To the Graduate Council:

I am submitting herewith a dissertation written by Matthew Alan Saxton entitled "Constraints on Primary Production in Lake Erie." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Steven Wilhelm, Major Professor

We have read this dissertation
and recommend its acceptance:

Alison Buchan

Erik Zinser

Mark Radosevich

Accepted for the Council:

Carolyn R. Hodges
Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

CONSTRAINTS ON PRIMARY PRODUCTION IN LAKE ERIE

A Dissertation

Presented for the Doctor of Philosophy Degree

University of Tennessee, Knoxville

Matthew Alan Saxton

May 2011

To Teresa and my family for being there

Acknowledgements

While I cannot thank everyone who, over the years, has contributed to the completion of this dissertation, I would like to recognize a few individuals with who's help I never could have succeeded.

I would like to thank my advisor, Dr. Steven Wilhelm, for giving me a chance to succeed when few others would. You have taught me there is a lot more to being a scientist than bench work. I cannot thank you enough.

Many thanks to all those who have served on my committee Dr. Alison Buchan, Dr. R. Michael McKay, Dr. Mark Radosevich, Dr. Jennifer Schweitzer and Dr. Erik Zinser, the insight and advice you all provided greatly enriched this work. I am thankful to the many collaborators and coauthors that contributed to this work. Thanks to all the great people I have worked with in the Wilhelm lab, especially the undergraduates I have had the privilege of mentoring. Thank you to all the graduate students of the 6th floor, especially Audrey Matteson and Jeff Morris and Dr. Janet Rowe. My deepest gratitude to Dr. Gary LeCleir and Dr. Johanna Rinta-Kanto, your guidance and mentorship throughout my graduate experience have been invaluable. I never could have gotten started or finished without of you.

Thanks to my family for supporting me unconditionally and always being there to listen. The love and support you have given me over the years has made this possible. Above all I thank my wife Teresa, your passion for learning inspired me to do more with my life. I could never have done any of this without you, and this degree is as much yours as it is mine.

Abstract

The Laurentian Great Lake, Lake Erie is an invaluable global resource and its watershed is home to over 11 million people. The pressures placed on the lake because of this high population caused Lake Erie to experience numerous environmental problems, including seasonal hypoxia and harmful algal blooms. While these topics have been widely studied in Lake Erie for over 40 years a more nuanced understanding of the interaction between phytoplankton and nutrient is needed to properly address the problems continuing to face the lake. In this study we combine classical limnological and cell growth experiments with modern molecular biological techniques and microscopy to more completely describe the aquatic microbial ecology of the lake.

We used an oxalate rinse technique to examine the surface absorbed P pool of the toxic cyanobacterium *Microcystis aeruginosa* grown under a range of P conditions, as well as the general Lake Erie plankton assemblage. Our results suggest that while *Microcystis* is plastic in its cellular P needs, the ratio of intracellular to extracellular P remains stable across growth conditions. We describe the effect of the phosphonate herbicide glyphosate on the Lake Erie phytoplankton community using laboratory cell growth studies, field microcosm experiments and PCR amplification of a gene implicated in the breakdown of this compound from the environment. Results from these experiments suggest that the presence of glyphosate can affect community structure in multiple ways and may explain areas of unexplained phytoplankton diversity in coastal areas of Lake Erie. We also show heterotrophic bacteria are likely critical to the breakdown of glyphosate and further illustrate that understanding the context of the larger microbial community is critical to understanding the ecology of the constituent members of the community. Finally, we investigate the activity of the phytoplankton community in winter months with a focus on diatoms abundant in Lake Erie under the ice. We show these diatoms are active and that the winter bloom is a likely source of carbon important

to seasonal hypoxia formation. Together, these studies significantly enrich our understanding of how phytoplankton influence important ecological processes in Lake Erie.

Table of Contents

SECTION I
LITERATURE REVIEW..... *1*
Objectives of Study..... *18*
List of References..... *19*
SECTION II
PLASTICITY OF TOTAL AND INTRACELLULAR PHOSPHORUS QUOTAS IN CULTURES AND NATURAL POPULATIONS OF *MICROCYSTIS AERUGINOSA*..... *29*
Abstract..... *31*
Introduction..... *33*
Materials and Methods..... *34*
Results..... *38*
Discussion..... *41*
Acknowledgements..... *47*
List of References..... *48*
SECTION III
GLYPHOSATE INFLUENCE OF PHYTOPLANKTON COMMUNITY STRUCTURE IN LAKE ERIE..... *54*
Abstract..... *56*
Introduction..... *57*
Materials and Methods..... *59*
Results..... 64
Discussion..... *69*
Acknowledgements..... *75*
List of References..... *76*
SECTION IV
SEASONAL SI:C RATIOS IN LAKE ERIE DIATOMS – EVIDENCE OF WINTER SILICIFICATION..... *80*
Abstract..... *82*
Introduction..... *83*
Materials and Methods..... *85*
Results..... *89*
Discussion..... *93*
Acknowledgements..... *96*
List of References..... *97*
SECTION V
Conclusions..... *101*
List of References..... *105*
VITA..... *106*

List of Tables

SECTION II

Table 2.1
Hydrological and biological parameters sampled from Lake Erie……..*35*

Table 2.2
Microcystis aeruginosa strains PCC 7806 and LE3 growth rates……*49*

Table 2.3
Pearson correlation analysis results comparing the extracellular percentage of total particulate P to hydrological and biological parameters……..*42*

SECTION III

Table 3.1
Growth of cyanobacterial cultures using glyphosate and breakdown products as sole sources of P and N……..*68*

SECTION IV

Table 4.1
Percentage of diatom cells and colonies exhibiting PDMPO fluorescence……*91*

Table 4.2
Oxygen evolution, Si deposition rates, as well as a of Si:C fixation ratio measured from Lake Erie seston collected in winter or summer 2010……..*92*

List of Figures

SECTION I

Figure 1.1
MODIS satellite image of Lake Erie taken 10-13-10……*3*

Figure 1.2
Global usage of Roundup Ready crops 1996-2003……*13*

Figure 1.3
Pathways of glyphosate breakdown in soils……*14*

SECTION II

Figure 2.1
Microcystis aeruginosa strains PCC 7806 and LE3 phosphorus quotas……*40*

Figure 2.2
Scatterplots depicting the relationship between the extracellular percentage of total particulate P to TP, TDP and SRP……*43*

SECTION III

Figure 3.1
Map of Western Basin of Lake Erie sampling sites……*60*

Figure 3.2
Lake Erie glyphosate amendment microcosm results……*65*

Figure 3.3
Glyphosate toxicity effects on cyanobacterial culture growth……*67*

Figure 3.4
Phylogenetic tree of *phnJ* amino acid sequences obtained from Lake Erie samples……*70*

SECTION IV

Figure 4.1
Map of Lake Erie sampling sites……*86*

Figure 4.2
Micrographs showing PDMPO deposition in winter and spring diatom communities……*90*

SECTION I

LITERATURE REVIEW

General Introduction to the Great Lakes and Lake Erie

The Laurentian Great Lakes are an unparalleled natural resource. Together these lakes comprise one -fifth of the surface freshwater in the world, over 22,684 km^3 total draining over 521,830 km^2 of land in the United States and Canada (Fuller, 1995). The Great Lakes system is comprised of five large lakes, their drainage basins and the river systems and several small lakes that connect the larger lakes to each other. Among the Great Lakes, Lake Erie is of particular interest to the international research community. Lake Erie is the shallowest and warmest (and coldest) of the lakes, and as a result it is also the most productive biologically, including a valuable fishery that drives tourism in the region.

Lake Erie (Fig. 1) is comprised of three distinct basins that function, for the most part, as distinct lakes. The western basin is the shallowest and warmest; it is usually dimictic, generally described as eutrophic, and has experienced algal blooms throughout recent history. The central basin has the largest surface area and has a maximum depth of 22m. This basin regularly becomes thermally stratified in the summer, though the depth and topography of this region results in a relatively shallow hypolimnion. The limited volume of the hypolimnion is a key factor in hypoxia formation in the central basin, resulting in areas of low dissolved oxygen (<2 mg O_2 L^{-1}) concentration. The eastern basin is the deepest of the three basins and is generally classified as oligotrophic. The Lake Erie watershed supports the largest population of all the Great Lakes at over 11,000,000 people (Fuller, 1995). Lake Erie is a large complex ecosystem that has experienced substantial change in the last 100 years and it also faces numerous serious problems currently, including invasive species, hypoxia, legacy chemical contaminants, and harmful algal blooms.



Figure 1.1. MODIS satellite image of Lake Erie taken 10-13-10 (NOAA)

Lake Erie is limnologically characterized as a dimictic lake, experiencing full mixing in the spring and fall with periods of thermal stratification in the summer and winter (Wetzel, 2001). In this paradigm, the microbial community of ice covered lakes is thought to be dormant (Agbeti and Smol, 1995). Due to the difficulties and dangers associated with large-lake limnology during the winter, studies investigations of Lake Erie during the winter have been rare. This dearth of recent studies examining the lake in the winter has led to the assumption that, similar to small ice covered lakes, Lake Erie is dormant in the winter. The previous studies of Lake Erie phytoplankton in the winter suggest the lake may be more active than assumed, with abundant diatoms observed in the western basin (Chandler, 1940; Holland, 1993). Indeed, a recent study confirmed the presence of a winter diatom community measuring high abundances across the lake (Twiss et al., 2010). The centric diatom *Aulacoseira islandica* is especially plentiful, with measured abundances as high as 4.93×10^6 cells L^{-1}. Notably, while phytoplankton are abundant and apparently active in the winter, heterotrophic bacteria are less active in the winter than in the summer (Wilhelm and Bullerjahn, unpublished). The combination of a high abundance phytoplankton community and low bacterial activity may result in the export of large amounts of carbon to the lake bottom, potentially influencing the formation of hypoxia. This prospect warrants further investigation, requiring study of both the winter phytoplankton and bacterial communities.

Human Impacts on Lake Erie: Eutrophication, Restoration and Invasion

Starting at the turn of the 19th century, the Lake Erie basin has been an important industrial and agricultural center of the United States and Canada. The permissive environmental controls of this era led to high levels of industrial and agricultural pollution contaminating the lake. Much of the pollution took the form of chemical contaminates. These contaminants included heavy metals, notably mercury, lead and copper, and organic chemicals such as polychlorinated biphenols

and polycyclic aromatic hydrocarbons (Marvin et al., 2004a; Marvin et al., 2004b; Marvin et al., 2004c). The state of chemical pollutants in the lake was typified by several instances in which the waters of a major tributary, the Cuyahoga River, caught fire (Stradling and Stradling, 2008).

Nutrient loading from agricultural, industrial, and sewage runoff led to eutrophication across Lake Erie, severely impacting in the ecology of the lake in several important ways. First, and most obviously, high dissolved nutrient levels supported large-scale algal blooms. These blooms were comprised of several nuisance algae including the diazatrophic cyanobacteria *Aphanizomenon* spp. and *Anabaena* spp., as well as the diatoms *Stephanodiscus* spp. and *Fragilaria* spp. (Davis, 1964; Makarewicz, 1993). High phosphorus loading during this period is usually understood to be the main driver of this increase in algal production (IJC, 1989). Lake phosphorus loads increased substantially in this period from 4000 metric tons in 1900 to 27,000 metric tons in 1970 (Sly, 1976). Nitrogen inputs did not increase at the same pace at this time resulting in a substantial shift in N:P ratio, from 35 to 9.2 between 1948 and 1962 (Sweeney, 1993).

A consequence of high algal biomass production during this period was the expansion of hypoxia in the central basin. Due to lake morphology and the relatively small volume of the hypolimnion during stratification, regions of hypoxia have been known to occur in the central basin of Lake Erie prior to eutrophication of the lake (Charlton, 1980; Rosa and Burns, 1987). The scope and severity of hypoxic events in the central basin increased upon the onset of eutrophication, growing in size from 300 km^2 in 1930 to over 10,000 km^2 (70% of the central basin) in the mid- 1970s (Herdendorf, 1980) with oxygen depletion rates increasing significantly between 1930 and 1980 (Rosa and Burns, 1987). Hypoxia in the lake had serious effects on lake ecology, starving benthic invertebrates and fish populations of O_2 and decimating their populations (Britt, 1955; Krieger, 1984).

Eutrophication and the deterioration of water quality in Lake Erie continued until the accumulation of problems led to the declaration in the popular press that the lake was "dead" (Sweeney, 1993). The resulting publicity for the degeneration of the Lake Erie ecosystem was a major driver for the formation of the International Joint Commission (IJC), which was tasked with advising the governments of the United States and Canada of the causes of the lake's problems and the most prudent path to restoration. Due in large part to Schindler's (1974) work identifying the influence of phosphorus loading in the eutrophication of freshwater, controls on point-source loading (such as industrial waste and sewage) of P to Lake Erie were instituted. A goal of limiting P loads to no more than 11,000 metric tons was set forth in the Great Lakes Water Quality Agreement (GLWQA) of 1972. Efforts to lower phosphorus loads, largely through the control of phosphate containing detergents, in Lake Erie were successful with loads dropping below the level set in the GLWQA (IJC, 1989) and dissolved phosphorus in the lake dropping with it (Bertram, 1993; Rosa, 1987). Consequently, improvements were observed in many indicators of lake health (Makarewicz and Bertram, 1991), including rebounds in the populations of important sport fish (Kutkuhn, 1976; Ludsin et al., 2001) and benthic invertebrates (Krieger, 1984). Phytoplankton blooms in the lake also abated following the implementation of phosphorus load limits (Nicholls et al., 1977), with marked biomass reduction in all basins, including a drop from 2.3 g phytoplankton biomass m^{-3} in the Eastern Basin in 1970 to 0.2 g m^{-3} in 1987 (Makarewicz, 1993). Eutrophic indicator species present from the 1950s through the early 70s showed significant reductions in abundance. For example, *Aphanizomenon flos-aquae* biomass dropped 92% in the western basin, *Stephanodiscus niagarae* showed over a 90% drop in the eastern and central basins, and *Fragilaria crotonensis* biomass reduced over 90% in all three Lake Erie basins (Makarewicz, 1993; Munawar and Munawar, 1976). Together these reductions in phytoplankton biomass were indicative of an overall improvement in the health of Lake Erie. Despite the significant progress in ecosystem health and water quality,

two important indicators of progress in Lake Erie health have not improved. Hypolimnetic oxygen demand and phosphorus release from the sediments have not improved since 1972 (Burns et al., 2005; Charlton, 1980; Charlton et al., 1993).

The next significant ecological event in Lake Erie was the introduction of *Dreissenid* (zebra and quagga) mussels in 1988. These filter-feeding bivalves are common in European waters and are believed to have arrived via the ballast water of seagoing vessels. They were first documented in Lake St. Clair in September 1988 and by 1990 mussels were reported to be present in all of the Great Lakes (Griffiths et al., 1991). The ecological impact of *Dreissenid* mussels on the Lake Erie ecosystem has been reviewed (Dermott and Munawar, 1993; Ludyanskiy et al., 1993). *Dreissenid* mussel filtration of the water column effects the environment in multiple ways. Mussels clear the water of particulate matter, allowing for light to penetrate deeper into the water column, effecting phytoplankton growth. Mussels have also been shown to affect lake nutrient chemistry by excreting more phosphorus than nitrogen, lowering the N:P ratio of the water body and potentially favoring the formation of cyanobacterial blooms (Arnott and Vanni, 1996; Conroy et al., 2005a).

Despite the apparent success of phosphorus control measures in limiting algal blooms, large blooms returned to the western basin of Lake Erie in 1995. In contrast to the blooms of eutrophic Lake Erie, which were primarily heterocyst-forming filamentous cyanobacteria, the blooms of the 1990s to today are largely composed of the colonial cyanobacteria *Microcystis* spp. (Brittain et al., 2000). *Microcystis* blooms are of concern not only for the fouling effects normally associated with cyanobacterial blooms, but also because this organism produces a hepatotoxic secondary metabolite, microcystin (Carmichael, 2001). Studies investigating the distribution of toxin-producing *Microcystis* have shown these organisms to be distributed across the lake (Ouellette et al., 2006; Rinta-Kanto et al., 2005). Though not a primary member of the phytoplankton assemblage of the eutrophic lake in the 1970s (Makarewicz, 1993), *Microcystis* was present, and was likely the primary producer of microcystin in Lake Erie during the 1970s (Rinta-

Kanto et al., 2009). It is not yet clear why these blooms occur, though the influence of *Dreissenid* mussels on *Microcystis* abundance has been extensively studied (Conroy et al., 2005a; Conroy et al., 2005b; Makarewicz et al., 1999; Vanderploeg et al., 2001)

A surprising result of surveys exploring the distribution of toxic *Microcystis* in Lake Erie was the discovery of areas in which high concentrations of microcystin toxin could be measured, but no toxic *Microcystis* could be identified, such as Sandusky Bay OH (Rinta-Kanto et al., 2005). Further investigation revealed that the filamentous cyanobacterium *Planktothrix* spp. was responsible for microcystin production in Sandusky Bay (Rinta-Kanto and Wilhelm, 2006). Other potentially toxic cyanobacteria are also present in hypereutrophic Sandusky Bay, including the recent identified invasive species *Cylindrospermopsis* spp (Conroy et al., 2007). Why *Planktothrix* and other filamentous cyanobacteria are successful in embayments whereas *Microcystis* is dominant elsewhere in the lake is not clear and warrants further study.

Cyanobacterial harmful algal blooms – history and ecological impacts

Harmful algal blooms (HABs), the overgrowth of certain algal species to the detriment to people and the environment, are a problem of worldwide scope and importance These microbes and the toxins they produce have impacted human health for much of recorded history. While there are numerous examples of HAB organisms among the eukaryotic algae, including the brown tide former *Aureococcus anophagefferens* (Gobler et al., 2002) and saxitoxin producing dinoflagellates (Anderson et al., 1990), HAB forming cyanobacteria represent a significant subset of HAB microbes. While many cyanobacteria are indispensable parts of balanced ecosystems, others are known for bloom formation and toxin production. Blooms occur naturally, often in the form of common pond scums but some cyanobacterial HABs are often also connected to anthropogenic sources such as industrial pollution or agricultural nutrient runoff.

Toxic cyanobacterial HABs have been reported on every inhabited continent making this a truly worldwide problem (Carmichael, 2001). Because of the obvious discoloration of water during a cyanobacterial bloom and the importance of fresh drinking water to life, it should be no surprise that cyanobacterial HABs have been reported throughout history. Among the earliest reports of a toxic cyanobacterial bloom is that of Chinese General Zu Ge-Ling whose troops fell ill after drinking green water in southern China (Chorus and Bartram, 1999). In 1175 Scottish monks named their home near Soulseat Loch *Monasterium Viridis Stagni* or Monastery of the Green Loch. Soulseat Loch is currently noted for blooms of toxic cyanobacteria *Planktonthrix agardhii,* including an event in 1994 that resulted in the loss of livestock (Dybas, 2003).

Australia has been a site of algal bloom related activity throughout its prehistory and history. Because Australia possesses a wide range of ecosystems, from tropical regions to more temperate and desert regions, the cyanobacterial threat is diverse and a variety of control strategies must be considered. Australian aboriginals demonstrated an appreciation of the presence of HAB organisms passing water through crude sand filters in an attempt to make it safe to drink (Heyman 1992). Australia also holds the distinction of being the site of the first toxic cyanobacterial bloom to be recorded in the scientific literature. This account describes an event in which livestock consumed water from Lake Alexandria in which the toxic cyanobacteria of the genus *Nodularia spp*. had bloomed, resulting in the death of the animals (Francis, 1878). A high profile example of the effect of HABs in Australia is the 1979 case of the Palm Island Mystery Disease. In this incident over 100 aboriginal children were admitted to the hospital showing various gastroenterological symptoms (Griffiths and Saker, 2003). Subsequent investigation revealed that the regional water source had experienced a bloom of the highly toxic cyanobacterial genus *Cylindrospermopsis*.

While there is controversy today as to whether the illness was caused by the cyanobacteria themselves or by the treatment of the water with copper sulfide aimed at killing the bloom, it can be agreed that this event illustrates the potential effects of HABs and the importance of considering the possible outcomes of treatment plans before they are used.

Modern study of cyanobacterial HABs has focused on taxa capable of producing toxic secondary metabolites. Toxic cyanobacteria are morphologically, genetically and metabolically diverse. They can be found in coccoid and a variety of filamentous forms; some are able to form heterocysts and fix atmospheric nitrogen and they are able to produce a variety of toxins. Interestingly, many genera of toxic cyanobacteria are able to produce multiple different types of toxin (Carmichael, 1997, 2001). A wide variety of cyanobacterial toxins have been described, including neurotoxins (anatoxin), hepatoptoxins (microcystin, nodularin, cylindrospermopsin), and dermaltoxins (lyngbyatoxin) (Carmichael, 2001). The physiological role of these compounds for the microbes that produce them is poorly understood. The toxins do not appear to be required for growth because strains of every potentially toxic genus exist that do not posses the genes necessary for toxin production (Zurawell et al., 2005). In fact, it has been observed that generally non-toxic individual cells outnumber toxic individuals, suggesting a high physiological cost for toxin production (Rinta-Kanto et al., 2005).

Eutrophication in freshwater – are P limits enough to control algal blooms?

Eutrophication via nutrient loading is a primary cause of algal bloom formation. Identification of phosphorus as a nutrient limiting algal growth in freshwater (Schindler, 1974, 1977) and the control of phosphorus loads into eutrophic bodies of water have been critically important to the rehabilitation of many systems, including Lake Erie. These studies also assert that similar controls of nitrogen loads are not necessary because nitrogen deficits can be compensated through the activity of nitrogen-fixing microbes (Schindler et al., 2008). However, a

growing body of evidence indicates that only controlling phosphorus inputs is not sufficient to successfully control eutrophication (Conley et al., 2009; Paerl, 2009; Paerl and Scott, 2010; Scott and McCarthy, 2010). Indeed, a recent study of an extremely eutrophic Chinese lake, illustrated that the phytoplankton community in this site were not limited by phosphorus alone but rather co-limited by phosphorus and nitrogen (Xu et al., 2010). As this debate continues, the return of algal blooms to Lake Erie has drawn the success of P-only and the focus on point source controls into question.

Analysis of P-input trends in Lake Erie tributaries gives insight into the effect of non-point source loads. Non-point pollution comes from diffuse sources, such as agricultural and urban runoff. Given that the land use of most of the Lake Erie drainage basin is agricultural, as high as 80% in the Maumee River basin, agricultural non-point sources such as fertilizers and pesticides are important. While the drop in total P loads in the Maumee River that started in the 1970 continue today, dissolved reactive phosphorus (DRP) has gone up 94% from the minimum recorded in the 1980s (Richards, 2009). It is interesting to note that the recent increase in DRP started in the mid 1990s corresponding to the widespread application of the herbicide glyphosate.

Glyphosate – mode of action and resistance

The phosphonate herbicide glyphosate (*N*-(phosphonomethyl)glycine) is the primary active ingredient in Roundup™, and the most widely used chemical herbicide globally (Baylis, 2000). Glyphosate has shown the ability to influence a microbial community positively as a nutrient source and, indeed, P, C and N mineralization from glyphosate by soil microbes has been observed (Dick and Quinn, 1995; Lancaster et al., 2010; Liu et al., 1991). Also, glyphosate has been observed to affect a microbial community negatively, acting as a toxic compound. The application of glyphosate has been observed to shift microbial community structure in freshwater (Perez et al., 2007), shifting the community from

glyphosate-sensitive green algae and diatoms to glyphosate-tolerant cyanobacteria. Additionally, changes in microbial community structure upon glyphosate exposure have been described in marine (Stachowski-Haberkorn et al., 2008) and soils systems (Lancaster et al., 2010).

Glyphosate is a broad-spectrum herbicide that inhibits the synthesis of aromatic amino acids acting on the enzyme 5-*enol*-pyruvylshikimate-3-phosphate (EPSP) synthase (Amrhein et al., 1980; Steinrucken and Amrhein, 1980). This enzyme promotes the transfer of an enolpyruvyl group from phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P). It has been shown that glyphosate binds the PEP binding site, preventing the binding of this molecule to the enzyme. Glyphosate does not prevent the binding of EPSP synthase to S3P; rather it facilitates the creation of a S3P-EPSP synthase-glyphosate complex, precluding S3P and EPSP synthase from further activity in the cell and blocking the shikimate pathway and the biosynthesis of aromatic amino acids (Dill, 2005; Schönbrunn et al., 2001).

The development of plants that harbor glyphosate resistance, known as Roundup Ready™ crops, has led to large increases in application levels (Fig. 2, Dill, 2005; Dill et al., 2008). Three types of genetic alterations have been used to attempt to impart glyphosate resistance. First, glyphosate resistance has been engineered through the modification of the EPSP synthase gene to produce a glyphosate tolerant enzyme. This alteration has been accomplished through the modification of the native EPSP synthase (Lebrun et al., 2003), or through the replacement of the native EPSP synthase with an insensitive version of the gene . The glyphosate insensitive EPSP synthase isolated from *Agrobacterium* spp. C4 is highly effective, and is widely used in Roundup Ready™ crops.

Glyphosate resistance has also been imparted on plants through genes encoding enzymes that are able to metabolize glyphosate. Two glyphosate degradation pathways have been observed (Fig. 3), both resulting in the eventual metabolism of the herbicide to phosphate, ammonia and carbon dioxide.

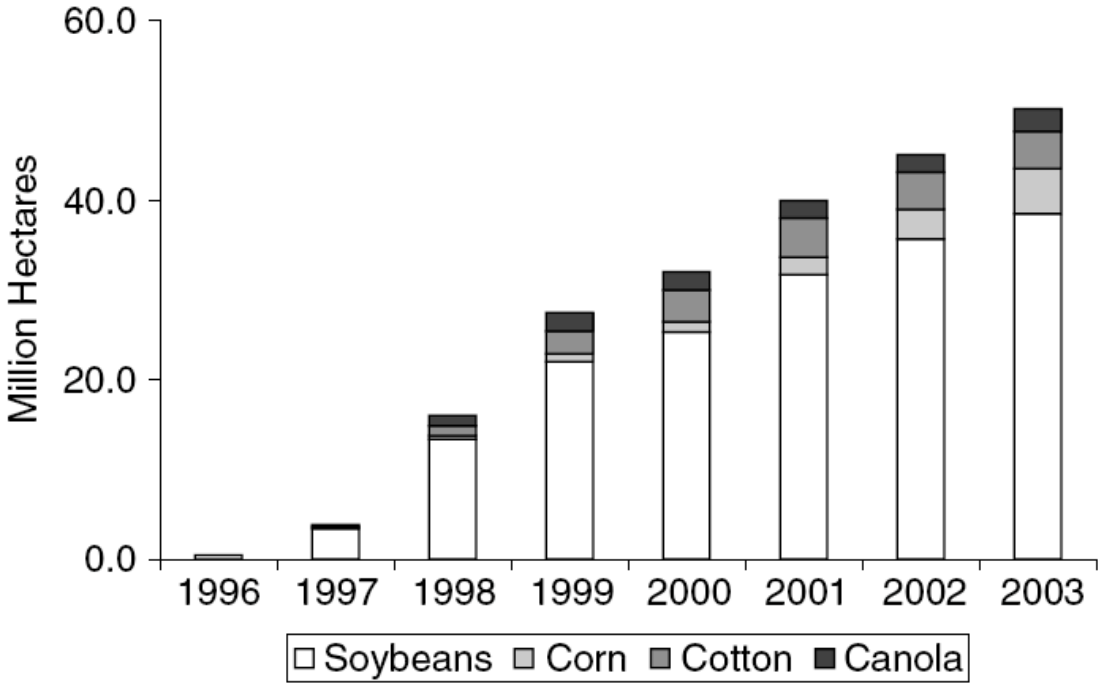


Figure 1.2. Global usage of Roundup Ready™ crops 1996-2003. Taken from Dill et al. 2005

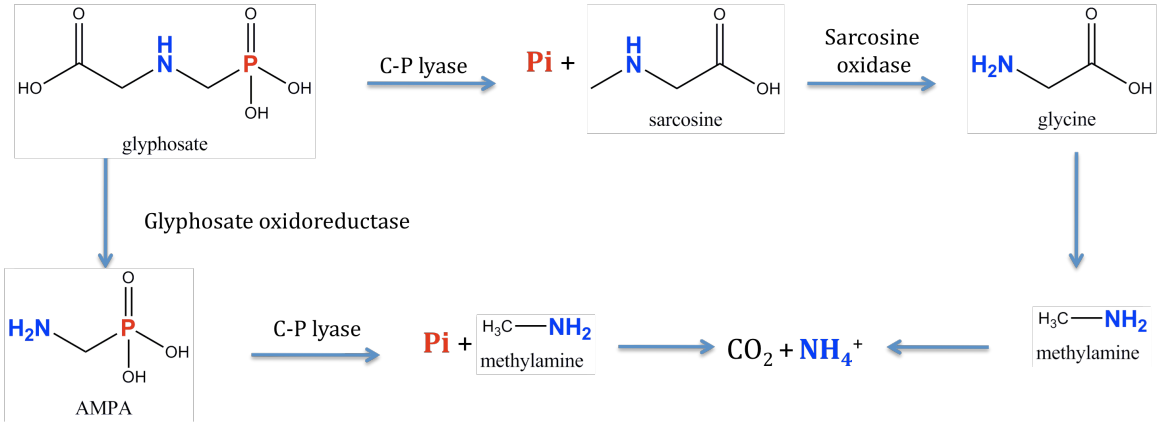


Figure 1.3. Pathways of glyphosate breakdown in soils. N highlighted blue, P highlighted red. Adapted from Borggaard and Gimsing 2008

One pathway begins with the metabolism of glyphosate to the intermediate aminomethylphosphonic acid (AMPA). The gene encoding the enzyme responsible for the N-C bond cleavage in this pathway is called glyphosate oxidoreductase (*GOX*) and was initially isolated from *Ochronobactrum anthropi* strain LBAA (Barry and Kishore, 1995; Pline-Srnic, 2006). *GOX* is commonly used commercially in conferring glyphosate resistance. Little is known about its activity in nature, though nearly all isolates known to posses the gene were obtained from glyphosate enrichments (Borggaard and Gimsing, 2008). *GOX* also appears infrequently in currently available metagenomic databases. As a result of the wide usage of *GOX*, AMPA is frequently observed in agricultural runoff, often at concentrations higher than those observed of glyphosate (Scribner et al., 2007).

The other known glyphosate metabolism pathway begins with the production of a sarcosine intermediate through the metabolism of the C-P bond by C-P lyase. A multi-gene system, C-P lyase has not been utilized commercially to confer glyphosate resistance because of the effectiveness and simplicity of the single gene glyphosate oxidoreductase. The C-P lyase system has been shown to be widely distributed and important to microbial degradation of these compounds in nature (Quinn et al., 1989; Wanner and Boline, 1990). Originally described in *E. coli*, the C-P lyase operon (*phnC-P)* in *E. coli* is a 10.4 kb multi-enzyme gene cluster under the control of a low P-nduced Pho promoter and encodes two major functional units. An ABC type phosphonate transporter is encoded by genes *phnC-E*, and *phnG-M* and makes up the C-P lyase responsible for the cleavage of the C-P bond. This enzyme has been show to be able to metabolize the C-P bond in many different phosphonate compounds. Evidence of frequent horizontal gene transfer has been observed in this gene cluster and gene order is not conserved between taxonomic groups, likely the result of repeated rearrangements (Huang et al., 2005).

Little is known about the specific mode of action of C-P lyase. A primary reason for this lack of understanding is because activity has not been detected in cell-free extracts *in vitro*, though this result suggests C-P lyase is associated with the

lipid bi-layer (Ternan et al., 1998). Also, the accumulation of gaseous alkene side products from C-P lyase active cell culture suggests a radical based dephosphorilation (Frost et al., 1987). Functions of some of the *phn* gene products have been elucidated through crystal structure and amino acid sequence analysis. The structure of *phnH* has been solved, though the function of this protein is still inconclusive, though the results suggest this protein interacs with another phn protein (Adams et al., 2008). Highly polar *phnM* has been proposed to be an the membrane bound component of the C-P lyase (Metcalf and Wanner, 1993). *PhnJ* is among the most highly conserved of the genes in the phn operon, it has been implicated in the metabolism of the C-P bond itself. Four conserved cystine residues may be involved in the coordination of a metal ion or iron sulfur complex critical to the suspected chemistry of C-P bond cleavage (Parker et al., 1999).

Much of our knowledge of glyphosate degradation in nature is based on what is known about the metabolism of the phosphonate bond. Phosphonates occur naturally, are common to the cell membrane components of many microbes (Kononova and Nesmeyanova, 2002) and compose up to 25% of high molecular weight P in marine systems (Clark et al., 1998; Kolowith et al., 2001). Phosphonate metabolism and the presence of *phn* genes have been recorded in cyanobacteria from freshwater and marine systems, specifically picocyanobacteria and filamentous cyanobacteria (Dyhrman et al., 2006; Ilikchyan et al., 2009; Stucken et al., 2010). Other phosphonate metabolism enzymes have been identified, many of which do not appear to be subject to the influence of exogenous P levels and are specific to particular phosphonate substrates, such as (2)-aminoethylphosphonate (Quinn et al., 2007).

Glyphosate usage in the Lake Erie Basin

Predictably, because of high application levels in the Lake Erie basin, glyphosate is present in measureable quantities in both tributaries and the main body of the lake (Kannan et al., 2006; Struger et al., 2008). Glyphosate observation in Lake Erie is transitory, with the highest concentrations being observed in conjunction with spring and fall agricultural glyphosate application (Byer et al., 2008, McKay and Bullerjahn, unpublished; Struger et al., 2008). At peak concentration, glyphosate can constitute a significant proportion of P in Lake Erie or its tributaries, ranging between 0.2-2% of total dissolved P in Maumee Bay and the Maumee River (McKay and Bullerjahn unpublished). This lack of glyphosate accumulation throughout the year is strongly indicative of microbial degradation of this compound in Lake Erie and its watershed.

OBJECTIVES OF STUDY

With the understanding that harmful algal bloom formation and seasonal hypoxia are two of the most critical problems associated with the Lake Erie ecosystem, our primary goal was to formulate and address a series of hypotheses that would enrich the greater understanding of how microbial activity impacts these problems. A secondary goal of the study was to utilize a wide variety of methods, combining laboratory and field studies, genetic analysis, cell culture based experiments and a variety of microscopic techniques. The use of a diversity of techniques allowed us to independently corroborate our findings in each case. In this study, we address the following hypotheses investigating how phytoplankton-nutrient interaction impact critical processes in Lake Erie:

1. Surface-bound P represents a significant proportion of the total cellular P pool in *Microcystis* and is potentially important to our understanding of *Microcystis* lifestyle in the environment.
    i. A parallel hypothesis is that, because previous cellular P measurements in *Microcystis* do not account for surface absorbed P, we have historically overestimated the P requirement of *Microcystis*.
2. Glyphosate exerts influence on phytoplankton community structure in Lake Erie, both through herbicidal effects on less tolerant members of the community and as a nutrient source to those able to metabolize it and its breakdown products.
3. The diatom assemblage observed in high concentrations in Lake Erie in the winter is active, and these diatoms are active at rates comparable to those observed in the summer and are producing biomass at rates that potentially impact hypoxia formation in the summer.

List of References

Adams, M.A., Luo, Y., Hove-Jensen, B., He, S.M., van Staalduinen, L.M., Zechel, D.L., Jia, Z.C., 2008. Crystal structure of *phnH*: an essential component of carbon-phosphorus lyase in Escherichia coli. J. Bacteriol. 190(3), 1072-1083.

Agbeti, M., Smol, J., 1995. Winter limnology: a comparison of physical, chemical and biological characteristics in two temperate lakes during ice cover. Hydrobiologia 304(3), 221-234.

Amrhein, N., Schab, J., Steinrücken, H.C., 1980. The mode of action of the herbicide glyphosate. Naturwissenschaften 67(7), 356-357.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104(3), 511-524.

Arnott, D.L., Vanni, M.J., 1996. Nitrogen and phosphorus recycling by the zebra mussel (*Dreissena polymorpha*) in the western basin of Lake Erie. Can. J. Fish. Aquat. Sci. 53, 646-659.

Barry, G.F., Kishore, G.M., 1995. Glyphosate tolerant plants, In: States, U. (Ed.). Monsanto Company, United States.

Baylis, A.D., 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pest Manag. Sci. 56(4), 299-308.

Bertram, P.E., 1993. Total Phosphorus and Dissolved Oxygen Trends in the Central Basin of Lake Erie, 1970-1991. J. Gt. Lakes Res. 19(2), 224-236.

Borggaard, O.K., Gimsing, A.L., 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag. Sci. 64(4), 441-456.

Britt, N.W., 1955. Stratification in Western Lake Erie in Summer of 1953: Effects on the Hexagenia (Ephemeroptera) Population. Ecology 36(2), 239-244.

Brittain, S.M., Wang, J., Babcock-Jackson, L., Carmichael, W.W., Rinehart, K.L., Culver, D.A., 2000. Isolation and characterization of microcystins, cyclic heptapeptide hepatotoxins from a Lake Erie strain of *Microcystis aeruginosa*. J. Gt. Lakes Res. 26, 241-249.

Burns, N.M., Rockwell, D.C., Bertram, P.E., Dolan, D.M., Ciborowski, J.J.H., 2005. Trends in temperature, Secchi depth, and dissolved oxygen depletion rates in the central basin of Lake Erie, 1983-2002. J. Gt. Lakes Res. 31, 35-49.

Byer, J.D., Struger, J., Klawunn, P., Todd, A., Sverko, E., 2008. Low Cost Monitoring of Glyphosate in Surface Waters Using the ELISA Method: An Evaluation. Environ. Sci. Technol. 42(16), 6052-6057.

Carmichael, W.W., 1997. The cyanotoxins. Advances in Botanical Research 27, 211-256.

Carmichael, W.W., 2001. Health Effects of Toxin-Producing Cyanobacteria: "The CyanoHABs". Human and Ecological Risk Assessment 7, 1393-1407.

Chandler, D.C., 1940. Limnological studies of western Lake Erie: I. Plankton and certain physical-chemical data of

589 the Bass Islands region, from September 1938, to November, 1939. Ohio J. Sci 40, 291-336.

Charlton, M.N., 1980. Oxygen depletion in Lake Erie: has there been any change. Can. J. Fish. Aquat. Sci. 37(1), 72-81.

Charlton, M.N., Milne, J.E., Booth, W.G., Chiocchio, F., 1993. Lake Erie Offshore in 1990 - restoration and resilience in the central basin. J. Gt. Lakes Res. 19(2), 291-309.

Chorus, I., Bartram, J.E., 1999. Toxic cyanobacteria in water: a guide to public health significance, monitoring and management. EF&N Spon, London.

Clark, L.L., Ingall, E.D., Benner, R., 1998. Marine phosphorus is selectively remineralized. Nature 393(6684), 426-426.

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(5917), 1014-1015.

Conroy, J.D., Edwards, W.J., Pontius, R.A., Kane, D.D., Zhang, H., Shea, J.F., Richey, J.N., Culver, D.A., 2005a. Soluble nitrogen and phosphorus excretion of exotic freshwater mussels (Dreissena spp.): potential impacts for nutrient remineralisation in western Lake Erie. Freshw. Biol. 50(7), 1146-1162.

Conroy, J.D., Kane, D.D., Dolan, D.M., Edwards, W.J., Charlton, M.N., Culver, D.A., 2005b. Temporal trends in Lake Erie plankton biomass: Roles of external phosphorus loading and dreissenid mussels. J. Gt. Lakes Res. 31, 89-110.

Conroy, J.D., Quinlan, E.L., Kane, D.D., Culver, D.A., 2007. Cylindrospermopsis in Lake Erie: Testing its association with other cyanobacterial genera and major linmological parameters. J. Gt. Lakes Res. 33(3), 519-535.

Davis, C.C., 1964. Evidence for the Eutrophication of Lake Erie from Phytoplankton Records. Limnol. Oceanogr. 9(3), 275-283.

Dermott, R., Munawar, M., 1993. Invasion of Lake Erie offshore sediments by Dreissena, and its ecological implications. Can. J. Fish. Aquat. Sci. 50(11), 2298-2304.

Dick, R., Quinn, J., 1995. Glyphosate-degrading isolates from environmental samples: occurrence and pathways of degradation. Appl. Microbiol. Biotechnol. 43(3), 545-550.

Dill, G.M., 2005. Glyphosate-resistant crops: history, status and future. Pest Manag. Sci. 61(3), 219-224.

Dill, G.M., CaJacob, C.A., Padgette, S.R., 2008. Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag. Sci. 64(4), 326-331.

Dybas, C.L., 2003. Harmful algal blooms: Biosensors provide new ways of detecting and monitoring growing threat in coastal waters. Bioscience 53(10), 918-923.

Dyhrman, S.T., Chappell, P.D., Haley, S.T., Moffett, J.W., Orchard, E.D., Waterbury, J.B., Webb, E.A., 2006. Phosphonate utilization by the globally important marine diazotroph Trichodesmium. Nature 439(7072), 68-71.

Francis, G., 1878. Poisonous Australian Lake. Nature 18, 11-12.

Frost, J.W., Loo, S., Cordeiro, M.L., Li, D., 1987. Radical based dephosphorilation and organophosphonate biodegradation. J. Am. Chem. Soc. 109(7), 2166-2171.

Fuller, K., Sherar, H., Wittig, J., 1995. The Great Lakes, An Environmental Atlas and Resource Book, Third edition ed. Government of Canada, Toronto, Ontario, Canada and United States Environmental Protection Agency, Great Lakes National Program Office, Chicago Illinois, p. 46.

Gobler, C.J., Renaghan, M.J., Buck, N.J., 2002. Impacts of nutrients and grazing mortality on the abundance of Aureococcus anophagefferens during a New York brown tide bloom. Limnol. Oceanogr. 47(1), 129-141.

Griffiths, D.J., Saker, M.L., 2003. The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin. Environmental Toxicology 18(2), 78-93.

Griffiths, R.W., Schloesser, D.W., Leach, J.H., Kovalak, W.P., 1991. Distribution and dispersal of the zebra mussel (*Dreissena polymorpha*) in the Great Lakes region. Can. J. Fish. Aquat. Sci. 48(8), 1381-1388.

Herdendorf, C.E., 1980. Lake Erie Nutrient Control Program An Assessment Of Its Effectiveness In Controlling Lake Eutrophication, In: E.P.A., U.S. (Ed.).

Holland, R.E., 1993. Changes in planktonic diatoms and water transparency in Hatchery Bay, Bass Island are, western Lake Erie since the establishment of the zebra mussel. J. Gt. Lakes Res. 19(3), 617-624.

Huang, J.L., Su, Z.C., Xu, Y., 2005. The evolution of microbial phosphonate degradative pathways. J. Mol. Evol. 61(5), 682-690.

IJC, 1989. International Joint Commission (IJC) United States and Canada 1989. Great Lakes Water Quality Agreement of 1978. http://www.ijc.org/agree/quality.html.

Ilikchyan, I.N., McKay, R.M.L., Zehr, J.P., Dyhrman, S.T., Bullerjahn, G.S., 2009. Detection and expression of the phosphonate transporter gene phnD in marine and freshwater picocyanobacteria. Environ. Microbiol. 11(5), 1314-1324.

Kannan, K., Ridal, J., Struger, J., 2006. Pesticides in the Great Lakes, In: Hites, R. (Ed.), Persistent Organic Pollutants in the Great Lakes. Springer Berlin / Heidelberg, pp. 151-199.

Kolowith, L.C., Ingall, E.D., Benner, R., 2001. Composition and cycling of marine organic phosphorus. Limnol. Oceanogr. 46(2), 309-320.

Kononova, S.V., Nesmeyanova, M.A., 2002. Phosphonates and their degradation by microorganisms. Biochemistry-Moscow 67(2), 184-195.

Krieger, K.A., 1984. Benthic Macroinvertebrates as Indicators of Environmental Degradation in the Southern Nearshore Zone of the Central Basin of Lake Erie. J. Gt. Lakes Res. 10(2), 197-209.

Kutkuhn, J., 1976. First technical report of the Great Lakes fishery commission of interagency management of the Walleye resource of western Lake Erie, In: Great Lakes Fisheries, C. (Ed.), Ann Arbor, MI.

Lancaster, S.H., Hollister, E.B., Senseman, S.A., Gentry, T.J., 2010. Effects of repeated glyphosate applications on soil microbial community composition and the mineralization of glyphosate. Pest Manag. Sci. 66(1), 59-64.

Lebrun, M., Sailland, A., Freyssinet, G., Esposito, J.B., Degryse, E., 2003. Mutated 5-enolpyruvylshikimate-3-phosphate synthase, gene coding for said protein and transformed plants containing said gene. Bayer CropScience S.A., United States.

Liu, C.-M., McLean, P.A., Sookdeo, C.C., Cannon, F.C., 1991. Degradation of the Herbicide Glyphosate by Members of the Family Rhizobiaceae. Appl. Environ. Microbiol. 57(6), 1799-1804.

Ludsin, S.A., Kershner, M.W., Blocksom, K.A., Knight, R.L., Stein, R.A., 2001. Life after death in Lake Erie: Nutrient controls drive fish species richness, rehabilitation. Ecol. Appl., 11:731-746.

Ludyanskiy, M.L., McDonald, D., Macneill, D., 1993. Impact of the zebra mussel, a bivalve invader. Bioscience 43(8), 533-544.

Makarewicz, J.C., 1993. Phytoplankton biomass and species composition in Lake Erie, 1970 to 1987. J. Gt. Lakes Res. 19(2), 258-274.

Makarewicz, J.C., Bertram, P., 1991. Evidence for the Restoration of the Lake Erie Ecosystem. Bioscience 41(4), 216-223.

Makarewicz, J.C., Lewis, T.W., Bertram, P., 1999. Phytoplankton composition and biomass in the offshore waters of Lake Erie: Pre- and post-Dreissena introduction (1983-1993). J. Gt. Lakes Res. 25, 135-148.

Marvin, C., Painter, S., Rossmann, R., 2004a. Spatial and temporal patterns in mercury contamination in sediments of the Laurentian Great Lakes. Environ. Res. 95(3), 351-362.

Marvin, C., Painter, S., Williams, D., Richardson, V., Rossmann, R., Van Hoof, P., 2004b. Spatial and temporal trends in surface water and sediment contamination in the Laurentian Great Lakes. Environ. Pollut. 129(1), 131-144.

Marvin, C.H., Sverko, E., Charlton, M.N., Thiessen, P.A.L., Painter, S., 2004c. Contaminants associated with suspended sediments in Lakes Erie and Ontario, 1997-2000. J. Gt. Lakes Res. 30(2), 277-286.

Metcalf, W.W., Wanner, B.L., 1993. Mutational analysis of an Escherichia coli fourteen-gene operon for phosphonate degradation, using TnphoA' elements. J. Bacteriol. 175(11), 3430-3442.

Munawar, M., Munawar, I.F., 1976. Lakewide study of phytoplankton biomass and its species composition in Lake Erie, April-December 1970. J. Fish. Res. Board Can. 33(3), 581-600.

Nicholls, K.H., Standen, D.W., Hopkins, G.J., Carney, E.C., 1977. Declines in the Near-Shore Phytoplankton of Lake Erie's Western Basin Since 1971. J. Gt. Lakes Res. 3(1-2), 72-78.

Ouellette, A.J.A., Handy, S.M., Wilhelm, S.W., 2006. Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molcular characterization of associated cyanobacterial communities. Microb. Ecol. 51, 154-165.

Paerl, H.W., 2009. Controlling Eutrophication along the Freshwater-Marine Continuum: Dual Nutrient (N and P) Reductions are Essential. Estuaries and Coasts 32(4), 593-601.

Paerl, H.W., Scott, J.T., 2010. Throwing Fuel on the Fire: Synergistic Effects of Excessive Nitrogen Inputs and Global Warming on Harmful Algal Blooms. Environ. Sci. Technol. 44(20), 7756-7758.

Parker, G.F., Higgins, T.P., Hawkes, T., Robson, R.L., 1999. Rhizobium (Sinorhizobium) meliloti phn Genes: Characterization and Identification of Their Protein Products. J. Bacteriol. 181(2), 389-395.

Perez, G.L., Torremorell, A., Mugni, H., Rodriguez, P., Vera, M.S., Do Nascimento, M., Allende, L., Bustingorry, J., Escaray, R., Ferraro, M., Izaguirre, I., Pizarro, H., Bonetto, C., Morris, D.P., Zagarese, H., 2007. Effects of the herbicide roundup on freshwater microbial communities: A mesocosm study. Ecol. Appl. 17(8), 2310-2322.

Pline-Srnic, W., 2006. Physiological Mechanisms of Glyphosate Resistance1. Weed Technol. 20(2), 290-300.

Quinn, J.P., Kulakova, A.N., Cooley, N.A., McGrath, J.W., 2007. New ways to break an old bond: the bacterial carbon-phosphorus hydrolases and their role in biogeochemical phosphorus cycling. Environmental Microbiology 9(10), 2392-2400.

Quinn, J.P., Peden, J.M.M., Dick, R.E., 1989. Carbon-phosphorus bond cleavage by Gram-positive and Gram-negative soil bacteria. Appl. Microbiol. Biotechnol. 31(3), 283-287.

Richards, R.P., 2009. Phosphorus Loads and Concentrations from the Maumee River. Detroit River-Western Lake Erie Basin Indicator Project, http://www.epa.gov/med/grosseile_site/indicators/maumee-p.html.

Rinta-Kanto, J.M., Ouellette, A.J.A., Boyer, G.L., Twiss, M.R., Bridgeman, T.B., Wilhelm, S.W., 2005. Quantification of toxic *Microcystis spp*. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. 39(11), 4198-4205.

Rinta-Kanto, J.M., Saxton, M.A., DeBruyn, J.M., Smith, J.L., Marvin, C.H., Krieger, K.A., Sayler, G.S., Boyer, G.L., Wilhelm, S.W., 2009. The diversity and distribution of toxigenic Microcystis spp. in present day and archived pelagic and sediment samples from Lake Erie. Harmful Algae 8(3), 385-394.

Rinta-Kanto, J.M., Wilhelm, S.W., 2006. Diversity of Microcystin-Producing Cyanobacteria in Spatially Isolated Regions of Lake Erie. Appl. Environ. Microbiol. 72(7), 5083-5085.

Rosa, F., 1987. Lake Erie central basin total phosphorous trend analysis from 1968 to 1982. Jounral of Great Lakes Research 13, 667-673.

Rosa, F., Burns, N.M., 1987. Lake Erie Central Basin oxygen depletion changes from 1929-1980. J. Gt. Lakes Res. 13, 684-696.

Schindler, D.W., 1974. Eutrophication and Recovery in Experimental Lakes: Implications for Lake Management. Science 184(4139), 897-899.

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. Proc. Natl. Acad. Sci. U. S. A. 105(32), 11254-11258.

Schönbrunn, E., Eschenburg, S., Shuttleworth, W.A., Schloss, J.V., Amrhein, N., Evans, J.N.S., Kabsch, W., 2001. Interaction of the herbicide glyphosate with its target enzyme 5-enolpyvuvylshikimate 3-phosphate synthase in atomic detail. Proc. Natl. Acad. Sci. U. S. A. 98(4), 1376-1380.

Scott, J.T., McCarthy, M.J., 2010. Nitrogen fixation may not balance the nitrogen pool in lakes over timescales relevant to eutrophication management. Limnol. Oceanogr. 55(3), 1265-1270.

Scribner, E.A., Battaglin, W.A., Gilliom, R.J., Meyer, M.T., 2007. Concentrations of glyphosate, its degradation product, aminomethylphosphonic acid, and glufosinate in ground- and surface-water, rainfall, and soil samples collected in the United States, 2001-06. U.S. Geological Survey Scientific Investigations Report 2007-5122.

Sly, P.G., 1976. Lake Erie and its basin. J. Fish. Res. Board Can. 33(3), 355-370.

Stachowski-Haberkorn, S., Becker, B., Marie, D., Haberkorn, H., Coroller, L., de la Broise, D., 2008. Impact of Roundup on the marine microbial community, as shown by an in situ microcosm experiment. Aquatic Toxicology 89(4), 232-241.

Steinrucken, H.C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic-acid 3-phosphate synthase. Biochem. Biophys. Res. Commun. 94(4), 1207-1212.

Stradling, D., Stradling, R., 2008. Perceptions of the Burning River: Deindustrialization and Cleveland's Cuyahoga River. Environmental History 13(3), 515-535.

Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., Marvin, C., 2008. Occurrence of Glyphosate in Surface Waters of Southern Ontario. Bulletin of Environmental Contamination and Toxicology 80(4), 378-384.

Stucken, K., John, U., Cembella, A., Murillo, A., Soto-Liebe, K., Fuentes-Valdé, J., Friedel, M., Plominsky, A., Vásquez, M., Glöckner, G., 2010. The Smallest Known Genomes of Multicellular and Toxic Cyanobacteria: Comparison,

Minimal Gene Sets for Linked Traits and the Evolutionary Implications. PLoS ONE 5(2), e9235.

Sweeney, R.A., 1993. "Dead" sea of North America? - Lake Erie in the 1960s and '70s. J. Gt. Lakes Res. 19(2), 198-199.

Ternan, N.G., Mc Grath, J.W., Mc Mullan, G., Quinn, J.P., 1998. Organophosphonates: occurrence, synthesis and biodegradation by microorganisms. World J. Microbiol. Biotechnol. 14(5), 635-647.

Twiss, M.R., McKay, R.M.L., Bourbonniere, R.A., Bullerjahn, G.S., Carrick, H.J., Smith, R.E.H., Winter, J.G., D'Souza, N.A., Furey, P.C., Lashaway, A.R., Saxton, M.A., Wilhelm, S.W., 2010. Diatoms abound in ice-covered Lake Erie: An investigation of offshore winter limnology in Lake Erie over the period 2007 to 2010 J. Gt. Lakes Res. Submitted.

Vanderploeg, H.A., Liebig, J.R., Carmichael, W.W., Agy, M.A., Johengen, T.H., Fahnenstiel, G.L., Nalepa, T.F., 2001. Zebra mussel (Dreissena polymorpha) selective filtration promoted toxic Microcystis blooms in Saginaw Bay (Lake Huron) and Lake Erie. Can. J. Fish. Aquat. Sci. 58(6), 1208-1221.

Wanner, B.L., Boline, J.A., 1990. Mapping and molecular-cloning of the phn (psid) locus for phosphonate utilization in Escherichia coli. J. Bacteriol. 172(3), 1186-1196.

Wetzel, R.G., 2001. Limnology. Academic Press, New York, NY.

Xu, H., Paerl, H.W., Qin, B.Q., Zhu, G.W., Gao, G., 2010. Nitrogen and phosphorus inputs control phytoplankton growth in eutrophic Lake Taihu, China. Limnol. Oceanogr. 55(1), 420-432.

Zurawell, R.W., Chen, H.R., Burke, J.M., Prepas, E.E., 2005. Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 8(1), 1-37.

SECTION II

PLASTICITY OF TOTAL AND INTRACELLULAR PHOSPHORUS QUOTAS IN CULTURES AND NATURAL POPULATIONS OF *MICROCYSTIS AERUGINOSA*

This section is a version of an article being prepared for submission to Harmful Algae under the same title by Matthew A. Saxton, Robert J. Arnold, Richard A. Bourbonniere R. Michael L. McKay and Steven W. Wilhelm.

My contribution to this paper was the collection of environmental samples and assistance with all aspects the *Microcystis* culture experiment. I also performed most of the data analysis and most of the background information gathering and writing of this section.

Abstract

Blooms of the toxic cyanobacterium *Microcystis* are common and as a result significant resources continue to be dedicated to monitoring and controlling these events. Recent studies have shown that a significant proportion total cell-associated phosphorus (P) in phytoplankton can be surface absorbed, and many of our current measurements do not accurately reflect the P demands of these organisms. In this study we measure the total cell-associated and intracellular P as well as growth rates of two toxic strains of *Microcystis aeruginosa* Kütz grown under a range of P concentrations. The results show that the intracellular P pool in *Microcystis* represents a percentage of total cell-associated P (50-90%) similar to what has been reported for actively growing cells in marine systems. Intracellular P levels (39-147 fg $cell^{-1}$) generally increased with increasing growth media P concentrations, but growth rate and the ratio of total cell-associated to intracellular P remained generally stable. Intracellular P quotas and growth rates in cells grown under the different P treatments illustrate the ability of this organism to successfully respond to changes in ambient P loads, and thus have implications for ecosystem scale productivity models

Introduction

Widespread nutrient loading has led to the eutrophication of fresh- and saltwater ecosystems throughout the developed world. A primary concern linked to eutrophication is the appearance of harmful algal blooms (HABs). HAB events are well-documented, ecosystem-wide problems that can impact human health, kill livestock, foul potable water supplies and compromise the integrity of both recreational and commercial fisheries (Carmichael, 2001). HABs have also been implicated in contributing to the formation of anoxic zones (Paerl et al., 1998). HAB-causing organisms are very diverse, including prokaryotic and eukaryotic algae; many cyanobacteria are particularly noted for bloom formation, especially in fresh- and brackish water systems.

Efforts to limit algal bloom formation have historically focused on reducing nutrient loading. Phosphorus (P) is understood to be the nutrient limiting phytoplankton growth in most freshwater systems (Schindler, 1977; Schindler et al., 2008). Controlling P has led to successes in controlling algal bloom formation in extreme eutrophic systems (Makarewicz and Bertram, 1991). Studies have also suggested that limiting nitrogen is important to controlling algal blooms, especially in terms of cyanobacterial speciation (Conley et al., 2009; Paerl, 1997). This developing understanding of eutrophication has been critical to the continued development of mathematical environmental models, which have become substantially more sophisticated with advances such as coupling hydrodynamic, ecological and watershed models (Jørgensen, 2010).

Microcystis spp. are among the most common bloom forming cyanobacteria in freshwaters and have a global bloom distribution (Chorus and Bartram, 1999). It is also known for the production of the hepatotoxin microcystin. The effects of P-limitation and uptake in *Microcystis* have been extensively studied. Like other cyanobacteria, *Microcystis* possesses the ability to store excess P intracellularly, as polyphosphate granules, and P starved cells have higher rates of P uptake than P-

sufficient cells (Jacobson and Halmann, 1982). *Microcystis* has been shown to respond to P limitation by initial incomplete rapid P uptake and is known for a notable increase in V_{max} in P-limiting conditions (Kromkamp et al., 1989). The recently sequenced *Microcystis* genomes indicate the presence of ATP driven P uptake similar to what has been described in *Synechococcus* (Frangeul et al., 2008; Kaneko et al., 2007; Ritchie et al., 1997, 2001).

Recently, an oxalate rinse developed to remove surface adsorbed iron (Tovar-Sanchez et al., 2003) was shown to also effectively remove surface adsorbed P (Fu et al., 2005; Sãnudo -Wilhelmy et al., 2004). Subsequently these studies have shown a significant proportion (15-45%) of total cell-associated P to be surface adsorbed in marine cyanobacteria. Arguably, this phenomenon, if universal in nature, is predicted to be of even greater relevance to freshwater ecosystems given their more universal P-limited nature. A consequence of this is that previous estimates of cyanobacterial P requirements may overstate the need for this nutrient. Alternatively, the ability of cyanobacteria to store P on their exterior surface may act as a novel strategy to allow persistence during periods of prolonged P deficiency (or to survive "boom-and-bust" cycles of nutrient availability). As such, a necessary first step is to determine the ability of cells to accumulate extracellular P and, further, to determine the contribution of this pool to estimated total P quotas.

In this study we determined the total cell-associated and intracellular P quotas and growth rates of two toxic *Microcystis* strains grown under a series of P concentrations. We similarly examined the ratio of total cell associated to intracellular P of natural communities in the western basin of Lake Erie where *Microcystis* forms an important part of the endemic plankton assemblage. Establishing accurate P-quotas (and their plasticity) for this potentially toxic bloom-forming cyanobacterium is a necessary step toward the development of realistic environmental phytoplankton growth models and management strategies for many important freshwater systems.

Materials and Methods

Cyanobacterial Culture Conditions.

Experiments were performed with *M. aeruginosa* PCC 7806 obtained from the Pasteur Culture Collection. While *M. aeruginosa* PCC 7806 is a globally used type strain, it is not representative of much of observed *Microcystis* diversity (Ouellette et al., 2006; Ouellette and Wilhelm, 2003; Ye et al., 2009). *M. aeruginosa* LE3, an isolate from Lake Erie (Brittain et al., 2000), has been shown to be representative of Lake Erie *Microcystis* (e.g., Rinta-Kanto and Wilhelm, 2006) and has been used as a standard in numerous studies (e.g., Rinta-Kanto et al., 2009b). Cultures were maintained in BG-11 medium (Rippka et al., 1979) with P concentrations ranging from 1.75-175 μM K_2HPO_4. Cultures were grown in 50 mL glass culture tubes (Kimble, Vineland, NJ) at 24° C under continuous illumination of ~30 μmol photons m^{-2} s^{-1}. Prior to use, all culture tubes were soaked overnight in 1% HCl and rinsed multiple times with MilliQ water. Samples were grown and transferred at experimental P concentrations two times prior to data collection. Multiple transfers were performed to ensure the stabilization of cellular P stores and the physiological equilibration of cell cultures to experimental P levels *vis a vis* Wilhelm et al. (1996). Cyanobacterial growth was monitored daily using a Turner Designs TD-700 fluorometer (Sunnyvale, CA, USA) equipped with an *in vivo* chl*a* filter set (excitation k = 340–500 nm; emission k = >665 nm). All experiments were performed in triplicate unless otherwise noted.

Field Sample Collection.

Field samples were collected at sites across Lake Erie (Table 1) between August 3-7 2010 aboard the *CCGS* Limnos. Surface water was collected at each site using 10L Niskin sampling bottles and subsequently stored in 3L opaque amber polycarbonate bottles until filtration. Following oxalate rinse procedure, samples were stored at -20° C until analysis.

Table 2.1 Hydrological and biological parameters sampled from Lake Erie August 2010. * indicates n=2

Station	478	970 (357)	973	882	1163	341	880 (84)
Location (Lat, Long)	41.65, -82.81	41.82, -82.97	41.79, -83.32	41.77, -83.31	41.47, -82.72	41.80, -82.29	41.94, -81.65
Extracellular P (%)	4.9	10.91	0.74	9.99	16.54	0	34.12
Total particulate P ($\mu g\ L^{-1}$)	8.2± 3.65	8.16± 3.65	27.05± 4.68	13.18± 0.91	96.97± 18.76	6.28± 0.86	6.25± 1.44*
Intracellular P ($\mu g\ L^{-1}$)	7.3± 2.78*	7.27± 2.78*	26.85± 0.80	11.86± 1.17	80.93± 13.20	6.30± 1.74	4.12± 0.099*
SRP ($\mu g\ L^{-1}$)	0.8	1.1	1.3	1.7	2.1	0.4	0.6
TDP ($\mu g\ L^{-1}$)	7.5	6.9	9.4	10.6	14.0	5.0	4.8
TP($\mu g\ L^{-1}$)	21.6	21.8	37.7	34.7	85.5	13.3	12.9
[a]NO_x^- ($mg\ L^{-1}$)	0.25	0.145	0.017	0.016	0.010	0.073	0.059
NH_3 ($mg\ L^{-1}$)	0.011	0.010	0.009	0.016	[b]BDL	0.012	0.018
TDN ($mg\ L^{-1}$)	0.526	0.338	0.301	0.410	0.393	0.361	0.291
PON ($mg\ L^{-1}$)	0.12	0.178	0.215	0.228	0.678	0.101	0.094
SiO_2 ($mg\ L^{-1}$)	1.46	1.53	1.77	2.00	3.61	1.53	0.28
POC ($mg\ L^{-1}$)	0.73	1.20	1.38	1.46	3.66	0.842	0.658
Chl*a* ($\mu g\ L^{-1}$±SD)	6.1± 0.13	15.82± 2.21	18.61± 0.61	16.09± 1.34	16.25± 0.52	5.52± 0.68	7.54± 0.74

[a]nitrate+nitrite. [b]BDL: below detectable limits (< 0.005 $mg\ L^{-1}$).

Quantification of total cellular and intracellular P.

Total cellular and intracellular P concentrations were independently measured. Surface bound P was removed using an oxalate wash reagent initially designed for removal of iron (Tovar-Sanchez et al., 2003) and subsequently demonstrated to be applicable for removal of surface-sorbed P (Sãnudo -Wilhelmy et al., 2004). Aliquots of 5 mL from individual cultures of 50-200 mL from field samples were harvested onto pre-combusted (450° C for 4 hours) 25mm Whatman (Kent, UK) GF/F glass fiber filters for the determination of total P in samples. For the determination of intracellular P from cultured isolates, aliquots were first mixed with an equal volume of oxalate wash and allowed to incubate for five minutes prior to harvest. Field samples were filtered until approximately 5 mL remained on the filter; 5 mL of oxalate rinse was then applied, incubated for culture isolates and harvested. A top-up solution containing equal parts oxalate rinse and filtered lakewater was applied as needed to counter losses due to residual vacuum. Following this incubation, the membrane was rinsed three times with 5 mL of P-free growth media or filtered lake water. To control for the removal of surface associated P by the washing process, samples for total cellular P were rinsed with P-free medium or filtered lake water four times as described above. Unused growth medium of each of the experimental P concentrations was either passed through filters and rinsed with P-free media or oxalate rinse solution was used to control for media associated P. Filtered station water was used as blanks for field samples. All treatments were performed in triplicate and blanks were performed in duplicate.

P Measurement.

Organic P was converted to inorganic P *via* persulfate oxidation (Menzel and Corwin, 1965; Wetzel and Likens, 2000). Freshly made 5% persulfate solution was added to samples that were then placed in a boiling water bath for 1 hour. Total P concentrations were measured spectrophotometricly (885 nm) using the ammonium molybdate method (Wetzel and Likens, 2000). All measurements for

field samples were performed on a Thermospectronic Genesys20 spectrophotometer (ThermoFisher Waltham, MA), whereas cultured samples were processed using a Biomate 5 spectrophotometer (ThermoFisher Waltham, MA).

Field samples were prepared by first digesting with 5% potassium persulfate, followed by autoclaving for 30 min. Total P was spectrophotometrically analyzed (880nm) with the molybdate ascorbic acid method (Strickland and Parsons, 1972) using a 10-cm quartz cell and a Cary 50 UV-vis spectrophotometer (Varian Palo Alto, CA). A calibration was conducted using phosphate reference standards of known concentration and National Institute of Standards & Technology NIST Standard Reference Material (Bovine Muscle Powder, NIST SRM 8414) processed in the same manner as sample material. Individual replicates were omitted from field sample analysis if one notably deviated from two others. Deviant replicates were not unexpected and were likely the result of the incomplete rinsing of cell aggregates.

Subsamples of cultured samples (1.5 mL) were also collected from each tube for direct counts of cell density. *Microcystis* cell densities were determined by epifluorescence microscopy with a Leica (Wetzlar, Germany) DMXRA epifluorescent microscope using the Texas Red filter set using accessory pigment (phycobillin) autofluorescence as previously described (Wilhelm et al., 2006). This was performed to normalize P measurements to *Microcystis* cell abundance.

Water Chemistry

Samples for nutrients, including total dissolved phosphorus (TDP), soluble reactive phosphorus (SRP), total dissolved nitrogen (TDN), NH_4^+, NO_3^- + NO_2^- silicate (SiO_2), particulate organic nitrogen (PON) and particulate orgaic carbon (POC), were collected and stored at 4 °C until analysis. Analysis was conducted at the National Laboratory for Environmental Testing (Environment Canada) using standardized techniques.

Extracted Chlorophyll a

Chlorophyll *a* (Chl*a*) concentration at field stations was determined as a proxy for total phytoplankton biomass. Seston was filtered through 0.2-µm nominal pore-size polycarbonate membranes. Chl*a* concentrations were determined using the non-acidification method (Welschmeyer, 1994) following extraction in 90% acetone overnight at -20° C using a Turner Designs 10AU fluorometer.

Results

P-constraints on Microcystis Growth.

The growth rates across a range of P concentrations are shown in Table 2. Paired samples *t*-tests were performed and significant differences ($p < 0.05$) reported. Growth rates in both strains were only slightly variable across different P concentrations in growth medium, especially for those cells maintained at higher P concentrations. Reported growth rates were more variable in *M. aeruginosa* LE3 than in *M. aeruginosa* PCC 7806. Although growth rates were stable across concentrations of P, biomass was notably lower in cultures maintained under the lower P concentrations (results not shown). It should also be noted that *Microcystis* PCC 7806 did not grow consistently at the lowest P concentrations in spite of repeated attempts.

Total cell-associated and intracellular P quotas in cultured Microcystis.

To obtain accurate intracellular P-quotas for *Microcystis aeruginosa*, we utilized the oxalate rinse technique to differentiate between the total cell-associated and intracellular P pools (Figure 1). The range of total cell-associated P values reported here (39-173 fg cell^{-1}) are within the range of previously reported values (29-433 fg cell^{-1}) (Rhee and Gotham, 1980; Sbiyyaa et al., 1998; Shen and Song, 2007; Tsukada et al., 2006). As was anticipated, intracellular P quotas (34-146 fg cell^{-1}) were smaller than the cell-associated values (39-173 fg cell^{-1}).

Table 2.2. *Microcystis aeruginosa* strains PCC 7806 and LE3 growth rates. * n=1 as replicates would not grow at these concentrations. Statically significant ($p < 0.05$) differences in growth rate denoted as a= > 8.75 µM; b= > 87.5 µM; c= > 175 µM.

Organism	Growth Media P concentration µM	Growth Rate (day^{-1}±sd)
M. aeruginosa PCC 7806	1.75	0.079*
	8.75	0.123±0.009
	17.5	0.149±0.005
	87.5	0.162±0.005[a]
	175	0.138±0.023
M. aeruginosa LE3	1.75	0.118±0.008
	8.75	0.142±0.001[b,c]
	17.5	0.0844*
	87.5	0.058±0.001
	175	0.108±0.001[b]

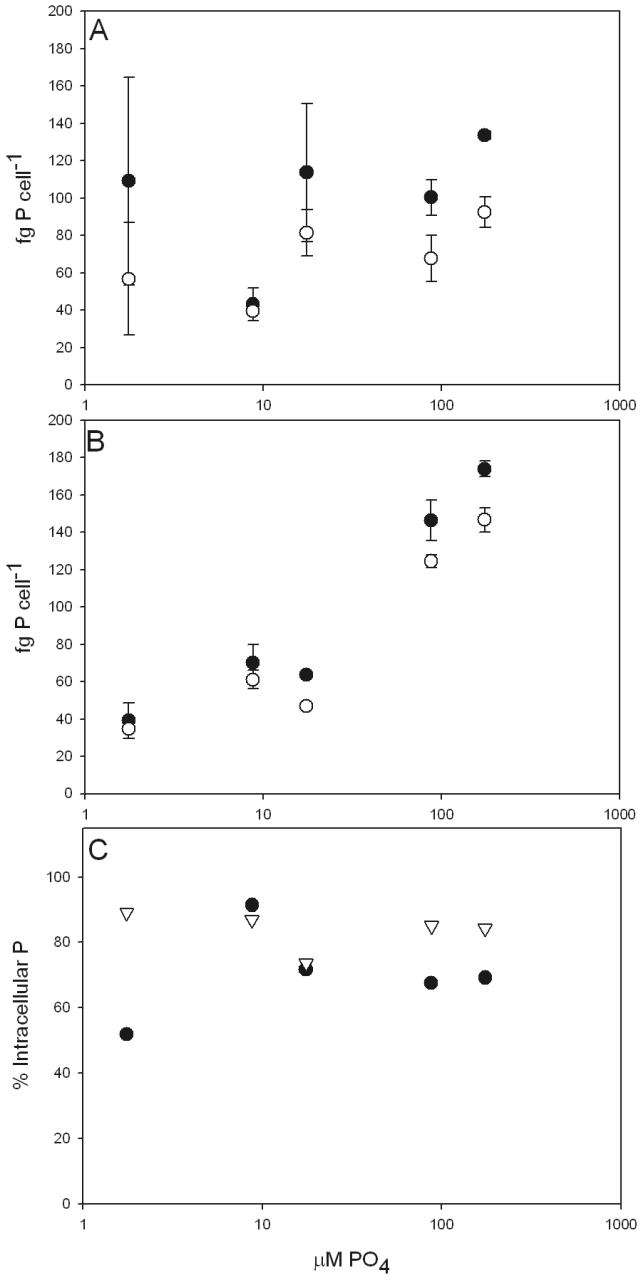


Figure 2.1. Total cell associated P (closed circles) and intracellular P (open circles) in fg cell^{-1} over growth media P concentration. Culture used in the individual figures: (A) *M. aeruginosa* PCC 7806 (B) *M. aeruginosa* LE3. (C) Percent intracellular P of total P pool in *M. aeruginosa* PCC 7806 (•) *M. aeruginosa* LE3 (Δ)

As had been previously reported for marine cyanobacteria (Fu et al., 2005), our results show that surface adsorbed P represents a significant portion (between 10-50%) of total cell-associated P in *Microcystis*. We also found that this percentage is stable across growth conditions in each of the strains tested (Figure 1C).

Total cell-associated and intracellular P in natural samples.

The ratio of total cell-associated P to intracellular P was also investigated in natural phytoplankton communities. Samples were collected for analysis across Lake Erie with specific emphasis on the Western Basin. This is because of a history of *Microcystis* blooms and a phytoplankton community at times dominated by *Microcystis* in this region (Moorhead et al., 2008; Rinta-Kanto et al., 2009a; Rinta-Kanto et al., 2005). Hydrological and biological parameters measured as part of this study were similar to those previously reported across Lake Erie (Table 2, DeBruyn et al., 2004). Intracellular P made up an average of 89±11% with a range of 0-65% of total particulate P in these samples (Table 1). Pearson correlation analysis of Western Basin samples showed weakly significant correlations ($p>0.1$) between the proportion of surface-associated extracellular P and several environmental variables, including SRP, POC, PON, and SiO_2 (Table 3). Notably, no significant correlation was observed between the proportion of extracellular P and either TP or TDP (Table 3, Figure 2).

Discussion

It is generally understood that P is the nutrient limiting phytoplankton biomass in many freshwater systems and that, by reducing P loading to eutrophic bodies of water, we can begin to control formation of algal blooms and biomass accumulation (Schindler et al., 2008). While the previous statement is generally true, it is important to note that individual HAB organisms have specific nutrient needs and

Table 2.3. Pearson correlation analysis results comparing the extracellular percentage of total particulate P to hydrological and biological parameters.

	r	p
Total particulate P	0.648	0.164
Intracellular P	0.619	0.189
SRP	0.784	0.065
TDP	0.714	0.111
TP	0.706	0.117
[a]NO_x	-0.169	0.748
NH_3	-0.508	0.304
TDN	0.144	0.785
PON	0.764	0.077
SiO_2	0.733	0.09
POC	0.757	0.081
chl*a*	0.451	0.369

[a]nitrate+nitrite

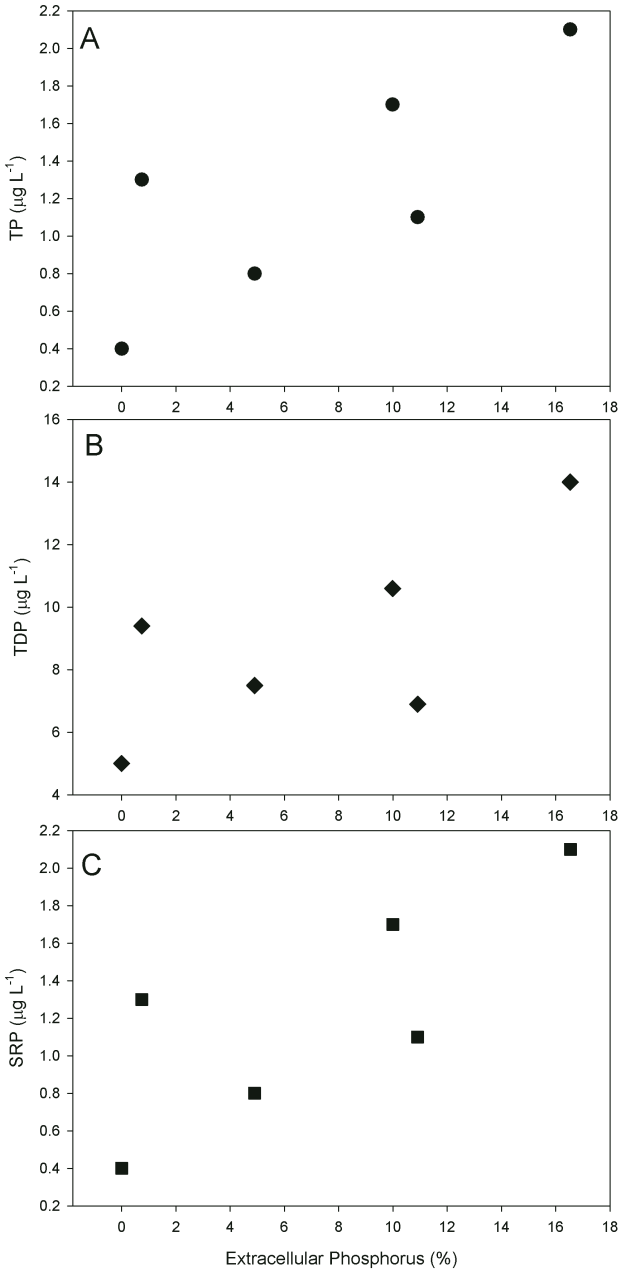


Figure 2.2. Scatterplots depicting the relationship between the extracellular percentage of total particulate P to TP (A), TDP (B) and SRP (C).

differ in their abilities to assimilate nutrients from different chemical species. For example, whereas the reduction of N in a eutrophic system may lead to an overall reduction in phytoplankton biomass, it can also lead to the proliferation of cyanobacteria capable of fixing N_2, many of which are potentially toxic (Chorus and Bartram, 1999). Moreover, it is now well established that some freshwater cyanobacteria possess the capacity to assimilate P from complex organic sources, such as phosphonates (Ilikchyan et al., 2009). To this end, we examined the P requirements of one of the most prominent bloom forming cyanobacteria, *M. aeruginosa*, in order to develop a dynamic cellular quota that can be used to inform credible data reporting. To do so, we applied recently developed techniques that allow for the individual analysis of total cell-associated *vs.* intracellular P-pools in phytoplankton.

Our results show plasticity in both cellular and surface absorbed P quotas. The ability to adjust intracellular P levels while maintaining a stable growth rate across a range of P concentrations is important for bloom forming cyanobacteria because it allows for persistence in conditions that do not allow for the accumulation of significant biomass. This nutritional flexibility is important for microbes in environments that experience large nutrient variability, such as large lakes and estuarine systems. In contrast to the plasticity of the cellular P quotas is the stability of the total cell-associated P to intracellular P ratio. Our data show this ratio to be stable, demonstrating the ability of *Microcystis* to adjust its P needs. These observations imply that surface associated P is a function of the external concentration in the environment, and thus probably not a mechanism to scavenge P for storage. One potential route for P adjustment has been described in marine cyanobacteria that have been observed to substitute phospholipids for nitrogen and sulfur containing lipids in response to P limitation (Van Mooy et al., 2009), though this ability has not yet been described in *Microcystis.*

Observed variability in P quota values in the tested strains is likely attributable to variance between strains. This variance is also observed in genetic differences between the sequenced *Microcystis* strains (Frangeul et al., 2008; Kaneko et al., 2007). In contrast to our observations in cultured *Microcystis,* the ratio of intracellular to extracellular P was not constant between Lake Erie stations. While this variability is correlated to differences in ambient SRP concentrations, dissimilarity in plankton communities between these sites may also contribute. The measured high proportion of intracellular P as compared to the total P pool in Lake Erie is indicative of an actively growing phytoplankton community as has been demonstrated previously (Sãnudo -Wilhelmy et al., 2004).

The fate of stored P on the cell surface remains elusive; it is possible that, when cells are shifted from P-replete to P-limiting conditions, this surface associated material can be assimilated by cells as opposed to the alternative of this P being lost back to the environment. Indeed, given that *Microcystis* can regulate its buoyancy (Brookes and Ganf, 2001) and is known to sink in response to P stress (Konopka et al., 1987), it is feasible that a benefit of moving "in and out" of the light field (from the upper water column to the deeper water column and back) may be to leave nutrient poor surface waters and access P released from the sediments. In this hypothetical scenario, it may be possible that cell-surface associated P gathered at depth becomes available for use in nutrient deplete upper waters when light conditions are more permissible.

Models of eutrophication and phytoplankton growth remain a critical tool for systems managers attempting to control frequent bloom formation and have been applied to Lake Erie since the middle of the 20th century (Di Toro et al., 1975; Léon et al., 2005; Schwab et al., 2009; Zhang et al., 2008). *Microcystis* growth has been specifically targeted in many of these models because it is a problematic, bloom-forming cyanobacterium. Physiological parameters such as cellular nutrient quotas, nutrient uptake rates and cellular growth rates are integral to generating accurate predictions of cellular growth and toxin production under specific environmental

conditions. Cellular P quota is a variable used (or is integral to variables used) in environmental models that have been applied to both *Microcystis* and Lake Erie (Burger et al., 2008; Léon et al., 2006; Robson and Hamilton, 2004; Trolle et al., 2008). This use of P quota as a modeling variable coupled with the fact that literature P quotas in *Microcystis* do not take into account the presence of externally bound P hinders the ability of these models to accurately project the occurrence of blooms and underestimates the algal biomass that can be supported by current P inputs. For example, the minimum internal P value applied to the CAEDYM model by Robson and Hamilton (2004) of 0.4 mg P (mg chl*a*)$^{-1}$ represents approximately a 40× overestimation of the P needs of this organism compared to the values reported here. The accurate prediction of natural conditions by environmental models is dependent on the use of precisely measured variables, so it is critical that the modeling community use the most appropriate values. Moreover, the significant metabolic plasticity with respect to P-quotas demonstrated by *Microcystis* in the present study is also not taken into account in current models. The current mathematical models use one or two values, often the minimum and maximum, to describe biological parameters such as growth rate or internal P quota (to limit the complexity of already complex models). *Microcystis* however has the ability to adapt to environmental challenge. As such, the ability of new hydrodynamic models, such as ELCOM, to more effectively describe changing physical conditions and nutrient concentrations in the water column suggest biological models would benefit from incorporating a more complex view of cyanobacterial growth over nutrient and temperature gradients.

Available sequence data from *Microcystis* genomes further demonstrate this microbe can adapt to a range of growth conditions. Genes encoding mechanisms to deal with cold, osmotic stress and darkness (Frangeul et al., 2008) are present as are multiple systems involved in phosphate acquisition (Kaneko et al., 2007). Both sequenced *Microcystis* genomes (*M. aeruginosa* PCC 7806 and NEIS-843) have a large percentage of long repeated sequences (11.7% in both) when compared to

other cyanobacterial genomes (<5%) (Frangeul et al., 2008; Kaneko et al., 2007). While the authors of these molecular studies highlight the data demonstrating the potential for these organisms to adapt to environmental changes, our current study goes beyond their work and demonstrates the range of this metabolic plasticity.

The importance of controlling the growth of HAB cyanobacteria cannot be understated because of the water quality impacts on both undeveloped and industrial countries. Previous studies examining P quotas in *Microcystis* measured only the total cell-associated P pool. In this study we differentiate between the total cell-associated P pools, including surface bound P, and the intracellular P pool; thus the P quotas reported here are a more representative examination of the P needs of *Microcystis* than has been previously reported. These results will be useful to resource managers attempting to model and control this nuisance alga.

Acknowledgements

We thank Michael Schultz for processing particulate organic phosphorus samples, and Jennifer DeBruyn for aid with statistical analyses. We would also like to thank the captain and crew of the *CCGS* Limnos. This research was supported by NOAA Oceans and Human Health external grant NA05NO54781251, Ohio Sea Grant College Program award R/ER-73 and NSF-IOS #0841918.

List of References

Brittain, S.M., Wang, J., Babcock-Jackson, L., Carmichael, W.W., Rinehart, K.L., Culver, D.A., 2000. Isolation and characterization of microcystins, cyclic heptapeptide hepatotoxins from a Lake Erie strain of *Microcystis aeruginosa*. J. Gt. Lakes Res. 26, 241-249.

Brookes, J.D., Ganf, G.G., 2001. Variations in the buoyancy response of *Microcystis aeruginosa* to nitrogen, phosphorus and light. J. Plankton Res. 23(12), 1399-1411.

Burger, D.F., Hamilton, D.P., Pilditch, C.A., 2008. Modelling the relative importance of internal and external nutrient loads on water column nutrient concentrations and phytoplankton biomass in a shallow polymictic lake. Ecol. Model. 211(3-4), 411-423.

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

Chorus, I., Bartram, J.E., 1999. Toxic cyanobacteria in water: a guide to public health significance, monitoring and management. EF&N Spon, London.

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(5917), 1014-1015.

DeBruyn, J.M., Leigh-Bell, J.A., McKay, R.M.L., Bourbonniere, R.A., Wilhelm, S.W., 2004. Microbial distributions and the impact of phosphorus on bacterial activity in Lake Erie. J. Gt. Lakes Res. 30, 166-183.

Di Toro, D.M., D.J., O.C., Thomann, R.V., Mancini, J.L., 1975. A preliminary phytoplankton-zooplankton-nutrient model of western Lake Erie, In: Patten, B.C. (Ed.), Systems Analysis & Simulation in Ecology. Academic Press, New York, NY, pp. 423-474.

Frangeul, L., Quillardet, P., Castets, A.-M., Humbert, J.-F., Matthijs, H., Cortez, D., Tolonen, A., Zhang, C.-C., Gribaldo, S., Kehr, J.-C., Zilliges, Y., Ziemert, N.,

Becker, S., Talla, E., Latifi, A., Billault, A., Lepelletier, A., Dittmann, E., Bouchier, C., Tandeau de Marsac, N., 2008. Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium. BMC Genomics 9(1), 274.

Fu, F.X., Zhang, Y.H., Leblanc, K., Sãnudo-Wilhelmy, S.A., Hutchins, D.A., 2005. The biological and biogeochemical consequences of phosphate scavenging onto phytoplankton cell surfaces. Limnol. Oceanogr. 50(5), 1459-1472.

Ilikchyan, I.N., McKay, R.M.L., Zehr, J.P., Dyhrman, S.T., Bullerjahn, G.S., 2009. Detection and expression of the phosphonate transporter gene *phnD* in marine and freshwater picocyanobacteria. Environmental Microbiology 11(5), 1314-1324.

Jacobson, L., Halmann, M., 1982. Polyphosphate metabolism in the blue-green alga *Microcystis aeruginosa*. J. Plankton Res. 4(3), 481-488.

Jørgensen, S.E., 2010. A review of recent developments in lake modelling. Ecol. Model. 221(4), 689-692.

Kaneko, T., Narajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., Nakamura, Y., Kasai, F., Watanabe, A., Kawashima, K., 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Res 14, 247 - 256.

Konopka, A., Kromkamp, J.C., Mur, L.R., 1987. Buoyancy regulation in phosphate-limited cultures of *Microcystis aeruginosa*. FEMS Microbiol. Lett. 45(3), 135-142.

Kromkamp, J., Vandenheuvel, A., Mur, L.R., 1989. Phosphorus uptake and photosynthesis by phosphate-limited cultures of the cyanobacterium *Microcystis aeruginosa*. British Phycological Journal 24(4), 347-355.

Léon, L.F., Smith, R.E.H., Romero, J.R., Heckey, R.E., 2006. Lake Erie hypoxia simulations with ELCOM-CAEDYM, In: Voinov, A., Jakeman, A.J., Rizzoli, A.E. (Eds.), Proceedings of the iEMSs Third Biennial Meeting: "Summit on

Environmental Modelling and Software". International Environmental Modelling and Software Society, Burlington, VT, USA.

Léon, L.K., Imberger, J., Smith, R.E.H., Hecky, R.E., Lam, D.C.L., Schertzer, W.M., 2005. Modeling as a tool for nutrient management in Lake Erie: a hydrodynamics study. J. Gt. Lakes Res. 31, 309-318.

Makarewicz, J.C., Bertram, P., 1991. Evidence for the restoration of the Lake Erie ecosystem. Bioscience 41(4), 216-223.

Menzel, D.W., Corwin, N., 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation. Limnol. Oceanogr. 10(2), 280-282.

Moorhead, D., Bridgeman, T., Morris, J., 2008. Changes in water quality of Maumee Bay 1928-2003, In: M., M., R., H. (Eds.), Checking the Pulse of Lake Erie. Backhuys Publishers, The Netherlands.

Ouellette, A.J.A., Handy, S.M., Wilhelm, S.W., 2006. Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molcular characterization of associated cyanobacterial communities. Microb. Ecol. 51, 154-165.

Ouellette, A.J.A., Wilhelm, S.W., 2003. Toxic cyanobacteria: the evolving molecular toolbox. Frontiers in Ecology and the Environment 7, 359-366.

Paerl, H.W., 1997. Coastal eutrophication and harmful algal blooms: Importance of atmospheric deposition and groundwater as ''new'' nitrogen and other nutrient sources. Limnol. Oceanogr. 42(5), 1154-1165.

Paerl, H.W., Pinckney, J.L., Fear, J.M., Peierls, B.L., 1998. Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse river estuary, North Carolina, USA. Marine Ecology-Progress Series 166, 17-25.

Rhee, G.Y., Gotham, I.J., 1980. Optimum N-P ratios and coexistence of planktonic algae. J. Phycol. 16(4), 486-489.

Rinta-Kanto, J.M., Konopko, E.A., DeBruyn, J.M., Bourbonniere, R.A., Boyer, G.L., Wilhelm, S.W., 2009a. Lake Erie Microcystis: Relationship between

microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae 8(5), 665-673.

Rinta-Kanto, J.M., Ouellette, A.J.A., Boyer, G.L., Twiss, M.R., Bridgeman, T.B., Wilhelm, S.W., 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. 39(11), 4198-4205.

Rinta-Kanto, J.M., Saxton, M.A., DeBruyn, J.M., Smith, J.L., Marvin, C.H., Krieger, K.A., Sayler, G.S., Boyer, G.L., Wilhelm, S.W., 2009b. The diversity and distribution of toxigenic *Microcystis* spp. in present day and archived pelagic and sediment samples from Lake Erie. Harmful Algae 8(3), 385-394.

Rinta-Kanto, J.M., Wilhelm, S.W., 2006. Diversity of microcystin-producing cyanobacteria in spatially isolated regions of Lake Erie. Appl. Environ. Microbiol. 72, 5083-5085.

Rippka, R., DeRulles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111, 1-61.

Ritchie, R.J., Trautman, D.A., Larkum, A.W.D., 1997. Phosphate uptake in the cyanobacterium *Synechococcus* R-2 PCC 7942. Plant Cell Physiol. 38(11), 1232-1241.

Ritchie, R.J., Trautman, D.A., Larkum, A.W.D., 2001. Phosphate limited cultures of the cyanobacterium *Synechococcus* are capable of very rapid, opportunistic uptake of phosphate. New Phytol. 152(2), 189-201.

Robson, B.J., Hamilton, D.P., 2004. Three-dimensional modelling of a *Microcystis* bloom event in the Swan River estuary, Western Australia. Ecol. Model. 174(1-2), 203-222.

Sãnudo -Wilhelmy, S.A., Tovar-Sanchez, A., Fe, F.X., Capone, D.G., Carpenter, E.J., Hutchins, D.A., 2004. The impact of surface-absorded phosphorus on phytoplankton Redfield stoichiometry. Nature 432, 897-901.

Sbiyyaa, B., Loudiki, M., Oudra, B., 1998. Nitrogen and phosphorus intracellular capacity in storage by *Microcystis aeruginosa* Kutz and Synechocystis sp.: toxic cyanobacteria occasionally forming blooms in Marrakesch area (Morocco). Annales De Limnologie-International Journal of Limnology 34(3), 247-257.

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. Proc. Natl. Acad. Sci. U. S. A. 105(32), 11254-11258.

Schwab, D.J., Beletsky, D., DePinto, J., Dolan, D.M., 2009. A hydrodynamic approach to modeling phosphorus distribution in Lake Erie. J. Gt. Lakes Res. 35(1), 50-60.

Shen, H., Song, L.R., 2007. Comparative studies on physiological responses to phosphorus in two phenotypes of bloom-forming *Microcystis*. Hydrobiologia 592, 475-486.

Strickland, J.D.H., Parsons, T.R., 1972. A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa.

Tovar-Sanchez, A., Sãnudo-Wilhelmy, S., Garcia-Vargas, M., Weaver, R.S., Popels, L.C., Hutchins, D.A., 2003. A trace metal clean reagent to remove surface -bound iron from marine phytoplankton. Mar. Chem. 82, 91-99.

Trolle, D., Skovgaard, H., Jeppesen, E., 2008. The Water Framework Directive: Setting the phosphorus loading target for a deep lake in Denmark using the 1D lake ecosystem model DYRESM-CAEDYM. Ecol. Model. 219(1-2), 138-152.

Tsukada, H., Tsujimura, S., Nakahara, H., 2006. Effect of nutrient availability on the C, N, and P elemental ratios in the cyanobacterium *Microcystis aeruginosa*. Limnology 7(3), 185-192.

Van Mooy, B.A.S., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblizek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappe, M.S., Webb, E.A.,

2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458(7234), 69-72.

Welschmeyer, N.A., 1994. Fluorometric analysis of chlorophyll-*a* in the presence of chlorophyll-*b* and pheopigments. Limnol. Oceanogr. 39(8), 1985-1992.

Wetzel, R.G., Likens, G.E., 2000. Limnological analyses. Springer, New York.

Wilhelm, S.W., Bullerjahn, G.S., Eldridge, M.L., Rinta-Kanto, J.M., Poorvin, L., Bourbonniere, R.A., 2006. Seasonal hypoxia and the genetic diversity of prokaryote populations in the central basin hypolimnion of Lake Erie: evidence for abundant cyanobacteria and photosynthesis. J. Gt. Lakes Res. 32, 657-671.

Wilhelm, S.W., Maxwell, D.P., Trick, C.G., 1996. Growth, iron requirements, and siderophore production in iron-limited *Synechococcus* PCC 7002. Limnol. Oceanogr. 41, 89-97.

Ye, W., Liu, X., Tan, J., Li, D., Yang, H., 2009. Diversity and dynamics of microcystin--Producing cyanobacteria in China's third largest lake, Lake Taihu. Harmful Algae 8(5), 637-644.

Zhang, H., Culver, D.A., Boegman, L., 2008. A two-dimensional ecological model of Lake Erie: Application to estimate dreissenid impacts on large lake plankton populations. Ecol. Model. 214(2-4), 219-241.

SECTION III

GLYPHOSATE INFLUENCE OF PHYTOPLANKTON COMMUNITY STRUCTURE IN LAKE ERIE

This section is a version of an article being prepared for submission to Aquatic Microbial Ecology under the same title by Matthew A. Saxton, Elizabeth A. Morrow, and Steven W. Wilhelm

My contribution to this paper was the collection of environmental samples, much of the design, setup, and collection of Lake Erie microcosms, the design, optimization and implementation of the *phnJ* PCR assay as well as the cloning, sequence editing, and tree building that followed. I also performed most of the data analysis, background information gathering and writing of this section.

Abstract

In this study we investigated the effect of loading of the phosphonate herbicide glyphosate (*N*-(phosphonomethyl)glycine) on the phytoplankton community structure in the Laurentian Great Lake, Lake Erie, using lake water incubations, laboratory growth experiments and phylogenetic analysis of phosphonate metabolism genes. The addition of glyphosate in Sandusky Bay microcosms resulted in a significant increase in phytoplankton abundance, specifically causing an increase in the abundance of *Planktothrix* spp. In Maumee Bay glyphosate did not stimulate phytoplankton growth but caused a decrease in *Microcystis* spp. abundance. The difference in the ability of *Planktothrix* spp. and *Microcystis* spp. to grow in the presence of glyphosate was confirmed in laboratory growth experiments. Further, an examination of the molecular pathways involved in phosphonate metabolism demonstrated that heterotrophic bacteria are a critical component of the community in Lake Erie. These results indicate glyphosate is positively and negatively influencing phytoplankton community structure in the lake, providing nutrient to microbes able tolerate the herbicidal effects of the compound while killing less tolerant microbes. Moreover, this work highlights that in natural environments microorganisms function as communities and the metabolic abilities of individual species are often less important than the collective ability of the community.

Introduction

The phosphonate herbicide glyphosate (*N*-(phosphonomethyl)glycine) is the primary active ingredient in Roundup™ and the most widely used chemical herbicide globally. Glyphosate is a broad-spectrum herbicide that acts as a glycine analogue, inhibiting the synthesis of aromatic amino acids acting on 5-*enol*-pyruvylshikimate-3-phosphate (EPSP) synthase (Steinrucken and Amrhein, 1980). The development of Roundup Ready™ crops that tolerate glyphosate exposure through the possession of a tolerant EPSP synthase and/or a glyphosate metabolism gene (Tan et al., 2006) has led to high application levels. Estimated application rates are between 103 and 113 million lbs in the United States, including high agricultural applications in the Lake Erie drainage basin (Kannan et al., 2006; Struger et al., 2008). Indeed, glyphosate has been measured in Lake Erie tributaries on both sides of the United States-Canadian border and has been measured in Lake Erie itself. Glyphosate detection in Lake Erie is transitory, with the highest concentrations being observed in conjunction with spring agricultural glyphosate application (Byer et al., 2008, McKay and Bullerjahn, unpublished; Struger et al., 2008). The lack of glyphosate accumulation throughout the year is strongly indicative of microbial degradation of this compound in Lake Erie.

Genes encoding for a microbial phosphonate metabolism system were originally described in *E. coli* (Wanner and Boline, 1990), subsequently this C-P lyase system has been shown to be important to microbial degradation of these compounds in nature (Quinn et al., 1989; Wanner and Boline, 1990). The C-P lyase operon (*phnC-P)* in *E. coli* is a 10.4 kb multi-enzyme gene cluster under the control of a low P induced Pho promoter and encodes two major functional units. An ABC type phosphonate transporter is encoded by genes *phnC-E*, and *phnG-M* make up the C-P lyase itself responsible for the cleavage of the C-P bond. This enzyme has been show to be able to metabolize a broad range of phosphonate substrates. Evidence of frequent horizontal gene transfer has been observed in this gene cluster and gene

order is not conserved between taxonomic groups, likely as a result of repeated rearrangements (Huang et al., 2005). Other phosphonate metabolism enzymes have been identified, many of which are not subject to the influence of exogenous P levels and are specific to particular phosphonate substrates (Quinn et al., 2007).

Glyphosate has shown to positively (as a nutrient source) and negatively (as a toxic compound) influence microbial communities. P, C and N mineralization from glyphosate by microbes in soils has also been observed (Dick and Quinn, 1995; Lancaster et al., 2010; Liu et al., 1991). Phosphonate metabolism and the presence of phosphonate metabolism genes have also been observed in cyanobacteria from freshwater and marine systems, particularly picocyanobacteria and filamentous cyanobacteria (Dyhrman et al., 2006; Ilikchyan et al., 2009; Stucken et al., 2010). The application of glyphosate has been observed to influence microbial community structure in freshwater (Perez et al., 2007), shifting the community from glyphosate-sensitive green algae and diatoms to glyphosate-tolerant cyanobacteria. Changes in microbial community structure upon glyphosate exposure have also been described in marine (Stachowski-Haberkorn et al., 2008) and soils systems (Lancaster et al., 2010).

While large scale blooms of the toxic cyanobacterium *Microcystis* have been documented in the Laurentian Great Lakes for over 15 years (Brittain et al., 2000; Rinta-Kanto et al., 2005), other cyanobacteria that are capable of producing the toxin microcystin have been documented and in some cases are dominant in regional embayments (Millie et al., 2009; Rinta-Kanto and Wilhelm, 2006). Given previous reports of the differential effects of glyphosate on cyanobacteria (Forlani et al., 2008), we tested the hypothesis that glyphosate loads from surrounding watersheds could simultaneously act as a negative selection, excluding *Microcystis* from these embayments, and a positive selection, providing nutrients to *Planktothrix*.

Given the need for molecular transformations of this compound, we further investigated a component of the pathway involved in glyphosate degradation (the phosphonate metabolism gene *phnJ*) to determine which members of the microbial community may be active in this process.

Materials and Methods

Sample collection

Experiments were performed using field samples collected during August 2007 aboard the *CCGS* Limnos at Environment Canada stations 973 (41° 47' 30" N, 82° 19' 56" W), 885 (41° 46' 59" N, 83° 02' 14" W) and 1163 (41° 43' 35" N, 83° 09' 00" W) in the western basin of Lake Erie (Fig. 1). Water was collected at each site using 10L Niskin sampling bottles.

Glyphosate amendment incubations were performed using surface water from stations 973 and 885. Station water was distributed into 1.2L polycarbonate bottles and premixed inorganic phosphate cocktail (1:4: mixture of KH_2PO_4:K_2HPO_4) or glyphosate was added to a final concentration of 1µM, unamended controls were also executed. All incubations were performed in triplicate. Microcosm bottles were sealed and incubated at *in situ* temperature using an on-deck incubator with constant surface water flow. Neutral density screening was used to reduce light levels to 37% surface radiation. Following a 48-hour incubation, bottles were removed from the incubator and samples were immediately collected for analysis.

Chlorophyll-a estimates and phytoplankton enumeration

Total community chl*a* was determined by filtration of whole water onto 0.2 µM pore-size 47mm polycarbonate filters (Millipore) after extraction (24h, 4°C) in 90% acetone. Extracted chl*a* was measured using a Turner Designs AU10 flourometer following the non-acidification protocol (Welschmeyer, 1994).

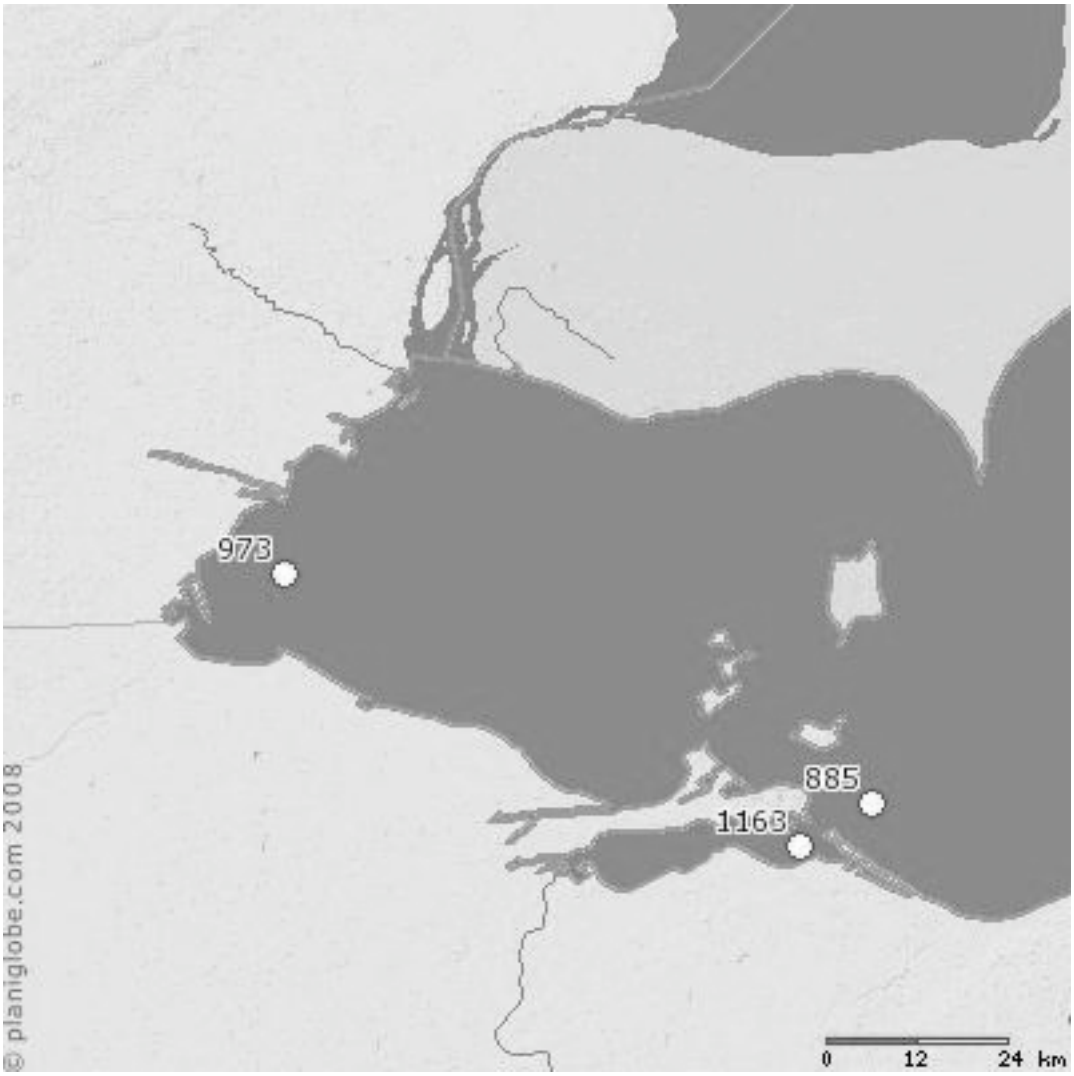


Figure 3.1. Map of Western Basin of Lake Erie sampling sites used in this study. Sampling sites are noted with Environment Canada station numbers.

Phytoplankton enumeration was performed using samples preserved in Lugol's Iodine as previously described (Anderson, 2005; Rodhe et al., 1958). 50 mL samples from each incubation were collected and immediately distributed into amber bottles with predespensed Lugol's Iodine resulting in a final concentration of 2% (vol/vol) fixative. Fixed-samples were then enumerated using a Sedgwick-Rafter counting slide (Wildco) on a Micromaster light microscope (ThermoFisher). Samples were allowed to settle in the counting chamber for 3-5 minutes prior to enumeration. Four horizontal passes were made across each slide encompassing the full height and width of the slide; no fewer than 40 fields of view were randomly selected across each slide. Each field of view was imaged and saved using Micron imaging software (Westover Scientific); the use of this software allowed for the measurement of the field of view.

Laboratory growth experiments

The ability of cyanobacterial cultures to use glyphosate and selected breakdown products as lone sources of P or N was tested by growing unialgal cultures in CT growth media in which the standard P or N sources (Na_2 β-glycerophosphate, KNO_3) were replaced with compounds known to be in the glyphosate breakdown pathway. Growth on P containing compounds was tested using 5 concentrations of each chemical ranging between 1.75 and 175 μM. Potential N sources were tested in concentrations ranging from 30μM - 3mM. Glyphosate and AMPA were tested as N and P sources, while sarcosine was only tested as N sources because this compound does not contain P. Experiments were performed with cyanobacterial strains *Microcystis aeruginosa* NIES 843 and *Planktothrix agardhii* PCC 7811. *M. aeruginosa* NIES 843 was obtained from the National Institute for Environmental Studies of Japan in 2009. This strain is capable of producing microcystin and is the only available isolate with a completed genomic sequence (Kaneko et al., 2007). *P. agardhii* PCC 7811 was acquired from the Pasteur culture collection in 2001; this strain is also known to possess microcystin synthase

genes (Ouellette and Wilhelm, 2003). Lake Erie strains *Microcystis aeruginosa* LE3 and *Planktothrix agardhii* LE9 were also tested to confirm type strain results (not shown). Cultures were grown in 50 mL glass culture tubes (Kimble, Vineland, NJ) at 24° C under 12h-12h day-night cycle with ~30 μmol photons m^{-2} s^{-1} illumination. Prior to use, all culture tubes were soaked overnight in 1% HCL and rinsed multiple times with MilliQ water. Samples were grown and transferred at experimental conditions two times prior to data collection. Multiple transfers were performed to ensure the physiological equilibration of cell cultures to experimental conditions *vis a vis* Wilhelm et al. (1996). Cyanobacterial growth was monitored daily using a Turner Designs TD-700 fluorometer (Sunnyvale, CA, USA) equipped with an *in vivo* chl*a* filter set (excitation λ = 340–500 nm; emission λ = >665 nm). All experiments were performed in triplicate.

Potential toxicity effects of the compounds used in the above growth experiments were tested on *M. aeruginosa* NIES 843 and *Planktothrix agardhii* PCC 7811. Cultures were grown in CT media containing the normal N and P sources as well as glyphosate and breakdown products at the concentrations described above. The toxicity of glyphosate was further investigated by testing 10 additional concentrations between 0.05 and 5 mM.

PCR, Construction of Clone Library, and Phylogenetic Analysis

Surface water collected from stations 973 and 1163 was filtered onto 0.22-μm nominal pore-size 25mm diameter polycarbonate filters (Millipore) and nucleic acids were extracted *via* the phenol-chloroform extraction method as previously described (Rinta-Kanto et al., 2005; Sambrook and Russell, 2001).
Primers targeting C-P lyase gene *phnJ* from heterotrophic bacteria and cyanobacteria were designed for this study using *phnJ* sequences acquired from NCBI GenBank (*phnJ*F-5'TSAARGTSATYGACCAGGG, *phnJ*R-5'GCARTARTCSGTRTCSGARCA). Primers were tested for amplification and specificity using cultured isolates. PCR reactions were performed using PuRe Taq

Ready To-Go PCR beads (GE Healthcare) according to specifications with ~1-µL of DNA, 20 *p*mol of each primer, and sterile water up to a total volume of 25 µL. Reaction mixes without template served as negative controls. Thermal cycling was performed as follows: initial denaturation of 95° for 5 minutes, followed by 40 cycles of 95° for 30 sec, 61° for 30 sec, 72° for 45 sec and by an extension step of 72° for 5 min. All PCRs were performed on a Mastercycler Gradient thermocycler (Eppendorf). PCR product was stored at 4° C until examined via electrophoresis on 1.5% agarose, with subsequent visualization and an Investigator digital imager (Fotodyne) with ethidium bromide filter using Foto/Analyst® PC Image v. 9.0.4 software. Following ethidium bromide staining target bands were excised and cleaned using the Wizard SV gel and PCR clean-up system (Promega). Eluted DNA was cloned using a TOPO TA cloning kit (Invitrogen), and resulting plasmids were extracted and purified using the QIAprep Spin Miniprep Kit (QIAGEN). DNA sequences were obtained from the University of Tennessee Molecular Biology Resource Facility.

Sequences were manually screened for quality at which time vector and primer sequences were removed. Translation from nucleotide to amino acid sequence was performed prior to phylogenetic analysis. Sequences were edited, translated and organized using Geneious bioinformatics software (Drummond, 2010). Sequences were aligned by ClustalW using ClustalX 2.0.8 software (Thompson et al., 1994). Phylogenetic relationships were investigated via Neighbor Joining analysis using the Poisson correction method. Bootstrap values were generated based on 5000 iterations. Maximum Parsimony and Maximum Likelihood tree building models resulted in the same general clustering pattern. Neighbor-Joining and Maximum Parsimony phylogenetic analyses were performed using MEGA 4.1 software. Maximum Likelihood analysis was performed through phylogeny.fr. One-tailed t-tests were used to determine significant differences between treatment variables. Statistical analysis was performed using SPSS v. 16

Results

Phosphonate addition microcosm

To determine the effect of glyphosate addition on Lake Erie, phytoplankton community microcosm experiments were performed in which lake water was amended with glyphosate or phosphate. These incubations were performed at stations 885 and 973; sta. 885 is located in the Sandusky sub-basin just outside of the opening to Sandusky Bay. We chose this location because phytoplankton in Sandusky Bay are too abundant for microscosm experiments to be consistently replicated. We have previously observed that the phytoplankton community at station 885 is influenced by "seeding" *via* outflow from the bay, resulting in a cyanobacterial community similar to the community in Sandusky Bay (although dilute and including both *Planktothrix* and *Microcystis*). At station 885, the glyphosate addition treatment resulted in a modest yet statistically significant increase (t-test $p < 0.05$) in chl*a* from 2.4-3 μg L^{-1} (Fig. 2A) when compared to the phosphate treatment and the unamended control. This chl*a* increase in the glyphosate treatment corresponded to a statistically significant increase in *Planktothrix spp.* cell abundance from 1.39 x10^7 to 2.56 x 10^7 cells mL^{-1} (Fig. 2B). A significant decrease in *Aulacoseira* spp. abundance was observed in both the phosphate and glyphosate treatments. No significant changes in chl*a* were observed in either treatment at station 973. In the Maumee Bay incubations ,a significant decrease in *Microcystis spp.* was observed in the glyphosate treatment (6.63 x 10^6 cells $^{-1}$mL) as compared to both the unamended control (7.97 x10^7 cells $^{-1}$mL) and the phosphate treatment (5.08 x10^7 cells $^{-1}$mL) (Fig. 2C).

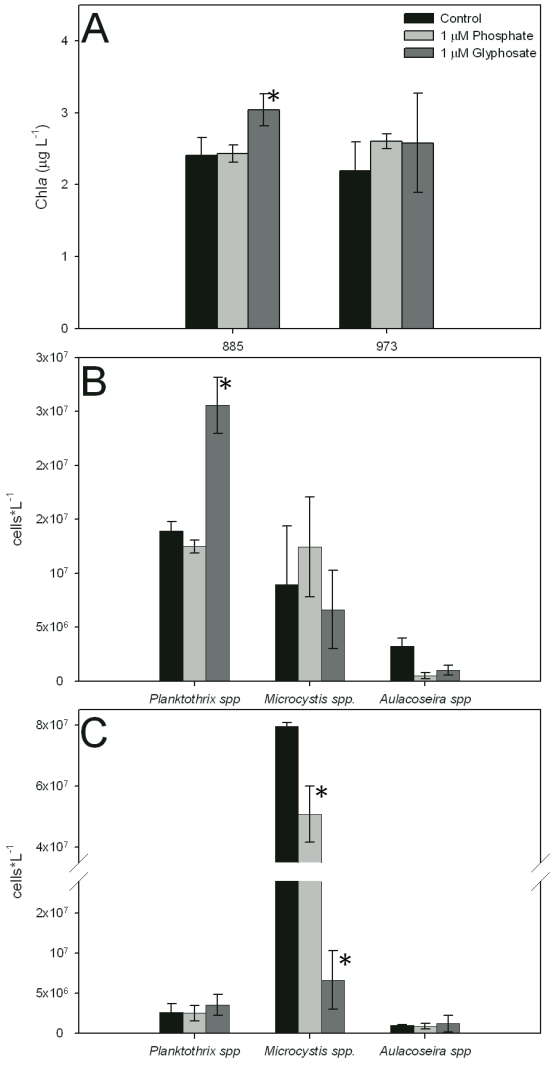


Figure 3.2. Lake Erie glyphosate amendment microcosm, black bar indicates no-addition control, light grey 1 µM phosphate addition and dark grey 1µM glyphosate addition. Statitcally significant differences ($p < 0.05$) are denoted *. (A) chl*a* concentrations from incubations performed at stations 885 and 973 as well as cell Other major taxa observed at both stations for which no significant changes in abundance were recorded include *Cyclotella* spp.*, Anabaena* spp. and *Scenedesmus* spp. count results from(B) sta. 885 and (C) sta. 973. All values are shown as mean ± standard deviation

Laboratory Growth Experiments

Toxicity of glyphosate to *Planktothrix agardhii* PCC 7811 and *Microcystis aeruginosa* NIES 843 was tested. *Microcystis* was observed to be less resistant to the toxic effects of glyphosate than *Planktothrix* (Fig. 3). *Microcystis* growth rate remained above 95% of the untreated control at 0.05 mM; however while *Planktothrix* maintained growth at above 90% of the control at 0.1 mM, *Microcystis* growth rate dropped to below 40% of the control. *Microcystis* were observed to be unable to grow at all over concentrations of 0.2 mM glyphosate. *Planktothrix* was unable to a maintain 90% growth rate at glyphosate concentrations over 0.15 mM and was unable to grow at concentrations over 0.35 mM.

In lab studies *Planktothrix* cultures were better able to use glyphosate and breakdown products known to accumulate in the environment as sources of nutrient than our model *Microcystis* strains. *Planktothrix* cultures were able to use glyphosate and AMPA as sources of P. These cultures were also able to use AMPA as an N source (Table 1). Not only is *Microcystis* unable to use glyphosate or AMPA as a source of P or N, but it is also unable to use any tested breakdown products (Table 1).

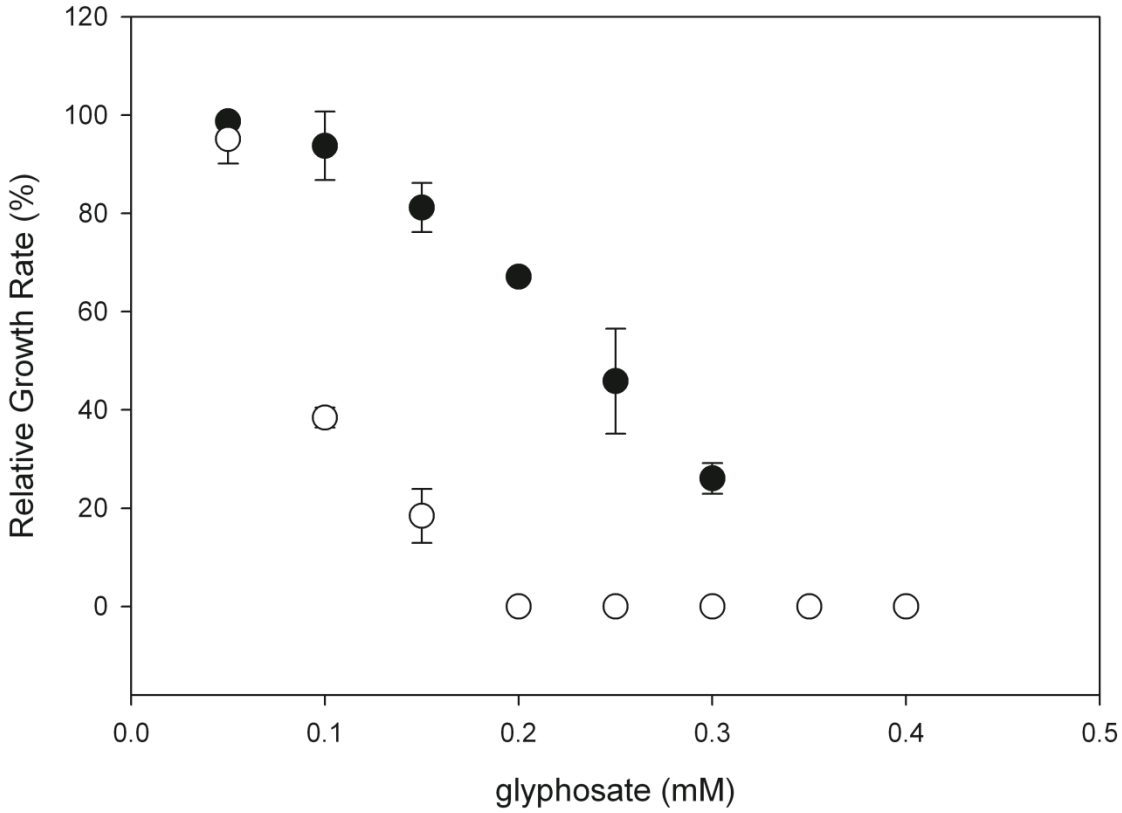


Figure 3.3. Glyphosate toxicity effects on cyanobacterial culture growth. Black circles: *Planktothrix agardhii* PCC 7811, open triangles: *Microcystis aeruginosa* NIES 843. Relative growth rate is growth rate as a percentage of growth in control media without glyphosate. Values shown as mean ± standard deviation.

Table 3.1 Growth of cyanobacterial cultures using glyphosate and select breakdown products as sole sources of P and N. Numerical values indicate maximum measured growth rate over a 21-day period as a percentage of control (Na_2 β-glycerophosphate, KNO_3), shown as mean ± standard deviation. Zero (0) indicate the culture was unable to grow using the experimental nutrient source in the tested period.

	Microcystis aeruginosa NIES 843	*Planktothrix agardhii* PCC 7811
Phosphorus		
Glyphosate	0	118.04% ±16.61 (175 μM)
AMPA	0	115.19% ±22.25 (44 μM)
Nitrogen		
Glyphosate	0	0
AMPA	0	123.66% ±5.17 (825 μM)
Sarcosine	0	0

Phylogenetic Analysis

To further investigate the bacterial (both heterotrophic and cyanobacteria) community involved in the metabolism of phosphonate in the environment, the phosphonate metabolism gene *phnJ* was amplified and sequenced from Lake Erie stations 973 and 1163. All (39 total) of the sequences obtained from both stations clustered most closely with heterotrophic bacteria (Fig. 4). At station 1163 nearly all (19 of 20) sequences are closely related to publicly available *E. coli phnJ* sequences. The other sequence obtained from station 1163 grouped with the soil γ-proteobacterial cluster. Sequences from sta. 973 group strongly with α and β-proteobacterial clusters. Also observed were sequences that grouped weakly with β-proteobacteria.

Discussion

Large agricultural application of the phosphonate herbicide glyphosate has led to the presence of this chemical in measurable quantities in Lake Erie and its tributaries. In this study we examined the effect of the occurrence of this chemical on phytoplankton community structure in Lake Erie. Our results demonstrate that glyphosate can suppress the growth of the cyanobacterium *Microcystis* but facilitate the growth of another potentially toxic cyanobacterium, *Planktothrix,* in regions of the lake. These results further suggest that glyphosate and its breakdown products are available to the microbial community as a source of nutrient, that the microbial community in the lake capable of phosphonate degradation is more diverse than previously observed, and that this degrading community is largely composed of heterotrophic bacteria.

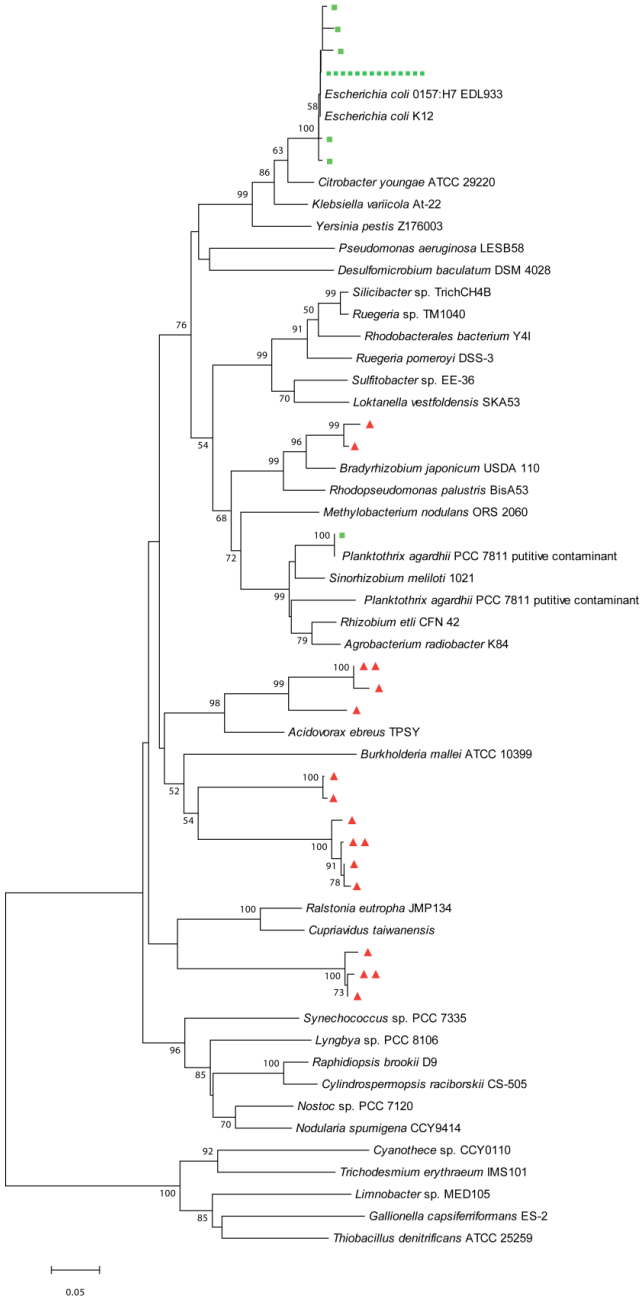


Figure 3.4. Neighbor Joining phylogenetic tree (5000 iterations) of *phnJ* amino acid sequences translated from DNA sequences PCR amplified from Lake Erie samples collected in 2007. Bootstrap values > 50% are shown. Red triangles are sequences obtained from sta. 973. Green squares were collected from sta. 1163. Number of icons indicates identical sequences.

Our observations indicate that controls of glyphosate loading in Lake Erie and its tributaries will be important to more successful management of harmful algal bloom events. These results also contribute to the growing body of literature showing that microbes in the environment do not exist as individuals, but rather are dependent on the metabolic abilities of their neighbors.

The significant increase in chl*a* at sta. 885 with the addition of glyphosate but not with phosphate is noteworthy because it gives an indication of which nutrients may be limiting phytoplankton growth at this site. Generally, phytoplankton in freshwater systems are considered to be phosphorus limited and limiting P in eutrophic systems has been critical to controlling algal blooms in multiple locations, including Lake Erie (Makarewicz and Bertram, 1991; Schindler et al., 2008). Recently this paradigm has been challenged with a growing body of evidence contesting that in many freshwater and estuarine systems controlling N inputs along with P is critical to controlling eutrophication (Conley et al., 2009). Our results indicate that Sandusky Bay was not P limited, but rather N-limited or N and P co-limited. Potential N limitation is further suggested at sta. 885 by a N:P ratio of 6.3 (Wilhelm and Bourbonniere, unpublished).Considering the significant resources dedicated to the control of eutrophication in Lake Erie, evidence that glyphosate, a chemical being applied in the Lake Erie watershed in large amounts, its a potential source of P and N is noteworthy.

This study also illustrates that the presence of glyphosate can exert negative control on a phytoplankton through the herbicidal effects of the chemical. These herbicidal effects are likely the reason why the significant decrease in *Microcystis* cell density was observed in glyphosate microcosms at sta. 973. Although, like all cyanobacteria, *Microcystis* possesses a glyphosate tolerant version of ESPS synthase, this cyanobacterium has been observed to be less tolerant of glyphosate *in vitro* than other tested cyanobacteria, both in this study and others (Forlani et al., 2008). While the glyphosate concentrations at which toxic effects on *Microcystis* are observed *in vitro* are higher (between 50 and 100 μM in culture) than the

concentration tested in this microcosm (1μM) or those observed in the field (0.001 μM in lake water, 0.01 μM in Maumee River, McKay and Bullerjahn, unpublished), the added stress of living in an environmental setting could lower the concentrations at which glyphosate effects growth in this organism. Also, despite the previously discussed drop in *Microcystis* cell number at sta. 973, there was no corresponding drop in chl*a.* This lack of a drop in chl*a* is not compensated by an increase in abundance of another measured taxa, but likely by an increase in *Synechococcus*, though this has not be tested. In fact, Lake Erie *Synechococcus* have been reported to possess phosphonate transport genes (Ilikchyan et al., 2009) that potentially allow them to utilize phosphonates such as glyphosate as a source of P.

To build on the results of the field microcosms, we examined the ability of *Planktothrix* and *Microcystis* cultures to grow in elevated concentrations of glyphosate as well as their ability to assimilate it (and its breakdown products) as P and N sources. Unialgal (but not axenic) cultures were used for this component: a growing body of evidence has clearly demonstrated that the interactions between phytoplankton and heterotrophic bacteria are critical to understanding how and why phytoplankton are successful in nature (Morris et al., 2008). Moreover, recent studies demonstrate that these cyanobacteria cultures are likely unavailabile axenically (Rounge et al., 2009). Notably, microbes in both *Microcystis* and *Planktothrix* cultures possessed the phosphonate metabolism gene *phnJ* (data not shown), and *phnJ* sequences obtained from the *Planktothrix* culture clustered most closely with soil α-proteobacteria, not cyanobacteria (Fig. 4). Indeed, with these glyphosate toxicity and nutrient results corroborating our Lake Erie microcosm findings, it is clear that glyphosate influences the distribution of *Microcystis* and *Planktothrix* in Lake Erie. This influence is enacted through the suppression of the population less able to withstand the toxic effects of glyphosate and the support of those microbes able to use it and its breakdown products as a source of nutrient.

Given the importance of glyphosate on phytoplankton community structure, we investigated the heterotrophic and cyanobacterial community potentially able to metabolize this compound in Lake Erie through the amplification of the C-P lyase gene *phnJ* from Sandusky and Maumee Bay. Obtained sequences show significant terrestrial influence in Sandusky Bay, with *E. coli* and rhizobium-like sequences likely from agricultural sources. Also observed were sequences without close relatives among available *phnJ* sequences, highlighting the general lack of freshwater bacterial genome sequences. It should be noted however that, because of high P-loads at this site, it is possible that the C-P lyase system is not active, though the varied gene arrangements that have been observed in sequenced genomes containing C-P lyase make non-Pho regulon controlled variants possible. Overall, these results illustrate the importance of heterotrophic bacteria in phosphonate metabolism in freshwater.

While both *Planktothrix* and *Microcystis,* as bloom forming cyanobacteria, thrive in warm eutrophic systems, these results illustrate how physiological and lifestyle differences between *Planktothrix* and *Microcystis* inform why these microbes are often successful in different environmental settings. *Microcystis* is known for a high P uptake rate and is successful in deeper eutrophic lakes. Observed to regulate buoyancy in response to nutrient stress, *Microcystis* reduces gas vesicle content within the cell when stressed for P, allowing it to migrate to nutrient-rich waters lower in the water column from where Pi from the sediments can be collected and stored (Konopka et al., 1987). In contrast, *Planktothrix* is successful in shallow well-mixed eutrophic lakes. While this success is often attributed to *Planktothrix* being better suited to the turbidity of such shallow bodies of water (Scheffer et al., 1997), algal growth itself is often responsible for much of this turbidity, likely making this explanation an oversimplification.

Another reason for the success of *Planktothrix* in shallow lakes is suggested by the results of this study; filamentous cyanobacteria may be better able to utilize complex nutrient forms cycled by the heterotrophic bacterial community. This possibility merits further study.

Applying the above observations to the Sandusky and Maumee Bay systems in Lake Erie, a clearer picture emerges concerning the differences in cyanobacterial community structure we see at these sites. In Sandusky Bay early season pulses of glyphosate impede *Microcystis* growth while glyphosate-tolerant filamentous cyanobacteria are allowed to proliferate. The enclosed nature of this embayment (depicted in Fig. 1) results in prolonged exposure of specific microbial communities. As the phosphonate metabolizing community mineralizes the post-application glyphosate pulse, the annual *Planktothrix* bloom sets up. This concentrated (a reported 2002 value of 75 μg L^{-1} (DeBruyn et al., 2004) is representative) bloom is supported by nutrients supplied by the Sandusky River and the sediments. High turbidity throughout the fully mixed water column favors the success of *Planktothrix* until the bloom collapses due to cold weather. Maumee Bay is large and open to mixing with the rest of Lake Erie. As a result the plankton of this site, though exposed to glyphosate, likely do not see continuous exposure but rather are exported elsewhere. Here, the phosphonate utilizing community metabolizes the spring glyphosate pulse. Following the drop in glyphosate levels, *Microcystis* can reestablish from the sediments or from an area not exposed to glyphosate.

The results of the above experiments indicate that glyphosate is a potentially important influence on phytoplankton community structure in Lake Erie and that glyphosate is a potential source of the important nutrient N and P to the Lake Erie system. Resource managers intending to control harmful cyanobacterial bloom occurrence and nutrient loading should carefully consider this compound and its impact on both of these important areas of concern to the health of Lake Erie. ™

Acknowledgements

We would like to thank the captain and crew of the *C.C.G.S.* Limnos. As well as George Bullerjahn, R. Michael McKay and Erik Zinser for helpful discussion, and Dinielle Truitt and Christina Kihm for help with cell culture assays. This research was supported by NOAA Oceans and Human Health external grant NA05NO54781251, NSF-IOS #0841918 and the International Association for Great Lakes Research.

List of References

Anderson, R.A., 2005. Algal culturing techniques. Academic Press, Boston.

Brittain, S.M., Wang, J., Babcock-Jackson, L., Carmichael, W.W., Rinehart, K.L., Culver, D.A., 2000. Isolation and characterization of microcystins, cyclic heptapeptide hepatotoxins from a Lake Erie strain of *Microcystis aeruginosa*. J. Gt. Lakes Res. 26, 241-249.

Byer, J.D., Struger, J., Klawunn, P., Todd, A., Sverko, E., 2008. Low cost monitoring of glyphosate in surface waters using the ELISA method: An avaluation. Environ. Sci. Technol. 42(16), 6052-6057.

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(5917), 1014-1015.

DeBruyn, J.M., Leigh-Bell, J.A., McKay, R.M.L., Bourbonniere, R.A., Wilhelm, S.W., 2004. Microbial distributions and the impact of phosphorus on bacterial activity in Lake Erie. J. Gt. Lakes Res. 30, 166-183.

Dick, R., Quinn, J., 1995. Glyphosate-degrading isolates from environmental samples: occurrence and pathways of degradation. Appl. Microbiol. Biotechnol. 43(3), 545-550.

Drummond, A.A., B. Buxton, S. Cheung, M. Cooper, A. Heled, J. Kearse, M. Moir, R. Stones-Havas, S. Sturrock, S. Thierer, T. Wilson, A., 2010. Geneious, 5.1 ed.

Dyhrman, S.T., Chappell, P.D., Haley, S.T., Moffett, J.W., Orchard, E.D., Waterbury, J.B., Webb, E.A., 2006. Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. Nature 439(7072), 68-71.

Forlani, G., Pavan, M., Gramek, M., Kafarski, P., Lipok, J., 2008. Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. Plant Cell Physiol. 49(3), 443-456.

Huang, J.L., Su, Z.C., Xu, Y., 2005. The evolution of microbial phosphonate degradative pathways. J. Mol. Evol. 61(5), 682-690.

Ilikchyan, I.N., McKay, R.M.L., Zehr, J.P., Dyhrman, S.T., Bullerjahn, G.S., 2009. Detection and expression of the phosphonate transporter gene *phnD* in marine and freshwater picocyanobacteria. Environmental Microbiology 11(5), 1314-1324.

Kaneko, T., Narajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., Nakamura, Y., Kasai, F., Watanabe, A., Kawashima, K., 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Res 14, 247 - 256.

Kannan, K., Ridal, J., Struger, J., 2006. Pesticides in the Great Lakes, In: Hites, R. (Ed.), Persistent Organic Pollutants in the Great Lakes. Springer Berlin / Heidelberg, pp. 151-199.

Konopka, A., Kromkamp, J.C., Mur, L.R., 1987. Buoyancy regulation in phosphate-limited cultures of *Microcystis aeruginosa*. FEMS Microbiol. Lett. 45(3), 135-142.

Lancaster, S.H., Hollister, E.B., Senseman, S.A., Gentry, T.J., 2010. Effects of repeated glyphosate applications on soil microbial community composition and the mineralization of glyphosate. Pest Manag. Sci. 66(1), 59-64.

Liu, C.-M., McLean, P.A., Sookdeo, C.C., Cannon, F.C., 1991. Degradation of the herbicide glyphosate by members of the family *Rhizobiaceae*. Appl. Environ. Microbiol. 57(6), 1799-1804.

Makarewicz, J.C., Bertram, P., 1991. Evidence for the restoration of the Lake Erie ecosystem. Bioscience 41(4), 216-223.

Millie, D., Fahnenstiel, G., Dyble Bressie, J., Pigg, R., Rediske, R., Klarer, D., Tester, P., Litaker, R., 2009. Late-summer phytoplankton in western Lake Erie (Laurentian Great Lakes): bloom distributions, toxicity, and environmental influences. Aquat. Ecol. 43(4), 915-934.

Morris, J.J., Kirkegaard, R., Szul, M.J., Johnson, Z.I., Zinser, E.R., 2008. Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by "helper" heterotrophic bacteria. Appl. Environ. Microbiol. 74(14), 4530-4534.

Ouellette, A.J.A., Wilhelm, S.W., 2003. Toxic cyanobacteria: the evolving molecular toolbox. Frontiers in Ecology and the Environment 7, 359-366.

Perez, G.L., Torremorell, A., Mugni, H., Rodriguez, P., Vera, M.S., Do Nascimento, M., Allende, L., Bustingorry, J., Escaray, R., Ferraro, M., Izaguirre, I., Pizarro, H., Bonetto, C., Morris, D.P., Zagarese, H., 2007. Effects of the herbicide roundup on freshwater microbial communities: A mesocosm study. Ecol. Appl. 17(8), 2310-2322.

Quinn, J.P., Kulakova, A.N., Cooley, N.A., McGrath, J.W., 2007. New ways to break an old bond: The bacterial carbon-phosphorus hydrolases and their role in biogeochemical phosphorus cycling. Environmental Microbiology 9(10), 2392-2400.

Quinn, J.P., Peden, J.M.M., Dick, R.E., 1989. Carbon-phosphorus bond cleavage by Gram-positive and Gram-negative soil bacteria. Appl. Microbiol. Biotechnol. 31(3), 283-287.

Rinta-Kanto, J.M., Ouellette, A.J.A., Boyer, G.L., Twiss, M.R., Bridgeman, T.B., Wilhelm, S.W., 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. 39(11), 4198-4205.

Rinta-Kanto, J.M., Wilhelm, S.W., 2006. Diversity of microcystin-producing cyanobacteria in spatially isolated regions of Lake Erie. Appl. Environ. Microbiol. 72(7), 5083-5085.

Rodhe, W., Vollenweider, R.A., Nauwerck, A., 1958. The primary production and standing crop of phytoplankton, In: Buzzati-Traverso, A.A. (Ed.), Perspectives in Marine Biology. University of California Press, San Francisco, pp. 299-322.

Rounge, T., Rohrlack, T., Nederbragt, A., Kristensen, T., Jakobsen, K., 2009. A genome-wide analysis of nonribosomal peptide synthetase gene clusters and their peptides in a *Planktothrix rubescens* strain. BMC Genomics 10(1), 396.

Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual. Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scheffer, M., Rinaldi, S., Gragnani, A., Mur, L.R., vanNes, E.H., 1997. On the dominance of filamentous cyanobacteria in shallow, turbid lakes. Ecology 78(1), 272-282.

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. Proc. Natl. Acad. Sci. U. S. A. 105(32), 11254-11258.

Stachowski-Haberkorn, S., Becker, B., Marie, D., Haberkorn, H., Coroller, L., de la Broise, D., 2008. Impact of Roundup™ on the marine microbial community, as shown by an *in situ* microcosm experiment. Aquatic Toxicology 89(4), 232-241.

Steinrucken, H.C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic-acid 3-phosphate synthase. Biochem. Biophys. Res. Commun. 94(4), 1207-1212.

Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., Marvin, C., 2008. Occurrence of glyphosate in surface waters of southern Ontario. Bull. Environ. Contam. Toxicol. 80(4), 378-384.

Stucken, K., John, U., Cembella, A., Murillo, A., Soto-Liebe, K., Fuentes-Valdé, J., Friedel, M., Plominsky, A., Vásquez, M., Glöckner, G., 2010. The smallest known genomes of multicellular and toxic cyanobacteria: Comparison, minimal gene sets for linked traits and the evolutionary implications. PLoS ONE 5(2), e9235.

Tan, S., Evans, R., Singh, B., 2006. Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. Amino Acids 30(2), 195-204.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL-W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22), 4673-4680.

Wanner, B.L., Boline, J.A., 1990. Mapping and molecular-cloning of the *phn* (*psid*) locus for phosphonate utilization in *Escherichia coli*. J. Bacteriol. 172(3), 1186-1196.

Welschmeyer, N.A., 1994. Fluorometric analysis of chlorophyll-*a* in the presence of chlorophyll-*b* and pheopigments. Limnol. Oceanogr. 39(8), 1985-1992.

Wilhelm, S.W., Maxwell, D.P., Trick, C.G., 1996. Growth, iron requirements, and siderophore production in iron-limited *Synechococcus* PCC 7002. Limnol. Oceanogr. 41, 89-97.

SECTION IV

SEASONAL SI:C RATIOS IN LAKE ERIE DIATOMS – EVIDENCE OF WINTER SILICIFICATION

This section is a version of an article being prepared for submission to Journal of Great Lake Research under the same title by Matthew A. Saxton, Nigel A. D'Souza, R. Michael L. McKay and Steven W. Wilhelm

My contribution to this study included the collection of some environmental samples and the optimization and implementation of the Si deposition assay for the Lake Erie system. I also performed most of the data analysis, and most of the background information gathering and writing of this section.

Abstract

Recent investigations of Lake Erie in the winter have demonstrated the occurence of significant phytoplankton blooms largely consisting of the diatom *Aulacoseira islandica* (O. Müller) Simonsen. The stoichiometric relationship between carbon and silica of this productive winter Lake Erie phytoplankton assemblage was examined and compared to the activity of the summer community. We measured O_2 evolution as proxy for C fixation and PDMPO fluorescence as a measure of Si deposition, the latter demonstrating conclusively that diatoms were active during winter months. Although the winter community was observed to be active, it was less active than the summer with lower measured rates of O_2 evolution and Si deposition. Our results confirm that diatoms are the primary drivers of winter productivity. These findings impact the current understanding of hypoxia in Lake Erie, providing insight into the influence of the winter diatoms on hypoxia formation.

Introduction

Diatoms are well described as significant drivers of C and Si cycling in freshwater and marine systems (Round et al., 1990). These phytoplankters are particularly noted for higher sinking rates than other phytoplankton as a result of their high-density Si frustules (Gibson, 1984; Smetacek, 1985). The role of diatoms in the export of carbon to the deep benthos has been well studied in marine systems (Billett et al., 1983; Smetacek, 2000), where interest in stimulating diatom blooms as a potential way to sequester excess atmospheric CO_2 in the deep ocean has been explored (Boyd et al., 2000). Their function in carbon export has also made diatoms of interest in areas experiencing hypoxia, such as the Gulf of Mexico and Lake Erie (Diaz and Rosenberg, 2008; Hawley et al., 2006; Rabalais et al., 2002). In these systems exported carbon is not sequestered in the benthos, but rather is consumed, along with O_2, by secondary producers resulting in low oxygen conditions.

Hypoxia in the central basin of Lake Erie is a well-described and ongoing problem (Rosa and Burns, 1987). The scale of hypoxia in Lake Erie has become quite large in recent years, with 10,000 km^2 region, nearly the entire central basin, becoming hypoxic in 2005 (Hawley et al., 2006). Although this phenomenon has long been a focus of the Great Lakes research community, many of the complex biological, chemical, and physical factors that contribute to hypoxia are not well understood. In a recent study, Lashaway and Carrick (2010) illustrated the importance of sedimentary algae to hypoxia development, showing that as much as 30% of oxygen usage in the sediments is the result of the oxidation of algal biomass. This study also noted the prevalence of diatom biomass in the sediments.

In Lake Erie, diatoms have historically been thought to be primary members of the spring phytoplankton community (Barbiero and Tuchman, 2001) though reported biomass estimates are modest, especially when compared to the scale of hypoxia in this system.

Large-scale winter diatom blooms are known to take place in Lake Baikal (Straškrábová et al., 2005) and intermittent investigations over the last half-century (Chandler, 1940; Holland, 1993) have suggested the occurrence of similar winter diatom blooms in Lake Erie.

Recent investigations have uncovered abundant phytoplankton across Lake Erie during the winter (Twiss et al., 2010). Remarkably high biomass was measured with chl*a* concentrations at some sites higher than those recorded in the summer. This assemblage was particularly noted for high diatom abundances, especially the centric diatom *Aulacoseira islandica* (O. Müller) Simonsen. *Aulacoseira* has also been identified as one of the primary members of the spring phytoplankton assemblage and this diatom has been observed in high abundances in benthos collected in the spring (Carrick et al., 2005). Substantial quantities of *Stephanodiscus* spp., *Asterionella* sp. and *Cyclotella* sp. were also present in the winter assemblage. In addition to diatoms, notable communities of *Synechococcus* spp. and picoeukaryotes such as *Chlorella* spp. were observed.

Of particular interest were high concentration diatom communities that appeared to be associated with the ice itself, described in detail by Twiss et al (2010). These communities may be associated with the underside of the ice, as has been illustrated in filamentous diatoms present on Arctic ice (Gutt, 1995), but this has yet to be confirmed in Lake Erie. It is not known how these ice-associated communities form or disperse or the time scale over which these events occur.

In this study, we investigated the hypothesis that the winter diatom community is active and thus contributes in a meaningful way to the accumulation of carbon in the benthos and hypoxia in the summer. At the onset of the study, our null hypothesis was that the diatom community observed in the winter is not active but rather the vestige of the late season diatom community surviving until conditions improve in the spring. To test this hypothesis, we needed to establish that the highly abundant diatoms were active. To specifically assess diatom activity in ice-covered Lake Erie, we used the fluorescent dye, PDMPO, which is co-deposited

with Si into newly synthesized diatom frustules (Leblanc and Hutchins, 2005). This technique allows for the diatom-specific analysis of primary production and for an estimation of the contribution of diatom biomass to the sedimentary carbon produced in the winter bloom. Here, we pair this measure of Si deposition with O_2 evolution as a measure of primary production to investigate the productivity of the winter bloom community and compare community Si:C ratios with those observed during an early summer sampling.

Material and Methods

Sample Collection

Winter samples were collected in February 2010 aboard the *CCGS* Griffon using a 153 µm mesh Wisconsin type plankton net. Summer samples were collected aboard the *CCGS* Limnos in June and July 2010 using a 20-µm mesh plankton net. Following collection, concentrated seston samples were maintained at 4° C in the dark until use. Stations are plotted in Figure 1. Silica deposition and O_2 evolution experiments were performed using the same plankton net collected seston.

Silica Deposition Assays

Si deposition experiments were performed using a modified method originally described by LeBlanc and Hutchins (2005) utilizing 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl)methoxy)-phenyl)oxazole (PDMPO), a fluorescent dye that is co-deposited with Si into newly synthesized diatom frustules (Shimizu et al., 2001). Incubations were performed in 250 mL polycarbonate bottles (Nalgene), and 5 mL of concentrated seston was added to 125 mL of 0.2µm filtered water from the station from which the seston was collected. PDMPO was then added to a final concentration of 0.125 µM. Following incubation, samples were dispersed for PDMPO quantification and microscopic taxonomic identification. All incubations were performed in triplicate.

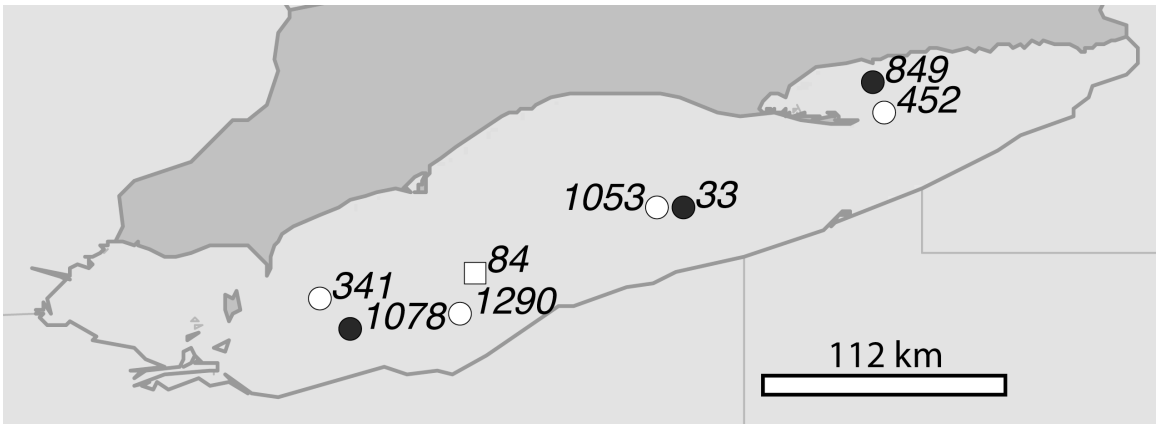


Figure 4.1. Map of sampling sites in occupied in this study. Sampling sites are noted with Environment Canada station number and indicated as sites February (°) or June/July (•). Station 84 was sampled in both seasons and is denoted as □.

Microscopy samples were filtered onto 0.1-µm nominal pore-size 25-mm diameter black polycarbonate filters (Millipore) then immediately frozen and stored at -80°C until analysis. Slides were viewed on a Leica DMXRA epifluorescent microscope with a Hammumatsu ORCA-ER camera using the Texas Red filter (λ_{ex} 520-600 nm; λ_{em} 570-720 nm) to visualize chlorophyll autofluorescence and a long-pass DAPI filter (λ_{ex} 340-380 nm; λ_{em} >425 nm) to visualize PDMPO fluorescence. No fewer than 200 cells were scored for PDMPO fluorescence on each slide. The percentage of active Si depositing diatom filaments/colonies and individual cells were determined, and taxonomic identifications were made. The camera was controlled and micrographs were taken using Simple PCI software (Hammumatsu). Following incubation, samples for the determination of Si deposition rate by PDMPO quantification were collected onto 47mm diameter 0.22-µm nominal pore-size polycarbonate filters. Following filtration, filters were washed with 0.2-µm filtered lake water to remove exogenous PDMPO. Next, a 2 min incubation with 10% HCl was followed by a 2 min incubation with MilliQ water (resistivity ≥ 18 MΩ-cm) to osmotically lyse the diatom cells releasing any PDMPO remaining in vacuoles. After a final rinse with filtered lake water, samples were flash frozen for transport. Next frustules were dissolved via a one hour incubation in 4 mL 0.2 M NaOH in the dark and then neutralized with 1 mL 1N HCl. PDMPO in this solution was quantified on a TD-700 laboratory flourometer (λ_{ex} 360-380nm; λ_{em} 522-542nm) (Andover). Standard Curves (r^2=0.99988) were constructed using dye diluted with NaOH-HCL frustule dissolution matrix. PDMPO concentration was converted to Si using the conversion factor 3230:1::Si:PDMPO (mol:mol) (Leblanc and Hutchins, 2005). Si deposition rates were normalized to chl*a.* Si deposition L^{-1} was calculating using extracted chl*a* filtered through 20 µM polycarbonate filter from 1m at the same station.

Measurement of Primary Productivity by Oxygen Evolution

O_2 evolution was measured using a Dissolved Oxygen electrode as a proxy for carbon-fixation. Concentrated seston was placed into a temperature controlled cuvette and exposed to a known light intensity during which the oxygen evolved in the chamber was to be measured by a Clark-type Dissolved Oxygen electrode (Qubit Systems) at the bottom of the cuvette. Seston was maintained in the dark for no less than 30 minutes and was sparged with N_2 gas to remove any dissolved O_2 in the sample immediately prior to experimentation. The material was exposed to light intensities ranging from 5 to 400 μmol photons m^{-2} s^{-1}. O_2 evolution rates were normalized to chl*a*. O_2 evolution rates were converted to C fixation assuming a photosynthetic quotient (PQ) of 1 (Reynolds, 2006; Williams, 1998). Though we realize the theoretical PQ of 1 is unlikely in this system, we have decided to use it in this study because of the wide range of PQs (0.3 - 1.3) measured in Lake Erie (Ostrom et al., 2005) and the lack of a published PQ for the winter Lake Erie assemblage.

Chlorophyll a estimation

Extracted chl*a* was determined by filtration onto 0.2-μM pore-size 47 mm diameter polycarbonate filters (Millipore) after extraction (24 h, 4°C) in 90% acetone. Extracted chl*a* was measured using a Turner Designs 10AU flourometer following the non-acidification protocol (Welschmeyer, 1994).

Statistical Analysis

Two-way repeated measures ANOVA analysis was used to determine significant differences between rates measured in the summer and winter. A 95% confidence interval was used. This analysis was performed for both O_2 evolution and Si deposition rates.

Results

Si-deposition microscopy

Lake Erie diatoms actively depositing Si were visualized microscopically by noting PDMPO fluorescence. Diatoms depositing PDMPO were visualized in incubations from all tested sites in both seasons (Fig. 2). In agreement with previous descriptions of the winter community, most of the diatoms observed to deposit Si were *Aulacoseira islandica* (Fig 2 A, C). PDMPO fluorescence was observed in no less than 90% of filaments or colonies (Table 1) and in between 25 and 10% of cells (Table 1) in the winter samples. Diatom assemblages in summer samples were dominated by *Fragilaria* spp. (Fig. 2B) and *Asterionella* spp. (Fig. 2D). 79% of total diatom cells exhibited PDMPO fluorescence, including 80% of *Fragilaria* spp., 65% of *Asterionella* spp., and 100% of *Stephanodiscus* spp. Over 90% of colonies observed in the summer samples exhibited PDMPO fluorescence.

O_2 Evolution and Si deposition rates

The rates of primary production, resolved as O_2 evolution, and Si deposition measured *via* PDMPO fluorescence were determined (Table 2). Measured rates of O_2 evolution were strongly significantly higher in the summer than in the winter ($p=0.019$). Si deposition rates were also higher in the summer than in the winter ($p=0.082$). Measured O_2 evolution rates ranged from 3.753 to 19.36 μmol O_2 μg chl*a* $^{-1}$ day $^{-1}$ in the February samples, and from 31.18 to 112.64 μmol O_2 μg chl*a* $^{-1}$ day $^{-1}$ in the summer. Si deposition rates were measured between 0.167 and 0.181 μmol Si μg chl *a* $^{-1}$ d $^{-1}$ in the summer and 0.208 and 0.342 Si μg chl *a* $^{-1}$ d $^{-1}$ in the winter. For incubations performed at summer stations 33 and 84, the PDMPO did not accumulate beyond background fluorescence, likely because of low diatom biomass in those samples. Low diatom densities observed microscopically confirm this assertion (data not shown).

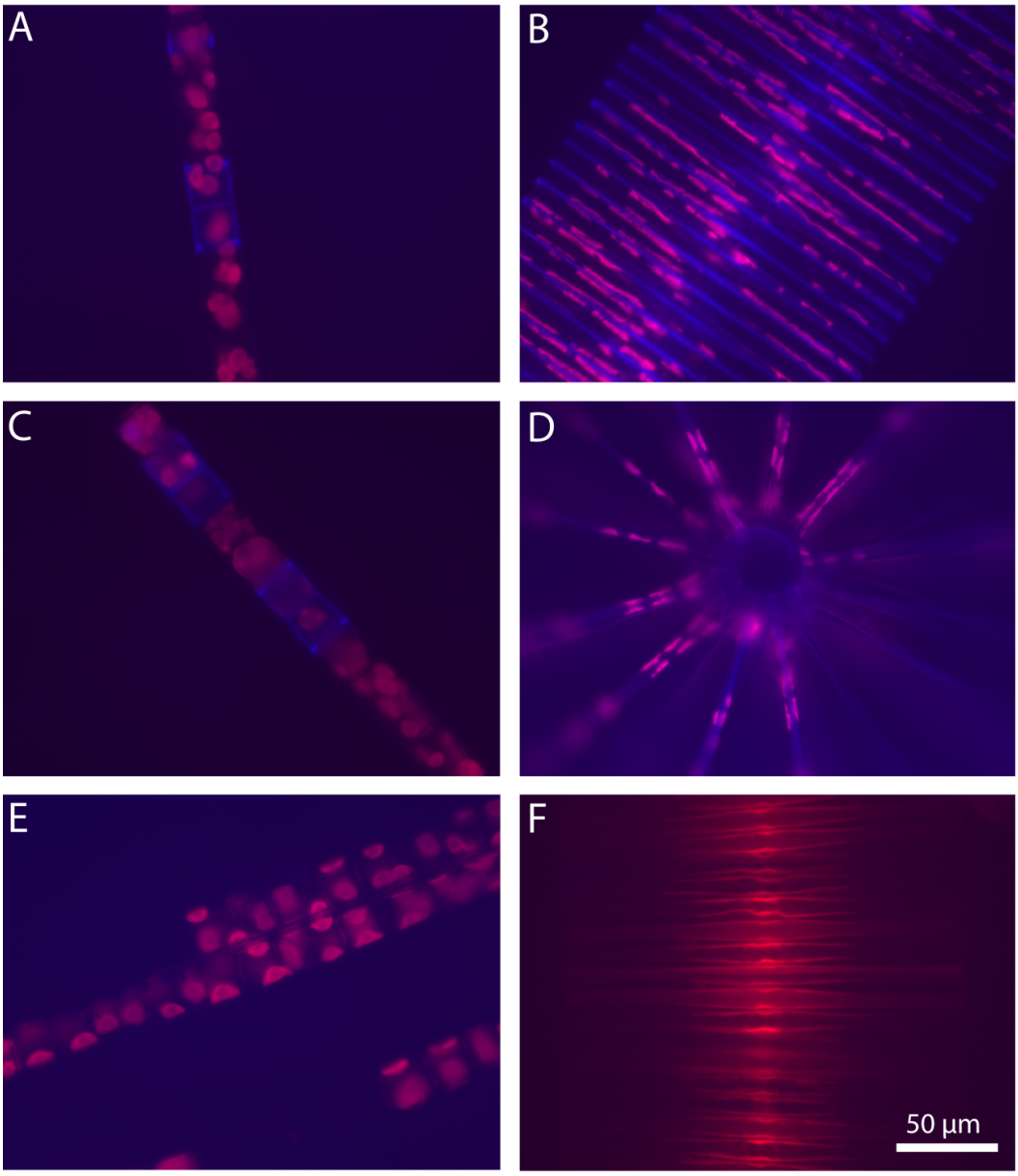


Figure 4.2. False color micrographs showing PDMPO deposition in winter and spring diatom communities. PDMPO flourescence is blue and chlorophyll autoflourescese is shown red. Samples A, C and E were collected in February 2010. Samples A and E were collected at station 84, sample C was collected at station 452. Samples B, D and F were collected in July at station 1053. Samples E and F are 2% glutaraldehyde killed controls.

Table 4.1. Percentage of diatom cells and colonies exhibiting PDMPO fluorescence. Values are presented as the mean ± SD. * indicates fewer than 200 cells were present and counted.

Station	% Fluorescent Cells	% Fluorescent Colonies
Winter		
452	17.5±2.2	91.8±4.8
84	10.4±0.5	90.6±2.3
1326	13.9±1.3	93.5±4.6
cache	25.6±4.5	96.2±0.2
Summer		
849	100*	100*
33	100*	100*
84	100*	100*
1078	78.5±6.6	96.9±5.2

Table 4.2. Oxygen evolution and Si deposition rates, as well as a ratio of Si:C fixation measured from Lake Erie seston collected in winter or summer 2010. Si deposition L^{-1} was calculating using extracted chl*a* filtered through 20 µM polycarbonate filter from 1m at the same station.

Station	µmol O_2 µg chl*a* $^{-1}$ day $^{-1}$	µmol Si µg chl*a* $^{-1}$ day $^{-1}$	µmol Si µg chl*a* $^{-1}$ L $^{-1}$	Si:C fixation mmol:mol
Winter				
452	3.8±0.1	0.18±0.1	0.13	48.2
84	19.4±1.9	0.14±0.1	0.24	7
1290	4.9+0.8	0.17	0.05	34
1053	17.3±7.2	N/A	N/A	N/A
341	9.7±0.006	N/A	N/A	N/A
Summer				
849	71.5±7.9	0.21±0.1	0.05	3
33	112.6±2.2	BDL	N/A	BDL
84	31.2±2.4	BDL	N/A	BDL
1078	108.9±8.0	0.34±0.1	0.04	3

Similarly, a Si deposition rate was not measured in the incubation performed using a concentrated ice-associated algal community. This result is likely the result of background fluorescence related to the sediment associated with these ice-associated diatoms.

Discussion

High percentages of diatom filaments/colonies showing PDMPO fluorescence observed in winter and summer samples are indicative of actively growing populations in both seasons. A high percentage of diatom colonies depositing Si is expected in an actively growing community because those diatoms that are not growing should sink from the surface layer to be deposited in the benthos; indeed, higher sinking rates have been observed during stationary growth of diatom cultures (Titman and Kilham, 1976). The influence of a highly productive community of phytoplankton that readily sinks upon cessation of growth on seasonal hypoxia is clear. These blooms are likely a major source of the diatom biomass observed in Lake Erie sediments.

Significantly higher primary productivity rates in the summer as compared to the winter were not surprising as this finding agrees with observations from Lake Baikal (Yoshida et al., 2003), a site in which seasonal *Aulacoseira* spp. blooms occur in the winter. Si deposition rates measured in Lake Erie were also higher in the summer than in the winter. This was a surprising result considering the relative concentration of diatoms in the winter is much higher in the summer. A likely reason for this result is due to differences in the diatom communities supported in the different seasons. The centric diatoms present in the winter grow in connected filaments maintaining established biomass at the surface for a longer period of time compared to pennate diatoms.

Our results illustrate this assertion, with a lower percentage of fluorescent cells in the winter (10-25%) than in the summer (78-100%) when pennate diatoms dominate. This extra diatom biomass held at the surface in the winter is still measured in chl*a* biomass estimates, thus skewing our results lower.

Although both the Si and C fixation rates were higher in the summer than winter, the calculated Si:C fixation ratio did not remain constant but rather was higher in the winter than the summer (Table 2). While this result is not surprising because of the high cellular Si:C ratio of *A. islandica* (Sicko-Goad et al., 1984), it has important implications to C export to the benthos and seasonal hypoxia formation. A consequence of this higher Si:C ratio is a diatom community disposed to higher sinking rates. Indeed, higher Si:C ratios have been connected to higher sinking rates in marine diatoms (Hutchins and Bruland, 1998). Pair this finding with others showing centric filamentous diatoms have the highest Si:C ratios (Sicko-Goad et al., 1984) and sinking rates (Gibson, 1984; Titman and Kilham, 1976) among freshwater diatoms and the winter Lake Erie assemblage, comprising primarily of these diatoms may be an efficient shuttle of C and Si to the benthos. Also contributing to the difference between Si:C fixation ratio differences in the summer and winter is the higher proportion of non-diatom primary producers in the summer samples.

The size and scope of the winter diatom bloom also appears to influence the composition of the summer assemblage through the sequestration of available silica, facilitating a shift from diatoms with high Si requirements in the winter and early spring to species with lower Si requirements in the summer. Twiss et al. (2010) described a drawdown in dissolved SiO_2 over the course of the winter, which is likely the result of high winter diatom activity. Indeed, Si limitation of diatom growth has been observed post-*Dreissena* in the central basin of Lake Erie (Moon and Carrick, 2007). Furthermore, *A. islandica* is noted for having a high Si requirement with a silica growth optima of 0.6 mg L^{-1} (Barbiero et al., 2006) and a Si:C ratio (mol:mol) >1 (Sicko-Goad et al., 1984); pennate diatoms common in the

summer, such as *Fragilaria* spp. and *Asterionella* spp., have lower Si optimums (Barbiero et al., 2006). It appears then that the winter bloom draws down Si concentrations, eventually causing Si limitation and resulting in a shift from centric to pennate diatoms. Though neither these results nor concurrent microcosm assays (data not shown) show evidence of Si limitation in either season, qualitative evidence of differing Si needs in the winter and summer assemblages was observed through higher per volume Si deposition rates (Table 2).
PDMPO fluorescence in the centric diatoms prevalent in the winter was bright and substantial (Fig. 2 A,C), while the fluorescence observed in the summer was noticeably less (Fig. 2 B,D).

With the exception of the high biomass ice associated communities, higher concentrations of diatom biomass have been in open water stations than in ice-covered sites (Twiss et al., 2010). This observation is critical considering the predicted temperature increases in current climate change models, because reduction in ice cover is predicted across the Great Lakes (Assel et al., 2003) as a result along with the rise in air temperature during the winter months. In this scenario, the reduction in ice cover would allow for increased diatom production, and potentially, for an earlier onset and more severe hypoxia in Lake Erie. A rough estimate of diatom production was calculated for an ice-free central basin. Using the previously measured *A. islandica* cellular Si:C ratio (1.54, mol:mol, Sicko-Goad et al., 1984), we estimate a diatom specific rate of 8,421 kg C solar day^{-1} in the surface 1 m of the maximum observed hypoxic area in the central basin (10,000 km^2). Clearly, not all diatom biomass represented in this estimation would export to the benthos. Nevertheless, this example illustrates the substantial impact winter diatom primary productivity can have in this system.

This study confirms the hypothesis that the diatom population observed in Lake Erie during winter is actively growing and provides insight into how it may shape spring events through the assimilation (drawdown) of Si. While this finding may seem evident based on the large concentration of phytoplankton biomass

observed in the winter, confirmation of this hypothesis is critical to the continuing studies describing how the activity of this diatom bloom affects phenomena observed in the spring and summer in the Lake Erie. Finally, these data contribute to the growing body of evidence concerning the potential for Lake Erie's winter diatom bloom to influence summer hypoxia and "*dead zone*" formation in Lake Erie.

Acknowledgements

We would like to thank Jan Ciborowski and Richard Bourbonniere for contributing ship time and Michael Twiss and George Bullerjahn for helpful discussion. We would like to thank the captains and crews of the *CCGS* Limnos *and CCGS* Griffon*.* We would also like to acknowledge the fanatical support of New York Sea Grant (to SWW), Ohio Sea Grant (to RMLM) and The University of Tennessee (M-CERV grant to SWW).

List of References

Assel, R., Cronk, K., Norton, D., 2003. Recent trends in Laurentian Great Lakes ice cover. Clim. Change 57(1), 185-204.

Barbiero, R.P., Rockwell, D.C., Warren, G.J., Tuchman, M.L., 2006. Changes in spring phytoplankton communities and nutrient dynamics in the eastern basin of Lake Erie since the invasion of *Dreissena* spp. Can. J. Fish. Aquat. Sci. 63(7), 1549-1563.

Barbiero, R.P., Tuchman, M.L., 2001. Results from the U.S. EPA's biological open water surveillance program of the Laurentian Great Lakes: I. Introduction and phytoplankton results. J. Gt. Lakes Res. 27(2), 134-154.

Billett, D.S.M., Lampitt, R.S., Rice, A.L., Mantoura, R.F.C., 1983. Seasonal sedimentation of phytoplankton to the deep sea benthos. Nature 302(5908), 520-522.

Boyd, P.W., Watson, A.J., Law, C.S., Abraham, E.R., Trull, T., Murdoch, R., Bakker, D.C.E., Bowie, A.R., Buesseler, K.O., Chang, H., Charette, M., Croot, P., Downing, K., Frew, R., Gall, M., Hadfield, M., Hall, J., Harvey, M., Jameson, G., LaRoche, J., Liddicoat, M., Ling, R., Maldonado, M.T., McKay, R.M., Nodder, S., Pickmere, S., Pridmore, R., Rintoul, S., Safi, K., Sutton, P., Strzepek, R., Tanneberger, K., Turner, S., Waite, A., Zeldis, J., 2000. A mesoscale phytoplankton bloom in the polar Southern Ocean stimulated by iron fertilization. Nature 407(6805), 695-702.

Carrick, H.J., Moon, J.B., Gaylord, B.F., 2005. Phytoplankton dynamics and hypoxia in Lake Erie: A hypothesis concerning benthic-pelagic coupling in the central basin. J. Gt. Lakes Res. 31, 111-124.

Chandler, D.C., 1940. Limnological studies of western Lake Erie: I. Plankton and certain physical-chemical data of the Bass Islands region, from September 1938, to November, 1939. Ohio J. Sci 40, 291-336.

Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science 321(5891), 926-929.

Gibson, C.E., 1984. Sinking rates of planktonic diatoms in an unstratified lake: a comparison of field and laboratory observations. Freshw. Biol. 14(6), 631-638.

Gutt, J., 1995. The occurrence of sub-ice algal aggregations off northeast Greenland. Polar Biol. 15(4), 247-252.

Hawley, N., Johengren, T.H., Rao, Y.R., Ruberg, S.A., Beletsky, D., Ludsin, S.A., Eadie, B.J., Schwab, D.J., Croley, T.E., Brandt, S.B., 2006. Lake Erie hypoxia prompts Canada-U.S. study. EOS 87(313-314).

Holland, R.E., 1993. Changes in planktonic diatoms and water transparency in Hatchery Bay, Bass Island area, western Lake Erie since the establishment of the zebra mussel. J. Gt. Lakes Res. 19(3), 617-624.

Hutchins, D.A., Bruland, K.W., 1998. Iron-limited diatom growth and Si:N uptake ratios in a coastal upwelling regime. Nature 393(6685), 561-564.

Lashaway, A.R., Carrick, H.J., 2010. Effects of light, temperature and habitat quality on meroplanktonic diatom rejuvenation in Lake Erie: implications for seasonal hypoxia. J. Plankton Res. 32(4), 479-490.

Leblanc, K., Hutchins, D.A., 2005. New applications of a biogenic silica deposition fluorophore in the study of oceanic diatoms. Limnology and Oceanography-Methods 3, 462-476.

Moon, J.B., Carrick, H.J., 2007. Seasonal variation of phytoplankton nutrient limitation in Lake Erie. Aquat. Microb. Ecol. 48(1), 61-71.

Ostrom, N.E., Carrick, H.J., Twiss, M.R., Piwinski, L., 2005. Evaluation of primary production in Lake Erie by multiple proxies. Oecologia 144(1), 115-124.

Rabalais, N.N., Turner, R.E., Wiseman, W.J., 2002. Gulf of Mexico hypoxia, aka "The dead zone". Annu. Rev. Ecol. Syst. 33, 235-263.

Reynolds, C.S., 2006. The ecology of phytoplankton. Cambridge University Press, Cambridge.

Rosa, F., Burns, N.M., 1987. Lake Erie central basin oxygen depletion changes from 1929-1980. J. Gt. Lakes Res. 13(4), 684-696.

Round, F.E., Crawford, R.M., Mann, D.G., 1990. The diatoms: biology & morphology of the genera. Cambridge University Press.

Shimizu, K., Del Amo, Y., Brzezinski, M.A., Stucky, G.D., Morse, D.E., 2001. A novel fluorescent silica tracer for biological silicification studies. Chemistry & Biology 8(11), 1051-1060.

Sicko-Goad, L.M., Schelske, C.L., Stoermer, E.F., 1984. Estimation of intracellular carbon and silica content of diatoms from natural assemblages using morphometric techniques. Limnol. Oceanogr. 29(6), 1170-1178.

Smetacek, V., 2000. The giant diatom dump. Nature 406(6796), 574-575.

Smetacek, V.S., 1985. Role of sinking in diatom life-history cycles: Ecological, evolutionary and geological significance. Marine Biology 84(3), 239-251.

Straškrábová, V., Izmest'yeva, L.R., Maksimova, E.A., Fietz, S., Nedoma, J., Borovec, J., Kobanova, G.I., Shchetinina, E.V., Pislegina, E.V., 2005. Primary production and microbial activity in the euphotic zone of Lake Baikal (Southern Basin) during late winter. Global and Planetary Change 46(1-4), 57-73.

Titman, D., Kilham, P., 1976. Sinking in freshwater phytoplankton: Some ecological implications of cell nutrient status and physical mixing processes. Limnol. Oceanogr. 21(3), 409-417.

Twiss, M.R., McKay, R.M.L., Bourbonniere, R.A., Bullerjahn, G.S., Carrick, H.J., Smith, R.E.H., Winter, J.G., D'Souza, N.A., Furey, P.C., Lashaway, A.R., Saxton, M.A., Wilhelm, S.W., 2010. Diatoms abound in ice-covered Lake Erie: An investigation of offshore winter limnology in Lake Erie over the period 2007 to 2010 J. Gt. Lakes Res. Submitted.

Welschmeyer, N.A., 1994. Fluorometric analysis of chlorophyll-*a* in the presence of chlorophyll-*b* and pheopigments. Limnol. Oceanogr. 39(8), 1985-1992.

Williams, P.J.L., 1998. The balance of plankton respiration and photosynthesis in the open oceans. Nature 394(6688), 55-57.

Yoshida, T., Sekino, T., Genkai-Kato, M., Logacheva, N.P., Bondarenko, N.A., Kawabata, Z., Khodzher, T.V., Melnik, N.G., Hino, S., Nozaki, K., Nishimura, Y., Nagata, T., Higashi, M., Nakanishi, M., 2003. Seasonal dynamics of primary production in the pelagic zone of southern Lake Baikal. Limnology 4(1), 53-62.

SECTION V

CONCLUSIONS

This body of work further illustrates how the interactions between microorganisms and nutrients influence the Lake Erie ecosystem. The international scientific community and the governments of the United States and Canada have identified harmful algal blooms and seasonal hypoxia as areas of ecological concern in Lake Erie (IJC, 1989) and have made research in these areas a priority. This priority is the result of the significant economic and ecological impacts these events have on Lake Erie. Hypoxia in the central basin of Lake Erie has detrimental effects on the success of the lake fishery through the pressure placed on small fish and benthic invertebrates (Rabalais et al., 2002; Wilhelm et al., 2006), and cyanobacterial bloom events foul beaches and produce potentially harmful toxins (Brittain et al., 2000; Carmichael, 2001). In this study we set out to understand how three specific phenomena influence these larger issues. First, we used a new understanding of how P is partitioned in phytoplankton (Sãnudo -Wilhelmy et al., 2004) cells to produce more representative P quotas in the bloom forming cyanobacterium *Microcystis*. Next, we examined the influence of agricultural herbicide use on the success of the potentially toxic cyanobacterial community in Lake Erie. Finally, we explored the activity of the Lake Erie winter diatom community, and its potential influence on seasonal hypoxia.

From these studies we have determined:

1. Surface bound P comprises a significant and stable proportion of the total cellular P pool in the cyanobacterium *Microcystis aeruginosa*, and that the cellular P quota of *Microcystis* is plastic. The metabolic plasticity we describe may contribute this cyanobacterium to survive in a range of environments until conditions improve and bloom formation can occur.

2. *Microcystis* cellular P quotas incorporated into currently harmful algal bloom modeling efforts overestimate the P quotas of this organism.

3. Glyphosate is able to influence phytoplankton community structure in Lake Erie, negatively through herbicidal effects, and positively as a nutrient source.
    a. *Planktothrix* is more resistant to glyphosate toxicity than *Microcystis.*
    b. Glyphosate and derived molecules can be used as sources of P and N by the microbial community.

4. Microorganisms capable of glyphosate metabolism are present in Lake Erie, and this community is largely made up of heterotrophic bacteria.

5. The winter Lake Erie diatom community is active and is a source of biogenic carbon to the benthos with a potentially significant impact on seasonal hypoxia.

These conclusions provide valuable insight into how microbes influence the flow of nutrients and, in turn, how nutrients affect plankton community structure in Lake Erie, but these results have also raised a new set of interesting questions. While we have shown that surface-absorbed P is a significant proportion of the total P pool in *Microcystis*, the fate of this P is not clear. Further study will be needed to determine if this P is accessible to the cell or if the presence of this P is solely a byproduct of the presence of dissolved P in the water.

We have shown glyphosate to be a potential nutrient source to microbes in the Lake Erie system, but the nature of glyphosate in this system is unknown. While we know that bacteria possessing C-P lyase are present in Lake Erie, it has not yet been determined if this enzyme is being actively used in the lake. It may be that another, as yet undetermined, glyphosate breakdown pathway is active in this system. We have also shown glyphosate community structure in Lake Erie, and have provided potential mechanisms for this control, but we can only speculate as to the mechanism in the environment. Future work should focus on detailing which nutrients glyphosate is providing in the lake, and the food chain through which these nutrients are passed.

Our study has shown the high abundances of *Aulacoseira islandica* found in Lake Erie in the winter to be active, but further characterization of this diatom is needed. Indeed, the specific details of how *Aulacoseira* is so successful under the ice in Lake Erie are unknown and detailed genomic and physiological studies are needed to characterize this organism.

Finally, while this study focused on specific details of how the interaction between plankton and nutrient leads to the formation, of hypoxia and algal blooms, it is critical to understand that these are part of a much larger problem. Given the overall importance of Lake Erie to the more than 11 million people who live near it, the successful rehabilitation of the lake-wide ecosystem is critical. With that it is our hope that this study is able to inform the decisions of lake managers in the United States and Canada and lead to the improved overall health of Lake Erie.

List of Refernces

Brittain, S.M., Wang, J., Babcock-Jackson, L., Carmichael, W.W., Rinehart, K.L., Culver, D.A., 2000. Isolation and characterization of microcystins, cyclic heptapeptide hepatotoxins from a Lake Erie strain of *Microcystis aeruginosa*. Journal of Great Lakes Research 26, 241-249.

Carmichael, W.W., 2001. Health Effects of Toxin-Producing Cyanobacteria: "The CyanoHABs". Human and Ecological Risk Assessment 7, 1393-1407.

IJC, 1989. International Joint Commission (IJC) United States and Canada 1989. Great Lakes Water Quality Agreement of 1978. http://www.ijc.org/agree/quality.html.

Rabalais, N.N., Turner, R.E., Wiseman, W.J., 2002. Gulf of Mexico hypoxia, aka "The dead zone". Annu. Rev. Ecol. Syst. 33, 235-263.

Sãnudo -Wilhelmy, S.A., Tovar-Sanchez, A., Fe, F.X., Capone, D.G., Carpenter, E.J., Hutchins, D.A., 2004. The impact of surface-absorded phosphorus on phytoplankton Redfield stoichiometry. Nature 432, 897-901.

Wilhelm, S.W., Bullerjahn, G.S., Eldridge, M.L., Rinta-Kanto, J.M., Poorvin, L., Bourbonniere, R.A., 2006. Seasonal hypoxia and the genetic diversity of prokaryote populations in the central basin hypolimnion of Lake Erie: evidence for abundant cyanobacteria and photosynthesis. Journal of Great Lakes Research 32, 657-671.

VITA

Matthew Saxton was born in Tiffin, Ohio on May 17th 1982. He graduated from Calvert High School in 2000. He received his Bachelor of Science degree with a major in Microbiology from Bowling Green State University in 2005. In August 2006, he entered the graduate program in Microbiology at the University of Tennessee, Knoxville in the lab of Dr. Steven Wilhelm. He completed the requirements for the Doctor of Philosophy degree in November 2010.