Tracking Microcystis N and C Uptake from Urea in Whole Lake Water

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Abstract

Lake Erie experiences annual summer cyanobacterial harmful algal blooms (cHABs) dominated by the cyanobacterium, *Microcystis*. *Microcystis* species are composed of a wide variety of strains, some of which produce the hepatotoxin microcystin. Lake Erie cHABs result from eutrophication driven primarily by agricultural runoff. About 60% of the world's nitrogen fertilizer is in the form of urea, which has increased ~100-fold in the last four years. Unlike nitrate and ammonia, which are inorganic sources of nitrogen, urea is an organic source. A previous study found that cultured *Microcystis* can use both nitrogen and carbon from dual-labeled (^{15}N, ^{13}C) urea. To analyze the potential of carbon and nitrogen uptake from urea in uncultured Western Lake Erie *Microcystis*, we incubated whole lake water samples with dual-labeled urea. The collection of *Microcystis* strains sampled from the lake did not incorporate carbon but did incorporate nitrogen. Overall, this work provides further evidence that Microcystis uses urea as a source of nitrogen, but that the use of urea as a carbon source may vary across different Microcystis strains and/or environmental conditions.

Keywords: Microcystis, Lake Erie, cHABs, urea, nitrogen

Introduction

Cyanobacterial harmful algal blooms (cHABs) cause severe damage to aquatic ecosystems and threaten human health worldwide (Binding et al., 2020; Yancey et al., 2023). The Laurentian Great Lakes have experienced increasing threats from cHABs in recent years. Lake Erie, specifically, experiences annual cHABs dominated by the cyanobacterium, *Microcystis* (Berry et al., 2017). *Microcystis*-dominated blooms are one of the most common algal blooms in freshwater environments (Wu et al., 2015). They have been documented in 108 countries and 6 continents (Harke et al., 2016; Krausfeldt et al., 2019). There is a large diversity of *Microcystis*

strains and many studies use lab cultures that are not necessarily indicative of the environment, especially when they were isolated in different locations and times. For example, the strain NIES 843 was isolated from Lake Kasumigaura, Ibaraki, Japan on August 28, 1997 (*NIES Collection MICROBIAL CULTURE COLLECTION | National Institute for Environmental Studies*, n.d.). Moreover, many cultured strains are axenic and non-colonial, unlike what we find in environmental samples.

Microcystis can produce the “fast-death factor” hepatotoxin, microcystin (MC), making it a deadly threat to our freshwater systems (Wu et al., 2015). The ingestion of microcystin can cause a multitude of health problems in humans and animals, like liver issues, cancer, and death (EPA, 2024). The production of microcystin makes it critical to understand the importance of nutrients to bloom formation and dynamics because they threaten drinking water and aquatic species' health. For example, in August 2014, Toledo, Ohio was without drinking water for more than 4 days due to elevated levels of microcystin in the city’s final treated drinking water leading to a state of emergency (Jetoo et al., 2015; Steffen et al., 2017). The 2014 August bloom led to a 65 million dollar loss for Ohio when taking into account property value, tourism, recreation (fishing, boating, and beach-going), and water treatment (Bingham et al. 2015).

While phosphorus is considered to be the main driver of *Microcysti*s bloom biomass in Lake Erie, and has been the focus of nutrient management policy, nitrogen plays an important role in determining the toxicity. Microcystin is a nitrogen-rich molecule with over 600 congeners. Congeners range in toxicities and have varying nitrogen requirements. Thus, understanding nitrogen uptake becomes important for understanding bloom toxicity. Almost a decade after the Toledo Water Crisis, cHABs in Lake Erie are still an annual event. At the same time, urea fertilizers have been replacing nitrate fertilizers (Krausfeldt et al., 2019; Lad et al., 2022). Due to urea’s popularity as an agricultural fertilizer, it is being linked to algal bloom and eutrophication events in freshwater ecosystems (Glibert et al., 2014; Krausfeldt et al., 2019). Currently, about 60% of the world’s nitrogen fertilizer is in the form of urea, which has increased ~100-fold in the last four years (Glibert et al., 2014; Krausfeldt et al., 2019; Zhou et al., 2019). This increase in urea greatly contributes to the availability of nitrogen to cyanobacteria, like *Microcystis,* in eutrophic lake waters because urea consists of two nitrogens (Bogard et al., 2012; Zhou et al., 2019). Nitrogen and phosphorus are not only essential nutrients for the growth of

Microcystis but large amounts are needed for the production of microcystin (Vézie et al., 2002; Zhou et al., 2019).

