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Analysis of cyanobacterial metabolites in surface and raw drinking waters reveals more than microcystin

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ABSTRACT

Freshwater cyanobacterial blooms are becoming increasingly problematic in regions that rely on surface waters for drinking water production. Microcystins (MCs) are toxic peptides produced by multiple cyanobacterial genera with a global occurrence. Cyanobacteria also produce a variety of other toxic and/or otherwise bioactive peptides (TBPs) that have gained less attention including cyanopeptolins (Cpts), anabaenopeptins (Apts), and microginins (Mgn). In this study, we compared temporal and spatial trends of four MCs (MCLR, MCRR, MCYR, MCLA), three Cpts (Cpt1020, Cpt1041, Cpt1007), two Apts (AptF, AptB), and Mgn690 in raw drinking water and at six surface water locations above these drinking water intakes in a eutrophic lake. All four MC congeners and five of six TBPs were detected in lake and raw drinking water. Across all samples, MCLR was the most frequently detected metabolite (100% of samples) followed by MCRR (97%) > Cpt1007 (74%) > MCYR (69%) > AptF (67%) > MCLA (61%) > AptB (54%) > Mgn690 (29%) and Cpt1041 (15%). Mean concentrations of MCs, Apts, and Cpts into two drinking water intakes were 3.9 ± 4.7, 0.14 ± 0.21, and 0.38 ± 0.92, respectively. Mean concentrations in surface water were significantly higher ($p < 0.05$) than in drinking water intakes for MCs but not for Cpts and Apts. Temporal trends in MCs, Cpts, and Apts in the two raw drinking water intakes were significantly correlated ($p < 0.05$) with measures of cell abundance (chlorophyll-*a*, *Microcystis* cell density), UV absorbance, and turbidity in surface water. This study expands current information about cyanobacterial TBPs that occur in lakes and that enter drinking water treatment plants and underscores the need to determine the fate of less studied cyanobacterial metabolites during drinking water treatment that may exacerbate toxicity of more well-known cyanobacterial toxins.

1. Introduction

Increased occurrence of cyanobacterial blooms, commonly known as blue-green algae, has created growing concerns over global freshwater sustainability. Cyanobacterial blooms negatively affect fisheries, recreational angling, boating and swimming, property values, and animal and human health. Recent evidence indicates that an increasing number of lakes and rivers in the United States are becoming hypereutrophic and thus, capable of supporting dense and/or frequent cyanobacterial blooms (Dodds et al., 2009). Furthermore, climate and land use change predictions suggest increased intensity and occurrence of cyanobacterial blooms in freshwater resources worldwide (Balbus et al., 2013; Paerl and Huisman, 2009).

Bloom-forming cyanobacteria produce a number of linear and circular peptides with varying degrees of potential toxicity in animals and humans. Microcystins (MCs) are cyclic heptapeptides that

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inhibit eukaryotic serine/threonine protein phosphatases 1 and 2A (MacKintosh et al., 1990), primarily in the liver, but also in other tissues (Feurstein et al., 2009; Fischer et al., 2005). At oral doses above 40 μg kg^{-1} body weight, MCs induce cellular lysis of liver cells and tissue necrosis (Fawell et al., 1999). In addition, some evidence suggests that MCs can cross the blood-brain barrier acting as a neurotoxin affecting learning and memory (Feurstein et al., 2009; Fischer et al., 2005; Li et al., 2014). In repeat oral dose rodent studies, MCs display toxicity to male reproductive organs (Chen et al., 2011). Ultimately, the tissue types affected by MCs are dictated by the distribution of organic anion transporting polypeptides responsible for MC uptake into eukaryotic cells (Feurstein et al., 2009; Hagenbuch and Gui, 2008; Meier-Abt et al., 2007).

In addition to their acute effects, MCs are tumor promoters and possible carcinogens as defined by the International Agency for Research on Cancer (Agudo et al., 2010). In chronic exposure studies, MCs have produced tumors in mice and rats (Andrew R. Humpage 2000, Falconer and Humpage, 1996, Nishiwaki-Matsushima et al., 1992), and an increasing number of human epidemiological studies have suggested that chronic, low-dose exposure to MC may contribute to the burden of liver and colorectal cancers in some populations (Fleming et al., 2002; Li et al., 2011; Lun et al., 2002; Svircev et al., 2009; Zhou et al., 2002). Mechanistic studies of phosphatase inhibitors have shown that tumor production is due to a disruption in cellular signaling pathways leading to unbalanced cell growth (reviewed in (Gehringer, 2004)).

Cyanobacteria that form blooms in lakes also produce hundreds of other peptides with molecular targets in eukaryotic cells that may or may not be considered toxic (Sivonen et al., 2010; Welker and von Dohren, 2006). For purposes of this study, we call these “toxic or otherwise bioactive peptides” or TBPs. Approximately 90% of the currently known TBPs can be classified as anabaenopeptins, aeruginosins, cryptophycins, cyanopeptolins, cyclamides, microginins, and microviridins (Chlipala et al., 2011). Many of these TBPs inhibit one or more proteases in eukaryotes and have been explored as potential sources of new drugs. For example, aeruginosins have been shown to inhibit trypsin, thrombin, and plasmin and therefore, may be useful in treating myocardial infarction and stroke (Ersmark et al., 2008; Radau et al., 2003). The linear pentapeptide microginin-690 (Mgn690) has been shown to inhibit angiotensin-converting enzyme (Okino et al., 1993) and thus, may be useful in treating high blood pressure in humans (Bagchi et al., 2015). Anabaenopeptins have been found to inhibit carboxypeptidase A and like MCs also inhibit protein phosphatases but with tenfold lower potency (Namikoshi and Rinehart, 1996; Sano et al., 2001). The cyanopeptolins (Cpt) inhibit serine proteases such as human kallikrein and chymotrypsin, which may be useful in treating certain cancers (Borgono and Diamandis, 2004) or as antiviral agents (Singh et al., 2011).

While some of these TBPs may be promising sources of new drugs, their occurrence in surface waters is potentially problematic for ecological and human health. For example, one of the most prevalent bloom forming genera are *Microcystis* species, which possesses at least 15 genetic loci coding for non-ribosomal peptide synthetases and polyketides (Kaneko et al., 2007). As such, in addition to MCs, a variety of other TBP classes of potential importance to human health have been isolated from *Microcystis* species. For example, recently cyanopeptolin-1020 (Cpt1020) isolated from *M. aeruginosa* UV-006 was shown to affect chitin synthesis in freshwater fairy shrimp (Gademann et al., 2010) and displayed neurotoxicity in a zebra fish study at levels similar to other acutely acting cyanotoxins (e.g. MCs) (Faltermann et al., 2014). Similarly, two new cyclamides isolated from *Microcystis* species display toxicity against the freshwater crustacean *Thamnocephalus platyurus* with a potency approximately an order of magnitude lower than that of MCs (Portmann et al., 2008). While anabaenopeptins inhibit protein phosphatases at lower potencies, in recent studies, they have been shown to co-occur with MCs at equal or higher levels (Beversdorf et al., 2017). Thus, multiple TBPs may be produced by bloom-forming *Microcystis* species in addition to the well-described MCs, and some of these TBPs may be considered emerging cyanotoxins.

