Effects of nitrogen source on the physiology and metal nutrition of *Emiliania huxleyi* grown under different iron and light conditions

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ABSTRACT: *Emiliania huxleyi*, a small oceanic coccolithophore, was isolated from the NE subarctic Pacific and maintained under oceanic conditions. Coccolith-forming cultures were grown with either NO_3^- or NH_4^+ as the primary nitrogen source. Fe-stress was induced experimentally, and physiological parameters including metal quotas (Fe, Mn, Zn, Cu) were measured for both NO_3^-- and NH_4^+-grown cells to determine whether it was advantageous for the cells to grow on NH_4^+ rather than NO_3^- under Fe-stressed conditions. The parameters used to observe the cell's physiological status were specific growth rate (μ), cell volume (CV), carbon (C), chlorophyll *a* (chl *a*), and nitrogen (N) per cell volume. Under Fe-replete conditions (100 nM Fe), no physiological parameters were significantly different (p < 0.05) between NO_3^-- and NH_4^+-grown cells. However, under Fe-stressed conditions, CV ($NH_4^+ > NO_3^-$), chl *a* CV^{-1} ($NH_4^+ < NO_3^-$), Mn CV^{-1} ($NH_4^+ < NO_3^-$), and Mn:C ($NH_4^+ < NO_3^-$) were all significantly different (p < 0.05) for NO_3^-- and NH_4^+-grown cells. Under Fe-stressed conditions, NO_3^--grown cells of *E. huxleyi* maintained the same or greater levels of chl *a*, N, Fe, Mn, and Cu as NH_4^+-grown cells, largely due to the drastic decrease in CV of NO_3^--grown cells under Fe-stress. Although NO_3^--grown cells substantially decreased their CVs under Fe-stressed conditions (whereas NH_4^+-grown cells did not), both NO_3^-- and NH_4^+-grown cells reduced their CVs equally under limiting-irradiance, Fe-replete conditions. A reduction in CV by the NO_3^--grown cells is particularly advantageous for cells living in a low Fe environment, since it reduces the cellular requirements for photosynthate, N, and Fe. The fact that these already small cells become smaller, along with their unique ability to maintain chlorophyll synthesis at Fe levels limiting to cell division, may help explain why *E. huxleyi* is a member of the numerically dominant size class in the NE subarctic Pacific.

KEY WORDS: Nitrogen · Ammonium · Nitrate · Coccolithophore · *Emiliania huxleyi* · Iron · Iron limitation · Metal quotas · Oceanic · Subarctic Pacific

INTRODUCTION

The Subarctic Pacific Ocean is one of the HNLC (high nutrient-low chlorophyll) regions of the world's ocean where high macronutrients (N, P, Si) and low chlorophyll concentrations are found year round. Fe is present in extremely low concentrations (<0.05 nmol kg^{-1} seawater) and thought to limit phytoplankton growth rates (Martin et al. 1989) as well as controlling species composition in combination with grazing

(Miller et al. 1991, P. Boyd et al. unpubl.). The region is dominated by small-sized phytoplankton species (non-diatoms), with large diatoms being present but not numerically abundant (Booth et al. 1982, Booth 1988, P. Boyd et al. unpubl.). One of the determining factors theorized to influence the species composition of phytoplankton in HNLC regions in general is the nitrogen source that phytoplankton use for their growth (Bruland et al. 1991, Morel et al. 1991, Price et al. 1991, 1994). This hypothesis is based on the theoretical calculations (Raven 1988) that nitrate utilization requires more Fe compared to ammonium utilization.

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Nitrate and nitrite reductase catalyze the reduction of nitrate to nitrite, and nitrite to ammonium, respectively. Both enzymes contain Fe, in a cytochrome molecule in the former, and in an Fe porphyrin molecule as well as ferredoxin in the latter (Goodwin & Mercer 1983). As a result, cells utilizing nitrate should theoretically have a higher Fe requirement than cells utilizing ammonium, and the difference has been calculated by Raven (1988) to result in a 60% higher Fe requirement for cells grown on nitrate compared to ammonium to achieve the same growth rates.

In addition, theoretically a cell must expend more reductant to grow on nitrate than on ammonium, because nitrate must be reduced to first nitrite and then to ammonium before it can be incorporated into amino acids. It has been calculated that due to the extra reducing steps required for nitrate incorporation, cells growing on nitrate require 22% more reductant than cells growing on ammonium (Thompson et al. 1989). In addition, it has been suggested that the energetic cost for the actual transport of nitrate across the cell membrane is higher than that for ammonium (Falkowski 1975, Turpin & Bruce 1990). Therefore, regardless of the Fe conditions, cells utilizing ammonium should have a theoretical advantage based on energy considerations over cells utilizing nitrate.

Fe is also required for chlorophyll synthesis (Spiller et al. 1982) and is a component of cytochrome and ferredoxin molecules, which are involved in electron transport for both photosynthesis and respiratory processes (Terry 1980, 1983, Sandmann 1985). Hence, a cell that is Fe-stressed could be analogous to one that is 'energy'-stressed, such as a light-limited cell. As a result, under light limitation, cells utilizing nitrate may not have as much energy to expend on other functions, perhaps resulting in lower growth rates or N quotas of nitrate-grown cells compared to ammonium-grown cells. Similarly, under Fe-stressed conditions, nitrate uptake could be more adversely affected than ammonium uptake due to the higher energy costs, resulting in nitrate-grown cells not being able to maintain their normal N quotas. In addition to Fe, Mn, Zn and Cu are also essential trace metals for phytoplankton metabolism and are involved in primary metabolic functions such as photosynthesis, respiration, N metabolism, and detoxification processes (Sandmann & Boger 1983, Sunda 1988/1989).

We present here experiments conducted with an oceanic coccolithophore, Emiliania huxleyi, isolated from the NE subarctic Pacific. E. huxleyi is a small (<5 μm diameter), ubiquitous oceanic coccolithophore occurring in almost all of the world's ocean except polar waters. Most noted are the very large blooms of this calcifying organism in the North Atlantic, span-

ning areas of 250000 km^2 or more (Holligan et al. 1993). This organism is receiving worldwide attention as a potential carbon sink due to its coccolith production and loss, as well as its influence on global climate via dimethylsulfoniopropionate (DMSP) production and carbon sequestration (Keller et al. 1989, Flynn 1990). In the North Pacific, large blooms of this species (or any other phytoplankter) do not occur, but E. huxleyi is a member of the most abundant size class present (Booth et al. 1982, Taylor & Waters 1982). In addition, cells <5.0 μm in diameter are responsible for 2/3 of the phytoplankton carbon biomass at Station Papa (Stn P), with E. huxleyi belonging in this size class (Booth 1988). E. huxleyi is also the most abundant coccolithophore found at Stn P (Honjo & Okada 1974). The important environmental parameters regulating phytoplankton growth rates in the North Atlantic include nitrogen and light, whereas in the subarctic Pacific, Fe must also be considered.

