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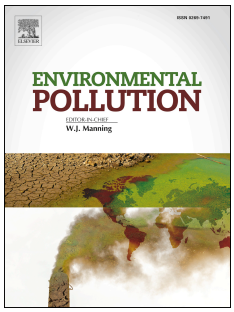


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PII: S0269-7491(18)31495-7

DOI: 10.1016/j.envpol.2018.07.008

Reference: ENPO 11312

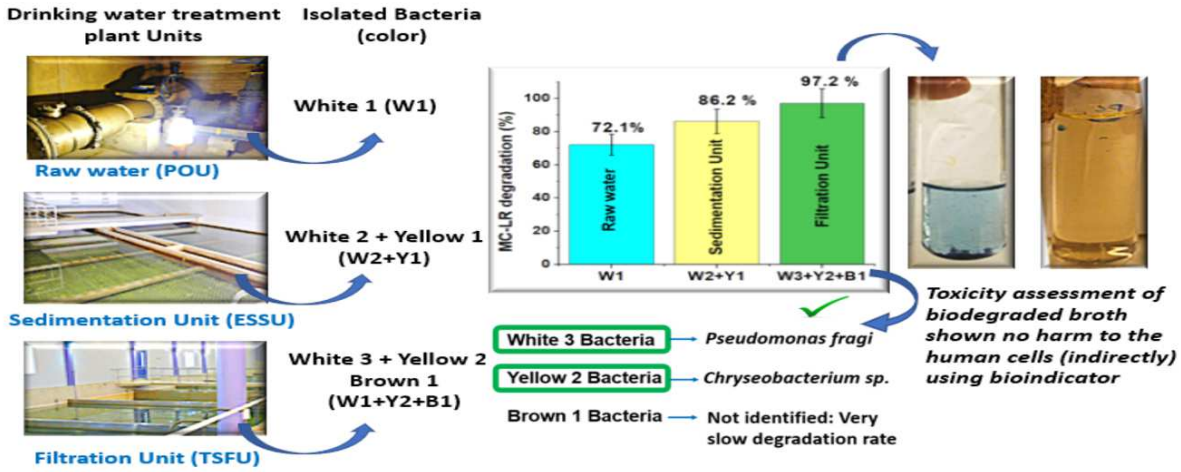
To appear in: *Environmental Pollution*

Received Date: 5 April 2018

Revised Date: 30 June 2018

Accepted Date: 2 July 2018

Please cite this article as: Kumar, P., Hegde, K., Brar, S.K., Cledon, M., Kermanshahi-pour, A., Roy-Lachapelle, A., Galvez-Cloutier, R., Biodegradation of microcystin-LR using acclimatized bacteria isolated from different units of the drinking water treatment plant, *Environmental Pollution* (2018), doi: 10.1016/j.envpol.2018.07.008.



# Biodegradation of microcystin-LR using acclimatized bacteria isolated from different units of the Drinking Water Treatment Plant

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## Abstract

Bacterial community isolated from different units of a Drinking Water Treatment Plant (DWTP) including pre-ozonation unit (POU), the effluent-sludge mixture of the sedimentation unit (ESSU) and top-sand layer water sample from the filtration unit (TSFU) were acclimatized separately in the microcystin-leucine arginine (MC-LR)-rich environment to evaluate MC-LR biodegradation. Maximum biodegradation efficiency of 97.2 ± 8.7% was achieved by the acclimatized-TSFU bacterial community followed by 72.1 ± 6.4 % and 86.2 ± 7.3 % by acclimatized-POU and acclimatized-ESSU bacterial community, respectively. Likewise, the non-acclimatized bacterial community showed similar biodegradation efficiency of 71.1 ± 7.37 %, 86.7 ± 3.19 % and 94.35 ± 10.63 % for TSFU, ESSU and POU, respectively, when compared to

the acclimatized ones. However, the biodegradation rate increased 1.5-folds for acclimatized versus non-acclimatized conditions. The mass spectrometry studies on MC-LR degradation depicted hydrolytic linearization of cyclic MC-LR along with the formation of small peptide fragments including Adda molecule that is linked to the reduced toxicity (qualitative toxicity analysis). This was further confirmed quantitatively by using *Rhizobium meliloti* as a bioindicator. The acclimatized-TSFU bacterial community comprised of novel MC-LR degrading strains, *Chryseobacterium sp.* and *Pseudomonas fragi* as confirmed by 16S rRNA sequencing.

***Capsule:***

**"Biodegradation of microcystin-LR by *in-situ* bacterial community present in the drinking water treatment plant without formation of toxic by-product"**

**Keywords:** Acclimatized bacteria, microcystin, degradation pathway, drinking water, toxicity

## 1. Introduction

The occurrence of cyanobacterial harmful algal bloom (CHABs) affects fresh and marine ecosystems (O`Neil et al., 2012). It is also a matter of public health concern, as the standard water treatments are not designed to target the removal of such compounds. (Hitzfeld et al., 2000). An algal bloom is a global issue, where CHAB genera, such as *Anabaena, Cylindrospermopsis, Nodularia, Microcystis* and other benthic species produce a variety of cyanotoxins, namely, cylindrospermopsin, nodularin, saxitoxin, microcystins (MCs), among others, affecting water quality (Carey et al., 2013). Among all, MCs are known to be stable in the natural aquatic environment due to their cyclic structure (Somdee et al., 2013). There are many variants of microcystin (MCs) produced by *Microcystis sp.*, such as MC-LR, MC-RR, MC-YR,

MC-WR, MC-LA, MC-LY, MC-LW, MC-LF, depending on the position of different peptide groups in the cyclic structure. Among all, MC-LR, produced by *Microcystis aeruginosa* is known to be one of the deadliest cyanotoxins, commonly found in an aquatic ecosystem. Further, the WHO recommends that the microcystin present in drinking water should not exceed 1 µg/L (WHO, 1999).

When microcystins enter the DWTPs (at concentrations above 10 µg/L), conventional treatment options, such as ozonation, chlorination, coagulation/flocculation, become ineffective in the long run due to the periodic change in the parameters such as pH, temperature, among others. They sometimes produce toxic byproducts too (Gagala et al., 2012). Also, the advanced methods of cyanotoxin treatment, such as photodegradation and RO membrane technique are not cost-effective and are energy-intensive. On the other hand, the biological approach is not only promising in degrading various cyanotoxins, but it is also sustainable, less energy-intensive and known to produce less toxic end-products than the parent compound (up to 160-fold less) (Somdee et al., 2013).

Many studies have reported biodegradation of MC-LR by native bacterial species isolated from various water streams, such as rivers, lakes, ponds, and sediments (Neilan et al., 2014; Chen et al., 2010). However, their applicability in DWTP is limited, given the fact that only a few studies have been reported on the interaction of MCs with the *in-situ* bacterial community present within the DWTP units. Hence, it is important to explore and compare the capability of such *in-situ*, naturally occurring microorganisms present in the DWTPs units for MC-LR degradation. These acclimatized microorganisms have the advantage of natural growth and therefore could be utilized without any modification in the existing treatment units. For example, the sand filtration system can be modified into a bio-sand filter to effectively degrade microcystins.

