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Seasonal occurrence and toxicity of *Microcystis* in impoundments of the Huron River, Michigan, USA

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ABSTRACT

Occurrence and toxicity of *Microcystis* spp. were measured by ELISA in three impoundments of the Huron River in southeast Michigan, USA. Assays were conducted weekly from June through October 2005. Additional samples were collected to assess microcystin concentrations throughout the drinking water treatment process at the Ann Arbor Water Treatment Facility. Water column stability, nutrient concentrations, and N:P ratios were examined as potential predictors of phycocyanin and microcystin. Microcystin was found in two of the impoundments at seasonally varying concentrations. *Microcystis* presence was associated with N:P ratios between 40 and 80 by moles, and toxin levels typically peaked one to two weeks after a peak in phycocyanin. The toxin was also detected at low levels at all stages of the drinking water treatment process. Freezing and thawing water samples prior to analysis yielded maximum microcystin assay concentrations. Experiments indicated that the competitive ELISA method is susceptible to false negative reporting. This is the first report of algal toxins in this catchment, and results demonstrate that sensitive and rapid analytical methods offer the chance to link the dynamics of toxin production with environmental conditions.

1. Introduction

Dense blooms of cyanobacteria are regular features of eutrophic lakes. Using intracellular gas vacuoles for buoyancy under thermally stratified conditions, cyanobacteria can form thick aggregations on lake surfaces (Paerl, 1988). The eyesores and ensuing foul odors associated with biomass decay lead to severe nuisance conditions and challenges to environmental management. As knowledge increases about the toxins produced by some of these algae, the public focus is shifting to encompass public health dimensions. The result is a fertile interdisciplinary interface among aquatic ecology, molecular methodologies, and environmental toxicology.

Microcystis is perhaps the most studied genus of the toxin-producing cyanobacteria. This genus can produce microcystin, an hepatotoxic heptapeptide with four main variants that have been implicated in both acute illness as well as chronic diseases such as hepatocellular carcinoma (Lam et al., 2000; Bischoff, 2001). The toxin causes permanent morphological changes in the mitochondria and cytoskeletal filaments of liver cells, leading to both apoptosis and necrosis (Batista et al., 2003; Majsterek et al., 2004). Reactions to the toxin occur mainly as a result of skin contact or ingestion. Though most documented cases of microcystin toxicity have occurred in domestic animals, there have been reports in humans. In one instance, the water in a hemodialysis center in Brazil became contaminated, resulting in the deaths of 60 patients due to acute liver failure (Pouria et al., 1998). Microcystins in drinking water have also been cited as one of the factors contributing to the elevated levels of primary liver cancer (PLC) in some areas in China (Lian et al., 2000; Yu et al., 2001). In 1996, the World Health Organization (WHO) listed toxic cyanobacteria

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among the pathogenic agents to be monitored in water, and in 1998 it determined that levels of microcystin above $1\,\mu g L^{-1}$, or 1 nM, were unsafe for long-term consumption (WHO, 1996, 1998).

Studies of *Microcystis* in the many small inland lakes of Michigan have been rare. The most extensive study to date examined 14 lakes in the lower peninsula for genetic variation in *Microcystis aeruginosa* (Wilson et al., 2005). The study found isolates of *M. aeruginosa* in 13 of the lakes, but samples were collected only once per lake during August of 2002. The study did not determine the magnitude or duration of any blooms.

The purpose of this study was to determine whether toxic strains of *Microcystis* occur in three important impoundments of the lower Huron River in southeast Michigan that are used intensively for recreation, including boating and fishing. One of the impoundments also serves as the major source of drinking water for the city of Ann Arbor, but no surveys of algal toxins had hitherto been undertaken. Understanding presence, seasonality, and environmental conditions associated with toxin production is prerequisite to developing management strategies for protecting water quality.

2. Materials and methods

2.1. Field sites

Water samples were collected weekly from June through October 2005. Surface grab samples were obtained from three impoundments of the Huron River in southeastern Michigan: Barton Pond (42.31° N, 83.75° W), Ford Lake (42.22° N, 83.58° W), and Belleville Lake (42.21° N, 83.49° W). Barton Pond was sampled at a single site (denoted BP) near its outlet dam. Ford Lake was sampled at three stations (denoted F1, F2 and F3) located respectively near the western inlet (5 m depth), the center (7 m depth), and near the eastern outlet dam (10 m depth). Belleville Lake was sampled at two stations (B1 and B2) located, respectively, in the center of the elongate lake and near the east end outlet dam. Water samples were transported to the laboratory for analysis. All samples were examined using optical microscopy for presence of *Microcystis* spp. Subsamples of the water were frozen until being tested for microcystin toxin.

