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Biodegradation of the cyanobacterial toxin microcystin LR in natural water and biologically active slow sand filters

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ABSTRACT

A bacterium (MJ-PV) previously demonstrated to degrade the cyanobacterial toxin microcystin LR, was investigated for bioremediation applications in natural water microcosms and biologically active slow sand filters. Enhanced degradation of microcystin LR was observed with inoculated (1×10^6 cell/mL) treatments of river water dosed with microcystin LR (>80% degradation within 2 days) compared to uninoculated controls. Inoculation of MJ-PV at lower concentrations (1×10^2—1×10^5 cells/mL) also demonstrated enhanced microcystin LR degradation over control treatments. Polymerase chain reactions (PCR) specifically targeting amplification of 16S rDNA of MJ-PV and the gene responsible for initial degradation of microcystin LR (*mlrA*) were successfully applied to monitor the presence of the bacterium in experimental trials. No amplified products indicative of an endemic MJ-PV population were observed in uninoculated treatments indicating other bacterial strains were active in degradation of microcystin LR. Pilot scale biologically active slow sand filters demonstrated degradation of microcystin LR irrespective of MJ-PV bacterial inoculation. PCR analysis detected the MJ-PV population at all locations within the sand filters where microcystin degradation was measured. Despite not observing enhanced degradation of microcystin LR in inoculated columns compared to uninoculated column, these studies demonstrate the effectiveness of a low-technology water treatment system like biologically active slow sand filters for removal of microcystins from reticulated water supplies.

1. Introduction

A range of cyanobacterial species produce a family of chemically stable cyclic hepatotoxins called microcystins (Carmichael 1994; Codd 1995; Harada, 1999; Chorus 2001; Zurawell et al., 2005). These toxins have the potential to persist for long periods in natural surface waters before degradation commences, though once commenced, degradation is usually complete within a couple of days (Jones and Orr, 1994; Harada and Tsuji, 1998; Hyenstrand et al., 2003). The acclimation period prior to degradation is suspected to be dependent on the conditioning or induction of an endemic microbial population capable of metabolising the microcystins (Jones and Orr, 1994; Cousins et al., 1996; Christoffersen et al., 2002). Other environmental factors including temperature, pH, and predation by protozoa also affect the length of

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this acclimation period (Alexander, 1994). The longer the acclimation phase the greater the risk of exposure of animals and humans to the dangers of these toxins. Livestock deaths (Francis, 1978; Negri et al., 1995; Sivonen and Jones, 1999), and isolated cases of human poisonings (Bourke et al., 1983; Hawkins et al., 1985; Jochimsen et al., 1998; Azevedo et al., 2002), have been attributed to consumption of cyanobacterial contaminated water.

Currently there is a significant risk that cyanobacterial toxins may enter drinking water supplies and pose a threat to public health. Conventional water treatment systems involving chemical coagulation, flocculation and rapid sand filtration result in only small decreases in cyanobacterial toxin concentrations (Hoffmann, 1976; Himberg et al., 1989; Keijola et al., 1998; Hoeger et al., 2005). Waterworks without chemical coagulation treatment have also been shown to be inefficient in removal of phytoplankton and especially cyanobacteria from raw water (Lahti, 1997; Lahti et al., 2001). However the removal of cyanobacteria in waterworks with powdered activated carbon, chemical coagulation, sedimentation and sand filtration has been demonstrated to be successful, though natural organic material can reduce its effectiveness (Lahti, 1997; Newcombe et al., 2003). Other water treatment technologies have also been investigated for microcystin toxin removal, including ozonation and photocatalytic degradation (Shephard et al., 1998, 2003; Rositano et al., 2001; Hoeger et al., 2005).

For small regional communities, biologically active slow sand filtration offers a cost-effective water treatment process which have previously demonstrated some removal of microcystins (Keijola et al., 1998; Lahti and Hiisvirta, 1989; Grützmacher et al., 2002). Biodegradation is mediated by the development of biological activity in the filters due to attachment and growth of biofilm microorganisms (Servais et al., 1994; Saitou et al., 2002; Saito et al., 2003a). Filters generally require a maturation phase, the time taken for a microorganism population to establish in the filter, which may be weeks or even months (Bruce and Hawkes, 1983; Zhang and Huck, 1996). For irregular cyanobacterial blooms, intermittent exposure to toxins may hinder the establishment or maintenance of an effective microbial population capable of degrading cyanobacterial toxins. One solution is to inoculate filters with a bacterial population to remove or shorten the maturation phase.

