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No observed effect of ocean acidification on nitrogen biogeochemistry in a summer Baltic Sea plankton community

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Abstract

Nitrogen fixation by filamentous cyanobacteria supplies significant amounts of new nitrogen (N) to the Baltic Sea. This balances N loss processes such as denitrification and anammox and forms an important N source supporting primary and secondary production in N-limited post-spring bloom plankton communities. Laboratory studies suggest that filamentous diazotrophic cyanobacteria growth and N_2-fixation rates are sensitive to ocean acidification with potential implications for new N supply to the Baltic Sea. In this study, our aim was to assess the effect of ocean acidification on diazotroph growth and activity as well as the contribution of diazotrophically-fixed N to N supply in a natural plankton assemblage. We enclosed a natural plankton community in a summer season in the Baltic Sea near the entrance to the Gulf of Finland in six large-scale mesocosms (volume $\sim 55\,m^3$) and manipulated fCO_2 over a range relevant for projected ocean acidification by the end of this century (average treatment fCO_2: 365–1231 µatm). The direct response of diazotroph growth and activity was followed in the mesocosms over a 47 day study period during N-limited growth in the summer plankton community. Diazotrophic filamentous cyanobacteria abundance throughout the study period and N_2-fixation rates (determined only until day 21 due to subsequent use of contaminated commercial ^{15}N-N_2 gas stocks) remained low. Thus estimated new N inputs from diazotrophy were too low to relieve N limitation and stimulate a summer phytoplankton bloom. Instead regeneration of organic N sources likely sustained growth in the plankton community. We could not detect significant CO_2-related differences in inorganic or organic N pools sizes, or particulate matter N : P stoichiometry. Additionally, no significant effect of elevated CO_2 on diazotroph activity was observed. Therefore, ocean acidification had no observable impact on N cycling or biogeochemistry in this N-limited, post-spring bloom plankton assemblage in the Baltic Sea.

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1 Introduction

Nitrogen (N) is an essential element for cell functioning in the biosphere due to its presence in many important biomolecules such as nucleic acids and proteins. However, in many marine ecosystems N is considered the limiting nutrient for important cellular processes in phytoplankton (Vitousek and Howarth, 1991), as indicated through stimulation carbon fixation and pigment synthesis through addition of inorganic N (e.g. Moore et al., 2008, 2013). This low N availability also prevails in post-spring bloom plankton communities in the Baltic Sea, as the nitrate pool is exhausted during the spring-bloom leaving behind an excess of dissolved inorganic phosphorus (Wasmund et al., 2001). Consequently, filamentous diazotrophic (N_2-fixing) cyanobacteria, in particular heterocystous *Nodularia spumigena* and *Aphanizomenon flos-aquae*, capitalise on this excess phosphate and increasing water column temperatures in summer months (Kononen et al., 1996; Pliński and Jóźwiak, 1999; Wasmund, 1997) and commonly form extensive blooms and surface aggregations (e.g. Kahru and Elmgren, 2014). The atmospheric nitrogen gas (N_2) fixed by these heterocystous cyanobacteria during the summer months forms a key N source for the wider plankton community in the Baltic Sea, since a significant fraction of the fixed N can be released as ammonium (Ohlendieck et al., 2000; Ploug et al., 2010; Stal et al., 2003; Wannicke et al., 2013) and dissolved organic N compounds (Ohlendieck et al., 2000, 2007; Wannicke et al., 2013). Thus in addition to N in diazotroph biomass, newly fixed N is also available for direct assimilation by phytoplankton and bacteria and is estimated to support up to 20–45 % of annual primary production in the Baltic Sea (Gustafsson et al., 2013). This new N input partly replenishes N loss processes such as anammox and denitrification in the deep anoxic basins (Vahtera et al., 2007). Furthermore, this fixed N can also be directly transferred to higher trophic levels through grazing by zooplankton (Engström-Öst et al., 2011; Hogfors et al., 2014; Wannicke et al., 2013).

Changes in seawater carbonate chemistry due to increased atmospheric CO_2 concentrations are expected to induce changes in phytoplankton physiology. The associ-

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ated decrease in seawater pH is called ocean acidification. Numerous single-strain culture studies have investigated the physiological responses of a variety of diazotrophic organisms and generally indicated increased N_2-fixation and diazotroph growth rates under elevated CO_2 (Barcelos e Ramos et al., 2007; Fu et al., 2008; Hutchins et al., 2007; Kranz et al., 2010; Levitan et al., 2007), with contrasting evidence under iron limitation (Shi et al., 2012) and with freshwater strains of *A. flos-aquae* (Yamamoto and Nakahara, 2005). Three studies on the common Baltic Sea species, *N. spumigena*, produced contrasting results with two studies under phosphate repletion suggesting a negative effect (Czerny et al., 2009; Eichner et al., 2014), and one study, under low inorganic phosphate availability, indicating a positive effect (Wannicke et al., 2012) of increased CO_2 on growth and N_2-fixation rates. This discrepancy may, however, be due to differences in phosphate availability (Eichner et al., 2014). Considering the contribution of diazotrophs to the N budget and primary productivity in the Baltic Sea, it is vital to understand the influence of future changes in pCO_2 on new N inputs by diazotrophs.

In this mesocosm study, our aim was to assess diazotroph growth and rates of N_2-fixation under a range of CO_2 concentrations in a natural plankton community. N limitation of phytoplankton growth was reported in the study area in the Finland Archipelago Sea (Kirkkala et al., 1997; Tamminen and Andersen, 2007). By utilizing the naturally occurring low N conditions in the Baltic Sea we wanted to examine the importance of new N inputs by diazotrophic organisms to the wider plankton community N supply under projected future ocean acidification scenarios.

2 Materials and methods

2.1 Experimental set-up and sampling

The study took place in the period between June and August 2012 in Tvärminne Storfjärden which is situated in the Archipelago Sea on the southwestern tip of Finland. Six pelagic mesocosms (total volume ~ 55 m^3, KOSMOS, Riebesell et al., 2013) were

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deployed on 12 June 2012 (day of experiment −10 = *t-10*, i.e. 10 days before CO_2 manipulation) and moored at 59°51.5′ N, 23°15.5′ E. The cylindrical mesocosm bags of 2 m in diameter extended from 1.5 m above to 19 m below the water surface and were closed at the bottom by a 2 m long sediment trap funnel on *t-5*. A 3 mm net was used to exclude larger organisms or particles before mesocosm closure.

A gradient of CO_2 treatments across the mesocosms was established over a four day period by additions of filtered (50 µm), CO_2-saturated seawater evenly distributed in the water column, as described by Riebesell et al. (2013). CO_2 additions were carried out in the afternoons of *t0* – *t4* not to interfere with the daily sampling. A CO_2 addition was also made in the upper 7 m on *t15* to counter strong outgassing in the upper water column. Initial fCO_2 ranged from ~ 240 µatm in the two ambient control mesocosms to up to 1650 µatm (Fig. 1a). Unenriched filtered (50 µm) seawater was added to the two control mesocosms (M1, M5). The seawater used for the additions to the mesocosms was collected from the Tvärminne Storfjärden from a depth of 10 m by a pipe connected to the laboratory at the research station.

