Final Report for the Lake Erie Protection Fund Small Grants Program Identification of Microcystin-Degrading Bacteria (SG 374-09)

Xiaozhen Mou and Robert Heath
Department of Biological Sciences, Kent State University, Kent OH 44242

Abstract

The main objective of this project is to investigate the role of heterotrophic bacteria in degrading one of the major cyanotoxins, e.g., microcystin, that are produced during harmful cyanobacterial algal bloom events (cyanoHABs) in the western basin of Lake Erie. Microcosm incubation experiments were set up to study response of lake bacterioplankton (cells that pass 1.0 μm-pore size filters) to elevated supply of microcystin LR (10 μg/L, final concentration). After 48 hr incubation, bacterial abundance in the microcystin amended microcosms (MCs) increased nearly 10 folds, while remained unchanged in the no-addition controls (CTRLs). At the same time, the microcystin concentration in the MCs decreased 70%, indicating active consumption of microcystin by bacterioplankton in the MCs. Genomic materials of bacterioplankton communities in the MCs and CTRLs were sequenced. Bioinformatic analysis identified a set of gene categories that were significantly enriched in the MCs relative to the CTRLs. Analysis of significantly enriched gene sequences indicated that microcystin degradation in Lake Erie may involve a diverse group of heterotrophic bacterioplankton, with *Betaproteobacteria* of particular importance. This was different from previous studies in other freshwater systems, which indicated bacteria of the Actinobacteria and Alphaproteobacteria taxa were most important in microcystin degradation.

INTRODUCTION

Lake Erie supplies drinking waters to millions of human and is home to numerous wild lives. Therefore, maintaining and improving the quality of Lake Erie water is of the top priority of local, state and federal management agencies. However, despite much research and restoration efforts, harmful cyanobacterial algal blooms (cyanoHABs) recur annually in Lake Erie, especially in the Western Basin, with increasing frequency, extent and affected areas. CyanoHABs produce a number of substances that are toxic to humans and lake organisms. One class of these cyanotoxins, microcystins, is particularly pernicious due to its damaging effects on animal liver. In warm months (June, July and August), microcystin concentrations in the Lake Erie Western Basin frequently exceed the World Health Organization (WHO) guideline values of drinking (1 μg/L) and recreational bath waters (low risk at 2-4 μg/L) (Figure 1), which is one of the major factors causing beach closing.

The toxicity of microcystins is determined by their concentrations in the water, so processes that produce and remove these toxins are equally important in controlling the hazardous effects of cyanoHABs. Early studies on the microcystin production rely on tracking their concentration inside of cyanobacterial cells under varied growth conditions (Chorus, 2001). Recent identification of the key genes involved in microcystin biosynthesis has promoted such studies to the genetic level (Kaebernick et al., 2000; Kaebernick et al., 2002). It is clear now that *Microcystis* cells regulate the production of microcystin under conditions of different cell growth phase, light quality and intensity, temperature and nutrient supplies (nitrogen, phosphorous and iron) (Ouellette and Wilhelm, 2003). What is still not clear is the opposite process, the degradation and removal of microcystins after they released into lake waters. This knowledge gap prohibits precise prediction of the overall harmfulness of cyanobacteria bloom events, which is necessary for efficient management efforts. Moreover, this knowledge gap limits our understanding on the mechanism of the ecosystem to naturally recover from a distorted food web, which has great implication to practical and environmental friendly bioremediation.

Studies have shown that abiotic reactions like photolysis are only responsible for part of the removal of microcystin, and these reactions usually take days or months under natural

