Reduced forms of nitrogen are a driver of non-nitrogen-fixing harmful cyanobacterial blooms and toxicity in Lake Erie

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ABSTRACT

Western Lake Erie (WLE) experiences anthropogenic eutrophication and annual, toxic cyanobacterial blooms of non-nitrogen (N) fixing *Microcystis*. Numerous studies have shown that bloom biomass is correlated with an increased proportion of soluble reactive phosphorus loading from the Maumee River. Long term monitoring shows that the proportion of the annual Maumee River N load of non-nitrate N, or total Kjeldahl nitrogen (TKN), has also increased significantly (Spearman's $\rho = 0.68$, $p = 0.001$) over the last few decades and is also significantly correlated to cyanobacterial bloom biomass (Spearman's $\rho = 0.64$, $p = 0.003$). The ratio of chemically reduced N to oxidized N (TKN:NO3) concentrations was also compared to extracted chlorophyll and phycocyanin concentrations from all weekly sampling stations within WLE from 2009 to 2015. Both chlorophyll (Spearman's $\rho = 0.657$, $p < 0.0001$) and phycocyanin (Spearman's $\rho = 0.714$, $p < 0.0001$) were significantly correlated with TKN:NO3. This correlation between the increasing fraction of chemically reduced N from the Maumee River and increasing bloom biomass demonstrates the urgent need to control N loading, in addition to current P load reductions, to WLE and similar systems impacted by non-N-fixing, toxin-producing cyanobacteria.

1. Introduction

Western Lake Erie (WLE) experiences anthropogenic eutrophication and annual, toxic cyanobacterial blooms (CHABs; Stumpf et al., 2012). A highly publicized example of the risks of CHABs was the shutdown of Toledo, Ohio's drinking water system in August 2014 (Steffen et al., 2017; Wilson, 2014). The economic impact of the 2014 bloom was estimated at $65 million, with a local impact over 30 years of $1.4 billion if blooms were to recur annually (Bingham et al., 2015). Reacting to toxin events is expensive and unsustainable and does not resolve the ultimate cause of CHABs, external nutrient loading, including both nitrogen (N) and phosphorus (P). For systems at human-aquatic interfaces to be sustainable, long-term strategies to reduce external nutrient loads must be developed to prevent CHABs (Paerl et al., 2016). Climate change is also forecast to exacerbate these harmful CHABs via the combined effects of higher water temperatures, evaporation rates, carbon dioxide, and altered flood/drought intensities (O'Neil et al., 2012; Paerl and Huisman, 2009).

Historically, most research and lake management efforts have focused on P in the Great Lakes and elsewhere (e.g., Schindler et al., 2016; Stumpf et al., 2012; Dove and Chapra, 2015). These management efforts are based on strong, significant relationships between P load and total bloom biomass (Kane et al., 2014; Scavia et al., 2014; Stumpf et al., 2012). The fraction of P as soluble reactive P (SRP) in the Maumee River P load has increased in recent decades, corresponding to increases in CHAB biomass (Baker et al., 2014; Stow et al., 2015). Models predicting bloom biomass in WLE are based on river discharge and nutrient load, as runoff from precipitation flushes P from soils into the Maumee River (Michalak et al., 2013; Stow et al., 2015; Ho and Michalak, 2017). August bloom biomass correlates with spring P load and discharge from the Maumee River beginning in the 1990s (Stumpf et al., 2012), but not with NO_3^- (Kane et al., 2014). There is a temporal disconnect between spring P loading and the onset of the CHAB, typically in late July and early August, however. The relationship between P load, and the SRP proportion of the total P load, and CHABs in WLE is well established and broadly acknowledged; thus, the focus of this study is on the impact of the composition of N loads to WLE. This study will not dwell further on these well established and acknowledged

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patterns for P and SRP, and the authors stress that continued efforts to reduce external P loading, in WLE and elsewhere, is prudent and necessary.

The focus on P as the primary nutrient for CHABs in WLE, and, to a lesser extent, studies evaluating oxidized N forms (e.g., NO_3^-; Kane et al., 2014; Dove and Chapra, 2015), have led to severe gaps in our understanding of the role of N cycling and loading in causing and maintaining CHABs and toxicity. A recent review suggested that management efforts to reduce P pollution, without simultaneously controlling N, have caused nutrient imbalances in eutrophic systems, which may favor CHABs that cannot fix atmospheric N_2 gas (Gobler et al., 2016). Modern CHABs (since the mid-1990 s) in Lake Erie are primarily comprised of the non-N-fixing *Microcystis*, while CHABs prior to P load reductions in the 1970 s were mostly potential N-fixers (e.g., *Aphanizomenon* and *Dolichospermum,* formerly *Anabaena*; Wacklin et al., 2009; Steffen et al., 2014). In WLE, *Microcystis* dominates summer and fall blooms (Berry et al., 2017; Steffen et al., 2017), while potential N-fixers typically represent a nominal (< 5%) proportion of the phytoplankton population (Berry et al., 2017), despite persistent N limitation (Chaffin et al., 2013; Chaffin and Bridgeman, 2014; Chaffin et al., 2014). Persistence of toxin-producing, non-N-fixing CHABs during N limited conditions is commonly observed in eutrophic lakes (e.g., Chaffin et al., 2014; McCarthy et al., 2016, 2013; Paerl et al., 2014).