Biological degradation of cyanobacterial toxins in surface waters has been investigated previously (Jones et al., 1995; Mazur and Plinski, 2001; Christoffersen et al., 2002; Jin et al., 2002; Hyenstrand et al., 2003) with bacterial isolates capable of mineralising the peptide compounds identified (Jones et al., 1994; Park et al., 2001; Maruyama et al., 2003; Saito et al., 2003b; Ishii et al., 2004; Rapala et al., 2005). Biological sediment and biofilm derived from conventional water treatment processes have also demonstrated enhanced degradation of microcystin toxins in laboratory trials (Miller and Fallowfield, 2000; Saitou et al., 2002; Holst et al., 2003; Saito et al., 2003b). To date however, the potential of using a characterised cultured bacterial strain capable of degrading microcystin toxins for enhanced removal of toxins from environmental water supplies and water treatment systems such as slow sand filters has not been investigated.

Previous studies described the isolation of a *Sphingomonas* sp. (MJ-PV), which degraded the cyanobacterial toxin microcystin LR (Jones et al., 1994) and characterised the pathway of degradation and genes involved (Bourne et al., 1996, 2001). Subsequent work developed molecular techniques for detection of the MJ-PV bacterium and the gene responsible for degradation of microcystin LR (*mlrA*) (Bourne et al., 2005). A natural progression is to investigate applications of this bacterium for remediation of water samples contaminated with microcystins. Therefore this study specifically aimed to investigate the addition of MJ-PV to river water microcosms with a view to the potential seeding of water bodies and also the possible water treatment applications using slow sand filtration columns inoculated with MJ-PV to enhance removal of microcystins.

2. Experimental methods

2.1. Origin of samples

2.1.1. Sand and water

River water (RW) is the term used to define all environmental water samples used in this study. The water was taken from a storage dam supplied from irrigation drainage water derived from the Murrumbidgee River (NSW, Australia), the same water source from which MJ-PV was originally isolated. Sand, rocks and pebbles used within sand filters (see Fig. 1), with no history of exposure to microcystin toxins, was obtained from the local gravel works.

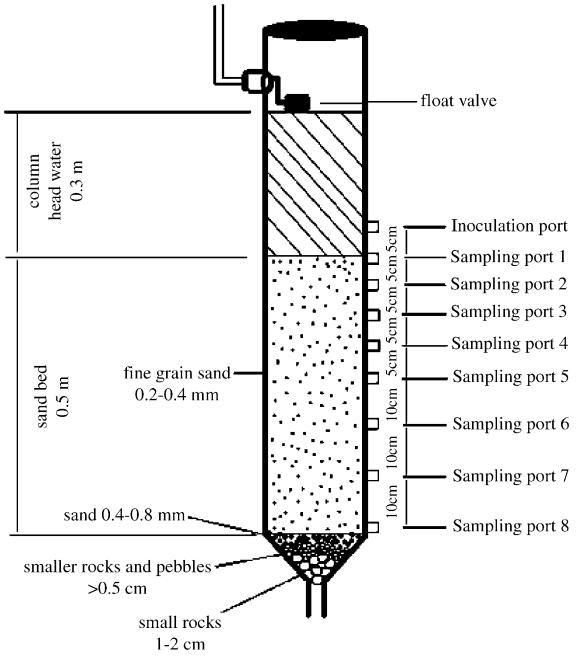

Fig. 1 – Detailed schematic representation of an individual slow sand filter column. Six replicate filters were constructed and the resulting design and set-up of the columns allowed a continuous flow of microcystin LR-dosed dam water to pass through the sand beds.

2.1.2. Microbial strain

The *Sphingomonas* sp. defined in this publication as MJ-PV (GenBank Accession No. AF411072) was grown and maintained as previously described (Bourne et al., 2001). Cultures used for inoculation were grown overnight in peptone yeast extract media at 30 °C. Cells were washed three times in ASM minimal media prior to inoculation.

