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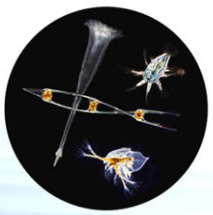

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Nutrient-specific responses of a phytoplankton community: a case study of the North Atlantic Gyre, Azores

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Nutrient concentrations are unevenly distributed in the oceans, influencing the abundance and composition of phytoplankton communities. Even so, the dominant driving factors responsible for variability between phytoplankton communities are still unclear. In the North Atlantic Gyre, the Azores present a good opportunity to study phytoplankton communities of oligotrophic areas that experience nutrient pulses. We followed the development of an enclosed natural phytoplankton community occurring off the coast of Terceira (Azores) and tested the effects of single (nitrate, phosphate, silicate and a mix of the trace metals Fe, Co, Cu, Mo, Zn and Mn) and combined nutrient enrichments on phytoplankton abundance, particulate organic matter (POM) build-up, nutrient drawdown and community composition. Towards the end of the microcosm-based incubation, biomass developed dramatically (430-fold) when all the nutrients considered were added simultaneously. Importantly, the community composition at the end of the incubation was dependent on the combination of nutrients supplied, with diatoms dominating most of the treatments; coccolithophores under Phosphate + Trace Metals; and organisms with characteristics of a nitrogen fixer such as low $\delta^{15}N$ under full nutrient enrichment. These results indicate group-specific nutrient requirements and limitations occurring near the Azores with a few taxa dominating the groups' response to nutrient pulses.

KEYWORDS: nutrients; phytoplankton; community composition; oligotrophic; North Atlantic

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INTRODUCTION

Phytoplankton abundance and community composition is determined by the interplay between biotic and abiotic factors. Nutrient availability varies spatially and temporally, influencing phytoplankton communities accordingly. Low-nutrient concentrations near the cell surface limit nutrient uptake, with consequences for growth rate (Blakman rate limit) and biomass yield (Liebig's Law) (Moore *et al.*, 2013). Often phytoplankton growth is simultaneously limited by several nutrients. Succinctly, co-limitations can be divided into three categories: Type I, in which pools of the nutrients involved in the co-limitation are depleted to equally limiting concentrations and the addition of each of these nutrients is subsequently required to induce a response; Type II, when one nutrient can be substituted for another; and Type III which involves the limitation of one nutrient that affects the ability to uptake other nutrients (Saito *et al.*, 2008; Moore *et al.*, 2013).

The most relevant macronutrients for most phytoplankton species are nitrate and phosphate, although diatoms additionally require silicate for constructing their frustules. In addition to macronutrients, the productivity and species composition of marine phytoplankton are also regulated by trace metals, since they are required for the biochemical function of various enzymes. However, the concentrations of certain elements can reach toxic levels for phytoplankton (Thomas *et al.*, 1980; Paytan *et al.*, 2009; Müller *et al.*, 2015). The limiting potential of each macro and micronutrient depends on many factors, such as phytoplankton species present, their nutrient uptake rates and interactions (Saito *et al.*, 2008). Indeed, species occurrence and succession in phytoplankton communities often result from resource competition, as a consequence of different nutrient utilization strategies (Litchman *et al.*, 2006, 2007). Frequently, coastal (turbulent and nitrate rich) phytoplankton communities are dominated by diatoms, while intermediate nutrient and turbulence conditions are dominated by coccolithophores and low-nutrient concentrations and turbulence conditions are often dominated by dinoflagellates (Margalef, 1978). Several factors have been suggested to be responsible for the observed general trends. Three strategies outlined in Sommer (1984) have been described for phosphorus competition: (i) "velocity-adapted" species with high growth rates and high V_{max} (maximum nutrient uptake rate); (ii) "storage specialists" able to store phosphorus for periods of depletion, with high V_{max} but lower growth rate and (iii) "affinity-adapted", with low half-saturation constants for phosphorus uptake. Rapid changes in light intensity and nutrient concentrations due to increased turbulence close to the coast, have been proposed to favour organisms with the capacity for rapid nutrient uptake, such as the "velocity-adapted" diatoms (Sommer, 1984; Barcelos e Ramos *et al.*, 2012). Additionally, coastal diatoms, such as *Phaeodactylum tricornutum*, have a higher capacity to dissipate excess photosynthetic energy than oceanic diatoms such as *Thalassiosira oceanica* (Lavaud *et al.*, 2007), potentially related to higher iron availability in coastal areas (Sunda *et al.*, 1991; Sunda and Huntsman, 1995). The dissipation of excess energy is very important since it reduces or even prevents the formation of potentially damaging reactive oxygen species. In this respect, open ocean species generally exhibit slower growth rates, decreased iron requirements and assume irregular shapes or reduced sizes to increase their surface to volume ratio (Sunda and Huntsman, 1995). It is also known that large diatoms are very efficient in taking up and storing available nutrients in vacuoles ("storage specialists"). Other species, such as some coccolithophores, have low half-saturation constants for phosphate uptake, giving them a competitive advantage at relatively low phosphate concentrations as "affinity-adapted" strategists (Sommer, 1984). Thus, the enrichment of a single nutrient may promote growth, but not necessarily of the initially dominant species, since it might be adapted to the previous conditions (Moore *et al.*, 2013). Interrelations between nutrients affect microbial dynamics, with consequences for the cycling and remineralization of nutrients. The community's response to nutrient amendment ultimately depends on each species' unique nutrient requirements, which are influenced by specific growth rates, morphology, species interactions, and existing grazers and parasites.

Vast areas of the ocean surface are characterized by simultaneous low concentrations of several nutrients and a year-round low abundance of phytoplankton and chlorophyll *a*. In areas with chlorophyll *a* concentration below 0.1 mg m^{-3}, the environment is considered oligotrophic (Ulloa and Grob, 2009). Nutrient enrichment of up to one order of magnitude into the ocean surface offshore Terceira (Narciso *et al.*, 2016) depends mainly on annual changes in the mixed layer depth and subsurface nutrient fields related to ocean circulation (Valente, 2013). Occasionally, it is also influenced by island runoff, aerosol deposition and island-related upwelling events. Much is still unknown about the phytoplankton communities occurring off the coast of the often oligotrophic Azores, in particular which nutrients limit phytoplankton abundance and succession. Moreover, shifts from "affinity to velocity-adapted" communities might be driven by nutrient pulses after increases in mixed layer depth (periodically reaching mesotrophic conditions) or nitrogen input through nitrogen fixation by diazotrophic cyanobacteria. Still, in oligotrophic

regions, even when nitrogen fixation occurs other nutrients might limit its rate and/or co-limit phytoplankton growth.

Important questions relate to which nutrient or combination of nutrients limit biomass development in oligotrophic areas such as the North Atlantic Gyre and whether there are individual species or group-specific responses to individual nutrients that may impact phytoplankton community composition. To date, little information has been obtained on the accessible waters off Terceira even though they offer an example of a frequently oligotrophic region that experiences natural nutrient pulses, thus providing an ideal ecosystem to address these questions.

Several nutrient manipulation studies have shown phytoplankton group and species-specific responses to nutrient enrichment, either by investigating clonal cultures (Sommer, 1984; Dyhrman and Palenik, 1999; Kaffes *et al.*, 2010; Matthiessen *et al.*, 2012) or natural communities from other regions, both utilizing enclosed bottles or *in situ* work (Schlüter, 1998; Rousseau *et al.*, 2002; Thingstad *et al.*, 2005; Assmy *et al.*, 2007; Boyd *et al.*, 2012; Moisander *et al.*, 2012; Moore *et al.*, 2013). However, there are still uncertainties concerning how natural oligotrophic communities respond to the abrupt addition of nutrients such as those occurring after pronounced vertical mixing, and its implications for phytoplankton community composition.

Here, an enclosed natural phytoplankton community of the North Atlantic Gyre off the Azores was studied to determine if single (nitrate, phosphate, silicate and trace metals) or combined/sequential high-nutrient pulse enrichments affect biomass build-up, taxa occurrence and microphytoplankton community structure. We tested whether: (i) phytoplankton overall biomass build-up of an oligotrophic community occurring off the Azores depends on the limiting nutrient or nutrients; and (ii) group-specific responses vary according to the nature of single or multiple nutrient addition.

