LIMNOLOGY and OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 9999, 2024, 1–9

doi: 10.1002/lno.12541

Switches between nitrogen limitation and nitrogen–phosphorus co-limitation in the subtropical North Atlantic Ocean

Zhongwei Yuan [1,2] **Eric P. Achterberg** [1] **Anja Engel** [1] **Minhan Dai** [2] **Thomas J. Browning** [1*]

[1]Marine Biogeochemistry Division, GEOMAR Helmholtz Centre for Ocean Research, Kiel, Germany
[2]State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, China

Abstract

Concentrations of bioavailable inorganic nitrogen (N) and phosphorus (P) are simultaneously depleted in the (sub)tropical North Atlantic Ocean, but it remains unclear if phytoplankton growth rates are N limited or N–P co-limited. Here we present findings from three bottle-scale experiments using a four-by-four matrix of low-level N and P additions, conducted at one site in the subtropical North Atlantic Ocean. Phytoplankton responses were assessed both in terms of bulk chlorophyll *a* (Chl *a*) concentrations and intracellular Chl *a* of dominant *Prochlorococcus* and *Synechococcus* groups. Two matrix experiments suggested that N was independently limiting in situ growth, with no co-limiting role for P, while the third showed co-limitation by both N and P in this region. This switch from N limitation to N–P co-limitation was attributed to an episodic wet deposition event that supplied N, thereby stimulating phytoplankton growth and consuming available P. Such rapid transitions in nutrient limitation in response to environmental forcing might be common in oceanic systems with multiple depleted nutrients.

Nutrient limitation constrains marine primary productivity and can affect phytoplankton community structure (Arrigo 2005; Moore et al. 2013). Accurate assessments of which nutrients are limiting phytoplankton growth are therefore key for understanding current ocean productivity and making projections under future climate conditions (Bindoff et al. 2019). The assessments have typically involved either of two approaches: (i) physiological assessments that imply limitation by specific nutrients (Ammerman et al. 2003; Saito et al. 2014; Ustick et al. 2021), or (ii) direct experimental supply of one or more nutrients and assessment of changes in phytoplankton biomass and/or primary production rates following incubation (Cullen et al. 1992; Beardall et al. 2001).

An important difference between these approaches is observed across the (sub)tropical North Atlantic Ocean (Moore et al. 2013; Ustick et al. 2021). In this region, and in contrast to much of the global ocean, phosphate is strongly depleted alongside biologically available nitrogen (N) (Martiny et al. 2019). Physiological assessments of nutrient stress in this system point towards phosphorus (P) limitation; specifically, (i) rates of alkaline phosphatase, an enzyme responsible for scavenging P from the organically bound pool, are strongly enhanced (Ammerman et al. 2003; Lomas et al. 2010; Mahaffey et al. 2014), and (ii) genomics analysis of *Prochlorococcus*, an abundant phytoplankton species in this region, suggests retention of a range of P stress genes to a greater extent than N stress genes (Ustick et al. 2021). In contrast to physiological assessments, bioassay experiments that directly add N and/or P to seawater have shown that community-level chlorophyll *a* (Chl *a*) biomass, primary production, and Chl *a* per cell of the dominant phytoplankton groups typically do not respond to P supply alone, but rather only increase following N addition, or occasionally following combined N and P addition (e.g., Graziano et al. 1996; Moore et al. 2008; Browning et al. 2017).

A recognized challenge with both approaches is that neither is directly related to limitation of in situ phytoplankton growth rates (Cullen et al. 1992; Moore et al. 2008; Ustick et al. 2021). Specifically, a range of physiological stress responses to nutrient shortage are invoked by phytoplankton to better cope with depleted P, which potentially could restrict

*Correspondence: tbrowning@geomar.de

Additional Supporting Information may be found in the online version of this article.

Author Contribution Statement: Z.Y. and T.J.B. conceived and designed the study. Z.Y. performed the experiments and analyzed the data. E.P.A. led the research cruise and oversaw the nutrient analyses. A.E. oversaw the flow cytometry analyses. Z.Y. and T.J.B. drafted the manuscript. E.P.A., A.E., and M.D. contributed to discussion and revision of the manuscript.

growth rates becoming P-limited despite the low P concentrations (Martiny et al. 2006; Van Mooy et al. 2009; Martin et al. 2014). In turn, the requirement for these physiological responses probably regulates whether the encoding genes are retained or lost within a population (Ustick et al. 2021). On the other hand, one of the inherent complications with the nutrient addition bioassay experiments is that a lack of biomass accumulation following P addition may not necessarily represent the absence of growth rate limitation in a system where the growth and loss terms are tightly coupled (Cullen 1991; Graziano et al. 1996; Moore et al. 2008). By extension, evaluation of multi-nutrient co-limitation of in situ growth rates, for example, by N and P simultaneously, could be similarly challenging (Saito et al. 2008).

Switches in limiting nutrients during bioassay incubations might also make it challenging to identify nutrient co-limitation. The "standard" nutrient addition experiments conducted so far have typically involved supply of high N and P concentrations (typically 2 μM N and 0.2 μM P, respectively) to phytoplankton communities and incubation over 2 d (e.g., Mills et al. 2004; Moore et al. 2008; Browning et al. 2017). In a system such as the (sub)tropical North Atlantic Ocean, where the concentrations of both N and P are simultaneously depleted to low levels, this means that, where not co-limiting, the second most deficient nutrient would likely be exhausted over the experimental timescale, generating a switch from growth limitation by one nutrient to growth limitation by the second (that is serial limitation of growth rates; Moore et al. 2008; Saito et al. 2008). Moreover, the supply of N + P in combination might increase in situ growth rates more than that of N alone but this is potentially difficult to decouple from serial limitation using observations of biomass changes alone (Moore et al. 2008).

