

# Nitrogen limitation of North Atlantic phytoplankton: analysis of physiological condition in nutrient enrichment experiments

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**ABSTRACT:** Nutrient enrichment experiments were conducted in May and June of 1993 at 8 stations along a North Atlantic transect, from Morocco to Nova Scotia, Canada. Variable fluorescence ( $F_v/F_m$ ) was measured in order to estimate the health or physiological state of the population as a whole. Low values across the transect indicated nutrient limited photosynthetic efficiency and probable growth rates ranging from 10 to about 50% of  $\mu_{max}$ . Where the lowest value was measured, over the Grand Banks of Newfoundland, Canada, nitrogen addition to incubated samples resulted in large, significant increases in photochemical efficiency. Numbers and cell-specific fluorescence of 3 major groups of picophytoplankton were studied using flow cytometry, in order to further quantify the physiological response to nutrient additions. Results indicated nitrogen limitation of physiology and/or abundance of small eukaryotes, cyanobacteria, and prochlorophytes. Abundance (cell numbers) and cellular fluorescence of the 3 groups responded differently to nutrient additions. Prochlorophytes showed the greatest response to incubation in terms of cell numbers, responding especially to nitrogen addition. By contrast, cyanobacterial numbers did not change from initial values or with treatment, although cell pigment content did. Cellular fluorescence as measured by the flow cytometer reflected cell pigment content in most experiments. Increased cellular fluorescence of all groups in nitrogen-amended treatments relative to unamended controls indicated physiological limitation by nitrogen.

**KEY WORDS:** Nitrogen limitation · Physiology · Fluorescence

## INTRODUCTION

Nutrient availability may affect phytoplankton productivity in 2 ways. First, fluxes of dissolved inorganic nutrients into, and organic nutrients out of, the euphotic zone may limit phytoplankton abundance (Dugdale & Wilkerson 1992). Second, the concentra-

tion of inorganic nutrients may limit phytoplankton growth rate (Dugdale & Goering 1967). The first type of limitation may be considered a modification of Liebig's Law of the Minimum while the second can be viewed as an extension of Blackman kinetics. The ability to distinguish between these 2 types of limitation has important implications for understanding oceanic productivity (Falkowski et al. 1992).

Diagnosis of nutrient limitation based on bioassay experiments is controversial and the application of concepts such as Liebig's Law is not straightforward (Cullen et al. 1992). For example, the absence of biomass accumulation following enrichment does not imply limitation of growth rate, due to complications arising from grazing and nutrient recycling (Goldman 1980). In addition, measurement of bulk parameters

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such as chlorophyll *a* (chl *a*) and cell numbers gives no information on physiological responses of a population, or on the responses of different taxa within an assemblage.

Interpretation of nutrient addition experiments and *in situ* measurements may be aided by recently achieved abilities to observe physiological changes independently of biomass changes using rapid fluorescence techniques (Greene et al. 1991, 1994), and to efficiently quantify the responses of different phytoplankton taxa, on a single-cell basis, using flow cytometry (Legendre & Yentsch 1989, DiTullio et al. 1993). Pump-and-probe and FRR (fast repetition rate) fluorometry provide measurements of variable fluorescence yield arising from photosystem II (Kolber et al. 1988, Kolber & Falkowski 1993). Several studies have established that low variable fluorescence may be equated to low photochemical efficiency (Kolber & Falkowski 1993). Variable fluorescence is directly related to the nutritional status of the cell (Kolber et al. 1988), although it may be affected by photoinhibition and photoadaptation (Geider et al. 1993a). A major advantage of this technique is that variable fluorescence has a physiological maximum achieved under nutrient-replete conditions, independent of the taxon under investigation, and therefore gives an absolute rather than a relative measure of photosynthetic efficiency. A disadvantage is that it provides a bulk population measurement, and does not distinguish among sub-populations which may respond differently to environmental conditions. Flow cytometry allows distinction amongst major taxonomic groups, based on size and fluorescence characteristics of individual cells (Legendre & Yentsch 1989). This allows observation of both physiological (cell pigment content) and biomass (cell number) changes within several major taxa during incubation experiments.

Here we describe 8 experiments conducted across the North Atlantic, in which pump-and-probe fluorometry and flow cytometry were used to infer the relative physiological state or health of *in situ* populations, and to observe changes in cell physiology and total biomass in response to 8 different nutrient addition treatments. The major objectives were: (1) to directly measure physiological responses, as well as changes in cell

numbers, of the phytoplankton to nutrient additions; (2) to separately quantify responses of the major taxonomic groups present; and (3) based on bioassay experiments and sampling of the water column, to infer *in situ* the physiological state of the North Atlantic phytoplankton populations. Our results indicate physiological limitation by nitrogen across much of the North Atlantic ocean.

## METHODS

Eight stations were sampled in May and June of 1993 for nutrient addition experiments (Fig. 1). A balanced design was used to examine the effects of nitrogen, phosphorus, and iron (N, P, and Fe) alone and in

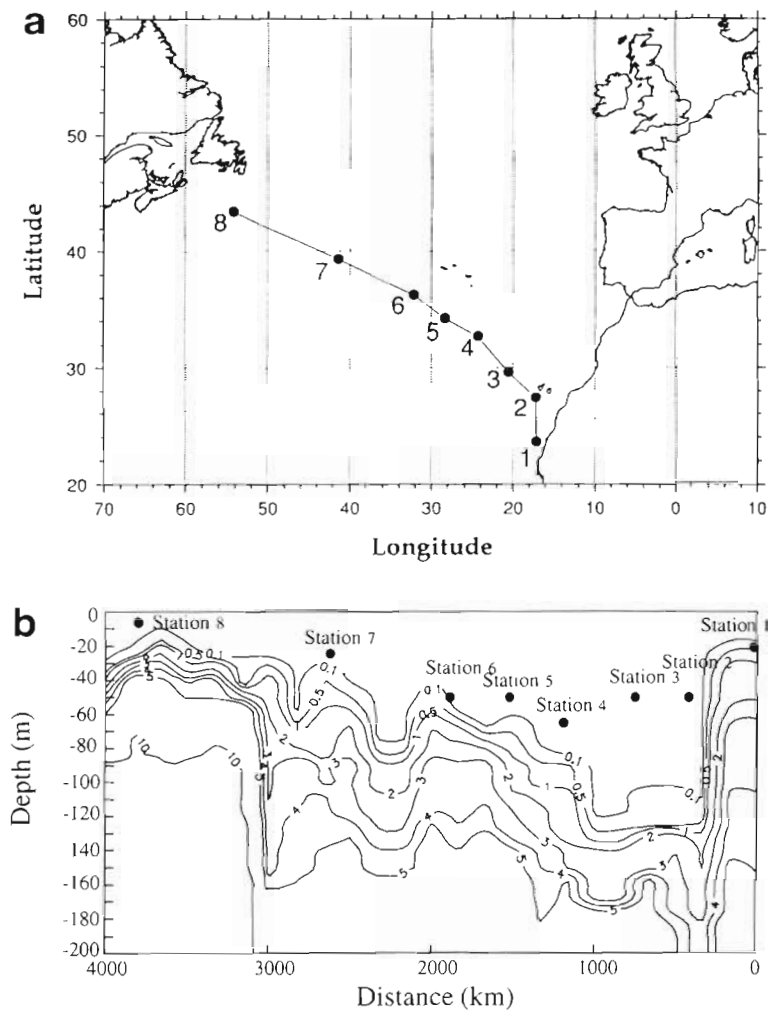


Fig. 1 (a) Cruise track of the CSS 'Hudson' May 21 to June 7, 1993, showing locations of stations where nutrient addition experiments were performed. (b) Nitrate contour plot, with stations indicated. Nitrate measurements courtesy W. G. Harrison and B. Irwin

combination. To this end 8 treatments were employed, each in duplicate: additions of (1) N alone; (2) P alone; (3) Fe alone; (4) N and P together; (5) N and Fe together; (6) P and Fe together; (7) N, P, and Fe; and (8) a control treatment with no additions, to distinguish enclosure effects from effects of treatment. At each station, water was collected with two 10 l Go-Flo bottles (Wu & Luther 1994) on Kevlar line at approximately 10:00 h local time, dispensed into trace-metal clean 500 ml polypropylene bottles, and enriched in 1 or more nutrients. Exposure to surface irradiance was avoided during collection and all subsequent steps.

