UREA IN LAKE ERIE: ORGANIC NUTRIENT SOURCES AS POTENTIALLY IMPORTANT DRIVERS OF PHYTOPLANKTON BIOMASS

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ABSTRACT

Significant evidence shows that nitrogen (N) supply may influence microbial community structure and, in some cases, the rate of primary productivity in fresh waters. To date, however, most focus has been on dissolved inorganic N (*i.e.*, ammonia and nitrate), or dinitrogen gas. Far less is known about the effects of dissolved organic N such as urea on plankton activity, although this compound is both produced by in-lake processes and is a significant component of external loading. We evaluated the urea distribution and the activity of the major enzyme responsible for its assimilation (urease) in Lake Erie, which has a significant history of eutrophication. During 2012 and 2013, lake-wide surveys estimated surface urea concentrations and urease activity, along with phytoplankton composition and biomass, cyanobacterial toxins (microcystin), major nutrients and other physico-chemical parameters. In parallel, *in situ* 48-hour microcosm experiments were executed to test whether different chemical forms of dissolved N could stimulate phytoplankton biomass. Results confirmed urea was a bioavailable form of N with *in situ* urea turnover times ranging from hours (for summer, *i.e.*, Aug 2012 and July 2013) to days (May 2013). Furthermore, we observed a positive correlation between urease activity and both microcystin concentrations and cyanobacterial dominance. Results also indicated a potential seasonal shift in the nutrient limiting phytoplankton biomass from phosphorus (P) to N. Our results reinforce the importance of both N and P in promoting phytoplankton growth and highlighted the need to consider organic nutrient sources as potentially important drivers of cyanobacterial blooms and toxin production.

INTRODUCTION

Phytoplankton blooms have become annual events in large freshwater systems, including Lake Erie and China's Lake Tai (or *Taihu*) (Qin et al., 2010; Rinta-Kanto et al., 2009b). The phytoplankton communities within these blooms consist of a diverse array of phototrophs, including diatoms, eukaryotic microalgae and cyanobacteria (Oliver et al., 2012). While phytoplankton are the base of the food web and drive processes and ecosystem services ranging from water quality to fisheries, large phytoplankton blooms are commonly referred to as harmful algal blooms (HABs) because they have a negative impact on human activities or the environment (Ho and Michalak, 2015; Zingone and Enevoldsen, 2000).

In the North American (Laurentian) Great Lakes, severe lake-wide blooms of diatoms and N-fixing cyanobacteria and associated deterioration in water quality and hypolimnetic anoxia became so dire in the mid- to late-1960s that Lake Erie was described as “dead” by the popular press (Ashworth, 1986; Charlton, 1980). Nutrient (phosphorus, P) abatement efforts led to a short-lived (1980s through to the mid-1990s) recovery in the overall health of the lake, but by the mid-1990s a reemergence of cyanobacterial blooms dominated by different (relative to the 1960s), non-N-fixing cyanobacteria species began to occur in Lake Erie (Conroy et al., 2005). Recent biomolecular evidence suggests that *Microcystis* blooms in Lake Erie during August 2012 actively transported major nutrients (various N and/or P-containing compounds) as well as inorganic carbon (Steffen et al., 2015). The co-expression of N and P transporters by the cells was interpreted to mean that *Microcystis* spp. were actively scavenging both elements, which *de facto* implies that neither element was in excess. The expression of genes associated with inorganic carbon concentrating mechanisms was further suggestive of a limited availability of CO₂ to this population (McGinn et al., 2003). This shift in phytoplankton community composition to cyanobacterial dominance can alter flow paths of energy and nutrients, reshaping the structure and trophic functionality of entire aquatic ecosystems (Oliver et al., 2012). This shift has also left researchers looking more closely at environmental drivers of community composition.

The observed historical transitions from a system dominated by N-fixing cyanobacteria (*Aphanizomenon flos-aquae* and *Anabaena spiroides*) to a “recovered” Lake Erie and on now to one where *Microcystis* spp. is a more dominant component have been linked to changes in nutrient flux and chemistry (Steffen et al., 2014a). Changes in nutrient loading (specifically P-

reductions) suggest a shift in conditions that determined the outcome of competition between cyanobacterial taxa, since the genetic richness (*i.e.*, which species are present) of the cyanobacterial community has remained fairly constant over the last 40 years (Rinta-Kanto et al., 2009b). Of particular interest are members of the genus *Microcystis*, spherical cells that are irregularly grouped into colonies of various sizes and densities, which may have a stratified, colorless mucilage (Šejnohová and Maršálek, 2012). While some cyanobacteria, including *Dolichospermum* (syn. *Anabaena*), *Aphanizomenon* and *Cylindrospermopsis*, have the capacity to produce the enzyme nitrogenase to fix atmospheric nitrogen gas (N_2), *Microcystis* does not possess the complete set of genes required for this process (Frangeul et al., 2008; Kaneko et al., 2007; Steffen et al., 2014a) and must assimilate N from other sources.

