Spatial analysis of toxic or otherwise bioactive cyanobacterial peptides in Green Bay, Lake Michigan

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Abstract

Cyanobacterial harmful algal blooms (cyanoHABs) are a growing problem in freshwater systems worldwide. CyanoHABs are well documented in Green Bay, Lake Michigan but little is known about cyanoHAB toxicity. This study characterized the diversity and spatial distribution of toxic or otherwise bioactive cyanobacterial peptides (TBPs) in Green Bay. Samples were collected in 2014 and 2015 during three cruises at sites spanning the mouth of the Fox River north to Chambers Island. Nineteen TBPs were analyzed including 11 microcystin (MC) variants, nodularin, three anabaenopeptins, three cyanopeptolins and microginin-690. Of the 19 TBPs, 12 were detected in at least one sample, and 94% of samples had detectable TBPs. The most prevalent TBPs were MCRR and MCLR, present in 94% and 65% of samples. The mean concentration of all TBPs was highest in the Fox River and lower bay, however, the maximum concentration of all TBPs occurred in the same sample north of the lower bay. MCs were positively correlated with chlorophyll and negatively correlated with distance to the Fox River in all cruises along a well-established south-to-north trophic gradient in Green Bay. The mean concentration of MC in the lower bay across all cruises was 3.0 +/- 2.3 µg/L. Cyanopeptolins and anabaenopeptins did not trend with the south-north trophic gradient or varied by cruise suggesting their occurrence is driven by different environmental factors. Results from this study provides evidence that trends in TBP concentration differ by congener type over a trophic gradient.

Keywords: cyanobacteria, microcystins, anabaenopeptins, cyanopeptolins, cyanoHABs, Green Bay

INTRODUCTION

Cyanobacterial harmful algal blooms, or cyanoHABs, are a growing problem in freshwater systems worldwide including the Laurentian Great Lakes due to excessive nutrient pollution (Boyer, 2008; Heisler et al., 2008; O'Neil et al., 2012a). Although cyanoHABs are naturally occurring, excess proliferation can have significant impacts on ecological health, as well as on the socioeconomics and human health of surrounding regions. Every year, toxins produced by cyanoHABs (cyanotoxins) are responsible for animal deaths, including pets and livestock (Backer et al., 2013) and in some cases have caused human illness and fatalities (Falconer, 1994; Pouria et al., 1998; Stewart et al., 2006). Furthermore, decaying cyanoHAB biomass creates hypoxic/anoxic conditions harmful to fish and other aquatic life (Lindholm et al., 1989; Vanderploeg et al., 2009).

Toxin-producing cyanoHABs have been described in some of the Great Lakes, although most studies have focused on the lower Lakes. Toxin-producing blooms are documented in Lake Erie (Rinta-Kanto et al., 2005; Steffen et al., 2014), Huron (Fahnenstiel et al., 2008; Vanderploeg et al., 2001) and Ontario (Hotto et al., 2007; Murphy et al., 2003), where *Microcystis* and *Planktothrix* have been shown to be the major genera producing microcystins (MCs) (Davis et al., 2015; Dyble et al., 2008). Lake Erie is often used as a model ecosystem for Great Lakes cyanoHAB events, but it is currently unknown if trends found in Lake Erie extend to other cyanoHAB impacted areas, such as Green Bay. Surprisingly, there is a lack of information on cyanotoxins in Green Bay, a highly productive region in the Laurentian Great Lakes (Klump et al., 2009)

One of the most commonly observed or measured cyanotoxins in the Great Lakes region is microcystin (MC), a peptide where more than 200 different variants have been detected

(Meriluoto et al., 2017). A potent liver toxin (Hooser et al., 1989; Konst et al., 1965), MC acts by inhibiting protein phosphatases 1 and 2A (Honkanen et al., 1990; MacKintosh et al., 1990). The general structure of MC is a cyclic heptapeptide containing the unique Adda (3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) side chain, plus four additional non-protein amino acids and two variable amino acids (Botes et al., 1982). Variations in the MC structure are numerous, due to substitutions and modifications of its amino acid residues although MC variants with leucine and arginine (MCLR) or arginine and arginine (MCRR) are often the dominant congeners. Nodularin is a peptide with similar structure to MCs primarily occurring in brackish waters, but is increasingly detected in freshwaters. It contains five amino acids, and has the same mode of toxicity as MCs (Chorus et al., 2000; Rinehart et al., 1988; Sivonen, 1999).

Cyanobacteria produce hundreds of other toxic or otherwise bioactive peptides (TBPs). These TBPs inhibit various proteases and may be beneficial for commercial or medicinal uses, such as antifungals, antimicrobials or antivirals (Patterson et al., 1994; Welker and Von Döhren, 2006). Microginins (Mgn), for example, are inhibitors of proteases including an angiotension converting enzyme and may be useful in treating high blood pressure (Okino et al., 1993). Anabaenopeptins (Apt) are also inhibitors of phosphatase 1 and 2A like microcystin (Sano et al., 2001) as well as inhibitors of carboxypeptidases (Halland et al., 2015; Murakami et al., 2000). At least 96 variants of Apts have been reported and as such, the pharmacological effects of these peptides is an emerging area of study (Bjorquist et al., 2004; Spoof et al., 2015). Cyanopeptolins (Cpt) are cyclic serine protease inhibitors and may have pharmaceutical value as they may be applied in treatment of asthma or viral infections (Singh et al., 2011). Alternatively, a Cpt variant, Cpt1020, has been shown in recent studies to be toxic to the crustacean *Thamnocephalus*

platyurus and a neurotoxin in zebrafish (Faltermann et al., 2014; Gademann et al., 2010). Ecologically, TBPs other than MCs including Apts, and Cpts have been implicated in a variety of phenomena including inhibiting parasitic infections from chytrid fungi (Rohrlack et al., 2013), preventing digestion of cyanobacteria by inhibiting zooplankton digestive enzymes (Baumann and Jüttner, 2008; Schwarzenberger et al., 2010), and allelopathic competition (Sedmak et al., 2008). Thus TBP diversity likely has implications for the ecology of cyanobacteria and their predators as well as for human health.

Despite decades of research, the causes, consequences and complexities of cyanoHABs remain too poorly understood to fully inform remediation, management and policy. As such, more information is needed about the occurrence of cyanotoxins, and collectively, TBPs. In this study, we focused on a suite of TBPs including eleven microcystins – MCLR, MCRR, MCYR, MCLA, desmethyl MCLR (dmMCLR), MCLF, MCLY, MCLW, MCWR, MCHtyR, MCHilR, three anabaenopeptins – AptB, AptF, and AptA, three cyanopeptolins – Cpt1007, Cpt1041, Cpt1020, one microginin analog – Mgn690 and nodularin. The spatial variability of these cyanotoxins was assessed in Green Bay, a large, shallow and eutrophic embayment in Lake Michigan. The bay experiences persistent nutrient pollution from point and nonpoint sources, including storm water and urban runoff, wastewater effluent and agriculture runoff, which can fuel cyanoHABs. There is a great need for information about cyanoHABs, their toxins, and other bioactive metabolites in this area that may pose recreational risk to swimmers, particularly children (D'Anglada, 2015; Weirich and Miller, 2014). While there are no recreational beach monitoring programs in lower Green Bay, the EPA does have provisional guidelines in place for recreation with regards to total microcystin concentrations (D'Anglada, 2016). Given the city of Green Bay plans to revitalize Bay Beach in lower Green Bay which may include reopening a

swimmable beach (Rodewald, 2015) in addition to the expansive size of Green Bay and its role as a popular recreational hub, assessing the spatial variability of cyanotoxins is crucial. This is the first study of its kind to assess the spatial diversity of cyanotoxins in Green Bay, Lake Michigan.

