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Identification of factors constraining nitrate assimilation in Lake Superior, Laurentian Great Lakes

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Abstract Despite a well-documented rise in nitrate concentration over the past century, Lake Superior has retained an oligotrophic character. In part, this status results from physical attributes of the lake including low temperatures and prolonged isothermy, resulting in deep-mixing and light limitation which constrain primary production. Lake Superior is also phosphorus deficient which limits phytoplankton growth. We conducted large (20 l) volume factorial bioassay experiments to assess the influence of light and nutrients (P, Fe) on nitrate assimilation by a Lake Superior chlorophyte alga. Bioassays seeded with the chlorophyte yielded a strong response to light resulting in the rapid depletion of nitrate. High light resulted in higher activities of the key N-assimilation enzyme nitrate reductase (NR) and increased algal biomass compared to low light treatments. NR activity was highly correlated with rates of nitrate incorporation in bioassays and field surveys suggesting that NR occupies a critical place in nitrate metabolism. In bioassays, the addition of nutrients (P, Fe) only slightly increased the rate at which nitrate became depleted. Parallel trials using a luminescent cyanobacterial bioreporter confirmed the lack of response by added nutrients supporting light as an important factor in constraining nitrate assimilation by phytoplankton in the lake.

Keywords Lake Superior · Light · Nitrate · Nitrate reductase · Phytoplankton

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Introduction

Eutrophication is widely recognized as a first-order threat to the ecological integrity of our freshwater and coastal marine resources (Schindler, 2006). The largest freshwater resource in the world, the Laurentian Great Lakes system, has followed the pattern of eutrophication occurring globally (Beeton, 1965). In particular, the lower Great Lakes (Erie, Ontario, and southern Lake Michigan) whose watersheds support large human populations as well as intensive agriculture

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and industry, have been profoundly impacted. In stark contrast, Lake Superior, which serves as the headwaters for the system, has remained, by and large, pristine. This is supported by historical data showing mainly flat profiles of total dissolved solids as well as concentrations of major ions which serve as indicators of anthropogenic impacts on the system (Beeton, 1965; Chapra et al., 2012).

Seemingly defying the static trends in major ions is the observation that Lake Superior has exhibited a continuous, century-long increase in nitrate levels (Sterner et al., 2007). This trend was initially reported in the years following implementation in 1968 of the Great Lakes Surveillance Program by Environment Canada but included historic data going back to early in the twentieth century (Weiler, 1978; Bennett, 1986). The trend of increasing nitrate has been confirmed through annual binational monitoring conducted by Environment Canada and the US EPA as well as sampling done by university investigators (Sterner et al., 2007). In fact, these monitoring efforts show that nitrate concentrations have increased across the entire Laurentian Great Lakes system (Neilson et al., 2003; Dove, 2009).

Globally, atmospheric deposition of reactive nitrogen is identified as a primary source of nitrate accumulating on land and in lakes and rivers (Galloway et al., 2003), yet patterns in regional nitrate ion wet deposition do not always support these trends. For instance, in Lake Erie's western basin where spring surveys showed nitrate levels had increased by 40% from 1983 to 1993, the increase was attributed in large part to changes caused by invasive *Dreissena* species (Makarewicz et al., 2000). Likewise, a 30% rise in nitrate observed in Lake Superior since the mid-1970s (Sterner et al., 2007; Kelly et al., 2011) cannot be explained by atmospheric deposition. Rather, nitrate is produced within the lake, derived primarily from microbial transformations dominated by nitrification (Finlay et al., 2007; Small et al., 2013a) catalyzed largely by ammonia-oxidizing archaea (Small et al., 2013a).

While nitrate levels in Lake Superior continue to increase, albeit at a lower rate (Sterner, 2011), a recent modeling effort suggests the system may be nearing its capacity to accumulate nitrate (McDonald et al., 2010). Several factors interact promoting the accumulation of nitrate in Lake Superior, although foremost are its oligotrophic state and strong oxic environment. Driving the oligotrophic character of the lake is its low phosphorus content with soluble reactive phosphorus (SRP) measured at low nanomolar levels (Anagnostou & Sherrell, 2008). The extreme depleted phosphorus character constrains primary production in the lake resulting in low export of organic carbon to the sediment (Sterner et al., 2004; Sterner, 2010). This, in turn, helps maintain the lakes oxic environment by limiting rates of bacterial remineralization that would otherwise consume oxygen. These processes also highlight the lack of strong biogeochemical "sinks" for nitrate in Lake Superior. Low levels of primary production will constrain biological assimilation of nitrate whereas the oxidizing environment, which extends deep into surface sediments (Carlton et al., 1989; Li et al., 2012; Small et al., 2013b), is not conducive to conventional dissimilatory sinks such as denitrification.

In contrast, the effect of stronger biogeochemical sinks can be observed downstream in Lake Erie, the eutrophic end member of the Great Lakes system. Present day nitrate levels in Lake Erie are variable with peak summer concentrations $\sim$30–40% lower compared with the upper lakes, despite known heavy fertilizer use in Erie's watershed (Richards & Baker, 1993). Higher rates of assimilation in Lake Erie are consistent with higher planktonic biomass measured in this lake relative to other lakes in the system (Barbiero & Tuchman, 2001; Conroy et al., 2005). Likewise, dissimilatory sinks are also relatively strong given the annual recurrence of Lake Erie's expansive hypoxic 'dead zone' (Hawley et al., 2006). Indeed, increasing sedimentary $\delta^{15}N$ values from $\sim$1910 to $\sim$1970 measured from sediment cores are consistent with the occurrence of respiratory denitrification in the lake's central basin (Lu et al., 2010) and moderate rates of potential denitrification ($\sim$150 μmol N m^{-2} h^{-1}) have been reported for nearshore Lake Erie sediments (McCarthy et al., 2007). Core flux studies indicate that Lake Superior sediments are a net source of nitrate, whereas Lake Erie sediments are a net sink (Small et al., 2013b).

