

AN EXAMINATION OF THE CELLULAR PARTITIONING OF PHOSPHORUS IN
FRESHWATER PHYTOPLANKTON

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A Thesis

Submitted to the Graduate College of Bowling Green
State University in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

MASTER OF ARTS IN TEACHING

December 2009

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ABSTRACT

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This study evaluated the wide-scale applicability of the Redfield ratio, which is a measure of the molar relationship of the macronutrients carbon, nitrogen, and phosphorus (C:N:P) in plankton. By measuring the elemental stoichiometry of phytoplankton, we can gain insights into the availability of nutrients in the water column. Several lines of evidence reported in recent years have given cause to reexamine the ubiquity of the Redfield ratio. Among these is the observation that a pool of cellular phosphorus is adsorbed to the cell exterior and may not be readily available for assimilation by the cells. With this study, we aimed to assess the cellular allocation of phosphorus along a trophic continuum of lakes in Ohio and Indiana including two inland lakes as well as coastal and pelagic locations in Lake Erie ranging from eutrophic to oligotrophic in status. Biomass as gauged by extractive chlorophyll was consistent with our designation of lake trophic status established prior to the survey with Nettle Lake and a nearshore station in Maumee Bay (MB20) supporting the highest chlorophyll levels whereas Crooked Lake and the eastern basin of Lake Erie were characterized as low chlorophyll sites. Despite the identifiable gradient in trophic status, total dissolved phosphate was relatively low at all locations yet not all indicators of cellular phosphorus status were indicative of P deficiency. For example, only at the two oligotrophic sites (Lake Erie station 452 and Crooked Lake, IN) were cellular N:P and C:P ratios suggestive of P deficiency using Redfield stoichiometry as a guide. Further, alkaline phosphatase activity was negligible at the Lake Erie stations sampled during February, 2007. In our limited survey, partitioning of cellular P correlated to lake trophic status with intracellular P representing a lower percentage of total P (14, 57%) at the two

oligotrophic sites (Crooked Lake and Erie station 452, respectively) compared to mesotrophic and eutrophic sites where intracellular P ranged from 70-95% (median: 82%) of the total cellular allocation. Our confirmation that substantial amounts of P are allocated to the cell surface and that the P allocation may vary with lake trophic status lends further evidence in support of a broader reexamination of the Redfield ratio in its broad scale application to assess nutrient status of phytoplankton.

I dedicate this thesis to my Dad.

ACKNOWLEDGMENTS

I want to express my gratitude to all of the people who helped me with my project and without whom this manuscript would not be possible.

I would like to also acknowledge individuals who provided sampling support:

Maumee Bay: Dr. Tom Bridgeman (University of Toledo Lake Erie Center), Lake Erie: Environment Canada and the captain and crew of *CCGS Griffon*, Crooked Lake: personnel from the Indiana DNR, Division of Fish and Wildlife, Columbia City District Office, and for Nettle Lake: Mr. Jim Short, participant with the Citizens Lake Awareness and Monitoring program of the Ohio Lake Management Society. Financial support for this project was provided by Ohio Sea Grant (R/ER-071).

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INTRODUCTION

Our freshwater supply is arguably the most important natural resource on this planet; its unequal distribution serving as the basis for present and possible future conflict (UNESCO, 2009). Even in areas with seemingly abundant supplies of freshwater, agricultural, residential and commercial activities can compromise water quality. Federal, state and municipal entities are charged with monitoring water quality. In doing so, they utilize multiple approaches, usually guided by the concerns specific to a particular region. Among these approaches, microorganisms can serve as bioindicators of water quality (Terrell and Perfetti, 1989). This study investigates the usage of the Redfield ratio in this capacity. The Redfield ratio is a measure of the relationship between the macronutrients carbon, nitrogen, and phosphorus (C:N:P) in aquatic ecosystems.

Phytoplankton, as important primary producers, are positioned at the base of the food web and can serve as important bioindicators of water quality (Lowe and Pillsbury, 1995). Fueled by their photosynthetic capacity, they are ascribed as converters of inorganic elements to essential organic forms. By investigating the resulting elemental stoichiometry of phytoplankton, we can gain insights into the efficiency of this process, and indirectly into the availability of nutrients in the water column. As early as the 1930's, researchers began to gain insight into the relationship between phytoplankton elemental stoichiometry and dissolved concentrations of nutrients in water (Redfield, 1934). From this work comes the *Redfield Ratio*, a stoichiometric representation of the molar ratios of dissolved macronutrients carbon, nitrogen, and phosphorus (106 C: 16 N: 1P) required for optimal phytoplankton growth (Redfield, 1958). While originally developed to constrain the relationship between open ocean phytoplankton and dissolved nutrients, the ratio has since been adopted “universally” serving as a proxy for determining the nutrient status of phytoplankton in diverse environments (Hecky et. al. 1993; Tyrell, 1999).

Whereas the Redfield Ratio still serves as an important tool, guiding our understanding of phytoplankton – nutrient relationships, several investigations in recent years have highlighted stoichiometric plasticity demonstrated on geological, spatial, temporal, and taxonomic scales (Falkowski 2000; Geider and La Roche 2002) thus sparking an interest in reexamining the foundations of the Redfield ratio.

Foundations of the Redfield Ratio

Alfred C. Redfield proposed that phytoplankton elemental ratios were proportionately constrained (1934) after conducting chemical analysis of ‘plankton’ in the Atlantic. He observed the ratio of dissolved fixed inorganic nitrogen (nitrate) was constant regardless of the bulk N:P ratio of plankton. The applied principle he used was that, since biota are built from nitrogen, hydrogen, carbon, and oxygen with metals (sodium, potassium, and iron) and nonmetals (chlorine, sulfur, and phosphorus), the quantities of nutrients in the ocean must be a result of the demand by the inhabiting biota exclusively in the euphotic zone (Redfield, 1934).

A summary of the conclusions drawn from Redfield’s work, combined with the work of others at the time, have suggested either that plankton responded adaptively to their environment (Darwinian), plankton determined their environment (non-Darwinian), or that the correlations between the macronutrients and plankton were coincidental (Klausmier et. al. 2008). Regardless of hypothetical posture, any nutrient supplied by the environment that influenced the growth of phytoplankton would influence its stoichiometric content (Sterner & Elser, 2002). When not nutritionally limited, the elemental stoichiometry of phytoplankton mirrors that which Redfield observed (Goldman et. al., 1979). Given that the basic needs are met for photoautotrophs (i.e.

nutrients, light, amenable temperature), the Redfield ratio has also been adopted for use with freshwater phytoplankton (Kilham, 1990; Hecky et. al., 1993).

General Considerations for Nutrient Cycles

Before a more detailed discussion of stoichiometric plasticity, it is important to consider how a water system is classified and how macronutrients are distributed within the system. One of the Laurentian Great Lakes, Lake Erie, was of particular interest for this study because it spans the trophic continuum, from the shallow, eutrophic (high nutrient) western basin and its associated embayments, to a mesotrophic (moderate nutrient) central basin and an oligotrophic (nutrient depleted) eastern basin (Fig. 1). Nutrient cycling is another important characteristic of inland water systems. All three study areas are influenced to varying extent by encroaching agriculture. The macronutrients carbon, nitrogen, and phosphorus receive a great deal of attention because these are the main components of the cell serving as the building blocks of the cellular machinery (e.g. RNA, DNA, ribosomes, proteins). Whereas carbon is derived ultimately from the atmosphere via photosynthetic carbon fixation, its cycling also includes degradation products of the food web from both autochthonous and allochthonous sources. Nitrogen inputs originate atmospherically, however, specialized organisms including diazotrophs such as cyanobacteria, facilitate its entry into the trophic pyramid. Other nitrogen sources are agricultural run-off and food chain recycling. Phosphorus is ultimately derived from weathering of rock. In the food web, its availability is augmented by recycling as well as agricultural run-off and industrial inputs.