METHODS

Study site

Lower Green Bay (an area of 55 km^2 of southern Green Bay) is listed as an Area of Concern (AOC) by the International Joint Commission and the State of Wisconsin (University of Wisconsin Sea Grant Institute, 2013). Unlike western Lake Erie and Saginaw Bay, very little is known about cyanobacterial bloom toxicity in this system. Previous studies have shown that the Lake Winnebago – lower Fox River – to – Green Bay corridor contributes approximately 1/3 of all phosphorus in Lake Michigan (Klump et al., 1997; Klump et al., 2009) while the Fox River contributes approximately 70% of the nutrient and sediment loading although most of this is entrained in the lower portion of the bay (Ahrnsbrak and Ragotzkie, 1970; Dolan and Chapra, 2012), giving Green Bay estuarine-like qualities as the transition zone from the Fox River to Lake Michigan. As such, the sampling sites in this study are spatially segregated along a series of east-west transects from north to south, divided into five geographic zones defined by water quality and trophic status (Qualls et al., 2007) (Fig. 1).

Sample collection

Green Bay was sampled from the RV *Neeskay* during three cruises – August 2014, and July and August 2015. The sites were based on a 5x5 km grid that has been used in previous Green Bay studies (Klump et al., 2009; LaBuhn and Klump, 2016; Lin et al., 2016). Samples were collected from the water column at 0 meter (m) and 1 m depths in 2014 and at 1 m depth

during both 2015 cruises. Samples collected at 0 m during the August 2014 cruise will be specifically referred to as such, whereas all other cruises with samples collected from 1 m will be referenced by their month and year (e.g. August 2014). Samples were collected via a submersible pump (flow rate ≈ 40 liters per minute) into 25 mL sterile plastic Vulcan® vials. Immediately following collection, 5 mL of sample water was pipetted out for shipboard fluorometer measurements using a Turner® handheld fluorometer. The remaining sample was sealed and placed in a freezer within 10 minutes of collection for TBP extraction and analysis.

Additional sites including the Fox River, East River (a tributary to the Fox River), and zones 1-3, were sampled from the Bay Guardian with NEW Water, the Green Bay Metropolitan Sewerage District, during the July 2015 cruise. These samples were taken from 1 m depth via a submersible pump into Nalgene bottles. Samples were kept on ice until processing immediately upon return to the lab. Samples were subsampled for TBP and chlorophyll analysis. For chlorophyll, water was filtered through 0.7 µm, 47 mm diameter Whatman GF/F filters (GE Healthcare, Pittsburgh, PA, USA). Filters were transferred to 15 mL tubes, amended with 90% acetone, sonicated and refrigerated overnight before spectrophotometric analysis (American Public Health Association, 1995; Wisconsin State Lab of Hygiene, 1991). Whole water was frozen at −20 °C until TBP extraction and analysis.

Extraction and analysis of TBPs

Frozen whole water samples (10 mL) were lyophilized and the dried mass was resuspended in 1 mL of 0.1% formic acid and subjected to three freeze-thaw cycles at -80 °C and 55 °C, respectively. After adding 2 mL of 100% methanol, samples were placed in a sonicating water bath at 45 °C for 10 minutes and then centrifuged at 10,000 x g for 15 minutes. One mL

portions of the supernatant were transferred to liquid chromatography (LC) vials and stored at -20 °C until analysis.

TBPs were measured via 20 µL injections using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization on an ABSciex 4000 QTRAP equipped with a Shimadzu Prominence HPLC. Cyanotoxins were separated using gradient elution on a reverse phase C18 column (Luna 3 µm C18 100 Å, LC Column 150 x 3 mm, Phenomenex, Torrance, CA, USA) where the mobile phase consisted of buffer A (0.1% formic acid and 5mM ammonium acetate in HPLC grade water) and buffer B (0.1% formic acid and 5mM ammonium acetate in 95% acetonitrile). The gradient began at 30% buffer B for 3 minutes, increasing over a linear gradient to 95% buffer B at 9 minutes, and held at 95% buffer B until 15 minutes at which point buffer B was returned to the starting condition until 20 minutes.

TBPs eluted from the column were detected on the mass spectrometer using a scheduled multiple reaction monitoring method. Compound specific parameters including ionization and collision energies were optimized for each compound by syringe infusion of reference standards at 1000 µg/L in 50% acetonitrile with 0.1% formic acid. Single charged ion species [M+H] were targeted for all MCs except MCRR, which preferentially takes on a double charge [M+2H]. Compound non-specific parameters including gas flows and ionization temperatures were optimized using flow injection analysis of standards in 70% methanol. Further details of the LC-MS/MS method are provided in Electronic Supplementary Material (ESM) Table S1 and have also been described previously (Beversdorf et al., 2017).

TBP standard materials

Whenever possible, certified reference standards were used. Nodularin, MCLR and dmMCLR were certified reference materials from the National Research Council of Canada

Biotoxins program (Halifax, Nova Scotia). Microcystin standards – MCLA (> 95%), MCRR (> 90%), and MCYR (> 90%) were purchased from Sigma-Aldrich (Milwaukee, WI) and MCLF (> 95%), MCLY (> 95%), MCWR (> 95%), MCLW (>95%), MCHtyR (> 95%), (> 95%), and MCHilR (> 95%) were purchased from Enzo Life Sciences (Farmington, NY, USA). AptA (> 95%), B (> 95%) and F (> 95%), Cpt1007 (> 95%), 1020 (> 95%), and 1041 (> 95%), and Mgn690 (> 95%) were purchased from MARBIONC (Wilmington, NC, USA).

Statistical Analysis

All statistics were performed using R statistical software (R Core Team, 2017). Pearson Moment correlations were used to compare the concentration of TBPs and chlorophyll to a spatial gradient (distance to the Fox River). Distance of sampling sites to the Fox River was calculated using the distCosine function in the R stats package 'geosphere' (Hijmans, 2017). Correlation matrices were visualized using the R stats package 'corrplot' (Hijmans, 2017). Correlations were considered significant at $P < 0.05$. Mann-Whitney U tests were used to test for significant differences in mean concentrations of TBPs, and an Analysis of Variance (ANOVA) was used to test for significant differences between the mean concentration of MCs by sampling zone.

RESULTS

Summary of TBPs Detected

Of the 19 TBPs targeted in this study, 12 were detected in at least one sample from Green Bay or the Fox River, including seven MCs, all three Apts, and two of three Cpts. The most prevalent TBPs were MCRR and MCLR, present in 94% and 65% of samples, respectively (Fig. 2). The average MCRR concentration (0.53 µg/L) was slightly higher than that of MCLR (0.47

µg/L), but the concentrations were not significantly different ($P > 0.05$) (Table 1). AptB was the most abundant of the three Apts followed closely in abundance by AptF, present in 30% and 27% of samples, respectively. The mean concentrations of these two Apts were similar at approximately 0.1 µg/L. The third Apt targeted, AptA, was detected in 12% of samples. Cpt1007 was the dominant Cpt, present in 24% of samples with an average concentration of 0.06 µg/L. The other Cpts targeted in this study were either detected infrequently (Cpt1041) or not detected (Cpt1020). The mean concentration for each Cpt was less than 0.1 µg/L. The maximum concentration for all TBPs was measured in the sample from site 17 on August 27, 2014. Site 17 is approximately 34 km northeast of the mouth of the Fox River and the location of the UW-Milwaukee Green Bay water quality data buoy (Great Lakes Observing System; station 45014).