Further insights have been obtained from the assessment of changes in intracellular fluorescence of individual phytoplankton groups following nutrient supply (Graziano et al. 1996; Davey et al. 2008; Moore et al. 2008). Whilst fluorescence per Chl *a* can be species-dependent and change in response to environmental conditions (Cullen 1982; Sosik et al. 1989), changes in within-species red fluorescence per Chl *a* under

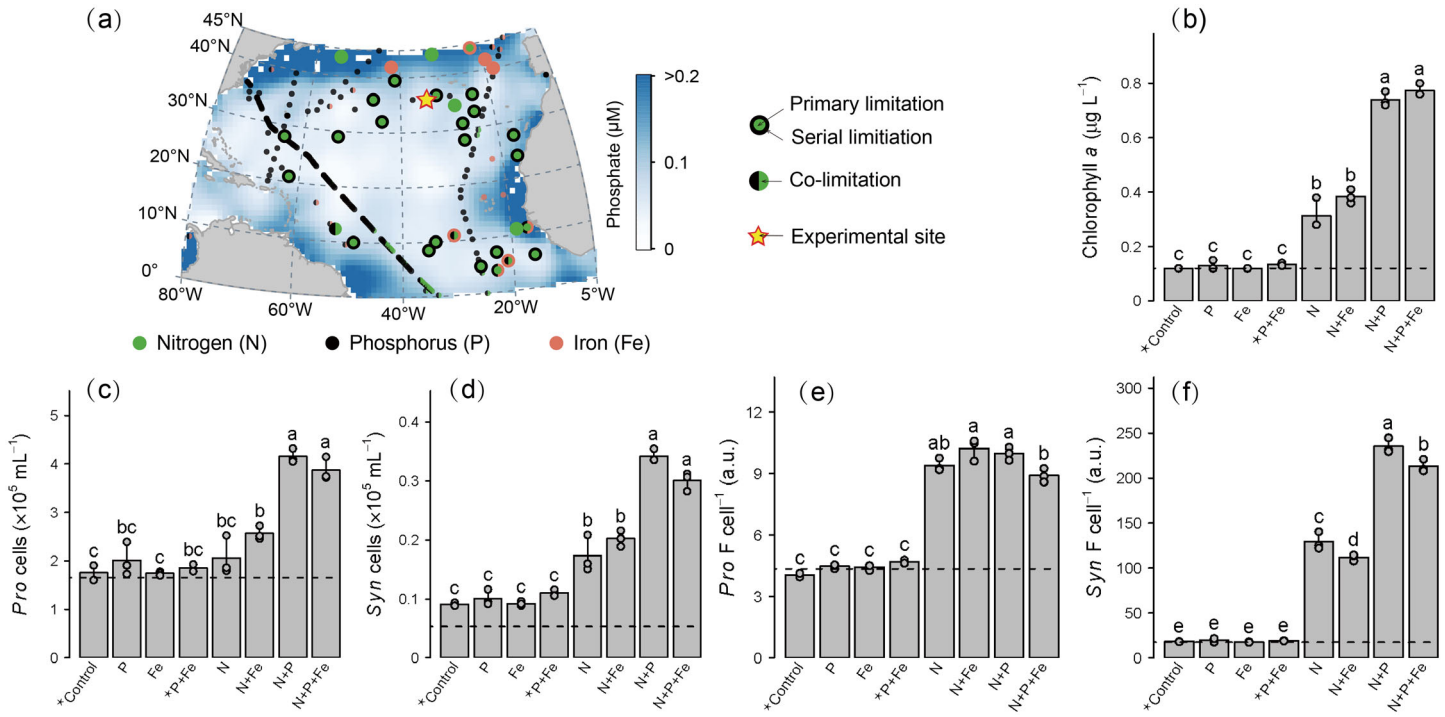

Fig. 1. Nutrient limitation in the North Atlantic Ocean. (**a**) Map showing the location of the nutrient addition bioassay experiments conducted in this study (star) on a background of surface annual average phosphate concentrations from the World Ocean Atlas (https://www.ncei.noaa.gov/products/world-ocean-atlas). Results from previous bioassay experiments are shown (*see* Supporting Information Table S1 for references), with central circle = primary limiting nutrient; outer circle = serial limiting nutrient; split circle = co-limiting nutrient; green = nitrogen; black = phosphorus; red = iron. The smaller circles represent nutrient stress patterns as revealed from an analysis of *Prochlorococcus* metagenomic data (Ustick et al. 2021). (**b**) Chl *a* responses to nutrient additions. Data were previously reported in Yuan et al. (2023). Responses of cell abundances of (**c**) *Prochlorococcus* and (**d**) *Synechococcus*, and intracellular red fluorescence (F cell^{-1}; arbitrary units; a.u.), a proxy for intracellular Chl *a*, of (**e**) *Prochlorococcus* and (**f**) *Synechococcus*, to nutrient additions. Bars are means of replicates with individual values shown as points, $n = 3$ for all except where indicated by an asterisk ($n = 2$). The horizontal dashed lines indicate initial time measurements. Treatment means have been compared using a one-way ANOVA followed by a post hoc Tukey HSD means comparison test (statistically indistinguishable means are labeled with the same letter, $p < 0.05$). The *F* values for the ANOVAs of (**b**–**f**) were 271.8, 45.8, 110.5, 236.9, and 701.0, respectively.

identical light and temperature conditions between nutrient addition treatments in bioassay experiments likely robustly reflect changes in intracellular Chl *a* (Graziano et al. 1996; Davey et al. 2008; Moore et al. 2008). In turn, this should be closely related to cell Chl *a* to carbon ratios (Chl : C; Li et al. 1993). Under fixed light and temperature conditions, culturing studies have found the latter to be closely related to phytoplankton specific growth rates regulated by nutrient (N or P) availability, with increased Chl : C reflecting enhanced light harvesting requirements for faster growth (Supporting Information Text S1). While changes in intracellular Chl *a* of the different phytoplankton groups measured in previous bioassay experiments have often matched changes in cell numbers and typically imply serial N then P limitation, decreases for some groups following N + P addition compared to N alone have also been observed (Davey et al. 2008). The latter has been hypothesized to result from the generation of smaller cells with lower intracellular Chl *a* under faster growth following N + P addition, but this was generally not consistent with changes in side scatter measurements, a proxy for cell size (Davey et al. 2008).

To further investigate the occurrence of either N limitation or N–P co-limitation of phytoplankton specific growth rates, we conducted experiments in the P-depleted subtropical North Atlantic Ocean using a replicated four-by-four matrix of low-level N and P additions. To avoid rapid drawdown of potential serial limiting nutrients (in this system, N or P), which could complicate decoupling of serial and co-limited growth, experiments were of relatively short duration (24–36 h). In addition to bulk Chl *a* changes, we focused on changes in intracellular Chl *a* (via red fluorescence) of *Prochlorococcus* and *Synechococcus*, two dominant picocyanobacteria in this system, which we cautiously interpret in terms of changes in specific growth rate. We compared the results from the low-level matrix experiments in the context of one "standard" nutrient addition experiment, widely deployed in the (sub)tropical North Atlantic Ocean, with high additions of N, P, and Fe in factorial combinations followed by 2-d incubation (Fig. 1a).

