

# Nutrient limitation of picophytoplankton photosynthesis and growth in the tropical North Atlantic

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## *Abstract*

Identification of the proximal nutrient limiting primary production is a necessary first step toward evaluating the physiological state of phytoplankton communities and the biogeochemical constraints on the current oceanic carbon cycle. We conducted 48-h nutrient addition bioassay experiments to evaluate nitrogen, phosphorus, and iron limitation of primary productivity, net chlorophyll synthesis, and net increase in cell numbers of the dominant picophytoplankton from the tropical North Atlantic. Our results indicate that N was the proximal limiting factor for primary production during the autumn of 2002, followed by P and then Fe. Net chlorophyll synthesis was significantly stimulated by addition of N alone and further stimulated by addition of P. Analysis of picophytoplankton populations by analytical flow cytometry revealed a more complex response. Cellular red fluorescence, an index of cell chlorophyll content, increased in *Prochlorococcus*, *Synechococcus*, and picoeukaryotes in response to addition of  $\text{NH}_4\text{NO}_3$  but was not affected by single or combined additions of P and Fe. In contrast, cell abundance in these picophytoplankton populations increased only after combined N and P (63% of comparisons) or N and Fe (21% of comparisons) additions. Thus, our experiments revealed that chlorophyll synthesis and primary production were limited by the availability of nitrogen alone, while net increase in cell abundance was colimited by N and P or N and Fe in the majority of these picophytoplankton populations.

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Nitrogen and phosphorus have long been considered the nutrients that limit primary productivity in the oligotrophic tropical and subtropical North Atlantic, with nitrogen assigned the role of the proximate limiting factor and phosphorus the ultimate limiting nutrient (Tyrrell 1999). This assertion has been challenged recently (Ammerman et al. 2003; Vidal et al. 2003) based on low soluble reactive phosphorus (Wu et al. 2000) and high inorganic N:P ratios in subtropical North Atlantic surface waters (Cavender-Bares et al. 2001). With the N:P of the dissolved inorganic, dissolved organic and particulate pools exceeding the Redfield ratio of 16 mol N:1 mol P (Ammerman et al. 2003; Vidal et al. 2003), it has been suggested that phosphate deficiency in the North Atlantic is an emerging paradigm (Ammerman et al. 2003). High aeolian inputs of dust to the North Atlantic have been hypothesized to relieve nitrogen fixation from limitation by Fe in this basin (Falkowski 1997), thus accentuating phosphate depletion in the subtropical North Atlantic relative to the subtropical

North Pacific (Wu et al. 2000) and driving the ecosystem to P limitation. Further evidence supporting the case for P deficiency includes high activity of alkaline phosphatase (Vidal et al. 2003) and expression of a high-affinity phosphate-binding protein in *Synechococcus* (Scanlan and Wilson 1999).

Despite the accumulating evidence for phosphate deficiency, nutrient addition bioassays conducted in this basin have shown that an input of dissolved inorganic nitrogen invariably leads to an increase in chlorophyll concentration and primary production in North Atlantic subtropical and tropical waters (Graziano et al. 1996; Paerl et al. 1999; Mills et al. 2004). Furthermore, phosphate stimulation of chlorophyll accumulation was observed only after alleviation of N limitation (Graziano et al. 1996; Mills et al. 2004; Moore et al. 2008), reinforcing the importance of fixed inorganic nitrogen as a primary limiting nutrient in this region.

Recent advances in understanding the physiological basis of elemental stoichiometry in marine unicellular photoautotrophs may help reconcile the discrepancies observed between the oceanographic measurements and bioassay incubations. The realization that cellular components have different N:P stoichiometric ratios (Sterner and Elser 2001) has contributed to explaining species-specific deviation from the Redfield ratio of 16:1 in phytoplankton (Geider and La Roche 2002; Quigg et al. 2003). Thus, the light and nutrient acquisition machinery is rich in C and N, while cellular P is found mainly in growth machinery such as ribosomal ribonucleic acid (RNA) (Klausmeier et al. 2004) and structural phospholipids of cell membranes. The cellular RNA content is itself higher in fast-growing cells and decreases with increase in cell size (Gillooly et al. 2005). It has been hypothesized that in oligotrophic surface waters, cell N must be invested in the proteins required to transport nutrients into the cell and for photosynthesis (Klausmeier et al. 2004). The high abundance of genes encoding for transporter and photosynthetic proteins in metagenomes of plankton communities from surface oligotrophic waters (DeLong et al. 2006) support this hypothesis. The light-harvesting pigment protein complexes and phycobilisomes are rich in protein and account for significant amounts of cell N. Significantly, chloroplast membranes are composed predominantly of glycolipids, and thus investment in these membranes does not significantly increase cell P content. Thus, nitrogen limitation will likely interfere with biochemical processes involved in nutrient and light energy acquisition, while phosphorus limitation likely blocks RNA synthesis, deoxyribonucleic acid (DNA) replication, and cell membrane synthesis, thus interfering directly with biochemical pathways involved in cell division.

Resource optimization models predict that exponential growth, such as in bloom-forming phytoplankton, leads to an allocation of N and P to the P-rich growth machineries (e.g., ribosomes and nucleic acids), while in nutrient-poor areas, the balance of nutrient utilization is shifted to increase the N-rich acquisition machinery (e.g., chloroplasts and protein) (Klausmeier et al. 2004; Arrigo 2005). If correct, this scheme would suggest that phytoplankton species growing in oligotrophic conditions will tend to have higher physiological requirements for N relative to P. Thus,

with the exception of diazotrophic cyanobacteria, oligotrophic phytoplankton populations may be proximally limited by N even when the ratio of dissolved inorganic nitrogen and phosphorus supply is much higher than Redfield.

In this paper, we focus on the response of four components of the picophytoplankton community to addition of inorganic N, P, and Fe. We present the results of chlorophyll and primary productivity measurements on six bioassay experiments conducted in the tropical North Atlantic. Within the frame of the bioassay experiments, we use analytical flow cytometry (AFC) to assess the change in the abundance and cellular red fluorescence of four picophytoplankton populations in response to the various treatments. The four groups studied are picoeukaryotes, *Prochlorococcus* sp., bright *Synechococcus*, and dim *Synechococcus* species. The light-harvesting pigments of the bright and dim *Synechococcus* have different ratios of phycourobilin (PUB) to phycoerythrobilin (PEB), suggesting that these are different strains of *Synechococcus* (Fuller et al. 2003). These measurements provide insight into the physiological mechanisms that lead to the cascade of nutrient limitation ( $N > P > Fe$ ), described earlier for this area (Mills et al. 2004). We show that alleviation of nitrogen limitation stimulates chlorophyll synthesis, with most of the increase in chlorophyll concentration attributed to an increase in chlorophyll per cell (increase in acquisition machinery) rather than to increased cell abundance. In addition, we show that addition of nitrogen alone does not lead to an increase in cell abundance, suggesting that net cell division, involving different biochemical pathways, is also limited by the availability of phosphorus.

## Methods

Six nutrient-addition bioassays (experiments I–VI), designed to investigate which nutrient (N, P, or Fe) limits daily primary production, increases in phytoplankton biomass, and phytoplankton physiological state, were conducted at sites in the tropical eastern Atlantic (I–VI) during October–November 2002 as part of German SOLAS cruise *Meteor* 55. Experiments were carried out during a west-to-east transect along 10–11°N, with a short transect to the equatorial upwelling region (Table 1) (Wallace and Bange 2004). The primary productivity and chlorophyll results for three of the experiments where  $N_2$  fixation rates were also measured were originally presented elsewhere (Mills et al. 2004).