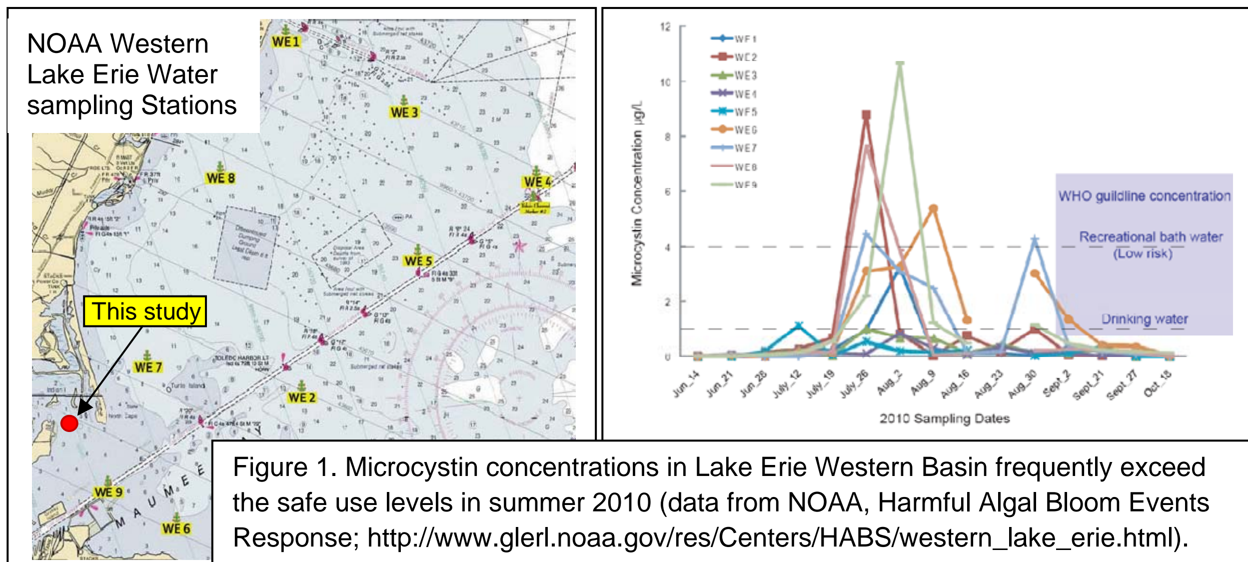


Figure 1. Microcystin concentrations in Lake Erie Western Basin frequently exceed the safe use levels in summer 2010 (data from NOAA, Harmful Algal Bloom Events Response; http://www.glerl.noaa.gov/res/Centers/HABS/western_lake_erie.html).

conditions (Welker and Steinberg, 2000). Heterotrophic bacteria are known for their strong ability to decompose a variety of naturally produced or synthetic organic compounds that are commonly found within their native habitat. The positive role of bacterial community in degrading microcystin has been indicated by several previous studies in other water bodies (Christoffersen et al., 2002; Ho et al., 2007; Lemes et al., 2008). However, clear linkage between bacterial taxa and *in situ* microcystin degradation is yet to be discovered. The lack of knowledge on genes and pathways that involved in the processes hinders our ability in sensitive and specific monitoring microcystin degradation in environmental samples.

The goal of this project is to solve these problems using an experimental metagenomic approach. Our results suggest that a diverse group of lake bacterioplankton are involved in microcystin degradation, with *Betaproteobacteria* play particularly important roles.

METHODS

Sample collection and processing

Surface water samples are collected in the Lake Erie Western Basin at station WB18 (Latitude 41.7423, Longitude -83.4019; Figure 1) on Aug. 27th, 2010. Water samples were first filtered by 1.0 µm-pore-size membrane filters to exclude big particles and bacterivores. Water filtrates were then amended with a mixture of inorganic nitrogen and phosphorus compounds (5 µM NH_4Cl, 5 µM $NaNO_3$, and 1 µM NaH_2PO_4, final concentrations) and incubated in the dark at room temperature with occasional agitation for 4 days to establish carbon-limited conditions. Microcosms were then established by filling 5L pre-incubated water to each of four 10L carboys. Microcystin LR (10 µg/L; Sigma Chem. Co.) was added to two of the microcosms (MCs), and the remaining two microcosms served as no-addition controls (CTRLs). Microcosms were incubated in the dark at room temperature with occasional agitation for a total of 48 h. Ten milliliter subsamples from each microcosm were taken at 12h, 24h and 48 h of incubation for microcystin concentration and bacterial growth measurements.