The N needed to fuel biomass and toxin production in non-N-fixing CHABs is delivered mostly from agriculture (e.g., fertilizer runoff; Robertson and Saad, 2011). Bioavailable N is present in chemically reduced (e.g., urea, NH_4^+, and DON) and oxidized forms (e.g., NO_3^- and NO_2^-), which are assimilated, transformed, and recycled at different rates. NH_4^+ is the preferred N source for most primary producers (Syrett, 1981), including cyanobacteria (Glibert et al., 2016; Monchamp et al., 2014), but urea is also an efficient N source for CHABs (Belisle et al., 2016). Non-N-fixing cyanobacteria are superior competitors for NH_4^+ relative to eukaryotic taxa (Blomqvist et al., 1994; Glibert et al., 2016; Hutchins et al., 2003; Monchamp et al., 2014). The inability of these cyanobacteria to fix atmospheric N and their strong affinity for chemically reduced N suggests an important role for external N loads from the watershed and internal N recycling mechanisms in modulating total biomass and, especially, CHAB community composition and toxicity. Indeed, *Microcystis* blooms in WLE respond quickly and strongly to N inputs, especially reduced N forms (Chaffin et al., 2013; Chaffin and Bridgeman, 2014; Chaffin et al., 2018). In addition, the toxin produced by *Microcystis* (and other cyanobacteria), microcystin, requires 10 N atoms per molecule (to 49 carbon atoms), and production of microcystins is strongly correlated with bioavailable N (Davis et al., 2015). Conversely, low NH_4^+ concentrations inhibit toxin production by blocking promoter sites for the microcystin gene operon, *mcy* (Kuniyoshi et al., 2011). Globally, CHABs producing toxins that interfere with resource utilization (e.g., drinking water, recreation, fisheries) in lakes are usually associated with non-N-fixing taxa in general, and *Microcystis* in particular (e.g., Lake Erie, Steffen et al., 2017; Lake Okeechobee, Rosen et al., 2017; Taihu Lake, Krausfeldt et al., 2017).

The Maumee River drains a mostly agricultural watershed and discharges into WLE, supplying ∼50% of the total nutrient loads (Maccoux et al., 2016; Verhamme et al., 2016), but only about 3% of the water volume, while about 95% is from the Detroit River (Joosse and Baker, 2011). The Maumee River discharge is episodic compared with the Detroit River: standard deviation of the monthly discharge compared with the mean is < 3% from March to October between 2008–2015 for the Detroit River (USGS 04165710 Detroit River at Fort Wayne at Detroit, MI), while the Maumee River discharge varied > 100% for the same time periods (USGS Gage Station 04193500 Maumee River at Waterville OH). Thus, the focus on the correlation between nutrient loadings and HAB development has largely centered on the Maumee River. Most farmers in the Maumee River watershed add N fertilizer as manure, anhydrous ammonia and/or urea, or some combination of multiple N forms, to cultivated fields (Millar et al., 2014). The average annual total Kjeldahl N (TKN; NH_4^+ + dissolved organic and bound N) load from the Maumee River to Lake Erie is about 9000 metric tons, compared to about 400 metric tons of dissolved reactive P (Richards et al., 2010). While not all of this TKN is immediately or even ultimately bioavailable to primary producers (Lewis and Wurtsbaugh, 2008), proportions of TKN not immediately bioavailable to primary producers can be expected to represent N that will likely become bioavailable after discharge and subsequent, likely rapid, remineralization (Gardner et al., 2017). Numerous studies confirm that the amount of rapidly recycled N, from phytoplankton biomass and other remineralizable, bound N, can often exceed external N loads and fuel continued biomass and toxin production (e.g., McCarthy et al., 2007; Paerl et al., 2011; McCarthy et al., 2013; Glibert et al., 2016; McCarthy et al., 2016), but the N available for internal recycling is ultimately controlled by external loading.

Dissolved organic N (DON), especially urea, is also a key driver of CHABs (Belisle et al., 2016), as it can be assimilated directly or after extracellular enzyme hydrolysis to NH_4^+ (Glibert et al., 2016). DON is often abundant in aquatic ecosystems, but little research has focused on its role in CHABs (Gobler et al., 2016), despite its direct and subsequent (via hydrolysis) bioavailability. The speciation of available N can thus strongly influence phytoplankton community structure, with reduced N (NH_4^+ and urea) promoting cyanobacteria (McCarthy et al., 2009). The proportion of fertilizer added as reduced N has increased since the mid 1990 s, both globally (Glibert et al., 2006) and in Ohio (Millar et al., 2014), exacerbating Lake Erie's eutrophication problem. Historically, little modeling or management effort has been expended to understand or quantify the fate of this N input into WLE or elsewhere.

