

Increasing *Microcystis* cell density enhances microcystin synthesis: a mesocosm study

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Abstract

An experimental protocol using mesocosms was established to study the effect of *Microcystis* sp. cell abundance on microcystin production. The mesocosms (55 L) were set up in a shallow eutrophic lake and received either no (control), low (to simulate a moderate surface accumulation), or high (to simulate a dense surface scum) concentrations of *Microcystis* sp. cells collected from the lake water adjacent to the mesocosms. In the low- and high-cell addition mesocosms (2 replicates of each), the initial addition of *Microcystis* sp. cells doubled the starting cell abundance from 500 000 to 1 000 000 cells mL⁻¹, but there was no detectable effect on microcystin quotas. Two further cell additions were made to the high-cell addition mesocosms after 60 and 120 min, increasing densities to 2 900 000 and 7 000 000 cells mL⁻¹, respectively. Both additions resulted in marked increases in microcystin quotas from 0.1 pg cell⁻¹ to 0.60 and 1.38 pg cell⁻¹, respectively, over the 240 min period. Extracellular microcystins accounted for <12% of the total microcystin load throughout the whole experiment. The results of this study indicate a relationship between *Microcystis* cell abundance and/or mutually correlated environmental parameters and microcystin synthesis.

Key words: cell abundance, cyanobacteria, cyanotoxins

Introduction

Microcystins are the most common and notorious toxins produced by cyanobacteria. Ingestion of water contaminated with microcystins has caused numerous human and animal poisonings (Kuiper-Goodman et al. 1999, Ransom et al. 2004). Microcystins are relatively large (about 1000 Da), cyclic peptides that are synthesized nonribosomally by large multifunctional enzyme complexes containing peptide synthetase and polyketide synthase domains. Cyanobacteria invest considerable resources into producing these toxins; the gene cluster encoding the biosynthetic enzymes accounts for about 1.5% of their genome, and up to 2% of the organism's dry weight can be comprised of toxin (Neilan et al. 2008). This energetic investment has prompted much speculation and research on the ecophysiological function of microcystins and parameters that regulate their production. Most of these

studies have been culture-based and have focused on environmental parameters typically encountered during cyanobacterial blooms (e.g., nitrogen, phosphorus, temperature, metal cations; Lee et al. 2000, Oh et al. 2000, Sevilla et al. 2008, Davis et al. 2009). While correlations with environmental parameters have been demonstrated, microcystin quotas typically vary by only 3- to 4-fold (Sivonen and Jones 1999), and the topic of microcystin regulation remains contentious.

In 2009 in Lake Rotorua (South Island, New Zealand), we demonstrated for the first time that microcystin synthesis is not continuous during a *Microcystis* sp. bloom, and that increases in cell abundance cause rapid changes in microcystin production (Wood et al. 2011). In the 2009 study, surface water samples were collected from Lake Rotorua every 4 h over a 2 d period. On the second day of sampling, a weak onshore wind caused a mass-accumulation of buoyant *Microcystis* colonies

(130 000–4 000 000 cells mL⁻¹) over a period of 5 h (0700 to 1300 h). During this increase in cell abundance there was a 28-fold increase in microcystin quotas. Gene expression (*mcyE*) was initially undetectable and became highly up-regulated over this time. As the cells dispersed during the afternoon there was a corresponding decline in microcystin quotas and *mcyE* gene expression. *Microcystis* sp. was the only microcystin-producing species present in the lake, and all *Microcystis* sp. genotypes were toxic (Wood et al. 2010), making this lake an ideal study site.

The aims of the present study were to (1) develop, using mesocosms, an experimental protocol to study the effect of varying *Microcystis* cell densities on microcystin production and, (2) determine if our 2009 observations, which were promoted by an *in situ* mass aggregation event, could be reproduced experimentally. Triggering significant up- and down-regulation in toxin production experimentally may allow cell concentration thresholds to be established as well as provide valuable insights into the biological function of these toxins.