Whereas many factors – chemical, physical and biological – can influence phytoplankton abundance, productivity is influenced mainly by light and nutrient availability, both of which can

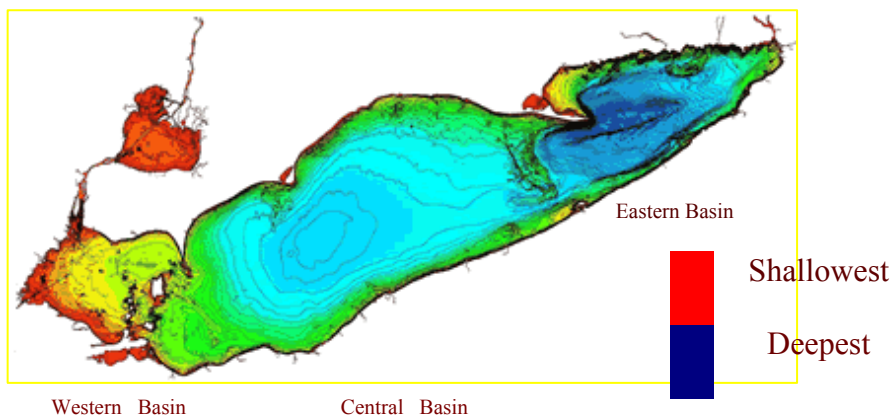


Figure 1. Lake Erie Bathymetry. A geospatial, two dimensional image showing the orientation of Lake Erie and the ridges which separate its three basins. Modified from: www.glerl.noaa.gov/.../1998/ppreid01-1.html.

be influenced by resource competition between species or water column dynamics (i.e. circulation and mixing, residence time, sedimentation, etc.). Although, the variability in elemental stoichiometry reported can be large, the Redfield ratio is commonly adopted because of its widespread use as a tool to model water biogeochemistry (Pahlow and Riebesell, 2000; Lenton and Klausmier, 2006). Goldman et al. (1979) found the elemental ratio, which approximated Redfield values, of marine plankton actually indicated nutrient-sufficient status and near optimal growth conditions. When phytoplankton are not stressed nutritionally, their molar elemental ratios are approximately the Redfield ratio of 106 C:16 N:1 P and, therefore, Redfield stoichiometry reflects optimal growth conditions for phytoplankton (Goldman, 1986; Goldman et al, 1979). The ratio of 16 N:1 P (>16 = P-limited growth, <16 = N-limited growth) was accepted as the threshold for optimal nutrient sequestration by phytoplankton (Falkowski, 1997; Tyrrell, 1999; Lenton and Watson, 2000). However, significant departures from this canonical threshold have been observed in phytoplankton as a response to nutrient stress (Guilford and Hecky 2000). Geider and La Roche (2002) found the critical ratio of N:P (transition point) to be as high as 50:1.

MATERIALS AND METHODS

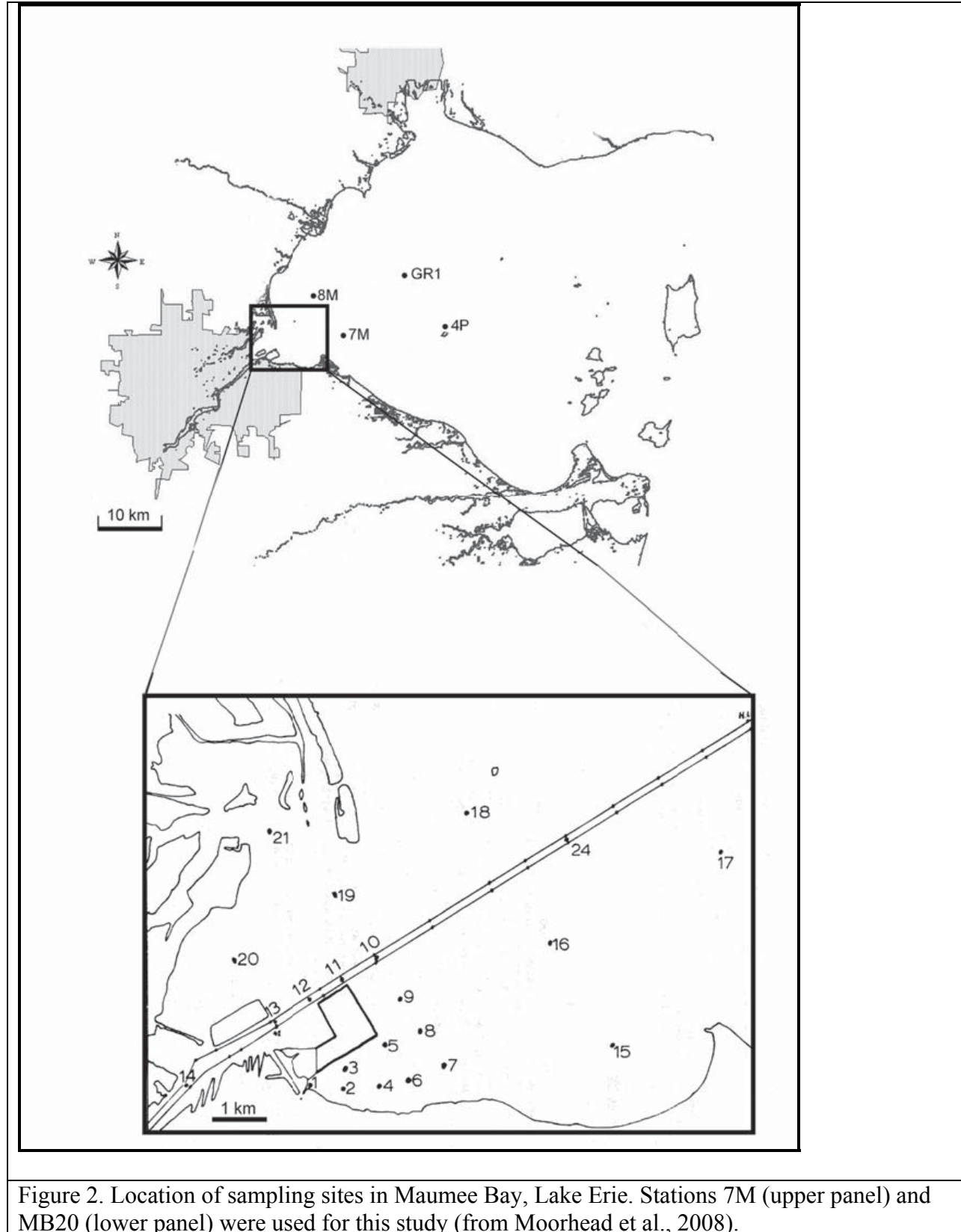
Sample collection.

Samples were collected from several freshwater lakes, chosen primarily for their trophic classification (Carlson and Smith, 2001). The smaller inland lakes chosen represent end members of a trophic continuum with Crooked Lake, IN serving as an oligotrophic member and Nettle Lake, OH representing a eutrophic lake. Sampling was also conducted through all three basins of Lake Erie which itself spans several trophic levels ranging from eutrophic in some embayments (Maumee Bay and Sandusky Bay) to verging on oligotrophic in the eastern basin. Water chemistry was analyzed by the National Center for Water Quality Research (Heidelberg University) to determine total dissolved (TD) nutrients: nitrogen (TDN), nitrate + nitrite (NO_3^-), ammonia (NH_3), phosphate (TDP) and silicic acid (SiO_2). Particulate nutrient data were derived from seston collected on filters for analysis of carbon, hydrogen, and nitrogen (CHN) and particulate phosphorus as orthophosphate (PP). Alkaline phosphatase activity (APase) was used as an indicator for phosphorus stress by the endemic phytoplankton.

Study Areas

Lake Erie: Maumee Bay

Sampling of Maumee Bay located at the western end of Lake Erie (Fig. 2) was conducted on June 12, 2007 through cooperation with Dr. Tom Bridgeman (University of Toledo Lake Erie Center) on board the R/V *Mayflyer*. Using a Van Dorn sampling bottle, three separate casts were made to 2 m depth. The water was passed through a 210 μm screen to remove larger zooplankton before filling 4 L plastic amber bottles. Water samples were stored in a cooler in darkness prior to processing later that same day.



