

Phosphorus and trace metal limitation of algae and bacteria in Lake Superior

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Abstract

We performed a set of nutrient-enrichment bioassay experiments using metal-clean protocols, testing for macronutrient and micronutrient limitation of algae and bacteria in western Lake Superior. Our analysis involved a novel approach to estimating the strength of nutrient limitation in factorial enrichment designs. We found that phytoplankton and bacterioplankton growth both increased with added phosphorus alone but not with iron, manganese, or zinc alone. However, even very small increases in growth rates following P supplementation (on the order of an additional 0.1 d^{-1}) induced iron limitation in the phytoplankton, indicating that the algae in this large lake may be on the cusp of micronutrient and macronutrient limitation. In contrast, and contrary to expectations, bacteria responded only to P and not to metals. Spatial and temporal variability in degree and quality of nutrient limitation was considerable.

The recent surge of interest in the role of bioactive metals in controlling production in the oceans (Morel and Price 2003) has created a need also to reassess the role of these elements in freshwaters. Recent advances in our understanding of the biogeochemistry of metals in aquatic systems derive in large part from improvements in sampling and analytical techniques, which now allow for measurement of subnanomolar concentrations in natural waters. To date, these improved techniques have been applied primarily to marine habitats. It is now clear that dissolved Fe limits offshore production in the equatorial Pacific Ocean (Coale et al. 1996), the Southern Ocean (Boyd et al. 2000), and subarctic regions of the Pacific (Martin and Fitzwater 1988). Iron also plays a role in regulating productivity in coastal regions, for example in the California (Hutchins et al. 1998) and Peruvian (Hutchins et al. 2002) upwelling regimes. Other bioactive metals are also being reevaluated and in some cases have been revealed as important in biological processes.

Although geochemical explanations of iron limitation in high-nutrient, low-chlorophyll (HNLC) open-ocean regions emphasize the importance of the very low wind-borne supply of iron to these remote locations (Duce and Tindale 1991), observations of metal limitation in coastal waters (described above) indicate that geochemical controls of metal limitation are more complex than simple distance from dust

sources. The dynamics of metal availability in natural waters is also influenced by upwelling (Wu and Boyle 2002), mixing of water masses (Gargett 1991), recycling by consumers (Landry et al. 1997), biological and chemical equilibria regulated by organism uptake (Maldonado et al. 2001), sorption to surfaces of particles (Ciffoy et al. 2001), and binding by biogenic and abiotic ligands of differing affinity and concentrations (Rue and Bruland 1997). High rates of uptake and high levels of organic complexation may reduce metal availability so that metal deficiency may occur even where total metal supply is high. In addition, whether trace metals or macronutrients limit primary productivity is a function of the relative supply of all potentially limiting substances. If P and N inputs are high enough, micronutrient deficiency may be induced even at high absolute metal loading rate.

Certain terms used here require operative definitions because they lack generally accepted meanings (Howarth 1988). Our focus is on the interactions between the growth rate of the extant biological community as a whole and the available nutrients in the water. Howarth (1988) referred to this as “limitation of net ecosystem production,” and others have referred to it as Blackman as opposed to Liebig limitation (*see, e.g., Thingstad et al. 1998*). We define limitation as a reduction in the growth rate of an *in situ* community due to low nutrient concentration. We define deficiency as a situation where there is insufficient nutrient to allow for maximum growth rate. One can also think of a stress in terms of physiological adjustment of a species to low nutrients that may precede, or occur with or without, deficiency or limitation.

Evidence that trace metals limit algal growth in lakes was

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Table 1. Concentrations of selected elements (nmol L^{-1} , total dissolved) in Lake Superior as measured by Nriagu et al. (1996) from sampling during August and September of 1991 and by Sherrell et al. (Unpub. data) during June, July, and September 2000. For comparison, values for the open ocean (Donat and Bruland 1994) also are presented. Lake Superior ranges exclude any very high values observed very near the lake bottom, and in the case of Sherrell et al., include a few nearshore observations that had considerably higher concentrations than offshore (n.d. = not determined).

	Nriagu	Sherrell	Open ocean
Fe	0.6–27	1.1–100	0.002–1.0
Mn	0.1–6	0.3–8	0.1–3.0
Zn	2–13	1–7	0.1–8.2
Cu	10–12	10–13	0.5–4.5
Cr	0.6–2	1.0–1.9	3–5
Pb	0.001–0.1	0.002–0.037	0.003–0.150
Ni	n.d.	1.2–37	2–12
Cd	n.d.	0.05–0.13	0.001–1.00
Co	n.d.	0.005–0.28	0.004–0.300

presented years ago (Schelske 1962). In fact, reports of stimulation of freshwater algal growth in response to metal amendments are common in the literature, even in such seemingly unlikely places as a meromictic urban lake (Swain 1984) or reservoirs with short hydraulic residence time (Sternier 1994). Large lakes have also been observed sometimes to have metal-limited or metal-deficient algae (e.g., Storch and Dunham 1986). These freshwater studies, however, preceded the analytical advances of recent years. It is widely accepted that metal concentrations in natural waters are easily contaminated by general laboratory conditions, calling into question the validity of early experiments. Experimental contamination of lake water by metals might bias an experiment against seeing metal limitation, but if macronutrient spikes used in early studies themselves had metal contaminants, responses attributed to macronutrients may have been incorrectly assigned. Recently, trace metal–clean techniques were used by Twiss et al. (2000) in incubation experiments in Lake Erie. Their work corroborated the earlier findings that freshwater algae may be metal limited. In one of their experiments, algae showed large growth stimulation resulting from iron addition alone. Apparently, trace nutrients play at least a supporting role in controlling algal growth rates in natural freshwater habitats as well as in the oceans.

This study evaluated trace metal interactions with both phytoplankton and bacterioplankton in Lake Superior. Lake Superior, Earth's largest lake by area, offers an interesting point of comparison both with oceanic studies of metal limitation, as well as with smaller freshwater lakes. Several early studies of Lake Superior pointed to the potential importance of bioactive metals to algal dynamics (Schelske et al. 1972; Shapiro and Glass 1975). The possibility of metal limitation in the Laurentian Great Lakes was made even more compelling through the work of Nriagu et al. (1996), who used metal-clean techniques and reported far lower concentrations of trace metals than previous, probably contaminated, studies (Table 1). Coincident with the work reported here, we used highly sensitive analytical techniques (Field

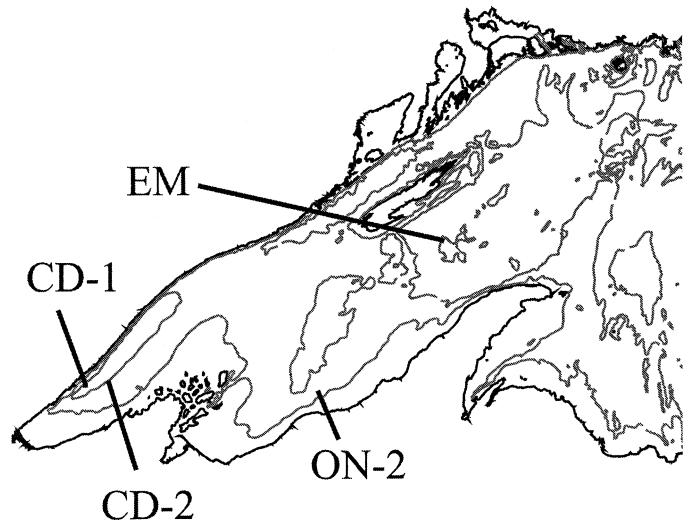


Fig. 1. Study region and sampling sites.