In this study, we aimed to identify factors constraining the biological assimilation of nitrate by phytoplankton in Lake Superior. Previous work by our group has demonstrated that phytoplankton growth in Lake Superior is co-limited by low available phosphorus and iron (Sterner et al., 2004). Subsequent studies using a luminescent cyanobacterial bioreporter

showed that nitrate utilization was stimulated in response to amendment of lake water by phosphorus and to a lesser extent by iron (Ivanikova et al., 2007a). Further, these studies highlighted the importance of light in nitrate assimilation demonstrating that the light climate during the period of spring mixing was insufficient to promote high rates of nitrate drawdown (Ivanikova et al., 2007a). Finally, shipboard- and in situ incubations demonstrated a strong temperature dependence of ^{15}N-nitrogen (nitrate, ammonium) uptake with maximum rates restricted to the warmer stratified surface mixed layer (Kumar et al., 2008).

The factors affecting nitrate assimilation can be species-specific. Patterns of nitrate incorporation vary with taxa (e.g., Needoba & Harrison, 2004), and at the extreme, some species are entirely incapable of using nitrate (e.g., Deyoe & Suttle, 1994; Moore et al., 2002). Thus, we used two approaches: making community-level measurements of a key enzyme in nitrate assimilation (nitrate reductase, NR: E.C. 1.7.7.1) in the field, as well as experimental measurements with a chlorophyte alga endemic to Lake Superior. This approach offers some parallels to the well-known Algal Assay Procedure (U.S. EPA, 1971) involving identification of a limiting nutrient based on the growth response of a standard test alga species to nutrient amendments of filtered water. In previous work on phytoplankton, NR has been shown to respond to changes in irradiance or iron limitation in step with changes in nitrate incorporation (e.g., Berges & Harrison, 1995a; Boyd et al., 1998); responses of NR to P-limitation are less well established, though nitrate incorporation is responsive to P in both phytoplankton and higher plants (e.g., Rhee, 1978; Rufty et al., 1990). Use of a luminescent cyanobacterial bioreporter strain responsive to nitrate assimilation (Ivanikova et al., 2005) offered an independent, but complementary approach. Based on previous work and on our understanding of phytoplankton community responses, we hypothesized that irradiance would have the strongest effects on nitrate assimilation.

Materials and methods

Sample collection

Samples were collected from multiple sites during three research surveys on Lake Superior in 2004 and 2005 aboard R/V *Blue Heron* (Fig. 1). The surveys were conducted during periods of weak inverse thermal stratification (18–22 May 2004) as well as strong summer thermal stratification (14–16 September 2004, 27–31 August 2005). At each hydrographic station, sampling was preceded by a conductivity–temperature–depth cast. In May 2004, during which the water column was mainly isothermal, water was sampled only from 5 m. During the late summer surveys, epi-, meta- and hypolimnetic samples were collected. At all locations, water was collected into acid-cleaned polycarbonate bottles from discrete depths using a metal-clean in situ pumping system following passage through a 0.45-μm capsule filter (GE Osmonics, GE Water and Process Technologies, Feasterville-Trevose, PA, USA) as described elsewhere (Hassler et al., 2009).

Subsamples for nutrient determination were frozen in polyethylene bottles on board ship for subsequent laboratory analysis. Nitrate was measured using an Alpkem autoanalyzer (OI Analytical, College Station, TX, USA) whereas measurement of total dissolved phosphate followed the ascorbic acid–molybdate method (Parsons et al., 1984). Samples for determination of particulate organic nitrogen (PON) and particulate phosphorus (PP) were collected by filtration (1–1.5 l) onto pre-combusted Whatman GF/F filters (GE Healthcare Life Sciences, Piscataway, NJ, USA). Filters for PP analysis were rinsed with 5 ml of 1% HCl and followed by deionized water prior to sample collection. Samples were transported frozen to the lab and then dried at 60°C for up to 48 h and prepared for analysis. PON samples were analyzed on a 2400 CHN analyzer (PerkinElmer, Waltham, MA, USA) calibrated with an acetanilide standard. PP samples were digested with potassium persulfate and total P analyzed spectrophotometrically using the ascorbic acid–molybdate method (Menzel & Corwin, 1965). Total chl *a* was measured on samples extracted overnight in 95% (v/v) acetone by fluorometry after Welschmeyer (1994).

Alkaline phosphatase activity (E.C. 3.1.3.1.)

Unfiltered water was dispersed to triplicate methacrylate cuvettes (2.5 ml) and incubated with 40 μmol l^{-1} 4-methylumbelliferyl phosphate (Sigma Inc., St. Louis, MO, USA) in darkness at ambient ship-laboratory temperature ($\sim$20°C). Sodium bicarbonate (4 mmol l^{-1})