The temporal and spatial distribution of TBPs other than MCs in lakes has not been well-described. Many studies to date have used non-targeted mass spectrometry approaches (e.g. MALDI-TOF) to characterize peptide diversity in cultures, single samples of bloom material, or cyanobacterial colonies (Welker et al., 2004a; Welker and Erhard, 2007; Welker et al., 2002; Welker and von Dohren, 2006); (Fastner et al., 2001). These studies have revealed that cyanobacteria produce a great diversity of bioactive peptides. Among those studies providing some spatial or temporal data, in a study of 165 individual colonies from seven lakes on two dates in Germany, Welker et al. identified 46 different cyanobacterial peptides, the majority of which were MCs, Apts, Cpts, microginins, and aeruginosins including some novel peptides (Welker et al., 2004b). Gkelis et al. surveyed MCs and Apts in 36 lakes using HPLC on either multiple dates or one date and found that in addition to MC-RR and MC-LR, Apt-B and Apt-A were dominant in some lakes, but relative to MCs occurred more sporadically (Gkelis et al., 2015). In a previous study of six eutrophic lakes using tandem mass spectrometry we showed that the production of Apts and Cpts in lakes is highly variable over time and do not necessarily follow the same temporal trajectory and spatial distribution as MCs (Beversdorf et al., 2017).

In this study, we describe the concentration and co-occurrence of MCs and a suite of TBPs at drinking water intakes and at six surface water locations in eutrophic Lake Winnebago, Wisconsin, with two of the locations sampled at depths above the drinking water treatment plant (DWTP) intake pipes (Fig. 1). We simultaneously monitored these MCs and TBPs in raw intake water prior to any chemical addition from the two DWTPs along with seventeen water quality parameters that are typically already measured by DWTPs. Our goal was to 1) compare lake water and raw intake drinking water concentrations of MCs and TBPs; 2) determine the rate of co-occurrence between MCs and TBPs in lake and intake water, and 3) identify any water quality parameters indicative of the occurrence of MCs and TBPs in raw intake water using multiple regression analysis. Indicators of particularly MCs in raw intake water may be useful given recent health advisories for these particular cyanotoxins in finished drinking water (D'Anglada and Strong, 2016). Our data suggest that multiple TBPs co-occur with MCs in lake and intake water. In this location, some standard water quality parameters were indicative of MCs and TBP concentration in raw intake water.

2. Materials and methods

2.1. Study site and sample collection

Lake Winnebago is a eutrophic lake located in eastern Wisconsin. It is the largest freshwater lake within Wisconsin at 557.3 km^2 (6.4 m mean depth) and is primarily fed by the upper Fox and Wolf River Basins through Lakes Poygan, Winneconne and Butte des Morts. Lake Winnebago suffers from chronic, noxious cyanobacterial blooms due to nonpoint source pollution and also contributes approximately 20% of Lake Michigan's annual phosphorus loading via the Green Bay and lower Fox River (Klump et al., 1997; Robertson and Saad, 2011). In addition to being a major hub for recreational activities and fisheries, including one of the world's largest lake sturgeon population, Lake Winnebago serves as a

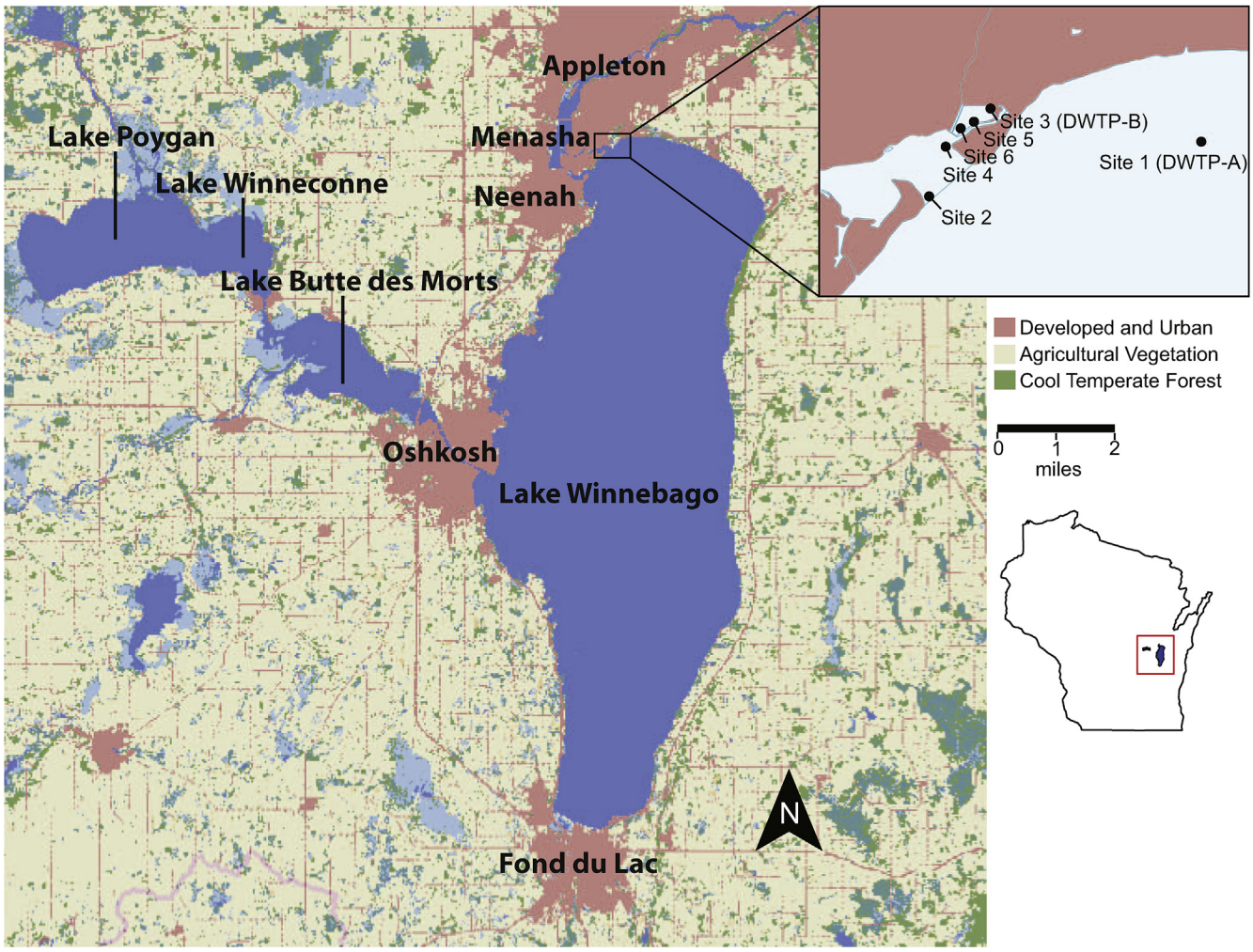

Fig. 1. Map of Lake Winnebago, WI, and the six sites surveyed in this study. Sites 1 and 3 are the locations of drinking water treatment plant (DWTP) intake pipes A and B, respectively.

drinking water source for over a quarter million people in the Oshkosh-Neenah-Menasha-Appleton metropolitan areas.