Methods

Voyage and seawater collection

Experiments were repeatedly conducted at a quasi-fixed site in September 2021 (M176/2 cruise; Fig. 1a; Yuan et al. 2023). Surface (~ 2–3 m depth) seawater was sampled from a custom-built towed-fish fitted with acid-washed tubing with suction provided by an acid-washed Teflon bellows pump (Dellmeco). Seawater was pumped directly into a purpose-built clean-air laboratory container with positive air pressure maintained via inward airflow passed through a high-efficiency particulate air filter.

Nutrient addition experiments

One "standard" nutrient addition experiment followed published protocols, as described in Yuan et al. (2023). Briefly, the experiment was carried out in 1-liter trace-metal-clean polycarbonate bottles (Nalgene). Natural seawater was collected randomly across 24 bottles at local nighttime and the filling time was ~ 20 min (total volume = 24 liters). Additional natural seawater was also collected at the beginning of the incubation bottle-filling procedure in order to obtain samples for analysis of initial dissolved macronutrient and trace element concentrations, Chl *a* concentrations, and flow cytometry. Incubation bottles were spiked in triplicate to final concentrations of 1.0 μM $NaNO_3$ + 1.0 μM NH_4Cl, 0.2 μM NaH_2PO_4, and 2.0 nM $FeCl_3$ (stabilized in 0.01 M HCl) in 8 factorial combinations (Control, N, P, Fe, N + P, N + Fe, P + Fe, N + P + Fe). Bottles were placed in an on-deck incubator with surface waters continuously supplied from the ship's underway flow-through system to maintain temperatures. The incubator was screened with Blue Lagoon screening (Lee Filters), which maintained irradiance at ~ 30% of that of the surface ocean. After 50-h incubation, the experiment was taken down and each triplicate was subsampled for Chl *a* and flow cytometry. Data for Chl *a* concentrations were previously reported in Yuan et al. (2023).

In addition, three four-by-four matrix of low-level N and P addition experiments were carried out in 125-mL trace-metal-clean polycarbonate bottles (Nalgene; Experiments 1, 2, and 3). Natural seawater was first collected into an 8-liter low-density polyethylene (LDPE) bottle (Nalgene), agitated to homogenize, and then immediately dispensed into the 125-mL bottles. Before filling the 8-liter LDPE bottle, additional natural seawater was also collected for initial measurements of dissolved macronutrients and trace elements, Chl *a*, flow cytometry, and diagnostic phytoplankton pigments. Incubation bottles were spiked in triplicate with a matrix of (i) N (0, 50, 100, and 150 nM NH_4Cl) and P (0, 10, 20, and 30 nM NaH_2PO_4) for Experiments 1 and 2, or (ii) N (0, 100, 300, and 500 nM NH_4Cl) and P (0, 10, 20, and 30 nM NaH_2PO_4) for Experiment 3. Increased concentrations of added N in Experiment 3 were conducted to assess the potential for clearer trends in phytoplankton responses. The incubation protocol followed that of the "standard" experiment except for reducing the incubation time to 24–36 h. The short durations of these experiments were conducted so that observed responses better reflected immediate physiological changes rather than the growth of the dominant species or a bloom of a rare species. The experiments were then taken down and each triplicate was subsampled for Chl *a* and flow cytometry.

Macronutrient concentrations

Samples for nitrate (determined as nitrate + nitrite) and phosphate were filtered (AcroPak 500 0.8/0.2-μm filter capsule, Pall) into 15-mL acid-washed polypropylene tubes, frozen immediately at –20°C and analyzed upon return to a land-based laboratory. Samples for nitrate and phosphate were measured using a SEAL QuAAtro nutrient autoanalyzer system (SEAL Analytical) equipped with 2 m waveguides (World

Precision Instruments Inc.) as described by Patey et al. (2008), with detection limits of 7 and 3 nM, respectively.

Trace element concentrations

Samples for dissolved iron (Fe) were filtered (AcroPak 500 0.8/0.2-μm filter capsule, Pall) into 125-mL acid-washed LDPE bottles (Nalgene) and acidified to pH 1.9 with ultrapure hydrochloric acid (UpA, Romil) onboard. Concentrations of dissolved Fe were analyzed following preconcentration on a SeaFAST device using inductively coupled plasma mass spectrometry (ICP-MS; Element XR, Thermo Scientific), with quantification by isotope dilution (Rapp et al. 2017). Certified values for GEOTRACES standards GSP and GSC matched well with our measured values (Yuan et al. 2023).

Chl *a* concentrations

Samples for Chl *a* were filtered (100 mL) onto 25-mm diameter glass microfibre filter (GF/F; Fisher MF300) filters, extracted in the dark for 16–24 h in 10 mL 90% (by volume) acetone at –20°C, and analyzed on a precalibrated fluorometer (Turner Designs Trilogy) (Welschmeyer 1994).

Flow cytometry

Flow cytometry samples (2 mL) were fixed with paraformaldehyde at a 1% final concentration (methanol-free 16% 10-mL glass ampules, Alfa Aesar/Thermo Fisher), mixed with a vortex, left for 10 min at room temperature in the dark, and then directly frozen at –80°C. Samples were analyzed upon return to land using a FACSCalibur flow cytometer (Becton Dickinson). Cell counts were carried out in CellQuest software (Becton Dickinson). Plots of orange fluorescence versus red fluorescence were used to identify and enumerate *Synechococcus* from other picophytoplankton, and plots of side scatter versus red fluorescence (with *Synechococcus* gated out) were used to enumerate *Prochlorococcus*. The division between picoeukaryotes and small nanoeukaryotes was difficult to assess in the gating analysis and therefore we focused on the two cyanobacteria groups that could be confidently identified.

Diagnostic phytoplankton pigment concentrations

Samples for diagnostic pigments were filtered (4 liters) onto 25-mm diameter GF/F filters (Fisher MF300) and frozen immediately at –80°C. Upon return to a land-based laboratory, pigments were extracted in 90% acetone in plastic vials by homogenization of the filters using glass beads in a cell mill, centrifuged (10 min, 5200 rpm, 4°C), then the supernatant was filtered through 0.2-μm polytetrafluoroethylene filters (VMR International) and subsequently quantified by reverse-phase high-performance liquid chromatography (HPLC, Dionex UltiMate 3000 LC system, Thermo Scientific) (Van Heukelem and Thomas 2001). Pigment standards were from Sigma-Aldrich (USA) and the International Agency for ^{14}C Determination (Denmark). Measured pigment assemblages were separated into three types of phytoplankton size groups (Uitz et al. 2006).

