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Cyanotoxin degradation activity and *mlr* gene expression profiles of a *Sphingopyxis* sp. isolated from Lake Champlain, Canada

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ABSTRACT: A bacterium capable of degrading five microcystin (MC) variants, microcystin-LR, YR, LY, LW and LF at an initial total concentration of 50 µg/l in less than 16 hours was

isolated from Missisquoi Bay, in the south of Quebec, Canada. Phylogenetic analysis of the 16S rRNA gene sequence identified the bacterium as *Sphingopyxis* sp., designated strain MB-E. It was shown that microcystin biodegradation activity was reduced at acidic and basic pH values. Even though no biodegradation occurred at pH values of 5.05 and 10.23, strain MB-E was able to degrade MCLR and MCYR at pH 9.12 and all five MCs variants tested at pH 6.1. Genomic sequencing revealed that strain MB-E contained the microcystin degrading gene cluster, including the *mlrA*, *mlrB*, *mlrC* and *mlrD* genes, and transcriptomic analysis demonstrated that all of these genes were induced during the degradation of MCLR alone or in the mixture of all five MCs. This novel transcriptomic analysis showed that the expression of the *mlr* gene cluster was similar for MCLR alone, or the mixture of MCs, and appeared to be related to the total concentration of substrate. The results suggested that the bacterium used the same pathway for the degradation of all MC variants.

Keywords: Microcystins; *Sphingopyxis* sp.; Transcriptomic analysis; Expression profiles

1. INTRODUCTION

The increasing frequency of toxic cyanobacterial blooms in surface waters worldwide comes with the potential to disrupt drinking water production systems and there are concerns for the health of users of these affected water resources.¹⁻³ Different genera of toxin producing cyanobacteria, such as *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, and *Planktothrix* have been detected in these water supplies.^{4,5} Among them, microcystin (MC) producing species are the most frequently reported and studied toxic cyanobacteria. MCs are cyclic heptapeptides composed of 3 D-amino acids; alanine (Ala), methylaspartic acid (MeAsp), glutamic acid (Glu), two unusual amino acids; N-methyldehydroalanine (Mdha) and 3-amino-9-

methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) and finally a pair of variable L-amino acids.⁶ As the result of structural variations at all seven amino acids' positions, 89 analogues of MCs have been identified to date.⁷ The hepatotoxicity of MC molecules is due to the presence of the unique β -amino acid, Adda, in their structure.⁸

Because of its small cyclic structure, MCs are resistant to enzymatic and physico-chemical breakdown.⁹ Among the processes controlling the fate of MCs in natural waters, biodegradation has been reported as a dominant mechanism.^{10, 11} As a result of the presence of bacterial communities in freshwater where cyanobacterial blooms are common, the concentration of MCs is usually lower than expected based on the numbers of cyanobacterial cells.¹² *Anabaena*, *Aphanizomenon* and *Cylindrospermopsis* species with the ability to produce anatoxin-A (ATX) and cylindrospermopsin (CYN)¹³, have been frequently detected in lakes that are a source of drinking water.^{4, 14} Consequently, mechanisms involved in the fate and disappearance of these neurotoxins and cytotoxins need to receive more attention, although at present, there is limited information available with regards to their biodegradability.

A dynamic change in the pH of water bodies during cyanobacterial blooms has been reported in several studies.^{3, 15} During photosynthesis, cyanobacterial cells cause a shift in the equilibrium of the carbonate system, increasing the pH of the environment, which allows them to outcompete other algal species.¹⁶ Under basic pH conditions, between 80-90% of the cyanotoxins are intracellular, but they are released into the aquatic phase following cell death.¹⁷ To better predict the fate and persistence of cyanotoxins in aquatic systems, the effect of pH on the biodegradation activity of degrader isolates needs to be elucidated. However, currently there is limited information in the literature regarding the effect of this parameter on cyanotoxin degradation.^{9, 18}