This study investigates the role of bacterial community, isolated from three distinct units of DWTP comprising samples before pre-ozonation step (POU) in the form of raw water, the effluent-sludge mixture from the sedimentation unit (ESSU) and top layer-sand particles from the filtration unit (TSFU). The degradation efficiency and rate of these bacterial communities were compared before and after acclimatization in the presence of MC-LR. Also, the toxicity test for the biodegraded broth was performed using a bioindicator. To the best of our knowledge, this is the first report exploring the ability of microcystin-acclimatized indigenous bacterial communities isolated from "*different units of the DWTP*" to degrade MC-LR.

## 2. Material and methods

### 2.1 Reagents and chemicals

Microcystin-LR was purchased from Cayman Chemicals, (Ann Arbor, Michigan, USA). $MgSO_4 \cdot H_2O$, $ZnSO_4 \cdot H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $KH_2PO_4$, $Na_2HPO_4 \cdot 7H_2O$, $CaCl_2$ and $FeCl_3$ was bought from Fisher Scientific, (Ontario, Canada). Millipore system (Milford, MA, USA) Milli-Q/Milli-RO was used to prepare mineral salt media (MSM) solutions spiked with MC-LR. Sodium chloride (NaCl), peptone and yeast extract were purchased from Fisher Scientific (Ottawa, ON, Canada) and used to prepare Luria-Bertani medium for bacterial culture and inoculation of the isolated bacteria. For the toxicity assay: Tris-HCl buffer (pH 7.5) was prepared using Tris-buffer and 6N HCl (Merck, US) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT ) was used for measuring cell viability, bought from Sigma Aldrich, (Ontario, Canada).

### 2.2 Microorganisms

*Sphingomonas sp.* (NRRL B-59555) and *Rhizobium meliloti* (NRRL L-84) were purchased from NRRL Agricultural Research Service (ARS) culture collection. They were respectively used as

the positive control in studying MC-LR degradation (Ishii et al., 2004) and as a bioindicator for determining the toxicity of the biodegraded broth (Botsford et al., 1997).

**2.3 Water sample collection for bacterial isolation**

Three unit operations of the DWTP at Chemin Ste-Foy, (Quebec City, Canada) were chosen for the water sample collection. The water sample from three different treatment stages *viz*., (1) influent stream (before pre-ozonation (POU) treatment), (2) effluent-sludge mixture from the sedimentation unit (ESSU) and, (3) top-sand water sample (biofilm+sand+water) from the filtration unit (TSFU) were collected for the microbial isolation. Henceforth, the study pertaining to these samples will be referred to as Unit-1, Unit-2, and Unit-3, respectively. Around 30 mL of the sample collected from Unit-1 was filtered using glass fiber filter (pore size: 0.45 µm) to separate out any solid particles and use filtrate as an inoculum for microbial culture. Likewise, around 30 mL of effluent-sludge collected from Unit-2 and sand biomass sample from Unit-3 was centrifuged at 8000 x g for 30 minutes and the supernatant was used as an inoculum.

**2.4 Bacterial Isolation**

Enriched culture method was used for culturing and isolation of bacteria from the water sample (Manage et al., 2009). In brief, 15 mL of a filtered water sample from Unit-1, Unit-2, and Unit-3 were individually added to 75 mL of Luria-Bertani (LB) media in 250 mL Erlenmeyer flask. The flasks were incubated at 30 ± 1 ºC and 150 rpm for 2 days. A 15 mL of enriched culture broth was sub-inoculated into the freshly prepared LB media (75 mL total). The procedure was repeated three times. The resulting culture broth (100 µl) was streaked on LB-agar plate and incubated at 30 ± 1 ºC for 2-3 days. The heterogeneous colonies (based on color, morphology, and dominance) for each of the three units were isolated and serially streaked onto the LB-agar plates. In brief, a total of six bacteria was isolated. One from (INRSW1; Unit-1 bacterial

117 community), two from ESSU (INRSW2+INRSY1= Unit-2 bacterial community) and three from
118 TSFU (INRSW3+INRSY2+INRSB1 = Unit-3 bacterial community) where W, Y, and B stands
119 for white, yellow and brown color, respectively.

120 **2.5. Bacterial growth study and viability test under MC-LR environment**

121 Growth characteristics of all the six isolated bacteria were studied individually for three cases
122 viz. a) without the presence of MC-LR; b) with a lower dose of MC-LR (10 µg/L) and; c) with a
123 higher dose of MC-LR (100 µg/L). Growth parameters, such as doubling time, lag phase, log
124 phase (not shown) and the relation between cell viability vs optical density: $A_{600nm}$ (UV-VIS
125 Cary-50) were determined for each bacterial variety. The viable cell count was determined by
126 colony forming unit (CFU) through serial dilution method as described by Gargouri et al, (2015)
127 and its relationship to $A_{600nm}$ was established. The viable count was measured by counting the
128 colonies on LB-agar Petri plate, expressing the result as CFU/mL. All experiments were
129 performed in triplicates. The experimental procedure and operating conditions were similar as
130 described in section 2.4 for the growth study.

131 Viability count test on LB-agar plate was performed for four exponential points (i.e. one
132 early, two mid and one late exponential point). The sum of viable colonies of all the four
133 exponential points was considered to determine viability variations. The control was assigned a
134 value of 100 (case a: control) and thus normalized values were reported to study the change in
135 viability that occurred both at lower (10 µg/L) and higher MC-LR (100 µg/L) concentration.

136 **2.6 Acclimatization and post-acclimatization biodegradation study of MC-LR**

137 Bacterial community derived from Unit-1, Unit-2 and Unit-3 were acclimatized using 200 µg/L
138 MC-LR and their degradation potential were evaluated. This study was performed to enrich the
139 bacterial community and acclimatize them under MC-LR environment (Bourne et al., 2001).

After acclimatization, the culture media (200 µL) was plated on LB-agar to isolate the enriched bacteria ($2^{nd}$ generation bacteria) individually. Both acclimatization and post-acclimatization of MC-LR degradation studies were carried out in MSM, as suggested by Valeria et al., (2006), with some modifications as follows. The composition of the media per liter included 100 mg $MgSO_4 \cdot H_2O$, 5 mg $ZnSO_4 \cdot H_2O$, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 300 mg $KH_2PO_4$, 650 mg $Na_2HPO_4 \cdot 7H_2O$, 20 mg $CaCl_2$ and 0.15 mg $FeCl_3$ as the nutrient and MC-LR as the sole carbon and nitrogen source.

Approximately, a 6 x $10^6$ cells/mL (exponential phase bacteria) were spiked to study MC-LR degradation for bacterial community derived from Unit-1, Unit-2, and Unit-3. For example, $6x10^6$ cells/mL of INRSW1 bacterial cells isolated from unit-1 sample, $3x10^6$ bacterial cells/mL each of INRSW2 and INRSY1 isolated from Unit-2 sample and $2x10^6$ bacterial cells/mL each of INRSW3, INRSY2, and INRSB1 from Unit-3 sample, were spiked in MSM to study MC-LR degradation. Hence, three flask studies were performed. Before spiking these bacterial cells, culture media (containing LB medium and bacterial cells) were centrifuged at 8000 rpm at room temperature for 30 minutes to obtain the bacterial pellets. These bacterial pellets were further rinsed and centrifuged twice with phosphate buffer (pH 6.91) to remove any residual carbon present in the solution (due to LB medium in the previous step). MSM solution (60 mL) containing 200 µg/L MC-LR was then spiked with bacterial pellets of known cell concentration (co-culture combination as discussed above), shaken well and incubated at 30 ± 1 ºC and 150 rpm.