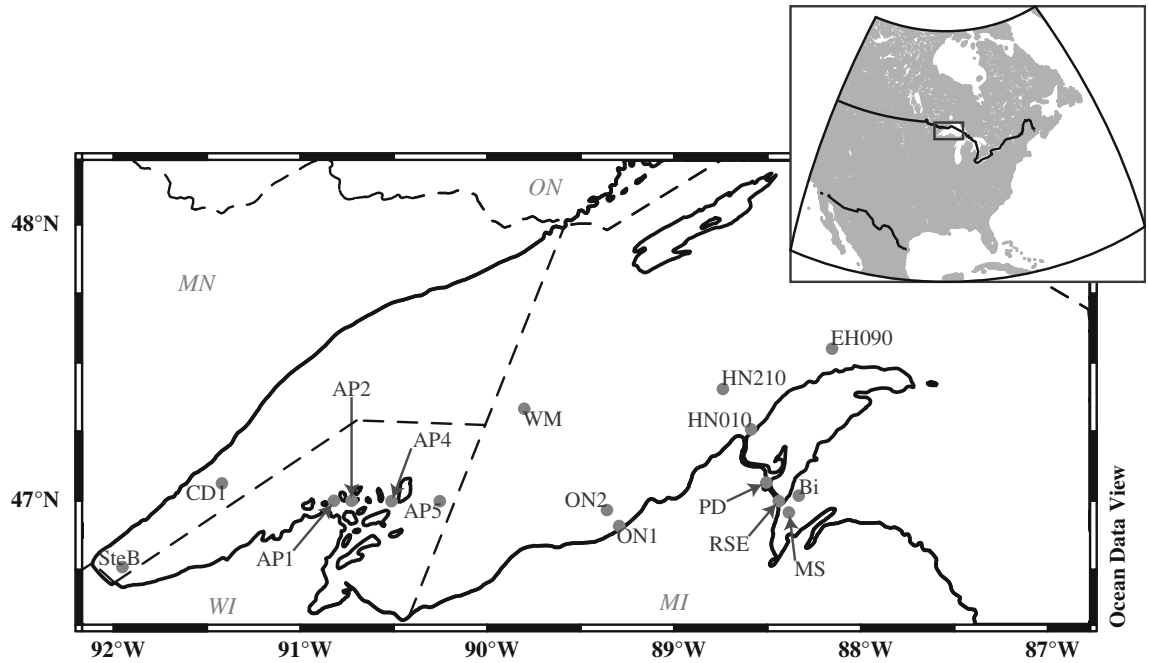

Fig. 1 Map of western Lake Superior showing the location of the hydrographic stations sampled. CD, Castle Danger; EH, Eagle Harbor (090: 9 km from shore); WM, Western Mid-lake; SteB, Sterner B; PD, Portage Deep; RSE, River South Entry; MS, McKay South; Bi, Bingman. Two nearshore–offshore transects along the Keweenaw Peninsula were sampled including Hancock North (HN; 210: 21 km from shore; 010: 1 km from shore) located north of the Keweenaw Waterway and Ontonagon (ON) adjacent to the Ontonagon River. In addition, a transect through the Apostle Islands was sampled (AP1–5). *Dashed lines* through the lake show geo-political boundaries. ON, Ontario (Canada); MN, Minnesota; WI, Wisconsin; MI, Michigan. *Inset*: Map of North America showing location of Lake Superior (*box*)

was substituted for Lake Superior water in substrate controls, whereas quench standards were prepared using unfiltered lake water and 1 µmol l^{-1} of 4-methylumbelliferone (Sinsabaugh et al., 1997). Enzyme activities were calculated using a reference standard containing 1 µmol l^{-1} of 4-methylumbelliferone. Alkaline phosphatase (APase)-catalyzed fluorescence was determined using a TD-700 laboratory fluorometer (Turner Designs, Sunnyvale, CA, USA) equipped with a near-UV lamp and a methylumbelliferyl filter set (excitation: 300–400 nm; emission: 410–610 nm). Enzyme activity was normalized to chl *a* biomass.

Nitrate reductase activity (E.C. 1.7.7.1)

Samples of up to 1 l were filtered through Pall Gelman type A/E 25 mm filters (Pall Corporation, Port Washington, NY, USA) and frozen immediately in liquid nitrogen in 2.0 ml cryovials. Assays followed Berges et al. (2004); frozen samples were homogenized in an extraction buffer (0.2 mol l^{-1} phosphate pH 7.9 with additions of 0.03% (w/v) dithiothreitol, 5 mmol l^{-1} EDTA, 3% (w/v) bovine serum albumin, 0.3% (w/v) polyvinyl pyrrolidone, and 0.1% (v/v) Triton X-100) and assayed (with final concentration of 0.2 mmol l^{-1} NADH, 10 mmol l^{-1} KNO_3, and 20 µmol l^{-1} FAD) for 60 min. The assay was stopped with the addition of zinc acetate and the production of nitrite (corrected for time zero) was expressed as nmol min^{-1} in the case of bioassay samples (Berges & Harrison, 1995a) or mmol m^{-3} d^{-1} for field samples. For field samples, a common assay temperature of 8°C was chosen and activities were corrected to in situ temperature using a Q_{10} of 2; data from Kumar et al. (2008) for ^{15}N-nitrate uptake rates indicated that such a temperature correction was appropriate.

Pigment analysis

For HPLC pigment analysis, 1,000 ml of water collected from 7-m depth from station ON2 in August 2005 was filtered onto a Whatman GF/F filter, wrapped

in Al foil and immediately frozen in liquid nitrogen and stored until analysis. HPLC pigment data were analyzed using the CHEMTAX (V 1.95) program (Mackey et al., 1996, 1998), obtained from S. Wright (Australian Antarctic Division). This program uses a steepest-descent algorithm to fit a matrix of expected pigment ratios determined from reference cultures to unknown samples. Methods followed those of Millie et al. (2009), using fucoxanthin (diatoms and chrysophytes), neoxanthin (chlorophytes), dinoxanthin (dinoflagellates), alloxanthin (cryptophytes), lutein (chlorophytes), and zeaxanthin (cyanobacteria). Final pigment allocation is expressed as percentages of total chl *a*.

Factorial bioassay experiments

Water sampled from the surface mixed layer was filtered (0.45 μm) and collected into acid-cleaned 20-l polycarbonate carboys. With water collected in August 2005, factorial experiments were performed using duplicate carboys and varying irradiance to mimic that encountered in the mixed layer during vernal (<25 μmol quanta m^{-2} s^{-1}) and seasonal (>100 μmol quanta m^{-2} s^{-1}) mixing (see Ivanikova et al., 2007a). Reflecting this, for the bioassays, irradiance was adjusted to 110 μmol quanta m^{-2} s^{-1} high light (HL) or 8 μmol quanta m^{-2} s^{-1} low light (LL), measured as the average of irradiance in front and behind the carboy, and adjusted by applying one or more layers of 3 mm black mesh as a neutral density filter. Experiments were conducted at 16°C. Carboys either received no nutrient additions (LN), or additions of 8 μmol l^{-1} phosphate (as KH_2PO_4) and 10 nmol l^{-1} iron (as $FeCl_3$ with equimolar Na_2EDTA) (HN). Carboys were continuously bubbled with filtered air and samples aseptically removed through a one-way sampling port; all tubing was silicone.