The trace-metal cleaning procedure for Go-Flo and polypropylene bottles involved a detergent wash followed by 50% nitric acid, followed by successive washes in decreasing HCl concentrations (50, 25, 10 and 5%), and a final acid-soak in Ultima trace-metal clean HCl. Polypropylene bottles were chosen because they are 100% translucent to light and are the cleanest plastic containers available, routinely used by trace-metal chemists for trace-element analyses.

Samples were incubated in flowing seawater on-deck incubators with light attenuated to either 4 or 10% of incident photosynthetically active radiation by blue plexiglass screening to approximate the *in situ* spectral quality. Incubations were carried out from approximately noon to noon to minimize potential diel variations in  $F_v/F_m$  and fluorescence yield. Nitrogen, phosphorus, and/or iron were added alone or in combination to increase nutrient concentrations by 1 to 5  $\mu\text{M}$  N, 0.05 to 0.5  $\mu\text{M}$  P, and/or 2 or 4 nM Fe. Table 1 summarizes the conditions of each experiment. A light meter was not available to measure irradiance at sam-

ple depths, so this has been calculated (Table 1). Nitrate (100 mM), ammonium (100 mM), and phosphate (10 mM) stock solutions contained less than 5 nM Fe after treatment with chelex resin. Since these were added to seawater in dilutions of about 1 to 100000, trace metal additions were negligible. Iron stock solutions (10  $\mu\text{M}$   $\text{FeCl}_3$ ) were prepared in 0.001 M HCl. Samples were dispensed and nutrient additions performed in a class 100 clean bench.

**Initial and final sampling.** Duplicate 100 ml samples were collected onto GF/F filters, extracted for 24 h at  $-20^\circ\text{C}$  in 90% acetone, and chlorophyll determined fluorometrically using a Turner Designs fluorometer calibrated against pure chl *a* (Strickland & Parsons 1972). Samples for pump-and-probe fluorescence measurements were dark-adapted for at least 30 min and then concentrated onto GF/F filters at low light ( $<50 \mu\text{E m}^{-2} \text{s}^{-1}$ ) under gentle vacuum filtration ( $<10 \text{ mm Hg}$ ) (Olaizola & Yamamoto 1994). Concentration was necessitated by the extremely low biomass. Filters were trimmed to fit into a 1 cm cuvette which contained a small amount of filtered seawater and measurements were generally completed within 2 min of filtration. Initial fluorescence of dark-adapted sample ( $F_0$ ), and maximum fluorescence following a saturating light flash ( $F_m$ ), were determined with a custom built pump-and-probe fluorometer (Kolber et al. 1988). Standard deviations for averages of 10 data points ( $F_0$  or  $F_m$ ) varied between 0.2 and 8.0% for all data collected, with the majority of cases being  $<4\%$ . A comparison of filtered sample with a cell suspension at a high biomass station gave the same average  $F_v/F_m$  (data not shown).

Table 1 Nutrient addition experiments. Eight treatments in duplicate were incubated in each case, using either no addition (control, = C) or phosphate, nitrate + ammonium, and iron singly or in combination (N, P, Fe, NP, NFe, PFe, All). Water column nutrient concentrations were measured by automated colorimetry within 1 h of sample collection

Stn	Hours	Collection depth (m)	% surface irradiance <sup>a</sup>	% incubation irradiance	Additions			Ambient concentrations <sup>c</sup>		
					N <sup>b</sup> ( $\mu\text{M}$ )	$\text{PO}_4^{3-}$ ( $\mu\text{M}$ )	Fe (nM)	Chl ( $\mu\text{g l}^{-1}$ )	$\text{SiO}_2$ ( $\mu\text{M}$ )	$\text{PO}_4^{3-}$ ( $\mu\text{M}$ )
1	48	20	39	10	5.0	0.5	4.0	0.11	0.93	0.01
2	24	50	10	10	5.0	0.5	4.0	0.10	0.22	0.05
3	24	50	22	4	5.0	0.5	4.0	0.04	0.73	0.04
4	48	65	8	4	2.0	0.1	2.0	0.07	0.50	0.01
5	48	50	16	4	2.0	0.1	4.0	0.06	0.85	0.08
6	48	50	16	4	1.0	0.05	2.0	0.06	0.36	0.10
7	24	25	28	10	2.0	0.1	4.0	0.13	0.15	0.02
8	24	5	62	10	5.0	0.5	4.0	0.59	0.30	0.54

<sup>a</sup> Calculated using the chlorophyll concentration and Morel's (1987) Eq. (5) [ $K_{\text{PAR}}(0, Z_e) = 0.121C^{0.428}$ , where  $K_{\text{PAR}}$  is the mean attenuation coefficient for the euphotic zone of depth  $Z_e$ , and  $C$  is the mean pigment concentration]. The fraction of surface downwelling irradiance was estimated by applying Smith & Baker's (1978) Eq. (2) relating surface irradiance,  $E(0)$ , to irradiance at depth [ $E(Z) = E(0)e^{-(KTZ)}$ , where  $KT = K_{\text{PAR}}$ ]

<sup>b</sup> N additions were in the form of equal parts nitrate and ammonium

<sup>c</sup>  $\text{NO}_3^-$  concentration was 0.08  $\mu\text{M}$  at Stn 1, but was below the detection limit of 0.05  $\mu\text{M}$  at all other stations



Flow cytometry analyses were completed within 3 h of sampling using a FACSort (Becton Dickinson, New Jersey, USA) with a sample volume of 0.25 to 0.5 ml. Abundances of 4 groups of ultraplankton were distinguished based on size (forward angle light scatter,  $<1$  to  $10^\circ$ ), relative chl *a* ( $>650$  nm, = 'red') fluorescence intensity, and phycoerythrin ( $585 \pm 21$  nm, = 'orange') fluorescence (Li 1994, 1995). Particles with red fluorescence weaker than emitted by *Prochlorococcus* sp. were rejected, to insure that only phytoplankton were counted. Forward light scatter was recorded in relative units based on measurements of beads of different sizes and composition (Polysciences #18859, 18860, 18604, 18861, 18862; Duke Scientific #R600, R0100, R0290, 268, 361; Coulter fluorosphere 5.43  $\mu\text{m}$ , 8.55  $\mu\text{m}$ , and 9.64  $\mu\text{m}$ ). The range of equivalent spherical diameter was from 0.44 to 6.8  $\mu\text{m}$ . The groups distinguished were prochlorophytes (*Prochlorococcus* spp.), chroococcoid cyanobacteria, and eukaryotes showing low or high intensity light scatter (referred to as small and large euks). The small euks were shown to pass through a 1  $\mu\text{m}$  polycarbonate membrane while the large euks were retained, although most passed through a 2  $\mu\text{m}$  membrane (Li 1994). Values are

reported as  $\text{cells ml}^{-1}$  and red (chl) or orange (PE) fluorescence  $\text{cell}^{-1}$ . Fluorescence  $\text{ml}^{-1}$  was derived from the product of fluorescence  $\text{cell}^{-1} \times \text{cells ml}^{-1}$  expressed in relative fluorescence units.