Weekly surface lake water samples were collected at 0.5 m depth from six Lake Winnebago sites near the cities of Neenah, Menasha, and Appleton between June 26th and September 25th, 2013 (Fig. 1). Two of the six surface lake water sites were sampled at depths above the intakes for two different DWTPs labeled DWTP-A and DWTP-B. The intake for DWTP-A is close to the surface while DWTP-B intake is approximately 2 m from the lake bottom. The mean intake rate in the summer for DWTP-A and DWTP-B is 75.7 and 30.3 million liters per day (Wisconsin Department of Natural Resources, 2003), respectively.

The drinking water treatment trains at DWTP-A and DWTP-B consist of podered activated carbon addition, lime softening, dual media filtration at DWTP-A, granulated activated carbon filtration at both plants, ultraviolet irradiation at DWTP-A or membrane filtration at DWTP-B followed by chlorine disinfection at both plants. Permanganate is added at the intake to raw drinking water at both plants to inhibit the growth of dreissenids in the crib and intake pipes.

Water samples were collected at the intakes of DWTP-A and DWTP-B, herein referred to as “raw water” on the same dates lake samples were collected. Samples at the intake were taken using the low lift pumps used to transfer water to the plant. Samples are collected using a sampling pump and piping, which extends out to the intakes prior to chemical addition. Samples from DWTP-A and -B were frozen in glass amber bottles and shipped on ice to the laboratory where they were immediately transferred to a −20 °C freezer for later analysis. Similarly, surface lake water samples were collected in amber bottles, placed in coolers on ice and then frozen at −20 °C once transported back to the laboratory.

2.2. Cyanotoxin and peptide standard materials

MCLR was purchased from the National Research Council of Canada Biotoxins program (Halifax, Nova Scotia) as a certified reference material. MCLA (purity > 95%), MCYR (>90%), and MCRR (>90%) were purchased from Sigma-Aldrich (Milwaukee, WI). Six TBPs were analyzed including anabaenopeptin (Apt) B (>95%) and AptF (>95%), cyanopeptolin (Cpt) 1007 (>95%), Cpt1020 (>95%), and Cpt1041 (>95%), and microginin (Mgn) 690 (>95%), all of which were purchased from MARBIONC (Wilmington, NC).

2.3. Extraction and analysis of cyanotoxins and TBPs

MCs and TBPs were quantified in samples of raw drinking water and all six surface water sites. All peptides were extracted from 10 mL water samples after lyophilization with 67% methanol as

previously described (Beversdorf et al., 2017). Thus concentrations reported represent the sum of both intracellular and extracellular toxin. Briefly, samples were partially thawed overnight at 4 °C and then to completion in a 50 °C water bath. Precisely 10 mL of sample was transferred to a 50 mL conical tube and frozen at −80 °C. Once completely frozen, the samples were lyophilized (Labconco FreeZone 6L) to dryness, and the dry mass then resuspended in 1 mL of 0.1% formic acid and subjected to three freeze/thaw cycles of −80 °C for 30 min and 50 °C for 5 min, respectively. After the final thaw, 2 mL of 100% methanol (MeOH) was added to each sample and sonicated for 10 min at 45 °C (SharperTEK Stamina XP Heated Ultrasonic Cleaner). The samples were vortexed and centrifuged for 15 min at 5000 × g. A 1 mL aliquot of sample was drawn from the top and stored at −20 °C in amber vials until analysis.

MCs and TBPs were analyzed by liquid chromatography tandem (MS/MS) mass spectrometry on an ABSciex 4000 QTrap mass spectrometer equipped with a TurboV electrospray ion source and a Shimadzu HPLC Model 20A. Peptides were separated on a reverse phase C18 column (Phenomenex Luna 3 μm, 100 Å, LC Column 150 × 3 mm) using mobile phases A) HPLC water with 0.1% formic acid and 5 mM ammonium acetate, and B) 95% acetonitrile with 0.1% formic acid and 5 mM ammonium acetate. Gradient elution was used for the C18 method consisting of 30% B for 3 min, increasing on a linear gradient to 95% B at 9 min, held at 95% B until 15 min, and returned to 30% B until 20 min.

Mass spectrometry conditions were optimized for each compound separately. A 1000 μg L^{-1} standard of each compound in 50% methanol/0.1% formic acid was syringe infused into the mass spectrometer at 10 μL min^{-1} and product ion spectra recorded (Figs. S1, and S2). The product ion spectra were compared to previously published spectra of each compound to confirm identity. Optimal compound specific parameters including collision energy, collision cell exit potential, entrance potential, and declustering potential (Table S3) were determined using Analyst 1.5 (Sciex, Concord, Ontario). Flow injection analysis was used to select optimal ion source gas flow rates, ionization energy, and temperature (Table S4). Two daughter ions were chosen for each compound based on maximum signal-to-noise ratios in chromatographic runs (Figs. S3 and S4). Total MC, total Apt, and total Cpt in a given sample is represented by the sum concentration of all congeners detected in those peptide classes.

2.4. Analytical and cell count measurements

Surface water quality parameters were measured in samples taken from the six lake surface water sites. Chlorophyll-a was measured spectrophotometrically after extraction of biomass on filters with 90% buffered acetone as previously described (Tett et al., 1975). Total phosphorous (TP) was measured as described by Valderrama (1981). All glassware and caps used were acid-washed with 10% HCl prior to analysis. Briefly, samples were thawed partially at room temperature and then to completion in a ~30 °C water bath. A 10% addition of "Valderrama reagent" was added to the sample and then autoclaved for 60 min (121 °C). After the samples reached room temperature, combined reagent was added and the color was allowed to develop for 30 min before standards and samples were read at 880 nm on a UV–Vis spectrophotometer (ThermoScientific, Genesys 10S).

Ammonium (Nessler/Hach method 8038), silica (Hach test kit SI-5), color (Hach method 8025), alkalinity, (Hach method 10239) and manganese (Hach method 8034) were measured using a Hach DR 2800 spectrophotometer. Turbidity was measured using a Hach 2100N benchtop turbidimeter. Conductivity was determined using a Hach HQ14d conductivity meter. Absorbance was measured at 254 nm (ThermoScientific, Genesys 10UV) in filtered samples and hardness was measured using the Hach test kit model HAC-DT.