As an organic nitrogen source, urea also contributes carbon to the ecosystem. The incorporation of carbon from urea is thought to be a competitive advantage for *Microcystis aeruginosa* NIES-843, particularly in times of low carbon availability during dense blooms (Krausfeldt et al., 2019). The incorporated ^{13}C was assimilated into pathways important for microcystin production such as central carbon metabolism and amino acid biosynthesis. However, this study, Krausfeldt et al., 2019, may not be representative of *Microcystis* nutrient uptake in Lake Erie due to differences in cultured vs uncultured experiments as previously discussed.

In this study, whole lake water was isolated during a visual cHABs bloom in Lake Erie to capture the complex microbial community. We hypothesized that a 4-hour incubation with dual-labeled urea would result in the direct assimilation of ^{15}N and ^{13}C from urea into *Microcystis* while limiting the transformation and secondary assimilation of ^{15}N and ^{13}C into *Microcystis* and other community members. We measured ^{15}N incorporation into *Microcystis* cell biomass but no measurable ^{13}C incorporation by *Microcystis*. Therefore, the length of the incubation and/or the Microcystis strain diversity may be important to consider when analyzing nutrient uptake.

Methods

Initial sample collection

Figure 1: Map of the Western Lake Erie basin with station locations (WE09 and WE16) indicated. Samples were taken from WE16 on August 3, 2021, and from WE09 on August 31, 2021.

Whole lake water samples were collected, using NOAA research vessels, from Lake Erie on August 3, 2021, from station WE16 (41.660, -83.143) and August 31, 2021, from station WE09 (41.718, -83.424) during a *Microcystis* bloom. Samples were collected from the surface to 1 m above the bottom (6.2m and 2.7m) using a peristaltic pump. Samples were kept in coolers until arrival back in the lab approximately 6 hours after initial collection.

Upon arrival at the lab, whole water was gently mixed and allocated into 50 mL sterile Falcon tubes. Urea additions were completed in the 50 mL canonical tubes before being aliquoted into 15 mL triplicate samples. Final dual-labeled urea concentrations were 0.5, 2, and 20 µM. One control was killed by the addition of formalin (final concentration 1%), dual-labeled urea was added (final concentration 20 µM), and then incubated for 4 hours. Sample triplicates were incubated for 4 hours at 200 µmol photon m^{-2} s^{-1} of light and 22℃ before being terminated by the addition of formalin (final concentration 1%). The 10 samples were stored at 4°C and shipped to Lawrence Livermore National Laboratory (LLNL) for further processing.

NanoSIMS

At LLNL, one replicate per concentration was filtered (15 mL) through Isopore Membrane 0.2um filters using a Millipore Blue Angel. To evaluate the incorporation of ^{15}N and ^{13}C in cells, stable isotope probing (SIP) using nanoscale imaging mass spectrometry (NanoSIMS) was used as described in Chaffin et al., 2022 and Mayali et al., 2023. This method allowed the quantification of nutrient assimilation of a single *Microcystis* cell in high resolution. The primary 133Cs+ ion beam was set to 2 pA with a 150 nm beam diameter at 16 keV. Images taken with a CAMECA NanoSIMS 50 at LLNL were 265x265 created over a 20x20 raster µm analysis area. An initial beam current of 90 pA to a depth of ~60 nm to achieve sputtering equilibrium. Therefore, guaranteeing the analysis of intracellular isotropic material. Secondary electron (SE) images and $^{12}C_2^-$, $^{13}C^{12}C^-$, $^{12}C^{14}N^-$, and $^{12}C^{15}N^-$ quantitative ion images were collected on individual electron multipliers.