Materials and methods

Study sites and sample collection

Lake Rotorua (42°24'05S, 173°34'57E) is a small (0.55 km²), shallow (max. depth 3 m), eutrophic lake in northeast South Island, New Zealand (Flint 1975). Mesocosm experiments were conducted on 18 March 2010 starting at 0900 h. Six mesocosms (in 2 columns of 3) were anchored to the lake bottom in approximately 2 m of water. Each mesocosm was made from low-density clear polyethylene of 60 µm thickness, attached to a float formed from a 40 m coil of high-density polyethylene pipe (15 mm external diameter) that extended 70 mm above the water surface. Each polyethylene bag was 570 mm deep with an internal diameter of 380 mm to give a total volume of approximately 55 L. Ten minutes prior to each cell addition, cyanobacterial cells in lake water adjacent to the mesocosms were concentrated using an 11 µm plankton net. The concentrated material was stored in 20 L plastic buckets and then divided into approximately equal proportions and poured into 4 mesocosms. The aim of these cell additions was to obtain a cell abundance similar to an actual surface accumulation event. In 2 of these mesocosms (high-cell additions), the cell collection and addition procedure was repeated immediately after sampling at 60 and 120 min post commencement. These cells were added to simulate a wind-accumulated surface bloom or scum, with a cell density similar to our 2009 study (Wood et al. 2010). Two mesocosms did not receive any cell additions (controls).

Surface water samples (200 mL) were collected from each mesocosm immediately prior to the start of the experiment and at 30, 60, 120, 240, and 360 min. Subsamples (100 mL) were preserved using Lugol's iodine for subsequent phytoplankton enumeration. Subsamples (50 mL, unfiltered) were frozen at -20 °C for analysis of total microcystins. A subsample of 1.5 mL was filtered (0.45 µm; Acrodisc Minisart, Sartoriusstedium Biotech, Germany) and the filtrate frozen for extracellular microcystin analysis. Subsamples (7–30 mL) were filtered (GF/C, Whatman) and filters stored in Mo Bio LifeGuard Soil Preservation Solution (Mo Bio Laboratories, Inc., Carlsbad, CA) at 4 °C for later DNA extraction. Water (one replicate about 5 mm below the water surface for each mesocosm type) and air (immediately above the water surface of the high-cell addition mesocosm) temperature and light intensity were recorded every 5 min (UA-002-08, HOBO, Onset Computer Corporation, MA).

Sample analysis

Cyanobacterial and algal enumeration was undertaken using an inverted Olympus microscope (CKX41, Olympus, Wellington, New Zealand). Samples were mechanically ground (Wheaton Tissue Grinder, Wheaton, NJ, USA) for about 30 s to break down *Microcystis* colonies and *Anabaena* filaments for accurate enumeration of individual cells. Subsamples (1 mL) were settled in Utermöhl chambers (Utermöhl 1958), and phytoplankton from 10 random fields were counted at 400× magnification. DNA was extracted from filters collected at 0, 120, and 360 min using the Mo Bio Power Soil kit (Carlsbad, CA, USA). Polymerase chain reaction (PCR) analysis was undertaken with *Anabaena mcyE* specific primers (*AnamcyE-F2-2/AnamcyE-R12-2*) using methods described in Gobler et al. (2007).

Water subsamples collected for toxin analysis were freeze-thawed, sonicated (20 min) and centrifuged (5000 × g, 10 min). The supernatant was diluted in Milli-Q water and used directly for enzyme-linked immunosorbent assay (ELISA). The total ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid)-containing microcystins in the samples were quantified with a commercially available ELISA kit (Abraxis LLC, USA) based on methods of Fischer et al. (2001). Each sample was analyzed in duplicate and an average value was reported. The limit of quantification for this assay ranges between 0.02 and 0.07 ng mL⁻¹ (Fischer et al. 2001), largely depending on the microcystin congeners present.