Concurrent with the increasing dominance of *Microcystis* as a bloom-forming taxon in the mid-1990s (Brittain et al., 2000; Conroy et al., 2005), agricultural practices in North America have also experienced a major shift. Use of ammonium nitrate as the preferred N-fertilizer in North America has waned in favor of urea-based fertilizers (USDA-ERS, 2013). Urea ($CO(NH_2)_2$) currently accounts for more than 50% of the N used globally for agricultural fertilizer, constituting more than a 100-fold increase in the past 4 decades (Glibert et al., 2006). It is a preferred N source for agricultural fertilizer because of its solubility in water, low cost of application, and the minimal damage it causes to root crops. Urea leaving the land and entering the aquatic system has been shown to be assimilated as an N source by some cyanobacteria and phytoplankton in both freshwater and marine environments (Donald et al., 2011; Finlay et al., 2010; Solomon et al., 2010).

There are a variety of natural biological sources of urea in lakes, including phytoplankton, bacteria, zooplankton and fish (Bogard et al., 2012). Previous estimates suggest urea concentrations in aquatic ecosystems are consistently less than those of NH_4^+ and NO_3^- , although urea may temporarily exceed these inorganic N-sources for short periods or even for prolonged period when runoff from either non-point or sustained point inputs of N occurs (Glibert et al., 2014; Solomon et al., 2010). Indeed recent estimates from Lake Tai (*Taihu*) in China indicate urea concentrations were higher in riverine inputs than in open lake waters: an observation that suggests urea is rapidly consumed in aquatic systems (Glibert et al., 2014; Han et al., 2014). In total, contributions, studies have suggested urea may provide 10 - 50% of the bioavailable N in lake and river surface waters (Bogard et al., 2012; Wiegner et al., 2006).

Despite the clear importance of urea in aquatic systems, no studies have evaluated its occurrence and few its potential for biological assimilation in Lake Erie (Chaffin and Bridgeman, 2014; Davis et al., 2015; Davis et al., 2010). The goal of this study was to address this knowledge gap. Most microorganisms use urease to assimilate urea; an enzyme that catalyzes the conversion of urea into carbon dioxide and ammonia. We therefore measured urease activity as a proxy for the ability of planktonic communities to assimilate this compound as an N source. We also investigated whether urea could act as an N-source for Lake Erie phytoplankton over the growing season. Measurements of urease enzymatic activity, nutrient concentrations and phytoplankton biomass were made during three lake-wide surveys (August 2012, May 2013, July 2013). This work was complemented by *in situ* microcosm experiments, where lake water was amended with various N species (urea, nitrate, ammonium) to determine if different chemical forms of N influenced the accumulation of phytoplankton biomass in a manner consistent with other recent studies (Chaffin and Bridgeman, 2014; Chaffin et al., 2013; Finlay et al., 2010). Our results contribute to the growing body of work implicating urea as an important N source in large freshwater systems.

METHODS AND MATERIALS

Environmental survey sample collection

Water for field samples surveys and microcosm experiments were collected from Lake Erie during three cruises of opportunity that occurred in August 13-17, 2012, May 27-31, 2013, and July 22-26, 2013 aboard the CCGS *Limnos*. Surface water (1 meter depth) was collected from each station (21 different sites in total, although due to operational limitations, not all stations were visited on all trips) using 10-L Niskin sampling bottles and transferred to the ship's laboratory in 4-L acid-cleaned polycarbonate bottles. Prior to use, all polycarbonate bottles were soaked in 1% HCl and rinsed thoroughly with Milli-Q[®] water (Millipore Corp., Billerica, MA, USA). Statistical relationships within the field data were determined using R version 3.02 (<http://cran.r-project.org/>). Principal components analysis was done using Primer v7.0.9 (Primer-E Ltd, Ivybridge, UK). Missing environmental data were estimated using an expectation maximum likelihood algorithm with 1000 iterations. Prior to PCA, variables were normalized

(the mean was subtracted from each value and the resultant then divided by the standard deviation) to account for the fact that they are measured on different scales.

