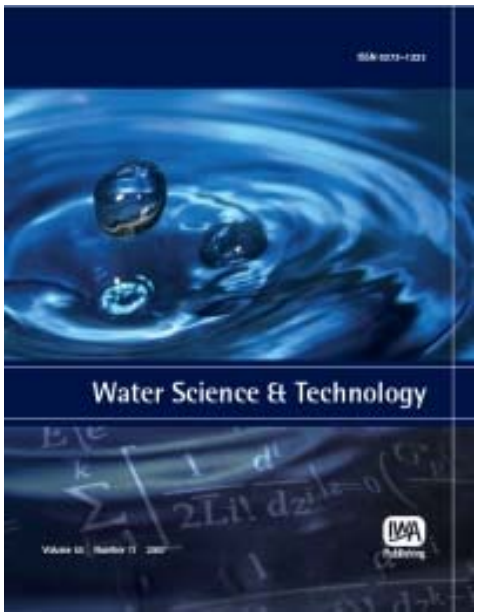

# Novel bacterial strains for the removal of microcystins from drinking water

L. A. Lawton, A. Welgamage, P. M. Manage and C. Edwards

## ABSTRACT

Microcystins (MC) and nodularin (NOD) are common contaminants of drinking water around the world and due to their significant health impact it is important to explore suitable approaches for their removal. Unfortunately, these toxins are not always removed by conventional water treatments. One of the most exciting areas that hold promise for a successful and cost effective solution is bioremediation of microcystins. Recent work resulted in successful isolation and characterisation of 10 novel bacterial strains (*Rhodococcus* sp., *Arthrobacter* spp. and *Brevibacterium* sp.) capable of metabolizing microcystin-LR (MC-LR) in a Biolog MT2 assay. The work presented here aims to further investigate and evaluate the metabolism and the degradation of multiple microcystins (MC-LR, MC-LF, MC-LY, MC-LW and MC-RR) and nodularin by the bacterial isolates. A total of five bacterial isolates representing the three genera were evaluated using Biolog MT2 assay with a range of MCs where they all demonstrated an overall metabolism on all MCs and NOD. Subsequently, the results were confirmed by observing the degradation of the range of toxins in a separate batch experiment.

**Key words** | abstract, algal toxins, blue-green algae, cyanobacteria, cyanotoxins

**L. A. Lawton** (corresponding author)
**A. Welgamage**
**C. Edwards**
Institute for Innovation, Design, and Sustainability Research,
Robert Gordon University,
Aberdeen, AB25 1HG,
UK
E-mail: *l.lawton@rgu.ac.uk*

**P. M. Manage**
Department of Zoology,
University of Sri Jayewardenepura,
Gangodawila,
Nugegoda,
Sri Lanka

## INTRODUCTION

Cyanobacteria are one of the most successful and widespread living organisms on earth. They can be found in almost all aquatic environments and many terrestrial environments. Blooms of cyanobacteria occur in late summer or early autumn in temperate countries, but in warmer climates, they can grow year round (Mur *et al.* 1999; Oliver & Ganf 2000). Blooms of cyanobacteria are often toxic, producing a wide range of potent hepatotoxic and neurotoxic secondary metabolites (Sivonen & Jones 1999). Toxic cyanobacteria have been reported from every continent of the world. The most commonly occurring cyanotoxins, microcystins, are potent liver toxins and can increase the risk of cancer following chronic exposure. Microcystins are cyclic peptides (Carmichael 1994) making them extremely stable during typical water treatment conditions. This has prompted the search for alternative removal methods which has included isolation of microcystin-degrading bacteria. Many studies have focused on isolating bacteria from water sources exposed to microcystin producing blooms (Edwards *et al.* 2008). These studies have resulted in the isolation of a small number of bacteria (especially *Sphingomonas* sp.) capable of metabolising the microcystins.

