A sea of change

Impacts of reduced nitrogen and phosphorus loads on coastal phytoplankton communities

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Chapter 2

Unbalanced reduction of nutrient loads has created an offshore gradient from phosphorus to nitrogen limitation in the North Sea
Unbalanced reduction of nutrient loads has created an offshore gradient from phosphorus to nitrogen limitation in the North Sea

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Abstract

Measures to reduce eutrophication have often led to a more effective decline of phosphorus (P) than nitrogen (N) concentrations. The resultant changes in riverine nutrient loads can cause an increase in the N:P ratios of coastal waters. During four research cruises along a 450 km transect, we investigated how reductions in nutrient inputs during the past 25 yr have affected nutrient limitation patterns in the North Sea. This revealed a strong offshore gradient of dissolved inorganic N:P ratios in spring, from 375:1 nearshore toward 1:1 in the central North Sea. This gradient was reflected in high nearshore N:P and C:P ratios of particulate organic matter (mainly phytoplankton), indicative of severe P deficiency of coastal phytoplankton, which may negatively affect higher trophic levels in the food web. Nutrient enrichment bioassays performed on-board showed P and Si limitation of phytoplankton growth nearshore, co-limitation of N and P in a transitional region, and N limitation in the outer-shore waters, confirming the existence of an offshore gradient from P to N limitation. Different species were limited by different nutrients, indicating that further reductions of P loads without concomitant reductions of N loads will suppress colonial *Phaeocystis* blooms, but will be less effective in diminishing harmful algal blooms by dinoflagellates. Hence, our results provide evidence that de-eutrophication efforts in northwestern Europe have led to a large imbalance in the N:P stoichiometry of coastal waters of the North Sea, with major consequences for the growth, species composition, and nutritional quality of marine phytoplankton communities.

Introduction

Although nutrient limitation of phytoplankton growth is well studied, our understanding of the “typical nutrient limitation” of aquatic environments is constantly being revised. Lakes have traditionally been considered phosphorus (P) limited (Vollenweider, 1976; Schindler, 1977; Hecky and Kilham, 1988), whereas oceans and coastal seas were considered primarily nitrogen (N) limited (Ryther and Dunstan, 1971; Blomqvist et al., 2004; Howarth and Marino, 2006). This traditional view has been challenged by reports on P limitation in different marine environments (Thingstad et al., 1998; Wu et al., 2000) and widespread N and P co-limitation in freshwater ecosystems (Elser et al., 2007). Moreover, due to increased anthropogenic nutrient inputs and subsequent nutrient reduction
efforts, this historical understanding is now blurred and more complex than ever (Artioli et al., 2008; Abell et al., 2010; Paerl et al., 2014). In recent decades, a global rise in the relative N to P content of the major riverine inputs to coastal zones has occurred, associated with an increased application of N fertilizers (Turner et al., 2003; Glibert et al., 2014) and more effective P removal from domestic and industrial wastewater during de-eutrophication efforts (Grizzetti et al., 2012). This increase in riverine N:P ratios may induce major changes in nutrient limitation of coastal waters. As Paerl et al. (2014) stated, the freshwater to marine environment exists as a continuum, where changes in the prevailing nutrient conditions “upstream” will inevitably influence nutrient conditions in the “downstream” coastal environment.

