Changing nutrient stoichiometry affects phytoplankton production, DOP build up and dinitrogen fixation – a mesocosm experiment in the eastern tropical North Atlantic

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Abstract

Ocean deoxygenation due to climate change may alter redox-sensitive nutrient cycles in the marine environment. The productive eastern tropical North Atlantic (ETNA) upwelling region may be particularly affected when the relatively moderate oxygen minimum zone (OMZ) deoxygenates further and microbially-driven nitrogen (N) loss processes are promoted. Consequently, water masses with a low N:P ratio could reach the euphotic layer, possibly influencing primary production in those waters. Previous mesocosm studies in the oligotrophic Atlantic Ocean identified N availability as controlling of primary production, while a possible co-limitation of nitrate and phosphate (P) could not be ruled out. To better understand the impact of changing N:P ratios on primary production and on N\(_2\) fixation in the ETNA surface ocean, we conducted land-based mesocosm experiments with natural plankton communities and applied a broad range of N:P ratios (2.67–48). Silicate was supplied at 15 µmolL\(^{-1}\) in all mesocosms. We monitored nutrient drawdown, bloom formation, biomass build up and diazotrophic feedback in response to variable nutrient stoichiometry. Our results confirmed N to be limiting to primary production. We found that excess P was channeled through particulate organic matter (POP) into the dissolved organic matter (DOP) pool. In mesocosms with low P availability, DOP was utilized while N\(_2\) fixation increased, suggesting a link between those two processes. Interestingly this observation was most pronounced in mesocosms where inorganic N was still available, indicating that bioavailable N does not necessarily has to have a negative impact on N\(_2\) fixation. We observed a shift from a mixed cyanobacterial/proteobacterial dominated active diazotrophic community towards diazotrophic diatom symbionts of the \textit{Richelia}-\textit{Rhizosolenia} symbiosis. We hypothesize that a potential change in nutrient stoichiometry in the ETNA might lead to a general shift within the diazotrophic community, potentially modifying primary productivity.
1 Introduction

Eastern boundary upwelling systems are characterized by cold, nutrient-rich water masses that are transported from intermediate water layers towards the surface. Resulting extensive primary production forms the basis for high biomass development and a productive food chain (Pennington et al., 2006). At the same time, biological degradation at depth and weak interior ventilation cause permanently low oxygen concentrations in intermediate water masses (100–900 m, Karstensen et al., 2008). These low oxygen conditions support denitrification and anammox that remove bioavailable nitrogen (N) from the water column (e.g. Codispoti et al., 2001; Lam et al., 2009; Kalvelage et al., 2011). Oxygen minimum zones (OMZs) also influence the availability of inorganic phosphorus (P), silicate (Si) and trace elements such as iron (Fe), which are released at the sediment–water interface under oxygen deficient conditions (Ingall and Jahnke, 1994; Hensen et al., 2006). Subsequently, the elemental stoichiometry of inorganic nitrogen to phosphorus (N : P) in upwelled water masses is below the Redfield ratio of 16 : 1 (Redfield, 1958), which manifests itself as an excess of P (P*) relative to N (P* = PO$_4^{3-}$ / NO$_3^-$ /16, after Deutsch et al., 2007).

In the Eastern Tropical North Atlantic (ETNA) nutrient concentrations within the euphotic layer cover a wide range. Water masses in coastal regions feature low N : P ratios mainly as a result of benthic N-loss along with P leaching from the sediment (Trimmer and Nicholls, 2009; Jaeschke et al., 2010; Schafstall et al., 2010) suggesting an N limitation of primary production in OMZ influenced surface waters (Deutsch et al., 2007). In the transition zone between coastal upwelling and open ocean, N : P ratios approach Redfield proportions (Moore et al., 2008). Nevertheless, the nitracline tends to be deeper than the phosphocline in the ETNA (Hauss et al., 2013; Sandel et al., 2015), which also points towards N being the limiting nutrient for primary production in the euphotic zone. In the Central and West Atlantic, N : P ratios beyond 30 : 1 can be reached (Fanning, 1992; Moore et al., 2008), suggesting a severe P limitation of primary producers (Ammerman et al., 2003; Mills et al., 2004). Oxygen concentrations
within the oxygen minimum in the ETNA are usually above 40 µmolkg⁻¹ and thus considered too high to support N loss processes in the water column (Karstensen et al., 2008; Löscher et al., 2012; Ryabenko et al., 2012). However, recent observations of very low oxygen levels just below the mixed layer associated to anticyclonic modewater eddies suggest a potential for denitrification – with an accompanied decrease in N : P levels – in the open ocean of the ETNA (Karstensen et al., 2015).