Microcosm Experiments

Nutrient enrichment experiments were conducted in microcosms maintained in on-deck incubators that simulated *in situ* water column conditions during three *CCGS Limnos* cruises. Microcosm experiments were performed at two ecologically relevant and historically well-studied stations, Station 885 (41°31'08" N, 82°38'29" W) and Station 973 (41°47'30" N, 83°20'00" W) (Figure 1). Station 885 was chosen because of the diversity of cyanobacteria historically present at this site, while Station 973 was chosen because of its proximity to the inflow of the Maumee River into Lake Erie, and a long history of *Microcystis*-dominated phytoplankton populations in the region (e.g., Rinta-Kanto et al., 2005; Rinta-Kanto et al., 2009b). Surface water (1 m) from each station was dispensed into acid-washed 1.2-L polycarbonate bottles and enriched with different nutrient amendments. Along with control (no nutrients added) treatments, a matrix of individual N additions (180 μ M final concentration added N, from either ammonium, nitrate or urea (*i.e.*, 90 μ M urea added)) or P (final concentration 1 μ M-PO₄, added from a mixture of 1:4, KH₂PO₄:K₂HPO₄) was deployed in triplicate. These concentrations were chosen to be consistent with those used in previous lab (Steffen et al., 2014b) and field (Davis et al., 2015) experiments, and provided non-limiting, but also non-toxic, concentrations of nutrients. Sealed bottles were incubated in the deck incubator for 48 h at ~37% incident radiation (approximating light at 1 m in depth, DeBruyn et al., 2004; Wilhelm et al., 2003). Following incubation, aliquots from each microcosm treatment were removed and analyzed for chlorophyll a (chl_a) concentrations as well as the microscopic enumeration of phytoplankton as described below.

Biological parameters measured

Chl_a concentrations were estimated using the non-acidification method (Welschmeyer, 1994), and served as a proxy for total phytoplankton biomass. For both surface waters and microcosms, 50 mL of water was filtered through 0.2- μ m nominal pore-size polycarbonate membrane filters (47 mm diam., Millipore) and stored in a 2.0 mL cryovials (Corning, NY) at -20° C until analysis. Filters were extracted into 90% acetone overnight (24 h) at -20° C and chlorophyll

concentrations estimated fluorometrically (Turner Designs 10-AU; Sunnyvale, CA, USA). For *in situ* estimates of community structure, the percentage of chlorophyll assigned to phycocyanin (PC)-rich cyanobacterial species was estimated fluorometrically using a bbe-Moldaenke GmbH Algal Online Analyzer (AOA, *aka* FluoroProbe; Schwentinental, Germany) on board the ship and at the time of sample collection. Separate samples were collected from 1 m depth and triplicate 100 ml samples were sequentially passed through the AOA at the start of each incubation. Chlorophyll fluorescence was assigned to green algae, heterokonts (dinoflagellates + diatoms), phycoerythrin-rich algae and PC-rich algae. The percent chlorophyll contributed by PC-rich species was calculated by dividing the PC-rich algal channel by the total bbe-chlorophyll channel (Kring et al., 2014).

Dissolved nutrient analyses for total nitrogen (TDN), total dissolved Kjeldahl nitrogen (TdKN), total phosphorous (TDP), soluble reactive phosphorus (SRP), $\text{NH}_3\text{-N}$ (ammonia), $\text{NO}_3/\text{NO}_2\text{-N}$ (nitrate/nitrite), and silicate (SiO_2) were performed on membrane-filtered (0.45- μm) samples at the National Laboratory for Environmental Testing (Environment Canada) using their standard techniques (NLET, 1994). Urea concentrations were measured using the ‘Direct Method’: a colorimetric assay based on the reaction of urea with diacetylmonoxime, as outlined by Revilla et al. (2005). Briefly, water samples were collected from each station and filtered through pre-combusted 25 mm Glass Fiber Filters (GF/F) (Kent, UK). Filtrate was stored at -20°C until analysis in a lab setting where they were measured in triplicate and analyzed using a BioMate 5 UV-Visible Spectrophotometer at 520 nm as previously described (Revilla et al., 2005). Standard curves were generated for each survey using a range of urea stock solutions (0, 0.5, 2.0, 5.0, 7.5, and 10.0 μM) with all concentrations expressed in molar N – equivalents. Environment Canada provided pH and surface temperature data for each station, at the time of sampling, from the ship’s automated water column profiler.

Urease activity (maximal) was determined on ship by measuring the accumulation of NH_4^+ , which is a product of urea metabolism. Surface water (50 mL) from each station was filtered through pre-combusted (450 $^\circ\text{C}$ for 4 h) 25-mm GF/F glass fiber filters. Urease activity was measured using the indophenol method optimized for aquatic environmental samples (Solomon et al., 2007). Each sample was assayed at room temperature (the first hour after collection) in triplicate and analyzed using a BioMate 5 UV-Visible Spectrophotometer at 640 nm. Calibration for each survey was performed using a range of freshly prepared NH_4^+ stock

solutions (0, 1.0, 2.5, 5.0, 7.5, and 10.0 μM). Due to the manner in which these data were collected, activity here should be considered substrate-saturated (20 μmol urea was added as substrate for each enzyme reaction) and thus the maximum possible rate.