*Water sampling and bottle filling*—Trace metal-clean techniques were strictly used throughout the preparation and execution of the experiments. The 1.18-liter incubation bottles, carboys, and plasticware used in the bioassay experiments were subjected to thorough cleaning based on the methods outlined in Graziano et al. (1996). Prior to use, the acid-cleaned bottles were rinsed repeatedly with the seawater collected for the bioassay incubations. Three sets of 1.18-liter incubation bottles were labeled and reused only for similar treatment regimes.

Table 1. The location and physical and biological parameters of seawater collected for each bioassay experiment. For all data, means are shown with  $\pm$  standard deviation for triplicate samples. Initial primary productivity was determined by  $^{14}\text{C}$  fixation during the first 24 h in unamended controls incubated alongside bioassay treatment bottles. Picoplankton community structure information was provided by analytical flow cytometry. n.a. = not available.

Site	Date (2002)	Location (lat., long.)	Chlorophyll (mg m <sup>-3</sup> )	Productivity (mg C m <sup>-3</sup> d <sup>-1</sup> )	Salinity	Temp (°C)	Mixed-layer depth (m)	1% light level (m)	<i>Prochlorococcus</i> (10 <sup>3</sup> cells mL <sup>-1</sup> )	<i>Synechococcus</i> (10 <sup>3</sup> cells mL <sup>-1</sup> )	<i>Prochlorococcus</i> : <i>Synechococcus</i>	% Bright <i>Synechococcus</i>	Picoeukaryotes (cells mL <sup>-1</sup> )
I	18 Oct	10°00.00'N, 49°28.80'W	0.36±0.03	21.8±1.0	32.40	29.20	8.5	63	69±20	50±6	1.4	65%	501±367
II	23 Oct	10°00.00'N, 34°43.26'W	0.18±0.02	17.9±0.8	34.53	28.30	27	50	101±18	11±0.8	9.7	32%	386±82
III	27 Oct	05°10.14'N, 26°20.46'W	0.13±0.005	9.9±0.4	34.77	28.50	32	86	72±18	16±0.8	4.4	5%	396±87
IV	31 Oct	03°50.52'N, 23°45.48'W	0.38±0.008	9.2±1.8	35.56	27.84	60	71	103±3	6.1±0.5	17	22%	375±155
V	06 Nov	11°01.56'N, 17°52.38'W	0.22±0.007	18.8±0.2	35.25	29.50	25	40	130±48	39±6	3.3	80%	428±10
VI	11 Nov	05°59.16'N, 15°34.68'W	0.18±0.01	13.7±0.6	34.29	28.80	24	n.a.	120±6	20±1	6.0	71%	343±23

Surface seawater was collected 2–3 h after sunset. Water was collected using a towed swim fish from a depth of 1–3 m in conjunction with a trace metal-clean Teflon diaphragm pump. Water was pumped directly into a 60-liter reservoir located in a clean laboratory container. The seawater was transferred simultaneously into the incubation bottles via a siphon to allow mixing of the seawater batch while minimizing bubble formation. Mixed-layer depths (MLDs) were estimated from conductivity, temperature, and depth station profiles surrounding the sites of water collection and defined as the depth where  $\sigma_t$  differed by 0.02 units from the surface value. The 1% light level was estimated from light profiles measured at the station nearest the site of collection using a spherical quantum sensor (Li-Cor).

*Nutrient and dust additions*—Nutrient amendments of P, N, and Fe were made alone and in combination to provide eight treatments (control, +P, +Fe, +PFe, +N, +NFe, +NP, and +NPFe). Additions were made under a laminar flow hood to give final concentrations of 1  $\mu\text{mol L}^{-1}$   $\text{NH}_4\text{NO}_3$ , 0.2  $\mu\text{mol L}^{-1}$   $\text{NaH}_2\text{PO}_4$ , and 2 nmol  $\text{L}^{-1}$   $\text{FeCl}_3$ . The dissolved nitrogen and phosphorus primary stock solutions were prepared using AnalaR-grade reagents and treated using Chelex-resin (1 g 50 mL<sup>-1</sup>), to minimize trace metal contamination in undiluted stocks. Working stock solutions were prepared immediately prior to use by diluting with Milli-Q water, then sterilized through 0.2- $\mu\text{m}$  syringe filters.

Saharan dust treatments were also conducted with final concentrations of 0.5 mg L<sup>-1</sup> (referred to as D1) and 2 mg L<sup>-1</sup> (referred to as D2). The dust consisted of the fine fractions of surface soils collected in the Hoggar region (southern Algeria) with a grain-size distribution and chemical composition typical for Saharan aerosols collected far from the source. The measured P and Fe content of the dust were 0.14  $\pm$  0.01% and 4.97  $\pm$  0.49%, respectively. The phosphate liberated from the dust treatments was 2.7  $\pm$  0.2 and 10.8  $\pm$  0.8 nmol L<sup>-1</sup>, while Fe released was 0.9  $\pm$  0.03 and 3.6  $\pm$  0.04 nmol Fe L<sup>-1</sup> for the 0.5- and 2.0-mg L<sup>-1</sup> dust treatments, respectively (Ridame and Guieu 2002; Bonnet and Guieu 2004; Mills et al. 2004).

*Incubation*—Simulated in situ incubations were conducted in Perspex flow-through incubators and cooled by surface seawater. Incident light was attenuated to approximately 20% of surface values using blue filters (Lagoon Blue, Lee Filters no. 172). For each treatment, parallel incubations for each variable were run in triplicate over 48 h with primary production measurements made during the final 24 h and chlorophyll concentration and analytical flow cytometry performed on samples collected at 48 h. An additional set of unamended control bottles were incubated alongside the treatments in order to determine primary productivity over the initial 24 h and to measure chlorophyll concentration and take AFC samples at 0 and 24 h.

*Primary production*—Primary production was measured using the method and calculations outlined in Ducklow and Dickson (1994). Before dawn, 3.7 MBq  $^{14}\text{C}$ -bicarbonate

were added to each bottle (1.18-liter volume), then incubated for 24 h in the on-deck incubators. After 24 h, the bottles were transferred predawn to a radioisotope laboratory container where they were stored in the dark prior to filtration. A 500-mL subsample from each incubation bottle was filtered onto GF/F filters and acidified in HCl fumes for 30–40 min. Filters were transferred to 20-mL glass scintillation vials to which 10 mL of Lumagel plus scintillation cocktail (by Lumac, supplied by PerkinElmer Life Sciences) were added. The vials were left for a minimum of 24 h at low light before counting using a Packard TriCarb liquid scintillation counter on board the ship. A further 250- $\mu$ L subsample was removed from each control bottle at 0 and 24 h to measure total activity (Tc) of the added  $^{14}$ C-bicarbonate. Ethanolamine (250  $\mu$ L) and scintillation cocktail (10 mL) were then added to the Tc samples, and all vials were counted with the filters intact.