The degradation study for the acclimatization (non-enriched bacteria) and post-acclimatization phase was carried out for 15 days and 10 days respectively using same experimental condition (30 ± 1 °C;150 rpm, 200 µg/L MC-LR) and same co-culture cell

suspension concentration (6 x 106 cells/mL). *Sphingomonas sp.* (NRRL B-59555) was spiked in MSM containing 200 µg/L MC-LR with the same cell suspension (6 x $10^6$ cells/mL) as the positive control, which was previously reported to degrade MC-LR (Ishii et al., 2004; Valeria et al., 2006). In addition, MSM media with 200 µg/L MC-LR without any bacteria was taken as the negative control.

Kinetics test was performed for the degradation study by following two equations given below. Equation 1 shows zero-order kinetics relation between substrate concentration (MC-LR here) and time whereas equation 2 fits into the first order kinetics relation.

$$C_o - C_t = kt \quad (1)$$

$$C_t = C_o . e^{-kt} \quad (2)$$

Putting log on both sides and on rearranging the parameters, we get: $\ln C_o - \ln C_t = kt$ (3)

Where $C_o$= Initial substrate concentration; $C_t$ = Substrate concentration at time t and k= kinetics constant.

**2.7 MC-LR biodegradation and by-products analysis**

The MC-LR was analyzed in samples collected after 5 days, 9 days, 12 days, and 15 days for acclimatization-degradation study and after 2 days, 5 days, 8 days, and 10 days for post-acclimatized degradation study. These samples (3 mL) were centrifuged at 10,000 x g for 15 minutes at 20 **± 1** ºC. The supernatant was extracted and filtered using 0.45 µm sterile filter, capped in black-colored microcentrifuge tubes (BCCT) to avoid MC-LR photodegradation and stored at -20 ºC, until High-Performance Liquid Chromatograph (HPLC) analysis following protocol as discussed in Fayad et al. (2015).

By-product fragments formed during degradation were analyzed by mass spectroscopy. Around 1 mg/L of microcystin-LR was used as an internal standard along with mobile phase of 50:50

(v/v) MeOH (A) and water (B) with 0.1% formic acid. The final parameters used to maximize the signal intensity were: capillary temperature: 350°C, vaporizer temperature. 450°C, sheath gas pressure, aux gas pressure and ion sweep gas pressure: 35, 10 and 0 arbitrary units, respectively. The scan time was adjusted to 0.02 sec, where the first and third quadrupoles were operated at the unit resolution with second quadrupole collision gas pressure at 1.5 mTorr. Overall, this method presents an optimized rapid chromatographic method using an on-line solid-phase extraction coupled to ultra-HPLC tandem mass spectrometry for the determination of seven different cyanotoxins including microcystin-LR.

**2.8 Toxicity assessment of the degraded MC-LR samples/broth**

The toxicity of biodegradation broth (biodegraded sample obtained for the $10^{th}$ day) for all three units (Unit-1, Unit-2, and Unit-3) were examined using a bio-indicator: *Rhizobium meliloti* . Soil media was used for the culture of *R. meliloti* as mentioned in Surange et al., (1997) with some modifications.. Finally, the solution was made up to 1 liter and was autoclaved at 121 ± 1 °C for 20 minutes. Lyophilized *R. meliloti* strain was cultured in the soil media and kept for 24-36 h in a shaking incubator at 150 rpm and 30 ± 1°C. Afterward, they were successively sub-cultured two times (5% v/v) and streaked on the LB-agar plate (1.8%) to obtain the pure colonies.

Toxicity-protocol described by Botsford et al (1999) was followed with some modifications as follows. A 1 mL each of Tris-HCl buffer (pH 7.5), toxic sample (DMSO and MC-LR at different concentration) and bacterial cell suspension (*R.meliloti*) were mixed in a glass test tube and allowed to stand for 60-120 seconds. This time lapse allowed sufficient exposure for partial or total mortality of the cell culture depending on the degree of toxicity of the compound (MC-LR and DMSO tested). Later, around 350 µL of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye solution (7 mg/10 mL) was added to

the mixture which converted the solution from initial yellow to blue precipitate (viable cells, if left any, dies and forms formazan (blue precipitate). A special study to evaluate the critical incubation period for the bioindicator was performed. at three-time periods (10h, 16h, and 24h). It was found that the overnight incubation (10h) time period was the most critical time period, as also observed by Bodsford et al., (1999) to obtain the most fitted absorbance trend graph with various concentration of toxic chemicals, i.e., without any ambiguity in trend (graph not included for different time periods of incubation). For the reference toxic chemical, three compounds were selected viz. methanol, ethanol and dimethyl sulfoxide (DMSO). Among these compounds, DMSO was finally chosen based on the uniformity in absorbance test achieved and devoid of any ambiguous observation in absorbance values. The relationship between MC-LR (concentration ranging: 92 µg/L to 1470 µg/L) and DMSO (10%-100% v/v) was then established to report toxicity of the biodegraded broth in terms of equivalent DMSO (% v/v) concentration.

The toxicity assessment was based on the absorbance measurement at 550 nm (UV-VIS Cary 50 Spectrophotometer) post color change from yellow to blue due to the addition of MTT dye. After addition of MTT dye (0.350 mL) in combination with Tris-buffer, the toxic sample and bacterial cell suspension (total 3.350 mL mixture), the glass test tubes were capped and incubated for 1.5-2.5 h at 35 ± 1 °C. All the tests were done in triplicates.

**2.9. Bacterial species identification by ribosomal sequencing**

Out of the three DWTP units viz. Unit-1, Unit-2 and Unit-3, the bacterial community, which showed the highest MC-LR degradation was selected to undergo the MC-LR degradation test (performed individually on each of its comprising isolates). Bacterial species were further identified by ribosomal sequencing.

The genomic DNA isolation was performed using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-Tek, USA) as per manufacturer's instructions. The isolated DNA was PCR amplified using a 27F forward primer (5'-AGAGTTTGATCCTGGCTCAG) and 1492R reverse primer (5'-GGTTACCTTGTTACGACTT). The PCR amplicon was further sequenced for 16s rDNA identification of the bacterial strain (sequencing service from Génome Québec Innovation Centre). The identified sequence was analyzed using NCBI BLAST service to identify the bacterial strain. The identified 16aS rDNA are deposited in NCBI GenBank.

### 2.10 Statistical Analysis

Statistical analysis related to the analyzed parameters such as standard deviation, student t-test, a p-value of all the data sets and other graphical presentations were performed in ORIGIN software (Version 8.5; OriginLab).

## 3. Results and discussion

### 3.1 Bacterial Culture and Isolation

Samples obtained from three different units of the DWTP were cultured and heterogeneous bacterial growth was obtained over the LB-agar plate for each case as discussed in section 2.4. The dominant bacteria as observed based on the color was chosen as the representative of the bacterial culture community for each of the three units. INRSW1 from Unit-1 with white color, INRSW1 and INRSY1 from Unit-2 with white and yellow color, respectively and INRSW1, INRSY2 and INRSB1 from Unit-3 with white, yellow and brown color respectively, were isolated. Each of these bacteria was further studied for their growth behavior under various conditions as discussed in the next section. However, the degradation study was carried out as a microbial community as discussed in later section 3.3.