Total microcystin activity in water samples, expressed as microcystin-LR equivalents, was determined using the protein phosphatase inhibition assay (PPIA). Samples (20 L) were collected on GF/F filters for toxin extraction as in our previous work (Rinta-Kanto et al., 2009a). Filters were extracted with 50% methanol using ultrasound, clarified by centrifugation and microcystins assayed in 96-well plates containing 0.1 milliunits enzyme (recombinant protein phosphatase 1A, catalytic subunit, Roche Applied Science), 1.05 mg para-nitrophenyl phosphate (Sigma) and 10 μL of sample or microcystin-LR (Sigma Biochemical) using the method of Carmichael and An (1999). The PPIA gives an integrated value for microcystin activity, and thus toxin concentrations are reported as microcystin-LR equivalents as other microcystin congeners were also likely present in these samples (Boyer, 2007).

Phytoplankton enumeration and identification in microcosm samples were performed on Lugol's Iodine preserved samples using microscopic morphometrics (Auinger et al., 2008; Wehr and Sheath, 2003). Samples (50 mL) were collected and preserved with 0.5 mL of Lugol's Iodine (100 g KI L^{-1} and 50 g $\text{I}_2 \text{ L}^{-1}$), and stored at 4° C (Auinger et al., 2008). Fixed samples were allowed to settle (3-5 min) in a 1 mL Sedgwick-Rafter counting slide then analyzed at 100 or 400 x magnification on a Micromaster light microscope (ThermoFisher). Each sample was analyzed by taking four horizontal transects, ten fields of view each, which encompassed the full height and width of the slide (Chorus and Bartram, 1999; Saxton et al., 2012). The resulting 40 randomly selected fields of view were digitally imaged and saved using Micron imaging software (Westover Scientific), for later identification using established taxonomic features (Guillard and Sieracki, 2005; Wehr and Sheath, 2003). Organisms observed for each sample were identified and counted from the 40 randomly selected fields of view. Differences in cell abundance were determined using Tukey's HSD comparison of means, with significance considered at $p \leq 0.1$.

RESULTS

Field surveys

Sites were surveyed on cruises during August 2012, May 2013, and July 2013 with collection across all three basins of Lake Erie at 21 individual stations (Figure 1). Extractable chlorophyll *a* (chl*a*) concentrations (a proxy for phytoplankton biomass) for August 2012, May 2013 and July 2013 ranged from 1.6 to 25.4 $\mu\text{g L}^{-1}$, 0.7 to 37.4 $\mu\text{g L}^{-1}$, and 1.0 to 72.5 $\mu\text{g L}^{-1}$ respectively (Table 1). Station 1163 exhibited the highest chl*a* concentration in May 2013 and July 2013 while for August 2012 Station 973 had higher biomass as estimated from chl*a*. The contribution of the cyanobacteria to the total chlorophyll was higher in July and August (~ 21%) than in the May samples (~ 8%), but varied across stations. Station 1163 had abundant cyanobacteria throughout the season, whereas station 973 showed a seasonal increase in the proportion of the population that was cyanobacterial (0%, 21% and 79% for the samples collected in May, July and August, respectively).

Nutrients (TDN, TDP and SRP) for Aug 2012 ranged from 1.5 to 11.6 μM , 0.13 to 4.94 μM mg L^{-1} , and below detectable limits (BDL) to 4.52 μM respectively, with the Station 741 (closest to the Maumee River inflow) exhibiting the highest measurements for all three (Electronic Supplementary Material (ESM) Table S1). Similarly, July 2013 concentrations for TDN, TDP, and SRP were highest at Station 741 and ranged from 2.49 to 26.6 μM , 1.55 to 4.97, and BDL to 2.67 μM (respectively). May 2013 station measurements for SRP ranged from BDL to 0.64 μM (TDN and TDP data were not collected).

Urea concentrations across the three cruises ranged widely, exceeding 6 μM (*i.e.*, ~0.2 $\mu\text{g urea-N L}^{-1}$) during observations in Aug 2012 (average = $2.08 \pm 1.56 \mu\text{M urea-N}$). Average concentrations were noticeably lower in May 2013 ($0.46 \pm 0.19 \mu\text{M urea-N}$) and July 2013 ($1.47 \pm 0.71 \mu\text{M urea-N}$) (Table 1), although this pattern largely reflected the low concentrations at 2 stations (741 and 1326). Higher average urease rates were observed during the May 2013 cruise ($1.25 \pm 0.43 \mu\text{M urea-N h}^{-1}$) relative to August 2012 ($0.16 \pm 0.17 \mu\text{M urea-N h}^{-1}$) and July 2013 ($0.16 \pm 0.11 \mu\text{M urea-N h}^{-1}$) (ESM Figure S1A, Table 1).