and Sherrell 2003) to determine trace metal concentrations in Lake Superior (Table 1). We found that total dissolved ($<0.45 \mu\text{m}$) iron, although not as low as reported for open-ocean environments (Johnson et al. 1997; Wu and Boyle 2002, Table 1), can be sufficiently low ($<2 \text{ nmol L}^{-1}$) to suggest the possibility of iron limitation. Both manganese and iron in Lake Superior are very dynamic spatially and temporally, whereas zinc shows a pronounced lake-wide seasonal epilimnetic drawdown and hypolimnetic buildup (Sherrell et al. unpubl. data). Nitrogen limitation in Lake Superior seems unlikely, at least for species capable of using nitrate, because of high nitrate concentrations in the lake (Bennett 1986) (*but see Discussion*). Dissolved phosphorus on the other hand is typically at the analytical limit of detection using the common soluble reactive phosphorus method (Baehr and McManus 2003; Weiler 1978). Total dissolved phosphorus generally runs between 20 and 30 nmol L^{-1} according to our measurements using ICP-MS (Inductively Coupled Plasma-Mass Spectrometer) techniques (Field and Sherrell 2003).

Bacteria may also be sensitive to low metal concentrations in the water. Cochlan (2000), for example, reported that bacterial abundance responded to large open-ocean iron additions in the IronEx II experiment, a response that may reflect either release from a direct iron limitation of bacterial growth or a coupling between enhanced algal growth and availability of other bacterial growth substrates such as organic carbon. Church et al. (2000) found that bacteria in the Southern Ocean responded to Fe additions in the dark when an organic carbon source (glucose) was also added, indicating that bacteria may be at least in iron deficit. Similar results have been reported for the California upwelling system (Kirchman et al. 2000), but until now freshwater bacterial response to metal availability has not been evaluated.

We assessed the role of several trace metals in limiting growth of phytoplankton and bacterioplankton in Lake Superior using metal-clean nutrient amendment bioassays conducted at multiple locations in the western half of the lake over three field seasons. We wished to gain an overall as-

Table 2. Experiments and conditions. Depth refers to the depth from which samples were taken. Light is reported as $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ or as percent of incident natural sunlight. Incubation locations refer to Saint Paul (SP), Bowling Green (BG), or on-deck (Deck). Treatments corresponds to the information given in Table 4. The treatments Fe, Mn, P+, and Zn were performed in a complete factorial design while EDTA, and 90:10 were single-factor treatments.

Date	Station	Depth (m)	Temp (°C)	Light	Incub. location	Incub. time (d)	Incub. volume (L)	Treatments	Initial Chl <i>a</i> ($\mu\text{M L}^{-1}$)	Initial bacteria (cells $\text{ml}^{-1} 10^6$)
27 Sep 99	CD-1	12	7	75	SP	6.5	1.0	Fe, Mn, P+ EDTA	1.33	—
27 Sep 99	CD-1	12	7	75	BG	6.5	10.0	Fe, Mn, P+	1.33	
9 Jun 00	EM	30	4	75	SP	7.5	1.0	Fe, Zn, P+, 90:10	0.81	1.3
21 Jul 00	CD-1	21	8	65	SP	6.6	1.0	Fe, Zn, P+, 90:10	2.3	1.9
18 Jul 00	EM	30	12	10%	Deck	2.8	1.24	Fe, Zn, P+, 90:10	1.7	1.4
18 Sep 00	ON-2	20	13	65	SP	8.8	1.24	Fe, Zn, P+	1.1	1.3
17 Sep 00	CD-2	20	13	10%	Deck	2.9	1.24	Fe, Zn, P+	1.7	1.6
29 May 01	CD-1	17	3	10%	Deck	3.4	1.24	Fe, Zn, P+	0.85	—

assessment of metal deficiency in the context of macronutrient (primarily P) limitation and to begin to assess some of the expected temporal and spatial variability of nutrient limitation. To do the latter with limited sampling ability, we focused on the regions in the lake where we expected the largest concentration gradients in macronutrients and micronutrients, based on previously published studies of Lake Superior chemistry (Weiler 1978). Theories about metal control of algal growth in the ocean emphasize isolation from continental dust and coastal riverine or sedimentary inputs, and we performed experiments both near to and far from shore (Fig. 1). One hypothesis tested is whether the largest expanse of fresh water on Earth has spatial nutrient deficiency patterns similar to the oceans. But in addition, our study is one of the first to use metal-clean methods to investigate metal limitation in freshwaters.

Methods

Eight experiments during five research cruises were conducted from September 1999 to May 2001. Conditions included both cold and isothermal water columns (June 2000 and May 2001) and thermal stratification (September 1999, July and September 2000) (Table 2). Dissolved Fe was $<10 \text{ nmol L}^{-1}$ during all but one experiment, dissolved Mn was $<2 \text{ nmol L}^{-1}$, dissolved Zn was $<3 \text{ nmol L}^{-1}$, and dissolved P was $<30 \text{ nmol L}^{-1}$ in all but one experiment (Table 3).

We used a metal-clean collection system consisting of 200 m of thoroughly cleaned one-half-inch inner diameter (ID) teflon-lined polyethylene tubing and a pneumatic pump. The depth of sampling coincided with the chlorophyll fluorescence maximum whenever this ubiquitous mid- to late-season feature (Barbiero and Tuchman 2001) was observed; at other times, water was collected from 20 m. Water was pumped to the surface and exposed to the atmosphere only within a laminar flow, HEPA (High Efficiency Particulate Air)-filtered clean bench. Analysis of numerous samples for a suite of trace metals collected with this system indicated negligible contamination (Field and Sherrell 2003). Polycarbonate incubation bottles were rinsed 5 times with Nanopure water, soaked in 5% reagent-grade HCl at 60°C overnight, rinsed 5 times with Nanopure, and double-wrapped in plastic

bags until use. Bottles were rinsed once with lake water before filling. After filling, nutrient spikes (Table 4) were added, and the bottles were capped, sealed with Parafilm, and mixed. Experiments were designed to emphasize detection of limitation by single metals (depending on the experiment, either Fe, Mn, or Zn). Our initial purpose was to remove all possibility that other micronutrients would become limiting; hence we included two other trace metals along with the phosphorus spike (referred to as “P+”, Table 4). Nevertheless, we judge the probability of growth limitation by any one of the metals in the phosphorus spike to be very unlikely, and in retrospect we have chosen to interpret responses to this treatment as a phosphorus effect. Experiments during September 1999 used Fe and Mn as the single metals of interest, whereas later experiments focused on Fe and Zn. Where filtered water was required (i.e., 90:10 dilution treatments), particles were removed with an in-line acid-cleaned all-polypropylene 0.45- μm capsule filter.