Stations MB20 (41° 42.90' N; 83° 27.32' W) and 7M (41° 43.97' N; 83° 17.78' W) were sampled as part of this effort. The sample sites were so chosen as to represent a gradient in decreasing influence from the Maumee River. Prior to sampling at each site, a YSI V2 sonde (model 6025; YSI Inc.) was deployed to provide a measure of conductivity, temperature, pH, turbidity, and *in situ* chlorophyll fluorescence. Upon returning to the lab, samples were assayed for alkaline phosphatase activity, elemental analysis of seston (CHN and PP) and size-fractionated chlorophyll (chl) *a*.

Lake Erie: Lakewide

Samples were collected at Environment Canada hydrographic stations between February 21-23, 2007 on the icebreaker CCGS *Griffon*. Stations occupied were distributed in each of the three basins of the lake (Fig. 3). Water column samples were collected using a 10 L Niskin bottle on a metered deck winch and by use of a submersible pump deployed 1 m below the surface. Triplicate samples from each station were filtered onto pre-combusted GF/F filters (Whatman) for CHN analysis and particulate phosphorus (PP) was measured as described and used previously (Tovar-Sanchez *et al.* 2003, Sañudo-Wilhelmy *et al.* 2004). Samples were processed for alkaline phosphatase activity on board immediately for determination of P nutrient stress.

Crooked Lake (Noble and Whitley County, IN)

Samples were collected from Crooked Lake during May, 2007 (Fig. 4) through coordination with scientists at the Indiana Department of Natural Resources (DNR; Columbia City District Office). Triplicate samples were collected at 3 m depth from a single site in Crooked Lake (Wetzel Site A). The samples were screened through a 153 μ m mesh in order to remove larger zooplankton. Samples were stored in a cooler in darkness prior to processing later the same day. Samples were assayed for alkaline phosphatase activity, elemental analysis of

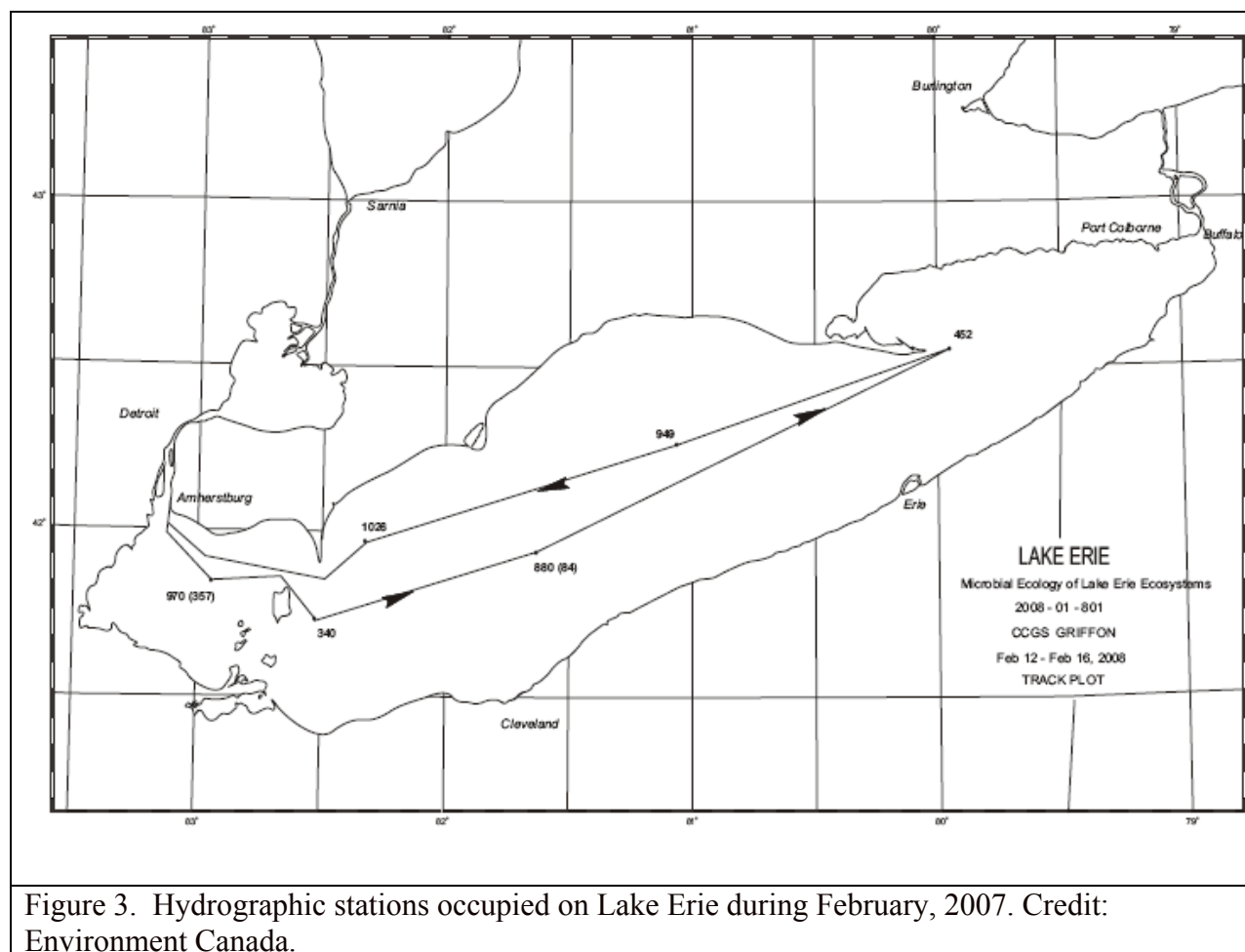
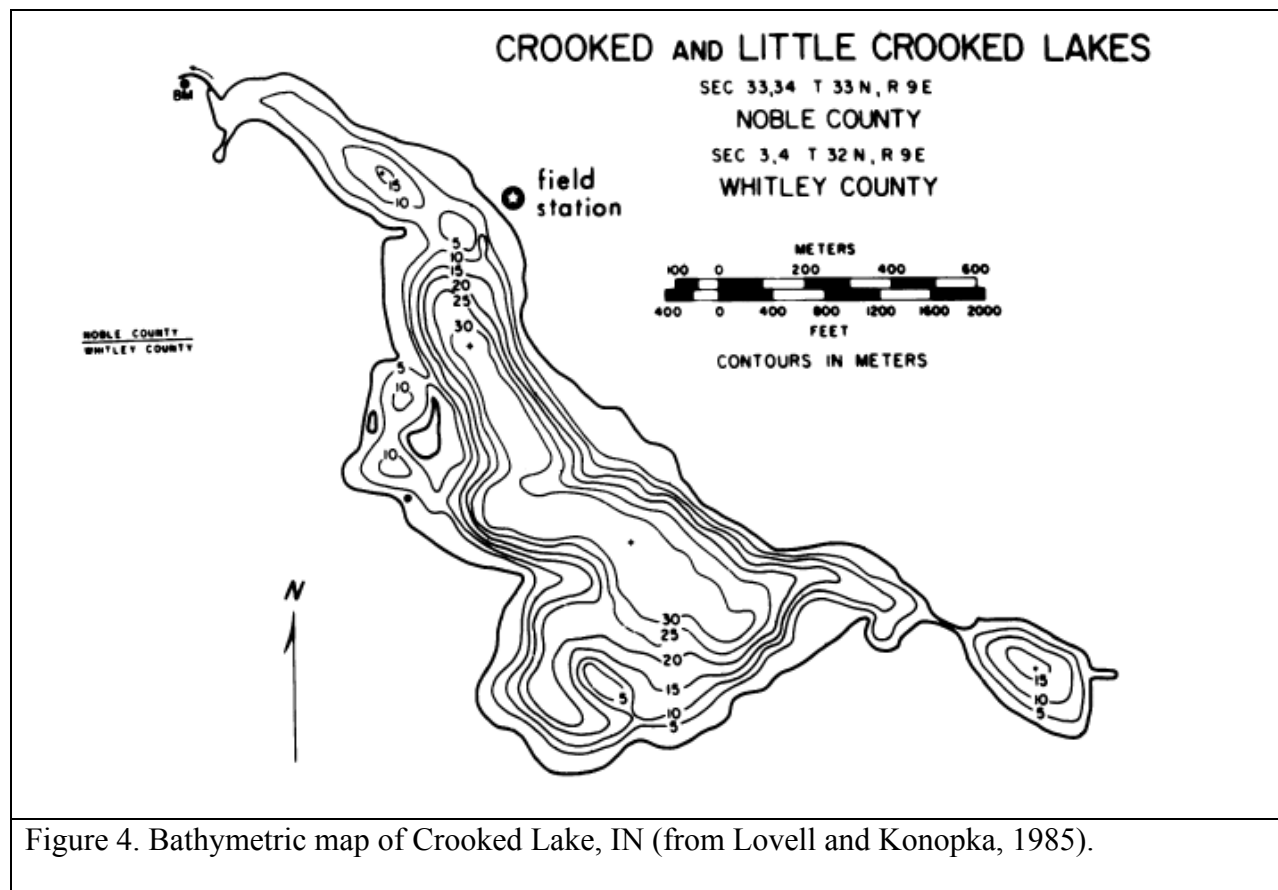