METHOD

Experimental Setup

Seawater for the experiment was collected offshore from the Azores (38°37.108′N/27°15.610′W, located on the 500 m depth contour at ~3 nautical miles south of Terceira) by filling vertically a 10 m long tube, which enabled collection of an integrated (mixed) sample of the upper 10 m of the water column. This water had a practical salinity of 36.3 and 21°C (annual variation between 14 and 24°C). The natural community was grown in a modified f/8 media (Guillard and Ryther, 1962), under a matrix of nutrient concentrations in triplicate (Table I). This allowed evaluation of the effects of the presence or absence of nutrients on the phytoplankton communities. Nutrients were added in excess to avoid limitation as well as considerable changes in nutrient availability during incubation. Trace metals bound to the chelator ethylenediaminetetraacetic acid (EDTA) were added at f/8 media concentrations (Guillard and Ryther, 1962). While EDTA increases trace metal solubility, at equilibrium it also considerably reduces the free ion concentrations to the low nano to picomolar range, preventing potentially toxic levels (Guillard and Ryther, 1962; Coale, 1991; Andersen, 2005; Sunda *et al.*, 2005). Overall trace metal additions were four times lower (f/8) than in typical phytoplankton culture media (Guillard and Ryther, 1962). All incubation bottles were acid cleaned (HCl 10% and rinsed three times with the seawater collected for the experiment) to prevent biological as well as iron contamination. At the onset (t0) of the experiment, ~20 L were sampled from the 96 L of seawater collected, to determine the initial

Table I: Treatments and correspondent nutrient concentrations

Treatments		Nutrient concentrations
First fertilization	*Second fertilization (after 5 days)*	
No addition	No addition	Control (~0)
	All nutrients	Nitrate ~220 μmol L^{-1}, Silicate ~26 μmol L^{-1}, Phosphate ~9 μmol L^{-1} and Trace Metals
Nitrate	Nitrate	~220 μmol L^{-1}
	Nitrate + Phosphate	*
Phosphate	Phosphate	~9 μmol L^{-1}
	Phosphate + Trace Metals	*
Silicate	Silicate	~26 μmol L^{-1}
	Silicate + Nitrate	*
Trace Metals	Trace Metals	Theoretical total concentration: iron ~3 μmol L^{-1}, EDTA ~3 μmol L^{-1}, copper ~10 nmol L^{-1}, molybdenum ~7 nmol L^{-1}, zinc ~19 nmol L^{-1}, cobalt ~11 nmol L^{-1} and manganese ~0.2 μmol L^{-1}
	Trace Metals + Silicate	*

*The second addition in the treatments with combined nutrients followed the concentrations added in the first fertilization.

nutrient concentrations, temperature, salinity, pH, community composition, phytoplankton abundance and POM. Part of the remaining water was used to fill 15 × 5 L bottles (triplicates of five treatments) for the first addition of nitrate, phosphate, silicate and trace metals as well as a control incubation without enrichment. After observing an increase in biomass in most bottles and within a short time frame for zooplankton growth (5 days), the 5 L bottles were sampled. From each 5 L bottle, 2 × 1.5 L were transferred into smaller bottles, one without further manipulation and another enriched with complementary nutrients to evaluate the effects of combined additions. As a result, triplicate 1.5 L bottles of each initial condition with and without additional manipulation were allowed to grow for a period of another 7 or 9 days according to biomass and sampling feasibility.

Bottles were placed randomly, so that all treatments were exposed to similar temperature (~20°C), light intensity (~2500 μmol m^{-2} s^{-1} on a sunny October day with an attenuation of on average ~81% at 2.4 m) and a 11/13 light/dark cycle since the bottles were incubated at 2.4 m depth in the marina of Angra do Heroísmo (Terceira), starting on 23 September 2010. There was no selection or size-fractionation of the initial community to minimize manipulation-related stress on the phytoplankton cells, so initial cell densities correspond to natural abundances. As a result, other organisms such as bacteria and zooplankton were included in the experiment and, at least partly, contributed to POM. All cultures were rotated 15 times, in the shade, twice a day (at 10:00 and 17:00) to avoid aggregation and sedimentation and, consequently, self-shading during the light phase (from ~7:00 to 19:00). During sampling, temperature, salinity, pH, nutrients, phytoplankton counts, phytoplankton community composition and particulate organic carbon (POC) and particulate organic nitrogen (PON) were measured. The pH_{total} measurements (glass electrode WTW, pH 340i, calibrated with a TRIS seawater buffer, supplied by A. Dickson) provided a proxy for autotrophic biomass development. Nutrient determination made it possible to obtain the exact initial and end concentrations in the treatments and to calculate nutrient drawdown between sampling points. POM (carbon and nitrogen) was measured to provide an additional metric of plankton biomass development of the fraction larger than 0.7 μm through time. It also provided a proxy for phytoplankton organisms with specific fractionation signatures such as low $\delta^{15}N$ of nitrogen fixers.

Potential limitation of more than one nutrient was assessed by comparing the increase in cell numbers or biomass after the addition of a second nutrient with those of the initial nutrient amendment. This is particularly relevant to determine Type III co-limitation while at the same time providing information on Type I co-limitation. For that purpose, phosphorus was added to the nitrate treatment to promote total growth; trace metals to the phosphorus treatment potentially favouring diazotrophs; nitrate to the silicate treatment and silicate to trace metals, testing for potential diatom growth; and a combination of all nutrients used in the experiment to the bottles that had previously not received nutrient enrichment.

Nutrients

Samples for nutrient determination were filtered through a polyethersulfone 0.2 μm syringe filter and stored at −20°C until analysis. Nutrient analyses were made from the original pool of collected seawater (in duplicate) and, from each bottle at the beginning and end of the incubations. Concentrations of nitrate (method range from 0.1 to 25 μmol L^{-1} ± 0.1 μmol L^{-1}), nitrite (0.01–2.5 μmol L^{-1} ± 0.02 μmol L^{-1}), silicate (0–80 μmol L^{-1} ± 2.5%) and phosphate (ranging from 0 to 10 μmol L^{-1} ± 0.02 μmol L^{-1}) were determined after dilution on a segmented flow analyser (SEAL QuAAtro) equipped with an autosampler (Hansen and Koroleff., 1999). Methods were modified for nitrate, where an ammonium chloride buffer was replaced by imidazole. Finally, a solution of Triton X-100 or sodium dodecyl sulphate was used to decrease surface tension and, therefore, improve segmented flow analysis.

Cell numbers

Samples (300–1000 mL) for phytoplankton counts were gently filtered through nitrate cellulose filters with 0.45 μm pore size and resuspended in 100 mL of sample. Resuspension of the cells had an associated error, but it did not affect the comparison between samples as demonstrated by the similarity between replicates and occasional repetitive counts of the same sample. Identification and abundance of microphytoplankton was determined (1100 cells counted on average per sample) from samples fixed with Lugol (2% final concentrations) by means of an inverted microscope (Nikon Eclipse TS100) at ×200 magnification and following Sournia (1986), Tomas (1997) and Kraberg *et al.* (2010).

Samples for analysis of coccolithophore abundance were gently filtered (200–1000 mL) through 0.45 μm nitrate cellulose filters and rinsed with a basic solution (pH 10, adjusted with NaOH) to remove all salts that would otherwise disturb microscopic visualization. The filters were mounted on a glass slide with Entellan® mounting media and coccolithophores identified and quantified (120 cells counted on average per sample)

at ×1250 magnification with a polarized light microscope (Olympus BX40).

Phytoplankton are presented in groups (diatoms, coccolithophores, dino- and other flagellates, and others, namely unidentified small spheres and silicoflagellates) to facilitate discussion and comparison with Margalef (1978).