2.2. Assessment of water treatment facility

In addition to the regular sampling schedule, water from the Ann Arbor Water Treatment Facility was examined at various stages of the treatment process. On 21 and 22 September 2005 grab samples were obtained from the BP sampling site. Additional samples were obtained on 21 September from five sequential processing stages of water treatment: (1) raw intake water, (2) secondary settling basin, (3) filter influent, (4) filter effluent, and (5) final drinking water. It is important to note that intake pipes for the water used in the treatment process are located at the bottom of the pond where the distribution of *Microcystis* may be different from the surface.

2.3. Algal biomass

Samples were collected for pigment determination. Both chlorophyll *a* and phycocyanin were analyzed but only the latter is reported here. To measure pigments, 250-ml aliquots were filtered through Whatman AH glass fiber filters, and were held frozen over desiccant until analysis. Filters were extracted in 0.05 M pH 7 phosphate buffer with tissue grinding. Phycocyanin was detected fluorometrically using a Turner Designs TD700 fluorometer with a 630 nm excitation filter and 660 nm emission filter.

2.4. N:P ratios

Total P was measured by persulfate oxidation followed by analysis as molybdate reactive P. Total N was measured as the sum of total dissolved N and particulate N using the alkaline persulfate oxidation method of D'Elia et al. (1977). Samples were oxidized to NO_3^-, acidified with HCl, and measured by second derivative UV spectroscopy (Crumpton et al., 1992).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The analytical method used for detection of microcystin was competitive enzyme-linked immunosorbent assay (ELISA) by colorimetric assay. This method is sensitive for low levels of microcystin (Pyo et al., 2005) and can be applied to aqueous samples without filtration or extraction. Thawed water samples were added, along with phosphate buffered saline (PBS) diluent to acrylic tubes that had been coated with polyclonal microcystin antibodies (EnviroLogix, Inc., Portland, ME, USA). After primary incubation with sample, excess antigen microcystin linked to horseradish peroxidase was added and incubated to adsorb to remaining vacant antibody sites. The liquid phase was discarded after 20 min and the tubes were washed copiously with deionized water, then air-dried by inversion of tubes with brisk tapping on a horizontal surface. Activity of the bound peroxidase was assayed as rate of hydrolysis of TMB (3, 3′, 5, 5′-tetramethylbenzidene), stopped with 1 N HCl, and measured spectrophotometrically at 450 nm (absorbance peak) and 650 nm (turbidity blank). All assays were performed on duplicate samples.

Samples were run in parallel with internal standard amendments. Measured aliquots of B2666 *Microcystis aeruginosa* Kütz em. Elenkin, a toxic strain obtained from the UTEX Culture Collection of Algae at the University of Texas at Austin, were added to deionized water as well as to field samples and were run alongside the unamended samples. To determine predicted values for the samples with additions, we summed the mean concentration of the deionized water with B2666 culture addition and the mean concentration of each unamended sample, with attention to volume proportions. These values were then compared with the observed concentrations of the samples after standard additions.

Experiments were performed to determine if freezing and thawing indeed releases additional microcystin for assay by ELISA compared with raw water directly from the field. Water was collected from the Barton Pond sampling station on 27 August 2005 using a conical plankton net with 64 μm mesh aperture, hauled from 2 m depth to the surface in order to

concentrate colonies of *Microcystis*. The assay was run comparing sample filtrate, raw water, and raw water that had been frozen and thawed. Filtrate was prepared by drawing sample through a GF/C glass fiber filter. Other than this experiment, and other similar ones using toxic strain B2666, all samples were frozen and thawed prior to analysis.