A level 1 bioassay approach was chosen (Hecky & Kilham, 1988) thus providing full experimental control of the parameters leading to population growth. Carboys were inoculated at an equal initial chl fluorescence with a *Chlamydomonas* species isolated from Lake Superior (clone CD-1 Red, Canadian Phycological Culture Centre [CPCC] strain 641). A ribosomal 18S rRNA sequence for the environmental isolate has been deposited to GenBank under accession number KC800695, and aligns perfectly with an 18S sequence in the database designated *Chlamydomonas sordida*. Before use, the chlorophyte was grown at the same irradiance as the LL treatment using modified Chu-10 medium (Andersen et al., 2005). Over the course of four grow-ups and dilutions (10 ml transfer in 1 l new medium), trace metal additions were eliminated and N and P were gradually reduced from full enrichment to half enrichment to no additions except those nutrients carried over in innocula for the last two additions. Cells were inoculated into the bioassay carboys when the increase in fluorescence of the stock cultures had ceased (early stationary phase). Experiments were followed for 8 d. Daily samples were taken for analysis of chl *a* (measured spectrophotometrically in 90% acetone extracts, Parsons et al., 1984), variable fluorescence emissions (measured in a Turner Designs TD-700 fluorometer before and after addition of 10 μmol l^{-1} 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; Cullen & Renger, 1979), nitrate and phosphate (measured in filtered samples, using the cadmium reduction method and the ascorbic acid–molybdate method, respectively, Parsons et al., 1984), and particulate C and N (measured in samples collected on precombusted 13 mm GF/F filters using a Flash EA 1112 elemental analyzer [Thermo Fisher Scientific, Waltham, MA, USA]). NR activity was measured in 500 ml samples collected on 25 mm Gelman A/E filters and assayed either immediately or after storage in liquid nitrogen for up to 2 weeks (see above).

All analyses were run on instruments that are calibrated at least annually. Replicate blanks and (where possible) standards were run alongside samples. No data were excluded from analyses; variation in replicate numbers indicates accidental loss.

Experiments were analyzed using two-way repeated-measures ANOVAs, with treatment (light and nutrient combination) and time as factors, treating each carboy as an experimental subject. Where found, significant differences at the 95% confidence level ($\alpha = 0.05$) were examined using the Holm–Sidak method using SigmaPlot (V. 12, Systat Software Inc., Chicago, IL, USA).

Cyanobacterial bioreporter

A bioluminescent nitrate bioreporter strain AND100 (Ivanikova et al., 2005, 2007a) was used on parallel samples in August 2005 (station ON2) to assess nitrate assimilation potential. Engineered using a construct containing the gene promoter for nitrite reductase fused to bacterial luciferase genes, the strain exhibits bioluminescence under physico-chemical conditions

that favor nitrate/nitrite assimilation with the intensity of luminescence serving as a measure of nitrate/nitrite uptake. *Synechocystis* sp. PCC 6803 strain AND100 was maintained in BG-11 medium containing reduced nitrate concentration (2.3 mmol l^{-1}) as described previously (Ivanikova et al., 2005). All cultures were bubbled with air and grown at 25°C in constant light (50 µmol quanta m^{-2} s^{-1} PAR) provided by cool-white fluorescent lamps. Growth of batch cultures was monitored daily by measuring light scattering at 750 nm (OD_{750nm}). Cells were harvested by centrifugation at 4,000×*g* for 15 min, washed twice in nitrate-, phosphate- and iron-free BG-11 and resuspended to a final $OD_{750nm} = 0.1$ in lake water or BG-11 of defined nitrate concentration which was used for calibration. For environmental samples, the AND100 reporter strain was seeded into filtered water from ON2, and luminescence was measured following amendment of samples with phosphate (8 µmol l^{-1} K_2HPO_4) and iron (10 nmol l^{-1} $FeCl_3$ chelated with Na_2EDTA in a ratio of 1:1.5), both individually and in combination. Samples were then incubated at 25°C with continuous gyratory shaking (100 rpm). During assays, irradiance was maintained at 50 µmol quanta m^{-2} s^{-1}, a light flux shown to yield maximum rates of nitrate assimilation using the cyanobacterial bioreporter (Ivanikova et al., 2005). Luminescence of AND100 cultures was measured with a Femtomaster model FB14 luminometer (Zylux Corp., Oak Ridge, TN, USA) immediately following the addition of 20 µl of methanol containing 27 mmol l^{-1} *n*-decyl aldehyde substrate (Sigma Inc., St. Louis, MO, USA) to 2 ml of the sample. Light emission from the bioluminescent AND100 shows strong positive correlation to the rate of nitrate assimilation (Ivanikova et al., 2005), thus nutrient factors that constrain nitrate assimilation can be identified.

Results

Physicochemical characteristics of Lake Superior

Surveys of western Lake Superior were conducted in May and September 2004 and August 2005 in support of studies to assess the potential of resident phytoplankton to assimilate nitrate. Inverse thermal stratification was recorded during the May survey with surface mixed layer temperatures at offshore stations below 3°C and low chl *a* biomass ranging from 0.82 to 1.82 µg l^{-1} at 5 m depth (Table 1). Surface mixed layer temperature as well as chl *a* biomass was higher in nearshore regions, especially at sites influenced by flow of the Ontanogan River (ON1, HN010). In summer, strong thermal stratification was evident at all sites during the September and August surveys with surface temperatures ranging between 16 and 18.5°C at station ON2 during 2005 (Fig. 2). There is evidence for a chlorophyll maximum at depth (located below the thermocline), identified by fluorometry during CTD profiles and supported by discrete measures of depth-resolved total chl *a* which peaked at 1.07 ± 0.42 µg l^{-1} at station ON2 (Fig. 2).