This is the first report on physiological, biochemical, and metal composition parameters obtained from an oceanic coccolithophore isolated from the NE subarctic Pacific. We examine both the effects of N source and Fe condition on the physiological status of Emiliania huxleyi in an attempt to determine whether it is advantageous for this organism to utilize ammonium rather than nitrate under low Fe conditions. Experiments are also presented on the effects of irradiance on both nitrate- and ammonium-grown cells, since under low irradiance levels competition for photosynthetically derived energy may become crucial, similar to Fe-stressed conditions. Metal quotas were measured using non-radiotracer ('cold-metal'), trace-metal clean techniques, allowing for the simultaneous measurement of multiple metals. Mn, Zn, and Cu were measured along with Fe to determine if their cellular levels varied with intracellular Fe levels, perhaps indicating additional utilization of these metals under Fe-stress. The physiological parameters measured were growth rate, CV, chlorophyll a (chl a) quota, in vivo fluorescence per chl a, N quota, and C quota.

MATERIALS AND METHODS

Emiliania huxleyi was isolated in November 1991 from the NE subarctic Pacific (Stn P, 145° W, 50° N). Cultures were maintained in natural Stn P water to retain the organism's ability to form coccoliths. Laboratory data were acquired shortly after isolation, with these particular experiments being conducted approximately 1 yr after isolation.

Experiments were carried out using Chelex-treated Aquil synthetic ocean water, SOW (Price et al. 1988/ 1989), made up with Nanopure water (Barnstead

Nanopure II distilled-deionized water system) and a.s.c. certified salts. The Chelex 100 resin was prepared according to Price et al. (1988/1989), with the exception of using Ultra HCl (Seastar Chemicals) for the final HCl soak. The total Fe remaining in the SOW after it was passed through the Chelex column at a rate of <1 ml min^{-1} was <0.8 nmol kg^{-1}, following the procedure of Yang (1993). Due to a limited supply of natural Stn P water, artificial seawater was chosen for these particular experiments.

The SOW was microwave-sterilized in acid-cleaned 2 l Teflon® bottles for a total of 12 min (Keller et al. 1988). All handling of the medium was done in a class 100 laminar flowhood to prevent metal contamination and contamination by bacteria. Macronutrient stocks were made up in Nanopure water and Chelex-treated at pH 3.0 for near 100% Fe removal (R. Chretien unpubl. data). Macronutrients were added to achieve a final concentration of 30 µM of either nitrate or ammonium, and 5.0 µM phosphate. This level of ammonium addition had no detrimental effect on the organism's growth rate or overall appearance. EDTA was added to yield a final concentration of 10 µM. Metal stocks were prepared in 0.01 M Ultra HCl in Nanopure water. Final metal concentrations added were: 8.0 nM Zn, 23 nM Mn, 1.0 nM Cu, 2.5 nM Co, 100 nM Mo, and 10 nM Se. 100 nM Fe final concentration was added for the Fe-replete cultures, and no Fe was added for the other treatments. Thiamine and biotin were added to give a final concentration of 1×10^{-4} g l^{-1} and 5×10^{-7} g l^{-1}, respectively.

Fe experiments. *Fe-replete experiments:* Semi-continuous cultures were grown in quadruplicate with either nitrate or ammonium as the N source in acid-cleaned 2.8 l polycarbonate Fernbach flasks. All plastics coming in contact with the medium or cultures were acid-cleaned in the following manner: an organic wash with Sparkleen detergent or acetone, a soak in 3 N reagent grade HCl for at least 24 h, 2 rinses with Nanopure water, and a final soak in 0.01 M Ultra HCl in Nanopure water for at least 1 wk. The flasks were equipped with Teflon® tubes and silicone stoppers. Cultures were not mechanically stirred, as this knocked off many of the coccoliths, but they were mixed once a day by swirling the flasks by hand. Cultures were maintained at 16°C on a 14 h light:10 h dark cycle with Vitalite fluorescent lights. The irradiance, as measured by a Biospherical Instruments model QSL-100 sensor, was 150 µE m^{-2} s^{-1} which was saturating for growth. Cultures were grown for 10 generations and harvested in log phase.

Fe-stressed experiments: Cultures were grown in triplicate with either nitrate or ammonium as the nitrogen source in identical conditions as above. However, when *Emiliania huxleyi* was semi-continuously diluted

with 'no Fe' medium (<0.8 nmol kg^{-1}), no physiological evidence of Fe limitation was observed. Therefore, we devised a culture protocol that resulted in Fe-limited cells after several transfers, similar to procedures used when comparing macronutrient deplete vs replete cells. Cultures were grown semi-continuously and sampled after 9 d (3 dilutions). These cells were in medium <0.8 nmol Fe kg^{-1} with 10 µM EDTA, but they showed little or no signs of Fe-stress (no drastic decrease in growth rate). Cells from these same cultures were again diluted and allowed to grow up until early stationary phase was reached as determined by cell counts. At this time, all macro- and micronutrients except Fe were added directly to the culture flasks (no new medium was added), forcing the cell number to increase with minimal additions of Fe to the medium (via contamination). This was repeated until the addition of macronutrients caused no increase in cell numbers (2 additions with macro- and micronutrients, 1 addition with N and P only). These 'Fe-stressed' cultures represent cells whose cell division was halted due to Fe limitation. After sampling, a final concentration of 5 nM Fe was added to the cultures and cell division resumed. The final cell densities of the Fe-stressed cultures were ca 6×10^5 cells ml^{-1}.

Growth rates were calculated from linear regressions of plots of the natural log of cell number vs time. Cell numbers and cell volumes were measured on a Coulter Counter model TAII. The pH was decreased to 5 with HCl before counting to dissolve the coccoliths and prevent interference, as the coccoliths overlapped with some of the same channels as the cell counts. The HCl was added directly to the counting chambers, allowed to sit for 1 min to completely dissolve coccoliths (time series were conducted), and then counted on the Coulter Counter. Chlorophyll a concentrations were determined by *in vitro* fluorometry (extracted in 90% ethanol; sonicated for 10 min; refrigerated for 24 h; fluorescence measured on a Turner Designs Fluorometer model 10-AU before and after acidification). The fluorometer was calibrated with chl a standards (Parsons et al. 1984).

Samples for particulate organic carbon and nitrogen were collected on precombusted 13 µm Gelman A/E filters and analyzed on a Carlo Erba CHN analyzer. Samples with and without coccoliths were measured. The C content of cells minus their coccoliths was determined by dissolving the coccoliths with HCl (pH 5) and filtering. This procedure did not appear to lyse the cells, as the N per cell remained the same for both non-acidified and acidified samples.