2.6. Theory

Competitive ELISA yields a reciprocal relationship between sample antigen concentration and enzyme activity (peroxidase in this case). Unknown sample concentrations were deduced by reference to standard concentrations of 0, 0.4, 0.5, 1.0, 2.5, or 3.0 nM microcystin (EnviroLogix, Inc), or serial dilutions of these. One of the most commonly used dose-response curves for competitive ELISA is the four-parameter log-logistic function (Rodbard and Hutt, 1974):

$$Y = \frac{a-d}{1+(X/c)^b} + d \quad (1)$$

where X is microcystin concentration (nM) and Y is the response variable (optical absorbance in this case). Rodbard and Hutt (1974) point out that the parameter b is usually indistinguishable from a value of 1; d is the expected response for an infinite dose of microcystin. In this case, an infinite dose would saturate all antibody binding sites and leave no room for binding enzyme conjugate. Hence, d approximates zero. Using these approximations and defining $\alpha = 1/ac$ and $\beta = 1/a$, Eq. (1) can be simplified to:

$$A = 1/(\alpha \bullet X + \beta) \quad (2)$$

where A is the difference between optical absorbance (1-cm path length) at 450 nm and 650 nm, and α and β are statistically determined parameters. Fig. 1 illustrates the fit of Eq. (2) to standard data.

Parameter values were obtained by application of the Microsoft Excel™ Solver routine and by the nonlinear regression option of SYSTAT version 10. The two methods yielded identical results.

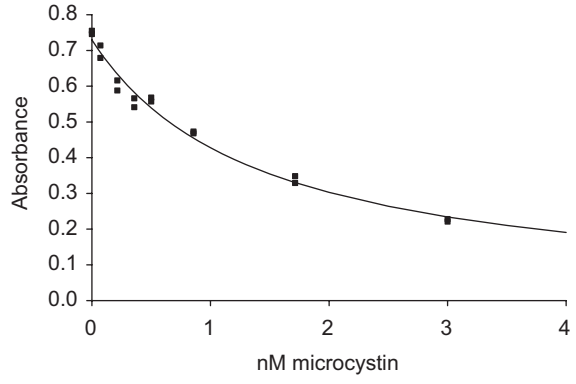

Fig. 1 – Eq. (2) fit to microcystin standard series; $\alpha = 0.967$, $\beta = 1.371$, $r^2 = 0.99$.

2.7. Limit of detection

Based on 117 replicate determinations of microcystin concentrations less than 1 nM, the mean deviation between replicates averaged 0.036 nM with a standard deviation of 0.028 nM. Various statistical conventions have been used to define method detection limit (MDL). One of the most conservative conventions aims to minimize the incidence of false positive detections by defining the limit of detection as 3 standard deviations greater than zero ($= 0.085$ nM in this case). The described approach is applied mainly to cases in which single, non-replicated determinations are performed. This method for confident detection of signal results in de facto confidence probability $= 0.001$ that the sample concentration exceeds the blank (de facto zero).

Our assays are performed in duplicate. Hence, the confidence that sample mean concentration exceeds the blank with probability $= 0.001$ occurs at MDL $= 0.06$ nM ($= 3 \times 0.028/\sqrt{2}$).

3. Results

3.1. Raw vs. Freeze-Thaw samples

Concentrations of microcystin from Barton Pond on 27 August 2005 (Table 1) exceeded the MDL in all cases. Microcystin detected by ELISA in the untreated raw sample was slightly elevated compared with filtrate. Freezing and thawing prior to analysis, however, elevated ELISA-detectable microcystin by 20-fold.

3.2. Algal biomass

Phycocyanin and microcystin in Barton Pond (Fig. 2) developed maxima in late August and September. Two phycocyanin peaks occurred; the first on 24 August with 184 $\mu g L^{-1}$, and the second, much smaller, on 21 September with 6.7 $\mu g L^{-1}$. These elevated pigment levels corresponded with the presence of *Microcystis*.