N loading reduction recommendations have not been included in documents urging additional P control in the Lake Erie watershed (e.g., Dove and Chapra, 2015; Michalak et al., 2013; Scavia et al., 2014; Stumpf et al., 2012; IJC, 2014), although there is mounting evidence that N control is also needed (Gobler et al., 2016). Indeed, arguments for N control on a global scale are gaining significant traction, since the massive increase in reactive N levels, primarily due to Haber-Bosch N fixation for fertilizer production, has been linked to loss of biodiversity, climate change, water and air pollution, and hypoxic zones (Erisman et al., 2015). Of great concern in Lake Erie is the connection between N and cyanotoxins (e.g., Gobler et al., 2016), and numerous studies show that the combination of N and P usually results in higher cyanobacteria biomass than either nutrient added singularly (Elser et al., 2007; Lewis and Wurtsbaugh, 2008; Paerl et al., 2016; Scott and McCarthy, 2010).

The importance of N as a potential driver of CHABs in Lake Erie has been largely overlooked, as total N loads have not increased significantly in the last two decades (Kane et al., 2014; Stow et al., 2015), and there was no significant correlation between annual NO_3^- load and increasing bloom biomass (Kane et al., 2014). Perhaps that should be expected as these relationships treat all N as equally bioavailable, or focus only on TN or NO_3^-, when cyanobacteria have a much stronger affinity for reduced N (in terms of oxidation state; e.g. non-NO_x). The proportion of non-nitrate N in the Maumee River load has increased significantly in recent decades. The focus of this paper is the hypothesis that this increase in non-nitrate N is significantly correlated with CHAB biomass on a long term (annual Maumee loads) and short term (weekly monitoring concentration) basis. This hypothesis was investigated from two long terms datasets: (1) the National Center for Water Quality Research (NCWQR) monitoring of the Maumee River loads; and (2) the NOAA Great Lakes Environmental Research Laboratory (GLERL) weekly summer/fall monitoring in WLE. The focus is on TKN because it includes immediately bioavailable N (e.g., NH_4^+ and urea) and readily remineralizable, bound N (including phytoplankton biomass) and has been reported since NCWQR monitoring began in the 1970s. NH_4^+, which is difficult to measure accurately in situ because of its high bioreactivity (McCarthy et al., 2013), and dissolved or particulate organic N values are not reported separately in these datasets.

2. Methods

For comparisons between N loading, availability, and bloom biomass, two long-term datasets were analyzed.

2.1. NCWQR Maumee loads

Nutrient concentration and loading data for the Maumee River to WLE is monitored and published through the NCWQR tributary monitoring program (Richards et al., 2010). Three water samples for nutrient analysis are collected daily using an automated system and stored refrigerated at the U.S. Geological Survey gaging station on the Maumee River at Waterville, OH. Only one sample per day is analyzed unless the river experiences high flow and high turbidity (Richards et al., 2010). The database can be accessed at http://www.heidelberg.edu/academiclife/distinctive/ncwqr/data. Nitrogen analyzed and reported by NCWQR includes oxidized (nitrate; NO_3^- + nitrite; NO_2^-) and reduced (TKN) forms. Nitrate and NO_2^- are analyzed using EPA method 300.1 using a Dionex ion chromatograph. Ammonium and dissolved or particulate organic N values are not reported separately, but TKN has been reported since monitoring began. The molar ratio of TKN to NO_3- (TKN:NO3) was calculated for every weekly monitoring sample reported in the NCWQR dataset. Zero or negative concentration values were assigned at the detection limit (0.005 mg/L) to maintain rational values. Weekly TKN:NO3 was averaged over each year for comparison to bloom biomass data. Annual loads of TKN and NO_3- based on flow-weighted discharge values were also calculated by the NCWQR template, and then the ratio of the total load of each N pool was correlated with the annual cyanobacterial bloom biomass values.