Statistical analyses

All statistical analyses were performed using R software (R version 4.1.2; R Development Core Team 2021). The significance of differences among treatments in the nutrient addition experiments were tested by one-way analysis of variance (ANOVA) followed by post hoc Tukey HSD (for the "standard" experiment) or Fisher's LSD (for the matrix experiments) means comparison tests using functions embedded in the "agricolae" package (version 1.3-7; de Mendiburu 2023).

Results and discussion

Initial concentrations of both nitrate and phosphate were always depleted (6–27 and 5–7 nM, respectively). However, derived P*, the excess available phosphate relative to nitrate assuming a 16 : 1 N : P requirement of phytoplankton, were 4–6 nM (P* = phosphate – nitrate/16; Table 1). This calculation does not include concentrations of ammonium (not determined), but assuming an upper ammonium concentration of 20 nM (Clark et al. 2008) and adding this to concentrations of nitrate, does not greatly change these values (P* range 3–5 nM). Regardless of other accessible organic forms of nitrogen (Zubkov et al. 2003, 2008; Berthelot et al. 2021), from a stoichiometric perspective based on a fixed N : P requirement of 16 (acknowledging the substantial potential variability in this ratio; Klausmeier et al. 2004; Martiny et al. 2013), dissolved P was therefore in excess of phytoplankton requirements relative to N. In line with the measured nutrient concentrations, the "standard" bioassay showed very similar responses to those previously conducted in the region (Fig. 1). The results of Chl *a* have been reported previously in Yuan et al. (2023).

Table 1. Initial conditions for bioassay experiments.

Experiment	Standard	1	2	3
Date	18 Sep	20 Sep	22 Sep	24 Sep
Incubation duration (h)	50	24	36	28
Sea surface temperature (°C)	24.7	24.8	24.9	24.6
Salinity	36.5	36.5	36.4	36.3
Nitrate (nM)	27	6	12	12
Phosphate (nM)	7	5	5	7
P* (nM)	5	5	4	6
Dissolved Fe (nM)	0.59	0.52	0.72	0.86
Chl *a* (μg L^{-1})	0.12	0.12	0.10	0.18
Prochlorococcus (cells mL^{-1})	165,383	125,025	127,438	124,232
Prochlorococcus F cell^{-1} (a.u.)	4.4	3.8	4.1	8.0
Synechococcus (cells mL^{-1})	5306	6291	9198	4141
Synechococcus F cell^{-1} (a.u.)	17.5	27.2	19.3	79.7

a.u., arbitrary units; *F*, intracellular red fluorescence.
P* represents excess available phosphate based on a 16 : 1 N : P requirement of phytoplankton (= phosphate – nitrate/16).

Specifically, no significant differences in Chl *a* were observed between the control and those amended with P alone, whereas the supply of N alone resulted in a significant increase (2.6-fold) in Chl *a*, and the combined supply of N + P further enhanced this (6.2-fold). Consistent with earlier experiments (Fig. 1a), and in line with the elevated dissolved Fe concentrations (0.59 nM; Table 1), supplying Fe in any nutrient combination did not stimulate any further significant increases.

The responses of bulk Chl *a* were also generally reflected in the cell abundances of *Prochlorococcus* and *Synechococcus*. Neither group responded to P addition but *Prochlorococcus* showed 1.2- and 2.4-fold increases in cell numbers following supply of N or N + P, and *Synechococcus* showed 1.9- and 3.8-fold increases. Intracellular Chl *a* also showed no responses to P addition, but serial responses to N then N + P supply for *Synechococcus* (7.1- and 12.9-fold increases, respectively), whilst responding the same to either N or N + P supply for *Prochlorococcus* (2.4-fold). As previously discussed (Moore et al. 2008), there appear two likely interpretations of the sequential enhancements following N then N + P: either (i) N is the single nutrient limiting the in situ growth rates of *Prochlorococcus* and *Synechococcus*, with phosphate concentrations being low but non-limiting and that only following artificial N supply in the experiment does the increase in phytoplankton biomass and associated artificial phosphate drawdown lead to P limitation, or (ii) N and P are co-limiting in situ growth rates, with N supply increasing growth rates to an extent, but this is further enhanced by supply of N + P.

The inconsistency between responses in *Prochlorococcus* cell abundances and intracellular Chl *a* has been observed previously, with reductions in intracellular Chl *a* following N + P (+ Fe) treatments in comparison to + N (+ Fe) treatments observed in four out of the six experiments in Davey et al. (2008), which were conducted in tropical Atlantic waters further to the south. Davey et al. (2008) hypothesized that this resulted from the generation of smaller cells with lower intracellular Chl *a* under faster growth following N + P addition, but this was not consistent with changes in side scatter measurements, a proxy for cell size, which generally show little change or small enhancements in N + P treatments over N alone (Supporting Information Fig. S1). An alternative explanation is that under the N + P treatment, both here and in Davey et al. (2008), *Prochlorococcus* occasionally ran out of experimentally supplied ammonium due to the enhanced growth of *Synechococcus* and other picoeukaryotic/nanoeukaryotic phytoplankton and this was reflected accordingly in reduced growth rates and intracellular Chl *a* (*Prochlorococcus* has been found to be incapable of using nitrate in previous experiments in this region; Moore et al. 2008). The latter groups, unlike *Prochlorococcus*, can utilize both the experimentally supplied ammonium and nitrate and thus potentially maintain enhanced growth rates and intracellular Chl *a* in the N + P treatment over the full experimental duration.

The low-level N and P matrix of additions provided further insights (Fig. 2). Experiments were conducted with low-magnitude nutrient amendments and relatively shorter durations in order to restrict preferential growth and nutrient utilization by initially rarer, larger phytoplankton with higher nutrient affinities and faster growth rates. For all experiments, picophytoplankton groups, especially *Prochlorococcus* and *Synechococcus*, were always the most abundant assemblages in initial surface waters (Table 1; Supporting Information Fig. S2). Over the short experimental durations (24–36 h), combined with the effects of grazing, changes in absolute cell abundances were restricted, and clear trends were not observed (Supporting Information Fig. S3), therefore preventing robust calculation of net growth rates for the different groups. We focus our analysis on changes in bulk Chl *a* concentrations, and intracellular Chl *a* of *Prochlorococcus* and *Synechococcus* (responding more rapidly and being less affected by grazing), which we cautiously interpret as a proxy for changes in Chl : C ratios and, under the identical temperature and light across experimental treatments, specific growth rates (Supporting Information Text S1; Fig. S4). Changes in side scatter for these groups, a proxy for cell size, were restricted, with generally only small increases in response to N amendments (Supporting Information Fig. S5); thus normalizing intracellular Chl *a* (fluorescence per cell) to cell size (side scatter) produced the same trends as intracellular Chl *a* alone (Supporting Information Fig. S6).