Particulate organic matter analysis

Samples (100–1000 mL) for total cellular POC and PON from the initial community (duplicate) and end of the incubations were gently filtered (200 mbar) through pre-combusted glass fiber filters (GF/Fs) (6 h, 450°C) and stored at −20°C until analyses. POC/PON samples were dried (4 h, 60°C) and sent for analysis at the Leibniz-Institute for Baltic Sea Research, where they were fumed 12 h in a desiccator (HCl 35%), dried, packed in tin boats and analysed in a Flash 2000 EA connected to a Thermo Delta Plus isotope ratio mass spectrometer via an open split Conflow IV, following Sharp (Sharp, 1974). The isotopic composition of POC and PON is reported relative to that of VPDB (Vienna Pee Dee Belemnite) and air as standards, respectively, with a precision better than 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, and calculated for POC as follows:

$$\delta^{13}C_{POC} = (({}^{13}R_{POC} / {}^{13}R_{STD}) - 1) \times 1000$$

with ${}^{13}R_{POC}$ and ${}^{13}R_{STD}$ denoting the ^{13}C to ^{12}C ratio in POC and the standard, respectively. The calculation for the isotopic composition of PON is similar

$$\delta^{15}N_{PON} = (({}^{15}R_{PON} / {}^{15}R_{STD}) - 1) \times 1000$$

with ${}^{15}R_{PON}$ and ${}^{15}R_{STD}$ denoting the ^{15}N to ^{14}N ratio in PON and the standard, respectively.

Finally, $\delta^{15}N$ of newly formed PON was calculated according to:

$$\delta^{15}N_{new} = (\delta^{15}N_{end} \times PON_{end} - \delta^{15}N_{start} \times PON_{start}) / PON_{new}, \quad (1)$$

where start and end values correspond to measurements done at the beginning and end of the experiment, respectively. PON_{new} refers to nitrogen assimilated by the phytoplankton grown during the experiment ($PON_{end} - PON_{start}$). This determination, together with measured $\delta^{15}N$ of the nitrate salt used to prepare the nitrate solution utilized in the experiment (17.25 ± 0.1‰), allowed the potential existence of organisms with characteristics of nitrogen fixers to be addressed.

Data analysis

The statistical significance of differences in the changes of POM build-up and phytoplankton abundance amongst treatments were tested for with an analysis of variance (significance determined as 99%, $P < 0.01$), using the program R (Development Core Team, 2011). Significant differences of phytoplankton communities based on the groups' relative abundances between treatments after 5 days and at the end of the experiment were analysed with analysis of similarities (ANOSIM). Moreover, Bray–Curtis dissimilarity (Bray and Curtis, 1957) was used to determine dissimilarities between phytoplankton groups at both sampling times for all treatments and in relation to the control that did not receive nutrient enrichment, while also providing information on similarities within each group. Finally, the species primarily driving the differences between the groups' relative abundances were identified using similarity percentages (SIMPER) analysis.

RESULTS

Initial conditions

Initial concentrations of nitrate, phosphate and silicate were below detection limits (Fig. 1) which coincided with relatively low biomass, specifically 44 μmol L^{-1} of POC, 3 μmol L^{-1} PON, 18 diatom cells mL^{-1}, 15 dino- and other flagellate cells mL^{-1}, 3 coccolithophore cells mL^{-1} and 12 cells mL^{-1} of other organisms.

Response of the overall phytoplankton community to single nutrient additions after 5 days

After the initial nutrient enrichment (Fig. 1) of a single macronutrient or Trace Metals there was an increase of POC ($P < 0.03$, highest 1.8-fold under trace metals enrichment) and PON in all treatments ($P = 0.03$) including the Control (Fig. 2). Total cell numbers (Fig. 3) did not show a concomitant overall increase in these first 5 days, decreasing on average 1.8-fold in all treatments, except for the Phosphate enrichment where total abundance did not change significantly (decreasing in average 1.1-fold). In fact, only the abundances of coccolithophores and dino- and other flagellates increased during this time period under enhanced Phosphate (Fig. 3), leading to dominance in the latter case. Diatom total abundances decreased 2.4-fold in all conditions

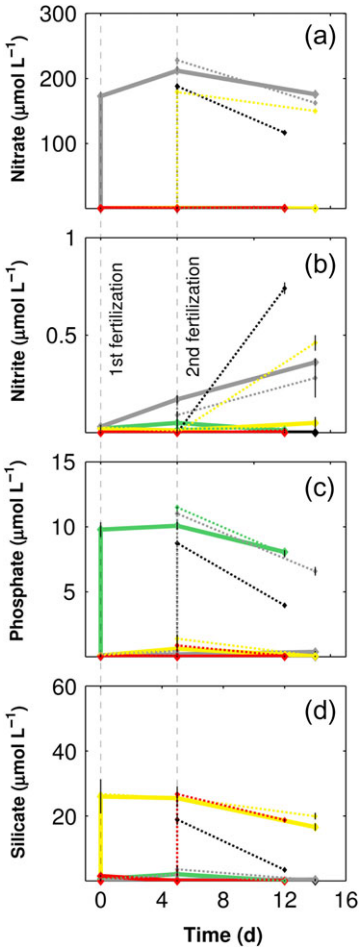

Fig. 1. Nutrient concentrations through time. Nitrate (**a**), nitrite (**b**), phosphate (**c**) and silicate (**d**). Solid lines refer to the first nutrient addition: Control (black); Trace Metals (red); Nitrate (grey), Phosphate (green); and Silicate (yellow) and the dashed lines to the second addition: Trace Metals + Silicate (red); Nitrate + Phosphate (grey); Phosphate + Trace Metals (green), Silicate + Nitrate (yellow) and All Nutrients (black). Lines connect the averages of triplicates and the vertical error bars represent standard errors of those means at each time point.

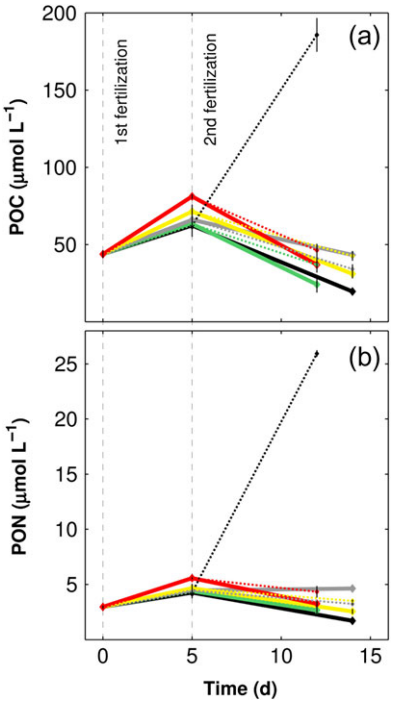

Fig. 2. Organic matter build-up through time. POC (**a**) and PON (**b**). Solid lines refer to the first nutrient addition: Control (black); Trace Metals (red); Nitrate (grey), Phosphate (green); and Silicate (yellow) and the dashed lines to the second addition: Trace Metals + Silicate (red); Nitrate + Phosphate (grey); Phosphate + Trace Metals (green), Silicate + Nitrate (yellow) and All Nutrients (black). Lines connect the averages of triplicates and the vertical error bars represent standard errors of those means at each time point. The vertical dotted line marks the fertilizations.

except under enhanced Trace Metals and Silicate where it decreased 1.4-fold in the first 5 days (Fig. 3). The apparent discrepancy between cell numbers and POM can be explained by increased overall cellular quotas (0.85 pmol C cell^{-1} in the initial community and on average 2.61 ± 0.13 pmol C cell^{-1} after the nutrient enrichments, with the addition of Phosphate leading to a lower carbon content of 1.3 pmol C cell^{-1}, data not shown) and shifted relative abundances towards larger diatom taxa. The latter is even more relevant since the two conditions with the highest POC increase in the first 5 days (Fig. 2) showed a shift from a community with similar relative abundances of the studied groups to a diatom-dominated community (Fig. 4). These differences between the condition without nutrient addition and the amended treatments Trace Metals (R = 0.41) and Silicate (R = 0.19) after 5 days corresponded to an R below 0.5, with a global R of 0.27. However, the significance level p already indicated differences between groups (P = 0.035). Simultaneously, there was an increase of δ^{13}C in POC, though this was less pronounced under Silicate than under Trace Metals addition (Fig. 5). Indeed, under enhanced Trace Metals (58% diatoms), the main diatom present *Guinardia* spp. (~50% of diatoms present) increased 10.6-fold from initial values (compared with the 1.6-fold average of the other treatments). In spite of having a smaller biomass increase, pH values increased the most in the Silicate treatment (Supplementary Fig. S1). This is related with the Silicate addition, which increased pH of the seawater as it altered total alkalinity. In this treatment the diatom abundance (56%) was shared by several taxa, namely *Melosira nummeloides* and *Rhizosolenia* spp. which made up 38% of the relative abundance of this group (Table II), being less abundant throughout the experiment (Supplementary Fig. S2). Additionally, Silicate addition showed almost exclusively *Bacteriastrum* sp., *Chaetoceros* spp.