TBP Dynamics by TBP Type and Cruise

Microcystins

Among all cruises, the 0 m samples in August 2014 had the greatest number of sites where the sum of all MCs detected (SumMCs) was higher than 4 µg/L (4.98 µg/L ± 5.90 standard deviation (S.D.)), the provisional EPA recreational guideline value. Within this set of samples at 0 m, the four sites with SumMCs above 4 µg/L were in zones 1, 2, and 3 following a northeasterly line from the Fox River to site 17 in mid-bay (Fig. 3). The greatest diversity of MC congeners was also observed in the 0 m samples from 2014 where 7 of the 11 MC congeners were detected. Interestingly, there were differences in the spatial distribution of individual MC congeners. dmMCLR was detected from zones 1, 2, and 3. MCWR, and MCHilR were also detected in zones 1, 2, and 3 only, whereas MCRR, MCLR, and MCYR were detected in all 5 zones. MCLA was detected twice, but only in zones further north, zones 3 and 5.

During the 2014 cruise, samples were taken at 1 m depth. Among these samples SumMCs in 2014 showed the greatest variability in concentration compared to all other samples and/or cruises (Fig. 4). Two samples, both from zone 2, had SumMCs greater than 4 µg/L. The overall mean concentration of SumMCs across all 1 m samples in 2014 was 1.38 µg/L ± 1.29 S.D. and ranged from 0.12 - 5.27 µg/L spanning zones 2 - 5., MCLR, MCYR, and MCWR were detected in all the zones, whereas MCLA was detected only in northern zones 4 and 5, and MCHilR was only detected in zone 2. Interestingly, dmMCLR was not detected in samples from 1 m, but was detected at 0 m. The max SumMCs was measured from zone 2 (5.27 µg/L) and the mean SumMCs were significantly different between zones ($P = 0.002$; ANOVA).

The July 2015 cruise included samples from all 5 zones and the Fox and East River (therein referred to as the river) (Fig. 5). SumMCs ranged from below detection limits to 4.70 µg/L. The overall mean concentration of SumMCs during the July 2015 cruise was 0.86 µg/L ± 1.16 S.D. across all sampling stations (Table 2). MCRR, MCLR, and MCYR were detected in all five zones and the river, whereas MCWR and MCHilR were not detected north of zone 1. As in 2014, MCLA was detected in only northern zones. The max SumMCs was measured from the river samples (4.70 µg/L), following a gradient of high SumMCs closest to the river with decreasing max concentrations further from the river. The mean SumMC between zones were significantly different ($P < 0.001$; ANOVA).

Samples from the August 2015 cruise had the lowest mean and max SumMCs (0.32 and 1.40 µg/L, respectively) (Table 2) of all cruises, which spanned zones 2 - 5 (Fig. 6). Similar to all other cruises, MCRR and MCLR were the dominant MC congeners with similar mean and max toxin concentrations (0.15 µg/L mean and 0.64 µg/L max for MCLR vs. 0.15 µg/L and 0.68 µg/L for MCRR). Unlike previous cruises MCLA was detected twice in zone 2 in addition to northern

zone 4. MCWR was detected once from zone 3. The max SumMC was measured from zone 3 (1.4 µg/L) and the mean SumMCs among zones were significantly different during the August 2015 cruise ($P = 0.002$; ANOVA).

Anabaenopeptins

Similar to SumMCs, the max sum of all Apts detected (SumApts) occurred in August 2014 from 0 m. This max (6.78 µg/L) was from a zone 4 sample, specifically at site 17 (Fig. 3) and was comprised of the three Apt congeners targeted in this study – AptB, F, and A. Among all 0 m samples, AptB was most dominant, detected in 58% of samples with a mean concentration of 0.42 µg/L followed by AptF (50% detection and 0.28 µg/L), and AptA (33% detection and 0.10 µg/L).

Of the three Apt congeners targeted, AptF was most abundant during the August 2014 cruise. AptF was detected in 69% of samples from all the zones sampled, zones 2 - 5, with a mean concentration of 0.19 µg/L (Fig. 4). AptB was also detected in zones 2 - 5 with a mean of 0.11 µg/L; whereas AptA was detected in zones 3 - 5 with a mean of only 0.02 µg/L. The mean SumApts was 0.32 µg/L ± 0.25 S.D. (Table 2).

During the July 2015 cruise, Apts were detected in 33% of samples, specifically in zones 2, 3, 4, and the river (Fig. 5). Specifically, AptB was the dominant congener detected in zones 2, 3, 4 and the river, AptF was detected in zone 3 and the river, and AptA was not detected. The mean SumApts was 0.06 µg/L ± 0.10 S.D. (Table 2)

Interestingly, no Apt congener was detected in samples from the August 2015 cruise (Fig. 6) even though they (SumApts) were detected frequently in the August 2014 (71% of samples) and July 2015 (17%) cruises.

Cyanopeptolins

Among all cruises, the 0 m samples in August 2014 had the greatest mean sum of Cpts (SumCpts) detected, equal to 0.30 µg/L ± 0.59 S.D. (Table 2). Max SumCpts was 0.53 µg/L and was measured from zone 4 (site 17) (Fig. 3). Cpt1007 was the dominant congener and detected in zones 1, 2, 4, and the river, while Cpt1041 was detected twice, in zones 2 and 4.

Cpt1007 was also the most abundant Cpt in samples collected during the August 2014 cruise, present in 35% of sites spanning zones 2 - 5 (Fig. 4). Cpt1041 was detected in one site from zone 4. The mean SumCpts was 0.06 µg/L ± 0.12 S.D. (Table 2).

During the July 2015 cruise, Cpt1007 was the only Cpt congener detected, present in 15% of sites spanning zones 1, 3 and the river (Fig. 5). The mean SumCpts was 0.04 µg/L ± 0.12 S.D. (Table 2). Cpt1007 was also the only congener detected in samples collected during the August 2015 cruise, present in 15% of sites spanning zones 2, 3, and 4 (Fig. 6). The mean SumCpts was 0.02 µg/L ± 0.06 S.D. (Table 2).

TBP Trends with Trophic Gradients

Previous research has established that Green Bay is characterized by a trophic gradient from a eutrophic or hypereutrophic environment in the Fox River and zone 1 (i.e. the AOC) transitioning to a mesotrophic environment in zone 2 and all zones north (Qualls, 2013; Rousar and Beeton, 1973). Our chlorophyll results confirmed a chlorophyll gradient was present on all three cruises (ESM Fig. S1). The July 2015 cruise included sites throughout all zones as well as the river in order to determine whether TBPs follow a similar gradient using chlorophyll as a trophic state indicator (Fig. 7). As expected, chlorophyll decreased significantly (R = -0.59, P = 0.0042) with increased distance from the mouth of the Fox River (lat = 44.53778 lon = -88.03889), as did MCs (R = -0.60, P = 0.00026) (Fig. 8). The August 2014 cruise (1 m samples) and August 2015 cruise (1 m samples) did not include samples from zone 1 or the river.

However, significant correlations were still observed between chlorophyll (R = -0.59, P = .002) and MC (R = -0.91, P = <0.0001) and distance to the Fox River in 2014 as well as in 2015 (R = -0.70, P = .0002 for MC; R = -.80, P = <0.0001 for chlorophyll). These correlations suggest that trends in MC concentration along the trophic gradient persist into zones beyond the AOC.