*Chlorophyll a*—Five-hundred-milliliter subsamples were collected from each of the triplicate bottles and filtered through 25-mm GF/F filters. Filtered chlorophyll samples were placed in a 10-mL tube, and 1 mL of Milli-Q water was added to it and frozen at  $-20^{\circ}\text{C}$ . After several hours, the samples were thawed, and 9 mL of 100% acetone were added. Extraction of the samples was then carried out in the dark at  $-20^{\circ}\text{C}$  for 24 h. Samples were equilibrated to room temperature before measurement. Fluorescence at 685 nm was measured using a Turner Designs 10-AU-005-CE Field Fluorometer, calibrated with chlorophyll *a* (Chl *a*) standard dissolved in 90% acetone (Welschmeyer 1994). Samples were taken from initial and control bottles at 0, 24, and 48 h; nutrient-amended bottles were sampled at 48 h.

*Analytical flow cytometry*—The abundance of picophytoplankton was measured by analytical flow cytometry (AFC). Samples (2 mL) for AFC were fixed with 1% formaldehyde (final concentration) for 24 h at  $2-4^{\circ}\text{C}$ , then transferred to  $-20^{\circ}\text{C}$  as described in Zubkov et al. (2003). The control incubations were sampled at 0, 24, and 48 h, while the nutrient-amended bottles were sampled only at 48 h. Samples were transported back to the laboratory and stored for 4 months at  $-80^{\circ}\text{C}$  prior to AFC analysis.

The samples were thawed at room temperature and analyzed using a Becton Dickinson FACSsort flow cytometer. The FACSsort counted particles and measured chlorophyll fluorescence ( $>650$  nm), orange fluorescence ( $585 \pm 21$  nm), and side scatter (light scattered at 90 degrees to the plane of the vertically polarized argon ion laser exciting at 488 nm). Data acquisition was triggered on red fluorescence, using laboratory cultures of *Prochlorococcus* sp. to help set rejection gates for background noise. Samples were analyzed for 3 min at a flow rate of  $122 \pm 4 \mu\text{L min}^{-1}$  and data stored in list-mode format. Cell counts were made by analyzing the list-mode data with WinMDI Version 2.8 (Joseph Trotter) flow cytometry analysis software. Scatter plots of side scatter vs. orange fluorescence were used to discriminate and enumerate *Synechococcus* sp. from other picophytoplankton, and plots of side scatter vs. red fluorescence (with *Synechococ-*

*cus* gated out) were used to enumerate *Prochlorococcus* sp. and picoeukaryotes. Four picophytoplankton groups were identified (Fig. 1). These were classified as bright and dim *Synechococcus*, which were identified in phycoerythrin fluorescence vs. side-scatter plots, and *Prochlorococcus* and picoeukaryotes. Bright and dim *Synechococcus* have high and low PUB:PEB, respectively, and high and low orange fluorescence, respectively, when analyzed by flow cytometry using blue light sources (Fig. 1). The four populations were clearly identifiable in all samples as illustrated in Fig. 1 for the time-zero (initial) samples and at 48 h in samples from control and nutrient amended treatments. Within *Synechococcus*, the bright subpopulation accounted for between 20% and 95% of the total. The picoeukaryote population ranged from 340 to 500 cells  $\text{mL}^{-1}$ ; however, underestimation of picoeukaryotes as a result of both the analytical method and the fixation technique employed cannot be ruled out.

## Results

Throughout the transect, dissolved reactive phosphorus and nitrate concentrations in surface waters were less than 0.03 and 0.3  $\mu\text{mol L}^{-1}$  respectively (P. Fritsche and F. Malien pers. comm.), below the limit of detection of the analytical techniques used in this study. Chlorophyll concentration ranged from 0.13 to 0.38  $\text{mg m}^{-3}$  and primary productivity from about 9 to 22  $\text{mg C m}^{-3} \text{d}^{-1}$  (Table 1) within the regions sampled. The assimilation number varied from a minimum of 21  $\text{g C (g Chl } a)^{-1} \text{d}^{-1}$  at site IV, which was located closest to the equator, to between 60 and 100  $\text{g C (g Chl } a)^{-1} \text{d}^{-1}$  at the other sites. The MLD calculated for these stations varied from less than 10 m to about 60 m. The first experiment was carried out with oceanic water influenced by the Amazon River plume (Kortzinger 2004), as confirmed from the lower salinity measured at this site and the resulting shallow MLD. Variations in the ratio of *Prochlorococcus* to *Synechococcus* ranged between 1.4 and 17 at these sites, reflecting the degree of oligotrophy along the transect. The ratio of *Prochlorococcus* to *Synechococcus*, has been shown before to increase with the degree of oligotrophy (Zubkov et al. 2003). In our study, the ratio of *Prochlorococcus* to *Synechococcus* was correlated with the MLD and suggested that site I was least nutrient impoverished and site IV most nutrient impoverished.

*Response of chlorophyll and primary productivity to nutrient additions*—The response of chlorophyll and primary production to nutrient addition showed similar patterns in all six experiments (Fig. 2). Chlorophyll concentrations in the control treatments at 48 h did not differ significantly from those measured at time zero when the experiments were initiated. Similarly, with the exception of experiment II (Fig. 2B), primary productivity in the control treatment did not differ significantly between the initial 0–24-h and final 24–48-h incubations; thus, all subsequent comparisons are relative to the unamended control incubations. Primary productivity and chlorophyll accumulation were stimulated in all the treatments with

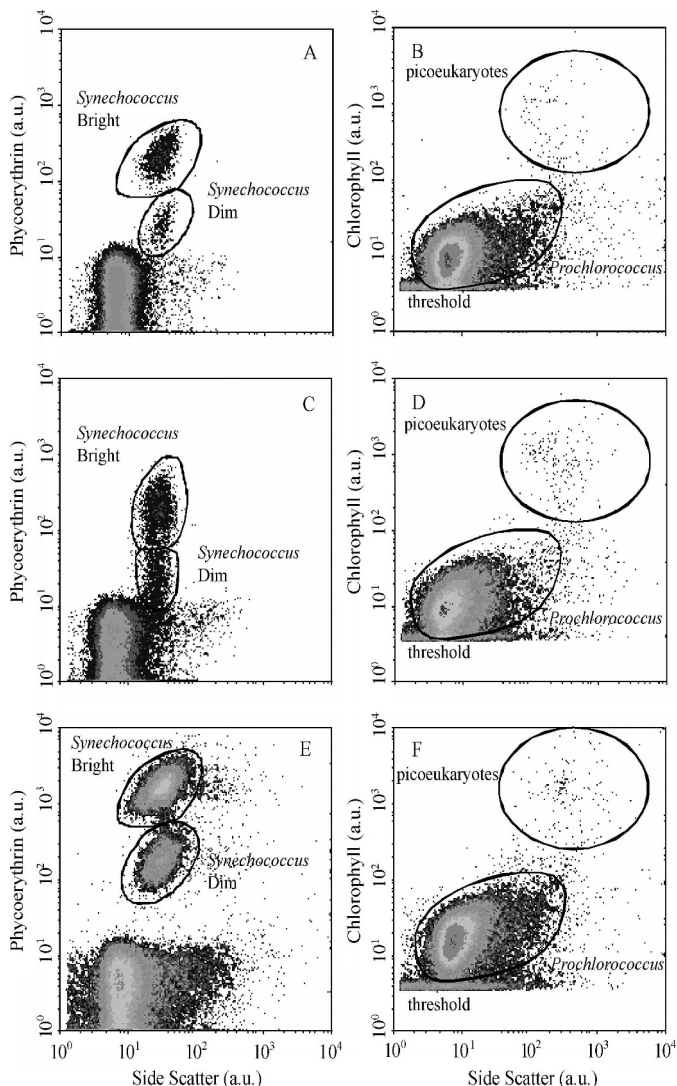


Fig. 1. (A–F) Density plots showing picoplankton groups analyzed by flow cytometry in experiment VI. (A, C, E) Discrimination of *Synechococcus* bright and dim populations using their side-scatter and orange (phycoerythrin) fluorescence properties. (B, D, F) Discrimination of picoeukaryotes and *Prochlorococcus* using their side scatter and red (chlorophyll) fluorescence (while *Synechococcus* was gated out of analysis). (A, B) Picoplankton populations at  $t = 0$ . (C, D) Control at 48 h. (E, F) +NPF treatment at 48 h. Chlorophyll, phycoerythrin, and side scatter are in arbitrary units (a.u.).