## RESULTS

### General characteristics of the transect and stations

The 8 stations sampled over the east-west transect are shown in Fig. 1a, and a nitrate contour plot is given in Fig. 1b. The depth of the nitricline decreased from east to west and reflected the presence of 2 water masses in the North Atlantic, apparent from hydrographic data: the eastern, North Atlantic Gyre-dominated basin and the Gulf Stream-dominated western basin (Olaizola et al. 1996). Stn 1 was taken in a nearshore, upwelling area while Stn 8 was taken in relatively eutrophic water near the Grand Banks of Newfoundland, Canada. Stns 1 and 8 therefore represent endpoints of higher nitrate concentration with extensive oligotrophic areas in between. Whereas most of the transect was numerically dominated by the

small prochlorophytes, Stn 1 was dominated by cyanobacteria, and prochlorophytes were absent at Stn 8. Large eukaryotic cells (retained by 1  $\mu\text{m}$  polycarbonate membrane) were abundant only at the last station. At all but the last station, the flow cytometer was optimized for picoplankton so that the weak fluorescence and light scatter from prochlorophyte cells could be detected. Fluorescence of large cells accumulated at the high end of the scale and could not be quantified. In the absence of prochlorophytes at Stn 8, these settings were changed to allow quantification of larger eukaryotic cells. Stn 6 exemplified the prochlorophyte-dominated, low-nitrate region of the transect, and data from this station are presented and discussed as being representative of the bulk of the transect. Complete data from all 8 stations are given in Tables 1 through 8.

To assess the variability of pump-and-probe measurements and the possibility of diel variability in  $F_v/F_m$  and chl *a* concentrations, a 20 l carboy was filled and incubated in constant, dim light ( $<50 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and

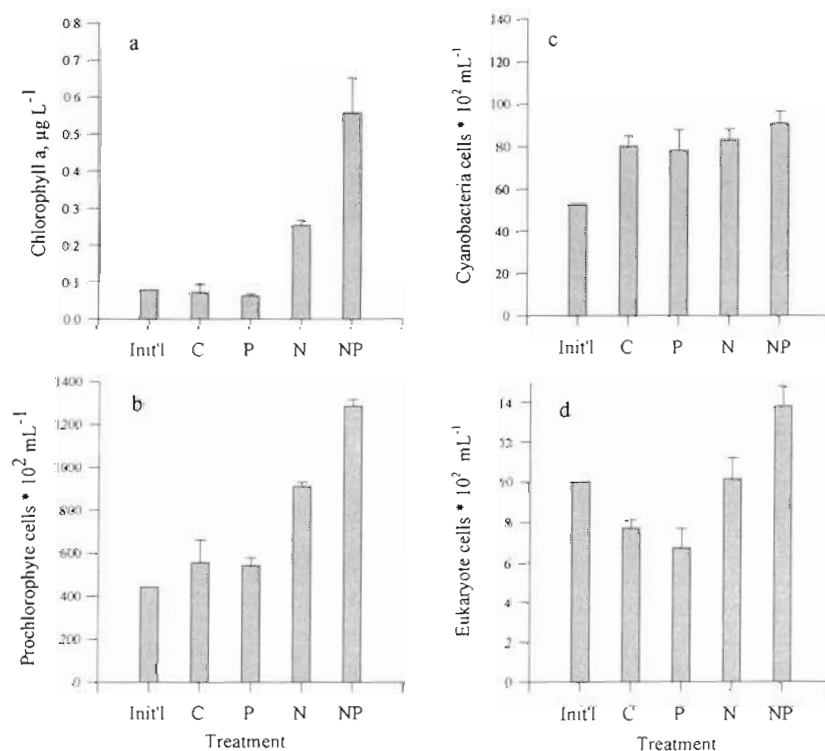


Fig. 2. Chlorophyll concentration and cell numbers from Stn 6. (a) Chl *a*; (b) cells  $\text{ml}^{-1} \times 10^2$  of prochlorophytes; (c) cyanobacteria; and (d) eukaryotes; note change of scale. Mean  $\pm 1$  SE of 4 values. C: control and iron addition treatments; P: phosphate and phosphate plus iron additions; N: nitrogen and nitrogen plus iron additions; NP: nitrogen plus phosphate and nitrogen plus phosphate plus iron additions

Table 2. Chlorophyll concentration ( $\mu\text{g chl l}^{-1}$ ), mean  $\pm$  1 SD of 4 values. Treatments: control (C); plus phosphorus (P); plus nitrogen (N); and plus nitrogen and phosphorus (NP). Note that the original 8 treatments in duplicate are here described as 4 in quadruplicate, since iron additions had no effect

Stn	Initial	Treatment			
		C	P	N	NP
1	-	0.07 $\pm$ 0.013	0.05 $\pm$ 0.006	0.18 $\pm$ 0.017	0.40 $\pm$ 0.123
2	0.10	0.07 $\pm$ 0.008	0.07 $\pm$ 0.005	0.17 $\pm$ 0.025	0.25 $\pm$ 0.022
3	0.04	0.03 $\pm$ 0.000	0.03 $\pm$ 0.005	0.02 $\pm$ 0.005	0.02 $\pm$ 0.005
4	0.07	0.05 $\pm$ 0.006	0.06 $\pm$ 0.009	0.09 $\pm$ 0.008	0.15 $\pm$ 0.040
5	0.06	0.08 $\pm$ 0.026	0.06 $\pm$ 0.023	0.11 $\pm$ 0.005	0.11 $\pm$ 0.031
6	0.06	0.07 $\pm$ 0.022	0.06 $\pm$ 0.009	0.25 $\pm$ 0.013	0.55 $\pm$ 0.108
7	0.13	0.11 $\pm$ 0.024	0.08 $\pm$ 0.022	0.16 $\pm$ 0.021	0.20 $\pm$ 0.022
8	0.59	0.45 $\pm$ 0.102	0.37 $\pm$ 0.064	0.63 $\pm$ 0.046	0.60 $\pm$ 0.036

chl *a* and  $F_v/F_m$  monitored over 24 h. There was good replication of filtered subsamples at a single time point (mean 0.07  $\pm$  0.00  $\mu\text{g l}^{-1}$  and 0.53  $\pm$  0.01 for chlorophyll and  $F_v/F_m$ , respectively;  $n = 5$ ) and no change over 24 h (mean 0.072  $\pm$  0.004  $\mu\text{g l}^{-1}$  and 0.51  $\pm$  0.02 for chlorophyll and  $F_v/F_m$ ;  $n = 7$ ).