Incubations were performed either in laboratory incubators at light and temperature conditions representative of the mixed layer for those dates (Table 2) or in an on-deck, flow-through incubator that kept the samples at the temperature of surface water in the lake at the time. For on-deck incubations, light was attenuated with neutral density screening, which reduced the light level to ca. 10% of incident (roughly equal to the 30-m depth level). Laboratory incubations incorporated a 14:10 h light:dark cycle. Bacteria and algae were incubated in the same bottles. In some experiments, chlorophyll was size-fractionated (1999, 0.2–5, 5–20, 20–80 μm ; 2000 and 2001, 0.2–2, 2–20, $>20 \mu\text{m}$) using polycarbonate membrane and Nitex filters. Chlorophyll measurement used 0.2- μm cellulose nitrate membranes, extraction in 90% acetone, and measurement using a narrow band fluorometer (Welschmeyer 1994). Bacteria were preserved in formalin (final concentration 2%) and counted using 0.2- μm black polycarbonate filters (Hobbie et al. 1977), acridine orange (1 mg ml^{-1}), 1,000 \times magnification and epifluorescence illumination. Growth rates were calculated from initial and final values assuming exponential change. Our experiments ranged in incubation duration, but we did not see any effect of incubation length in our results.

Primary treatments (P+, Fe, and either Mn or Zn during

Table 3. Lake concentrations (total dissolved, nmol L^{-1} , <0.45 , $\mu\text{mol L}^{-1}$) of selected elements corresponding to date and depth of sampling for experiments.

Date	Station	Depth (m)	Fe	Mn	Zn	P
27 Sep 99	CD-1	12	5.5	1.2	1.9	27
9 Jun 00	EM	30	3.4	0.46	2.6	23
21 Jul 00	CD-1	21	5.2	0.75	1.6	25
18 Jul 00	EM	30	1.5	0.37	2.3	24
18 Sep 00	ON-2	20	6.1	1.5	1.6	26
17 Sep 00	CD-2	20	5.3	0.88	1.7	42
29 May 01	CD-1	20	18.4	1.8	2.4	22

1999 and 2000–2001, respectively) were applied in a complete factorial design, with all possible combinations of treatments performed in triplicate (with one exception being an unreplicated experiment conducted in 20-liter carboys in September 1999). We switched from Mn to Zn treatments after our initial year because we observed that Zn concentration had substantial epilimnetic drawdown and hypolimnetic buildup, and we wanted to see whether it was reduced to limiting levels. Furthermore, zinc interactions with P—arising from the fact that alkaline phosphatase is a Zn-requiring enzyme—and with N (Wang et al. 2001) may be important. Several additional treatments were imposed without all possible treatment combinations. We performed one experiment with ethylenediaminetetraacetic acid (EDTA) to examine the effect of changed chelator concentration, where significance was judged via a two-sample comparison (*t*-test) with the controls. We also performed a number of 90:10 filtered:unfiltered water treatments to examine herbivory and bacterivory (Landry and Hassett 1982), again comparing growth in these treatments to controls. All treatments were applied with a single level. Analysis of the primary treatments began with a three-way analysis of variance (ANOVA) (which found significance at $p < 0.001$ in all cases), followed by a post hoc least significant difference (LSD) analysis. The LSD test identifies homogeneous groups, or treatment combinations not statistically different from one another; letters on the figures indicate these.

Our interpretations are based principally on an examination of effect sizes. Effect sizes are absolute differentials of growth between specified treatments. We tested whether these differentials differed from zero (Kuehl 1994), but effect sizes emphasize the magnitude of response, whereas strict hypothesis testing using ANOVA alone does not (Cohen 1994; Johnson 1999). Effect sizes as calculated here correspond to the Δr measure as used by Downing et al. (1999) in their review.

We examined in particular two types of effects of nutrients on growth. The first of these was the response of algae and bacteria to single addition of any treatment. For example, response to Fe is given by the growth rate in the presence of the Fe alone treatment minus growth in controls. We refer to such an effect as +Fe. The second was the response of algae and bacteria to a treatment in the presence of all other treatments. For example, response to Fe in the presence of high levels of other nutrients was given by the growth in the

Table 4. Spike concentrations used in P and metal enrichment incubation studies in 1999, 2000, and 2001. The phosphate treatment was made from K_3PO_4 dissolved to 50 mmol L^{-1} , and precleaned by ion exchange chromatography using Chelex-100 resin. All metal spikes were 10 or 1000 ppm ultrapure single-element ICP-MS standards (High Purity Standards), made from pure metal solids and provided in 2% HNO_3 (final NO_3 concentrations given in table). The Co and Mo spikes were added separately at time of experiment.

Spike	Final concentrations in bottle (nmol L^{-1})	NO_3 additions ($\mu\text{mol L}^{-1}$)
P+	1000 P, 4 Co, 3 Mo	15
Fe	50	0.9
Zn	10	21
Mn	50	0.9
EDTA	50	0
Cd	10	7

bottles receiving all treatments minus the growth in bottles receiving everything but Fe. We refer to such an effect as -Fe (the minus sign is merely a mnemonic aid to allow us to discuss these two kinds of contrasts concisely). We presume the first kind of effect, e.g., +Fe, measures the instantaneous, ongoing, limitation of the extant biological community by that nutrient. The latter kind of effect, e.g., -Fe, we presume measures a potential limitation, or what we define as a deficiency, once all other nutrients are made abundant. Where multiple nutrients limit growth either of individual species or of different species, one should see both kinds of effects, with the minus effects expected to be the same magnitude or larger than the plus effects because

Table 5. Growth rates (d^{-1}) for control (no nutrient amendment) bottles.

Date	Station	Fraction	Whole	Diluted
Sep 99	CD-1	Total chl	-0.0069	—
		Pico	0.0057	—
		Micro	-0.035	—
		Nano	-0.010	—
Jun 00	CD-1	Total chl	0.038	—
	EM	Total Chl	-0.025	0.14
Jul 00	CD-1	Bacteria	-0.012	0.29
		Total chl	-0.044	0.11
Jul 00	EM	Bacteria	-0.67	0.66
		Total chl	-0.12	0.061
		Pico	0.0026	0.12
		Micro	-0.18	-0.15
		Nano	-0.29	-0.40
Sep 00	ON-2	Bacteria	-0.067	0.66
		Total chl	0.0098	—
Sep 00	CD-2	Bacteria	-0.023	—
		Total chl	-0.0081	—
		Pico	0.17	—
		Micro	0.043	—
		Nano	-0.20	—
May 01	CD-1	Bacteria	0.0042	—
		Total chl	-0.000085	—
		Pico	-0.00016	—
		Micro	0.000046	—
		Nano	-0.000065	—

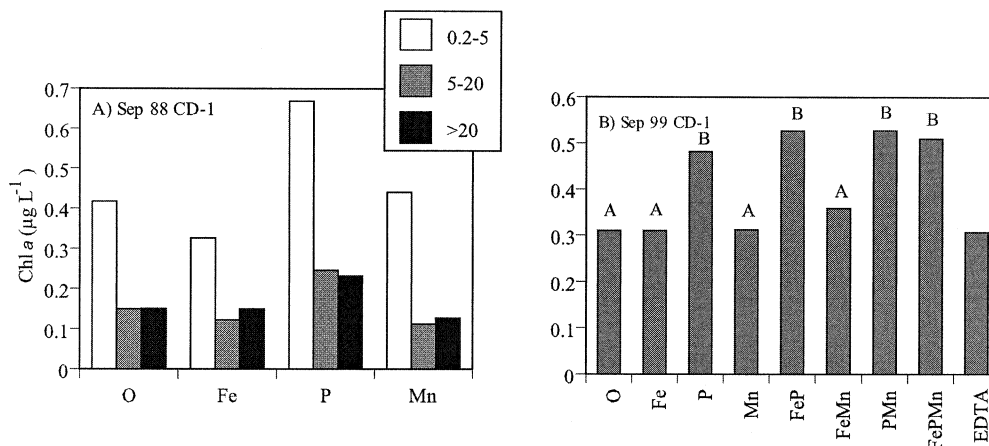


Fig. 2. Chlorophyll *a* concentrations at the end of incubation for experiments performed during 1999. Bars in A represent size-fractionated values for unreplicated bottles. Bars in B represent means of three bottles per treatment combination. Letters above bars in B indicate homogeneous groups by LSD test at $p = 0.05$. In this and the following figures, letters above bars indicate groups of treatments not statistically different from one another. The EDTA treatment, was tested with a *t*-test against the control bottles, and was found not to be significantly different ($p = 0.99$).

growth reductions due to other nutrients are removed in the minus comparisons. We invoke growth limitation in the lake only when both the plus and the minus effects are statistically different from zero.