added  $\text{NH}_4\text{NO}_3$ . Addition of P and/or Fe failed to stimulate chlorophyll production or primary production, unless  $\text{NH}_4\text{NO}_3$  was added as well. Thus, only treatments amended with nitrogen ( $\pm$  other nutrients) stimulated chlorophyll and primary production (Table 2). Once nitrogen limitation was relieved, additional increases in primary productivity were observed in treatments containing both N and P (Table 2), suggesting a secondary role for P as a limiting nutrient. An additional effect of Fe on increased chlorophyll was observed in three of the six experiments (Table 2), confirming the cascading effect of

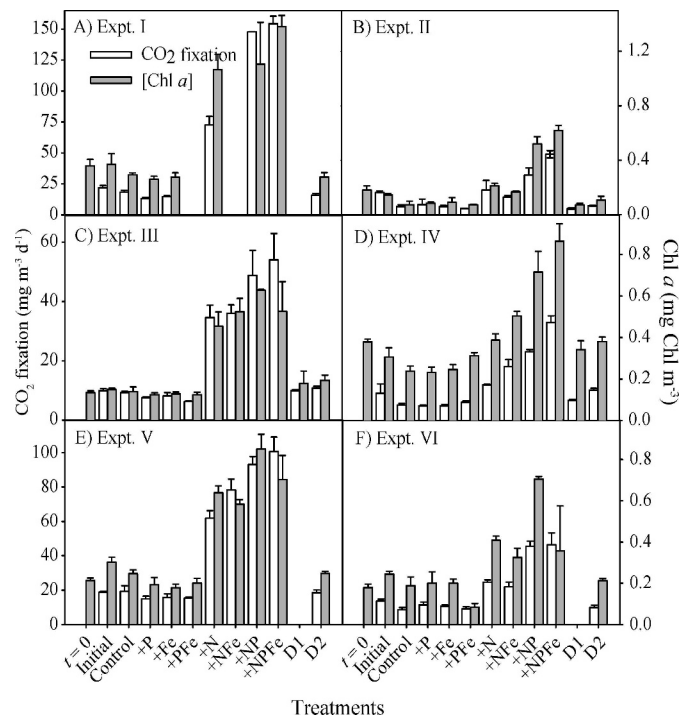


Fig. 2. (A–F) The effect of nutrient additions on primary production (net  $\text{CO}_2$  fixation) and chlorophyll concentration. Two Saharan dust treatments were included in some experiments at  $0.5 \text{ mg L}^{-1}$  (D1) and  $2 \text{ mg L}^{-1}$  (D2). Chl *a* concentrations in control incubations were determined at  $t = 0, 24$ , and  $48 \text{ h}$ , while all treatments were measured at  $t = 48 \text{ h}$ . Initial primary productivity was determined between  $0$  and  $24 \text{ h}$ , while all other measurements were made between  $24$  and  $48 \text{ h}$ . Separate triplicate bottles were sampled, and means are shown  $\pm$  standard deviation ( $n = 3$ ). Treatment means were compared using a one-way ANOVA and differences identified using a post hoc Tukey means comparison test. The results from the six replicate experiments conducted at the sites listed in Table 1 are summarized in Table 2.

N, P, and Fe as limiting nutrients for our study region (Mills et al. 2004).

Dust addition caused a slight but statistically significant stimulation (at the 95% level) of primary productivity in only one of the six experiments (site IV, Fig. 2D). The new results that we report here are consistent with the conclusion of Mills et al. (2004) that the major effect of dust was on the diazotrophic community.

*Response of the picophytoplankton community to nutrient additions*—In contrast to the total phytoplankton community, the response of the four groups of picophytoplankton analyzed was more variable between bioassay experiments. The data for two experiments (II =  $03^\circ 50.52' \text{N}$ ,  $23^\circ 45.48' \text{W}$  and III =  $05^\circ 10.14' \text{N}$ ,  $26^\circ 20.46' \text{W}$ ), each showing a different response of the *Synechococcus* group to the nutrient addition treatments, are presented here (Figs. 3, 4). At both experimental sites, cellular red fluorescence of all picophytoplankton groups increased in the treatments with added  $\text{NH}_4\text{NO}_3$ . At site III, the increase was about 2- to 2.5-fold in *Synechococcus*

Table 2. Summary of effects of nutrient additions on primary production and net chlorophyll (Chl *a*) synthesis in each bioassay experiment. CO<sub>2</sub> fixation and chlorophyll were measured from separate triplicate bottles. The response of the phytoplankton community to different treatments was compared with controls at 48 h using one-way ANOVA ( $\alpha = 0.05$ ) followed by a post hoc Tukey test to compare means between eight treatments ( $n = 3$  replicates per treatment throughout, degrees of freedom = 23). Significant differences between individual treatments are indicated by < or >; = indicates no significant differences. N, P, and Fe refer to the nutrient addition as initially described in the "Methods" section. No significant differences were observed in productivity or chlorophyll between control and P, Fe, PFe, and all but one of the dust treatments (D2). In the composite summary (pooled), the effects of the different nutrient treatments on primary productivity and chlorophyll synthesis were assessed across all six sites. In each experiment, the relative differences of each treatment to the unamended control at 48 h were determined. The relative responses were compared by one-way ANOVA ( $n = 6$ ,  $\alpha = 0.05$ ) and means compared by a post hoc Tukey means comparison test. Significant differences are expressed as < or >; = indicates no significant difference.

