Switching toxin production on and off: intermittent microcystin synthesis in a *Microcystis* bloom

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Summary

Toxic cyanobacterial blooms are increasing in prevalence. Microcystins are the most commonly produced cyanotoxin. Despite extensive research the variables regulating microcystin production remain unclear. Using a RT-QPCR assay that allowed the precise measurement of *mcyE* transcriptional gene expression and an ELISA that enabled small changes in total microcystin concentrations to be monitored, we demonstrate for the first time that microcystin production is not always constitutive and that significant up- and downregulation in microcystin synthesis can occur on time scales of 2–6 h. Samples were collected over 3 days from a small eutrophic lake during a dense microcystin-producing *Microcystis* bloom. *McyE* gene transcripts were detected in only four out of 14 samples. Vicissitudes in both microcystin quotas and extracellular microcystin levels corresponded with changes in *mcyE* expression. During the period of exalted microcystin synthesis *Microcystis* sp. cell concentrations increased from 70 000 cells ml^{-1} to 4 000 000 cells ml^{-1}. These data provide compelling evidence that changes in *Microcystis* cell concentrations influence microcystin production.

Introduction

Anthropogenic eutrophication, catchment modification and climate change have been linked to a global intensification of cyanobacterial blooms (Paerl and Huisman, 2008; Conley *et al.*, 2009). The cyanobacteria responsible for these blooms commonly produce toxic compounds and contact with, or consumption of contaminated water poses a serious health risk to humans, livestock, pets and wildlife (Codd *et al.*, 2005). Toxic blooms have resulted in immeasurable costs to tourism, agriculture, farming and human health worldwide.

Of the known cyanotoxins the hepatotoxic microcystins are the most notorious. Microcystins, of which more than 80 different congeners have been identified (Zurawell *et al.*, 2005), are found ubiquitously worldwide. They are well known for their role in the human fatalities in 1996 when patients of dialysis clinics in Brazil were treated intravenously with microcystin-contaminated water (Azevedo *et al.*, 2002). Microcystins irreversibly inhibit eukaryotic serine/threonine protein phosphatases (e.g. 1 and 2a; MacKintosh *et al.*, 1990; Honkanen *et al.*, 1991) resulting in liver disease as well as nephro- and neurotoxicity (Feurstein *et al.*, 2009). Recently, microcystins have been implicated in increased incidences of human liver cancer in China (Yu, 1995) and carcinogenic responses in rodents (Falconer and Buckley, 1989; Falconer, 1991; Nishiwaki-Matsushima *et al.*, 1992; Ito *et al.*, 1997). Microcystins have therefore been evaluated by the International Agency for Research on Cancer of the World Health Oganization whereby microcystin-LR was placed into category 2B, i.e. 'probably carcinogenic for humans' (Grosse *et al.*, 2006).

The complex interactions among physical, chemical and biological variables that lead to the proliferation of cyanobacteria in lentic systems have been the topic of many decades of research. There is still only limited knowledge, however, of the variables that regulate toxin synthesis, e.g. microcystins, at a cellular level. Studies on *Microcystis* spp. (the most common microcystin producing and bloom forming species) have shown correlations between microcystin quotas (total intracellular microcystins per cell), and a multitude of physiochemical variables including: nutrients (Orr and Jones, 1998; Lee *et al.*, 2000; Oh *et al.*, 2000), temperature (Van der Westhuizen and Eloff, 1985) and pH (Van der Westhuizen and Eloff, 1983). However, results of these studies are often contradictory and environmental factors usually only induce changes in microcystin quotas three or fourfold (Sivonen and Jones, 1999).

Most studies of the regulation of microcystins have been undertaken *in vitro* using single strains of

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microcystin producing cyanobacteria. Such studies can allow single variables to be manipulated and effects on microcystin production to be closely monitored (e.g. Orr and Jones, 1998; Oh *et al.*, 2000; Schatz *et al.*, 2007). Studying cyanobacteria in these 'artificial' environments may, however, alter or remove the variables that regulate the production of microcystins. Changes in cyanobacteria maintained in culture for extended periods are also well known, for example, loss of colonial morphology in *Microcystis* (Zhang *et al.*, 2007) and loss of toxin production (Schatz *et al.*, 2005). In contrast, in-lake studies are challenging because of the plethora of interacting biotic and abiotic variables that may be critical in regulating growth of cyanobacteria and microcystin production.