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Bacteria with the ability to degrade multiple cyanotoxins have been isolated from a variety of water sources in different geographical locations (Table S1, Supporting Information). The majority of studies that have focused on MC degradation have identified *Sphingomonas* sp. as the most commonly encountered degraders.¹⁹⁻²² *Sphingopyxis* is one of the strains that has been reported as a promising bacterium with the ability to degrade multiple MCs.^{10, 18, 23, 24} Other bacteria, such as *Paucibacter toxinivorans*, *Arthrobacter*, *Brevibacterium*, *Bordetella* sp and *Rhodococcus* sp, with the ability to degrade a variety of microcystins and nodularins have also been isolated.²⁵⁻²⁷ However, the diversity and number of identified toxin degraders remain low.

Bourne et al.¹⁹ identified the enzyme encoded by the *mlrA* gene in *Sphingomonas* sp. as the initiator for the biodegradation of MCLR through the cleavage of its cyclic structure at the arginine-Adda peptide bond. Imanishi and colleagues²¹ also highlighted the importance of the arginine-Adda peptide bond by demonstrating that only MCs containing this bond are degraded by *Sphingomonas* sp. B9. In contrast, Ishii et al.²⁸ and Ho et al.¹⁰ showed that other MCs such as MCLW, LY and LF, which do not have the arginine-Adda peptide bond, are also degraded by bacteria belonging to the genus *Sphingomonas*. These differing observations on the site of cleavage of the cyanotoxin variants suggest the possibility that different pathways or different degradation genes may exist for MC degradation.

The advent of next generation sequencing methods has made it possible to sequence whole microbial genomes at a much lower cost and provides opportunities to examine gene expression patterns under different environmental conditions. To the best of our knowledge, genomic and transcriptomic analyses have not yet been used to characterize bacteria that have the ability to degrade multiple microcystin variants.

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The aim of this study was to isolate and identify a bacterium with the ability to degrade multiple cyanotoxins from a drinking water production site that experiences frequent and severe cyanobacterial blooms. The specific objectives were: a) to evaluate the capacity of the novel isolate to degrade multiple MCs, ATX and CYN, b) to determine the effects of pH on MC degradation activity, c) to identify the genes involved in the degradation of microcystins and using transcriptomic analysis, determine whether the same genes are involved in the degradation of a single microcystin or a mixture of different MC variants.

2. MATERIALS AND METHODS

2.1. Cyanotoxins and Reagents

MCLR, YR, LY, LW, LF, ATX and CYN were purchased from a commercial supplier (Enzo Life Science, Farmingdale, NY, USA). Individual stock solutions were prepared by dissolving cyanotoxins in a mixture of methanol and sterilized Milli-Q (Millipore, Etobicoke, ON) water according to the instructions of the supplier. To minimize the concentration of methanol in the stock solution, 100 µg of cyanotoxin was dissolved in 100 µl methanol and then diluted 100 times with Milli-Q water. Due to the minimized amount of methanol and lack of evidence in the literature that methanol can induce the degradation of microcystins authors refrained to test methanol effect as a control on the gene expression. In addition, the very low concentration of methanol and the fact that methanol was present under all test conditions over time, allowed authors to rule out any involvement. All reagents used were of analytical or HPLC grade.

2.2. Isolation of microcystin degraders from lake water

Water samples were collected from Missisquoi Bay, in the south of Quebec, Canada. Missisquoi Bay is a shallow bay of Lake Champlain, bordered by the states of New York and

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Vermont (U.S.A.) and the province of Quebec (Canada). Cyanobacterial blooms in the bay have been severe and the breakthrough of toxins into treated drinking water has been observed.²⁹ Microcystin producing genera such as *Microcystis* have been reported as the major part of the cyanobacterial community in the bay during the summer and early fall since 2002.¹⁴ However other species known to produce ATX, saxitoxins (STX) and CYN have also been identified, including *Anabaena* and *Aphanizomenon*.³⁰