Site	Nutrient limiting primary production	Mean comparisons	Nutrient limiting Chl <i>a</i>	Mean comparisons
I	N	(NP=NPFe)>N	N	NPFe>(N=NP)
II	N	NPFe>NP>(N>NFe)	N	NPFe>NP>(N=NFe)
III	N	(NP=NPFe)>(N=NFe)	N	NPFe=N=NFe=N
IV	N	NPFe>NP>(N=NFe)>D2	N	NPFe>NP>(N=NFe)>D2
V	N	(NP=NPFe)>NFe>N	N	NP>(N=NFe=NPFe)
VI	N	(NP=NPFe)>(N=NFe)	N	NP>(N=NFe=NPFe)
Pooled	N	NPFe>NP>(N=NFe)	N	(NP=NPFe)>(N=NFe)

and *Prochlorococcus* and about 1.5-fold in the picoeukaryotes (Fig. 3). In both the dim and the bright *Synechococcus* populations, cellular red fluorescence decreased in the control at 48 h from that measured initially (time zero) (Fig. 3A,B). Similar magnitudes of decline were also observed in +P, +Fe, and +PFe treatments in *Synechococcus* (Fig. 3A,B). In contrast, cellular red fluorescence was stable in the control, +P, +Fe, and +PFe treatments in both *Prochlorococcus* and the picoeukaryotes (Fig. 3C,D). A similar response of cellular chlorophyll to nutrient addition was also observed for site II (Fig. 4). These results suggest (1) that relief of N limitation by NH<sub>4</sub>NO<sub>3</sub> led to chlorophyll synthesis and an increase in chlorophyll per cell in all four picophytoplankton populations and (2) that N limitation was exacerbated in the *Synechococcus* populations in treatments without added NH<sub>4</sub>NO<sub>3</sub>, while the *Prochlorococcus* and picoeukaryote populations were unaffected.

At site III, cell abundance showed marked changes in the controls and treatments relative to the initial values (Fig. 3E–H); however, different picophytoplankton groups varied in their response to nutrient treatments. *Prochlorococcus* cell abundance declined in control and treatments without added NH<sub>4</sub>NO<sub>3</sub>; however, it remained stable in the treatments +NP and +NFe. The net population growth rate of *Prochlorococcus* ranged from about  $-0.4 \text{ d}^{-1}$  in the control to  $0.1 \text{ d}^{-1}$  in the +NP treatment. Picoeukaryote cell abundance at site III increased in the control and in all the nutrient addition treatments relative to the initial (time zero) value. The increase was about fourfold in the +NP and +NPF treatments, about threefold in the +N, +NFe, and +PFe treatments, and about twofold in the control, +P and +Fe treatments. Thus, net population growth rates of the picoeukaryotes varied from  $0.45 \text{ d}^{-1}$  in the control to  $0.8 \text{ d}^{-1}$  in the +NPF treatment.

At sites III (Fig. 3E,F) and IV (result not shown), the abundance of dim *Synechococcus* populations in treatments with and without nitrogen showed an opposite trend with the abundance of bright *Synechococcus*, thus marking an

exception to the otherwise similar response of these two groups to the nutrient treatments in the rest of the experiments (e.g., Fig. 4E).

Cellular abundance of dim *Synechococcus* increased markedly in treatments without added N, with a maximum increase in the +PFe treatment. The increase in cell abundance observed in the +PFe treatment suggests that perhaps a component of the dim *Synechococcus* group is capable of diazotrophic growth. In contrast, abundance of bright *Synechococcus* increased dramatically in the +N treatment and to even greater extents in the +NP, +NFe, and +NPF treatments. The maximum net population growth rate of the bright *Synechococcus* was  $1.1 \text{ d}^{-1}$  in the +NPF treatment.

In all other experiments (I, II, V, VI), the response of both groups of *Synechococcus* to the various nutrient additions paralleled each other as represented by experiment II (Fig. 4), with cell fluorescence being stimulated by all N treatments and with an increase in cell abundance on the joint addition of N and P. These two opposite responses of the dim *Synechococcus* account for part of the large variations seen in this group when comparing the mean response of the picophytoplankton in all six experiments (Fig. 5A,B,E,F).

We used ANOVA to assess the interaction of N, P, and/or Fe limitation on cellular red fluorescence in each of the six bioassays. These analyses showed that N alone limited the increase of cellular red fluorescence in the four picoplankton groups in 21 out of 24 comparisons (Fig. 5; Table 3). Cellular red fluorescence was not affected by addition of +P, +Fe, +PFe, or dust in any of the four picophytoplankton groups. In *Prochlorococcus*, a second nutrient affected cellular red fluorescence in four of the six experiments (Table 3). In this group, cellular red fluorescence tended to be lower in the +NP and +NPF treatments than in the +N and +NFe treatments. Thus, in *Prochlorococcus*, the addition of phosphate could reduce the magnitude of the increase of cellular red fluorescence that occurred when N limitation was alleviated. This may be

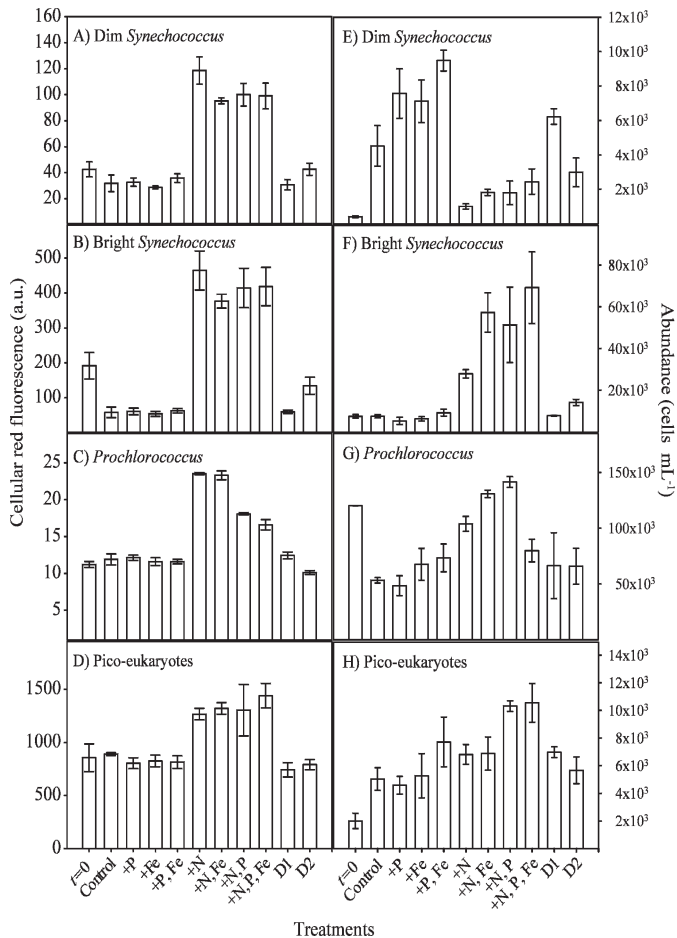


Fig. 3. Experiment III: (A–D) The effect of nutrient additions on the cellular red fluorescence (indicative of chlorophyll per cell) and (E–H) cell abundance of the different picoplankton groups. Samples were taken from separate triplicate bottles. (A, E) Dim *Synechococcus*. (B, F) Bright *Synechococcus*. (C, G) *Prochlorococcus*. (D, H) Picoeukaryotes. Means are shown  $\pm$  standard deviation ( $n = 3$ ). Treatment means were compared using a one-way ANOVA and differences identified using a post hoc Tukey means comparison test. The results of all six replicate experiments (Table 1) are summarized in Table 3.

because P stimulated cell division (*see below*) and smaller cells contain less chlorophyll and hence reduced red fluorescence compared to larger cells.

Although a single nutrient, namely, nitrogen, limited chlorophyll synthesis (Table 3), net growth rate, estimated from the increase in absolute cell concentration, was often colimited by N and P, or N and Fe. N and P colimitation of net increase in cell number was evident in 18 out of 24 comparisons (Table 3), whereas N and Fe colimitation was evident in 5 out of 24 comparisons (Table 3). For the bright and dim *Synechococcus* groups, N and P or N and Fe could sometimes simultaneously colimit net increase in cell number, suggesting that these groupings may be heterogeneous between and within stations and may be composed of several species of cyanobacteria.