Alkaline phosphatase activity (APase, E.C. 3.1.3.1) was measured in samples of whole water collected using the clean pump system at station EM (Fig. 1) during July 2000 (30 m), station ON-2 during September 2000 (10 m), and station CD-1 during May 2001 (17 m). Enzyme activity was also measured in subsamples from the large bottle amendments (CD-1, September 1999) at the completion of the incubation. Whole water was dispersed to triplicate methacrylate cuvettes (2.5 ml) and incubated with $40 \mu\text{mol L}^{-1}$ 4-methyl umbelliferyl phosphate (Sigma) in a deck-top flow-through incubator at ambient surface water temperature. Assays associated with the large bottle amendments were conducted at ambient laboratory temperature ($\sim 20^\circ\text{C}$). Sodium bicarbonate (4 mmol L^{-1}) was substituted for whole water in substrate controls, whereas quench standards were prepared using whole water and $1 \mu\text{mol L}^{-1}$ of 4-methylumbelliferone (Sinsabaugh et al. 1997). Enzyme activities were calculated using a reference standard containing $1 \mu\text{mol L}^{-1}$ of 4-methylumbelliferone. APase was normalized to a chlorophyll-specific value, although we recognize that other contributors (such as bacteria) probably played some role.

APase-catalyzed fluorescence was determined using a TD-700 laboratory fluorometer (Turner Designs) equipped with a near-UV lamp and a methylumbelliferyl filter set (ex, 300–400 nm; em, 410–610 nm). Sensitivity of detection of the fluorescent products of methylumbelliferyl substrates is pH dependent with maximum fluorescence obtained at pH 10.3 (Hoppe 1993). Recognizing this, measures of mixed layer pH were made at various stations using a calibrated pH meter with values ranging from 7.4 (September 2000) to 7.8 (May 2001). End-point addition of NaOH (20 mmol L^{-1}) as part of several experiments resulted in increased fluores-

cence of only $\sim 4\%$. As a result, sample pH was not adjusted prior to reading fluorescence.

Results

Lake temperatures were generally cold, and the phytoplankton in the lake mainly inhabited dimly illuminated strata (Table 2). When combined with low dissolved nutrient concentrations, these conditions produced only slow growth of both algae and bacteria in all treatments. In addition, the extant community may have been dominated by species with low maximal growth rates. Most of our experimental treatments were performed with whole (unfiltered) lake water; this minimized water handling and potential risk of contamination. However, it is apparent from a comparison of growth rates in whole water compared to the growth in the dilution treatments (Table 5) that measured growth in whole water is often substantially less than a true maximum. In almost all cases, growth in the diluted water (which reduced encounters between consumers and particles and thus grazing losses) was considerably higher than growth in the whole water controls (for further examples for Lake Superior, see Schampel 2001). Significant herbivory and bacterivory likely occurred in the whole lake water incubations. Our further analysis is based on the assumption that herbivory and bacterivory rates are independent of nutrient treatment.

Significant differences among final chlorophyll levels as a function of nutrient amendments were observed in all experiments (ANOVA: $p < 0.001$). Experiments in 1999 were performed with Fe and Mn as well as the phosphorus treatment (Table 4). Two experiments from September 1999 (Fig. 2) demonstrated responses to the phosphorus treatment; statistical analysis was possible in only one of these experiments. It appeared that all size fractions of algae responded to P, though we could not test that aspect of the data statis-

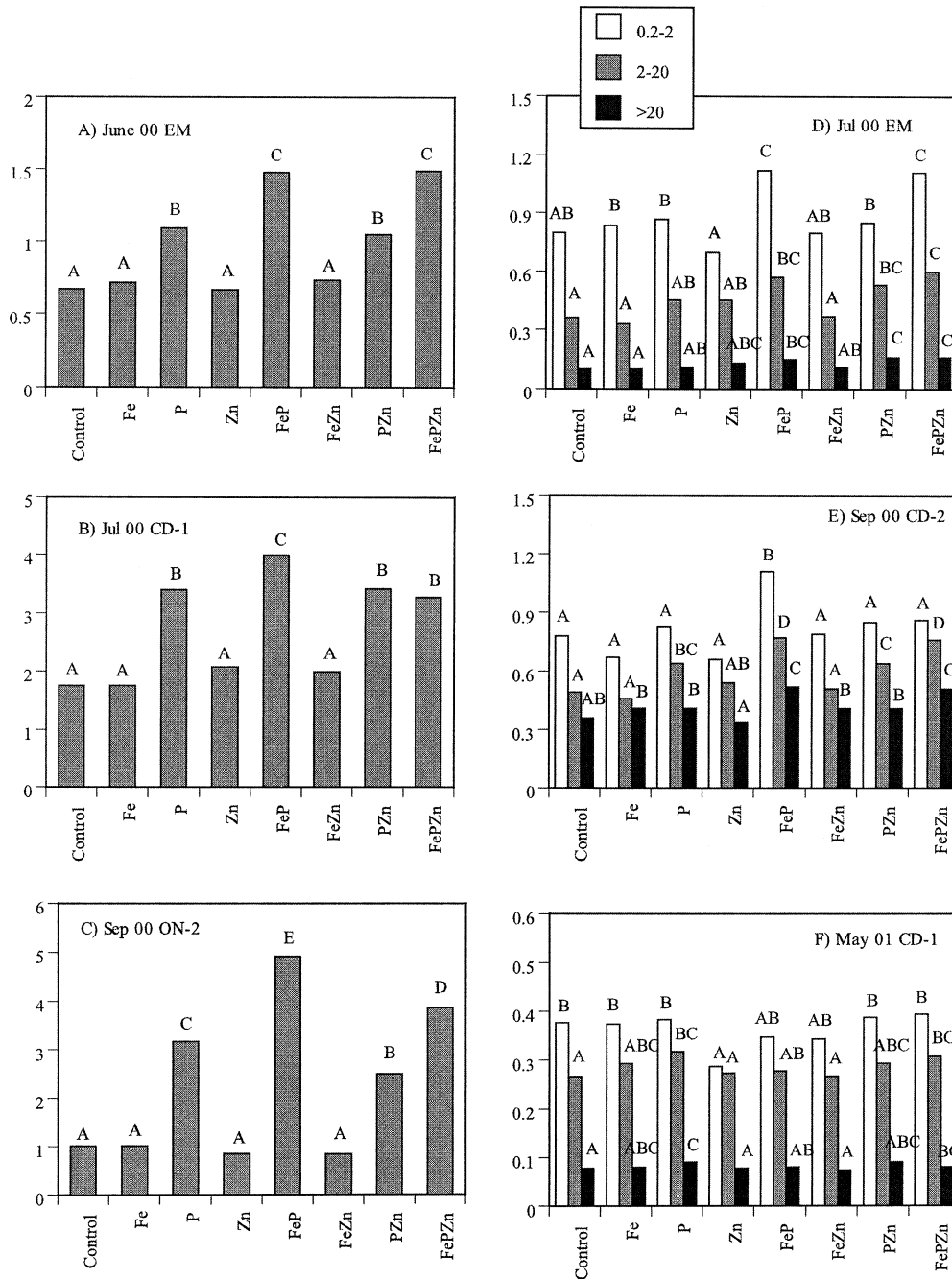


Fig. 3. Chlorophyll *a* concentrations at the end of incubation (mean of three bottles per treatment combination) for experiments performed during 2000–2001. Letters above bars indicate homogeneous groups within particular size fractions. Six separate experiments are represented. In three of them (right panels), chlorophyll was size fractionated. In the other three, only total chlorophyll was measured.

tically. No effects of Fe or Mn or interactions between those metals were observed in 1999. EDTA alone had no measurable effect on algal growth.