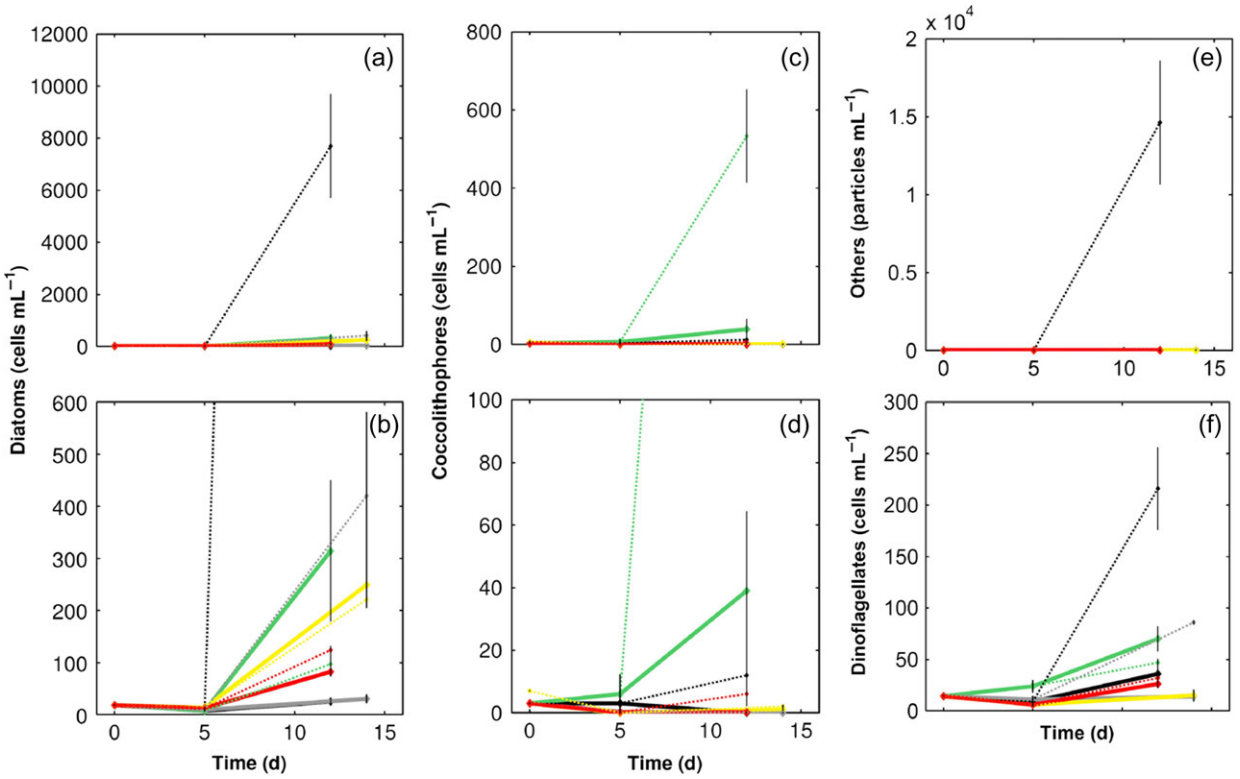

Fig. 3. Abundance of phytoplankton as determined by microscopic counts through time. Diatoms (**a** and **b**), coccolithophores (**c** and **d**), others (**e**) and dinoflagellates (**f**). All abundances (top) and excluding highest abundance for improved visualization (bottom). Solid lines refer to the first nutrient addition: Control (black); Trace Metals (red); Nitrate (grey), Phosphate (green); and Silicate (yellow) and the dashed lines to the second addition: Trace Metals + Silicate (red); Nitrate + Phosphate (grey); Phosphate + Trace Metals (green), Silicate + Nitrate (yellow) and All Nutrients (black). Lines connect the averages of triplicates and the vertical error bars represent standard errors of those means at each time point.

and *Leptocylindrus* sp., with increases of ~20, 40 and 30-fold, respectively. The Phosphate enrichment showed a shift to a dinoflagellate-dominated community, reaching 58% of the community in comparison to only ~18% coccolithophores, within the first 5 days of incubation (0.22 R statistic, Fig. 4). Finally, Nitrate amendment did not result in differences among the phytoplankton groups relative abundances (ANOSIM $R < 0$).

Similarity between replicates of each treatment after 5 days of incubation ranged from 66% under Silicate to 87% under Trace Metals enrichment. When comparing the control with the other treatments, the differences (dissimilarities) were still low (<33%).

Response of the overall community to longer exposure (12–14 days) to single and sequential nutrient additions

After the additional (at the end of the 5 day incubations) nutrient enrichments, POC and PON decreased until Day 12 or 14 (both $P < 0.01$) in all except the treatment replenished with All Nutrients (Fig. 2). The increase of POM in this treatment was associated with the strongest increase in total cell numbers (Fig. 3) and lowest stable carbon isotope fractionation (Fig. 5a). Despite the observed organic matter decrease in all other treatments (Fig. 2), there was an increase in total phytoplankton abundance ($P < 0.01$). In fact, total abundance increased from only ~1.4-fold under the Nitrate enrichment and the treatment without addition to 23- and 430-fold for the Phosphate + Trace Metals and All Nutrients, respectively (Fig. 3). In the latter this increase was associated with a concomitant pH increase (Supplementary Fig. S1) and C:N decrease (data not shown) between Day 5 and the end of the experiment. After the whole incubation period, diatom-dominance was observed in all treatments, except under the enrichment with Phosphate + Trace Metals (9 ± 7%,), with All Nutrients (34 ± 12%), and when no nutrients (35 ± 17%) were added (Supplementary Fig. S3).

Under prolonged (end of experiment) limitation of all nutrients, coccolithophore numbers became virtually non-existent, while diatoms represented ~35% (±17), dinoflagellates ~43% (±20) and other taxa ~22% (±4) of the

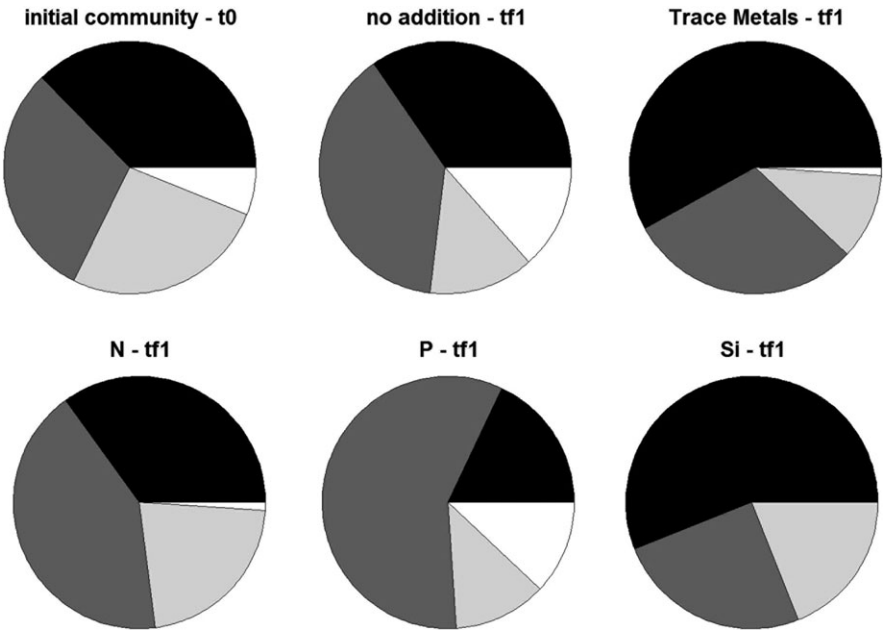

Fig. 4. Relative abundance of diatoms (black), coccolithophores (white), dinoflagellates (dark grey) and others (light grey) in each treatment at the beginning (t0) and after 5 days of incubation (tf1).