The North Sea is a prime example of a coastal zone experiencing pronounced shifts in nutrient limitation. Coastal waters of the southern North Sea are strongly influenced by the discharge of several European rivers, including the Scheldt, Maas, Rhine, Weser, and Elbe. From the 1960s to the mid-1980s a large increase of riverine P and N loads (Pätsch and Radach, 1997) led to coastal eutrophication of the North Sea, signified by enhanced primary production (Cadée and Hegeman, 2002), changes in phytoplankton species composition (Philippart et al., 2000), a marked increase in the intensity and duration of nuisance blooms of *Phaeocystis* (Cadée and Hegeman, 1986; Lancelot et al., 1987), and the development of hypoxia in the German Bight (Westernhagen and Dethlefsen, 1983). In response, member states of the OSPAR Convention agreed to reduce river nutrient loads by at least 50% compared with the reference year 1985 (OSPAR, 1988). By 2002, total P inputs to the continental coastal waters of the North Sea were down 50–70%, but the N load was reduced by only 20–30% (Lenhart et al., 2010; Passy et al., 2013). This unbalanced de-eutrophication has yielded riverine inputs to the coastal zones with increasing N:P ratios that currently greatly exceed the Redfield ratio of 16:1 (Radach and Pätsch, 2007; Thieu et al., 2010; Grizzetti et al., 2012).

Ecological theory predicts that a reduction in nutrient loads and changes in ambient N:P ratios may cause major changes in the productivity and species composition of phytoplankton communities (Tilman, 1982; Smith and Bennett, 1999; Brauer et al., 2012), with possible consequences for the entire marine food web (Sterner and Elser, 2002; Philippart et al., 2007). The effect of reduced nutrient loads is expected to be most pronounced in nearshore regions.
with high fluvial inputs (Skogen and Mathisen, 2009). In the North Sea, this region of freshwater influence (ROFI) extends along the Dutch, German, and Danish coastal zone reaching up to 30–50 km offshore (Simpson et al., 1993). A bioassay study with an isolate of Skeletonema costatum revealed co-limitation by P and Si in the ROFI region, although this study did not investigate the response of the natural phytoplankton assemblage (Peeters and Peperzak, 1990). Furthermore, model analyses point to an increased potential for P limitation in the coastal waters of the North Sea (Loebl et al., 2009; Passy et al., 2013; Troost et al., 2014), and field studies have already indicated changes in phytoplankton species composition and a shift from N to P limitation of the phytoplankton spring bloom in the adjacent western part of the Wadden Sea (Philippart et al., 2007; Ly et al., 2014). However, a comprehensive understanding of the implications of changing riverine nutrient inputs for the North Sea at large is still lacking.

In this study, we aim to investigate to what extent the reductions in riverine nutrient loads have affected nutrient limitation patterns in the North Sea. Four research cruises were performed during the spring and summer seasons to investigate ambient N:P ratios and phytoplankton community composition along a 450 km transect extending from the Dutch coast to the central North Sea. We used the molar Redfield ratio of 16:1 as a preliminary guide to indicate whether phytoplankton growth was limited by N or P. However, comparison of ambient nutrient concentrations against the Redfield ratio provides, at best, a coarse indication of the potentially limiting nutrient. For instance, many phytoplankton species show a high degree of physiological plasticity in their cellular C:N:P ratios, particularly in response to changes in nutrient and light availability (Droop, 1973; Ducobu et al., 1998; Sterner and Elser, 2002). Furthermore, the optimal N:P ratio of phytoplankton cells varies among species, from ratios as low as 10:1 for species with low N requirements to as high as 45:1 for species with low P requirements (Geider and La Roche, 2002; Klausmeier et al., 2004).

Therefore, we also carried out on-board nutrient enrichment bioassays with natural phytoplankton assemblages to obtain a better understanding of the actual nutrient limitation realised by the phytoplankton community (Hecky and Kilham, 1988; Tamminen and Andersen, 2007). A key advantage of nutrient bioassays is that a factorial design can be applied in which nutrients are added alone and in different combinations to distinguish between single nutrient
limitations and co-limitation by different nutrients (Arrigo, 2004; Harpole et al., 2011).

Our hypothesis is that the unbalanced reduction of nutrient loads and concomitant increase in N:P ratios of the riverine input will have shifted phytoplankton growth in coastal waters of the North Sea toward more P-limited conditions. To address this hypothesis, we focus on the following three related questions: (1) to what extent has P limitation become more pronounced over the past decades, (2) how does nutrient limitation vary during the seasons and along an offshore gradient from the coast toward the central North Sea, and (3) how do these shifts in nutrient limitation affect the phytoplankton species composition and their nutritional quality as a potential food source for higher trophic levels?