Consistent with the "standard" experiment, adding a range of low-level P (10, 20, and 30 nM) alone led to no increases in bulk Chl *a* or intracellular Chl *a* of *Prochlorococcus* or *Synechococcus*, with all remaining close to initial conditions (Fig. 2). In contrast, supply of N alone in Experiments 1 and 2, and N + P in combination in Experiment 3 generally led to both increased bulk Chl *a* concentrations and intracellular Chl *a* of the two phytoplankton groups. That the increase in bulk Chl *a* and intracellular Chl *a* in Experiments 1 and 2 generally only responded to N addition at the lowest supplied N additions (100 nM for *Prochlorococcus* and 50 and 100 nM for *Synechococcus*) pointed towards primary N limitation of specific growth rates in these two experiments, with P not playing a direct co-limiting role. In contrast to the lowest level N additions in Experiments 1 and 2 (50 and 100 nM), subsequent enhancements in both bulk Chl *a* and intracellular Chl *a* were observed with increasing P addition at the highest N addition (150 nM). This suggests that, with this artificial N addition, the stimulated biomass accumulation (of larger phytoplankton, as changes in *Prochlorococcus* and *Synechococcus* numbers were not clear; Supporting Information Fig. S3) and/or intracellular phosphate uptake of potentially all phytoplankton groups (e.g., Zubkov et al. 2007), depleted the initial available P (5 nM in both cases) to the extent that P became serially growth-limiting. Furthermore, the accentuated increases following progressively elevated P supply at the highest N treatment (150 nM) in Experiment 2 in comparison to Experiment 1 potentially reflected the longer incubation duration of this experiment (36 vs. 24 h) and therefore more time for P drawdown.

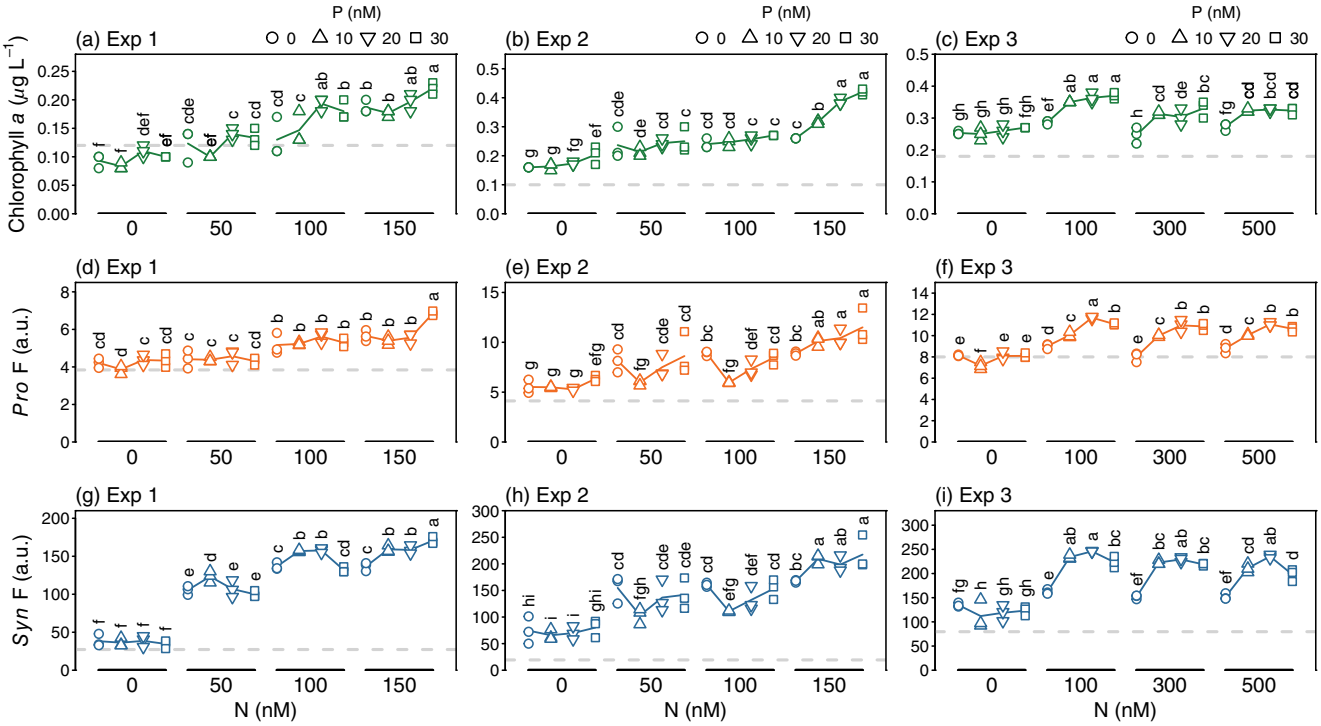

Fig. 2. Low nutrient addition matrix experiments. Responses of (**a**–**c**) bulk chlorophyll *a* and intracellular red fluorescence (F cell^{-1}; arbitrary units; a.u.), a proxy for intracellular Chl *a*, of (**d**–**f**) *Prochlorococcus* and (**g**–**i**) *Synechococcus*. Circles, right-side up triangles, upside-down triangles, and squares denote added phosphate concentrations at 0, 10, 20, and 30 nM, respectively. The incubation durations of Experiments 1, 2, and 3 were 24, 36, and 28 h, respectively. All observations from the triplicate biologically independent replicates are shown. The solid lines indicate the treatment means. The horizontal dashed lines indicate initial time measurements. Treatment means are compared using a one-way ANOVA followed by a post hoc Fisher's LSD means comparison test (statistically indistinguishable means are labeled with the same letter, $p < 0.05$). The *F* values for the ANOVAs of (**a**–**i**) were 19.8, 33.1, 22.7, 20.0, 13.9, 68.8, 207.5, 19.2, and 60.5, respectively.