Discrepancies from the canonical N : P ratio are known to influence productivity and composition of primary producers (Grover, 1997). Since the average elemental composition of C, N and P in seawater as well as in phytoplankton is 106 : 16 : 1, a deviation of dissolved inorganic nutrients from this ratio could indicate which nutrient is ultimately limiting for phytoplankton growth (Lagus, 2004; Moore et al., 2013). Transferring this concept to upwelling regions with inorganic N : P ratios below Redfield, one would expect that the limiting nutrient for phytoplankton growth in those areas is N. It has been shown, however, that certain functional ecotypes of phytoplankton differ in their required nutrient ratio, as specific cellular entities (e.g. chlorophyll, proteins or rRNA) of primary producers have a unique stoichiometric composition deviating from the classical Redfield stoichiometry (Geider and La Roche, 2002; Quigg et al., 2003; Arrigo, 2005). Thus, surface waters adjacent to OMZs potentially provide a niche for certain types of primary producers, whose growth strategy and metabolic requirements are favored by low ratios of N : P. Arrigo (2005) refers to them as “bloomers” and characterizes them as organisms adapted to exponential growth, which contain high amounts of ribosomes and P-rich rRNA. Those organisms build their biomass in non-Redfield-proportions and exhibit low cellular N : P ratios. The deficit in inorganic N of water masses adjacent to OMZs would thus be reduced by this non-Redfield production and N : P ratios further offshore would approach Redfield conditions.

Another concept of phytoplankton growth in N deficient waters is that inorganic nutrients are taken up in Redfield proportion by primary producers, which leaves the surface water masses enriched in P. Excess phosphate presence has been hypothesized to favor N₂-fixation (Deutsch et al., 2007). The conversion of readily available dissolved N₂
into bioavailable forms of fixed N by diazotrophs could replenish the N-deficit in surface waters adjacent to OMZs.

Previous bioassay studies that were conducted to identify controlling factors for primary production in the eastern Atlantic using inorganic N, P and dissolved Fe addition, determined N as the key limiting nutrient (e.g. Graziano et al., 1996; Mills et al., 2004; Moore et al., 2008). These findings are in accordance with an on-board mesocosm study from the same area, where phytoplankton growth depended on the initial supply of N rather than on the N : P ratio and where a combined addition of N and P did not further increase biomass production compared to the addition of N sources alone (Franz et al., 2012). Additionally, the authors deduced that at low N : P ratios excess P was assimilated by non-diazotrophic phytoplankton and was channeled into dissolved organic phosphorus (DOP). As DOP might serve as an additional source of P for bacteria and phytoplankton (Mahaffey et al., 2014 and references therein) and is preferentially taken up by the filamentous diazotrophic cyanobacterium *Trichodesmium* (Dyhrman et al., 2006; Sohm and Capone, 2006), it has been proposed that N\(_2\) fixation might be stimulated by an enhanced DOP supply under low N : P ratios (Franz et al., 2012).

Until recently, oceanic N\(_2\) fixation was mainly attributed to phototrophic cyanobacteria, such as *Trichodesmium* or *Crocosphaera*, which are restricted to nutrient depleted surface to subsurface waters due to their light demand (Capone et al., 1997; Zehr and Turner, 2001). However, several groups of non-cyanobacterial diazotrophs and cyanobacterial symbionts have been detected in various oceanic regions, thus demonstrating the ubiquity and high diversity of diazotrophs (Foster et al., 2009; Farnelid et al., 2011; Loescher et al., 2014). Despite the growing awareness of diazotrophic diversity and distribution, the environmental conditions controlling diazotrophy are still not well understood. However temperature, Fe and P availability and dissolved oxygen concentrations are regarded key factors for diazotrophic distribution and partly for active N\(_2\) fixation (e.g. Sohm et al., 2011). The presence of high amounts of fixed N is thought to inhibit N\(_2\) fixation (Weber and Deutsch, 2014), since diazotrophs are either outcompeted by fast growing phytoplankton species such as diatoms (Bonnet et al.,
2009; Monteiro et al., 2011), or they themselves take up bioavailable forms of N rather than use the energy consuming process of N\textsubscript{2} fixation (Mulholland and Capone, 2001; Mulholland et al., 2001; Dekaezemacker and Bonnet, 2011).

In the ETNA, upwelling of N depleted waters along with high Fe input via Saharan dust deposition (Gao et al., 2001) sets a classical niche for N\textsubscript{2} fixation, while high N : P ratios beyond the upwelling region of the ETNA point towards P limitation of diazotrophs (Ammerman et al., 2003; Mills et al., 2004). Nevertheless, a diverse community of cyanobacterial diazotrophs such as *Trichodesmium* (Capone et al., 1997; Tyrrell et al., 2003), a variety of unicellular cyanobacterial diazotrophs (Groups A, B, C, diatom-symbionts) (Falcon et al., 2002; Langlois et al., 2005) as well as non-cyanobacterial diazotrophs such as different clades of proteobacteria are abundant and widely distributed (e.g. Langlois et al., 2005, 2008). Those diazotrophs have previously been demonstrated to actively fix N\textsubscript{2} in the ETNA (Langlois et al., 2005, 2008; Foster et al., 2009), showing highest rates in nutrient depleted surface to subsurface waters (Großkopf et al., 2012).