Figure 3. Hydrographic stations occupied on Lake Erie during February, 2007. Credit: Environment Canada.



seston (CHN and particulate phosphorus (PP)) and size-fractionated chlorophyll (chl) *a*.

Nettle Lake (Williams County, OH)

Sampling at Nettle Lake was coordinated in May 2007 with a citizen volunteer associated with the Citizens Lake Awareness and Monitoring (CLAM) program through the Ohio Lake Management Society (<http://dipin.kent.edu/CLAM/clampage.htm>). Site 1 was located in the north embayment of the lake (Fig. 5). Samples were processed for alkaline phosphatase activity, seston elemental analysis (CHN and PP) and chl *a*. In addition, an Endogenous Phosphatase Detection kit (ELF 97; Molecular Probes) was used to conduct an enzyme labeled fluorescent assay in order to visualize cell-specific phosphatase activity.

Chemical Analysis

Particulate Phosphorus Determination

GF/F filters (25 mm, Whatman) were ashed at 400° C for 4 h prior to use. Pre-combusted filters were prepared for filtration by first rinsing the filter cup with 1% HCl followed by a milli-Q water rinse. The filters were incubated with oxalate reagent (2-3 mL; Appendix A) for 1 min followed by another rinse with milli-Q H₂O. Water samples (50 ml- 300 ml) were filtered until approximately 5 ml of volume remained at which time an equal volume of oxalate reagent was added and incubated for 5 min to remove surface bound phosphate as described elsewhere (Sanudo-Wilhelmy *et al.* 2004). The oxalate rinse was prepared based on that described in Tovar-Sanchez *et al.* (2003, 2004) but with reduced salinity for use with freshwater plankton. As necessary, a ‘top-up’ solution (1:1 mixture of oxalate reagent and filtered lakewater) was added to prevent the filter from drying during incubation. Treated samples were stored at -20 °C until they were analyzed. Parallel samples were processed in the same manner but without incubation with oxalate reagent. For each set of samples, several filter blanks were processed by using

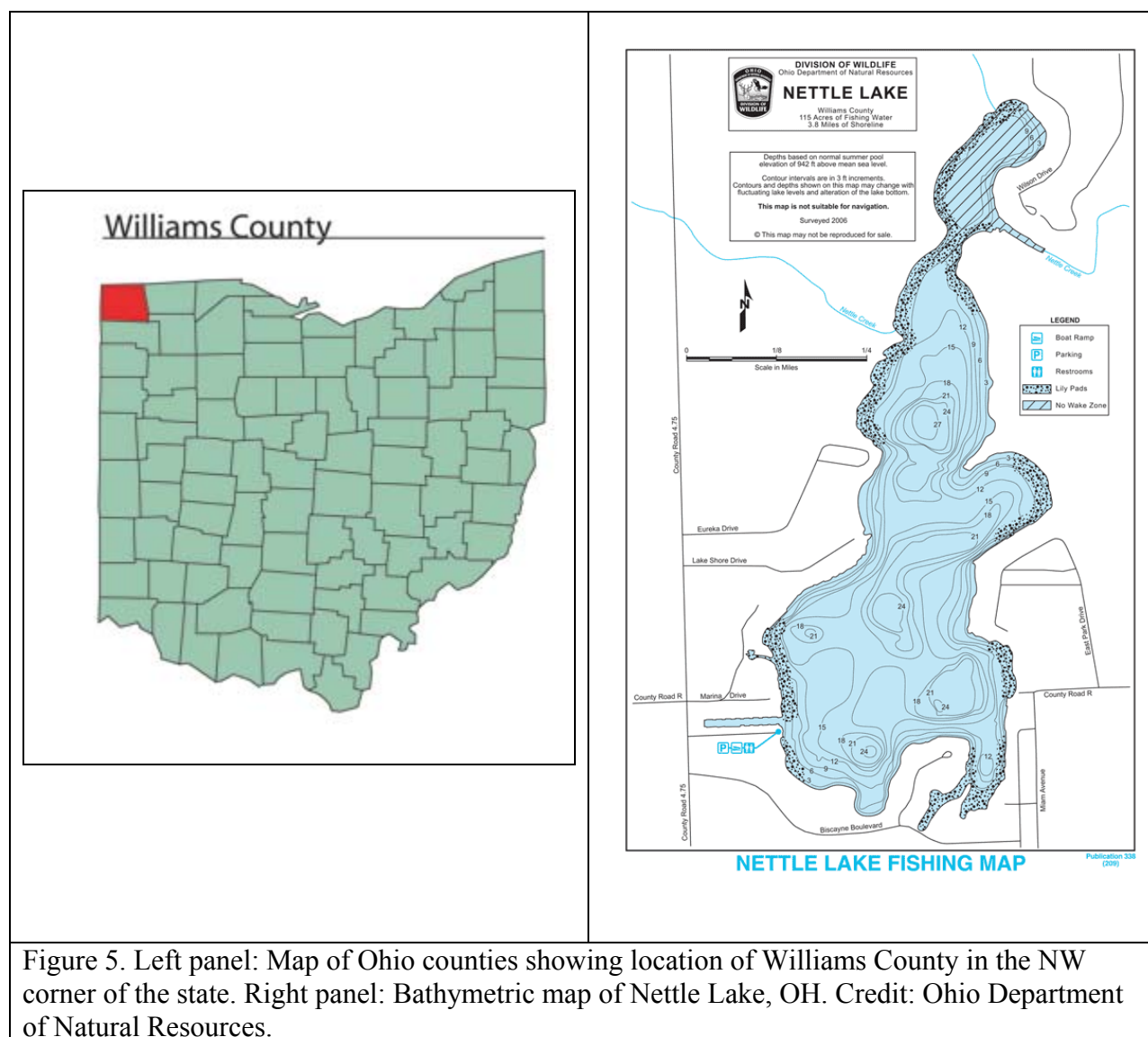


Figure 5. Left panel: Map of Ohio counties showing location of Williams County in the NW corner of the state. Right panel: Bathymetric map of Nettle Lake, OH. Credit: Ohio Department of Natural Resources.

filtered lakewater in place of the sample.

Prepared samples collected on GF/F filters were digested by adding 5% potassium persulfate and autoclaving for 30 min (Appendix B). Liberated SRP was analyzed colorimetrically using the molybdate ascorbic acid method (Strickland & Parsons, 1972) using a 10-cm path length quartz cell and a Cary 50 UV-Vis spectrophotometer (Varian) scanning from 870 nm to 890 nm in order to capture maximum absorption of orthophosphate at ~ 880 nm. For calibration, phosphate reference standards of known concentration were measured along with NIST Standard Reference Material (Bovine Muscle Powder, NIST SRM 8414).