The lack of response of *Prochlorococcus* to the smallest N addition (50 nM) in Experiments 1 and 2, whilst *Synechococcus* showed 2.9-fold increases in intracellular Chl *a*, is surprising. Although *Synechococcus* might generally be expected to be under stronger nutrient limitation than *Prochlorococcus*, due to its moderately larger cell size (up to 1.5 μm cf. *Prochlorococcus* diameter of ~ 0.6 μm) and subsequent smaller surface area to volume ratio (Moore et al. 2013; Lomas et al. 2014), both groups, if N limited, would be expected to respond to this nutrient treatment. One hypothesis is that because *Prochlorococcus* is less nutrient limited in situ than *Synechococcus*, this group had invested less resources into ammonium uptake transporters (García-Fernández et al. 2004) and therefore could not respond as rapidly to increases in intracellular Chl *a* (and by extension, growth rates). Alternatively, *Prochlorococcus* may have been optimized to use more abundant organic forms of nitrogen such as urea (Painter et al. 2008; Shilova et al. 2017) and amino acids (Zubkov et al. 2003; Berthelot et al. 2021).

In contrast to Experiments 1 and 2, in Experiment 3, where the supplied N concentrations were higher (100, 300, and 500 nM respectively), P appeared to be co-limiting with N (Fig. 2c,f,i). This was identified via N supply alone (any concentration) not enhancing either bulk Chl *a* concentrations or intracellular Chl *a* of the two phytoplankton groups, but required supplementary addition of at least the lowest P addition (10 nM). Furthermore, for this experiment with elevated N supply concentrations, maximal bulk Chl *a* concentrations and intracellular Chl *a* were already observed at the lowest N (100 nM) and P (10 nM) additions, with subsequent higher concentrations producing no further enhancement. This suggested that these lowest additions were enough to saturate requirements over the relatively short experimental timescale (Lomas et al. 2014). The subsequent small decreases in intracellular Chl *a* at the highest combined N + P additions, also previously observed in multi-nutrient treatments in some "standard" bioassay experiments (Davey et al. 2008; Browning et al. 2022), presumably represent more rapid depletion of the supplied nutrients to growth-limiting levels over the experimental timescale, due to a combination of the higher initial phytoplankton concentrations (indicated by higher Chl *a* concentrations; Table 1) and the increased growth of larger phytoplankton, with capability for enhanced nutrient uptake and storage, stimulated by larger nutrient additions at this experimental site.

In summary, the low-level nutrient matrix of additions suggested that, depending on the specific experimental conditions at this single site, (i) N was independently limiting in situ growth rates of *Prochlorococcus* and *Synechococcus* with no co-limiting role for P (Experiments 1 and 2), or (ii) N and P were simultaneously co-limiting growth rates (Experiment 3). While the former matched many of the previous "standard" nutrient addition experiments (Fig. 1; Davey et al. 2008; Moore et al. 2008) in that N was exclusively limiting the growth rates of *Prochlorococcus* and *Synechococcus* in the natural, undisturbed seawater, the latter scenario of N–P co-limitation could potentially be attributed to a shift in environmental conditions. Specifically in our field campaign, an intense rainfall event occurred immediately before the initiation of Experiment 3, marked by a precipitation rate of up to 61 mm d^{-1} on 23–24 September (Yuan et al. 2023). In this event, atmospheric aerosols, typically characterized by an N : P ratio of > 500 (Baker et al. 2010; Zamora et al. 2013), were deposited into surface seawater. Yuan et al. (2023) showed that this extra N supply was quickly exhausted to support a ~ 2-fold increase in Chl *a* biomass (also see Table 1). Making the reasonable assumption that P supply from the rainfall event was minimal (Baker et al. 2010; Zamora et al. 2013), we hypothesize that this external N supply and the subsequent phytoplankton biomass enhancement depleted bioavailable P in seawater (including phosphate and bioavailable dissolved organic P). Although noting that any reduction in the residual phosphate was too low to be discernible (measured as 7 nM; Table 1), alongside high levels of plasticity in phytoplankton N : P requirements (Klausmeier et al. 2004; Martiny et al. 2013), we expect this N addition to have resulted in an intensification of P stress, as is often observed following the artificial N addition in bioassay experiments in low P settings (Mahaffey et al. 2014; Browning et al. 2017). Therefore, N limitation versus N–P co-limitation appears to be sensitive to changes in external supply ratios of N versus P in a N–P co-depleted system such as the (sub)tropical North Atlantic Ocean.

Bioassay experiments can be challenging to unambiguously interpret in terms of nutrient co-limitation of in situ specific growth rates due to the potential for drawdown of low concentration, but not initially limiting, nutrients over experimental timescales. Here, we attempted to address this by conducting a matrix of low-level nutrient additions with relatively short incubation durations and detecting intracellular Chl *a* as a relative indicator for specific growth rates. As previously discussed, an important assumption of this approach is assuming co-variability between intracellular fluorescence of either *Prochlorococcus* or *Synechococcus* and their specific growth rates. With this caveat, our results agree with previous "standard" bioassay experiments in suggesting N limitation, or occasional N–P co-limitation, of phytoplankton growth rates in the subtropical North Atlantic Ocean (e.g., Graziano et al. 1996; Moore et al. 2008; Browning et al. 2017). In this case, our experimental results would point toward strong phytoplankton P stress, as observed in this ocean region in Ustick et al. (2021), presumably reflecting enhanced physiological adjustments to low P availability, while not directly reflecting proximal P limitation of growth.

To our knowledge, our results present the first field experimental observation of very rapid (~ 1 d) temporal shifts in ocean phytoplankton nutrient limitation to transient environmental forcing. The transition from N limitation to N–P co-limitation observed here following a major rainfall event underscores the susceptibility of systems where multiple nutrients are depleted to switches in limiting nutrients, and can potentially be extended to other nutrient combinations elsewhere in the global ocean, for example, Fe and either N, manganese, or vitamin B_{12} (Browning and Moore 2023). Our study therefore furthermore prompts a deeper consideration of the ensuing biogeochemical implications of nutrient limitation shifts in oceanic ecosystems.

Data availability statement

Data are stored and available on Zenodo (https://zenodo.org/doi/10.5281/zenodo.10476992).