A recent study carried out by Manage *et al.* (2009) successfully isolated a group of novel bacteria capable of rapidly degrading microcystin-LR. A novel method of rapid screening for bacterial biodegradation activity was applied. Biolog MT2 plates, a 96 well format containing a tetrazolium redox dye provide a rapid indication of metabolic activity, indicating if a bacterial isolate can use microcystin. The novel bacterial isolates have so far only been shown to degrade microcystin-LR however many microcystin variants occur. Therefore it is essential to explore a number of chemical variants. The Biolog MT2 method was implemented as a rapid tool for examining multiple bacterial strains with multiple microcystin variants along with different toxin concentrations with a view to establishing the suitability of these organisms for the removal of microcystins from drinking water.

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## MATERIALS AND METHODS

### Evaluation of the metabolism of a range of Microcystins and Nodularin by bacterial isolates using the Biolog MT2 assay

In previous studies, ten bacterial isolates from three Scottish waters were found to metabolize and degrade MC-LR (Manage *et al.* 2009). Five of these isolates, *Rhodococcus* sp. C1, *Arthrobacter* sp. C6, (River Carron); *Brevibacterium* sp. F3, *Arthrobacter* sp. F7 (Forfar Loch) and *Arthrobacter* sp. R4 (Loch Rescobie) were selected either because they were from a different genus or isolated from a different location. Isolates, maintained on nutrient agar slopes, were transferred into 8 ml of liquid nutrient broth and incubated in a shaker, (25°C, 24 hrs, and 150 rpm; Stuart scientific-Orbital incubator SI 50) in the dark. *Paucibacter toxinivorans* DSMZ-16998 (Braunschweig, Germany) was used as the positive control bacterial strain as it has been found to degrade MC-LR, MC-YR and nodularin (Rapala *et al.* 2005). The exponentially growing bacterial cultures were washed three times with 0.01 M phosphate buffered saline (PBS) by centrifugation at $3000 \times g$ for 15 min with re-suspension of the pellet in sterile 0.01 M PBS and cultures were incubated at 25°C for 24 hours to deplete residual carbon.

To allow the evaluation of the metabolism of a range of microcystins and nodularins (MC-LR, MC-LF, MC-LW, MC-LY, MC-RR and NOD) individual purified toxins was added to the Biolog MT2 plates (Technopath, Limerick, Ireland) in triplicates at a final concentration of 0.1, 1.0, 10 $\mu g\ ml^{-1}$. Before the inoculation of bacterial isolates in MT2 plates, all suspensions in PBS were adjusted to $A_{590} = 0.35$ using a spectrophotometer (Pharmacia biotech Nova Spec II). The control wells in the Biolog MT2 plate contained bacterial isolates plus sterile PBS in triplicates.

The colour development in the plate was measured with a Dynex microplate reader (Jencons, Leighton Buzzard, UK) at a wavelength of 595 nm immediately after the inoculation of bacteria (0 h) and again after 24 hours. The oxidization of the analyte (microcystin or nodularin) would result in a colour reaction taking place in the well by the reduction of the tetrazolium dye which can be measured and quantified spectrophotometrically (Garland & Mills 1991).

### Evaluation of Microcystin and Nodularin degradation by isolated bacteria

It was observed that all the selected isolates metabolized all microcystins and nodularin in the Biolog MT2 assay. Prior to further development of isolates for use in water remediation it is important to confirm that the microbes can degrade the toxins in batch shake flasks. The bacterial inoculums (C1, C6, F3, F7, R4 and *P. toxinivorans*) were prepared as before with overnight incubation (25°C, 24 hrs, 150 rpm), washing $\times 3$ with 0.01 M PBS and centrifugation at $3000 \times g$ for 15 min. The bacterial suspension was adjusted to a turbidity of $A590 = 0.35$ and 0.5 mL was added to glass universal bottles (triplicates) containing 9 ml of 0.2 μm filter sterilized water from the original location where each bacterium was isolated. Sterile, aqueous MC-LR, MC-LF, MC-LW, MC-LY, MC-RR and NOD were added under aseptic conditions at a final concentration of 10 $\mu g\ ml^{-1}$. The positive control, *P. toxinivorans* was inoculated in Rescobie water. Controls containing sterile water plus microcystin were also monitored to confirm that degradation was due to microbial activity alone. The sample bottles were incubated for 10 days at 25°C and shaken at 150 rpm. At 24 h intervals, aliquots (0.5 ml) were removed into microcentrifuge tubes (1.5 ml) under sterile conditions, frozen (−20°C) immediately and freeze dried. Samples for HPLC-MS analysis were prepared by freeze drying, reconstituted in 200 μl of 50% aqueous methanol followed by centrifuged at $15{,}000 \times g$ for 10 min. Supernatant (100 μl) was removed for HPLC-MS analysis.