2.2. Bloom biomass

Annual cyanobacterial bloom biomass values were reported by Kane et al (2014) through 2010 and updated with data from the Annex 4 Objectives and Targets Task Team Final Report to the Nutrients Annex Subcommittee (Annex 4, 2015) based on Fig. 3 in Stumpf et al. (2012). WLE sampling sites and protocols are described in Conroy et al. (2005), and cell counts were determined based on microscope counts of cyanobacterial cells collected from integrated water sampling from the surface to twice the Secchi depth. Stumpf et al. (2012) cyanobacterial cell counts were calculated from satellite images. Briefly, cyanobacterial biomass was calculated for 10-day composite images, and intensity was derived by summing the value of the Cyanobacterial Index at all pixels within the potential bloom area of each composite. Stumpf et al. (2012) calculated the Cyanobacterial Index as opposite of the product of the spectral shape around 681 nm band (definition from Wynne et al., 2008). The annual bloom severity was determined by averaging the highest three consecutive 10 day composites (Stumpf et al., 2012). Total bloom cell counts were converted to wet weight per mL for equivalency with Kane et al. (2014) by dividing by the volume of WLE and assuming a cell wet weight of 200 pg (the most conservative value from Long et al. (2001)).

2.3. NOAA GLERL western basin monitoring

In WLE, NOAA GLERL, in collaboration with Cooperative Institute for Great Lakes Research (CIGLR), monitors eight stations bi-weekly in May/June and weekly from July-October (with some variance between years) to quantify *Microcystis* abundance and toxicity, nutrient concentrations, pigments, and other environmental parameters, since 2008; however, this manuscript only incorporates the data collected between 2009–2015 as the 2008 sampling was very limited. Chlorophyll *a*-inferred biomass was measured by concentrating lake water on a GF/F filter (Whatman, 47 mm), extracting with acetone or *N,N*-dimethylformamide under low light, and analyzing with a 10 AU fluorometer (Turner Designs; Speziale et al., 1984). Filters for phycocyanin-inferred biomass were collected as described above and extracted in phosphate buffer (Ricca Chemical, pH 6.8) using two freeze–thaw cycles, followed by sonication (Horváth et al., 2013). Relative fluorescence was measured on a Turner Aquafluor fluorometer and converted to phycocyanin concentration using a calibration curve from a series of dilutions of a commercial standard (Sigma-Aldrich).

For dissolved nutrients, duplicate whole water samples were collected in a triple rinsed (ultrapure water) 20 mL syringe, filtered in the lab through a sample-rinsed 0.22 μm nylon filter into a 15 mL vial, and stored at $-20\,°C$ until analysis. Nutrient concentrations were determined using standard automated colorimetric procedures as modified by Davis and Simmons (1979) on a Seal QuAAtro according to methods detailed by the manufacturer. NH_4^+ was determined by the Berthelot reaction in which NH_4^+ ions react with salicylate and free chlorine to form a blue-green complex. $NO_3 + NO_2$ was determined by the cadmium reduction method.

Particulate microcystins (MCs) were measured by filtering whole lake water onto a 25 mm, 3 μm polycarbonate membrane and kept at $-20\,°C$ until analysis (Filtrate was frozen for dissolved MC). Particulate MCs were extracted from samples using a combination of physical and chemical lysis techniques. All samples were resuspended in 1 mL of molecular grade water (pH 7; Sigma-Aldrich, St. Louis, MO) and subjected to three freeze/thaw cycles before the addition of QuikLyse reagents (Abraxis LLC; Warminster, PA) per manufacturer's instructions. The samples were then centrifuged for 5 min at $2000 \times g$ to pellet cellular debris. The concentrations of microcystins (reported as microcystin-LR equivalents) were measured using a microcystin enzyme-linked immunosorbent assay (ELISA; Abraxis LLC) following Fischer et al. (2001). This assay is largely congener-independent since it detects the ADDA moiety, which is found in almost all MCs. These analyses yielded a detection limit of $0.04\,\mu g\,L^{-1}$ for microcystins.

Values for all stations (excluding any surface scum samples) were averaged by week. Although NOAA GLERL does not monitor TKN or DON, ratios of reduced N (NH_4^+ + PON) to oxidized N (NO_3^- + NO_2^-) concentrations were compared, for consistency with NCWQR data, to bloom biomass proxies (extracted chlorophyll *a* and phycocyanin).

2.4. Statistics

Each dataset was analyzed for normality using the Anderson-Darling test. Data were determined to be non-normal, so nonparametric statistical tests were used to analyze differences between datasets, variable correlations, and significance of paired data within datasets using JMP13 (SAS). Nonparametric correlations between paired data were analyzed using Spearman's rank correlation (ρ). The Kruskal-Wallis Rank Sum Test was used to determine if groups of data were significantly different (similar to ANOVA). The Wilcoxin Rank Sign Test for paired data was used to determine if groups of paired data were significantly different (similar to Student's *t*-test). Correlations were evaluated with a Spearman's ρ rank correlation (significant at $p < 0.05$). There is some disagreement regarding the appropriate use of non-parametric tests, so linear regressions of natural log-transformed data have been included in the supplementary information, where applicable. Linear regressions and Michaelis-Menton correlations were determined in KaleidaGraph 4.5.2 (Synergy).