2.1.3. Crude microcystin LR: source and analysis

A thick scum of *Microcystis aeruginosa* was collected from a bloom in a lagoon off the Murrumbidgee River as described in Jones et al. (1995). The scum was dried, ground with a mortar and pestle before being stored dried. Crude extraction of microcystins was performed by ultrasonication (10×30 s Braun Labsonic L sonicator, 150 W output) of dried cyanobacterial powder (1 g) in distilled–deionised water (50 mL). Cell debris was removed by centrifugation and filtration with the filtrate stored frozen. Previous studies of extracts derived from this source demonstrated the presence of the dominant variant (>95%) microcystin LR (Jones et al., 1995).

Microcystin LR was quantified by reverse-phase HPLC (Waters) using a Spherisorb ODS-2 C_{18} column (5 μm; 250×4.6 mm; Alltech). The mobile phase consisted of 20% acetonitrile in 8mM aqueous ammonium acetate. The acetonitrile content was raised to 35% with a 20-min linear gradient. Microcystin LR was identified by co-elution with standard microcystin LR (CalBiochem, La Jolla, CA) and by comparison of UV spectra obtained using a Spectra-Physics rapid spectral scanning detector. Identity was confirmed by FAB-MS analysis (M^+ 995). Further details of the HPLC system have been described previously (Jones et al., 1994). Due to the low levels of microcystin LR used in this study (~ 50–100 μg/L), concentrations were quantified based on HPLC peak height rather than peak area to provide greater reproducibility in recorded values. The response of peak height was linear over microcystin LR concentrations (2–100 μg/L) (results not shown) and the detection limit was 2 μg/L.

2.2. Experimental design

2.2.1. Enhanced microcystin LR degradation

Flasks (250 mL) were filled with 100 mL of RW and dosed with microcystin LR at a final concentration of 50 μg/L. Four treatments of three replicates were set up as follows; Treatment 1: pre-sterilised (0.2 μm filtered) uninoculated RW, Treatment 2: uninoculated RW, Treatment 3: pre-sterilised (0.2 μm filtered) MJ-PV inoculated RW, Treatment 4: RW inoculated with MJ-PV. MJ-PV was added at 2.2×10^5 cells/mL and removal of microcystin LR was followed over time by HPLC analysis. In addition, samples (1 mL) were removed from each treatment at times $T = 0$, 1, 2, 4, 6 and 12 days for DNA extraction and PCR analysis.

2.2.2. MJ-PV cell dilution remediation

Flasks (250 mL) were filled with 100 mL of RW and dosed with microcystin LR at a final concentration of 110 μg/L. The added microcystin was approximately twice the previous experiment to test the ability of MJ-PV to degrade the toxin over a range of concentrations. MJ-PV was inoculated into the flasks at final concentrations of 10^5, 10^4, 10^3, and 10^2 cells/mL and an uninoculated control was also set-up. Treatments were run in triplicate and microcystin LR removal followed using HPLC analysis.

2.2.3. Biologically active slow sand filter design

The design of slow sand filters was based closely on industrially designed filters, though for practical reasons, scaled down. Parameters including hydraulic loading rate, retention time, effective sand size and water head were determined to be within standard operating conditions (Rachwal et al., 1988; Bourgine et al., 1994; Ellis and Aydin, 1995). Sand filters were constructed of PVC piping, 100 mm in diameter and 1 m in length (see Fig. 1 for a schematic representation). Opaque PVC was used in preference to Perspex to avoid algal growth within the columns and preliminary tests established negligible absorption of microcystin toxins to the plastics. Sand of defined size was obtained via a series of wire mesh sieves to give an effective size of 0.20–0.40 mm. A sand bed depth of 0.50 m was constructed in each column. The supporting gravel system was composed of layers ranging from small rocks at the bottom to pebbles and coarse sand of effective size 0.40–0.80 mm. Eight sampling ports were spaced vertically down the column (Fig. 1) and one inoculation port just above the sand bed. Sampling ports consisted of drilled holes in the PVC tubing, filled and sealed with rubber septa to prevent leakage.