The relationship between chl*a* and urea concentrations was not significant when combining data from August 2012, May 2013, and July 2013 (ESM Figure S1), even after log transformation. There was, however, a significant (but weak) correlation between maximal urease activity and chl*a* for the August 2012 cruise (Figure 2A, August 2012, $p = 0.034$, $R^2 = 0.282$; Figure 2B, May 2013, $p = 0.403$, $R^2 = 0.050$; Figure 2C, July 2013, $p = 0.312$, $R^2 = 0.093$).

Across all three surveys a series of statistical relationships emerged from our Spearman analyses (Figure 3, ESM Table S2). Significant correlations were observed between microcystin concentrations and urease ($r_s = 0.47$, $p < 0.05$) and the resulting relationship with urea turnover (hours, $r_s = -0.51$, $p < 0.05$). While there was a significant relationship between urease and NH_3 estimates ($r_s = 0.55$, $p < 0.05$), all other relationships between urea or urease and nutrients were non-significant. Microcystin concentrations were significantly related ($p < 0.05$) to both TdKN ($r_s = 0.66$) and TDP ($r_s = 0.44$).

Combining all these data, a principle component analysis revealed that stations generally clustered according to sampling dates, with May 2013 stations clustering away from August 2012 and July 2013 (Figure 4). Urease activity and temperature emerged as important discriminators between these dates, with highest urease activity seen in conjunction with the lowest temperatures. Indeed, overall there was a strong inverse relationship ($r_s = -0.49$, $p < 0.05$) between temperature and urease activity (ESM Figure S2, ESM Table S2). It should be noted that two of the stations that appeared as outliers on the PCA plot included samples collected within the warm and shallow Sandusky Bay (station 1163) and Maumee Bay (741) (Figure 4). The third (station 1326) is off the coast of Cleveland (OH) and potentially influenced by the regional urban environment.

Microcosm Experiments

Nutrient amendment experiments were conducted at stations 973 and 885 during August 2012, May 2013, and July 2013. August 2012 samples showed no significant difference between the non-amended control treatment and the phosphorus amendment for either station (Figure 5A-B). The addition of nitrogen ($180 \mu\text{M-N}$), however, significantly stimulated *chl a* concentrations across all N treatments compared to the control treatment at both stations (Figure 5A-B).

Chlorophyll biomass within the experimental bottles for May 2013 was not significantly different from the controls for all treatments at both stations (Figure 5C-D). Biomass accumulation for July 2013 experiments was statistically similar to respective controls for all treatments at station 973 (Figure 5E). The same was true for station 885, with the exception of the P treatment (Figure 5F).

Given our interest in the influence of nitrogen on the phytoplankton community composition in this system, we investigated the densities of several cyanobacterial populations in

August 2012 microcosms. For both stations 885 and 973, *Microcystis* cell densities were statistically similar to the respective controls for all treatments (ESM Figure S3 A-B, E-F). Similar results were observed for *Dolichospermum* cell counts for Station 885 (ESM Figure S3 B) and Station 973 (ESM Figure S3 F), with the NO₃-N treatments being the only treatments that were statistically different from the control. It should be noted that *Planktothrix* and *Cylindrospermopsis/Raphidiopsis* were only observed at station 885, but that both increased in the ammonium treatment.

DISCUSSION

The proliferation of blooms of potentially toxic cyanobacteria over the past 20 years has led to a re-evaluation of our efforts to constrain bloom occurrence *via* nutrient management. Our observations indicate that the accumulation of biomass in phototrophs can be limited at different locations and different times of the year by the concentration of either N or P-substrates. Urea is an important N-containing substrate, and its potential contribution as a N-source at different times in the growing season can be described by estimates of urea concentration and the activity of the enzyme (urease) responsible for its consumption.

Solomon *et al.*, (2010) reported that not all phytoplankton possess the same ability to use urea as a source of nitrogen, and some even lack enzymes for the uptake and/or catabolism of urea. It is well-documented, however, that cyanobacteria such as *Microcystis* spp. (McLachlan and Gorham, 1962) as well as *Anabaena* spp. (Mackerras and Smith, 1986) have the capability to use urea as a nitrogen source. This ability to use urea potentially contributes to the success of some bloom-forming cyanobacterial species, such as *M. aeruginosa* (Han et al., 2014; Huang et al., 2014). Multiple field studies have provided evidence to support this idea in freshwater systems around North America (Davis et al., 2010; Donald et al., 2011; Finlay et al., 2010), and recent observations have documented the activity of urea acquisition and metabolism genes in water samples collected concurrent with our current observations (Steffen et al., 2015). In this study, we build on this previous genetic evidence through direct measurements of the presence and metabolism of urea across Lake Erie as well as its influence on phytoplankton biomass in the eutrophic western basin and Sandusky sub-basin of Lake Erie.