We investigated the effect of variable N and P supply on phytoplankton growth and addressed the diazotrophic response to changes in N : P stoichiometry over time in two consecutive mesocosm experiments. In order to extend the design of previous mesocosm experiments (Franz et al., 2012), N and P supply ratios were varied while keeping either nitrate or phosphate at constant concentrations. High N : P ratios were applied to investigate potential inhibition of marine N\textsubscript{2} fixation, while low N : P supply ratios were applied to unravel the role of excess P and consecutively formed DOP on primary production and diazotrophy. Direct N\textsubscript{2} fixation rate measurements as well as determination of *nifH* gene and transcript abundances were carried out to characterize the diazotrophic community and their response to the chosen treatment levels. The experimental design and response variables were chosen in order to assess responses of the phytoplankton community to possible changes in oceanic nutrient stoichiometry as a consequence of ocean deoxygenation.
2 Methods

2.1 Experimental setup

In October 2012 we conducted two 8 day mesocosm experiments at the Instituto Nacional de Desenvolvimento das Pescas (INDP), Mindelo, Cape Verde. The night before the start of each experiment, surface water was collected with RV Islândia south of São Vicente (16°44.4′ N, 25°09.4′ W) and transported to shore using four 600 L food safe intermediate bulk containers. The experimental setup comprised sixteen plastic mesocosm bags, which were distributed in four flow-through water baths. Blue, transparent lids were added to reduce the light intensity to approximately 20 % of surface irradiation. The collected water was evenly distributed among mesocosm bags by gravity, using a submerged hose to minimize bubbles. The volume inside each mesocosm was calculated after adding 1.5 mmol silicate and measuring the resulting silicate concentration. The volume ranged from 105.5–145 liters. Experimental manipulation was achieved by adding different amounts of inorganic N and P. In the first experiment, the P supply was changed at constant N supply (varied P) in thirteen of the sixteen units, while in the second experiment the N supply was changed at constant P supply (varied N) in twelve of the sixteen units. In addition to this, “cornerpoints” were chosen, where both the N and P supply was changed. These treatments were repeated during both experiments (see Fig. 1 for experimental design). Four cornerpoints should have been repeated, but due to erroneous nutrient levels in mesocosm 10 during varied N, this mesocosm also was adjusted to the center point conditions. Experimental treatments were randomly distributed between the four water baths. Initial sampling was carried out immediately after filling of the mesocosms on day 1. After nutrient manipulation, sampling was conducted on a daily basis between 09:00 and 10:30 LT for days 2 to 8. Nutrient levels were set between 2 and 20 µmol L\(^{-1}\) for nitrate, 0.25 and 1.75 µmol L\(^{-1}\) for phosphate and 15 µmol L\(^{-1}\) for silicate. Table S1 gives the target nutrient concentrations and corresponding measured concentrations in the mesocosms.
It has to be noted, that no algal bloom developed in mesocosm 5 during varied N (target concentrations: 17.65 µmolL$^{-1}$ NO$_3^-$, 0.40 µmolL$^{-1}$ PO$_4^{3-}$). Thus, it was not included in the analysis and data are not presented.

Although we refer to our experimental approach as mesocosm experiment, this label might be disputable depending on the definition of the term mesocosm. Sometimes, experimental enclosures are only defined by size, where our approach would fall into the range of a microcosm experiment (< 1 m$^3$; Riebesell et al., 2010). Independent of its size, a mesocosm can also be defined as a confined body of water, where environmental factors are manipulated at the community or ecosystem level (Stewart et al., 2013). In contrast, microcosm experiments are usually used to manipulate factors at the population level and often lack the realism to extrapolate results to natural systems (Stewart et al., 2013). Although our experimental enclosures are limited in size, we feel justified in using the term mesocosm, as we conducted our experiment with a natural community consisting of at least 3 different trophic levels (bacteria, phytoplankton, microzooplankton).

### 2.2 Nutrients

Samples for dissolved inorganic nutrients NO$_3^-$, NO$_2^-$, PO$_4^{3-}$, Si(OH)$_4$) were taken daily from each mesocosm and measured directly using a QuAAtro Autoanalyzer according to Grasshoff et al. (1999).

### 2.3 Chlorophyll a

For chlorophyll $a$ (Chl $a$) analyses, water samples (0.5–1 L) were vacuum-filtered (200 mbar) onto Whatman GF/F filters (25 mm, 0.7 µm) before adding 1 mL of ultrapure water. Filters were immediately stored frozen for at least 24 h. 9 mL acetone (100 %) was then added to each sample and the fluorescence was measured with a Turner Trilogy fluorometer, which was calibrated with a Chl $a$ standard dilution series (Ana-
cystis nidulans, Walter CMP, Kiel, Germany). Chl a concentrations were determined according to Parsons et al. (1984).

2.4 Dissolved organic phosphorus

Water samples for analyses were filtered through pre-combusted (450 °C, 5 h) Whatman GF/F filters (25 mm, 0.7 µm). The filtrate was stored in acid-clean 60 mL HDPE bottles (5 % HCL for at least 12 h) and frozen at −20 °C until further analysis.

Prior to analysis of total dissolved phosphorus (TDP) one metering spoon of the oxidizing reagent Oxisolv (Merck) was added to 40 mL of sample, which was hereupon autoclaved for 30 min. Samples were then analysed spectrophotometrically (Autoanalyzer QuAAtro Seal Analytic), following Bran and Luebbe AutoAnalyzer Method No. G-175-96 Rev. 13 (PO$_4^{3-}$).