Image Processing

Data collected from NanoSIMS was processed using L'Image to create 15N/14N and 13C/12C permil images, which indicates the level at which ^{13}C and ^{15}N are incorporated into the *Microcystis'* biomass. *Microcystis* were identified using the SE channel. The secondary electron images show 3D versions of *Microcystis'* allowing us to identify them using L'image. Specifically, we calculated the 12C/13C permil and 15N/14N permil to quantify how much ^{13}C and ^{15}N were used from dual-labeled urea. 37 images were analyzed and 538 data points were generated for *Microcystis.* It is also important to note that due to an error in labeling, data from WE16 20 µM was not reported and is missing in the analysis.

Results

To get a better understanding of the environment during the bloom we looked at extracted phycocyanin and particulate microcystin values measured by NOAA GLERL. Extracted phycocyanin is a proxy for *Microcystis*. It is a pigment, like chlorophyll, but more specific to cyanobacteria (Almuhtaram et al., 2021). We assumed that the amount of phycocyanin correlated reasonably well with how much *Microcystis* is present.

Results show that at these stations, an early bloom occurred in early August, peak bloom occurred in late August, and the post-bloom occurred in late September (Figure 2). High values of extracted phycocyanin were measured at the location and timing of sample collection, indicating both sites had high amounts of *Microcystis* compared to other times in the season (Figure 2). WE09 (peak bloom) had a slightly higher value than WE16 (early bloom). While our samples did not capture the peak identified on September 13, 2021, our efforts were limited due to boat scheduling. Particulate microcystin concentrations, however, did peak at sampling points around August 2nd at station WE16 and August 30, 2021 at station WE09 (Figure 3).

There is little to no particulate microcystin leading up to the first date in August (Figure 3). The peak value of particulate microcystin at WE09 is in early September and decreases in late September (post-bloom). Both dates of collection were at the highest values of particulate microcystin concentration for each station. (Figure 3).