Surface nitrate remained high during surveys in this study (median: 24.3 µmol l^{-1}; Table 1). Dissolved phosphorus data are available only for the September survey where mixed layer SRP was measured at low nanomolar concentrations (1–5 nmol l^{-1}; Ivanikova et al., 2007a). Dissolved iron was higher in May (mean = 11.7 nmol l^{-1}) than in September (mean = 4.0 nmol l^{-1}) and was elevated at nearshore sites compared with offshore locations during the May survey (Hassler et al., 2009).

In August 2005 coinciding with the factorial bioassay trials, high resolution sampling conducted at ON2 showed that dissolved major nutrients varied only modestly with depth (Fig. 3). During summer, total dissolved phosphate was depleted through the water column, whereas nitrate maintained high concentrations of >23 µmol l^{-1}. Despite the static dissolved nutrient profiles, stoichiometry in particles was variable with particulate N:P being twofold higher in the epilimnion than in the hypolimnion and consistently higher than Redfield stoichiometry (N:P = 16:1 by moles) throughout the water column (Fig. 3). Depth-resolved measurement of APase activity tracked the profile of seston N:P stoichiometry (Fig. 3).

Pigment analysis

HPLC analysis of phytoplankton collected from the mixed layer at station ON2 in August 2005 showed dominance of diatoms and chrysophytes, with a moderate representation of cyanobacteria (23%), some chlorophytes (6%) and a small biomass of dinoflagellates (1%).

Table 1 Water quality parameters from western Lake Superior in May and September, 2004

Station	Sample depth (m)	Temperature (°C)	Chl *a*[a] (μg l^{-1})	NO_3^-[a] (μmol l^{-1})	NR (±SE) (mmol m^{-3} d^{-1})
May					
CD1	5	2.7	0.86	26.97	0.0126 (0.0008)
WM	5	2.4	0.82	26.16	0.0130 (0.0083)
EH090	5	2.4	0.86	25.61	0.0105 (0.0034)
HN010	5	7.0	1.76	24.14	0.0062 (0.0051)
HN210	5	2.6	0.93	26.16	0.0100 (0.0141)
ON1	5	7.0	1.82	24.95	0.0181 (0.0080)
ON2	5	3.8	1.37	26.55	0.0317 (0.0080)
AP1	5	6.9	1.27	24.09	0.0067 (0.0038)
AP2	5	6.5	1.27	24.12	0.0050 (0.0003)
AP4	5	4.6	1.23	25.44	0.0055 (0.0016)
AP5	5	3.5	0.98	19.92	0.0192 (0.0174)
September					
CD1	5	12.2	1.49	22.05	0.0222
CD1	15 (metalimnion)	11.4	1.16	21.83	0.0128
WM	5	12.7	0.91	22.30	0.0085
WM	30 (metalimnion)	7.3	1.78	24.33	0.0105
SteB	5	12.75	1.38	24.50	0.0162
PD	5	18	6.41	11.15	0.0195 (0.0032)
RSE	Surface	ND	6.16	10.13	0.0441 (0.0169)
MS	5	12.1	0.89	23.17	0.0051 (0.0040)
Bi	5	10.4	0.60	23.37	0.0131 (0.0006)

Station abbreviations are as shown in Fig. 1

NR nitrate reductase activity

[a] From Ivanikova et al. (2007a)

NR measurements in the field

NR activity in the field varied from 0.005 to 0.044 mmol m^{-3} d^{-1} (median: 0.013 mmol m^{-3} d^{-1}, $n = 20$) with little discernible pattern (Table 1). NR was not significantly correlated with chl *a* or nitrate ($P > 0.2$ in both cases), nor were there obvious differences between seasons.

Factorial bioassay experiments

The effects of light treatment were clearly observed in bioassays seeded with a Lake Superior chlorophyte in August 2005 (Fig. 4). Cultures incubated at HL exhausted nitrate between days 5 and 8, with no significant differences observed between low nutrient (LN) and high nutrient (HN) treatments ($P > 0.5$). By contrast, in LL cultures, nitrate remained above 15 μmol l^{-1} over the duration of the 8 d experiment (Fig. 4B). Accumulation of chl *a* and particulate C indicated that HL–HN cultures grew faster than HL–LN cultures (chl *a* significantly greater in HN than LN by day 4, Fig. 4A and particulate C by day 8, Fig. 4D, $P < 0.05$ in both cases), though significant differences were not seen in particulate N (Fig. 4G, $P > 0.05$). Phosphate did not change over time in LN cultures ($P > 0.05$), but declined significantly in HN cultures ($P < 0.05$), with HL–HN cultures showing greater declines than LL–HN culture after 5 d (Fig. 4E, $P < 0.05$). On a volumetric basis, NR activity was uniformly low in LL cultures, but significantly higher in HL–HN cultures until day 5 and HL–LN cultures until day 4 (Fig. 4F, $P < 0.05$). The decline in NR activity in HL bioassays coincided with the depletion of nitrate in each experiment. F_v:F_m was relatively high in all cultures, but declined over time in all cases ($P < 0.003$); by day 8 HL cultures had lower values than LL cultures (Fig. 4C, $P < 0.05$).