To isolate cyanotoxin degrading bacteria, water samples were collected during cyanobacterial blooms in July, August and September. Water samples were collected in the littoral area of Philipsburg, Quebec, throughout the water column from the surface to a depth of 2 m. Samples were collected in 1 liter sterilized plastic bottles, transferred to the lab on the same day and kept in a refrigerator (4°C). The mixture of seven cyanotoxins including MCLR, YR, LY, LW, LF, CYN and ATX at individual concentrations of 10 µg/l was spiked aseptically into sterilized amber glass bottles containing 100 ml of the lake water. Bottles were shaken at 175 rpm aerobically on a rotary shaker at room temperature (22-23°C). After one week, a 10 ml sub-sample was transferred to new bottles containing the same cyanotoxin mixture as the sole carbon sources in 90 ml sterilized mineral salt medium (MSM- 0.25 g K₂HPO₄, 0.5 g KH₂PO₄, 0.25 g Na₂HPO₄, 5 mg NH₄Cl, 25 mg CaCl₂, 20 mg MgSO₄.7H₂O, 0.25 mg FeCl₃.6H₂O; 0.5 mg MnCl₂.4H₂O per litre). The pH of the medium was adjusted to 7.4 using a solution of 0.1 M HCl or 0.1 M NaOH. This enrichment procedure was continued for six weeks with sub-culturing into the same medium at weekly intervals. Control bottles containing only lake water without cyanotoxins were also prepared and incubated under the same conditions. All incubations were performed in duplicate. At the end of the incubation, 1 ml samples from each bottle was plated onto solid peptone-yeast extract (PY) medium (1 g peptone, 0.5 g yeast extract, 18 g agar per

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liter) and incubated at 32°C for 96 hours. Based on shape, size and color, colonies that grew on the solid medium were streaked several times on fresh plates to obtain pure cultures. To confirm whether individual isolates possessed the ability to degrade cyanotoxins, each isolate was incubated overnight in PY medium (0.2% (w/v) peptone, 0.1% (w/v) yeast extract, pH 7.4). Bacteria in the logarithmic growth phase were removed by centrifugation at 1000 x g for 15 min and the bacterial pellet was washed 3 times with 0.05 M phosphate buffer saline (PBS). The isolates were incubated in PBS at room temperature overnight to reduce any residual carbon. Bottles containing sterilized MSM and the mixture of the seven cyanotoxins at individual concentrations of 10 µg/l were spiked with an isolate to reach an optical density OD (660 nm) of 0.05. Sub samples of 5 ml were taken daily under aseptic conditions, filtered through 0.22 µm nylon filters and frozen at -20°C to monitor residual cyanotoxin concentrations. In parallel, control bottles containing only MSM and cyanotoxins without bacteria were prepared as negative controls. An isolate (MB-E) with the ability to degrade MCs at the highest biodegradation rate was selected for further analysis.

2.3. Total DNA extraction, PCR amplification and sequencing of 16S rRNA

One colony from strain MB-E was grown for 2 days at 30°C in PY medium and 175 rpm. DNA was extracted with the DNeasy Blood and Tissue kit following the protocol for Gram-negative bacteria (QIAGEN, Mississauga, ON). PCR amplification of the whole 16S rRNA gene was performed using the F1 and R13 primers published by Dorsch and Stackebrandt.³¹ Additional information regarding the method can be found elsewhere (Text S1, Supporting Information).

A phylogenetic tree was constructed based on available 16S rRNA gene sequences (950 bases or longer) of bacteria that have the ability to degrade several microcystin variants. Phylogenetic

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relationships were constructed with evolutionary distances (Jukes-Cantor distances) and the neighbor-joining method using the MacVector software package (Accelrys, Inc., San Diego, CA, USA). The bootstrap analyses were calculated by running 1000 replicates to assign confidence levels to the tree topology.