Escherichia coli and coliform counts were determined using the standard Colilert-18® assay with Quanti-Tray®/2000 (IDEXX Laboratories). For cyanobacterial identification and cell counts, 10 mL of unfiltered water was pressure "crushed" in a syringe and then spun down in a centrifuge at 10,000 rpm for 10 min (Eppendorf 5804R). The supernatant was decanted, and the pellet was resuspended in 1 mL BG11 media. For cell counts, 980 μL was loaded into a Sedgewick-Rafter chamber and counted on a Nikon Eclipse E200 microscope, following Sukhanova (1978). The remaining 20 μL was reserved in case a higher magnification was needed to identify cyanobacteria, which were identified to genus focusing on the dominant groups: *Dolichospermum*, *Aphanizomenon*, and *Microcystis*.

2.5. Statistical analysis

All analyses were conducted in MATLAB. Site comparisons were analyzed using the Kruskal-Wallis test and analysis of variance (ANOVA), and comparisons explicitly between raw water and drinking water were compared using the Wilcoxon test. Pearson correlations were used to compare temporal variability of cyanotoxins to TBPs and to environmental variables. Prior to the analysis anon-detects were set to one-half of the estimated detection limit and all variables log-transformed.

3. Results

3.1. Spatiotemporal variation in lake surface water characteristics

All six surface water sites were located at the north end of Lake Winnebago (Fig. 1); some sites were pelagic with others near shore, and water depths ranged from 1.0 to 4.5 m. Despite these differences, little spatial variability was observed in the 18 environmental parameters and nine MCs/TBPs detected and quantified between the six sites sampled (Fig. 2, Tables S1 and S2). Only manganese and *E. coli* concentrations had significant differences between the six sites ($p < 0.05$; Kruskal Wallis; Table S1). Similarity between sites could likely be due to the lake being shallow and polymictic (i.e. well-mixed). Water temperature was above 20 °C at all sites for the duration of the study, with the exception of the last date sampled (September 25th) (Fig. 2). Over the same time period, chlorophyll-*a* increased to 135 μg L^{-1} on August 21st and then declined, which significantly correlated with trends in *Aphanizomenon* ($R^2 = 0.58$; $p < 0.01$), *Microcystis* ($R^2 = 0.23$; $p < 0.05$), and total cyanobacterial abundance ($R^2 = 0.72$; $p < 0.001$). Surprisingly, ammonium concentrations were relatively high, despite high biomass, and ranged from 0.22 to 1.25 mg L^{-1} suggesting phytoplankton growth was most likely P limited. Absorbance (254 nm), turbidity, and water color trended with cyanobacterial abundance, while other water quality indicators such as alkalinity, conductivity, hardness, and pH were highly variable throughout the study (Fig. 2). Similarly, no obvious trends existed for total coliforms measured or *E. coli* concentrations.

At surface water sites, at least one MC congener was detected on all dates with mean and max total MC concentrations of 10.2 and 107 μg L^{-1}, respectively, across all sites. On average, total MC was greater than the United States Environmental Protection Agency's (EPA) draft recreational water quality criteria guideline level of 4 μg L^{-1} (D'Anglada and Strong, 2016) on 57% of sampling dates. Based on these criteria, Lake Winnebago should have been closed for recreation for nearly half the summer. MCLR was the most abundant MC congener with mean and max levels ranging from 7.3 to 10.2 and 42–103 μg L^{-1}, respectively, depending on surface water location (Fig. 3, Table S2). MCLR was detected in 100% of all samples

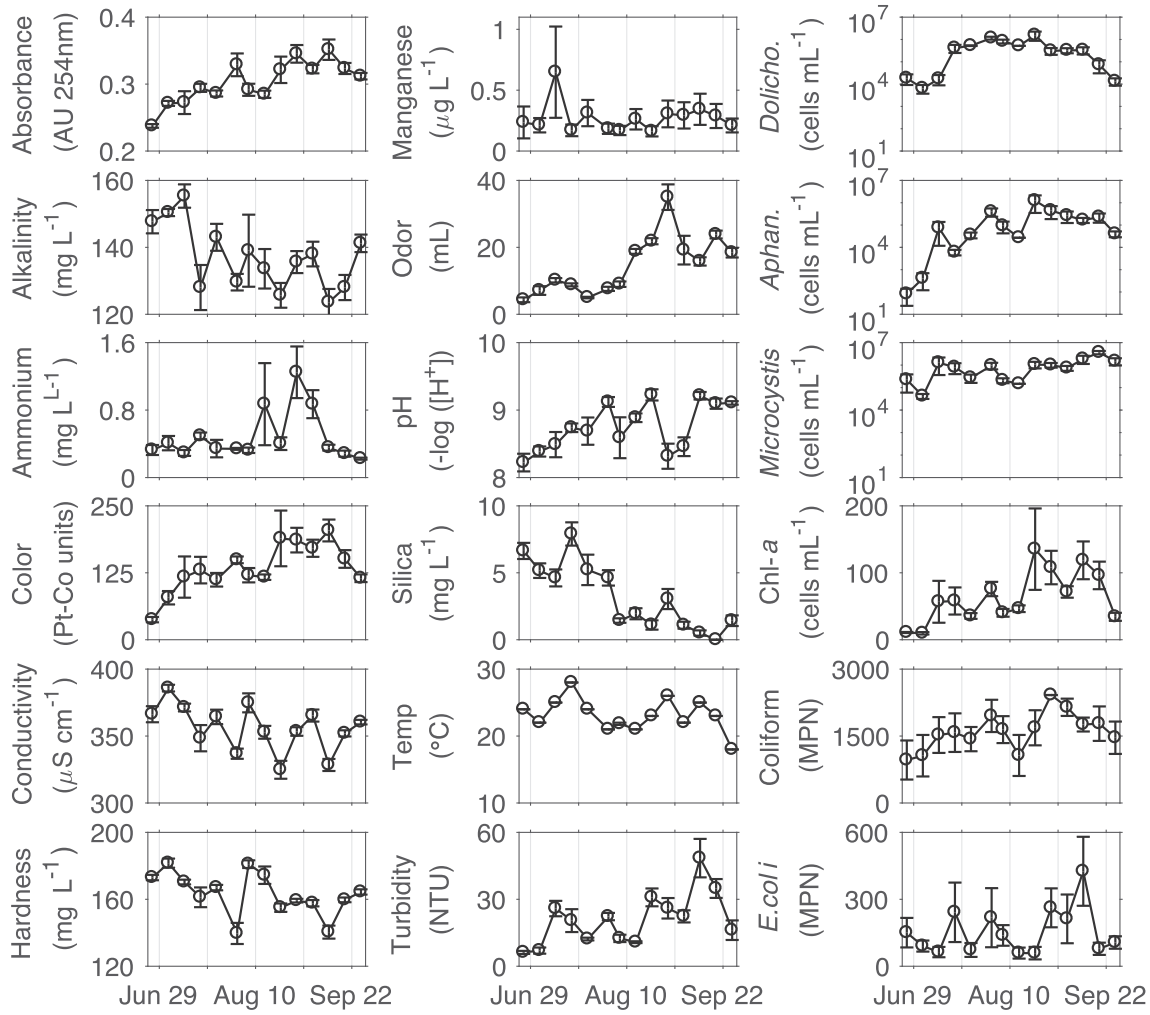

Fig. 2. Average water quality parameters measured in lake surface water across six sites in Lake Winnebago. Concentrations were not signicantly different across sites except for measures of manganese and *E. coli*. Error bars represent the standard error of the mean.

from all sites. MCRR was also detected in 100% of all samples, except in a few samples at one site, and average concentrations (0.73 μg L^{-1}) were approximately ten-fold lower than MCLR. MCYR and MCLA were detected less frequently and at average concentrations approximately 100-fold lower than MCLR.