Cell size (side scatter) increased in the +N treatment only in the two *Synechococcus* groups but not in the picoeukar-

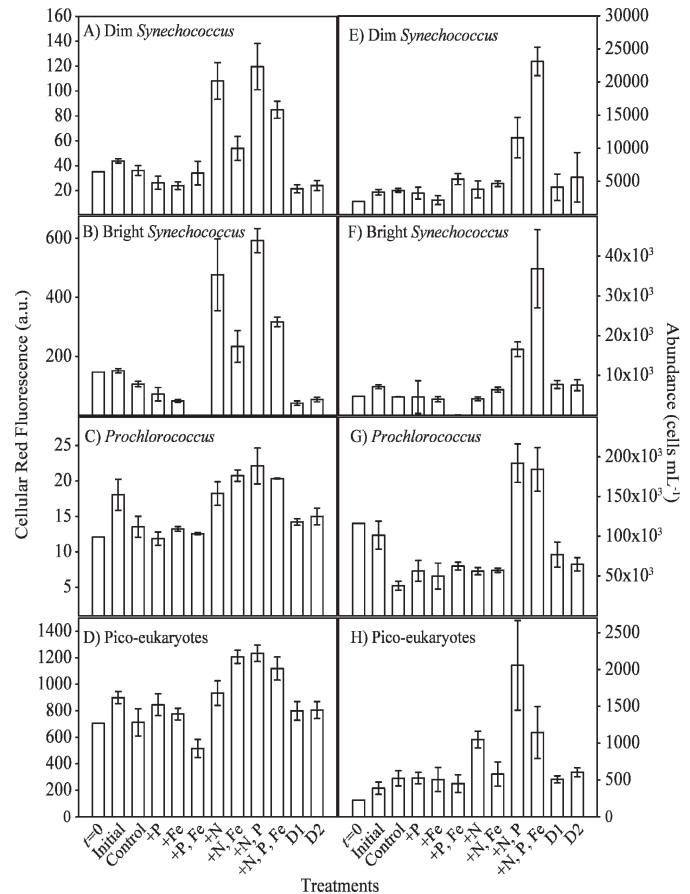


Fig. 4. Experiment II: (A–D) The effect of nutrient additions on the cellular red fluorescence (indicative of chlorophyll per cell) and (E–H) cell abundance of the different picoplankton groups. Samples were taken from separate triplicate bottles. (A, E) Dim *Synechococcus*. (B, F) Bright *Synechococcus*. (C, G) *Prochlorococcus*. (D, H) Picoeukaryotes. Arithmetic means are shown  $\pm$  standard deviation ( $n = 3$ ). Treatment means were compared using a one-way ANOVA and differences identified using a post hoc Tukey means comparison test.

yotes and *Prochlorococcus* (Fig. 6; Table 3). These observations suggest that in *Synechococcus*, the relief of N limitation in the N addition treatment may lead to an increase in cell volume due to biochemical synthesis of chlorophyll and proteins but that the residual dissolved P (or sometimes Fe) would be insufficient for the cells to undergo division. Thus, our data suggest that P and/or Fe may have limited cell division in *Synechococcus* when sufficient N was present.

## Discussion

Now that an important role has been established for iron as a limiting nutrient in several oceanic regions (Martin et al. 1994; Moore et al. 2006), one current hypothesis suggests that the balance between  $N_2$  fixation, denitrification and P limitation is set by the availability of Fe for diazotrophs (Wu et al. 2001). High iron deposition in the tropical North Atlantic, in contrast to the North Pacific

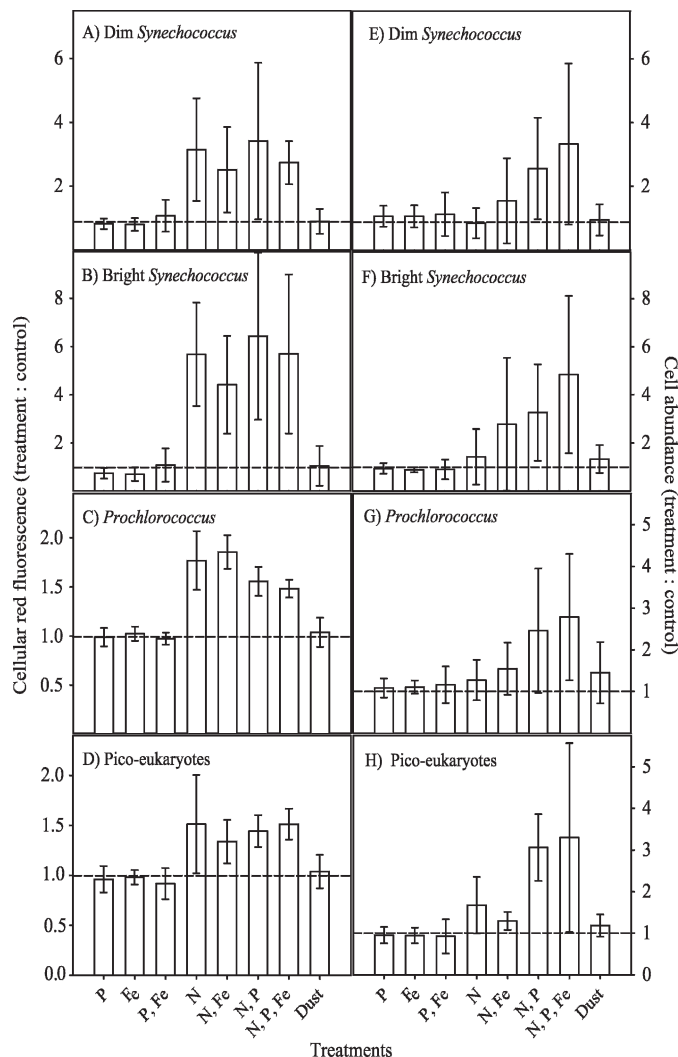


Fig. 5. (A–D) The effect of nutrient additions on cellular red fluorescence and (E–H) cell abundance in the different picoplankton groups when assessed across all six experiments. (A, E) Dim *Synechococcus*. (B, F) Bright *Synechococcus*. (C, G) *Prochlorococcus*. (D, H) Picoeukaryotes. In each experiment, the relative responses of each treatment were calculated from the ratio of the treatment to the unamended control at 48 h. The average relative responses for each treatment was calculated and are shown  $\pm$  standard deviation ( $n = 6$ ). The dust treatment represents pooled results for D1 and D2 dust additions. The dashed line at a value of 1 indicates the unamended control. The relative responses were compared by one-way ANOVA ( $n = 6$ ,  $\alpha = 0.05$ ) and significant differences identified using a post hoc Tukey means comparison test. The results of the individual experiments and of the pooled response are presented in Table 3.