Methods
Offshore transect

Four research cruises were conducted with the Dutch research vessel RV Pelagia along a 450 km transect in the North Sea (Fig. 2.1). The most nearshore station was 7 km from the Dutch coast north of the island of Terschelling. This shallow station (8 m depth) is located in the Region of Freshwater Influence (ROFI) of the river Rhine, which stretches out along the Dutch coast (Simpson et al., 1993; De Ruijter et al., 1997) with a salinity varying between 30 and 34. The water gradually deepens along the transect to ~45 m depth at 150 km offshore, where the water mass is dominated by southern North Sea water with a salinity of 34–35. Subsequently, the transect passes the relatively shallow area of the Dogger Bank (30 m depth) at 200–300 km offshore, and then enters the deeper central North Sea which is strongly influenced by mixing with water from the North Atlantic Ocean (Fig. 2.1). During summer, the central North Sea is thermally stratified and a deep chlorophyll maximum may develop (van Haren et al., 1998; Weston et al., 2005).

The cruises took place on 15–30 August 2011, 08–12 May 2012, 15–25 March 2013 and 24 April 2013–04 May 2013. The timing of the cruises coincided with the early onset (March 2013), the peak (April 2013) and the decline (May 2012) of the phytoplankton spring bloom, and the late summer period (August 2011) (see Fig. S2.1). Eight stations were selected for sampling during the cruises and are designated by their distance from shore: stations 7 km, 70 km, 100 km, 180 km, 250 km, 300 km, 360 km, and 450 km.
Vertical distributions of light, temperature, and salinity were obtained using a CTD sampler (Sea-Bird SBE911+, Sea-Bird Electronics, Bellevue, Washington) with a sensor for photosynthetically active radiation (PAR; Satlantic logarithmic PAR-sensor). Water for nutrient analysis and bioassay experiments was collected using a sampling rosette equipped with 24 Niskin water samplers of 12 L each.

Figure 2.1 Location of the transect with the eight sampling stations in the North Sea. Station names indicate the distance from shore. Arrows indicate residual currents; ROFI, region of freshwater influence.

**Nutrient and phytoplankton analysis**

**Nutrients**

Samples for dissolved inorganic nutrients were collected from all eight stations at depths of 2 m, 7 m, 10 m and then every 5 m to the bottom of the water column, and transferred into acid-washed 125 mL polypropylene bottles. In the on-board lab, nutrient samples were gently syringe filtered over a 0.22 μm polycarbonate filter into 5 mL polyethylene vials, rinsing three times, and stored
in the dark at 4°C until analysis. Nutrient concentrations were analyzed with a SEAL QuAAtro autoanalyzer (SEAL Analytical, Fareham, UK) for nitrate and nitrite (NOx; Grasshoff et al., 1983), ammonium (Helder and de Vries, 1979), dissolved inorganic phosphate (DIP; Murphy and Riley, 1962) and silicate (DSi; Strickland and Parsons, 1972). Dissolved inorganic nitrogen (DIN) was defined as the sum of nitrate, nitrite, and ammonium.