All TBPs except Cpt1020 were detected in at least one surface water sample, in some cases at relatively high maximum concentrations compared to MC congeners. For example, while mean levels of Cpt1007 (0.84 μg L^{-1}) were low and nearly the same as MCRR, the maximum concentration of Cpt1007 detected (14.4 μg L^{-1}) was higher than all MC congeners except for MCLR. In addition, the maximum concentration of Cpt1041 at Site 1 was similar to that of MCRR, or over three times that of MCLA and MCYR. Average concentrations of Apts F and B were similar to MCLA and MCYR. However, on average across all surface water sites, AptF was the third most frequently detected cyanobacterial metabolite. Thus, Apts persisted at a low, but constant background level. Mgn960 was detected infrequently and never exceeded 0.5 μg L^{-1} with a mean of 0.04 μg L^{-1} across all sites.

No significant differences existed between the six sites with regard to abundance of MCs or TBPs using ANOVA. However, some obvious temporal differences did exist for total MC, Apt, and Cpt (Fig. S5). For example, on day 191 (July 10th), lake water at site 1 had approximately three times the concentration of observed MC, Apt, and Cpt than any of the other lake water sites. Conversely, on days 254 (September 11th) and 261 (September 18th), Site 4 lake water contained the maximum amount of MC, Apt, and Cpt concentrations observed of any site. Thus, while no statistically significant differences were observed between sites over the course of the entire sampling season, it was clear that some days at the beginning and end of the season experienced major, daily differences.

3.2. MCs and TBPs in raw DWTP water

Of the four MC variants measured, MCLR was the most prominent in the DWTP raw water, followed by MCRR, MCYR, and MCLA at both intakes (Fig. 4). In total, 74% of all raw DWTP samples had MC concentrations above 0.3 μg L^{-1} (D'Anglada and Strong, 2016). There were 11 sample dates (79% of samples) at DWTP-A where the total MC concentration in raw water was higher than 0.3 μg L^{-1}. Similarly, MC concentrations were greater than 0.3 μg L^{-1} on 9 dates (69% of samples) at DWTP-B. No regulations or preliminary guideline values currently exist for Mgn690, Apts, and Cpts; however, Cpt1007 concentrations did average above 1 μg L^{-1} at both DWTPs (Table S2, Fig. 4). Cpt1007 was the most abundant and commonly detected TBP in the DWTPs raw water occurring in 62% and 92% of samples collected from DWTP-A and B, respectively.

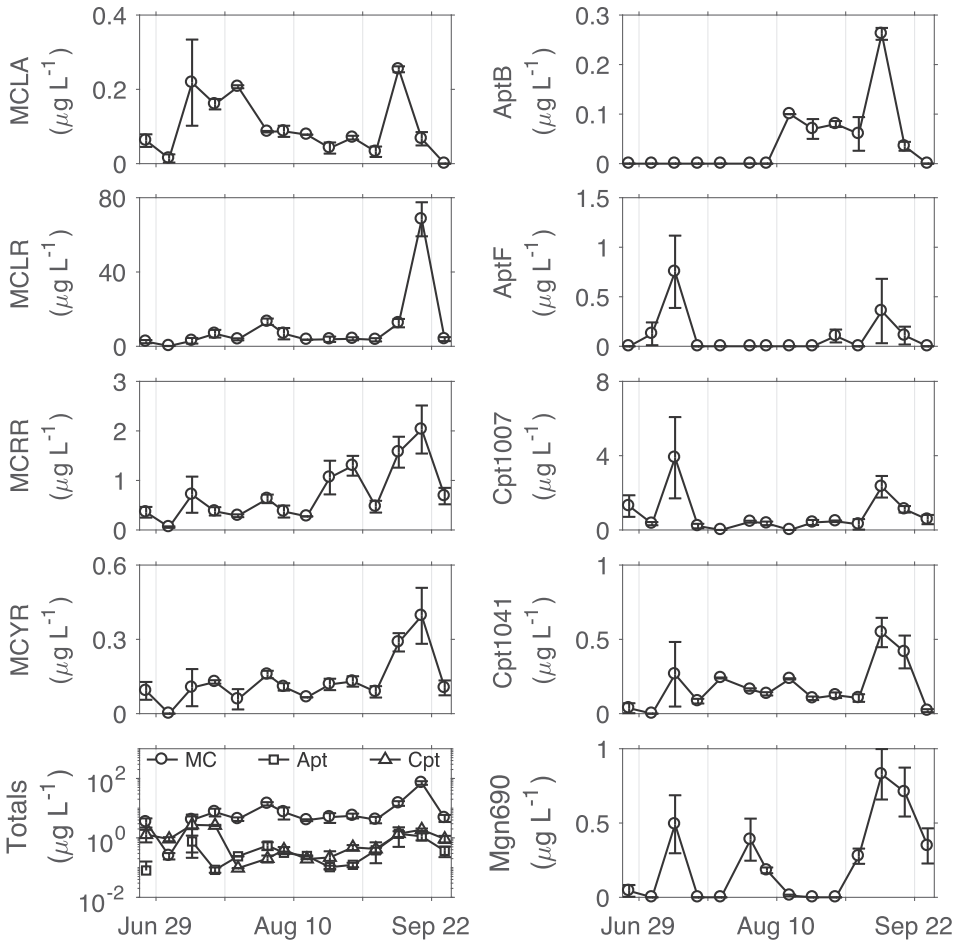

Fig. 3. Average concentration of microcystins (MC), anabaenopeptins (Apts), and cyanopeptolins (Cpts) measured at six surface water sites in Lake Winnebago. Mean concentrations were not signicantly different between sites. Error bars represent the standard error of the mean.