Depth-integrating water samplers (IWS, HYDRO-BIOS, Kiel) were used to collect water from 0–17 m depth in each mesocosm for analysis of particulate matter, dissolved inorganic and organic matter, phytoplankton pigments, phytoplankton abundances, carbonate chemistry variables. Samples for carbonate chemistry variables were taken directly from the IWS on board the sampling boat whereas all other samples were pooled in 10 L plastic carboys and stored on board in the dark until sub-sampling on shore (Paul et al., 2015). Particulate matter collected in the sediment trap was pumped to the surface and collected in sampling bottles (Boxhammer et al., 2015).

Particulate matter (C, N, P) was collected onto GF/F filters (nominal pore size of 0.7 µm, 25 mm diameter, Whatman) by gentle vacuum filtration (pressure < 200 mbar). Filters and glass petri dishes were combusted at 450 °C for 6 h before use. Collected particulate sediment material was concentrated, freeze-dried and ground to a homogenous powder, while supernatant subsamples were filtered and subsequently analysed as for water column material. Total particulate carbon and nitrogen (TPC and PON)

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content and isotopic composition were analysed according to Sharp et al. (1974) using an elemental analyser (EuroEA) coupled by either a Conflo II to a Finnigan Delta^{Plus} isotope ratio mass spectrometer or by a Conflo III to a Thermo Finnigan Delta^{Plus} XP isotope ratio mass spectrometer. Stable N isotope composition of particulate N is reported in permil (‰) relative to the atmospheric N_2 standard (AIR). Total particulate phosphorus (TPP) concentrations were determined spectrophotometrically following sample digestion as described in Hansen and Koroleff (1999). Samples for biogenic silica (BSi) analyses were collected on cellulose acetate filters (pore size of 0.65 µm, 25 mm diameter, Whatman) by filtration as described above for particulate matter. Concentrations were determined spectrophotometrically following sample digestion according to Hansen and Koroleff (1999). Samples for determination of nanomolar concentrations of dissolved inorganic nutrients were filtered (GF/F, nominal pore size of 0.7 µm, Fisher Scientific). Nitrate and nitrite (hereafter nitrate) and dissolved inorganic phosphate concentrations were then analysed colorimetrically using a 2 m liquid waveguide capillary cell (Patey et al., 2008; Zhang and Chi, 2002) and a miniaturised detector (Ocean Optics Ltd). Concentrations of ammonium (NH_4^+) were determined fluorimetrically (Trilogy, Turner) according to Kérouel and Aminot (1997). Total dissolved nitrogen (TDN) was analysed using a high-temperature catalytic combustion technique with a Shimadzu TOC-TN V analyser as described by Badr et al. (2003). Samples were filtered (GF/F, nominal pore size of 0.7 µm, Fisher Scientific) to remove particulate material and collected in clean glass vials, acidified with HCl to pH 1.9 and flame sealed. Filters and vials were combusted for 6 h at 450 °C before use. Dissolved organic nitrogen (DON) concentrations were calculated by subtracting the inorganic N concentrations from TDN. Phytoplankton pigments were extracted in acetone (90 %) and after homogenisation and centrifugation, the supernatant was filtered (0.2 µm PTFE filters, VWR International) and concentrations were determined by reverse phase high performance liquid chromatography (HPLC; WATERS HPLC with a Varian Microsorb-MV 100-3 C8 column; Barlow et al., 1997; Derenbach et al., 1969). A library of premeasured commercial standards was used to calibrate peaks.

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Phosphate excess (P*, Deutsch et al., 2007) was calculated from the dissolved inorganic phosphate, nitrate and ammonium concentrations according to:

$$P^* = \left[PO_4^{3-}\right] - \frac{\left[NO_3^-\right] + \left[NH_4^+\right]}{16} \quad (1)$$

Dissolved silicate (DSi) drawdown was calculated as the difference in DSi concentration on a given sampling day (*tx*) and *t1*:

$$\text{DSi drawdown} = [\text{DSi}]_{t1} - [\text{DSi}]_{tx} \quad (2)$$

A comprehensive description of mesocosm deployment, set-up and sampling procedures including sample collection, handling and analyses for particulate matter, dissolved inorganic and organic matter, phytoplankton pigments, and sediment trap particulate matter is covered in Paul et al. (2015), also in this Special Issue. An overview table of sampled variables for the entire experiment, including sampling frequency, is also presented in this accompanying manuscript.

2.2 N_2-fixation rate incubations

Incubations for determination of N_2-fixation rates were carried out according to Mohr et al. (2010). Seawater used for $^{15}N\text{-}N_2$ enrichments was filtered (polycarbonate Isopore™ filter, pore size of 0.22 µm, 47 mm diameter) before being pumped through a degassing membrane (Membrana Mini Module G542) attached to a water-jet pump to remove ambient N_2. The degassing system was cleaned with 5 % HCl before and after use, followed by cycling with deionised water (MilliQ, Millipore) to remove any traces of acid. Seawater from the Tvärminne Storfjärden was collected from a depth of 10 m and cycled once through the degassing system before collection in an air-tight, acid-cleaned bag with septum (SKC Tedlar® Bag with single polypropylene fitting) without exposure to the atmosphere. 1 mL of $^{15}N\text{-}N_2$ gas (98 at % ^{15}N, Sigma Aldrich, Lot no.: CX0937 until *t21*, SZ1670V after *t21*) was injected through the septum into the

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bag for every 100 mL of sample. The resulting bubble was dissolved and the ^{15}N-N_2-enriched seawater was stored at in situ temperature of the mesocosms until addition to incubation bottles. Seawater for the blank incubations was prepared in a separate bag using the same process however ambient air was added instead of isotopically labelled ^{15}N-N_2 gas.