To gauge the potential for urea to play a role in lake function, its concentration in the lake as well as the capacity of the community (*via* an assay for enzyme activity) to process (*i.e.*,

assimilate) this compound was measured. As noted, August 2012 measurements of urease activity and *chl a* concentration exhibited a direct statistical correlation (Figure 2). However, no significant statistical correlation was observed within the May and July 2013 samples or when these data were pooled (ESM Table S2). In previous field measurements of urease activity, researchers had difficulty in identifying which phytoplankton species contributed the largest percentage of community urease activity (Solomon, 2006), and this unfortunately remains the case here. In samples from the Kranji Reservoir (Singapore), Penn and colleagues (2014) attributed all of the potential urease gene activity to *Microcystis*. While we do not have the data to reproduce this observation, the positive relationship between urease activity and microcystin concentrations supports this observation (Figure 3B). However, the lack of a correlation between urease activity and *chl a* in the current study supports the idea that non-photosynthetic members of the community (*e.g.*, heterotrophic bacteria) may be contributors to the observed urease activity (*vis a vis* Collier et al., 2009). Results from other published field experiments in two locations found that measurements of urease activity decreased as *chl a* increased (Solomon et al., 2007). While these relationships are important, they also suffer from several potentially confounding factors, including (1) a temporal disconnect, as use of urea must occur prior to the accumulation of biomass if it is acting as a N source stimulating growth of the bloom (*i.e.*, the relationship we are seeing is not “cause” but “effect”) and (2) biomass accumulation may stimulate internal urea cycling due to increased protein turnover: internal recycling (driven by new protein biosynthesis in bacteria) would mean that less nitrogen is available to enter the urea cycle.

Estimates of urea concentrations across Lake Erie follow an inverse relationship relative to measurements of urease activity (*i.e.*, higher urease activity occurs at lower urea concentrations, ESM Figure S1A). Urea concentrations were lowest during sampling cruises conducted during the early summer months (May/June), and highest during warmer sampling cruises occurring in the late summer and fall months (July/August) resulting in a cross seasonal relationship with temperature that is significant ($r_s = 0.71$, $p < 0.05$; Table S2). Using this information, maximal urease activity rates in May (0.66 to 1.96 $\mu\text{M-N h}^{-1}$) and *in situ* concentrations during that period imply turnover times for urea of 0.2 – 1.2 h during that period. In contrast, turnover times for pools of urea during July and August were longer (up to > 150 h). Previously published reports have shown that higher temperatures during summer months can

increase heterotrophic bacteria activity and result in increased urea production from purines, compared to other seasons (Berg and Jorgensen, 2006). While our measured concentrations of urea in Lake Erie are consistent with this pattern, we did not observe an increase in urease activity with the increase in water temperature (ESM Figure S2), strongly suggesting another level of regulation of urease activity (*e.g.*, substrate or total N supply rates, changes in microbial community structure, the overall N-status of cells (*e.g.* Figure 5), *etc.*) is more important. This is in some ways surprising, as enzyme activity generally shows a positive relationship with temperature, (Juan et al., 2010). However, this relationship between temperature and urease needs to be interpreted with some caution as we experienced relatively minor changes in water temperature within a given cruise (2-5°, Table 1) and the changes in temperature between the spring and late summer cruises (as well as different locations) were also associated with changes in planktonic community structure that may have a much greater effect on this observation.

To test the potential for urea to be a nutrient (*i.e.*, nitrogen) source for Lake Erie phytoplankton, we completed a series of microcosm assays designed to evaluate nutrient limitation of biomass production and the potential use of different N sources (Figure 5). During the August 2012 experiment, addition of P alone did not stimulate *chl a* accumulation, while the addition of all N (nitrate, urea, and ammonia) sources resulted in statistically increased *chl a* concentrations, suggesting the community was N-limited and that phytoplankton were able to access all forms of N we provided. This observation further suggests that urea was as efficient as other N sources at stimulating primary producers. In contrast, neither the addition of N or P in May 2013 stimulated *chl a* production, implying that other factors (temperature, light, virus-lysis or grazing) may limit primary producers at this time of year. The July 2013 experiments demonstrated biomass production was stimulated by added P but not added N, consistent with findings from the previous decade (Wilhelm et al., 2003). These observations are in agreement with the seasonal shifts in nutrient-limitation seen in other large lakes such as Taihu (Xu et al., 2010). The results in our experiments and those of others (Chaffin et al., 2013; Davis et al., 2015) provide additional evidence for consideration of both N and P as important limiting nutrients, and that both N concentrations and seasonal effects need be considered for models aiming to predict late season bloom events (*e.g.*, *Microcystis*).