Recent advances in analytical methods (e.g. ELISA and liquid chromatography-mass spectrometry) have allowed accurate monitoring of microcystins (e.g. Fischer *et al.*, 2001; Dell'Aversano *et al.*, 2004). Microcystins are extremely stable compounds (Tsuji *et al.*, 1995; Harada *et al.*, 1996) and although there is strong evidence that microcystins can be actively transported out of cells (Pearson *et al.*, 2004), it is likely that over time there is some continuous accumulation of these compounds within cells (Young *et al.*, 2005). Thus, measuring minor alterations in intra- or extracellular microcystins may be difficult, or impossible, when relying solely on toxin analysis.

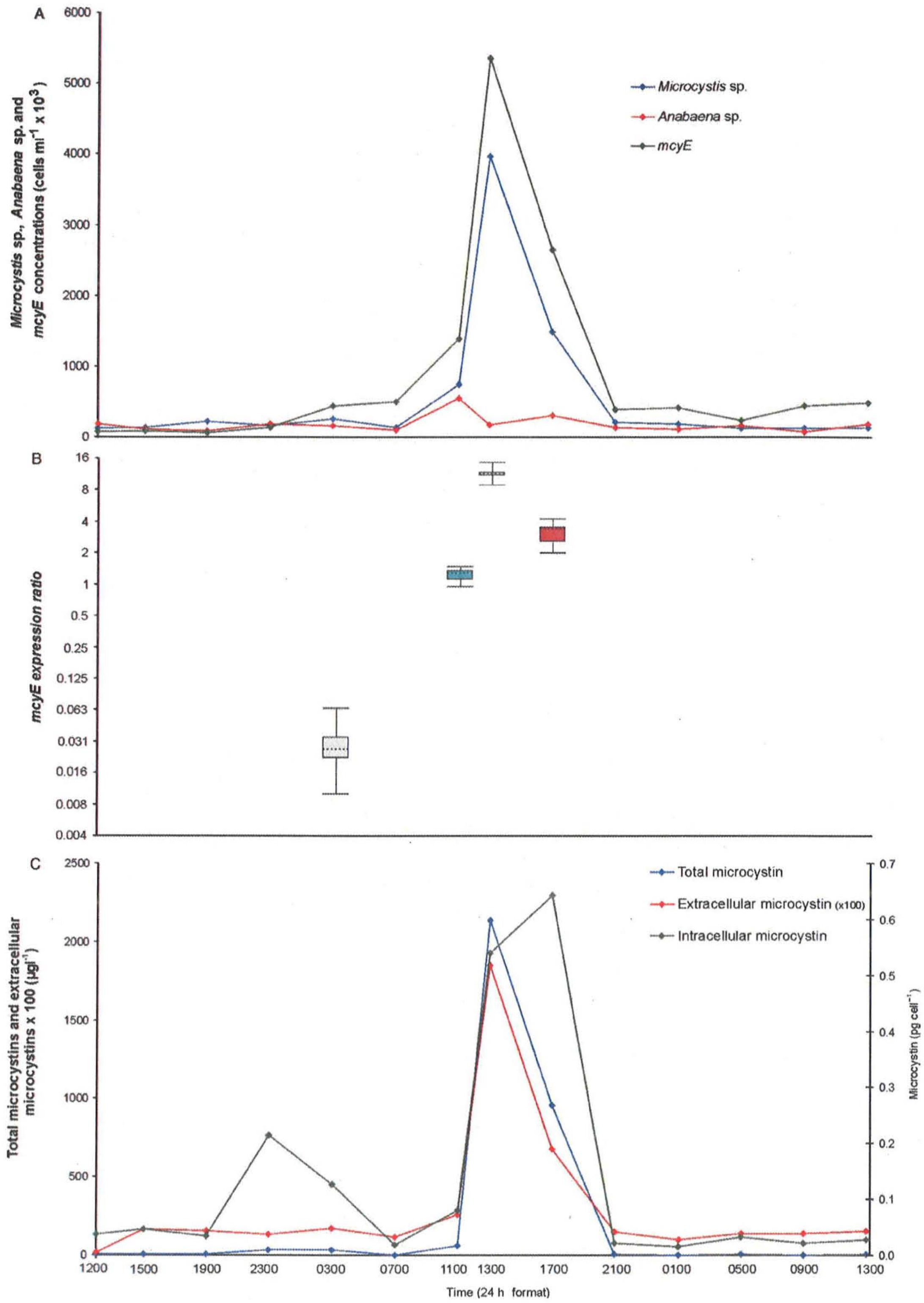
Microcystins are synthesized non-ribosomally by a large (55 kb) peptide synthetase and polyketide synthase enzyme complex. The gene cluster involved in microcystin synthesis (*mcyA–J*) has been identified and sequenced (Nishizawa *et al.*, 2000; Tillett *et al.*, 2000), providing the opportunity to employ molecular techniques to study microcystin production at a genetic level. Moreover, the most recently developed pseudo-viral controlled reverse-transcriptase quantitative PCR (RT-QPCR) assay (Rueckert and Cary, 2009) allows for the accurate assessment of *mcyE* gene expression in toxic *Microcystis* and *Anabaena*. In prokaryotic organisms mRNA is continuously degraded during translation (Voet and Voet, 2004). Using the latter RT-QPCR assay, analysis can be undertaken of temporary transcripts with an average half-life of a few minutes, thus allowing measurement of the transcriptional regulation of microcystin production almost simultaneously with the signal that induces it. The RT-QPCR assay described in Rueckert and Cary (2009) includes an internal armoured RNA standard with the same primer binding sites as the *mcyE* target gene, as well as a unique internal probe binding sequence. This technique provides, for the first time, a method that can be used to quantitatively measure microcystin transcriptional gene expression.