Water samples for N_2-fixation rate incubations were directly transferred in a gentle manner from the integrating water sampler into 2.3 L polycarbonate bottles on board the sampling boat using silicon tubing. The bottles were stored in a closed cool box to control temperature and to block sunlight until return to the on shore laboratory. Each bottle was weighed and homogenised by gentle rotation before 70–90 mL of water was removed to make space for the ^{15}N-enriched seawater. Enriched or "blank" seawater was transferred from the Tedlar® bags to the respective bottles through Tygon™ tubing, immersed in the sample bottle, using a peristaltic pump to minimise tracer loss through exposure to atmosphere. Incubation bottles were filled with no headspace. After addition, the caps were immediately screwed on to seal the bottles air tight. During these procedures, the bottles were reweighed at each step in order to determine the exact amount of isotope label inside each bottle. The final ^{15}N-enrichment of dissolved N_2 gas in each bottle was between 1.0–3.5 at %. The bottles were then mixed by gentle rotation and placed in a climate chamber at in situ temperature and under controlled light conditions (~ 73 ± 1 µmol photons m^{-2} s^{-1}, mean ± SD). Irradiance was measured using a LI-COR LI-192 quantum sensor. Measured irradiance were within the range of average depth-integrated (0–17 m) irradiance in the mesocosms taken from daily CTD profiles at between 13:30 and 14:30 LT (20 to 300 µmol photons m^{-2} s^{-1}). The light-dark cycle followed the natural sunrise-sunset variation which on the summer solstice (21 June 2012, *t-1*) was 19:5 h (L : D). Climate chamber temperature was programmed to follow the daily integrated water column temperature as recorded by the afternoon CTD sampling and thus is reported as in situ temperature. Consistency between irradiance conditions at each bottle position were achieved by a rotation regime. Bottles were rotated gently to mix and the bottle position rotated systematically approximately

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every three hours during the light cycle. Time of rotation was recorded allowing the calculation of average irradiance between each individual bottle.

Incubations were terminated after 24 h by filtration through a combusted (6 h at 450 °C) and acid rinsed (1 % HCl) GF/F filter (0.7 µm pore size, 25 mm diameter, Whatman) under reduced vacuum (< 200 mbar). Filters were placed in glass petri dishes (combusted 6 h, 450 °C), frozen immediately and stored at −20 °C until analysis on a mass spectrometer as described for particulate C and N analyses above and also in Paul et al. (2015). Rates were calculated according to Montoya et al. (1996). Estimated internal analytical uncertainty in calculated N_2–fixation rates was less than ±10 % when rates were above the detection limit. The detection limit was determined as a difference in $\delta^{15}N$ between initial and final values of larger than 1.0 ‰. This corresponded to a calculated rate of more than 0.15 nmol N L^{-1} d^{-1}.

2.3 Enrichment of mesocosms with ^{15}N-N_2 gas

Four of six mesocosms spanning the range of fCO_2 treatments were enriched with the isotopically labelled ^{15}N-N_2 gas to investigate the fate of newly fixed N in this plankton community under future ocean acidification conditions. A similar approach to Mohr et al. (2010), as described for the N_2-fixation incubations (see Sect. 2.2), was employed on a larger scale. A total of approximately 1500 L of unfiltered seawater was collected from the Baltic at ca. 10 m depth and pumped into the laboratory building at Tvärminne Zoological Station. Mesocosm enrichment occurred in two pulses on *t22* and *t26*. We added this in two steps because of the limited number of bags available for preparing the ^{15}N-N_2 enriched seawater. For the first step, seawater was filtered and collected as for the N_2-fixation incubations in bags (thermoplastic polyurethane, ~ 30 L capacity) with a tap and a crimp sealed septum (N20 grey butyl rubber plugs, Macherey and Nagel) on opposite ends of the bag. The large physical effort required to dissolve the gas by "bag-slapping", as commonly done for small volumes using the method described by Mohr et al. (2010), led to a modification of the enrichment method for the second enrichment step. Water was collected and degassed as previously de-

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scribed through the degassing membrane. Instead of collecting the water directly after this step, the water then passed through a second membrane that was flooded with $^{15}N\text{-}N_2$ gas and was connected to an overflow system which allowed monitoring of gas dissolution (Fig. 2). The high surface area in the membrane enhanced the labelled gas dissolution. This enriched water was then pumped directly into the empty collection bags using a peristaltic pump without contact with the atmosphere. One complete cartridge of gas (500 mL, nitrogen – $^{15}N\text{-}N_2$, 98 at % ^{15}N, Sigma Aldrich, Lot no.: SZ1670V, SZ1423V, CX0937) was added per bag through the septum. A total of 150 L of enriched seawater prepared was added to four mesocosms (M3, M5, M6, M8), and 100 L unenriched filtered seawater was added to the other two mesocosms (M1, M7) as isotope label controls on *t22* and *t26*.

2.4 Phytoplankton counts

Counts of phytoplankton cells > 20 µm were made from 50 mL samples fixed with acidic Lugol's iodine solution (1 % final concentration). Samples were concentrated using gravitational settling and counted under an inverted microscope (ZEISS Axiovert 100) after Utermöhl (1958) and following the guidelines for determination of phytoplankton species composition, abundance and biomass for the COMBINE programme provided by HELCOM (Annex C-6). The cells were counted either on half of the chamber at 100 fold or on 3 to 4 strips at 200 fold magnification. Filamentous cyanobacteria were counted in 50 µm length units. Plankton were identified where possible to the species level according to Hoppenrath et al. (2009), Kraberg et al. (2010) and Tomas (1997). Biovolumes of counted plankton cells were calculated according to Olenina et al. (2006) and converted to cellular organic carbon quotas by the equations of Menden-Deuer and Lessard (2000).

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2.5 Statistical analyses

A linear regression analysis was applied to determine the relationship between mean fCO_2 and the mean response of each variable for the three experimental phases (Phase I, II and III), as described in Paul et al. (2015). Linear regression analyses were undertaken using R (R Core Team, 2015).

3 Results

Three experimental phases after initial CO_2 manipulation on *t0* were defined in Paul et al. (2015) using temperature and chlorophyll *a* (Chl *a*) fluctuations: Phase I (*t1–t16*), Phase II (*t17–t30*) and Phase III (*t31–t43*). These phases are also used to assist with data interpretation in this manuscript.

3.1 Inorganic nutrient availability and nutrient limitation

There were low concentrations of inorganic N present throughout the study period with inorganic nitrate concentrations in the range of 3–107 nmol L^{-1} (Fig. 1c). Ammonium was the dominant source of inorganic N with concentrations ranging between 20 and 289 nmol L^{-1}. Hence NH_4^+ was also included in the calculations of P^* (excess phosphate) and inorganic nutrient elemental stoichiometry according to the Redfield ratio (Fig. 3, Eq. 1).

There was an excess of inorganic phosphate to inorganic N in all mesocosms ($P^* > 0$ nmol L^{-1}, Fig. 3a) and the surrounding waters throughout the study period, with phosphate concentrations ranging between 72 and 214 nmol L^{-1} in the mesocosms and up to 410 nmol L^{-1} outside the mesocosms in the surrounding Archipelago Sea. Inorganic phosphate concentrations decreased during Phase I, followed by an increase during Phase II with more stable concentrations in Phase III. Nitrate concentrations increased throughout the experiment with a possible small drawdown after *t39* in all treatments, whereas NH_4^+ concentrations were variable. Samples for NH_4^+ analyses

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were lost on *t27* and *t29*. There did not appear to be any remarkable relationship linking accumulated precipitation (between sampling days), and the increase in nitrate (Fig. 1c and e), indicating that wet atmospheric deposition of nitrate into the mesocosms was effectively prevented by the mesocosm roofs and did not affect the nitrate pool. Precipitation data for the Hanko weather station (ID no.: GHCND:FIE00142025, latitude: 59.8439, longitude: 23.2517) were obtained from the National Oceanographic Data Center (NOAA).