DNA extraction and metagenomic sequencing

After 48 h incubation, cells in the MC and CTRL microcosms were collected onto 0.2 µm-pore-size membrane filters by filtration. DNA was extracted from the filters PowerMax Soil Mega Prep DNA Isolation Kit (Mobio Inc) and sequenced by Georgia Genomics Facility at UGA using 454 pyrosequencing with the titanium chemistry.

Pyrosequence data annotation.

16S rRNA genes and protein-encoding sequences were identified and categorized using criteria established previously (Mou et al., 2008). Briefly, pyrosequences were screened to remove identical reads generated as artifacts during pyrosequencing. The remaining sequences were analyzed by BLAST against the RDPII database to identify putative 16S rRNA gene sequences (cutoff value of $E < 10^{-5}$), and hits were subsequently removed from the library. The putative protein-encoding sequences were annotated by BLASTX against the NCBI RefSeq protein database. These protein-encoding sequences were further categorized into Clusters of

Orthologous Groups (COG) and Kyto Encyclopedia of Genes and Genomes (KEGG) pathways by BLAST against the NCBI COG database and the KEGG database. Sequences not meeting any of the above criteria were not considered in further analyses.

The taxonomic composition of the metatranscriptomic libraries was obtained by querying protein-encoding sequences against the NCBI RefSeq database using the BLASTx algorithm in the MEGAN program (Huson et al., 2007).

Statistical analysis of metagenomic data.

A multivariate comparison was performed to compare the overall composition of metagenomic data sets (with independent replicates) using non-metric multidimensional scaling (MDS; Primer5; Primer-E Ltd). The square-root-transformed Bray–Curtis similarity matrix of COG assignments was normalized for size differences between the data sets.

To identify specific gene categories enriched or depleted in the MC treatments, comparison was made at two levels by pair-wise bootstrapping using Xipe (Rodriguez-Brito et al., 2006). One comparison was between the two metagenome replicates for any given treatment (MC or CTRL) and the other comparison was between pooled metagenomes of different treatments (MC vs. CTRL). Pair-wise comparisons were performed on the relative abundance of COG categories in each query metatranscriptomes. In each comparison, a total of 20,000 resamplings were made, with the sample size equal to the average number of the sequences in two metatranscriptomic libraries being compared. Significant differences between the metatranscriptomic datasets were reported at the level of $P < 0.05$. COG and KEGG categories with significant within-treatment differences were removed from the final list of gene categories that showed significant differences between treatments.

Flow cytometric analysis (FCM)

Flow-cytometric analysis was performed with a FACSAria (Becton Dickinso). Subwater samples for FCM analysis were preserved with 1% paraformaldehyde at room temperature immediately after sampling and stored at 4 °C. Prior to running on the instrument, preserved bacterioplankton were stained with Sybr Green II (1:5,000 dilution of the commercial stock; Molecular Probes Inc.) in the dark at room temperature for 20 min and then mixed with for 20 min and then mixed with an internal bead standard (1-µm-diameter yellow-green fluorescent beads; Fluoresbrite YG Microspheres; Polysciences, Warrington, Pa.) with a known bead density. Data acquisition was triggered by green fluorescence (FL1). All signals were collected with logarithmic amplification.

Microcystin concentration measurement

Water samples taken from the microcomsms were filtered through 0.2-µm-pore size filters and the filtrates were stored at -20°C or preceded immediately for microcystin concentration measurement using a Microcystins/Nodularins (ADDA) ELISA Kit (Abraxis BioScience) following the manufacturer's instruction.

RESULTS

Surface water samples were taken near the Maumee Bay in the Western Basin of Lake Erie on Aug 27th, 2010 (Figure 1). Although the water is relatively turbid, no obvious cyanobacterial bloom was visible at the time of sampling, which is in accordance with relatively low chlorophyll concentration measured (Table 1). However, periodic cyanobacterial blooms had been reported in Maumee Bay and other nearby locations in July and early Aug 2010 (Figure 1, NOAA, Harmful Algal Bloom Events Response; http://www.glerl.noaa.gov /res/ Centers/HABS/western_lake_erie.html).