Assessing Cellular Phosphorus Stress

Alkaline phosphatase (APase, E.C. 3.1.3.1.) is an extracellular enzyme capable of cleaving the phosphate oxyanion so that free phosphate may be acquired for cellular metabolism. Alkaline phosphatase activity of field samples was measured fluorometrically using 4-methylumbelliferyl phosphate (MUBP; Sigma) as substrate for the enzyme (Sinsabaugh *et al.* 1997; DeBruyn *et al.*, 2004; Sterner *et al.*, 2004). When cells under P deficiency were incubated with MUBP, the enzyme cleaves the phosphate group and results in an increased fluorescence of methylumbelliferone. The field samples were distributed to triplicate methacrylate cuvettes and incubated with 50 μ M MUBP in darkness. Sodium bicarbonate (4 mM) was substituted for sample in substrate controls whereas quench samples were prepared using sample and 4-methylumbelliferone. APase-catalyzed fluorescence was monitored at timed intervals of incubation (ambient lab temperature, ~23 °C) using a TD-700 laboratory fluorometer (Turner Designs) equipped with a near UV lamp and a methylumbelliferyl filter set (λ_{ex} : 300-400 nm; λ_{em} : 410-610 nm). Enzyme activities (normalized to chl *a*) were calculated using a 4-methylumbelliferone reference standard.

Enzyme-Labeled Fluorescence

Individual cells were analyzed for phosphorus deficiency by enzyme-labeled fluorescence (Dyhrman and Palenik, 1999). Endogenous phosphatase activity was detected using the ELF[®] 97 kit (Molecular Probes), which allowed for cell-specific visualization of alkaline phosphatase activity by way of enzyme-labeled fluorescence (ELF). A suitable aliquot of sample was taken to achieve a representative pellet of phytoplankton by gentle filtration on 0.22 μm polycarbonate filters (Millipore). The cells were resuspended and transferred into an Eppendorf tube and preserved using 70% EtOH at 4 °C in complete darkness overnight. The following day, the cells were resuspended and centrifuged at $5,000 \times g$ for 5 min. The supernatant (EtOH) was removed and cells were rinsed with filtered (0.2 μm) lakewater. The ELF[®] 97 buffer-stain mix (100 μl) was added and allowed to incubate with cells for 30 min. At the end of the incubation period, the cells were centrifuged and excess stain removed. The cells were rinsed with filtered lakewater 3 \times and spread on poly L-lysine-coated slides (Sigma) using a slide centrifuge (StatSpin; Iris Sample Processing). The prepared cells were examined using a Zeiss Axiophot microscope equipped with a mercury lamp and DAPI filter set. Cells were recorded as either fluorescing or not fluorescing (Fig. 6).

Seston Stoichiometry: Carbon, Hydrogen and Nitrogen (CHN)

Samples for CHN analysis were collected by gentle vacuum filtration (< 65 mm Hg) onto pre-combusted GF/F filters and stored frozen at -20° C. Blanks were processed alongside the field samples. Samples were analyzed via collaboration with Dr. R. Sterner (University of Minnesota) using a Perkin Elmer 2400 CHN analyzer.

Chlorophyll a

Extractive chlorophyll (chl) *a* pigment is used as a proxy for measuring planktonic biomass in a sample. Seston was size-fractionated through polycarbonate membranes of 0.2, 2 and 20 μm pore size. The seston-containing filters were transferred to polyethylene tubes and extracted overnight at -20°C using 10 ml of 90% acetone (in MgCO_3 -saturated water).

Chlorophyll *a* content was determined fluorometrically (Welschmeyer, 1994) using a TD-700 fluorometer (Turner Designs).

RESULTS

Biomass as gauged by extractive chlorophyll was consistent with our designation of lake trophic status established prior to the survey with Nettle Lake ($32.6 \mu\text{g chl L}^{-1}$) and a nearshore station in Maumee Bay (MB20; $43.7 \mu\text{g chl L}^{-1}$) supporting the highest chlorophyll levels whereas Crooked Lake ($2 \mu\text{g L}^{-1}$) and the eastern basin of Lake Erie ($< 1 \mu\text{g chl L}^{-1}$) were characterized as low chlorophyll sites (Table 1). Despite the identifiable gradient in trophic status, total dissolved phosphate was relatively low at all locations ranging from $1\text{-}16 \mu\text{g L}^{-1}$ (Table 2).

The inland lakes sampled included a eutrophic representative (Nettle Lake) and an oligotrophic representative (Crooked Lake). Of all the lakes sampled, eutrophic Nettle Lake was most depleted of TDP whereas oligotrophic Crooked Lake was depleted of nitrate and silicic acid (Table 2). Community alkaline phosphatase activity was moderate-high at the two lakes suggestive of a P-deficient planktonic assemblage (Table 3). At Nettle Lake, alkaline phosphatase activity was also resolved at the level of individual taxa using enzyme-labeled fluorescence (ELF). ELF staining was widespread among the algal community with representative diatoms and green algae (Fig. 6) as well as dinoflagellates (not shown) clearly stained by ELF.

Seston stoichiometric ratios calculated for Crooked Lake were also consistent with the characterization of a P-stressed assemblage with both N:P and C:P ratios elevated over Redfield (Table 3). Despite the depleted nitrate measured in this lake, seston C:N ratios were only marginally higher than the Redfield ratio of 6.6 (Table 3). Assessment of the allocation of cellular P using the oxalate rinse procedure confirmed the existence of separate surface-adsorbed and intracellular pools of P associated with seston collected from Nettle Lake ($PP > PP_{\text{oxalate}}$; $P < 0.005$, one-tailed t -Test) and from Crooked Lake ($PP > PP_{\text{oxalate}}$; $P < 0.01$, one-tailed t -Test). Further, there existed large differences in the cellular allocation of P between the two inland lakes with intracellular P

Table 1. Collection parameters for winter and spring 2007 sampling.

Lake	Station	Latitude (N)	Longitude (W)	Z _m (m)	Date (2007)	Chl ($\mu\text{g L}^{-1}$)
Erie	357	41° 49.30'	82° 58.30'	11.9	21 Feb	1.2
	340	41° 45.25'	82° 24.08'	11.2		8.4
	84	41° 56.07'	81° 39.11'	22.3	22 Feb	2.4
	452	42° 30.24'	79° 54.00'	64		0.6
Nettle	Site 1	41° 04.43'	85° 08.31'	6	7 May	32.6
Crooked	Wetzel A	41° 15.74'	85° 28.76'	6.4	22 May	2.0
Maumee Bay	7M	41° 43.97'	83° 17.78'	6	12 June	14.3
	MB20	41° 42.90'	83° 27.32'	1.7		43.7

Table 2. Dissolved nutrient data for stations sampled.

Lake	Station	TDP ($\mu\text{g L}^{-1}$)	TP ($\mu\text{g L}^{-1}$)	^a NO ₃ ⁻ (mg L ⁻¹)	NH ₃ ($\mu\text{g L}^{-1}$)	TDN (mg L ⁻¹)	Silica ^b (mg L ⁻¹)
Erie	357	1.6	9.9	0.68	BD ^c	0.87	2.29
	340	1.6	9.9	0.18	BD ^c	0.40	0.46
	84	7.9	16	0.25	BD ^c	0.56	0.89
	452	6.5	15.4	0.29	BD ^c	0.50	0.98
Nettle	Site 1	1	-	0.64	70	0.73	2.77
Crooked	Wetzel A	9.1	-	0.04	17.7	0.37	0.30
Maumee Bay	7M	7.7	-	0.49	4.0	0.55	0.78
	MB20	16	117	0.34	32	0.87	1.61

^anitrate + nitrite^breactive SiO₂^cbelow detection**Table 3.** Particulate nutrient data from seston

Station	POC	PON	PP ($\mu\text{mol L}^{-1}$)	PP _{ox}	C:P(P _{ox})	C:N (mol:mol)	N:P(P _{ox})	APase ^a
357	18.9	1.5	0.17	0.12	115(157.5)	13	8.8(12.5)	0
340	33.7	3.3	0.27	0.22	125(153.18)	10.2	12.3(15)	0
84	18.7	1.4	0.19	0.16	97.6(116.88)	13.5	7.2(8.75)	0
452	9.9	1.2	0.07	0.04	151(247.5)	8	18.9(30)	0
Nettle (Site 1)	-	-	2.4	1.95	-	-	-	22.2 ± 0.9
Crooked (Wetzel A)	25.48	2.82	0.02	0.002	1274(12740)	9.0	141(1410)	9.0 ± 0.6
7M	77.85	8.28	1.08	0.89	72.1(87.47)	9.4	7.7(9.30)	8.6 ± 0.8
MB20	-	-	49.5	47.0	-	-	-	9.4 ± 0.3

^a alkaline phosphatase activity ± SD. (nmol MUF-P $\mu\text{g chl}^{-1} \text{h}^{-1}$)

comprising 81% of the total cellular P pool in Nettle Lake but only 14% in the oligotrophic Crooked Lake (Table 3; Fig. 7).