### HPLC-MS analysis of Microcystins and Nodularin

Analysis of microcystins and nodularin was based on the HPLC methods described by Lawton *et al.* (1994) using HPLC-MS (Waters Alliance 2695 solvent delivery system) with photodiode array (2996 PDA) equipped with mass detector (ZQ 2000 MS) in series (Waters, Elstree, UK). The separation was performed on a Sunfire C18 column (2.1 mm i.d. X 150 mm long; 5 μm particle size) maintained at 40°C. Mobile phase consisted of MilliQ water (A) and acetonitrile (Rathburn, Walkersburn, UK) (B) both containing 0.05% trifluoroacetic acid (TFA) (Fisher Scientific, Leicestershire, UK). Samples were separated using a gradient increasing from 15% to 60% B over 25 minutes at a flow rate of 0.3 ml $min^{-1}$ followed by ramp up to 100% B and re-equilibration over the next 10 minutes. Eluent was monitored from 200–300 nm with a resolution 1.2 nm with the toxins quantified by external standard at 238 nm. Mass spectrometry analysis was performed in positive ion electro-spray mode, scanning from *m/z* 100 to 1200 with a scan time of 2 s and inter-scan delay of 0.1 s. ion source parameters; sprayer voltage, 3.07 kV; cone voltage, 80 V; de-solvation temperature, 300°C; and source temperature, 100°C. Instrumental control, data acquisition and processing were achieved using Masslynx v4.0.

## RESULTS AND DISCUSSION

### Evaluation of the metabolism of a range of microcystins and nodularin by bacterial isolates using the Biolog MT2 assay

All of the five selected isolates and the positive control were found to successfully metabolize the range of microcystins and nodularin, although there were some differences between the level of response observed for different bacteria and different toxins (Figure 1). Isolates *Rhodococcus* sp. C1 and *Arthrobacter* sp. C6 were shown to utilize most microcystins to a similar degree, although C1 indicated slightly higher utilization of MC-RR compared to the other microcystin variants. The *Arthrobacter* sp. F7 showed metabolism of all the toxin variants with the highest metabolic activity, typical absorption of over 0.8, whereas *Brevibacterium* sp. F3 and *Arthrobacter* sp. R4 demonstrated much lower metabolism (around 0.45). The positive control, *P. toxinivorans* was found to show higher utilize MC-LY (greater than 0.8 of absorption difference) compared to other microcystins. Metabolism of NOD by *P. toxinivorans* was distinctly lower (~0.3 absorbance) which is interesting since this is one of the initial toxins used in its isolation and characterisation (Rapala *et al.* 2005). Two of the isolates, one *Brevibacterium* sp. (F3) and *Arthrobacter* spp. (R4), show no notable difference in utilization of toxin variants, demonstrating a similar capability to utilize the five microcystin variants and nodularin. These characteristics might justify using these bacteria in future experiments to explore their ability to degrade a mixture of microcystins and nodularin in contaminated water. Amongst all six bacterial strains, F7 (which is an