3.2 Diatom abundance, silicate dynamics and dissolved N utilisation

Diatoms were mostly abundant at the beginning of the experiment with the species *Chaetoceros* sp. and *Skeletonema marinoi* present in the large size class (> 20 µm, Fig. 4). Fucoxanthin marker pigment concentrations in this size class and suspended BSi concentrations (> 0.65 µm) declined markedly during the first few days in Phase I and the dynamics fitted well to the microscopy counts of both *Chaetoceros* sp. and *Skeletonema marinoi*. Dissolved silicate (DSi) concentrations continued to decrease up until *t*13. No statistically significant difference between CO_2 treatments was detected for diatom abundance (microscopy counts), DSi drawdown or BSi concentrations (Table 1, Fig. 4c and e), apart from BSi in Phase II where a positive effect was detected ($p = 0.034$, see Paul et al. (2015) for statistical analyses).

Dissolved organic nitrogen (DON) concentrations ranged between 20 and 25 µmol L^{-1} (Fig. 4a). DON concentrations appeared to decrease during Phase I, however considerable variability in the data meant this DON drawdown could not be accurately quantified.

3.3 Diazotroph abundance and N_2-fixation rates, $\delta^{15}N$ in particulate N

The abundance of filamentous diazotrophic cyanobacteria remained low throughout the experiment with no significant bloom development (< 6 µg C L^{-1}, Fig. 5a). The most dominant species, *A. flos-aquae*, had a maximum biomass of 4.9 µg C L^{-1} in the meso-

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cosms (M1, *t27*), whereas the next most abundant species, *Anabaena* sp., had a maximum biomass in the water column of 0.18 µg C L^{-1} (M1, *t17*). Aphanizophyll, a pigment present in *A. flos-aquae* and *Anabaena* sp. (Schluter et al., 2004), was detected in both suspended material in the water column, and in the sinking material collected in the sediment trap. Concentrations of this pigment increased at the end of Phase I concurrent with an increase in N_2-fixation rates (Fig. 5). Although numbers in the mesocosms remained generally low, *A. flos-aquae* abundances based on microscopy counts and phytoplankton pigment analyses, were highest in Phases II/III and lowest in Phase I (Fig. 5). *A. flos-aquae* biomass outside the mesocosms was up to 30 µg C L^{-1} on *t15* and is supported by high Aphanizophyll pigment concentrations of 109 ng (mg TPC)$^{-1}$ also on *t15* (data not shown).

Rates of N_2-fixation until *t21* ranged from below the detection limit at the beginning of the experiment, up to 4.4 nmol N L^{-1} d^{-1} inside the mesocosms and up to 37.9 nmol L^{-1} day^{-1} in the waters outside. We observed a substantial increase in the N_2-fixation rates from 2.6 to 4.4 nmol L^{-1} day^{-1} up to 50 to 60 nmol L^{-1} day^{-1} between *t21* and *t23* without any remarkable change in diazotroph abundance of the same magnitude (Fig. 5). This is also evident in *A. flos-aquae* biomass-related N_2-fixation rates (Fig. 6). This increase coincided with the use of a new ^{15}N-N_2 gas bottle with a lot number which was reported two years later as contaminated with ^{15}N-labelled NH_4^+ and NO_3^- by Dabundo et al. (2014) (Sigma Aldrich, Lot no. SZ1670V). The measured rates from *t23* on are therefore not exclusively N_2-fixation and are not reliable thus they were excluded from analyses. In addition to the bottle assays, the ^{15}N-N_2 isotope tracer was also added directly to all mesocosms except for M1 (control) and M7. Therefore these two mesocosms were not affected by this contamination issue. Hence, the natural abundance $\delta^{15}N$ data from the suspended material in the water column and the sinking material from the sediment trap is reported for the entire experiment (*t-3* until *t43*) for M1 and M7 mesocosms (Fig. 5e and f) but only until *t21* for M3, M5, M6 and M8. Any NH_4^+ or nitrate added to the four mesocosms with the isotope tracer was highly

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isotopically enriched in ^{15}N but was in very low concentration and so was insignificant for the nutrient budget.

The natural abundance $\delta^{15}N$ in suspended particulate N in the mesocosms decreased during the period of higher Chl *a* in Phase I from 6.0±0.5 ‰ on *t1* to 2.6±0.5 ‰ on *t15* (mean ± S.D.). This indicated potential input of atmospheric N with a low $\delta^{15}N$ into particulate matter via N_2-fixation during this period. A sharp decrease in $\delta^{15}N$ in the sinking particulate material occurred on *t17*, the same day that considerable amounts of Aphanizophyll and Fucoxanthin were found in the sediment trap material (Fig. 5d and f, Fucoxanthin not shown). This was one day after the mesocosm walls were cleaned indicating that there were likely diazotrophic species and diatoms attached to the mesocosm walls. Identification from microscope photos revealed the presence of filamentous cyanobacteria with heterocysts in the collected sediment trap material. Aside from this, there were no remarkable fluctuations in $\delta^{15}N$ in either the suspended or sinking particulate matter pools, including after *t21* in M1 and M7 (Fig. 5e and f).

Assessment of in situ N_2-fixation rates based on ^{15}N -uptake from the combined dissolved N pool of NO_3^-, NH_4^+ and N_2 was abandoned due to high uncertainty in initial ^{15}N enrichment and concentrations of the combined dissolved N pool, and fast saturation of label uptake after ca. four days (two successive sampling days). To assess the contribution of diazotrophy to N supply in the mesocosms, we calculated a theoretical cumulative diazotrophic N input using measured N_2-fixation rates from bioassays up until *t21* (M1 = 20 nmol N L^{-1}), and then assumed a constant N_2-fixation rate of 4 nmol N L^{-1} d^{-1} into particulate N between *t23* and *t43* (total = 80 nmol N L^{-1}). The assessment for between *t23* and *t43* is based on the premise of continued elevated *A. flos-aquae* biomass and assuming 50 % exudation of fixed N as DON or NH_4^+ (< *t21* = 20 nmol N L^{-1}, > *t21* = 80 nmol L^{-1}, total = 100 nmol N L^{-1}). This yielded a theoretical new N input from *A. flos-aquae* of only 200 nmol N L^{-1}, amounting to ∼ 5 % of mean PON pool standing stock (∼ 3 µmol L^{-1}) and is clearly at the higher end of

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estimations. We calculated corresponding N requirement of the plankton community of 27.2 nmol N L^{-1} d^{-1} from the average phosphorus uptake rate across all treatments of 1.7 nmol PO_4^{3-} L^{-1} d^{-1} from *t1–t30* as reported by Nausch et al. (2015), by assuming Redfield nutrient uptake stoichiometry (16N : 1P). This is almost seven times larger than estimated daily diazotrophic N inputs of ~ 4 nmol N L^{-1} d^{-1}, corresponding to 14 % of calculated community N requirement.