Iron addition had no significant effect on any parameter measured, at any of the 8 stations ( $p < 0.05$ ). Results from the iron addition treatments were therefore used as additional replicates for other treatments; these are C (= control and +Fe), +P (= +P and +PFe), +N (= +N and +NFe), and +NP (= +NP and +NPFe; see Table 2).

### Chlorophyll, cell abundance, and *in vivo* fluorescence: Stn 6

#### Chlorophyll and cell abundance

Chl *a* concentration in control and P addition treatments did not differ from the initial value (Fig. 2a, Table 2), but increased 4-fold with N addition and nearly 10-fold with addition of both N and P in this experiment. The response of cell numbers to treatment (Fig. 2b–d, Table 3) was much smaller. The numerically dominant prochlorophytes doubled in number with N addition and tripled with NP addition. Cyanobacterial numbers increased by about 50% over the initial value regardless of treatment, while eukaryotic cells responded positively only to a combined N and P addition.

#### *In vivo* chlorophyll fluorescence

Fig. 3 shows relative fluorescence due to chlorophyll (red fluorescence) per ml of sample, and the relative contributions of the 3 taxa, for Stn 6. Total fluorescence per ml covaried with bulk chlorophyll, giving an  $r^2$  of 0.91 (Fig. 4). Despite their 1.5 to 2 orders of magnitude lesser abundance than prochlorophytes, the much larger eukaryotic cells contributed an equal amount to the total fluorescence signal. As with chlorophyll, fluorescence per ml did not covary with cell numbers. This is especially noticeable for cyanobacteria, whose contribution to bulk fluorescence increased by nearly 10 times with N addition and by 20 times with NP addition, although cell numbers changed very little and did not differ between N and NP treatments (see Figs. 2c & 3).

Table 3. Cell numbers ( $\times 10^2 \text{ ml}^{-1}$ ) for 8 experiments; Pro: prochlorophytes; Cyan: cyanobacteria; Euk: eukaryotes  $\leq 2 \mu\text{m}$  diameter. Mean  $\pm$  1 SD of 4 values. Treatments as in Table 2

Stn	Initial	Treatment				
		C	P	N	NP	
1	Pro	-	69 $\pm$ 16	52 $\pm$ 21	38 $\pm$ 10	79 $\pm$ 40
	Cyan	-	1390 $\pm$ 200	1000 $\pm$ 140	1420 $\pm$ 240	2350 $\pm$ 410
	Euk	-	85 $\pm$ 9	65 $\pm$ 14	23 $\pm$ 6	179 $\pm$ 45
2	Pro	800	460 $\pm$ 300	570 $\pm$ 100	800 $\pm$ 110	880 $\pm$ 120
	Cyan	74	79 $\pm$ 4	76 $\pm$ 3	76 $\pm$ 3	77 $\pm$ 1
	Euk	27	9 $\pm$ 0.5	10 $\pm$ 0.6	12 $\pm$ 1	13 $\pm$ 1
3	Pro	130	480 $\pm$ 13	420 $\pm$ 87	210 $\pm$ 56	170 $\pm$ 13
	Cyan	40	45 $\pm$ 2	41 $\pm$ 3	39 $\pm$ 3	36 $\pm$ 1
	Euk	8	5 $\pm$ 0.5	4 $\pm$ 0.8	5 $\pm$ 0.6	4 $\pm$ 0.5
4	Pro	620	770 $\pm$ 120	820 $\pm$ 190	790 $\pm$ 15	720 $\pm$ 92
	Cyan	60	73 $\pm$ 2	72 $\pm$ 5	67 $\pm$ 2	72 $\pm$ 6
	Euk	10	6 $\pm$ 1.5	8 $\pm$ 0.3	7 $\pm$ 1	7 $\pm$ 2
5	Pro	480	1250 $\pm$ 190	1060 $\pm$ 510	1110 $\pm$ 60	980 $\pm$ 260
	Cyan	25	39 $\pm$ 15	32 $\pm$ 32	39 $\pm$ 3	31 $\pm$ 9
	Euk	15	14 $\pm$ 0.7	11 $\pm$ 5	14 $\pm$ 0.4	12 $\pm$ 4
6	Pro	440	580 $\pm$ 100	540 $\pm$ 35	910 $\pm$ 19	1290 $\pm$ 31
	Cyan	53	80 $\pm$ 5	78 $\pm$ 10	83 $\pm$ 5	91 $\pm$ 5
	Euk	10	8 $\pm$ 0.4	7 $\pm$ 1	10 $\pm$ 1	14 $\pm$ 1
7	Pro	110	170 $\pm$ 12	180 $\pm$ 18	150 $\pm$ 3	160 $\pm$ 4
	Cyan	290	350 $\pm$ 5	330 $\pm$ 12	320 $\pm$ 12	340 $\pm$ 9
	Euk	30	25 $\pm$ 1	25 $\pm$ 1	26 $\pm$ 2	28 $\pm$ 0.7
8	Cyan	270	370 $\pm$ 12	370 $\pm$ 6	360 $\pm$ 14	350 $\pm$ 20
	Euk	85	96 $\pm$ 5	96 $\pm$ 6	110 $\pm$ 4	120 $\pm$ 7
	Euk (>2 $\mu\text{m}$ )	20	22 $\pm$ 1	20 $\pm$ 1	21 $\pm$ 1	22 $\pm$ 1

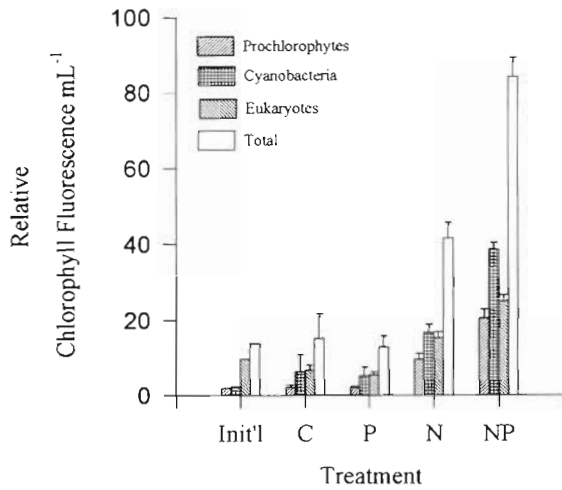


Fig. 3. Relative red (chlorophyll) fluorescence per ml due to prochlorophytes, cyanobacteria, eukaryotes, and in total, Stn 6. Fluorescence is in arbitrary units. Treatments as in Fig. 2

Fluorescence per cell

Since total chlorophyll concentration varied independently of cell numbers, the chlorophyll content of cells must necessarily have changed. In fact the variation in fluorescence per cyanobacterial cell, numbers of which changed very little, could be used to explain most of the variation ( $r^2 = 0.995$ , slope = 0.96) in bulk chlorophyll concentration at Stn 6 (Fig. 5a, d). Cellular red fluorescence of cyanobacteria responded much more dramatically to N additions than did fluorescence of prochlorophytes or eukaryotes (Fig 5b, c).

The eukaryotic phytoplankton were characterized by cellular chlorophyll fluorescence values 100 to 200 times greater than those measured in prochlorophytes (Table 4, Fig. 5b, c). Therefore, despite their low abundance these picoplankton were an important component of the red fluorescence signal and by inference of the chlorophyll standing stock. The smaller magnitude of cellular fluorescence response in the eukaryotes than in either prokaryotic taxon may reflect a longer generation time of these cells, or more rapid response of the prokaryotes to increased nutrient availability.