DOP concentrations were calculated as:

DOP = total dissolved phosphorus (TDP) − dissolved inorganic phosphorus (DIP) (1)

2.5 Particulate organic matter

Particulate organic matter concentrations were determined by filtering 0.5–1 L seawater through pre-combusted (450 °C for 5 h) Whatman GF/F filters (25 mm, 0.7 µm) under low pressure (200 mbar). Filters were immediately frozen and stored until analysis.

Prior to analysis, particulate organic carbon (POC) and nitrogen (PON) filters were fumed with HCL (37 %, for 24 h) in order to remove inorganic carbon. After drying, filters were wrapped in tin cups (8 mm × 8 mm × 15 mm) and measured according to Sharp (1974) using an elemental analyzer (Euro EA, EuroVector, Milan, Italy).

For particulate organic phosphorus (POP) measurements, filters were autoclaved with the oxidation reagent Oxisolv (Merck) and 40 mL of ultrapure water for 30 min in a pressure cooker. Then, orthophosphate was analyzed photometrically according to Hansen and Koroleff (1999).
2.6 Molecular methods

Samples for the extraction of DNA/RNA were taken by filtering a volume of 1–2 L (exact volumes and filtration times were determined and recorded continuously) of seawater through 0.2 µm polyethersulfon membrane filters (Millipore, Billerica, MA, USA). The filters were frozen and stored at −80°C until analysis. Nucleic acid extraction was performed using the Qiagen DNA/RNA All prep Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The extracted RNA was reverse transcribed to cDNA using the Superscript III First Strand synthesis Kit (Invitrogen) following the manufacturer’s protocol with primers nifH2 and nifH3 (Langlois et al., 2005; Zani et al., 2000). NifH clusters were quantified from DNA and cDNA by quantitative Real Time PCRs as previously described by Church et al. (2005) and Langlois et al. (2008). TaqMan® qPCRs were set up in 12.5 µL reactions and were performed in technical duplicates in an ABI ViiA7 qPCR system (Life technologies, Carlsbad, CA, USA). For each primer and probe set, standard curves were obtained from dilution series ranging from 10^7 to 10 gene copies per reaction; standards were constructed using plasmids containing the target nifH gene. Sequences of primers and probes are given in Table 1. To confirm purity of RNA, non-template qPCRs were performed using the corresponding RNA.

2.7 ^15N2 seawater incubations

Seawater incubations were performed in triplicates from each mesocosm on day 2 and day 8 of both experiments as previously described by Mohr et al. (2010) and Großkopf et al. (2012). Seawater samples were filled headspace-free; 100 mL of seawater were exchanged with previously degassed seawater containing a defined concentration ^15N2 and ^13C-NaCO. Incubations were performed in 4.5 L polycarbonate bottles closed with Teflon®-coated butyl rubber septum caps. Water samples were incubated for 24 h in the mesocosm water baths, thus at the same temperature and light regime, followed by a filtration on Whatman GF/F filters, which were analyzed using mass spectrometry as previously described in Loescher et al. (2014).
2.8 Statistical evaluation

Relationships of dissolved and particulate organic matter build up to the inorganic nutrient supply ratios were determined using regression analyses (SigmaPlot, Systat).

Data selection

Statistical modelling was performed with a subset of the dataset generated by removal of (i) variables with missing measures, (ii) incomplete observations, (iii) variables left with all-zero data. In order to minimize collinearity of explanatory variables in the statistical models, a set of environmental variables was chosen according to their variance inflation factor (VIF): starting with a linear model that included all variables of interest, the variable with the highest VIF was iteratively determined and removed from the model until all remaining explanatory variables had a VIF < 2.5. The set of explanatory variables with minimal collinearity was used in model selection (see Supplement).

Model selection

Concentrations of genes and transcripts, respectively, as determined by qPCR, were fitted to the selected explanatory variables in generalized linear additive mixed models employing functions gam and gamm of the R package mgcv v1.8-4 (Wood, 2004, 2011). Multivariate analysis of counts was realized by a factor of gene/transcript names in the model. Temporal variation of gene/transcript counts was modeled by cubic spline smoothers. Each combination of gene/transcript and Run_ID (i.e. varied N or varied P) was given its own smoothing function. Other explanatory variables were standardized to zero mean and unit variance and added as covariates, or – if a non-linear response was to be expected – as cubic spline smoothers.

Candidate models were compared by means of the Akaike Information Criterion (AIC) and validated following the protocol of Zuur et al. (2009). Briefly, this comprised: (i) choice of an appropriate variance structure for the full model containing all selected
3 Results

3.1 Bloom development and nutrient dynamics in the mesocosms

In both consecutive experiments (varied \(P\) and \(N\)) a bloom formation was observed following nutrient manipulation. \(\text{NO}_3^-\) and \(\text{PO}_4^{3-}\) were readily taken up by the plankton community and nutrient concentrations thus declined until the end of the experiment (Fig. 2). \(\text{NO}_3^-\) was fully depleted in all mesocosms at days 6–8 in both runs, except in the mesocosms with highest \(N: P\) ratios (12.00\(N/0.25P\) in varied \(P\) and 17.65\(N/0.40P\) in varied \(N\)). Residual \(\text{PO}_4^{3-}\) was still detectable at the end of the experiments (day 8) in all mesocosms with initial \(N: P\) values < 10 (varied \(P\): 6.35\(N/1.10P\), 12.00\(N/1.25P\), 12.00\(N/1.75P\); varied \(N\): 2.00\(N/0.75P\), 4.00\(N/0.75P\), 6.00\(N/1.03P\)) indicating a limitation of primary productivity dependent on the \(N: P\) stoichiometry.