Results

The bloom contained 2 cyanobacterial species: *Anabaena* sp. *Nova* (<3% of total cyanobacterial cells in all samples) and *Microcystis* sp. No *Anabaena*-specific *mcyE* genes were detected in the 3 samples tested, indicating that all microcystins in the samples were produced by *Microcystis* sp. Eukaryotic algae accounted for <0.5% of the total number of cells in all samples. *Microcystis* sp. concentrations in the mesocosms prior to the cell additions ranged between 410 000 and 640 000 cells mL⁻¹ (Fig. 1A). After the first cell additions, *Microcystis* sp. concentrations in both the low- and high-cell addition mesocosms were 1 100 000 cells mL⁻¹. No additional cells were added to the control and low-addition mesocosms; however, in

both mesocosms *Microcystis* sp. concentrations at the surface continued to increase throughout the experiment, reaching maxima of 2 200 000 and 2 600 000 cells mL⁻¹, respectively, after 240 min (Fig. 1A). In the high-cell addition mesocosms after the second cell addition (immediately post 60 min sampling) *Microcystis* sp. concentrations increased to 2 900 000 cells mL⁻¹. After the third cell addition (immediately post 120 min sampling) the concentrations in these mesocosms were 7 000 000 cells mL⁻¹ (Fig. 1A).

The microcystin quotas at the start of the experiment in all mesocosms ranged between 0.10 and 0.13 pg cell⁻¹. In the control and low-cell addition mesocosms, the microcystin quotas decreased to between 0.02 and 0.05 pg cell⁻¹ after 120 min. Quotas then gradually increased to