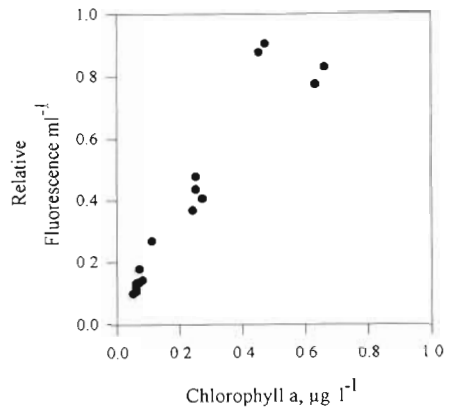


Fig. 4. Total relative chlorophyll fluorescence per ml versus chlorophyll concentration for the initial sample and final samples from 16 bottles, Stn 6. Fluorescence is in arbitrary units. Linear regression analysis gives  $r^2 = 0.91$

Overall, as exemplified for Stn 6, the cyanobacteria exhibited the greatest physiological response to N addition by accumulation of photosynthetic pigments (Tables 4 & 5), but were unable to increase in numbers. The prochlorophytes were also able to increase their pigment content when N was added, although to a lesser extent. These cells were also able to increase in

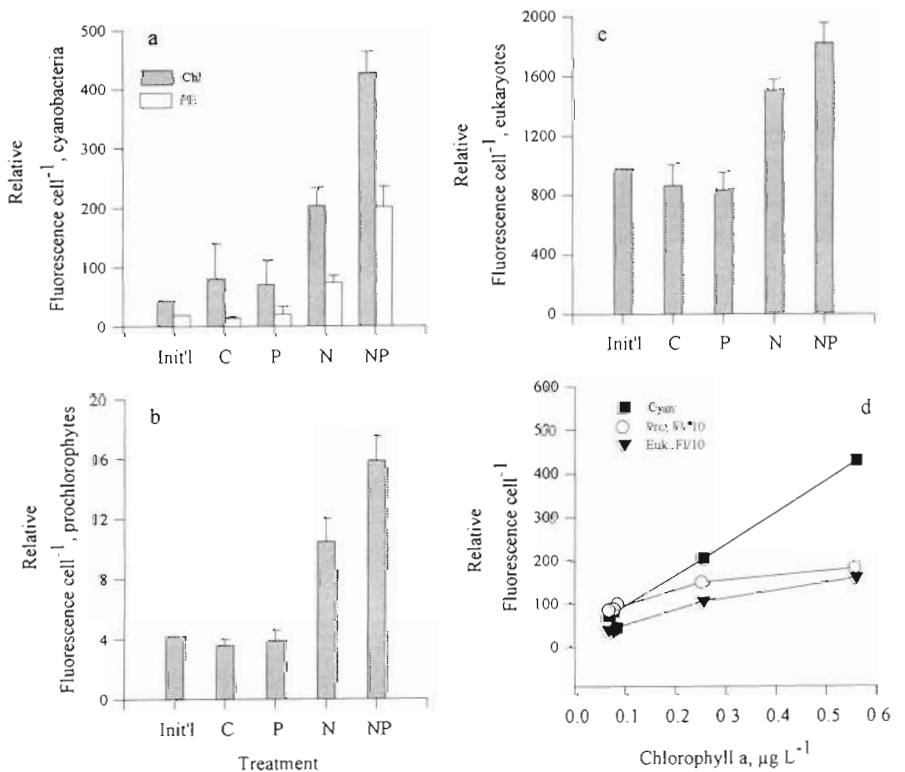


Fig. 5. Relative red (chlorophyll) and orange (phycoerythrin, PE) fluorescence per cell for (a) cyanobacteria, (b) prochlorophytes, and (c) eukaryotes, Stn 6. Treatments as in Fig. 2. (d) Chlorophyll fluorescence per cell for the same 3 groups, plotted against chl a. Fluorescence in arbitrary units

Table 4. Chl *a* (red,  $\lambda > 630$  nm) fluorescence cell<sup>-1</sup> in relative fluorescence units for prochlorophytes, cyanobacteria, and eukaryotes. Mean  $\pm$  1 SD of 4 values. Treatments as in Table 2

Stn	Initial	Treatment				
		C	P	N	NP	
1	Pro	–	6 $\pm$ 0.8	6 $\pm$ 0.8	13 $\pm$ 0.5	13 $\pm$ 0.6
	Cyan	–	33 $\pm$ 15	30 $\pm$ 13	170 $\pm$ 10	220 $\pm$ 21
	Euk	–	276 $\pm$ 14	256 $\pm$ 15	827 $\pm$ 110	489 $\pm$ 20
2	Pro	4	3 $\pm$ 0.05	3 $\pm$ 0.3	4 $\pm$ 0.3	4 $\pm$ 0.2
	Cyan	25	50 $\pm$ 21	45 $\pm$ 2	125 $\pm$ 20	158 $\pm$ 21
	Euk	696	490 $\pm$ 44	517 $\pm$ 28	883 $\pm$ 49	943 $\pm$ 97
3	Pro	4	6 $\pm$ 0.3	5 $\pm$ 0.3	4 $\pm$ 0.1	4 $\pm$ 0.1
	Cyan	22	118 $\pm$ 19	120 $\pm$ 10	114 $\pm$ 8	115 $\pm$ 5
	Euk	1075	633 $\pm$ 57	622 $\pm$ 50	510 $\pm$ 71	512 $\pm$ 81
4	Pro	4	4 $\pm$ 0.4	4 $\pm$ 0.3	6 $\pm$ 0.1	7 $\pm$ 0.3
	Cyan	30	96 $\pm$ 40	133 $\pm$ 96	221 $\pm$ 78	344 $\pm$ 17
	Euk	1110	710 $\pm$ 99	861 $\pm$ 114	836 $\pm$ 65	1134 $\pm$ 186
5	Pro	5	7 $\pm$ 4	8 $\pm$ 3	12 $\pm$ 0.6	14 $\pm$ 0.3
	Cyan	39	153 $\pm$ 74	184 $\pm$ 58	317 $\pm$ 13	392 $\pm$ 14
	Euk	974	1230 $\pm$ 129	1270 $\pm$ 161	1420 $\pm$ 69	1620 $\pm$ 106
6	Pro	4	4 $\pm$ 0.4	4 $\pm$ 0.7	11 $\pm$ 2	16 $\pm$ 2
	Cyan	43	80 $\pm$ 60	70 $\pm$ 41	203 $\pm$ 31	427 $\pm$ 37
	Euk	974	864 $\pm$ 142	835 $\pm$ 119	1500 $\pm$ 72	1820 $\pm$ 135
7	Pro	6	6 $\pm$ 0.3	6 $\pm$ 0.7	9 $\pm$ 0.3	11 $\pm$ 0.5
	Cyan	37	51 $\pm$ 18	52 $\pm$ 6	82 $\pm$ 4	137 $\pm$ 4
	Euk	701	577 $\pm$ 86	498 $\pm$ 37	752 $\pm$ 66	951 $\pm$ 41
8	Cyan	32	30 $\pm$ 2	32 $\pm$ 6	49 $\pm$ 2	46 $\pm$ 1
	Euk	284	284 $\pm$ 11	272 $\pm$ 29	364 $\pm$ 18	355 $\pm$ 10
	Euk (>2 $\mu$ m)	6640	5020 $\pm$ 82	4740 $\pm$ 624	6180 $\pm$ 520	5970 $\pm$ 293

abundance by as much as 3-fold. The small eukaryotic cells showed a mixed response but appeared less able than the prokaryotes to take advantage of nutrient additions.