A *Microcystis* bloom comprising of almost entirely toxic genotypes in Lake Rotorua (42°24′05 S, 173°34′57 E, South Island, New Zealand) provided a valuable opportunity to elucidate how interactions among abiotic and biotic variables influence microcystin synthesis in a cyanobacterial bloom. In this study an RT-QPCR assay (Rueckert and Cary, 2009) and an ADDA-ELISA (Fischer *et al.*, 2001) were used in concert with measurement of a range of physiochemical parameters, microscopic cell counts and QPCR assays to demonstrate for the first time that microcystin synthesis is not always continuous and that changes in *Microcystis* cell concentrations influence microcystin production.

Results and discussion

Surface water samples were collected from a cyanobacterial bloom in Lake Rotorua (South Island, New Zealand) every 4 h over a 49 h period between 6 and 8 May 2009, hereafter known as days 1, 2 and 3. Using microscopy three cyanobacterial species were identified in surface water samples: *Aphanothece* sp. (< 0.5 cell ml^{-1}), *Anabaena* sp. *Nova* and *Microcystis* sp. (Fig. 1A). No *Anabaena*-specific *mcyE* genes were detected in the DNA extracted from the preserved filters using QPCR (Vaitomaa *et al.*, 2003), indicating that all microcystins in the samples were produced by *Microcystis* sp. Microcystin and non-microcystin producing strains of *Microcystis* can coexist in lake populations (Kurmayer *et al.*, 2002), with this difference attributed to the presence or absence of the microcystin synthetase gene cluster (Nishizawa *et al.*, 2000). QPCR (Vaitomaa *et al.*, 2003) was used to determine the *Microcystis*-specific *mcyE* copy numbers in all samples and the results showed high congruence with the microscopically determined cell concentrations (Fig. 1A), indicating that most, if not all, cells within the population had the ability to produce microcystins.

In this study we used a recently developed RT-QPCR assay (Rueckert and Cary, 2009) to monitor *mcyE* gene transcripts. This assay incorporates a ribonuclease-resistant armoured RNA standard that consists of a non-contagious MS2 bacteriophage virus, in which the genome has been altered to harbour the *mcyE* gene, with a unique internal probe binding sequence for its differential detection. The advantage of this design is that the target and standard sequences are virtually identical, resulting in both sequences undergoing reverse transcription and amplification with the same efficiency. The armoured RNA is used to spike each individual sample, allowing monitoring for the integrity of all nucleic acid processing steps including extraction, reverse transcription, amplification and detection. Using this RT-QPCR assay, *mcyE* gene transcripts were detected in only four out of 14 samples (Fig. 1B). Among the positive samples there were significant up- and downregulations in *mcyE* gene expression over short time periods (2 to 5 h) that



A
B
C
Microcystis sp., Anabaena sp. and mcyE concentrations (cells ml-1 x 10³)
Microcystis sp.
Anabaena sp.
mcyE
mcyE expression ratio
Total microcystins and extracellular microcystins x 100 (µgl-1)
Total microcystin
Extracellular microcystin (x100)
Intracellular microcystin
Microcystin (pg cell-1)
Time (24 h format)

corresponded with changes in microcystin quotas. The highest *mcyE* expression was observed at 1300 h on day 2, and this was 462-fold greater than values from a sample collected 12 h earlier (0300 h) and 12-fold higher than a sample collected just 2 h earlier (Fig. 1B). By 1700 h expression levels had decreased but were still substantially elevated (115-fold) compared with the 0300 sample (Fig. 1B). Previous studies (Kaebernick *et al.*, 2000; Sevilla *et al.*, 2008) have noted variations in the magnitude of *mcy* gene expression; however, these studies have always been undertaken using cultured strains, and changes in *mcy* expression have been small and over longer time frames. Furthermore *mcy* gene expression has always been observed to be continuous rather than extremely variable as observed in our study. Our study is the first to demonstrate that microcystin production is not always continuous over the duration of a cyanobacterial bloom.