3. Results

3.1. Maumee River N load

Weekly monitoring of the Maumee River since the 1970 s from the NCWQR shows that the annual average of the fraction of reduced (TKN) to oxidized N (TKN:NO3) from all NCWQR weekly sampling events shows a significant positive increase over time since the 1970 s (Fig. 1; Spearman's ρ = 0.45, p = 0.02) and a stronger increase since 1995
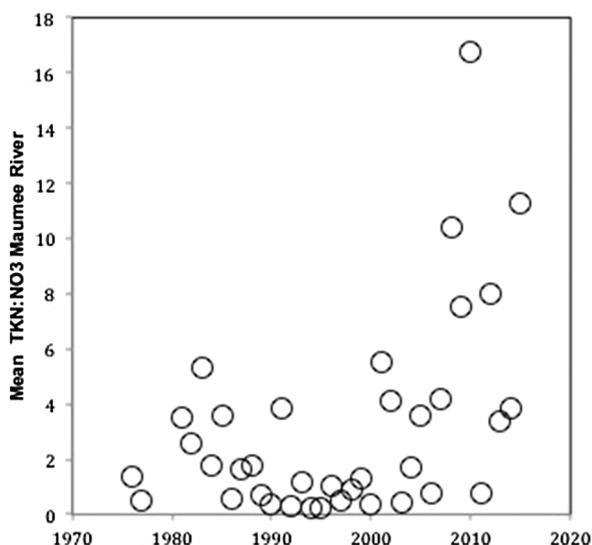
Fig. 1. The Maumee River non-nitrate fraction of the N load (TKN:NO3) has increased over time (time-weighted loads from the Heidelberg NCWQR monitoring data.) Values are annual averages of weekly sampling ratios from 1976 to 2015.
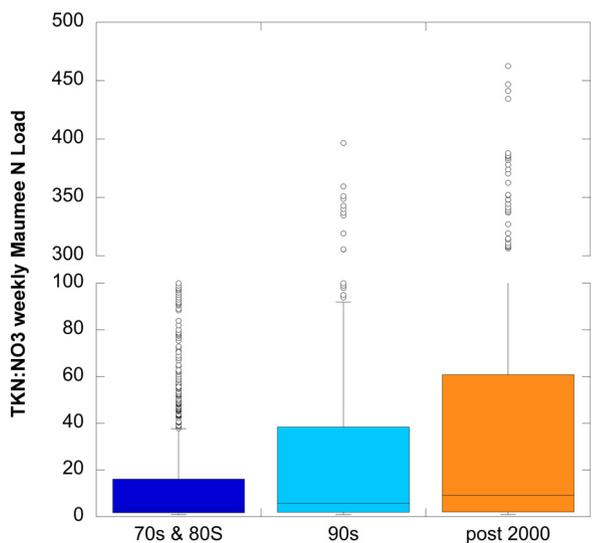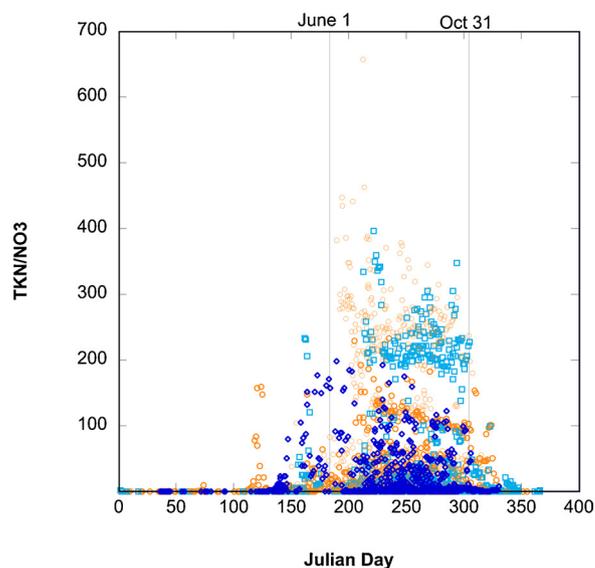

Fig. 2. Weekly TKN:NO_3 ratio of Maumee River N load in the 1980 s (dark blue), 1990 s (light blue), and post-2000 (orange). Values below 1 are not displayed (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Spearman's ρ = 0.68, p = 0.001). After 2000, TKN:NO_3 values are significantly greater than those from the 1970 s, 1980 s, and 1990 s (Kruskal-Wallis Rank Sum Test, p < .0001; Wilcoxon Rank Sign Test, p < 0.0001 between all groupings; values above 1 shown in Fig. 2). Increases in the weekly TKN:NO_3 occur between the months of May and October (Fig. 3). There is a strong, significant correlation between the total WLE cyanobacterial bloom biomass and the annual average TKN:NO_3 from weekly sampling from the Maumee River between 1996–2015 (Spearman's ρ = 0.64, p = 0.003; Fig. 4; log-transformed linear relationship also shown in Supplementary Fig. S1).