RW was settled in a 3000 L settling tank for the duration of the experiment to remove any suspended solids. Settled water (20 L) was transferred daily to a feed water reservoir tank, dosed with microcystin LR (~50 μg/L) and gravity fed into the columns. Two feed water reservoir tanks were rotated to provide continuous flow of microcystin LR-dosed water and rinsed and dried on days of non-use. A float valve on each column maintained a constant level of water over the sand bed (water head of 0.30 m) and prevented column overflow. A constant effluent flow rate of 130 mL/h (2.2 mL/min) was maintained by a peristaltic pump. The hydraulic loading rate on the columns was 0.0165 m/h, which equates to an empty bed contact time (EBCT) of 30 h. The water head in the columns took 18 h before passing into the sand bed, resulting in the total time for water to enter the column before exiting being approximately 48 h.

2.2.4. Sand filter inoculation and sampling

RW (containing no microcystin LR) was washed through the sand filters for 24 h to remove all small soil particles before being drained prior to inoculation. Microcystin degradation activity of strain MJ-PV was confirmed prior to column inoculation using standard extraction and activity analysis (Bourne et al., 1996). Three replicate sand filters (1, 3 and 5) were inoculated with a MJ-PV population of 5×10^8 cells (in a volume of 10 mL) by injection through the inoculation port above the sand bed. Three replicate sand filters (2, 4 and 6) were left uninoculated. After addition of cells to the top of the sand bed, an adhesion period of 1 h was allowed before microcystin LR-dosed water was re-added to fill the columns. The peristaltic pump was turned on (the time defined as $T = 0$), and experimental sampling of the columns commenced.

The concentration of the microcystin LR in the water entering the columns (dosed with 50 μg/L toxin) and water eluting from the columns was measured by HPLC analysis daily. Sand cores (1 g) were removed from the sampling ports through a 14 gauge needle for DNA extraction and PCR amplification. Cores were removed from sampling port 1 at $T = 0$ and 24 h and from all ports down the column (1–8) at $T = 14$ days. Samples for PCR analysis were also taken from the column header waters, feed water reservoir, settling tank and crude microcystin extract.

2.3. DNA extraction and PCR

DNA was extracted from water and sand samples as outlined previously (Bourne et al., 2005). Two PCR primer sets were used for specific detection of MJ-PV; *Sph*-f1008/*Sph*-r1243 amplified a 252 bp product from the 16S rDNA and *mlrA*-f1/*mlrA*-r1 amplified a 1086 bp fragment including the *mlrA* gene involved in degradation of microcystin LR. The design, optimisation and PCR amplification conditions for these primer sets were reported previously (Bourne et al., 2005).

2.4. Bacterial cell counting

The concentration of MJ-PV in culture was determined by direct counting of 4′,6′-diamidino-2-phenylindole (DAPI) stained cells. MJ-PV was diluted and filtered onto black 0.25 μm polycarbonate filters (Millipore) under a low vacuum. Bacterial cells were covered with 10 μg/mL solution of DAPI for 2 min before washing with 2×5 mL of sterile Milli-Q water. Cell counts were performed by epifluorescence microscopy using a Leitz DIAPLAN microscope with fluorescence incident-light and appropriate filter sets. Ten replicate fields of view containing between 30 and 300 cells were counted and cells/mL determined.

3. Results and discussion

3.1. MJ-PV detection limit

The PCR detection limit of inoculated cells was determined by serial dilution (10^6 cells/mL to 0) of MJ-PV into sand (1 g) and RW (1 mL) samples. Using the 16S rDNA MJ-PV specific primers (*Sph*-f1008/*Sph*-r1243) the detection limit was 10^2 cells for both sand and RW. The *mlrA* gene PCR detection limit with the specific primers *mlrA*-f1/*mlrA*-r1 was also 10^2 cells per sample. These results were comparable to previous studies (Bourne et al., 2005). The 16S rDNA molecule is a multi-copy gene and therefore a lower detection limit would be expected for this target over the single copy *mlrA* gene. However the detection limit evaluation was performed on 10-fold dilutions of the two gene targets and a smaller dilution threshold (e.g. 2 fold) may discriminate between the detection limits and explain this paradox.