In August 2014, Cpt and chlorophyll were not significantly correlated (R = 0.28, P = 0.16) nor were Cpts significantly correlated with respect to distance from the Fox River (R = -0.22, P = 0.27) (Fig. 8). However in 2015 Cpt was correlated with distance to the Fox River in samples from the July 2015 and August 2015 cruises, (R = 0.84, P = <0.0001 and R = 0.76, P = <0.0001, respectively), and strongly correlated with chlorophyll (R = -0.37, P = 0.04 and R = -0.62, P = 0.002, respectively). Apts did not decrease significantly with distance to the Fox River on any cruise and was not correlated with chlorophyll (P > 0.05). Thus only MCs showed a consistent trend with trophic gradients in Green Bay on these cruises whereas other TBPs did not trend with the trophic gradient or showed a variable response. This suggests that the production of MCs and other TBPs are not driven by the same ecological conditions.

DISCUSSION

CyanoHABs have long been observed in Green Bay (Beeton, 1969; Vanderhoef et al., 1974; Wiley et al., 1957; Wisconsin State Committee on Water Pollution, 1939) fueled by excessive nutrient runoff from the Fox-Wolf watershed. While much is known about the biogeochemistry and phytoplankton ecology in Green Bay, this is the first spatial analysis of cyanoHAB toxins and other metabolites (i.e. TBPs) of human health concern in Green Bay, from the mouth of the Fox River to south of Chambers Island. To date, very few cyanotoxin studies have taken place in Green Bay, despite this being the largest freshwater estuary in the world and

highly eutrophic. The influx of nutrients combined with shallow waters in the lower bay creates an ideal environment for the proliferation of cyanobacteria and formation of cyanoHABs. This study describes congener- specific changes in cyanotoxin profiles over a trophic gradient.

One limitation of this study is the lack of data on cyanobacterial community composition. In Green Bay, early reports from 1939 described blooms of *Aphanizomenom* beginning in early June followed by *Microcystis* dominance in mid-July with *Anabaeana* (now *Dolichospermum*) present but in low abundance (Wisconsin State Committee on Water Pollution, 1939). More recent work confirms all three genera are still the dominant cyanobacteria taxa seasonally in Green Bay in moderate to high abundance (De Stasio et al., 2014). All three genera are known to produce a variety of TBPs (O'Neil et al., 2012b). Of those TBPs targeted in this study, MCs, Cpts, Apts, and microginins have been detected in both *Microcystis* and *Dolichospermum* taxa as well as genes for their biosynthesis (Botes et al., 1982; Humbert et al., 2013; Namikoshi and Rinehart, 1996; Rinehart et al., 1988) while *Aphanizomenomen* taxa have been shown to produce Apts (Murakami et al., 2000; Welker and Von Döhren, 2006). Whether these genera are responsible for production of the TBPs targeted in this study in Green Bay is unknown. Answering that question is complicated by the fact that multiple genera have been shown to produce individual TBP congeners, the genes for TBP synthesis can be mutated and/or lost, potentially gained through horizontal gene transfer, and transcriptional/translational regulation may increase or decrease TBP synthesis according to cyanobacterial physiological status. Thus TBP producers cannot be identified through a microscopic examination. An analysis of TBP RNA transcript abundance may provide one avenue for the identification of TBP producers, but was beyond the scope of this study.

Data from this study informs the development of beneficial use impairments in Green Bay. Green Bay is an important recreational resource, supporting many sport fisheries and is a popular destination for summer water activities. EPA's draft recreational water quality criteria state water should not exceed 4 µg/L MCs for safe recreation. In 2014, 16% of samples exceeded 4 µg/L and in 2015, 2% exceeded the guideline. Most of the exceedances were located in the AOC.

Use of Lower Green Bay as a drinking water resource is considered impaired under the AOC guidelines. According to the Lower Green Bay and Fox River Area of Concern remedial action plans from 1988 to 2017, beneficial use of Green Bay for recreation and drinking water is impaired due to cyanobacteria and recent action plans cite an absence of sufficient data on concentration and type of toxins present. Thus the results of this study directly addresses this need.

Currently, 1 µg/L of MCLR equivalents (MCs) is used as the standard for listing lower Green Bay as impaired for use as a drinking water resource under the AOC listing (WI Department of Natural Resources, 2009), which is the same guideline established by the World Health Organization (WHO) (World Health Organization, 2004) for drinking water. However, historically there has been a lack of data describing MC concentrations in Green Bay including the lower Green Bay AOC making this beneficial use impairment questionable. This study provides some baseline data to inform the AOC guidelines. We report here that of all the samples, 50% exceeded 1 µg/L MC in 2014 and 14% of samples exceeded the threshold in 2015, for samples from all sites in the study, not just those in the AOC. Thus impairment of the Lower Green Bay AOC for drinking water production is warranted. While one municipality (Marinette) uses Green Bay as a drinking water source, it is located far north of the AOC. However, it is

important to consider that cyanoHAB toxicity is highly variable from site to site and from year to year (Beversdorf et al., 2017; Hotto et al., 2008; Sinang et al., 2013). Indeed, the highest TBP concentrations were measured in a sample well north of the AOC.

MC concentrations reported here in Green Bay are comparable to other eutrophic water bodies. SumMCs in Green Bay ranged from <1 – 20 µg/L, with an average of 1.27 ± 2.52 µg/L, which is similar to Bay of Quinte, Lake Ontario (2.40 ± 0.5 µg/L) (Watson, 2009) and Sodus Bay, Lake Ontario (<1 – 20 µg/L) (Perri et al., 2015). MCs in Lake Erie vary from extreme concentrations of 3,144 µg/L and 570 µg/L measured from surface or shallow water scum samples, to an average of 1-3 µg/L in open water (as reviewed in (Miller et al., 2017)). A robust study in the early 2000's describes MC concentrations in New York lakes (including Lakes Erie, Ontario and Champlain) ranging from not-detected to > 20 µg/L (Boyer, 2008). Thus MC concentrations in Green Bay are similar to other eutrophic environments in the Great Lakes region that have been impacted by cyanoHABs.

Currently, recreational and drinking water guideline values do not exist for Cpts and Apts in the United States. These bioactive peptides are considered "nontoxic" and little is known about the pharmacological effects of these peptides either from exposure to individual TBPs or as a mixture of Apts, Cpts, and MCs, which is common in nature and in this study. Some of these TBPs exhibit similar modes of toxicity as MCs, but yet do not exhibit similar toxicity, one example being AptF (Sano et al., 2001). From an ecological standpoint, AptF and AptB are interesting because they have been shown to lyse certain cyanobacterial species (Sedmak et al., 2008), and as protease inhibitors it has been suggested that they may function to inhibit digestive enzymes in crustaceans, making cyanobacteria that produce them a poor food source. Indeed, Cpts have been found to be highly toxic to freshwater crustaceans, and they have also been

classified as a potential neurotoxin in zebra fish. Cpts were detected in approximately 24% of samples from 2014 and 2015. The co-occurrence of Apts, Cpts, and MCs was common at these Green Bay sites, although it is interesting that the different TBP classes either correlated (MC) or did not (Cpt and Apt) with the trophic gradient. Future work to elicit TBP-specific drivers is needed.