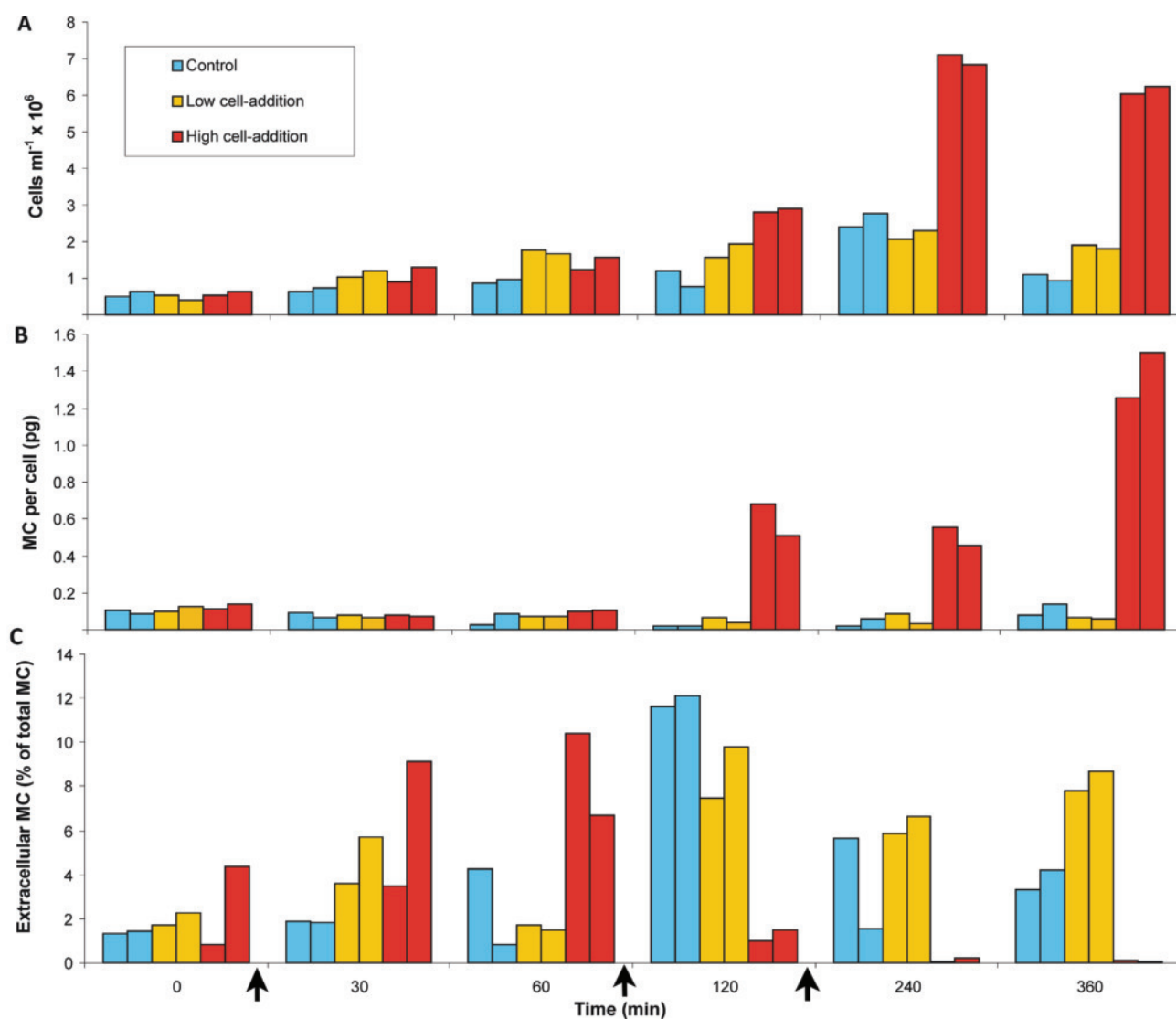


Fig. 1. (A) *Microcystis* sp. cell concentrations, (B) microcystin (MC) quotas, and (C) extracellular microcystins in surface samples collected from 6 mesocosms in Lake Rotorua (South Island, New Zealand) March 2010. The arrow represents addition of cyanobacterial cells to each mesocosm. Second and third cell additions were made only to high-cell addition mesocosms.

between 0.04 and 0.11 pg cell⁻¹ at the conclusion of the experiment. The high-cell addition mesocosms followed a similar trend until the second cell addition, when microcystin quotas rose markedly to 0.60 pg cell⁻¹. The third cell addition did not have an immediate effect, with little change in microcystin quotas at 240 min. At the 360 min sampling, the microcystin quotas had risen considerably to 1.38 pg cell⁻¹ (Fig. 1B), equal to an 18-fold increase in the microcystin quotas from the lowest values measured 330 min earlier.

Extracellular microcystins accounted for <12% of the total microcystin concentration in the mesocosms (Fig. 1B). There was no relationship between the extracellular concentrations and *Microcystis* cell concentrations. In the control and low-cell addition mesocosms, the highest extra-

cellular percentage of microcystins occurred after 120 min. In the high-cell addition, the extracellular percentage of microcystins peaked after 60 min (Fig. 1B).

Water temperature gradually increased in all mesocosms, reaching a peak of 22.7 °C after 300 min in the control and low-cell addition mesocosms. The maximum temperature was lower (21.5 °C) and more constant in the high-cell addition mesocosms (Fig. 2A). The changes in water temperature were most likely related to the increasing air temperature (9.9 °C initially, peaking at 34.5 °C after 305 min; Fig. 2A) and solar radiation. The light at water depth 5 mm in the high-cell addition mesocosm experiments remained relatively constant (Fig. 2B) and after the second cell addition was consistently lower than in either the low-cell addition or control mesocosms (Fig. 2B).