References

Ammerman, J. W., R. R. Hood, D. A. Case, and J. B. Cotner. 2003. Phosphorus deficiency in the Atlantic: An emerging paradigm in oceanography. Eos. Trans. Am. Geophys. Union **84**: 165–170. doi:10.1029/2003EO180001

Arrigo, K. R. 2005. Marine microorganisms and global nutrient cycles. Nature **437**: 349–355. doi:10.1038/nature04159

Baker, A. R., T. Lesworth, C. Adams, T. D. Jickells, and L. Ganzeveld. 2010. Estimation of atmospheric nutrient inputs to the Atlantic Ocean from 50°N to 50°S based on large-scale field sampling: Fixed nitrogen and dry deposition of phosphorus. Global Biogeochem. Cycl. **24**: GB3006. doi:10.1029/2009GB003634

Beardall, J., E. Young, and S. Roberts. 2001. Approaches for determining phytoplankton nutrient limitation. Aquat. Sci. **63**: 44–69. doi:10.1007/PL00001344

Berthelot, H., S. Duhamel, S. L'Helguen, J. F. Maguer, and N. Cassar. 2021. Inorganic and organic carbon and nitrogen uptake strategies of picoplankton groups in the northwestern Atlantic Ocean. Limnol. Oceanogr. **66**: 3682–3696. doi:10.1002/lno.11909

Bindoff, N. L., and others. 2019. Changing ocean, marine ecosystems, and dependent communities. p. 447–587. *In* H.-O. Pörtner and others [eds.], IPCC special report on the ocean and cryosphere in a changing climate. Cambridge University Press. doi:10.1017/9781009157964.007

Browning, T. J., E. P. Achterberg, J. C. Yong, I. Rapp, C. Utermann, A. Engel, and C. M. Moore. 2017. Iron limitation of microbial phosphorus acquisition in the tropical

North Atlantic. Nat. Commun. **8**: 15465. doi:10.1038/ncomms15465

Browning, T. J., X. Liu, R. Zhang, and others. 2022. Nutrient co-limitation in the subtropical Northwest Pacific. Limnol. Oceanogr. Lett. **7**: 52–61. doi:10.1002/lol2.10205

Browning, T. J., and C. M. Moore. 2023. Global analysis of ocean phytoplankton nutrient limitation reveals high prevalence of co-limitation. Nat. Commun. **14**: 5014. doi:10.1038/s41467-023-40774-0

Clark, D. R., A. P. Rees, and I. Joint. 2008. Ammonium regeneration and nitrification rates in the oligotrophic Atlantic Ocean: Implications for new production estimates. Limnol. Oceanogr. **53**: 52–62. doi:10.4319/lo.2008.53.1.0052

Cullen, J. J. 1982. The deep chlorophyll maximum: comparing vertical profiles of chlorophyll *a*. Can. J. Fish. Aquat. Sci. **39**: 791–803.

Cullen, J. J. 1991. Hypotheses to explain high-nutrient conditions in the open sea. Limnol. Oceanogr. **36**: 1578–1599. doi:10.4319/lo.1991.36.8.1578

Cullen, J. J., X. Yang, and H. L. MacIntyre. 1992. Nutrient limitation of marine photosynthesis, p. 69–88. *In* P. G. Falkowski, A. D. Woodhead, and K. Vivirito [eds.], Primary productivity and biogeochemical cycles in the sea. Springer.

Davey, M., G. A. Tarran, M. M. Mills, C. Ridame, R. J. Geider, and J. LaRoche. 2008. Nutrient limitation of picophytoplankton photosynthesis and growth in the tropical North Atlantic. Limnol. Oceanogr. **53**: 1722–1733. doi:10.4319/lo.2008.53.5.1722

de Mendiburu, F. (2023). agricolae: Statistical Procedures for agricultural research. R package version 1.3-7. Available from https://CRAN.R-project.org/package=agricolae

García-Fernández, J. M., N. T. de Marsac, and J. Diez. 2004. Streamlined regulation and gene loss as adaptive mechanisms in *Prochlorococcus* for optimized nitrogen utilization in oligotrophic environments. Microbiol. Mol. Biol. Rev. **68**: 630–638. doi:10.1128/mmbr.68.4.630-638.2004

Graziano, L. M., R. J. Geider, W. K. W. Li, and M. Olaizola. 1996. Nitrogen limitation of North Atlantic phytoplankton: Analysis of physiological condition in nutrient enrichment experiments. Aquat. Microb. Ecol. **11**: 53–64. doi:10.3354/ame011053

Klausmeier, C. A., E. Litchman, T. Daufreshna, and S. A. Levin. 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature **429**: 171–174. doi:10.1038/nature02454

Li, W. K. W., T. Zohary, Y. Z. Yacobi, and A. M. Wood. 1993. Ultraphytoplankton in the eastern Mediterranean Sea—Towards deriving phytoplankton biomass from flow cytometric measurements of abundance, fluorescence and light scatter. Mar. Ecol. Prog. Ser. **102**: 79–88. doi:10.3354/meps102079

Lomas, M. W., A. L. Burke, D. A. Lomas, D. W. Bell, C. Shen, S. T. Dyhrman, and J. W. Ammerman. 2010. Sargasso Sea phosphorus biogeochemistry: An important role for dissolved organic phosphorus (DOP). Biogeosciences **7**: 695–710. doi:10.5194/bg-7-695-2010

Lomas, M. W., J. A. Bonachela, S. A. Levin, and A. C. Martiny. 2014. Impact of ocean phytoplankton diversity on phosphate uptake. Proc. Natl. Acad. Sci. USA **111**: 17540–17545. doi:10.1073/pnas.1420760111

Mahaffey, C., S. Reynolds, C. E. Davis, and M. C. Lohan. 2014. Alkaline phosphatase activity in the subtropical ocean: Insights from nutrient, dust and trace metal addition experiments. Front. Mar. Sci. **1**: 73. doi:10.3389/fmars.2014.00073

Martin, P., S. T. Dyhrman, M. W. Lomas, N. J. Poulton, and B. A. S. Van Mooy. 2014. Accumulation and enhanced cycling of polyphosphate by Sargasso Sea plankton in response to low phosphorus. Proc. Natl. Acad. Sci. USA **111**: 8089–8094. doi:10.1073/pnas.1321719111

Martiny, A. C., M. L. Coleman, and S. W. Chisholm. 2006. Phosphate acquisition genes in Prochlorococcus ecotypes: Evidence for genome-wide adaptation. Proc. Natl. Acad. Sci. US A. **103**: 12552–12557. doi:10.1073/pnas.0601301103

Martiny, A. C., C. T. A. Pham, F. W. Primeau, J. A. Vrugt, J. K. Moore, S. A. Levin, and M. W. Lomas. 2013. Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. Nat. Geosci. **6**: 279–283. doi:10.1038/ngeo1757

