

Evidence for phosphorus, nitrogen, and iron colimitation of phytoplankton communities in Lake Erie

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Abstract

Three nutrient enrichment experiments involving the addition and removal of iron (Fe) alone, as well as in combination with phosphorus (P) and/or nitrogen (N), were conducted in the offshore and nearshore waters of the eastern basin of Lake Erie. Lake Erie phytoplankton are at times colimited by P, N, and Fe. This was most clearly demonstrated in the offshore, strongly stratified waters, where the Fe concentration was below detection ($DL = 2.0 \text{ nmol L}^{-1}$), and nutrient limitation indicators (APA, P debt, C:P, N:P, N debt, and C:N) indicated strong P and moderate N limitation. Enrichment with Fe alone did not result in a significant increase in phytoplankton biomass, but the combined addition of Fe, P, and N yielded greater biomass increases than the addition of P and N alone. Phosphorus and Fe were both required to stimulate biomass increases. Nutrient limitation indicators and dissolved nutrient measurements showed that P and Fe additions facilitated the uptake of nitrate (NO_3^-), which is the most abundant source of N because of the low ammonium (NH_4^+) concentrations in Lake Erie. Our results support a colimitation hypothesis wherein the addition of Fe reduces N limitation by allowing the phytoplankton communities to use NO_3^- , take up more P, and become more strongly P limited. Phytoplankton communities of the eastern basin of Lake Erie and numerous other lakes experience colimitation by P, N, and Fe during the summer period of thermal stratification.

Iron may be an important limiting nutrient in some freshwaters as well as in some areas of the oceans. Recent studies have reported that levels of dissolved Fe can decline to low concentrations in offshore waters of both Lake Erie (2 nmol L^{-1} ; Twiss et al. 2005) and Lake Superior (1.1 nmol L^{-1} ; Sterner et al. 2004). Also, the addition of Fe to lake water has been demonstrated at times to stimulate phytoplankton biomass (Twiss et al. 2000; Guildford et al. 2003). Iron limitation of phytoplankton growth rates in oceanic high-nutrient, low-chlorophyll (HNLC) regions is well documented (Tsuda et al. 2003).

However, recent studies have shown that Fe is not the single limiting factor. Mills et al. (2004) suggest that Fe and P colimit N_2 fixation in the N-limited eastern tropical North Atlantic, while de Baar et al. (1990) postulated that light and large grazers are additionally major factors controlling phytoplankton in the Weddell and Scotia seas. In fact, colimitation by Fe and light best describes the HNLC regions in 40% of the world oceans (de Baar et al. 2005).

Iron limitation is not expected to occur in lakes because of the proximity of terrestrial influences, but several investigators have seen a response by phytoplankton to Fe in lakes of various sizes (Sakamoto 1971; Storch and Dunham 1986). The nearshore regions of lakes in particular are not expected to exhibit signs of Fe limitation because of the reductive dissolution of Fe oxides in the sediments that become remobilized and diffuse into the water column (Schoemann et al. 1998). In the well-mixed nearshore, this mechanism occurs together with elevated concentrations of Fe from fluvial inputs (Martin 1990). Nevertheless, coastal marine environments have also responded to Fe enrichment (Hutchins and Bruland 1998; Hutchins et al. 1998).

In diagnosing the nutrient factors controlling phytoplankton, we are specifically interested in limitation of growth rates (Blackman limitation). The variations in such limitation and the selective advantages and disadvantages thereby incurred among species are among the fundamental controls on primary production and community composition (Tilman 1976). As defined by Arrigo (2005), multi-nutrient colimitation of growth rates can occur when two nutrients are below optimal concentrations for uptake and the simultaneous addition of both nutrients increases

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Acknowledgments

We acknowledge the technical assistance of D. Depew, T. Nelson, A. Desellas, and S. Ronzio. This research was funded by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and additional funding from the National Science Foundation (Chem Oce 0327730) to M.R.T. R. L. North was supported by an Ontario Graduate Scholarship and an NSERC postgraduate scholarship. We acknowledge the following analytical support: the Chemical Engineering Analytical Chemistry Services in the Department of Chemical Engineering at the University of Waterloo for the 2002 and 2003 NO_3^- data; M. N. Charlton and the National Laboratory for Environmental Testing (NLET) at the Canada Centre for Inland Waters for the TP and TDP analyses and the 2001 $\text{NO}_3^- + \text{NO}_2^-$ chemistry; M. Bridoux for the 2002 Fe analysis; the Analytical Laboratory at the Freshwater Institute for the 2001 particulate P and particulate C:N analyses; and the Environmental Isotope Lab of the University of Waterloo for the DOC analyses. This manuscript benefited from the constructive comments of R. E. Hecky, V. H. Smith, and H. J. W. de Baar.

growth. Cellular biochemical colimitation is a particular case of colimitation in which a trace element facilitates the assimilation of a major nutrient, as in Fe-facilitated assimilation of NO_3^- . At the community level, colimitation can occur when one nutrient is below a threshold concentration for one species and another nutrient is below a threshold concentration for another species, as in the classic case of diatom competition for P and silica (Tilman 1976). We may therefore expect potentially complex patterns of nutrient limitation in nature, with simple control by a single nutrient being only one of several possibilities. Iron is particularly likely to exert its effects via colimitation.

Iron plays a catalytic role in many biochemical reactions as a cofactor of enzymes and proteins involved in chlorophyll synthesis, detoxification of reactive oxygen species, electron transport, and N assimilation. In order to utilize nitrite (NO_2^-) and NO_3^- , phytoplankton must first reduce them to NH_4^+ , which utilizes the nitrate and nitrite reductase enzymes (Paerl and Zehr 2000). Iron limitation has been shown to decrease nitrate reductase activity in phytoplankton (Milligan and Harrison 2000), as Fe is a principal component of these enzymes. Because of the high energetic cost of NO_3^- reduction, NH_4^+ is the preferred inorganic N source (Harrison et al. 1996). Iron is typically not limiting to biomass directly, yet NO_3^- uptake has high Fe requirements. Therefore, phytoplankton NO_3^- metabolism and thus growth rates may be reduced (Timmermans et al. 2004) by low bioavailable concentrations of Fe.

Historically, the simplest approach for demonstrating nutrient limitation in phytoplankton was to measure a biomass response to the addition of a single nutrient. Nutrient limitation can also be investigated using indicators of the bioavailability of nutrients as sensed by the cells. Nutrient limitation indicators are based on the premise that the cellular constituents, nutrient uptake, and certain enzymatic activities will vary in predictable ways depending on the nutritional state of the phytoplankton cell (Healey and Hendzel 1979b).

Lake Erie is the twelfth-largest lake in the world by area, provides drinking water for 11 million people (Environmental Protection Agency 2000), and is one of the largest and most productive freshwater fisheries in the world with annual landings of 21,900 MT (Nepszy 1999). The shallowest of the Laurentian Great Lakes with a maximum depth of 64 m in the eastern basin, it was once referred to as a "dead lake." However, its trophic status has reverted from eutrophic to meso-oligotrophic as a result of the implementation of the Great Lakes Water Quality agreement in 1972 that led to a decrease in total phosphorus (TP) (Charlton et al. 1993). Lake Erie currently has low TP ($0.2 \mu\text{mol L}^{-1}$), high NO_3^- and NO_2^- ($13.2 \mu\text{mol L}^{-1}$) (Charlton and Milne 2004), and low NH_4^+ concentrations ($0.37 \mu\text{mol L}^{-1}$; this study). Lake Erie phytoplankton are most frequently characterized as P limited (Lean et al. 1983; Wilhelm et al. 2003; Guildford et al. 2005), although recently in the eastern basin, there has been evidence of multinutrient colimitation due to N limitation, as indicated by N bioreporters and N limitation indicators (Wilhelm et

al. 2003; Guildford et al. 2005). This is surprising, as NO_3^- and NO_2^- concentrations are already high and increasing, representing one of the largest anthropogenic changes detected in Lake Erie (Charlton and Milne 2004). Human activities have led to major increases in global emissions of N to the atmosphere, nearly fourfold greater than before the industrial revolution, and total atmospheric deposition of reactive N is currently an order of magnitude greater than in preindustrial times (Phoenix et al. 2006). Increased loading from Lake Erie agricultural tributaries has also been documented, with increases in NO_3^- and NO_2^- concentrations from 0.71 to $10 \mu\text{mol L}^{-1}$ per year (Richards and Baker 1993).