3.2. Enhanced microcystin LR degradation trial

The potential for enhanced degradation of microcystin LR was tested by inoculation of the bacterial strain MJ-PV (2.2×10^5 cells/mL) into microcystin LR-dosed RW. In a 0.2 μm filtered and uninoculated control (treatment 1), no microcystin LR degradation was observed over the period of the experiment (12 days). In the unfiltered and uninoculated control (treatment 2), rapid removal of microcystin LR after an initial acclimation phase which varied between 4–6 days for the replicate trials (as reflected by the large standard deviation observed for day 6 plotted data) was recorded (Fig. 2). Variability in the length of the acclimation phase has previously been observed for biodegradation studies of microcystins (Jones and Orr, 1994; Cousins et al., 1996; Christoffersen et al., 2002; Holst et al., 2003; Hyenstrand et al., 2003; Ishii et al., 2004). For MJ-PV inoculated treatments of both filtered (treatment 3) and unfiltered RW (treatment 4), ~ 90% removal of microcystin LR was observed within 2 days of inoculation for all replicates (Fig. 2). Once degradation commenced, removal of microcystin LR was rapid, characteristic of classic biodegradation profiles (Mirgain et al., 1993; Alexander, 1994) and similar to other studies of microcystin degradation (Hyenstrand et al., 2003; Ishii et al., 2004). Mineralisation of microcystin-LR by MJ-PV was through a series of enzymatic steps previously documented (Bourne et al., 1996, 2001) and no trace of transformed LR by-products were observed in HPLC analysis. The result demonstrated the potential of strain MJ-PV for shortening the acclimation phase and facilitating rapid removal of microcystin LR from RW.

In non-filtered RW, irrespective of inoculation (treatments 2 and 4), no residual microcystin LR could be detected at the end of the experiment. In contrast, residual microcystin LR (~2–5 μg/L) remained in each replicate of filtered RW inoculated with MJ-PV (treatment 3). This indicates that although MJ-PV facilitates 90–95% degradation, complete degradation of microcystin LR may require the presence of a consortium of bacteria. Jones and Orr (1994) observed bi-phasic degradation kinetics in field studies of microcystin LR breakdown and attributed this to different populations of bacteria degrading the toxins at high and low concentration, respectively. Similarly, metabolism of the low concentrations of microcystin LR by an alternate bacterial population may have occurred within treatments 2 and 4.

Samples taken for molecular analysis immediately after inoculation of MJ-PV ($T = 0$), yielded 16S rDNA and *mlrA* PCR products for inoculated treatments 3 and 4 only (Table 1). No

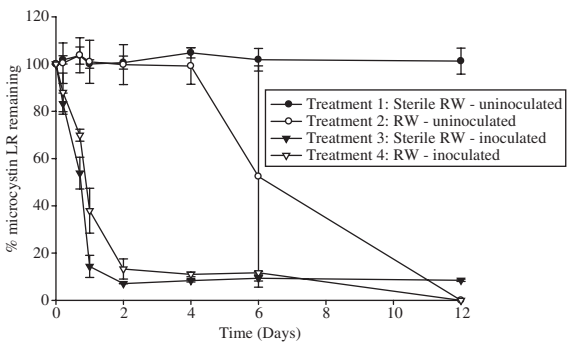

Fig. 2 – Degradation profile of the flask inoculation experiment showing percentage loss of microcystin LR (vs.) time for each treatment.

change in the PCR results was observed until day 6 when PCR signals for both primer sets disappeared for treatment 4 (inoculated non-filtered RW) (Table 1), indicating that the MJ-PV population had been eliminated or dropped below the detection limit. In contrast, the inoculated but 0.2 μm filtered treatment 3 resulted in strong amplification of PCR products throughout the experiment (Table 1). The filtering step removes all indigenous bacteria and protozoa and the role of protozoa in controlling bacterial populations and eliminating slow-growing members of a community in natural waters is well documented (Sherr and Sherr, 1987; Tso and Taghon, 1999; Tadonléké et al., 2005). Therefore it is likely that protozoan grazing reduced the MJ-PV population below PCR detection limits in treatment 4.