During 2000 and 2001, we replaced the Mn treatment with a Zn treatment, retaining Fe and P treatments (Table 4). Responses to the phosphorus treatment continued to be very noticeable. However, in most experiments metals also had effects in some treatment combinations (Fig. 3). In June

2000, algae responded to phosphorus, but two of the treatments including both P and Fe gave even larger algal yield (Fig. 3A). Similarly, experiments from July and September 2000 demonstrated the largest responses in treatments combining phosphorus and one or more of the metals (Fig. 3B–E). The experiment from May 2001 had the lowest incubation temperature of all experiments (Table 2), and algal abundance also was lowest. Some small and subtle differ-

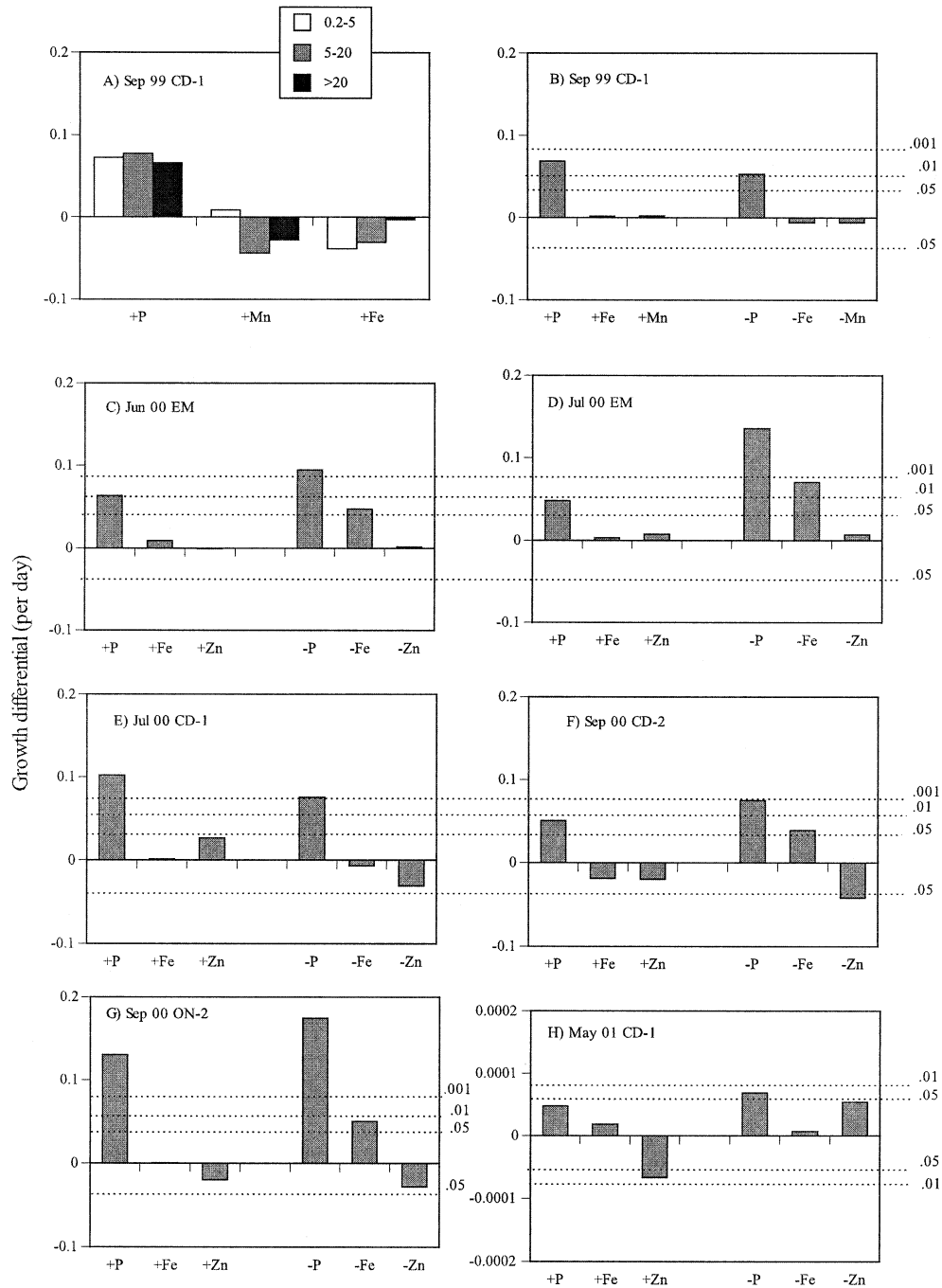


Fig. 4. Differences in chlorophyll *a*-determined growth rates between selected treatment combinations. Factors marked with + correspond to the difference in growth rates between single factor additions and controls lacking nutrient amendments. Factors marked with - correspond to the difference in growth rate between treatments receiving all nutrients and treatments receiving all but one nutrient; thus, - treatments measure the effect of one treatment in the presence of supplementation by all other nutrients. Size-fractionated values are given in panel A; the other panels show results for total chlorophyll. Horizontal dotted lines indicate critical single comparison values at the indicated *p* values. To calculate these critical values, the mean square for error was pooled among the results given in panels B-G, where growth rates were in a similar range. Critical values for panel H were calculated separately. A more conservative test would consider the six effect sizes to be independent contrasts and the critical *p* value for experiment-wide error of 0.05 would correspond to a level of 0.009 on these graphs (close to the 0.01 dotted lines).