The objective of this study was to examine the role of Fe in modifying the response to P and N of Lake Erie phytoplankton. We hypothesize that under certain situations P and N limitation detected in Lake Erie phytoplankton is the result of colimitation by P and Fe and that the addition of Fe with P will relieve Fe and P limitation and allow NO_3^- assimilation, thereby alleviating N limitation. A corollary of this hypothesis is that ambient lake concentrations of P and NH_4^+ are at times too low to satisfy biological demand (multinutrient colimitation) and that the NO_3^- levels, although high, represent an unavailable source of N because of low Fe bioavailability (biochemical colimitation). We suggest that low Fe concentrations in the offshore stratified regions of Lake Erie may prevent the uptake of NO_3^- that is present in excess during this period. We tested this hypothesis by measuring the biomass, nutrient concentrations, and physiological nutrient limitation response of the phytoplankton community to P, N, and Fe enrichment in both the nearshore and the offshore regions of the lake. Three experiments were conducted over 3 yr: two in the offshore area and one in the nearshore zone. The in situ conditions were characterized through physical parameters, lake water chemistry, and nutrient limitation indicators, after which we conducted nutrient enrichment experiments using trace metal-clean techniques in which specific combinations of nutrient treatments were applied, including the removal of Fe.

Materials and methods

Study area and field sampling—Three nutrient enrichment experiments were performed in the eastern basin of Lake Erie during the summer (July–September) from 2001 to 2003 (Table 1). The offshore station was located at $42^\circ 41.808' \text{N}$, $79^\circ 56.650' \text{W}$, while the nearshore station was located at $42^\circ 46.934' \text{N}$, $79^\circ 59.045' \text{W}$. A six-station grid was also sampled monthly from June to September 2003 in order to determine the seasonal and spatial Fe and NH_4^+ concentrations in the eastern basin (Table 2). The grid contained three nearshore sites and three offshore sites. All water samples were taken from the epilimnion of nearshore and offshore sites. For the purposes of this paper, offshore represents stations ≥ 20 m, and nearshore represents stations < 20 m.

Physical measurements on the lake included temperature and pH profiles. For the 2001 experiment, these parameters

Table 1. Experimental conditions for the three enrichment experiments conducted in the eastern basin of Lake Erie.

Date	Location	Depth of station (m)	Depth of sample (m)	Experimental treatment	Nutrient amendment concentrations ($\mu\text{mol L}^{-1}$)	Days subsampled
Sep 2001	Offshore	30	0–10	Control NO_3^- and P Fe NO_3^- and Fe and P DFB	$\text{NaNO}_3 = 10$ $\text{K}_2\text{HPO}_4 = 1$ $\text{FeCl}_6 \cdot \text{H}_2\text{O} = 0.5$ DFB = 0.1	3, 6
Aug 2002	Offshore	30	7.5	Control NO_3^- and P Fe NO_3^- and Fe and P DFB NO_3^- and Fe Fe and P	$\text{NaNO}_3 = 27$ $\text{K}_2\text{HPO}_4 = 0.8$ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O} = 0.1$ DFB = 0.08	2, 4, 6, 8
Jul 2003	Nearshore	5	2.5	Control NO_3^- and P Fe NO_3^- and Fe and P	$\text{NaNO}_3 = 27$ $\text{K}_2\text{HPO}_4 = 0.8$ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O} = 0.1$ DFB = 0.08	4

were collected utilizing a SeaBird™ SBE-19 profiler, and the 2002 and 2003 experiments employed a Hydrolab. In 2002 and 2003, pH was also measured using a handheld pH meter. Stratification was assumed to have occurred when there was a vertical gradient of $>1^\circ\text{C}$ per meter. Vertical profiles of photosynthetically active radiation (PAR) were measured with a Li-Cor cosine underwater quantum sensor and a Li-Cor LI-1000 data logger. The vertical attenuation coefficient for PAR: K_d , was determined from the linear regression of the natural logarithm of irradiance versus depth. The Z_{mix} and mean water column light intensity as a percentage of surface irradiance were calculated according to Guildford et al. (2000). Secchi disc depths were also recorded. The experimental water was collected using a tube sampler in 2001 and a hand pump with tubing in 2002 and 2003 using trace metal-clean protocols (see below for details). The survey water (Table 2) was also collected using trace metal-clean techniques (see below). All of the water was prescreened through a 200-micron nylon (Nitex) filter to remove macrozooplankton.

Experimental design—Lake water was collected in 10-liter low-density polyethylene trace-clean level 3 containers

(VWR) in triplicate, with the exception of the 2001 experiment, which was conducted in duplicate. Prior to enrichment, samples were collected for all parameters herein referred to as collection samples. Chlorophyll *a* (Chl *a*) samples were taken prior to enrichment to ensure that the autotrophic biomass was equal among treatments.

The containers were enriched with a combination of the nutrients: Fe, P, and N (Table 1). There was a change in the solute concentrations used in the treatments and in the N:P supply ratios added between 2001 and the following experiments due to a restructuring of the experimental design. A specific Fe chelator, desferrioxamine mesylate (desferal, DFB) was added as a treatment in the two offshore experiments to effectively remove all of the bioavailable Fe. Bioavailable Fe is difficult to quantify, and specific assays for Fe limitation were not used in these experiments. However, Fe removal using a fungal siderophore (DFB) allowed us to explore the effect of Fe limitation on phytoplankton biomass. After the additions were made, the containers were incubated for 2–8 d, after which time the biomass, water chemistry, and nutrient limitation were assessed and compared with that of a control treatment to which nothing was added.

Table 2. Iron and NH_4^+ concentrations shown for stations in the eastern basin of Lake Erie, June–September 2003. Offshore is designated as stations at a depth of ≥ 20 m, whereas nearshore is stations < 20 m. Data shown represent the range (minimum value–maximum value) of concentrations where $n = 3$.

Date	Station location	Total dissolved Fe (nmol L^{-1})	Particulate Fe (nmol L^{-1})	NH_4^+ ($\mu\text{mol L}^{-1}$)
10 Jun 2003	Nearshore	117.3–196.5	NA*	0.35–0.53
	Offshore	8.1–11.1	NA	0.22–0.25
23 Jul 2003	Nearshore	22.3–108.1	270.1–341.6	0.36–0.71
	Offshore	3.1–7.1	14.9–45.2	0.32–0.64
13 Aug 2003	Nearshore	4.1–63.0	92.9–116.2	0.51–0.63
	Offshore	2.9–37.7	7.5–50.6	BD–0.30†
16 Sep 2003	Nearshore	25.4–31.1	104.4–496.5	BD–0.88
	Offshore	3.4–7.4	22.1–36.3	0.63–0.72

* NA, not available.

† BD = below detection (limit = $0.20 \mu\text{mol L}^{-1}$).

Containers were incubated in a growth chamber on a diel cycle (16 : 8 light : dark photoperiod) under cool white fluorescent lights at the ambient light level and water temperature at the time and depth of collection.

Trace metal clean protocols—The experimental water was collected using either a tube sampler in 2001 or a hand pump in 2002 and 2003. The survey work conducted in 2003 (Table 2) employed a Go-Flo bottle (General Oceanics), or the hand pump at specified depths within the photic zone, or a surface grab was taken. Once the water arrived back at the University of Waterloo, it was taken directly to a clean room, and the water was filtered for total dissolved Fe (TDFe) and particulate Fe in a HEPA laminar flow hood. The P and N additions were pretreated by passing through an ion-exchange resin (Chelex-100; Bio-Rad) to remove any metallic impurities. All materials used in this study were acid cleaned to reduce the incidence of trace metal contamination. The deionized, distilled water contained concentrations of TDFe that were below detection limits (see below) and thus was used for all solution preparations and washing purposes.