3.2. NOAA GLERL WLE weekly monitoring data

Weekly seasonal monitoring at the NOAA GLERL sampling stations in WLE from 2009 to 2015 shows similar trends (Table 1). TKN is not measured in this monitoring program, so the ratio of reduced N (sum of

Fig. 3. Weekly TKN:NO_3 ratio of Maumee River N load by Julian day in the 1980 s (dark blue diamonds), 1990 s (light blue squares), and post-2000 (orange circles). Values below 1 are not displayed (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
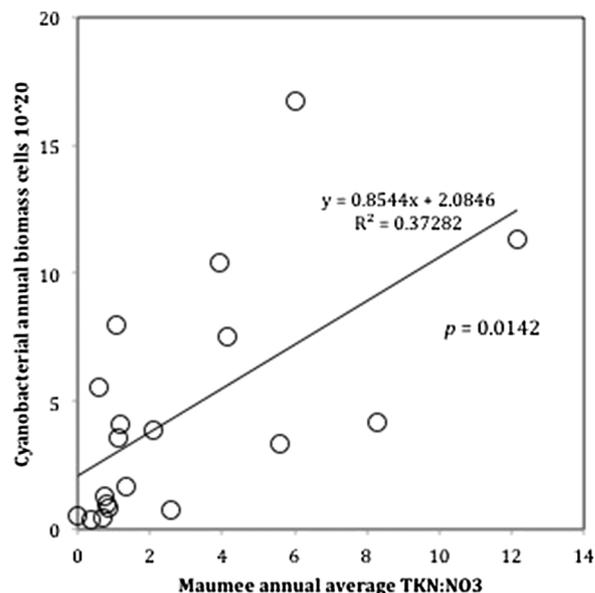
Fig. 4. Average annual TKN:NO3 in the Maumee River vs. annual total cyanobacterial bloom biomass from 1996 to 2015.

Table 1
Correlations (Spearman's ρ) between concentrations of geochemical parameters from NOAA GLERL weekly monitoring across all stations from 2009 to 2015. Significant p values are bolded.

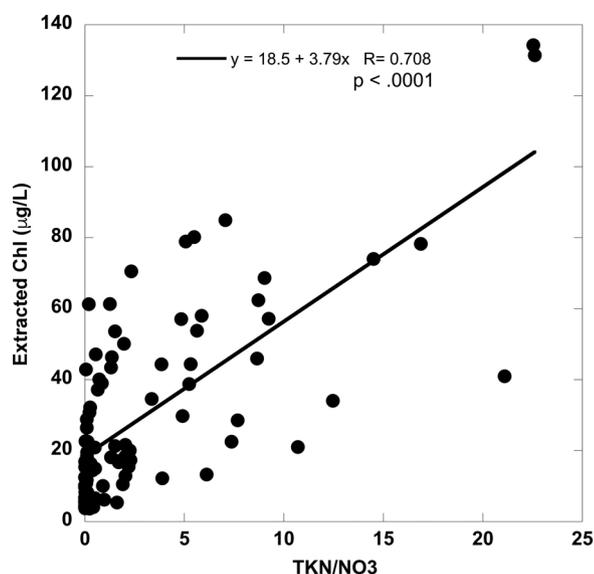|	Extracted chlorophyll	Extracted phycocyanin
TKN/NO3	0.657	0.548
p	< .0001	< 0.0001
NH_4^+	−0.527	−0.465
p	< .0001	< 0.0001
PON	0.650	0.714
p	< .0001	< .0001

Fig. 5. TKN:NO$_3$ versus extracted chlorophyll across all stations averaged by week (from NOAA GLERL monitoring in WLE 2009–2015).

NH$_4^+$ and PON) to oxidized N (NOx) concentrations from the NOAA GLERL monitoring data was used as a similar measure. Here, this ratio is referred to as TKN:NO$_3$ for consistency with the NCWQR dataset. There was a significant positive relationship between TKN:NO$_3$ and extracted chlorophyll (linear regression, R = 0.71; Fig. 5), and non-parametric analysis showed a highly significant correlation (Spearman's $\rho = 0.657$, $p < 0.0001$). Linear regression of log-transformed data showed a similar, significant trend (Supplementary Fig. 2). TKN:NO$_3$ was also significantly correlated with extracted phycocyanin (Spearman's $\rho = 0.548$, $p < 0.0001$). PON was also highly correlated with extracted chlorophyll (Spearman's $\rho = 0.650$, $p < 0.0001$) and extracted phycocyanin (Spearman's $\rho = 0.714$, $p < 0.0001$). NH$_4^+$ concentrations were negatively correlated with both extracted chlorophyll (Spearman's $\rho = -0.527$; $p < 0.0001$) and extracted phycocyanin (Spearman's $\rho = -0.465$, $p < 0.0001$ for NH$_4^+$).