One objective of this study was to observe relationships between a trophic state indicator (i.e. chlorophyll) in Green Bay and TBP concentrations. In addition to being a trophic state indicator, chlorophyll data were also used for the context of cyanobacterial bloom presence. MC showed the strongest correlations with chlorophyll and both were significantly negatively correlated with distance from the Fox River. In the three separate cruises, MCs followed the strong south-north trophic gradient previously described in Green Bay (De Stasio and Richman, 1998).

A concurrent study to the August 2014 cruise using the same samples at 1 m examined phosphorus species from the same spatial gradient (Lin et al., 2016). By August, all forms of phosphorus (P) measured (dissolved inorganic P, dissolved organic P, particulate inorganic P, particulate organic P) were in highest abundance in the lower bay, localized to the eastern shore. Similarly, in this study TBPs were also most abundant in the lower bay and along the Eastern shore. Indeed our analysis of TBPs at 1 m showed the highest concentrations of toxins were measured in the lower bay, specifically in zone 2 (samples were not collected south of zone 2 at 1 m in 2014) with two samples exceeding 4 µg/L. Thus P species, like chlorophyll and MC, follow a south-north gradient. Both P and MC showed extensive entrainment in the lower bay with pockets of accumulation along the eastern coast of the bay.

In conclusion, this study provides a necessary baseline on spatial distribution of TBPs in Green Bay. We identified the most abundant TBPs and congener- specific changes in TBP diversity along a trophic gradient. Future studies should examine the most abundant TBPs identified here alongside a compendium of limnological variables (e.g. taxonomic community composition) in order to identify a suite of possible environmental drivers of TBP production.

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Table 1. Statistics for TBPs detected of 19 targeted in 2014 and 2015 from 3 cruises with samples collected at 0 and 1 m (2014) and 1 m only (2015). TBPs not detected include MCLF, MCLY, MCHtyR, MCLW, Cpt1020, Mgn690, and NOD. SumMC = the sum of all microcystin congeners; SumApt = the sum of all anabaenopeptin congeners; SumCpt = the sum of all cyanopeptolin congeners; MC = Microcystin; Apt = Anabaenopeptin; Cpt = Cyanopeptolin; Mgn = Microginin; NOD = Nodularin.

TBP	**Mean (µg/L)**	**Max (µg/L)**	**Frequency of Detection**	**Coefficient of Variation**
SumMC	1.28	19.97	94%	1.97
SumApt	0.20	6.78	38%	3.45
SumCpt	0.07	1.92	24%	3.20
MCLR	0.47	7.76	65%	1.91
MCRR	0.66	7.74	53%	1.97
MCYR	0.19	2.40	18%	2.03
MCLA	0.04	0.65	2%	4.17
dmMCLR	0.01	0.42	1%	6.62
MCWR	0.07	0.63	4%	2.63
MCHilR	0.03	0.37	2%	2.80
AptA	0.08	1.12	2%	6.44
AptB	0.09	3.54	10%	3.74
AptF	0.10	2.12	9%	2.70
Cpt1007	0.06	1.07	6%	2.63
Cpt1041	0.02	0.85	2%	5.93

Table 2. Max and mean concentrations of all TBPs measured from samples taken on 0 m from August 2014 and 1 m from August 2014, July 2015, and August 2015 cruises. SumMC = the sum of all microcystin congeners; SumApt = the sum of all anabaenopeptin congeners; SumCpt = the sum of all cyanopeptolin congeners.

Transect	**Analyte**	**Mean (µg/L)**	**Max (µg/L)**
August 2014	SumMCs	4.98	20.0
0 meter	SumApts	0.92	6.78
n = 12	SumCpts	0.36	1.92
	Chl-*a*	NA	NA
August 2014	SumMCs	1.38	5.28
1 meter	SumApts	0.32	0.73
n = 26	SumCpts	0.06	0.53
	Chl-*a*	15.3	32.5
July 2015	SumMCs	0.86	4.70
1 meter	SumApts	0.06	0.31
n = 33	SumCpts	0.04	0.58
	Chl-*a*	15.8	84.3
August 2015	SumMCs	0.32	1.40
1 meter	SumApts	0.00	0.00
n = 26	SumCpts	0.02	0.17
	Chl-*a*	15.0	43.6

Fig. Captions

Fig. 1. Sampling sites in Green Bay, Lake Michigan. Colors indicate sampling zones.

Fig. 2. Concentration of toxic or otherwise bioactive peptides (cyanotoxins) detected on all cruises. The central line represents the median. The top and bottom of the box represents the 25th and 75th quartiles, respectively. The whiskers extend to data points that are not considered outliers, and solid circle symbols are outliers. ND = not detected.

Fig. 3. Spatial distribution of toxic or otherwise bioactive peptides during the August 2014 cruise at 0 meters.

Fig. 4. Spatial distribution of toxic or otherwise bioactive peptides during the August 2014 cruise at a depth of 1 meter.

Fig. 5. Spatial distribution of toxic or otherwise bioactive peptides during the July 2014 cruise at a depth of 1 meter. Samples denoted with an 'N' were collected by NEW Water, the Green Bay Metropolitan Sewerage District. Samples from river sites include the Fox and East rivers, collectively referred to as the 'river' in the text.

Fig. 6. Spatial distribution of toxic or otherwise bioactive peptides during the August 2014 cruise at a depth of 1 meter.

Fig. 7. Concentration of the sum of all congeners of MC, Apts, and Cpts for samples taken at a depth of 1 m; A. August 2014, B. July 2015, and C. August 2015. The central line represents the median. The top and bottom of the box represents the 25th and 75th quartiles, respectively. The whiskers extend to data points that are not considered outliers, and solid circle symbols are outliers. MC = Sum Microcystins, AP = Sum Anabaenopeptins, CP = Sum Cyanopeptolins

Fig. 8. Results from pair-wise correlations among the variables: SumMCs, SumApts, Sum Cpts, chlorophyll (Chl-*a*), and Distance to the Fox River for samples taken at a depth of 1 m. An 'X' indicates

the two variables are not significantly correlated ($P<0.05$). Positive correlations are represented in blue and negative correlations in red; the correlation coefficient is represented by the size and color of the pie.

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Figure 1:

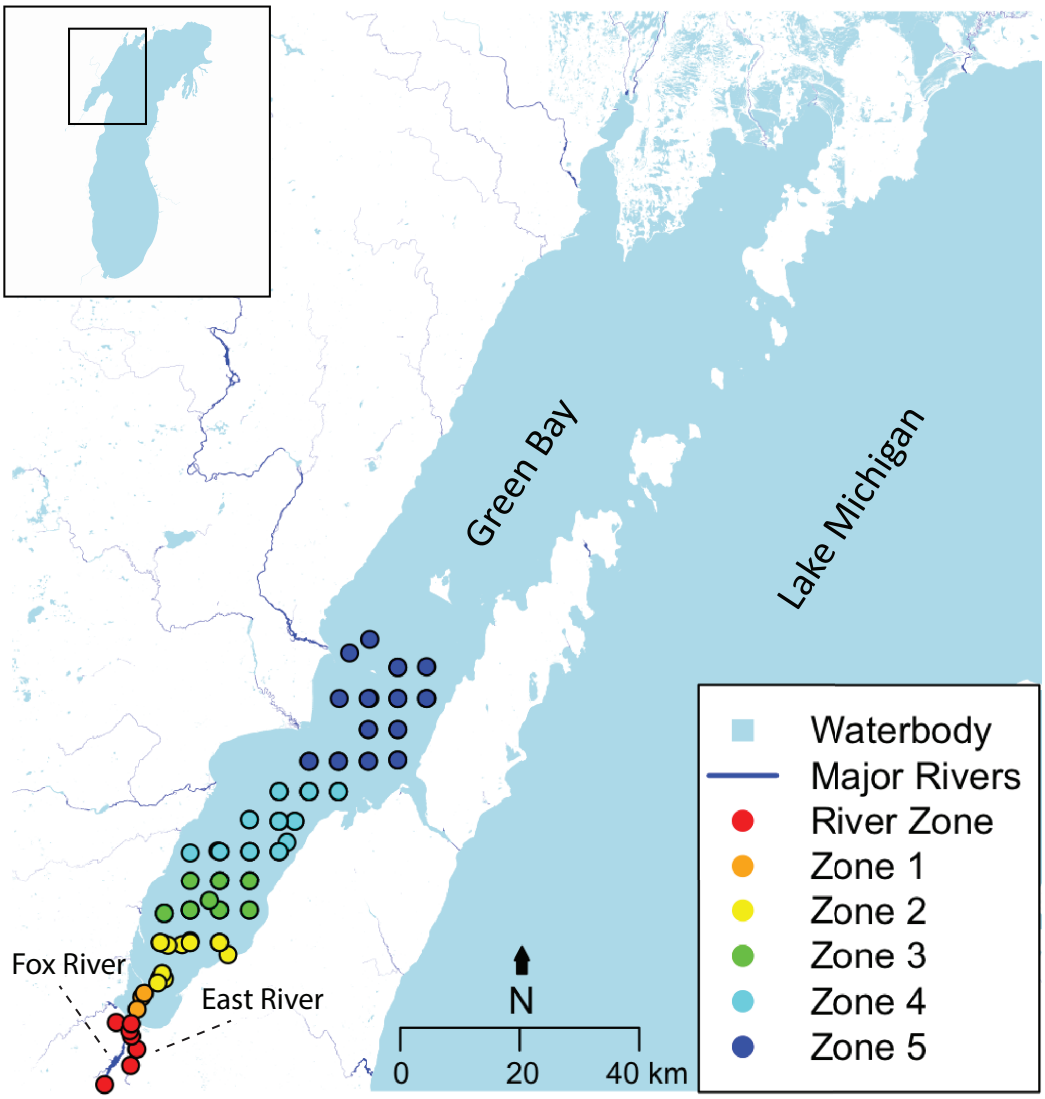

Green Bay
Lake Michigan
Fox River
East River
N
0
20
40 km
Waterbody
Major Rivers
River Zone
Zone 1
Zone 2
Zone 3
Zone 4
Zone 5

Figure 2:

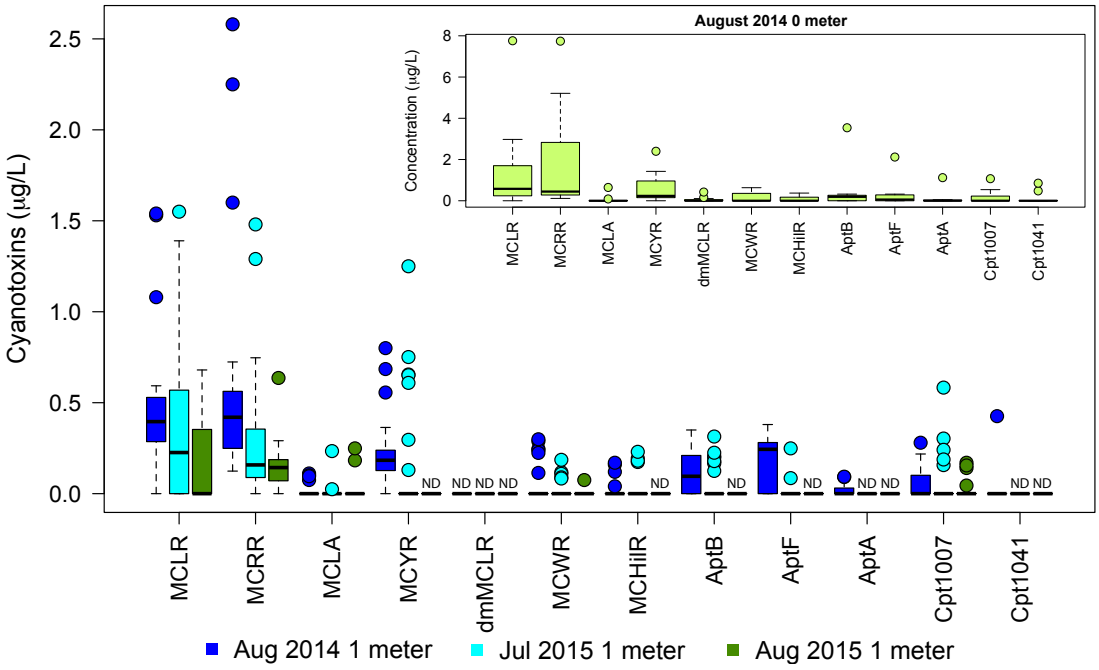

August 2014 0 meter
Concentration (µg/L)
MCLR
MCRR
MCLA
MCYR
dmMCLR
MCWR
MCHilR
AptB
AptF
AptA
Cpt1007
Cpt1041
Cyanotoxins (µg/L)
ND
Aug 2014 1 meter
Jul 2015 1 meter
Aug 2015 1 meter