Water chemistry—Particulate C and N samples were analyzed by the methods described by Stainton et al. (1977). For the 2001 enrichment experiment, the particulate C samples were measured at the Analytical Laboratory at the Freshwater Institute (Stainton et al. 1977). For the 2002 and 2003 enrichment experiments, the samples were processed at the University of Waterloo. The dried filters were placed in a desiccator containing hydrochloric acid and fumed for 24 h and then analyzed on an Exeter Analytical Inc. CEC-440 (combustion 980°C, reduction 700°C) autoanalyzer. As the 2002 and 2003 particulate C filters were fumed with acid prior to analysis, they represent organic C only, and thus any increase in particulate C can be regarded as an estimate of autochthonous production because the containers were all sealed for the duration of the incubations with no exogenous source of organic carbon.

Total dissolved Fe was not measured in 2001; however, we believe the concentration to be slightly higher than found at the same station for 2002 based on the response to Fe removal. Samples for TDFe were filtered and stored at 4°C until analyzed for dissolved Fe using a graphite furnace atomic absorption spectrophotometer (Perkin Elmer AA-analyst 600) at Clarkson University. Samples were acidified with trace metal clean HNO₃ (Baseline; Seastar) to pH 2. Subsamples were analyzed in replicate by direct injection with 15 µg Mg(NO₃)₂ of matrix modifier. Accuracy was assured by SLRS-4 certified standard freshwater reference solution (National Research Council of Canada) appropriately diluted to be within expected range of Fe content. Particulate Fe filters and retained seston were deposited in Teflon jars and digested with concentrated HNO₃ (Seastar) for 2–3 d at 20°C. Iron concentration was subsequently determined using the same procedure as TDFe following appropriate dilution. Blank values have been subtracted from measured concentrations. The detection limit for the samples was 2.0 nmol L⁻¹.

Samples for TP and total dissolved P (TDP) were analyzed following preservation and analytical procedures of NLET (1994). Soluble reactive P (SRP) samples for all years were analyzed according to Stainton et al. (1977). The 2001 and 2002 particulate P samples were analyzed using the muffle furnace digestion method according to Stainton et al. (1977). In 2003, particulate P was measured using the persulfate digestion method in an autoclave (Parsons et al. 1984).

Nitrate and NO₂⁻ from the 2001 collection container were analyzed following preservation and analytical procedures of NLET (1994). The 2002 and 2003 NO₃⁻ samples were analyzed on a Dionex DX500 chromatography system (ion chromatograph).

The NH₄⁺ samples were analyzed using the indophenol blue method (Stainton et al. 1977) in 2001 and the fluorometric method in 2002 and 2003 (detection limit = 0.2 µmol L⁻¹) (Holmes et al. 1999).

The dissolved organic carbon (DOC) samples were submitted for analysis to the Environmental Isotope Lab of the University of Waterloo using a Rosemount Dohrmann DC-190 High-Temperature TOC analyzer (Hinton et al. 1997).

Chl a analysis—Sample water was filtered onto glass fiber (GFF: nominal pore size 0.7 µm, 47 mm) filters that were kept in the dark and stored frozen (-20°C) before passive extraction with 90% acetone. Picoplankton Chl *a* samples were filtered onto a 2-µm pore-size polycarbonate membrane filter. The filtrate was then treated exactly as the sample water. The extracts were quantified by fluorometry (Turner Designs 10-AU) that was calibrated annually with pure Chl *a*.

Phytoplankton nutrient limitation indicators—Phosphorus and N limitation were determined by particulate C:P, N:P, and C:N composition ratios, P and N debt assays (Healey and Hendzel 1979b), and alkaline phosphatase activity (APA; Healey and Hendzel 1979a; Table 3). The P debt assay measured the phosphate removed over a 24-h period per unit of Chl *a*. The determination of limitation was then assessed according to the criteria developed by Healey and Hendzel (1979b). In the P debt assay, KH₂PO₄ was added (final concentration 5 µmol L⁻¹) to an unfiltered water sample. The SRP concentrations were measured at the beginning and end of a 24-h incubation in the dark at room temperature. The N debt assay follows the same methodology as P debt, except that it is the NH₄⁺ removed over a 24-h period. In the N debt assay, NH₄Cl was added (final concentration 5 µmol L⁻¹) to an unfiltered water sample. N debt was calculated as the N removed over a 24-h period per unit of Chl *a* (Healey and Hendzel 1979b).

Alkaline phosphatase is an enzyme localized on the cell surface of algal and bacterial cells that is produced when the organisms are P limited. This enzyme removes the phosphate molecules from dissolved organic P compounds, therefore utilizing an otherwise unavailable source of P. APA was measured fluorometrically (Healey and Hendzel 1979a), using 5 µmol L⁻¹ of o-methyl-fluorescein-phos-

Table 3. Nutrient limitation indicators. Values shown are indicative of presence or absence or degree of nutrient limitation for indicators used in this study. Criteria for nutrient limitation are based on Healey and Hendzel (1979b) and adapted from Guildford et al. (2005).*

Indicator	Nutrient	No limitation	Moderate limitation	Extreme limitation	Limited
C: Chl <i>a</i> ($\mu\text{mol C } \mu\text{g Chl } a^{-1}$)	N or P	<4.2	4.2–8.3	>8.3	
N:P (atomic ratio)	P	<22			>22
C:P (atomic ratio)	P	<129	129–258	>258	
P debt ($\mu\text{mol P } \mu\text{g Chl } a^{-1}$)	P	<0.075			>0.075
APA ($\mu\text{mol P } \mu\text{g Chl } a^{-1} \text{ h}^{-1}$)	P	<0.003	0.003–0.005	>0.005	
C:N (atomic ratio)	N	<8.3	8.3–14.6	>14.6	
N debt ($\mu\text{mol NH}_4^+ \mu\text{g Chl } a^{-1}$)	N	<0.15			>0.15

* C, particulate carbon; N, particulate N; P, particulate P; APA, alkaline phosphatase activity.

phate as the substrate. Parallel determinations were made of total and soluble activities to distinguish between APA associated with particles and APA in solution, the soluble activity being that passing through 0.2- μm pore-size polycarbonate membrane filters. The difference was reported as particulate APA.

Statistical analyses and data presentation—One-way analysis of variance (ANOVA) was used to determine the difference between the experimental treatments for all variables and the survey data, and a Tukey–Kramer post hoc test was employed with a significance value of $p < 0.05$. The results shown in Figures 1–4 illustrate the mean and