Samples for the elemental composition of the plankton community were collected from 7 m depth at the four stations that were also selected for the bioassay experiments (7 km, 100 km, 250 km, and 450 km). Particulate matter was concentrated on-board, in triplicate, on pre-combusted and pre-weighed 0.45 μm pore size glass fiber filters (25 mm Ø; GF/F, Millipore International Ltd., Maidstone, England) under gentle vacuum, until filters were visibly clogged to ensure adequate collection of biomass. The filters were quickly rinsed under vacuum with 5 mL of 0.22 μm filter sterilized milli-Q water to remove excess salts and stored at −20°C until further processing onshore. Prior to analysis, filters were dried at 60°C for 24 h. Particulate organic nitrogen (PON) and particulate organic carbon (POC) were analyzed using an elemental analyzer (VarioEL Cube, Elementar Analysensysteme GmbH, Hanau, Germany). Particulate organic phosphorus (POP) was analyzed using the ignition method followed by acid digestion to ortho-phosphate (Anderson, 1976), which was then measured using standard colorimetric methods (Murphy and Riley, 1962). Based on the typical biochemical composition of algae and cyanobacteria, Geider and La Roche (2002) estimated that the critical cellular N:P ratio marking the transition from N- to P-limited growth ranges from 15 to 30, and that cellular C:N ratios from 3 to 17 and C:P ratios from 27 to 135 are typical for nutrient-replete conditions. We used these ratios as benchmarks for comparison against our PON, POP, and POC measurements, assuming that PON:POP ratios < 15 and POC:PON ratios > 17 are indicative of N limitation, whereas PON:POP ratios > 30 and POC:POP ratios > 135 are indicative of P limitation.

**Phytoplankton**

Phytoplankton samples were collected from 7 m depth at all stations. Water samples (45 mL) were transferred to pre-rinsed 50 mL centrifuge tubes, preserved with 1% Lugol's iodine, and stored in the dark at 15°C until microscopic analysis. Furthermore, 4.5 mL samples were preserved with 0.5 mL
formaldehyde (18% v/v)-hexamine (10% w/v) solution in 5 mL cryogenic vials. These samples were placed in 4°C for 30 min, flash frozen in liquid nitrogen, and stored at −80°C until analysis via flow cytometry.

Large phytoplankton (>5 μm) were identified to genus level and enumerated using light microscopy. Lugol's iodine preserved samples were settled in 10 mL Utermöhl chambers (Hydro-Bios, Kiel, Germany). The entire chamber was counted at 40X magnification using an inverted microscope (DM IRB, Leica Microsystems, Wetzlar, Germany). To investigate whether different functional groups within the phytoplankton community responded differently to nutrient limitation, the phytoplankton genera were sorted into four major functional groups: diatoms, dinoflagellates, nanoflagellates (excluding Phaeocystis), and Phaeocystis spp. The cell counts included both autotrophic and mixotrophic dinoflagellates and nanoflagellates, whereas heterotrophic species (e.g., Protoperidinium spp.) were excluded from our analyses.

Picocyanobacteria and picoeukaryote abundances were determined by flow cytometry (Marie et al., 2001), using an Accuri C6 flow cytometer (BD Biosciences, San Jose, California) equipped with a blue laser (488 nm) and red laser (640 nm). Frozen samples were thawed at 4°C, pre-filtered through a 5 μm polycarbonate filter, and then analyzed. Picocyanobacteria and picoeukaryotes were distinguished based on bivariate plots of side scatter against the autofluorescent properties of the key phytoplankton pigments chlorophyll (excitation with 488 nm laser, autofluorescence with 670 nm filter), phycoerythrin (excitation with 488 nm laser, autofluorescence with 585 nm filter) and phycocyanin (excitation with 640 nm laser, autofluorescence with 675 nm filter).

Biovolumes of the picocyanobacteria, picoeukaryotes and larger phytoplankton were calculated from cellular dimensions and geometry according to Hillebrand et al. (1999). Subsequently, C-biomass of the phytoplankton was calculated from the cell counts and biovolumes of the cells according to Menden-Deuer and Lessard (2000). The colony matrix of Phaeocystis was not included in the phytoplankton biomass calculations.