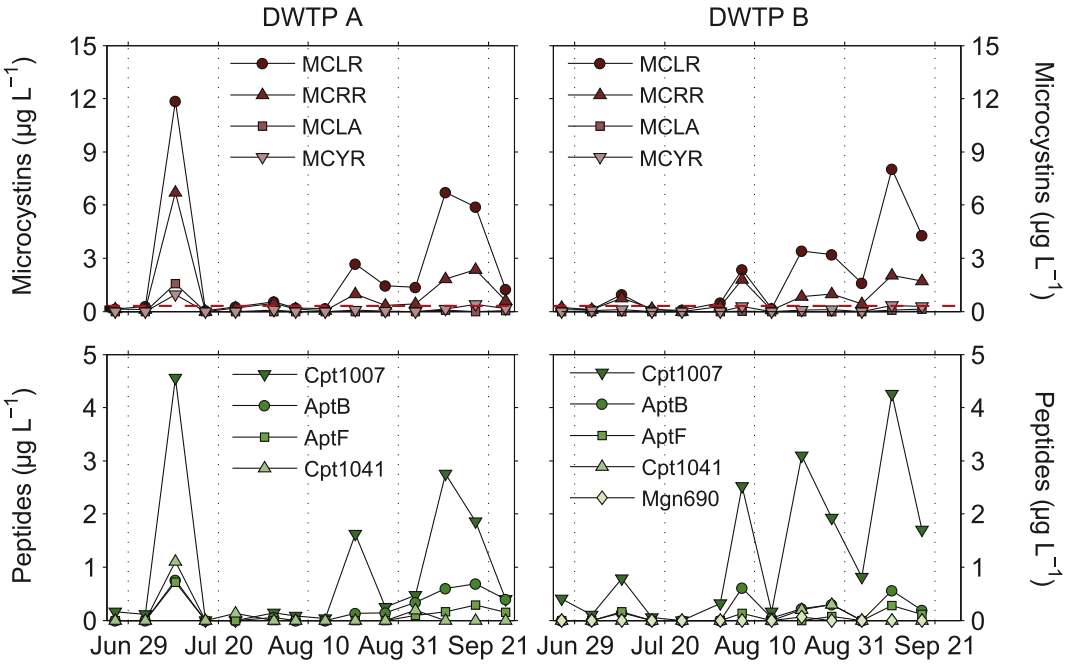

Fig. 4. Raw water cyanotoxin and peptide concentrations measured at drinking water treatment plants (DWTP) A and B. MC = microcystin; Cpt = cyanopeptolin; Apt = anabaenopeptin; Mgn = Microginin. The red dashed line indicates 0.3 μg L-1, the EPA limit for safe drinking water. Error bars represent the standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

AptB was the next most abundant TBP, which was observed in 62% and 54% of samples from DWTP-A and B, respectively. Mgn690 was only detected once in DWTP-B at 0.06 μg L^{-1}, but interestingly, it was not detected in the lake surface water above this intake on the same day.

We examined differences in MC and TBP concentrations between raw water and surface water Sites 1 and 3 above these intakes. On average, total MC was significantly higher in lake water compared to raw water at both DWTP-A ($p = 0.03$) and DWTP-B ($p = 0.04$) with average concentration differences of 7.89 (31.5%) and 6.29 (30.4%) μg L^{-1}, respectively (Fig. 5). On one day, MC in raw water at DWTP-A was 7.5 μg L^{-1} higher in raw water than lake surface water concentrations at Site 1 (July 10th), and MC in raw water at DWTP-B was 1.1 μg L^{-1} higher than surface water concentrations at Site 3 (August 7th). On average, total Apt was higher in lake water than raw water at DWTPs A and B by concentrations of 0.25 (52.7%) and 0.27 (45%) μg L^{-1}, respectively. The average total Cpt was higher in lake water than raw water at DWTP-A (0.83 μg L^{-1}, 50.3%), but not at DWTP-B (−0.38 μg L^{-1}, 142%). Neither of those relationships with Cpt was significant ($p > 0.05$) following a Wilcoxon test.

3.3. Environmental indicators of MCs and TBPs at two DWTP intake sites

We compared all surface water and meteorological parameters to total MC, Apt, and Cpt concentrations at the two DWTPs using a Pearson correlation coefficient and a significance cutoff of $p < 0.05$ (Table S5). Not surprisingly, the majority, but not all of the parameters that were correlates of MCs and TBPs are indicators of algal biomass. At both drinking water intakes, total MC was significantly correlated with the largest number of parameters compared to total Cpt and Apt (Fig. 6). *Aphanizomenon*, *Microcystis*, chlorophyll-*a*, UV absorbance, water color, silica, odor, turbidity, and manganese all significantly correlated with total MC at DWTP-A. Except in a few cases, variables significantly correlating with total MCs at DWTP-B overlapped with variables that significantly correlated with total MC at DWTP-A. Total Apt had similar, but less

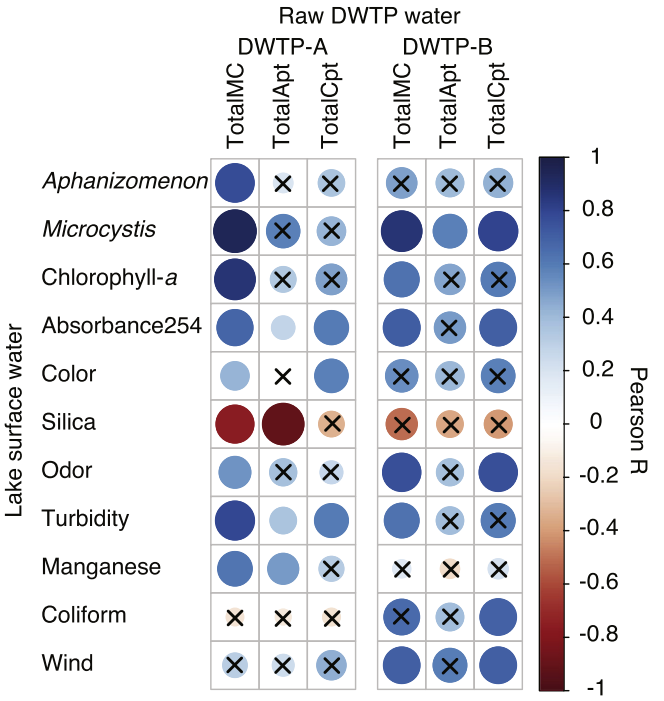

Fig. 6. Results of pairwise Pearson correlations between total microcystins (MC), anabaenopeptins (Apt) cyanopeptolins (Cpt) in DWTP intake water of two plants versus environmental water quality and meterological conditions in Lake Winnebago surface water above the intakes. X = not signi_cant p > 0.05.