Figure 3: August 2014 0 meter

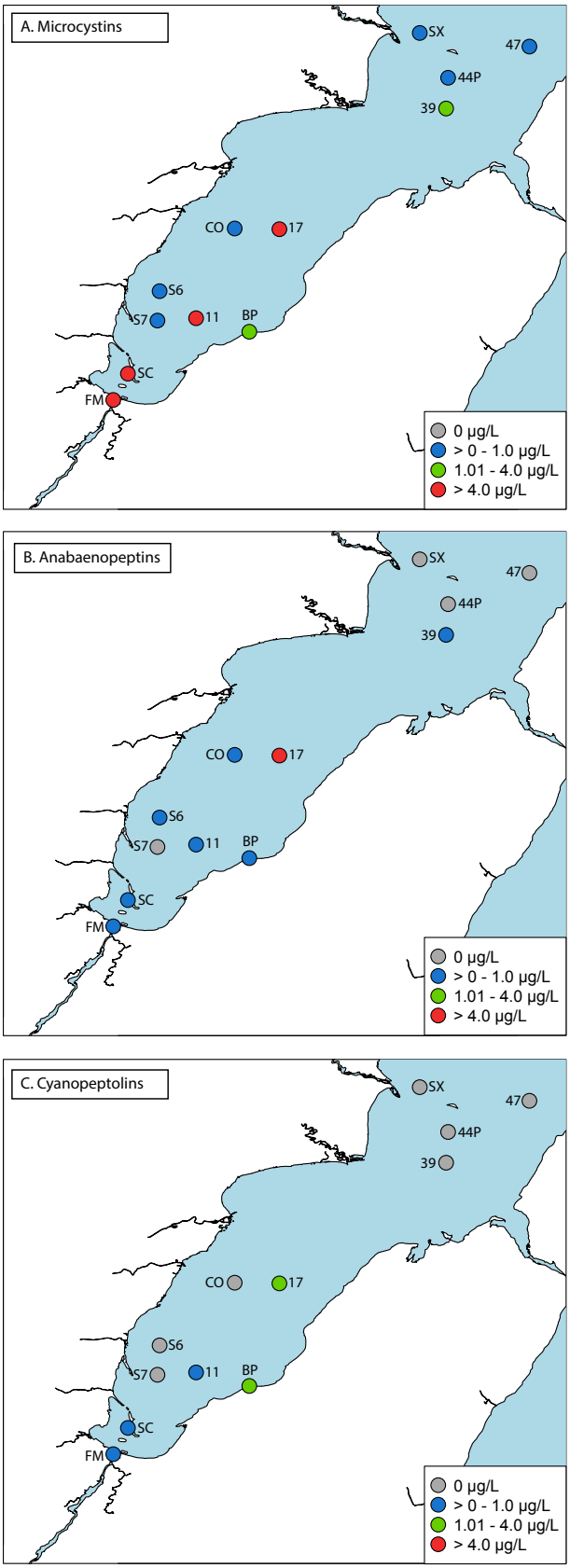


Figure 4: August 2014 1 meter

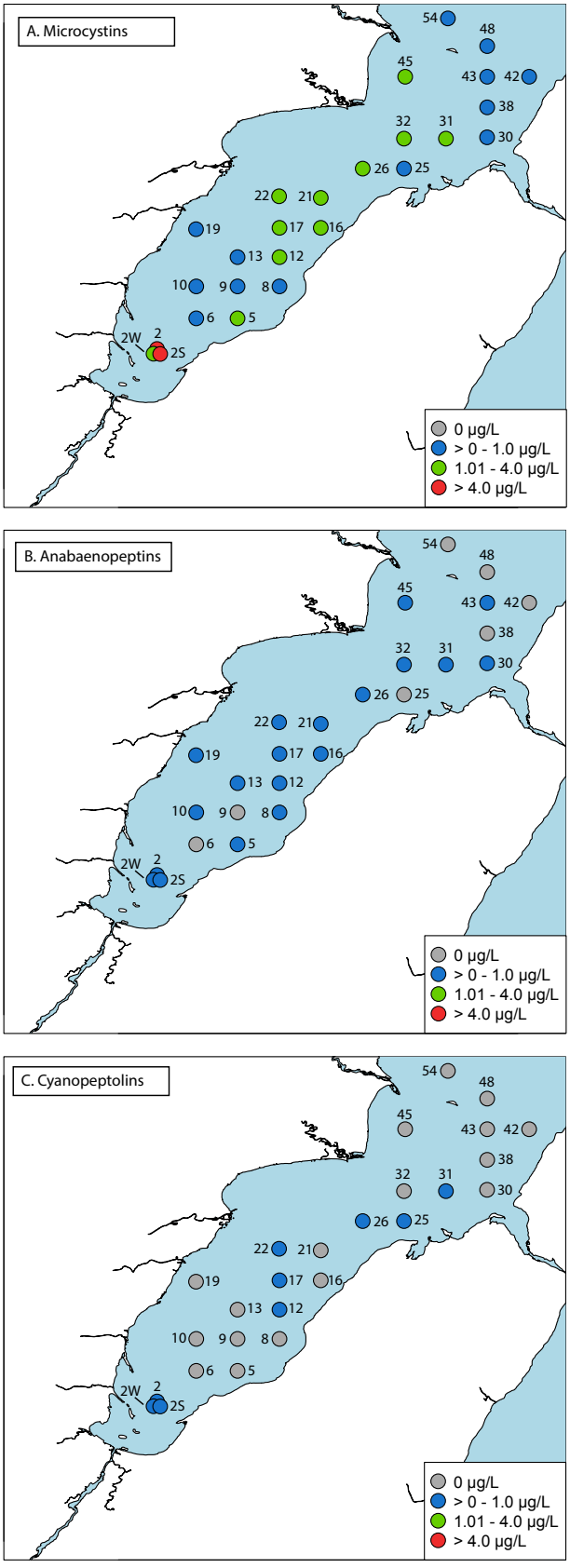


Figure 5: July 2015 1 meter

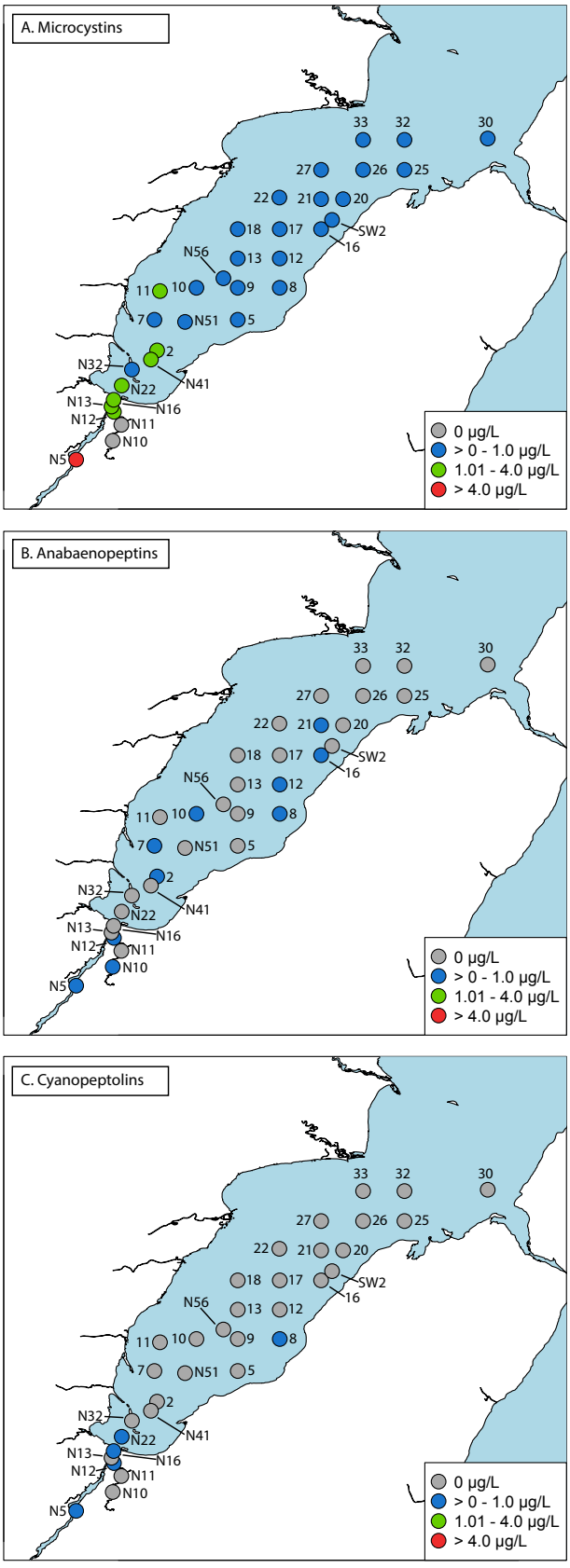


Figure 6: August 2015 1 meter

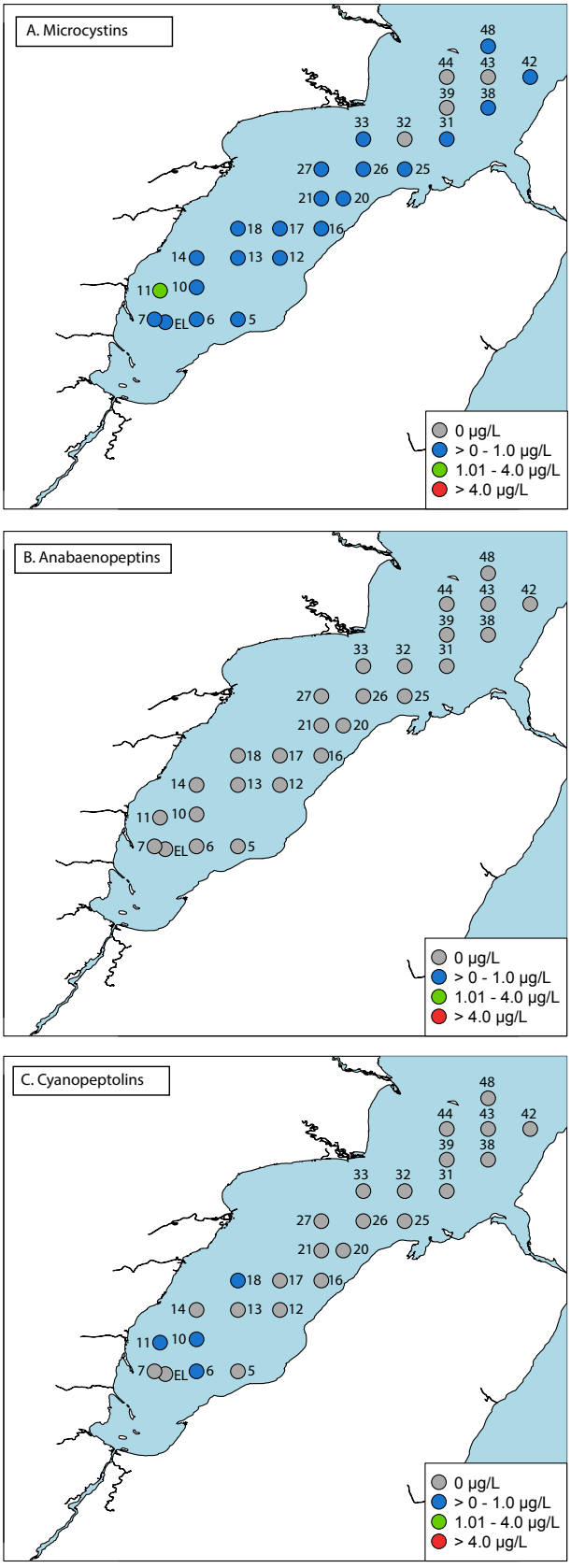


Figure 7:

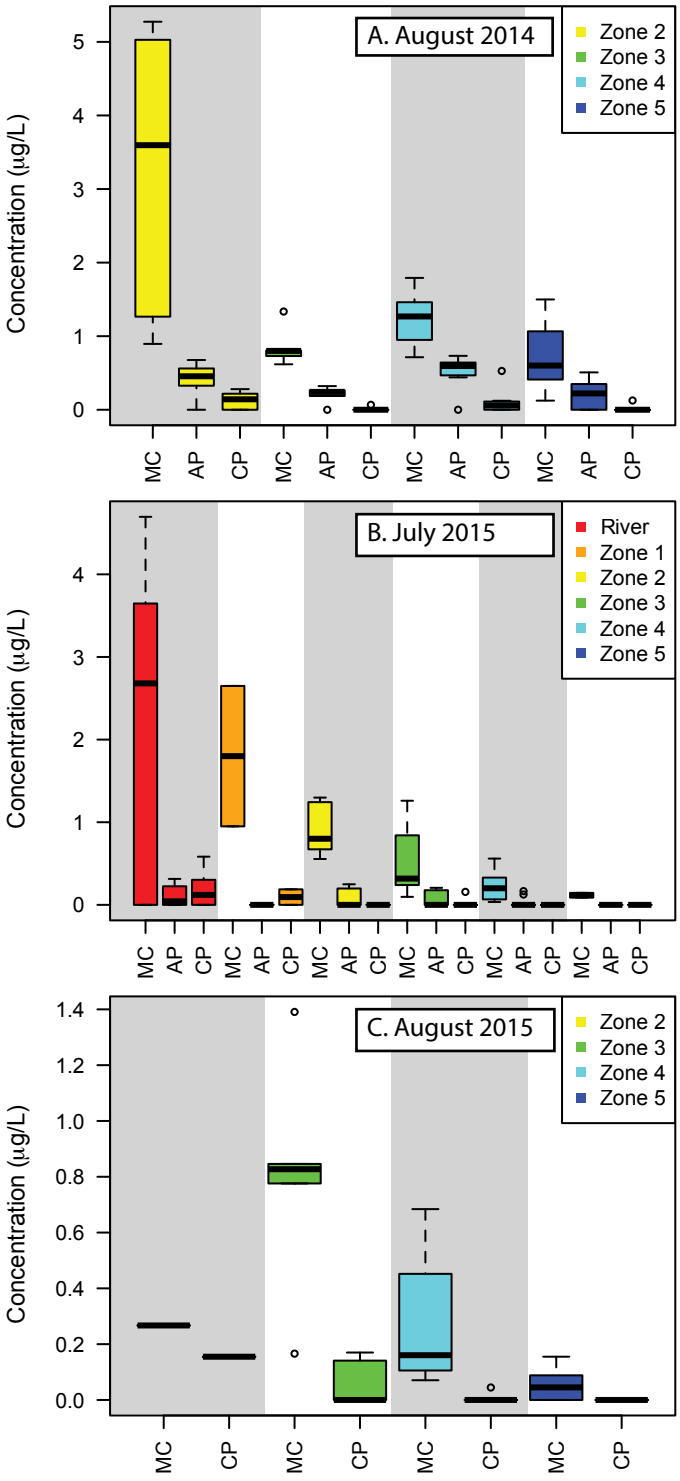
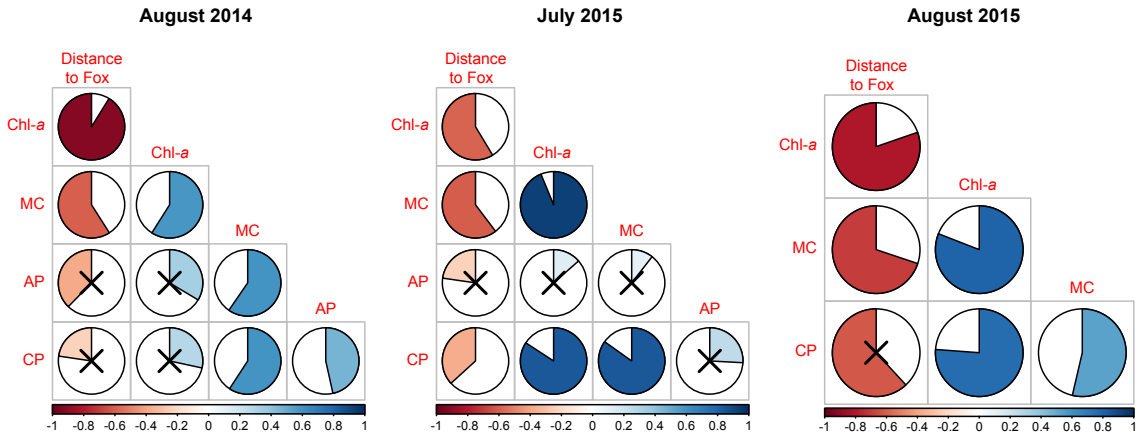

A. August 2014
Zone 2
Zone 3
Zone 4
Zone 5
Concentration (µg/L)
0
1
2
3
4
5
MC
AP
CP
B. July 2015
River
Zone 1
Zone 2
Zone 3
Zone 4
Zone 5
C. August 2015
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

Figure 8:


August 2014
July 2015
August 2015
Distance to Fox
Chl-a
MC
AP
CP
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1

Supporting Figure 1: