

December 2012

Freshwater Phytoplankton Populations Detected Using High Pressure Liquid Chromatography (HPLC) of Taxon-Specific Pigments

Lauren Jeanne Simmons
University of Wisconsin-Milwaukee

Follow this and additional works at: <http://dc.uwm.edu/etd>

 Part of the [Aquaculture and Fisheries Commons](#), [Biology Commons](#), and the [Fresh Water Studies Commons](#)

Recommended Citation

Simmons, Lauren Jeanne, "Freshwater Phytoplankton Populations Detected Using High Pressure Liquid Chromatography (HPLC) of Taxon-Specific Pigments" (2012). *Theses and Dissertations*. Paper 341.

This Thesis is brought to you for free and open access by UWM Digital Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of UWM Digital Commons. For more information, please contact kristinw@uwm.edu.

FRESHWATER PHYTOPLANKTON POPULATIONS DETECTED USING HIGH
PRESSURE LIQUID CHROMATOGRAPHY (HPLC) OF
TAXON-SPECIFIC PIGMENTS

by

Lauren Simmons

A Thesis Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Master of Science
in Biological Sciences

at

The University of Wisconsin-Milwaukee

December 2012

ABSTRACT
FRESHWATER PHYTOPLANKTON POPULATIONS DETECTED USING HIGH
PRESSURE LIQUID CHROMATOGRAPHY (HPLC) OF
TAXON-SPECIFIC PIGMENTS

by

Lauren Simmons

The University of Wisconsin-Milwaukee, 2012
Under the Supervision of Professors Craig D. Sandgren and John A. Berges

Phytoplankton are key primary producers in aquatic ecosystems, and the principle food source for primary consumers. Individual phytoplankton species respond to different physical, chemical and biological parameters, so monitoring taxonomic composition of the phytoplankton community is a means to monitor changes in environmental conditions. Phytoplankton community changes have frequently been monitored by estimating biomass (using chlorophyll *a*, measured fluorometrically), and taxonomic data obtained from cell counts. While such methods are useful, they are time-consuming. I hypothesized that high pressure liquid chromatography (HPLC) methods, which have been frequently used in marine systems, would allow separation and identification of key pigments. These pigments could then be used to allocate chlorophyll *a* to particular taxa using an optimization algorithm (CHEMTAX), providing simultaneous biomass estimates and taxonomic information.

The present study tested the applicability of HPLC/CHEMTAX methods in Lake Michigan phytoplankton communities. During Summer 2008, six synoptic cruises were

conducted aboard the *R/V Neeskay*, sampling communities at nearshore and offshore sites and carrying out grazing and nutrient enrichment experiments. HPLC/CHEMTAX methods were compared with conventional methods over the seasonal cycle from June to August. Estimates of chlorophyll *a* biomass measured with the HPLC method agreed well with conventional fluorometric measurements of extracted pigments. Chlorophyll *a* reached maxima (averaging $5.0 \mu\text{g L}^{-1}$ nearshore and $1.5 \mu\text{g L}^{-1}$ offshore) in late June/early July, with the exception of the offshore metalimnion where chlorophyll *a* peaked ($3.0 \mu\text{g L}^{-1}$) in late July. Nutrient enrichment experiments demonstrated that the algal communities were phosphorus-limited, while grazing experiments showed declines in phytoplankton biomass with increasing grazer abundance. Taxonomic groups were consistently misidentified by HPLC/CHEMTAX, relative to microscope methods; confusion between diatoms and chrysophytes was particularly serious. Specific improvement to the HPLC method that might help overcome the problems include faster sample processing techniques to prevent pigment degradation, eluting fewer critical pigments to improve resolution, and use of flow cytometric measurements in parallel. Although the HPLC method is faster and more efficient, it seems unlikely that microscopy to verify the specific taxa within the phytoplankton community can be avoided.

Dedicated to my husband and daughter who have supported and believed in me through this journey; my parents to whom I am forever grateful, and all of those who have been there for me through it all. I would not have been able to do this without you.

What we do for ourselves dies with us.
What we do for others is, and remains, immortal.
-Albert Pine

Scientific translation: If it's not published, you didn't do it!
-Craig "Doc" Sandgren

TABLE OF CONTENTS

Introduction	1
Methodology	7
Results	19
Discussion	38
References	49
Appendices	56

LIST OF FIGURES

Figure 1. Schematic of nutrient and herbivore grazing experimental design used during the 2008 field season.....12

Figure 2. Relationship of HPLC-detected and fluorometrically-determined pigments in three regions of Lake Michigan, June-August, 200820

Figure 3. Seasonal comparison of the phytoplankton community at Linnwood station (43° 04.58' N, 87° 50.29' W), Lake Michigan26

Figure 4. Seasonal comparison of the phytoplankton community at Fox Point, epilimnetic station (43° 11.67' N, 87° 47.26' W), Lake Michigan28

Figure 5. Seasonal comparison of the phytoplankton community at Fox Point, metalimnetic station (43° 11.67' N, 87° 47.26' W), Lake Michigan30

Figure 6. Comparison of cell count and HPLC/CHEMTAX distributions of taxa from Lake Michigan field samples collected June-August, 200832

Figure 7. Example of herbivore gradient experimental data from Fox Point, June 2536

Figure 8. Example of nutrient enrichment data from Fox Point, July 737

LIST OF TABLES

Table 1. Representative algal taxa and genera used for calculation of pigment extinction coefficients	8
Table 2. CHEMTAX input pigment ratios	18
Table 3. Summary of statistical analyses of changes in chl <i>a</i> biomass and HPLC/CHEMTAX taxon estimates during Lake Michigan herbivore grazing experiments in 2008	34
Table 4. Summary of statistical analyses of changes in chl <i>a</i> biomass and HPLC/CHEMTAX taxon estimates during Lake Michigan nutrient enrichment experiments in 2008	35

ACKNOWLEDGEMENTS

I want to begin by thanking both of my advisors, Craig “Doc” Sandgren and John Berges, for granting me the opportunity to study all things water (and in Lake Michigan no less!) and helping me to grow as a scientist. I also want to thank Doc for sharing his love of algae, and introducing me to the Friday Harbor Labs. In addition to my work out on Lake Michigan, some of my fondest memories from my time in the Sandgren/Berges Lab were in Friday Harbor. I also want to thank John for stepping in following Doc’s death to be my advisor. John made the transition very easy, and has been nothing short of amazing. You have both provided me with an excellent foundation to continue building my love of algae from.

I would like to thank my graduate committee of Drs. Craig “Doc” Sandgren, John Berges, Erica Young and Art Brooks for all of their guidance and support throughout the entire process while working on my thesis. What we have accomplished in the last year in regards to this project and the program is unreal, and I do not think that I could not have accomplished this without your help. Each of you found time to provide feedback, support and help me get my thesis completed quickly, and in the right way. It has been a crazy few years for all of us, but I am glad that we made it through this together. Thank you all!

I also want to thank my labmates (past and present), Carter Ingwell, Paul Engevoid and Chrissy Kozik. We are kind of like our own family in 574, and you are true friends. Thank you for being there to vent about anything and everything, talk sports, and anything else under the sun. Thank you Paul for always knowing where everything is, and if you don’t, you look until you find it. You have provided so much knowledge and

support throughout this process. I wish you the best as you too complete your thesis work and prepare for the future. Thank you Chrissy for being a great friend. It's been nice to have another woman in the lab (No offense guys!). Also, thank you helping out with Sydney when she tries to help us out. You have been amazing. Carter, thank you for keeping me sane, even if from the halfway across the country.

In closing, I would like to thank the Dr. David Millie who has lent his expertise in all things CHEMTAX and HPLC. His help has allowed me to make sense of the nuances of the methods used for the work contained within. Also, thank you to the Biological Sciences staff. Everyone has been so helpful and accommodating throughout my years here, especially Phyllis, Kim and Cynthia who have all the answers when you need them. You have all been wonderful!

INTRODUCTION

Background/Literature Review

Phytoplankton communities have been monitored for over 200 years in a variety of ways. These communities are dynamic, responding to environmental conditions (light and nutrient availability, grazing pressure) resulting in shifts in species composition and cell size/shape distribution (Berquist & Carpenter 1986, Carpenter & Kitchell 1993). Understanding changes within the phytoplankton community is important as these organisms play an integral part in global carbon cycles (Jeffrey et al. 1997, Li et al. 2002) and as primary producers, are a food source for aquatic grazers and key contributors to the oxygen cycle (Reynolds 2006).

Biomass estimated as chlorophyll *a* (chl *a*), is a common method of monitoring algal communities. All algae contain this pigment, although the presence of chl *a* biomass does not provide resolution of individual taxa of algae present within a community (Kruskopf & Flynn 2005, Falkowski & Raven 2007). To identify the taxa comprising algal communities, chl *a* samples are often paired with preserved samples to be enumerated using time-consuming microscopic methods (Li et al. 2002, Havskum et al. 2004). Even with modified cell counting techniques to correct for non-random settling of cells (Sandgren & Robinson 1984), accuracy is largely dependent upon the skills of the observer. Recently, more molecular-based methods have been introduced using 18s rRNA genes to determine the groups present within the community (Countway et al. 2005). This method has the ability to increase the resolution of community studies as it can be used to identify cryptic species (Beszteri et al. 2005), though the method is

currently limited by the number of available reference sequences in databases for taxa found in “environmental” samples (Countway et al. 2005).

An alternative strategy for whole community monitoring is to use pigment analysis, which can most accurately be carried out using high pressure liquid chromatography (HPLC). HPLC methods have been commonly used to resolve phytoplankton pigments present in marine systems, identifying representative algal taxa to accompany satellite-based chlorophyll data (reviewed by Jeffrey et al. 1997). HPLC analyses can be completed in much less time than microscopic cell counting, while providing an accurate quantification of the pigments present (Pinckney et al. 1996). In addition to chl *a*, HPLC can detect taxon-specific accessory pigments (e.g. additional chlorophylls, carotenoids, xanthophylls) found within the chloroplast that can be used to identify the algal taxa present in a sample, including some that may be difficult to identify with microscopy (Jeffrey et al 1999). HPLC methods also allow for the detection of pigment degradation products (pyropheophorbide *a*, phaeophorbide *a*) that can be used as indicators of grazing pressure (Jeffrey et al. 1997, Jeffrey et al. 1999). HPLC techniques have been applied in diverse analyses of seasonal and spatial shifts in phytoplankton community composition (e.g. Li et al. 2002, Descy et al. 2005, 2009), but less commonly in analyzing responses of phytoplankton community composition to nutrient and herbivore manipulations (Meyer-Harms and von Bodungen 1997, Pinckney et al. 2001, Thys et al. 2003, De Wever et al. 2008).

HPLC can be used to identify which algal taxa are in an environment (Wilhelm et al. 1995), providing greater resolution of algal biomass by taxon-specific accessory pigments. Although the HPLC method does lack specificity (i.e. some pigments are

shared among groups, Gieskes et al. 1988), the taxon-specific pigments are better indicators of biomass than chl *a* detected with conventional extractive fluorometric assays for chl *a*. In a 1998 study, Schmid and colleagues found that the dominating class in natural samples determined with HPLC by taxon-specific pigments had a high correlation ($r = 0.96$) with biovolume estimates made using the Utermöhl microscopy for cells present (Schmid et al. 1998). Along with the taxon-specific pigments, chl *a* is also detected, and therefore potentially allows continuation of the historical records of conventional biomass estimates. It is important to note however, that HPLC methods are more reproducible compared to those of cell counts, even though HPLC methods can lead to errors in estimating the groups present (Pinckney et al. 2001, Havskum et al. 2004).

HPLC methods have been used to monitor taxon-specific pigments in the Great Lakes. Millie et al. (2002) used HPLC to study pigment changes during early spring water column mixing in the late 1990s in Lake Michigan and found that the community was dominated by diatoms and cryptophytes, with little variation in diagnostic pigment concentrations at various locations. This suggested that cells were relatively unaffected by changing light and nutrient conditions. In contrast, total chl *a* had varied among locations; the differences were ascribed to varying turbidity levels due to mixing events (Millie et al. 2002).

In order to test the usefulness of HPLC pigment analyses in community studies, environments where relative abundance of the taxa present vary widely are needed. We can exploit seasonal changes in nutrient availability since we know that nitrogen, phosphorus and silica are essential macronutrients required to support the growth of phytoplankton, as they are necessary components of organelles and cell coverings

(Sterner and Elser 2002, Reynolds 2006). Limitation of these nutrients can occur throughout the summer as nutrients that were resuspended during spring turnover are used up or settle below the metalimnion. In most temperate lakes, phosphorus is limiting during the summer (Schindler 1974, Kalff and Knoechel 1978), with another nutrient possibly becoming limiting as seasonal succession of the phytoplankton occurs. Nutrient bioassays are done to determine which nutrient is limiting phytoplankton growth, with algal growth responses determined as net chl *a* biomass (Fairchild et al. 1985, Lehman & Sandgren 1985, and others). The nutrient that causes the largest increase in biomass is then determined to be limiting. The nutrient dynamics can influence the community, altering the size structure and phytoplankton abundance (Vanni 1987) although they can not explain all of the variation within the phytoplankton community as it is controlled only in part by nutrient availability (Tilman et al. 1982, Lehman and Sandgren 1985, Carpenter et al. 1995).

In addition to nutrient availability, herbivore grazing also affects the community both directly via selective grazing (Berquist et al. 1985, Lehman and Sandgren 1985) and indirectly via nutrient regeneration (Sterner 1986). Selective grazing, where zooplankton preferentially feed upon select particle sizes/shapes (Brooks & Dodson 1965), can affect algal succession and community composition (Sarnelle 1993), as well as net phytoplankton abundance (Vanni 1987). Grazing can decrease the abundance of some phytoplankton – unicellular forms and cryptophytes – while increasing the abundance of others via nutrient regeneration and recycling (Lehman & Sandgren 1985, Carpenter et al. 1995), which can fulfill the nutrient requirements of some algal assemblages (Lehman 1980), particularly in areas with low phytoplankton production (Suthers and Rissick

2009). The nutrients regenerated from grazing come from zooplankton waste excretion (ammonia, dissolved phosphorus) or from sloppy feeding habits (Lehman 1980), with egested silica, sinking out of the water column (Lampert and Sommer 2009). This regeneration may be the most important source of nutrients for phytoplankton during the summer (Lampert and Sommer 2009), potentially offsetting zooplankton interactions with phytoplankton (i.e. grazing of phytoplankton by herbivores; Tilman et al. 1982, Sterner 1990).

To study such interactions between phytoplankton and zooplankton, herbivore grazing experiments can be used. Herbivore abundance gradients have been used to look at the effects of natural zooplankton assemblages on phytoplankton growth (reproduction), and to determine preferred prey taxa (e.g. Lehman and Sandgren 1985, Carrick et al. 1991). Using varying concentrations of collected herbivores, both studies found that smaller algae were preferentially grazed over larger species. Dilution experiments, set-up by incubating samples with varying proportions of whole lake water (including grazers) and screened water (excluding grazers), have also been used to study phytoplankton/zooplankton interactions. For example, Dobberfuhl et al. (1997) showed that decreasing the abundance of the copepod *Diacyclops thomasi* decreased ciliate abundance and consequently increased chl *a* and phytoplankton and bacterial growth. Thus the copepod regulated the phytoplankton community by controlling the abundance of its ciliate predators.

Overview and Objectives

The overall objective of this thesis was to assess how accurately data obtained using HPLC analysis of algal pigments can estimate biomass and taxonomic composition

of phytoplankton communities in Lake Michigan. My central hypothesis was that the HPLC/CHEMTAX method developed would allow measurement of changes in the relative abundance of individual taxa in field samples over time scales ranging from a few days (in incubation experiments) to few weeks (for seasonal samples). By validating HPLC/CHEMTAX use in freshwaters, I hoped to provide a better tool that could rapidly provide information about lake phytoplankton communities.

In order to test my hypothesis I developed three specific goals:

- 1) To correlate HPLC-determined chl *a* with estimates based on chl *a* detected by a fluorometer (“conventional extractive fluorometric assays for chl *a*- based” method) to validate HPLC as a general indicator of biomass.
- 2) To compare the taxonomic composition estimated using the HPLC/CHEMTAX software with that based on cell counts and volume estimates in seasonal samples from nearshore and offshore Lake Michigan sites to determine whether HPLC can provide valid taxonomic data.
- 3) To use the HPLC/CHEMTAX method to infer changes in the relative abundance of algal taxa in response to manipulations of nutrients and grazing pressures in incubation experiments in order to assess practicality and ease of use of these methods in practice.

METHODOLOGY

HPLC Method Development

The HPLC method to detect taxon-specific pigments in Lake Michigan was adapted from Pinckney et al. (1996) (Appendix A), using a Shimadzu Prominence series HPLC system, outfitted with a Shimadzu Premier C-18 column and a Vydac 201TP C-18 column, and a Shimadzu diode array detector (Model SPD-M20A) set to produce an output with detection at 435 nm and 440 nm. An 82-minute binary gradient was selected (A: 80:20 methanol:ammonium acetate 0.5M, B: 80:20 methanol:acetone) with a 1.25 mL min⁻¹ flow rate and 500 µL injection volume. The column oven temperature was kept at 40°C and the autosampler at 4°C. All pigments detected have detection wavelengths between 375 and 700 nm. A system calibration of chl *a* was done with a standard from *Anacystis nidulans* (Sigma-Aldrich, USA).

Following calibration of chl *a*, cultures of representative algal taxa (Table 1) were used to validate the method. All cultures were grown at 18°C in liquid DY-V growth medium (Lehman 1976), with the exception of the Cyanobacteria which were grown in liquid BG-11 growth medium (Stanier et al. 1971). Cultures were harvested after at least one week, with a minimum of 50 mL of algal culture filtered onto Whatman GF/F filters (47 mm, 0.7µm nominal pore size). Filters were placed into cryotubes and frozen in liquid nitrogen and stored at -70°C storage until pigments could be extracted (> 3 months). Filters were removed from storage, and extracted in 3 mL of 100% acetone overnight at -20°C. Extracts were sonicated on ice for 1 minute (Fisher Scientific Sonic Dismembrator Model 100, 15 W), and filtered through a 0.2 µm syringe filter. A final volume of 800 µL was placed into an autosampler vial. Immediately prior to injection,

Table 1. Representative algal taxa and genera used for calculation of pigment extinction coefficients (Appendix B).

Taxon	Genus, species/clone	Diagnostic pigments
Euglenophyceae	<i>Euglena</i> sp. #02	chlorophyll <i>b</i>
Chlorophyceae	<i>Chlamydomonas reinhardtii</i>	chlorophyll <i>b</i> lutein violaxanthin
Chrysophyceae	<i>Dinobryon</i> sp. #11	fucoxanthin
Dinophyceae	<i>Peridinium inconspicuum</i>	peridinin dinoxanthin
Cryptophyceae	<i>Cryptomonas ozollini</i>	alloxanthin
Raphidophyceae	<i>Gonyostomum</i> sp. #2	chlorophyll <i>c</i> fucoxanthin
Cyanophyceae	<i>Microcystis</i> sp. LE 3 <i>Nostoc</i> sp. PCC 7120 <i>Synechococcus</i> sp. PCC 7942	lutein echinenone zeaxanthin

200 μ L of 0.5 M ammonium acetate was added to aid in the separation of the pigments (Pinckney et al. 1996).

Taxon-specific pigments were identified by HPLC column retention time and absorbance spectra using previously published data (Jeffrey et al. 1997). Peak areas on the chromatogram of absorption versus time were converted to molar concentrations using extinction coefficients at the detection wavelength of 440 nm (see Appendix B).

Field Work

Sampling sites

Field sampling and experimental manipulations were performed twice per month (June, July, August) during the summer of 2010. Field sampling was completed aboard the R/V Neeskay on Lake Michigan, at two sites – Linnwood (43° 04.58' N , 87° 50.29' W, 20m depth, nearshore) and Fox Point (43° 11.67' N , 87° 47.26' W, 100m depth, offshore), which have served as reference locations for studies conducted at the Great Lakes Water Institute (see <http://waterbase.glwi.uwm.edu>).

Water column measurements

At each site, CTD casts (Sea-Bird Electronics Model 25 SeaLogger CTD) were taken for conductivity, dissolved oxygen, chl *a* fluorescence, quantum irradiance, and temperature. Using the irradiance-depth profiles, sampling depths were identified as a percentage of surface light intensity, with epilimnetic field and experimental samples collected from a depth with 30% surface light intensity, and metalimnetic samples collected from the depth of the deep chlorophyll maximum (identified from the chl *a* fluorescence versus depth profile).

Water for chemical analyses and biomass determination was collected with a 30 L Niskin bottle, and stored in brown polypropylene bottles on ice filtered. Filtrations for biomass determination and HPLC pigment analysis (see below) were performed onboard the ship. Chemical analyses were performed the following day in the laboratory. The epilimnetic samples served as both field and initial samples for incubation experiments. Whole water samples were collected for phytoplankton cell counts (500 mL) and preserved using Lugol's iodine. 200 mL subsamples for chl *a* were collected in replicate for each sampling site and depth onto Pall Supor filters (47 mm, 0.20 μm pore size). Filters were wrapped in aluminum foil, stored on ice until return to the laboratory, and transferred to -20°C until extraction. Subsamples for HPLC (≥ 2000 mL of screened (153 μm Nitex mesh) water) were filtered onto Whatman GF/F filters (47 mm, 0.45 μm pore size). Filters were placed into cryotubes and stored in liquid nitrogen. Samples were stored in an ultra-cold freezer (-70°C), for up to 12 months until pigment extraction.

Experimental Treatments

Herbivore grazing and nutrient enrichment experiments were established shipboard using water collected with the Niskin bottle at the depth of 30% surface light irradiance. Control treatments (NC) contained unfiltered Lake Michigan water. For zooplankton grazing treatments, triplicate bottles were filled with water that was prescreened (153 μm Nitex mesh) to remove macrozooplankton. Triplicate treatments included no herbivores (0X), controls with ambient concentrations of grazers (1X), or samples with multiples of grazers added (2X through 6X) (Fig. 1). Grazer gradient bottles (8 L) were inoculated with a sample of a zooplankton concentrate collected using a Puget Sound-type plankton net (1 m diameter, 135 μm mesh), as used previously by Lehman (1987).

In order to identify nutrient limitations for the phytoplankton communities, replicate nutrient manipulation experiments (Fig. 1) were done with whole Lake Michigan water by adding silica (5 μ M as sodium silicate), nitrate (5 μ M as sodium nitrate) or phosphorus (1 μ M as potassium phosphate) to 4 L polycarbonate bottles. All bottles were placed in the dark on ice until return to the laboratory.

Upon returning to the laboratory, experimental bottles were placed onto a water-jacketed plankton wheel (Prepejchal et al. 1981). The bottles were exposed to near-ambient lake temperatures, with a light period of 16 hours light: 8 hours dark. Cool white fluorescent lamps were turned on in 4 equal steps at 15 min intervals. With the entire light bar on, irradiance varied from 94 to 263 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ from the bottom to top of a rotation (P. Engevoold, pers. comm.). Linnwood experiments were incubated for 3-4 days under these conditions, and Fox Point experiments incubated for 4-5 days, as growth rates tend to be lower offshore due to temperature differences and decreased nutrient availability. After the 3-5 day incubations, bottles were removed from the wheel and the contents passed through a 153 μm mesh to remove macrozooplankton for preservation with sugar formalin or ethanol for later cell counts (enumeration). The remaining water was then used to measure phosphorus (TP/TDP), and pigments (both using fluorescence and HPLC).

Nutrient and chlorophyll analyses

In the laboratory, pigments were extracted from chlorophyll filters and analyzed using EPA Method 445 (EPA 1997), except that pigments were extracted in 90% buffered (1 g magnesium carbonate) acetone for a minimum of 1 month at -20°C. Filters and pigment extracts were brought to a final volume of 10 mL and centrifuged.

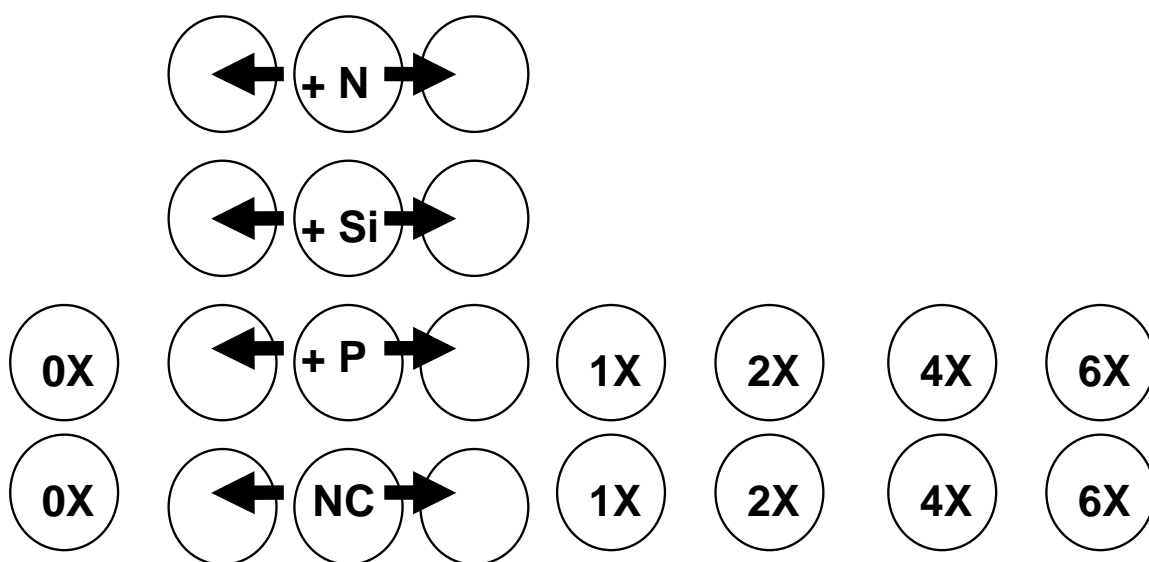


Figure 1. Schematic of nutrient and herbivore grazing experimental design used during the 2008 field season. Nutrient experiments are shown vertically with abbreviations as follows: “+N” for nitrogen as NH_4NO_3 , “+Si” for silica as $\text{Na}_2\text{SiO}_3 \cdot 9 \text{H}_2\text{O}$, and “+P” for phosphorus as NaHPO_4 . The herbivore grazing experiment is shown horizontally, with varying concentrations of herbivores present. Abbreviations for the herbivore experiments are as follows: “0X” meaning screened Lake Michigan water only; “1X” treatments were to mimic ambient herbivore concentrations, with “2X-6X”: treatments representing an increase in abundance from ambient herbivore concentrations. “NC” is the control for both experiments.

Fluorescence of supernatants was read using a Turner Designs TD 700 fluorometer (configured with a daylight lamp, excitation filter 10-050R and emission filter 10-051R, and red-sensitive PMT tube), using the acidification correction for phaeopigments (Parsons et al. 1984).

Silicate, ammonium, nitrate and total phosphorus were analyzed on filtered samples. For silicate, the molybdate method was used (Parsons et al. 1984; 815 nm, 4 cm flow-through cell). Ammonium was measured using the phenol hypochlorite method (Solórzano 1969; reading at 640 nm with a 4 cm flow-through cell). Nitrate was analyzed with the Brucine method (Holty and Patworowski 1972; 410 nm in a 5 cm flow-through cell). Absorption measurements were made with a Thermospectronic Gensys 6 spectrophotometer. Total particulate and dissolved phosphorus samples were processed with the persulfate oxidation method (Menzel and Corwin 1965) and read using a LKB BioChrom UltraspecII (870 nm, 10 cm cell).

Phytoplankton and macrozooplankton analyses

Phytoplankton samples were counted and measured biovolume estimates calculated, and converted to chl *a* for comparison with HPLC/CHEMTAX estimates. Phytoplankton were settled overnight using a 50 mL Utermöhl chamber and counted using an Olympus IX70 inverted microscope at magnification levels of 100-400x, using appropriate taxonomic keys (Prescott 1951, Nygaard 1976, Dillard 2007). Samples were counted using random fields, multiple transects, or half-chamber scans, depending on the abundance of the taxon. Dimensions of the first 5 individuals of a given taxon were measured in order to calculate cell biovolume (using simple geometric approximations,

Hillebrand et al. 1999). Biovolume was converted to chl *a* using the chl *a*:volume equation found in Montagnes et al. (1994).

Zooplankton data were collected to determine dominant zooplankton functional groups and to calculate mortality rates of phytoplankton and grazing rates of the herbivores of the representative algal taxa along the herbivore grazing gradient.

Zooplankton were enumerated and biomass determined following the procedure outlined in the EPA LG403 (EPA 2003) using descriptions and identification keys by Balcer et al. (1984) and the USGS (www.glsc.usgs.gov/greatlakescopepods/default.asp). Enumeration of zooplankton was completed by Paul Engevoold.

Experimental HPLC samples

Each filter extract was analyzed with analytical replicates (i.e. two separate runs were done for every filter collected). Peak areas for pigments were obtained and analytical replicates were averaged, and converted to molar quantities (see HPLC method development, above).

CHEMTAX analyses

HPLC pigment data (expressed as ratios to chl *a*, hereafter “pigment ratios”) were analyzed using the CHEMTAX (V 1.95) program (Mackey et al. 1996), obtained from S. Wright (CSIRO, Australia). This program uses the steepest-descent algorithm to fit a matrix of pigment ratios that define key taxa (determined from reference cultures) to the pigment ratios in an unknown sample.

With assistance from D. Millie (PI-Informatics, Florida, USA), a ratio matrix for Lake Michigan samples was created using a spreadsheet that he provided. An input table of pigment ratios (Table 2) was used to standardize pigment:chl *a*, using pigments

identified from the pigment separation trials (Appendix A) and the extinction coefficients (Appendix B) calculated for the HPLC method. Diagnostic pigments included: fucoxanthin (diatoms and chrysophytes), neoxanthin (chlorophytes), dinoxanthin (dinoflagellates), alloxanthin (cryptophytes), lutein (chlorophytes), and zeaxanthin (cyanobacteria). The input table of pigment ratios (Table 2) was derived from reference cultures, except for the diatoms for which pigment ratios were obtained from D. Millie (pers. comm.). Other program parameters were set as recommended in the program software (Mackey et al. 1996).

Statistical analyses

Conventional extractive fluorometric chl *a* was compared to HPLC-based chl *a*, as well as to HPLC chl *a* + chlorophyllide *a* and analyzed with Microsoft Excel 2010 using regression analyses of epilimnetic (Linnwood and Fox Point) and metalimnetic field samples. A similar regression was used to compare phaeophytin measured with conventional extractive fluorometric assays for chl *a* and chlorophyllide *a* measured using the HPLC method. T-tests were used to determine whether regression slopes were different from 1.

Proportions of different taxa estimated with HPLC/CHEMTAX were compared to allocations based on phytoplankton counts and biovolume data using Chi-square analysis of a 2x6 (i.e. 2 estimates, one based on counts, one on HPLC/CHEMTAX x 6 algal taxa) contingency table (Zar 2009) for each station and sampling date. Herbivore grazing experiments were analyzed with regression analyses to look for overall relationships of chl *a* biomass with increasing herbivore abundance. For dates with significant responses (i.e. decreases in chl *a* with increased herbivore abundance), HPLC/CHEMTAX data for

the “control” and “6X” grazer abundance treatments were treated with a Chi-square analysis to determine if significant changes in distribution between taxa occurred among the treatments. Nutrient enrichment experiments were analyzed with a one-way analysis of variance (ANOVA) to determine if there were significant change in chl *a* among nutrient additions. Like the analyses for grazing experiments, nutrient treatments showing significant chl *a* responses were further compared with Chi-square analysis of HPLC/CHEMTAX estimates of relative distribution among the major taxa among control and nutrient addition treatments. Analyses were done with Sigmaplot (Version 12.3).

Table 2. CHEMTAX input pigment ratios. Abbreviations: Peri- peridinin; Fuco- fucoxanthin; Neo- neoxanthin; Viol- violaxanthin; Dino- dinoxanthin; Diad- diadinoxanthin; Allo- alloxanthin; Lut- lutein; Zeax- zeaxanthin; Chl *b*- chlorophyll *b*; Chl *a*- chlorophyll *a*; β,β - β,β -carotene; β,ϵ - β,ϵ -carotene

Taxon	Pigment												
	Peri	Fuco	Neo	Viol	Dino	Diad	Allo	Lut	Zea	Chl <i>b</i>	Chl <i>a</i>	β, β	β, ϵ
Diatoms		0.520		0.003		0.067			0.007		1.000	0.002	
Cyanobacteria											1.000	0.194	
Cryptophytes							0.495				1.000	0.006	0.036
Chrysophytes		0.334		0.125		0.042					1.000	0.045	0.031
Chlorophytes			0.088	0.047				0.528	0.101	0.321	1.000	0.047	0.027
Dinoflagellates	0.443				0.060	0.425					1.000	0.024	0.020

RESULTS

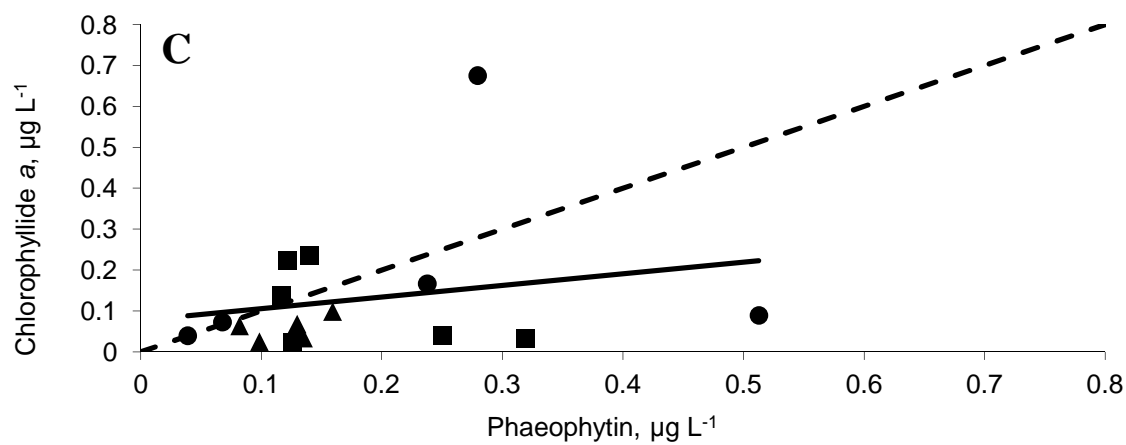
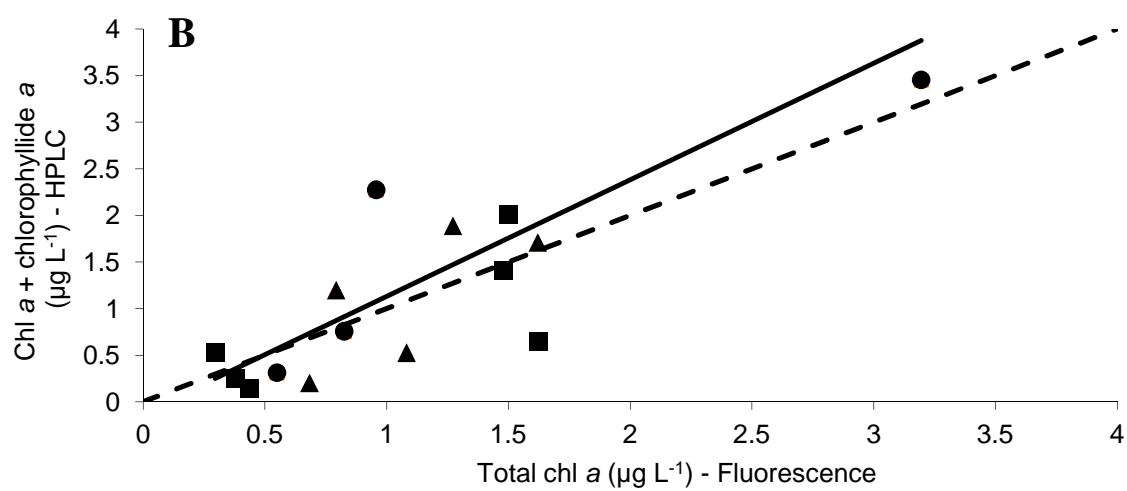
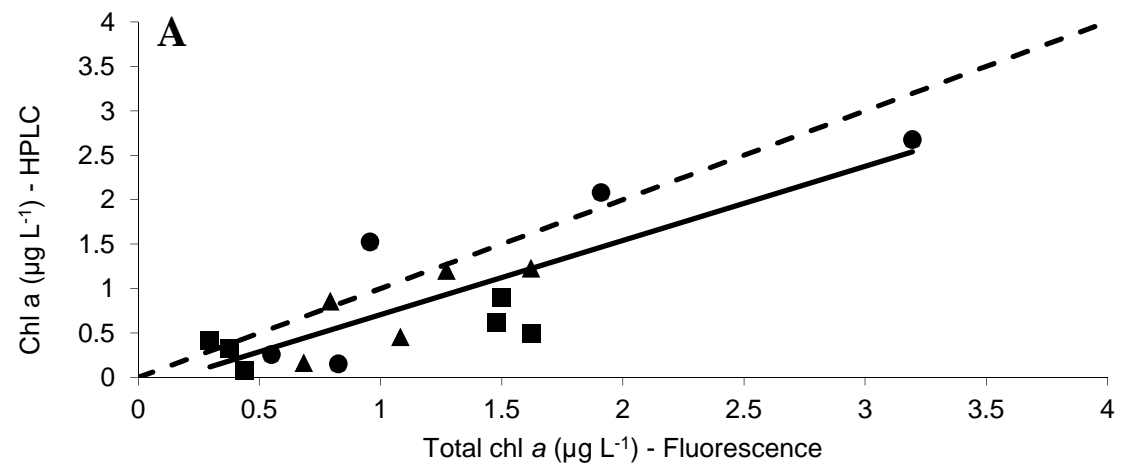
HPLC & conventional extractive fluorometric assays for chl a

Chl *a* determined by HPLC was significantly, linearly related to that measured using conventional extractive fluorometric assays for chl *a* ($p < 0.0001$, $r^2 = 0.68$, Fig. 2a). HPLC chl *a* + chlorophyllide *a* was also significantly related to chl *a* determined using conventional methods ($p < 0.001$, $r^2 = 0.62$, Fig. 2b). Neither linear regression was significantly different from the 1:1 relationship (Fig. 2a: $\{t(15) = 1.09\}$, Fig. 2b: $\{t(15) = 0.96\}$, $p > 0.1$ for both). In contrast, no significant relationship was found between HPLC chlorophyllide *a* and fluorescence phaeophytin ($p > 0.43$, $r^2 = 0.04$, Fig. 2c).

Phytoplankton cell counts and CHEMTAX

Water column phytoplankton biomass and composition varied throughout the season at both sampling locations, and at depth (Figs. 3, 4, 5).

Linnwood. Chlorophyll *a* biomass at Linnwood was below $2 \mu\text{g L}^{-1}$ for the entire season, with the exception of late June (day 177), when biomass was approximately $5 \mu\text{g L}^{-1}$. The community composition determined by cell count and biovolume estimates at this station showed dominance of diatoms throughout the sampling season; diatoms represented between 45-95% of chl *a* (Fig. 3b). Chrysophytes also contributed up to 25% in early July (day 189) decreasing to 5% throughout the rest of the season (late July-August). Cyanobacteria chl *a* increased from 5 to 25% at the end of the season (August, days 220-240), and dinoflagellates showed a similar pattern. Cryptophytes were present but in relatively low amounts (maximum pigment contribution 20% decreasing to 5%) throughout the summer.



▲ Linnwood epilimnion ■ Fox Point epilimnion Fox ● Point metalimnion

Figure 2. Relationship of HPLC-detected and fluorometrically-determined pigments in three regions of Lake Michigan from June-August, 2008. Solid lines indicate linear regression while dotted lines show the 1:1 relationship. Linnwood (43° 04.58' N , 87° 50.29' W) epilimnion samples were taken from depths ranging 2-5 m (average depth 3.5 m). Fox Point (43° 11.67' N , 87° 47.26' W) samples were collected from depths ranging 4-7 m (average depth 5.2 m) while metalimnetic samples were collected from depths ranging 12-46 m (average depth 28.5 m; Sampling depth dependent upon maximum fluorescence signal detected near metalimnion by CTD cast. A. HPLC-detected chl *a* compared with fluorometrically-determined chl *a* ($y = -0.12 + 0.83X$). B. HPLC-detected chl *a* combined with the degradation product chlorophyllide *a* compared with fluorometrically-determined chl *a* ($y = -0.12 + 1.25X$). C. Relationship of the degradation products HPLC-detected chlorophyllide *a* and fluorometrically determined phaeophytin ($y = 0.08 + 0.28X$).

In contrast, HPLC/CHEMTAX estimates (Fig. 3c) found chrysophytes to be the dominant taxon throughout the season, contributing 40-75% of the total chl *a*, with the exception of early June (day 154) when cryptophytes were dominant. HPLC/CHEMTAX estimated diatoms were present throughout the summer and contributing only 5-10% to total chl *a*. In closer agreement with count/biovolume-based data, cyanobacteria accounted for less than 5% of the chl *a* and cryptophytes accounted for less than 20% of the measured chl *a*, decreasing throughout the season.

Fox Point. Epilimnetic Fox Point chl *a* (HPLC) varied throughout the season with a maximum measured at approximately $1.5 \mu\text{g L}^{-1}$. Count/biovolume data showed that diatoms dominated the community on three dates (early June- day 154, late July-early August- days 206 and 220) with over 75% of the estimated chl *a* being accounted for in this taxon. Chrysophytes became dominant (60%) in late June-early July (Fig. 4b) and dinoflagellates were the dominant taxon in late August (day 240). Cryptophytes were present on all dates, though in decreasing amounts during the summer (15% maximum allocation) and cyanobacteria were only present in early July and late August (days 189 and 240).

HPLC-CHEMTAX community estimates (Fig. 4c) again differed from count data (Fig. 4c). Chrysophytes appeared to be the dominant taxon on all dates (>50% of chl *a*), with the exception of early June where diatoms (45%) and cryptophytes (40%) were co-dominant. Throughout the season, cryptophytes followed a similar pattern to that observed with count data, as did dinoflagellates, though they represented lower proportions of biomass compared to cell count and biovolume data (Fig. 4c).

Metalimnetic (DCM) Fox Point chl *a* increased steadily throughout the season, reaching a maximum biomass of 3 $\mu\text{g L}^{-1}$ in early August (day 220). The community composition determined by cell count and biovolume estimates was dominated by diatoms (>50%) on all dates except for late June-early June when chrysophytes were dominant (70%, and 50%, respectively) (Fig. 5b). Cyanobacteria (<10%) were observed in samples at the end of August only. Cryptophytes accounted for less than 20% of the chl *a* on any date.

Again, in contrast to counts, HPLC/CHEMTAX estimates (Fig. 5c) estimated the community to be dominated by chrysophytes (>90%) July-August. Diatoms were the dominant taxon in late June (day 177, 40%), but scarce (<5%) on any date the rest of the season. HPLC/CHEMTAX estimated Cyanobacteria were present July-August (5-20%), although count data only show cyanobacteria present at the end of August (day 240).

Statistical comparison of estimation methods

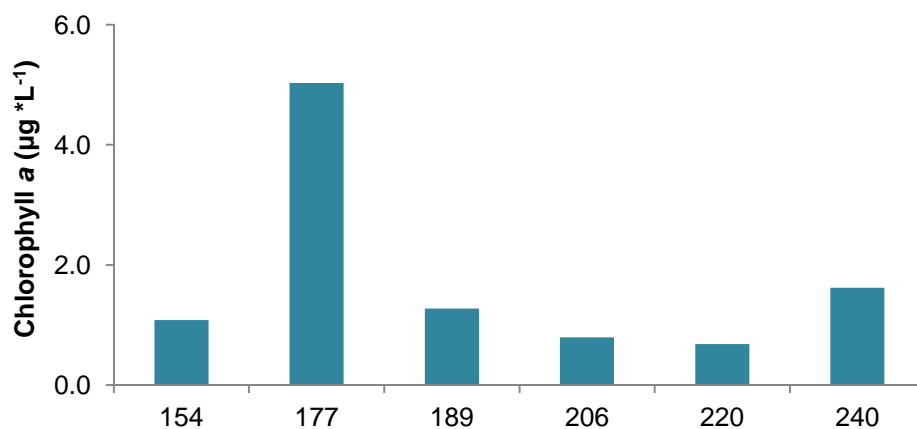
Of 18 dates and locations on which direct comparisons of pigment allocations by counts and biovolume versus HPLC/CHEMTAX were possible, only one comparison (Fox Point metalimnetic sample, late June- day 177) was not significantly different ($p>0.05$). If we combined chrysophytes and diatoms as a single category, 5 of the 18 comparisons were not significantly different. Subjectively, estimates were in better agreement at the offshore Fox Point sampling station. The most and least similar data sets were selected from each of the sampling stations (Fig. 6). In all but one example (Fig. 6e), HPLC/CHEMTAX overestimated the proportion of chrysophytes present where cell count and biovolume estimates clearly indicated diatoms to be the dominant taxon in most samples selected. Cryptophytes were occasionally overestimated by

HPLC/CHEMTAX compared to cell count and biomass estimates as well, while cyanobacteria, chlorophyte and dinoflagellate estimates were in relatively good agreement.

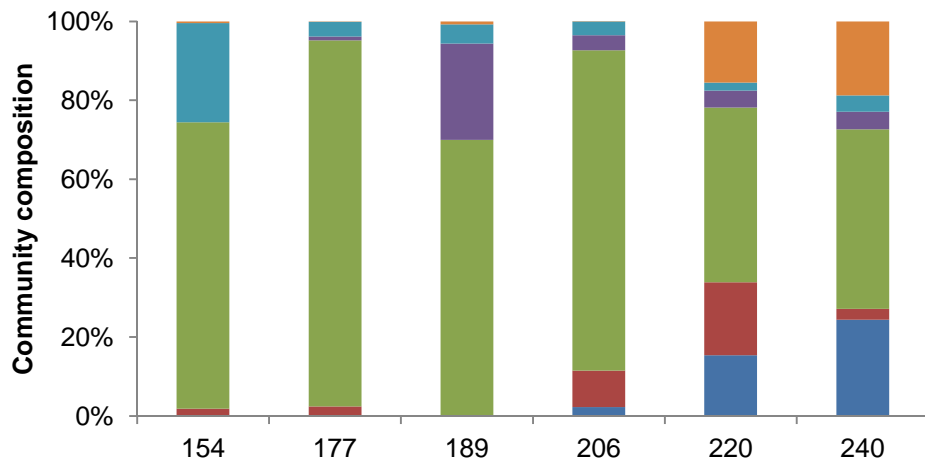
Experimental response estimates

Chrysophytes and diatoms were the taxa with the greatest HPLC/CHEMTAX-estimated responses to treatments. With regard to grazing experiments, 5 of the 7 experiments were found to have significant changes in chl *a* with increasing grazer abundance but only 3 were found to have significant differences in taxon composition (Table 3). In herbivore grazing experiments showing significant biomass decline, HPLC/CHEMTAX estimated that cryptophytes increased in the 6X treatment compared to the control. For example, the offshore Fox Point community (late June) had a decrease in chl *a* biomass, with about half of the biomass being removed in the 6X treatment compared to the control (Fig. 7). HPLC/CHEMTAX estimated that chrysophytes were removed entirely over the gradient suggestive of selective grazing, while Diatoms were lightly grazed and cryptophytes became more visible within the community. Chlorophytes were estimated to increase as the herbivore abundance increased, suggesting that they were not grazed (Fig. 7). In enrichments experiments, 6 of the 7 experiments had significant growth responses, and of these 6, 3 had significant differences in taxon composition (Table 4). In most enrichments, diatom biomass tended to increase, while chrysophytes decreased. Figure 8 illustrates one example of a nutrient enrichment where there was an increase in chl *a* biomass with phosphorus enrichment of the sample (Fox Point, early July). This response was estimated to be from diatoms and

chlorophytes responding to the enrichment. Chrysophytes responded to the silica enrichment, along with dinoflagellates who also responded to nitrogen.

A. Total Chlorophyll *a*

B. Cell counts



C. HPLC/CHEMTAX

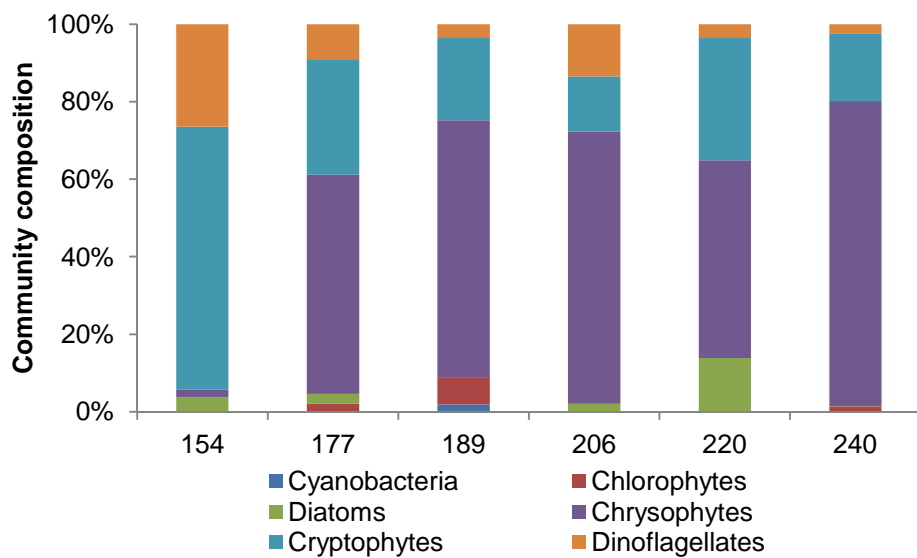
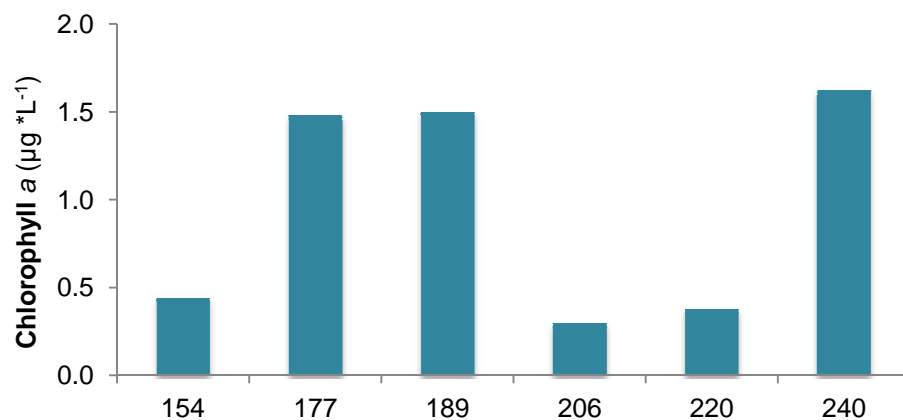
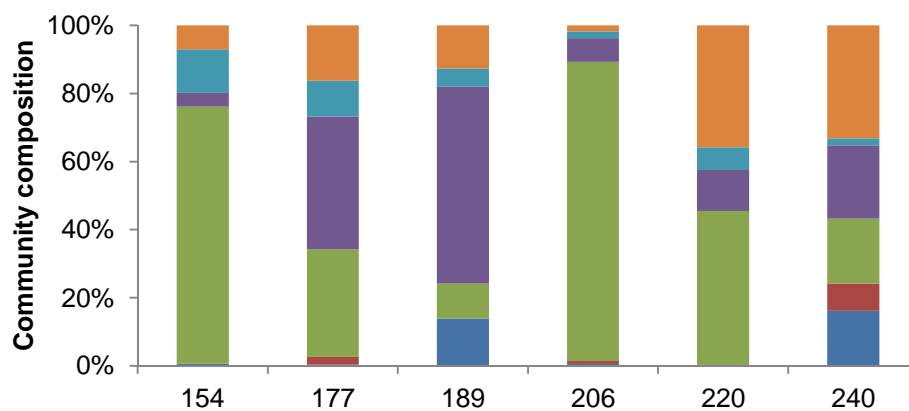


Figure 3. Seasonal comparison of the phytoplankton community at Linnwood station (43° 04.58' N, 87° 50.29' W), Lake Michigan. A: Total chl *a* measured using HPLC on acetone extracts. B: Community composition based on percentage of biovolume based on cell counts expressed as a proportion of chl *a*. C: Community composition determined using CHEMTAX analysis of HPLC data, as a proportion of total chl *a*. Samples cover the period from June 2 (day 154) to August 27 (day 240).

A. Total Chlorophyll *a*

B. Cell counts



C. HPLC/CHEMTAX

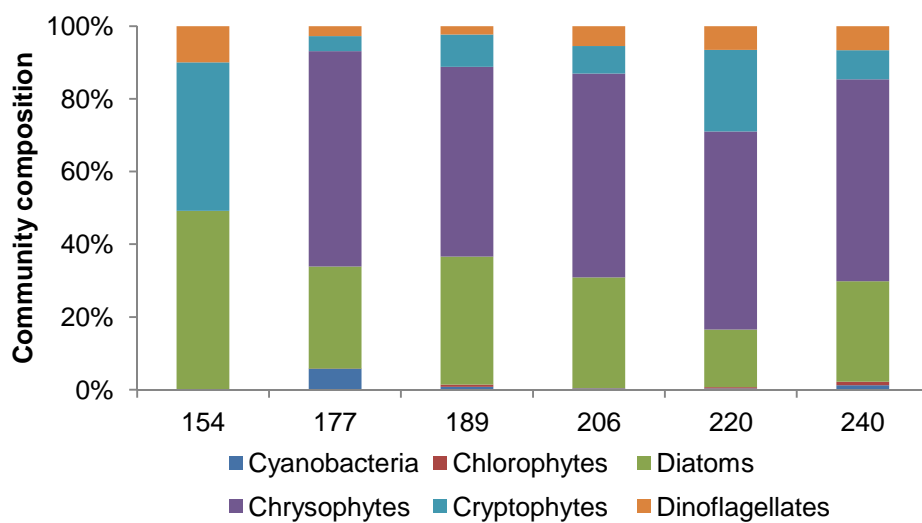
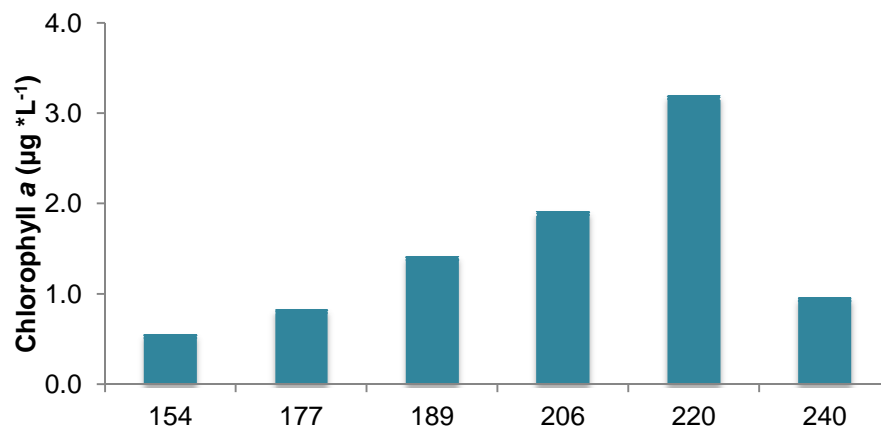
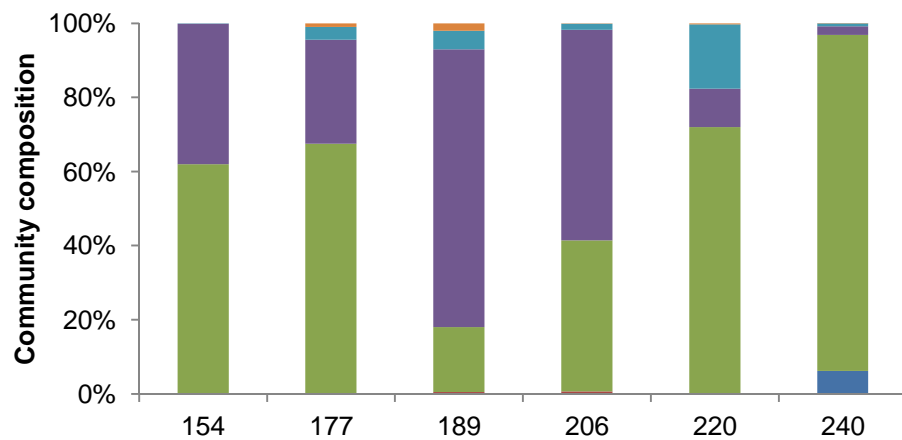


Figure 4. Seasonal comparison of the phytoplankton community at Fox Point, epilimnetic station (43° 11.67' N, 87° 47.26' W), Lake Michigan. A: Total chl *a* measured using HPLC on acetone extracts. B: Community composition based on percentage of biovolume based on cell counts expressed as a proportion of chl *a*. C: Community composition determined using CHEMTAX analysis of HPLC data, as a proportion of total chl *a*. Samples cover the period from June 2 (day 154) to August 27 (day 240).

A. Total Chlorophyll *a*

B. Cell counts



C. HPLC/CHEMTAX

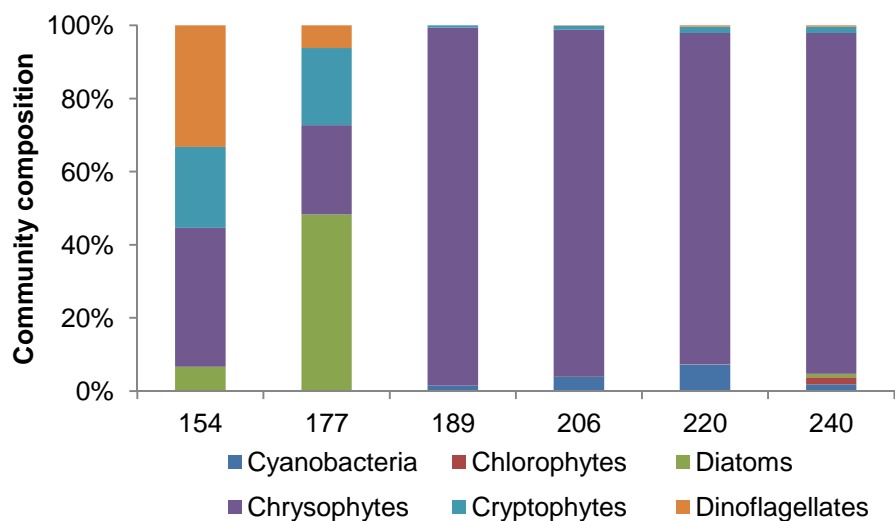


Figure 5. Seasonal comparison of the phytoplankton community at Fox Point, metalimnetic station (43° 11.67' N, 87° 47.26' W), Lake Michigan. A: Total chl *a* measured using HPLC on acetone extracts. B: Community composition based on percentage of biovolume based on cell counts expressed as a proportion of chl *a*. C: Community composition determined using CHEMTAX analysis of HPLC data, as a proportion of total chl *a*. Samples cover the period from June 2 (day of year 154) to August 27 (day of year 240).

Best relationship

Worst relationship

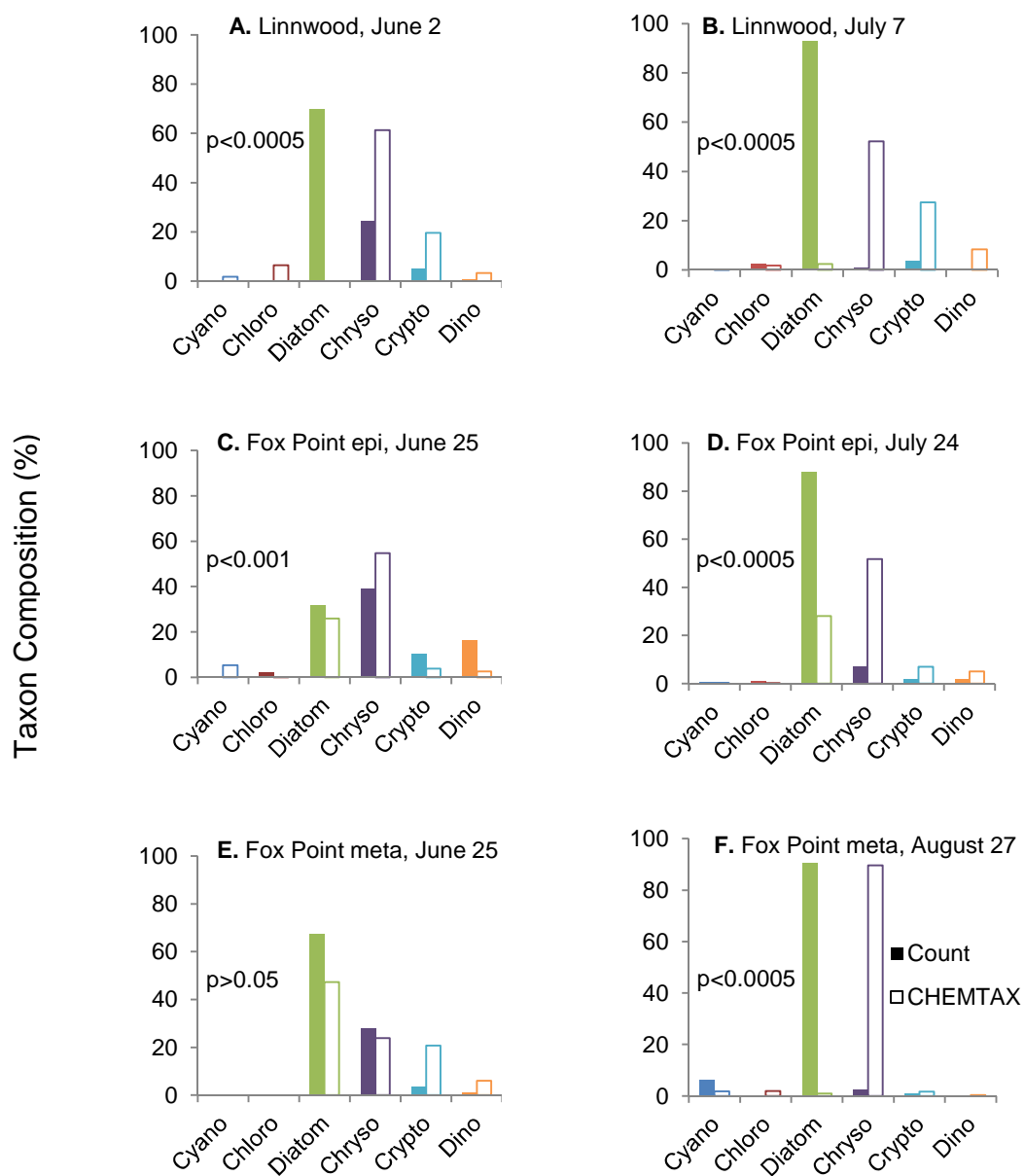


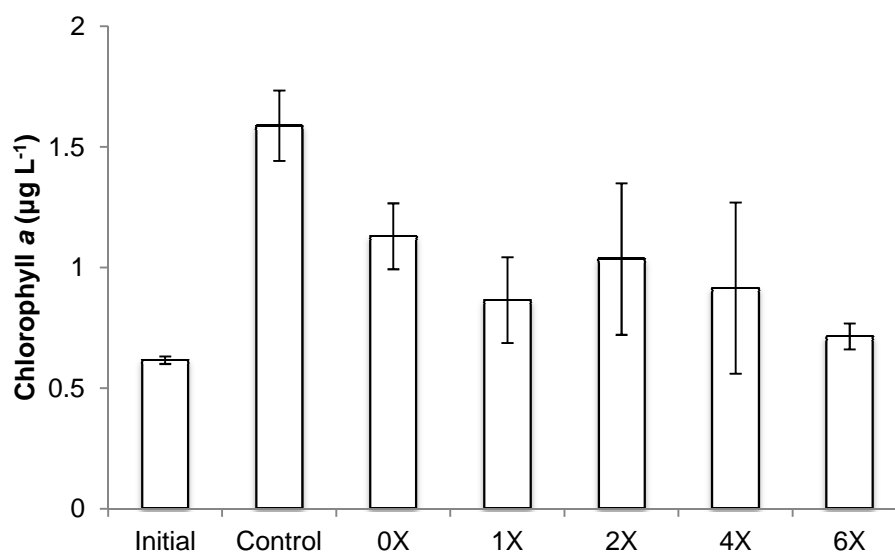
Figure 6. Comparison of cell count and HPLC/CHEMTAX distributions of taxa from Lake Michigan field samples collected June-August, 2008. Data were selected by comparison of Chi-square values for each sampling date and location. For each location, the “best relationship” data shows data from the date with the lowest Chi-square value, indicative of most significant similarity between the HPLC/CHEMTAX and cell count data, while the “Worst relationship” data had the highest Chi-square values, indicative of the least similar estimates. Abbreviations: Cyano- Cyanobacteria; Chloro- Chlorophytes; Diatom- Diatom; Chryso- Chrysophytes; Crypto- Cryptophytes; Dino- Dinoflagellates.

Table 3. Summary of statistical analyses of changes in chl *a* biomass and HPLC/CHEMTAX taxon estimates during Lake Michigan herbivore grazing experiments in 2008. Regression analyses tested whether significant changes in chl *a* had occurred with increasing grazer concentrations. For significant changes in chl *a* (bold), X² analyses were done for HPLC/CHEMTAX data between the control and 6X treatment to determine whether there were significant differences in taxon composition. Abbreviations: N.D.- no data; “—“ = analysis not performed.

Station/ Sampling date	Regression Significance of chl <i>a</i> changes (p-value)	CHEMTAX community composition (X ²) (p-value)
Linnwood		
June 25	0.0017	>0.05
July 7	0.02	>0.05
July 24	0.025	<0.001
August 27	0.8476	—
Fox Point		
June 25	0.04	<0.001
July 7	0.00019	<0.001
July 24	0.25	—
August 27	N.D.	N.D.

Table 4. Summary of statistical analyses of changes in chl *a* biomass and HPLC/CHEMTAX taxon estimates during Lake Michigan nutrient enrichment experiments in 2008. One-way ANOVA analyses tested to find significant differences among nutrient enrichments treatments. For significant changes in chl *a* (bold), X^2 analyses were done for HPLC/CHEMTAX data to determine whether there were significant differences in taxon composition. Abbreviations: Si- Silica; N- Nitrogen; P- Phosphorus; N.D.- no data; “—“ analysis not performed.

Station/ Sampling date	Treatment (ANOVA) p-value	Treatments significantly different	CHEMTAX community composition (X^2) (p-value)
Linnwood			
June 25	0.221	None	—
July 7	0.014	P	<0.001
July 24	<0.001	Si	0.076
		N	0.097
		P	0.500
August 27	<0.001	P	0.860
Fox Point			
June 25	<0.001	Si	0.017
		N	0.037
		P	<0.001
July 7	<0.001	N	0.028
		P	<0.001
July 24	0.005	Si	0.119
		N	0.470
August 27	N.D.	N.D.	N.D.

A. HPLC-measured chlorophyll *a*

B. HPLC/CHEMTAX-determined community composition

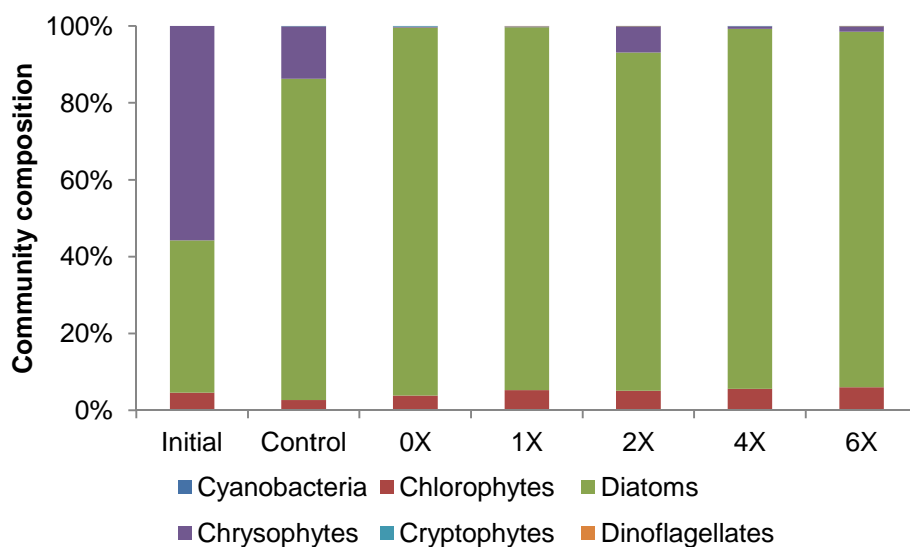
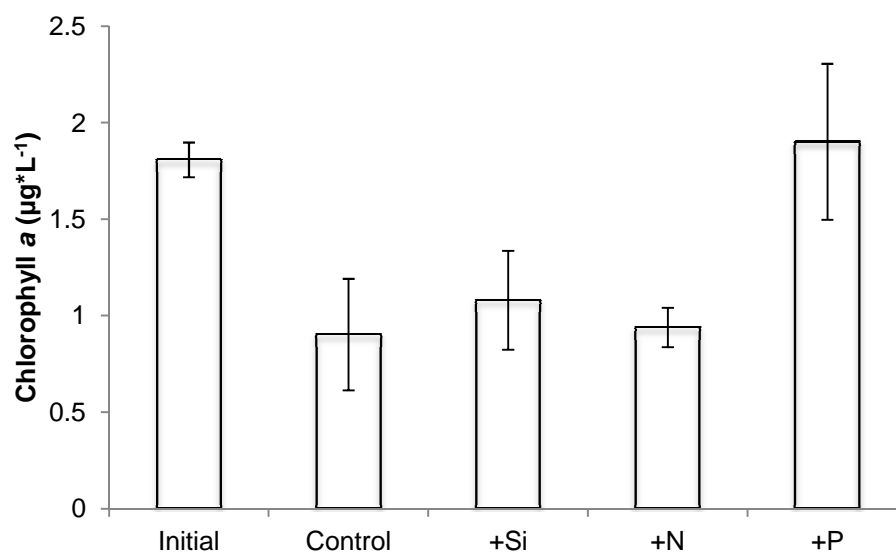


Figure 7. Example of herbivore gradient experimental data from Fox Point, June 25.
 A. HPLC-determined chl *a* to determine significance of relationship among treatments.
 B. CHEMTAX-derived community composition as a proportion of chl *a* in response to herbivore grazing. See Table 3 for statistical analyses.

A. HPLC-measured chlorophyll *a*

B. HPLC/CHEMTAX-determined community composition

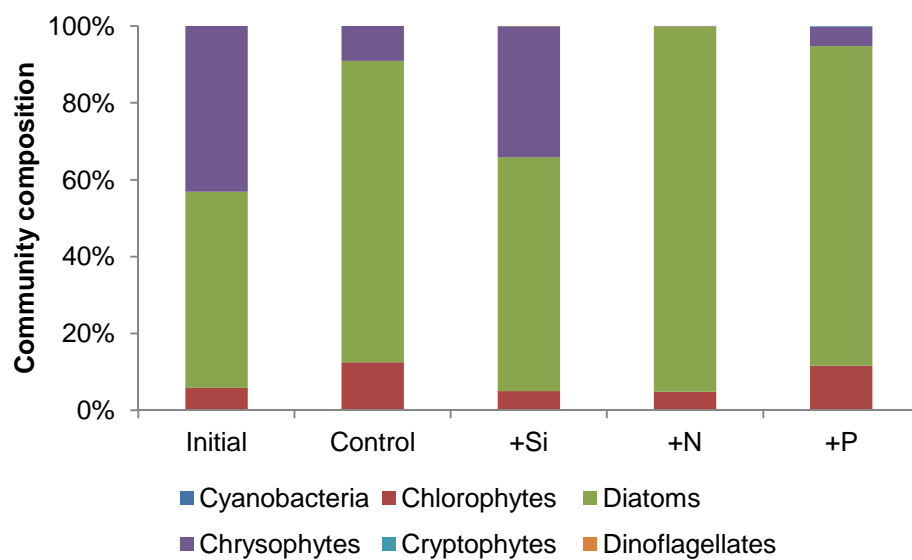


Figure 8. Example of nutrient enrichment data from Fox Point, July 7. A. HPLC-determined chl *a* to determine significance of relationship among treatments. B. CHEMTAX-derived community composition as a proportion of chl *a* in response to nutrient enrichments. See Table 4 for statistical analyses.

DISCUSSION

*HPLC and conventional extractive fluorometric assays for chl *a**

Chl *a* detected by the HPLC method was strongly correlated to fluorometrically measured pigments, validating the use of the methods as an alternative to fluorometry. The relationship changed little with the addition of measured chlorophyllide *a* to HPLC chl *a* values. Chlorophyllide *a* is a degradation product of chl *a* which can be indicative of algal cell senescence or sample degradation (Jeffrey 1974, Suzuki and Fujita 1986). This was expected as chl *a* and chlorophyllide *a* are separated using the HPLC method, but conventional extractive fluorometric assays for chl *a* include an uncertain component of chlorophyllide *a*. Because the HPLC method separates degradation products, if degradation products arise due to storage/handling issues, the chl *a* estimate may be less robust than conventional extractive fluorometric assays for chl *a*. Indeed, it has been argued that although the HPLC method provides a more accurate estimation of chl *a*, it may not be a good replacement for fluorometry as most indices that use chl *a* are based upon the conventional (inaccurate) method (see discussion in Chapter 8 of Peters 1991).

In contrast, the relationship between the chl *a* degradation products of chlorophyllide *a* (measured with HPLC) and phaeopigments (phaeophorbide and phaeophytin, measured with the fluorometer using acidification) was poor. This suggests that the chlorophyllide *a* results from degradation of samples during storage/processing, rather than the presence of degraded chl *a* in the field. Such degradation is likely due to the activation of the chlorophyllase enzyme, which degrades chl *a* to chlorophyllide *a* during sample processing (Suzuki and Fujita 1986, Jeffrey and Hallegraeff 1987; note there is also some small possibility that phaeophytin could be a product). Interestingly,

Diatoms and Chrysophytes, dominant taxa within Lake Michigan samples (Figs. 3b, 4b, 5b), are taxa with naturally high chlorophyllase activity (Jeffrey and Hallegraeff 1987). Samples for extractive fluorometric assays were extracted for at least one month (an unusually long time) in buffered 90% acetone before analysis, while HPLC samples were extracted overnight in unbuffered 100% acetone. This could result in further sample degradation as chlorophyllase may still be active in the fluorometric extracts which would eventually degrade chl *a* to phaeophytin *a*. Also, pigment samples were collected by filtration which can damage cells, causing them to lyse and release endogenous chlorophyllase into the sample (Suzuki and Fujita 1986, Jeffrey and Hallegraeff 1987). Furthermore, the pigments can also be affected by the presence of cell debris which can activate chlorophyllases while extracting in an organic solvent such as acetone.

To prevent activation of chlorophyllases, extracting pigments in 100% acetone has been suggested (Jeffrey 1974), as was done in the present study for HPLC pigment samples. This has not always been found to be effective however, as other sample processing techniques (e.g. filtration, centrifugation) can damage the cells and activate the enzyme. To avoid cells being exposed to debris collected on filters that could accelerate the degradation process, Suzuki and Fujita (1986) recommend collecting cells with centrifugation and extracting by grinding samples at room temperature for 1 minute; this was effective in most cases. For one species, *Skeletonema costatum*, extraction in 100% hot acetone (65°C, 2 min.) was necessary to inhibit chlorophyllase (Suzuki and Fujita 1986), but such high temperatures can also degrade pigments. In future studies, comparisons of collection and storage methods for Lake Michigan samples seem worthwhile.

HPLC-measured phytoplankton biomass

Algal biomass was dynamic throughout the summer of 2008 at sampling locations, ranging from <0.5 (offshore Fox Point epilimnion) to $5 \mu\text{g L}^{-1}$ (nearshore Linnwood, June 25 (day 177)) (Figs. 3a, 4a, 5a). The highest values at the Linnwood station are likely a result of land-based detritus and chlorophyll that entered the system following a storm even that occurred earlier in June (NOAA 2008, USGS 2008). At the offshore Fox Point epilimnetic site, increases of biomass to $1.5 \mu\text{g L}^{-1}$ on days 177 and 189 (June 25 and July 7, Fig. 4a) could also be in due to the nutrient and land-based chlorophyll pulses following the storm earlier in June. At offshore Fox Point, metalimnetic biomass increased throughout the season (Fig. 5a), reaching a maximum of $3.0 \mu\text{g L}^{-1}$ on August 7 (day 220). This increase likely reflects sedimentation of phytoplankton from higher in the water column, or growth in response to nutrients being mixed back into the metalimnion from the upper layer of the hypolimnion as has been previously been described by Fahnenstiel and Scavia (1987). The biomass estimates are typical of recent trends in Lake Michigan, with net annual chl *a* biomass decreasing over the past few decades to $<2.0 \mu\text{g L}^{-1}$ due to invasive dreissenid mussels that have cleared the water of small plankton (Mida et al. 2010; Fahnenstiel et al. 2010).

Phytoplankton cell counts and CHEMTAX

Phytoplankton community composition estimates determined by biovolume conversion to chl *a* estimates or HPLC/CHEMTAX analyses showed seasonal variation of the key phytoplankton taxa, but were not in good agreement (Figs. 3-5). In comparison with count-based estimates, HPLC/CHEMTAX estimates overestimated the chrysophyte

contribution to the community while underestimating diatoms (see examples in Fig. 6a, 6b, 6f). CHEMTAX biases similar to the current study have been seen in Lake Erie (D. Millie, pers. comm.) where Chrysophytes were estimated to be the dominant using HPLC/CHEMTAX methods. Millie (pers. comm.) has previously noted this issue in unpublished Lake Erie datasets and recommends combining diatom and chrysophyte data, since the two taxa have few functional differences. In freshwaters, the two taxa can only truly be differentiated by separating chlorophyll *c* forms (i.e. diatoms have chl *c*₁ and chl *c*₂ and chrysophytes have chl *c*₂); we were not able to resolve these chlorophyll *c* forms in the present study. CHEMTAX also overestimated cryptophytes and dinoflagellates, though not as frequently.

There are several reasons why the CHEMTAX community composition and count-based allocation of pigment estimates may not agree – the particular diagnostic pigments we selected, complex co-variations of pigments in response to environmental factors in field samples, errors in sample cell counting, or the most likely – errors with the CHEMTAX ratio matrices used for analysis. To begin, the pigments selected for the current study (Table 2) reflect those that we felt were most useful and representative of the sample analyzed. These pigments included photoprotective pigments such as diadinoxanthin and neoxanthin, both of which have been removed from input matrices in previous studies to avoid pigment ratio conflicts in response to light regimes (Buchaca, et al. 2005 for Chrysophyte input ratios, Llewellyn et al. 2005). The photoprotective pigments respond to changing light conditions (e.g. diadinoxanthin is converted to diatoxanthin and violaxanthin is converted to zeaxanthin in high light), leading to changes in pigment:chl *a* ratios (Dimier et al. 2007). Two field projects similar to the

present study provide contrasting conclusions about the accuracy of HPLC/CHEMTAX methods. Buchaca et al. (2005) found that HPLC/CHEMTAX estimates of Chrysophytes, Dinoflagellates and Cryptophytes from Lake Redon were in good agreement with biovolume data from microscopy, while Chlorophyte estimates were poorly correlated probably due to cell count biovolume errors because the representative species were colonial and had small cell sizes. In contrast, Llewellyn et al. (2005) sampled populations from the English Channel and found no clear relationship among the HPLC/CHEMTAX and cell count biomass estimates, noting that there was a decrease in the fucoxanthin:chl *a* ratio of Diatoms during the summer when cells were exposed to high light conditions. They recommended refining the HPLC method to separate more of the minor taxonomic pigments to better resolve the community.

Environmental factors can also affect the pigment ratios within a sample. Irradiance can activate the xanthophyll cycle, causing the photoprotective pigments to change to another form, changing the pigment:chl *a* ratios. For example, in high light conditions, the violaxanthin:chl *a* ratio has been found to increase in cells closer to the surface (Buchaca et al. 2005). Violaxanthin is found in Diatoms, Chrysophytes and Chlorophytes, all which had estimation errors by HPLC/CHEMTAX in the present study. If Chrysophyte cells in the epilimnion were acclimating to higher irradiance, they may have a lower violaxanthin:chl *a* ratio, resulting in an over-estimation of the taxon compared to Diatoms and Chlorophytes which tended to be underestimated by CHEMTAX here (Figs. 3-5). In addition to changing irradiance, nutrient availability can also affect the pigment ratios of the taxa. Under nutrient limitation, N limitation will reduce chlorophyll synthesis, which will decrease light harvesting and subsequently

biomass. Nutrient limitation will also change the pigment:chl *a* ratios for all taxa, but the degree of variation changes by taxon (Henriksen et al. 2002). Lake Michigan is strongly P-limited and the effects of P on pigments are much less clear. P-limited cells can have an increase in xanthophyll pigment:chl *a* ratios similar to that seen with other nutrient limitations (Geider et al. 1993).