During the Crooked Lake survey, we also collected water from Little Crooked Lake, a small basin located at the SE extreme of Crooked Lake and connected by a narrow channel (Fig. 4). Little Crooked Lake is eutrophic in character with total chlorophyll measured at $14.5 \mu\text{g L}^{-1}$, and thus about $10 \times$ higher than Crooked Lake. Whereas assay of community alkaline phosphatase suggested a similar degree of P deficiency between both basins, the cellular allocation of P was markedly different with much higher intracellular P measured in cells collected from Little Crooked Lake (87%; data not shown).

Also chosen for sampling was Lake Erie, the smallest of the Great Lakes by volume. Among the Great Lakes, Lake Erie is impacted most heavily by urbanization and agriculture and was the first Great Lake to show evidence of lake-wide eutrophic imbalance characterized by large-scale algal blooms and oxygen depletion. Further, Lake Erie is naturally divided into three basins with each basin occupying a distinct position along the trophic continuum. Since the US EPA (Great Lakes National Program Office) began monitoring the lake in the early 1980's, the shallow western basin, including Maumee Bay has been characterized as varying between eutrophy and mesotrophy whereas the central basin is characterized as oligomesotrophic and the deeper eastern basin as mainly oligotrophic.

Sampling in Lake Erie included two sites in Maumee Bay that have been monitored for several years for water quality and algal blooms and have long term sampling regimens and data sets (Moorhead et al., 2008). Pelagic sites in Lake Erie were sampled as part of a February, 2007 research cruise during which dissolved nutrient levels were high through the entire lake, yet a concentration gradient existed in terms of nitrate (nitrate + nitrite) and silica (dissolved reactive

SiO₂) with levels in the western basin (Sta. 357) exceeding those measured in the central (Sta. 84) and eastern basins (Sta. 452) by 2-3 times (Table 2). Seston stoichiometric ratios indicated a P-sufficient assemblage. N:P ratios were generally <16 whereas C:P ratios were only modestly higher than the Redfield ratio (Table 3). Community alkaline phosphatase activity was negligible (Table 3) further supporting the existence of a P sufficient assemblage. A pool of surface adsorbed phosphorus was evident with the pool varying from 9% of the total cellular P quota in the central basin to as much as 36% in the western basin (Table 3; Fig. 7).

Whereas seston C:N ratios were uniformly higher than the Redfield ratio of 6.6 (Table 3), they are within range of values reported for N-sufficient phytoplankton in lakes. Average dissolved nitrate was $\sim 25 \mu\text{mol L}^{-1}$, which further argues against the likelihood of N deficiency.

Biomass, as gauged by total POC, was ~ 2 -fold lower at the eastern basin site (Sta. 452) in 2007 compared to western and central basin sites (one-way ANOVA; $P < 0.0001$), a trend that was paralleled by changes in chl *a* (Table 3).

In Maumee Bay, there was a gradient in dissolved nutrients with elevated levels of NH₃, TDP and silicic acid at Station MB20, located near the mouth of the Maumee River compared with Station 7M located several miles offshore (Table 2). Seston stoichiometric ratios calculated for the offshore 7M sample indicated a P-sufficient assemblage with both N:P ratios and C:P ratios below Redfield values (Table 3). Contrary to the stoichiometric relationships, community alkaline phosphatase activity was measured at a similar moderate-high level at both Maumee Bay stations (Table 3) with the values corresponding to P deficiency according to the scale introduced by Healey and Hendzel (1979). Assessment of the allocation of cellular P using the oxalate rinse procedure confirmed the existence of separate surface-adsorbed and intracellular pools of P associated with seston collected from the offshore station 7M ($PP > PP_{\text{oxalate}}$; $P <$

0.005, one-tailed *t*-Test), but not from the nearshore site MB20 ($P = 0.064$; $df = 4$). Intracellular P measured in seston collected from Sta. 7M made up more than 80% of total particulate P in the sample (Table 3; Fig. 7).

Similar to the pelagic Lake Erie sites, seston C:N ratios at Station 7M were higher than the Redfield ratio of 6.6 (Table 3), although were within range of values reported for N-sufficient phytoplankton in lakes. High dissolved nitrate at both Maumee Bay sampling sites argues against the likelihood of N deficiency.

Biomass, as gauged by total chl *a*, was $\sim 3\times$ higher at the nearshore site (Sta. MB20), consistent with the elevated nutrient pools measured at this site.