Metal quotas (Fe, Mn, Zn, Cu) were measured using non-radiotracer (cold-metal), trace-metal clean techniques. For the Fe-replete experiment, cultures were filtered onto acid-cleaned 3.0 µm pore size cellu-

lose acetate filters using acid-cleaned Nalgene fil-
tration holders. Acid-cleaned cellulose acetate filters
gave similar blanks to acid-cleaned polycarbonate fil-
ters, and were used instead of polycarbonate filters for
their ease in digestion. For total (intra- plus extra-
cellular) Fe measurements, filters were rinsed twice
with 5 ml of Chelex-treated Stn P water to wash off any
loosely associated metals. Filters were carefully
removed, placed in acid-cleaned locking petri dishes
and frozen. The filters were then digested in concen-
trated Ultra HNO_3^- (Seastar Chemicals) using a CEM
Corporation microwave digestion apparatus. The
digestate was completely clear. A subsample was
taken and diluted appropriately for injection into a
Varian graphite furnace atomic absorption spectrome-
ter with Zeeman background correction. A minimum
of 3 filter blanks for all treatments were taken and sub-
tracted from the samples. Internal or intracellular Fe,
Fe_{int}, was determined by Ti(III) treatment (Hudson &
Morel 1989). Cultures were first treated with Ultra HCl
to remove the coccoliths prior to Ti(III) treatment. This
was to avoid any complications involving adsorption of
Fe onto the coccoliths and to eliminate the extra barrier
to the cell membrane. The acid and Ti(III) treatment
did not increase the cellulose acetate filter blanks.
Acid treated, Ti(III) washed filter blanks were sub-
tracted from the identically treated culture samples.
However, even though the acid and Ti(III) treatment
did not increase the filter blanks, the treatment did
cause an increase in the Fe retained on the filters for
culture samples of Fe-stressed cells. We can think of 3
possible reasons for this increase: (1) Any contaminat-
ing Fe present in the Ti(III) reagent could have bound
to empty Fe-uptake sites on the cell surface of the Fe-
stressed cells; (2) the extra particulate matter present
on culture samples prevented complete rinsing with
the Chelex-treated Stn P water; or (3) Fe-stressed cells
may be producing organic compounds, possibly to aid
in Fe acquisition and transport, which may cause
retention of Fe on the filters. Due to these complica-
tions at low particulate Fe concentrations (Fe-stressed
cells), we estimated the Fe_{int} using Hudson & Morel's
(1990) finding that 75 to 90% of the total Fe in Fe-
stressed diatom cultures was intracellular. Further-
more, at external Fe concentrations below 100 nM, the
Ti(III) treatment does not appear to be necessary, as it
was found to not remove any extracellular Fe at very
low external Fe concentrations (Sunda et al. 1991).

All statistical comparisons were done using a Stu-
dent's t-test at the 95% confidence level ($p < 0.05$).
Comparisons were made between ammonium and
nitrate-grown cultures for a given Fe treatment.

Irradiance experiments. Cultures were grown in
triplicate in Silinized-treated (Pierce) 50 ml glass tubes
with either 30 µM nitrate or ammonium as the N source.

Medium preparation and additions were identical to
the Fe-replete experiments (100 nM Fe, 10 µM EDTA).
Cultures were maintained in quasi-steady state (diluted
at 1/3 of the maximal fluorescence) for a minimum of
6 generations using Vitalite tubes as the irradiance
source, at irradiances of 24, 40, 70, 119, and 176 µE m^{-2}
s^{-1}. Growth rates were obtained by regressions of the
natural log of *in vivo* fluorescence versus time. All other
methods were the same as for the Fe experiments.

RESULTS

Fe experiments

Growth rate

The specific growth rates of nitrate- and ammonium-
grown cells were not significantly different under the
high Fe (100 nM Fe) treatment (Table 1). Under the low
Fe (0.8 nM Fe) treatment, nitrate-grown cells grew
significantly faster than ammonium-grown cells
(Table 1). For the Fe-stressed cultures, cell numbers
were increased until the external Fe concentration
reached levels limiting to growth, resulting in no cell
division. After sampling, a final concentration of 5 nM
Fe was added to the cultures, and cell division resumed
at a growth rate of 0.0128 and 0.0095 h^{-1} measured over
a 2 d period for ammonium and nitrate-grown cultures,
respectively (Table 1). Ammonium-grown cells grew
significantly faster than nitrate-grown cells after this Fe
addition.

Cell volume

The cell volume, CV, of nitrate and ammonium-
grown cells was not statistically different under the
high and low Fe treatments (Fig. 1, Table 1). However,
nitrate-grown cells had significantly smaller CVs than
ammonium-grown cells under Fe-stressed conditions
($p = 0.007$), with the mean CVs being 24.9 and
33.8 $µm^3$, respectively. In general, the CV of both
nitrate- and ammonium-grown cells were larger under
the high Fe treatment than under the low Fe treatment
(Fig. 1). However, the CV of ammonium-grown cells
between the low Fe and Fe-stressed treatments
remained identical, whereas the CV of nitrate-grown
cells decreased 30% under Fe-stress (Fig. 1, Table 1).

Biochemical composition

Chlorophyll a. Chl *a* cell^{-1} was not statistically differ-
ent for nitrate- and ammonium-grown cells under the

Table 1. *Emiliania huxleyi*. Physiological parameters for nitrate- and ammonium-grown cultures under 3 Fe treatments: high Fe (100 nM Fe), low Fe (0.8 nM Fe), and Fe-stressed conditions (≪0.8 nM Fe). Values are means ± 1 SE (n = 4 for 100 nM Fe, n = 3 for other Fe treatments). *Significant difference at p < 0.05 between NO_3^-- and NH_4^+-grown cells for a particular Fe treatment with the asterisk placed next to the greater value. μ: growth rate; CV: cell volume; C_t: total cell carbon, including coccoliths; C_a: cell carbon excluding coccoliths (coccoliths removed by acidification, see 'Methods'); Fl: fluorescence