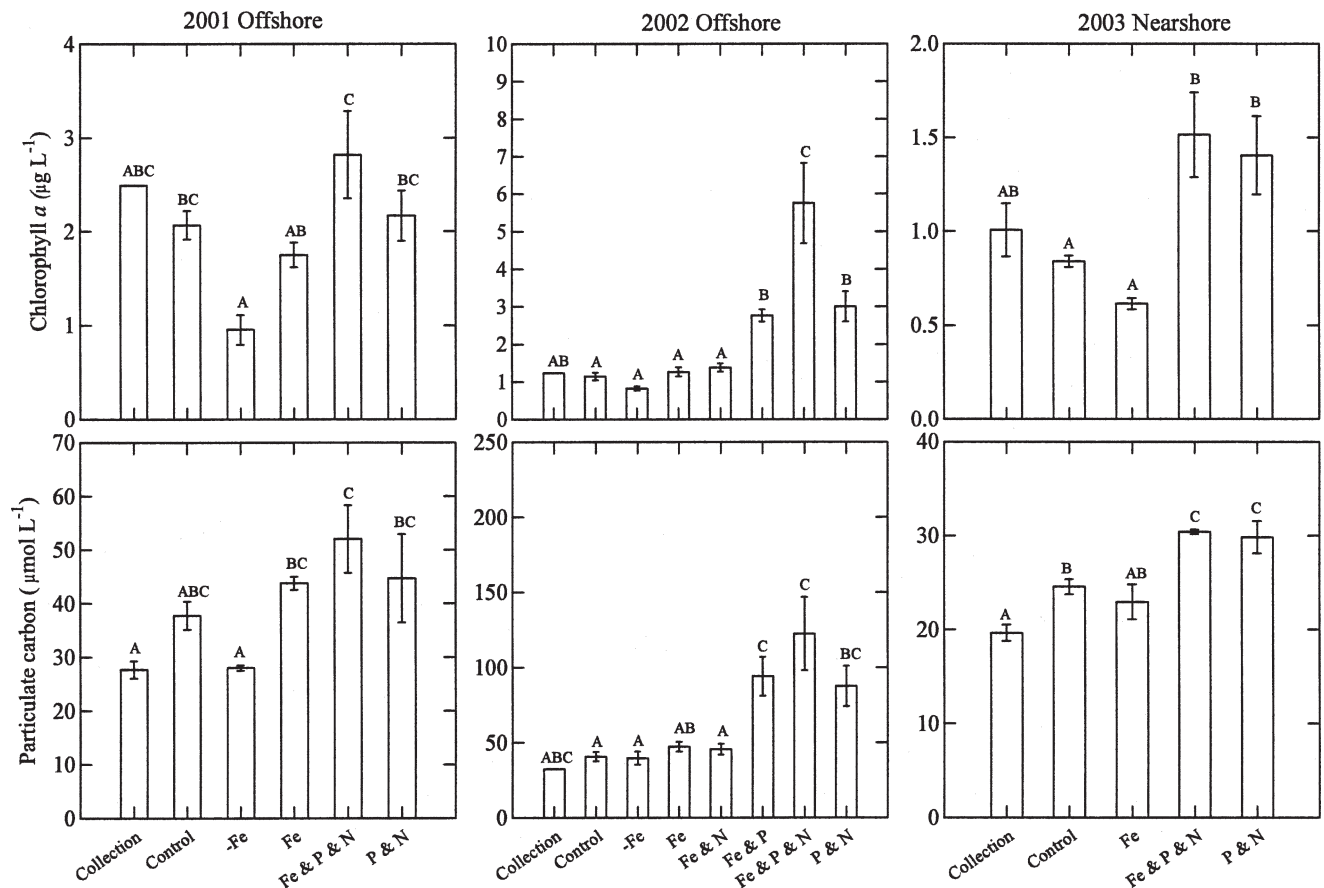


Fig. 1. Biomass response. Chlorophyll *a* and particulate C concentrations shown for the three experiments. The 2001 offshore experiment was subsampled for both parameters on days 3 and 6 ($n = 4$ per treatment). The 2002 offshore experiment was subsampled for both parameters on days 2, 4, 6, and 8 ($n = 12$ per treatment). The 2003 nearshore experiment was subsampled for both parameters on day 4 ($n = 3$ per treatment). For all graphs, the collection was sampled on day 0. The concentrations added for each treatment are listed in Table 1. Error bars represent the standard error of the mean. In this and the following figures, the letters above bars indicate statistical significance at a significance level of $p < 0.05$. The relationship between identical letters is not statistically significant, whereas the relationship between different letters is significant. For example, in the 2001 offshore experiment, the relationship between Chl *a* for the collection and the control treatment is not statistically significant, while the relationship between the control and the -Fe treatment is statistically significant.

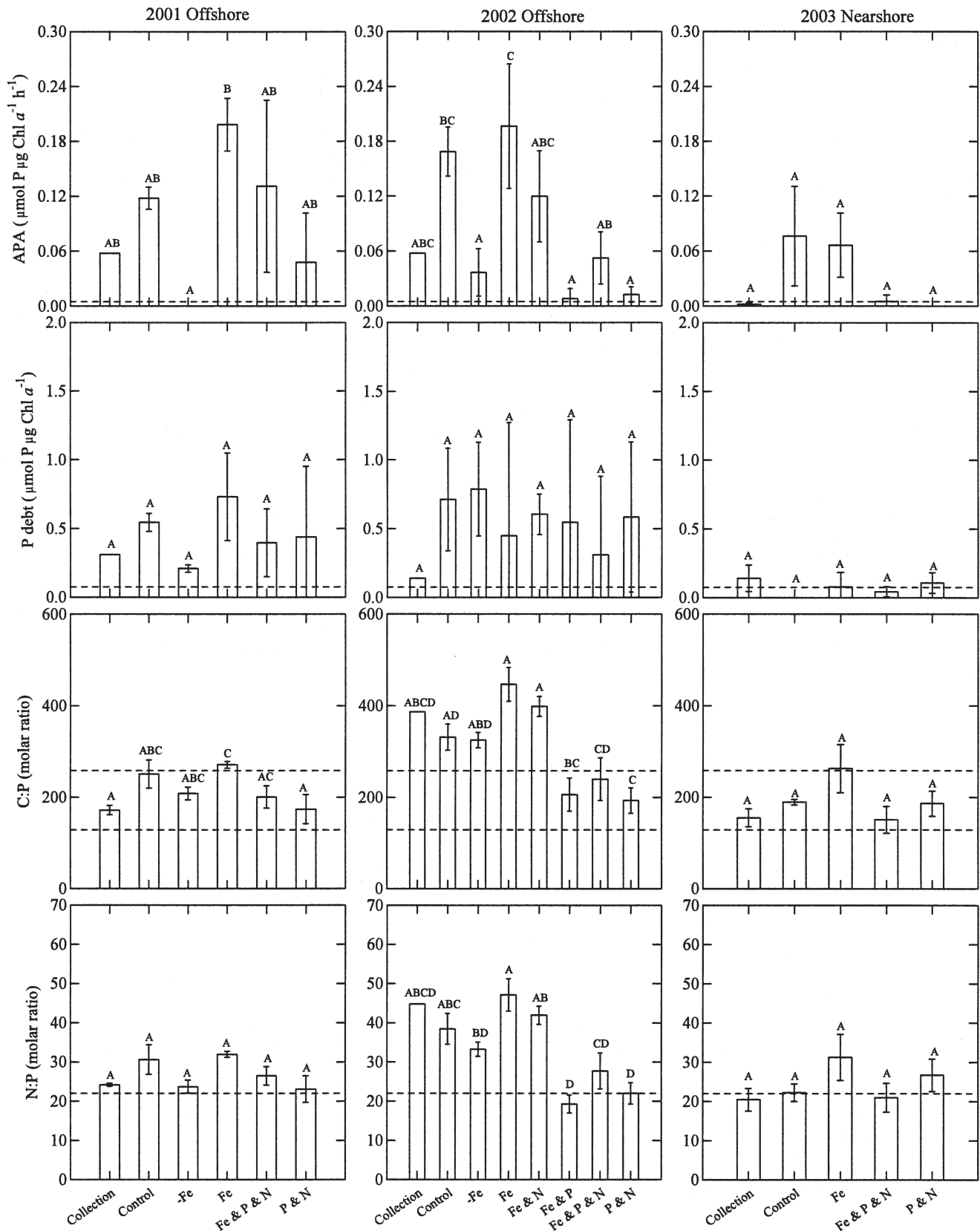


Fig. 2. Indicators of P limitation. Alkaline phosphatase activity (APA) normalized to Chl *a*, P debt normalized to Chl *a*, particulate C : particulate P (C : P), and particulate N : particulate P (N : P) ratios shown for the three experiments. The 2001 offshore experiment was subsampled for APA and P debt on day 3 ($n = 2$ per treatment) and on days 3 and 6 ($n = 4$ per treatment) for the C : P