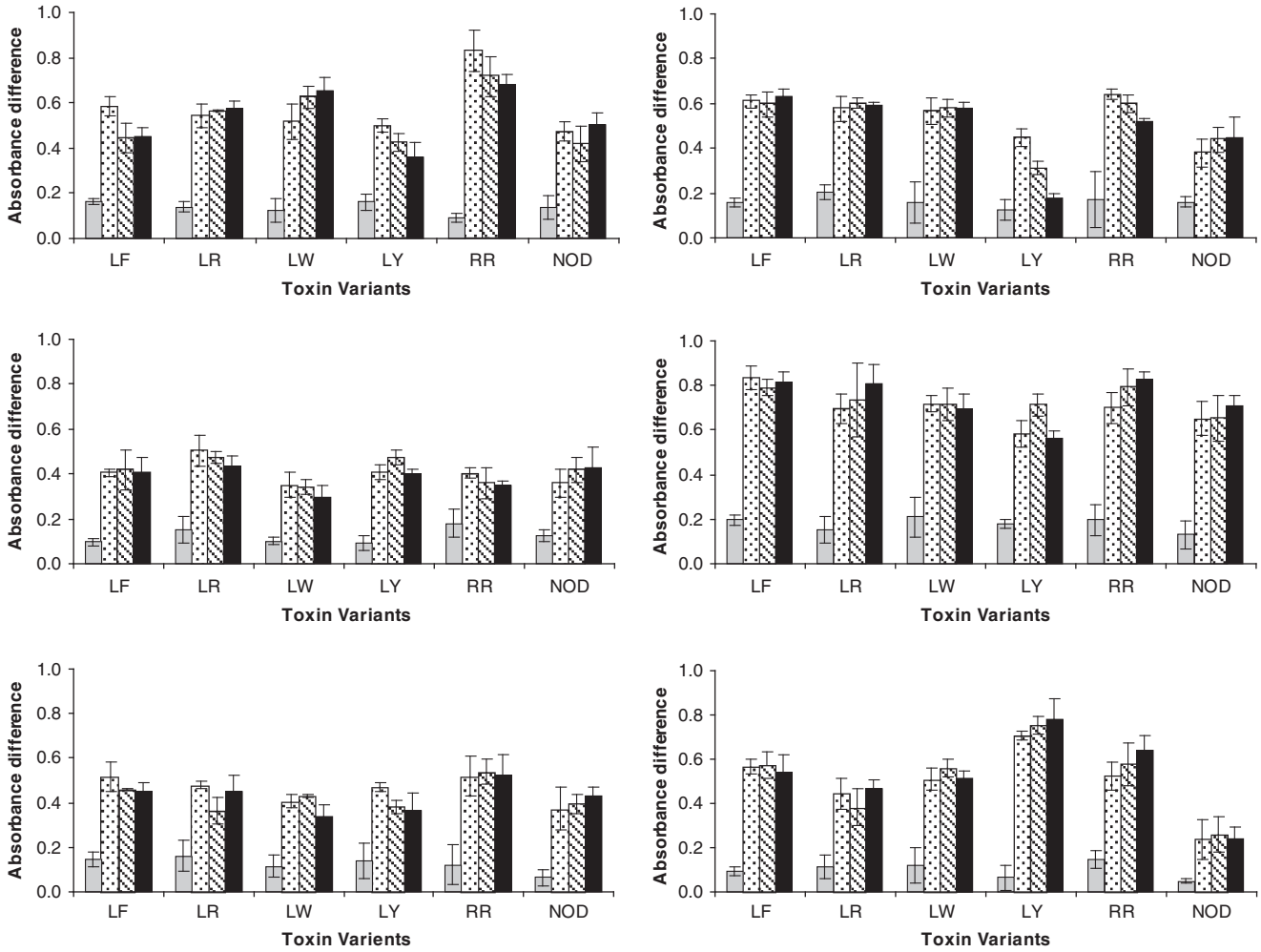


**Figure 1** | Biolog screen of MC-LF, MC-LR, MC-LW, MC-LY, MC-RR and NOD metabolism by the six bacterial isolates at 24 hours of incubation. Control (■) contains no added carbon. Final MC-LR concentrations in wells were 0.1 μg ml$^{-1}$ (⬚), 1 μg ml$^{-1}$ (▨), 10 μg ml$^{-1}$ (▩). Error bars represent one standard deviation (n = 3).

Arthrobacter sp.) has demonstrated the highest utilization of all toxin variants, confirming the previous results showing its ability source (Manage *et al.* 2009).