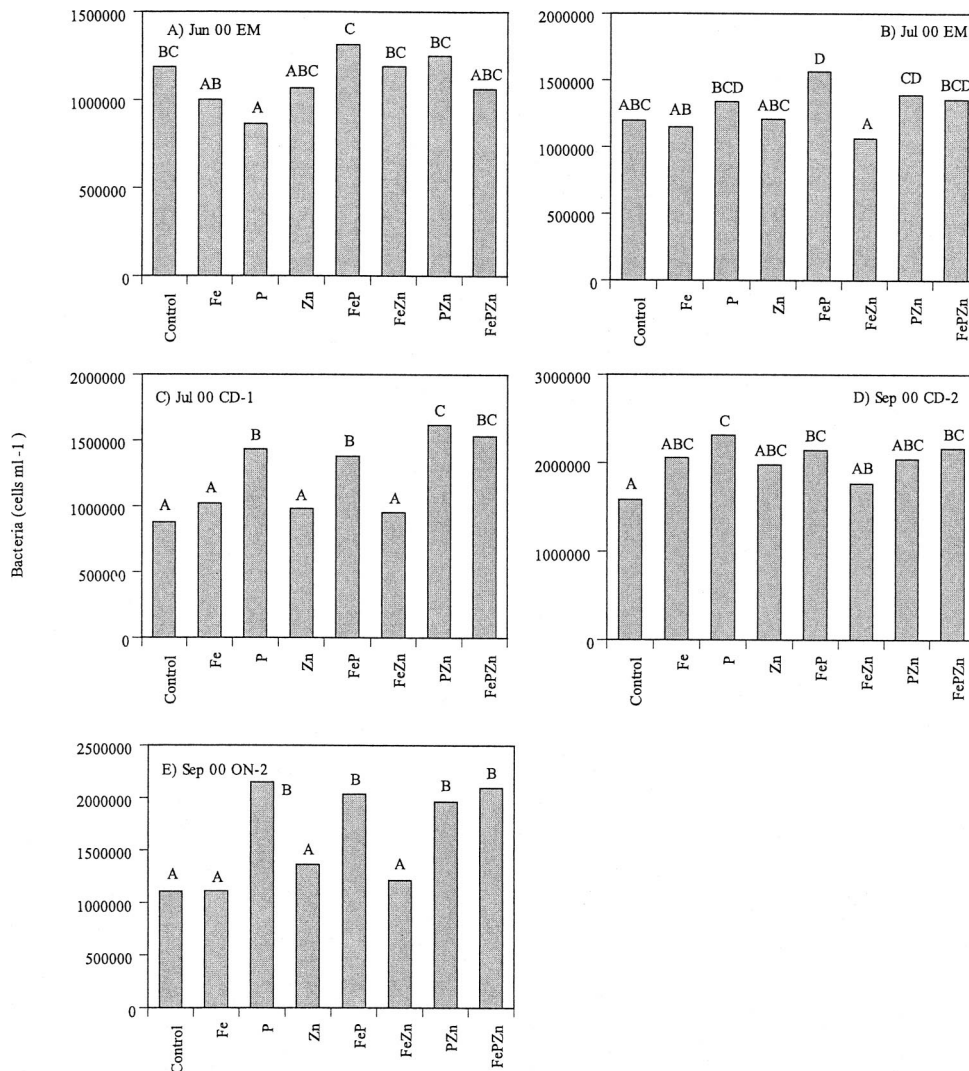


Fig. 5. Cell densities of bacteria at the end of incubation (mean of three bottles per treatment combination). Letters above bars indicate homogeneous groups.

ences among treatments nevertheless manifested themselves (Fig. 3F). Algal size fractions behaved similarly to one another, although not identically (note homogeneous groups identified by letters over data bars). In general, responses to phosphorus plus additional response to one or more metals was observable in all size fractions to varying degrees during lake stratification. No size fractions responded to metals alone compared to controls.

Multiway ANOVA has the advantages both of a large level of replication of main effects and the presence of all possible treatment combinations so that numerous interactions can be revealed. However, biological hypotheses about limitation are best addressed with specific contrasts. We found consistent evidence for P limitation from both plus and minus contrasts (as defined in Methods) in all experiments (with only the May 2001 experiment lacking significance of the +P treatment) (Fig. 4). As expected, -P effects were generally larger than +P effects, which indicate a larger effect of phosphorus in the presence of supplemented metals. The -P treatments were significant in all experiments based

on single comparisons at an error rate of 0.05. All but one -P treatment was significant using a more conservative experiment-wide error rate of 0.05 for six independent contrasts (see legend to Fig. 4). We conclude that algal growth in the lake was in fact limited by phosphorus during most if not all of the study period. Growth increments in response to the phosphorus treatment were mainly in the range of 0.05 to 0.15 d⁻¹.

In no cases did Fe alone stimulate algal growth, as can be seen in the +Fe treatments (Fig. 4) as well as the homogeneous groups determined from final algal abundances (Fig. 3). Zinc alone had a negative effect on algal growth rates in the experiment in May 2001 (Fig. 4H). One may conclude from these results either that (1) algae in the lake were well adapted to low trace nutrients or had access to sufficient manganese, iron, and zinc so that they did not limit growth, or that (2) any growth limitation was too weak to be detected in our experiments, or that (3) in situ growth limitation by micronutrients occurs but is not detectable because of a simultaneous deficit of phosphorus.

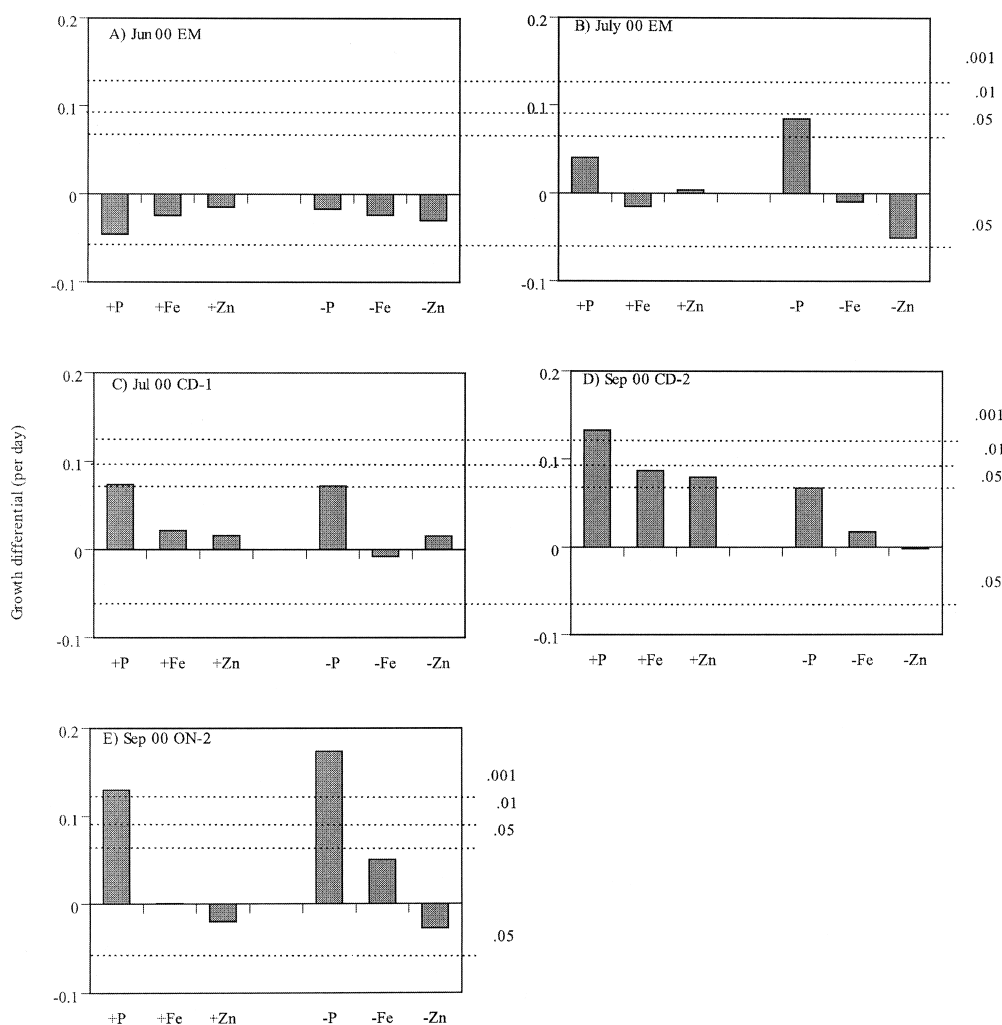


Fig. 6. As in Fig. 4, but for bacterial abundances based on counts of cell numbers. Critical values using pooled errors are indicated by horizontal dashed lines. See legend to Fig. 4 for discussion of experiment-wide error rate.