Degradation of microcystin LR within treatment 2 (uninoculated) commenced between 4 and 6 days with complete removal after 12 days (Fig. 2). PCR results indicated that the MJ-PV population was not endemic to the RW and that other bacteria mediated degradation of the toxin. The water used within the trials was derived from a source similar to that which the MJ-PV strain was originally derived. However during initial isolation of strain MJ-PV, other morphologically distinct bacterial species with microcystin LR degrading capacity were also isolated (Jones et al., 1994). Recent studies have isolated additional bacteria capable of degradation of cyanobacterial toxins (Park et al., 2001; Ishii et al., 2004; Rapala et al., 2005). The PCR detection techniques used in this study appear specific to the MJ-PV strain with no detection of other related strains active in microcystin degradation (Bourne et al., 2005).

3.3. *MJ-PV dilution remediation trial*

Effective biodegradation of xenobiotics requires a critical bacterial biomass to prevent significant delays before onset of measurable biodegradation (Corseuil and Weber, 1994). To investigate the concentration of bacteria required to facilitate enhanced biodegradation of microcystin, serial dilutions of MJ-PV were inoculated into microcystin LR-dosed RW. All bacterial treatments demonstrated enhanced degradation of microcystin LR over the uninoculated control (Fig. 3). Even inoculation at 10^2 cells per treatment resulted in enhanced degradation of microcystin LR with the acclimation period decreasing by 24 h compared with uninoculated controls. The result indicates that if MJ-PV is above the PCR detection limit it is capable of enhanced microcystin LR degradation. In addition, the concentration of microcystin LR was approximately twice that of the previous experimental trials and demonstrated the ability of MJ-PV to degrade the toxin over a range of environmentally relevant concentrations.

3.4. *Bioremediation within biologically active slow sand filters*

In a study of six biologically active sand filtration columns, complete removal of microcystin LR was observed in all sand

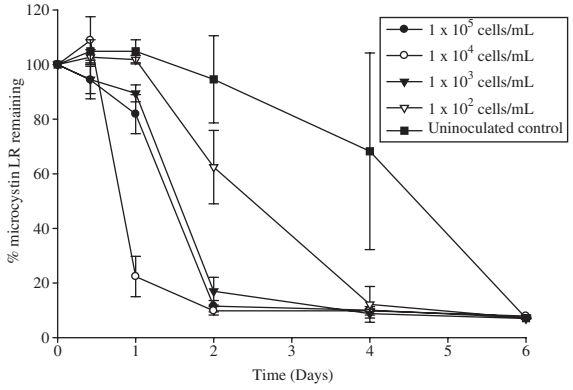

Fig. 3 – Degradation profile of the MJ-PV dilution inoculation experiment showing percentage loss of microcystin LR (vs.) time for each cell dilution treatment.

Table 1 – Summary of the PCR amplification results for microcosm microcystin LR bioremediation trials

	PCR product amplification[a]							
	16S rDNA gene Primer set: (*Sph*-f1008/*Sph*-r1243)				*mlrA* gene Primer set (*mlrA*-f1/*mlrA*-r1)			
Day	Trt 1	Trt 2	Trt 3	Trt 4	Trt 1	Trt 2	Trt 3	Trt 4
0	–	–	+	+	–	–	+	+
1	–	–	+	+	–	–	+	+
2	–	–	+	+	–	–	+	+
4	–	–	+	+	–	–	+	+
6	–	–	+	–	–	–	+	–
12	–	–	+	–	–	–	+	–

Trt 1: Treatment 1: pre-sterilised (0.2 μm filtered) uninoculated RW.
Trt 2: Treatment 2: uninoculated RW.
Trt 3: Treatment 3: pre-sterilised (0.2 μm filtered) MJ-PV inoculated RW.
Trt 4: Treatment 4: RW inoculated with MJ-PV.
[a] –No amplified product; +positive amplified product (Results consistent for all replicated treatments).

columns within 6 days and sampling up to 15 days did not detect any further breakthrough of the toxin (Fig. 4). However no enhanced degradation of microcystin LR was observed in the inoculated columns over the uninoculated columns. All columns displayed a similar degradation profile which included an acclimation phase of between 2 and 4 days before the commencement of microcystin LR degradation (Fig. 4). Prior to inoculation ($T = 0$), sand sampled through port 1 demonstrated no amplified 16S rDNA or *mlrA* PCR products, indicative of the presence of MJ-PV, for any column (Table 2). After inoculation and 24 h of passage of microcystin LR-dosed RW ($T = 24$ h), samples from port 1 resulted in strong PCR detection of MJ-PV (both 16S rDNA and *mlrA*) for the inoculated columns (1, 3 and 5). Interestingly, uninoculated columns 2, 4 and 6 also resulted in positive, though weak, signals for the 16S rDNA gene (Table 2). A corresponding *mlrA* amplified signal was not observed for these uninoculated columns.