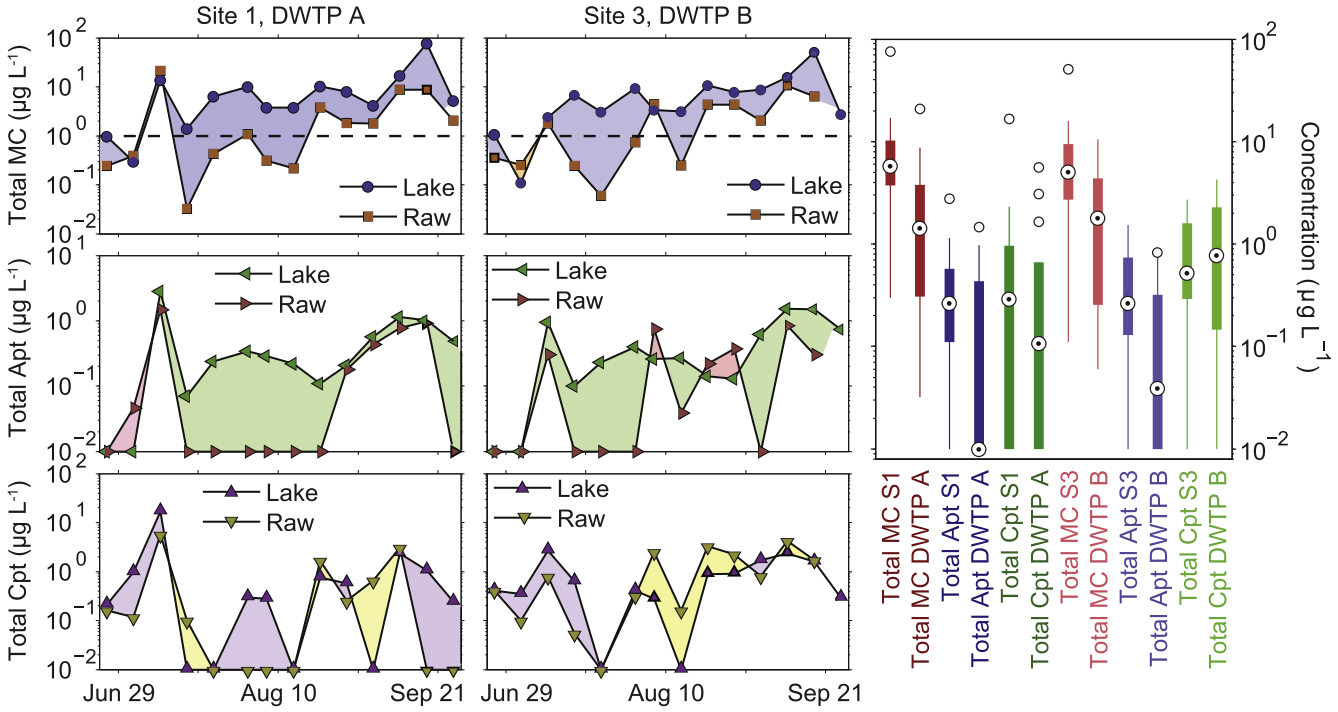

Fig. 5. Left panels) Difference in total microcystin (MC), anabaenopeptin (Apt), and cyanopeptolin (Cpt) between lake water and drinking water treatment plant (DWTP) raw water. Right panel) Boxplot comparison between total MC, Apt, and Cpt concentrations in lake surface water versus DWTP water. DWTP A raw water comes from Site 1, and DWTP B raw water comes from Site 3. The black dot indicates the median value, whiskers indicated 25th and 75th percentiles, and circles indicate outliers.

correlating variables than total MC, and at DWTP-B, only *Microcystis* cell counts were significantly correlated to total Apt. Total Cpt was significantly correlated to *Microcystis*, absorbance, odor, coliforms, and wind at DWTP-B, whereas at DWTP-A total Cpt was significantly correlated with only absorbance, color, and turbidity.

4. Discussion

To our knowledge, this is the first study to investigate individual MC congeners plus multiple TBPs in DWTP raw water and surface water. Some TBPs display toxicity in animal models or as in the case of Apts also inhibit protein phosphatases like MCs, albeit at lower toxicity. Much attention has been paid to the occurrence of MCs in lakes and drinking water for good reason, but by comparison, TBPs have been largely ignored with respect to drinking water treatment. Results presented here show that cyanobacterial blooms produce a mixture of peptide classes that enter DWTPs. The combined toxicity of this mixture is unknown. Furthermore, we have likely only measured a small percentage of the diversity of this peptide mixture. More research is needed to delineate the range of TBPs produced by cyanobacterial blooms, their combined toxicity thresholds, and removal efficiency by DWTP processes.

One of the goals of this study was to survey several sites along northern Lake Winnebago for future drinking water intake locations. Currently, DWTP-A has an intake in the pelagic area of Lake Winnebago where the max depth is around 4 m, and the intake sits about 2 m above the lake bottom. DWTP-B has an intake pipe at the surface of a baffled embayment of the lower Fox River Basin. To our surprise, very little spatial variability was observed at these sites despite having different lake depths and physical characteristics.

More concerning may be that each surface water site had MC concentrations greater than the EPA draft water quality advisory for recreational usage for half the season. All MC and TBP concentrations were highly temporally variable, both in the lake surface water locations and especially in the raw drinking water. For example, a sample taken at DWTP-A on day 184 (July 3rd) was $<1\ \mu g\ L^{-1}$ for total MC measured in raw water, but was $>20\ \mu g\ L^{-1}$ only a week later (Fig. 5). Total MC dropped to near detection days after. Thus, in order to determine public health risk and respond appropriately, daily measurements would be needed, putting a huge economic and energetic cost on the DWTP. For example, the extraction and quantification of even a single cyanotoxin can be extremely costly (e.g. > $200 a sample using LC-MS/MS) and take several hours to analyze appropriately. For this reason, several studies have identified environmental parameters that are indicative of toxin concentrations (reviewed in (Neilan et al., 2013)).

The concentration of MCs reported here are comparable or higher than those reported in other similar lakes in this region. In this study, the average concentration of MC across all surface water sites and in raw drinking water was $11 \pm 19\ \mu g\ L^{-1}$ and $3.2 \pm 4.7\ \mu g\ L^{-1}$. Studies of Lake Erie, Green Bay, and various sites in Ohio show that the concentration of MCs varies from 1 to $3\ \mu g\ L^{-1}$ on average in surface water, with large outliers of $>1000\ \mu g\ L^{-1}$ common under heavy bloom scenarios and $0.1–6\ \mu g\ L^{-1}$ in raw drinking water (as reviewed by (Miller et al., 2017)). It is not clear how concentrations of the other TBPs reported here compare to other lakes. Most studies of cyanobacterial peptide diversity have used semi-quantitative approaches with exact mass instruments, or have quantified peptides in biomass alone whereas in this study we quantified the total peptide concentration in both the biomass and dissolved phase.

In this study, we describe temporal variability in MCs and TBPs in surface lake water at DWTP intake sites and simultaneously in DWTP raw water on the same day. It should be noted that these measurements include total or bulk water concentrations representing the sum total of both dissolved and particulate TBP. Few studies to our knowledge have conducted this type of analysis targeting individual congeners of MCs plus multiple other TBPs. The concentration of MCs in raw water was almost always less than that of lake surface water on the same day (Fig. 5). This might be expected since cyanobacteria typically present positive buoyancy until late autumn when the rate of carbon ballast metabolism slows resulting in cells sinking to the benthos. This could be one factor driving higher total MC in surface water compared to raw drinking water at the same location. Physical forces (e.g. wind/waves) pushing cells toward the surface could be another factor. In addition, a higher rate of MC catabolism by heterotrophic bacteria might also occur in the benthos. However, complicating these explanations is the fact that the intake at DWTP-B was close to the lake surface and yet total MC was consistently lower in the raw water. Thus, other factors at the intake may alter the loading of toxin into the DWTP. Both DWTP intakes were sampled prior to permanganate addition, but mechanical screens may alter the type and/or amount of cyanobacterial biomass and perhaps the amount of MC entering the intake pipe. In contrast, Cpt and Apt concentrations were not significantly lower in raw water compared to lake surface water on the same date (Fig. 5), which might be related to differences in species responsible for producing these compounds. While *Microcystis* is the most obvious producer identified in this study, *Dolichospermum* has also been shown to produce Cpt and Apt (Repka et al., 2004). In addition, while it is generally understood that the majority of MCs remain inside the cell, less is known about whether Apt and Cpt are actively released from the cells producing them, or remain in the intact cell similar to MCs.