Parameters	High Fe (100 nM)		Low Fe (0.8 nM)		Fe-stressed (≪0.8 nM)	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
μ (h^{-1})	0.0175 ± 0.0010	0.0183 ± 0.0010	0.0134 ± 0.0002	0.0191* ± 0.0010	0 [0.0128]*,a ± 0.0006	0 [0.0095]a ± 0.0007
CV (μm^{-3})	53.0 ± 1.6	48.9 ± 2.5	36.6 ± 0.6	34.9 ± 0.7	33.8* ± 1.5	24.9 ± 0.9
Chl a cell^{-1} (fg cell^{-1})	120 ± 8	120 ± 3	110 ± 6	110 ± 13	130* ± 3	110 ± 4
Chl a CV^{-1} (fg μm^{-3})	2.2 ± 0.2	2.5 ± 1.0	3.1 ± 0.3	3.2 ± 0.3	4.0 ± 0.2	4.5* ± 0.2
N cell^{-1} (pg cell^{-1})	1.9 ± 0.1	1.8 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.6* ± 0.1	1.3 ± 0.1
N CV^{-1} (fg μm^{-3})	36 ± 2	37 ± 1	38 ± 6	43 ± 1	47 ± 2	52 ± 4
C_t cell^{-1} (pg cell^{-1})	31.9* ± 0.8	27.6 ± 1.1	25.2 ± 6.2	21.2 ± 3.4	28.3* ± 1.3	13.4 ± 0.1
C_t CV^{-1} (pg μm^{-3})	0.60 ± 0.01	0.56 ± 0.02	0.69 ± 0.17	0.61 ± 0.09	0.84* ± 0.02	0.52 ± 0.02
C_a cell^{-1} (pg cell^{-1})	13.9 ± 0.3	13.9 ± 0.9	17.7 ± 2.6	16.5 ± 1.8	19.8* ± 1.1	12.9 ± 0.8
C_a CV^{-1} (pg μm^{-3})	0.26 ± 0.01	0.28 ± 0.03	0.48 ± 0.07	0.47 ± 0.04	0.59 ± 0.02	0.53 ± 0.01
C_a:N (mol:mol)	8.3 ± 0.3	8.9 ± 0.7	14.1 ± 0.5	12.5 ± 1.3	14.0 ± 0.8	11.2 ± 1.2
In vivo Fl:chl a (ng chl a^{-1})	0.30 ± 0.02	0.28 ± 0.01	0.32 ± 0.01	0.35 ± 0.03	0.35 ± 0.03	0.36 ± 0.01
C_a:chl a (wt)	120 ± 9	120 ± 11	160 ± 46	150 ± 5	150 ± 11	120 ± 5

aGrowth rates achieved over a 2 d period after 5 nM Fe was added to the Fe-stressed cultures

high or low Fe treatment, but it was significantly higher for ammonium-grown cells under Fe-stress (Table 1). However, when normalized to CV, chl a CV^{-1}, the chl a content was significantly higher for nitrate- than for ammonium-grown cells under Fe-stressed conditions (Fig. 2A, Table 1). There was no significant difference in chl a CV^{-1} between nitrate- and ammonium-grown cells under the other Fe conditions (Fig. 2A, Table 1). Because of the large difference in CV between nitrate- and ammonium-grown Fe-stressed cells, all physiological parameters were normalized to CV.
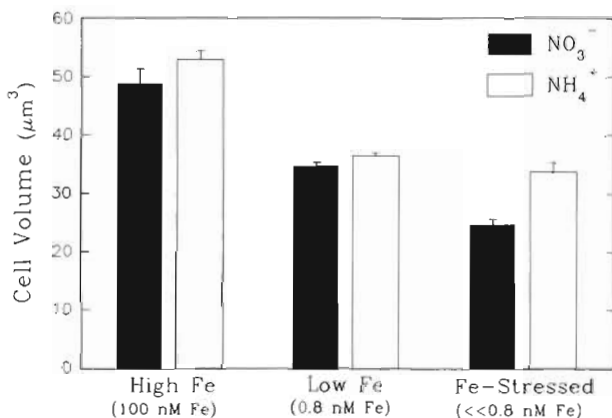

Fig. 1. *Emiliania huxleyi*. Cell volume vs Fe treatment for NO_3^-- and NH_4^+-grown cells. Error bars represent standard error (n = 4 for 100 nM Fe, n = 3 for other Fe treatments)
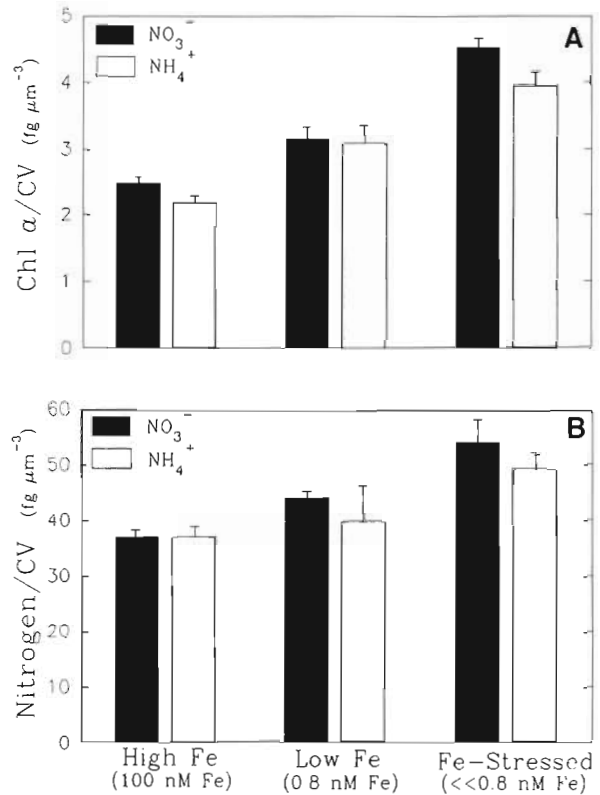

Fig. 2. *Emiliania huxleyi*. (A) Chlorophyll a per CV and (B) N per CV vs Fe treatment for NO_3^-- and NH_4^+-grown cells. (For error bars see Fig. 1)

In vivo fluorescence : chl a. The *in vivo* Fl:chl a, a measure of the efficiency of photosynthetic electron transport, was not significantly different between nitrate- and ammonium-grown cells for any of the Fe treatments (Table 1).

Nitrogen. The N CV^{-1} of nitrate- and ammonium-grown cells was not significantly different under any of the 3 Fe treatments, including the condition when Fe was limiting cell division (Fig. 2B, Table 1). In addition, cells under Fe-stress had higher N CV^{-1} values than cells under the other Fe treatments (Fig. 2B, Table 1).

Carbon. The total carbon (C_t) CV^{-1}, which includes coccolith carbon, was not statistically different for nitrate- and ammonium-grown cells under the high and low Fe treatments (Table 1). Similarly, cellular C excluding coccolith carbon (C_a) CV^{-1} was not different for ammonium- and nitrate-grown cells under these Fe treatments. Moreover, the amount of coccolith carbon, estimated by subtracting C_a from C_t, remained fairly constant regardless of nitrogen source or Fe treatment. The exception was nitrate-grown cells under Fe-stress. However, due to the very small size of these cells, some of the coccoliths could have been lost through the filter.

Carbon : chlorophyll a. The C_a:chl a ratio was not statistically different for nitrate- and ammonium-grown cells under any of the Fe conditions (Table 1).