We did, however, see multiple cases of an effect of iron on Lake Superior algae in the presence of other treatments, particularly phosphorus. Significant $-Fe$ responses, which indicate a response to Fe when other nutrients were added, were observed in June 2000 and July 2000, both at the EM site, and in September 2000 at the ON-2 site (Fig. 4C,D,G). These are the most easterly stations (Fig. 1). It was also observed at the CD-2 site in September 2000 (Fig. 4F). In these instances one may conclude that the lake water contained insufficient iron to support phosphorus-enhanced growth and that although there was no metal limitation, there was metal deficiency. Alternatively, iron may have been limiting to algal growth, but the simultaneous large phosphorus limitation made it impossible to observe an iron limitation. The Fe required for a doubling of biomass (1 week of growth at an extra 0.1 d^{-1}) is approximately $0.4 \text{ nmol L}^{-1} \text{ Fe}$, which comes from estimating $8 \mu\text{mol L}^{-1}$ particulate organic carbon (POC) and a C:Fe ratio of 20,000. Given the observed Fe concentrations (Tables 1 and 3) and the likelihood that a small percentage of total iron is bioavailable, growth demand

may well have outstripped availability. At a minimum, these results indicate that Lake Superior phytoplankton are near a true Fe limitation and that the lake has insufficient iron in the water to support even the modest enhanced growth rates generated by amended phosphorus. Zinc had an inhibitory effect in the presence of other nutrient spikes in the experiment in September 2000 at site CD-2 (Fig. 4F).

Phosphorus was the predominantly important factor in the bacteria growth experiments. As seen for algae, abundances of bacteria at the end of the incubation period were significantly related to treatment in all experiments (Fig. 5). Differences in abundance were small, however. Inspection of the + and - effects (Fig. 6) shows significant stimulation of bacteria by P in four of five experiments. The exception was the first experiment from the unstratified period in June 2000, when, somewhat mysteriously, bacterial abundances were reduced by addition of nutrients singly or in combination with other nutrients (Fig. 6A). Perhaps nutrients stimulated bacterivorous consumers in this experiment. The July 2000 EM experiment indicated P limitation, but this was

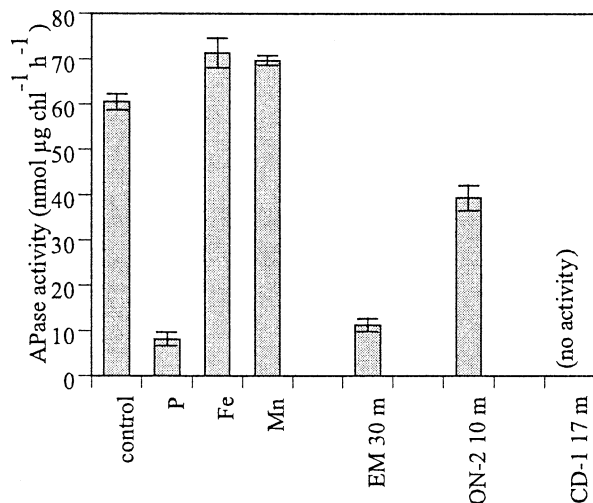


Fig. 7. Alkaline phosphatase enzyme activity level in experiments and in the lake. From left to right, values originate from experimental treatments from station CD, September 1999 (first four bars, treatments as marked); station EM, July 2000; station ON-2, September 2000; and station CD-1, May 2001.

statistically significant only when Fe and Zn were present (Figs. 5, 6), possibly indicating a secondary role of metals in that experiment. The July 2000 CD-1 experiment (Fig. 6C) showed an effect only of P, and the similarity of +P and -P treatments indicated no effect of either Fe or Zn. The September 2000 CD-2 experiment (Fig. 6D) showed a significant +P but no significant -P effect; this is difficult to interpret. Further, in the same experiment, the +Fe treatment was only marginally significant even with a single comparison test ($p = 0.046$). Considering the small effect sizes and lack of consistency of effects of metals on bacterial growth, this apparently significant response to iron is not strong evidence for Fe limitation of bacteria in the lake. Finally, the September 2000 ON-2 experiment (Fig. 6E) provided strong evidence for P limitation of bacteria; these phosphorus effects were significant under the more conservative experiment-wide test as well. In general, evidence for participation of metals in bacterial growth dynamics is much weaker than we saw for algae, and the most appropriate conclusion seems to be that neither Fe nor Zn were limiting, or even deficient, to bacteria.

Alkaline phosphatase activity also indicated P deficiency (Fig. 7). When we applied the indices described by Healy and Hendzel (1979), rates of APase activity measured in all samples except for the May 2001 sampling at station CD-1 indicated severe P deficiency. Lack of activity in May 2001 is consistent with our bioassay results, which also failed to find significant growth stimulation of P for this particular station and date (Fig. 4H). As expected, P addition greatly reduced APase activity compared to controls and metal addition treatments in the large volume bioassay conducted in September 1999.

Discussion

Several characteristics make Lake Superior intriguing from the standpoint of potential metal limitation. Because of

its large surface area and relatively small ratio of watershed to lake area, direct atmospheric inputs are generally very important to the lake's biogeochemistry. Although hardly as removed from continental influence as the open ocean, Lake Superior may well represent the closest freshwater analog. It also exhibits surplus nitrate (but not phosphate), and low algal abundance, further similarities to the high-nutrient, low-chlorophyll (HNLC) regions of the ocean.

Our results are consistent with a metal deficiency, if not a metal limitation of algal growth. The most conservative interpretation of our results is that even very small P-induced growth demands of only about 0.1 d^{-1} (doubling time $\sim 7 \text{ d}$) cannot be met by the available iron. Dissolved iron in Lake Superior is highly dynamic in space and time (Nriagu et al. 1996). In particular locations, there is a pronounced spatial gradient from nearshore to offshore, with concentrations in excess of 100 nmol L^{-1} near river outflows but consistently low concentrations of $<5 \text{ nmol L}^{-1}$ offshore (Sherrell et al. unpubl. data). Given these gradients, it is interesting to note that we observed -Fe effects (Fig. 4) at all sites except the most westerly one (CD-1, Fig. 1). Westerly wind-borne iron in dust may induce nearshore-offshore gradients similar to those hypothesized for portions of the ocean, but little is known about spatial patterns of nutrient delivery, or even lake-wide circulation patterns in this water body. We hypothesize that there is considerable spatial variability in nutrient limitation patterns, likely driven in part by the nearshore delivery of iron with a rather rapid depletion of iron as water moves offshore. Precise mapping of nutrient limitation is beyond the resolution of this study, but the results are suggestive of such a trend.

Neither Zn nor Mn had a stimulatory effect on algal or bacterial growth. In contrast, zinc supplements inhibited algal growth in two experiments. Reasons for such responses are somewhat elusive. Zinc levels were sufficiently low (Tables 1 and 3) that direct toxicity seems a remote possibility. Inhibitory effects of zinc on algae were observed both in May and in September, and in the eastern and western portions of the sampling area (Fig. 4G,H), but were not observed in most experiments, and we are unable to interpret these responses further.