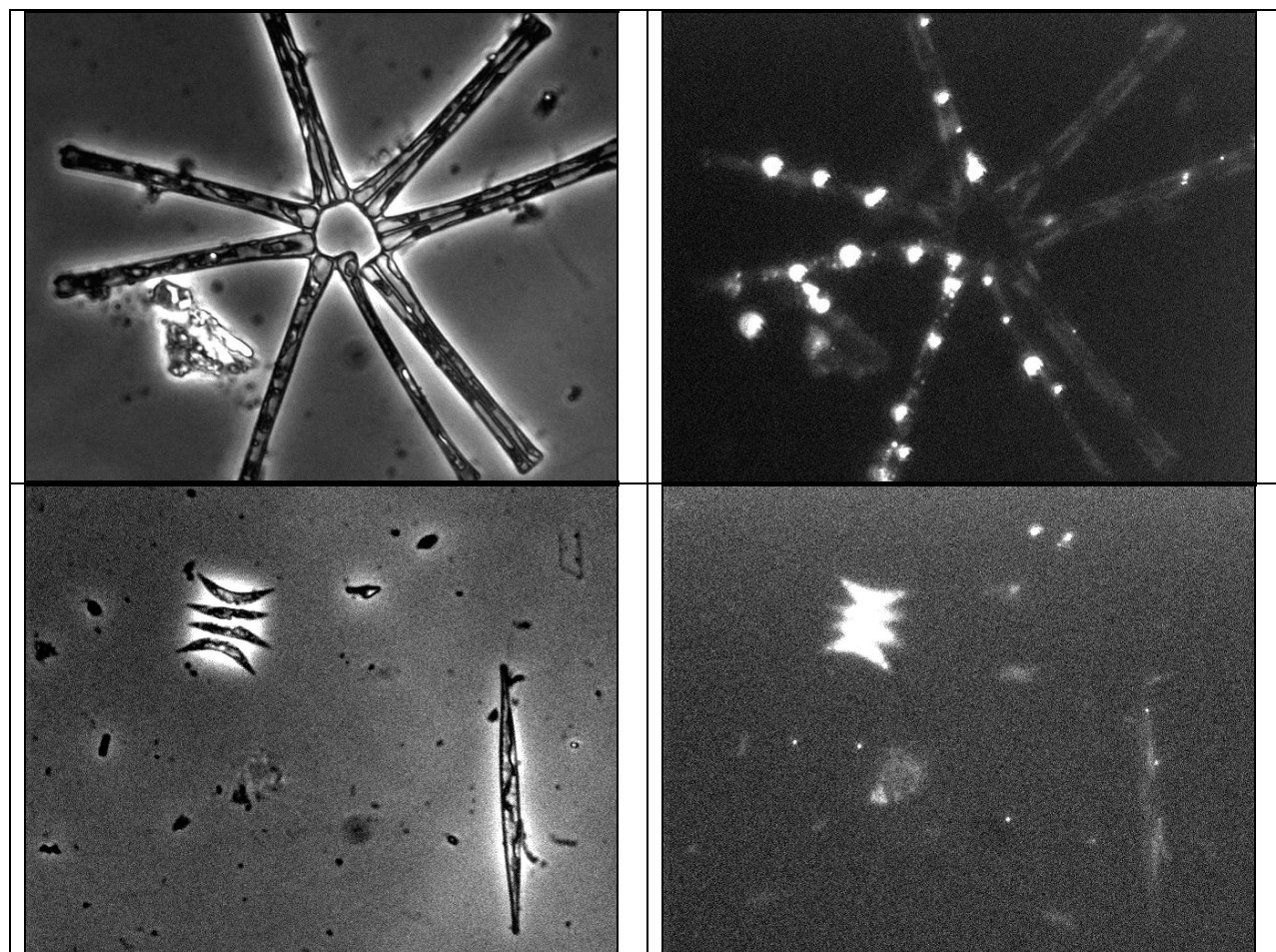


Figure 6. Cells collected from Nettle Lake stained using ELF to visualize cell-surface alkaline phosphatase activity. Top row: The diatom *Asterionella* spp. photographed using bright field (left) and under epifluorescence (right). Staining of alkaline phosphatase activity is shown in discrete patches along some, but not all, cells. Bottom row: The colonial green alga *Scenedesmus* spp. photographed using dark field (left) and under epifluorescence (right). ELF staining is associated with all cells in the colony.

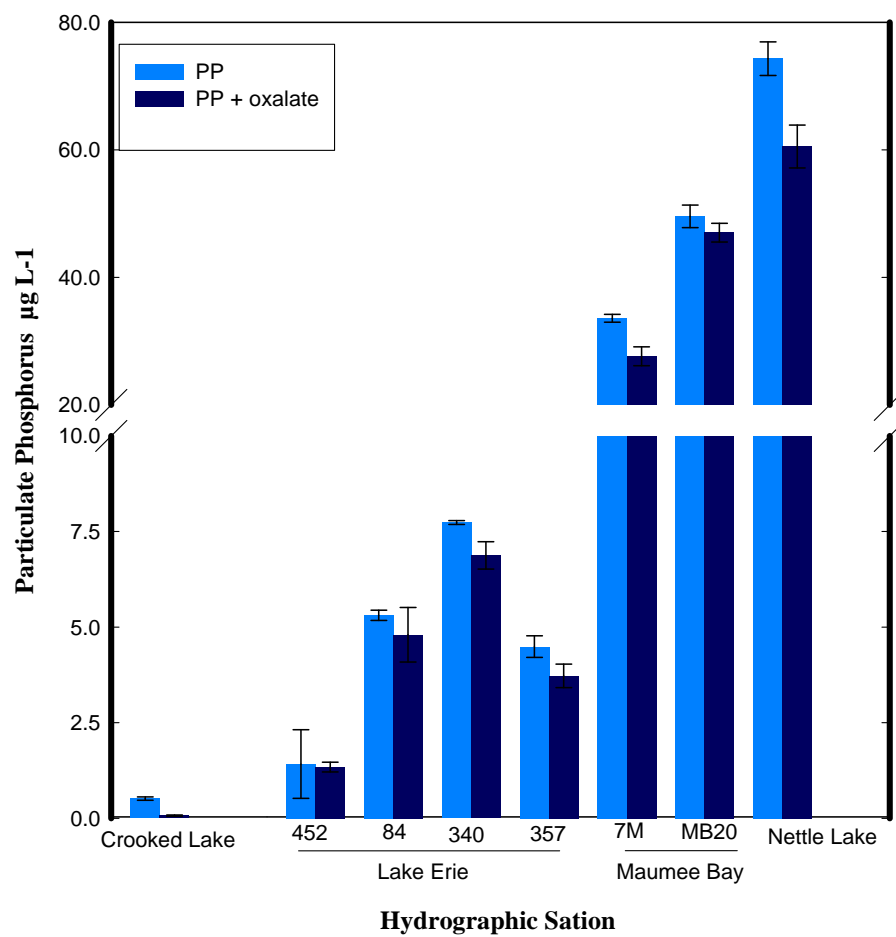


Figure 7. Analysis of particulate P from samples obtained during 2007.

DISCUSSION

The Redfield Ratio (106 Carbon: 16 Nitrogen: 1 Phosphorus, by moles) (Redfield, 1958) relating the stoichiometry of macronutrients in phytoplankton, is one of the foundations of modern day aquatic biogeochemistry (Falkowski, 2000). Redfield observed a nearly constant stoichiometry among the C, N and P of seston in the surface waters of the offshore ocean (1958). Redfield's findings have since been extended to many other habitats including nearshore oceans and freshwater lakes (Elser and Hassett, 1994; Hecky *et al.*, 1993). The prevailing paradigm is that seston C:N:P is at or near Redfield proportions in most of the offshore ocean, but that inland waters often exhibit departures (higher C:P and C:N) (Hecky *et al.*, 1993; Sterner and Elser, 2002). Existence of Redfield stoichiometry in particles contributed greatly to discussions about nutrient limitation status in marine algae because cultured algae have been observed to have Redfield stoichiometry only when they are not strongly nutrient limited (Goldman *et al.*, 1979).

This study was motivated primarily by the recently published observation that cellular phosphorus can be partitioned into separate surface-adsorbed and intracellular pools and that allocation to the surface-adsorbed pool may account for substantial amounts of particulate phosphorus (PP); the surface-adsorbed pool can reach 90% of total PP (Sañudo-Wilhelmy *et al.*, 2004). The observations of Sañudo-Wilhelmy *et al.* (2004) have been particularly intriguing. The oxalate reducing reagent they used in determining cellular P allocation was initially developed to remove surface-adsorbed Fe from cells for the purpose of determining intracellular Fe quotas (Sanchez-Tovar *et al.*, 2003). The redox potential of oxalic acid ($E^0_{\text{H}} = -631 \text{ mV}$) is expected to facilitate the reduction of most of the amorphous Fe ($E^0_{\text{H}} = -295 \text{ mV}$) and Mn ($E^0_{\text{H}} = -50 \text{ mV}$) hydroxides bound to phytoplankton cell surfaces. Using the oxalate reagent, they demonstrated that a substantial pool of phosphorus was also adsorbed to the cell surface yielding

an elevated internal molar ratio of 316-434 C: 59-83 N: 1 P for the marine diazotrophic cyanobacterium *Trichodesmium* (Sañudo-Wilhelmy *et al.*, 2004). P-limitation is thought to be much more characteristic of freshwater systems than marine systems making the results of Sañudo-Wilhelmy *et al.* (2004) of great relevance to our understanding of P dynamics in lakes.

We assessed the cellular allocation of P at various locations throughout Ohio and NE Indiana including two inland lakes as well as coastal and pelagic locations in Lake Erie (Table 1). By no means was sampling intended to be comprehensive; rather, our goal was to sample several representative locations that span the trophic continuum. As a representative eutrophic lake, we sampled Nettle Lake, located in Williams County, in the NW corner of Ohio (Fig. 5). The choice of Nettle Lake was guided largely by results compiled by the Citizens Lake Awareness and Monitoring (CLAM) program, sponsored by the Ohio Lake Management Society (<http://www.olms.org/clam.php>). CLAM provides an opportunity for lake stakeholders to take an active role in learning about aquatic ecology, lake and stream water quality, and watershed management. As part of the program, each year CLAM releases an annual report indexing the trophic state of a subset of Ohio's lakes and reservoirs (i.e. those "adopted" by CLAM volunteers). The most recent annual report issued by CLAM is for 2001 (Carlson and Smith, 2001). Of the 38 lakes for which data are contained in this report, 12 lakes were classified as hypereutrophic (Secchi depth < 20 inches), 25 lakes were classified as eutrophic (Secchi depth of 20-78 inches) and only a single lake, Lake Buckhorn located in Holmes County, was classified as mesotrophic. In each case, the trophic status was confirmed by lake color analysis (Custar Color Strip) indicating algal growth and not suspended sediment, as the primary factor behind light attenuation.