2.4. Genomic sequencing

Genomic sequencing was performed on isolate MB-E. One microgram of DNA was treated with RNase If (New England Biolabs, Whitby, ON) according to the instructions of the manufacturer. The DNA was purified with the QIAEX II Kit (QIAGEN) and quantified with the Quant-iT PicoGreen dsDNA assay (Invitrogen, Burlington, ON). One hundred nanograms of DNA were fragmented for 20 min using the Ion X press Plus gDNA kit (Life Technologies, Burlington, ON). The DNA was quantified with a BioAnalyzer, high sensitivity DNA chip (Agilent Technologies, Mississauga, ON). A total of 4.7×10^8 molecules were used in the emulsion PCR using the Ion OneTouch Template kit (Life Technologies) and the OneTouch instrument (Life Technologies). Sequencing was performed on an Ion Torrent personal genome machine (PGM) using the Ion 316D chip and the Ion PGM Sequencing 200 kitV2 (Life Technologies) following the instructions of the manufacturer.

2.5. Time course degradation of MCs, CYN and ATX by isolate MB-E

To validate the degradation of multiple MCs, CYN and ATX by strain MB-E and for transcriptomic sequencing, three solutions were prepared; a) a mixture of the five MCs (MCLR, YR, LY, LW and LF), b) the mixture of CYN and ATX and c) MCLR was prepared and spiked at a concentrations of 10 µg/l in separate amber glass bottles containing 150 ml of sterilized MSM (autoclaved at 121°C for 15 min). Strain MB-E was grown overnight in PY medium and

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3 178 then washed with PBS as described previously, before inoculating the media to an optical density
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5 179 (660 nm) of 0.05. Sub-samples of 5 ml and 1 ml were taken every 2 hours over 28 hours using
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7 180 aseptic conditions. One ml samples were flash frozen in liquid nitrogen and kept in -80°C for
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9 181 transcriptomic analysis. To check for any losses of cyanotoxins because of abiotic processes,
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11 182 bottles containing sterilized MSM medium with cyanotoxins were used as controls.
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183 **2.6. Transcriptomic analysis of microcystin degradation genes**

184 **2.6.1 Total RNA extraction**

185 RNA was extracted using a modified hot phenol method from Rhodius.³² The main modification
186 involved the addition of fresh lysozyme (3mg/ml) to the frozen cultures and an incubation at
187 64°C for 1 min. The RNA was purified using the RNeasy Mini Elute clean up kit (QIAGEN)
188 then treated with DNase using the TURBO DNA free kit (Ambion, Burlington, ON) following
189 the instructions of the manufacturers.

190 **2.6.1. Transcriptome sequencing**

191 The total RNA was subjected to ribosomal 16S and 23S RNA subtraction following the
192 procedure of Stewart et al.³³ Total rRNA-subtracted RNA was reverse-transcribed using the
193 SuperScript III kit (Thermo Fisher Scientific Inc., MA, USA). Illumina libraries were prepared
194 following the protocol of Meyer and Kircher³⁴, with tags 1 to 24. The indexed libraries were
195 pooled in an equimolar ratio and sequenced (300 cycles) using a MiSeq Reagent Kit v2 (Illumina
196 Inc., CA, USA) on the MiSeq Illumina sequencer.

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197 **2.7. Bioinformatic analyses**

198 **2.7.1. Genomic analysis**

199 Sequencing data was assembled using the de novo assembly function of the software suite CLC
200 Genomics Workbench 7 (<http://www.clcbio.com/products/clc-genomics-workbench/>). All ORFs
201 were then given a functional qualifier through a blastp (1e-10) search against the refseq_protein
202 database (<http://www.ncbi.nlm.nih.gov/refseq/>). The detailed method is presented in Supporting
203 Information (Text S2).

204 **2.7.2. Transcriptomic analyses**

205 Raw transcriptomic sequence pairs were first merged into individual contigs using pear³⁵ with
206 default settings. Assembled and unassembled sequences were then quality controlled and freed
207 of remaining adapter sequences using Trimmomatic.³⁶ The setting and the procedure are
208 presented in the Supporting Information (Text S3). The statistical significance of the difference
209 in *mlr* gene/cpn60 ratios between the toxin mix and MC-LR cultures was tested for each time
210 point with T-tests, using the t.test function of R version 3.2.1 (<http://www.r-project.org>) (p value
211 cutoff 0.05). The statistical significance of differences in *mlr* gene/cpn60 ratios between time
212 points was also tested for each culture with ANOVAs using R's anova function and a linear
213 model (p value cutoff 0.05). Post hoc analyses were then conducted using R's aov and tukeyHSD
214 functions (p value cutoff 0.05).