A luminescent cyanobacterial bioreporter (Ivanikova et al., 2005) was used to assess the effects of nutrients on nitrate assimilation potential. In the absence of added nutrients, the AND100 bioreporter underestimated the nitrate concentration at station ON2 in August 2005 (Table 2). Further, amending water sampled from

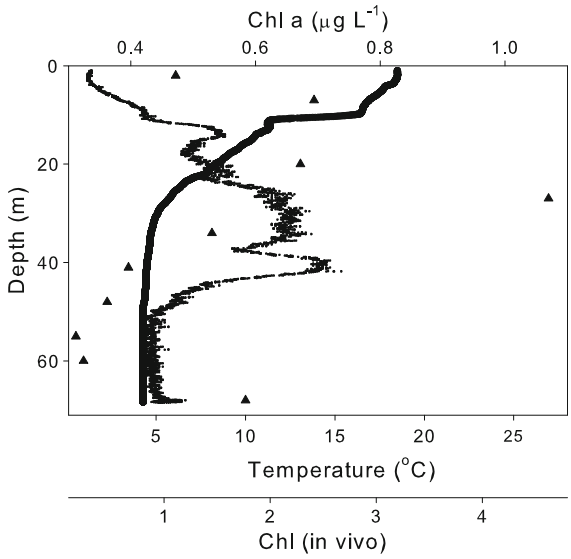

Fig. 2 Temperature (*solid line*) and relative chlorophyll fluorescence (*dotted line*) from the CTD profile at station ON2 in August 2005. The lake was thermally stratified in August with surface water temperatures between 16 and 18.5°C. Discrete measurements of depth-resolved Chl *a* (*filled triangle*) are superimposed on the plot supporting the presence of a deep chlorophyll maximum located below the thermocline at this site

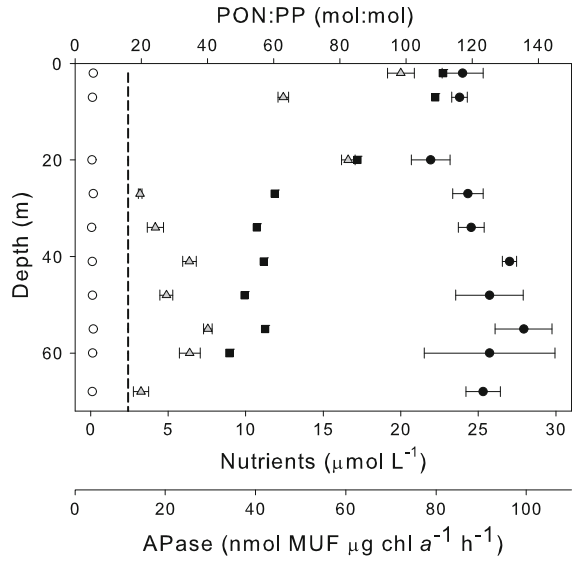

Fig. 3 Dissolved nutrients and seston nutrient stoichiometry at station ON2 in August 2005. Nutrient profiles were static with abundant NO_3^- (*filled circle*), whereas total dissolved phosphorus (*circle*) was depleted. Molar ratios of particulate organic nitrogen to particulate phosphorus (PON:PP; *filled square*) showed that seston was enriched in nitrogen relative to phosphorus throughout the water column. Whereas this ratio decreased with depth, it was always higher than the Redfield ratio (shown by *vertical hashed line*). Measures of alkaline phosphatase activity (*filled triangle*) tracked the decline in PON:PP of seston. Error bars represent data range (n = 3–8)

station ON2 with phosphate and iron demonstrated that nitrate assimilation was not constrained by nutrient deficit, since no increase in luminescence could be seen following nutrient addition.

Across field and laboratory measurements, when NR activity was compared to nitrate incorporation rates, either based on measurements of nitrate depletion from cultures (lab) or averaged rates of ^{15}N uptake measured in Lake Superior across several sites in 2005 (Kumar et al., 2008), a strong relationship was found (Fig. 5). The overall linear regression ($Y = 0.3446 + 0.5857\ X$) was highly significant ($P < 0.001$, $R^2 = 0.59$). The relationship is nearly identical if only the bioassay data are considered ($Y = 0.3671 + 0.5822\ X$, with $P < 0.001$ and $R^2 = 0.54$ (Online Resource 1).

Discussion

Lake Superior during the study period

Physicochemical characteristics of survey sites occupied in 2004 were reported previously (Ivanikova et al., 2007a; Hassler et al., 2009), and similar spatial–temporal variation has been reported previously for dissolved iron in Lake Superior (Nriagu et al., 1996; McKay et al., 2005).

In August 2005 coinciding with the factorial bioassay trials, high resolution sampling conducted at ON2 showed that dissolved major nutrients varied only modestly with depth (Fig. 3). During summer, total dissolved phosphate was depleted through the water column, whereas nitrate maintained high concentrations of >23 μmol l^{-1}. Despite the static dissolved nutrient profiles, stoichiometry in particles was variable with particulate N:P being twofold higher in the epilimnion than in the hypolimnion and consistently higher than Redfield stoichiometry (N:P = 16:1 by moles) throughout the water column (Fig. 3), a pattern described previously during summer (Sterner, 2011). A similar trend has been documented tracking particulate C:P (Barbiero & Tuchman, 2004; Sterner, 2011). Depth-resolved measurements of APase activity (Fig. 3) were indicative of moderate-to-severe phosphorus deficiency according to the indices of Healey &