Microcystin quotas varied throughout the study period. Analysis using LC-MS (Wood *et al.*, 2010) identified the microcystin variants; -YR, -LR, desmethyl-LR, didesmethyl-LR and WR. When microcystin concentrations were low LC-MS was not suitable for assessing minor changes in microcystin concentrations because individual variants were often below the reliable limits of detection (0.2 $\mu g\ l^{-1}$). The ADDA-ELISA (Fischer *et al.*, 2001) measures total ADDA-containing compounds and in this study proved to be an accurate method for assessing small fluctuations in total microcystins (LOD, 0.02–0.07 ng ml^{-1} dependent on variants present). At 1200, 1500 and 1900 h (day 1) the microcystin quotas ranged between 0.035–0.046 pg $cell^{-1}$. This increased approximately fourfold in the two subsequent samples (2300 h on day 1 and 0300 h on day 2; to 0.214 and 0.126 pg $cell^{-1}$, respectively), and then decreased to 0.019 pg $cell^{-1}$ at 0700 h before some recovery to 0.080 pg $cell^{-1}$ by 1100 h (Fig. 1C). At 1300 and 1700 h (day 2) there was an approximate 15-fold increase in microcystin quotas to 0.539 and 0.644 pg $cell^{-1}$, respectively (Fig. 1C), i.e. a 28-fold increase over a 5 h period (1300–1700). To our knowledge no previous studies have shown such a dramatic increase in microcystin quotas of *Microcystis*, and certainly not within 5 h (Sivonen and Jones, 1999). The observed increase in microcystin quotas could have resulted from an influx of 'more toxic' strains, as some *Microcystis* strains grown in culture are known to always produce greater quantities of microcystins (Bolch *et al.*, 1997). However, the increase in microcystin quotas coupled with the upregulation of *mcyE* provides compelling evidence that this increase is due to some external or internal stimulus. Furthermore, given the continuously high concentrations of *Microcystis* cells present throughout the study (> 115 000 cells ml^{-1}) it is likely that any variations in microcystin quotas between strains would be averaged among the population.

The ecophysiological basis for microcystin production has been the topic of many decades of research, with various hypotheses postulated including: defence against grazers (Lüring, 2003), gene regulation (Dittmann *et al.*, 2001), allelopathic interactions (Sukenik *et al.*, 2002), intra-specific regulation (Schatz *et al.*, 2007) and siderophoric scavenging of, and binding to metals such as iron (Martin-Luna *et al.*, 2006; Saito *et al.*, 2008). The data presented in this study provide some further evidence to support and refute some of these hypotheses. The most noteworthy change in a biotic variable that coincided with elevated microcystin quotas and *mcyE* expression was the dramatic increase in the cell concentrations of *Microcystis* between 0700 h and 1300 h (day 2) when there was a 30-fold increase in *Microcystis* concentrations (Fig. 1A). This coincided with a period of gentle onshore winds (data not shown). *Microcystis* colonies are extremely buoyant, commonly forming wind-blown scums (Znachor *et al.*, 2006), and we surmise it was the onshore wind that caused the dramatic increase in cell concentrations. The role of microcystins as a signalling or quorum sensing molecule has recently received increased attention (e.g. Dittmann *et al.*, 2001; Schatz *et al.*, 2007). In our study, as microcystin quotas escalated in Lake Rotorua, there was a corresponding increase in extracellular microcystin concentrations (Fig. 1C), possibly indicating that the microcystins were being exported out of the cell and could be functioning as signalling compounds. However, we have no way of distinguishing between extracellular