Low filamentous diazotrophic cyanobacteria abundances exacerbated the inherent sampling error in both microscopy and pigment analyses due to patchy distribution and the tendency of filaments to aggregate. Hence, unfortunately no reliable statistical analyses on the effect of higher fCO_2 on diazotroph abundance or marker pigment concentration could be undertaken, for any phase of the experiment. Any potential CO_2 effect on diazotroph abundance was also not obvious on visual data inspection, and no effect could be detected on N_2-fixation rates or $\delta^{15}N$ natural abundance in suspended particulate matter from the water column or sediment trap particulate matter up until *t21* (Table 1), when rates were reliable and there was data from a sufficient number of CO_2 treatments.

4 Discussion

4.1 Effects of elevated CO_2 on diazotrophic N inputs

Bioavailable N was present in low concentrations and was probably the limiting macronutrient in the plankton community. Hence, higher phytoplankton biomass and lower phosphate concentrations at higher CO_2 observed in this same mesocosm study (Paul et al., 2015), may have suggested relief of N limited growth by potentially increased N_2-fixation. However we have no strong evidence to support this hypothesis based on N pool standing stocks and estimated diazotrophic N inputs. The only statistically significant, but very minor, correlation was a positive relationship between CO_2 and PON concentrations (Fig. 3b, 0.08 µmol L^{-1}, 3 % difference in PON, slope

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$= 1.75 \times 10^{-4}$ µmol L^{-1} µatm^{-1}, data from Paul et al., 2015). No significant difference in N_2-fixation rates (until *t21*) or *A. flos-aquae* abundance at elevated CO_2 compared to the ambient treatments was detected (Table 1, Fig. 5). Phosphate turnover rates, a potential indicator of P demand for N_2-fixation, were also unaffected by CO_2 in Phases I or II (Nausch et al., 2015). These variables (N_2-fixation and phosphate uptake rates) provide a more sensitive measure of turnover rates of N and P than assessing changes in N pool standing stocks in this tightly-coupled regenerative plankton community. Unfortunately, we only have reliable N_2-fixation rates from incubations until *t21* due to contamination of ^{15}N-N_2 gas with bioavailable N compounds (Dabundo et al., 2014) and not after ~ *t25* when significant CO_2-related differences in C and P pools were apparent. Hence, in the later stages of the experiment (Phase II and III), it is possible that there was a divergence in N_2-fixation rates between treatments that was missed, despite low abundances of *A. flos-aquae*, the dominant filamentous diazotrophic cyanobacterium present. Nonetheless we estimate that the contribution of diazotrophy to N supply in the mesocosms over the study duration of 43 days was small (~ 200 nmol L^{-1}). Maximum measured N_2-fixation rates of 4.4 nmol N L^{-1} d^{-1} were low compared to reported for the Baltic Sea in mid-summer which range from 1.7 up to 550 nmol N L^{-1} d^{-1} (Farnelid et al., 2013; Ohlendieck et al., 2000, 2007; Wasmund et al., 2001). This is due to the rather low *A. flos-aquae* biomass in the mesocosms compared to literature values (this study: maximum biomass = 5 µg C L^{-1} integrated over 0–17 m; Gulf of Finland: 22–26 µg C L^{-1} in the surface 5 m, 6–7 µg C L^{-1} at 20 m deep in July, Laamanen and Kuosa, 2005). Thus even if all newly-fixed N by diazotrophs was transferred to diazotroph and plankton biomass (i.e PON pool), this small accumulation would most likely remain below the detection limits in the suspended PON pool (~ 10 % = 0.3 µmol L^{-1}). On top of this, any CO_2–related differences in N_2-fixation would be near impossible to resolve in this small contribution by diazotrophs.

The absence of any detectable effect may of course be influenced by the relatively low abundances of filamentous diazotrophic cyanobacteria in this study, as temperatures were mostly below temperatures thought to stimulate bloom development (16 °C,

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Wasmund et al. (1997); this study 8–16 °C, Paul et al., 2015). Nevertheless our results from this CO_2 manipulation study are in agreement with studies from both the marine (Böttjer et al., 2014; Law et al., 2012) and freshwater (Shapiro, 1997; Yamamoto, 2009) realms which detected no significant effect of decreased pH/increased CO_2 on diazotroph abundance and/or activity in natural plankton communities. These four independent studies all contradict physiological investigations in single-strain culture experiments where diazotroph growth and activity was modulated by CO_2 availability (e.g. Barcelos e Ramos et al., 2007; Czerny et al., 2009; Eichner et al., 2014; Fu et al., 2008; Hutchins et al., 2013; Wannicke et al., 2012). Diazotrophic organisms typically have slower growth rates than other organisms. Hence any potential influence of ocean acidification on their physiology may take longer to become apparent in biogeochemical parameters sampled in larger-scale field studies. However to the best of our knowledge, there are no direct N_2-fixation rate measurements from CO_2-manipulation studies with *A. flos-aquae* in the field which could shed light on any underlying physiological response of this diazotroph and confirm laboratory findings in the field. Furthermore, high grazing pressure, hence top-down control, particularly after *t17* (Lischka et al., 2015) may have overridden any potential CO_2 effect of bottom-up control on diazotroph growth.

In addition to these highly visible filamentous N_2-fixers, there is growing evidence to support the role of heterotrophic and non-phototrophic N_2-fixation by smaller unicellular organisms in diverse ecosystems (Halm et al., 2012; Loescher et al., 2014; Moisander et al., 2010; Zehr et al., 2008) including in the Baltic Sea and Kattegat (Bentzon-Tilia et al., 2015; Farnelid et al., 2009), which cannot be quantified by common microscopic methods used in this experiment. Hence, while there appeared to be a good correlation between *A. flos-aquae* abundance and N_2-fixation rates until *t21* in this study, we cannot rule out the contribution of heterotrophic organisms to the measured rates. However, regardless of the diazotroph community present, N_2-fixation rates were low and diazotrophy made only a small contribution ($< 200\,nmol\,L^{-1}$) to the N cycle in this study. Thus we have no direct evidence from observations in this study that N_2-fixation or di-

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azotroph abundance (Fig. 5) were significantly influenced by CO_2 nor that this could explain the observed higher particulate matter concentrations or lower phosphate concentrations in the higher CO_2 treatments (Paul et al., 2015) based on hypothesised relief of N-limitation.