Ford Lake (Fig. 3) witnessed two distinct blue-green algal blooms. The first was almost exclusively dominated by *Aphanizomenon flos aquae*, accounting for the major peak in phycocyanin at all three stations during late August. The two smaller peaks that follow in early September can be attributed to *Microcystis*, based on optical microscopy. The first peak occurred on 12 September for F1 and F2 (96 and 48 $\mu g L^{-1}$, respectively). For F3, the peak began on 6 September and remained high through 15 September, with the highest level being 97 $\mu g L^{-1}$. The second peak associated with *Microcystis* occurred on 26 September for F1 and F2 (340 and

Table 1 – Microcystin concentrations (nM) of Barton Pond surface water detected by ELISA

Sample	nM microcystin
Filtrate	0.22
Unfiltered sample	0.28
Freeze-Thaw sample	4.99

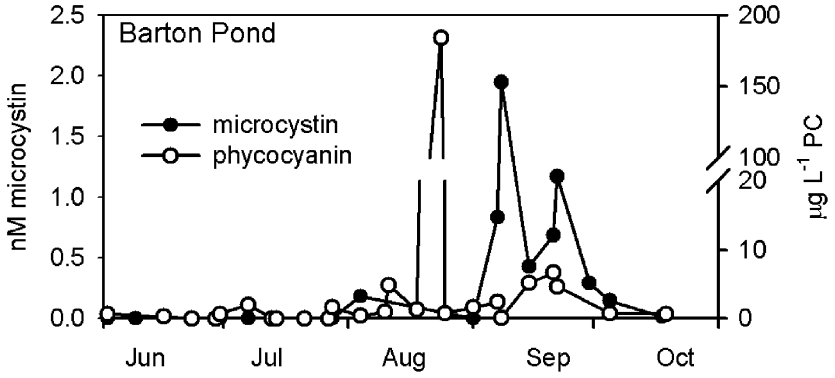

Fig. 2 – Microcystin concentrations (nM) and phycocyanin (PC, $\mu g L^{-1}$) of Barton Pond surface water, June to October 2005.

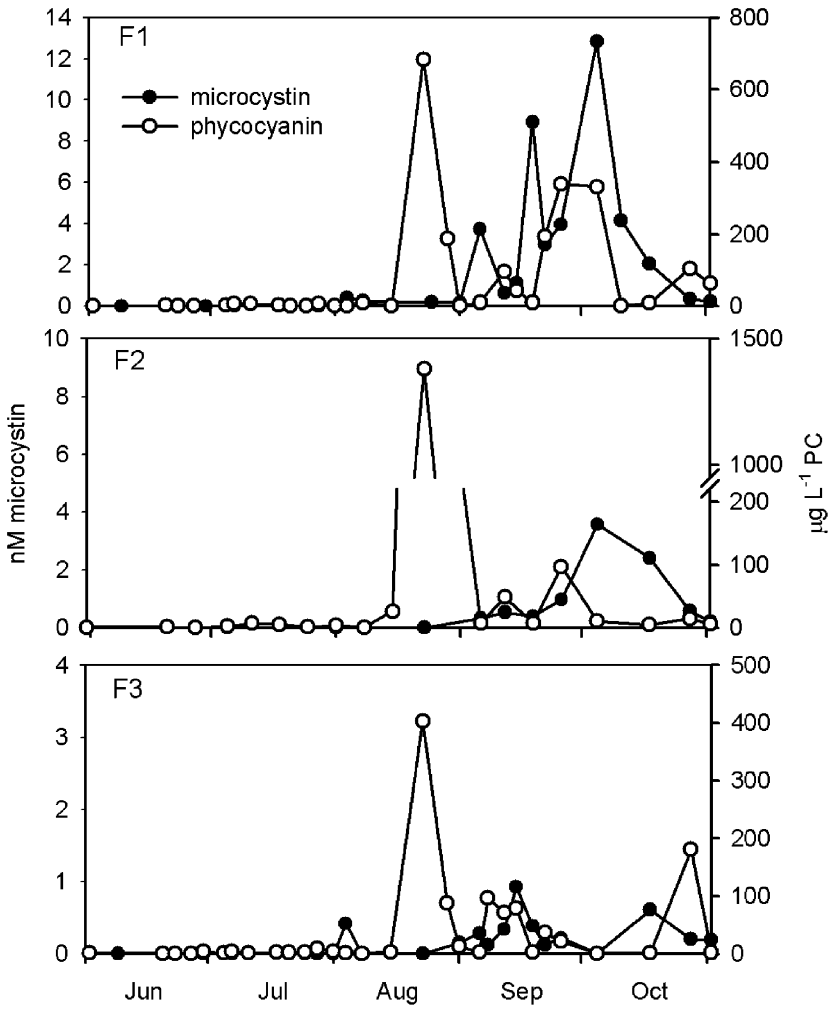

Fig. 3 – As Fig. 2, but for Ford Lake surface water, stations F1, F2, and F3. The phycocyanin maxima on 23 August are attributable to *Aphanizomenon*; subsequent maxima in September and October are *Microcystis*.