Although initial Chl \(a\) concentrations were slightly higher in varied \(P\) than in varied \(N\) (≈ 0.38 and 0.2 \(\mu g\) \(L^{-1}\), respectively), the increase in Chl \(a\) concentration was 5–10-fold until days 5/6 in varied \(P\) compared to 10–50-fold in varied \(N\). After the bloom at days 5 and 6 Chl \(a\) declined again to 0.05–0.7 and 0.6–1.7 \(\mu g\) \(L^{-1}\) in varied \(P\) and varied \(N\), respectively (Fig. 2). In both runs, bloom formation was initially independent from nutrient supply, however, the applied statistical model showed a slight positive correlation between Chl \(a\) and initial \(N: P\) (see Supplement, Fig. S1).
3.2 Particulate organic matter (POM) build-up and stoichiometry

Temporal dynamics of POM were similar during both experiments. Initial concentrations of POC, PON and POP were 10–17, 1.5–2, and 0.05–0.12 μmol L\(^{-1}\), respectively (Fig. 2). In varied \(P\), POC and PON reached a maximum on day 6, while POP increased until the end of the experiment. In varied \(N\) POM build up also peaked on day 6 or 7 in most mesocosms, but differences between N : P treatments were more pronounced in varied \(N\) compared to varied \(P\). Our results indicate that POM build-up was independent of the initial nutrient supply ratio in both experiments (Fig. 3). We observed a significantly positive regression coefficient between maximum POC and PON concentrations (defined as peak POC and PON concentration subtracted by the initial (day 1) POC and PON concentration) to the initial N supply (POC: \(r^2 = 0.64, p = 0.006\); PON: \(r^2 = 0.80, p < 0.0001\)) while POP build-up showed a significantly positive regression coefficient to initial P supply (\(r^2 = 0.31, p = 0.048\)).

Mean PON : POP ratios during the exponential growth phase appeared to be independent of the initial N : P supply ratio in both experimental runs (Fig. 4). With ratios between 16.6 and 22.8, the PON : POP ratios were above, but close to Redfield proportion in all treatments during the first 5 days of the experiments, consistent with an observed initial uptake of N and P in Redfield proportions in all mesocosms. During the post bloom phase, mean PON : POP ratios were positively correlated with the initial nutrient supply ratio (\(r^2 = 0.73, p < 0.0001\)). Nevertheless, stoichiometry of POM (N : P between 15.9 and 31.9) mostly exceeded Redfield proportions, even in treatments with lowest N : P ratios.

3.3 Dissolved organic phosphorus dynamics

Initial DOP concentrations during varied \(P\) were 0.14 (±0.009) μmol L\(^{-1}\) and increased in all mesocosms, except in the one with lowest initial P supply (12.00N/0.25P). Maximum DOP concentrations of around 0.4 μmol L\(^{-1}\) were determined in mesocosm 12.00N/0.75P on day 5 and decreased again afterwards. In all other mesocosms DOP
concentrations increased progressively until the end of the experiment (Fig. 5). Maximum DOP build up (defined as described for maximum POM build up, Sect. 3.2) was significantly correlated to the initial P supply (Fig. 5; $r^2 = 0.63$, $p = 0.0007$), which was also in accordance with the applied statistical model (Fig. S1).

In varied $N$ initial DOP concentrations in the mesocosms were 0.2 (±0.038) µmol L$^{-1}$ and increased slightly until day 3. Afterwards DOP concentrations remained rather constant, although with considerable variability in the data (Fig. 5).

### 3.4 Importance of the Richelia–Rhizosolenia symbiosis for diazotrophy

Directly measured rates of N$_2$ fixation showed an increase with time in varied $P$, while no statistically significant increase could be observed in varied $N$ (Fig. 6).

A molecular screening of the diazotrophic community in the initial water batch used for varied $P$ using the $nifH$ gene as functional marker gene showed a dominance of filamentous cyanobacterial diazotrophs related to *Trichodesmium* accounting for ~ 54% of the diazotrophic community (results from qPCR), followed by proteobacterial diazotrophs (~ 36%) in varied $P$. The high abundance of filamentous cyanobacterial diazotrophs indicated the presence of a bloom in the initial water batch in varied $P$. In varied $N$, the initial community consisted mainly of proteobacterial diazotrophs (~ 88%), followed by UCYN-B (9%) and filamentous cyanobacteria (3%).