Metal composition

Iron. The intracellular Fe (Fe_{int}) CV^{-1} was not significantly different between nitrate and ammonium-grown cells under any of the Fe treatments tested (Table 2). When Fe quotas were normalized to C, Fe_{int}:C_t or Fe_{int}:C_a, there was still no significant difference between
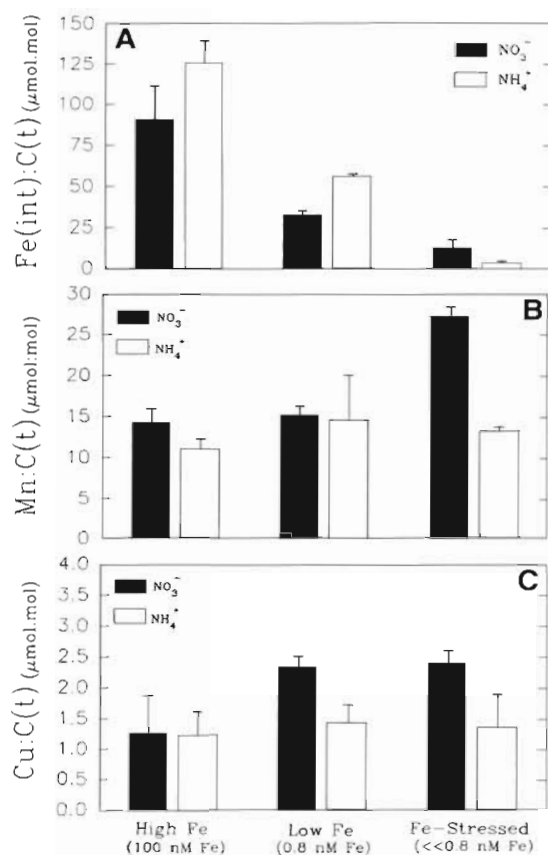

Fig. 3. *Emiliania huxleyi.* (A) Intracellular Fe per total C, (B) Mn per total C, and (C) Cu per total carbon vs Fe treatment for NO_3^-- and NH_4^+-grown cells. (For error bars see Fig. 1)

nitrate- and ammonium-grown cells under the high Fe and the Fe-stressed treatments, but ammonium-grown cells had higher Fe_{int}:C_t and Fe_{int}:C_a than nitrate-grown cells under the low Fe treatment (Fig. 3A, Table 3).

Table 2. *Emiliania huxleyi.* Metal quotas normalized per cell and per cell volume (CV) for nitrate- and ammonium-grown cultures under 3 Fe treatments. Fe_{int}: intracellular Fe; Fe_t: total Fe (both intra- and extracellular); amol = 1×10^{-18} moles; NA: data not available. The Fe_{int} values for the low Fe and Fe-stressed treatments are 75 to 90% of the measured Fe_t (see 'Methods'). *Significance as in Table 1

Parameters	High Fe (100 nM)		Low Fe (0.8 nM)		Fe-stressed ($\ll 0.8$ nM)	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
Fe_{int} cell^{-1} (amol cell^{-1})	330 ± 38	210 ± 44	94–110 ± 26–32	49–59 ± 4–5	9–11 ± 3	14–17 ± 6–7
Fe_{int} CV^{-1} (amol µm^{-3})	6.3 ± 0.6	4.3 ± 1.0	2.5–3.1 ± 0.7–0.8	1.4–1.7 ± 0.08–0.09	0.27–0.33 ± 0.07–0.08	0.57–0.68 ± 0.02–0.03
Mn cell^{-1} (amol cell^{-1})	27 ± 1	30 ± 1	24 ± 2	24 ± 1	32 ± 0.4	31 ± 1.2
Mn CV^{-1} (amol µm^{-3})	0.52 ± 0.03	0.61 ± 0.05	0.65 ± 0.07	0.69 ± 0.03	0.94 ± 0.03	1.2* ± 0.1
Zn cell^{-1} (amol cell^{-1})	45 ± 4	41 ± 5	NA	NA	61 ± 3	15 ± 1
Zn CV^{-1} (amol µm^{-3})	8.4 ± 0.6	8.3 ± 0.1	NA	NA	1.8 ± 0.9	0.6 ± 0.04
Cu cell^{-1} (amol cell^{-1})	4.4 ± 0.7	2.8 ± 1.2	2.2 ± 0.02	3.6 ± 0.80	3.1 ± 1.2	2.7 ± 0.3
Cu CV^{-1} (amol µm^{-3})	0.08 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.10* ± 0.02	0.09 ± 0.03	0.11 ± 0.01

Table 3. *Emiliania huxleyi*. Metal quotas normalized to cell carbon for nitrate- and ammonium-grown cultures under 3 Fe treatments. *Significance as in Tables 1 & 2

Parameters	High Fe (100 nM)		Low Fe (0.8 nM)		Fe-stressed (≪ 0.8 nM)	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
$Fe_{int}:C_t$ (µmol:mol)	130 ± 14	90 ± 21	56–68* ± 1.3–1.5	33–40 ± 2.5–2.9	3.9–4.7 ± 1.0–1.2	12–15 ± 5.5–6.6
$Fe_{int}:C_a$ (µmol:mol)	80 ± 32	160 ± 54	66–80* ± 2.3–2.8	38–46 ± 3.6–4.3	5.6–6.7 ± 1.4–1.7	12–14 ± 5.2–6.2
$Mn:C_t$ (µmol:mol)	10 ± 0.7	13 ± 0.8	15 ± 5.7	15 ± 1.1	13 ± 0.5	27* ± 1.2
$Mn:C_a$ (µmol:mol)	25 ± 2.5	26 ± 1.5	17 ± 3.3	17 ± 1.0	19 ± 0.9	27* ± 2.3
$Zn:C_t$ (µmol:mol)	17 ± 1.5	18 ± 1.9	NA	NA	26 ± 14	13 ± 0.9
$Zn:C_a$ (µmol:mol)	41 ± 7.2	30 ± 5.6	NA	NA	38 ± 21	13 ± 0.7
$Cu:C_t$ (µmol:mol)	1.2 ± 0.4	1.3 ± 0.6	1.4 ± 0.3	2.3 ± 0.2	1.4 ± 0.6	2.4 ± 0.2
$Cu:C_a$ (µmol:mol)	3.8 ± 0.5	1.4 ± 0.5	1.3 ± 0.4	2.3 ± 0.5	2.0 ± 0.8	2.4 ± 0.1

Manganese. The Mn CV^{-1} was the same for nitrate- and ammonium-grown cells under the high and low Fe treatments (Table 2). However, under Fe-stress, nitrate-grown cells had significantly higher Mn levels per CV than ammonium-grown cells (Table 2). When normalized to C, these relationships were even more pronounced (Fig. 3B, Table 3).

Zinc. The Zn CV^{-1} was the same for nitrate- and ammonium-grown cells under the high Fe treatment (Table 2). Similarly, when normalized to C, ammonium- and nitrate-grown cells were not different under high Fe or under Fe-stressed conditions (Table 3).

Copper. The Cu CV^{-1} was not different between nitrate- and ammonium-grown cells under high Fe or under Fe-stress (Table 2). However, nitrate-grown cells had higher Cu CV^{-1} values than ammonium-grown cells under the low Fe treatment (Table 2). The same relationships hold true when normalized to C (Fig. 3C, Table 3).

Irradiance experiments

Growth rate

The growth rates of both nitrate- and ammonium-grown cells increased with irradiance (Fig. 4A). At the lowest irradiance level which supported growth (24 µmol m^{-2} s^{-1}), nitrate-grown cells had significantly higher growth rates than ammonium-grown cells (Fig. 4A). At all other irradiance levels the growth rates of nitrate- and ammonium-grown cells were not statistically different.