Table 1. Basic parameters of water samples used in the project (data from Dr. T. Bridgeman).

Parameter	Measurement
Sampling depth	0.345 m
Temperature (T)	23.37 °C
Dissolved oxygen (DO)	9.06 mg/L
DO saturation	106.5%
pH	8.57
Turbidity	14.9 NTU
Chlorophyll a	3.6 μg/L
Secchi depth	0.6 m

After transported back to the lab (water samples stored at RT during the 2-h transportation), bacterioplankton fraction was obtained by removing bacterivores and other big particles by filtration (1.0 μm filtration). To obtain a carbon-limited condition that would promote consumption of added MC-LR, excessive inorganic N and P compounds were supplied to the bacterioplankton. After 4 days incubation, bacterioplankton were amended with MC-LR at 10 μg/L of final concentration (MCs). While the bacterioplankton cell number remained nearly stable in the controls that received no extra microcystin (CTRLs), the total cell number increased

Figure 2. Average bacterial cell density and standard deviation (error bar, n = 12) in MC and CTRL microcosms.

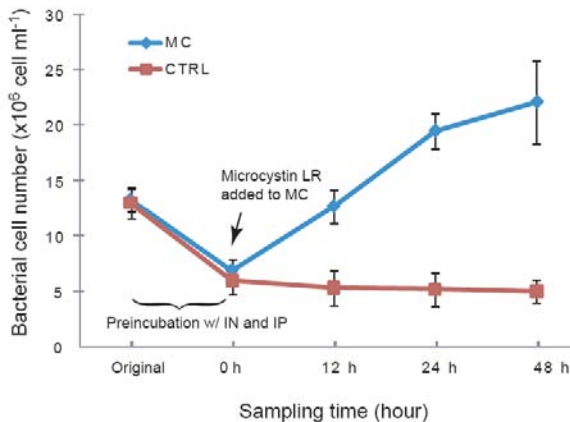


Figure 3. Average microcystin LR concentration and standard deviation (error bars, n = 12) in MC and no cell CTRL samples.

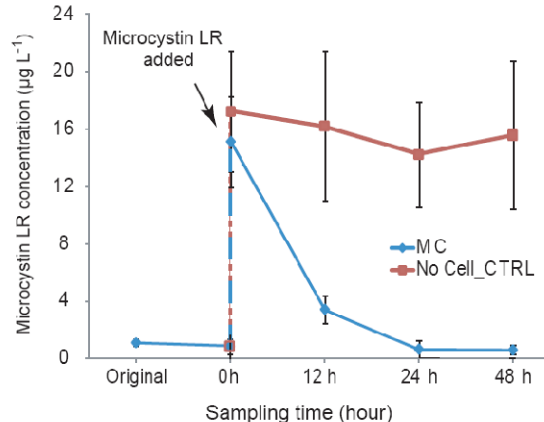
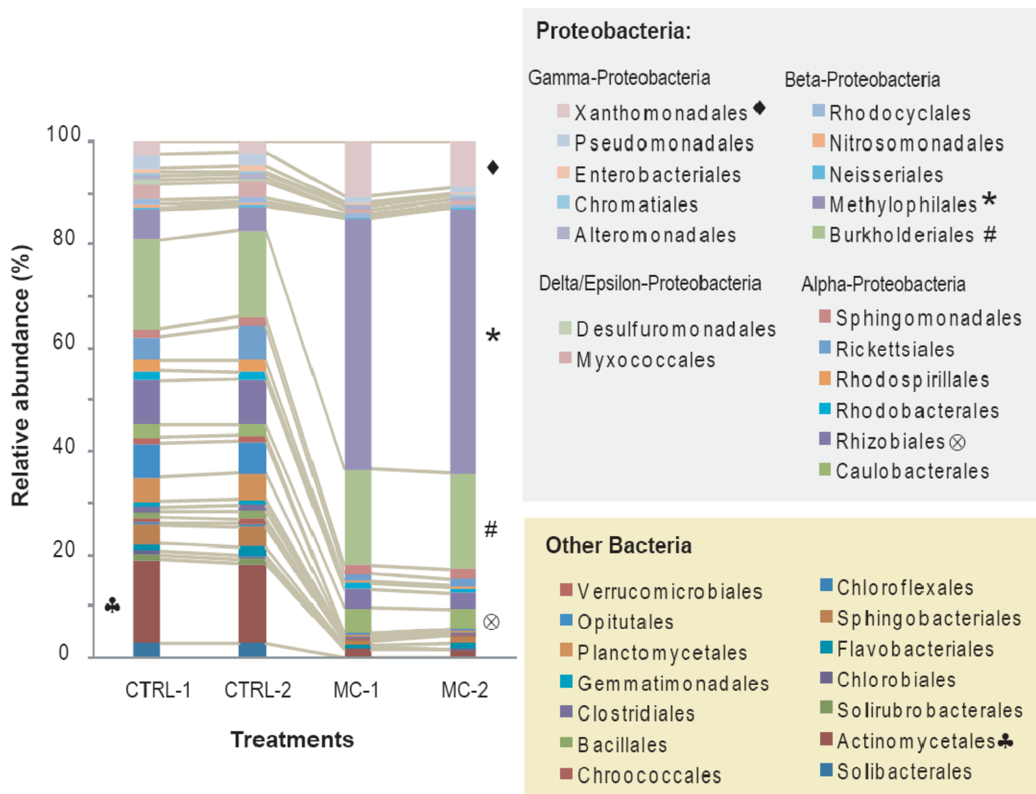