#### Photochemical efficiency

The ratio of variable to maximum fluorescence ( $F_v/F_m$ ) varied little throughout most of the transect and with nutrient additions (Table 6). Initial values ranged from 0.42 to 0.45, except at Stn 8 where the lowest value, 0.27, was observed. Values for each treatment from Stns 6 and 8 are shown in Fig. 6. In most experiments an increase over initial values occurred in all bottles regardless of treatment, while slightly higher values were measured in plus N treatments. These differences were not significant at  $p < 0.05$ , however. The exception to this was Stn 8, where N addition resulted in significantly higher values than those measured either initially or in the control treatment.

## DISCUSSION

### Measurement of red fluorescence as a substitute for chl *a*

Phytoplankton populations throughout the bulk of the transect were numerically dominated by prochlorophytes, with cyanobacteria being less abundant and relatively low numbers of the mixed pico-eukaryote assemblage present. In our use of red fluorescence signals as an analog for chlorophyll, we have assumed that summed total red fluorescence included most of the phytoplankton present, and that larger cells were not major contributors to bulk chlorophyll. This assumption is supported by the regression analyses of summed red fluorescence on bulk chlorophyll (Table 7), with  $r^2$  values ranging from 0.59 to 0.97, although in one case (Stn 3,  $r^2 = 0.41$ ) the slope was not significantly different from zero. Veldhuis et al. (1996) noted a similar linear relationship between flow cytometer fluorescence measurements and total chlorophyll in Indian Ocean picoplankton communities. Along this North Atlantic transect, side scatter versus red fluorescence plots (4 decade scale) showed nearly identical results before and after filtration through a 2  $\mu$ m PC membrane (Li 1994), with only the rare cells of greater than 200 units side scatter being retained on the filter. In addition, summed <sup>14</sup>C uptake per ml from sorted samples accounted for >90% of the total <sup>14</sup>C uptake in unsorted samples (Li 1994), although it

Table 5. Phycoerythrin (orange,  $\lambda = 564$  to 606 nm) fluorescence cell<sup>-1</sup> in relative fluorescence units for cyanobacteria. Mean  $\pm$  1 SD of 2 replicates. Treatments as in Table 2

Stn	Initial	Treatment			
		C	P	N	NP
1	–	45 $\pm$ 24	44 $\pm$ 24	274 $\pm$ 32	312 $\pm$ 42
2	12	28 $\pm$ 18	16 $\pm$ 3	51 $\pm$ 6	59 $\pm$ 9
3	7	44 $\pm$ 8	34 $\pm$ 8	42 $\pm$ 5	34 $\pm$ 2
4	14	49 $\pm$ 27	62 $\pm$ 63	115 $\pm$ 4	171 $\pm$ 14
5	18	11 $\pm$ 42	79 $\pm$ 35	148 $\pm$ 13	193 $\pm$ 18
6	19	29 $\pm$ 29	20 $\pm$ 16	73 $\pm$ 14	203 $\pm$ 37
7	9	11 $\pm$ 5	9 $\pm$ 2	18 $\pm$ 2	32 $\pm$ 4
8	24	24 $\pm$ 2	24 $\pm$ 4	38 $\pm$ 2	36 $\pm$ 1



Table 6.  $F_v/F_m$  (ratio of variable fluorescence to maximum fluorescence yield). Mean  $\pm$  1 SD of 4 values. Treatments as in Table 2

Stn	Initial	Treatment			
		C	P	N	NP
1	-	0.43 $\pm$ 0.04	0.39 $\pm$ 0.02	0.48 $\pm$ 0.01	0.48 $\pm$ 0.04
2	0.48 $\pm$ 0.03	0.44 $\pm$ 0.03	0.36 $\pm$ 0.04	0.35 $\pm$ 0.09	0.45 $\pm$ 0.04
3	0.44 $\pm$ 0.03	0.49 $\pm$ 0.02	0.52 $\pm$ 0.02	0.43 $\pm$ 0.04	0.45 $\pm$ 0.03
4	0.47 $\pm$ 0.01	0.47 $\pm$ 0.02	0.49 $\pm$ 0.01	0.50 $\pm$ 0.02	0.50 $\pm$ 0.02
5	0.44 $\pm$ 0.02	0.51 $\pm$ 0.02	0.51 $\pm$ 0.03	0.51 $\pm$ 0.02	0.52 $\pm$ 0.03
6	0.42 $\pm$ 0.01	0.44 $\pm$ 0.04	0.45 $\pm$ 0.02	0.51 $\pm$ 0.03	0.50 $\pm$ 0.03
7	0.44 $\pm$ 0.01	0.46 $\pm$ 0.03	0.46 $\pm$ 0.02	0.48 $\pm$ 0.02	0.48 $\pm$ 0.03
8	0.27 $\pm$ 0.02	0.32 $\pm$ 0.03	0.34 $\pm$ 0.06	0.45 $\pm$ 0.04	0.44 $\pm$ 0.03

was pointed out that large, rare cells may be under-represented in small volume samples. At Stn 8 ( $r^2 = 0.59$ ) chlorophyll fluorescence was almost certainly underestimated, due to the presence of larger cells. With the exception of Stn 8, red fluorescence measurements of sorted samples therefore accounted for most of the chlorophyll present in our experiments.

Changes in cyanobacterial red fluorescence were highly correlated with changes in orange (PE) fluorescence. This may be due in part to tailing of the phycoerythrin fluorescence peak into the red region, which is not compensated for such spillover, such that part of the red fluorescence signal displayed by cyanobacteria

is attributable to phycoerythrin. The overlap in emission peaks of these pigments is small (see for example Yentsch & Phinney 1990, Fig. 5), so the percent contribution of PE fluorescence to total fluorescence above 650 nm depends on the relative magnitude of the 2 peaks, which is very difficult to determine by flow cytometry. Based on fluorescence emission spectra from several PE-containing species (Yentsch & Phinney 1990, D. A. Phinney & T. Cucci unpubl.), it is estimated that between 5 and 25% of fluorescence over 650 nm may be

attributed to PE, with stationary phase DC2 having the lowest relative chlorophyll fluorescence. Kana et al. (1992) noted that fluorescence emission spectra of 3 strains of *Synechococcus* sp. in log phase showed distinct PE and chlorophyll peaks of about equal magnitude. The contribution of PE to red fluorescence in that case would be small (less than 5%). Red fluorescence values in the present study are therefore contaminated by PE fluorescence to an unknown extent, but within the bounds estimated above. It is probable that for mixed oceanic populations, the contribution of PE to the red fluorescence measured is at the low end of the range, closer to 10 than to 25%.