### 3.2 Bacterial growth study and viability test under MC-LR environment

Figure 1 (A, B, C, D) shows the growth curve (48 h period) for all the six bacteria isolated from different units of the DWTPs studied for four cases viz. a) in absence of MC-LR (Fig 1A), b) with 10 µg/L MC-LR (Fig 1B), c) with 100 µg/L MC-LR (Fig 1C) and, d) acclimatized bacteria ($2^{nd}$ generation) without MC-LR.

The growth characteristics were measured as lag phase and doubling time (time needed to double the bacterial cells during exponential phase). Table S1 shows doubling time (DT) and lag phase values for above four cases for all the six isolated bacteria. Their ability to grow under the MC-LR environment (case "b" and "c") were judged based on the change observed in lag phase and doubling time. It was found that the doubling time and lag phase increased for all the bacteria except for the yellow isolates (INRS Y1 and INRS Y2) and INRSB1. This showed that the viable bacterial population under MC-LR environment decreased but continued their natural growth. However, from student`s t-test analysis which was carried out for all six bacteria to compare their growth under MC-LR (in terms of $A_{600}$) to case "a" (no MC-LR), it was found that there was no significant difference (p-value greater than t-value) between the case "a" and "b" (average p-value 0.73 as compared to a t-value of 0.33 for all six bacteria) and "a" and "c" (averaging p-value 0.98 as compared to a t-value of 0.02 for all six bacteria ). This signifies that the growth characteristics did not change in response to the presence of MC-LR even at 100 µg/L.

To further confirm these observations qualitatively, the sum of viable cells (CFU/mL) of four exponential points (one early, two mid and one late exponential points) for the case "b" and "c" were compared to the case "a" (no-MC-LR; control and assigned value 100). Figure S1 (supplementary files) shows the bar graph for the survival test of these bacteria under MC-LR

environment. It was found that sum of viable cells decreased (normalized value<100) for all the six bacteria showing an overall obvious maximum effect at 100 µg/L (case c). Even the worst viability among all the six variety of bacteria was shown to be above 75% (77% for INRS W2) (also proved statistically as discussed in the previous paragraph) This indicates the bacterial potential to survive even in presence of high MC-LR concentration.

Acclimatized bacteria (case "d") showed a decrease in doubling time for all bacteria when compared with the case "a" (non-acclimatized without MC-LR) except INRS W3 (which anyways showed minor change). This indicated that after acclimatization, the growth rate of bacteria gets enhanced. A study by Hu et al., (2009) indicated that MCs degradation by *Methylobacillus sp.*, (isolated from cyanobacteria-salvaged sludge) required initial lag period which after acclimatization accelerated MC-LR degradation without any lag phase, even when new MCs extract was added (highlighting the importance of acclimatization).

Table S1 (supplementary files) shows the relationship between viable cells (CFU/mL) and $A_{600}$ for acclimatized bacteria. This relationship helped in obtaining the known concentration of bacterial cells for the degradation study (discussed in the next section).

### 3.3. Acclimatization biodegradation study of MC-LR

Degradation ability of co-cultured bacterial mix as found in Unit-1 (INRS W1), Unit-2 (INRS W2 + INRS Y1) and Unit-3 (INRS W3 + INRS Y2 + INRSB1) were tested with 200 µg/L MC-LR for both non-acclimatized bacteria (no previous MC-LR exposure) and acclimatized bacteria (previous exposure with MC-LR; case "d" as discussed in section 3.1). Figure 2 (A) shows the degradation study of MC-LR for non-acclimatized bacterial culture for each unit: Unit-1, Unit-2, and Unit-3 along with the trend for the cell viability. Total MC-LR degradation achieved by

298 bacterial community mixture derived from POU (Unit-1), ESSU (Unit-2) and TSFU (Unit-3)
299 was observed to be 71.1 ± 7.4 %, 86.7 ± 3.2 % and 94.3 ± 10.6 %, respectively after 15 days. As
300 depicted in Figure 2(A), the heterogeneous bacterial cell count decreased from initial count of 6
301 x $10^6$ CFU/mL to 5.2 x $10^6$, 5.7 x $10^6$ and 5.3 x $10^6$ CFU/mL for Unit-1, Unit-2 and Unit-3
302 respectively after 15 days. The viable cell count decreased after 5 days followed by attaining
303 maximum viability of 7.8 x $10^6$, 7.2 x $10^6$, 7.8 x $10^6$ CFU/mL on the 9th day for Unit-1, Unit-2,
304 and Unit-3, respectively (Figure 2(A)). The initial decrease in cell count might be due to the
305 toxic-shock of MC-LR to the bacterial community, which gradually recovered later, by
306 metabolizing MC-LR (present as the sole carbon and nitrogen source in MSM). Kansole et al
307 (2016) reported degradation of MC-LR (100 µg/L: by *Bacillus sp.* for 12 days) with a continuous
308 decrease in bacterial population from 85 x $10^5$ CFU/mL to 8 x $10^5$ CFU/mL. However, in the
309 present study, the decrease did not persist longer and after 5 days, the bacterial population
310 increased 1.25 folds than the starting cell count (as discussed above), clearly showing the
311 positive sign of bacterial adaptation utilizing MC-LR to maintain their metabolic activity.

312 **3.4 Post-acclimatization biodegradation study of MC-LR**

313 Figure 2 (B) shows the MC-LR degradation profile for the post-acclimatization phase ($2^{nd}$
314 generation bacteria) along with the cell viability tested between 2 to 10 days. The final MC-LR
315 degradation efficiency was similar to the degradation in acclimatization phase. Acclimatized-
316 TSFU (Unit-3) bacterial community achieved the highest degradation efficiency of 97.2 ± 8.7 %
317 followed by ESSU (Unit-2) (86.2 ± 7.3 %) and POU (Unit-1) (72.1 ± 6.4 %) acclimatized
318 bacterial community. However, steady state was achieved after 10 days (as compared to 15 days
319 for the non-acclimatized case) where corresponding degradation rate increased to 14.46
320 µg/L/day,17.32 µg/L/day,19.45 µg/L/day for Unit-1, Unit-2 and Unit-3, respectively (Table 1).

These degradation rates were around 1.5-fold higher than the degradation under the non-acclimatized bacterial case. The degradation rates were higher than some previously reported studies on microcystin variants (MC-LR/MC-RR) with the same growth medium (MSM) used as shown in Table S3.

Table 1 shows the comparison between pre-acclimatization and post-acclimatization degradation study in terms of kinetics constant, degradation efficiency and overall degradation rate for all the three units. Zero-order reaction was obtained for Unit-1 (14.07 μg/L/day) and Unit-2 bacterial community (20.81 μg/L/day). However, first order reaction was observed for the Unit-3 bacterial community (0.443/day). The kinetic constant for acclimatized bacterial community showed an increase of 44.4%, 68.9% and 140.7% for Unit-1, Unit-2, and Unit-3, respectively as compared to the non-acclimatized case. This indicated that the metabolic activity of this *in-situ* bacterial community enhanced through acclimatization. Also, it signified that the bacterial community derived from Unit-3 holds better potential as compared to Unit-1 and Unit-2 bacterial community in effectively metabolizing MC-LR. Under similar experimental conditions, the first order kinetic constant for both acclimatized and non-acclimatized bacterial community present in Unit-3 was found to be higher than the study by Kansole et al., (2016) (0.180/day for the non-acclimatized case (this study) and 0.443/day (this study) for the acclimatized case as compared to k= 0.026/day). These differences might be due to isolated bacteria utilized in the non-acclimatized-form as compared to our study which utilized bacterial isolates in the acclimatized-form. Some studies even reported incomplete degradation of microcystin variants. Ramani et al. (2011) reported incomplete degradation of both MC-LR (84%) and MC-RR (63.28%) at the end of 30 days with enriched bacteria (unidentified) in MSM. Kansole et al. (2016) also reported an incomplete MC-LR degradation study after 12 days (around 74%) with *Bacillus sp.* in autoclaved