Cultures of *Rhodococcus* sp. (C1) and *Arthrobacter* sp. (C6) indicated slight inhibition with increasing concentrations of MC-LY whereas *P. toxinivorans* gave the best response with this toxin. The most important finding in this experiment is the diversity of bacteria that can utilize a range of toxin concentrations and toxin variants. MC-RR may be more stable (Takenaka & Tanaka 1995) with its two arginine groups however C1, C6, F7, and R4 have shown a pronounced utilization of MC-RR showing the possibility of diverse biochemical pathways available in these bacteria to utilize microcystins and nodularin. The positive control *P. toxinivorans*, which was previously reported to degrade MC-LR, MC-YR and NOD (Rapala *et al.* 2005) has shown an active metabolism in the presence of MC-LF, MC-LW, MC-LY and MC-RR but comparatively low metabolism on NOD.

This is the first report to demonstrate a wide range of bacterial isolates capable of utilizing five chemically diverse, commonly occurring microcystin variants and nodularin. So far, the number of bacterial species reported to be capable of degrading microcystins is very limited. Jones & Orr (1994), Bourne *et al.* (1996), and Saitou *et al.* (2003) have reported microcystin degradation by several strains of *Sphingomonas* sp. and were only reported to degrade a limited number of microcystin variants. Only *Sphingomonas* strain B9 and *P. toxinivorans* were recorded to degrade nodularin (Imanishi *et al.* 2005; Rapala *et al.* 2005) indicating the limited diversity of microcystin degrading bacteria available. But the present study has shown the potential capability of these bacterial isolates to utilize a wider range of microcystins and nodularin (Figure 1). Due to the high diversity of microcystin variants distributed worldwide (Dietrich & Hoeger 2005) this is a promising approach which could provide a reliable method to degrade microcystins in future water treatment strategies. The bacteria that have demonstrated "universal" microcystin degradation have better potential in such applications, compared to single variant degraders.

The Biolog experiments revealed the bacterial metabolism in the presence of different microcystins and nodularin but could not confirm that actual degradation occurred in Biolog MT2 plates. Hence, further investigation to confirm the actual degradation by these isolates was performed by HPLC-MS using the remaining samples in the wells of the Biolog plate. It was found that degradation of MC-LR had taken place in the wells of Biolog MT2 plates in which the *Rhodococcus* sp. (C1) bacteria were incubated. The characteristic degradation products of MC-LR were present in the HPLC-MS analysis (Table 1), revealing that hydrolysis had taken place as explained by Bourne *et al.* (1996).

Surprisingly, the previous studies carried out by Manage *et al.* (2009) on the same bacterial isolates have revealed the absence of *mlrA*, *mlrB* and *mlrC* genes responsible for producing hydrolysing enzymes to carry out the degradation. However the MC-LR intermediate products identified in the current study (Table 1) are similar to the MC-LR intermediate products revealed by Bourne *et al.* (1996), suggesting the possible presence of similar but not identical genes and a similar degradation pathway. This would suggest the possibility of reporting a clear degradation pathway of microcystin for the first time by the bacteria belonging to the family of *Actinobacteria* although further work is required.

## Examination of microcystin and nodularin degradation by isolated bacteria in batch experiments

All six of the bacterial isolates were shown to degrade the five microcystin variants and nodularin (Table 2). This confirmed the Biolog findings although relative degree of metabolism observed in the Biolog did not reflect the rate of degradation. It is clear from the results that the most significant factor influencing degradation is the chemistry of the compound, not the bacterial isolate itself. The two toxins which were found to degrade particularly slowly were MC-RR and NOD.

**Table 1** | The MS degradation products from *Rhodococcus* sp C1 incubated in Biolog plates showing the characteristic bacterial breakdown products (Bourne *et al.* 1996) of MC-LR

| *m/z* | Ion/Degradation product |
|---|---|
| 1013 | M + H (Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH + H) |
| 879 | M + H – $PhCH_2CHOMe$ |
| 726 | CO-Glu-Mdha-Ala-Leu-Masp-Arg-OH + 2H |
| 571 | Mdha-Ala-Leu-Masp-Arg-OH + 2H |
| 486 | Ala-Leu-Masp-Arg-OH |
| 304 | MeAsp-Arg-OH + 2H |
| 286 | MeAsp-Arg + H |
| 213 | Glu-Mdha + H |
| 175 | Arg-OH + 2H |
| 155 | Mdha-Ala + H |
| 135 | $PhCH_2CHOMe$ |