$97\,\mu g L^{-1}$) and on 19 September for F3 ($36\,\mu g L^{-1}$); the elevated levels lasted into October.

Belleville Lake also experienced two peaks in phycocyanin over the summer of 2005, but optical microscopy did not detect abundant cyanobacteria. These peaks occurred on 31 August and 14 September with 85 and $71\,\mu g L^{-1}$, respectively. The predominant algae at these times were Cryptophyta, a division known to produce three forms of phycocyanin (Clay et al., 1999). No microcystin was ever detected; results are not shown.

3.3. *Microcystin by ELISA*

In both Ford Lake and Barton Pond local maxima in concentrations of microcystin followed peaks in phycocyanin

by about 1–2 weeks. In Barton Pond, microcystin was first detected 4 August 2005, and subsequently increased. During September there were two distinct peaks in toxin levels, with the highest concentration reaching a value of 1.95 nM on 8 September, approximately two weeks after a prominent appearance of phycocyanin and *Microcystis* on 24 August. After 30 September, toxin levels began a steady decline, and by 18 October concentrations fell below the MDL (Fig. 2).

The pattern in Ford Lake was similar to that of Barton Pond. Microcystin was first detected at stations F1 and F3 on 4 August 2005, but not until September did toxic *Microcystis* begin to dominate the lake's surface (Fig. 3). There were three distinct peaks in concentration at F1 between 6 September and 5 October, with the third peak being the highest at 12.8 nM. Concentrations finally declined at F1 as the water chilled in October. At F2, microcystin levels rose contemporaneously with F1, but only one major peak developed, on 5 October with a concentration of 3.6 nM, much lower than that of F1 on the same date. Levels of toxin at station F3 never reached the same potency as at the other two upstream and shallower stations. Toxin levels followed the patterns of F1 and BP, but maximum microcystin concentration was only 0.93 nM on 15 September. A second maximum occurred later in October, and appeared to be caused by downstream flushing of the bloom through the lake. As with Barton Pond, toxin levels typically succeeded cyanobacteria biomass maxima, measured as phycocyanin, by between one and two weeks. At the time of these observations, the hydrologic residence time (lake volume/water income) was about 50 days (unpublished data).

Belleville Lake did not experience *Microcystis* blooms despite its being directly downstream from Ford Lake. At no time was the organism conspicuously present by microscopic inspection. ELISA assays were performed, nonetheless, to assess the sensitivity of the competitive ELISA, but the negative results are not displayed. Because the assay never reported microcystin in any of these samples, it is likely that this test has a negligible false positive rate.

3.4. *N:P ratio*

Figs. 4 and 5 present the molar TN:TP ratio and phycocyanin concentrations for each site. Barton Pond maintained a relatively high ratio throughout the summer except for a few weeks in August and September, which were interrupted by a mixing event (Fig. 4). The lowest value was 49.5 and occurred on 21 July. Another low point occurred on 24 August (N:P = 50.4), corresponding with the highest peak in phycocyanin (184 $\mu g L^{-1}$). In general, N:P ratios remained in the 50–70 range from mid-July through the end of September. Such moderate N:P ratios are consistent with *Microcystis* blooms (Downing et al., 2005).

In Ford Lake, rapid declines in N:P occurred from June through July at all three sampling sites (Fig. 5), then rose slightly before a second decline in August. The August decline in N:P was caused by a storm-driven mixing event that stirred 12 μM phosphate from the hypolimnion into an epilimnion that originally had less than 1 μM TP. The result was a massive *Aphanizomenon* bloom. This lakewide phenomenon resulted in simultaneous maxima in phycocyanin and minima in N:P at all three sampling sites on the same date (23 August). The N:P ratios were 18.5, 21.1, and 28.0 at stations F1, F2, and F3, respectively. Subsequent peaks in phycocyanin attributed to *Microcystis* were associated with more moderate N:P ratios, similar to Barton Pond. The peaks in phycocyanin associated with *Microcystis* rather than *Aphanizomenon* at F1 occurred at N:P molar ratios of 63.6 on 12 September and 50.8 on 26 September. Ratios at F2 were 67.2 on 12 September and 77.3 on 26 September, and at F3 they were 47.8 on 6 September and 62.4 on 19 September.

