0 to 48 h incubation. (Control, 0.1 $\mu\text{g/ml}^{-1}$, 1 $\mu\text{g/ml}^{-1}$ and 10 $\mu\text{g/ml}^{-1}$ at each incubation are shown from left to right.) Control has no addition of carbon source. MC-LR was added as the carbon source at different concentrations 0.1 $\mu\text{g/ml}^{-1}$, 1 $\mu\text{g/ml}^{-1}$ and 10 $\mu\text{g/ml}^{-1}$. Error bars represent standard deviations from triplicate measurements.

pronounced responses of the utilization of a high concentration of MCLR (Fig. 2). Interestingly 9 bacterial isolates from Loch Rescorbie which has a long history of the occurrence of microcystin-producing blooms gave little to no metabolic increase with the exception of R1, R4, R6 and R9 (Fig. 3). In fact, some of the isolates might have experienced inhibition of metabolism in the presence of MCLR. Three isolates from River Carron (C1, C3 and C6) metabolized MCLR as the sole carbon source (Fig. 4). Thus, the present observation is worthy of further research and it would be interesting to observe inhibition of responses with alternative carbon sources. Therefore, it was clear that the isolates responded differently at a range of concentrations at different time intervals (Haack *et al.*, 1995; Garland, 1996; Winding and Hendriksen, 1997). The ability to degrade MCLR appears to be widespread and not dependent on prior exposure. Microcystins were degraded by microbes in all water samples, but not significant in the River Carron water. This suggests that different microbes with differing degradation capabilities exist in different habitats. As seen by retarded degradation in Loch Rescorbie and enhanced degradation in Forfar Loch it may be suggested that

MCLR has a significant influence on the degradation by natural microbial population. The identification of bacterial biodegradation intermediates further supports the hypothesis that there is a much broader array of micro-organisms and/or enzymatic pathways capable of degrading microcystins than previously known. Characterization of microcystin degrading bacteria from three Lochs are currently in progress using traditional and molecular approaches, together with further elucidation of the degradation products and assessment of their stability and toxicity.

The presence of cyanobacteria throughout the world has been well recognized as a major problem in water treatment and risk to zooplankton, aquatic and terrestrial plants, animals and human being. Although a large variety of toxins have been identified, their function, toxicity and stability are still unclear and remain to be determined.

In the present study, it is very clear that Biolog MT2 plates are useful as a quick and easy method for screening active isolates utilizing microcystin-LR as sole source of carbon or energy. The use of Biolog MT2 plates provides the capability of selecting microbial system adapted to metabolite conscripted chemicals as their sole carbon and/or energy source. Several concentrations of the target chemical may be added to the same plate so that a range of concentrations can be tested. Simultaneously, this result indicates that Biolog MT2 plates are useful tools for screening bacterial isolates and consortia for their ability to survive, metabolize and potentially degrade selected organic chemicals.

The findings of this study have clearly shown that various genera of bacteria are able to degrade microcystin-LR. In order to elucidate the microbial degradation of microcystin-LR in water, we focused on the synergistic and individual activities of the microflora in natural water. It was found that the degradation was slow in case of the isolates in their original condition but high when exposed to microcystin-LR exposed individual isolates. It is concluded that complete degradation of microcystin-LR was observed by natural microflora from the aquatic environment which implies that degradation of microcystin-LR is possible but the rate of degradation was slow in natural water environment. So far, most of the researches centered on only a few bacteria such as *Sphigomonas*, *Pseudomonas*, *Paucibacter* sp. and *Sphingosinicella* sp. But there are many bacterial species which can degrade MC-LR more effectively than those studied. It was detected that biodegradation is the most effective and inexpensive way of destruction of microcystin-LR in water so operational and viable approaches should be exercised to minimize the risk of cyanotoxins.

Acknowledgments—The work was supported by the grants from the Leverhulme Trust Fund UK. The authors would like to thank the School of Pharmacy and Life Sciences, The Robert Gordon University, UK for their full support throughout the study period.