Cell volume

The CV of both nitrate- and ammonium-grown cells was larger under high (and growth saturating) ir-

radiances than under low irradiances (Fig. 4B). Under the 2 lowest irradiance levels there was no significant difference in CV between nitrate- and ammonium-grown cells (Fig. 4B). However, ammonium-grown cells were significantly larger than nitrate-grown cells at growth-saturating irradiances.

Chlorophyll *a*

Chl *a* CV^{-1} increased with decreasing irradiance, with the exception of the lowest irradiance level, where chl *a* CV^{-1} decreased (Fig. 4C). At 47 and 70 µE m^{-2} s^{-1}, the chl *a* CV^{-1} was significantly greater for nitrate-grown cells compared to ammonium-grown cells (Fig. 4C).

DISCUSSION

This was the first study to report basic physiological and multi-element metal composition parameters for an oceanic coccolithophore isolated from the NE sub-arctic Pacific. In addition, the effect of nitrogen source on the basic physiology of an oceanic coccolithophore has not previously been examined, nor the interactions that Fe or irradiance may have in conjunction with nitrogen source. Specific results from the experiments will first be discussed, followed by discussions pertaining to the ecological scenario at Stn P.

Effect of N source under Fe-replete conditions

Under the Fe-replete (100 nM) treatment in the Fe experiments, there appeared to be no physiological differences between nitrate- and ammonium-grown cells in any of the parameters that we measured. Other
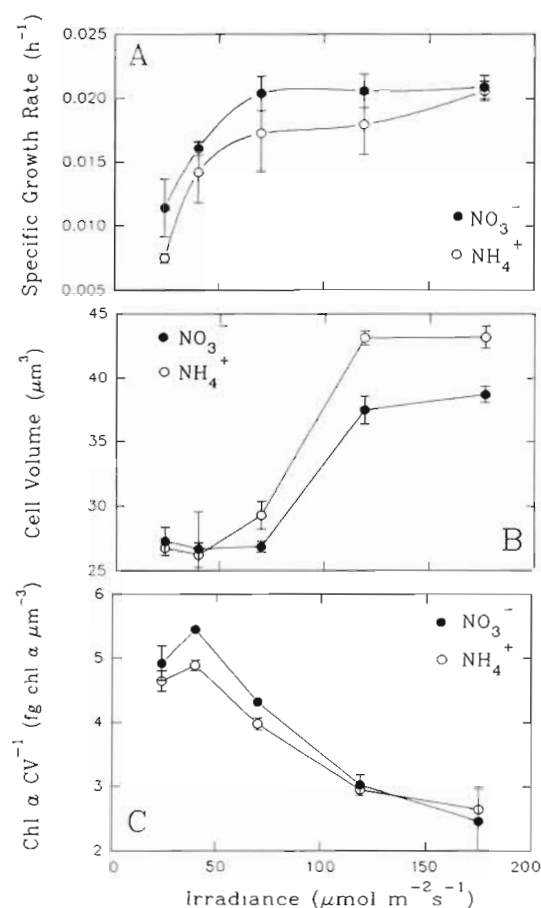
Fig. 4. *Emiliania huxleyi*. (A) Specific growth rate, (B) cell volume, and (C) chl a per CV for NO_3^-- and NH_4^+-grown cells vs irradiance. Error bars represent standard error (n = 3)

rating light and Fe conditions. This may have been due to the lower energetic cost of ammonium transport and utilization compared to nitrate. In our Fe experiments, however, no such difference was observed under Fe-replete conditions; nitrate-grown cells were able to maintain similar N quotas compared to ammonium-grown cells. Thompson et al. (1989) found that nitrate-grown cells had 21% lower C quotas than ammonium-grown cells, and they attributed this to the possibility that competition for reductant may reduce the C quotas (and growth rate) of nitrate-grown cells. However, again, nitrate-grown cells of *Emiliania huxleyi* in the present study maintained the same C quotas, CV, chlorophyll, and N levels per CV as ammonium-grown cells under Fe-replete conditions (Table 1, Fig. 2).

Similar to the physiological parameters, there was no different in the metal composition of nitrate- and ammonium-grown cells under Fe-replete conditions (Table 3). Despite the prediction that nitrate-grown cells should maintain higher Fe quotas than ammonium-grown cells under saturating conditions (Raven 1988), we did not find this in the Fe-replete cultures, even though the growth rates were similar. Sunda & Huntsman (1995) also did not find a difference in Fe_{int}:C ratios between nitrate- and ammonium-grown cultures of *E. huxleyi* (their isolate was from the Gulf of Mexico). This expected relationship was also absent in Fe-replete cultures of an oceanic diatom (*Actinocyclus* sp.) from the NE subarctic Pacific (Muggli et al. in press).

We can compare our Mn, Zn, and Cu quotas with the few studies that have reported these parameters previously. Ours is the first study, however, to measure multiple metals simultaneously on an oceanic coccolithophore. The range of $Mn:C_t$ values that *Emiliania huxleyi* maintains under replete conditions is between 10 to 14 µmol $Mn:mol$ C_t (Fig. 4B, Table 3). The previously reported Mn:C ratios are for 2 diatom species, both *Thalassiosira* sp., and range from 22 to 32 µmol Mn:mol C under saturating conditions maximal for growth (Sunda 1988/1989). The oceanic diatom *Actinocyclus* sp. maintained a Mn:C ratio of 34 µmol Mn:mol C for Fe-replete conditions (Muggli et al. in press). It appears, then, that *E. huxleyi* maintains a lower Mn:C ratio than other previously reported species (although all diatoms), including one diatom from the subarctic Pacific. This is in agreement with the finding of Brand et al. (1983) that oceanic coccolithophores have a low requirement of Mn for growth relative to other phytoplankton.

The Zn:C ratio maintained by Fe-replete cells of *Emiliania huxleyi* was ca 17 µmol Zn:mol C_t (Table 3). Previous reports of Zn:C are from 3 diatom species (all *Thalassiosira* sp.) and 2 isolates of *E. huxleyi* (1 from the Sargasso Sea, 1 from the Gulf of Mexico) and range

researchers have previously found differences in growth rates between nitrate- and ammonium-grown cells under saturating conditions. Thompson et al. (1989), using a marine diatom (*Thalassiosira pseudonana*) under saturating light and Fe-replete conditions, found that the growth rates of ammonium-grown cells were significantly higher (8%) than the growth rates of nitrate-grown cells. Similarly, Levasseur et al. (1993) found higher growth rates for ammonium-grown cells than for nitrate-grown cells of several species under the same conditions. However, an oceanic diatom isolated from the subarctic Pacific was found to have no difference in the growth rates of nitrate- and ammonium-grown cells under Fe-replete conditions (Muggli et al. in press). Despite the possible energetic advantages, higher growth on ammonium does not always occur.