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2.8. Effect of pH on growth of the isolate MB-E and its degradation activity of multiple MCs

To assess the effect of pH on biodegradation of multiple MCs by the isolate MB-E, individual bottles containing autoclaved MSM medium were spiked with a mixture of five microcystins (MCLR, YR, LY, LW and LF) at individual concentrations of 10 µg/l, inoculated with the isolate MB-E at an initial concentration of 0.05 OD (660 nm) and incubated on a rotary shaker (175 rpm) at 22-23°C. The pH was adjusted to 5.05, 6.10, 7.21 and 8.05 using potassium phosphate buffer, at 9.12 with boric acid buffer and 10.23 with NaOH. All the pH values were measured again at the end of the experiment to check for stability. To monitor the growth of isolate MB-E at different pH values, it was added to the PY medium to obtain a final bacterial density of 0.02 (660 nm).

2.9. Analysis of cyanotoxins

A measurement system composed of an Ultra High Performance Liquid Chromatography (UHPLC-Thermo Finnigan, San Jose, CA) coupled to a TSQ Quantum Ultra AM Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) with a Heated Electrospray (HESI) source has been developed to quantify multiple cyanotoxin concentrations. Full details of the analytical method are provided elsewhere³⁷, and instrumental operating conditions are summarized in Tables S2-S3 and Text S4 of the Supporting Information.

3. RESULTS AND DISCUSSION

3.1. Isolation of the cyanotoxin degrading bacterium

A total of 22 strains were initially isolated with the ability to degrade cyanotoxins from the water samples collected during and after the bloom season. Among the isolated strains, four were able

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to degrade all five microcystin variants (MCLR, YR, LY, LW and LF) in less than three days (data not shown). Sequencing analysis of the 16S rRNA gene, showed that one of the isolate demonstrated 99% identity with the *Sphingopyxis* genus. The phylogenetic position of the isolate is presented in Figure 1. Several subgroups were generated based on 16S rRNA gene sequences available in the NCBI database including two different *Sphingopyxis* clusters. The analysis showed that our isolate MB-E was 99% similar to *Sphingopyxis* sp. C1. The 16S rRNA gene sequence of strain MB-E has been deposited in the GenBank database (KP096395).

Figure 1. Phylogenetic tree of microcystin-degrading bacteria. This tree was analyzed by the neighbor-joining method. The sequence of *Aquifex pyrophilus* was used as an out-group to root the tree. Bootstrap values with 1000 replicates were used to assign confidence levels to the tree topology.

3.2. Biodegradation of multiple cyanotoxins

Sphingopyxis sp. MB-E produced yellow-pigmented colonies on nutrient agar and exhibited the highest rate of microcystin degradation by completely degrading all five microcystin variants

with pseudo first order rate constants ranging from 0.29 h^{-1} to 0.39 h^{-1} . Biodegradation rate constants for each MC are presented in Table S4 in Supporting Information. The biodegradation profiles are shown in Figure 2. To the best of our knowledge there are few studies of species that are able to rapidly degrade a wide range of microcystins. Manage et al.²⁶ isolated a group of bacteria from three Scottish water bodies that were able to degrade only MCLR and Lawton et al.²⁵ demonstrated that five of those bacteria were able to degrade a combination of five microcystins (MCLR, YR, LY, LW, and LF) and nodularin. Strain MB-E was able to completely degrade all five microcystin variants at a total initial concentration of $50 \text{ }\mu\text{g/l}$ in less than 16 hours. There was no loss of toxins in the control bottles.