In this area of the Baltic Sea, plankton communities, containing filamentous diazotrophic cyanobacteria, are exposed to large diurnal and seasonal changes in pH (Almén et al., 2014; Brutemark et al., 2011). In addition, filamentous cyanobacteria form characteristic surface aggregations, similar to the tufts and puffs formed by *Trichodesmium*. Inside these aggregations, microenvironments can create substantially different conditions compared to the surrounding water with large diurnal fluctuations in pH (7.4 vs. 9.0) and O_2 concentrations (~ 150–450 µmol O_2 L^{-1}) and thus also inorganic carbon availability (Ploug, 2008). Hence natural exposure to highly variable carbonate chemistry conditions may have also played a role in dampening any potential influence of ocean acidification in this plankton community.

4.2 Evidence from N pools of the importance of regenerative production and effects of CO_2

Productivity in this plankton community appeared to be dominated by regenerative production (sensu Dugdale and Goering, 1967) under low nitrate availability during Phase I, as has been observed in summer plankton communities in the Baltic Sea (Kuparinen, 1987; Sahlsten and Sörensson, 1989; Tamminen, 1995). DON appeared to be a more important N source than N derived from N_2-fixation. Any relatively fresh and labile N-rich dissolved organic matter (DOM) present after the decline of the spring bloom was likely remineralised by the bacterial community. Here, simultaneous drawdown of DSi and DON between *t-1* and *t15* suggests that in particular diatoms, also persisting from the spring bloom, were beneficiaries of this organic N turnover. Available NH_4^+ (~ 100 nmol L^{-1}) could not have supported the DSi uptake (~ 0.4 µmol L^{-1}) as the sole N source based on ~ 1 : 1 molar Si : N requirement by diatoms, thus suggesting instead potential rapid resupply of NH_4^+ through remineralisation of organic N

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by the heterotrophic community particularly in Phase I and Phase II. Although there is no indication of a high level of NH_4^+ production above the variability in the data set, we presume this bioavailable NH_4^+ would have been very quickly assimilated into particulate N in the N-limited plankton community. This rate of N regeneration probably limited net phytoplankton growth such that significant phytoplankton biomass could not accumulate in the water column. Nevertheless, neither the readily available NH_4^+ nor the nitrate pool were fully exploited by the plankton assemblage with up to 50 nmol L^{-1} of nitrate and 170 nmol L^{-1} of NH_4^+ remaining at the end of the study period on *t43*. In fact, nitrate concentrations continually increased throughout the experiment at an average net rate of 1 nmol N L^{-1} day^{-1} (Fig. 1c) despite proportionally high phosphate availability. This suggests a small net imbalance in N cycle processes and may be connected to ammonium inhibition of nitrate uptake during spring-bloom decline and post-bloom period in the study area (Tamminen, 1995), leading to this small accumulation of nitrate in the water column.

No significant effect of CO_2 was detected on the DON pool, nor DSi drawdown, or PON or BSi cumulative sinking fluxes (see also Paul et al., 2015 in this Special Issue). Likewise, if there was any difference in uptake of N from the N-rich DOM pool (N : P ~ 80 : 1) between CO_2 treatments, we could not detect the small signal (nmol L^{-1}) outside of the analytical precision (µmol L^{-1}) of the DON measurements. Thus this organic N drawdown via regenerative production in diatoms in this study appeared to be either unaffected or immeasurable by simulated ocean acidification.

5 Summary

Plankton biomass build-up in this study was limited by low inorganic N availability therefore organic N pools were utilised supporting regenerative production during the more productive period in Phase I, with diatoms benefitting from this N turnover. Estimated N_2-fixation rates and abundances of the most dominant filamentous diazotroph, *A. flos-aquae*, remained very low, therefore diazotrophs probably made only a minor contribu-

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tion to overall N supply in this plankton community. Hence we did not observe relief of N limitation and stimulation of a summer plankton bloom by non-diazotrophic organisms. Indeed, dissolved inorganic nitrate present increased throughout the experiment indicating higher supply than consumption, despite a considerable phosphate excess present.

We detected no significant differences in N pool sizes between CO_2 treatments apart from the PON pool. However, the detected positive effect of CO_2 on PON standing stocks was minor (< 3 % difference in PON concentration). Thus N uptake rates were well balanced with supply or any net differences were too small to be detected in N pool sizes across the range of simulated ocean acidification scenarios. In addition, we found no conclusive evidence from our data until *t21* (N_2-fixation rates, *A. flos-aquae* abundances, natural $\delta^{15}N$ abundances) that CO_2 had a measurable impact on N inputs via diazotrophy. The absence of any detectable effect may have been influenced by the low abundances of filamentous diazotrophic cyanobacteria in this study. However, the lack of response was consistent with other studies of diazotrophic organisms in natural plankton communities where resource competition with other plankton functional groups and top-down control may also play important roles in mediating the physiological response of N_2-fixing organisms.

Nonetheless, it appears that increased CO_2 may have slightly enhanced the ability of the N-limited plankton community in the Baltic Sea to exploit the low N sources available thereby potentially explaining lower phosphate concentrations, higher particulate matter concentrations and Chl *a* observed under higher CO_2 (Paul et al., 2015). However, we have no direct evidence of increased new N inputs via diazotrophy or changed N biogeochemistry within the first three weeks and no conclusive indirect evidence from N pool sizes up to six weeks after CO_2 manipulation. Therefore we conclude that elevated CO_2 had no observable impact on the N cycle in this summer Baltic Sea plankton community.

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Table 1. Summary of linear regression analyses of fCO_2 and nutrient stoichiometry, dissolved silicate drawdown, abundance of large (> 20 µm) dominant diatom species present (*Chaetoceros* sp., *Skeletonema marinoi*), N_2-fixation rates and stable nitrogen isotope natural abundance. Numbers in bold indicate variable had a negative correlation with average fCO_2. Dashes indicate no regression was completed to avoid any bias in the conclusions because either no data or no complete data set is available. Degrees of freedom, $n = 4$.