Bioassay experiments

We performed on-board bioassay experiments to test for nutrient limitation in natural phytoplankton assemblages sampled from the North Sea. During the
March 2013 and April 2013 cruises, stations 7 km, 100 km, 250 km, and 450 km were selected for the bioassay experiments. During the May 2012 cruise only station 7 km was selected due to time restrictions. During the August 2011 cruise, stations 180 km and 450 km were selected. Water samples for the bioassay experiments were always collected at 08:00 from 7 m depth. Immediately after sampling 3 L of water was transferred into 3.2 L polyethylene carboys which had been thoroughly rinsed with sample water prior to the filling. Nutrients were added (1 mL additions) in accordance with the nutrient treatments described below, and within 45 min after sampling the carboys were placed in four flow-through incubation containers on the aft deck of the research vessel. Sea water was continuously pumped through all incubation containers to maintain consistent ambient temperature. Incubations continued for 96–144 h depending on the cruise length.

Given space limitations on-board, we selected seven different treatments: Control, +N (addition of 80 μM NaNO₃), +P (5 μM K₂HPO₄), +Si (80 μM Na₂SiO₃), +N+P, +N+P+Si, and +light (Table 2.1). Neutral density filters covered the top of the incubators to obtain the same irradiance (PAR range; measured with a LI-250 LI-COR light meter; Lincoln, Nebraska) as at the sampling depth (7 m) from which the phytoplankton assemblage originated. For the +light treatment, we reduced the neutral density filters to obtain a 25% higher irradiance level. The bioassay experiments during the May 2012 and August 2011 cruise had five replicates per treatment, while we used four replicates per treatment in the March 2013 and April 2013 cruise.

Progress was monitored by taking a 250 mL sample from each carboy every 48 h, which was filtered onto 0.45 μm pore size glass fiber filters (GF/F, Whatman, Maidstone, England) using a vacuum manifold (model 1225, Millipore, Eschborn, Germany). The loaded filters were dark adapted for 10 min, after which chlorophyll fluorescence was measured with a mini-PAM fluorometer (Walz, Effeltrich, Germany).

At the beginning and end of the incubation period, from each carboy a water sample (15 mL) was collected and preserved for identification, cell counts and C-biomass calculations of the phytoplankton at the genus level, according to the methods described above. Furthermore, samples of 500 mL (beginning) and 250 mL (end of incubation) taken from the carboys were filtered onto 0.45 μm pore size glass fiber filters (GF/F, Whatman). Filters were folded into aluminium
foil and stored at −20°C until analysis. Chlorophyll a (Chl a) was extracted with 90% acetone and measured using standard fluorometric analysis (Welschmeyer, 1994).

**Table 2.1 Nutrient additions in the bioassay experiments.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrate (µM)</th>
<th>Phosphate (µM)</th>
<th>Silicate (µM)</th>
<th>Light level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>collection depth</td>
</tr>
<tr>
<td>+N</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>collection depth</td>
</tr>
<tr>
<td>+P</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>collection depth</td>
</tr>
<tr>
<td>+Si</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>collection depth</td>
</tr>
<tr>
<td>+N+P</td>
<td>80</td>
<td>5</td>
<td>0</td>
<td>collection depth</td>
</tr>
<tr>
<td>+N+P+Si</td>
<td>80</td>
<td>5</td>
<td>80</td>
<td>collection depth</td>
</tr>
<tr>
<td>+ Light</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25% more</td>
</tr>
</tbody>
</table>

**Statistical analysis**

**Particulate organic nutrients**

We performed a two-way analysis of variance to test whether the PON:POP, POC:POP, and POC:PON ratios varied significantly with distance from shore (stations 7 km, 100 km, 250 km, and 450 km) and month of the cruise (March, April, and May/August). To improve homogeneity of variance, as tested by Levene's test, dependent variables were log-transformed prior to analysis. Type IV sum of squares was used to account for missing data at station 100 km in May/August. Post-hoc comparison of the means was based on Tukey’s HSD test using a significance level (α) of 0.05.