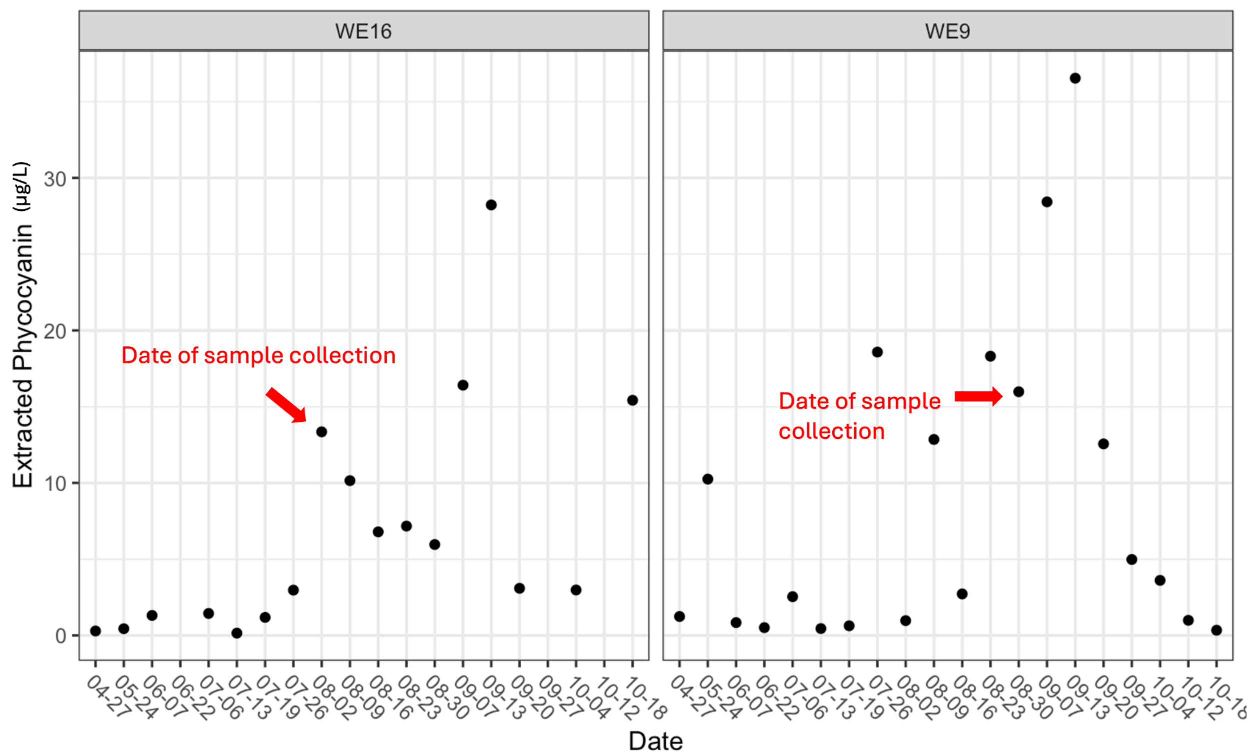

Figure 2: Extracted phycocyanin (µg/L) samples during the 2021 (April-October) bloom in Lake Erie at sites WE16 and WE09. The red arrow points to when whole water samples were taken during the bloom (roughly the same date).

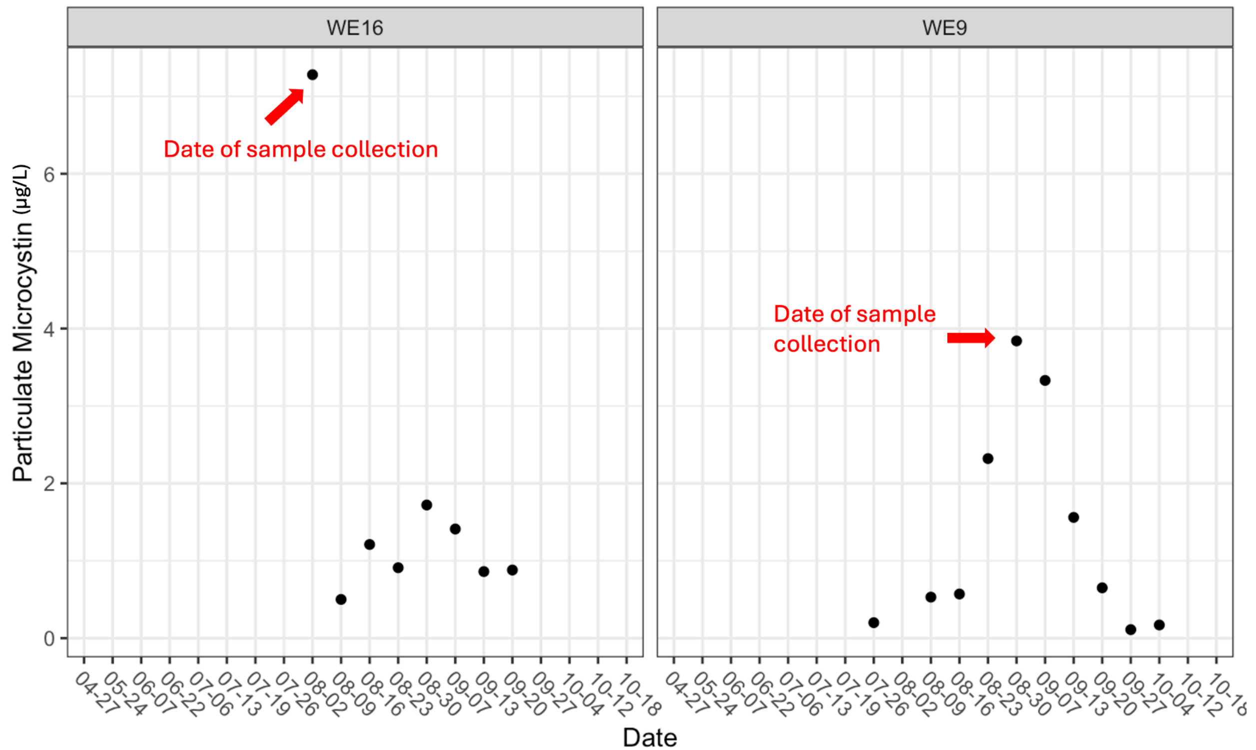

Figure 3: Particulate microcystin (µg/L) in the field during the 2021 (April-October) bloom in Lake Erie at sites WE16 and WE09. Missing values correspond to <0.1µg/L amounts of particulate microcystin. The red arrow points to when whole water samples were taken during the bloom (roughly the same date).