community (Supplementary Fig. S3). Thus, under this condition, relative abundances remained similar throughout the experiment for diatoms ~36% (±1), dinoflagellates ~36% (±8) and other taxa ~24% (±3) of the community. Furthermore, the enrichment of All Nutrients to the initially un-manipulated Control treatment increased the abundance of an otherwise rare, unidentified species (spherical, ~4 μm diameter, possibly a unicellular nitrogen-fixing cyanobacterium) within the category "others" towards dominance (65 ± 12%), at the expense of dinoflagellates (1%, with the highest relative contribution (9%) belonging to *Scripsiella* sp.) and coccolithophores (0.1%). Relative abundance of diatoms did not vary dramatically in relation to 5 days earlier (~34%), with *Cylindrotheca closterium* and *fusiformis* and a small and thin unidentified diatom (potentially *Nanoneis* sp.) being well represented within this group under All Nutrients. The potential presence of nitrogen fixers in this treatment is supported by stable nitrogen isotope signatures of POM, δ^{15}PON (here, −1.2‰ of final PON and −2.2‰ (equation (1)) of PON formed during the experiment, in comparison to the signature of nitrogen fixers of δ^{15}PON −2 to −1‰) (Fig. 5b).

The complementary addition of trace metals to the Phosphate treatment increased the abundance of coccolithophores (mostly small coccospheres, such as *Emiliania huxleyi*) by 4- to 5-fold (Fig. 3), shifting the community towards coccolithophore dominance (~86 ± 7%).

Similarities of phytoplankton groups at the end of the experiment were still high, ranging from 55% under phosphate enrichment and 98% under Nitrate + Silicate. At the same time there were significant differences in species composition between the amended treatments and the control even though the statistical analysis is less robust due to the low number of replicates ($n = 3$). The highest dissimilarity (77%) was found for Phosphate + Trace Metals with 50% being explained by coccolithophores. This was followed by ~50% for Silicate, Silicate + Nitrate, Phosphate and for All Nutrients with >40% of the dissimilarity being explained by diatoms in the first three treatments and 43% explained by others in the latter. Dissimilarity was below 50% for the remaining treatments. Finally, the differences between amended treatments and the condition without nutrient manipulation were statistically significant (global $R = 0.63$, $P = 0.001$, ANOSIM) and showed high separation ($R > 0.5$) for Phosphate ($R = 1$), Silicate ($R = 0.96$), All Nutrients ($R = 0.93$), Nitrate + Silicate ($R = 0.89$), Silicate + Trace Metals ($R = 0.78$) and Trace Metals ($R = 0.63$).

As phytoplankton abundance increased, nitrate and phosphate concentrations decreased (Supplementary Fig. S4). The highest drawdown of both nitrate and phosphate was observed under Nitrate + Phosphate and when All Nutrients were added, followed by the individual nutrient additions (Supplementary Fig. S4). However, the strong drawdown of nitrogen was not translated to a similar increase in PON (cells bigger than the retention capacity of 0.7 μm of GF/F) in any of the treatments (Fig. 2), not even when all nutrients were

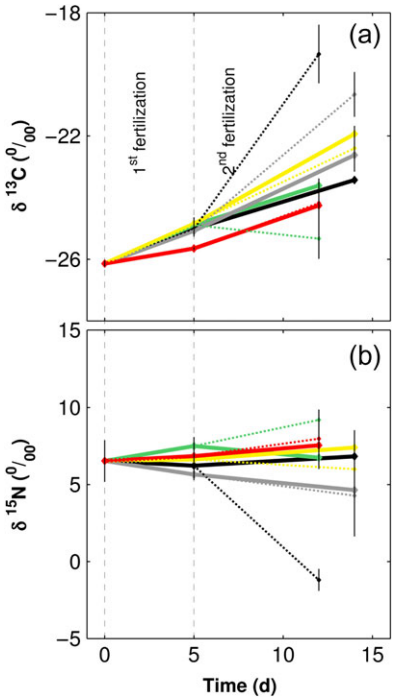

Fig. 5. Organic matter fractionation through time. Carbon (**a**) and nitrogen (**b**). Lines connect the averages of triplicates and the vertical error bars represent standard errors of those means at each time point. Solid lines refer to the first nutrient addition: Control (black); Trace Metals (red); Nitrate (grey), Phosphate (green); and Silicate (yellow) and the dashed lines to the second addition: Trace Metals + Silicate (red); Nitrate + Phosphate (grey); Phosphate + Trace Metals (green), Silicate + Nitrate (yellow) and All Nutrients (black). Lines connect the averages of triplicates and the vertical error bars represent standard errors of those means at each time point. The vertical dotted line marks the fertilizations.

added since PON build-up was ~21 μmol L^{-1} and nitrate drawdown on average 72 (±19.1) μmol L^{-1}. This nitrate loss that might have occurred by dissolved organic nitrogen exudation, bacterial uptake (0.7 μm is the cut-off size of GF/F filters used for POM filtration) or adsorption to bottle walls, was higher in the conditions without the addition of all nutrients considered. The treatment, enriched with Nitrate + Phosphate showed a nitrate drawdown of on average 66 (±13) μmol L^{-1} but build-up of organic N was not measurable. Excluding what was lost, drawdown per total abundance decreased under the full replenishment in comparison to the other treatments to an average of 3.31 ± 0.55 pmol $cell^{-1}$ (other treatments with nitrate addition were all above 180 pmol $cell^{-1}$). The carbon to nitrogen ratio (mol:mol) of organic matter was close to Redfield in all treatments, decreasing after the addition of All Nutrients simultaneously (data not shown). Finally, increasing community growth rate by nutrient additions can explain increasing stable carbon isotope signatures in the POM with the exception of the Phosphate + Trace metals enrichment (Fig. 5a).

Taxon-specific responses

A few taxa were always associated with specific nutrient treatments. All Phosphate enrichments showed a significant increase in relative abundance of the dinoflagellate *Prorocentrum* spp., reaching 16% after only 5 days under Phosphate enrichment and ~52% of all dinoflagellates at the end of the experiment compared with just 7% in the other treatments (Supplementary Fig. S2a). Indeed, *Prorocentrum* spp. accounted for 42% of the dissimilarities found by SIMPER for dinoflagellates and other flagellates between the Phosphate and the Control treatment, 49% between the Nitrate + Phosphate and the Control treatment, and 40% between the Phosphate + Trace Metals and the Control treatment. The dinoflagellate *Scripsiella* sp. was relatively rare in most treatments, with the exception of the addition of All Nutrients, where it accounted for 15.75% of dissimilarities between this treatment and the Control. Likewise, most treatments showed low relative abundances of the diatom *Guinardia* spp., but after 5 days of the Trace Metals addition *Guinardia* spp. represented ~45% of the existing diatoms and explained 20% of the dissimilarities found for diatoms between this treatment and the Control. However, the additional enrichment with Trace Metals to the Phosphate treatment did not result in a *Guinardia* spp. increase. Under enhanced Silicate, alone and in combination with nitrate, the diatom *Chaetoceros* spp. increased in abundance, reaching ~34% (±19) at the end of the experiment (Supplementary Fig. S2c). *Chaetoceros* spp. accounted for 19% of the dissimilarities found for diatoms among the Control and the Silicate treatment and 21% between the Control and the Silicate + Nitrate treatment. Finally, after the addition of All Nutrients the unidentified spherical cell abundance increased from below detection limit in most treatments and ~2 cells mL^{-1} under Trace Metals to ~20 × 10^3 cells mL^{-1} where it explained 50% of dissimilarities of the group others.