Martiny, A. C., M. W. Lomas, W. Fu, and others. 2019. Biogeochemical controls of surface ocean phosphate. Sci. Adv. **5**: eaax0341. doi:10.1126/sciadv.aax0341

Mills, M. M., C. Ridame, M. Davey, J. La Roche, and R. J. Geider. 2004. Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. Nature **429**: 292–294. doi:10.1038/nature02550

Moore, C. M., M. M. Mills, R. Langlois, A. Milne, E. P. Achterberg, J. La Roche, and R. J. Geider. 2008. Relative influence of nitrogen and phosphorus availability on phytoplankton physiology and productivity in the oligotrophic sub-tropical North Atlantic Ocean. Limnol. Oceanogr. **53**: 291–305. doi:10.4319/lo.2008.53.1.0291

Moore, C. M., M. M. Mills, K. R. Arrigo, and others. 2013. Processes and patterns of oceanic nutrient limitation. Nat. Geosci. **6**: 701–710. doi:10.1038/ngeo1765

Painter, S. C., R. Sanders, H. N. Waldron, M. I. Lucas, and S. Torres-Valdes. 2008. Urea distribution and uptake in the Atlantic Ocean between 50° N and 50° S. Mar. Ecol. Prog. Ser. **368**: 53–63. doi:10.3354/meps07586

Patey, M. D., M. J. A. Rijkenberg, P. J. Statham, M. C. Stinchcombe, E. P. Achterberg, and M. Mowlem. 2008. Determination of nitrate and phosphate in seawater at nanomolar concentrations. Trends Anal. Chem. **27**: 169–182. doi:10.1016/j.trac.2007.12.006

R Development Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Rapp, I., C. Schlosser, D. Rusiecka, M. Gledhill, and E. P. Achterberg. 2017. Automated preconcentration of Fe, Zn, Cu, Ni, Cd, Pb, Co, and Mn in seawater with analysis using high-resolution sector field inductively-coupled plasma mass spectrometry. Anal. Chim. Acta **976**: 1–13. doi:10.1016/J.ACA.2017.05.008

Saito, M. A., T. J. Goepfert, and J. T. Ritt. 2008. Some thoughts on the concept of colimitation: Three definitions and the importance of bioavailability. Limnol. Oceanogr. **53**: 276–290. doi:10.4319/lo.2008.53.1.0276

Saito, M. A., M. R. McIlvin, D. M. Moran, T. J. Goepfert, G. R. DiTullio, A. F. Post, and C. H. Lamborg. 2014. Multiple nutrient stresses at intersecting Pacific Ocean biomes detected by protein biomarkers. Science **345**: 1173–1177. doi:10.1126/science.1256450

Shilova, I. N., and others. 2017. Differential effects of nitrate, ammonium, and urea as N sources for microbial communities in the North Pacific Ocean. Limnol. Oceanogr. **62**: 2550–2574. doi:10.1002/lno.10590

Sosik, H. M., S. W. Chisholm, and R. J. Olson. 1989. Chlorophyll fluorescence from single cells: Interpretation of flow cytometric signals. Limnol. Oceanogr. **34**: 1749–1761. doi:10.4319/lo.1989.34.8.1749

Uitz, J., H. Claustre, A. Morel, and S. B. Hooker. 2006. Vertical distribution of phytoplankton communities in open ocean: An assessment based on surface chlorophyll. J. Geophys. Res. Ocean. **111**: C08005. doi:10.1029/2005JC003207

Ustick, L. J., A. A. Larkin, C. A. Garcia, and others. 2021. Metagenomic analysis reveals global-scale patterns of ocean nutrient limitation. Science **372**: 287–291. doi:10.1126/science.abe6301

Van Heukelem, L., and C. S. Thomas. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. J. Chromatogr. A **910**: 31–49. doi:10.1016/S0378-4347(00)00603-4

Van Mooy, B. A. S., and others. 2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature **458**: 69–72. doi:10.1038/nature07659

Welschmeyer, N. A. 1994. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. Limnol. Oceanogr. **39**: 1985–1992. doi:10.4319/LO.1994.39.8.1985

Yuan, Z., E. P. Achterberg, A. Engel, Z. Wen, L. Zhou, X. Zhu, M. Dai, and T. J. Browning. 2023. Phytoplankton community response to episodic wet and dry aerosol deposition in the subtropical North Atlantic. Limnol. Oceanogr. **68**: 2126–2140. doi:10.1002/lno.12410

Zamora, L. M., J. M. Prospero, D. A. Hansell, and J. M. Trapp. 2013. Atmospheric P deposition to the subtropical North Atlantic: Sources, properties, and relationship to N deposition. J. Geophys. Res. Atmos. **118**: 1546–1562. doi:10.1002/jgrd.50187

Zubkov, M. V., B. M. Fuchs, G. A. Tarran, P. H. Burkill, and R. Amann. 2003. High rate of uptake of organic nitrogen compounds by *Prochlorococcus* cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Appl. Environ. Microbiol. **69**: 1299–1304. doi:10.1128/AEM.69.2.1299-1304.2003

Zubkov, M. V., G. A. Tarran, I. Mary, and B. M. Fuchs. 2008. Differential microbial uptake of dissolved amino acids and amino sugars in surface waters of the Atlantic Ocean. J. Plankton Res. **30**: 211–220. doi:10.1093/plankt/fbm091

Zubkov, M. V., I. Mary, E. M. S. Woodward, P. E. Warwick, B. M. Fuchs, D. J. Scanlan, and P. H. Burkill. 2007. Microbial control of phosphate in the nutrient-depleted North Atlantic subtropical gyre. Environ. Microbiol. **9**: 2079–2089. doi:10.1111/j.1462-2920.2007.01324.x

Acknowledgments

We thank the captain, crew, and scientists of the RV Meteor M176/2 cruise. Tania Klüver, Kerstin Nachtigall, André Mutzberg, Xunchi Zhu, Tim Steffens, and Dominik Jasinski are thanked for assistance with sample analyses and Zuozhu Wen for assistance at sea. Xuechao Wang is thanked for assistance and advice with cruise preparations. The research cruise was funded by Deutsche Forschungsgemeinschaft (DFG) grant "RainbowPlume" to E.P.A. and T.J.B. Z.Y. was supported by the Chinese Scholarship Council (File No. 202106310003). Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

None declared.

Submitted 26 September 2023
Revised 10 January 2024
Accepted 12 February 2024

Associate editor: Laura Bristow