REFERENCES

- Babica, P., L. Bláha and B. Marsálek (2005): Removal of microcystins by phototrophic biofilms. *Environ. Sci. Pollut. Res.*, **12**, 369–374.

- Bourne, D. G., R. L. Blakeley, P. Riddles and G. J. Jones (2006): Biodegradation of the cyanobacterial toxin microcystin LR in natural water and biologically active slow sand filters. *Water Res.*, **40**, 1294–1302.
- Christine, E., G. Douglas, F. Nicholas and A. L. Linda (2008): Biodegradation of microcystins and nodularin in freshwaters. *Chemosph.* (in print).
- Christoffersen, K., S. Lyck and A. Winding (2002): Microbial activity and bacterial community structure during degradation of microcystins. *Aquat. Microbial. Ecol.*, **27**, 125–136.
- Garland, J. L. (1996): Analytical approaches to the characterization of sample microbial communities using patterns of potential C source utilization. *Soil Biol., Biochem.*, **28**, 213–221.
- Haack, S. K., H. Garchow, M. J. Klug and L. J. Forney (1995): Analysis of factors affecting the accuracy, reproducibility and interpretation of microbial community carbon source utilization patterns. *Appl. Environ. Microb.*, **61**, 1458–1468.
- Himberg, K., A. M. Keijola, L. Hiisvirta, H. Pyysalo and K. Sivonen (1989): The effect of water treatment processes on the removal of hepatotoxins from *Microcystis* and *Oscillatoria cyanobacteria*: a laboratory study. *Water Res.*, **23**, 979–984.
- Ho, L., T. Meyn, A. Keegan, D. Hoefel, J. Brookes, C. P. Saint and G. Newcombe (2006): Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Res.*, **40**, 768–774.
- Lawton, L. A. and P. K. J. Robertson (1999): Physico-chemical treatment methods for the removal of microcystins (cyanobacterial hepatotoxins) from potable waters. *Chem. Soc. Rev.*, **28**, 217–224.
- Lawton, L. A., C. Edwards and G. A. Codd (1994): Extraction and high performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst.*, **119**, 1525–1530.
- Lawton, L. A., E. Christine, K. A. Beattie, S. Pleasance, G. J. Dear and G. A. Codd (1995): Isolation and characterization of microcystins from laboratory cultures and environmental samples of *Microcystis aeruginosa* from an associated animal toxicosis. *Nat. Toxins.*, **3**, 50–57.
- Rapala, J., K. Lathi, K. Sivonen and S. I. Niemela (1994): Biodegradation and absorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. *Lett. Appl. Microbiol.*, **19**, 423–426.
- Richard, D. S., K. A. Beattie and G. A. Codd (1983): Toxicity of cyanobacterial blooms from Scottish freshwaters. *Environ. Technol. Lett.*, **4**, 377–382.
- Tsuji, K., M. Asakawa, Y. Anzai, T. Sumino and K. Harada (2006): Degradation of microcystins using immobilized microorganism isolated in a eutrophic lake. *Chemosphere*, **65**, 117–124.
- Winding, A. and N. B. Hendriksen (1997): Biolog substrate utilization assay for metabolic fingerprints of soil bacteria: incubation effects. In *Microbial Communities: Functional versus Structural Approaches*, ed. by H. Insam and A. Rangger, 195 pp.
- World Health Organization (1998): Guidelines for drinking water quality, 2nd ed. Addendum to vol. 2. Health criteria and other supporting information. World Health Organization, Geneva, p. 95–110.
- Yamamoto, Y. and K. Suzuki (1990): Distribution and algal-lysing activity of fruiting Myxobacteria in Lake Suwa. *J. Phycol.*, **26**, 457–492.
- Zurawell, R. W., H. Chen, J. M. Burke and E. E. Prepas (2005): Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. *J. Toxicol. Environ. Health.*, **8**, 1–37.