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# Investigations into the biodegradation of microcystin-LR in wastewaters

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ABSTRACT

Microcystins are potent hepatotoxins that can be produced by cyanobacteria. These organisms can proliferate in wastewaters due to a number of factors including high concentrations of nutrients for growth. As treated wastewaters are now being considered as supplementary drinking water sources, in addition to their frequent use for irrigated agriculture, it is imperative that these wastewaters are free of toxins such as microcystins. This study investigated the potential for biodegradation of microcystin-LR (MCLR) in wastewaters through a biological sand filtration experiment and in static batch reactor experiments. MCLR was effectively removed at a range of concentrations and at various temperatures, with degradation attributed to the action of microorganisms indigenous to the wastewaters. No hepatotoxic by-products were detected following the degradation of MCLR as determined by a protein phosphatase inhibition assay. Using TaqMan polymerase chain reaction, the first gene involved in bacterial degradation of MCLR (*mlrA*) was detected and the responsible bacteria shown to increase with the amount of MCLR being degraded. This finding suggested that the degradation of MCLR was dependent upon the abundance of MCLR-degrading organisms present within the wastewater, and that MCLR may provide bacteria with a significant carbon source for proliferation; in turn increasing MCLR removal.

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## 1. Introduction

The microcystins are a group of hepatotoxins which can be produced by cyanobacteria, including species of *Microcystis*, *Anabaena*, *Nostoc* and *Planktothrix* [1–3]. There are currently over seventy known analogues of the microcystin toxins each with a specific nomenclature. For example, microcystin-LR (MCLR) has a **L**eucine amino acid and an a**R**ginine amino acid in the variable positions. Microcystins have been detected in drinking water sources and have the potential to severely compromise human health; in some cases fatalities have been reported through excess consumption of these toxins [4]. The current guideline level for the microcystins is 1 $\mu g L^{-1}$ as MCLR-equivalents, as issued by the World Health Organization [5]. MCLR is one of the most toxic and well studied of the microcystin analogues; consequently, derivation of this guideline level evolved through the numerous toxicity studies on MCLR.

Studies have shown that the microcystins are biodegradable in water bodies and sand filters, where in many cases specific *Sphingomonadaceae* bacteria have been documented to be responsible for their degradation [6–14]. However, recent studies have reported microcystin degradation by bacteria other than the *Sphingomonadaceae*. Manage et al. [15] identified three isolates, *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp., as having the capability to degrade microcystin. Similarly, Hu et al. [16] isolated a *Methylobacillus* sp. from a cyanobacterial sludge which could effectively degrade two microcystin analogues (MCLR and microcystin-RR). Nevertheless, most of the genotypic studies on microcystin degradation have focused on the *Sphingomonadaceae* as this group of bacteria has been shown to contain the genes required for microcystin degradation.

A specific set of genes, *mlrA*, *mlrB*, *mlrC* and *mlrD*, were identified by Bourne et al. [6,7] as a gene cluster involved in microcystin degradation. The authors determined that the *mlrA* gene encoded an enzyme that hydrolytically cleaved the cyclic structure of MCLR. The resultant linear MCLR molecule was then sequentially hydrolysed by peptidases encoded by the *mlrB* and *mlrC* genes. The final gene, *mlrD*, encoded for a putative transporter protein that allowed for microcystin uptake into the cell. Since then, various studies have designed qualitative polymerase chain reaction (PCR) assays for the detection of these genes, in particular, *mlrA*, the first gene involved in cleaving the cyclic structure of microcystin [12,13,17]. More recently, Hoefel et al. [18] designed and optimized a quan-

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titative real-time PCR (qPCR) assay for the detection of the *mlrA* gene.

