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# Urea as an Effective Nitrogen Source for Cyanobacteria

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# Abstract

Urea use has grown substantially in the past half-century, with urea now accounting for > 50% of nitrogen fertilizer applications worldwide. The shift from inorganic nitrogen fertilizers to urea-based sources has coincided with the reappearance of cyanobacteria blooms in freshwaters. Here, we examined urea as a nitrogen source for three bloom-forming cyanobacteria species. We found that (1) urea was consumed more rapidly relative to inorganic nitrogen substrates, suggesting that cyanobacteria exhibit a preference for urea; (2) urea was consumed in excess of cellular requirements; and (3) urea may offer cyanobacteria a competitive edge over eukaryotic algae by enhancing light absorption capabilities. These findings build on the growing body of literature demonstrating the importance of urea in freshwater eutrophication and satisfying the nitrogenous needs of cyanobacteria. As society's reliance on urea is projected to escalate, it is important that we understand the unintended consequences of urea discharge on receiving freshwaters.



**Keywords:** freshwater, cyanobacteria, cyanobacteria harmful algal blooms (cyanoHABs), eutrophication, fertilizer, nitrogen, urea

# Co-Authorship Statement

This M.Sc. thesis is part of a research project funded by NSERC CREATE on Algal Bloom Abatement through Technology and Education (ABATE), awarded to Dr. Irena Creed and Dr. Charles Trick. This thesis has been formatted into two manuscripts that will be submitted to the journal *Freshwater Biology* and *Harmful Algae*. I will be the lead author, as I contributed to the conceptual design, completion of experiments, analyses of data, and writing of the manuscript. Co-authors, Dr. Irena Creed and Dr. Charles Trick, contributed the conceptual design, interpretation of results, the writing and editing of the manuscripts and provided the financial resources to complete the study.

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# Abbreviations

| | |
|---|---|
| α | Initial slope of photosynthesis (photosynthetic efficiency) |
| APC | Allophycocyanin |
| ATP | Adenosine Tri-Phosphate |
| BG-11 | Blue-green medium #11 |
| chl-*a* | Chlorophyll a |
| CPCC | Canadian Phycological Culture Centre |
| CyanoHAB | Cyanobacteria Harmful Algal Bloom |
| dd$H_2O$ | Double distilled water |
| DIN | Dissolved inorganic nitrogen |
| DON | Dissolved organic nitrogen |
| N | Nitrogen |
| NA | Nitrogenase |
| $N_2$ | Atmospheric nitrogen |
| $NH_3$ | Ammonia |
| $NH_4^+$ | Ammonium |
| $NO_3^-$ | Nitrate |
| NR | Nitrate Reductase |
| NiR | Nitrite Reductase |
| $O_2$ | Oxygen |
| PI Curve | Photosynthesis-Irradiance response curve |
| PC | Phycocyanin |
| PE | Phycoerythrin |
| $P_{max}$ | Maximum rate of photosynthesis (light saturated point) |

# Chapter 1

# 1 Introduction

## 1.1 Problem Statement

Freshwater algal blooms formed by cyanobacteria have been on the rise in Canada (Winter *et al*., 2011; Pick, 2016), and other north-temperate regions have seen an analogous trend (Taranu *et al*., 2015). The drivers of enhanced bloom frequency and duration are not fully understood, but conventional wisdom implicates increased nutrient availability combined with elevated temperatures and longer growing seasons (O'Neil *et al*., 2012; Paerl & Otten, 2013; Pick, 2016). The widespread use of chemical fertilizers linked to modern agricultural practices has enhanced the fertility of surface waters promoting the expansion of harmful cyanobacteria blooms (cyanoHABs) (Oliver *et al*., 2012; O'Neil *et al*., 2012). While phosphorus (P) has been recognized as the principal agent regulating phytoplankton productivity in inland waters (Schindler, 1977), elevated P is not a universal trigger for bloom initiation (Paerl & Otten, 2013; Paerl *et al*., 2016). P fertilizer applications across the globe have now been outpaced by nitrogen (N) fertilizer use. The associated change in nutrient loads to freshwaters has created a scenario by which lakes are now saturated with excess N relative to P (Elser *et al*., 2009; Glibert *et al*., 2014).

Not only has the supply of N entering surface waters increased, but the chemical composition has also been altered (Glibert *et al*., 2006; Paerl *et al*., 2016). The use of inorganic N fertilizers ($NO_3^-$, $NH_4^+$) has declined in favour of urea-based products; with urea now accounting for more than half of the total N-fertilizer applications worldwide (Finlay *et al*., 2010; Glibert *et al*., 2014). This contemporary shift in fertilizer consumption patterns has coincided with the extensive re-emergence of cyanoHABs in freshwaters (Glibert *et al*., 2014; Belisle *et al*., 2016). As cyanobacteria are the phytoplankton group of greatest concern in inland waters, this thesis aims to: (1) predict how freshwater cyanobacteria may respond to current and future urea pollution by

examining the extent to which urea is incorporated into growth and photosynthetic processes, and (2) investigate whether cyanobacteria exhibit preferential use of urea over inorganic N forms. Society's reliance on urea-based fertilizers is projected to increase further (Finlay *et al*., 2010), and it is important that we improve our understanding of the potential unintended consequences urea pollution may have on the ecology of primary producers in receiving water bodies.

## 1.2 Cyanobacteria: Pioneers of Primitive Earth

Cyanobacteria are a primitive group of photosynthetic prokaryotes and have been a natural part of the Earth's oceans and lakes for about 3.5 billion years (Paerl *et al*., 2001). As one of the earliest of inhabitants known to exhibit oxygenic photosynthesis, cyanobacteria's diversification and subsequent expansion during the Earth's early history was responsible for converting the Earth's previous anoxic atmosphere into an oxygen oasis—a phenomenon referred to as the great oxygenation event (Nisbet, 1985; Kasting, 1993). Cyanobacteria also played an important role in the origin of plants. Cyanobacteria forged an endosymbiotic relationship with eukaryotic algae and higher plants, with the incorporation of cyanobacteria functioning as a precursor to the chloroplast (Kulasooriya, 2011). The evolution of higher plants resulted in the "greening" of the terrestrial world, which played a pivotal role in molding the Earth's climate and shaping the evolutionary trajectory of life on planet earth (Kenrick & Crane, 1997). Furthermore, some cyanobacteria have the capacity to process "inert" atmospheric N ($N_2$) to biologically accessible forms thereby loosening N constraints that frequently limit ecosystem productivity (Paerl *et al*., 2001). Although cyanobacteria have made numerous beneficial contributions to re-engineering the planet into its current productive state, these tiny microbes also harbour other, more sinister characteristics that can lead to series of unfortunate circumstances when dense aggregates accumulate in surface waters (Explored in Section 1.5).

## 1.3 Characteristics of Cyanobacteria

Often regarded as physiologically simple due to their prokaryotic cell organization, the diversification of cyanobacteria has given rise to a variety of cellular arrangements. The two dominant configurations that exist include: (1) coccid cells that exist either as free-floating unicellular organisms or colonial forms that produce aggregated masses; and (2) filamentous varieties that form intricate linkages of individual cells (Paerl *et al*., 2001). Being prokaryotic organisms, cyanobacteria share striking structural similarities to bacteria, in that they both lack membrane-bound organelles and a defined nucleus (Paerl & Otten. 2013). However, unlike most other prokaryotes, cyanobacteria contain chlorophyll-*a* (chl-*a*) and undergo oxygenic photosynthesis. In addition to chl-*a*, cyanobacteria have phycobilins, accessory pigments which function as light absorbing complexes (Gantt, 1975; Oliver & Ganf, 2000). Phycobilins, phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE), work in combination with chl-*a* to extend the range of light attenuation and act as photo-protectants, which help minimize photo-damage to the primary photosynthetic apparatus (Nisbett, 1985). Phycobilins are universally present in both freshwater and marine representatives. However, freshwater cyanobacteria tend to be more PC-rich. When present in sufficiently high concentrations, PC contributes to their distinctive blue-green appearance and colloquial name, the blue-green algae (Brient *et al*., 2007).

## 1.4 Cyanobacteria: Champions of the Pelagic

Over their long evolutionary history, cyanobacteria have endured major environmental changes. Evolving in a continentally changing world has endowed cyanobacteria with a range of highly effective ecophysiological traits for ensuring their long-term success under both natural and anthropogenically-mediated change (Paerl *et al*., 2001; Paerl & Otten, 2013). Some of these adaptations that have allowed cyanobacteria to survive and dominate over their eukaryotic competitors include: (1) a small surface area to volume ratio that enhances nutrient sequestrating capabilities under low-nutrient regimes (Finkel *et al*., 2010; Carey *et al*., 2012); (2) luxury P uptake and storage, concentrating P in polyphosphate granules and subsequently releasing P when it is in low supply (Paerl &

Otten, 2013); (3) opportunistic consumers of N, with some species capable of fixing $N_2$ (Flores & Herrero, 2005; Chaffin & Bridgeman, 2014); (4) production of organic complexing ligands that scavenge micronutrients (e.g., Fe) in low supply (Murphy *et al*., 1976; Wilhelm & Trick, 1994; Molot *et al*., 2014); (5) buoyancy that enables regulation of their vertical position in the water column to access nutrient-rich hypolimnetic waters as well as illuminated surface waters to drive photosynthesis (Ganf & Oliver,1982; Carey *et al*., 2012); and (6) higher temperature optimums enabling their success under warming conditions (Paerl & Huisman, 2008; O'Neil *et al*., 2012). The ecological "success" of cyanobacteria is in large part due to the suite of innovative strategies they have acquired over their long evolutionary history that has allowed this group to colonize nearly every conceivable habitat, spanning from the frigid waters of the Antarctic to the scorching heat of tropical deserts (Paerl *et al*., 2001).

## 1.5 Cyanobacteria: Ecological and Health Impacts

The term harmful algal bloom (HAB) is often used loosely in the wider literature with the "harmful" label often used exclusively to describe episodes tied to toxin production. However, the presence of toxins is not a prerequisite necessary for an algal bloom to be deemed "harmful," as all blooms have the potential to jeopardize ecosystem or human health in some fashion (Paerl *et al*., 2001; Backer, 2002). Aside from being aesthetically unpleasant, cyanobacteria blooms are considered a major threat to freshwater resources due to the multitude of water-quality concerns that can quickly ensue following bloom initiation (Paerl & Otten, 2013; Brooks *et al*., 2016). For example, cyanobacteria blooms often manifest as a reduction in water transparency that inhibits the growth of aquatic macrophytes due to restricted light availability and subsequently disrupts invertebrate and fish habitat (Paerl et al., 2001; Pick, 2016). Cyanobacteria blooms can also impair food webs because their essential fatty acid composition is considered of a lower quality compared to eukaryotic algae (Schmidit & Jónasdóttir, 1997; Gearhart *et al*., 2017). When cyanobacteria outcompete more beneficial phytoplankton varieties, they create alternative food webs hampering energy transfer, which may lead to a series of negative effects on higher trophic states (Hixson & Arts, 2016; Gearhart *et al.*, 2017). During bloom die-offs, bacterial abundance typically increases, and these elevated bacterial loads

are frequently coupled with lower oxygen availability and in extreme cases cause fish kills (Smith *et al*., 1999; O'Neil *et al*., 2012).

However, of greatest concern is the ability of some cyanobacteria to produce potentially harmful compounds (Paerl *et al*., 2001; de Figueiredo *et al*., 2004). These toxic secondary metabolites are referred to as cyanotoxins and can be broadly categorized into neurotoxins and hepatotoxins. Among the variety of toxins produced, microcystins are by far the most widespread and frequently encountered in freshwaters worldwide (de Figueiredo *et al*., 2004). Microcystins are hepatotoxins, targeting the liver, and are becoming increasingly recognized as a potential contaminant of concern due to the rise in cyanoHAB reports (Carmichael, 2001; O'Neil *et al*., 2012). The capacity of some cyanobacteria genera to produce cyanotoxins has led the Ontario Ministry of the Environment and Climate Change (OMOECC) to classify all algal blooms containing cyanobacteria as harmful, indicating the potential risk they pose to society (OMOECC, 2014).

## 1.6 Eutrophication: Enrichment of Surface Waters

Eutrophication is a naturally occurring phenomenon by which water bodies gradually age and become increasingly more productive (Smith *et al*., 1999). Human activities have accelerated this natural phenomenon by increasing nutrient loads to surface waters, and freshwater ecosystems may experience eutrophic conditions within decades of human encroachment (Smith & Schindler, 2009; O'Neil *et al*., 2012). As nutrient availability rises, the relative abundance of phytoplankton groups associated with higher nutritional content (e.g., cryptophytes and diatoms) begin to diminish in importance, but the relative abundance of cyanobacteria increases. Cyanobacteria blooms are a cardinal symptom of eutrophication in freshwater environments, and these high biomass events often manifest as unsightly and potentially harmful blooms (Finlay *et al*., 2010; O'Neil *et al*., 2012; Paerl & Otten, 2013).

Two elements, N and P, have long been recognized as the major growth determinants governing the spatial and temporal distribution of cyanobacteria blooms in inland waters

(Schindler, 1977; Paerl *et al*., 2001). Traditionally, P abatement has been the prescription to control nuisance algal growth. P abatement was successful in slowing down rates of eutrophication and cyanobacteria blooms (Schindler, 1977; Sterner, 2008), but a recent resurgence in cyanoHAB reports has sparked controversy over whether controlling cyanobacteria biomass requires reducing inputs of P, N or a dual nutrient approach (Schindler *et al*., 2008; Lewis *et al*., 2011; Paerl *et al*., 2016). P abatement efforts have been widely implemented since the late 1960s, resulting in total P loads to stabilize or decrease over time (Paerl *et al*., 2016). Whereas P is an important driver controlling cyanobacteria growth, focusing on P alone has increasingly driven many lakes out of stoichiometric balance (Elser *et al*., 2009; Glibert *et al*., 2014). The significant cost associated with N abatement together with the physiological ability of some cyanobacteria to thrive under N-deprived conditions has meant that N abatement has often been overlooked to control cyanoHAB formation resulting in N to enter surface waters virtually unregulated (Glibert *et al*., 2014; Gobler *et al*., 2016).

## 1.7 The Importance of Nitrogen

N is an essential macronutrient required for all forms of biological life and functions as a key component for many organic biomolecules, such as proteins, nucleic acids, and chlorophyll. Although N is found in relatively high abundance in the Earth's atmosphere as $N_2$, this form of N is largely inaccessible to most living organisms (Fields, 2004; Galloway *et al*., 2008). Consequently, N is relatively scarce in most biological systems and thus is one of the major limiting nutrients regulating phytoplankton community composition of freshwater ecosystems (Galloway *et al*., 2004). For N to become available to much of the biological world, $N_2$ must be converted to a more chemically available form, such as ammonia ($NH_3$). The industrial fixation of $N_2$ to $NH_3$, also known as the Haber-Bosch process (Smil, 1999), is often referred to as the single most important experiment of the 20$^{th}$ century (Erisman *et al*., 2008; Glibert *et al*., 2014). This reaction revolutionized the agricultural sector by allowing for mass production of synthetic N that was used to produce food for billions (Smil, 1999; Fields, 2004). However, this production of food has resulted in a 500% increase in the use of N fertilizers. Today, global N fertilizer use has seen a seven-fold increase since the 1970s, while P fertilizer

use has seen only a three-fold increase (Glibert *et al*., 2014). The increased reliance on N-based fertilizers over the past century has been universally acknowledged, but little attention has been focused on the recent shift in its composition from inorganic N forms to organic (urea) N forms (Glibert *et al*., 2006; Bogard *et al*., 2012).

## 1.8 Global Reliance on Urea

Prior to the 1960s, global urea use was minimal, representing less than 5% of total N fertilizer applications. However, urea use has grown such that it now accounts for over half of N fertilizer applications worldwide (Fig 1.1) (Glibert *et al*., 2014). This increase in urea fertilizer use stems from the advantages urea offers over its inorganic counterparts, including: (1) urea fertilizers are more cost-effective both because they have lower production costs and their higher N content means lower application rates; (2) urea fertilizers are more water-soluble increasing the likelihood of N percolating through the soil profile; and (3) urea fertilizers are less explosive, making them safer for transportation and storage, and less likely to be converted into explosives (Glibert *et al*., 2006; Paerl *et al*., 2016). The global shift towards higher urea fertilizer use now represents a potentially significant source of urea pollution to freshwaters (Finlay *et al*., 2010; Donald *et al*., 2011; Glibert *et al*., 2014; Belisle *et al*., 2016).

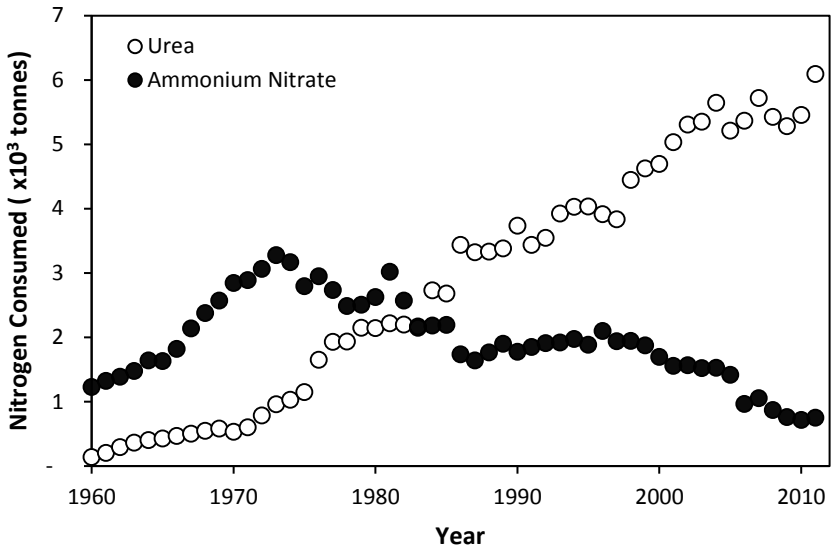


**Figure 1.1** Change in N fertilizer use in the United States between 1960 and 2011. Data show a shift from ammonium nitrate to urea as the dominant N-fertilizer (data replotted from USDA-ARS; Pearl *et al*., 2016).

It was once assumed that urea applied to agricultural soils would either be incorporated into plant biomass or degrade into its decomposition products and thus would not enter waterways (Glibert *et al*., 2006). However, there is growing evidence to suggest that up to 40% of applied urea fertilizer enters waterways (Bogard *et al*., 2012; Glibert *et al*., 2014). Urea may "bypass" the soil system. For example, the application of urea fertilizer is frequently paired with rainfall or irrigation. As urea is highly soluble in water, it readily moves across and through landscapes, increasing the likelihood of urea entering adjacent surface waters. Alternatively, urea may "avoid" degradation. For example, the hydrolysis of urea depends primarily on soil temperature and pH; relatively cool temperatures and low pH reduce urea decomposition efficiency by suppressing microbial metabolism and thus allowing urea to accumulate in the soil (Glibert *et al*., 2006). Furthermore, the hydrolysis of urea may be inhibited by the application of chemical inhibitors (e.g., Argotain$^{TM}$ – a commercial additive used to limit urea hydrolysis). These chemical additives temporarily restrict microbial activity, specifically targeting the urease enzyme, which is responsible for the hydrolysis of urea into $NH_4^+$ (Glibert *et al*., 2006; Belisle *et al*., 2016).

In aquatic environments, urea levels are largely dependent on the catchment to waterbody area ratio, and how the surrounding landscape is managed (Bogard *et al*., 2012). Urea concentrations may be elevated in waters adjacent to heavily fertilized regions, whereas concentrations near non-agricultural lands are generally below detection (Glibert *et al*., 2014). Urea concentrations in freshwater lakes commonly range from undetectable to 150 µmol-N $L^{-1}$ (Berman, 1974; Siuda & Chorst, 2006; Bogard *et al*., 2012). However, in extreme cases, urea in downstream waters may exceed > 1000 µmol-N $L^{-1}$ under conditions favouring its export (Finlay *et al*., 2010; Davis *et al*., 2016).

## 1.9 Nitrogen Metabolism and Assimilation

N is found in a variety of forms in freshwaters, with $NO_3^-$, $NH_4^+$, and urea being the most common in eutrophic waters (Glibert *et al*., 2016). Generally, $NO_3^-$ makes up the largest fraction of the N pool, followed by $NH_4^+$. Historically, concentrations of urea were negligible because natural inputs were low (Glibert *et al*., 2006). However, urea may represent the largest fraction of the N pool in regions with intensive agriculture (Bogard *et al*., 2012; Glibert *et al*., 2014). Dissolved inorganic N (DIN), including $NH_4^+$ and $NO_3^-$, has been the primary focus of researchers investigating the link between N and phytoplankton productivity. The role of dissolved organic nitrogen (DON), including urea, has received comparatively little attention (Finlay *et al*., 2010; Fiedler *et al*., 2015). DON was initially thought to be largely refractory and therefore inaccessible to most phytoplankton, only becoming biologically available through bacterial mineralization or other degradative processes (Bronk *et al*., 2007). This assumption lead scientists to believe urea could not function as an important nutritional substrate. This assumption was reinforced by laboratory-based studies that used urea concentrations far exceeding ecologically relevant levels; high concentrations yielded poor phytoplankton growth, leading investigators to conclude that urea did not stimulate cyanobacteria blooms (Xu, 2015). When scientists began to explore concentrations well below urea's inhibitory threshold, it became clear that urea could satisfy the nitrogenous demands of phytoplankton and in some cases, alter the distribution of common algal groups (Berman & Chava, 1999; Berman & Bronk, 2003; Finlay *et al*., 2010; Solomon *et al*., 2010; Glibert *et al*., 2014).

Cyanobacteria can use a variety of inorganic and organic N substrates (Berman & Chava, 1999; Finlay *et al*., 2010; Chaffin & Bridgeman, 2014). It has long been assumed that cyanobacteria typically favor $NH_4^+$ over other N forms, as $NH_4^+$ is energetically simple to incorporate into the cell and can be used directly upon intake (Flores *et al*., 2005; Oliver *et al*., 2012). Although $NH_4^+$ is energetically favourable, there is a threshold at which $NH_4^+$ becomes toxic to cells and this response has been shown to be species-specific (Dai *et al*., 2012). Therefore, $NH_4^+$ functions as a "paradoxical" nutrient, stimulating algal growth at lower concentrations, whereas higher levels may suppress algal productivity (Dai *et al*., 2012; Collos & Harrison, 2014).

$NH_4^+$ is also believed to function as a regulatory agent, controlling N assimilation and metabolism within the cell. $NH_4^+$ concentrations greater than 1 µM result in repression of the synthesis of enzymes involved in N assimilation, whereas $NH_4^+$ concentrations lower than 1 µM result in activation of the genes that regulate the uptake of alternative N compounds (Flores *et al*., 2005; Glibert *et al*., 2016). The energy required to assimilate these alternative N forms and convert them into $NH_4^+$ differs greatly. Consequently, the chemical form of N that is assimilated affects other cellular processes by reallocating energy reserves (Fig 1.2) (Herrero *et al*., 2001).

$N_2$ fixation is by far the most energetically demanding pathway used by cyanobacteria in their attempt to satisfy N requirements (Flores *et al*., 2005; Finlay *et al*., 2010). $N_2$ fixation is the enzymatic conversion of "inert" $N_2$ into two $NH_4^+$ molecules. This N sequestration mechanism requires 16 adenosine triphosphate (ATP) molecules and a constant supply of electrons ($8e^-$) and protons ($8H^+$) (Paerl, 2017). The triple covalent bond shared between the two N atoms creates a very stable element resistant to decomposition. The high ATP requirements can be attributed to the high-energy demands necessary to break down the strong triple bond (Howarth *et al*., 1988; Flores *et al*., 2005). Only a few cyanobacteria genera–the diazotrophs–can fix $N_2$ (Herrero *et al*., 2001). Diazotrophic cyanobacteria have specialized structures called heterocysts that facilitate $N_2$ fixation thus allowing them to thrive in environments enriched with other essential nutrients (e.g., P and trace metals) but deprived of N. However, $N_2$ fixation comes at a cost—the development and maintenance of heterocysts and the subsequent conversion of

$N_2$ to $NH_4^+$ requires complex enzyme systems (nitrogenases) that further increases the energy burden imposed (Finlay *et al*., 2010; Paerl, 2017). The conversion of $NO_3^-$ into $NH_4^+$ is energetically less costly, requiring active transport and a two-step $NO_3^-$ reduction system. First, $NO_3^-$ is reduced to nitrite ($NO_2^-$) via the enzyme nitrate reductase (NR). Second, $NO_2^-$ is reduced to $NH_4^+$ ferredoxin-dependent nitrite reductase (NiR). $NO_3^-$ reduction requires a steady supply of electrons ($8e^-$) and protons ($9H^+$) (Flores *et al*., 2005).

The conversion of urea into $NH_4^+$ could be the most energetically advantageous, as the hydrolysis of urea produces a two-fold increase in $NH_4^+$ (Herrero *et al*., 2001). The hydrolysis of urea can occur through two mutually exclusive pathways involving the production of either ATP-urea amidolyase or urease (Leftley& Syrett, 1973). ATP-urea amidolyase production is restricted to some orders within the Chlorophyceae, whereas urease production occurs in all phytoplankton, including cyanobacteria (Berns *et al*., 1966; Bekheet & Syrett, 1977). Despite requiring energy to produce the urease enzyme, urea hydrolysis results in the formation of two $NH_4^+$ molecules (Finlay *et al*., 2010). Additionally, urea hydrolysis produces carbon dioxide ($CO_2$) as a by-product, which can then be incorporated into photosynthesis reducing the cells reliance on active uptake (Berman & Chava, 1999; Glibert *et al*., 2014).

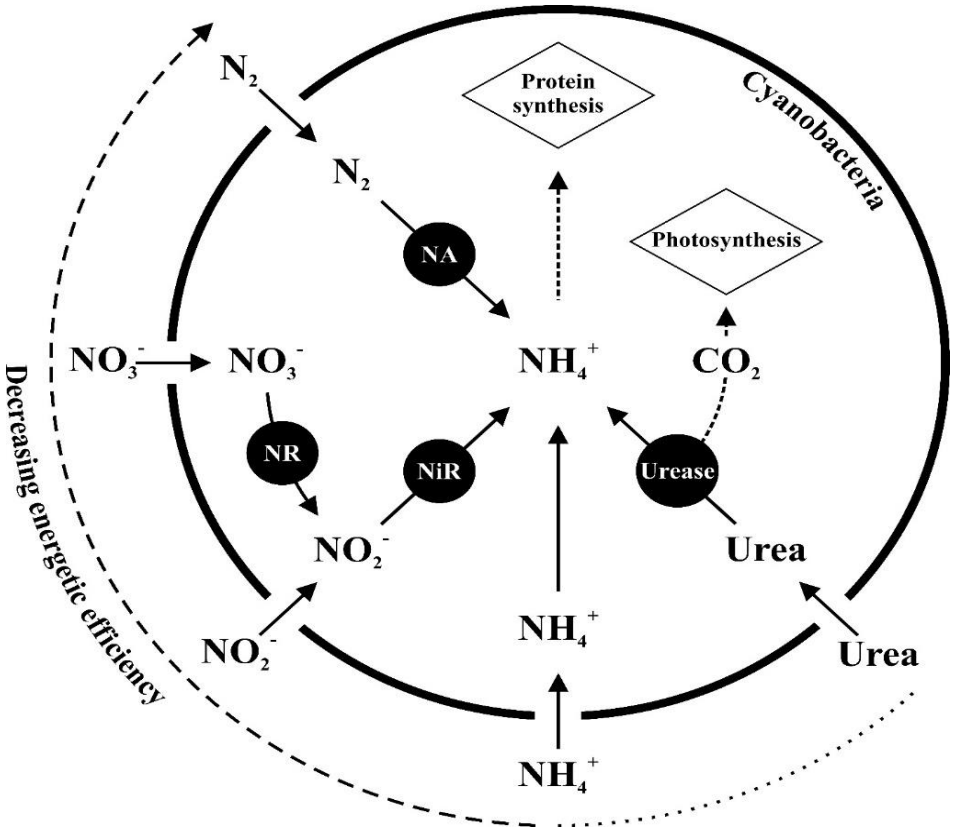


**Figure 1.2** Cell model describing the various pathways in which cyanobacteria obtain N. Pathways that require enzymatic reactions are indicated with black circles; nitrogenase (NA), nitrate reductase (NR) and nitrite reductase (NiR). The outer dashed line indicates the traditional energetic assumption on nitrogen use in cyanobacteria, while the dotted line represents our prediction.

## 1.10 Nitrogen Speciation Influences Community Composition

It has been known for some time (e.g., Redfield *et al*., 1963; Dugdale & Goering, 1967) that individual species of marine phytoplankton have different preferences for N sources. In the marine ecological model, $NO_3^-$ is "new" N, originating from terrestrial runoff, whereas $NH_4^+$ is a "recycled or regenerated" nutrient from bacterial decomposition, viral lysis of phytoplankton, or zooplankton metabolic waste. As a result, waters dominated with $NO_3^-$ will form a distinctly different phytoplankton community composition compared with waters containing $NH_4^+$ (Syrett, 1981). The competition of marine phytoplankton for $NO_3^-$ or $NH_4^+$ has been the foundation for marine HAB models

(Sournia 1974; Smayda 1979; Glibert *et al*., 2016). While $NO_3^-$ and $NH_4^+$ are the dominant N-forms in marine waters (excluding the reduction of $N_2$ by N-fixing bacteria and cyanobacteria), urea historically played a minor ecological role, as historical concentrations of urea were low even in coastal waters (even though many marine phytoplankton can utilize the N from urea for growth (McCarthy, 1972).

In freshwater systems, the supply of urea cannot be dismissed (Finlay *et al*., 2010; Donald *et al*., 2011; Glibert *et al*., 2014). As the waters sit adjacent to natural terrestrial and agricultural lands, $NO_3^-$, $NH_4^+$, and urea all serve as sources of "new production", influencing productivity, production, biomass, and species composition (Berman & Chava, 1999; Donald *et al*., 2011). In general, cyanobacteria dominate eukaryotic phytoplankton when grown under elevated $NH_4^+$ concentrations, likely reflecting a competitive advantage for the low energetic costs associated with $NH_4^+$ uptake and assimilation (Oliver & Ganf, 2000; Glibert *et al*., 2016). In contrast, eukaryotic phytoplankton show dominance over cyanobacteria under elevated $NO_3^-$ concentrations, as NR is more readily stimulated in eukaryotes compared to cyanobacteria (Blomqvist *et al*., 1994; Donald *et al*., 2011). Diatoms, in particular, have been described as $NO_3^-$ specialists due to their proportionally higher abundance of $NO_3^-$ transporters and non-saturating uptake kinetics when silica is abundant (Lomas & Glibert, 1999; Glibert *et al*., 2016). In contrast, other eukaryotic phytoplankton, such as the chlorophytes, grow particularly well when supplied with either reduced or oxidized N, reflecting the wide diversity of N transporters they possess (Galvan & Fernandez, 2001; Glibert *et al*., 2016). At present, there is a limited understanding of the effects of urea on natural phytoplankton communities. One exception is that urea additions to P-saturated freshwaters promote the growth of non-fixing cyanobacteria and chlorophytes over $N_2$-fixing cyanobacteria (Finlay *et al*., 2010; Donald *et al*., 2011).

Phytoplankton possess two dominant N transporter types, with N transport activity dependent on external N concentrations. High-affinity transporters (HATs) are saturable and expressed under N-deprived environments, whereas low-affinity transporters (LATs) are non-saturable and expressed under N-replete conditions (Howitt & Udvardi, 2000). LATs are often referred to as low affinity, high capacity systems. Their high capacity

results in uptake kinetics that perform in linear or biphasic fashion. LATs are more frequently encountered for $NO_3^-$ uptake and result in greater N uptake capabilities under N-replete conditions (Collos *et al*., 1997; Lomas & Glibert 1999). In contrast, $NH_4^+$ uptake often exceeds that of $NO_3^-$ under N-limited conditions (Glibert *et al*., 2013). The regulation of N transporters is not only influenced by N availability, but also N speciation. $NO_3^-$ functions as a positive signaling molecule, with increased $NO_3^-$ availability promoting higher rates of $NO_3^-$ uptake (Dugdale *et al*., 1981). In contrast, $NH_4^+$ acts as a negative signaling agent, with increased $NH_4^+$ availability downregulating the uptake and assimilation of itself and alternative N compounds (Dugdale *et al*., 1981; Flynn *et al*., 1997; Glibert *et al*., 2016).

Cyanobacteria exhibit great physiological plasticity in response to different N forms, and have evolved an assortment of transporters to capitalize on this limiting substrate (Flores *et al*., 2005; Glibert *et al*., 2016). The vast majority of DIN and DON uptake moves against a concentration gradient and therefore requires an energy input (Dagenais-Bellefeuille & Morse, 2013). For example, $NO_3^-$ uptake requires the participation of ATP to fuel transport (Flores *et al*., 1983). While all cyanobacteria can obtain N through active transport, $NH_4^+$ and urea transport can also occur without an energetic investment. $NH_4^+$ and urea can enter cells via diffusion (Valladares *et al*., 2002; Flores *et al*., 2005; Finlay *et al*., 2010) and urea, being a small-uncharged molecule, may rapidly enter cells via aquaporins (Valladares *et al*., 2002; Kojima *et al*., 2006). As reduced N forms may alternatively be transported in a less energetically demanding manner, this further supports why cyanobacteria prefer reduced N sources over oxidized forms (Finlay *et al*., 2010; Donald *et al*., 2011).

## 1.11 Thesis Hypothesis and Predictions

To evaluate whether widespread use of urea has promoted cyanobacteria dominance in freshwater lakes, three bloom-forming cyanobacteria were selected to understand the effect of different N forms on growth, photosynthesis, and N preference. The goal of this thesis is to understand if urea loading has contributed to increased cyanobacteria abundance in inland waters.

In nature, organisms strategically select pathways that require the least amount of energy to accomplish a task. It follows that the untapped energy reserves can then be allocated to other essential functions to improve cellular fitness (Schluter, 1996). As Raven et al. (1992) stated: "If the use of the resource needing more manipulation ($NO_3^-$) ... to achieve the same rate of product formation, then the cell doubling time will be significantly increased since more energy for manipulation will be required to double the cell mass …" Here I hypothesis that the magnitude of response exhibited by cyanobacteria would be greatest on N forms that provide the greatest energetic return or least amount of manipulation, reflecting energetic efficiency for cellular N assimilation. As a result, cells would selectively uptake less energetically intensive N substrates and cellular performance would be enhanced on these energetically favourable N forms.

Based on this simple assumption the following predictions were set:

I. Photosynthetic activity and growth of cyanobacteria would be enhanced on reduced N forms, specifically urea, as reduced N forms lower the energetic constraints of N acquisition or offer the benefit of additional nutrient building blocks.

II. Selected cyanobacteria species will transport energetically simple reduced N forms over energetically demanding oxidized forms; and (2) the transport of urea is independent of the availability of either of the two inorganic N-sources. Specifically, I predicted the sequence of N assimilation would be the independent utilization of urea, and the sequential utilization of $NH_4^+$, prior to the use of $NO_3^-$.

III. Different cyanobacteria species will exhibit varying capacities to utilize urea and that uptake will be direct rather than indirect.

## 1.12 References


Anderson D.M., Glibert P.M. & Burkholder J.M. (2002) Harmful algal blooms and eutrophication: Nutrient sources, composition and consequences. *Estuaries*, **25**, 704-726.

Backer L.C. (2002) Cyanobacteria harmful algal blooms (CyanoHABs): Developing a public health response. *Lake and Reservoir Management*, **18**, 20-31.

Bekheet I.A. & Syrett P.J. (1977) Urea-degrading enzymes in algae. *British Phycological Journal*, **12**, 137-143.

Belisle B.S., Steffen M.M., Pound H.L., DeBruyn J.M., Watson S.B., Bourbonniere R.A., Boyer G.L. & Wilhelm S.W. (2016) Urea in Lake Erie: organic nutrient sources are potentially important drivers of phytoplankton biomass. *Journal of Great Lakes Research*, **42**, 599-607.

Berman T. (1974) Urea in waters of lake Kinneret (Sea of Galilee) *Limnology and Oceanography*, **19**, 977-980.

Berman T. & Chava S. (1999) Algal growth on organic compounds as nitrogen sources. *Journal of Plankton Research*, **21**, 1423-1437.

Berman T. & Bronk D.A. (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquatic Microbial Ecology*, **31**, 279-305.

Berns D.S., Holohan P. & Scott E. (1966). Urease activity in blue-green algae. *Science*, **152**, 1077-1078.

Bogard M.J., Donald D.B., Finlay K. & Leavitt P.R. (2012) Distribution and regulation of urea in lakes of central North America. *Freshwater Biology*, **57**, 1277-1292.

Brient L., Lengronne M., Bertrand E., Rolland D., Sipel A., Steinmann D., Baudin I., Legeas M., Le Rouzic B. & Bormans M. (2007) A phycocyanin probe as a tool

for monitoring cyanobacteria in freshwater bodies. *Journal of Environmental Monitoring*, **10**, 248-255.

Bronk D.A., See J.H., Bradley P. & Killberg L. (2007) DON as a source of bioavailable nitrogen for phytoplankton. *Biogeosciences*, **4**, 283-296.

Brooks B.W., Lazorchak J.M., Howard M.D., Johnson M.V., Morton S.L., Perkins D.A., Reavie E.D., Scott G.I., Smith S.A. & Steevens J.A. (2016) Are harmful algal blooms becoming the greatest inland water quality threat to public health and aquatic ecosystems? *Environmental Toxicology and Chemistry*, **35**, 6-13.

Carey C.C., Ibelings B.W., Hoffmann E.P., Hamilton D.P. & Brookes J.D. (2012) Ecophysiological adaptations that favor freshwater cyanobacteria in a changing climate. *Water Research*, **46**, 1394-1407.

Carmichael W.W. (2001). Health effects of toxin-producing cyanobacteria: "The CyanoHABs". *Human and Ecological Risk Assessment*, **7**, 1393-1407.

Chaffin J.D. & Bridgeman T.B. (2014) Organic and inorganic nitrogen utilization by nitrogen-stressed cyanobacteria during bloom conditions. *Journal of Applied Phycology*, **26**, 299-309.

Collos Y. & Harrison P.J. (2014) Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin*, **80**, 8-23.

Dai G., Shang J. & Qiu B. (2012) Ammonia may play an important role in the succession of cyanobacteria blooms and the distribution of common algal species in shallow freshwater lakes. *Global Change Biology*, **18**, 1571-1581.

Dagenais-Bellefeuille S. & Morse D. (2013) Putting the N in dinoflagellates. *Front Microbiology*, **4**, 369.

de Figueiredo D.R., Azeiteiro U.M., Esteves S.M., Gonçalves F.M. & Pereira J.M. (2004) Microcystin-producing blooms: A serious global public health issue. *Ecotoxicology and Environmental Safety*, **59**, 151-163.

Donald D.B., Bogard M.J., Finlay K. & Leavitt P.R. (2011) Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. *Limnology and Oceanography*, **56**, 2161-2175.

Dugdale R.C. & Goering J.J. (1976) Uptake of new and regenerated forms of nitrogen adaptation of nutrient assimilation. *Limnology and Oceanography*, **12**, 196-206.

Dugdale R.C. Jones B. H., MacIsaac J.J. & Goering J.J. (1981) Adaptation of nutrient assimilation. *Canadian Bulletin of Fisheries and Aquatic Sciences*, **210**, 234-250.

Elser J.J., Andersen T., Baron J. S., Bergstrom A.K., Jansson M., Kyle M., Nydick K. R., Steger L. & Hessen D.O. (2009) Shifts in lake N:P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science*, **326**, 835-837.

Erisman J.W., Sutton M.A., Klimont Z., Galloway J. & Winiwarter W. (2008) How a century of ammonia synthesis has changed the world. *Nature Geoscience*, **1**, 636-639.

Fiedler D., Graeber D., Badrain M. & Kohler J. (2015) Growth response of four freshwater algal species to dissolved organic nitrogen of different concentration and complexity. *Freshwater Biology*, **60**, 1613-1621.

Fields S. (2004) Global nitrogen: cycling out of control. *Environmental Health Perspectives*, **112**, 556-563.

Finkel Z.V., Beardall J., Flynn K.J., Quigg A., Rees T.A.V. & Raven J.A. (2009) Phytoplankton in a changing world: cell size and elemental stoichiometry. *Journal of Plankton Research*, **32**, 119-137.

Finlay K., Patoine A., Donald D.B., Bogard M.J. & Leavitt P.R. (2010) Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the Northern Great Plains. *Limnology and Oceanography*, **55**, 1213-1230.

Flores E., Gurrero M.G. & Losada M. (1983) Nitrate uptake and its regulation in the cyanobacterium, *Anacystis nidulans*. *Biochimica et Biophysica Acta*, **896**, 103-108.

Flores E. & Herrero A. (2005) Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochemical Society Transactions*, **33**, 164-167.

Flynn K.J., Fasham M.J.R. & Hipkin. C.R. (1997) Modelling the interactions between ammonium and nitrate uptake in marine phytoplankton. *Philosophical Transaction of the Royal Society*, **352**, 1625-1645.

Galloway J.N., Dentener F.J., Capone D.G., Boyer E.W., Howarth R.W., Seitzinger S., Asner G.P., Cleveland C.C., Green P.A., Holland E.A., Karl D.M., Michael A.F., Porter J.H., Townsend A.R. & Vörösmarty C.J. (2004) Nitrogen cycles: past, present and future. *Biogeochemistry*, **70**, 153-226.

Galloway J.N., Townsend A.R., Erisman J.W., Bekunda M., Cai Z., Freney J.R., Martinelli L.A., Seitzinger S.P. & Sutton. M.A. (2008) Transformation of the nitrogen cycle: recent trends, questions and potential solutions. *Science*, **320**, 889-892.

Galvan A. & Fernandez E. (2001) Eukaryotic nitrate and nitrite transporters. *Cell and Molecular Life Sciences*, **58**, 225-233.

Gearhart T.A., Ritchie K., Nathan E., Stockwell J.D. & Kraft J. (2017) Alteration of essential fatty acids in secondary consumers across a gradient of cyanobacteria. *Hydrobiologia*, **784**, 155-170.

Ganf G.G. & Oliver R.L. (1982) Vertical separation of light and available nutrients as a factor causing replacement of green algae by blue-green algae in the plankton of a stratified lake. *Journal of Ecology*, **70**, 829-844.

Gantt E. (1975) Phycobilisomes: light-harvesting pigment complexes. *Bioscience*, **25**, 781-788.

Glibert P.M., Harrison J., Heil C. & Seitzinger S. (2006) Escalating worldwide use of urea—a global change contributing to coastal eutrophication. *Biogeochemistry*, **77**, 441-463.

Glibert P. M. Kana T.M. & Brown K. (2013) From limitation to excess: Consequences of substrate excess and stoichiometry for phytoplankton physiology, trophodynamics and biogeochemistry, and implications for modeling. *Journal of Marine Systems*, **125**, 14-28.

Glibert P.M., Maranger R., Sobota D.J. & Bouwman L. (2014) The Haber Bosch–harmful algal bloom (HB–HAB) link. *Environmental Resource Letters*, **9**, 105001.

Glibert P.M., Wilkerson F.P., Dugdale R.C., Raven J.A., Dupont C., Leavitt P.R., Parker A.E., Burkholder J.M. & Kana T.M. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnology and Oceanography*, **61**, 165-197.

Gobler C.J., Burkholder J.M., Davis T.W., Harke M.J., Johengen T., Stow C.A. & van de Waal D.B. (2016) The dual role of nitrogen supply in controlling the growth and toxicity of cyanobacteria blooms. *Harmful Algae*, **54**, 87-97.

Herrero A., Muro-Pastor A.M. & Flores E. (2001) Nitrogen control in cyanobacteria. *Journal of Bacteriology*, **183**, 411-425.

Hixson S.M. & Arts M.T. (2016) Climate warming is predicted to reduce omega-3, long-chain, polyunsaturated fatty acid production in phytoplankton. *Global Change Biology*, **22**, 2744-2755.

Howarth R.W., Marino R., Lane J. & Cole J.J. (1988) Nitrogen fixation in freshwater, estuarine, and marine ecosystems.1: Rates and importance. *Limnology and Oceanography*, **33**, 669-687.

Howitt S.M. & Udvardi M.K. (2000) Structure, function and regulation of ammonium transporters in plants. *Biochimica et Biophysic Acta*, **1465**, 152-170.

Kasting J. F. (1993) Earth's early atmosphere. *Science*, **259**, 920-926.

Kenrick P. & Crane R.R. (1997) The origin and early evolution of plants on land. *Nature*, **389**, 33-39.

Kojima S., Bohner A. & Von Wiren N. (2006) Molecular mechanisms of urea transport in plants. *Journal of Membrane Biology*, **212**, 83-91.

Kulasooriya S.A. (2011) Cyanobacteria: Pioneers of Planet Earth. *Ceylon Journal of Science*, **40**, 71-88.

Leftley J.W. & Syrett P.J. (1973) Urease and ATP: urea amidolyase activity in unicellular algae. *Journal of General Microbiology*, **77**, 109-115.

Lewis W.M., Wurtsbaugh W.A. & Paerl H.W. (2011) Rationale for control of anthropogenic nitrogen and phosphorus to reduce eutrophication of inland waters. *Environmental Science & Technology*, **45**, 10300-10305.

Lomas, M. W. & Glibert P.M. (1999) Interactions between $NH_4^+$ and $NO_3^-$ uptake and assimilation: Comparison of diatoms and dinoflagellates at several growth temperatures. *Marine Biology*, **133**, 541-551.

McCarthy J.J. (1972) The uptake of urea by marine phytoplankton. *Limnology and Oceanography*, **17**, 738-748.

Molot L., Watson S.B., Creed I.F., Trick C.G., McCabe S.K., Verschoor M.J., Sorichetti R.J., Powe C., Venkiteswaran J. & Schiff S.L. (2014) A novel model for cyanobacteria bloom formation: The critical role of anoxia and ferrous iron. *Freshwater Biology*, **59**, 1323-1340.

Murphy T.P., Lean D.R.S. & Nalewajko C. (1976) Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. *Science*, **192**, 900-902.

Nisbet E.G. (1985) The geological setting of the earliest life forms. *Journal of Molecular Evolution*, **21**, 289-298.

O'Neil J.M., Davis T.W., Burford M.A. & Gobler C.J. (2012) The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae*, **14**, 313-334.

Oliver R.L. & Ganf, G.G. (2000) Freshwater blooms. In: B. A. Whitton and M. Potts (Ed.), *The Ecology of Cyanobacteria* (pp. 149-194). Dordrecht: Springer Netherlands.

Oliver R.L., Hamilton D.P., Brookes J.D. & Ganf, G.G. (2012) Physiology, Blooms and Predictions of Planktonic Cyanobacteria. In: B. A. Whitton (Ed.), *The Ecology of Cyanobacteria II: Their Diversity in Space and Time* (pp. 115-187). Dordrecht: Springer Netherlands.

Ontario Ministry of the Environment and Climate Change (2014) *Information about blue-green algae: Background, potential impacts to human health and safety of drinking water*. Toronto: Queen's Printer for Ontario.

Paerl H.W., Fulton R.S., Moisander P.H. & Dyble J. (2001) Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal*, **1**, 76-113.

Paerl H.W. & Huisman J. (2008) Blooms like it hot. *Science*, **320**, 57-58.

Paerl H.W. & Otten T.G. (2013) Harmful cyanobacteria blooms: Causes, consequences, and controls. *Microbial Ecology*, **65**, 995-1010.

Paerl H.W., Scott T., McCarthy M.J., Newell S.E., Gardner W.S., Havens K.E., Hoffman D.K., Wilhelm S.W. & Wurtsbaugh W.A. (2016) It Takes Two to Tango: When and Where Dual Nutrient (N & P) Reductions Are Needed to Protect Lakes and Downstream Ecosystems. *Environmental Science & Technology*, **50**, 10805-10813.

Paerl H.W. (2017) The cyanobacteria nitrogen fixation paradox in natural waters. *F1000 Research*, **6**, 224.

Pick F.R. (2016) Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria. *Canadian Journal of Fisheries and Aquatic Sciences*, **73**, 1-10.

Raven J.A., Wollenweber B. & Handley L.L. (1992) A comparison of ammonium and nitrate as nitrogen sources for photolithotrophs. *New Phytologist*, **121**, 19-32.

Redfield A.C., Ketchum B.H. & Richards F.A. (1963) The influence of organisms on the composition of sea-water, In: Hill, M.N. (Ed.) *In The sea* (vol 2: pp. 26-77). John Wiley & Sons, New York.

Schindler D.W. (1977) Evolution of phosphorus limitation in lakes. *Science*, **195**, 260-262.

Schindler D.W., Hecky R.E., Findlay D.L., Stainton M.P., Parker B.R., Paterson M.J., Beaty K.G., Lyng M. & Kasian S.E.M. (2008) Eutrophication of lakes cannot be controlled by reducing nitrogen input: Results of a 37-year whole-ecosystem experiment. *Proceedings of the National Academy of Sciences*, **105**, 11254-11258.

Schluter D. (1996) Adaptive radiation along genetic lines of least resistance. *Evolution*, **50**, 1766-1774.

Schmidit K. & Jónasdóttir S.H. (1997) Nutritional quality of two cyanobacteria: how rich is 'poor' food? *Marine Ecological Progress Series*, **151**, 1-10.

Siuda W. & Chrost R.J. (2006) Urea and ureolytic activity in lakes of different trophic status. *Polish Journal of Microbiology*, **55**, 211-225.

Smil V. (1999) Detonator of the population explosion. *Nature*, **400**, 415.

Smith V.H., Tilman G.D. & Nekola J.C. (1999) Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution*, **100**, 179-196.

Smith V.H. & Schindler D.W. (2009) Eutrophication science: where do we go from here? *Trends in Ecology and Evolution*, **24**, 201-207.

Solomon C.M., Collier J.L., Berg G.M. & Glibert P.M. (2010) Role of urea in microbial metabolism in aquatic systems: a biochemical and molecular review. *Aquatic Microbial Ecology*, **59**, 67-88.

Sournia A. (1974) Circadian periodicities in natural populations of marine phytoplankton. *Advances in Marine Biology*, **12**, 325-389.

Sterner R.W. (2008) On the phosphorus limitation paradigm for lakes. *International Review of Hydrobiology*, **93**, 433-445.

Smayda T.J. (1997) Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnology and Oceanography*, **42**, 1137-1153.

Syrett P.J (1981) Nitrogen metabolism in microalgae. *Canadian Bulletin of Fisheries and Aquatic Sciences*, **210**, 196-210.

Taranu Z.E., Gregory-Eaves I., Leavitt P.R., Bunting L., Buchaca T., Catalan J., Domaizon I., Guilizzoni P., Lami A., McGowan S., Moorhouse H., Morabito G., Pick F.R., Stevenson M.A, Thompson P.L. & Vinebrooke R.D. (2015) Acceleration of cyanobacteria dominance in north temperate-subarctic lakes during the Anthropocene. *Ecology Letters*, **18**, 375-384.

Valladares A., Montesinos M.L., Herrero A. & Flores E. (2002) An ABC-type, high-affinity urea permease identified in cyanobacteria. *Molecular Microbiology*. **43**, 703-715.

Wilhelm S.W. & Trick, C.G. (1994) Iron-limited growth of cyanobacteria: Multiple siderophore production is a common response. *Limnology and Oceanography*, **39**, 1979-1984.

Winter J.G., DeSellas A.M., Fletcher R., Heintsch L., Morley A., Nakamoto L. and Utsumi K. (2011) Algal blooms in Ontario, Canada: increases in reports since 1994. *Lake and Reserve Management*, **27**, 107-114.

Xu Y. (2015) Molybdenum and Iron Interactions as Micronutrients for Growth of a Freshwater Cyanobacterium, *Microcystis aeruginosa*. Electronic Thesis and Dissertation Repository. 3293. http://ir.lib.uwo.ca/etd/3293.

# Chapter 2

# 2 The efficiency of different nitrogen forms on the growth and photosynthetic efficiency of three bloom-forming cyanobacteria

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## 2.1 Introduction

Excess nutrients entering surface waters can disrupt the existing ecological balance, giving rise to an environment where some organisms thrive at the expense of others (Anderson, Glibert & Burkholder, 2002; Glibert, Maranger, Sobota, & Bouwman, 2014). In freshwaters, a telltale sign of nutrient over-enrichment is a shift in the phytoplankton assemblage towards cyanobacteria dominance (Smith, Tilman, & Nekola, 1999; Paerl & Otten, 2013). Cyanobacteria are notable bloom formers in inland waters, and the development of high biomass events can wreak havoc on aquatic ecosystems and adjacent shoreline communities (Paerl, Fulton, Moisander, & Dyble, 2001; Pick, 2016). Under these conditions, when excessive cyanobacteria growth jeopardizes human or ecosystem health they are coined cyanobacteria harmful algal blooms (cyanoHABs) (O'Neil, Davis, Burford, & Gobler, 2012).

Widespread P removal efforts were introduced following the universal recognition that phosphorus (P) was the primary nutrient linked to the proliferation and expansion of phytoplankton blooms in freshwaters (Schindler, 1977; Sterner, 2008). Abatement efforts targeting P have stabilized or decreased P inputs, which has contributed to elevated N:P ratios in neighboring waterways (Glibert et al., 2014; Paerl et al., 2016). In addition, many lakes are receiving elevated loads of bioavailable N relative to P, as N inputs have now surpassed P inputs due to increased use of N-based fertilizers (Elser et al., 2009; Glibert, Harrison, Heil, & Seitzinger, 2006). As a consequence, phytoplankton are now

receiving more N than their stoichiometrically-estimated requirements, and these higher N: P loads can alter the structure of phytoplankton communities (Elser et al., 2009; Glibert et al., 2014).

Freshwater cyanobacteria are particularly well-suited to increased N: P loading, as this microbial group has evolved a suite of physiological adaptations to withstand low ambient P concentrations (Glibert et al., 2014; Gobler et al., 2016). Some of these adaptations include: (1) small surface area to volume ratio, typical of most cyanobacteria taxa, enhances P sequestering capabilities under low P conditions; (2) luxury P uptake and storage, concentrating P in polyphosphate granules, when P is in large supply; and (3) substitution of P-rich lipids with alternative elements (e.g., sulfolipids) when P is scarce. The combination of these adaptive strategies provides cyanobacteria with a strong competitive advantage in surface waters enriched with N (Glibert et al., 2014).

While higher N: P loads to freshwaters have been widely acknowledged, less attention has been given to the changing chemical composition of N in freshwaters. N is an important control on phytoplankton growth and functions as a key component for many organic biomolecules, such as proteins, nucleic acids and pigments (Fields, 2004; Glibert et al., 2016). Cyanobacteria can use a variety of N substrates, with each substrate possessing its own unique energetic investment (Herrero, Muro-pastor, & Flores, 2001; Solomon, Collier, Berg, & Glibert, 2010; Donald, Bogard, Finlay, & Leavitt, 2011). N entering the cell must be metabolized into $NH_4^+$, as $NH_4^+$ is the basic building block for protein synthesis (Fig. 1.2) (Finlay et al., 2010). $N_2$ fixation is by far the most energetically demanding pathway used by certain cyanobacteria genera in their attempt to satisfy N requirements (Flores & Herrero, 2005; Finlay et al., 2010). While less energetically costly, the conversion of $NO_3^-$ into $NH_4^+$ requires a two-step $NO_3^-$ reduction system (Herrero et al., 2001). $NH_4^+$ is energetically favorable to $NO_3^-$, as $NH_4^+$ is energetically simple to uptake into the cell and can be used directly upon intake (Flores & Herrero, 2005; Donald et al., 2011). Due to low energetic demands for $NH_4^+$ utilization, $NH_4^+$ is considered the preferred N form among cyanobacteria (Herrero et al., 2001; Finlay et al., 2010). However, urea may offer the greatest energetic advantage, given that urea hydrolysis results in the production of two $NH_4^+$ molecules, thereby providing a

two-fold increase in N compared to inorganic sources (Finlay et al., 2010; Donald et al., 2011). Furthermore, the breakdown of urea results in the release of $CO_2$ as a by-product, which can then be incorporated into photosynthesis, reducing the cells reliance on active uptake (Berman & Chava, 1999; Glibert et al., 2014). However, the relationship between urea and the occurrence of cyanoHABs remains largely unexplored.

Historically, N in fertilizers were based on nitrate ($NO_3^-$) and ammonium ($NH_4^+$), but these have since been replaced by urea ($CO(NH_2)_2$)-based fertilizers composition (Glibert et al., 2006; Belisle et al., 2015). Urea now accounts for more than half of the total N-fertilizer applications worldwide and this contemporary shift in fertilizer consumption patterns has coincided with the extensive reemergence of cyanoHABs in inland waters (Finlay, Patoine, Donald, Bogard, & Leavitt, 2010; Glibert et al., 2014). In this study, we examined the relative performance of cyanobacteria grown with urea, $NO_3^-$ or $NH_4^+$ as an N source. We hypothesized that the magnitude of response would be greatest on N forms that provide the greatest energetic return or least amount of manipulation, reflecting energetic efficiency for cellular N assimilation (Raven, Wollenweber, & Handley, 1992; Glibert et al., 2016). Based on this simple energetic assumption, we predicted that growth and photosynthetic performance of cyanobacteria would be enhanced on urea, as urea lowers the energetic constraints of N acquisition and can offer the benefit of additional nutrient building blocks (urea supplying both N and C to the cell).

## 2.2 Material and Methods

### 2.2.1 Experimental Design

Three bloom-forming species of cyanobacteria (*Microcystis aeruginosa, Dolichospermum flos- aquae,* and *Synechococcus* sp.) were used to evaluate whether cyanobacteria performed better in terms of growth and photosynthetic efficiency when supplied with N substrates that are energetically cheaper to assimilate and metabolize. Species selection was based on two major criteria: 1) organisms with cosmopolitan distributions; and 2) studied organisms represent the range of morphological diversity documented within cyanobacteria (e.g., filamentous, colonial, and picocyanobacteria).

*M. aeruginosa* (CPCC 300) and *D. flos- aquae* (CPCC 67) isolates were obtained from the Canadian Phycological Culture Centre (CPCC), and *Synechococcus* sp. was isolated from a sample taken from Lake Erie in 2015 at Western University. The isolates were maintained as non-axenic, uni-algal strains in BG-11 media (adjusted to pH 7.4). The basal medium for each experimental treatment was adjusted to contain a different N form: $NO_3^-$ (sodium nitrate), $NH_4^+$ (ammonium chloride), or urea and four different concentrations of each N form were selected: 1, 3, 5, and 7 mmol-N $L^{-1}$. For each treatment, cell densities were logged daily, beginning from the initial inoculation (Day 0) and followed until cells reached the stationary growth phase (Day 10). Oxygen evolution measurements were recorded during the mid-exponential phase, and pigments were extracted and quantified during the early stationary phase of growth.

Prior to conducting experimental trials, cultures in stationary growth were centrifuged (2000 g for 5 min). The supernatant was aspirated, and the pelleted cells were washed three times with N-free medium. After the final wash, cells were resuspended in N-free media and preconditioned for a period of 5 days to ensure cells had been exhausted of any intracellular N stores. Experiments were conducted in 125 mL Erlenmeyer flasks containing 50 mL of the mixture (i.e., medium and inoculum). Temperature was kept at 23°C (±1°C), and cultures were sustained by a continuous light flux of 60–70 μmol photons $m^{-2}$ $s^{-1}$.

### 2.2.2 Growth (*k*)

Culture densities were monitored daily at an absorbance of 750 nm on a spectrophotometer. Optical density measurements at 750 nm correspond to particle density and are less prone to interference by pigments, as this wavelength extends beyond the range of photosynthetic pigments. Therefore, measurements of 750 nm address changes in relative turbidity. Optical density measurements were calibrated to hemocytometer (*D. flos-aque*) or flow cytometry (*M. aeruginosa* and *Synechococcus* sp. ) counts, which were used to estimate cell concentrations for normalizing photosynthetic measurements on a $cell^{-1}$ basis. Optical density measurements were plotted over time and

the exponential portion of the curves was used to calculate *k* (division day$^{-1}$) as defined by Guillard (1973):

$$k \text{ (divisions day)} = \ln(N_1/N_0)/0.6931(t_1-t_0)$$

where $N_1$ is the final cell concentration, $N_0$ is the initial cell concentration, and $t_1$-$t_0$ is the time elapsed in days.

### 2.2.3 Pigment Extractions

Pigments were analyzed using a modified version described in Morgan-Kiss *et al*. (1998). Aliquots (5 mL) of late-exponential phase cells were collected on Whatman GF/F filters and stored at -80°C prior to analysis. For each group of pigments, the extraction protocol was kept constant, only differing by the extraction solvent and incubation temperature. 90% (*v*/*v*) acetone was used for chl-*a* extractions (Jeffery & Humphrey, 1975), whereas phosphate buffer (0.1M, pH of 6.8) was used for PC extractions (Lawrenz *et al*., 2011). Filters were suspended in solvent (2 mL) and were mechanically disrupted using a bead beater (3 × 10 sec cycles) fractioned with 0.1 silica beads. The resulting "slurry" was stored for 24 hr at -20°C for chl-*a* and 4°C for PC extractions. Clarification of extracts was carried out through centrifugation (6000 *g* for 5 min). Supernatant (1 mL) was then passed through a 0.22 µM syringe filter and measured on a spectrophotometer in a 1mL glass cuvette with 1-cm path length. Total chl-*a* concentrations were calculated using the method of Jeffery and Humphrey (1975), and PC concentrations were calculated determined using the method of Lawrenze *et al*. (2011). Chl-*a* and PC concentrations were normalized to the cellular level (pg cell $^{-1}$).

### 2.2.4 Oxygen Evolution

Photosynthesis-Irradiance response (PI) curves were constructed using a series of light intensities ranging from 0 to 800 µmol photons $m^{-2}$ $s^{-1}$. PI curves were used to calculate two photosynthetic parameters, which were calculated to represent the photosynthetic capacity of each species: (1) $P_{max}$ represents the maximum (light-statured) rate of photosynthesis; and (2) α is the initial slope (photosynthetic efficiency) under light-

limiting conditions (Talling, 1957). $O_2$ evolution was measured *in vivo* using a Clark-type $O_2$ electrode at room temperature (20 ºC ± 2 ºC). Measurements were performed on 1.5 mL aliquots of mid-exponential-phase cells and were transferred into the reaction vessel with a magnetic stirrer to ensure a homogenous suspension. Prior to measurements, cells were dark adapted for 4 min, and sodium bicarbonate ($NaHCO_3$) was introduced to a final concentration of 4 mM to avoid $CO_2$ deficiencies (Maxwell, Falk, Trick, & Hüner, 1994).

### 2.2.5 Statistical Analysis

Experiments were run three times, with each experiment comprised of three replicates. Two-way ANOVAs with post-hoc Tukey's analyses were used to assess differences in growth, pigment content and photosynthetic efficiency among N sources ($NO_3^-$ , $NH_4^+$ and urea) and N concentrations (1, 3, 5, 7 mmol-N $L^{-1}$). Statistical analyses were performed using SigmaPlot 12 (Systat Software, San Jose, CA) and significance was assessed at $p < 0.05$. All graphs were generated with Origin 9.0.

## 2.3 Results

### 2.3.1 Growth

All N substrates were suitable for growth, with all species displaying similar growth kinetics when supplied with the same N source (Fig. 2.2). However, the highest urea concentration (7 mmol-N $L^{-1}$) showed inhibitory properties in two of the species, with lower growth kinetics observed in *Synechococcus* and complete inhibition in *M. aeruginosa* (Fig. 2.1*)*. With the exception of the 7 mmol-N $L^{-1}$-urea treatment, $NO_3^-$ and urea displayed comparable *k* values for all three species. In contrast, when cells were supplied with $NH_4^+$ growth values were halved compared to $NO_3^-$ or urea treatments ($p<0.05$) (Fig. 2.1).

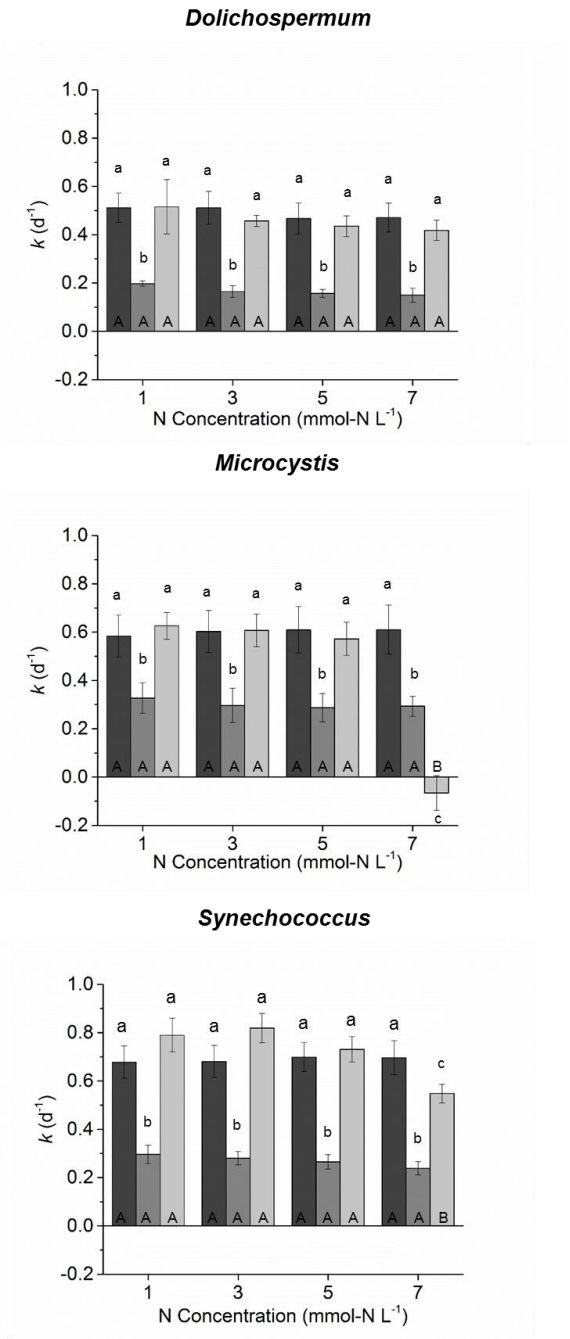


**Fig. 2.1** Divisions per day (*k*) under different N sources. Values are expressed as means ± SD, n=3. Values are expressed as means ± SD, N=3. N sources selected: $NO_3^-$ (■), $NH_4^+$ (■), and urea (■). Same uppercase letters indicate no significant effect of N concentration

with the same N source. Same lowercase letters indicate no significant effect of N source with the same N concentration. Significance tested at $p < 0.05$ level.

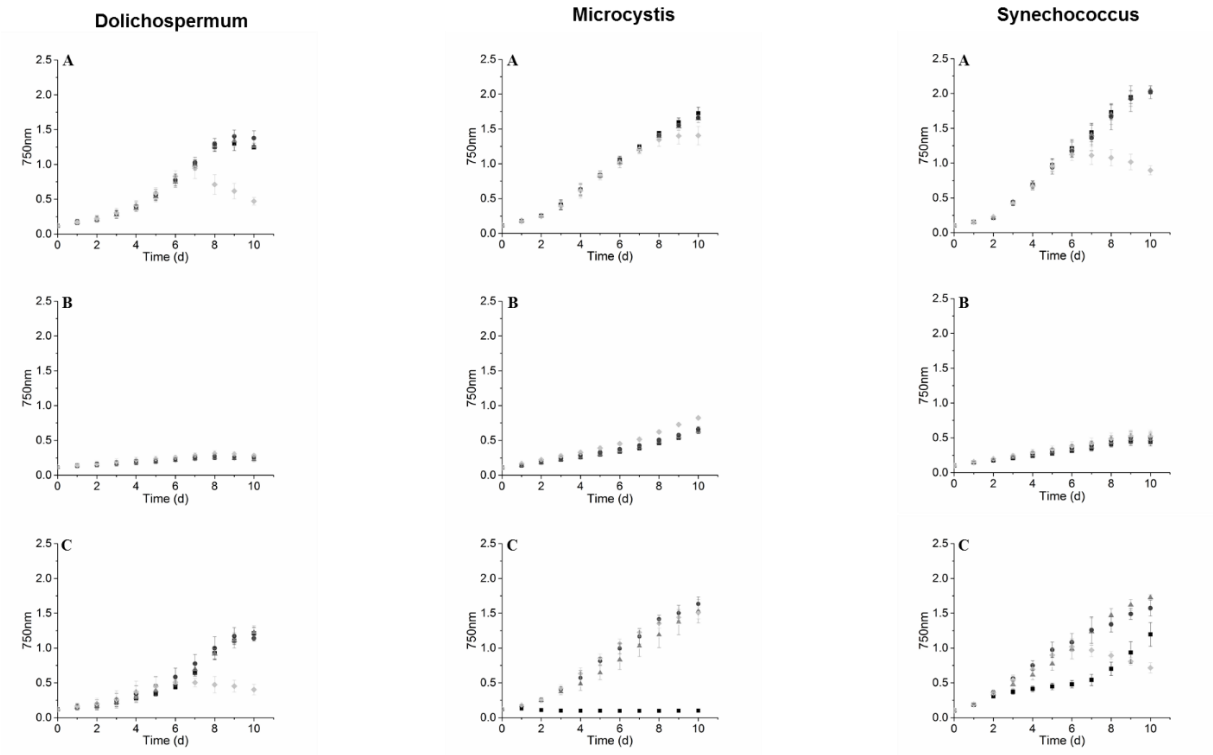


**Fig. 2.2** Growth of N-stressed cyanobacteria to additions of inorganic N (nitrate, ammonium) and urea. A) $NO_3^-$, B) $NH_4^+$ and C) urea. Values are expressed as means ± SD, N=3. Concentrations selected: 7 mmol-N $L^{-1}$ (■), 5 mmol-N $L^{-1}$(▲), 3 mmol-N $L^{-1}$ (●), and 1 mmol-N $L^{-1}$ (♦).

## 2.3.2 Pigments

The lowest pigment content was observed under the $NO_3^-$ and urea treatments at the lowest concentration (1 mmol-N $L^{-1}$), relative to higher N concentrations of the same N source (**Fig. 2.3**). This decline was especially evident in PC, with significant reductions observed among all three species ($p<0.05$). Although both pigments increased under urea amendments, the response observed in PC was more pronounced with mid-concentrations of urea (3 and 5 mmol-N $L^{-1}$) showing significantly higher PC levels in *Synechococcus* and *M. aeruginosa* ($p<0.05$), and higher concentrations (5 and 7 mmol-N $L^{-1}$) being more

suitable for *D. flos-aquae*. Similar to growth, *Synechococcus* and *M. aeruginosa* displayed significantly lower pigment concentrations when grown on the highest urea treatment ($p<0.05$). Significant effects were observed for both chl-*a* and PC for cells grown on the lowest concentration of $NH_4^+$ (1 mmol-N $L^{-1}$) compared to $NO_3^-$ or urea at the same concentration ($p<0.05$).

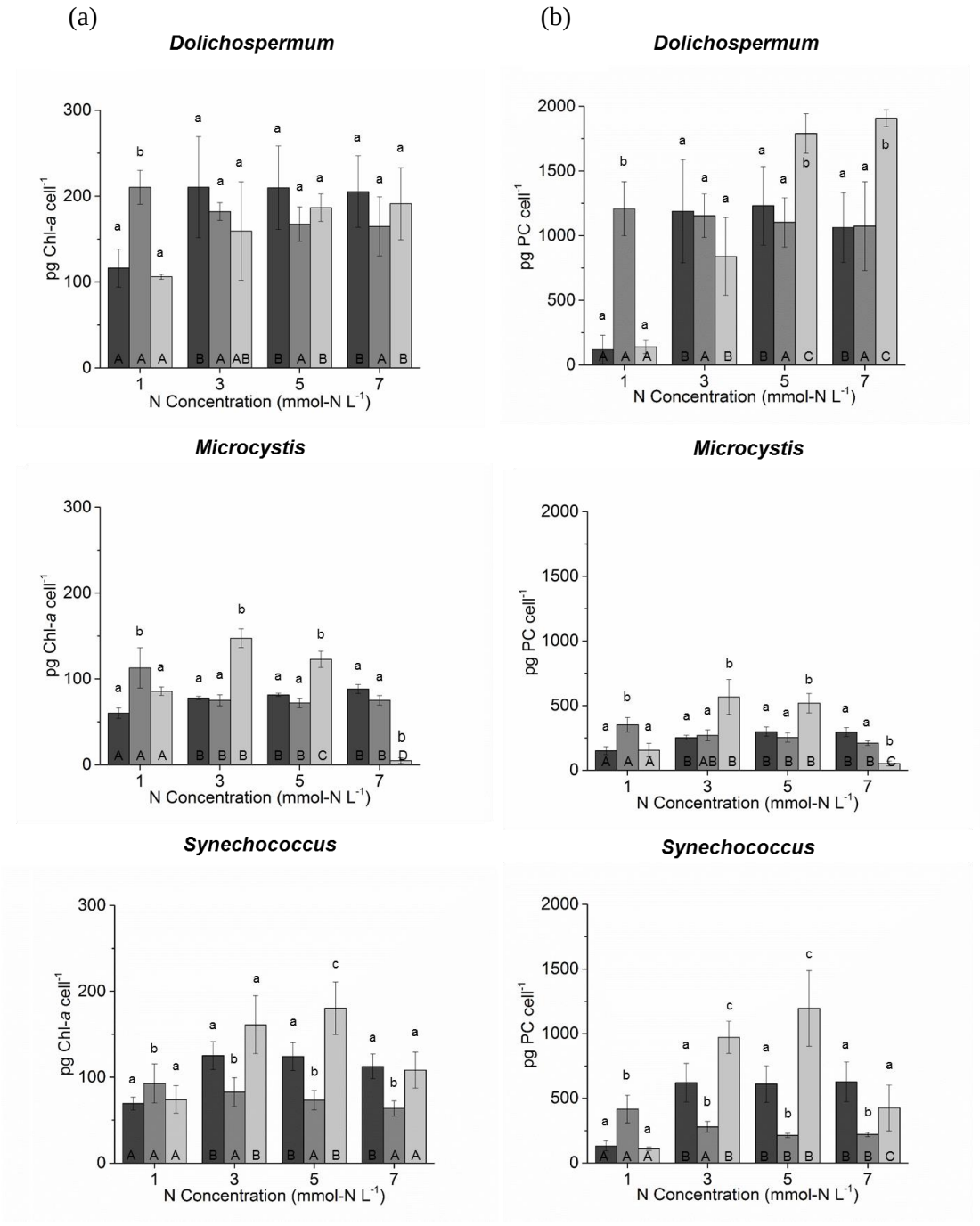


**Figure 2.3** (a) Chlorophyll-*a* (chl-*a*), and (b) Phycocyanin (PC) concentrations under different N sources. Values are expressed as means ± SD, N=3. N sources selected: $NO_3^-$ (■), $NH_4^+$ (■), and urea (■). Same uppercase letters indicate no significant effect of N concentration with the same N source. Same lowercase letters indicate no significant effect of N source with the same N concentration. Significance tested at $p < 0.05$ level.

### 2.3.3 Oxygen Evolution

Significant effects were not observed when cells were grown on varying concentrations of $NO_3^-$; however, *Synechococcus* displayed lower α (Fig. 2.4a) and $P_{max}$ (Fig. 2.4b) when supplied with the lowest $NO_3^-$ concentration (1 mmol-N $L^{-1}$). The highest α and $P_{max}$ values occurred under mid-urea concentrations (3 to 5 mmol-N $L^{-1}$) among all species ($p<0.05$). Again, the highest urea treatment (7 mmol-N $L^{-1}$) restricted photosynthetic activity in *Synechococcus* and *M. aeruginosa* ($p<0.05$). However, this hindering effect was not observed in *D. flos-aquae*, which had an elevated α (Fig. 2.4a) and $P_{max}$ (Fig. 2.4b) value at the highest urea concentration. Lower $NH_4^+$ concentrations yielded significantly higher α and $P_{max}$ values compared to higher $NH_4^+$ concentrations for all cyanobacteria sp. While $P_{max}$ declined in a dose-dependent fashion, only *Synechococcus* and *M. aeruginosa* were shown to have significant N concentration effects ($p<0.05$).

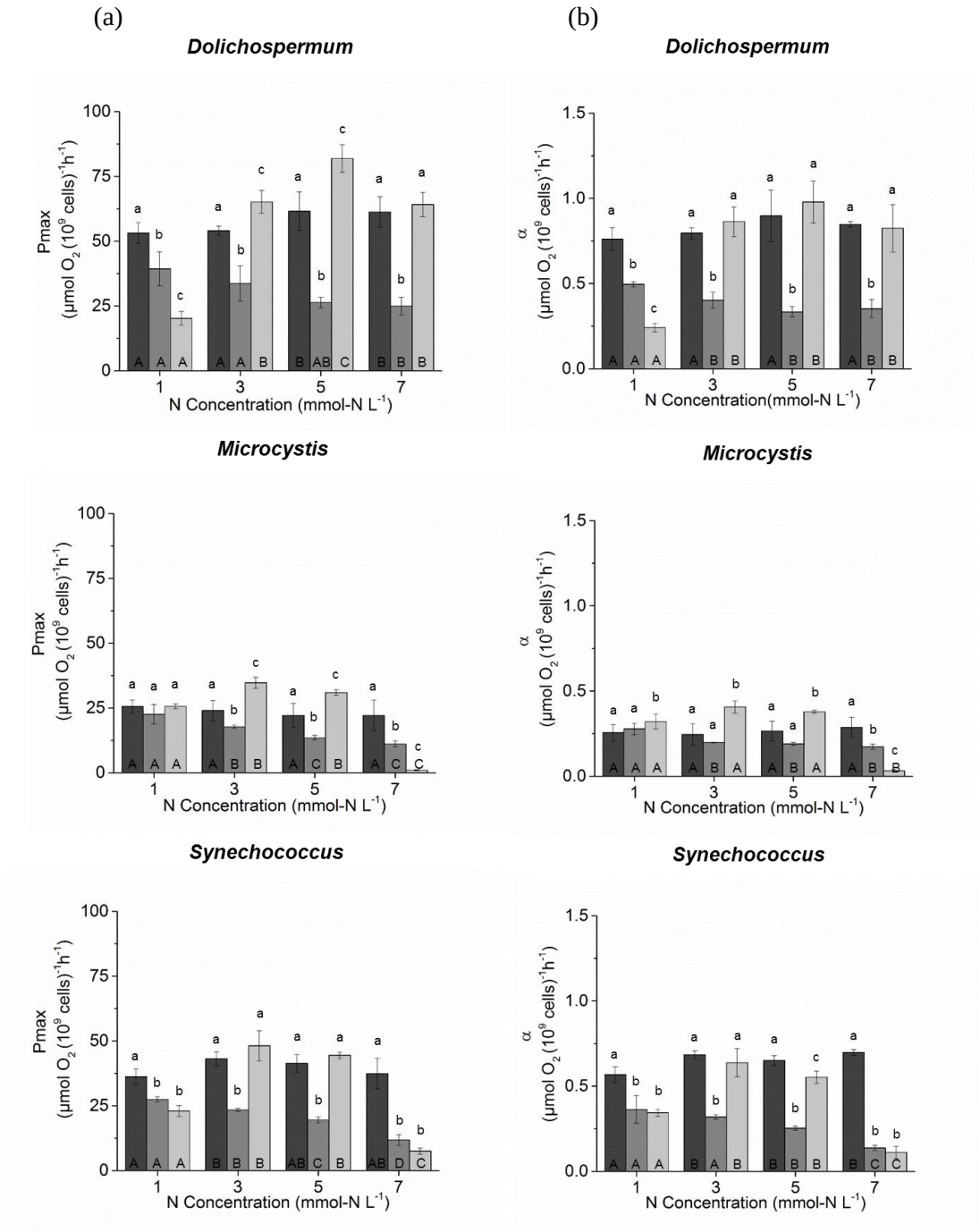


**Figure 2.4** (a) Maximum Photosynthetic rate ($P_{max}$), and (b) Photosynthetic efficiency (α) under different N sources. Values are expressed as means ± SD, N=3. N sources selected: $NO_3^-$ (■), $NH_4^+$ (■), and urea (■). Same uppercase letters indicate no significant effect of N concentration with the same N source. Same lowercase letters indicate no significant effect of N source with the same N concentration. Significance tested at $p < 0.05$ level.