The 2001 CLAM report recorded a Secchi depth reading of ca. 30 inches for Nettle Lake. This lake is situated reasonably close to BGSU and is also in close proximity to the Oliver and

Walters chain of low productivity, marl lakes located in NE Indiana. With no oligotrophic inland lakes reported for Ohio in the 2001 CLAM report, we extended our sampling into nearby Indiana to sample Crooked Lake whose oligotrophic status is the result of the co-precipitation or absorption of ortho- or hydrogen phosphate to calcium carbonate as groundwater, supersaturated with dissolved calcium carbonate, enters the lake bottom (Otsuki and Wetzel, 1972). This lake hosts a field station owned by Indiana University-Purdue University Fort Wayne and has been the subject of study (productivity and water chemistry) in recent decades (Wetzel 1966; Wetzel, 1973; Lovell and Konopka 1985a, 1985b; Konopka et al., 1999).

Sampling was also extended to Lake Erie, the smallest of the Great Lakes by volume. Lake Erie is impacted most heavily by urbanization and agriculture and was the first Great Lake to show evidence of lake-wide eutrophic imbalance characterized by large-scale algal blooms and oxygen depletion. Further, Lake Erie is naturally divided into three distinct basins with each basin occupying a distinct position along the trophic continuum. Since the EPA's Great Lakes National Program Office began monitoring the lake in the early 1980's, the shallow western basin, including Maumee Bay has been characterized as varying between eutrophy and mesotrophy, whereas the central basin is characterized as oligomesotrophic and the deeper eastern basin as mainly oligotrophic.

Samples collected from all locations during late spring exhibited some degree of P stress, either as assessed by alkaline phosphatase activity or inferred from elemental ratios and their deviation from Redfield stoichiometry. Further, we confirmed the results of Sañudo-Wilhelmy et al. (2004) as we routinely observed a partial allocation of P to the cell surface. Great Lakes seston show between 5-15% of cellular P allocated to the cell surface, regardless of physiological P status (alkaline phosphatase activity) whereas our limited sampling of inland lakes indicates

that even higher levels of P, up to 90%, can be absorbed to the cell surface as demonstrated for seston collected from Crooked Lake. Thus our results are consistent with those shown for marine environments in terms of the cellular partitioning of P. Fu *et. al.* (2005) found similar trends between their cultured cells of representative species (e.g. *Trichodesmium* IMS101) of surface adsorbed P and natural bloom samples of diatoms, dinoflagellates, and cyanobacteria with surface P ranging from 14% to 57%, and from 15% to 46% of respectively, by using the same oxalate method as described by Tovar-Sanchez *et. al.*(2003). However, the amount of surface associated P we observed in freshwater phytoplankton was less than that observed in marine species.

The mechanism behind surface adsorption is presently not known, although Sañudo-Wilhelmy *et al.* (2004) invoke inorganic scavenging processes rather than active biogenic uptake. At first consideration, this mechanism appears to run counter to our knowledge of the surface charge of cells. That is, the surfaces of nearly all cells are negatively charged because of the presence of proteins and other wall and cell membrane components that contain phosphate, carboxyl and other acidic groups. How then does the phosphate oxyanion become scavenged by a typical cell? Two mechanisms, might address this apparent anomaly:

- 1) small, but significant amounts (20%) of phosphorus species are present as cationic compounds (Chamberlain, 1968; Lean, 1973: as reported in Francko and Heath, 1979), and
- 2) ligand exchange offers the possibility for oxyanionic elements to complex with hydrous oxides on biogenic surfaces and minerals (Sigg and Stumm, 1981)

With reference to the latter mechanism, Sañudo-Wilhelmy *et al.* (2004) present evidence suggesting that surface adsorbed Mn hydroxides appear to be the main factor facilitating the adsorption of phosphorus to the cell surface, at least for *Trichodesmium*. Another possible mechanism explaining the occurrence of surface associated P was provided by Olson *et al.* (2006) who proposed that this phenomenon is a by-product of algal photosynthesis by algae through perturbation of the total inorganic carbon system. They suggest that elevated pH resulting from photosynthesis facilitates the precipitation of P onto surfaces by precipitation reactions involving formation of CaCO_3 and Mg(OH)_2 .

Implications for Lake Management

Reference to the Redfield Ratio, in particular N:P ratios, is commonplace in documents intended to guide management decisions related to cultural eutrophication of bodies of water in the USA and abroad (US EPA, 1998; WHO, 1999; Claussen *et al.*, 2004). For example, the Redfield Ratio figures prominently in the US EPA's "National Strategy for the Development of Regional Nutrient Criteria" (US EPA, 1998), a blueprint intended to guide States and Tribal authorities in adopting nutrient criteria as part of water quality standards that will result in attainment of a designated use of a body of water (e.g. fishing, swimming) as determined by the State. The Redfield Ratio is described in the Introduction of this document and is listed as one of the environmental indicators (N:P ratios) for the establishment of scientifically defensible nutrient criteria for lakes and reservoirs as well as wetlands.

Likewise, the World Health Organization invokes the Redfield Ratio as an indicator of ecosystem carrying capacity to support blooms of toxic cyanobacteria (WHO, 1999). For planning and management, it is important to be able to estimate which of the key resources

(light, nitrogen or phosphorus) is likely to control phytoplankton biomass in a given system and examining the Redfield Ratio serves as an important approach for doing so.

The Redfield Ratio is also invoked in drafts of a Pan-European Eutrophication document applicable to both inland and coastal waters (Claussen et al., 2004). The draft adopts guidelines currently included in the Oslo-Paris Convention (OSPAR), a regional convention governing practices aimed at protecting the marine environment of the NE Atlantic Ocean. The OSPAR Convention includes a "Common Procedure for the Identification of the Eutrophication Status" where nutrient ratios are included as one of the key assessment parameters (OSPAR, 1997). In adopting the OSPAR "Common Procedure", the Steering Group charged with drafting the proposed legislation invoke the Redfield Ratio in interpreting the importance of dissolved nutrient ratios. In light of accumulating evidence that the Redfield Ratio is not universally applicable, it is clear that classical ideas of C:N:P ratios in the water column need revision, especially if we are to continue advocating the use of the Redfield Ratio as an environmental indicator of lake nutrient status.

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APPENDIX A.

Composition and preparation of the oxalate reagent for freshwater plankton (based on Tovar-Sanchez <i>et al.</i> 2003).			
Chemicals	Basic reagent	Trace metal clean reagent	Concentration (mol L ⁻¹)
a) EDTA (EDTA-Na ₂ H ₂ O)	1.86 g	2.23 g	0.05
b) Sodium citrate (C ₆ H ₅ Na ₃ O ₇ 2H ₂ O)	1.47 g	1.76 g	0.05
c) KCl	0.074 g	0.089 g	0.01
d) NaCl	0.12 g	0.14 g	0.02
e) NaOH (10 M)	drops until pH 6– 7		
f) Oxalic acid (C ₂ H ₂ O ₄ 2H ₂ O)	1.26 g	1.51 g	0.1
g) NaOH (10 M)	buffer to pH 8		
*Mix consecutively from (a) to (f) in 60 ml MQ water and take to 100ml with MQ water.			

APPENDIX B.

Color Reagents for spectrophotometric analysis of PP.	
Reagents	Volume
5N Sulfuric Acid	50ml
Potassium Antimony Tartrate	5ml
Ammonium Molybdate	15ml
Ascorbic Acid	30ml
Combine reagents and add to sample (0.16ml ml ⁻¹ of sample). Allow to develop 10-30 min before reading.	