(Jickells et al. 2005), should thus drive this oceanic ecosystem toward P limitation (Wu et al. 2000). According to this hypothesis, by limiting the rate of  $N_2$  fixation, Fe and P are the ultimate limiting nutrients that determine the availability of dissolved inorganic nitrogen (DIN). Based largely on geochemical evidence of low dissolved inorganic phosphorus (DIP) concentrations and high DIN:DIP, Ammerman et al. (2003) have made the case that phosphorus deficiency is an emerging paradigm in the

North Atlantic. Although they do not differentiate between ultimate and proximal limiting factor, Ammerman et al. (2003) imply that P is the proximal limiting nutrient. In contrast, short-term bioassay experiments with natural phytoplankton populations have suggested that N rather than P is the proximal limiting nutrient throughout the oligotrophic and mesotrophic tropical and subtropical North Atlantic (Mills et al. 2004; Moore et al. 2006; this study). Although we cannot rule out that P limitation may be more pronounced in another region and at another time of the year, short-term bioassays indicate that proximal P limitation, should it exist in the oligotrophic North Atlantic, is not universal.

Use of bioassay experiments to assess nutrient limitation has sometimes been questioned on the basis of perceived flaws in the experimental design and/or perceived limitations imposed by confining natural phytoplankton communities in bottles (Beardall et al. 2001). Although bioassay experiments cannot be extrapolated to predict the effect of changes in nutrient supplies on the pelagic ecosystem, the factorial design incorporated in our bioassay approach provides stringent criteria by which to evaluate the proximal nutrient-limiting chlorophyll synthesis and growth of the phytoplankton community. In addition, the use of AFC allowed us to differentiate between community-level and cellular responses, overcoming several of the perceived limitations in bioassay experiments. In order to identify which nutrient limits phytoplankton, the results of factorial nutrient bioassays must be consistent for all the treatments within an experiment. Thus, to conclude that N is the proximal limiting factor, a positive response to all the treatments with added N and no response in the treatments from which N was omitted must be recorded (Cullen 1991). Despite some variability in magnitude, the nutrient addition bioassays demonstrated that total primary productivity of the phytoplankton community and bulk chlorophyll concentrations increased only in treatments in which nitrogen was added. Conversely, no significant increases relative to the unamended samples were observed when N was omitted from the nutrient additions. This pattern was consistent across the west-to-east transect in the tropical North Atlantic, thus identifying N as the proximal nutrient-limiting carbon fixation and chlorophyll accumulation in this region at the time of our study. The additional increase in primary production and chlorophyll brought on by P addition after the initial relief of N limitation demonstrates the important but secondary role played by P in this region.

Our experimental results also indicate that Fe is not far in excess of the phytoplankton growth requirements. In fact, small but significant increases in chlorophyll and primary productivity were sometimes observed in treatments where iron was added incrementally together with additions of N and P, suggesting that dissolved Fe can be drawn down to limiting levels once N and P limitations are relieved (Fig. 2B,D; Table 2). Consistent with this, diazotrophs can be colimited by Fe and P in this region despite the high iron deposition fluxes and comparatively high Fe concentrations in the surface waters (Mills et al. 2004).

Table 3. Summary indicating the limiting nutrients governing cellular red fluorescence (FL) (a proxy of Chl *a* cell<sup>-1</sup>), cell number (providing information on net cell division), and degree of particle side scatter (a proxy of cell size) for each of the four picoplankton groups analyzed by flow cytometry. The response of the different treatments was compared with the unamended control at 48 h (C<sub>48</sub>) using one-way ANOVA ( $\alpha = 0.05$ ) and followed by a post hoc Tukey test to compare means. Treatments in which means differed significantly from the control at 48 h are grouped according to the relationship between the responses (i.e., <, >, or =); nsd indicates the occasions when no significant difference was observed between treatments or C<sub>48</sub> (i.e.,  $F < F_{crit}$  and  $p > 0.05$ ).

Experiment	Nutrient limiting FL cell <sup>-1</sup>	Mean comparison for FL cell <sup>-1</sup>	Nutrient limiting cell abundance	Mean comparison of cell abundance	Comparison of mean cell size (side scatter)
<i>Taxon dim Synechococcus</i>					
I	N	NPFe=NP*=N	NP	NP*=NPFe	nsd
II	N	(N=NP=NPFe)>NFe	NP	NPFe>NP	N>(NFe=NP=NPFe)
III	N	N>(NFe=NP=NPFe)	PFFe	N inhibits $\mu_{net}$	N>(NFe=NP=NPFe)
IV	nsd	nsd	nsd	N inhibits $\mu_{net}$	nsd
V	N	(N=NP)>(NFe=NPFe)	NP and NFe	NFe=NP=NPFe	N>(NFe=NP)
VI	N	(NP=NPFe) >(N=NFe)	nsd	nsd	N=NP =NPFe
Pooled	N	N=NP	NP	NP=NPFe	N>(NP=NPFe)
<i>Taxon bright Synechococcus</i>					
I	N	N>NP=NPFe	NP	NP*=NPFe	N=NPFe
II	N	(N=NP)>(NFe=NPFe)	NP	NPFe>NP	N>(NFe=NP)>NPFe
III	N	N=NFe=NP=NPFe	NP and NFe	(NFe=NP=NPFe)>N	N>(NFe=NP=NPFe)†
IV	N	(N=NP=NPFe)>NFe	NFe	NPFe>NFe	(N=NP)>(NFe=NPFe)‡
V	N	NP>(N=NFe=NPFe)	NP and NFe	(NFe=NP=NPFe)	(N=NP)>(NFe=NPFe)
VI	N	(NP=NPFe)>(N=NFe)	nsd	nsd	(N=NFe=NP)>NPFe
Pooled	N	N=NP=NPFe	NPFe	NPFe	N>(NP=NPFe=Dust)
<i>Taxon Prochlorococcus</i>					
I	N	N>(NP*=NPFe)	NP	NP*=NPFe	nsd
II	N	N=NFe=NP=NPFe	NP	NP>(NPFe=N)	C <sub>48</sub> >(P=NP=NFe=NPFe)
III	N	(N=NFe)>(NP=NPFe)	N	(NFe=NP)>(N=NPFe)	nsd
IV	N	N=NFe=NP=NPFe	NP	NP=NPFe	N>C <sub>48</sub> >(PFe=NPFe)
V	N	(N=NFe)>(NP=NPFe=D1)	NP	NP	All treatments>C <sub>48</sub>
VI	N	(N=NFe)>(NP=NPFe)	NP	NP	nsd
Pooled	N	N=NP=NPFe	NP	NP=NPFe	nsd
<i>Taxon picoeukaryotes</i>					
I	N	N>(NP*=NPFe)	NP	(NP*=NPFe)>N	nsd
II	N	(NFe=NP)>(N=NPFe)	NP	NP>NPFe	NP >(N=DI=D2)
III	N	N=NFe=NP=NPFe	NP	NP=NPFe	N=NP
IV	N	(NP=NPFe)>(N=NFe)	NFe	NP=NPFe	nsd
V	N	N>(NFe=NP=NPFe)	NP and NFe	NP>(NFe=NPFe)	NFe=N
VI	NP	(NP=NPFe)>(N=NFe)	NP	NP>N	(N=NFe=NP=NPFe)‡
Pooled	N	N=NP=NPFe	NP	NP=NPFe	nsd

\*  $n = 2$ ;  $n = 3$  elsewhere.  
 † C<sub>48</sub> > (P = Fe).  
 ‡ C<sub>48</sub> > PFe.