Hulupi Lake water. This may be due to the involvement of different bacterial degradation mechanisms for the effective breakdown of microcystin (Neilan et al., 2014). Further, in some cases, certain conditions favor the production of the *mlrA* gene (gene responsible for microcystin breakdown) (Dexter et al., 2018), such as supportive metabolites, and by-products, etc. indicating the complete death of toxins in some cases. Other studies, where MSM media was used for evaluating MC-LR degradation with bacteria, such as *Sphingomonas isolate NV-3* (Somdee et al., 2013) and *Pseudomonas aeruginosa* (Lemes et al., 2015), achieved 100% degradation in 3 days and 24 days, respectively. However, their degradation rate of 8.33 µg/L/day for *Sphingomonas isolate NV-3* and 0.05 µg/L/day for *Pseudomonas aeruginosa* was lower than the biodegradation rate of 20 µg/L/day using bacteria derived from Unit-3 in this study as mentioned in Table S3*)*.

On another note, Kang et al., (2012) reported a degradation rate of 2.2 µg/L/day using $10^5$ cells/mL of *Pseudomonas aeruginosa.* Likewise, this study maintained the cell viability > $10^6$ cells/mL (comprising a bacterial strain: INRS W3, a *Pseudomonas* member too, see section 3.7) till the end and enhanced MCs degradation rate by 9 times (19.5 µg/L/day). This further highlights the importance of acclimatization for achieving effective MC-LR degradation. Thus, the acclimatization of *in-situ* bacterial community present in the DWTPs unit is key to degrade MC-LR effectively and faster.

Contrary to the non-acclimatization degradation phase, an increase in the cell viability was observed during the initial period (5 days) for all the three unit study (increasing from approx. 6 x $10^6$ CFU/mL to a minimum of 8 x $10^6$ CFU/mL. However, the viability decreased for all of them after 5 days of degradation, which can be linked to the decrease in the substrate concentration (MC-LR) that happened with time. This increasing trend followed by a decrease in

the bacterial population was also observed by Lemes et al., (2015) who studied MC-LR degradation with bacteria isolated from the beach sediment, where highest bacterial growth occurred after 12 days of degradation ($117 \times 10^5$ CFU/mL) that lasted for 24 days. The final viability count after 24 days was 80 x $10^5$ cells/mL when compared with the initial cell viability of 71 x $10^5$ cells/mL. Similar findings were observed in the present study (started from 6 x $10^6$ cells/mL and ended at 4.25 x $10^6$, 5.0 x $10^6$ and 5.5 x $10^6$ cells/mL for Unit-1, Unit-2 and Unit-3, respectively). However, the initial increase in cell viability of MC-LR-degrading bacteria can be related to the enhancement of energy metabolism (by the acclimatized bacteria) that helped to break down the complex, stable and cyclic microcystin-LR molecule. This might be a reason for the significant increase in the degradation rate as discussed above (1.5 fold). Likewise, this fact can also be attributed to the increase in the kinetic constant values in comparison to pre-acclimatization degradation phase.

### 3.5. By-product fragments analysis

The highest MC-LR degradation of 97.2 ± 8.7 % was achieved by the acclimatized-TSFU bacterial community (Unit-3). Thus, the qualitative toxicity assessment which depends on the formed by-products, their characterization (in terms of m/z value and change of chemical bond in structure) was needed. Figure S2 (supplementary files) illustrates the chromatograms for zero day, 2$^{nd}$ day and 5$^{th}$ day of MC-LR degradation by the Unit-3 bacterial community. The intensity (measured in absolute value) decrease with time corresponded to 69.75% and 94% degradation after day 2 and day 5, respectively. The other peaks observed are degradation by-products (Bourne et al., 1996). These by-products were further analyzed using mass spectra, which revealed several accompanied ions at m/z= 155.99, 162.9, 213.14, 268.24, 292.84, 315.19, 332.93, 375.25, 398.18, 470.29, 507.31, 553.29, 571.27, 599.34 and 862.48. Among them, m/z

value of 862.5, 507.31, 571.27, 599.34 were identified as the biotransformed products, namely, $[M - NH_2 - PhCH_2CHOMe + H]^+$, $[M + H_2O + 2H]^{2+}$, [Mdha-Ala-Leu-Masp-Arg + H]+, and $[M^{\#} - NH2 + H]+$ respectively, where M is a cyclo MC-LR Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH and M# is tetrapeptide Adda-Glu-Mdha-Ala-OH (Bourne et al., 1996). Also, smaller m/z value of 332.93 and 315.19 were identified as the biotransformed products as $[M^*+ H]^+$ and $[M^* - NH_3 + H]+$ respectively where M* = Adda molecule (Figure 3).

Figure 3 shows the proposed and hypothesized mechanism for the breakdown of the MC-LR compound by the bacterial community derived from Unit-3. The hypothesis predicts that the linearization of closed and complex MC-LR structure occurred after the formation of a biotransformed product having m/z value of 862.5. This linearization was due to the elimination of the terminal phenylethylmethoxy group and $NH_2$ group from Adda group through radical fragmentation (N-terminal Adda) (Bourne et al., 1996; Imanishi et al., 2005). Adda is one of the constituent amino acid compounds and is considered essential for the characteristic biological activity of microcystins as the toxicity disappears due to oxidation of Adda portion. It has been also reported that Adda is non-toxic up to 10 mg/kg in mice and it did not exhibit protein phosphate inhibition even at 10mM (Schmidt et al., 2014; Fujiki et al., 1996).

From Figure 3, a fragment with m/z value of 553 showed the presence of carboxy-terminal arginine similar to the fragment ion with m/z value of 571(Mdha-Ala-Leu-Masp-Arg-OH + 2H) and 488 (Ala-Leu-Masp-Arg-OH + 2H) as determined by Bourne et al., (1996) which corresponded to the C-C fragmentation at the N-terminal Adda representing tetrapeptide fragments. These tetrapeptides were further known to be cleaved by *mlrB* and *mlrC* genes into smaller peptides (as shown in Figure 3; m/z= 268, 213,155 and found in our study too). From mass spectra analysis, Adda fragment (m/z= 314) was formed for all the three biodegraded

broths (Unit-1, Unit-2 and Unit-3) followed by these small peptide fragments indicating further oxidation of Adda might have occurred leading to non-toxicity.