We further explored patterns in algal effect size by first examining whether effect size related to temperature, likely to be an important factor in this low-temperature environment. We found (Fig. 8A) a statistically significant, positive effect of temperature (analysis of covariance [ANCOVA], temperature effect: $t = 2.64$, $p = 0.02$) and a significant difference between the effect sizes for the two nutrients ($t = 2.96$, $p = 0.01$). This temperature effect is likely caused by an expanded dynamic range of growth rate at higher temperature, simply because maximal growth rate is higher at warmer temperature. We then explored the role of lake chemistry on temperature-corrected effect sizes by plotting the residuals from (A) against the associated lake concentration of either Fe or P from Table 3. For Fe (Fig. 8B), effect sizes were greatest at the lowest lake concentrations. For P, the data were more equivocal: a small effect was observed in the one experiment with high lake concentration but no other trend was apparent.

Our data suggest that there is a contrast in the growth

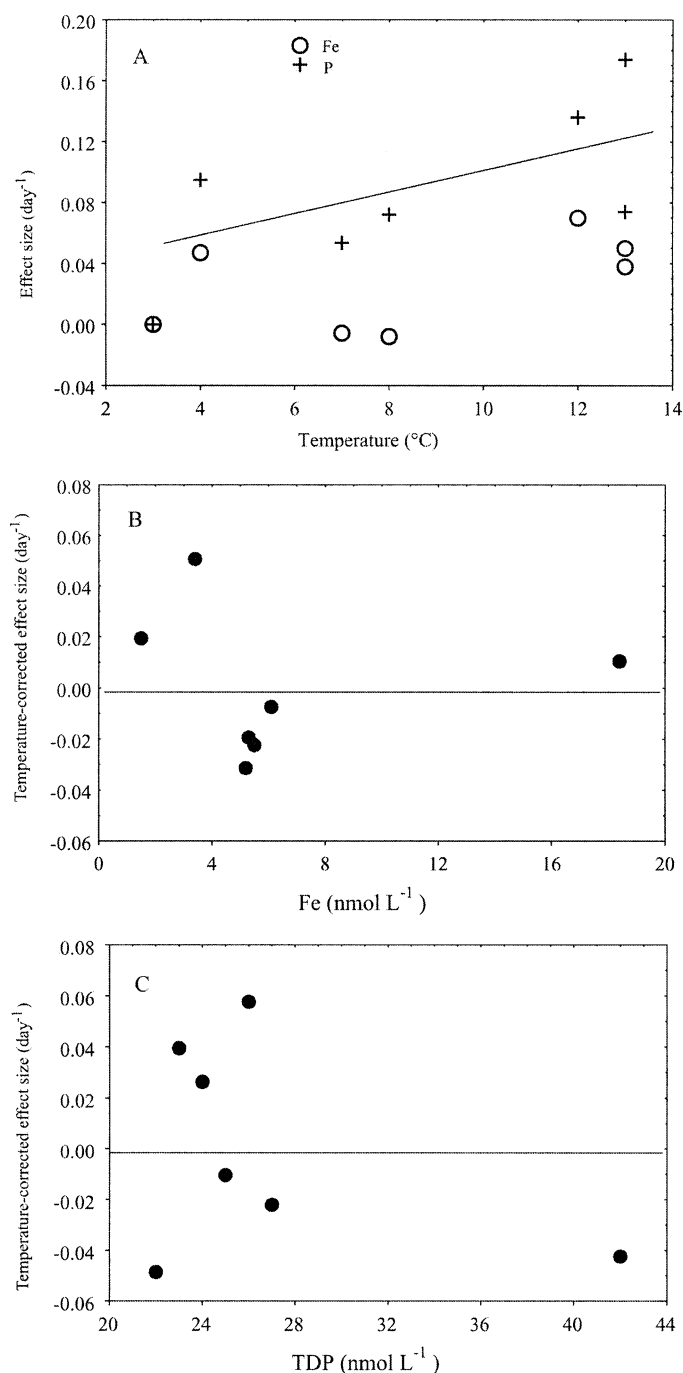


Fig. 8. Relation of effect size to incubation temperature and lake chemical concentration. (A) Effects were larger at warmer temperature. The lines are described by the equations $y = 0.0013 + (0.0068 \times \text{temp}) \pm 0.0295$ (the plus branch is for P, the minus branch is for Fe). (B, C) Examination of temperature-corrected effect sizes (the residuals of the plots in A) indicated that larger Fe effects occurred when Fe concentrations were less than 4 nmol L⁻¹ (both from station EM, see Table 3), whereas it was less clear whether temperature-corrected effect size depends on P concentration. Although suggestive of a trend associated with Fe, neither the relationship in B nor that in C was statistically significant.

limitation patterns of algae versus bacteria relative to iron. Algae (as measured by chlorophyll) of all size classes showed iron deficits in some experiments, whereas bacterial growth, even when supplemented by P, was best interpreted as being independent of supplemented iron. Apparently bacteria had access to sufficient iron in the water column to support extant as well as P-enhanced growth rates. This contrast between algae and bacteria is surprising in light of difference in Fe contents in the two groups. Limited data indicate that bacteria have higher Fe requirements than algae. Tortell et al. (1996) found molar C:Fe of 23,000 to 130,000 in bacteria, while C:Fe of approximately 300,000 in phytoplankton under similar conditions was found by Maldonado and Price (1996). Both of these studies used a titanium wash to minimize extracellular Fe in their measurements. Two interpretations seem plausible to explain our observed contrast. First, bacteria may have greatly superior Fe uptake capabilities. Second, bacteria in the lake may possess large iron stores such that over the duration of a single growth assay experiment, they do not experience iron deficit. We are unable to distinguish between these alternatives with these data, but this biological difference in metal interactions warrants future consideration.

An iron deficit, if not overt iron limitation, could have considerable biological significance in this lake. Iron availability is well known to influence the ability of organisms to assimilate nitrate, both due to the role of metals as cofactors in relevant enzymes and due to the provision of reductant in this pathway (McKay et al. 2001). An iron deficit then is expected to lessen biological demand on nitrate, which may play some role in the decades-long trend of increasing levels of nitrate in this lake (Bennett 1986).

Our results are qualitatively similar to those of other studies on the Laurentian Great Lakes, in particular the incubation experiments reported by Twiss et al. (2000). These investigators also found spatially and temporally varying degrees of iron deficiency, stress, or limitation layered on top of a generally strong macronutrient (P) limitation. Given the known huge variations in supply rates of macronutrients and micronutrients to natural waters, to find that Fe availability is neither much larger nor much smaller than that required by phytoplankton to meet their growth needs seems to beg for an explanation. Freshwater systems like these are on the cusp of macronutrient and micronutrient (metal) limitation. It is not likely that the iron and phosphorus cycles would separately and independently arrive at this configuration. Macronutrients, particularly P and N, are thought largely to control biomass levels in waters. Micronutrient availability is under direct biological control via production of ligands, which can enhance metal concentrations by keeping them in solution, but may also compete with the biota for metal binding. Dissolved bioavailable metals may largely be regulated by biomass levels due to ligand production. A full picture of metal limitation in lakes and oceans will require a synthesis of regional scale processes such as dust deposition and smaller scale biotic and chemical interactions.

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