Figure 4. Major taxa at the order level in the MC and CTRL metagenomes



significantly in response to the addition of microcystin in the MC samples (*t* test, $P < 0.05$), indicating bacterioplankton were using added microcystin as carbon source (Figure 2).

Microcystin consumption by the heterotrophic bacterioplankton was tracked by measuring the concentration of microcystin LR in the MC samples. To eliminate the effects of spontaneous degradation of MC, no-cell controls were established (No cell_CTRLs) by adding microcystin LR into filter sterilized (0.2 µm pore size) lake water. No cell_CTRLs were parallel incubated with the MCs at room temperature in the dark. In oppose to significant decrease of microcystin LR concentration in the MC samples; microcystin LR concentration in the no cell_CTRL did not change during the course of incubation (Figure 3).

Metagenomes of bacterioplankton in the MC and CTRL samples were sequenced by high throughput pyrosequencing, which, after removing low quality and artificial sequences, contained a total of 815,435 sequences of averagely 386 bp in length (Table 2). Nearly twice as much of sequences were recovered for the MC libraries than the CTRLs, probably because the starting DNA materials for the former (~10 µg each by picogreen fluorescence assay) were a lot more than the latter (~2µg each). About 0.2% and 0.4% of the metagenomes of the CTRL and MC samples were 16S rRNA genes, in accordance with the expected frequency in prokaryotes (Mou et al., 2008).

Based on annotation by the MEGAN program (Huson et al., 2007), diverse bacterial taxa consisted of the MC and CTRL metagenomes (Figure 4). Major taxa (relative abundance in MC plus CTRL libraries >5%) at the phylum level included *Acidobacteria, Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteoacteria* (alpha, beta and gamma lineages), and *Verrucomicrobia*. At the order level, the MC metagenomes were predominated by *Methylophiales* (*Betaproteobacteria*), which accounted for nearly half of the metagenomic sequences. Other major orders (> 5% sequences) in MC metagenomes included *Burkhoderiales* (18%, *Betaproteobacteria*), *Xanthomonadales* (10%, *Gammaproteobacteria*). CTRL metagenomic sequences were mainly (> 5% sequences) distributed in orders of *Burkholderiales* (*Betaproteobacteria*, 16%), *Actinomycetales* (*Actinobacteria*, 15%), *Rhizobiales* (*Alphaproteobacteria*, 8%), *Opitutales* (*Verrucomicrobia*, 6%) and *Methylophilales* (*Betaproteobacteria*, 5%) (Figure 4). Statistic comparison of major taxa between MCs and CTRLs using the *Chi* square analysis identified that *Methylophiales* (10 folds) and *Xanthomonadales* (4 folds) were significantly enriched, while *Opitutales* (1/20 folds), *Planctomycetales* (*Planctomycetes,* 1/11 folds), *Actinomycetales* (*Actinobacteria,* 1/11 folds) and *Richettsiales* (*Alphaproteobacteria,* 1/4 folds) were significantly depleted in the MC metagenomes, relative to the CTRL metagenomes ($P < 0.05$). Microcystin LR-degrading bacteria have been studied before in other lake systems and representative isolates were mainly in the orders of *Sphigomonadales* (*Alphaproteobacteria*) and *Actinomycetales* (*Actinobacteria*) (Maruyama et al., 2006; Ho et al., 2007; Manage et al., 2009). Taxa of these two orders, however, were not in the list of significantly enriched taxa in the MC metagenomes relative to the CTRL.