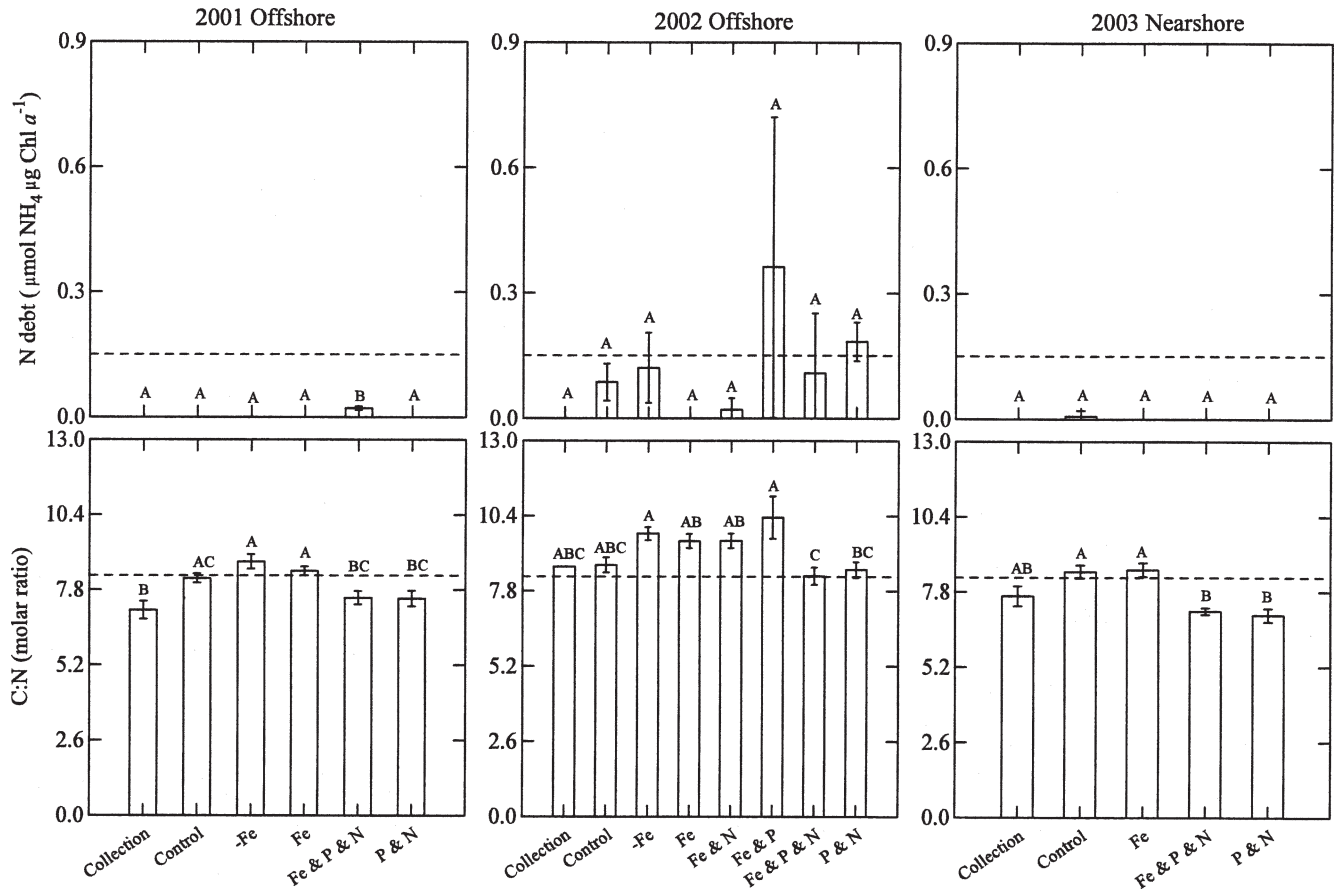


Fig. 3. Indicators of N limitation. N debt normalized to Chl *a* and particulate C: particulate N (C:N) ratios are shown for the three experiments. The 2001 offshore experiment was subsampled for N debt on day 3 ($n = 2$ per treatment) and on days 3 and 6 ($n = 4$ per treatment) for the C:N ratio. The 2002 offshore experiment was subsampled for N debt on days 2, 6, and 8 ($n = 3$ per treatment) and on days 2, 4, 6, and 8 ($n = 12$ per treatment) for the C:N ratio. The 2003 nearshore experiment was subsampled for both parameters on day 4 ($n = 3$ per treatment). The dashed line represents criteria for N limitation as defined by Healey and Hendzel (1979b). Values below the dashed line are considered not to be N limited, while values above the dashed line are considered to be N limited. The concentrations added for each treatment are listed in Table 1. Error bars represent the standard error of the mean. Letters above bars indicate statistical significance.

standard error for all of the days subsampled for that parameter (Table 1). One-way ANOVA determined that there was no significant difference in the Chl *a* concentrations (used here as a proxy of photoautotrophic biomass) between the times subsampled in the two offshore experiments. Length of incubation had no significant effect on biomass.

Results

In situ conditions at the initiation of experiments—When comparing the in situ conditions at the offshore sampling

location for the experiments conducted in 2001 and 2002, the water column was thermally stratified in both years. However, in 2002 the mixing depth was shallower; the epilimnion had better light conditions for algal growth, undetectable TDFe concentrations, and the lowest TP and NO₃⁻ of all three experiments (Table 4). Although in both of the offshore experiments, phytoplankton communities were P limited according to all four P limitation indicators (C:P, N:P, APA and P debt), 2002 was the most strongly P limited (Table 4). In 2002 and 2003, the C:N ratio was indicative of moderate N limitation, even though NO₃⁻ concentrations were high (Table 4). The well-mixed near-

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and N:P ratios. The 2002 offshore experiment was subsampled for APA on days 2, 6, and 8 ($n = 3$ per treatment); on days 2 and 8 ($n = 2$ per treatment) for P debt; and on days 2, 4, 6, and 8 ($n = 12$ per treatment) for the C:P and N:P ratios. The 2003 nearshore experiment was subsampled for all parameters on day 4 ($n = 3$ per treatment). The dashed lines represent criteria for P limitation as defined by Healey and Hendzel (1979b). Values above the dashed lines are indicative of P limitation, while values below the dashed lines are not considered to be P limited. When two lines are present, values between the upper and lower dashed lines are indicative of moderate P limitation, and values greater than the upper dashed lines are indicative of severe limitation. The concentrations added for each treatment are listed in Table 1. Error bars represent the standard error of the mean. Letters above bars indicate statistical significance.

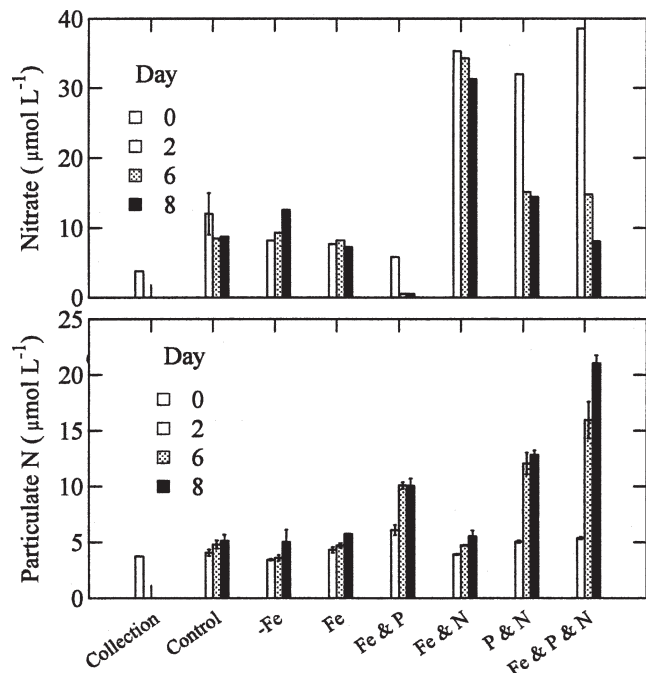


Fig. 4. Nitrate and particulate N concentrations for the 2002 offshore experiment. The NO_3^- data were collected on days 2, 6, and 8 ($n = 1$). The concentrations added for each treatment are listed in Table 1. The particulate N data was collected on days 2, 4, 6, and 8 ($n = 12$). Error bars represent the standard error of the mean.

shore station sampled in 2003 also had low TP and NH_4^+ concentrations, but the TDFe concentration was much higher (Table 4). The initial Fe conditions of the experiments correlate well with those found in both the nearshore and the offshore environments of the eastern basin, where there is significantly lower TDFe in the offshore than the nearshore (Table 2). Chlorophyll *a* was lowest ($0.79 \mu\text{g L}^{-1}$) at the nearshore station in 2003, but the phytoplankton community did not appear nutrient limited, as indicators of P and N limitation were low and inconsistent (Table 4). Light was never low enough to be limiting at any of the stations, and mean PAR was 25% or more of surface irradiance in all years (Table 4).