**Bioassay experiments**

Net growth rates (d⁻¹) were calculated as $\mu = \ln(B_t/B_0)/t$, where $B_0$ and $B_t$ are the initial and final total phytoplankton biomass or Chl a concentrations determined from the bioassay experiments and $t$ is the incubation time in days. Net growth rates based on phytoplankton biomass were also determined for each phytoplankton group separately (diatoms, dinoflagellates, nanoflagellates, *Phaeocystis* spp., picoeukaryotes, and picocyanobacteria). One-way analysis of variance with post-hoc comparison of the means (Tukey's HSD test) was used to assess significant differences in net growth rates among the treatments.
Based on the statistical results, we distinguished between three different types of co-limitation (Harpole et al., 2011). “Independent co-limitation” was defined as the stimulation of phytoplankton growth by two or more single nutrient treatments (e.g., +N only and +P only) as well as by the combined nutrient treatment (+N+P). “Simultaneous co-limitation” was defined as the stimulation of phytoplankton growth by the combined nutrient treatment, but not by the single nutrient treatments. Finally, “serial co-limitation” was defined as the stimulation of growth by only one of the single nutrient treatments (e.g., +N only, but not +P only) and an even stronger stimulation by the combined nutrient treatment (+N+P).

**Results**

**Dissolved nutrients**

In March, DIN concentrations were highest (∼30 μmol L⁻¹) in nearshore waters and gradually decreased offshore, with a local minimum at station 300 km (Fig. 2.2a). In April, during the phytoplankton spring bloom, nitrogen depletion occurred throughout most of the transect, except in the nearshore waters where the DIN concentration was still relatively high (Fig. 2.2b). During late spring and summer, DIN concentrations were low throughout the entire transect, with slightly higher concentrations below the thermocline in the outer-shore region (Fig. 2.2c,d; see Fig. S2.2c,d).

In March, DIP concentrations were also highest (∼0.63 μmol L⁻¹) in nearshore waters (Fig. 2.2e). In April, however, nearshore DIP concentrations were strongly depleted to < 0.05 μmol L⁻¹, while outer-shore concentrations were depleted but to a lesser extent (∼0.18 μmol L⁻¹ at station 450 km) (Fig. 2.2f). Nearshore DIP concentrations remained below 0.05 μmol L⁻¹ in late spring and summer (Fig. 2.2g,h). During summer stratification, outer-shore DIP concentrations were low in the top 40 m, but higher (up to ∼1 μmol L⁻¹) below the thermocline (Fig. 2.2h).

Like DIN and DIP, in March, silicate (Si) concentrations were highest (∼12 μmol L⁻¹) in nearshore waters (Fig. 2.2i). Silicate concentrations were strongly depleted along the entire transect during the phytoplankton spring bloom in April, and slowly increased into the summer (Fig. 2.2j–l).
Figure 2.2 Concentrations of (a–d) DIN, (e–h) DIP and (i–l) DSi along the offshore transect, during research cruises in March 2013, April 2013, May 2012, and August 2011. Dots indicate depths from which samples were collected. Shading indicates depths below the euphotic zone (i.e., light levels < 1% of surface light intensity). Graphs were generated using Ocean Data View software (http://odv.awi.de).