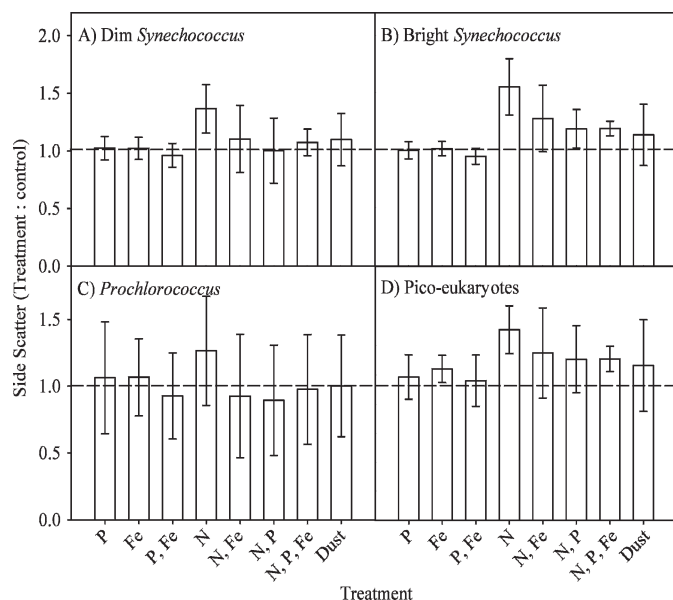


Fig. 6. The effect of nutrient additions on particle side scatter (cell size) in the different picoplankton groups when assessed across all six experiments. (A) Dim *Synechococcus*. (B) Bright *Synechococcus*. (C) *Prochlorococcus*. (D) Picoeukaryotes. In each experiment, the relative response of each treatment was calculated from the ratio of the treatment to the unamended control at 48 h. The dust treatment represents pooled results for D1 and D2 dust additions. The average relative responses for each treatment were calculated and are shown  $\pm$  standard deviation ( $n = 6$ ). The dashed line at a value of 1 indicates the unamended control. The relative responses were compared by one-way ANOVA ( $n = 6$ ,  $\alpha = 0.05$ ) and differences identified post hoc using the Tukey means comparison test.

The conclusions drawn from the bioassay experiments reported here are not unique. Proximal N limitation of chlorophyll accumulation and of  $^{14}\text{C}$  productivity has been obtained along longitudinal transects in oligotrophic regions of the North Atlantic (Graziano et al. 1996; Moore et al. 2006, 2008). These transects included waters with differing picophytoplankton community structure. In particular, the *Prochlorococcus*:*Synechococcus* ratio, which can be used as an index of the degree of oligotrophy (Zubkov et al. 2003), varied widely in our study. Thus, proximal N limitation of net chlorophyll synthesis and NP colimitation of net population growth appear to be widely distributed conditions in picophytoplankton communities of the tropical and subtropical North Atlantic.

Is there a biochemical basis that can reconcile the phytoplankton response observed in our experiments and the interpretation based on dissolved nutrient measurements? Bulk parameters such as chlorophyll concentration and primary production yield little information that can be used to understand the mechanisms operating at the cell or community level. Here we address this concern by examining the response of various picophytoplankton populations to the nutrient amendments. Picophytoplankton are abundant in phytoplankton assemblages of the tropical North Atlantic (Perez et al. 2005) and accounted on average for 72% (range 35–95%) of the phytoplankton

biomass in our experiments (results not shown). Thus, changes in the cellular characteristic of the picophytoplankton are likely to dominate the response of the phytoplankton as a whole in our experiments.

In AFC, fluorescence per cell can be used as a proxy for cellular pigment content, and side scatter per cell can be used as a proxy of cell size (DuRand et al. 2002). Measuring these cellular properties enables us to assess the physiological response of different picophytoplankton populations. The results from the AFC shed light on the physiological processes affected by N and P limitation in tropical phytoplankton assemblages. In particular, cellular red fluorescence is a sensitive indicator of nitrogen status of the population, with increases of red fluorescence accompanying release of primary productivity from nutrient limitation. Although chlorophyll accounts for only a small proportion of the N cellular demand (Geider and La Roche 2002), the response of N-limited populations to addition of N is observed because of the high nitrogen content of pigment-binding proteins and photosynthetic electron transfer chain components (Raven 1990) that must be synthesized to allow net chlorophyll accumulation. In our experiments, significant increases in inferred cell sizes for the *Synechococcus* groups were often observed in the nitrogen treatment (Table 3), suggesting that the increase in chlorophyll was sometimes accompanied by a buildup in cellular components.

The contrasting elemental stoichiometries of different components of the phytoplankton cell (Geider and La Roche 2002) may explain why primary productivity and chlorophyll synthesis were proximally N limited in our experiments, whereas net cell division required P in addition to N (Fig. 6; Table 3). Phosphorus is required for RNA and DNA synthesis and thus for biomass synthesis and cell division. Ribosomes are the organelles richest in P with a N:P of approximately 6 in prokaryotes (Sterner and Elser 2001). In addition, phospholipids are a component of most intracellular membranes and the plasma membrane, and thus the proportion of phospholipids is expected to be higher in rapidly growing cells. *Prochlorococcus* is an exception to this generality with sulfolipids instead of phospholipids in its membranes, making this group especially adapted to grow in low dissolved phosphorus conditions (Van Mooy et al. 2006). In contrast, photosynthetic components contain very little P with an average N:P ratio of 80 (Sterner and Elser 2001). Significantly, thylakoid membranes contain glycolipids rather than phospholipids, and thus, in the short term, it appears that a supply of phosphorus is not essential for increasing chloroplast components and photosynthesis rates.

Nitrogen limitation of primary production is expected in the oligotrophic open sea based on the recent model of Klausmeier et al. (2004) in which resource allocation to two classes of cellular machinery (assembly or resource acquisition) is used to predict optimal N:P stoichiometric ratios under diverse environmental conditions. Their model predicts that in nutrient-poor, oligotrophic conditions, the N:P ratio of phytoplankton will range between 35 and 45, much higher than the Redfield proportion of 16. In their

model, this result is due to the larger proportion of N-rich resource acquisition machinery (e.g., proteins involved in photosynthesis and nutrient uptake) required for phytoplankton growing in oligotrophic conditions relative to P-rich assembly machinery (ribosomes) required for protein synthesis. The Klausmeier et al. (2004) model implies that phytoplankton communities will tend to be nitrogen limited in nutrient-poor environments, even when the N:P of the nutrient-rich deep-water supply from below is higher than the Redfield ratio of 16 and the surface dissolved phosphate concentrations are low (1–3 nmol P L<sup>-1</sup>), as is often observed in the North Atlantic (e.g., N:P of 17–20; Zubkov et al. 2007).

Although our experimental results and oceanographic observations can be reconciled through the stoichiometric arguments presented here (Sterner and Elser 2001; Geider and La Roche 2002; Klausmeier et al. 2004), we cannot yet explain the close balance between N, P, and Fe supply that leads to the cascade effect of N, P, and Fe limitation observed in our experiments. Perhaps part of the explanation lies indeed with the supply rate of iron and the role of this nutrient in controlling nitrogen fixation (Falkowski 1997). Diazotrophs, for which growth is independent of the supply of dissolved inorganic nitrogen (e.g., ammonium, nitrate), will be important in preventing the accumulation of dissolved P and Fe concentrations in the surface waters of these regions.

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