## 2.4 Discussion

Phosphorus is recognized as an important nutrient regulating cyanoHAB development in freshwaters (Schindler, 1977). While P mitigation measures are important for minimizing the risk of nuisance algal growth, some scientists recommend a shift towards a paradigm focused on dual nutrient control—both N and P (Conley et al., 2009; Lewis et al., 2011; Paerl et al., 2016). Unlike P, N occurs in various bioavailable forms in natural freshwaters. Therefore, targeting specific N substrates, such as those linked to the proliferation of cyanoHABs, may be particularly useful rather than concentrating abatement efforts on all bioavailable forms (Chaffin & Bridgeman, 2014). This study assessed the potential for growth and photosynthetic activity of cyanobacteria supplemented with diverse N forms to determine the role of urea on cellular fitness relative to inorganic N sources.

### 2.4.1 Growth

We predicted that cyanobacteria would experience maximum growth on N forms that lower energetic constraints for N acquisition. Thus, growth would sequentially increase from $NO_3^-$, followed by $NH_4^+$ and lastly urea. However, cells supplied with $NO_3^-$ did follow energetic expectations, as growth values were twice that of $NH_4^+$ and comparable to urea. The poor response in $NH_4^+$ likely reflects the "paradoxical" nature of this substrate (Glibert et al., 2016). Depending on substrate availability, phytoplankton growth can either be enhanced or suppressed by the presence of $NH_4^+$ (Britto & Kronzucker, 2002; Dugdale, Wilkerson, Parker, Marchia, & Taberski, 2012). At the lower end of the availability spectrum, $NH_4^+$ is frequently reported as the preferred N source of most phytoplankton due to its superior uptake kinetics, whereas at the higher end of the availability spectrum, $NH_4^+$ has been shown to inhibit growth (Dai, Shang, & Qiu, 2012; Glibert et al., 2016). Concentrations of $NH_4^+$ exceeding several 0.1 mmol-N $L^{-1}$ have been shown to suppress algal growth, with these toxic effects not easily alleviated (Britto & Kronzucker, 2002; Dai et al., 2012; Collos & Harrison, 2014). $NH_4^+$ functions as a negative signaling agent, with increased $NH_4^+$ availability downregulating the uptake and assimilation of itself (Flynn, Fasham, & Hipkin, 1997; Glibert et al.,

2016). Consequently, $NH_4^+$ is more bioavailable to phytoplankton at lower concentrations and concentrations selected for this experiment were likely too high to support optimal growth (Glibert et al., 2016).

Marked differences in growth were not observed when cyanobacteria were supplied with urea relative to inorganic N forms, as growth on urea was matched by cells grown on $NO_3^-$. Our original prediction was that urea would stimulate higher growth responses compared to inorganic substrates due to: (1) low energetic investment; and (2) extra nutrient building blocks (Herrero et al., 2001; Finlay et al., 2010). The additional C and N generated from urea hydrolysis may have not been allocated towards growth, but rather redirected to other key physiological processes requiring high N quotas such as pigment synthesis or toxin production (Allen & Smith, 1969; Harke, Davis, Watson, & Gobler, 2016). Therefore, urea may not enhance cell quantity, but rather cell "quality" and these higher quality cells would have a higher likelihood of survival due to increased N storage or enhanced light absorption capabilities.

Even though urea was a suitable N source of cyanobacteria growth, cyanobacteria cultured on high urea concentrations ($\geq$7 mmol-N $L^{-1}$) lead to various degrees of inhibition among the cyanobacteria species. *M. aeruginosa* displayed complete inhibition, *Synechococcus* sp. exhibited signs of impairment followed by recovery and *D. flos-aquae* appeared to be unaffected (Fig. 2.2). The high variability in responses suggests a range of tolerance, and that cyanobacteria exhibit varying capacities to exploit this organic N source. Mackerras and Smith (1986) and Sakamoto, Delgaizo, & Bryant (1998) triggered a similar response when growing cyanobacteria under elevated urea concentrations (> 10 mmol-N $L^{-1}$ ). Their findings suggest that cyanobacteria have a high affinity for urea and will hydrolyze urea in excess of their biosynthetic requirements, but the hydrolysis of urea resulted in high concentrations of $NH_4^+$ accumulating in the medium, which led to cellular impairment (Mackerras & Smith, 1986). Hence, rates of urea hydrolysis are far greater than the incorporation of $NH_4^+$ into cellular components, and excess $NH_4^+$ is excreted externally when cellular requirements are satisfied, periodically creating conditions unsuitable for growth (Mackerras & Smith, 1986; Sakamoto et al., 1998).

## 2.4.2 Photosynthetic Activity

Nitrogen is an essential structural element for pigment synthesis (Allen & Smith, 1969) and therefore we predicted that N forms that require the lowest energetic investment or supply additional N sources would enhance pigment production. Under this basic energetic principle, urea was projected to enhance the photosynthetic capacity of cyanobacteria relative to inorganic N source due to the additional nutrient N and C sources supplied following urea hydrolysis. Nitrogen speciation influenced cyanobacteria pigment composition by changing the relative abundance of the primary photosynthetic pigment (chl-*a*) and the dominant accessory pigment in freshwater cyanobacteria (PC). In general, we found cyanobacteria displayed the strongest increase in pigment content when grown on urea relative to inorganic N sources. Elevated pigment synthesis likely reflects the additional N and C resulting from hydrolysis of urea, which is absent when inorganic N sources are metabolized.

Chl-*a* is often used as a proxy for phytoplankton biomass due to the time-consuming nature and taxonomic expertise required for microscope counts. However, variability in chl-*a* may reflect changes in chl-*a* content per cell rather than total algal biomass (Geider, MacIntyre, & Kana, 1997). For instance, growth values remained constant for cells grown on $NO_3^-$ and urea, whereas chl-*a* and PC concentrations were enhanced under urea amendments (Fig. 2.3). This finding suggests that excess N produced from the hydrolysis of urea was not used towards active growth, but rather accumulated in secondary pools to increase production of N-rich compounds, such as PC. PC functions as an accessory pigment as well as a nitrogen reserve becoming mobilized under times of N stress (Allen & Smith, 1969; Boussiba & Richmond, 1980). Cells grown on $NO_3^-$ and urea at 1 mmol-N $L^{-1}$ displayed symptoms of N deprivation, with signs of chlorosis appearing midway during growth. Interestingly, these cells yielded similar growth values compared to cells grown on higher N concentrations of the same N source. However, pigment values were significantly reduced at lower N concentrations relative to higher N concentrations, suggesting that cells grown in lower N concentrations began to degrade pigment pools and use these N-rich compounds to fuel active growth. This finding suggests that under

N-stressed conditions, cyanobacteria may tap into pigment reserves and reutilize the liberated N to sustain growth (Allen & Smith, 1969).

Following bloom initiation, light availability declines due to the shading effect created by dense surface aggregates. This shadow exerts competition among phytoplankton and only the strongest competitors for light can flourish (Hutchinson, 1961; Paerl et al., 2001). PC-rich cyanobacteria tend to thrive in turbid freshwaters, as they are superior competitors under low-light regimes (Stomp et al., 2007). A steeper attenuation of light due to the presence of PC improves photosynthetic efficiency, whereas the capacity to achieve higher growth rates under low light conditions further increases the competitive edge cyanobacteria possess under reduced water transparency (Tilzer, 1987; Scheffer, Rinaldi, Gragnani, Mur, & van Nes, 1997). Although cyanobacteria are well equipped to thrive under conditions of low light, their presence also promotes such conditions, as their biomass influences light attenuation in surface waters (van Liere, Mur, Gibson, & Herdman, 1979; Scheffer et al., 1997). Urea pollution could further improve the competitive advantage cyanobacteria possess under eutrophied environments by: (1) enhancing light absorption capabilities through elevated chl-*a* and PC content; and (2) further promoting conditions favorable for sustaining cyanobacteria growth by further reducing light availability through bloom development, thereby encouraging the dominance of this shade-tolerant group.

## 2.5 Conclusion

Urea has become the backbone of modern agriculture, and its ubiquitous use and high mobility support the mounting evidence of urea export into neighboring freshwaters. While urea did not increase cyanobacteria abundance, this organic N source enhanced the production of N-rich pigments. The physiological capacity of cyanobacteria to incorporate urea into pigment synthesis may offer a competitive edge by enhancing light absorption capabilities and N storage, thus making freshwaters more prone to cyanobacteria dominance. Thus, in a future with more intense and widespread agriculture, urea could become a key player in the formation of blooms by supplying cyanobacteria with an N form that enhances their adaptive capacities to environmental

change. Future work should focus on the potential role of urea stimulating other N-rich compounds, such as cyanotoxins, which have gained widespread attention as a contaminant of concern in inland waters due to the global increase in cyanoHAB events. As urea has the potential to alter physiological processes to enhance the storage of N-rich pigments, perhaps urea may also favor the synthesis of N-rich toxins, such as microcystins.

## 2.6 Reference


Allen M. M., & Smith, A. J. (1969). Nitrogen chlorosis in blue-green algae. *Archives of Microbiology*, **69**, 114–120.

Anderson, D., Glibert, P., & Burkholder, J. (2002). Harmful algal blooms and eutrophication: nutrient sources, compositions, and consequences. *Estuaries*, **25**, 704–726.

Belisle, B. S., Steffen, M. M., Pound, H. L., … Wilhelm, S. W. (2015). Urea in Lake Erie: Organic nutrient sources as potentially important drivers of phytoplankton biomass. *Journal of Great Lakes Research*, **42**, 599–607.

Berman, T., & Chava, S. (1999). Algal growth on organic compounds as nitrogen sources. *Journal of Plankton Research*, **21**, 1423–1437.

Boussiba S., & Richmond A.E. (1980). C-phycocyanin as a storage protein in the blue-green alga Spirulina platensis. *Archives of Microbiology*, **125**, 143-147.

Britto, D. T., & Kronzucker, H. J. (2002). $NH_4^+$ toxicity in higher plants: A critical review. *Journal of Plant Physiology*, **159**, 567–584.

Carmichael, W.W. (2001). Health effects of toxin-producing cyanobacteria: "The CyanoHABs". *Human and Ecological Risk Assessment*, **7**, 1393–1407.

Chaffin, J. D., & Bridgeman, T. B. (2014). Organic and inorganic nitrogen utilization by nitrogen-stressed cyanobacteria during bloom conditions. *Journal of Applied Phycology*, **26**, 299–309.

Collos, Y., & Harrison, P. J. (2014). Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin*, **80**, 8–23.

Conley, D. J., Paerl, H. W., Howarth, R. W., … Likens, G.E. (2009). Controlling eutrophication: nitrogen and phosphorus. *Science*, **323**, 1014–1015.

Dai, G. Z., Shang, J. N., & Qiu, B. S. (2012). Ammonia may play an important role in the succession of cyanobacteria blooms and the distribution of common algal species in shallow freshwater lakes. *Global Change Biology*, **18**, 1571–1581.

Donald, D. B., Bogard, M. J., Finlay, K., & Leavitt, P. R. (2011). Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. *Limnology and Oceanography*, **56**, 2161–2175.

Dugdale, R. C., Wilkerson, F. P., Parker, A. E., Marchia, A., & Taberski, K. (2012). River flow and ammonium discharge determine spring phytoplankton blooms in an urbanized estuary. *Estuarine, Coastal and Shelf Science*, **115**, 187–199.

Elser, J. J., Andersen, T., Baron, J. S., … Hessen, D. O. (2009). Shifts in lake N: P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science*, **326**, 835–837.

Fields, S. (2004). Global nitrogen: cycling out of control. *Environmental Health Perspectives*, **112**, 556–563.

Finlay, K., Patoine, A., Donald, D. B., Bogard, M. J., & Leavitt, P. R. (2010). Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the Northern Great Plains. *Limnology and Oceanography*, **55**, 1213–1230.

Flores, E., & Herrero, A. (2005). Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochemical Society Transactions*, **33**, 164–167.

Flynn, K. J., Fasham, M. J. R., & Hipkin, C. R. (1997). Modelling the interactions between ammonium and nitrate uptake in marine phytoplankton. *Philosophical Transaction of the Royal Society B*, **352**, 1625–1645.

Geider, R. J., MacIntyre, H. L., & Kana, T. M. (1997). Dynamic model of phytoplankton growth and acclimation: responses of the balanced growth rate and the

chlorophyll a: carbon ratio to light, nutrient-limitation and temperature. *Marine Ecology Progress Series*, **148**, 187–200.

Glibert, P. M., Harrison, J., Heil, C., & Seitzinger, S. (2006). Escalating worldwide use of urea - A global change contributing to coastal eutrophication. *Biogeochemistr*y, **77**, 441–463.

Glibert, P. M., Maranger, R., Sobota, D. J., & Bouwman, L. (2014). The Haber Bosch–harmful algal bloom (HB–HAB) link. *Environmental Resource Letters*, **9**, 105001.

Glibert, P. M., Wilkerson, F. P., Dugdale, R. C., … Kana, T. M. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnology and Oceanography*, **61**, 165–197.

Gobler, C. J., Burkholder, J. M., Davis, T. W., Harke, M. J., Stow, C. A., & Van de Waal, D. B. (2016). The dual role of nitrogen supply in controlling the growth and toxicity of cyanobacterial blooms. *Harmful Algae*, **54**, 87–97.

Guillard, R. R. L. (1973). Division rates. In: Stein JR, editor. Handbook of phycological methods-culture methods and growth measurements. Cambridge University Press, pp. 289–311.

Harke, M. J., Davis, T. W., Watson, S. B., & Gobler, C. J. (2016). Nutrient-controlled niche differentiation of western Lake Erie cyanobacteria populations revealed via metatranscriptomic surveys. *Environmental Science and Technology*, **50**, 604–615.

Herrero, A., Muro-pastor, A. M., & Flores, E. (2001). Nitrogen control in cyanobacteria. *Journal of Bacteriology*, **183**, 411–425.

Hutchinson, G. E. (1961). The paradox of the plankton. *The American Naturalist*, **95**, 137–145.

Jeffrey, S. W., & Humphrey, G. F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher-plants, algae and natural phytoplankton. *Biochemie Und Physiologie Der Pflanzen*, **167**, 191–194.

Lawrenz, E., Fedewa, E. J., & Richardson, T. L. (2011). Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extract. *Journal of Applied Phycology*, **23**, 865–871.

Lewis, W. M., Wurtsbaugh, W. A., & Paerl, H. W. (2011). Rationale for control of anthropogenic nitrogen and phosphorus in inland waters. *Environmental Science & Technology*, **45**, 10030–10035.

Mackerras, A. H., & Smith, G. D. (1986). Urease activity of the cyanobacterium *Anabaena* cylindrica. *Journal of General Microbiology*, **132**, 2749–2752.

Maxwell, D. P., Falk, S., Trick, C. G., & Hüner, N. P. A. (1994). Growth at low temperature mimics high-light acclimation in Chlorella vulgaris. *Plant Physiology*, **105**, 535–543.

Morgan, R. M., Ivanov, A. G., Priscu, J. C., Maxwell, D. P., & Huner, N. P. A. (1998). Structure and composition of the photochemical apparatus of the Antarctic green alga, *Chlamydomonas subcaudata*. *Photosynthesis Research*, **56**, 303–314.

O'Neil, J. M., Davis, T. W., Burford, M. A., & Gobler, C. J. (2012). The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae*, **14**, 313–334.

Paerl, H. W., Fulton, R. S., Moisander, P. H., & Dyble, J. (2001). Harmful freshwater algal blooms, With an emphasis on cyanobacteria. *The Scientific World Journal*, **1**, 76–113.

Paerl, H. W., & Otten, T. G. (2013). Harmful cyanobacterial blooms: causes, consequences, and controls. *Microbial Ecology*, **65**, 995–1010.

Paerl, H. W., Scott, J. T., McCarthy, M. J., … Wurtsbaugh, W. A. (2016). It Takes Two to Tango: When and Where Dual Nutrient (N & P) Reductions Are Needed to Protect Lakes and Downstream Ecosystems. *Environmental Science & Technology*, **50**, 10805–10813.

Pick, F. R. (2016). Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria. *Canadian Journal of Fisheries and Aquatic Sciences*, **10**, 1–10.

Raven, J.A., Wollenweber, B., & Handley, L. L. (1992). A comparison of ammonium and nitrate as nitrogen sources for photolithotrophs. *New Phytologist*, **121**, 19–32

Sakamoto, T., Delgaizo, V. B., & Bryant D. A. (1998). Growth on urea can trigger death and peroxidation of the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Applied Environmental Microbiology*, **64**, 2361–2366.

Scheffer, M., Rinaldi, S., Gragnani, A., Mur, L., & van Nes, E. (1997). On the dominance of filamentous cyanobacteria in shallow, turbid lakes. *Ecology*, **78**, 272–282.

Schindler, D. W. (1977). Evolution of phosphorus limitation in lakes. *Science*, **195**, 260–262.

Smith, V. H., Tilman, G. D., & Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution*, **100**, 179–196.

Solomon, C. M., Collier, J. L., Berg, G. M., & Glibert, P. M. (2010). Role of urea in microbial metabolism in aquatic systems: A biochemical and molecular review. *Aquatic Microbial Ecology*, **59**, 67–88.

Sterner, R. W. (2008). On the phosphorus limitation paradigm for lakes. *International Review of Hydrobiology*, **93**, 433–445.

Stomp, M., Huisman, J., Vörös L., Pick, F.R., Laamanen, M., Haverkamp, T., & Stal L.J. (2007). Colourful coexistence of red and green picocyanobacteria in lakes and seas. *Ecology Letters*, **10**, 290–298.

Talling, J. F. (1957). The phytoplankton population as a compound photosynthetic system. *New Phytologist*, **56**, 133–149.

Tilzer, M. M. (1987). Light-dependence of photosynthesis and growth in cyanobacteria: Implications for their dominance in eutrophic lakes. *New Zealand Journal of Marine and Freshwater Biology*, **21**, 401–412.

van Liere, L., Mur, L. R., Gibson, C. E., & Herdman, M. (1979). Growth and physiology of *Oscillatoria agardhii* and some related species, a survey. *Developments in Hydrobiology*, **2**, 67–77.

# Chapter 3

# 3 The differential utilization of ammonium, nitrate and urea by three bloom-forming cyanobacteria

This chapter will be submitted as a manuscript for publication to the journal *Harmful Algae*.