Response to enclosures—The control enclosures during the experiments were reflective of the initial conditions with the understanding that enclosure isolates the phytoplankton communities from external sources of nutrients and recycling mechanisms normally present in lake systems. The Chl *a* concentrations in the control enclosures decreased from the time of collection in all three experiments, while the particulate C concentrations increased (Fig. 1). The enclosure effect alone exacerbated P limitation in the offshore waters of both years (Fig. 2). In the offshore water examined in 2001, the increase in P limitation was particularly pronounced and consistent. The offshore water collected in 2002 was already very P limited, so the enclosure response was not as pronounced; however, the P limitation indicator values were highest in the control treatments. Enclosure of the nearshore water in

2003 stimulated APA and resulted in a higher C:P ratio (Fig. 2). Enclosure resulted in slightly but consistently higher C:N ratios in all three experiments and detectable NH_4^+ uptake in two of the experiments (Fig. 3), suggesting that the phytoplankton in the enclosures were unable to use ambient NO_3^- and that the phytoplankton were on the cusp of N as well as P limitation.

Response to Fe removal—The response to Fe removal in the two offshore experiments were quite different and suggests that in 2002 Fe was already colimiting with P and N at the time of collection yet not in 2001. In 2001, Fe removal (indicated by -Fe) brought about by the addition of the strong Fe-binding ligand DFB resulted in significantly less Chl *a* than the control (Fig. 1), a reduction in P limitation (Fig. 2), and stimulation of N limitation according to the C:N ratio (Fig. 3). The muted response to Fe removal in 2002 suggests that Fe was already limiting the phytoplankton community, as Fe removal had no effect on Chl *a* concentrations (Fig. 1). Phosphorus limitation was reduced but not as dramatically as in 2001 (Fig. 2), N limitation increased according to the C:N ratio, and NH_4^+ uptake was evident in the N debt assay (Fig. 3). In addition, the NO_3^- concentration in the Fe removal enclosure actually increased over the course of the experiment (Fig. 4), unlike in the other treatments where NO_3^- decreased or stayed the same.

Response to enrichment—The response of the offshore phytoplankton communities to multiple enrichment with Fe, P, and N indicated that all three nutrients were colimiting at the time of collection in 2002 and were close to colimiting in 2001. Fe was not colimiting in the nearshore phytoplankton community sampled in 2003. The addition of Fe, P, and N stimulated phytoplankton biomass to a greater extent than P and N addition without Fe in the offshore experiments (Fig. 1). In the nearshore waters, the addition of P and N with and without Fe stimulated phytoplankton biomass to the same degree (Fig. 2). This was consistent with the relatively high TDFe concentration of 38.5 nmol L^{-1} in the nearshore water, compared to the undetectable concentration in the offshore water in 2002 (Table 4).

Colimitation of the phytoplankton community was most clearly observed in the 2002 experiment because TDFe was undetectable (Table 4), and the largest variety of treatments was applied. Iron added alone or in combination with N did not stimulate biomass because P was also limiting. Phytoplankton in enclosures enriched with Fe or with Fe and N remained P limited or became more strongly P limited according to all of the indicators for P limitation applied (Fig. 2). We believe that the increased P limitation was not a result of the addition of Fe removing the bioavailable P through a chemical complexing event. There was no significant difference between P fractions (SRP, TDP, TP, particulate P), time, or treatment at the end of the incubations (data not shown). Also, when we added Fe in all three experiments, the SRP concentrations were higher than the control.

The addition of NO_3^- did not relieve the moderate N limitation indicated by the C:N ratio (Fig. 3). However, in

Table 4. Initial conditions for the three enrichment experiments. The 2001 NO_3^- data include NO_2^- concentrations. Bolded values indicate P or N limitation according to the nutrient limitation indicators (Table 3).*

Parameter		2001: Offshore	2002: Offshore	2003: Nearshore
	Date	September	August	July
Physical	Surface water temp. ($^{\circ}\text{C}$)	21.6	22.6	20.5
	Z_{max} (m)	30	30	5
	Z_{mix} (m)	16	11	5
	K_d (m^{-1})	0.25	0.27	0.45
	pH	NA	8.19	7.82
	Secchi (m)	4.25	5.3	3
	Mean PAR (%)	25	32	40
Chemical	TDFe (nmol L^{-1})	NA	BD	38.5
	Particulate Fe (nmol L^{-1})	NA	NA	734
	DOC (mg L^{-1})	NA	2.5	2.8
	TP ($\mu\text{mol L}^{-1}$)	0.35	0.19	0.27
	Particulate P ($\mu\text{mol L}^{-1}$)	0.16	0.08	0.13
	Particulate N ($\mu\text{mol L}^{-1}$)	4.0	3.7	2.4
	NO_3^- ($\mu\text{mol L}^{-1}$)	9.14	3.8	13.04
	NH_4^+ ($\mu\text{mol L}^{-1}$)	0.55	0.55	0.29
Biological	Chl <i>a</i> ($\mu\text{g L}^{-1}$)	2.48	1.2	0.79
	Particulate C ($\mu\text{mol L}^{-1}$)	30	32	20
	C : Chl <i>a</i> ($\mu\text{mol C } \mu\text{g Chl } a^{-1}$)	12.1	26.1	25.3
	N : P (atomic ratio)	24.2	44.8	18.9
	C : P (atomic ratio)	186	387	160
	APA ($\mu\text{mol P } \mu\text{g Chl } a^{-1} \text{ h}^{-1}$)	0.058	0.058	0.002
	P debt ($\mu\text{mol P } \mu\text{g Chl } a^{-1}$)	0.31	0.14	0.09
	C : N (atomic ratio)	7.1	8.6	8.5
	N debt ($\mu\text{mol NH}_4^+ \mu\text{g Chl } a^{-1}$)	0	0	0

* NA, not available; BD, below detection (limit = 2.0 nmol L^{-1}); K_d , attenuation coefficient for PAR; C, particulate carbon; N, particulate N; P, particulate P; APA, alkaline phosphatase activity; Z_{max} , maximum depth of station; Z_{mix} , mixed layer depth; mean PAR (%), mean water column light intensity as a percent of surface irradiance; DOC, dissolved organic carbon.

the enclosures enriched with Fe and P but not N, a higher biomass was observed (Fig. 1), indicating that the phytoplankton community was able to access ambient NO_3^- once Fe and P were available. In these enclosures, P limitation was relieved (Fig. 2), N limitation became strongest (Fig. 3), NO_3^- was reduced to undetectable concentrations, and particulate N increased (Fig. 4). In the same experiment, P and N addition without Fe also stimulated biomass (Fig. 1) and reduced indicators of both P and N limitation (Figs. 2, 3). Added NO_3^- was taken up by the extant phytoplankton communities but not to the extent that it was in the Fe and P enriched container or the container enriched with all three nutrients (Fig. 4). Particulate N concentrations reflected the changes in NO_3^- concentrations that may be viewed as uptake by the extant phytoplankton and heterotrophic communities. The addition of all three nutrients resulted in the highest particulate N concentrations relative to the other treatments (Fig. 4).

Drawdown of Fe added alone or in combination with P and/or N is shown for the 2002 experiment (Fig. 5). Every treatment that was given an Fe addition drew it down on average by 88%, indicating again that there was essentially no excess of Fe relative to demand (Fig. 5). The addition of Fe in combination with P and N resulted in the fastest drawdown of TDFe; however, a large drawdown in the Fe alone treatment was also observed (Fig. 5).