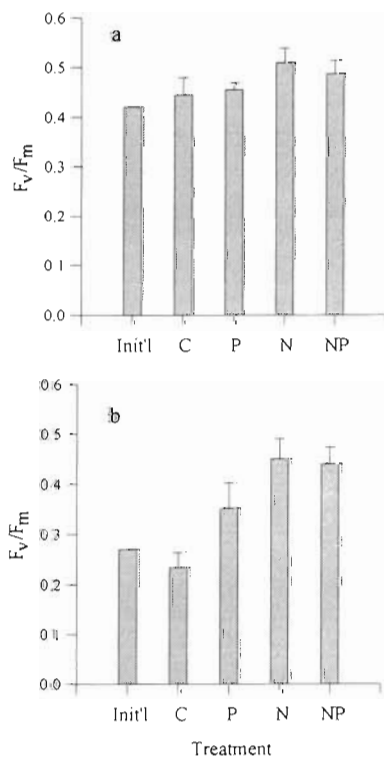


Fig. 6.  $F_v/F_m$  for (a) Stn 6 and (b) Stn 8. Treatments as in Fig. 2

#### Physiological and ecological components of pigment increase

Observed changes in cellular red fluorescence and cell abundance can be used to infer the nutrient status of the prochlorophytes, cyanobacteria, and eukaryotic picoplankton. Since cellular fluorescence provides a relative rather than absolute index of nutrient status, initial observations revealing the *in situ* conditions were compared with observations on control and nutrient amended samples. In 6 of the 8 experiments discussed here, total cellular fluorescence ( $F_I$ ) covaried significantly with chlorophyll (Table 7), while cell numbers did not. Therefore changes in fluorescence may be interpreted as indicative of changes in cellular chlorophyll content. In general, then, chlorophyll concentration changed in response to physiological effects of treatment rather than with changing cell abundance. This was especially true for cyanobacteria which showed the largest response to incubation with added nitrogen, while cell numbers changed relatively little.

Phycoerythrin (PE), here measured as an 'orange' fluorescence signal and covarying with chlorophyll fluorescence, is the major light-harvesting pigment of



Table 7. Comparison of  $F_0$ ,  $F_I$  (total cellular fluorescence), and chl  $a$  concentration by Model I linear regression analysis

Stn	$F_I$ vs $F_0$ $r^2$	$F_I$ vs chl $a$ $r^2$	$F_0$ vs chl $a$ $r^2$
1	0.88	0.97	0.94
2	0.78	0.71	0.72
3	0.05*	0.41*	0.68
4	0.26*	0.79	0.44*
5	0.77	0.96	0.85
6	0.93	0.91	0.86
7	0.74	0.82	0.73
8	0.01*	0.59	0.002*

\*Slope not significantly different from zero

cyanobacteria. Repressed synthesis and/or active degradation of cyanobacterial phycobilisomes under nitrogen starvation has been described by many authors (e.g. Collier & Grossman 1992). In the present study cellular PE fluorescence responded to N addition at all but Stn 3, as did chlorophyll fluorescence. In several experiments both PE and chlorophyll fluorescence increased in control treatments (Tables 4 & 5), and we suspect this may have been a response to low light. At most stations, the irradiance used for on-deck incubations was lower than at the depth from which samples were taken, perhaps leading to active pigment synthesis as a consequence of photoadaptation to low light. The greater ability of cells, when supplied with additional N, to compensate for decreased irradiance by increasing pigment content would further indicate *in situ* nitrogen limitation. It should be noted, however, that the change in PE fluorescence (initial to final values, all treatments) did not covary with change in irradiance (from collection to incubation depth)

### Nutrient limitation and fluorescence parameters

$F_v/F_m$  is calculated from 2 measurements: the minimum fluorescence ( $F_0$ ) from dark-adapted cells with all functional reaction centers 'open' ( $Q_a$  is oxidized), and the maximum fluorescence ( $F_m$ ) after all reaction centers have been closed by a saturating flash of light (Kolber et al. 1988). The difference between these ( $F_m - F_0 = F_v$ ), normalized to  $F_m$ , is a measure of photochemical efficiency.  $F_0$  and  $F_m$  normalized to chlorophyll also give physiological information; patterns of  $F_0$  and  $F_m/\text{chl } a^{-1}$  across the transect and with depth are discussed in detail by Olaizola et al. (1996).

Cellular fluorescence emission, designated  $f$ , can be divided into 2 components: fluorescence efficiency ( $\phi_f$ , dimensionless), and a light absorption coefficient ( $a_{\text{cell}}$ , with units of  $\text{m}^2 \text{cell}^{-1}$ ). The coefficient  $a_{\text{cell}}$  can be fur-

ther divided into cell chlorophyll content ( $\text{pg chl } a \text{ cell}^{-1}$ ) and a chlorophyll-specific light absorption coefficient,  $a_{\text{chl}}$  ( $\text{m}^2 \text{pg chl } a^{-1}$ ), giving:

$$f = \phi_f (\text{chl cell}^{-1})(a_{\text{chl}})$$

All of the parameters on the right hand side of this equation have been observed to change with nutrient limitation, complicating interpretation of cell fluorescence. Several studies have demonstrated that  $a_{\text{chl}}$  increases under nitrogen limitation although cell chlorophyll and accessory pigment content declines (Osborne & Geider 1986, Kolber et al. 1988, Herzig & Falkowski 1989). This results from an increase in accessory pigment:chlorophyll ratio and therefore an increase in light-harvesting antennae size relative to PSII reaction center chlorophyll (Herzig & Falkowski 1989), as well as from a decrease in the package effect (Geider et al. 1993b).

Effects of nutrient limitation on fluorescence efficiency are more complex. As the amount of PSII antennae pigment declines, total fluorescence emission also declines. However, N limitation also causes a loss of proteins required for photochemical energy conversion, and a resultant increase in fluorescence from the photochemically inactive reaction centers (Kolber et al. 1988). Effects of nutrient limitation which may be seen as an increase in  $F_0$  therefore include a loss of functional PSII reaction centers and/or inefficient energy transfer to reaction centers, due to loss of key proteins (Kolber et al. 1988, Falkowski et al. 1989); increased chlorophyll-specific absorbance due to decreased cell chlorophyll content (Berner et al. 1989); and changes in the accessory pigment:chlorophyll ratio (Sosik & Mitchell 1991).  $F_m$  will respond primarily to changes in chlorophyll-specific absorption and relative pigment content. At Stn 8,  $F_0/\text{chl } a^{-1}$  was significantly lower in N-amended treatments while  $F_m/\text{chl } a^{-1}$  was not ( $p = 0.05$ ). This reduction is consistent with a decrease of  $\Phi_{F_0}$  and implies repair of damaged PSII reaction centers following N addition. At all other stations,  $F_0/\text{chl } a^{-1}$  varied more closely with  $F_m/\text{chl } a^{-1}$ , indicating little or no change in fluorescence efficiency with treatment, as expressed in the  $F_v/F_m$  values.