Levasseur et al. (1993) also found that nitrate-grown cells had lower N quotas than ammonium-grown cells for the diatom *Thalassiosira pseudonana* under satu-

from 11 to 14 for the diatoms and as low as 1 µmol Zn:mol C for the *E. huxleyi* isolates (Sunda & Huntsman 1992). An oceanic diatom from the subarctic Pacific maintained a ratio of 3 to 7 µmol Zn:mol C under Fe-replete conditions (Muggli et al. in press). Our *E. huxleyi* from the NE subarctic Pacific appears to have a requirement for Zn that lies in the upper range of previously reported values, being at least double the requirement for the diatom from the same location.

There have been no recently published estimates of Cu:C ratios in phytoplankton. However, Price & Morel (1994) report 2 studies on *Chlorella* sp. for which the estimated values were 0.8 and 9 µmol Cu:mol C for 90% of maximum growth (Walker 1953, Manahan & Smith 1973). Our values for *E. huxleyi* are between 1 and 2 µmol Cu:mol C_t, which lies within the range of previously reported values.

The intracellular Fe:C ratios as a function of growth for this isolate of *Emiliania huxleyi* will be reported in detail elsewhere (Muggli & Harrison unpubl.).

Effect of N source under Fe-stressed conditions

Theoretically, the competition for reductant should become more severe under Fe-stressed conditions, and therefore ammonium-grown cells should be able to maintain even higher growth rates, N quotas and C quotas than nitrate-grown cells. Rueter & Ades (1987) examined the possibility of Fe-stressed cells being limited by reducing power using cultures of the green alga *Scenedesmus quadricauda*. They found that under Fe-stress, ammonium-grown cultures had higher C fixation rates than nitrate-grown cells under a range of irradiance levels (ca 20 to 180 µmol m^{-2} s^{-1}). This observation along with other information allowed them to conclude that Fe-stressed cells can exhibit the same response as energy-limited cells (Rueter & Ades 1987). However, in our Fe experiments, ammonium-grown cells of *Emiliania huxleyi* did not show signs of any energy advantage over nitrate-grown cells under Fe-stress. For example, nitrate-grown cells maintained the same or greater chlorophyll, N and C levels as ammonium-grown cells under Fe-stressed conditions (Fig. 2, Table 1). This was primarily the result of the nitrate-grown cells substantially reducing their CVs under Fe-stress (Fig. 1).

Growth (Fe-stress vs low light)

Since Fe-stress could lead to energy-limited cells similar to light-limited cells, it is interesting to compare the effects of low irradiance levels on nitrate- and ammonium-grown cells (Fig. 4). Under low irradiance levels where the competition for reductant is most severe, nitrate-grown cells of *Emiliania huxleyi* grew significantly faster than ammonium-grown cells, in contrast to what would be theoretically expected (Fig. 4A). In the Fe experiments, before the cessation of cell division by Fe-stress was achieved, nitrate-grown cells were also growing faster than ammonium-grown cells (data not shown), as was the case for the 0.8 nM Fe treatment. The smaller CV of nitrate-grown cells compared to ammonium-grown cells could have partially accounted for the faster division rate (lower requirement of C, N, metals, etc.), but why this was also found in the irradiance experiments when the CVs were identical under low light is puzzling.

Cell volume (Fe-stress vs low light)

The most contrasting finding between the low irradiance cultures and the Fe-stressed cultures was that no difference in CV was found between nitrate- and ammonium-grown cells in the low irradiance cultures, whereas there was a large difference in CV between nitrate- and ammonium-grown cultures under Fe-stress (Figs. 2 & 4B). The decrease in CV of nitrate-grown cells and not ammonium-grown cells in response to severe Fe-stress in the Fe experiments indicates that somehow the nitrate-grown cells were more severely affected by Fe-stress than the ammonium-grown cells, and that more than just photosynthetically derived products and energy may have been the cause of this change. Fe-stress will also affect non-photosynthetic energy deriving processes such as respiration, as well as possibly hindering the synthesis of nitrate and nitrite reductase (although a difference on the scale of atoms of Fe would probably not cause a difference). Somehow the ammonium-grown cells under Fe-stress did not require a reduction in CV to meet their metabolic needs, whereas the nitrate-grown cells acclimated to Fe stress by reducing their CV.

A decrease in CV supports the prediction by Hudson & Morel (1990) that oceanic phytoplankton cannot increase their Fe transport kinetics, and consequently their only means of acclimation to Fe-stressed conditions is a reduction in Fe-requirement or a reduction in cell size. This reduction in CV in response to Fe-stress has also been found to occur in a dinoflagellate (Doucette & Harrison 1990), but nitrate was the only nitrogen source used. The only other study to-date that has examined the effects of both nitrate and ammonium on CV under Fe-stress is for an oceanic diatom, and no difference in CV was found under Fe-stress between nitrate- and ammonium-grown cells (Muggli et al. in press).

Chlorophyll *a* quotas (Fe-stress vs low light)

The difference in chlorophyll quotas between nitrate- and ammonium-grown cultures in the irradiance experiments mimics the results obtained in the Fe experiments. Under 2 of the sub-saturating irradiance levels (45 and 70 µmol m^{-2} s^{-1}), nitrate-grown cells maintained higher chlorophyll per CV ratios than ammonium-grown cells, similar to that which was found under Fe-stress (Figs. 4C & 2A). Under low irradiance, where the competition for reductant is the most crucial, it is surprising to observe nitrate-grown cells maintaining higher chlorophyll levels than ammonium-grown cells. However, since nitrate-grown cells would need more reductant to transport and utilize their N source, they may require more chlorophyll than ammonium-grown cells to meet these needs. Thompson et al. (1989) came to the conclusion that nitrate-grown cells may be more efficient in the production of reductant than ammonium-grown cells, and nitrate-grown cells could achieve this by being more efficient in light absorption or by improving their quantum yield in some other way. One of these ways may be to increase their chlorophyll levels. The same arguments can be applied to the fact that nitrate-grown cells of *Emiliania huxleyi* under Fe-stress had higher levels of chlorophyll than ammonium-grown cells. Apparently the expected added strain of Fe limitation on nitrate-grown cells does not severely affect the cell's ability to synthesize chlorophyll relative to ammonium-grown cells.

The generally expected observation of decreased chlorophyll quotas under Fe-stress that has been found in other studies (Glover 1977, Doucette & Harrison 1990, Greene et al. 1991, 1992, Rueter & Unsworth 1991) was not evident in *Emiliania huxleyi* (Fig. 2A, Table 1). Despite a greater than 10 times reduction in the cellular Fe quota, Fe-stressed cells maintained relatively constant C:chlorophyll ratios (Table 1). This oceanic coccolithophore appears truly unique in this regard. In contrast, an oceanic diatom (*Actinocyclus* sp.) from the NE subarctic Pacific exhibited significant decreases in chlorophyll levels under Fe-stress (Muggli et al. in press). *E. huxleyi* must have some unique metabolic characteristics which allow it to maintain chlorophyll synthesis under severe Fe-stress (when Fe was limiting cell division completely). This may be one of the ways in which this organism survives at Stn P continually.