In the 2002 experiment where P, N, and Fe were colimiting from the outset, we observed a shift in the size

distribution of the phytoplankton community in response to the different treatments. In enclosures with no enrichment or only one available form of the three limiting nutrients, picoplankton ($0.2\text{--}2 \mu\text{m}$) comprised on average 28% of the total Chl *a*. In contrast, in enclosures able to

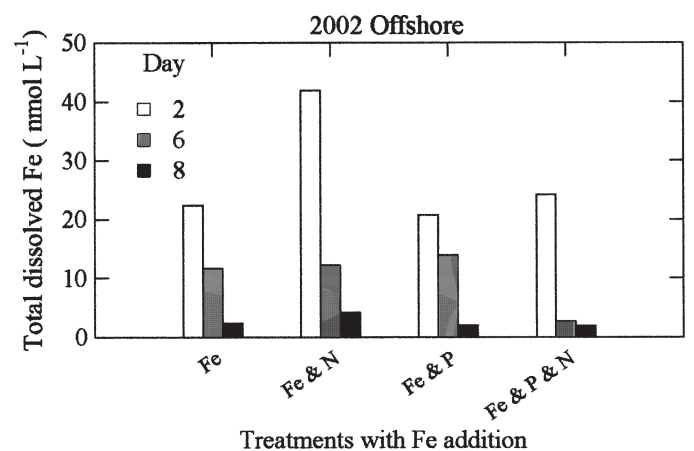


Fig. 5. Total dissolved Fe concentrations over time. The bars represent the TDFe concentrations for the 2002 offshore experiment that was subsampled on days 2, 6, and 8 ($n = 1$). Only treatments to which Fe was added are shown. Initially, Fe was added to all treatments shown at a concentration of 110 nmol L^{-1} .

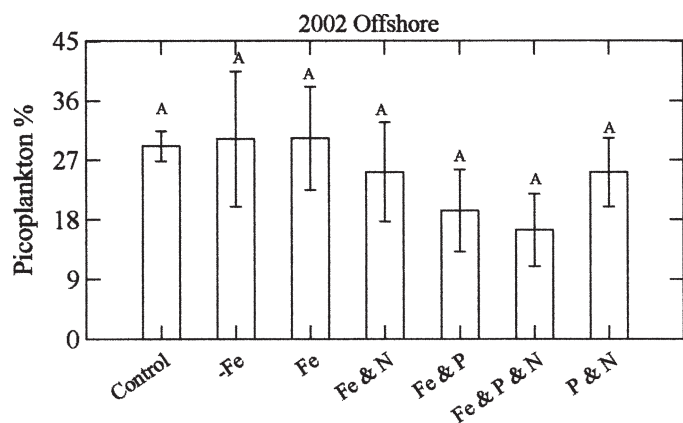


Fig. 6. Picoplankton (0.2–2 μm) percentage of total Chl *a*. The bars represent the picoplankton percentage of total Chl *a* for the 2002 offshore experiment that was subsampled on days 2, 6, and 8 ($n = 3$ per treatment). The concentrations added for each treatment are listed in Table 1. Error bars represent the standard error of the mean. Letters above bars indicate statistical significance.

access P, N, and Fe, picoplankton became less important (Fig. 6).

Evidence for colimitation of P, N, and Fe is not as clear in the offshore experiment conducted in 2001 as it is for 2002. However, the biomass and nutrient limitation measurements support the conclusion that once the water was enclosed, all three nutrients were unavailable, and although sufficient ambient NO_3^- was present, it was not taken up until P and Fe were supplied. The addition of Fe alone did not stimulate biomass or relieve N limitation presumably because P was limiting. The addition of P and N without Fe also stimulated biomass (Fig. 1) and relieved both P and N limitation (Figs. 2, 3) but not to the same extent as P and N with Fe.

The nearshore water exhibited only slight P and N limitation at the time of collection (Figs. 2, 3) even though the ambient TP and NH_4^+ concentrations were in the same range as the more strongly P- and N-limited offshore stations (Table 4). The nutrient enrichments of the water collected from the nearshore did not demonstrate colimitation by Fe; however, the addition of Fe alone increased P limitation relative to the control. The P and N treatment generated the same biomass response and a similar release from P and N limitation as P and N with Fe.

Discussion

The Lake Erie phytoplankton community can be colimited by P, N, and Fe in the offshore, stratified waters of the eastern basin. The reason that N can be limiting despite the abundance of NO_3^- is that the Fe that is required for NO_3^- assimilation is evidently not bioavailable. This phenomenon was clearly demonstrated in the 2002 experiment when the biomass of the P- and N-limited phytoplankton community was stimulated by the addition of Fe with P. Phytoplankton were able to access the ambient NO_3^- only following the supply of exogenous Fe and P. In the waters of Lake Erie where NO_3^- is the most

abundant source of N because of low NH_4^+ concentrations, our enrichment experiments show that Fe can facilitate the assimilation of NO_3^- . Once the phytoplankton are no longer N limited, they take up more P, causing them to exhibit stronger indications of P limitation.

Fe limitation has been documented in several small lake systems (Haphey-Wood and Pentecost 1981; Priscu et al. 1982). These previous studies were initiated because Fe was thought to be unavailable to the phytoplankton. In natural waters, complexation by dissolved organic compounds allows Fe to remain in the euphotic zone, where it is accessible to the phytoplankton communities. The availability of these Fe complexes for assimilation by phytoplankton depends on many factors: the kinetic lability of the complex, rates of photoreduction and photo-oxidation, and the uptake strategies employed by phytoplankton, which varies among species (Wells et al. 1995). Therefore, it is not just the measurable concentration of Fe that is important but also the availability. The original Fe work on freshwater systems by Schelske (1962), Schelske et al. (1962), and Wetzel (1966, 1972) observed increased primary production as a result of Fe enrichment experiments in water from marl lakes. Whiting events, characteristic of marl lakes (Wetzel 1966), are caused by supersaturation of calcium carbonate (CaCO_3) and occurred regularly in summer and fall from 1972 to 1975 in Lake Erie. In 1969, Lange (1971) reported a stimulation of cell numbers with the addition of Fe in Lake Erie. Whiting events precipitate Fe, as CaCO_3 crystal formation can scavenge trace metals by coprecipitation and surface sorption (Strong and Eadie 1978). Therefore, Fe limitation detected in the past in Lake Erie may be due to this scavenging. Recently, in the eastern basin of Lake Erie, a decline in spring alkalinity, combined with a decreased frequency of whiting events, has been observed. Barbiero and Tuchman (2004) attribute the decline in marl events to Ca uptake by dreissenid populations. However, Fe limitation is still occasionally reported on Lake Erie (Twiss et al. 2000; this study).

Although most temperate, freshwater lakes are considered to be P limited (Schindler 1977), studies report that N and P limitation in lakes is not mutually exclusive and have documented simultaneous limitation of N and P (Davies et al. 2004). In a survey of enrichment experiments conducted on 60 North American lakes, Elser et al. (1990) concluded that total algal biomass production was commonly limited by the availability of both N and P. It has also been reported that the combined addition of Fe with either N and/or P in nutrient enrichment experiments brought about a greater biomass response than either N and/or P (Schelske 1962; Sakamoto 1971). Our study has provided an explanation wherein Fe is needed to be able to assimilate the NO_3^- .

In eastern Lake Erie, the nearshore region is different and more variable compared to the offshore region in terms of Chl *a* and nutrient concentrations (Hecky et al. 2004; North unpubl. data). In our experiments, the Chl *a* concentration was lower in the nearshore than the offshore, likely because of the high density of exotic dreissenid mussels in this region that have been implicated in Chl *a* reduction and changes in the nutrient cycling of the

nearshore environment (Nicholls and Hopkins 1993; Hecky et al. 2004). Dissolved Fe concentrations in Lake Erie are highly heterogeneous (Twiss et al. 2000; Porta et al. 2005; this study) and range from 2 to 404 nmol L⁻¹ in the eastern basin during thermal stratification. Both the dissolved and the particulate Fe concentrations were higher in the nearshore than the offshore regions of Lake Erie and Lake Superior (McKay et al. 2004, 2005). In Lake Superior, through the application of an immunoblotting approach for flavodoxin accumulation in diatoms and a cyanobacterial Fe bioreporter, Fe was found to be more available nearshore than offshore (McKay et al. 2004). Iron is rapidly depleted by various scavenging mechanisms (Twiss and Campbell 1998) as water moves offshore. Also, the nearshore is typically not stratified, which allows mixing to the bottom, thereby enhancing the flux of Fe from sediments during early diagenesis. An indication of such an Fe source is the lower pH values in the water column as the pH tends to be lower within sediment pore waters (Schoemann et al. 1998). There are lower pH values at the nearshore station than the offshore (Table 4); thus, reductive dissolution and subsequent mixing in the nearshore could explain the higher Fe concentrations in the nearshore.