DISCUSSION

Initial environment and phytoplankton community

Conditions in the Eastern North Atlantic Subtropical Gyre are often oligotrophic with chlorophyll *a* concentrations below 0.1 mg m^{-3} (Teira *et al.*, 2005). Close to the Azores, chlorophyll *a* reaches mesotrophic concentrations

Table II: Colour coding for the matrix of nutrient additions and the corresponding dominant taxa of each treatment

Nutrient added	Dominant diatoms	Dominant coccolithophores	Dominant dinoflagellates
Initial water	Pennate and *M. nummeloides*	*Emiliania huxleyi* and small unidentified coccospheres	*Alexandrium* sp.
First fertilization			
No addition	Pennate and *Climacosphenia* sp.	*E. huxleyi*	*Alexandrium* sp.
Nitrate	Pennate and *M. nummeloides*	*E. huxleyi*	*Alexandrium* sp.
Phosphate	Pennate and *Guinardia* spp.	*E. huxleyi*	*Alexandrium* sp.
Silicate	*Rhizosolenia* spp. and *M. nummeloides*		*Alexandrium* sp.
Trace Metals	Pennate and *Guinardia* spp.	*Calcidiscus leptoporus*	*Alexandrium* sp.
Second fertilization (after 5 days)			
No addition	Pennate and *C. closterium*		*Alexandrium* sp.
All nutrients	*C. closterium* and unidentified thin diatom	*E. huxleyi*	*Scripsiella* spp.
Nitrate	Pennate and *C. closterium*		*Alexandrium* sp. and *Prorocentrum* spp.
Nitrate + Phosphate	*Leptocylindrus* sp. and *C. closterium*	Small unidentified coccospheres	*Prorocentrum* spp.
Phosphate	*Rhizosolenia* spp. and unidentified thin diatom	*E. huxleyi*	*Prorocentrum* spp.
Phosphate + Trace Metals	*C. closterium* and unidentified thin diatom	*E. huxleyi*	*Prorocentrum* spp.
Silicate	*Chaetoceros* spp. and unidentified thin diatom	*Syracosphaera* spp.	*Alexandrium* sp.
Silicate + Nitrate	*Chaetoceros* spp. and unidentified thin diatom	*Syracosphaera* spp. *and E. huxleyi*	*Alexandrium* sp.
Trace Metals	*Guinardia* spp. and *Pseudo-Nitzschia* spp.	*Helicosphaera* sp. and *Umbilicosphaera sibogae*	*Alexandrium* sp.
Trace Metals + Silicate	*Guinardia* spp., pennate and unidentified thin diatom	*E. huxleyi*	*Alexandrium* sp.

seasonally, with a maximum average chlorophyll *a* of ~0.4 mg m^{-3} (Teira *et al.*, 2005; Valente, 2013). Accordingly, nutrient concentrations are low in the proximities of the Azores, varying with mixed layer depth and subsurface nutrient fields (Valente, 2013). The mixed layer depth increases from autumn to winter due to increasingly stormy weather and heat loss at the surface, and decreases from spring to summer. The initial community of this experiment was sampled at the beginning of autumn, i.e. after a period of higher sea surface temperatures, with increased stratification and consequently low-nutrient availability. At the time of water collection the initial nutrient concentrations were extremely low, markedly at the lower range of data collected in the region between September 2010 and December 2014 (0–3.96 µmol L^{-1} nitrate, 0–2.41 µmol L^{-1} phosphate and 0–9.07 µmol L^{-1} silicate). Nutrient-depleted conditions are typical for surface waters in the summer offshore the Azores and are interrupted by nutrient pulses due to enhanced mixed layer depth at the beginning of autumn. Hence, the initial community enclosed in this experiment might have been adapted to respond to abrupt nutrient enrichments rather than long limitation periods. In this scenario, bacteria most likely played an important role in nutrient recycling and to a smaller extent in biomass build-up.

General biomass development

Five days after the single nutrient enrichments, POC and PON rose in all treatments, including the Control. This was, hypothetically, a consequence of higher mean light intensity during the experiment, since the water collected resulted from a mixture of the upper 10 m and bottles were incubated at 2.4 m. The biomass increase in all treatments within 5 days could also be related to the scarcity of grazers in the bottles, in particular migrating zooplankton, since sampling occurred close to midday (Coale, 1991). The initial biomass increase was not reflected in phytoplankton cell numbers which might be explained by overall enhanced cellular element quotas, changes in relative abundances towards bigger diatom taxa and, to a broader size class considered when filtering (GF/F nominal pore size of ~0.7 µm) for POM than the one used for microscope counts (>~2–4 µm). Indeed, larger diatoms are known to thrive under pulse situations (Goldman, 1993; Litchman *et al.*, 2009) analogous to the nutrient increase within the first 5 days of the experiment. This might be related to their capacity to complement the single nutrient enrichments, as a result of their higher storage capacity for nutrients in comparison to most other taxa. Bacteria also have the ability to grow after nutrient pulses through the utilization of nutrient pools such as amino acids not easily available to phytoplankton (Thingstad *et al.*, 2005). However, bacteria are not responsible for the observed trend, since their typical size range will mostly pass through the GF/F filters.

At the end of the experiment (Day 12 or 14), POC decreased in relation to Day 5 in all treatments except in the All Nutrients treatment. Considering that abundances of microphytoplankton increased in several

treatments, this paradox could be explained by a stronger demise of picophytoplankton abundance (hypothetically due to grazing by micro or nanograzers).

Nutrient-specific responses of a phytoplankton community

No addition (control)

Smaller cells typically dominate oligotrophic systems due to their high surface to volume ratio (Moore *et al.*, 2013). In the present study, picophytoplankton quantification was unresolved and only partly accounted for in POM. However, the initial community structure of nano and microphytoplankton while enclosing a natural community without nutrient manipulation was maintained, with the exception of coccolithophores that declined to barely detectable numbers at the end of the experiment. A rationale for the coccolithophore demise might be related to higher sensitivity of this group to the enclosed environment which concentrated cells at the bottom of the bottle due to sedimentation and increased predator-prey encounter rate there. This is even more relevant under nutrient-limiting conditions, since mixotrophs might enhance their grazing activity. A further possibility is that the dominant coccolithophore species (*E. huxleyi*), commonly occurring as the diploid calcified form is known to respond to stresses such as virus presence, by changing to its flagellate haploid life-cycle phase (Frada *et al.*, 2008). Thus, the decrease of coccolithophores abundance could in theory be explained by a life-cycle change.

Trace Metals

In spite of potential limitation by other nutrients, Trace Metals replenishment induced relatively strong changes in the community composition. Within 5 days and throughout the experiment, Trace Metals promoted a shift to diatom-dominance as previously observed in iron-limited areas such as the Southern Ocean and other HNLC (high nutrient low chlorophyll) areas (Lam *et al.*, 2001; Boyd *et al.*, 2007). Likewise, the addition of iron-rich dust from the Sahara to Antarctic seawater resulted in increased growth rate of two Antarctic diatom species (Visser *et al.*, 2003) and augmentation of iron-rich aerosol induced dominance of species with higher growth rates, such as the diatoms *Guinardia* spp., *Chaetoceros* spp., *Pseudonitzchia* spp., *Thalassionema* spp. and *Skeletonema* spp. (Guo *et al.*, 2012). In the present study, *Guinardia* spp. also increased dramatically, representing ~50% of diatoms under enhanced Trace Metals enrichment. This observed shift in community composition after Trace Metals enrichment could be related to storage capacity for macronutrients, which would enable diatoms to respond quickly to the micronutrients. Moreover, most *Guinardia* spp. observed here belong to the species *Guinardia striata*, known to have lower silicate requirements (Schapira *et al.*, 2008) and, due to their large size, potentially having high phosphorus and nitrate reserves, a determinant feature in oligotrophic environments.

Various oligotrophic/upwelling regions of the ocean benefit from atmospheric deposition of nutrients and trace metals (Wu and Boyle, 2002; Morel *et al.*, 2003; Eker-Develi *et al.*, 2006) resulting in phytoplankton blooms. Iron availability has been considered one of the major factors controlling nitrogen fixation in nature (Schlosser *et al.*, 2014), together with phosphate (Sanudo-Wilhelmy *et al.*, 2001; Mills *et al.*, 2004). The nitrogen-fixing diazotrophs which can achieve maximum net growth rates under iron or iron plus phosphorus amendments (Moisander *et al.*, 2012) could potentially have benefitted from the trace metal additions in the present study. However, there was no evidence for significant increase of the 4 μm spherical cells under Trace Metal addition.