Variable	Phase	p	F-statistic	R^2	Variable	Phase	p	F-statistic	R^2
N_2-fixation rate	I	0.764	0.104	0.025	*Chaetoceros* sp. abundance	I	0.737	0.129	0.031
	II	–	–	–		II	–	–	–
	III	–	–	–		III	0.075	5.726	0.589
$\delta^{15}N$ in suspended particulate matter	I	0.417	0.819	0.170	*Skeletonema marinoi* abundance	I	0.772	0.097	0.024
	II	–	–	–		II	–	–	–
	III	–	–	–		III	–	–	–
$\delta^{15}N$ in sinking particulate matter	I	0.289	1.494	0.272	Excess phosphate (P^*)	I	0.493	0.569	0.125
	II	–	–	–		II	0.783	0.086	0.021
	III	–	–	–		III	**0.004**	**37.56**	**0.904**
DSi drawdown	I	0.927	0.010	0.002	DIN:DIP (includes NH_4^+)	I	0.647	0.569	0.125
	II	0.520	0.496	0.110		II	0.556	0.412	0.093
	III	0.966	0.001	0.002		III	0.797	0.076	0.019

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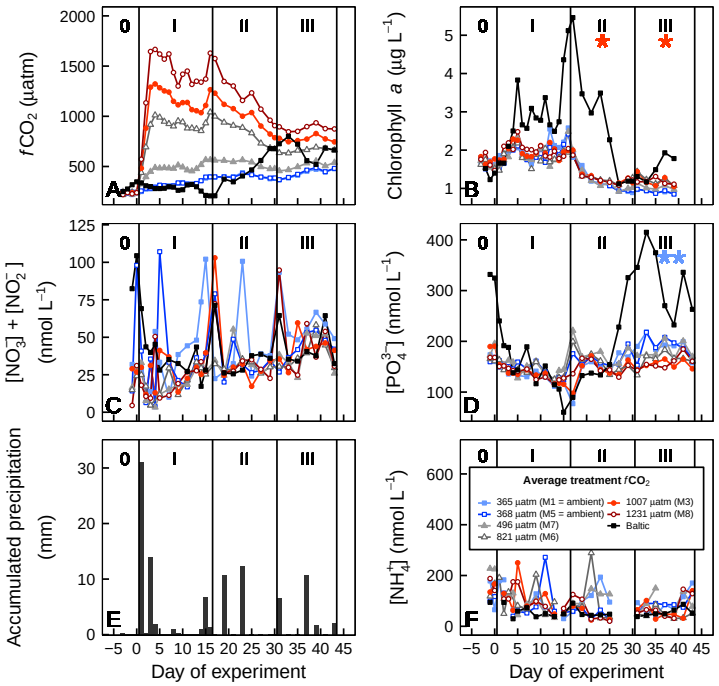

Figure 1. Temporal development in **(a)** calculated fCO_2 using measured DIC and pH_T, **(b)** chlorophyll *a* concentrations, **(c)** dissolved inorganic nitrate concentrations, **(d)** dissolved inorganic phosphate concentrations over the study period, **(e)** accumulated precipitation between sampling days recorded at the Hanko weather station (ID no.: GHCND:FIE00142025, latitude: 59.8439, longitude: 23.2517), and **(f)** measured dissolved ammonium concentrations. Data for **(a–d)**, and **(f)** is from Paul et al. (2015) and for **(e)** from National Oceanographic Data Center, NOAA. $^{*} = p < 0.05$, $^{**} = p < 0.01$ where red indicates positive and blue a negative detected effect of fCO_2. Legend indicates colours and symbols for each mesocosm. Average treatment fCO_2 was calculated for each mesocosm between *t1* and *t43*.

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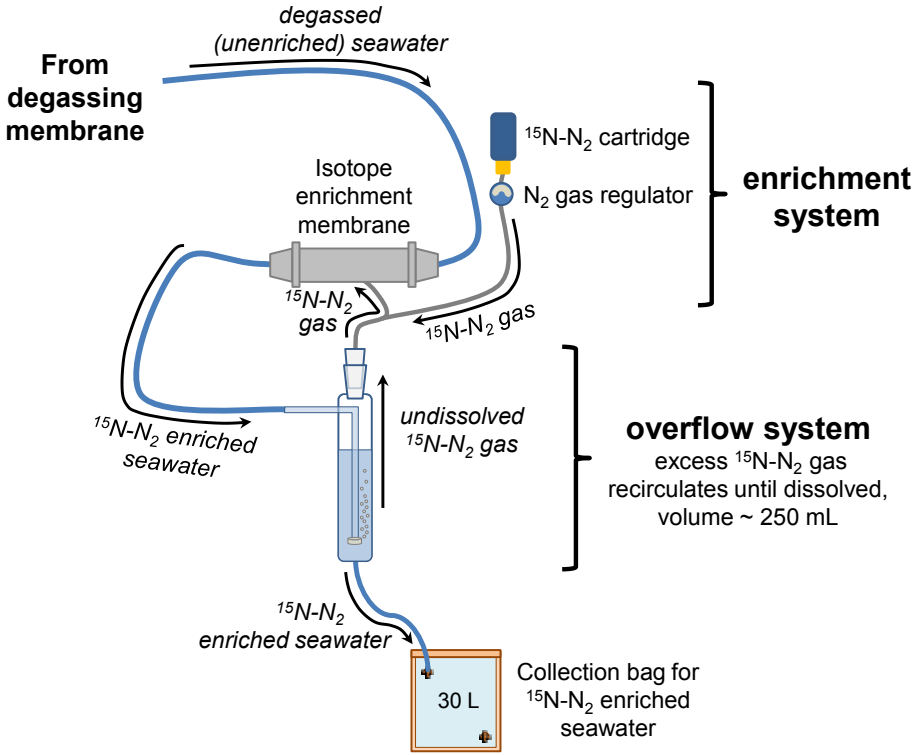

Figure 2. Diagram of set-up used for large-scale preparation of ^{15}N-N$_2$ enriched seawater which was added to selected mesocosms.

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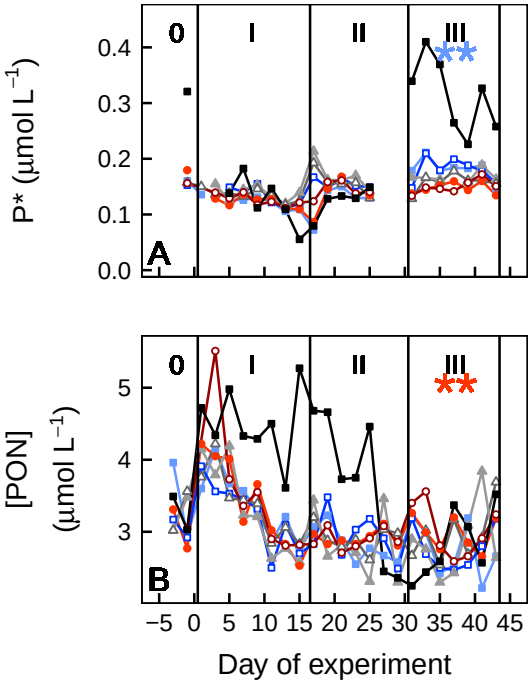

Figure 3. Temporal development in **(a)** excess dissolved inorganic phosphate (P^*) calculated according to Eq. (1), and **(b)** suspended particulate organic nitrogen (PON) concentration. Data and statistical significance is from Paul et al. (2015). Colours and symbols are the same as for Fig. 1. ** = $p < 0.01$ where red indicates positive and blue a negative detected effect of fCO_2.