Fig. 1. Data from surface samples collected at Lake Rotorua (South Island, New Zealand) over a 49 h period (6–8 May 2009). Water samples were preserved (Lugol's Iodine) prior to identification and enumeration and frozen or filtered and frozen, for total and extracellular microcystin analysis. Samples for DNA and RNA extraction were filtered through Whatman GF/C glass microfibre filters and filters were placed immediately in LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Carlsbad, CA).
A. *Microcystis* sp., *Anabaena* sp. *Nova* concentrations determined using Utermöhl settling chambers (Utermöhl, 1958). Subsamples (1 ml) where settled and 10 random fields counted at 400× original magnification. *McyE* concentrations were assessed using QPCR (Vaitomaa *et al.*, 2003) and data are an average of two separate analyses.
B. Relative *mcyE* gene expression. Statistical data analysis was performed using the Relative Expression Software Tool (REST 2005 V1.9.12; http://www.gene-quantification.info, Pfaffl *et al.*, 2002). The boxes represent the interquartile range or the mid-point of 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.
C. Total and extracellular microcystins, and microcystin quotas as determined by ADDA-ELISA (Fischer *et al.*, 2001). Data represent an average of two separate analyses.

microcystins that have been exported from those that result from cell lysis. Interestingly, although *mcyE* expression and extracellular microcystin levels had decreased by 1700 h on day 2, microcystin quotas remained elevated, perhaps suggesting that cells maintain some microcystins for short periods until their requirement for microcystin abates. Microcystins are extremely stable compounds (Tsuji *et al.*, 1995; Harada *et al.*, 1996) and the rapid decrease in extracellular concentrations (Fig. 1C) is surprising. The most like explanation is water movement within the lake transported the microcystins out of our sampling area. Bacterial degradation may also have contributed.

The water temperatures measured in Lake Rotorua (8.2–12.5°C; Fig. S1A) are well below those optimal for *Microcystis* growth (Imai *et al.*, 2009). The cooler temperatures may have contributed to the rapid and pronounced changes in *mcyE* expression and microcystin quotas. We speculate that at these low temperatures the cells are already stressed and their response to external or internal stimuli is therefore enhanced.

No other measured abiotic variables changed markedly within the 2 h period (1100–1300 h), when we observed the greatest rate of change in *mcyE* expression and microcystin synthesis. Water temperature increased by only 1.3°C (Fig. S1A) and had changed little by 1700 h when *mcyE* expression had already decreased. Previous culture-based studies have shown only a onefold to twofold increase in microcystin quotas when temperatures have been increased in > 5°C increments (e.g. Watanabe and Oishi, 1985). Significant spikes in light intensity were recorded but these did not start until after 1300 h (Fig. S1A), by which time microcystin quotas and *mcyE* expression levels had already increased dramatically. Despite considerable evidence from culture-based studies showing the role of nutrients in regulating microcystin concentrations (e.g. Orr and Jones, 1998; Vezie *et al.*, 2002) changes observed in dissolved nutrients in Lake Rotorua did not correlated with periods of up- or downregulation of microcystins production (Fig. S1B). *Microcystis* and *Anabaena* accounted for almost the entire phytoplankton community; therefore allelopathy does not seem like a viable explanation for the biological role of microcystins in this lake. Although *Anabaena sp. Nova* concentrations did fluctuate (Fig. 1A) these shifts did not align with the marked changes in microcystin synthesis.

Culture-based studies have been the mainstay of research exploring the role and regulation of microcystins. They have typically focused on environmental factors that differ throughout the duration of a cyanobacterial bloom (e.g. light, temperature and nutrients). Often only one parameter is changed while others are maintained at optimal levels. Few, if any, culture-based studies have changed conditions with the speed and intensity observed in the lake environment, for example, the 30-fold increase in cell concentrations recorded within 6 h in this study. The elucidation of the microcystin biosynthetic pathway has started a new era in the search to understand the function and regulators of microcystins, and this study highlights the benefits of combining highly sensitive molecular and analytical techniques. Using a state-of-the-art technique that allowed the precise measurement of *mcyE* transcriptional gene expression and a sensitive method capable of measuring small changes in total microcystin concentrations, we have demonstrated for the first time that in a cyanobacterial bloom microcystin production is not always constitutive. The significant and rapid up- and downregulation of *mcyE* expression corresponding to variations in microcystin quota, extracellular microcystin levels and cell concentrations adds further evidence to support the hypothesis that microcystins may act as signalling compounds. Increased understanding of the regulation of microcystins in the environment will assist in assessing periods of greatest health risk and may ultimately lead to the development of models enabling real-time predictions of microcystin synthesis to be undertaken.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Physicochemical data at sampling site at Lake Rotorua (South Island, New Zealand) over a 49 h period (6–8 May 2009).

A. Water temperature and light intensity (approximately 2 cm below water surface) recorded every 5 min (UA-002-08, HOBO®, Onset Computer Corporation, MA).

B. Dissolved reactive phosphorous, ammonium, nitrate and nitrite concentrations determined using standard methods (APHA, AWWA, and WEF, 2005).