Functional gene contents were compared between the MC and CTRL metagenomes using a bootstrap resampling method (Rodriguez-Brito et al., 2006). There were 13 KEGG pathways and 80 COG groups significantly enriched in the MC metagenomes, relative to the CTRL metagenomes ($P < 0.01$). While, some significantly enriched KEGG pathways were for cellular motility and processing of environmental signals, most were for biosynthesis and catabolism of carbon, nitrogen and sulfur substrates (Table 3).

Table 2. Sequence annotation statistics for CTRL and MC metagenomes.

Parameter	**CTRL-1**	**CTRL-2**	**MC-1**	**MC-2**
Number of unique sequences	164,026	198,712	251,154	201,543
Average sequence length (bp)	414	377	386	366
Number (per cent) of rRNA genes	381 (0.2%)	359 (0.2%)	1000 (0.4%)	771 (0.4%)
Number (per cent) of predicted functional genes	83,621 (51%)	91,778 (46%)	182,251 (73%)	140,869 (70%)
Number (per cent) of functional genes categorized by COG groups	53,754 (64%)	61,742 (67%)	121,807 (67%)	92,719 (66%)
Number (per cent) of functional genes categorized by KEGG pathways	72,542 (87%)	83,391 (91%)	168,118 (92%)	129,469 (92%)

Conclusion.

Experimental metagenomic analysis indicated a number of lake heterotrophic bacteria may be involved in microcystin degradation. Unlike previous findings in other lakes, which indicated the importance of *Alphaproteobacteria* and *Actinobacteria* in microcystin degradation (Ho et al., 2007; Manage et al., 2009), our study indicated another taxon, namely *Betaproteobacteria*, especially those affiliated with the order of *Methylophiale,* may be main members that drive the natural degradation of microcystin in Lake Erie.

Acknowledgement.

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Table 3. Significantly enriched KEGG pathways in the MC metagenomes relative to the CTRL (P < 0.01).

KEGG	General Processes	Functional Description
	Cellular Processes	
2030	Cell Motility	Bacterial chemotaxis
2040	Cell Motility	Flagella assembly
	Environmental Information Processing	
2020	Signal Transduction	Two-component system
3070	Membrane Transport	Bacterial secretion system
	Metabolism	
61	Lipid Metabolism	Fatty acid biosynthesis
253	Tetracycline Metabolism	Tetracycline biosynthesis
480	Metabolism of Other Amino Acids	Glutathione metabolism
540	Glycan Biosynthesis and Metabolism	Lipopolysaccharide biosynthesis
550	Glycan Biosynthesis and Metabolism	Peptidoglycan biosynthesis
561	Lipid Metabolism	Glycerolipid metabolism
564	Lipid Metabolism	Glycerophospholipid metabolism
680	Energy Metabolism	Methane metabolism
780	Metabolism of Cofactors and Vitamins	Biotin metabolism
860	Metabolism of Cofactors and Vitamins	Porphyrin and chlorophyll metabolism
910	Energy Metabolism	Nitrogen metabolism
920	Energy Metabolism	Sulfur metabolism
980	Xenobiotics Biodegradation	Xenobiotics metabolism by cytochrom P450

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