Successful application of HPLC/CHEMTAX relies heavily on the initial input matrix as it estimates the community composition from HPLC pigment data (Henriksen et al. 2002), and assumes that pigment ratios do not change significantly within a dataset (Mackey et al. 1996). The method also assumes that all species within a taxon will have similar pigment ratios, which is not always true as the ratios can respond to changing environmental conditions (Schlüter et al. 2000, Henriksen et al. 2002). Thus, the input matrix could be a significant source of error. As previously mentioned, the CHEMTAX input table of pigment ratio matrix (Table 2) was derived from representative cultures (Table 1) grown under controlled conditions (same lighting regime and nutrient availability). These growth conditions are not representative of those at the sampling sites, so it should be anticipated that culture estimates may not provide an accurate estimate of natural pigment:chl *a* ratios. The genera selected for the current study were chosen as they seemed to be most representative of what was expected to be found in the field. For the CHEMTAX input table of pigment ratios, we included most of the pigments separated by the HPLC solvent and method employed, and included more of the accessory pigments than others have used (Schlüter and Møhlenberg 2003, Buchaca et al. 2005 and others). By increasing the number of accessory pigments to the input table of pigment ratios, it has been suggested that biomass estimate errors by CHEMTAX would

decrease (Llewellyn et al. 2005). However, our use of 12 diagnostic pigments compared to 11 by Llewellyn et al. (2005), found a similar outcome of poor agreement among between HPLC/CHEMTAX and cell count biomass estimates.

Given these limitations, it seems unwise to use HPLC/CHEMTAX methods as a “shortcut” to obtain detailed taxonomic data, but instead to use in combination with other methods. One example is provided by Irigoien et al. (2004)’s study of phytoplankton in the English Channel using HPLC/CHEMTAX and microscopy estimates (2004). Data were treated with a “blind” analysis, where samples were analyzed with CHEMTAX using input pigment ratios from literature without knowledge of the community composition, followed by directed analysis, where samples were enumerated with microscopic methods first, and then input ratios were adjusted based upon the representative community before CHEMTAX analysis. Blind analyses found CHEMTAX overestimated Diatoms during two blooms, one being of the Dinoflagellate *Karenia mikimotoi* and the other of a Haptophyte, *Phaeocystis pouchetii*; like Diatoms, both of these species contain fucoxanthin. These results reinforce the need for HPLC-CHEMTAX methods to be used in combination with others to verify community composition.

Experimental response estimates

In the grazing and nutrient addition experiments, significant differences among grazer treatments were detected (Tables 3 and 4). Chrysophyte biomass declined, possibly in response to either selective grazing or a “bottle effect” (i.e. selective mortality in the container), in late June (Fig. 7), while Diatoms and Chlorophytes remained in the treatments. Selective grazing of Chrysophytes would make sense if cladocerans, which

selectively feed on Chrysophytes, were abundant (Quiblier-Llobéra et al. 1996).

However, zooplankton enumeration data (Engevd, unpublished) indicate cladocerans accounted for only about $0.20 \mu\text{g L}^{-1}$ biomass in this period, less than 3% of the total zooplankton by mass.

In nutrient enrichment treatments, the increase in chl *a* biomass when phosphorus was added (Fig. 8a) suggests that the community was phosphorus-limited, a common occurrence in temperate lakes during the summer (Schindler 1974). Chrysophytes responded to both phosphorus and silica enrichments in some cases (Fig. 8b). Some Chrysophytes produce silicate scales as an anti-grazing strategy, and thus can become silicate-limited or compromised if silicate is low (Sandgren et al. 1996). Thus, a growth response to silicate is not unreasonable.

Limitations of HPLC/CHEMTAX

HPLC/CHEMTAX clearly provides a rapid method for estimating taxa in the phytoplankton community. Caution must be taken in analysis interpretation as using the method without knowledge of the taxa present may result in major identification errors (Iriogoien et al. 2004). Microscopy can resolve issues in misidentification (Iriogoien et al. 2004, Sarmiento and Descy 2008), but sample analysis using HPLC/CHEMTAX cannot be used as a shortcut or as a full replacement for microscopic analysis. Prior to electing the HPLC/CHEMTAX method it is important to consider the information needed to answer the study question – highly detailed taxonomic information for a few samples or low-level taxonomic detail for many samples (Sarmiento and Descy 2008). For the present study, we wanted both types of information for seasonal data, in order to test the feasibility of using HPLC/CHEMTAX to accurately estimate the taxa within Lake

Michigan to eliminate the need for full phytoplankton enumeration. We found major errors in identification of taxa that likely originate with the input table of pigment ratios.

In future studies where HPLC/CHEMTAX methods are used to analyze phytoplankton responses, we recommend that the solvent system and separation gradient could be modified to separate only the taxonomically-important pigments. This will substantially shorten processing times and allow more samples to be processed. For example, if you eliminated photoprotective pigments such as violaxanthin or zeaxanthin and the carotenoids β,β -carotene and β,ϵ -carotene, the run time could be about 50 min versus 82 min. The methods developed for the current study were designed to separate as many pigments as possible, initially without real evaluation of their diagnostic importance. Perhaps modifying the solvent system and separation gradient to something similar to that of Zapata et al. (2000), where they were able to separate all three chlorophyll *c* forms (marine species), would provide better biomass estimates of the Diatoms and Chrysophytes. This may be an expensive solution however, as their methods used a C-8 column (we use C-18 columns), as well as the new solvents that would be required of the method. Another change to the HPLC method would be to use methanol as an extraction solvent instead of acetone in order to extract the phycobilin pigments of the Cyanobacteria and Cryptophytes, though this would also require a change in the HPLC solvent system and separation gradient. Again, this would allow for greater resolution of HPLC/CHEMTAX data. An alternative suggestion would be to try to eliminate errors with HPLC/CHEMTAX and cell counts by using methods such as flow cytometry, as suggested by Llewellyn et al. (2005). Flow cytometry would allow for the

separation of particles based on fluorescence and more accurate measurement of particles providing better estimates of biovolume for the community.

Summary

This study does not support the hypothesis that an HPLC/CHEMTAX method could be used as a way to rapidly provide detailed information about phytoplankton populations in Lake Michigan. Although HPLC chl *a* was good at estimating biomass in line with the conventional extractive fluorometric assays for chl *a*, pigment:chl *a* ratios analyzed with HPLC/CHEMTAX compared with microscopic count data were in poor agreement. Errors found within the HPLC/CHEMTAX data set included over and under estimation of dominant algal taxa found in Lake Michigan. These errors have caused me to question the validity of experimental results estimated by HPLC/CHEMTAX as no count data exists to verify the results. In future studies, it is recommended that when using an HPLC method phytoplankton samples be archived in order to verify community composition estimated by the pigment:chl *a* ratios. By doing so, if HPLC estimates are questionable, methods can be corrected with verification of dominant taxa.

Even with the estimation errors found within the current study, HPLC would still be beneficial as a general biomass indicator based on chl *a* concentrations, albeit an expensive one. The HPLC method could be modified to decrease sample run times by adjusting the methods for separating only chl *a* and degradation products. By having shorter run times, samples can be processed more efficiently and automatically decreasing the need for laboratory assistants to process chl *a* conventional extractive fluorometric assays for chl *a* samples.

The use of HPLC/CHEMTAX for examining community composition could still prove to be useful in Lake Michigan, with modification of the overall method. Changes could include limiting the pigments being separated by the HPLC method to those that are of the greatest usefulness taxonomically (i.e. fucoxanthin- diatoms), or by compiling an average pigment:chl *a* ratio for the pigments of interest from published literature for similar studies. The later may be difficult to do at this time since HPLC pigment analysis depends on the methods for extraction, and solvent and column system used. This causes a lot of variability within ratios, as elution times will vary with solvent systems and extraction of particular pigments may be limited to a particular solvent that is not optimal for the rest of the study questions.

References

- Balcer, M.D., Korda, N.L. and Dodson, S.L. 1984. *Zooplankton of the Great Lakes: A guide to the identification and ecology of the common crustacean species*. The University of Wisconsin Press.
- Berquist, A.M., Carpenter, S.R. and Latino, J.C. 1985. Shifts in phytoplankton size structure and community composition during grazing by contrasting zooplankton assemblages. *Limnology and Oceanography* 30: 1037-1045.
- Berquist, A.M. and Carpenter, S.R. 1986. Limnetic herbivory: Effects on phytoplankton populations and primary production. *Ecology* 67(5): 1351-1360.
- Beszteri, B., Acs, E. and Medlin, L.K. 2005. Ribosomal DNA sequence variation among sympatric strains of the *Cyclotella meneghiniana* complex (Bacillariophyceae) reveals cryptic diversity. *Protist* 156: 317-333.
- Brooks, J.L. and Dodson, S.I. 1965. Predation, body size, and composition of plankton. *Science* 150: 28-35.
- Buchaca, T., Felip, M. and Catalan, J. 2005. A comparison of HPLC pigment analyses and biovolume estimates of phytoplankton groups in an oligotrophic lake. *Journal of Plankton Research*. 27: 91-101.
- Carpenter, S.R., Kitchell, J.F. and Hodgson, J.F. 1985. Cascading trophic interactions and lake productivity. *Bioscience* 35: 634-639.
- Carpenter, S.R. and Kitchell, J.F. 1993. *The trophic cascade in lakes*. Cambridge University Press, London, England.
- Carrick, H.J., Fahnenstiel, G.L., Stoermer, E.F. and Wetzel, R.G. 1991. The importance of zooplankton-protzoan trophic couplings in Lake Michigan. *Limnology and Oceanography* 36(7): 1335-1345.
- Countway, P.D., Gast, R.J., Savai, P. and Caron, D.A. 2005. Protistan diversity estimates based on 18S rDNA from seawater incubations in the Western North Atlantic. *Journal of Eukaryotic Microbiology* 52(2): 95-106.
- De Wever, A., Muylaert, K., Langlet, D., Alleman, L., Descy, J.P., Andre, L., Cocquyt, C. and Vyverman, W. 2008. Differential response of phytoplankton to additions of nitrogen, phosphorus and iron in Lake Tanganyika. *Freshwater Biology* 53: 264-277.
- Descy, J.P., Higgins, H.W., Mackey, D.J., Hurley, J.P. and Frost, T.M. 2000. Pigment ratios and phytoplankton assessment in northern Wisconsin lakes. *Journal of Phycology*. 36: 274-286.

- Descy, J.P., Hardy, M.A., Stenuite, S., Pirlot, S., Leporcq, B., Kimirei, I., Sekadende, B., Mwaitega, S.R. and Sinyenza, D. 2005. Phytoplankton pigments and community composition in Lake Tanganyika. *Freshwater Biology* 50: 668-684.
- Descy, J.P., Sarmiento, H. and Higgins, H.W. 2009. Variability of phytoplankton pigment ratios across aquatic environments. *European Journal of Phycology* 44(3): 319-330.
- Dillard, G.E. 2007. *Freshwater Algae of the Southeastern United States. Part 8: Chrysophyceae, Xanthophyceae, Raphidophyceae, and Dinophyceae.* Bibliotheca Phycologia Band 112. J. Cramer, Berlin.
- Dimier, C., Corato, F., Tramontano, F. and Brunet, C. 2007. Photoprotection and xanthophyll-cycle activity in three marine diatoms. *Journal of Phycology*. 43: 937-947.
- Dobberfuhl, D.R., Miller, R. and Elser, J.J. 1997. Effects of a cyclopoid copepod (*Diacyclops thomasi*) on phytoplankton and the microbial food web. *Aquatic Microbial Ecology* 12: 29-37.
- Dyble, J., Fahnenstiel, G.L., Litaker, R.W., Millie, D.F. and Tester, P.A. 2008. Microcystin concentrations and genetic diversity of *Microcystis* in the lower Great Lakes. *Environmental Toxicology* 23: 504-516.
- EPA National Exposure Research Laboratory Office of Research and Development. 1997. *In vitro* determination of chlorophyll *a* and phaeophytin *a* in marine and freshwater algae by fluorescence. 445, Revision 1.2 September 1997.
- EPA Great Lakes National Program Office. 2003. Standard operating procedure for zooplankton analysis. LG403, Revision 03 February 2003.
- Fahnenstiel, G.L. and Scavia, D. 1987. Dynamics of Lake Michigan phytoplankton: the deep chlorophyll layer. *Journal of Great Lakes Research*. 13: 285-295.
- Fahnenstiel, G., Pothoven, S., Vanderploeg, H., Klarer, D., Nalepa, T. and Scavia, D. 2010. Recent changes in primary production and phytoplankton in the offshore region of southeastern Lake Michigan. *Journal of Great Lakes Research*. 36: 20-29.
- Falkowski, P.G. and Raven, J.A. 2007. *Aquatic photosynthesis, 2nd edition.* Princeton University Press, New Jersey.
- Fairchild, G.W., Lowe, R.L. and Richardson, W.B. 1985. Algal periphyton growth on nutrient-diffusing substrates: An in situ bioassay. *Ecology* 66(2): 465-472.