To our knowledge, no studies have investigated the removal of TBPs from raw drinking water. This study shows that the amount of TBP entering drinking water plants is comparable to MCs (Table 1). For example, based on average intake flow rates for the summer, mean loadings per day for total MC are $2.75–3.62\ g\ day^{-1}$, whereas those for total Cpt and Apt are only about two-fold less and 6–10 fold less, respectively. Thus, a significant amount of TBPs are entering these DWTPs.

In a previous study of these plants, MC was removed to below $0.3\ \mu g\ L^{-1}$ in finished drinking water (Karner et al., 2001). However, at that time mean concentrations in raw water was less than $1\ \mu g\ L^{-1}$, whereas mean concentrations reported here are approximately $3\ \mu g\ L^{-1}$. Still, it is likely that treatment strategies in both plants are able to remove MCs. Both DWTP-A and DWTP-B use

Table 1
Average loading per day of total MC, Apt, and Cpt at each DWTP.

Average Characteristic	DWTP-A	DWTP-B
Intake Rate (million liters day^{-1})	75.7	30.3
Mean Total MC ($\mu g\ L^{-1}$)	3.62	2.75
Mean Total Cpt ($\mu g\ L^{-1}$)	1.46	1.28
Mean Total Apt ($\mu g\ L^{-1}$)	0.65	0.22
Mean Total MC load ($g\ day^{-1}$)	274	83
Mean Total Cpt load ($g\ day^{-1}$)	110	38
Mean Total Apt load ($g\ day^{-1}$)	49	6.5

powdered activated carbon (PAC), which has been shown to adsorb microcystins in an exponential manner over time (He et al., 2016). For example, Ho et al. show that 22 μg/L MC was removed within 30 min when at least 100 mg L^{-1} of PAC was used. Both plants also use GAC, which has been shown to remove MC by biodegradative and absorption processes at loading concentrations higher than reported here (Wang et al., 2007). Finally, ozone and membrane filtration, which are implemented at plants A and B, respectively, have also been shown to remove residual MCs as tertiary treatment processes (Chang et al., 2014). The degree to which these processes are effective in practice may vary with characteristics of the raw water including organic carbon load, and pH, which has been reviewed extensively elsewhere (He et al., 2016; Hitzfeld et al., 2000; Hoeger et al., 2005). It is not clear if Cpts, and Apts would be removed by these same processes. Given that they are also circular peptides like MC, it may be that they have similar fate and transport pathways both in the environment as well as during drinking water treatment. On the other hand, some cyanopeptolins like Cpt-1041 are chlorinated, which may make them more recalcitrant to oxidative processes (e.g. chlorination, ozonation). Future research is needed in this regard.

A great deal of research has been conducted on cyanobacterial metabolites such as MC, anatoxin-a (ATX), saxitoxin, and cylindrospermopsin (CYL), but recently, much has been learned about the numerous "bioactive" compounds that accompany the more well known "toxins" (Briand et al., 2015; Elkobi-Peer and Carmeli, 2015; Gademann et al., 2010; Gkelis et al., 2015; Pereira and Giani, 2014; Sadler et al., 2014). There are no case studies of human toxicity caused by these compounds, but all three are protease inhibitors of eukaryotic enzymes that may alter human physiology (Silva-Stenico et al., 2012). In addition, like MCs, some Apts also inhibit protein phosphatases (Sano et al., 2001). Likewise, it has been shown that MC toxicity is not always accounted for by MC concentrations alone (Chorus, 2001), suggesting these additional bioactive compounds could act synergistically with MC to affect human health. To what extent Apt, Cpt, and/or Mgn concentrations would have to reach to affect human health is not known, nor do any regulations or advisories exist for these compounds in recreational or drinking waters. This study highlights the need for more information about DWTP processes that may be effective at removing the diversity of bioactive secondary cyanobacteria metabolites from drinking water. MCs and other TBPs quantified were not always correlated with each other suggesting that MCs are not necessarily indicators for the presence of other potentially harmful TBPs. It should be noted that while the TBPs targeted in this study have been found to be readily detectable in natural populations (Gkelis et al. 2006, 2015; Welker et al., 2004a), these represent only a small portion of the number of TBPs that cyanobacteria are capable of producing (reviewed in (Chlipala et al., 2011)).

In this study, we asked whether any surface water characteristics correlated to total MC, Cpt, and/or Apt concentrations in raw drinking water (Fig. 6). Many of the parameters that were significantly correlated with TBPs may be considered indicators of biomass (cell density, chlorophyll-*a*, absorbance, turbidity) and/or dissolved organic carbon. Other studies have shown correlations between measures of cyanobacterial biomass and cyanotoxins (Francy et al., 2015; Rinta-Kanto et al., 2009; Wicks and Thiel, 1990). It has even been suggested that monitoring algal pigments at DWTP intakes in Lake Erie may serve as early warning signals for the presence of toxins.

Using biomass as a direct indicator of toxin concentration is complicated by a number of interacting factors. The concentration of MC and TBPs in aquatic systems is under the control of at least four factors including the rate of synthesis (e.g. transcription, translation, enzymatic rates), the rate of destruction or half-life of each compound, the proportion of cells that contain MC or TBP biosynthetic genes, as well as cyanobacterial abundance, among others (e.g. predation). Accordingly, our previous studies as well as those of others in other lakes have shown little to no significant correlations between cell density, chlorophyll, or even toxin gene abundance and actual MC concentration, likely due to these interacting factors (e.g. transcriptional regulation and percent toxicogenic cells) (Beversdorf et al., 2015; Ye et al., 2009). More likely, the correlating variables found here are indicators of a bloom, and while they were indicators of MC and TBPs in this particular study, it remains to be determined whether these could serve as reliable indicators from season to season.

5. Conclusions

- In this study (June through September 2013), all but one sample date was conducted when the temperature was >20 °C, *Microcystis* and *Dolichospermum* were the most abundant cyanobacteria measured, every date had an MC detect, and 74% of raw drinking water samples had MC concentrations above the new EPA health advisory of 0.3 μg L^{-1} (D'Anglada and Strong, 2016), while 57% of surface waters were over the EPA draft recreational water quality criteria of 4 μg L^{-1}.
- All TBPs were detected at least once, except Cpt1020, and loadings into drinking water treatment intakes of Cpts and Apts were comparable to that of MCs.
- These results suggest that removal efficiency of TBPs by drinking water treatment processes should be investigated.
- Surface water quality characteristics that significantly correlated with MCs and TBPs in raw water were measures of cyanobacterial biomass (*Microcystis* cell density and chlorophyll-*a*) and/or dissolved organic carbon (UV absorbance and turbidity).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.04.032.

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