Predictability of gene abundances by time and Run ID (i.e. varied $N$ or varied $P$) was assessed at the response scale by plotting the values fitted by the model with their 95% confidence bands (Fig. 7). Except for $nifH_{Fil}$, which displayed no temporal changes at all, gene count levels were generally higher in varied $P$ compared to varied $N$. This was especially true for all time points in case of the gamma proteobacterial $nifH_{AO}$ (significance bands overlapped by less than one half, Cumming et al., 2007). In unicellular cyanobacterial clusters $nifH_{UA}$ (UCYN-A) and $nifH_{CR}$ (UCYN-B), gene abundances differed only intermittently between the two experimental runs: while $nifH_{UA}$ abundances were higher in varied $P$ at days 3–4 and 6–8, $nifH_{CR}$ abundances were higher in varied $P$ at days 2 and 4–6.
Changes in transcript abundance over time and Run ID (Fig. 8) showed mainly different response patterns between nifH_Het I transcripts. At day 2, nifH_Het I transcript abundances were higher in varied N conditions compared to varied P. This relation changed over the course of the experiment, with a pronounced increase of nifH_Het I transcript abundances between day 6 and 8 in varied P.

Thus, all classical nifH clusters (filamentous cyanobacteria, UCYN-A, -B, -C and proteobacteria diazotrophs) decreased in abundance of genes and gene transcripts down to the detection limit in both experiments (Figs. 7, 8, S3, and S4), whereas diazotrophs of the Richelia-Rhizosolenia symbiosis (Het I) were the only diazotrophs that showed an increase in nifH transcripts over the course of the experiment, exclusively in varied P (Figs. 8, S2).

In varied P, we observed an accumulation of DOP. In contrast, during varied N, nifH gene and transcript abundance of the Richelia-Rhizosolenia cluster was close to the detection limit and DOP build-up was rather negligible, thus a potential impact of DOP on diazotrophy was hypothesized. To unravel a potential impact of DOP on N₂ fixation, we investigated temporal DOP patterns, which appeared strongly non-linear. At standard scores below −1 (~0.19 µM) DOP tended to contribute negatively to overall nifH transcript abundance levels, whereas at standard scores around −0.5 (~0.24 µM) the effect of DOP on nifH transcript abundance was positive. At higher concentrations, DOP tended again to a neutral or negative impact on transcript abundance indicating an optimum of DOP concentration on nifH transcript abundance.

In varied P, mesocosms with a significant increase in N₂ fixation (12.00N/0.25P and 12.00/0.75P) were also the ones where DOP was used as P-source for biomass build up after PO₄³⁻ was depleted (Fig. 9). In mesocosm 12.00N/0.75P, PO₄³⁻ concentrations were below the detection limit after day 5. This coincided with a decrease of DOP after day 5, while POP concentrations increased until the end of the experiment. In mesocosm 12.00N/0.25P, POP also increased beyond the point of PO₄³⁻ depletion and highest POP build up exceeded values that could be explained by P incorporation alone. In mesocosms without a significant increase in N₂ fixation, POP and DOP con-
centrations increased until the end of the experiment and no apparent uptake of DOP could be observed.

4 Discussion

4.1 Controls on primary production

In order to understand potential consequences of changes in nutrient regimes, it is necessary to determine the factors that control and limit primary production. In our experiments, amendments of N significantly increased chlorophyll concentrations and enhanced the buildup of POM, indicating the ability of the plankton community to rapidly and intensively react to N availability. These results indicate that the ultimate limiting nutrient for the phytoplankton community in our experiment was N. N$_2$ fixation was measurable in all initial samples, which indicates the presence of a niche for diazotrophs in the Cape Verde region. For the upwelling region as well as for the oligotrophic open ocean of the ETNA, N limitation of the phytoplankton community has previously been reported (Davey et al., 2008; Moore et al., 2008; Franz et al., 2012). Additionally, Moore et al. (2008) observed a co-limitation of N and P during nutrient addition bioassay experiments in the ETNA. In our experiment, however, only POP build up was positively affected by P supply. This argues against a secondary limitation by P, but rather points towards a mechanism of accumulating and storing phosphate as polyphosphate within the cell (Schelske and Sicko-Goad, 1990; Geider and La Roche, 2002; Martin et al., 2014).

There is a large difference between the supply ratio of inorganic nutrients and the PON : POP ratio of primary producers in our study. Although initial N : P ratios in our mesocosms covered a wide range, PON : POP ratios reached maximum values of $\sim$ 21 in both experiments during the exponential growth phase. During stationary growth, maximum PON : POP values of 38.8 in varied N and 21.9 in varied P were measured. However, during growth phases in both experiments PON : POP ratios did never drop
below 15.9. Very similar results were obtained by Franz et al. (2012) off the Peruvian coast. However, two experiments conducted by Franz et al. (2012) in the ETNA and WA showed a different response of the phytoplankton community. In these two cases, N : P supply ratio and PON : POP were highly correlated and PON : POP ratios as low as 6.0 (±1.4) were observed in the stagnant phase. This shows that the stoichiometry of phytoplankton communities is flexible to a certain extent, but sometimes does not reach dimensions observed in laboratory experiments (Hecky et al., 1993) and implied by theoretical approaches (e.g. Geider and La Roche, 2002; Klausmeier et al., 2004). Reasons could be differences in the initial community composition that might lack organisms capable of assembling a P-rich growth machinery (Klausmeier et al., 2004; Arrigo, 2005) or other, yet unresolved factors. It has been reported that cellular N contents seems relatively inflexible in parts of the phytoplankton community, thus restricting the maintenance of metabolic processes at low dissolved inorganic nitrogen (DIN) concentrations (Moore et al., 2013). In contrast, P requirements seem to be comparably flexible, as certain cellular components containing P (e.g. phospholipids) can be replaced by non-phosphorus containing compounds (Moore et al., 2013). This can also be deduced from our experiments, where higher N : P ratios lead to increasing PON : POP ratios, possibly due to the flexibility to substitute P compounds within the biomass. In contrast, lower N : P ratios lead to lower biomass accumulation, as the plasticity of PON : POP seems to be constrained by the availability of N in our experiments.