Reduced bioavailability of trace metals in the ocean is aggravated by the formation of metal-ligand complexes (Saito and Moffett, 2002). As a response, some phytoplankton have evolved specialized uptake systems to utilize complexed metals (Shaked *et al.*, 2005) and higher plasticity in relation to these micronutrients which enables their substitution (Morel *et al.*, 2003; Saito *et al.*, 2008). However, at high concentrations certain trace metals may be toxic to marine phytoplankton (Nayar *et al.*, 2004; Paytan *et al.*, 2009). In our study, trace metals (e.g. total copper), were added at high concentrations compared to their natural concentrations in this region (Moore *et al.*, 2008), even though they are similar to other areas such as the Narragansett Bay and Saanich Inlet (Thomas *et al.*, 1980). Nevertheless, trace metals bioavailability (e.g. copper and zinc) is much lower than what would be toxic to phytoplankton (Crawford *et al.*, 2003; Nayar *et al.*, 2004; Paytan *et al.*, 2009).

Phosphate

Phosphate has been considered to limit phytoplankton biomass on short timescales in the subtropical North Atlantic, based on high activity of alkaline phosphatase (Lomas *et al.*, 2004) and N:P ratios higher than Redfield in the dissolved and particulate pools (Wu *et al.*, 2000; Ammerman *et al.*, 2003). Marine phytoplankton and bacteria have diverse phosphate acquisition systems and storage capacities (Thingstad *et al.*, 1993; Talarmin *et al.*, 2015 and

references therein), which affect their response to phosphorus increments. Phosphate was the only treatment resulting in dominance of dinoflagellates and other flagellates in the first 5 days of our study. Dinoflagellates would be expected to be dominant under calm, oligotrophic conditions (Margalef, 1978), such as those at the start of the experiment. Indeed, they were reasonably represented in the initial community (~36%), but also in the treatments without nutrient addition (Control) and under Nitrate replenishment alone.

After a longer incubation period with Phosphate recycling of other nutrients required for the observed growth of diatoms and coccolithophores may have occurred. In contrast, relative abundance of dinoflagellates and other flagellates decreased (except when no nutrients were added). Species responses to macronutrient enrichments depend on their requirements for micronutrients. Coccolithophores (Boyd *et al.*, 2010) and diatoms (Ho *et al.*, 2003), especially open ocean species (Strzepek and Harrison, 2004), have been considered to have lower iron requirements. In the Mediterranean Sea, diatom abundance has been shown to increase under enhanced phosphate concentrations and comparatively lower carbonate and pH (Oviedo *et al.*, 2015). Moreover, groups that occur under oligotrophic conditions, such as coccolithophores (Oviedo *et al.*, 2015) and diazotrophs have been shown to correlate with phosphate concentrations. The growth of diazotrophs could have been favoured by the decrease of the molar Redfield ratio (Redfield, 1934) below 16:1 nitrogen: phosphorus after the increasing phosphate concentrations. The absence of response by the diazotrophs could be related to a quicker response of other phytoplankton groups to the phosphate replenishment.

Nitrate

The upper water column of low latitude regions such as the Mediterranean Sea (Tanaka *et al.*, 2011) and oligotrophic subtropical and tropical oceans (Moore *et al.*, 2008) is mostly nitrate limited (Moore *et al.*, 2013). In accordance, nitrate plus nitrite in the Mediterranean Sea have been shown to be positively correlated with calcareous nanoplankton such as *Florisphaera profunda* and *Gladiolithus flabellatus* (Oviedo *et al.*, 2015). Contrary to previous studies in oligotrophic areas (Tyrrell, 1999) and the South Pacific Ocean (Moisander *et al.*, 2012), but in accordance with Davey *et al.* (2008) single addition of Nitrate in seawater collected offshore the Azores during this study did not result in increased POM or phytoplankton abundances, potentially due to the lack of other nutrients such as phosphate which is required for cell division (Müller *et al.*, 2008).

Silicate

Silicate often occurs at similar or higher concentrations than nitrate in the oceans as well as in cellular diatom quotas (Brzezinski, 1985). It is reasonable to hypothesize that the lack of an increase in diatom abundance in the first 5 days after silicate addition resulted from co-limitation of two or more nutrients. Nevertheless, already in this short time frame there was a clear shift in the community composition towards diatom-dominance (56%), being even more severe after 14 days (~90%). In particular, diatom relative abundance shifted from a community distributed proportionately between larger diatoms to a community dominated by colonies of *Chaetoceros* spp. and thin, unidentified chain forming diatoms (probably *Nanoneis* sp.). *Chaetoceros* spp. is known to form colonies that provide a physical substrate for other microbes, which potentially might supply other macronutrients through remineralisation. Interestingly, both treatments that followed the initial Silicate treatment (Silicate and Silicate + Nitrate) also showed a strong increase in the relative abundance of the coccolithophores *Syracosphaera* spp. often associated with nutrient-depleted waters (Ziveri *et al.*, 2004), but its meaning is still unclear.

Effects of combined nutrient enrichment (Days 5–12 or 14): co-limitations of a natural phytoplankton community

While the response of phytoplankton to the addition of single nutrients has been studied in laboratory and field experiments, and biogeochemical models, co-limitation is harder to access (Arrigo, 2005; Saito *et al.*, 2008). However, vast areas of the ocean are oligotrophic, harbouring phytoplankton communities simultaneously depleted by several nutrients. The resulting decreased primary production depends on cellular requirements and cell size of the phytoplankton taxa, which are related to ratios of the macromolecular pools, reserves and energy. As in previous studies (De Baar *et al.*, 2005; Henjes *et al.*, 2007), the addition of macro and micronutrients increased the abundances of the "velocity-adapted" diatoms in most nutrient treatments. This might be due to higher growth rates of diatoms than taxa from other groups and their lower grazing pressure in comparison to the smaller prokaryotic algae (Landry *et al.*, 2000; Henjes *et al.*, 2007).

Silicate + Nitrate and Trace Metals + Silicate

Silicate co-limitation has been largely overlooked. Here, potential co-limitation was hindered by the response of Silicate alone. Indeed, Silicate triggered an increase in the abundance of diatoms at the end of the experiment,

possibly due to the remineralisation of other nutrients, potentially overcoming co-limitation. Thus, it is hard to disentangle the relative effects of Trace Metals from Silicate on diatom relative abundance since the addition of Silicate or Trace Metals alone already resulted in an abundance increase by this group. Nitrate addition to seawater previously enriched with Silicate also increased diatom abundance with high relative abundance of *Chaetoceros* spp. similar to the single Silicate treatment. Thus, additional nitrate did not produce a shift in the community nor enhanced abundance in relation to the Silicate treatment. Supported by low nitrate drawdown (data not shown, value similar to the nitrate treatment) and unchanged stable carbon isotope signature of the organic matter, we hypothesize that remineralised nitrate was sufficient to increase diatom abundance in the Silicate treatments.

It is relevant to note that the observed dominance of the diatom *Guinardia* spp. under Trace Metals + Silicate addition was related to the initial addition of Trace Metals rather than Silicate as demonstrated by: (i) *Guinardia* spp. abundance increased following initial single Trace Metals fertilization; (ii) *Guinardia* spp. showed enhanced abundance after longer incubation period under Trace Metals amendment and (iii) lack of response from this taxa under other treatments with silicate enrichment.

Phosphate + Trace Metals

In principle, the addition of trace metals to phosphate-enriched water could increase the abundance of nitrogen fixers as observed in the Western North Atlantic Ocean (Wu *et al.*, 2000), since both are considered the main limiting nutrients of this group (Hutchins and Fu, 2008). The main reason is that the nitrogen-fixing enzyme (nitrogenase) requires more iron than the nitrate reductase used for the uptake of nitrate. Moreover, other enzymes such as carbonic anhydrase that are important for carbon concentrating mechanisms also require trace metals (Morel *et al.*, 2003). However, complementary addition of trace metals to the Phosphate treatment strongly increased coccolithophore abundances, leading to the only treatment with coccolithophore dominance. In fact, coccolithophores, such as *E. huxleyi* (the dominant species of this treatment, though at lower relative abundance than in other treatments), have been shown to be limited by phosphorus (Egge and Heimdal, 1994), but less relevance has been given to trace metal requirements except when related to the fertilizing effect of the Saharan dust in *E. huxleyi* blooms (Guerzoni *et al.*, 1999). *Emiliania huxleyi* has the ability to use organic phosphorus by means of several alkaline phosphatase enzyme systems (Riegman *et al.*, 2000). More importantly it also has high affinity for inorganic phosphorus, resulting in a faster uptake per cell surface (Sunda and Huntsman, 1995) as well as the capacity to up-regulate glutamine synthetase activity and increase NH_4^+ affinity under limiting nitrate conditions (Maurin and Le Gal, 1997). Hence, with the biomass decrease between Days 5 and 12, NH_4^+ could theoretically have increased giving this group the competitive advantage under Phosphate + Trace Metals.