## 3.1 Introduction

Increased nutrient availability has been identified as one of the dominant drivers responsible for the recent upsurge in cyanobacteria harmful algal bloom (cyanoHABs) reports (O'Neil et al., 2012; Paerl and Otten, 2013; Pick, 2016). Of particular concern is the loading of macronutrients, phosphorus (P) and nitrogen (N), which have been recognized as strong risk factors linked to the formation of cyanoHABs (Schindler, 1977; Paerl et al., 2001; Smith, 2003). While P has been recognized as the key limiting nutrient regulating cyanoHABs in freshwaters (Schindler, 1977; Sterner, 2008), the importance of N in mediating bloom dynamics is emerging (Conley et al., 2009; Gobler et al., 2016; Paerl et al., 2016). N has emerged as a pollutant of concern due to: (1) its increasing presence in inland waters; (2) accumulating evidence illustrating the importance of combined N and P reductions over single nutrient controls (Conley et al., 2009; Lewis et al., 2011; Paerl et al., 2014); and (3) the influence of N speciation on phytoplankton community structure (Finlay et al., 2010; Glibert et al., 2014; Glibert et al., 2016).

In freshwater environments, much research has been devoted to investigating the effects of growth-limiting nutrients (e.g., specifically P) on phytoplankton productivity (Schindler et al., 2008; Sterner, 2008). However, there is growing evidence to suggest that even nutrients at non-limiting concentrations (e.g., N) can shape phytoplankton community structure (Finlay et al., 2010; Chaffin and Bridgeman, 2014; Glibert et al., 2016). Taxon-specific differences in the assimilation and metabolism of reduced (ammonium ($NH_4^+$) and urea) and oxidized ($NO_3^-$) N play an important role in determining which phytoplankton members will succeed (Finlay et al., 2010; Donald et al., 2011; Glibert et al., 2014). For example, cyanobacteria blooms frequently occur in

waters where chemically reduced N dominants, whereas diatoms flourish in surface waters enriched with $NO_3^-$ (Glibert et al., 2016).

Higher N concentrations relative to P in freshwaters have become an emerging trend, with elevated N: P ratios arising from the disproportionate use of N and P fertilizers (Elser et al., 2009; Galloway et al., 2008; Glibert et al., 2014) and nutrient reduction efforts aimed almost exclusively on P (Gobler et al., 2016; Paerl et al., 2016). Aside from receiving excess N, there has also been a shift in the dominant N form delivered to freshwaters. Recent modifications to the chemical composition of fertilizers have altered the primary N form entering surface waters, with inorganic-N fertilizers now replaced with fertilizers containing urea (Glibert et al., 2006; Finlay et al., 2010; Davis et al., 2016). There is mounting evidence to suggest that the contemporary increase in urea fertilizer use may favor the formation and maintenance of cyanoHABs in inland waters (Berman and Chava, 1999; Finlay et al., 2010; Donald et al., 2011; Glibert et al., 2014; Harke et al., 2016).

Traditionally, dissolved inorganic N (DIN), including $NH_4^+$ and $NO_3^-$, has been the primary focus of researchers investigating the link between N and phytoplankton productivity. The role of dissolved organic nitrogen (DON), including urea, has received comparatively little attention (Finlay *et al*., 2010; Fiedler *et al*., 2015). A heavy research emphasis on DIN suggests a preference for $NH_4^+$ over $NO_3^-$among cyanobacteria, as $NH_4^+$ lowers the energetic constraints of N acquisition (Herrero *et al*. 2001; Flores and Herrero, 2005; Glibert *et al*. 2016). However, what remains unclear is how urea, a growing N form of concern, fits in this energetic hierarchy. While the hydrolysis of urea requires an initial energetic investment to drive the enzymatic reaction required breakdown urea, this reaction generates two $NH_4^+$ molecules for every urea molecule assimilated (Finlay et al., 2010; Donald et al., 2011). The hydrolysis of urea also provides an additional carbon source, circumventing the need for active uptake to drive photosynthesis (Finlay et al., 2010; Flores and Herrero, 2005). Due to these additional benefits, urea may rank at the top of the energetic hierarchy (Finlay et al., 2010).

In nature, phytoplankton are rarely exposed to only one bioavailable form of N; however single nutrient additions are a common practice in culture studies. This single nutrient approach makes understanding nitrogenous preference challenging, as one cannot predict the synergetic effects of oxidized and reduced N forms, typical of most aquatic systems (Glibert et al., 2016). For example, classical physiological research points to the delayed or repressed uptake of alternative N forms in the presence of $NH_4^+$ (Morris and Syrett, 1963; Dortch, 1990; Flores and Herrero, 2005). While $NH_4^+$ has been demonstrated to reduce the uptake of $NO_3^-$, the possible synergetic effects on urea remain in question (Singh, 1990; Glibert et al., 2016).

To elucidate the effects of co-existing N forms on urea uptake and metabolism, $NO_3^-$, $NH_4^+$ and urea were supplied in series of paired-combinations to three bloom-forming freshwater cyanobacteria (*Microcystis*, *Dolichospermum*, and *Synechococcus*) and N concentrations were monitored to track N uptake. Experiments were designed to understand the preferential utilization of N and the potential synergetic effects of different N forms. High urea concentrations were supplied in one of the treatments to understand how excess urea influenced growth. The prediction was that: (1) the sequence of N assimilation would be, from most to least energetically efficient: urea, then $NH_4^+$, and ultimately $NO_3^-$; (2) the uptake of urea would be independent of the availability of either of $NO_3^-$ or $NH_4^+$; and (3) cyanobacteria would rapidly consume urea when supplied in abundance. As urea fertilizer use is projected to continue to escalate, it is essential to better understand whether urea could be promoting the growth of phytoplankton species more harmful to shoreline communities and wildlife. The development of N reduction schemes depend on both, understanding the N requirements of cyanobacteria, and identifying N forms that pose the greatest risk to propagating and maintaining cyanoHABs.

## 3.2 Methods

### 3.2.1 Experimental Design

This investigation focused on three bloom-forming cyanobacteria, maintained as non-axenic, unialgal strains. *Microcystis aeruginosa* (CPCC 300) and *Dolichospermum flos-aquae* (CPCC 67) were obtained from the Canadian Phycological Culture Centre (CPCC) and *Synechococcus* sp. was isolated from Lake Erie in 2015 at Western University. Cyanobacteria species were supplied with BG-11 medium (adjusted to pH 7.4) and maintained at 23°C (±1°C) under a continuous light flux of 60 -70 µmol photons $m^{-2}$ $s^{-1}$. Before initiating experiments, cells in stationary growth were collected via centrifugation (2000 g for 5 min). The resulting supernatant was discarded and pelleted cells were then washed three times with N-free BG-11 medium. Washed cells were inoculated into N-free BG-11 media and grown for a 5-day period to eliminate internal N reserves. Experiments were conducted in 125 mL Erlenmeyer flasks and experimental culture was started at a low concentration (OD750 ~ 0.08) to lower the likelihood of nutrient carryover.

For the preferential N experiment, the basal medium was adjusted to contain two N forms at a total of 1500 µmol-N $L^{-1}$ of each substrate. Three different treatments were selected: $NO_3^-$ + $NH_4^+$, urea + $NO_3^-$, and urea + $NH_4^+$. For the urea inhibition experiment, cells were supplied with 7000 µmol-N $L^{-1}$ representing the high urea treatment. Whereas the control treatment contained 3000 µmol-N $L^{-1}$ urea (optimal growth condition (Erratt Unpublished)) was tested simultaneously. For both experiments, N concentrations in the culture medium were monitored via a suite of colourmetric microplate techniques and measurements were recorded every two days, beginning from the initial inoculation (day 0) and followed until cells reached the stationary growth phase (day 10).

In addition to the preferential N source and urea inhibition experiments, the potential influence of external factors on N utilization was addressed. Cultures were grown in the absence of light to ensure algal-associated bacteria were not contributing to N

consumption. The culture medium without cells was also exposed to experimental growth conditions to verify that light and temperature were not influencing N uptake.

Experiments were run three times and each experiment comprised of three replicates. For the preferential experiment, t-tests were used to determine whether the means of the two N treatments were statistically. Statistical analyses were performed using SigmaPlot 12 (Systat Software, San Jose, CA) and significance was assessed at $p < 0.05$ level. Graphs were generated with Origin 9.0 (OriginLab Corporation, Northampton, MA).

### 3.2.2 Colorimetric Assays

In general, 1 mL of culture was collected in a microcentrifuge tube and centrifuged (6000 g for 5 min) to remove cellular extracts. The resulting supernatant was either used directly or diluted with $ddH_2O$ before being seeded into 96-well microplates. All reagents were analytical grade and were prepared in glassware that had been prewashed with 0.1 M hydrochloric acid (HCl). Reagents remained stable for one month and were stored at 4 ºC in the dark, with the exception of the vanadium trichloride ($VCl_3$) solution which was kept at -20ºC. All absorption measurements were read spectrophotometrically and clear bottom polystyrene 96-well microplates were used for all measurements.

#### 3.2.2.1 Nitrate

$NO_3^-$ concentrations were determined using a single reagent procedure. $VCl_3$ in an acidified solution was used as a reduction agent, reducing $NO_3^-$ to nitrite ($NO_2^-$). Griess reagents (sulfanilamide and N-(1-naphthyl)-ethylenediamine (NED)) were used to detect the total amount of $NO_2^-$ by forming a red coloured product. Reagent solutions were prepared as described in Doane and Horwath (2003). 1 M HCl was prepared by combining 84 mL concentrated HCl into 916 mL $ddH_2O$. For the $VCl_3$ solution, 0.35 g of $VCl_3$ was combined with 50 mL of 1 M HCl. Griess reagents were prepared separately, with the 2% sulfanilamide reagent prepared by adding 0.2 g sulfanilamide to 10 mL of 1 M HCl, and the 0.2% NED solution prepared by adding 0.02 g NED to 10 mL $ddH_2O$. The above solutions were mixed to form a working reagent solution (50 mL $VCl_3$ solution, 3.3 ml 2% sulfanilamide solution, 3.3 mL 0.2 % NED solution and 400 mL

dd$H_2O$) which was separated into 10 mL aliquots and stored in the dark at -20ºC prior to analysis. During the analysis, samples (20 µL) were seeded into microplate wells followed by 180 µL of the working reagent. Microplates were incubated in the dark at 37ºC for 50 min. Absorbance values were read at 540 nm.

### 3.2.2.2 Ammonium

$NH_4^+$ determination was based on the reaction of $NH_4^+$ with salicylate and hypochlorite in the presence of sodium nitroprusside to produce a coloured reaction ranging from pale green to dark blue. Three separate solutions were prepared according to Baethgen and Alley (1989): salicylate solution (6.8 g sodium salicylate, 5 g sodium citrate, 5 g sodium tartrate and 0.025 g sodium nitroprusside was added to 100 mL of dd$H_2O$), sodium hydroxide solution (6 g sodium hydroxide was added to 100 mL of dd$H_2O$), and a bleach solution (0.1 mL commercial bleach and 4.9 mL sodium hydroxide solution) were made fresh prior to analysis. Samples (20 µL) were added to microplate wells followed by 90 µL of salicylate solution and 90 µL of bleach solution. Microplates were mixed between reagent additions and kept in the dark at room temperature (22 ± 2°C) during a 60 min reaction period. Absorbance values were read at 650 nm.

### 3.2.2.3 Urea

Dissolved urea concentrations were measured based on a method that involves the reaction of two reagents, diacetyl monoxime and thiosemicarbazide, in an acidified solution to form a pink coloured product (Revilla et al., 2005). The reagents were prepared according to Revilla *et al*. (2005). Diacetyl monoxime (6.8 g in 100 mL of dd$H_2O$) and thiosemicarbazide solution (0.38 g in 40 mL of dd$H_2O$) were prepared separately and mixed at a 5:1 ratio (diacetyl monoxime to thiosemicarbazide) to prepare Reagent A. Reagent B consisted of 300 mL of concentrated sulfuric acid (>98% grade) with 535 mL dd$H_2O$ and 0.5 mL ferric chloride solution (0.15 g in 10 mL dd$H_2O$). Reagent A and B were mixed at 3.4: 1 ratio (Reagent B to Reagent A) to create the working reagent and was used within 15 min of analysis. Samples (200 µL) were seeded into microplate wells followed by 60 µL of working reagent. Samples were incubated for

72 hr in the dark at room temperature (22 ± 2°C). Absorbance values were read at 520 nm.

### 3.2.3 Formulae

Utilization efficiency (η) of N substrates was defined by:

$\eta = (C_0\text{-}C_t/C_0) \times 100$.

Where $C_0$ is the total N concentration at the initial time point, and $C_t$ is the total N concentration at the end time point (Li *et al*. 2016). η values were calculated at day 6, representing approximately the half-way point during the experimental run.

N drawdowns were plotted vs. time; the greatest slope of the curves were used to calculate the N assimilation rate (K):

$K\ (day^{-1}) = \ln (N_1/N_0) / (t_1\text{-}t_0)$.

Where $N_0$ is the cell concentration at the initial time point ($t_0$), $N_1$ is the cell concentration at the end of the time period ($t_1$), and $t_1\text{-}t_0$ is the time elapsed between the time points.

## 3.3 Results

### 3.3.1 Preferential Use

#### 3.3.1.1 Utilization Efficiency (η)

To understand if preferential use of N occurs in cyanobacteria, two nitrogenous compounds were supplemented to the medium and N levels were monitored to determine whether any N form was preferentially uptaken (Fig. 3.1). Significant effects were observed for all cyanobacteria when grown on urea and $NO_3^-$ ($p<0.05$). A significant decline in urea was observed, with η values reaching 96.7 % and 91.7% for *Synechococcus* and *Microcystis* respectively, whereas η values for *Dolichospermum* were lower achieving 53.4 % ($p<0.05$) (Table 3.1). All isolates demonstrated superior uptake kinetics for urea relative to $NO_3^-$, which had η values for $NO_3^-$ ranging from 32.7% to

8.3%. When urea and $NH_4^+$ were offered in combination, a similar response was observed with urea utilization efficiency rates, reaching 98.4% for *Synechococcus* and 94.7% for *Microcystis*, and again *Dolichospermum* reaching lower values of 51.2% ($p<0.05$). However, $NH_4^+$ levels remained relatively constant with only slight declines observed after urea reserves had become exhausted (Fig. 3.1). When both inorganic N substrates were supplied, $NO_3^-$ and $NH_4^+$, $NH_4^+$ appeared to be the preferred N source for *Microcystis* and *Dolichospermum.* $NH_4^+$ concentrations decreased gradually, with η values of 30.7 % for *Dolichospermum* and 41.2% for *Microcystis* ($p<0.05$). However, for these two isolates, $NO_3^-$ uptake appeared to be delayed by the presence of $NH_4^+$, as $NO_3^-$ levels remained relatively constant during the duration of the experiment. *Synechococcus* did not follow this pattern; rather it displayed simultaneous drawdown of both N substrates at equivalent rates, 52.1% for $NO_3^-$ and 58.0% for $NH_4^+$.

**Table 3.1** Utilization efficiency of cyanobacteria grown on different N combinations. Values are expressed as means ± SD, n=3. Same uppercase letters indicate no significant differences among N sources with a species. Significance tested at $p < 0.05$ level.

| Treatment | N substrate | Utilization Efficiency (η) | Utilization Efficiency (η) | Utilization Efficiency (η) |
|---|---|---|---|---|
| | | *Dolichospermum flos-aquae* | *Synechococcus* sp. | *Microcystis aeruginosa* |
| Urea + $NO_3^-$ | Urea | $53.4 \pm 7.7_A$ | $96.7 \pm 4.6_A$ | $91.7 \pm 11.2_A$ |
| | $NO_3^-$ | $8.3 \pm 8.7_B$ | $32.7 \pm 8.5_B$ | $26.8 \pm 11.0_B$ |
| $NO_3^-$ + $NH_4^+$ | $NO_3^-$ | $-2.0 \pm 19.9_A$ | $52.2 \pm 4.8_A$ | $-6.0 \pm 11.2_A$ |
| | $NH_4^+$ | $30.7 \pm 2.5_B$ | $58.0 \pm 2.9_A$ | $41.2 \pm 0.9_B$ |
| Urea + $NH_4^+$ | Urea | $51.2 \pm 8.3_A$ | $98.4 \pm 1.1_A$ | $94.7 \pm 6.2_A$ |
| | $NH_4^+$ | $2.8 \pm 9.5_B$ | $10.7 \pm 9.2_B$ | $5.6 \pm 4.9_B$ |

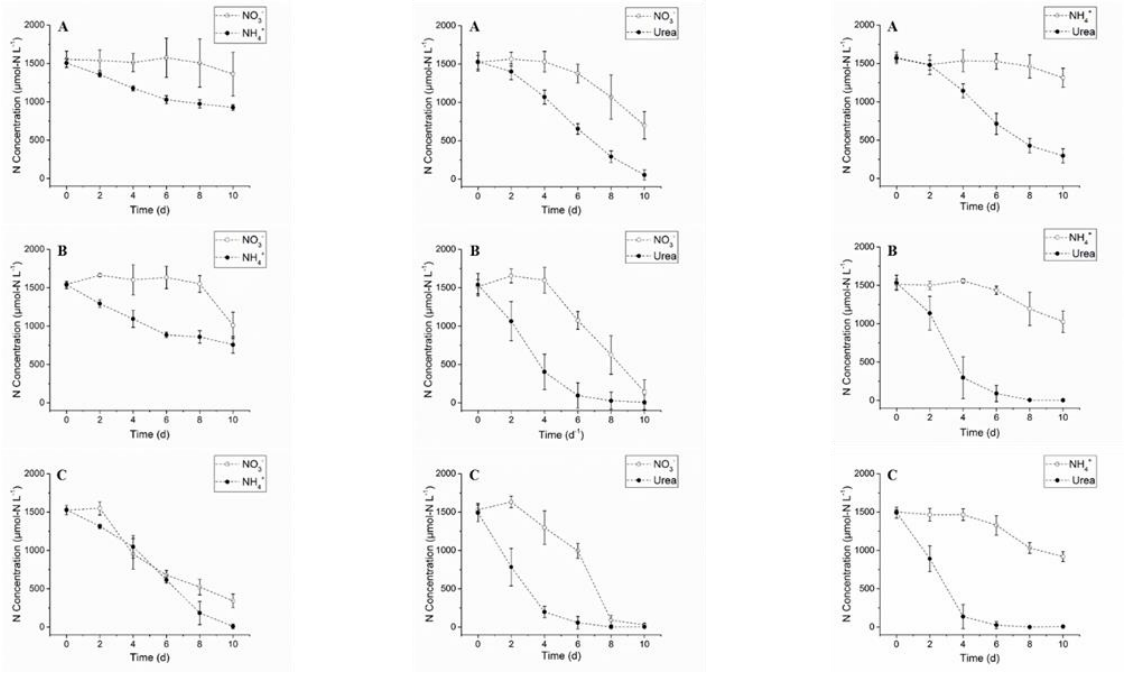


**Figure 3.1** N utilization of cyanobacteria grown on different combinations of nitrogen. A) *Dolichospermum flos-aquae,* B) *Microcystis aeruginosa and* C) *Synechococcus*. Values are expressed as means ± SD, n=3.

### 3.3.1.2 Slope (K)

When urea and $NO_3^-$ were supplied, uptake rates for urea were slightly higher than $NO_3^-$. Although this response was significant for one of the isolates, *Dolichospermum* ($p<0.05$), it was not significant for two of the isolates, *Microcystis* and *Synechococcus* (Table 3.2). When both urea and $NH_4^+$ were supplied, all isolates demonstrated significantly higher assimilation rates for urea relative to $NH_4^+$ ($p<0.05$). On average, urea uptake rates were up to 10-fold higher compared to uptake rates for $NH_4^+$ (Table 3.2). When both $NO_3^-$ and $NH_4^+$ were supplied, there was no significant difference in assimilation rates; K values for $NO_3^-$ and $NH_4^+$ remained relatively consistent among all isolates.