We aimed to quantify the amount of ^{13}C and ^{15}N from dual-labeled urea incorporated into *Microcystis* cells during different bloom phases. To address our question we added dual-labeled urea to whole lake water that contains a diversity of *Microcystis* strains. After a 4-hour incubation, no uptake of ^{13}C was observed by any of the *Microcystis* at any of the tested dual-labeled concentrations of urea addition (Figures 4 and 5). An experimental error precluded the use of the 20 µM treatment for WE16. The 12C/13C permil for both sites was predominantly negative, ranging from -254 to 13 indicating no carbon was incorporated from the added labeled urea (Figure 5). Additionally, these values are similar to or lower than that of the kill control which did not allow for microbial uptake of urea.

However, there was an obvious incorporation of ^{15}N into *Microcystis* cells (Figures 4 and 6). The incorporation at 0.5 and 2 µM was slightly lower at WE09 than the WE16 (Figure 6). At station WE09 there was noticeably less incorporated ^{15}N at 20 µM than other concentrations (Figure 6). At the medium urea concentration (0.2 µM) ^{15}N incorporation was highest, while ^{15}N incorporation was lowest at the highest urea concentration (20 µM) for WE09. The lowest

concentration (0.5 μM) had the least incorporation of ^{15}N at WE16. There is a separation of clusters at site WE09 and not at WE16 (Figure 5). Specifically, at 0.5 μM there are 3 distinct clusters. The highest cluster is at the same 15N/14N ratio as the 0.5 μM concentration at WE16 (Figure 5).

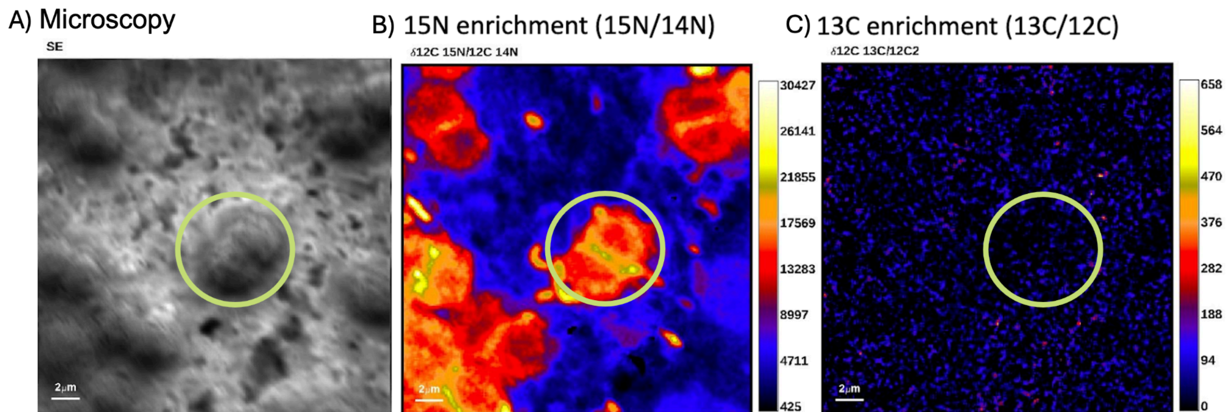

Figure 4: Images of a 4-hour incubation with dual-labeled urea ($^{15}N^{13}C$). Image A) shows the secondary electron channel (SE) spotlighting where Microcystis are located (green circle). Image B) illustrates nitrogen enrichment (highlighted in red and orange). Warmer colors correspond to higher enrichment of urea. Image C) exhibits carbon enrichment (there is almost none in this image).