4.2 The impact of bioavailable N on N₂ fixation

The ability of diazotrophs to grow independent of a fixed N source gives them in principle an advantage to thrive under conditions where their competitors are limited by N availability. At the same time, diazotrophs are considered disadvantaged when competing with faster growing non-diazotrophs for nutrients under N replete conditions (Tyrrell, 1999; Ward et al., 2013). Contrary to this classical view, we could not detect a direct influence of reactive N compounds on N₂ fixation in our experiments. Despite a wide
spectrum of applied N concentrations in varied N, no significant change in N\(_2\) fixation rates could be detected. Evidence from culture experiments also suggests that inorganic N compounds do not universally repress N\(_2\) fixation. While NO\(_3^-\) addition in Trichodesmium spp. (Mulholland et al., 2001; Holl and Montoya, 2005) and NH\(_4^+\) addition in Crocosphaera watsonii (Dekaezemacker and Bonnet, 2011) reduced N\(_2\) fixation rates, NO\(_3^-\) addition did not reduce N\(_2\) fixation rates in C. watsonii and Nodularia spp. cultures (Sanz-Alférez and del Campo, 1994; Dekaezemacker and Bonnet, 2011). Moreover, recent field surveys demonstrated the occurrence of N\(_2\) fixation in nutrient rich water masses of the eastern tropical South Pacific (ETSP) and equatorial Atlantic upwelling regions (Fernandez et al., 2011; Subramaniam et al., 2013; Loescher et al., 2014) and also modelling efforts predict high N\(_2\) fixation rates in waters containing measurable amounts of fixed N (Deutsch et al., 2012; Weber and Deutsch, 2014). Clearly, the degree of feedback concerning the inhibition of N\(_2\) fixation by reactive N compounds is not universal and there is evidence that the absence of P and Fe in seawater is a stronger indicator for limitation of N\(_2\) fixation than the presence of inorganic N compounds (Weber and Deutsch, 2014).

### 4.3 The role of excess P and DOP as controls on N\(_2\) fixation

Deutsch et al. (2007) suggested that N\(_2\) fixation is favored in upwelling regions, where N loss in adjacent OMZ waters and P leaching from the sediment lead to upwelling of waters enriched in P. This excess P is thought to be consumed by diazotrophs, thus replenishing the N-deficit in the vicinity of upwelling regions.

As nutrients were taken up in Redfield or above Redfield proportions in our experiments we would have expected excess P in mesocosm with N:P supply ratios below Redfield. Instead, excess P was absent and our data point towards a channeling of P through the particulate pool into DOP, as an increase in P supply significantly increased the concentration of DOP. Why phytoplankton synthesize and excrete higher levels of DOP under excess P conditions remains unclear, but enhanced P uptake (followed by DOP accumulation) is thought to hamper P limitation when sudden boosts in N are en-
countered (Mackey, 2012). In accordance with our study, mesocosm experiments from the ETNA and eastern tropical south Pacific (ETSP) open ocean (Franz et al., 2012) and measurements from shelf regions of the ETNA (Reynolds et al., 2014) showed the accumulation of DOP under excess P supply. Although the composition and bioavailability of the DOP pool needs to be further evaluated, DOP may act as a source of P for prokaryotic primary producers, either exclusively or in addition to DIP (Björkman and Karl, 2003; Dyhrman et al., 2006; Mahaffey et al., 2014; Reynolds et al., 2014). This indicates that the ability to utilize DOP may give diazotrophs a competitive advantage when bioavailable forms of N are depleted and either P* or DOP concentrations are sufficient.

In our experiments a significant increase in N\textsubscript{2} fixation rates was only measured in varied P. In mesocosms with highest N\textsubscript{2} fixation rates, DIP was depleted after day 5 or 6 while POP increased until the end of the experiment. After DIP depletion, DOP concentrations declined, which indicates that DOP served as P source until the end of the experiment. Increasing diazotrophic transcript abundances of *Richelia intracellularis* in symbiosis with the diatom *Rhizosolenia* (Het I) were detected over the course of the same experiment. While the diatom abundance was probably favored by replete amounts of silicate added at the beginning of the experiment, no increase in diatom-diazotroph associations (DDAs) was detected in the varied N experiment. This leads us to speculate that DDAs were favored in the varied P experiment, where diazotrophs in the mesocosms utilized DOP resources in order to supply P to themselves and/or their symbiont. The ability to utilize DOP has previously been shown for *R. intracellularis* (Girault et al., 2013) and our observations suggest that they may not only provide their symbionts with N via N\textsubscript{2} fixation but also with P via DOP utilization.