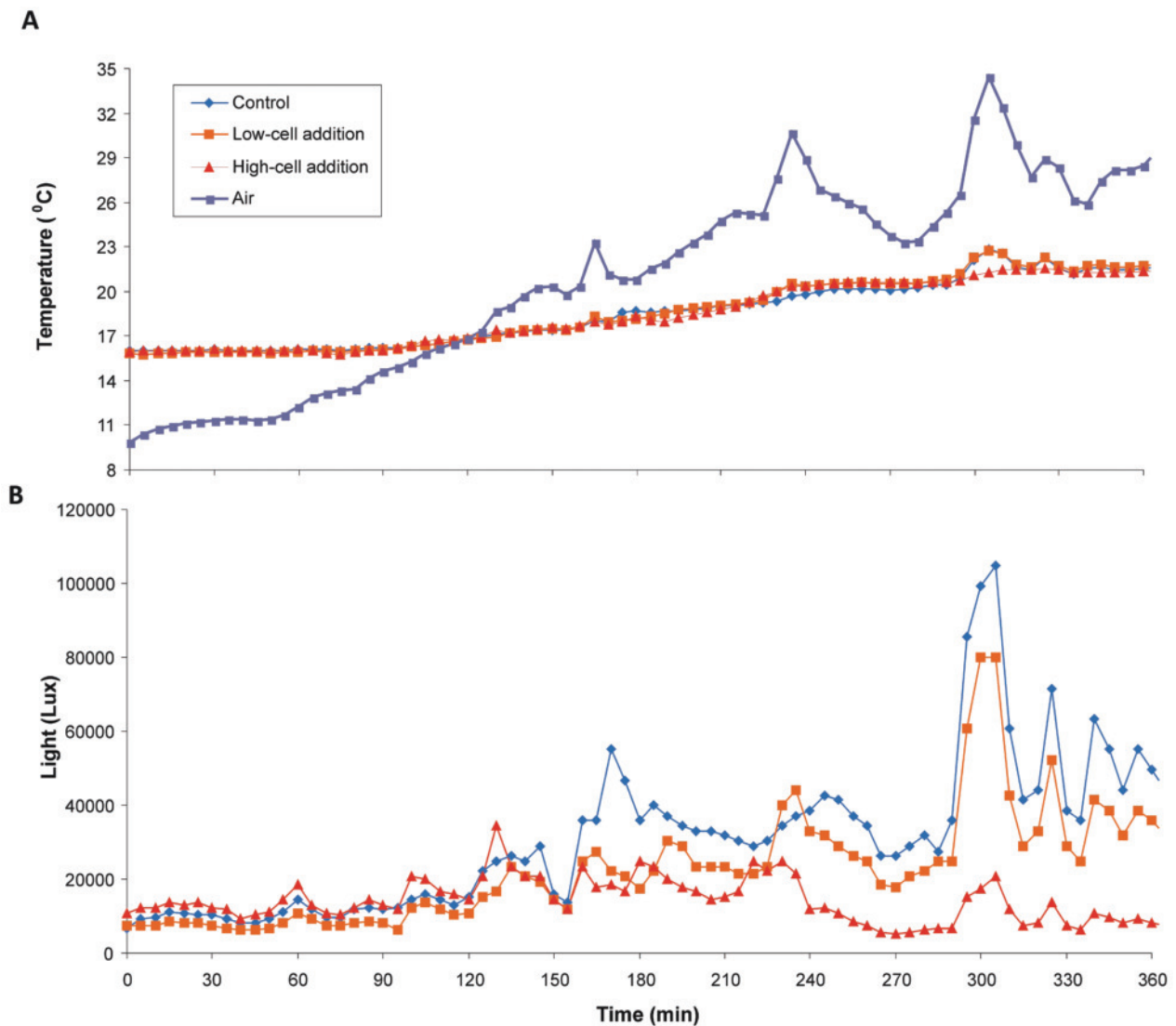


Fig. 2. (A) Temperature and (B) light for water loggers placed about 5 mm below the surface in one replicate of each mesocosm type, and for an air logger immediately above the water surface of the high-cell addition mesocosm in Lake Rotorua (South Island, New Zealand) starting 0900 h 18 March 2010.

Discussion

The results of this study confirm earlier observations in 2009 in Lake Rotorua (Wood et al. 2010) and indicate a link between high *Microcystis* sp. cell concentrations and/or stressors induced by high cell abundance (e.g., oxygen supersaturation) and the up-regulation of microcystin synthesis. The response in the high-cell addition mesocosm once the cell abundance exceeded about $2\,800\,000\text{ cell mL}^{-1}$ was relatively rapid, with the microcystin quotas increasing 5- to 6-fold within 60 min. The final cell addition, which took cell densities to $>7\,000\,000\text{ cell mL}^{-1}$, caused a further increase in microcystin quotas; however, unlike the earlier addition, there was a delay in the response, with an increase in microcystin quotas after 240 min but not in the sample collected 120 min after the addition.

The changes in microcystin quotas in this study (18-fold) were not as pronounced as observed in the initial 2009 Lake Rotorua study (28-fold; Wood et al. 2010) despite a higher final cell abundance in this experiment. It is possible that microcystin quotas continued to increase after the third cell addition at 240 min after the initiation of the present experiment. Despite being lower, an 18-fold increase in microcystin quotas, which occurred after 330 min, is notably higher and occurred in a shorter time frame than that obtained in laboratory studies manipulating a range of parameters (Sivonen and Jones 1999).