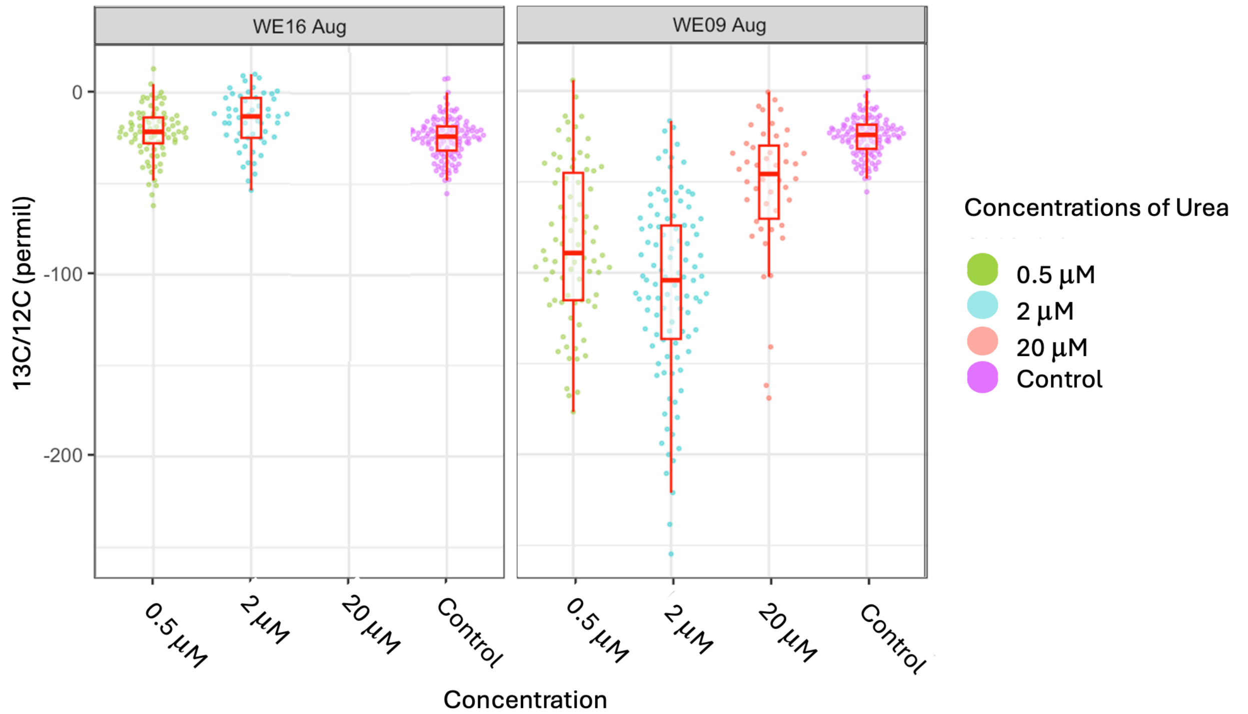

Figure 5: Incorporated ^{13}C into cell biomass from dual labeled urea in 13C permil. Each dot represents a *Microcystis* cell. There are negative values of ^{13}C in all samples. Therefore, no ^{13}C was used by cells. The control acts as a baseline measurement where cells are killed before incubation time begins. Due to an experimental error, data from WE16 20 µM was not included.