To date, the majority of studies investigating microcystin biodegradation have focused on drinking water, with little information in the literature regarding the biodegradation of this toxin from wastewater. Nutrient levels are considerably higher in wastewaters than drinking water sources, and with increasing ambient temperatures, there is potentially greater risk of cyanobacterial blooms [19–21]. These may result in greater levels of toxin production, including the microcystins. With the onset of climate change and drought, particularly in countries such as Australia and the United States, there is a need to seek alternative water sources, which would allow water authorities to manage water security more effectively. Treated wastewater is one of these sources; hence, there is a need to ensure that the quality of this water is of a standard that can be used appropriately. In many countries, including Australia, treated wastewater is used in the agricultural sector for irrigation purposes. As the microcystins have been reported to accumulate in crops, in addition to adversely affecting the growth of crops, it is imperative that treated wastewater is free of these toxins to ensure that exposure to humans via crop-based foods is minimized [22–24]. Consequently, there is a need to ensure that microcystins can be efficiently removed from treated wastewater and to determine whether biodegradation could be a feasible treatment option. It should not be assumed that the previous observations for drinking water can be directly translated to treated wastewaters due to the different chemical composition, matrix and microorganism populations.

The major objective of this study was to investigate microcystin degradation in treated wastewaters by the indigenous organisms present, and to determine if any potentially toxic by-products were generated in the process. Furthermore, TaqMan qPCR targeting the *mlrA* gene was used to investigate the abundance of microcystin degraders within the treated wastewaters, how these numbers changed during MCLR biodegradation and if MCLR was degraded as a primary substrate.

## 2. Experimental procedures

### 2.1. Materials and reagents

Tertiary treated effluent (TTE) water was obtained from the Bolivar wastewater treatment plant (WWTP) in Adelaide, South Australia. This effluent water was sampled prior to the sand filters at the dissolved air flotation filtration (DAFF) plant and used for a laboratory-scale sand column experiment and batch degradation experiments. The treatment processes upstream of TTE water include preliminary grit removal, primary sedimentation, secondary activated sludge treatment and detention in waste stabilization lagoons (for ~20 days) prior to the tertiary DAFF plant. In addition, activated sludge treated effluent (ASTE) water was used for batch degradation experiments. Characteristics of these waters are presented in Table 1. Both waters did not contain any levels of MCLR at the time of this study, as determined by high performance liquid chromatography (HPLC).

MCLR (≥95% purity) was purchased from a commercial supplier (Sapphire Bioscience Pty. Ltd., Australia) and dissolved in methanol and ultrapure water (Millipore Pty. Ltd., USA) to prepare a stock solution. Aliquots from this stock solution were then dosed into sample waters at the desired concentrations.

### 2.2. Laboratory sand column experiment

A biological sand filtration experiment was conducted using a laboratory-scale glass column. Sand samples, collected from the Bolivar DAFF plant, were packed into the columns (length 30 cm, I.D. of 2.5 cm) at a bed height of 15 cm. The effective particle size of the sand was 0.9 mm. The column was fed with TTE water (from the Bolivar DAFF plant) which was spiked with MCLR at a target concentration of 6 $\mu g L^{-1}$. In addition, an inlet MCLR concentration of approximately 14 $\mu g L^{-1}$ was trialed for a short period. The column was operated at an empty bed contact time of 15 min at room temperature (22 °C). Water samples were taken routinely (every 2–3 days) from the column inlet and outlet and analysed for MCLR. Sand was periodically sampled (every 2–3 days) from the top of the column bed and DNA from the biofilm extracted for *mlrA* determinations as described by Hoefel et al. [18].

### 2.3. Batch degradation experiments

Batch experiments were conducted to assess the degradation of MCLR by indigenous microorganisms within the treated wastewaters (TTE and ASTE). In these experiments, 2 L or 5 L Schott Pyrex bottles, containing MCLR-spiked treated wastewaters, were adopted as reactors. In addition, parallel control reactors containing sterilized (autoclaved at 121 °C for 15 min) treated wastewater were prepared to assess losses of MCLR due to physical processes. The reactors were spiked with MCLR at specific concentrations (6 $\mu g L^{-1}$ or 20 $\mu g L^{-1}$) and incubated aerobically at temperatures of either 10 °C or 22 °C with continuous stirring. Samples were taken aseptically from the reactors at regular intervals (every 1–3 days) for HPLC, protein phosphatase 2A (PP2A) and qPCR analyses.