Microcystin- and nonmicrocystin-producing *Microcystis* genotypes can coexist in lake populations, and differences in microcystin concentrations, particularly over the duration of a bloom, have been attributed to differences in their relative abundances (Kurmayer et al. 2002). In our study the abundance of different genotypes was not directly monitored; however, the cells used for the additions were collected adjacent to the mesocosms, and the concentration of *Microcystis*, even in the controls, was very high ($>400\,000\text{ cells/mL}$). It is unlikely that the proportion of toxic to nontoxic cells would differ among treatments or from the initial populations in the mesocosms. Additionally, there was a marked increase in microcystin quotas between 240 and 360 min (Fig. 1B). During this period no additional cells were added to the mesocosms, demonstrating that the change in microcystin quotas was not caused by differences in abundances of *Microcystis* genotypes introduced in the cell additions.

In the 2009 Lake Rotorua study (Wood et al. 2010), as microcystin quotas escalated there was a corresponding increase in extracellular microcystin concentrations. We speculated that microcystins were being exported out of the cell and could be functioning as signaling compounds. The same pattern was not observed in this study, suggesting that the *Microcystis* sp. cells retained the mi-

crocytins or that transport out of the cells had not begun when our sampling period stopped. The absence of a change in extracellular microcystins could also support the notion of an intracellular function for microcystins. Zilliges et al. (2011) recently presented data to support an intracellular protein-modulation function for microcystins. They observed that responses were most pronounced in *Microcystis* sp. during acclimation to high light and oxidative stress conditions. The dense *Microcystis* sp. concentrations in the high-cell addition mesocosms could have caused changes in multiple environmental parameters (e.g., pH, nutrients, and oxygen saturation), which could have placed the cells under oxidative stress, ultimately leading to microcystin synthesis.

Studying microcystin regulation in the laboratory using pure cultures has provided valuable data (e.g., Orr and Jones 1998, Oh et al. 2000, Schatz et al. 2007); however, under these conditions, variables that regulate the production of microcystins in cyanobacteria may be altered substantially from *in situ* conditions. Additionally, changes occur in cyanobacteria maintained in culture for extended periods, such as loss of colonial morphology or toxin production in *Microcystis* sp. (Schatz et al. 2005, Zhang et al. 2007). The mesocosms used in the experiment proved to be a useful and practical method to study microcystin synthesis *in situ*. The main limitations were our inability to rapidly concentrate and determine the abundance of the cells used for the cell additions. Using larger plankton nets and an on-site method for cell enumeration could resolve these issues. The results of Wood et al. (2010) and this study indicate that manipulating cell abundance affects microcystin production in *Microcystis* sp. The changes in temperature and light that occurred during this study did not seem to be directly related to microcystin production in the Lake Rotorua mesocosms. However, we cannot rule out the possibility that these and other parameters (i.e., pH, nutrients, and oxygen supersaturation) that are mutually correlated with cell abundance could also play a critical role in regulating microcystins. Further experiments are planned with the aim to understand the mechanisms involved in the increased microcystin synthesis and to assess a wider spectrum of parameters.

Microcystins are produced by a multitude of distantly related genera that are present not only in water blooms, but also on substrates and in extreme environments, such as hot and cold benthic mats (Wood et al. 2008, Krienitz et al. 2003) and terrestrial soils (Prinsep et al. 1992). Phylogenetic studies have suggested a common and ancient origin for microcystins that may have pre-dated the metazoan lineage (Rantala et al. 2004). If the function of microcystins is universal, then attempts to explain why cyanobacteria produce these peptides must take these

factors into consideration. Our finding that cell abundance and/or mutually correlated environmental parameters affects microcystin synthesis supports hypotheses such as signaling, gene regulation, or protein modulation (Dittmann et al. 2001, Schatz et al. 2007, Zilliges et al. 2011), all of which could be applicable to cyanobacteria inhabiting other niches.

Acknowledgements

This research was funded by the New Zealand Ministry of Science and Innovation (UOWX0505) and a German Science Foundation grant to DRD (DI 698/18). Beth and John Wood are thanked for field assistance.

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