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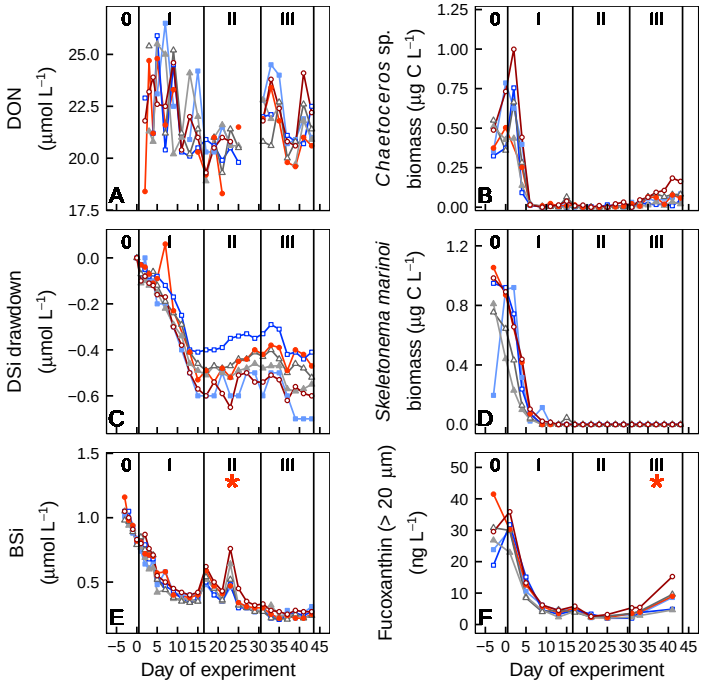

Figure 4. Temporal development in **(a)** dissolve organic nitrogen concentrations (DON), **(c)** dissolved silicate (DSi) drawdown and **(e)** particulate biogenic silicate (BSi) concentrations (data from Paul et al., 2015), the abundances of the two dominant diatom species determined by microscopy (**b**, **d** and **f**), Fucoxanthin marker pigment concentrations (> 20 µm), a key pigment in diatoms. Colours and symbols are the same as for Fig. 1. Red asterisk denotes significant positive effect of CO_2 ($* = p < 0.05$).

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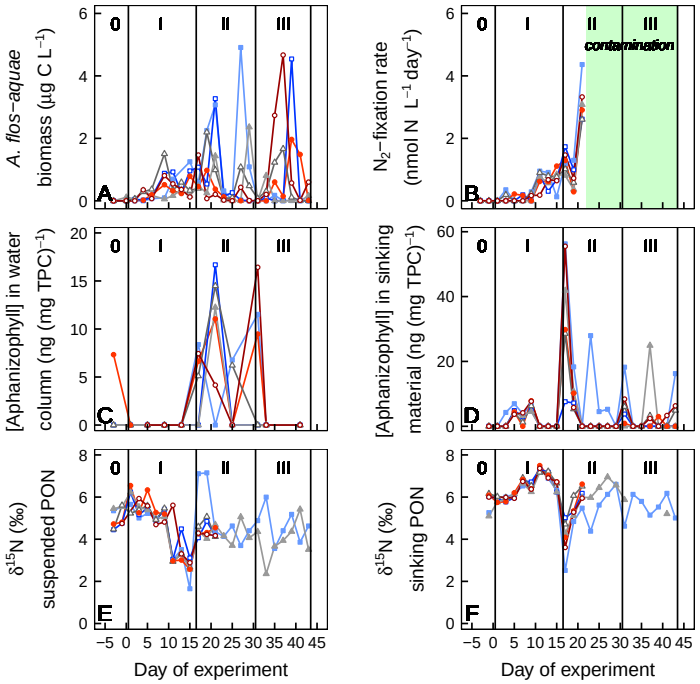

Figure 5. Variables indicating abundance and activity of filamentous diazotrophic cyanobacteria: **(a)** biomass of *A. flos-aquae* calculated from microscopy abundance data, **(b)** N_2-fixation rates determined by stable isotope incubations, **(c)** carbon-normalised Aphanizophyll marker pigment concentration relative as a proxy for *A. flos-aquae* abundance in the water column and **(d)** in the sediment trap material, **(e)** natural abundance $\delta^{15}N$ of particulate organic nitrogen (PON) in the water column and **(f)** natural abundance $\delta^{15}N$ in the sinking particle organic nitrogen collected in the sediment trap determined by analyses on an isotope ratio mass spectrometer. The green shaded area in **(b)** between *t23* and *t43* indicates when contaminated ^{15}N-N_2 gas was used in incubations (see Dabundo et al., 2014). Colours and symbols are as described in Fig. 1.

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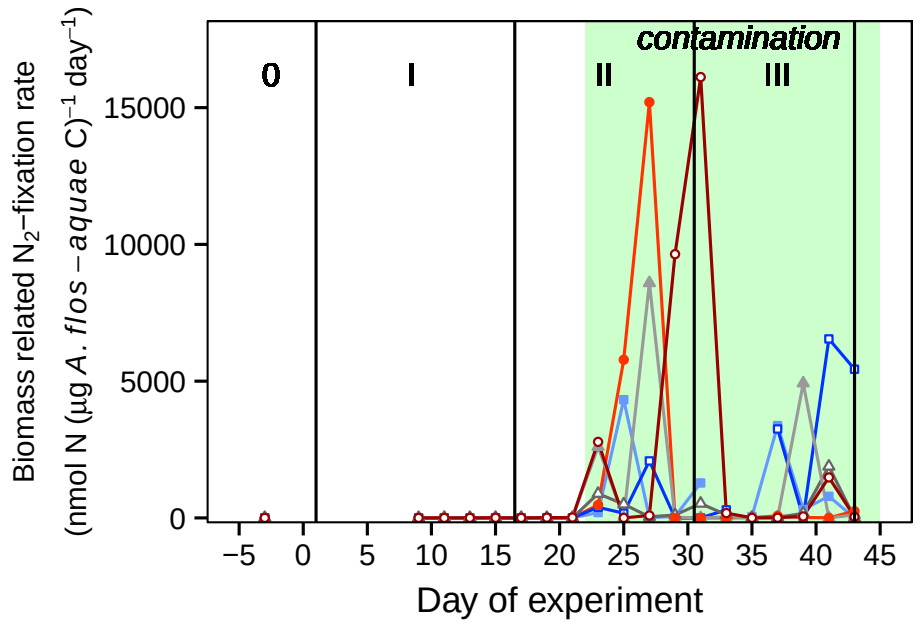

Figure 6. *A. flos-aquae* carbon-normalised N_2-fixation rates over the study period. Where data points are missing before *t9*, rates were either below detection limit (0.15 nmol N L^{-1} d^{-1}) or did not coincide with sampling for phytoplankton abundance counts. Green shaded area between *t23* and *t43* indicates when contaminated ^{15}N-N_2 gas was used in incubations (see Dabundo et al., 2014) and added to mesocosms. Colours and symbols are as described in Fig. 1.

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