Generally, hydrolysis of MC-LR is responsible for the linearization of the structure where m/z value increases by 18 (i.e. 995 (basic $[M+H]^+$ value) +18 $[H_2O]$=1013) (Figure 3). However, Edwards et al., (2008) and Dziga et al., (2012) indicated that further loss from hydrolyzed state due to loss of a portion from Adda group and amino groups incurs a total loss of 151 in m/z value resulting in a product with m/z value of 862 (Figure 3). This by-product is also related to the hydrolysis of parent ions by a *mlrA* gene which further suggested that the bacterial community present in TSFU (Unit-3) might contain *mlrA* gene which is responsible for the biotransformation of the cyclic and complex MC-LR compound. However, this hydrolyzed product was found in all the three units (Unit-1, Unit-2, and unit-3 bacterial community), which could be due to the induction of certain genes, such as *mlrA* as discussed above. Further, the *mlrA* has also been known to detect the presence of MC-degrading bacteria (Saito et al., 2003; Hoefel et al., 2009). Some researchers also showed that MC-LR degrading bacteria containing microcysatinase *mlrA* encodes a hydrolytic enzyme capable of initiating MCs degradation by cleaving the Adda-arg peptide bond (shown by the dashed arrow in Figure 3) (Bourne et al., 2001). Moreover, these hydrolyzed linear by-products formation is also linked to the reduced toxicity (Hoefel et al., 2009) which is quantitatively studied using a bioindicator in the next section.

### 3.6 Toxicity assessment of the degraded MC-LR samples

The qualitative toxicity analysis based on mass spectra results depicted the formation of small peptide fragments and amino groups, thereby suggesting the non-toxicity nature of the degraded samples/broth. However, quantitative toxicity assay was also performed using a bioindicator:

436 *Rhizobium meliloti* to confirm the findings. Figure 4 (A) shows the absorbance (at 550 nm) vs
437 concentration graph for the reference toxic solution, i.e., dimethyl sulphoxide (DMSO) ranging
438 from 10%-100% (v/v) at critical time-period of 10 h using *Rhizobium meliloti* as a bioindicator.

439 As the DMSO concentration increased from 10% (v/v) to 100%, (v/v), a decrease in absorbance
440 was observed. This is because all survived bacterial cells, after getting exposed to DMSO (a
441 toxic substance) precipitated to blue color (formazan) post MTT (yellow color dye) addition. If
442 the cell viability increased (less toxic compound), more precipitate would have been formed
443 (hence more absorption value) and vice-versa, as reported by Botsford et al., (1997) too. In this
444 study, a similar toxicity behavior trend was observed for MC-LR also. The color of the solution
445 remained mostly yellow even at least MC-LR concentration tested (92 µg/L) which indicated the
446 toxic behavior of MC-LR (visuals: Supplementary files: Figure S4). The trend followed a good
447 quadratic curve fit with $R^2$ value 0.9591 for the critical incubation period of 10 h (supplementary
448 files: Figure S3). Figure 4(B) shows the equivalent DMSO concentration (% v/v) for the various
449 MC-LR concentrations (92 to 1470 µg/L) being studied. Based on the absorbance values
450 obtained for the biodegraded broth, their equivalent DMSO concentration has been plotted. The
451 equivalent DMSO concentration (% v/v) is the value proportional to the absorbance shown by
452 various MC-LR concentrations when compared to the DMSO absorbance (shown in Figure 4 A).
453 Hence, DMSO acted as a surrogate reference toxic solution to simulate MC-LR toxicity in the
454 biodegraded broth.

455 Equivalent DMSO toxicities of the biodegraded broth for all three cases studied viz. Unit-1,
456 Unit-2 and Unit-3 were found to be –8.4 (% v/v), -16.5 (% v/v) and -19.37 (% v/v), respectively.
457 Negative concentration means the biodegraded broth was safe enough to be compared with
458 DMSO potential toxicity. The blue color observed for three samples illustrated non-toxicity and

459 unchanged yellow color illustrated toxicity for DMSO sample, as tested (supplementary file,
460 Figure S4).

461 Many researchers have used DMSO as the reference solution to observe the toxic effects on
462 various human cells as well as other species. For example, *in vitro* toxicity in a retinal neuronal
463 cell line from rats was observed at DMSO concentration higher than 1% (v/v) (Galvao et al.
464 2013). DMSO was also shown to affect red blood cells, platelets and vascular endothelial cells *in*
465 *vitro* at a concentration > 0.6% v/v and bacterial strains, such as *S.epidermidis* and *S.paratyphia*
466 at a concentration >5% (v/v) (Yi et al., 2017). Another study revealed that DMSO with 0.5%–
467 2% v/v significantly suppressed the expression of many pro-inflammatory cytokines/chemokines
468 (Proost et al., 2016). In fact, at 0.1-1.0 % v/v, it not only affected the phenotypic characteristics
469 but also induced a significant alteration in the gene expression, protein content, and
470 functionality of the differentiated hepatic cells. As compared to the literature, the biodegraded
471 broth in the present study showed equivalent DMSO toxic level that did not affect any living
472 cells.

473 **3.7 Bacterial species identification by ribosomal sequencing**

474 The best MC-LR degrading bacterial community was found to be from TSFU (listed in Table S1
475 and Table S2) which comprises three bacterial strains: INRSW3, INRSY2, and INRSB1. Before
476 their identification, MC-LR degradation potential was evaluated for each of them individually
477 (Supplementary files: Figure S5). Results showed maximum degradation of 85.3% and 84.6%
478 for INRSW3 and INRSY2, respectively and thus was further screened for identification through
479 16S rRNA PCR sequencing.

The BLAST analysis of the 16S rDNA sequencing of INRSW3 strain showed 99% homology to the *Pseudomonas fragi* and the INRSY2 strain showed 99% homology to the *Chryseobacterium sp.* The nucleotide sequence of 16S rDNA of the identified INRSW1 and INRSY2 bacterial strains has been deposited in NCBI with Acc. No. MH150821 and MH150822, respectively. However, it is interesting to note that there are very few literature reports available on the microcystin degradation studies from *Pseudomonas fragi sp*. However, there are no reports available on the degradation of microcystin by *Chryseobacterium sp*. Nevertheless, the present study showed that *Chryseobacterium sp*. and *Pseudomonas fragi sp*. degrade microcystin at 250 µg/L (Supplementary files: Figure S5) that is remarkably more than the other microorganisms in our study (more than 80%: supplementary files: Table S3). The comparison of the microcystin degradation of various microorganisms is listed in Table S3. As shown in Table S3, some studies on MC-LR degradation achieved lower degradation rate than our study. Most importantly, they have been characterized for their toxicity and this suggests that the by-products formed are non-toxic. Thus, these microorganisms could be of interest for efficient degradation of microcystin. Further studies on phylogenetic analysis and in-silico identification of gene cluster responsible for degradation of microcystin in these organisms would shed more light on their genetic characteristics with respect to microcystin degradation. This will aid in cloning and characterization of microcystin degrading enzymes from *Chryseobacterium* sp. and *Pseudomonas fragi* sp.

## 4. Conclusion

The microcystin-LR-enriched bacterial community showed enhanced degradation rate as compared to the non-acclimatized (no-MCLR enrichment) bacterial community isolated from different units of the drinking water treatment plants viz. raw water entering the pre-ozonation

unit (POU), effluent-sludge sedimentation unit (ESSU) and top-sand filtration unit (TSFU). Acclimatized-TSFU bacterial community showed the best result achieving 97.2 ± 8.7% MCLR degradation. Based on the best MC-LR degradation results, two strains comprising TSFU community revealed over 99% homology to *Pseudomonas fragi* and *Chryseobacterium spp.* and were found to be novel MC-LR degrading species. Mass spectra result depicted hydrolysis of complex MCLR molecule into smaller peptide molecules along with Adda molecule formation (m/z= 314) which qualitatively suggested decreased toxicity of the final biodegraded broth. Furthermore, *Rhizobium meliloti* used as a bioindicator qualitatively confirmed these by-products as non-toxic. This study gives a lead to utilize these identified novel strains in DWTP for effective degradation of MC-LR ensuring safe and toxin-free drinking water.