To examine the August stimulation of *chl a* by N addition further, we enumerated phytoplankton cells from samples collected during the August 2012 experiment. While all

treatments amended with N demonstrated increased total chlorophyll concentrations, surprisingly the only increase in cell density was in treatments where ammonia was added; additionally, only the *Planktothrix* and *Cylindrospermopsis* / *Raphidiopsis* groups increased. Increased chl_a in the absence of increased cell density may be due to the short time course of the experiments: microcosm experiments with natural communities are by necessity kept short to reduce potential bottle effects (DeBruyn et al., 2004; Hare et al., 2005; Wilhelm et al., 2003). It is quite likely that we observed rapid physiological changes within the phytoplankton community (*i.e.*, a rapid increase in chl_a as nutrient limitation is relieved) that outpaced the slower net growth rate of phytoplankton, making it difficult to detect any accumulation of new cells. To address this limitation, future microcosm experiments may benefit from a longer incubation period under *in situ* conditions, and the use of other metrics (*e.g.*, F_v/F_m - a standard metric of photosystem II stress, specific RNA/DNA ratios, *etc*) that could markedly augment such observational studies.

One other observation that arose from this study was the linkage between microcystin concentrations and various environmental parameters. Microcystin concentrations correlated positively with chl_a, the proportion of cyanobacteria, urease activity, and with both TdKN and TDP concentrations (ESM Table S2). The relationship between microcystin and N-cycling is of interest, as it builds upon observations from similar enrichment studies in Sandusky Bay (Davis et al., 2015). In addition, linkages between nitrogen-status of the cell and toxin production have been inferred through genetic regulatory mechanisms for toxin production (Neilan et al., 2013). To this end, not only does the present study add to the gathering wealth of data on N-loading and phytoplankton species succession and seasonal biomass in Lake Erie (Chaffin and Bridgeman, 2014; Chaffin et al., 2013), it also supports the general idea that nitrogenous chemical species need to be considered as potentially important drivers of cyanobacterial toxin production.

Overall our observations provide insight into the ambient concentrations of urea in Lake Erie, and the potential for the microbial community to hydrolyze this N source. We also add to other studies showing the markedly different effects of added nutrients on biomass (chlorophyll *a*) accumulation at different times of year. While much of the load of urea to the system is potentially from internal cycling (Bogard et al., 2012), the nearshore to offshore gradients we observed suggest there was significant contribution to in-lake levels from the catchment (although the long residence time of Lake Erie water relative to the rapid turnover time estimated for the enzyme activity suggest internal sources must also be active). The continued reduction of

phosphorus loading into eutrophic systems remains an important focal point for future management practices; however, failure to address point and nonpoint sources of N pollution may result in the continued biomass accumulation in these systems. Additionally, the ability of Lake Erie bloom community to use urea as a N source, with the near 100-fold increase of urea use as a nitrogen fertilizer (Glibert et al., 2006), supports the ecological importance of the linkages between urea fertilization and transport to downstream waters, and clear need to better characterize these processes in future work

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FIGURE LEGENDS

Figure 1: Lake Erie map of environmental sampling stations visited during the August 2012, May 2013, and July 2013 sampling cruises, aboard the CCGS *Limnos*. Stations where microcosm assays were performed (885 and 973) are shown in bold font.

Figure 2: Relationship between chlorophyll *a* concentrations and urease activity for August 2012 (A), May 2013 (B) and July 2013 (C). Error bars are the range of duplicate measurements for each sample. Where not shown, error bars are within the size of the symbol.

Figure 3. Volcano plots of Spearman's statistical relationships between A) urea concentrations, B) urease activity and C) urea turnover rate (h) for stations sampled in Lake Erie. Plots demonstrate statistical significance ($p < 0.05$) of relationships on the Y-axis and the strength of the relationship (as Spearman's rho) on the X-axis. Floating X-axis denotes $p = 0.05$ (points above this area considered statistically significant). Data are reported in ESM Table S2.

Figure 4. Principle component analysis of stations from all three cruises. $\triangle$ – Aug 2012; ∇ - May 2013; $\blacksquare$ - July 2013. Stations generally cluster by sampling date – outliers at stations 741, 1326 and 1163 are labeled.

Figure 5. Biomass estimates for microcosms at stations 973 (A, C, E) and 885 (B, D, F) for August 2012 (A, B), May 2013 (C, D) and July 2013 (E, F). Controls (open bars) are compared to +1 μM -P (grey) or + 180 μM -N (black) as ammonium, nitrate or urea. Statistically similar treatments ($n = 3$ biological replicates, $\pm$ SD) are denoted with similar letters above bars.

Table 1: Data for surface waters from cruises (Z = 1 m). ND indicates measurements were not determined. For microcystin concentrations, samples outside standards are reported as < or > limits. The percent chlorophyll assigned to cyanobacteria are for PC-rich cells only (see methods). Urea concentrations are presented both as those that were directly measured (mass per volume) and as the conversion to molar concentration.