During early to mid September, while *Microcystis* populations were increasing, inorganic phosphate was plentiful in the epilimnion of Ford Lake (Fig. 6). Although the inlet, and main source of external loading, is nearest F1 (5 m depth), the soluble reactive P (SRP) levels were highest at F3 (10 m depth).

3.5. *Internal Standard–False Negative Reports*

On nine separate occasions the values predicted for internal standard additions to lake water was significantly higher than the values observed, suggesting that the ELISA assay method is susceptible to reporting false negative values. Eight of these occurrences happened in Ford Lake, five of them at station F3. The anomalies are not correlated with unamended levels of microcystin observed in the lake ($R = 0.27$, $P = 0.51$ with one

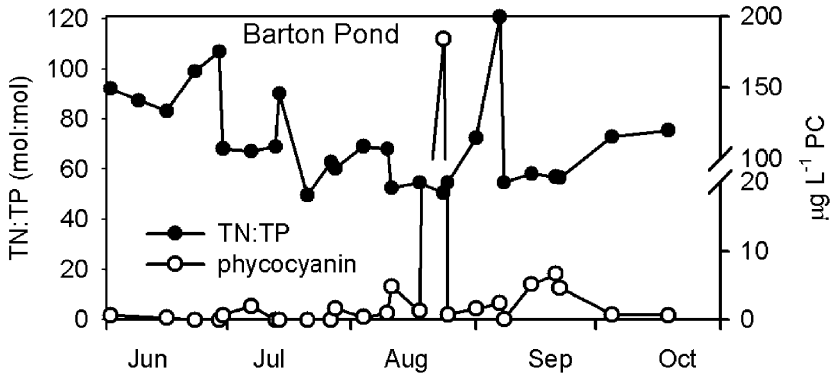

Fig. 4 – TN:TP ratio and phycocyanin concentrations ($\mu g L^{-1}$) of Barton Pond surface water, June–October 2005.

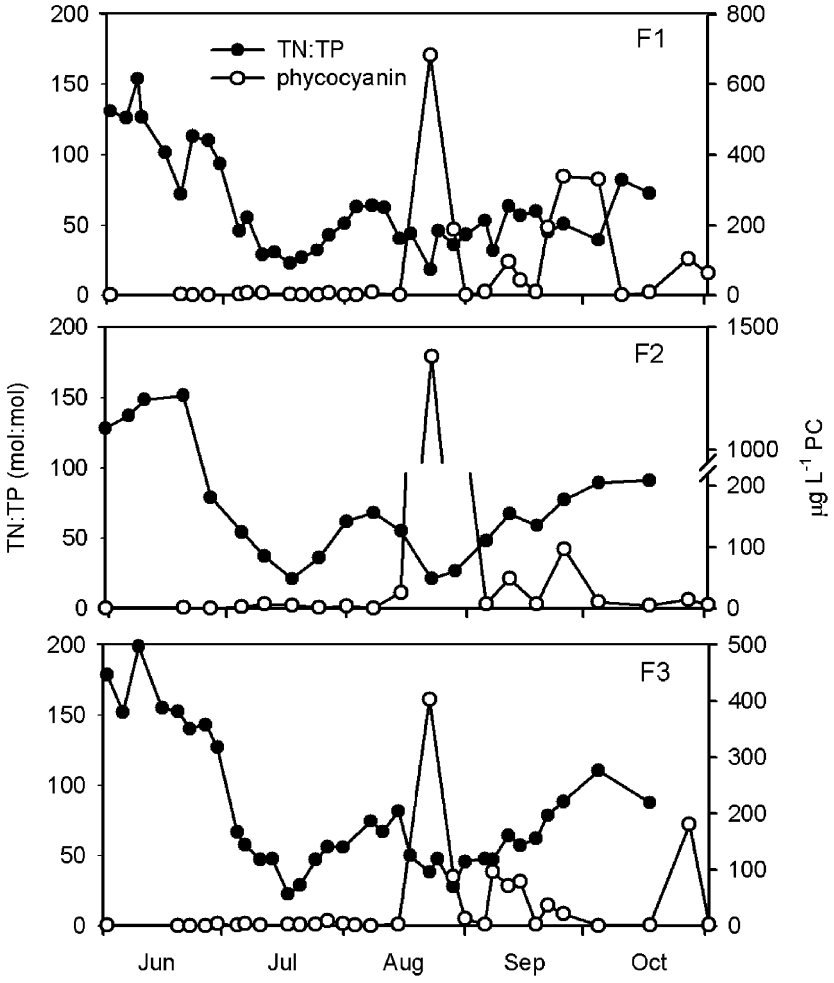

Fig. 5 – As Fig. 4, but for Ford Lake surface water, stations F1, F2, and F3.

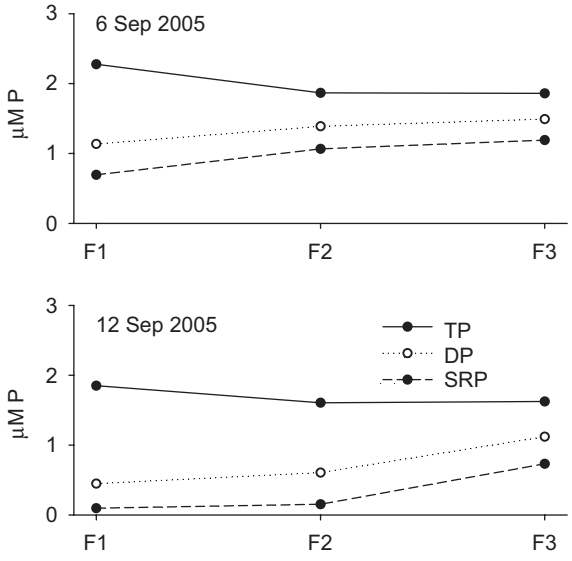

Fig. 6 – Surface concentrations (µM) of total P (TP), dissolved P (DP), and soluble reactive P (SRP) at three stations in Ford Lake.

outlier removed), or with the amount of standard toxin added ($R = 0.17$, $P = 0.66$). The anomalies were large enough to mask the presence of, on average, 0.40 (SD = 0.22) nM microcystin. Moreover, there was one instance when the assay produced a value lower (i.e., higher optical absorbance) than the blank. This occurred in Barton Pond on 1 September with an inferred concentration 0.37 nM less than blank.

3.6. *Assessment of water treatment facility*

Measured concentrations of microcystin are reported in Table 2. All empirical determinations exceed the MDL. Nominal 95% confidence estimates for all determinations are mean microcystin ± 0.04 nM. Overall the levels throughout the treatment process are low and consistent with one another.

4. Discussion

4.1. *Field observations*

This is the first report of toxic strains of *Microcystis* in any of the impoundments of the Huron River watershed. They seem

Table 2 – Microcystin concentrations (nM) of Ann Arbor Water Treatment Facility samples detected by ELISA

Sample collection date	Sample	nM microcystin
21 September 2005	Barton Pond surface water	0.69
22 September 2005	Barton Pond surface water	1.17
21 September 2005	Raw water intake	0.21
21 September 2005	Secondary settling basin	0.18
21 September 2005	Filter influent	0.10
21 September 2005	Filter effluent	0.11
21 September 2005	Final drinking water	0.14

likely to be a recurring feature, especially in Ford Lake where nuisance algal blooms have been a problem for many years.

Levels of microcystin in Barton Pond were relatively low compared to Ford Lake, and the measured toxin concentrations at different process stages within the Water Treatment Plant are well below 1 nM (1 $\mu g L^{-1}$), a level that the World Health Organization has deemed unsafe for long term consumption (WHO, 1998). Nevertheless, the toxin is present at concentrations within one order of magnitude of public health concern, and future developments in the watershed might easily promote elevated concentrations. This becomes evident by comparison with downstream Ford Lake, where concentrations exceeded 12 nM.

Microcystin levels were not consistent across Ford Lake. Although maxima were contemporaneous, it appears the toxicity was greatest in the western, shallow end of the lake, near the inlet, and became less toxic at each eastward station. This suggests there may be a combination of factors contributing to the degree of bloom development in Ford Lake. By September, Ford Lake had become isothermal (vertical profiles available at http://www.umich.edu/~hrstudy/) and mixing depth increased progressively from F1 to F3. Water transparency, as measured by Secchi disk visibility, was similar at all three sites: between 1 and 2 m. This combination of factors produced an increase in optical depth from F1 to F3, and therefore increased likelihood of light limitation under well mixed conditions.