### 2.4. Analysis of microcystin

Prior to HPLC analysis, MCLR was concentrated from sample waters by C18 solid phase extraction according to the methods of Nicholson et al. [25]. A HPLC system consisting of a 600 pump controller, 717plus autosampler and 996 photodiode array detector (Waters Pty. Ltd., Australia) was employed using methods described by Ho et al. [12]. Concentrations of MCLR were determined by calibration of the peak areas (at 238 nm) with that of an external standard (Sapphire Bioscience Pty. Ltd., Australia). The HPLC method has a detection limit of 0.1 $\mu g L^{-1}$. MCLR recoveries were greater than 95% with a relative precision of 10%.

Samples were also analysed for hepatotoxicity using a PP2A assay according to the method described in Heresztyn and Nicholson [26]. Inhibition of serine/threonine PP2A (Promega Corporation, Australia) was determined using *p*-nitrophenol phosphate (pNPP) as the substrate. The PP2A comprised the 36–38 kDa

**Table 1**
Characteristics of the treated wastewaters employed in this study.

| Parameter | Tertiary treated effluent (TTE) water | Activated sludge treated effluent (ASTE) water |
|---|---|---|
| Nitrate (mg $L^{-1}$) | 7.1 ± 2.6 | 18.7 ± 1.8 |
| Ammonia (mg $L^{-1}$) | 2.3 ± 1.9 | 0.7 ± 0.2 |
| Phosphorus (mg $L^{-1}$) | 2.4 ± 1.6 | 5.3 ± 0.7 |
| Dissolved organic carbon (DOC) (mg $L^{-1}$) | 8.4 ± 0.8 | 11.3 ± 0.7 |
| Chemical oxygen demand (COD) (mg $L^{-1}$) | 107 ± 41 | 143 ± 30 |
| Biochemical oxygen demand (BOD) (mg $L^{-1}$) | 4 ± 5 | 1 ± 2 |

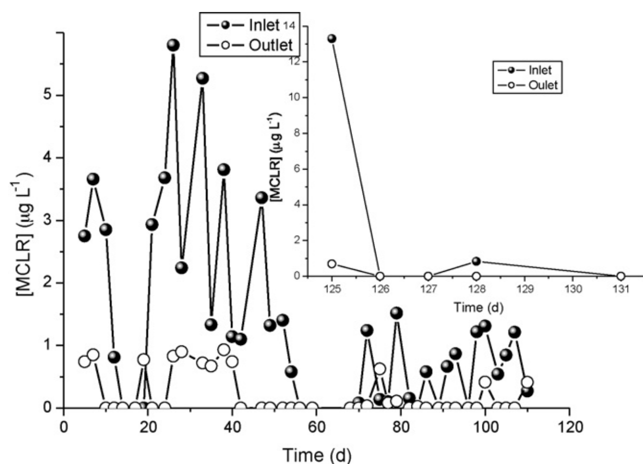


**Fig. 1.** Removal of microcystin-LR (MCLR) through a laboratory sand filter fed with tertiary treated effluent water. Inset: higher MCLR inlet concentration of ~14 $\mu$g L$^{-1}$ spiked into the laboratory sand filter on day 125.

catalytic subunit isolated from rabbit skeletal muscle and was supplied in lots of 25 units, 1 unit being defined as the amount of enzyme required to hydrolyse 1 nmol of pNPP min$^{-1}$ at 30 °C under the specified assay conditions (Promega Technical Bulletin 537).

### *2.5. Detection of mlrA gene involved in microcystin degradation*

A qPCR assay was implemented for the detection of the *mlrA* gene during the laboratory sand column experiment and selected batch degradation experiments. Full details of this assay have been documented by Hoefel et al. [18]. Briefly, reactions were carried out in triplicate in a Rotor Gene 6000 thermal cycling system (Corbett Research, Australia). Each 25 $\mu$L reaction mixture contained 200 $\mu$M of each deoxynucleoside triphosphate, 2.0 mM of $MgCl_2$, 1× PCR buffer, 0.5 $\mu$M of primers qmlrAf and qmlrAr, 0.25 $\mu$M of TaqMan probe qmlrA-tm, 400 $\mu$g mL$^{-1}$ bovine serum albumin, 0.5 U of Platinum Taq DNA polymerase (Invitrogen, USA), and 2.5 $\mu$L of either a DNA standard or sample template. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 5 s and annealing/extension at 62 °C for 25 s. Data were collected in the FAM channel (gain set to 6) at the end of each annealing/extension step.