Station	Date	Extracted Chl <i>a</i> ($\mu\text{g L}^{-1}$)	Cyanobacteria (% of chl <i>a</i>)	urea ($\mu\text{M} - \text{N}$)	urea (mg L^{-1})	urease ($\mu\text{M-N h}^{-1}$)	urea turnover (h)	microcystin ($\mu\text{g/ L}^{-1}$)	Temp ($^{\circ}\text{C}$)
879	8/16/12	2.31	0%	1.225	0.037	0.018	68.06	0.004	23.2
478	8/15/12	6.62	11%	1.544	0.046	0.414	3.73	0.017	23.9
589	8/16/12	9.43	24%	1.624	0.049	0.013	124.92	0.042	23.9
741	8/15/12	14.90	11%	6.203	0.186	0.298	20.82	0.048	23.7
880	8/14/12	3.08	0%	1.065	0.032	0.007	152.14	0.006	23.9
882	8/15/12	16.05	42%	0.932	0.028	0.440	2.12	0.50	23.4
885	8/14/12	12.85	28%	0.932	0.028	0.029	32.14	0.19	24.5
937	8/16/12	1.56	4%	1.225	0.037	0.024	51.04	0.008	22.5
966	8/13/12	5.65	11%	1.735	0.052	0.082	21.16	0.030	24.5
970	8/13/12	4.99	17%	1.708	0.051	0.069	24.75	0.080	24.1
971	8/13/12	2.06	10%	2.536	0.076	0.133	19.07	0.025	23.2
972	8/15/12	7.41	17%	1.735	0.052	0.015	115.67	0.007	23.3
973	8/15/12	25.4	79%	2.269	0.068	0.486	4.67	0.37	23.7
974	8/15/12	9.02	14%	1.495	0.045	0.215	6.95	0.10	22.8
1163	8/14/12	15.60	60%	1.495	0.045	0.025	59.80	0.62	23.9
1326	8/14/12	3.16	0%	5.605	0.168	0.299	18.75	0.026	23.9
67	5/29/13	8.87	2%	0.823	0.025	0.656	1.25	<0.02	14.9
222	5/28/13	3.40	0%	0.415	0.012	0.766	0.54	<0.00	11.2
478	5/30/13	6.56	8%	0.431	0.013	ND	NA	<0.00	17.2
879	5/29/13	0.73	0%	0.183	0.005	0.870	0.21	<0.00	10.4
880	5/30/13	3.80	0%	0.375	0.011	ND	NA	<0.00	10.7
881	5/27/13	13.00	0%	0.383	0.012	1.724	0.22	<0.01	14.9
885	5/28/13	5.43	17%	0.534	0.016	1.024	0.52	<0.01	15.2
937	5/29/13	1.45	0%	0.175	0.005	0.985	0.18	<0.00	11.4
958	5/28/13	4.31	35%	0.606	0.018	1.591	0.38	<0.01	15.6
966	5/28/13	2.79	0%	0.327	0.010	1.572	0.21	<0.00	15.0
970	5/30/13	3.30	0%	0.431	0.013	ND	NA	<0.00	17.3
971	5/30/13	1.55	0%	0.447	0.013	ND	NA	<0.01	16.8
973	5/27/13	2.61	0%	0.574	0.017	1.559	0.37	<0.03	16.8
1156	5/27/13	5.35	0%	0.327	0.010	0.948	0.34	<0.01	ND
1163	5/28/13	37.35	65%	0.510	0.015	1.395	0.37	0.418	ND
1326	5/28/13	3.33	0%	0.877	0.026	1.963	0.45	<0.00	11.4
452	7/25/13	1.39	0%	0.734	0.022	0.112	6.55	<0.00	21.3
478	7/23/13	4.80	9%	1.244	0.037	0.115	10.82	0.03	26.5

741	7/24/13	18.00	11%	3.333	0.100	0.494	6.75	<0.03	26.9
880	7/25/13	1.44	0%	1.069	0.032	0.149	7.17	<0.00	23.6
882	7/24/13	6.54	49%	1.427	0.043	0.106	13.46	0.13	25.2
885	7/23/13	21.00	55%	0.710	0.021	0.225	3.16	0.235	25.7
937	7/22/13	4.46	64%	1.148	0.034	0.054	21.26	<0.00	19.8
958	7/24/13	1.06	63%	1.364	0.041	0.104	13.12	0.04	25.5
970	7/23/13	3.47	6%	0.893	0.027	0.049	18.22	0.03	25.5
971	7/23/13	4.24	16%	1.643	0.049	0.173	9.50	<0.00	25.9
973	7/24/13	3.44	21%	2.113	0.063	0.157	13.46	0.07	25.2
1163	7/23/13	72.45	28%	2.041	0.068	0.200	10.21	0.11	26.4
1326	7/24/13	3.04	0%	1.388	0.046	0.187	7.42	<0.00	23.6

Figure 1

Figure 2.

Figure 3.

Figure 4.

Figure 5.