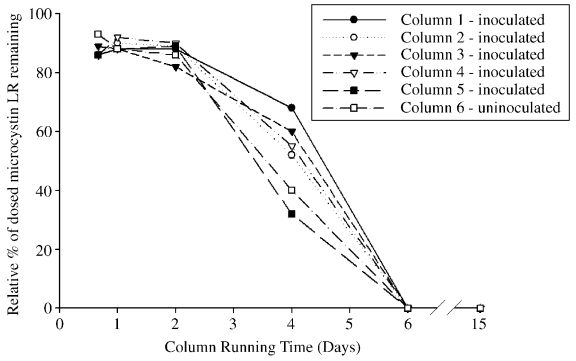

Fig. 4 – Degradation profile demonstrating relative concentration of microcystin LR (vs.) time for each biologically active slow sand filter. The concentration of microcystin LR exiting the columns was related to the known concentration dosed in the feed water to obtain a value of the percentage of incoming LR toxin remaining after passage through the columns. The column running time is taken from $T = 0$ and is equivalent to the time the peristaltic pump was turned on after addition of bacteria and microcystin LR-dosed source water was added to the columns and experimental sampling of the columns commenced.

After 14 days of continuous passage of microcystin LR dosed water, sand removed from each port spaced down the six columns demonstrated positive 16S rDNA amplified signal for all samples, though the PCR product became progressively weaker as the samples moved down the sand bed (Table 2). The *mlrA* gene was detected down to port 6 corresponding to a 0.3 m penetration into the sand beds for all columns. Water samples eluting from each column was collected at 24 h and 14 days and also demonstrated positive detection of the MJ-PV 16S rDNA product but negative results for the *mlrA* gene. The head water (above the sand bed) within each column was tested for the presence of a MJ-PV population. No PCR products were detected at $T = 0$ though products for both primers sets were detected at $T = 14$ days. An identical result was observed for the feed water reservoir bringing water into the columns. The water settling tank (3000 L) and crude microcystin extract demonstrated no detection of a MJ-PV population at any time during the experiment.

The likely scenario explaining the column degradation profiles and PCR results was that uninoculated columns were contaminated by MJ-PV cells from inoculated columns colonising the feed water delivery lines and the feed water reservoir. This was supported by PCR detection of a MJ-PV population in both the column head water and feed water reservoir late in the experiment. Once present in the feed water reservoir, MJ-PV distribution to all columns would be rapid as observed with 16S rDNA detection of the strain in all columns after 24 h (Table 2). The long retention times and

Table 2 – Summary of PCR results from sampling ports within biologically active slow sand filters

Sample Location	PCR product amplification[a]						
	16S rDNA gene			*mlrA* gene			
	Primer set: (*Sph*-f1008/*Sph*-r1243)			Primer set (*mlrA*-f1/*mlrA*-r1)			
	Time			Time			
	$T = 0$[b] Port 1	$T = 24$ h Port 1	$T = 14$ days Ports 1–8	$T = 0$[b] Port 1	$T = 24$ h Port 1	$T = 14$ days Ports 1–6	$T = 14$ days Ports 7–8
Column 1 (inoculated)	–	+	+	–	+	+	–
Column 2 (uninoculated)	–	+	+	–	–	+	–
Column 3 (inoculated)	–	+	+	–	+	+	–
Column 4 (uninoculated)	–	+	+	–	–	+	–
Column 5 (inoculated)	–	+	+	–	+	+	–
Column 6 (uninoculated)	–	+	+	–	–	+	–

[a] –No amplified product;+positive amplified product.
[b] Prior to inoculation of MJ-PV bacterium.

stable water environment within the sand filters would have contributed to MJ-PV colonising the feed water reservoir. The sand filters used a drip intake with control float valve and as the water reached a specific height above the sand bed (0.3 m), the float valve would seal the water inlet. Although effective for stopping water overflow, it did not stop contamination by bacteria.