**Conflict of interest**

None

**Acknowledgment**

The authors are sincerely thankful to the Natural Sciences and Engineering Research (Discovery Grant 23451), Genome Québec, Genome Canada) and ATRAPP (Algal blooms, treatment, risk assessment, prediction, and prevention) for financial support. Special thanks to Prof. Sébastien Sauvé for allowing the sample receipt and analysis in his laboratory. Authors would also like to thank the team for constant support and timely suggestions. We would like to thank Genome Quebec for timely sequencing for the bacterial identification. The views or opinions expressed in this article are exclusively those of the authors.

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**List of Tables:**

**Table 1:** Degradation efficiency and degradation rate for pre-acclimatization and post-acclimatization phase

| Case | Degradation rate before acclimatization | Removal efficiency before acclimatization | Kinetics constant (k-value) [order] Before acclimatization | Degradation rate after acclimatization | Removal efficiency after acclimatization | Kinetics constant (k-value) [order] After acclimatization |
|---|---|---|---|---|---|---|
| Raw water (POU) | 9.46 µg/L/day | 71.1 ± 7.37 % | 9.74 µg/L/day [0] | 14.46 µg/L/day | 72.1 ± 6.4 % | 14.07 µg/L/day [0] |
| Sedimentation unit (ESSU) | 11.56 µg/L/day | 86.7 ± 3.19 % | 12.32 µg/L/day [0] | 17.32 µg/L/day | 86.2 ± 7.3 % | 20.81 µg/L/day [0] |
| Filtration unit (TSFU) | 12.58 µg/L/day | 94.35 ± 10.63 % | 0.184/day [1] | 19.45 µg/L/day | 97.2 ± 8.7 % | 0.443/day [1] |

[1] -19.38 µg/L/day is kinetics zero-order constant for positive control

[1]

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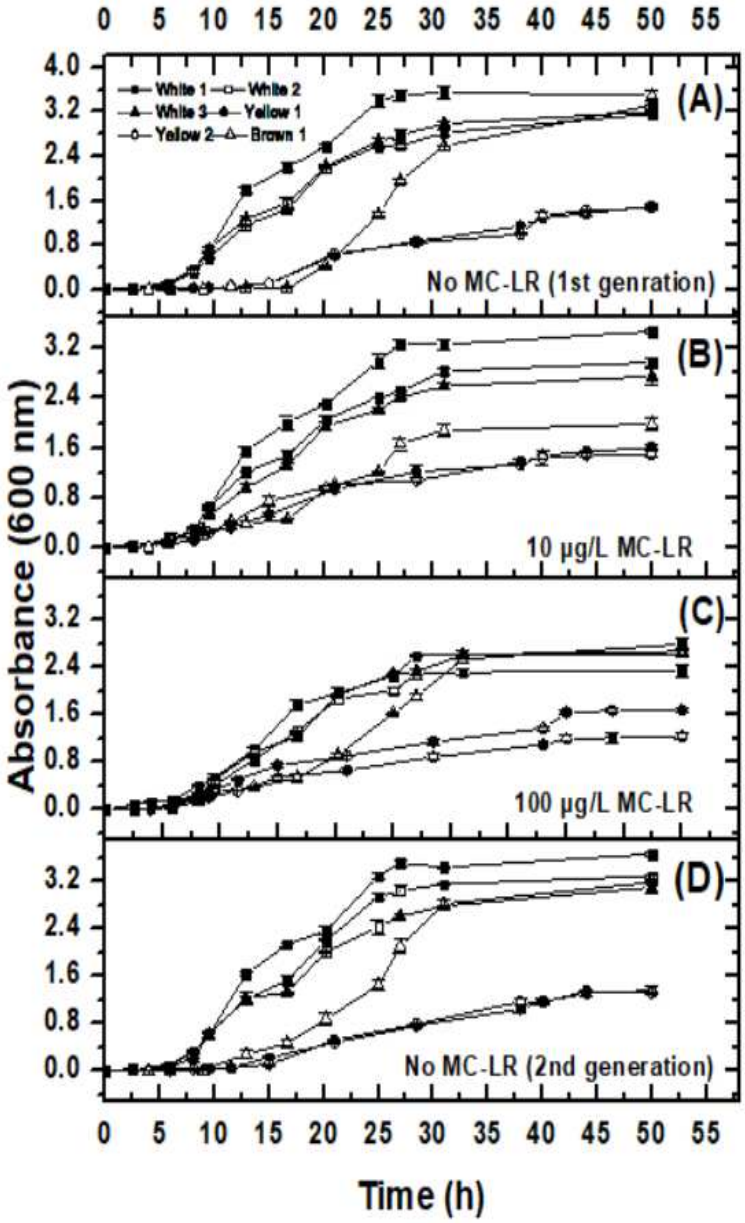
684 **List of Figures:**

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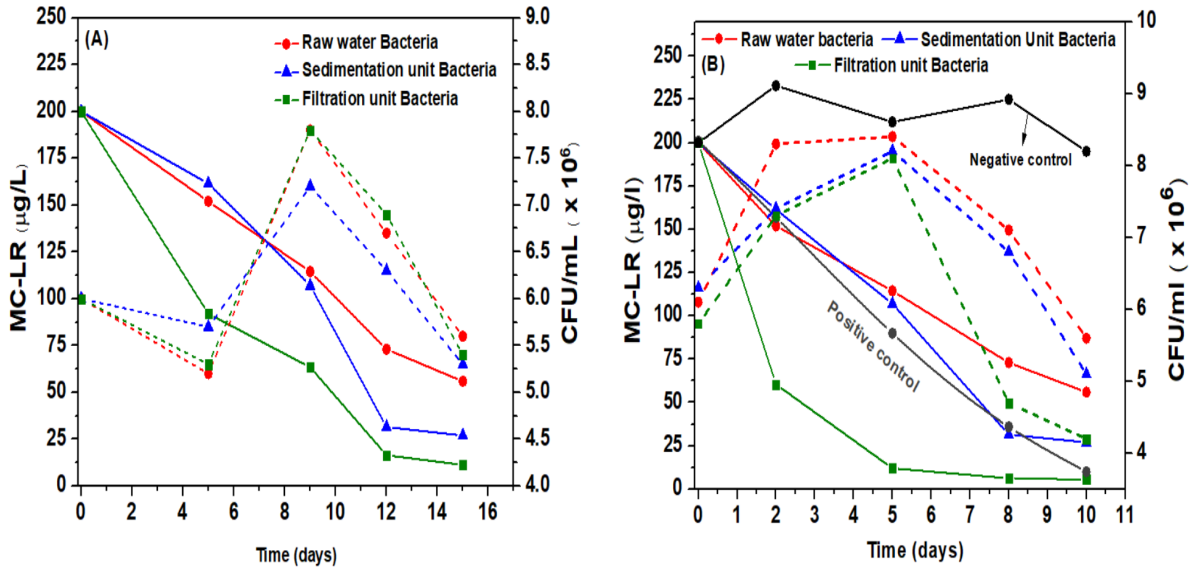
687 **Figure 1:** Growth curve (Optical density $OD_{600}$ Curve vs time) for all six bacteria isolated from
688 different units of drinking water treatment plant (DWTP) with spiked microcystin-LR (MC-LR)
689 (A) No MC-LR;–(B) 10 µg/L MC-LR;(C) 100 µg/L MC-LR and; (D) No MC-LR ($2^{nd}$
690 generation: acclimatized bacteria)