**Table 3.2** Assimilation rates of cyanobacteria grown on different N combinations. Values are expressed as means ± SD, N=3. Same uppercase letters indicate no significant differences among N sources. Significance tested at $p < 0.05$ level.

| Treatment | N substrate | Assimilation rate (K) | Assimilation rate (K) | Assimilation rate (K) |
|---|---|---|---|---|
| | | *Dolichospermum flos- aquae* | *Synechococcus* sp. | *Microcystis aeruginosa* |
| $NO_3^- + NH_4^+$ | $NO_3^-$ | $0.04 \pm 0.06_A$ | $0.16 \pm 0.03_A$ | $0.12 \pm 0.07_A$ |
| | $NH_4^+$ | $0.06 \pm 0.01_A$ | $0.25 \pm 0.07_A$ | $0.09 \pm 0.01_A$ |
| Urea + $NO_3^-$ | Urea | $0.28 \pm 0.05_A$ | $0.94 \pm 0.25_A$ | $0.85 \pm 0.25_A$ |
| | $NO_3^-$ | $0.14 \pm 0.03_B$ | $0.73 \pm 0.15_A$ | $0.43 \pm 0.22_A$ |
| Urea + $NH_4^+$ | Urea | $0.22 \pm 0.05_A$ | $0.97 \pm 0.16_A$ | $0.88 \pm 0.30_A$ |
| | $NH_4^+$ | $0.01 \pm 0.01_B$ | $0.08 \pm 0.04_B$ | $0.08 \pm 0.03_B$ |

### 3.3.2 Effect of High Urea Levels on N Assimilation

No significant effects ($p<0.05$) in η values were observed between cyanobacteria grown under optimal conditions (3000 µmol-N $L^{-1}$) and high urea treatments (7000 µmol-N $L^{-1}$). Thus, urea utilization remained constant regardless of urea availability. When cyanobacteria were exposed to 3000 µmol-N $L^{-1}$ urea, only trace amounts of urea were detected in the medium (Fig. 3.2). Whereas cells grown on 7000 µmol-N $L^{-1}$ urea, had urea drawdowns accompanied by an increase in $NH_4^+$ in the medium (Fig.1.3). $NH_4^+$ concentrations recorded on day 10 were significantly higher ($p<0.01$) compared to optimal conditions, with values ~ 100-200 times higher (Table 3.3). The amount of $NH_4^+$ produced at 7000 µmol-N $L^{-1}$ urea corresponded with the varying degrees of inhibition observed among the cyanobacteria isolates (Table 3.3). *Dolichospermum* exhibited the lowest increase in ambient $NH_4^+$ concentrations and appeared to be the less sensitive to the high urea concentrations. Whereas *Microcystis* showed the highest peak in $NH_4^+$, and these high concentrations lead to complete inhibition.

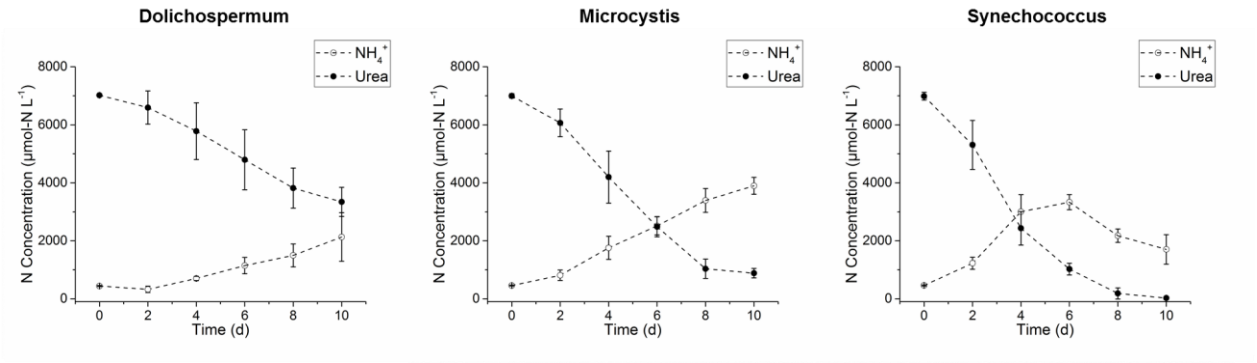


**Figure 3.2** Ammonium production of cyanobacteria grown on 7000 µmol-N $L^{-1}$ - urea. Values are expressed as means ± SD, n=3.

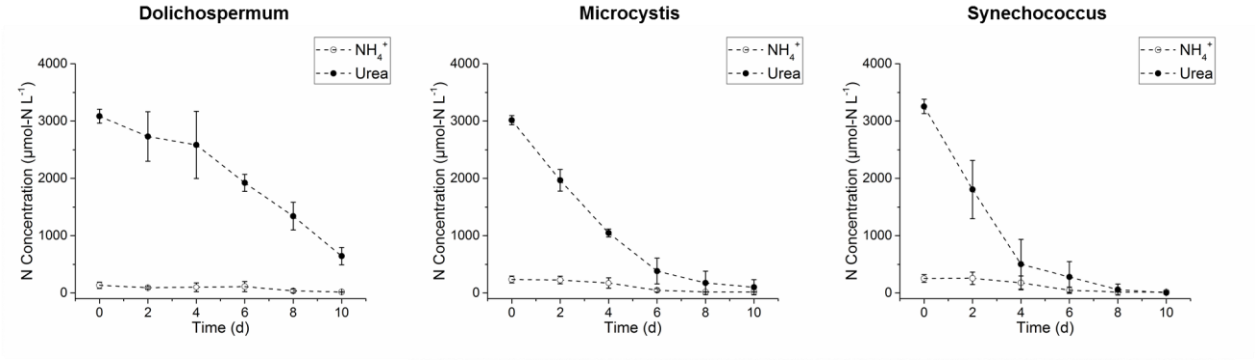


**Figure 3.3** Ammonium production of cyanobacteria grown on 3000 µmol-N $L^{-1}$ - urea. Values are expressed as means ± SD, n=3.

**Table 3.3** Growth and ammonium production of cyanobacteria grown on 7000 µmol-N $L^{-1}$ and 3000 µmol-N $L^{-1}$ - urea. Values are expressed as means ± SD, n=3. Same uppercase letters indicate no significant differences among N sources within a species. Significance tested at $p < 0.05$ level.

| Cyanobacteria Isolates | Urea Concentration (7000 µmol-N $L^{-1}$) | Growth (*k*) | Utilization Efficiency (η) | $NH_4^+$ Production Day 10 (µmol-N $L^{-1}$) |
|---|---|---|---|---|
| *Dolichospermum* | 3000 | $0.32 \pm 0.03_A$ | $36.6 \pm 2.30_A$ | $10.8 \pm 1.3_A$ |
| | 7000 | $0.28 \pm 0.04_A$ | $31.7 \pm 14.65_A$ | $2130.4 \pm 836.5_B$ |
| *Synechococcus* | 3000 | $0.82 \pm 0.08_A$ | $91.5 \pm 7.92_A$ | $17.0 \pm 1.9_A$ |
| | 7000 | $0.55 \pm 0.04_B$ | $85.3 \pm 2.93_A$ | $1702.9 \pm 507.9_B$ |
| *Microcystis* | 3000 | $0.61 \pm 0.07_A$ | $81.9 \pm 7.89_A$ | $18.9 \pm 2.7_A$ |
| | 7000 | $-0.07 \pm 0.07_B$ | $69.5 \pm 4.82_A$ | $3893.3 \pm 291_B$ |

### 3.3.3 Influence of External Factors on Urea Uptake

To confirm urea drawdowns were attributed entirely to cyanobacterial activity, the possibility of heterotrophic bacteria contributing to urea utilization had to be ruled out. Cultures were grown in the absence of light to suppress photosynthetic activity. Under dark conditions, minimal urea uptake was observed (Fig. 3.4), with N utilization efficiency ranging between 0.9 and 8.9% (Table 3.4). Cyanobacteria grown in the dark at 7000 µmol-N $L^{-1}$ urea had significantly lower ($p<0.05$) η values compared to cells grown with light at the same concentration (Table. 1.4). These results confirm that bacterial interference played little role in N consumption.

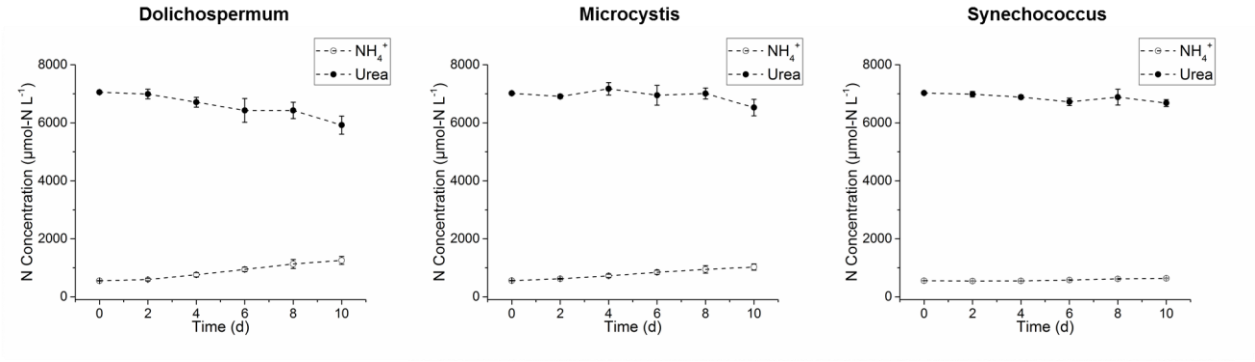


**Figure 3.4** Urea consumption by cyanobacteria grown on 7000 µmol-N $L^{-1}$ – urea in the dark. Values are expressed as means ± SD, n=3.

**Table 3.4** Comparison between light and dark responses of cyanobacteria grown on 7000 µmol-N $L^{-1}$. Values are expressed as means ± SD, n=3. Same uppercase letters indicate no significant differences among N sources within a species. Significance tested at $p < 0.05$ level.

| Cyanobacteria Isolates | Urea Concentration (7000 µmol-N $L^{-1}$) | Utilization Efficiency (η) | $NH_4^+$ Production Day 10 (µmol-N $L^{-1}$) |
|---|---|---|---|
| *Dolichospermum* | Light | $31.7 \pm 14.65_A$ | $2130.4 \pm 836.5_A$ |
| | Dark | $8.9 \pm 5.48_B$ | $1255.8 \pm 137.1_A$ |
| *Synechococcus* | Light | $85.3 \pm 2.93_A$ | $1702.9 \pm 507.9_A$ |
| | Dark | $4.3 \pm 1.73_B$ | $637.2 \pm 16.2_B$ |
| *Microcystis* | Light | $69.5 \pm 4.82_A$ | $3893.3 \pm 291_A$ |
| | Dark | $0.9 \pm 5.22_B$ | $1024.9 \pm 5.22_B$ |

In addition to heterotrophic bacteria interference, the possibility of light and temperature influencing N degradation had to be eliminated. Culture media without cells was exposed to experimental growth conditions (Fig. 3.5). Throughout the 10-day exposure period, no significant degradation of the three N substrates was observed, with utilization efficiency ranging between -6.7 to 0.4 % for all three N substrates (Table 3.5). Thus, confirming that no abiotic transformations of the available N sources occurred throughout the course of the experiments.

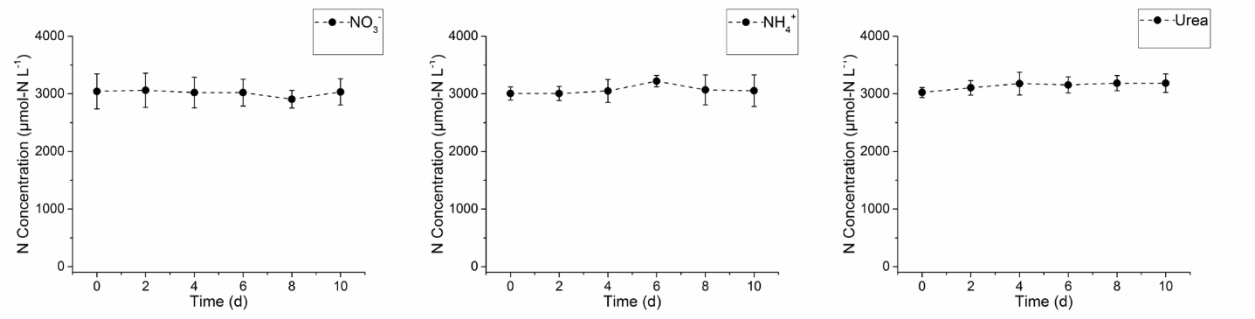


**Figure 3.5** Different N substrates grown on 3000 µmol-N $L^{-1}$ without cells exposed to experimental growth conditions. Values are expressed as means ± SD, n=3.

**Table 3.5** Utilization efficiency of different N sources without cells exposed to growth conditions. Values are expressed as means ± SD, n=3.

| N Concentration (3000 µmol-N $L^{-1}$) | $NO_3^-$ | $NH_4^+$ | Urea |
|---|---|---|---|
| Utilization Efficiency (η) | 0.4 ± 5.51 | -6.7 ± 4.77 | -4.3 ± 3.09 |

## 3.4 Discussion

Urea is now the dominant form of N pollution in agriculturally impacted regions, and its growing presence in freshwaters has increased concomitantly with the intensity and duration of cyanoHAB events (Finlay et al., 2010; Glibert et al., 2014; Davis et al., 2016). In the marine realm, urea has been branded as a N source of concern based on correlative evidence presenting an emerging trend between higher incidences of shellfish poisoning in regions experiencing elevated urea inputs. Although this evidence is indirect, this link

is further supported by the physiological capacity for urea uptake by many important marine HAB species (Glibert et al., 2006; Kudela et al., 2008). The importance of urea in contributing to freshwater HAB formation is now emerging. Here, we investigated whether cyanobacteria, the most notable bloom-forming algal group in freshwaters, exhibit preferential selection of urea over other nitrogenous compounds.

Earlier observations suggest that urea uptake varies considerably within and among freshwater phytoplankton, with cyanobacteria achieving higher growth rates when grown on urea compared to inorganic N forms (Berman and Chava, 1999). Contrary to Berman and Chava (1999), there is growing evidence to suggest that preferential uptake of urea by freshwater cyanobacteria may not be a universal characteristic shared among all genera. Non-diazotrophic cyanobacteria, such as *Microcystis* and *Planktothrix*, are more readily stimulated by this organic N substrate than $N_2$-fixing cyanobacteria (Finlay et al., 2010; Donald et al., 2011). In this study, all cyanobacteria isolates consumed urea more rapidly than inorganic N, but the non-$N_2$-fixing taxa, *Microcystis* and *Synechococcus*, exhibited higher utilization efficiencies than the $N_2$-fixing taxa, *Dolichospermum*.

$NH_4^+$ control has been well established in the classical physiological literature, with a delayed or repressed uptake of $NO_3^-$ observed in the presence of $NH_4^+$ (Dortch,1990; Glibert *et al*., 2016). This classical assumption played out for two of the studied cyanobacteria, *Microcystis* and *Dolichospermum*. In the presence of $NH_4^+$, *Microcystis* and *Dolichospermum* showed delayed $NO_3^-$ uptake, only tapping into $NO_3^-$ reserves late into the experimental run. However, *Synechococcus* challenged this well-grounded notion, exhibiting simultaneous drawdowns of both inorganic N forms. This response goes to show that cyanobacteria species cannot be lumped into one physiological grouping, as different physiological traits may exist among species.

When urea was coupled with inorganic N forms, urea appeared to be the superior N source for all isolates, being drawn down at higher rates relative to inorganic N sources. Contrary to earlier assumption, the repressive effects of $NH_4^+$ on urea were absent, in fact urea was shown to potentially hinder $NH_4^+$ uptake. It was long assumed that most cyanobacteria preferred $NH_4^+$ over alternative N forms, due to its favorable energetics

(Flores and Herrero, 2005; Finlay et al., 2010). However, urea may be more energetically efficient and therefore can override the repressive nature $NH_4^+$. Aside from functioning as a superior N source, offering a two-fold increase in N. Urea may also function as readily available carbon source, thus lowering the cells reliance for active uptake to drive photosynthesis (Finlay et al., 2010; Donald et al., 2011).

Although utilization efficiency values were significantly elevated for urea compared to $NO_3^-$, assimilation rates were similar between the two N forms for two of the isolates, *Microcystis* and *Synechococcus*. Hence, assimilation rates for each substrate are comparable, but a significant lag period was observed prior to $NO_3^-$ uptake. This extended lag period was also observed for all species supplemented with urea and $NH_4^+$, with $NH_4^+$ appearing to be assimilated once urea had been exhausted.

Cyanobacteria exposed to high urea concentrations lead to a range of inhibition among cyanobacteria species. To understand this response, changes in N forms in the medium were measured to determine if the transformation of urea into alternative N sources was hindering growth. Findings were consistent with Mackerras and Smith (1986) and Sakamoto et al., (1998), who detected elevated $NH_4^+$ production when cyanobacteria were exposed to high urea concentrations. Cyanobacteria engaged in a "gluttonous" behavior, rapidly consuming urea in excess of their N requirements. Rather than accumulating excess $NH_4^+$ intracellularly, cyanobacteria expelled excess N into the external environment to avoid $NH_4^+$ toxicity. However, due to confining space and lack of dispersal within the culture vessel, $NH_4^+$ accumulated to dangerously high levels resulting in growth impairment. Interestingly, cells grown on lower urea concentrations (3 mmol-N $L^{-1}$) did not exhibit this trend, and only transient levels of $NH_4^+$ were found.

The range of inhibition observed among the three isolates corresponded with the level of $NH_4^+$ detected. *Dolichospermum* being the least sensitive to high urea concentrations accumulated the lowest levels of $NH_4^+$ in the medium, whereas *Microcystis* showed complete inhibition and the highest $NH_4^+$ burden. $NH_4^+$ concentrations in freshwaters have been proposed as a regulatory factor influencing phytoplankton community structure and cyanoHAB potential (Dai et al., 2012; Glibert et al., 2016). As a group,

cyanobacteria are relatively tolerant of high $NH_4^+$ concentrations compared to eukaryotic algae, with the exception of the chlorophytes. However, certain cyanobacteria genera, such as *Microcystis*, have been shown to be quite intolerant to elevated $NH_4^+$ levels with blooms appearing only at very low concentrations (Dai et al., 2012; Collos and Harrison, 2014). Hence, *Microcystis* poor performance under high urea amendment is likely attributed to its sensitivity to $NH_4^+$.

Given the increasing trends in the use of the urea as a chemical fertilizer, the incorporation of isolated strains of other dominant phytoplankton groups (e.g., diatoms and chlorophytes) into preferential N uptake studies could provide a stronger understanding of how urea pollution may be driving phytoplankton community dynamics. The effect of urea on vulnerable freshwater systems (i.e., oligotrophic and mesotrophic lakes) also needs to be assessed at larger scales. Currently, many oligotrophic and mesotrophic systems are not neighboring large agricultural operations and therefore do not experience elevated urea loads. However, with climate change, the land suitable for agriculture will expand into northern regions, and these vulnerable systems will likely become more susceptible to urea pollution. Exploring how urea may influence natural phytoplankton assemblages in low-nutrient waters could offer insight into how lake primary production will change in response to future urea loading.

## 3.5 Conclusion

The loss of urea into freshwaters has been linked to the contemporary rise in cyanoHAB outbreaks. There is amassing evidence to suggest the importance of N in freshwater eutrophication and that the composition of the N pool may function as a regulatory factor determining the distribution of common phytoplankton species. Urea was consistently drawndown at higher rates relative to inorganic N substrates, indicating cyanobacteria exhibit a higher affinity for urea. Furthermore, when supplied in excess, cyanobacteria rapidly consumed urea in excess of their biosynthetic requirements suggesting a form of urea "gluttony". The results of this study illustrate the importance of urea in freshwater eutrophication and satisfying the nitrogenous nutrition of cyanobacteria. As society moves forward into an era where influxes of urea in freshwaters will become increasingly

common, the need to understand how urea influences phytoplankton community composition is stronger than ever.