In general, Lake Erie phytoplankton are considered to be limited by P (Lean et al. 1983; Guildford et al. 2005). However, N limitation has also been detected (Wilhelm et al. 2003; Guildford et al. 2005; North, unpubl. data). Evidence for N and P colimitation in the eastern basin of Lake Erie was reported by DeBruyn et al. (2004) where P enrichment of water from an offshore stratified station induced N limitation. Our experiments demonstrate that in offshore stratified waters where Fe is frequently low (Porta et al. 2005; this study), N was colimiting with P. Iron enrichment experiments at a nearshore site in the western basin of Lake Erie were conducted by Lange (1971) and consisted of biweekly experiments during one growing season using filtered lake water inoculated singly with four algal cultures. He reported a stimulation of cell numbers with the addition of unchelated Fe in 22% of the experiments conducted, although chelated Fe yielded a higher growth response seen in 35% of the experiments. Storch and Dunham (1986) also found that chelated Fe yielded a higher cell yield compared to unchelated Fe, which frequently inhibited algal growth in their experiments. They conducted 18 experiments over 4 yr using phytoplankton collected from the nearshore of eastern Lake Erie. At lower concentrations of chelated Fe additions, photosynthesis was enhanced in 67% of the experiments. There was also evidence of colimitation in these experiments, as the addition of Fe in combination with N and P increased cell yield compared to Fe alone (Storch and Dunham 1986). Recent experiments by Twiss et al. (2000, 2005) showed that the addition of unchelated Fe caused an increase in biomass in only 5% of the 20 experiments conducted. However, one experiment conducted on the strongly stratified offshore waters of the eastern basin showed a dramatic 180% and 30% increase in biomass of picoplankton and nanoplankton, respectively. In addition, Fe stressed phytoplankton exhibited draw-

down of Fe as a result of uptake (Twiss et al. 2000). They also demonstrated a colimitation of P and Fe to phytoplankton growth (Twiss et al. 2000, 2005), as the addition of Fe and P combined yielded a higher biomass than the addition of P alone. Iron bioreporter results also indicated Fe limitation in Lake Erie (Durham et al. 2002). In these previous experiments, primary production and algal biomass were the typical measured responses to nutrient additions, and the preexisting nutrient limitations of the in situ phytoplankton communities were often not considered. In this study we demonstrated that P and N limitation indicators were more sensitive than biomass to the addition of Fe and emphasize the importance of the interaction of Fe and phytoplankton P and N limitation.

Sterner et al. (2004) performed eight nutrient enrichment experiments in western Lake Superior from September 1999 to May 2001 and reported no stimulation of Chl *a* with the addition of Fe. It is likely that the simultaneous P limitation of the phytoplankton made it impossible to observe a direct response to Fe in these experiments. Colimitation of P and Fe was observed as the addition of P resulted in increased growth rates and induced Fe limitation, and Fe additions increased APA, an indicator of P limitation. This colimitation was also evidenced, as the simultaneous additions of P and Fe yielded the greatest biomass response (Sterner et al. 2004). In several experiments, the percentage of picoplankton present relative to total Chl *a* was higher for the control and treatments where only Fe was added (Sterner et al. 2004). In the treatments where Fe and P and where P alone were added, the picoplankton percentage was smaller (Sterner et al. 2004), which is what we observed in our Lake Erie experiments where larger phytoplankton dominated when all of the colimiting nutrients were supplied. McKay et al. (2005) also investigated Fe limitation in Lake Superior through the use of a cyanobacterial Fe bioreporter. Results show that the Fe bioreporter gave an Fe-deficient response at offshore stations (McKay et al. 2005). In Lake Huron, Lin and Schelske (1981) conducted nutrient enrichment experiments monthly from April to December 1975. They found that the simultaneous additions of P and Fe resulted in large increases in chlorophyll production and concluded that chelated Fe was an important secondary limiting nutrient after P during the summer months (Lin and Schelske 1981).

Guildford et al. (2003) conducted Fe enrichment experiments in two N-limited African Great Lakes: Malawi and Victoria. In both lakes, the response to enrichment was assessed using Chl *a*, photosynthesis, and nutrient limitation indicators. Three Fe enrichment experiments were conducted in the offshore of Lake Malawi from 1998 to 1999 during two different stratification regimes. Although the addition of Fe alone caused an increase in phytoplankton biomass in only one of the three experiments, when Fe was added with P and N, the Chl *a* response was four times the response to P and N alone. They also reported that rates of P uptake were higher in Fe amended samples and that Fe additions stimulated N uptake. In Lake Victoria, two experiments were conducted in both the nearshore and the offshore during the early stratified season of 1998. The addition of Fe did not result in an increase in Chl *a* in either

experiment; however, at the offshore station, Fe additions stimulated N uptake, and at the nearshore station, the addition of Fe stimulated N_2 fixation rates (Guildford et al. 2003).

Several examples of colimitation by Fe can be found in the marine literature in both coastal and open ocean environments (de Baar et al. 2005). The results of Fe enrichment experiments in the coastal California upwelling region vary from dramatically enhanced particulate C production to no increase at all (Hutchins et al. 1998). However, Fe additions did stimulate NO_3^- drawdown to almost complete depletion (Hutchins and Bruland 1998) and resulted in increased particulate N concentrations (Hutchins et al. 1998). In the open ocean, bottle incubations conducted by Timmermans et al. (1998) from HNLC waters showed that although the addition of Fe did not result in a change in Chl *a* concentrations, a drawdown of the total and dissolved Fe was observed. In addition, on Fe enrichment, a physiological N stimulation was observed, as the addition of Fe increased the NO_3^- uptake rates by a factor of 1.06, although NH_4^+ uptake remained unaltered (Timmermans et al. 1998). Mills et al. (2004) conducted nutrient enrichment experiments at three stations in the eastern tropical North Atlantic. Response to P, N, and Fe enrichment was assessed using Chl *a*, C, and N_2 fixation rates. Evidence of P, N, and Fe colimitation was observed, as the largest biomass increase was found when P, N, and Fe were added simultaneously. Although community primary productivity was N limited, N_2 fixation was colimited by Fe and P, as the addition of P and Fe together resulted in a two- to threefold enhancement of N_2 fixation rates at all three stations (Mills et al. 2004). In other HNLC regions, it was found that the addition of Fe increased the NO_3^- uptake rates by a factor of 5–7 (Martin and Fitzwater 1988) and 2 (Timmermans et al. 2004). In some cases, the addition of Fe caused a complete drawdown of NO_3^- (Martin and Fitzwater 1988; Tsuda et al. 2003) and an increase in particulate N concentrations (Price et al. 1991).

Communities that are N limited are often colimited by Fe at low NH_4^+ concentrations (Price et al. 1991; de Baar et al. 2005). It should be possible to ameliorate this multiple nutrient limitation by adding either Fe or an N source that does not require Fe for its uptake, such as NH_4^+ , provided no additional colimitation prevents a response. Guildford et al. (2005) have pointed out that the eastern basin of Lake Erie is not as P limited as expected for such oligotrophic waters. Although moderate P limitation was detectable throughout the summer, it is hypothesized that phytoplankton may be controlled by factors additional to P. The present results point specifically to low available Fe and NH_4^+ as factors preventing development of strong P limitation in the eastern basin. Furthermore, N limitation in the eastern basin (Wilhelm et al. 2003; Guildford et al. 2005; North, unpubl. data) is likely due more to the availability of Fe than to N concentrations.

The three multiresponse experiments conducted in the eastern basin of Lake Erie provide evidence that under conditions of strong stratification, the phytoplankton communities may become colimited by P, N, and Fe

despite high NO_3^- concentrations. Phytoplankton need Fe when NO_3^- is the source of N for growth; thus, the availability of Fe can limit the uptake of NO_3^- . By alleviating the N limitation, the phytoplankton communities thus become more P limited, leading to a greater response to changes in P concentrations. Therefore, by alleviating N limitation, Fe can affect P limitation in this Laurentian Great Lake. The data presented in this paper support the conclusion that the phytoplankton communities of the eastern basin of Lake Erie exhibit colimitation of P, N, and Fe and suggest that this condition may be more widespread in large areas of the Laurentian Great Lakes.

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Received: 19 June 2006

Accepted: 20 July 2006

Amended: 14 September 2006