## 3. Results and discussion

### *3.1. Biological sand filtration of microcystin*

Fig. 1 shows the removal of MCLR through a laboratory sand filter fed with TTE water. MCLR was effectively removed through the sand filter; the World Health Organization guideline value for MCLR is 1 $\mu$g L$^{-1}$ [5], and at no stage during the experiment did the sand filter outlet concentrations reach or exceed this level. A MCLR inlet concentration of 14 $\mu$g L$^{-1}$ was also spiked into the filter inlet for a short period of time on day 125, but the sand filter continued to effectively remove MCLR during this challenge (see Fig. 1 inset). The inlet MCLR concentrations varied throughout the experiment due to biodegradation of MCLR within the inlet reservoir; in some instances no MCLR was detected in the inlet water and this is consistent with previous studies where degradation of MCLR within the inlet water occurred prior to the column [12,27]. Removal of MCLR through the sand filter was presumed to be through biological processes as previous studies utilizing sterilized sand media showed negligible removal to physical processes, such as adsorption [12,27].

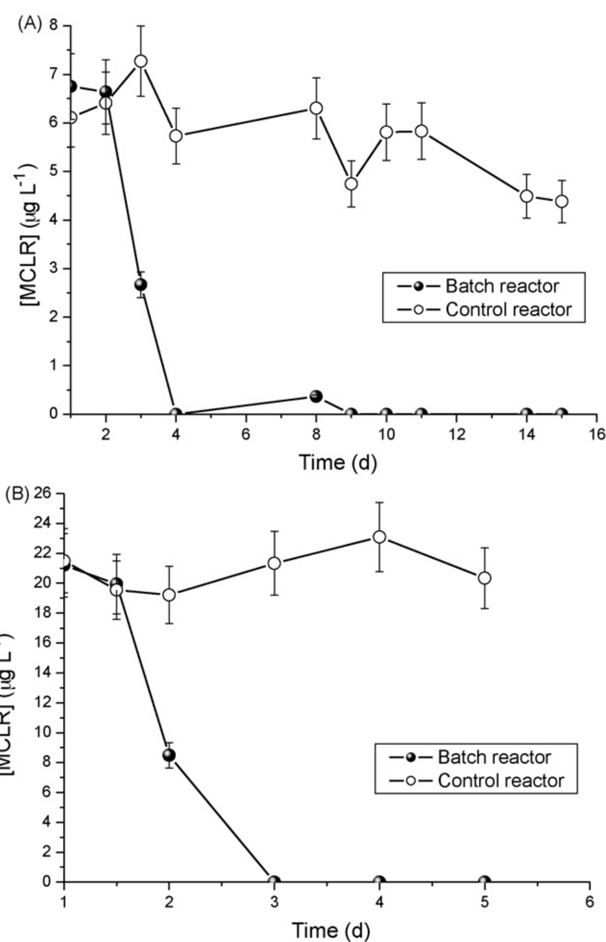


**Fig. 2.** Batch degradation of microcystin-LR (MCLR) in tertiary treated effluent (TTE) water at initial MCLR concentrations of: (A) 6 $\mu$g L$^{-1}$ and (B) 20 $\mu$g L$^{-1}$. Error bars represent 95% confidence intervals.

Analysis by qPCR confirmed the presence of the *mlrA* gene within the biofilm of the sand filter throughout the experiment (data not shown) providing strong evidence that removal of MCLR through the sand filter was by biodegradation processes. Similar findings have been reported by Ho et al. [12] and Hoefel et al. [18].

These results reveal for the first time that biological sand filtration may be an effective treatment barrier in WWTPs for the removal of microcystin toxins. To date, studies have only shown the effectiveness of biofiltration for the removal of microcystins from drinking water using sand taken from WTPs [12,13,18]. Results here suggest that biofiltration of MCLR is still effective despite the differences in water quality characteristics and differing bacterial populations within the biofilms of drinking water treatment plant and WWTP sand filters.