## 3.6 References


Baethgen W.E. & Alley M.M. (1989) A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. *Communications in Soil Science and Plant Analysis*, **20**, 961-969.

Belisle B.S., Steffen M.M., Pound H.L., DeBruyn J.M., Watson S.B., Bourbonniere R.A., Boyer G.L. & Wilhelm S.W. (2016) Urea in Lake Erie: organic nutrient sources are potentially important drivers of phytoplankton biomass. *Journal of Great Lakes Research*, **42**, 599-607.

Berman T. & Chava S. (1999) Algal growth on organic compounds as nitrogen sources. *Journal of Plankton Research*, **21**, 1423-1437.

Bogard M.J., Donald D.B., Finlay K. & Leavitt P.R. (2012) Distribution and regulation of urea in lakes of central North America. *Freshwater Biology*, **57**, 1277-1292.

Chaffin J.D. & Bridgeman T.B. (2014) Organic and inorganic nitrogen utilization by nitrogen-stressed cyanobacteria during bloom conditions. *Journal of Applied Phycology*, **26**, 299-309.

Collos Y. & Harrison P.J. (2014) Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin*, **80**, 8-23.

Conley D.J., Paerl H.W., Howarth R.W., Boesch D.F., Seitzinger S.P., Havens K.E., Lancelot C. & Likens G.E. (2009) Controlling eutrophication: nitrogen and phosphorus. *Science*, **323**, 1014-1015.

Dai G., Shang J. & Qiu B. (2012) Ammonia may play an important role in the succession of cyanobacteria blooms and the distribution of common algal species in shallow freshwater lakes. *Global Change Biology*, **18**, 1571-1581.

Davis A.M., Tink M., Rohde K. & Brodie J.E. (2016) Urea contributions to dissolved 'organic' nitrogen losses from intensive, fertilized agriculture. *Agriculture, Ecosystems & Environment*, **223**, 190-196.

Doane T. A. & Horwath W.R. (2003) Spectrophotometric determination of nitrate with a single reagent. *Analytical Letters*, **36**, 2713-2722.

Donald D.B., Bogard M.J., Finlay K. & Leavitt P.R. (2011) Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. *Limnology and Oceanography*, **56**, 2161-2175.

Dortch Q. (1990) The interaction between ammonium and nitrate: Variation with growth rate, nitrogen source and species. *Marine Ecology Progress Series*, **61**, 183-201.

Elser J.J., Andersen T., Baron J. S., Bergstrom A.K., Jansson M., Kyle M., Nydick K.R., Steger L. & Hessen D.O. (2009) Shifts in lake N:P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science*, **326**, 835-837.

Finlay K., Patoine A., Donald D.B., Bogard M.J. & Leavitt P.R. (2010) Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the Northern Great Plains. *Limnology and Oceanography*, **55**, 1213-1230.

Flores E. & Herrero A. (2005) Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochemical Society Transactions*, **33**, 164-167.

Galloway J.N., Townsend A.R., Erisman J.W., Bekunda M., Cai Z., Freney J.R., Martinelli L.A., Seitzinger S.P. & Sutton. M.A. (2008) Transformation of the nitrogen cycle: recent trends, questions and potential solutions. *Science*, **320**, 889-892.

Glibert P.M., Harrison J., Heil C. & Seitzinger S. (2006) Escalating worldwide use of urea—a global change contributing to coastal eutrophication. *Biogeochemistry*, **77**, 441-463.

Glibert P.M., Maranger R., Sobota D.J. & Bouwman L. (2014) The Haber Bosch–harmful algal bloom (HB–HAB) link. *Environmental Resource Letters*, **9**, 105001.

Glibert P.M., Wilkerson F.P., Dugdale R.C., Raven J.A., Dupont C., Leavitt P.R., Parker A.E., Burkholder J.M. & Kana T.M. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnology and Oceanography*, **61**, 165-197.

Gobler C.J., Burkholder J.M., Davis T.W., Harke M.J., Johengen T., Stow C.A. & van de Waal D.B. (2016) The dual role of nitrogen supply in controlling the growth and toxicity of cyanobacteria blooms. *Harmful Algae*, **54**, 87-97.

Harke M.J., Davis T.W., Watson S.B. & Gobler C.J. (2016) Nutrient-controlled niche differentiation of western Lake Erie cyanobacteria populations revealed via metatranscriptomic surveys. *Environmental Science & Technology*, **50**, 604-615.

Healey F.P. (1977) Ammonium and urea uptake by some freshwater algae. *Canadian Journal of Botany*, **55**, 61-69.

Herrero A., Muro-Pastor A.M. & Flores E. (2001) Nitrogen control in cyanobacteria. *Journal of Bacteriology*, **183**, 411-425.

Kudela R., Lane J. & Cochlan W. (2008) The potential role of anthropogenically derived nitrogen in the growth of harmful algae in California, USA. *Harmful algae*, **8**, 103-110.

Li J.H., Zhang J.B., Huang W., Kong F.L., Li Y., Xi M. & Zheng Z. (2016) Comparative bioavailability of ammonium, nitrate, nitrite and urea to typically harmful cyanobacterium *Microcystis aeruginosa*. *Marine Pollution Bulletin*, **110**, 93-99.

Lewis W.M., Wurtsbaugh W.A. & Paerl H.W. (2011) Rationale for control of anthropogenic nitrogen and phosphorus to reduce eutrophication of inland waters. *Environmental Science & Technology*, **45**, 10300-10305.

Mackerass A.H. & Smith G.D. (1986) Urease activity of the cyanobacterium Anabaena cylindrical. *Journal of General Microbiology Reviews*, **132**, 2749-2752.

Morris I. & Syrett P.J. (1963) The development of nitrate reductase in chlorella and its repression by ammonium. *Archiv Fur Mikrobiologie*, **47**, 32-41.

O'Neil J.M., Davis T.W., Burford M.A. & Gobler C.J. (2012) The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae*, **14**, 313-334.

Paerl H.W., Fulton R.S., Moisander P.H. & Dyble J. (2001) Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal*, **1**, 76-113.

Paerl H.W. & Otten T.G. (2013) Harmful cyanobacteria blooms: Causes, consequences, and controls. *Microbial Ecology*, **65**, 995-1010.

Paerl H.W., Xu H., McCarthy M.J., Zhu G., Qin B., Li Y. & Gardner W.S. (2014) Controlling harmful cyanobacteria blooms in a hyper-eutrophic lake (Lake Taihu, China): the need for a dual nutrient (N & P) management strategy. *Water Research*, **45**, 1973-1983.

Paerl H.W., Scott T., McCarthy M.J., Newell S.E., Gardner W.S., Havens K.E., Hoffman D.K., Wilhelm S.W. & Wurtsbaugh W.A. (2016) It Takes Two to Tango: When and Where Dual Nutrient (N & P) Reductions Are Needed to Protect Lakes and Downstream Ecosystems. *Environmental Science & Technology*, **50**, 10805-10813.

Pick F.R. (2016) Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria. *Canadian Journal of Fisheries and Aquatic Sciences*, **73**, 1-10.

Revilla M., Alexander J. & Glibert P.M. (2005) Urea analysis in coastal waters: Comparison of enzymatic and direct methods. *Limnology and Oceanography Methods*, **3**, 290-299.

Ria A.K. & Singh S. (1987) Kinetic and regulation of urea uptake in *Anabaena doliolum* and *Anacystis nidulans*. *Journal of Applied Microbial Ecology*, **31**, 111-125.

Rippka R., Deruelles J., Waterbury J.B., Herdman M. & Stanier (1979) Generic assignments, strain histories and properties of pure culture of cyanobacteria. *Journal of Microbiology*, **111**, 11-61.

Sakamoto T., Delgaizo V.B & Bryant D.A. (1998) Growth on urea can trigger death and peroxidation of the cyanobacterium Synechococcus sp. strain PCC 7002. *Applied Environmental Microbiology*, **64**, 2361-2366.

Schindler D.W. (1977) Evolution of phosphorus limitation in lakes. *Science*, **195**, 260-262.

Schindler D.W., Hecky R.E., Findlay D.L., Stainton M.P., Parker B.R., Paterson M.J., Beaty K.G., Lyng M. & Kasian S.E.M. (2008) Eutrophication of lakes cannot be controlled by reducing nitrogen input: Results of a 37-year whole-ecosystem experiment. *Proceedings of the National Academy of Sciences,* **105**, 11254-11258.

Singh S. (1990) Regulation of urease activity in the cyanobacterium *Anabaena doliolum*. *FEMS Microbiology Letters*, **67**, 79-84.

Smith, V. H. (2003). Eutrophication of freshwater and coastal marine ecosystems: A global problem. *Environmental Science and Pollution Research,* **10**, 126-139.

Sterner R.W. (2008) On the phosphorus limitation paradigm for lakes. *International Review of Hydrobiology*, **93**, 433-445.

# Chapter 4

# 4 Conclusion

## 4.1 Main Findings

Eutrophication of freshwater lakes remains a growing threat to water security despite more than a half-century of research. This is due to the increased flux of growth-limiting nutrients, most notably P and N, entering surface waters (Smith *et al*., 1999; Conley *et al*., 2009; Paerl *et al*., 2016). Eutrophication is accompanied by an expansion of cyanoHABs (Smith, 2003; O'Neil *et al*., 2012; Brooks *et al*., 2016). Although our knowledge of cyanobacteria-related water quality concerns has advanced over the last few decades, knowledge gaps still exist, including the factors that initiate and maintain toxin-producing taxa. Even our most "scientifically sacred" nutrient paradigms (P-limitation) are under intense scientific scrutiny (Lewis *et al*., 2011; Molot *et al*., 2014; Paerl *et al*., 2016). While some scientists strongly defend a single nutrient approach focused exclusively on P to reduce cyanoHABs (Schindler, 1977; Sterner, 2008), other scientists argue that P reductions together with N reductions could further reduce and the frequency and intensity of cyanoHAB events (Conley *et al*., 2009; Lewis *et al*., 2011; Paerl *et al*., 2016).

In Chapter 2, I examined the effects between the supply of various nitrogenous compounds ($NO_3^-$, $NH_4^+$, and urea) on the growth and photosynthetic characteristics of three bloom-forming cyanobacteria species. Urea was predicted to be the superior N source, largely due to its low energetic costs for N acquisition and the additional benefit of extra nutrient substrates. Although, urea did not significantly increase cyanobacteria abundance relative to inorganic N forms. Urea did yield elevated pigment concentrations. The extra N generated from the hydrolysis of urea was not incorporated into active growth, but rather accumulated in secondary pools to increase production of N-rich pigments. Elevated pigment content provides cyanobacteria with a competitive edge by improving light absorption capabilities, while also functioning as N reserve, which can be

mobilized under times of N stress. Thus, urea produced “higher-quality” cells that may be more adapted to a changing world.

In Chapter 3, I examined the effect of high urea concentrations (>7 mmol-N $L^{-1}$) on cyanobacteria growth. A range of sensitivity was observed among the three cyanobacteria species, with some showing complete inhibition while other remained unaffected. Interestingly, urea was not directly contributing to growth impairment, but rather high extracellular concentrations of $NH_4^+$ hindered growth. The range of inhibitation among the three species corresponded with the level of $NH_4^+$ detected. For example, complete inhibition matched the highest $NH_4^+$ burden, whereas the lowest detected $NH_4^+$ levels did not suppress growth. All cyanobacteria isolates hydrolysed urea in excess of their N requirements, with excess $NH_4^+$ expelled into the extrenal environment to avoid $NH_4^+$ toxcitiy. When supplied with lower concentrations of urea, cells generated trace amounts of $NH_4^+$. This finding suggests that cyanobacteria display a form of “luxury uptake” when urea is in excess.

In Chapter 3, I also tested whether cyanobacteria would preferentially select urea over inorganic N substrates. Consistent with energetic expectations, urea was consistently drawn down at higher rates compared to inorganic N forms. However, preferential uptake of urea was not evident, as inorganic N were simultaneously drawn down, but experienced either extended delays or significantly lower rates of uptake relative to urea. In chapter 3, the repressive nature of $NH_4^+$ on the uptake of alternative N forms was also explored (Dai *et al*., 2012; Glibert *et al*., 2016). Our research suggests that this statement is not entirely true, while $NH_4^+$ inhibitory effects were reported from cells grown on $NH_4^+$ and $NO_3^-$ for two isolates, the other exhibited simultaneous uptake of each substrate at equivalent rates. Furthermore, urea appeared to be unaffected by the presence of $NH_4^+$ and this result may reflect the superior energetics of this organic N substrate.

## 4.2 Significance

The crops that humanity depend on for survival demand more N than nature can provide. Thus, N fertilizers are perceived as a modern miracle, fueling the agricultural sector and

providing sustenance to a hungry world (Smil, 1999; Erisman *et al*., 2008). With an additional three billion more mouths to feed by 2050, global reliance on N is projected to escalate concurrently with N concentrations in the world's freshwaters (Galloway *et al*., 2004; Glibert *et al*., 2014). The dominant N source applied to agricultural landscapes is urea (Glibert *et al*., 2006; Finlay *et al*., 2010) and as society continues to release massive quantities to secure a steady food supply, inevitably some urea is lost and seeps into neighboring freshwaters acting as energy source for phytoplankton (Donald *et al*., 2011; Glibert *et al*., 2014).

While initially overlooked as a contaminant of concern half-century ago, urea has now surpassed and nearly replaced inorganic N fertilizers (Glibert *et al*., 2006; Glibert *et al*., 2014). This global shift in fertilizer consumption habits has brought about new questions surrounding the potential negative effects of urea pollution on aquatic environments. While urea has been recognized as N source of concern in marine systems (Glibert *et al*., 2006; Kudela *et al*., 2008), it was only until recently that the urea enrichment to freshwaters was feared to be promoting the growth cyanoHABs (Finlay *et al*., 2010; Donald *et al*., 2011; Glibert *et al*., 2014; Belisle *et al*., 2016). The practical significance of this study lays in understanding environmental factors that may initiate and prolong cyanoHABs. Determining how different chemical forms of N may influence cyanobacteria abundance could offer insight regarding the nutritional requirements of cyanobacteria. A stronger understanding on the nitrogenous nutrition of this nuisance algal group could assist legislatures and scientists in developing effective polices and mitigation efforts for suitable water reclamation. For instance, if the dominant P paradigm is expanded to include N, then concentrating N abatement efforts of N species of greatest concern could be of potential interest, rather than tackling all bioavailable forms.

The research findings build on the growing body of literature demonstrating the importance of urea in freshwater eutrophication and satisfying the nitrogenous nutrition of cyanobacteria. Cyanobacteria's voracious appetite for urea is worrying, as urea content is freshwaters is only projected to increase in upcoming years. As society moves forward into an era where influxes of urea in surface waters will become increasingly common,

the need to understand how urea influences phytoplankton community composition is stronger than ever. By uncovering conditions that render freshwaters more susceptible to cyanobacteria dominance, knowledge obtained could help establish effective mitigation measures aimed at combating this significant threat to global water security.

## 4.3 References


Belisle B.S., Steffen M.M., Pound H.L., DeBruyn J.M., Watson S.B., Bourbonniere R.A., Boyer G.L. & Wilhelm S.W. (2016) Urea in Lake Erie: organic nutrient sources are potentially important drivers of phytoplankton biomass. *Journal of Great Lakes Research*, **42**, 599-607.

Brooks B.W., Lazorchak J.M., Howard M.D., Johnson M.V., Morton S.L., Perkins D.A., Reavie E.D., Scott G.I., Smith S.A. & Steevens J.A. (2016) Are harmful algal blooms becoming the greatest inland water quality threat to public health and aquatic ecosystems? *Environmental Toxicology and Chemistry*, **35**, 6-13.

Conley D.J., Paerl H.W., Howarth R.W., Boesch D.F., Seitzinger S.P., Havens K.E., Lancelot C. & Likens G.E. (2009) Controlling eutrophication: nitrogen and phosphorus. *Science*, **323**, 1014-1015.

Dai G., Shang J. & Qiu B. (2012) Ammonia may play an important role in the succession of cyanobacteria blooms and the distribution of common algal species in shallow freshwater lakes. *Global Change Biology*, **18**, 1571-1581.

Donald D.B., Bogard M.J., Finlay K. & Leavitt P.R. (2011) Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. *Limnology and Oceanography*, **56**, 2161-2175.

Erisman J.W., Sutton M.A., Klimont Z., Galloway J. & Winiwarter W. (2008) How a century of ammonia synthesis has changed the world. *Nature Geoscience*, **1**, 636-639.

Finlay K., Patoine A., Donald D.B., Bogard M.J. & Leavitt P.R. (2010) Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the Northern Great Plains. *Limnology and Oceanography*, **55**, 1213-1230.

Galloway J.N., Dentener F.J., Capone D.G., Boyer E.W., Howarth R.W., Seitzinger S., Asner G.P., Cleveland C.C., Green P.A., Holland E.A., Karl D.M., Michael A.F., Porter J.H., Townsend A.R. & Vörösmarty C.J. (2004) Nitrogen cycles: past, present and future. *Biogeochemistry*, **70**, 153-226.

Glibert P.M., Harrison J., Heil C. & Seitzinger S. (2006) Escalating worldwide use of urea—a global change contributing to coastal eutrophication. *Biogeochemistry*, **77**, 441-463.

Glibert P.M., Maranger R., Sobota D.J. & Bouwman L. (2014) The Haber Bosch–harmful algal bloom (HB–HAB) link. *Environmental Resource Letters*, **9**, 105001.

Glibert P.M., Wilkerson F.P., Dugdale R.C., Raven J.A., Dupont C., Leavitt P.R., Parker A.E., Burkholder J.M. & Kana T.M. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnology and Oceanography*, **61**, 165-197.

Kudela R., Lane J. & Cochlan W. (2008) The potential role of anthropogenically derived nitrogen in the growth of harmful algae in California, USA. *Harmful algae*, **8**, 103-110.

Lewis W.M., Wurtsbaugh W.A. & Paerl H.W. (2011) Rationale for control of anthropogenic nitrogen and phosphorus to reduce eutrophication of inland waters. *Environmental Science & Technology*, **45**, 10300-10305.

Molot L., Watson S.B., Creed I.F., Trick C.G., McCabe S.K., Verschoor M.J., Sorichetti R.J., Powe C., Venkiteswaran J. & Schiff S.L. (2014) A novel model for cyanobacteria bloom formation: The critical role of anoxia and ferrous iron. *Freshwater Biology*, **59**, 1323-1340.

O'Neil J.M., Davis T.W., Burford M.A. & Gobler C.J. (2012) The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae*, **14**, 313-334.

Paerl H.W., Scott T., McCarthy M.J., Newell S.E., Gardner W.S., Havens K.E., Hoffman D.K., Wilhelm S.W. & Wurtsbaugh W.A. (2016) It Takes Two to Tango: When and Where Dual Nutrient (N & P) Reductions Are Needed to Protect Lakes and Downstream Ecosystems. *Environmental Science & Technology*, **50**, 10805-10813.

Schindler D.W. (1977) Evolution of phosphorus limitation in lakes. *Science*, **195**, 260-262.

Smil V. (1999) Detonator of the population explosion. Nature, **400**, 415.

Smith V.H., Tilman G.D. & Nekola J.C. (1999) Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution*, **100**, 179-196.

Smith, V. H. (2003). Eutrophication of freshwater and coastal marine ecosystems: A global problem. *Environmental Science and Pollution Research,* **10**, 126-139.

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Erratt, K. J., Creed, I. F., and Trick, C. G. (2015). Do cyanobacteria have an allelopathic effect? An assessment of lakes in Ontario. NSERC CREATE ABATE, Ontario Lakes Workshop, Toronto, ON, Nov. 13.