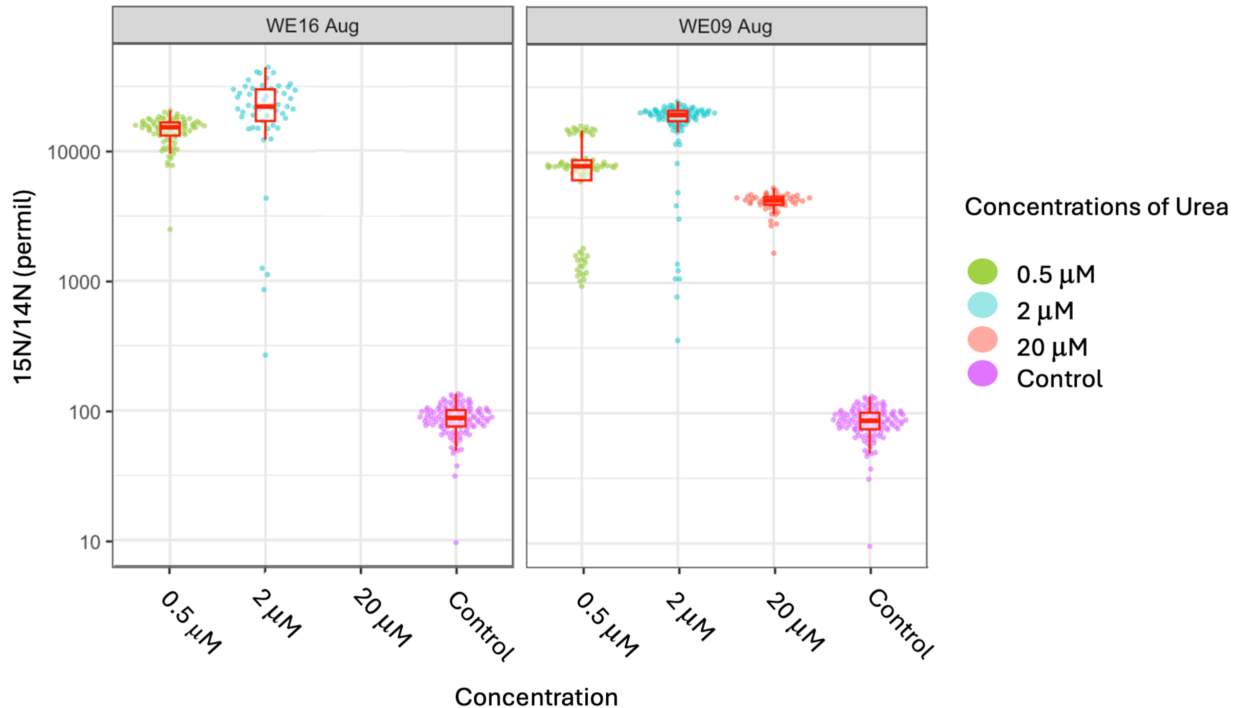

Figure 6: Incorporated ^{15}N into cell biomass from dual labeled urea. Measurements are in 15N permil and are displayed on a logarithmic scale. Each dot represents a *Microcystis* cell. At medium concentration, we see the most incorporation, while at the highest we see the least at both sites. The control acts as a baseline measurement where cells are killed before incubation time begins. Due to an experimental error, data from WE16 20 µM was not included.

Discussion

Western Lake Erie experiences annual cHABs, but the severity of the bloom is hard to predict. Eutrophication resulting from agricultural runoff contributes to the ongoing cHABs (Nie et al., 2018). With the increasing use of urea by the agricultural community, it is increasingly important to understand how organic sources of nitrogen like urea are utilized compared to inorganic sources such as nitrate and ammonia (Glibert et al., 2014). Because the lake environment is complex and hard to replicate, this study used integrated whole lake water samples from Lake Erie to address the question of nitrogen and carbon uptake by *Microcystis*. A recent study showed that *Microcystis* can assimilate both carbon and nitrogen from urea and several have hypothesized it (Cai et al., 2021; Krausfeldt et al., 2019). This is contrary to our work that found only nitrogen was incorporated from urea.

Cyanobacteria use a catalytic enzyme called urease to assimilate urea. Urease converts urea into ammonia and carbon dioxide, which can then be used by cyanobacteria (Belisle et al., 2016). It is assumed that the *Microcystis* in the whole water samples have the urease gene necessary to use urea as a nutrient because several Lake Erie *Microcystis* strains contain the gene (Yancey et al., 2023). *Microcystis* can thrive in low CO_2 conditions because of its CO_2-concentrating mechanism (CCM) (Krausfeldt et al., 2019; Sandrini et al., 2015). The CCM requires ATP, so the CO_2 byproduct of urease may provide an easier source of CO_2 to the *Microcystis* because dissolved CO_2 gets rapidly converted to bicarbonate. However, usually, *Microcystis* use already present bicarbonate as a carbon source in the water column because even less energy is needed to use it than CCM or urease (Ji et al., 2020; Wilhelm et al., 2020). Therefore, accessible bicarbonate may be preferentially used (Wu et al., 2015).