### *3.2. Microcystin degradation in batch experiments*

Batch experiments were conducted to confirm the biodegradability of MCLR in TTE water. MCLR was spiked into two separate reactors containing TTE water at initial concentrations of 6 $\mu$g L$^{-1}$ and 20 $\mu$g L$^{-1}$; these MCLR concentrations were selected to be representative of both low and high concentrations in WWTPs. The indigenous organisms within this water were shown to be responsible for the degradation of MCLR, confirmed by the lack of removal in the parallel sterile controls (Fig. 2A and B). More rapid degradation of MCLR was evident in TTE water containing the higher MCLR concentration with complete degradation by day 3, compared with complete degradation by day 4 for TTE water containing

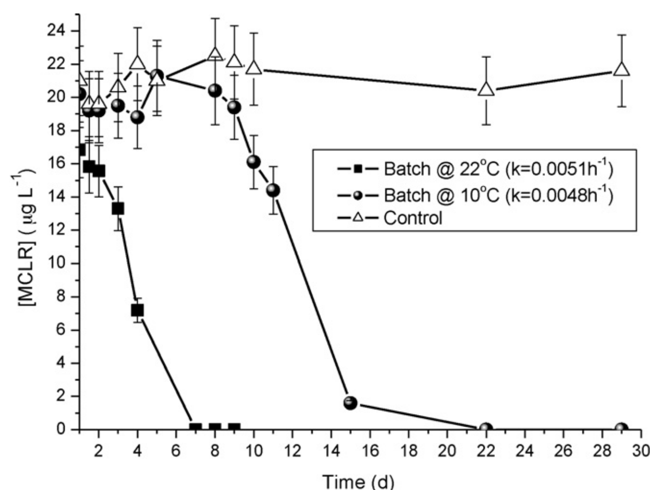


**Fig. 3.** Batch degradation of microcystin-LR (MCLR) in activated sludge treated effluent (ASTE) water at temperatures of 22 °C and 10 °C. Pseudo-first-order decay rate constants are presented in the figure legend. Error bars represent 95% confidence intervals.

the lower MCLR concentration. These observations are consistent with previous studies where Smith et al. [28] and Hoefel et al. [29] reported more rapid degradation of cyanobacterial metabolites cylindrospermopsin (CYN) and geosmin when present at higher concentrations in reservoir waters. Smith et al. [28] showed a strong linear relationship between the initial CYN concentration and the rate of CYN degradation and hypothesized that this was due to CYN acting as an inducer, where a minimum concentration of CYN (the inducer) would be required to activate the genes responsible for CYN degradation. This may also be the case during microcystin degradation where MCLR induces expression of the *mlrA* gene.

Batch experiments were also conducted using ASTE water spiked with MCLR at 20 $\mu$g L$^{-1}$. This water was sampled upstream of the TTE water at the Bolivar WWTP and had different water quality characteristics, in particular, greater levels of nutrients and organic carbon (Table 1). Fig. 3 shows the degradation of MCLR in ASTE water at temperatures of 22 °C and 10 °C. Degradation of MCLR in ASTE was not as rapid as in TTE water (Fig. 2). This difference may be explained by the higher and more assimilable organic carbon and nutrient levels in ASTE which were utilized in preference to MCLR [30]. Furthermore, ASTE may have had a greater abundance and diversity of indigenous organisms which resulted in substrate competition for the MCLR-degrading organisms [31,32]. Predation of MCLR-degrading organisms by higher eukaryotic organisms may have also varied between ASTE and TTE waters which influenced the degradation of MCLR in the respective wastewaters [33].

Whilst it appeared more rapid degradation of MCLR occurred at 22 °C (complete removal by day 7) compared with 10 °C (complete removal by day 22), the pseudo-first-order decay rate constants at the two temperatures were similar (0.0051 h$^{-1}$ at 22 °C, and 0.0048 h$^{-1}$ at 10 °C). The only difference between the two temperatures was the lag period prior to the onset of degradation which was only observed at the lower temperature. Similar findings have been documented by Ho et al. [13]. That study suggested that the lower temperature may have delayed expression of MCLR-degrading genes or decreased the activity of the enzymes involved in the degradation of microcystins. It is also possible that the lower temperature may have decreased the rate of MCLR diffusion into the cell.