- Geider, R.J., Laroche, J., Greene, R.M. and Olaizola, M. 1993. Response of the photosynthetic apparatus of *Phaeodactylum-tricornutum* (Bacillariophyceae) to nitrate, phosphate, or iron starvation. *Journal of Phycology*. 29: 755-766.
- Gieskes, W.W.C., Kraay, G.W., Nontji, A., Setiapermana, D. and Sutomo. 1988. Monsoonal alteration of a mixed and layered structure of a euphotic zone of the Banda Sea (Indonesia): A mathematical analysis of algal pigment fingerprints. *Netherlands Journal of Sea Research*. 22: 123-137.
- Havskum, H., Schluter, L., Scharek, R., Berdalet, E. and Jacquet, S. 2004. Routine quantification of phytoplankton groups- microscopy or pigment analyses? *Marine Ecology Progress Series* 274: 31-42.
- Henriksen, P., Riemann, B., Kaas, H., Sørensen, H.M. and Sørensen, H.L. 2002. Effects of nutrient-limitation and irradiance on marine phytoplankton pigments. *Journal of Plankton Research*. 24: 835-858.
- Higgins H.W., Wright S. W. and Schlüter L. 2011. Quantitative Interpretation of Chemotaxonomic Pigment Data, Chapter 6, *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography*, Suzanne Roy, Einar Skarstad Egeland, Geir Johnsen and Carole Anne Llewellyn (eds.) Cambridge University Press.
- Hillebrand, H., Dürselen, C., Kirschtel, D., Pollinger, U. and Zohary, T. 1999. Biolvolume calculation for pelagic and benthic microalgae. *Journal of Phycology*. 35: 403-424.
- Holty, J.G. and Potworowski, H.S. 1972. Brucine analysis for high nitrate concentrations. *Environmental Science & Technology*. 6 : 835-837.
- Irigoién, X., Meyer, B., Harris, R. and Harbour, D. 2004. Using HPLC pigment analysis to investigate phytoplankton taxonomy: the importance of knowing your species. *Helgoland Marine Research*. 58: 77-82.
- Jeffrey, S.W. 1974. Profiles of photosynthetic pigments in the ocean using thin-layer chromatography. *Marine Biology*. 26: 101-110.
- Jeffrey, S.W. and Hallegraeff, G.M. 1987. Chlorophyllase distribution in ten classes of phytoplankton: a problem for chlorophyll analysis. *Marine Ecology Progress Series*. 35: 293-304.
- Jeffrey, S.W., Mantoura, R.F.C. and Wright, S.W. 1997. *Phytoplankton pigments in oceanography: guidelines to modern methods*. UNESCO Publishing, France.
- Jeffrey S.W., Wright, S.W. and Zapata, M. 1999. Recent advances in HPLC pigment analysis of phytoplankton. *Marine and Freshwater Research*. 50: 879-896.

- Kalff, J. and Knoechel, R. 1978. Phytoplankton and their dynamics in oligotrophic and eutrophic lakes. *Annual Review of Ecology and Systematics*. 9: 475-495.
- Kruskopf, M. and Flynn, K.J. 2005. Chlorophyll content and fluorescence responses cannot be used to gauge reliably phytoplankton biomass, nutrient status or growth rate. *New Phytologist*. 169: 525-536.
- Lampert, W. and Sommer, U. 1997. *Limnoecology: The ecology of lakes and streams*. Oxford University Press, New York.
- Lehman, J.T. 1976. Ecological and nutritional studies on *Dinobryon* Ehrenb.: Seasonal periodicity and the phosphate toxicity problem. *Limnology and Oceanography* 21: 646-658.
- Lehman, J.T. 1980. Release and cycling of nutrients between planktonic algae and herbivores. *Limnology and Oceanography*. 25: 620-632.
- Lehman, J.T. and Sandgren, C.D. 1985. Species-specific rates of growth and grazing loss among freshwater algae. *Limnology and Oceanography*. 30: 34-46.
- Lehman, J.T. 1987. Palearctic predator invades North American Great Lakes. *Oecologia*. 74: 478-480.
- Li, H., Gong, G. and Hsiung, T. 2002. Phytoplankton pigment analysis by HPLC and its application in algal community investigations. *Botanical Bulletin of Academia Sinica*. 43:283-290.
- Lionard, M., Mulaert, K., Tackx, M. and Vyverman, W. 2008. Evaluation of the performance of HPLC-CHEMTAX analysis for determining phytoplankton biomass and composition in a turbid estuary (Schelde, Belgium). *Estuarine, Coastal and Shelf Science*. 76: 809-817.
- Llewellyn, C.A., Fishwick, J.R. and Blackford, J.C. 2005. Phytoplankton community assemblage in the English Channel: a comparison using chlorophyll *a* derived from HPLC-CHEMTAX and carbon derived from microscopy cell counts. *Journal of Plankton Research*. 27: 103-119.
- Mackey, M.D., Mackey, D.J., Higgins, H.W., and Wright, S.W. 1996 CHEMTAX – a program for estimating class abundances from chemical markers: application to HPLC measurements of phytoplankton. *Marine Ecology Progress Series*. 144: 265-283.
- Meyer-Harms, B. and von Bodungen, B. 1997. Taxon-specific ingestion rates of natural phytoplankton by calanoid copepods in an estuarine environment (Pomeranian

- Bight, Baltic Sea) determined by cell counts and HPLC analyses of marker pigments. *Marine Ecology Progress Series*. 153:181-190.
- Mida, J.L., Scavia, D., Fahnenstiel, G.L., Pothoven, S.A., Vanderploeg, H.A. and Dolan, D.M. 2010. Long-term and recent changes in southern Lake Michigan water quality with implications for present trophic status. *Journal of Great Lakes Research*. 36: 42-49.
- Millie, D.F., Fahnenstiel, G.L., Lohrenz, S.E., Carrick, H.J., Johengen, T.H. and Schofield, O.M.E. 2003. Physical-biological coupling in southern Lake Michigan: influence of episodic sediment resuspension on phytoplankton. *Aquatic Ecology*. 37: 493-408.
- Millie, D.F., Fahnenstiel, G.L., Dyble-Bressie, J., Pigg, R.J., Rediske, R.R., Klarer, D.M., Tester, P.A. and Litaker, R.W. 2009. Late-summer phytoplankton in western Lake Erie (Laurentian Great Lakes): Bloom distributions, toxicity, and environmental influences. *Aquatic Ecology*. 43: 915-934.
- Montagnes, D.J.S., Berges, J.A., Harrison, P.J. and Taylor, F.J.R. 1994. Estimating carbon, nitrogen, protein, and chlorophyll *a* from volume in marine phytoplankton. *Limnology and Oceanography*. 39: 1044-1060.
- Nygaard, G. 1976. *Tavlerne fra "Dansk Planteplankton"*. Gylendal, Denmark.
- NOAA. 2008. Updated heavy rainfall and flooding information from early June. http://www.crh.noaa.gov/news/display_cmsstory.php?wfo=mkx&storyid=14030&source=0. Accessed November 26, 2012.
- Parsons, T.R., Miata, Y. and Lalli, C.M. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press.
- Pinckney, J.L., Millie, D.F., Howe, K.E., Paerl, H.W. and Hurley, J.P. 1996. Flow scintillation counting of ¹⁴C-labeled microalgal photosynthetic pigments. *Journal of Plankton Research*. 18: 1867-1810.
- Pinckney, J.L., Richardson, T.L., Millie, D.F. and Paerl, H.W. 2001. Application of photopigment biomarkers for quantifying microalgal community composition and in situ growth rates. *Organic Geochemistry*. 32: 585-595.
- Prepejchal, W., Marshall, J.S., Hall, G.B. and Thommes, M.M. 1981. Radiological and environmental research division annual report (ANL-80-115 Part III). Illinois: Argonne National Laboratories.
- Prescott, G.W. 1951. *Algae of the western Great Lakes area*. Otto Koeltz Science Publishers, West Germany.

- Quiblier-Llobéras, C., Bourdier, G. and Amblard, C. 1996. A qualitative study of zooplankton grazing in oligo-mesotrophic lake using phytoplanktonic pigments as organic markers. *Limnology and Oceanography*. 41: 1767-1779.
- Reynolds, C. 2006. *The ecology of phytoplankton*. Cambridge University Press, New York.
- Sandgren, C.D. and Robinson, J.V. 1984. A stratified sampling approach to compensating for non-random sedimentation of phytoplankton cells in inverted microscope settling chambers. *British Phycological Journal*. 19: 67-72.
- Sandgren, C.D., Hall, S.A. and Barlow, S.B. 1996. Siliceous scale production in Chrysophyte algae. I. Effects of silica-limited growth on content, scale morphology, and the constant scale layer of *Synura petersenii*. *Journal of Phycology*. 32: 675-692.
- Sarmiento, H. and Descy, J.P. 2008. Use of marker pigments and functional groups for assessing the status phytoplankton assemblages in lakes. *Journal of Applied Phycology*. 20: 1001-1011.
- Sarnelle, O. 1993. Herbivore effects on phytoplankton succession in a eutrophic lake. *Ecological Monographs*. 63: 129-149.
- Schindler, D.W. 1974. Eutrophication and recovery in experimental lakes: Implications for lake management. *Science*. 184: 897-899.
- Schlüter, L., Møhlenberg, F., Havskum, H. and Larsen, S. 2000. The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll *a* ratios. *Marine Ecology Progress Series*. 192: 49-63.
- Schlüter, L. and Møhlenberg, F. 2003. Detecting presence of phytoplankton groups with non-specific pigment signatures. *Journal of Applied Phycology*. 15: 462-476.
- Schmid, H., Bauer, F. and Stich, H.B. 1998. Determination of algal biomass with HPLC pigment analysis from lakes of different trophic state in comparison to microscopically measured biomass. *Journal of Plankton Research*. 20: 1651-1661.
- Stanier, R.Y., Kunisawa, R., Mandel, M. And Cohen-Bazire, G. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews*. 35: 171-205.
- Sterner, R.W. 1986. Herbivores' direct and indirect effects on algal populations. *Science*. 231: 605-607.

- Sterner, R.W. 1990. The ratio of nitrogen to phosphorus resupplied by herbivores: Zooplankton and the algal competitive arena. *The American Naturalist*. 136: 209-229.
- Sterner, R.W. and Elser, J.J. 2002. *Ecological stoichiometry: The biology of elements from molecules to the biosphere*. Princeton University Press, New Jersey.
- Suthers, I.M. and Rissick, D. 2009. *Plankton: A guide to their ecology and monitoring for water quality*. CSIRO Publishing, Australia.
- Suzuki, R. and Fujita, Y. 1986. Chlorophyll decomposition in *Skeletonema costatum*: a problem in chlorophyll determination of water samples. *Marine Ecology Progress Series*. 28:81-85.
- Thys, I., Leporcq, B. and Descy, J.P. 2003. Seasonal shifts in phytoplankton ingestion by *Daphnia galeata*, assessed by analysis of marker pigments. *Journal of Plankton Research* 25(12): 1471-1484.
- Tilman, D., Kilham, S. and Kilham, P. 1982. Phytoplankton community ecology: the role of limiting nutrients. *Annual Review of Ecology and Systematics*. 13: 349-372.
- USGS. 2008. USGS surface-water monthly statistics for the nation. http://waterdata.usgs.gov/nwis/monthly?site_no=04087170&agency_cd=USGS&por_04087170_10=1353063,00060,10,1994-04,2011-09&referred_module=sw&format=html_table. Accessed December 6, 2012.
- Vanni, M.J. 1987. Effects of nutrients and zooplankton size on the structure of a phytoplankton community. *Ecology* 68: 624-635.
- Wilhelm, C., Volkmar, P., Lohmann, C., Becker, A. and Meyer, M. 1995. The HPLC-aided pigment analysis of phytoplankton cells as a powerful tool in water quality control. *Journal of Water Supply: Research and Technology - Aqua*. 44: 132-141.
- Zar, J.H. 2009. *Biostatistical analysis, 5th ed.* Prentice Hall.

Appendix A

HPLC methods used in development

- A. Original method of Pinckney et al. (1996). Solution A: 80% methanol:20% 0.5M ammonium acetate; Solvent B: 80% methanol:20% acetone.

Time (minutes)	% Solution A	% Solution B	Flow rate (mL min ⁻¹)
0	100	0	0.80
5	50	50	0.80
35	0	100	1.25
40	0	100	1.5
41	0	100	0.80
44	100	0	0.80
55	100	0	0.80

- B. Method used in the present study. This method was modified from the method shown above. Full details of method described in main body of thesis under “Methodology.”

Time (minutes, post-injection)	% Solution A	% Solution B	Process
0	100	0	Injection; equilibration
2	100	0	Start gradient
7	72	28	
17	56	44	
30	48	52	
64	0	100	HOLD
66	0	100	Start recovery gradient
70	25	75	
75	100	0	End gradient; start re-equilibration
82	100	0	STOP

Appendix B

Comparison of published and calculated extinction coefficients

Prior to analyzing pigment data with CHEMTAX, data must be treated with a correction for extinction coefficient to accommodate the detection wavelength of the method. Values below are provided for comparison of published values found within Jeffrey et al. pigment data sheets (1997) with the exception of the starred datum, found in Higgins et al. (2011) and those calculated from reference culture spectra. The calculation for extinction coefficient used was:

$$\text{Published extinction coefficient} \times \frac{\text{maU at 440 nm}}{\text{maU at published coefficient } \lambda}$$

Pigment	Solvent for published value	Published coefficient (L *g ⁻¹ *cm ⁻¹)	Calculated at 440 nm (L *g ⁻¹ *cm ⁻¹)
Chlorophyll <i>c</i> ₁ / <i>c</i> ₂	90% acetone + 1% pyridine	318	297.76
Chlorophyll <i>c</i> ₂	90% acetone + 1% pyridine	374	348.21
Peridinin	Methanol	136	110.21
Fucoxanthin	Acetone	166	161.11
Neoxanthin	Ethanol	227	218.97
Violaxanthin	Acetone	240	264.35
Dinoxanthin	Acetone	210	231.00
Diadinoxanthin	Methanol	225	210.94
Lutein*	Methanol	22.1	20.90
Alloxanthin	Acetone	250	220.49
Zeaxanthin	Acetone	234	204.38
Chlorophyll <i>b</i>	90% acetone	51.36	68.48
Chlorophyll <i>a</i>	90% acetone	87.67	69.03
β,ε-carotene	Acetone	250	222.50
β,β-carotene	Acetone	270	264.13