### Application of $F_v/F_m$ as a diagnostic of nutrient limitation

The parameter  $F_v/F_m$  is physiologically constrained to a narrow range independent of taxon, with a maximum value of about 0.64 in nutrient replete cultures, and a minimum of about 0.3 in N-limited cultures growing at 10% of their nutrient saturated rate (Kolber et al. 1988). Thus the absolute value of  $F_v/F_m$  is an absolute measure of the maximum efficiency of photo-

system II photochemistry, and is proportional to the maximum quantum efficiency of photosynthesis,  $\Phi_m$  (Geider 1993). This biophysical index is fundamentally different from physiological indices such as chl *a* cell<sup>-1</sup>, which vary with taxon as well as physiological state of the cell. Since variable fluorescence is virtually independent of growth temperature and irradiance under nutrient sufficiency, it is an excellent, and relatively non-intrusive, biophysical approach to examining nutrient limitation of phytoplankton photosynthesis (Geider 1993). Low values of  $F_v/F_m$  have been consistently correlated with reduced growth rates in nitrogen-, iron-, and phosphorus-limited cultures (Kolber et al. 1988, Greene et al. 1992, Geider et al. 1993b, Graziano et al. 1996) and low photosynthesis rates in the field (Kolber & Falkowski 1993, Kolber et al. 1994).  $F_v/F_m$  is a non-linear function of growth rate and does not decline significantly until the relative growth rate falls below 50% of  $\mu_{max}$  (Kolber et al. 1988, Graziano et al. 1996). Measurements made during the same transect as a basin-wide study of variations in  $\Phi_m$ , and a more in-depth discussion of the use of  $F_v/F_m$  in field studies of nutrient limited photosynthesis and growth, are presented in Olaizola et al. (1996). Our observations of low values of  $F_v/F_m$  are consistent with nutrient limitation of photochemical energy conversion and therefore of growth rates of North Atlantic picoplankton.

At Stns 1 through 7 in this study, water was taken from below the surface but above the chlorophyll maximum (20 to 65 m). Addition of nitrogen did not significantly ( $p < 0.05$ ) increase  $F_v/F_m$  at any of these stations although enclosure did, while the highest value measured after incubation (0.52) was still lower than that found in coastal, nutrient-rich waters. Water column values were low compared with the physiological maximum of 0.64. Assuming a strict proportionality between  $F_v/F_m$  and fraction of functional reaction centers, and that all reaction centers are functional when  $F_v/F_m = 0.64$  (Greene et al. 1994), between 25 and 35% of PSII reaction centers were non-functional over our transect. Other investigators have observed low  $F_v/F_m$  values in the ocean, including the equatorial Pacific (Greene et al. 1994, Kolber et al. 1994), the Sargasso Sea (Geider et al. 1993a) and the Gulf of Maine (Kolber et al. 1990). It is significant that incubations with nutrient additions fail to result in full recovery (Greene et al. 1994), even over 5 to 6 d incubations. The exception to this finding is the *in situ* iron fertilization experiment carried out in the equatorial Pacific (Kolber et al. 1994), where values of  $F_v/F_m$  increased from about 0.3 to over 0.6 within 2 d of water column iron enrichment.

It seems that some factor in addition to nitrogen availability in the North Atlantic causes the depressed

$F_v/F_m$ , and that this was partially alleviated by enclosure in bottles. Values tended to increase with N addition, although only at Stn 8 was this increase significant at  $p < 0.05$ . Other factors which may affect this measurement are discussed by Geider et al. (1993b). Confounding effects of xanthophyll cycling are eliminated by a 30 min dark-adaptation time prior to fluorescence measurements and by low incubation irradiance, so non-photochemical quenching should not have caused a reduction in  $F_v/F_m$ . It is possible that the presence of cyanobacteria may reduce  $F_v/F_m$ , due to their relatively high zeaxanthin:chlorophyll ratios which may result in non-photochemical fluorescence quenching (J. La Roche unpubl. data). However, the numerical dominance of cyanobacteria at our Stns 1, 7, and 8 did not correspond to low initial variable fluorescence or to the magnitude of increase in our incubations.

Despite the failure of N-amended samples to recover in terms of photochemical efficiency, N addition clearly resulted in increased pigmentation. It is possible that the time-course of incubation allowed an increase in pigment synthesis under N sufficiency, but was too short to allow repair of protein components of the photosynthetic apparatus. This differs from previous findings (Geider et al. 1993b, Kolber et al. 1994) that an increase in both  $F_v/F_m$  and chl *a* cell<sup>-1</sup> occurs within 24 h of nutrient addition.

The last experiment (Stn 8) was conducted with near-surface water where the algae were severely nutrient stressed *in situ* as judged by the low value of  $F_v/F_m$  (0.27). A much greater response of variable fluorescence to nitrogen addition was observed at this station than at any other. Despite the presence of many larger cells, however,  $F_v/F_m$  was only 0.45 in N-enriched samples, lower than the maximum reached in all but the most oligotrophic station. The 24 h incubation time used may have been insufficient to allow full physiological recovery of this assemblage. However, partial recovery of  $F_v/F_m$  provides strong evidence for physiological limitation by nitrogen *in situ* at Stn 8.

## CONCLUSIONS

The principal experimental tool used to determine the nutrient(s) limiting phytoplankton productivity has been the nutrient addition bioassay. Such experiments have provided evidence for nitrogen limitation of phytoplankton abundance in subtropical gyres and coastal areas (Fisher et al. 1992, DiTullio et al. 1993). However, interpretation of results is controversial due to artifacts associated with confining a natural assemblage in bottles for a day or longer. We employed pump-and-probe measurements of photochemical effi-

ciency ( $F_v/F_m$ ) and flow cytometric measurements of abundance and cellular fluorescence, in order to examine physiological responses independently of cell abundance in nutrient addition experiments. Observations have been interpreted within the context of documented variability in chlorophyll content and photosynthetic efficiency of phytoplankton under nutrient limited conditions (Kolber et al. 1988, Geider 1993).

Overall, these experiments suggest *in situ* nitrogen limitation of cell biomass (numbers and/or pigment content) and photosynthetic physiology of the 3 taxa investigated. The results are summarized in Table 8. In several cases the prokaryotes appeared to outcompete the eukaryotes for added nutrients, as indicated by a decline in number and cell fluorescence of eukaryotes. In other instances, eukaryotic cell chlorophyll was enhanced by N addition but numbers declined, either because of increased grazing or decreased division rate. Cyanobacterial numbers did not respond to treatment, although cellular fluorescence increased over initial values in all experiments, indicating increased pigment content and possibly faster division rates. The most oligotrophic station (Stn 3) was exceptional in that some factor other than nutrient availability appeared to constrain algal biomass and cellular chlorophyll.

Pump-and-probe fluorescence measurements indicated low photosynthetic efficiency across the entire transect. It is interesting that within a 1 or 2 d incubation, photochemical efficiency was significantly improved only at the relatively eutrophic station, where an extremely low initial value was measured. Large increases in cellular pigment content, and lesser increases in cell numbers with nitrogen addition, strongly imply both physiological and biomass limitation by nitrogen.

Table 8. Summary of results of nitrogen addition in 8 experiments. +N treatments include N, NFe, NP, and All. Response is indicated by +, -, or 0 relative to control. \*Indicates a decrease from initial value. np: not present

Stn	Chl ( $\mu\text{g l}^{-1}$ )	$F_v/F_m^a$	Cell red Fl			Cells $\text{ml}^{-1}$		
			Pro	Cyan	Euk	Pro	Cyan	Euk
1	+	+	+	+	++	-	0	0
2	+	0	+	+	+	+	0	+
3	-*	-	-	0	-*	-	-	0*
4	+	+	+	+	+	0	0	0*
5	+	0	+	+	+	-	0	0
6	++	+	+	+	+	+	0	+
7	+	+	+	+	+	0	0	0*
8	+	+	np	+	+	np	0	+

<sup>a</sup>Increase was significant at  $p < 0.05$  only at Stn 8

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