A qPCR assay was employed to investigate the changes in the abundance of microcystin-degrading bacteria during MCLR degradation. Samples were taken from the ASTE batch experiments (Fig. 3) and the DNA extracted for quantitative assessment of the

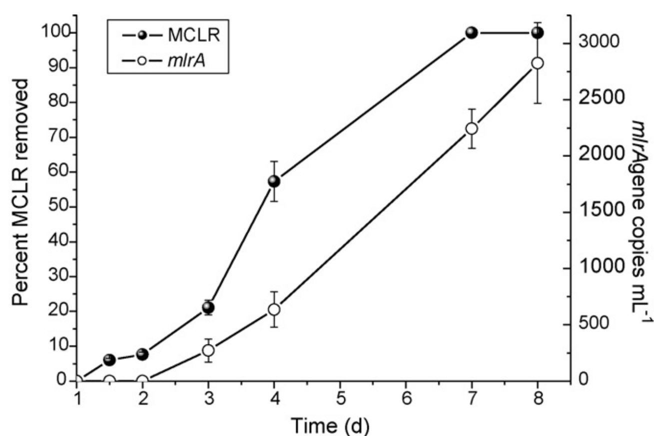


**Fig. 4.** Percentage removal of microcystin-LR (MCLR) and abundance of *mlrA* gene copies as a function of time in activated sludge treated effluent (ASTE) water. Error bars represent 95% confidence intervals.

*mlrA* gene. Fig. 4 shows that the removal trend of MCLR in ASTE water closely followed the abundance of the *mlrA* gene, suggesting that the removal of MCLR was related to the number of MCLR-degrading organisms. However, it was unclear if the degradation of MCLR resulted in an increase in the abundance of the MCLR-degrading bacteria, or if the increase in MCLR-degrading bacteria (from degradation of other organic carbon) resulted in additional degradation of MCLR. To determine this, an additional experiment was conducted in ASTE water where the abundance of the *mlrA* gene was monitored both in the presence (20 $\mu$g L$^{-1}$) and absence of MCLR, in addition to a sterilized control spiked with MCLR. Results, shown in Fig. 5, revealed that the abundance of the *mlrA* gene was below the limit of detection of the qPCR assay for each suspension at the beginning of the experiment. However, the presence of MCLR resulted in an increase in the abundance of *mlrA* gene (to over 2500 copies mL$^{-1}$), whereas in the absence of MCLR, the *mlrA* gene reached a plateau at approximately 500 copies mL$^{-1}$ (Fig. 5). This result demonstrates for the first time that $\mu$g L$^{-1}$ concentrations of MCLR may be a primary substrate for the proliferation of microcystin-degrading bacteria compared with the mg L$^{-1}$ concentrations of other organic carbon within the wastewater. It has been suggested previously that cyanobacterial metabolites are degraded within water as secondary substrates [18,28,29]; however, the data here potentially suggests that this may not always be the case. This

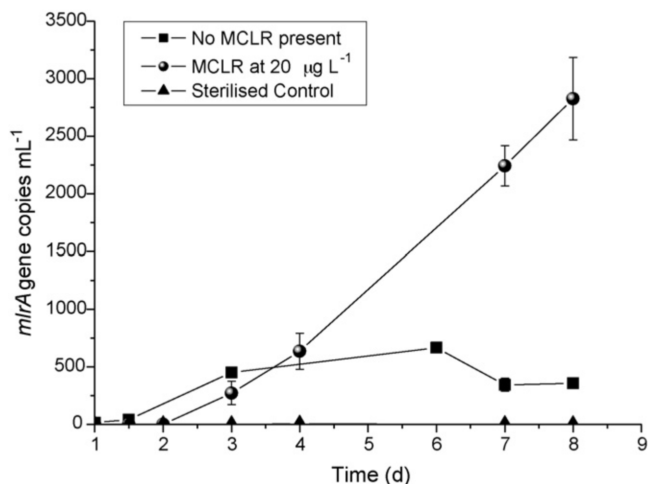


**Fig. 5.** Abundance of *mlrA* gene during batch degradation experiments in activated sludge treated effluent (ASTE) water in the absence and presence of microcystin-LR (MCLR). Error bars represent 95% confidence intervals.