Figure 2. Biodegradation of a mixture of MCs, CYN and ATX by *Sphingopyxis* sp. MB-E in mineral salt medium at pH 7.4 and 22-23°C. Error bars represent the standard deviation of triplicate samples and duplicate analyses.

In this study, similar biodegradation patterns of different MCs were observed with *Sphingopyxis* sp. MB-E in lake water (Figure 2). Ishii et al.²⁸ demonstrated that the degradation half-life of MCLR by their microcystin degrader (7CY) was shorter than the half-life of MCLY, LW and LF. Similar observations were made with regards to the degradation of MCLR and MCLA using *Sphingomonas* ACM-3962 by Ho and colleagues²³, in which MCLR was more easily degraded than MCLA at a concentration of 10 µg/l. We also demonstrated previously that MCLR and MCYR follow the same biodegradation pattern and were more rapidly degraded as compared to MCLY, MCLW and MCLF in Missisquoi Bay water and the clarifier sludge of a water drinking treatment plant³⁸. In contrast, Lawton et al.²⁵ demonstrated that the degradation half-life of MCLY, LF and LW was lower than for MCLR and MCRR by six different toxin degrading isolates. Interestingly, they reported that although all of their strains belonged to the *Proteobacteria* phylum, none contained the *mlrA*, *mlrB* and *mlrC* genes, which have been identified in most toxin degraders to date, although all these degrader strains belonged to the. It

is possible that different degradation pathways exist in bacteria from different phyla, such as the *Actinobacteria*²⁶, and that degradation kinetics for different toxin variants may therefore be a consequence of these different pathways. Interestingly, in the study by Manage et al.²⁶, although the known microcystin degradation genes were not detected, the metabolites produced by the known degradation pathway were identified.

The isolated *Sphingopyxis* sp. MB-E was not able to degrade CYN. Previous observations of CYN biodegradation have generally only occurred in water bodies that experienced blooms of CYN-producing species. At this time, the presence of CYN has not been regularly reported in Missisquoi Bay. Strain MB-E was also not able to degrade ATX during 28 hours of incubation. To confirm whether time was a limiting factor for CYN and ATX biodegradation, the experiment was continued for 4 weeks, but no degradation was observed. Little information is available regarding the biodegradability of ATX.¹³ There is only one study that reported the biodegradation of CYN by an isolated bacterium.³⁹ The authors isolated a *Bacillus* strain (AMRI-03) from cyanobacterial blooms capable of degrading CYN with a high biodegradation rate, which correlated positively with the initial CYN concentration.

3.3. *mlr* gene cluster and microcystin degradation activity

Metagenomic sequencing of *Sphingopyxis* sp. MB-E revealed a *mlr* gene cluster in a 5.8 kb sequence fragment. The cluster consisted of four genes, *mlrA*, *mlrB*, *mlrC*, and *mlrD* that are involved in the degradation of microcystin (Figure 3). The *mlrA* gene encoded the enzyme microcystinase and was present between bases 1955 and 2962. The *mlrD* gene encoded a transporter protein (between bases 2971- 4239) that contained 1268 bases. It was located immediately downstream of *mlrA* with the same direction of transcription. The *mlrB* and *mlrC*

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genes encoding peptidases were in the opposite direction of transcription. The *mlrB* gene was a 1610 bp fragment downstream of *mlrD*. The *mlrC* was identified upstream of *mlrA* from 281 to 1864 bp within the 5.8 kb fragment. The organization of *mlr* genes in this cluster is identical to the organization determined for several *Sphingomonas* strains that degrade microcystins (19, 40). The highest similarities for the *mlrA*, *mlrB*, and *mlrC* genes of *Sphingopyxis* sp. MB-E were with the microcystin-degrading genes of *Sphingopyxis* sp. USTB-05⁽⁴¹⁾ with values of 86%, 99% and 95%, respectively (data not shown). The *mlrD* gene was 96% similar to the *mlrD* gene of *Sphingopyxis* sp. C1 (GenBank: BAL02997).