4. Discussion

This study used two long-term datasets to better understand how changes in the Lake Erie N pool may be influencing bloom size, duration, and toxicity. Previous studies have clearly demonstrated links between N and bloom toxicity in microcystin-producing blooms (Gobler et al., 2016; Bullerjahn et al., 2016). Here, this study shows a link between changing ratios of N forms (TKN:NO$_3$) and increases in bloom size. The importance of N as a driver of bloom biomass has been largely overshadowed by P, as TN loading has not increased, and NO$_3^-$ concentration and average monthly loads from the Maumee River have decreased since the early 2000's (Kane et al., 2014; Stow et al., 2015). Monitoring data from the NCWQR, however, show that the Maumee River N load has become significantly more reduced, in terms of oxidation state, over time (Fig. 1). That is, the TKN proportion of the total N load has increased. Not only has the TKN:NO$_3$ from all NCWWR weekly sampling events significantly increased since the 1970 s ($p = 0.02$), but the correlation is stronger with time since 1995 (Spearman's $\rho = 0.45$ vs. $\rho = 0.68$, $p = 0.001$), when CHABs returned to Lake Erie. Likewise, current (post-2000) TKN:NO$_3$ values are significantly greater than those from the 1970 s, 1980 s, and 1990 s ($p < .0001$). This increase in chemically reduced N forms has led to a more favorable environment for cyanobacterial growth by taxa not capable of fixing atmospheric N. Previous work has shown that increased TN promotes cyanobacteria growth (Downing et al., 2001) and toxicity (Scott et al., 2013; Davis et al., 2015; Harke et al., 2016), but an

increase in the proportion of reduced N (NH$_4^+$ and urea) also favors cyanobacterial growth over diatoms (Blomqvist et al., 1994; McCarthy et al., 2009; Glibert et al., 2016).

The timing of the TN shift to predominantly non-NO$_3$ is also critical, as those increases occur between the critical months of May (fertilization is recommended after spring planting; Millar et al., 2014) and October (Fig. 3). With regard to onset of the bloom, there is a disconnect between the delivery of SRP with spring rain (March to June; Stumpf et al., 2016) and the onset of the bloom in July (Bridgeman et al., 2013). This disconnect in the timing of the SRP delivery and HAB onset is particularly puzzling given that the residence time of WLE is, on average, 51 days (Millie et al., 2009) and likely much shorter during higher discharges typically observed in spring. Thus, some proportion of spring P loads likely passes through WLE to the central basin during the critical spring loading period. Presumably, the majority of the TP is maintained in WLE within biomass and subsequently recycled, given the strong relationship between total annual bloom size and TP load. In direct contrast, the timing of the *Microcystis* bloom (which may also be tied to temperature or other factors) is very similar to the timing of the shift in the Maumee N load from predominantly NO$_3^-$ to predominantly TKN, where the highest ratios of TKN:NO3 typically occur after July 1, around July 10 or Julian Day 191 (Fig. 3). This coincides very closely with the onset of the annual summer HAB. Increased N turnover from bloom biomass may further increase the TKN:NO3 ratio as nitrate is drawn down. However, Maumee loading data from July through October already shows a median TKN:NO3 ratio of 16:1 and a mean ratio of 66:1 since 2000, so the external N source is already heavily weighted toward reduced N.

As mentioned previously, the resurgence of cyanobacterial blooms in WLE significantly and positively correlates with increased SRP loading from the Maumee River (Kane et al., 2014; Baker et al., 2014; Michalak et al., 2013), but no similar correlation was found for the TN or NO$_3^-$ loads and annual bloom biomass (Kane et al., 2014). There is, however, a strong, significant correlation between WLE cyanobacterial bloom biomass and the annual average TKN:NO3 from weekly Maumee River sampling from 1996 to 2015 ($p = 0.003$). TKN includes organic, particulate N in cells and therefore could have a tautologous relationship with cyanobacterial cell biomass (e.g., Lewis and Wurtsbaugh, 2008). Similarly, the relationship between TP and chlorophyll has frequently been used to argue that P is the main driver and limiting factor for bloom biomass (Schindler et al., 2008; Paterson et al., 2011). In the case of TKN, though, internal and repeated recycling of biomass to readily bioavailable N suggests a potentially compounding effect for the biomass component of TKN.

Remineralization of organic matter and, therefore, recycling of NH$_4^+$ happens very quickly within cyanobacterial blooms (Gardner and Lee, 1975; Paerl et al., 2011; McCarthy et al., 2013). For example, recycling rates were an order of magnitude higher during the cyanobacterial bloom in WLE in 2015 than before or after the bloom (Gardner et al., 2017). Reduced N (NH$_4^+$) concentrations also remained high enough during the 2010 bloom to sustain high biomass (Chaffin et al., 2011). In Lake Taihu and Missisquoi Bay, Lake Champlain, regeneration of NH$_4^+$ from organic matter has vastly exceeded total annual N loads and was an important factor supporting summer CHABs (McCarthy et al., 2007; Paerl et al., 2011; Hampel et al., 2018; McCarthy et al., 2013). Thus, TKN includes a pool of N that is both immediately bioavailable (e.g., NH$_4^+$ and urea) and very likely to become bioavailable over short time periods (e.g., seasonal).