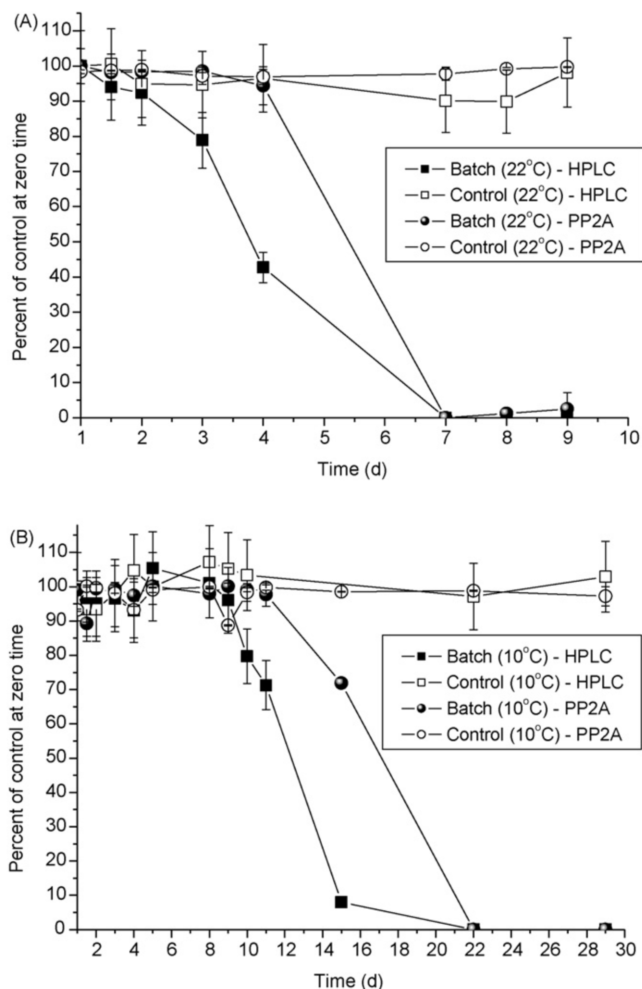


**Fig. 6.** (A and B) Comparison of microcystin-LR (MCLR) detection by high performance liquid chromatography (HPLC) and protein phosphatase 2A (PP2A) during batch degradation experiments conducted in activated sludge treated effluent (ASTE) water. Results presented as percent of control at zero-time. Error bars represent 95% confidence intervals.

data also suggests that the increase in *mlrA* abundance shown in Fig. 4 was primarily due to the degradation of MCLR rather than other organic carbon.

A PP2A assay was also undertaken to detect any potential hepatotoxic by-products of MCLR degradation within the batch experiments. Results for experiments conducted in ASTE water at 22 °C and 10 °C are shown in Fig. 6A and B, respectively. Similar trends were observed between the PP2A results and the HPLC results, where the decrease in PP2A response paralleled the decrease in MCLR concentrations. This strongly suggested that no hepatotoxic by-products were generated from the degradation of MCLR in this treated wastewater based on the lack of PP2A inhibition. Similar findings have been reported by Ho et al. [34].

## 4. Summary and conclusions

Studies on the degradation of microcystin toxins in wastewaters are sparse, with little information documented in the literature. In this study, effective biofiltration of MCLR was observed through a WWTP sand filter fed with TTE, and MCLR was also shown to be readily degraded by the action of indigenous planktonic microorganisms within two treated wastewaters (TTE and ASTE). Further investigations also revealed for the first time how MCLR may be used as a primary substrate by MCLR-degrading bacteria, resulting in an increase in the abundance of those organisms within a planktonic state. Furthermore, a PP2A assay determined that no hepatotoxic by-products were generated during the degradation of MCLR in ASTE.

The results from this study suggest that biological treatment of MCLR can be an effective process in WWTPs, similar to the previous findings in drinking water treatment plants, despite the differences in chemical and microbial compositions of the waters from the respective plants. This is particularly important as wastewaters are highly susceptible to cyanobacterial blooms due to greater levels of nutrients. If wastewaters can be shown to be free of cyanobacterial toxins, then this will ensure that these waters are of a standard that can be used appropriately (such as irrigation purposes), which will ultimately allow water authorities to manage water security more effectively.

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