DDAs in our experiment were favored by replete amounts of silicate and DOP and were – in contrast to the classical view – not restrained by reactive N compounds. These findings suggest that DDAs have the potential to actively fix nitrogen in shelf waters of upwelling regions. Therefore, the N-deficit of upwelled water-masses could already be replenished locally prior offshore transport.
5 Conclusions and future implications for ETNA

Our findings add to the growing evidence that diminished N : P ratios in upwelling waters in the ETNA will either decrease the biomass of non-diazotrophic primary producers, specifically due to the decline of bioavailable N, or lead to a community shift towards primary producers that are able to adapt to changing N : P conditions. As a considerable amount of DOP was produced under excess P conditions, changes in the N : P ratio of waters could exert profound control over DOP production rates in the ETNA. Our results indicate that enhanced DOP production in upwelling regions will likely fuel N\textsubscript{2} fixation, with an advantage for those diazotrophs capable of DOP utilization. We propose that N\textsubscript{2} fixation in the ETNA might not only be restricted to the oligotrophic open ocean but can occur in nutrient-rich upwelling regions as previously demonstrated for the tropical Pacific (Löscher et al., 2014) and the Atlantic equatorial upwelling (Subramanian et al., 2013), as N\textsubscript{2} fixation in DDAs seems to be favored by the presence of silicate and DOP, and not by the absence of fixed N compounds.

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Table 1. Primers and Probes used in *nifH* TaqMan qPCR assays.

| Target Group | Reverse Primer (5′–3′) | Forward Primer (5′–3′) | Probe (5′–3′) |
|--------------|------------------------|------------------------|--------------|
| Filamentous (Fil) | GCAAATCCACCCGAAACAAC | TGGCGTGTTATTACTGCTATC | AAGGAGTTTACAGATCTA |
| UCYN-A | TCAGGACCCCAGAATTTACTAA | TAGCTGCGAAAGAGAAGGAGAAGGAGAG | TAACTCGGGCTTAAAAAC |
| UCYN-B | TCAGGACCCCAGAATTTACTAA | TGCTGAAATGTGGGTTGCTTGGAA | GAAGACGTATGCT |
| UCYN-C | GGTTGCTTACAGATTTCTCCGCTCTGTAA | TCTACCCGTTTTGATGCTACACCTA | AACTCGGCATTCTCCTTAGCAG |
| GamA | ATGCATGATTTCCTGAGCCTATTTC | TTATGATGGTTGCTGATGTT | TGCAATGCGCATTTCG |
| Het I (Rich-Rizo) | ATACCACGACCGCGACAAC | CCGTTTCCGAGGTGACGTT | TCCGCTGGCTGCTGAGCCTGGT |
| Het II (Rich-Hemi) | AATGCGCGACCAGCACAAC | TGGTACCGGATGATCAGT | TCTGGTGGCTGGAGCGCTGGT |
**Figure 1.** Experimental design and initial nutrient supply conditions during varied $P$ (blue circles) and varied $N$ (red diamonds). “Cornerpoints” during varied $P$ and varied $N$ are depicted as grey circles and white diamonds, respectively.
Figure 2. Temporal development of (a) NO$_3^-$ and NO$_2^-$, (b) PO$_4^{3-}$, (c) Chl a, (d) POC, (e) PON and (f) POP within all treatments of both experimental runs. Standard errors are depicted as shaded error bands.
Figure 3. Maximum POC, PON and POP build-up as a function of the initial supply of N, P and N/P. Maximum δPOM is defined as peak POM concentration subtracted by the initial (day 1) POM concentration. Treatments in varied P are depicted as blue circles; treatments in varied N are depicted as red diamonds.
Figure 4. PON/POP stoichiometry during (a) the exponential growth phase and (b) the stationary growth phase of the experiment. The grey line visualizes the Redfield Ratio. The color code is the same as in Fig. 3.
Figure 5. Temporal development of DOP with standard errors depicted as shaded error bands.
Figure 6. Maximum DOP build-up (defined as peak DOP concentration subtracted by the initial DOP concentration) as a function of initial P supply during varied P (blue circles) and varied N (red diamonds).
Figure 7. \(N_2\) fixation rates measured on day 2 and day 8 of both experiments. Because of the high variance between replicates we omitted \(N_2\) fixation rates from un-replicated treatments. Asterisks indicate a significant difference between day 2 and day 8.
Figure 8. Selected gene count models over time. Predicted counts (solid lines) with 95% confidence intervals (CI; dashed lines) are plotted along with measured count data. The predictive model is based on the original (untransformed) gene counts. For better visualization, values were square root-transformed prior to plotting.
Figure 9. Selected transcript count models over time. Predicted counts (solid lines) with 95% confidence intervals (CI; dashed lines) are plotted along with measured count data. The predictive model is based on the original (untransformed) transcript counts. For better visualization, values were square root-transformed prior to plotting.
Figure 10. Dynamics of $\text{PO}_4^{3-}$, POP and DOP in all mesocosms. Because of the high variance between replicates we omitted $\text{N}_2$ fixation rates from un-replicated treatments.