Even though the Phosphate treatment had the second highest relative abundance of coccolithophores, the single addition of Trace Metals did not benefit this group. Hence, Phosphate seems to be the main limiting nutrient while complementary Trace Metals enrichment enabled their dominance. Similarly, coccolithophores did not respond to Trace Metals amendment in HPLC areas, potentially due to their low iron requirements (Sunda and Huntsman, 1995; Lam *et al.*, 2001), their ability to use siderophores to scavenge iron from mineral forms as observed for *E. huxleyi* (Sunda and Huntsman, 1995) and/or their relatively lower response time than other groups when all nutrients are available.

Nitrate + Phosphate

Nitrogen is considered to be the predominant limiting resource in oligotrophic regions in the Atlantic and Pacific oceans according to some studies (Graziano *et al.*, 1996; Davey *et al.*, 2008; Moisander *et al.*, 2012). However, the Northern Hemisphere oligotrophic gyres have lower phosphate concentrations than Southern Hemisphere Gyres, which makes nitrate limitation in relation to phosphate unlikely (Moore *et al.*, 2013). In the oligotrophic North Atlantic it has been hypothesized that after the supply of nutrients with enhanced N:P ratios, phosphorus concentrations are close to limiting, inducing enhanced use of nitrogen for the synthesis of nutrient acquisition proteins to a point of co-limitation (Moore *et al.*, 2008). Initial nutrient concentrations below the detection limit made it impossible to determine the ultimate limiting nutrient. The community responded to the phosphate addition to the Nitrate treatment by increasing the relative abundance of diatoms while decreasing overall dinoflagellate importance, but increasing both diatom (*C. closterium* accounting for ~69% of the diatoms) and dinoflagellate (*Prorocentrum* spp. 58% of dinoflagellates) abundances. The increase of phytoplankton abundances in the Nitrate + Phosphate treatment was very similar to single Phosphate addition, while the Nitrate treatment remained unaltered. This suggests the absence of nitrate and phosphate co-limitation Type I as observed in a community from the oligotrophic subtropical North Atlantic (Moore *et al.*, 2008)

and tropical North Atlantic (Davey *et al.*, 2008), potentially related to an additional nitrogen source here.

Full nutrient replenishment

Full nutrient replenishment (All Nutrients treatment) shifted the community to dominance by unidentified autotrophic spheres of ~4 µm in diameter. At the same time C:N ratios of the POM decreased and $\delta^{15}N$ values were of −2 to −1‰ (Zakrisson *et al.*, 2014). Picoplankton (picophytoplankton and bacterioplankton) is often associated with oligotrophic or ultraoligotrophic areas such as the eastern Mediterranean (Thingstad *et al.*, 2005) due to their lower surface to volume ratio (Irigoien *et al.*, 2004), being responsible for a great portion of carbon fluxes through the microbial loop (Azam *et al.*, 1983). The Azores region can be characterized as alternating between oligo and mesotrophic conditions seasonally. Moreover, some unicellular diazotrophs and *Prochlorococcus* together with *Synechococcus*, often dominant in oligotrophic gyres (Boyd *et al.*, 2010), have shown positive response to dissolved organic carbon (Moisander *et al.*, 2012). However, further information would be required for conclusive reasoning for the observed response, since unicellular diazotrophs are very diverse (Zehr *et al.*, 1998), diverging in their physiology and nutrient requirements for instance (Bullerjahn and Post, 2015).

Finally, the replenishment of All Nutrients also induced an increase in coccolithophore abundance and the strongest response of diatoms and dinoflagellates (Fig. 3). Thus, following the clear dominance of an unidentified sphere (65%) were the small to medium diatoms (~34%, e.g. *C. closterium* and a thin unidentified species, probably *Nanoneis* sp.), followed by dinoflagellates and other flagellates (1%), showing high abundance of the otherwise rare *Scripsiella* sp., and finally coccolithophores (0.1%, dominated by *E. huxleyi*). The overall increase in phytoplankton abundance and inorganic carbon demand decreased carbon fractionation (increased $\delta^{13}C$), potentially due to enhanced growth rates of the dominant species and, therefore, lower uptake of the lighter isotope. In addition, stable carbon isotopic fractionation values depend on environmental conditions and organism physiology, shown by species variable fractionation capacities (Wong and Sackett, 1978), but here it was the overall response that was quantified.

Group/taxon-specific responses to nutrients

Species respond differently to nutrient oscillations, with so-called stable species not following the variation in nutrient concentrations and oscillating species responding to each nutrient replenishment (Sommer, 1984). When phytoplankton reaches high biomass after a nutrient enrichment event, the resulting community is often dominated by diatoms, dinoflagellates or haptophyte species, due to a combination of shading and selective grazing at high biomass (Irigoien *et al.*, 2004).

The abundance of the dinoflagellate *Prorocentrum* spp. increased during the first 5 days (potentially showing a "velocity-adapted" behaviour) under enhanced Phosphate, and was high in several treatments with phosphate replenishment in the second incubation period. An important factor behind the response to the single addition of phosphate might be the ability to store high amounts of nitrogen as shown in *Prorocentrum minimum* (Sciandra, 1991) and to produce a siderophore under iron limiting conditions (Trick, 1989; Sunda and Huntsman, 1995). Natural populations of *Prorocentrum* spp. from different locations might be able to store phosphate, since this genus has been seen to follow peaks of dissolved inorganic phosphorus, thus being negatively correlated with phosphorus concentrations (Li *et al.*, 2011) and positive with nutrient N:P ratio (Li *et al.*, 2011; Glibert *et al.*, 2012). This genus can additionally utilize organic phosphate when inorganic phosphorus becomes scarce due to the enzyme alkaline phosphatase (Dyhrman and Palenik, 1999), enabling it to thrive under phosphate limiting conditions (Li *et al.*, 2011).

The diatoms *Chaetoceros* spp. occurred in small abundances in most treatments, except after the Silicate addition where cell numbers increased, showing, on the one hand, high requirements for silicate and, on the other hand, storage capacity for the other macronutrients. Finally, the replenishment of this macronutrient was also associated with aggregates (~30% of total diatoms present) of a thin diatom, most likely *Nanoneis* sp. even though *C. closterium* is known to form dense aggregates in the natural environment and in the laboratory, which made identification difficult.

Conclusions and implications for natural phytoplankton communities

Phytoplankton communities naturally occurring in oligotrophic areas are often limited by a combination of nutrients. While the Eastern North Atlantic Gyre is often considered nitrogen limited other areas of the Atlantic depend on iron input via Saharan dust. Here, most treatments shifted to the dominance of "velocity-adapted", diatoms. If this response to high-nutrient concentrations could be translated into the environment around the Azores it might have potential impacts for carbon cycling. Phytoplankton biomass build-up and community composition depended on different nutrients, with microphytoplankton abundances responding strongly to the sequential addition of several nutrients

while showing group-specific responses to single nutrients. The addition of Phosphate + Trace Metals, but not Phosphate alone, shifted the community to coccolithophore dominance. Nitrate replenishment increased diatom and dinoflagellate abundances, but not dramatically, indicating that phosphorus (together with trace metals) more than nitrate is the limiting nutrient in this region in October. In spite of being often treated independently, combined nutrient limitation in oligotrophic areas may play a relevant role in phytoplankton community structure. Finally, the addition of All Nutrients increased the biomass of all groups (except coccolithophores) dramatically, being potentially dominated by diazothrophs, which shows the competitive advantage of nitrogen fixers under nutrient replenishment after long limitation periods.

SUPPLEMENTARY DATA

Supplementary data is available at *Journal of Plankton Research* online.

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