Metal quotas

The only difference in metal quotas between nitrate- and ammonium-grown cells when cell division had just ceased due to Fe-stress was that nitrate-grown cells had significantly higher Mn:C ratios than ammonium-grown cells (Table 3). Both nitrate- and ammonium-grown cultures in the Fe-stressed treatment were exposed to greater Mn levels than in the other 2 Fe treatments due to the addition of Mn in the process of inducing Fe-stress. However, ammonium-grown cells did not increase their cellular Mn levels, whereas nitrate-grown cells did (Fig. 4B, Tables 2 & 3). One factor that could possibly explain this, in part, was that the nitrate-grown cells decreased their CVs whereas the ammonium-grown cells did not. This reduction in CV would increase the surface area to volume ratio, possibly resulting in more surface uptake sites per volume than in ammonium-grown cells. The phenomenon of increased cellular Mn levels under Fe-stress for nitrate-grown cells has been found previously; Fe-limited cells of *Thalassiosira weissflogii* grown on nitrate had higher Mn quotas than Fe-replete cells at fixed concentration of free Mn ion (Harrison & Morel 1986). In addition, an oceanic diatom from the subarctic Pacific also exhibited this response (Muggli et al. in press). In the present study, the nitrate-grown cells under Fe-stress contained more Mn and chlorophyll per carbon than ammonium-grown cells, indicating that photosystem II may somehow be more active in Fe-stressed nitrate-grown cells than Fe-stressed ammonium-grown cells.

Ecological relevance

Comparison of *Emiliania huxleyi* isolates from different locales

It is known that genetic variation is prevalent among *Emiliania huxleyi* isolates from different locations (Brand 1982, van Bleijswijk et al. 1991, Young & Wesbrook 1991), and this may explain some of the variation in physiological characteristics observed between isolates from different areas of the ocean. Growth rate can be an indicator of the overall physiology of a cell, and it is a parameter frequently measured in other studies. Although there are differences in culturing conditions, our *E. huxleyi* isolate from the subarctic Pacific had a much lower maximal growth rate than other reported isolates. Paasche & Klaveness (1970) found that the coccolith-forming cells of *E. huxleyi* from Oslo fjord, Norway had a maximum growth rate of 1.68 doublings d^{-1} (21°C, continuous light). Similarly, Brand (1982) examined 73 clones of *E. huxleyi* isolates from the Sargasso Sea and the Gulf of Maine and found growth rates ranging from 1.5 to 1.7 divisions d^{-1} (20°C, 14 h light:10 h dark cycle). In contrast, our isolate of *E. hux-*

leyi from Stn P grows maximally at ca 0.6 doublings d^{-1} at 16°C with a 14 h light:10 h dark regime. This is also the maximal growth rate achieved by this isolate when grown on artificial seawater under continuous illumination (Lecourt et al. in press). Since all other isolates reported here are from the Atlantic Ocean while ours is from the Pacific, it would be expected that genetic differences are present. However, our isolate is the only one from an HNLC area. Brand (1994) hypothesized that some coccolithophores in oligotrophic areas may exhibit extreme *K*-selection; that is, grow extremely well at low nutrient concentrations and not grow any faster at higher nutrient concentrations.

The subarctic Pacific

Since dissolved Fe concentrations in the surface waters of the NE subarctic Pacific have been measured to be 0.05 to 0.4 nmol kg^{-1} seawater (Martin & Gordon 1988, P. Boyd et al. unpubl., I. Kudo unpubl. data), our experimental conditions of 'low Fe' (total Fe = 0.8 nmol kg^{-1}) may be representative at Stn P at some times. The external Fe concentration in the Fe-stressed culture flasks in which cell division had actually ceased was likely extremely small, as this organism has been found previously to be difficult to Fe-limit in the laboratory (Brand et al. 1983). However, in all of the present experiments (as well as in all other previously reported trace metal-phytoplankton studies), EDTA was present in the culture medium (10 µM EDTA in our case), making a direct comparison with entirely natural seawater difficult. Not only does EDTA affect the speciation of trace metals, it may also interfere with naturally produced organic compounds that may serve as biological aids for metal acquisition. Results of an experiment conducted with both *Emiliania huxleyi* and an oceanic diatom in natural Stn P water without EDTA present will be reported elsewhere (Muggli & Harrison unpubl.). For the purposed of this study, then, we will focus our discussion on the possible effects of nitrogen source.

Under the experimental low Fe conditions (0.8 nmol kg^{-1}), ammonium-grown cells did not appear to have a physiological advantage over nitrate-grown cells. In fact, nitrate-grown cells had significantly higher growth rates than ammonium-grown cells under these conditions (Table 1). This would indicate that if fast cell division is ecologically favored and cells would acclimate to this, *Emiliania huxleyi* may be utilizing nitrate *in situ* to meet its nitrogen requirements. ^{15}N experiments conducted at Stn P have indicated that the small size fraction (<5.0 µm) is utilizing nitrate to some extent (30 to 50% of nitrogen require-

ment; D. Varela unpubl. data). Similarly, Price et al. (1994) in the equatorial Pacific (another HNLC region) found that nitrate was being utilized by the indigenous small phytoplankton. Attributing nitrate uptake to single species in the field is difficult, as mixed assemblages are always present. Furthermore, species assemblages can vary dramatically at a single locale, let alone in different regions of the ocean. However, these field data would indicate that there is a possibility that *E. huxleyi*, and possibly other small prymnesiophytes like them, are indeed utilizing nitrate *in situ*. The irradiance experiments also support the notion that *E. huxleyi* is utilizing nitrate *in situ*, as faster growth was found for nitrate-grown cells under low light, and ammonium-grown cells never grew faster than nitrate-grown cells (Fig. 4A).

Hence, results from our experiments would indicate that under normal, low Fe conditions in the subarctic Pacific, *Emiliania huxleyi* is probably utilizing nitrate as its primary N source. In the event of Fe addition to the system (via atmospheric deposition), our data does not indicate that one N source over the other is more beneficial to this organism. Assessing which N source is actually taken up under ecologically relevant concentrations in the laboratory is the next step in understanding the ecophysiology of this species at Stn P. Wells et al. (1995) point out the complete absence of laboratory studies on organisms from the field locations in question. Care must especially be taken when working with *E. huxleyi*, as there is much genetic variation between isolates (Brand 1982), and the origin of the isolate should be clearly identified in all reports. In addition, if truly ecological questions are to be asked, the culture conditions in the laboratory need to be carefully considered (e.g. omitting artificial chelators; see Muggli & Harrison unpubl.). Physiological laboratory studies on organisms from HNLC regions need to be conducted if we are to truly understand the ecology of these systems and how the individual organisms present would respond to perturbations to the system.

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