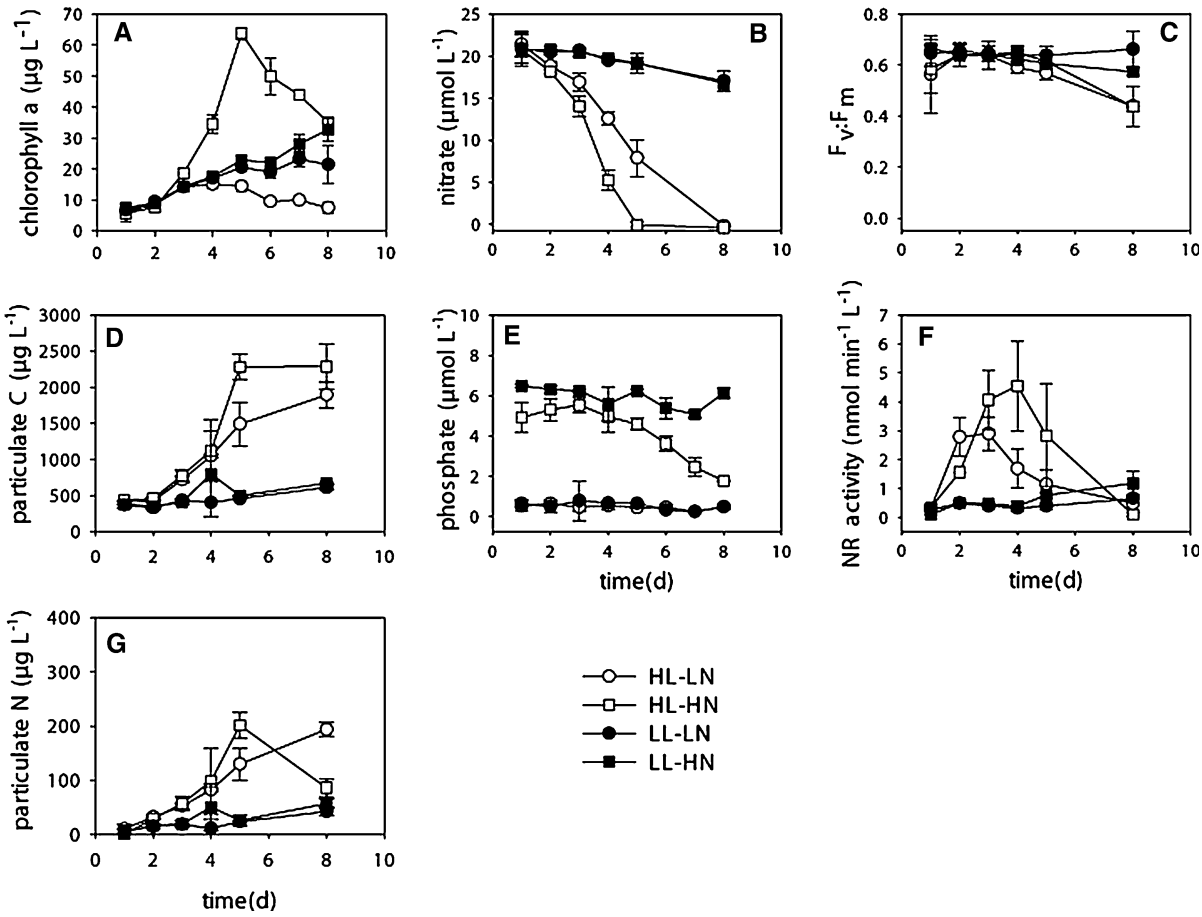

Fig. 4 Reponses of a chlorophyte alga to factorial light and nutrient treatments in water collected from Lake Superior in August 2005. Treatments are combinations of irradiance (HL = 110 µmol quanta m^{-2} s^{-1}, LL = 8 µmol quanta m^{-2} s^{-1}) and enrichment with 8 µmol l^{-1} phosphate and 10 nmol l^{-1} iron (HN) or ambient nutrients (LN). Chlorophyll *a* (**A**, measured fluorometrically in acetone extracts), nitrate and phosphate (**B**, **E**, measured colorimetrically), particulate carbon and nitrogen (**D**, **G**, measured using an elemental analyzer), variable florescence emissions (F_v:F_m, **C**, measured using a fluorometer and DCMU), and nitrate reductase activity (**F**, measured in cell extracts, based on time-dependent nitrite production). Each symbol represents the mean of measurements from two 20 l carboys and error bars represent standard deviations, or where not seen are smaller than the symbol

Table 2 Nitrate concentration for surface mixed layer (7 m) at station ON2 estimated using AND100 bioreporter

Treatment	Bioreporter [NO_3^-] (µmol l^{-1})
Control	18.72 ± 2.5
+Fe	17.80 ± 1.4
+P	18.98 ± 1.2
+Fe/P	18.14 ± 1.3

Chemical analysis reported nitrate at 24.20 ± 0.5 µmol l^{-1}

Hendzel (1979). Overall, profiles of nutrients and seston stoichiometry were consistent with the well-documented characterization of Lake Superior as phosphorus deficient during the summer stratified period (Nalewajko & Voltolina, 1986; Guildford & Hecky, 2000; Guildford et al., 2000; Sterner et al., 2004).

In agreement with the HPLC findings, previous characterization of seasonal phytoplankton communities in Lake Superior has shown summer assemblages dominated by diatoms and chrysophytes (Barbiero & Tuchman, 2001). The cyanobacterial signal is likely due to phycoerythrin-rich picoplankton as reported elsewhere (Ivanikova et al., 2007b). The sizeable contribution of this class was likely missed in the analysis by Barbiero & Tuchman (2001) which relied on microscopic identification and enumeration.

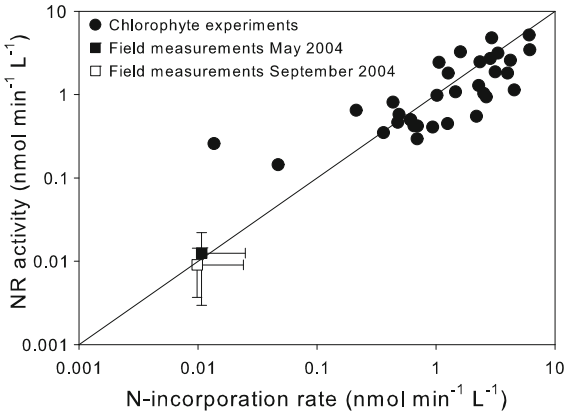

Fig. 5 Relationship between nitrate reductase (NR) activity and nitrate incorporation rate in field samples and experimental samples. Field samples represent averages of NR activities from Table 1, plotted against ^{15}N uptake rates averages from 2005 to 2006 data presented in Kumar et al. (2008). Experimental data is derived from data presented in Fig. 4, plotting measured NR against nitrate uptake from day to day of the experiment. *Solid line* represents the 1:1 relationship. The data are fit by a regression line of equation $Y = 0.3446 + 0.5857\,X$ ($R^2 = 0.59$, $P < 0.001$)