The strain type may also be a factor in the *Microcystis* cells' ability to use the carbon from urea. Lake Erie hosts a variety of *Microcystis* strains (Yancey et al., 2023). Different strains have diverse and sometimes distinct genomes that require various nutrients and concentrations (Yancey et al., 2023). For example, microcystin-producing strains require more nitrogen to sustain the microbe than a non-microcystin strain (Vézie et al., 2002). Therefore, these strains could quickly cycle through the available urea and have to take advantage of both the carbon and nitrogen in urea to maintain growth. This may explain why toxic strains such as NIES 843 used in previous work took up ^{13}C after 7 days (Krausfeldt et al., 2019). However, we also likely had microcystin-producing strains in our water sample based on previous results (Yancey et al., 2023). Additionally, our samples were collected when the bloom had high levels of microcystin (Figure 3). Another potential explanation for the different results in our study versus Krausfeldt et al. may be the shorter incubation time; over a 4-hour incubation period, the *Microcystis* did not have time to incorporate the $^{13}CO_2$ released by urease. In the 7-day incubation conducted by Krausfeldt et al., time was likely not a limiting factor.

During the early and peak bloom, there was an abundant ^{15}N in cell biomass for all concentrations (Figure 5). The highest concentration of urea had the least amount of ^{15}N in cells, while the medium concentration had the highest amount (Figure 5). Other studies have shown similar results, lower concentrations of urea promoted more growth and incorporation of ^{15}N (Krausfeldt et al., 2019; Wu et al., 2015). Additionally, several studies have discovered similar

outcomes of *Microcystis* using ^{15}N from urea to acquire nitrogen (Cai et al., 2021; Wu et al., 2015; Zhou et al., 2019).

We also see large variations in our data for each concentration. This is likely due to the use of whole lake water, which consists of several strains. This may explain why we see 3 distinct clusters during the peak bloom for 0.5 µM concentration (Figure 6). Each cluster could represent distinct strains with different nitrogen uptake requirements or rates. Urea as a nitrogen source has also been thought to be advantageous to *Microcystis.* Urea is more suitable for the growth and development of *Microcystis* than other organic and inorganic nitrogen sources, including NH_4^+, $NO3^-$, ornithine, glucosamine, hypoxanthine, and guanine (Berman & Chava, 1999; Wu et al., 2015). Naturally, urea occurs in a range of concentrations but the increase in urea-based fertilizers may impact the available urea in the future (Cai et al., 2021; Wu et al., 2015). One study found that planktonic communities' uptake rates of Urea-nitrogen contribute to more than 50% of the nitrogen they assimilate (Solomon et al., 2010; Wu et al., 2015). Therefore even at low concentrations of urea, *Microcystis* can take advantage of ^{15}N.

Another concern regarding the use of urea by *Microcystis* is its impact on the toxicity and production of microcystins. Low concentrations of urea promote the production of microcystins, while high concentrations decrease toxicity (Huang et al., 2014). Zhou found a similar result, that low concentrations of urea create a more toxic environment due to the increase in microcystins (Zhou et al., 2019). Therefore in lower concentrations of urea in the natural environment, there may be an increase in *Microcystis* growth and production of microcystin, while high concentrations would mute the impact of urea entering the waterways. We found similar results in our data. Lower concentrations of urea had more ^{15}N uptake which would likely mean there is more growth occurring (Figure 6).

Conclusions

The importance of understanding the use of urea as a nutrient source by *Microcystis* in Lake Erie is timely and critical because lake environments are complex and hard to replicate, and the use of urea as a fertilizer is projected to double by 2050 (Bogard et al., 2012). In this study, integrated whole water samples from Lake Erie were used to address the question of nitrogen and carbon uptake by *Microcystis*. After a 4-hour incubation with dual-labeled urea, *Microcystis* did not incorporate ^{13}C into their biomass but did incorporate ^{15}N. This finding contradicts

previous studies that hypothesized or found that *Microcystis* can and will use urea as a carbon and nitrogen source (Cai et al., 2021; Krausfeldt et al., 2019; Wu et al., 2015). We hypothesize that *Microcystis* strain and ability to produce microcystin may be factors in determining the use of ^{13}C. Additionally, this study reinforces that urea is a nitrogen source for *Microcystis*. Future work should focus on how strain diversity may impact the incorporation of ^{15}N and ^{13}C from urea into *Microcystis*. Understanding this within the context of environmental diversity present in freshwater will be crucial in the control and mitigation of fertilizer run-off responsible for cHABs. The consequence of small amounts of urea entering our waterways may increase bloom intensity and toxicity affecting wildlife and human activities.

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