No *mlrA* PCR product was detected in uninoculated columns after 24 h despite successful 16S rDNA detection of the inoculated bacterial strain. Although a similar detection level was previously demonstrated for both gene targets, this was based on 10-fold serial dilutions and a finer scale of dilutions may discriminate the detection limit for the single-copy *mlrA* gene and the multi-copy 16S rRNA gene. In addition the limits determined in the initial experiments were based on bacterial spiked samples that were immediately extracted for PCR. Sand and soil particles provide bacteria with a surface to attach creating shelter for microbial colonies protecting them from predation but making them more difficult to release for the purpose of isolating DNA (Picard et al., 1992; Herrick et al. 1993). For sand samples that have been colonised it is likely that DNA extraction efficiencies are reduced and as a result the detection limit of the PCR method increased.

An acclimation period was observed for all columns before measurable biodegradation of microcystin LR. A large initial inoculum of 5×10^8 MJ-PV cells was added on top of the sand beds to ensure inoculated bacteria attained a critical biomass and exceeded levels of indigenous bacteria resulting in an increased chance of survival in the biological filter systems (Corseuil and Weber, 1994). However, the method for inoculation of cells onto the top layer of the sand effectively inoculates only a thin cross section of each column. Presumably, passage of water through the column would carry bacteria inoculating lower sections of the sand bed. The actual contact time between microcystin LR passing in the water and the bacteria attached to the sand would have been short, potentially only a few hours or less in the early stages of the experiment. As a result, the acclimation period may be due to the time for the MJ-PV population to establish and disperse within the sand bed such that a longer contact time between bacteria and toxin can occur. It is possible the MJ-PV population may not have attained a critical biomass required within the sand filters, thus leading to a delay before microcystin LR degradation. However, this is unlikely since microcosm experiments demonstrated enhanced microcystin LR degradation when MJ-PV bacteria were inoculated at 10^2 cells/mL.

PCR analysis demonstrated the presence of MJ-PV at all sample sites through the columns, and in water eluting from the columns after 14 days of the experiment, indicating the MJ-PV strain survived within the columns. It cannot be ruled out that the feed water harboured an endemic MJ-PV population which established within the columns and was responsible for positive PCR detection since the water used in this study was from the similar source from which the strain was originally derived. However the previous microcosm experiments and other environmental surveys (Bourne et al., 2005) failed to detect an endemic population in this RW and therefore it is considered unlikely.

4. Conclusions

The success of MJ-PV to survive in semi-natural conditions and degrade realistic environmental microcystin toxin concentrations provides encouragement for the use of this bacterium for wider bioremediation applications. As a water treatment option, inoculation with a MJ-PV population may provide the initial rapid degradation of microcystins, shortening the acclimation phase and allowing water resources to be returned to supply more rapidly. The combined activity of MJ-PV and endemic bacteria facilitated removal on microcystin LR in microcosm experiments to below 1 μg/L, a threshold guideline level for human exposure based on 'worse case' long-term exposure scenarios (WHO, 1998). Animals could safely tolerate much higher levels of microcystins in water supplies and therefore the use of a MJ-PV population for bioremediation of small farms dams represents a valid option. The complete degradation of cyanobacterial toxins within the sand filters demonstrated the effectiveness of this low technology infrastructure which may act as a cost-effective mechanism to ensure safe reticulated supplies. Once rapidly matured, the sand filters were effective in removal of microcystin LR to below detection level, though no enhanced degradation was observed for inoculated compared against uninoculated columns.

Future experiments need to investigate the fate of a seeded bacterial population to periodic exposure of cyanobacterial toxins and determine if the population can be maintained during these periods. In addition, although previous studies demonstrated strain MJ-PV was able to degrade microcystin LR and RR (Jones et al., 1994) further studies need to be performed to evaluate the applicability of this strain on a wider array of cyanobacterial toxins in ecological relevant simulations. This includes investigation on the applicability of this strain for large scale use in water supplies and large scale biologically active sand filters.

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