Given the significant relationship between TKN:NO$_3$ in the Maumee River load to annual cyanobacterial bloom biomass in WLE, the relationship for the weekly NOAA GLERL sampling stations in WLE from 2009 to 2015 was also examined. There was a significant positive relationship between TKN:NO$_3$ and extracted chlorophyll (Fig. 5) and phycocyanin. PON alone was also signficantly correlated with extracted chlorophyll ($p < 0.0001$) and phycocyanin ($p < 0.0001$), although this relationship is tautalogous (Lewis and Wurtsbaugh, 2008) and

expected to strongly correlate with biomass. There was a significant, positive relationship between PON and particulate microcystins, although the correlation was not as strong, but this relationship may also be tautalogous, as microcystin includes 10 N atoms (Davis et al., 2015).

NH_4^+ concentrations were negatively correlated with both extracted chlorophyll and extracted phycocyanin (p < 0.0001 for both). Given the high bioavailability of NH_4^+, any increase in cyanobacterial biomass would be expected to coincide with a strong drawdown of NH_4^+. Despite this, the importance of NH_4^+ is often overlooked (e.g., Dove and Chapra, 2015) because concentrations are often low or undetectable during traditional, 'snapshot' sampling. Snapshot concentration measurements do not accurately reflect availability; rather, turnover rates (e.g., uptake and regeneration) must be measured to determine its actual availability to primary producers. TKN includes PON bound in cells, which are often readily remineralized to dissolved inorganic forms (e.g., (Gardner and Lee, 1975)), and is a better reflection of N that is either immediately bioavailable or can be remineralized quickly (Gardner et al., 1989; Gardner et al., 2017).

These data show strong, significant relationships between increases in the reduced fraction of TN (both in the annual load from the Maumee and in the measured concentrations at WLE monitoring sites) and annual cyanobacterial bloom biomass, total chlorophyll, and phycocyanin. Consideration of only NO_3^- or TN is not sufficient to monitor or predict bloom severity and toxicity. While others have shown a clear relationship between N concentrations and toxicity (Gobler et al., 2016), and TN and cyanobacterial biovolume (Rigosi et al., 2014), this study is, to our knowledge, the first documentation of a relationship between N form and annual bloom biomass in WLE. These data show that the often-overlooked reduced N fraction is also a significant driver of bloom biomass. This correlation between the reduced N fraction from the Maumee River and bloom biomass demonstrates the urgent need to control N loading, in addition to current P load reductions, to WLE and similar systems impacted by toxin-producing, non-N-fixing cyanobacteria.

Numerous studies have shown that the combination of N and P often results in higher cyanobacteria biomass than either nutrient added singularly (e.g., Elser et al., 2007; Lewis and Wurtsbaugh, 2008; Scott and McCarthy, 2010). Unfortunately, the current reality is that most impaired ecosystems have already achieved the often simulated conditions of simultaneous over-enrichment of N and P. It is often claimed that N is too expensive to control in watersheds of eutrophic, cyanobacteria-impacted systems (e.g., Schindler et al., 2008; Schindler, 2012). Within the Lake Erie watershed, existing educational and diffuse loading reduction programs are already being implemented for P to achieve the new Annex 4 target of an additional 40% reduction beyond previously established targets. These strategies will likely also contribute to N loading reductions, and there is evidence that N (and P) loads to WLE are decreasing already (Stow et al., 2015), but the proportion of TKN in those N loads is increasing, as reported here. In addition, bloom severity continues to increase, and climate change will likely result in 'moving targets' for nutrient thresholds for the foreseeable future. As such, scientists and managers should focus more effort on educating the public and agriculture interests about the need to control N loads in combination with P (i.e., adopt comprehensive nutrient management programs including both N and P) within existing educational and management programs. Utilizing existing outreach programs means that these additional education and management efforts can be implemented at little or no cost. Scientists and regulators also need to act quickly to implement and quantify thresholds for N reductions to WLE and other non-N-fixing CHABs impacted systems. It is critical to begin quantifying the extent to which these reduction strategies simultaneously reduce N without being specifically targeted. A complete N budget for Lake Erie needs to be formulated and assessed to determine if additional N reductions are needed to further limit bloom biomass and toxicity. Organizations such as the International Joint Commission (IJC) also should be encouraged to adopt and include

similar recommendations to those recently advocated by the USEPA (https://www.epa.gov/sites/production/files/documents/nandpfactsheet.pdf), which advocates for comprehensive management of both N and P to reduce eutrophication and cyanoHABs.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2018.11.003.

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