NR as an indicator of N-incorporation

The range of NR values corresponds quite closely to the range of ^{15}N nitrate uptake rates (approximately 0.001–0.037 mmol m^{-3} d^{-1}) measured in Lake Superior in 2005 and 2006 (Kumar et al., 2008). In marine waters, NR has often been measured and compared with nitrate uptake and incorporation, though earlier work likely suffered from assays that underestimated activity (see Berges & Harrison, 1995a). Good relationships have been observed in a number of environments, especially those with diatoms, under conditions including iron and light limitation (Berges et al., 1995, 2004; Boyd et al., 1998; Hung et al., 2000). In contrast, despite a long history of NR measurement in cultures of freshwater algal genera such as *Chlorella* and *Chlamydomonas* (e.g., Morris & Syrett, 1963) there are relatively few studies of NR in freshwaters (Wynne & Berman, 1990; Aleya, 1992; Mallet et al., 1998; Gordillo et al., 2001), and these have generally been unable to relate NR activity to rates of assimilation or environmental variables. Some of the issues may be methodological (see Hochman et al., 1986; Berges & Harrison, 1995a), but another factor may be the species in the systems. NR activity has been especially difficult to relate to environmental variables in dinoflagellate-dominated systems (Wynne & Berman, 1990), and Berges & Harrison (1995a) noted weaker relationships between NR activity and nitrate incorporation in a dinoflagellate species. Moreover, using polyclonal antibodies raised against NR purified from the marine diatom *Thalassiosira weissflogii* (Vergara et al., 1998), we detected cross-reacting proteins of appropriate size on Western blots containing protein extracts of bulk net seston collected from multiple sites in May 2004 (J. Berges, unpublished). These results suggest that NR assays developed for marine waters are applicable to freshwaters and that rates of NR activity agree with independent measures of nitrate uptake, even when it is poorly correlated with environmental measurements.

We found a strong relationship between NR activity and N-incorporation across field and bioassay samples. Some of the lowest nitrate incorporation rates measured (0.01–0.2 nmol min^{-1} l^{-1}) were not well-correlated with NR activity, but occurred in bioassays at the point where cultures exhausted nitrate. This likely reflects the fact that it takes some period of time before NR activity responds to removal of substrate (see Berges et al., 1995). Such a robust relationship across species, limiting factor and environment suggest that NR occupies a critical place in nitrate metabolism, if not controlling incorporation, then at least acclimating quickly enough to be useful as an indicator (Berges & Harrison, 1995a, Berges, 1997).

Bioassays of limiting factors in Lake Superior

The chlorophyte used in these experiments was able to completely remove ambient nitrate in Lake Superior water only if provided high irradiance. This suggests that most other resources are present at sufficient levels to support production sufficient for nitrate drawdown and that light is a key factor. These findings reinforce the observations of Ivanikova et al. (2005, 2007a). In these experiments, temperature was maintained at 16°C guided by the in situ temperatures encountered in the mixed layer at station ON2 from where the water was collected (Fig. 2). While this temperature was somewhat higher than what is normally recorded for surface waters in late summer in the lake (cf. Table 1), this may have alleviated the temperature limitation on nitrate assimilation described previously (Kumar et al., 2008). If this were the case; however, it seems more likely that temperature would

change the time scale of responses rather than the pattern. We found little evidence in the bioassay experiments of phosphorus or iron limitation, though nitrate was exhausted earlier in HL–HN than in HL–LN carboys. This is also supported by uniformly high F_v:F_m values, that did respond to eventual nitrate depletion. However, it is important to recognize that chlorophytes were grown for a period of weeks before the experiments in artificially enriched media containing higher nutrients than lake water, and carryover of dissolved P from the inocula may have further contributed to P availability in the bioassay. We did see some differences in growth and incorporation of nutrients between LN and HN treatments indicating some residual nutrient deficiency even with possible carryover factored in. However, even though we attempted to minimize these effects by gradually diluting cultures before inoculation, we cannot reject the possibility that cells had internal and external pools of nutrients higher than what phytoplankton would have had in situ. Thus, while our work affirms the importance of irradiance, the significance of limited nutrients is less straightforward to assess. Further, it is clear from the literature that species-specific responses are important to understanding nutrient dynamics in the field (e.g., Needoba & Harrison, 2004; Berges et al., 2004), so the response of the chlorophyte is not necessarily indicative of all species in the lake.

We did not normalize NR activity to algal biomass because the focus of the study was nitrate dynamics in the lake. Since we could independently measure uptake and incorporation on a volumetric basis, we chose this approach. Normalizing to chl *a* was considered inappropriate because the cells were acclimating to different irradiances and so chl *a* per cell would be changing; normalizing per unit C or N face similar issues. Berges & Harrison (1995b) demonstrated that scaling to biomass typically increased the variance of measurements. In fact, in this study, scaling to chl *a* or C, while it increased variance, did not change the basic patterns in NR activity, nor did it alter the statistical results (Online Resource 2).

Whereas the bioassay approach can provide useful information regarding nutrient limitation of phytoplankton, the assays are of long duration over which time increases in algal biomass may yield changes in water chemistry. Thus, complementing the factorial bioassay approach was use of a luminescent cyanobacterial bioreporter (Ivanikova et al., 2005) to rapidly (6–12 h) assess the effects of nutrients (P, Fe) on nitrate assimilation. This approach also offered analysis using a separate taxon, important considering recognized differences in nutrient assimilation mechanisms existing among phytoplankton. Indeed, assimilatory nitrate transport and reduction fundamentally differs between eukaryotes and cyanobacteria (Berges, 1997; Needoba & Harrison, 2004).

Added nutrients had no effect on the AND100 bioreporter assay (Table 2), similar to trials conducted throughout western Lake Superior in 2004 (Ivanikova et al., 2007a). This suggests that nitrate assimilation was not constrained by nutrient deficit, since no increase in luminescence could be seen following nutrient addition. At first glance, this runs counter to our characterization of the epilimnion at this site as phosphorus deficient based on seston N:P ratios and APase activity (Fig. 3). This may; however, reflect taxonomic differences in susceptibility to phosphorus deficiency and highlights the importance of extending multiple approaches in addressing community-level phenomena.

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