**Table 2** | Degradation half-life for five microcystin variants and nodularin incubated with individual toxins and monitored daily by HPLC to determine the amount of toxin remaining. Values of >10 indicate that degradation has occurred but the amount remaining is greater than 50%

| | Time taken to degrade 50% of microcystin or nodularin (Days) | | | | | |
|---|---|---|---|---|---|---|
| **Bacterial Isolate** | **MC-LR** | **MC-LY** | **MC-LF** | **MC-LW** | **MC-RR** | **NOD** |
| *Rhodococcus* sp. C1, | 9 | 5 | 8 | 6 | 10 | >10 |
| *Arthrobacter* sp. C6, | 9 | 5 | 8 | 8 | >10 | >10 |
| *Brevibacterium* sp. F3, | 9 | 6 | 7 | 9 | >10 | 10 |
| *Arthrobacter* sp. F7 | 9 | 6 | 6 | 7 | >10 | >10 |
| *Arthrobacter* sp. R4 | 9 | 7 | 7 | 6 | >10 | 10 |
| *P. toxinivorans* | 9 | 6 | 6 | 7 | >10 | >10 |

A possible explanation for this could be that they are structurally most different to the other four, all of which contain Leucine. MC-RR contains two arginines in the variable positions which increases its polarity and may cause steric hindrance, reducing the rate of enzymic attack. On the other hand NOD is a much smaller and tighter ring structure containing only five amino acids compared to seven in the microcystins and it has been suggested that this makes it harder to open the ring structure to facilitate degradation. Interestingly MC-LR, the most commonly detected microcystin, was also relatively slowly degraded with MC-LY being generally the most rapidly removed (Table 2).

A study carried out by Edwards *et al.* 2008 has also reported the greater stability of NOD, compared to microcystins, in a mixed population of bacteria naturally present in water from the River Carron and Forfar Loch. For example, the report indicated a considerably slower rate of NOD degradation in Loch Rescobie water where they found a complete degradation after 18 days compared to 11 days for MC-LR. In future studies it will be important to ensure that a panel of microcystins and nodularin are used to evaluate the efficacy of this application for water treatment.

It is also worth noting that degradation time for the isolates is significantly slower than when first reported (Manage *et al.* 2009) where the half-life for MC-LR was typically below 2 days with most toxin undetected at 3 days. The cultures used in the study have been maintained in lab culture for over a year and it may be that their ability to biodegrade is diminished over time. It is essential that this aspect of their behaviour be investigated fully to ensure optimum performance.

Another aspect that needs to be explored before implementing biodegradation technologies (Huang *et al.* 2008) is the availability of nutrients necessary to support bacterial growth and activity ultimately promoting rapid degradation. This in turn may influence the position of a biodegradation system in the complete water treatment process. For example, water which has undergone full treatment ready for distribution will be much lower in many nutrients than water earlier in the treatment cycle. It may be important for the toxin bioremediation system to be located early in water treatment process. It is therefore essential to develop a greater understanding of the influence of nutrients on degradation before these microbes are employed in future water treatment processes.

## CONCLUSIONS

This study reveals a number of novel findings on bacterial bioremediation of microcystins and nodularin. This is the first report on the multiple degradation of a wide range of microcystins and nodularin by a number of bacterial isolates belonging to *Actinobacteria*. All six bacterial isolates were capable of performing non-specific toxin degradation on microcystins. Previously identified *P. toxinivorans*, capable of degrading MC-LR, MC-LY and NOD, was found to degrade MC-LF and MC-LW. Further studies need to be carried out to evaluate the optimum conditions for toxin removal and the applicability of the bacterial strains to water remediation. There is high demand for an effective and low cost approach to removing cyanobacterial toxins from potable water due to the significant health risk and prevailing water scarcity. Bacterial bioremediation is a promising, low cost and potentially effective means for removing cyanotoxins (Bourne *et al.* 1996; Ho *et al.* 2007; Manage *et al.* 2009) and these novel bacterial strains may contribute to treatment strategies.

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