Elevated concentrations of SRP at the deeper, western stations (Fig. 6) and increased biomass development represented as particulate P (difference between TP and DP) as well as higher levels of phycocyanin at the shallow eastern station (F1) are consistent with light limitation.

Microcystis blooms were associated with higher N:P ratios than those seen during the *Aphanizomenon* bloom in August. This is consistent with Downing et al. (2005) who found the growth rate and microcystin production of *Microcystis aeruginosa* were partly modulated by medium N:P ratios.

It appears that there is a lag time between peak phycocyanin concentrations and peak microcystin toxicity. High levels of microcystin typically followed a surge in phycocyanin by approximately 1–2 weeks. Other workers have also reported higher toxicities at the end of the growth phase and in the stationary phase (Watanabe et al., 1989; Orr and Jones, 1998), but this pattern has not been reported universally (Lee et al., 2000; Long et al., 2001). If senescing cells are more prolific producers of toxin, phycocyanin maxima can be used to predict ensuing episodes of toxicity, which would be helpful in monitoring a water supply.

Microcystin concentrations differ significantly between surface water and the water entrained in the drinking water intake pipes of the Ann Arbor Water Treatment Facility. There are also statistically significant decreases in microcystin concentrations along the sequential treatment process, which includes ozone treatment to destroy organic material. Interpretations must be tempered, however, by a potential compounding of temporal effects.

Microcystis is known to engage in a diel vertical migration linked to changes in colony buoyancy caused by carbohydrate content (van Rijn and Shilo, 1985; Visser et al., 1997; Ha et al., 2000). Colonies become laden with photosynthetic carbohydrate during the day and then they sink; over the night they respire carbohydrate and become positively buoyant, rising again to the surface at dawn. Without time series measurements of entrained microcystin concentration, inferences about the efficacy of treatment stages are not reliable. In other words, without a time series of measurements of the toxin concentrations entering the intake pipes, it is not reliable to deduce how much, if any, was removed during processing, which itself takes time.

4.2. *Experiment observations*

It is clear that freezing and thawing releases more microcystin for detection by ELISA compared to raw sample directly from the field. The freezing process ruptures cell walls and releases intracellular toxin into solution, where it can react with antibody. A freeze-thaw treatment is likely the most representative method for assessing microcystin health risks, because ingestion or water treatment processes are likely to cause cell lysis, as well.

4.3. *Potential limitations*

The competitive ELISA analysis appears susceptible to reporting false negatives with greater frequency than false positives. At no time were positive results obtained from samples without *Microcystis*, whether they were deionized water or lake samples, nor did internal standard addition ever predict values lower than those observed. This is not to say that the test is perfect on this account, but rather that false positive events must be so rare that we have not observed them thus far. There were a number of times, however, when expected concentrations based on internal standard additions were clearly higher than those observed. This means it is possible that this assay could test negative for microcystin when, in reality, microcystin is present in the sample. Since lake water contains numerous hydrolytic enzymes, it is possible that one or more of them is capable of adsorbing onto the tubes and surviving the wash step of the ELISA test. Such enzymes may hydrolyze the substrate in addition to the enzyme-linked antigen. This could result in stronger color development and, hence, an underestimate of the true value.

This is consistent with Sim and Mudge (1993) who found that high levels of phosphorylase phosphatase activity completely masked the presence of microcystin.

5. Conclusion

Increasing attention to algal toxins in surface waters and water supplies by use of sensitive and inexpensive analytical methods offers the chance to explore the dynamics of toxin production as distinct from population dynamics. Peak toxin expression always seemed to follow peak cyanobacterial abundance, and the two were never contemporaneous. ELISA is a well-established tool that can detect microcystin toxin at levels well below those of concern to public health. However, the method is subject to false negative reporting, and practitioners should recognize the possibility that levels may be underreported. Moreover, it appears that freeze-thaw treatment liberates substantial quantities of microcystin to the assay antibodies that would otherwise remain undetected. That said, the freeze-thaw treatment is a better analog of exposure through ingestion than by skin contact alone.

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