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692 **Figure 2** Drawdown curve of MC-LR and cells viability trend for: (A) pre-acclimatization phase
693 and; (B) Post-acclimatization phase

694

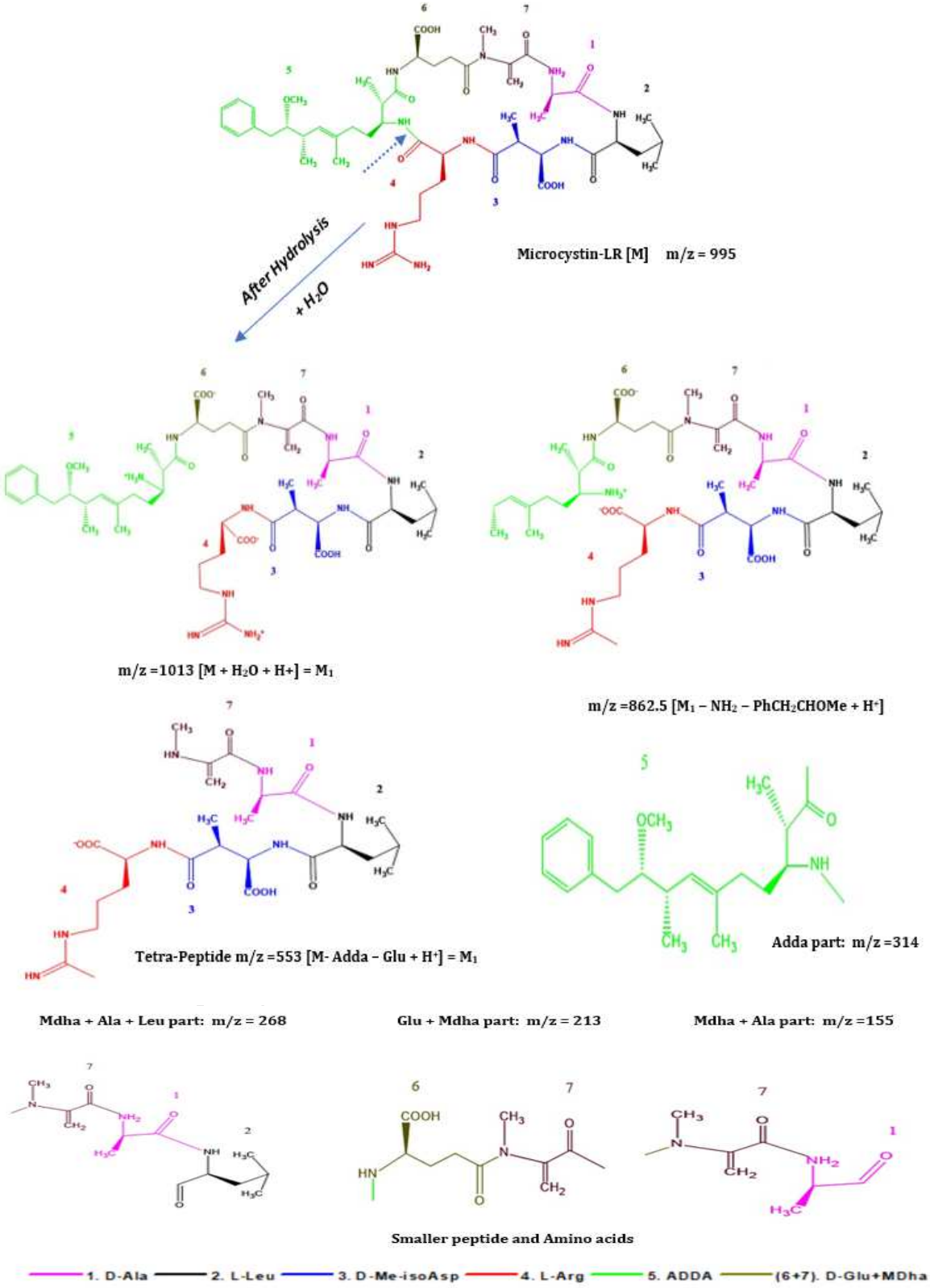
After Hydrolysis
+ H2O
Microcystin-LR [M] m/z = 995
m/z =1013 [M + H2O + H+] = M1
m/z =862.5 [M1 – NH2 – PhCH2CHOMe + H+]
Tetra-Peptide m/z =553 [M- Adda – Glu + H+] = M1
Adda part: m/z =314
Mdha + Ala + Leu part: m/z = 268
Glu + Mdha part: m/z = 213
Mdha + Ala part: m/z =155
Smaller peptide and Amino acids
1. D-Ala
2. L-Leu
3. D-Me-isoAsp
4. L-Arg
5. ADDA
(6+7). D-Glu+MDha

**Figure 3:** Proposed degradation pathway (based on identified fragments by mass spectra) for the breakdown of MC-LR by the co-culture bacterial community isolated from Top-sand filtration sand Unit (TSFU)

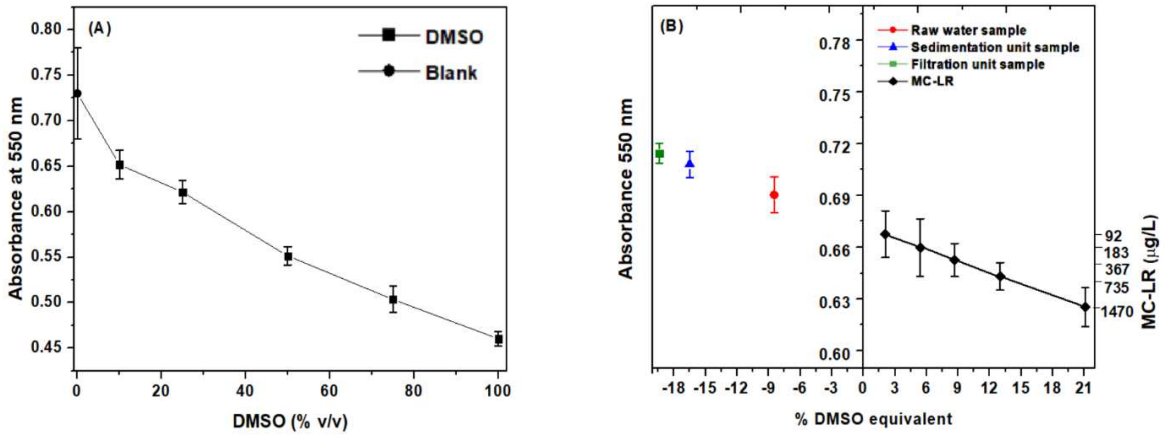


**Figure 4**: (A) Toxicity assay colorimetric test for various concentration of DMSO (% v/v) vs absorbance at 550 nm and; (B) Equivalent Dimethyl sulfoxide (DMSO) (% v/v) for microcystin-LR compound and biodegraded broth

**Highlights:**

- Filtration unit bacterial community showed maximum MC-LR degradation of 97%.
- Acclimatized bacteria (MC-LR enriched) increased degradation rate by over 52%.
- Degraded samples showed toxicity level well below 2.5% v/v DMSO (using Bioassay).
- *Pseudomonas fragi* and *Chryseobacterium sp.* were identified as the novel MC-LR degraders.