The ability of *Sphingopyxis* sp. MB-E to degrade MCLR and a mixture of MCs was validated by performing a time course biodegradation experiment followed by transcriptomic sequencing. The ratio of *mlr/cpn60* gene transcripts is presented in Figure 3. Statistically indistinguishable transcription profiles were observed in *Sphingopyxis* sp. MB-E at times 0 and 4 hours into the degradation of MCLR and the toxin mixture. A statistically significant spike in the production of *mlrC*, *mlrA*, and *mlrB* transcripts was observed after 12 hours in the case of the mixture of MCs. A similar spike was observed in the expression of the *mlrC* gene after 8 hours in the presence of MCLR. After 20 hours, all of the enzymes involved in the degradation of microcystins were transcribed at levels that were statistically indistinguishable to the beginning of the experiment. The same transcription profiles were obtained when the ratio of *mlr/rpoA* gene expression was calculated in order to normalize each dataset to a value approximating the average single cell expression profile (Figure S1, Supporting Information).

The *mlr* gene cluster identified in this study was similar to the cluster of the MC-degrading bacterium *Sphingomonas* sp. strain ACM-3962.¹⁹ In their study, *mlr* gene sequences were compared with other microorganisms. The authors suggested that that the genes in the *mlr* cluster

may all be involved in the cycling of cell wall peptidoglycan and that the hydrolysis of MCLR may simply be a fortuitous event. The gene expression profiles observed in our study at the beginning of the experiment supports this hypothesis. All of the genes were being transcribed at a basal level prior to the addition of the microcystin substrates. The activity that we identified at time zero could also be the result of the degradation of peptides and proteins found in the PY growth medium. Our results revealed that the presence of microcystin in the medium triggered the transcription of the enzyme encoded by the *mlrA* gene which is responsible for the opening of the cyclic structure. For example, 8 hours into the experiment an increase in the expression of the *mlrA* and *mlrC* genes was observed suggesting that the newly linearized microcystin was being degraded into a tetrapeptide. The transcriptomic approach also revealed that the amount of induction appears to be related to the total amount of substrate. The highest expression levels were obtained in 8 hours with MCLR alone compared to 12 hours for the toxin mixture, which contained five times the amount of substrate. These results are consistent with Li et al.⁴² who studied the correlation between MCLR-biodegradation kinetics and *mlrA* and *mlrD* gene expression dynamics in a *Novosphingobium* sp. THN1 strain. They observed similar trends in expression of *mlrA* and *mlrD* under various nutrient conditions (in the absence and presence of nitrogen, sodium nitrate or phosphorus sources). Li et al.⁴² also reported a positive correlation between degraded MCLR and the induction ratio of *mlrA* and *mlrD*. Our transcriptomic results demonstrated that all three enzymes *mlrA*, *mlrB* and *mlrC* were induced sequentially in the presence of MCLR and in the five MCs mixture. Our results suggest that these enzymes are catalyzing the degradation of all the tested microcystins through a linear, three-step biodegradation process.

Figure 3. *mlr* gene cluster of *Spingopyxis* sp. MB-E and expression of the *mlr* genes during growth on microcystin LR and the mixture of five cyanotoxins, in comparison to the expression of the *cpn60* reference gene. Error bars represent the standard deviation of duplicate samples and duplicate analyses.

The next generation sequencing methods used in this study proved to be useful in characterizing the *mlr* gene cluster of strain MB-E and its corresponding gene expression profiles when provided with microcystin-LR or a mixture of microcystins. These results are novel and suggest that *Spingopyxis* sp MB-E uses the same pathway in the degradation of all MC variants.

3.4. Effect of pH on MC biodegradation

Analysis of the 16S rRNA gene revealed that isolate MB-E was 99% identical to *Sphingopyxis* sp. C1, an alkali-tolerant microcystin-degrading bacterium.¹⁸ This strain was isolated by enrichment culture using MCLR as the carbon and nitrogen source and was shown to degrade MCLR between pH 6.52 and pH 10.

Cyanobacterial blooms are often associated with a high pH (between 8.5-11) as a consequence of photosynthesis.^{43, 44} The effect of pH on the degradation of multiple microcystins by *Sphingopyxis* sp. MB-E over time is presented in Figure 4. The strain was able to degrade all the five microcystin analogues efficiently at pH values of 6.10, 7.22 and 8.05 in 20 hours; however, no biodegradation was observed at the more acidic and basic pH values, 5.05 and 10.23. The alteration of pH in control groups without the MB-E isolate was less than 8% (data not shown).

The degradation activity of *Sphingopyxis* sp. MB-E was higher under weakly basic and neutral pH conditions with the highest biodegradation rate being observed at pH 7.22. These results are consistent with the Saitou et al.⁹ study where the activity of their MCLR degrader was reduced by increasing the basicity of the growth medium, and the strain had the highest degradation capacity at pH 7.2. Okano et al.¹⁸ also isolated an alkali-tolerant *Sphingopyxis* sp. which was able to grow at pH 11 and had the highest degradation activity between pH 6.72 and 8.45. All the microcystin variants followed the same trend with higher degradation activity at a more neutral pH.

Figure 4. The effect of pH on the biodegradation of microcystin variants by *Sphingopyxis* sp. MB-E. Error bars represent the data range from duplicate samples and duplicate analyses.

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3 380 The highest growth rate of isolate MB-E in PY medium was observed at pH 7.42 with no growth
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5 381 at pH 10.10 (Figure S2A, Supporting Information). These results support the biodegradation data
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7 382 and show that the strain was not able to grow under basic conditions.

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9 383 Understanding the effect of pH on the biodegradation activities of cyanotoxin degrading bacteria
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11 384 is crucial as a result of the dramatic changes of the pH in drinking water sources during blooms
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13 385 of cyanobacteria. The pH of Missisquoi Bay water was measured during the bloom season in
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15 386 2011 and 2012 (Figure S3, Supporting Information). The highest pH values were 9.40 and 9.49
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17 387 in 2011 and 2012, respectively. The experimental studies performed at different pH values
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19 388 demonstrated that *Sphingopyxis* sp. MB-E may have had limited biodegradation activity for
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21 389 microcystin variants during a bloom of cyanobacteria in Missisquoi Bay. Further work studying
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23 390 the impact of shifts in the equilibrium of carbonate, ammonia and pH on *Sphingopyxis* MB-E
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25 391 could provide insight into the persistence and degradation activity of this strain during severe
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27 392 cyanobacterial blooms.

28 393 3.5. Conclusions

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- 30 395 • A new *Sphingopyxis* sp with the ability to degrade multiple MCs was isolated for the first
31 396 time from a Canadian drinking water source
 - 32 397 • The *mlr* gene cluster including: *mlrA*, *mlrB*, *mlrC* and *mlrD* was identified as the
33 398 producer of enzymes that are responsible for MCs degradation
 - 34 399 • Next-generation sequencing methods were successfully applied in this study to
35 400 characterize the *mlr* gene cluster of strain MB-E and also the expression of the *mlr* genes
36 401 during growth on MCLR and the mixture of MCLR, MCYR, MCLY, MCLW and MCLF
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- 401 • A novel approach was applied in determining the expression profiles of the MC degrader
402 genes in a timely manner. The approach revealed similar expression profiles from the
403 isolated *Sphingopyxis* MB-E *mlr* gene cluster over time in proportion to the concentration
404 of substrate of both individual and mixtures of microcystins. The results suggested that
405 the same degradation pathway was used by the isolate to degrade all MC variants.
- 406 • The new *Sphingopyxis* sp. was shown to have its highest degradation activity at pH 7.22;
407 however, MCLR and MCYR were also degraded at a pH of 9.12 that is closer to the
408 natural pH of drinking water sources during cyanobacterial blooms.

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Transcriptomic analysis has been used to compare the expression profiles of *mlr* gene cluster in newly isolated *Sphingopyxis* sp. MB-E.