During most cruises, the molar DIN:DIP ratio decreased with distance from the coast (Fig. 2.3). The high DIN:DIP ratio nearshore is associated with freshwater influence, as supported by a negative correlation between salinity and molar DIN : DIP ratios (Pearson correlation coefficient = −0.69, n = 29, p < 0.001; see Fig. S2.2e–h for the salinity gradient). In March, the DIN:DIP ratio ranged from 54:1 in the nearshore region to 4:1 in the outer-shore region. The Redfield isocline (DIN:DIP ratio of 16:1) occurred at ∼130 km from shore (Fig. 2.3a). In April, the spatial gradient in DIN:DIP was most extreme, with ratios of 375:1 in nearshore and 1:1 in outer-shore waters. The Redfield isocline was also the furthest offshore, at ∼200 km from the Dutch coast (Fig. 2.3b). In May, the DIN:DIP gradient was slightly less extreme, ranging from 152:1 to 1:1, and the Redfield isocline moved landward to ∼75 km from shore (Fig. 2.3c). Finally, in August, DIN:DIP ratios were below Redfield throughout the transect and the offshore DIN:DIP gradient had disappeared (Fig. 2.3d).
Figure 2.3 Molar ratios of (a–d) DIN:DIP and (e–h) DSI:DIP along the offshore transect, during research cruises in (a, e) March 2013, (b, f) April 2013, (c, g) May 2012 and (d, h) August 2011. The Redfield ratio of 16:1 for DIN:DIP and 15:1 for DSI:DIP is indicated by the black contour line. Shading indicates depths below the euphotic zone (i.e., light levels < 1% of surface light intensity). Arrows on x-axis indicate the four stations (7 km, 100 km, 250 km and 450 km) of the nutrient bioassay experiments.

The molar DSI:DIP ratio showed a similar offshore gradient as the DIN:DIP ratio in March, with higher than Redfield ratios at the nearshore region and lower than Redfield ratios further offshore (Fig. 2.3e). The DSI:DIP ratio dropped below Redfield along the entire transect during the phytoplankton spring bloom in April (Fig. 2.3f), showed an offshore gradient in May (Fig. 2.3g), and was near or above Redfield in the surface layer in August (Fig. 2.3h).

Particulate organic nutrients

POC, PON, and POP were measured at four stations along the transect. Microscopic inspection showed that the particulate matter was dominated by phytoplankton at all four stations, except for station 7 km of the March 2013 cruise which also contained a lot of detritus and sediment particles. Comparison of the POC measurements against the total C-biomass of the phytoplankton community calculated from cell counts, after removal of the 7 km station of March 2013, confirmed that the particulate organic matter largely consisted of
phytoplankton (linear regression through the origin: C-biomass = 0.977 × POC; $R^2 = 0.93$, $N = 10$, $p < 0.001$). The PON:POP, POC:POP, and POC:PON ratios all varied significantly with distance from shore (Station) and season (Month), and also the interaction terms (Station × Month) were significant (Table 2.2).

**Table 2.2** Two-way analysis of variance, with the particulate organic nutrient ratios as dependent variable, and station and month as independent variables.

<table>
<thead>
<tr>
<th>Effect</th>
<th>$df_1$, $df_2$</th>
<th>$F$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PON:POP ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Station</td>
<td>3, 22</td>
<td>52.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Month</td>
<td>2, 22</td>
<td>12.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Station × Month</td>
<td>5, 22</td>
<td>5.59</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>POC:POP ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Station</td>
<td>3, 22</td>
<td>33.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Month</td>
<td>2, 22</td>
<td>44.96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Station × Month</td>
<td>5, 22</td>
<td>3.81</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>POC:PON ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Station</td>
<td>3, 22</td>
<td>3.46</td>
<td>0.034</td>
</tr>
<tr>
<td>Month</td>
<td>2, 22</td>
<td>189.50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Station × Month</td>
<td>5, 22</td>
<td>13.73</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The PON:POP ratio showed a similar offshore gradient as the DIN:DIP ratio, with values high above the transition zone between N- and P-limited growth (Geider and La Roche, 2002), indicative of P limitation, at the nearshore station 7 km (Fig. 2.4a). PON:POP ratios were significantly lower further offshore, with values below the transition zone between N- and P-limited growth, indicative of N limitation, at station 250 km in April and stations 250 km and 450 km in May/August (Fig. 2.4a). The POC:POP ratio followed a similar pattern, with high nearshore values indicative of P limitation in April and May/August and significantly lower values indicative of P replete conditions offshore (Fig. 2.4b). The POC:PON ratio showed a reverse pattern, with low values indicative of N replete conditions nearshore and significantly higher values indicative of N limitation at offshore station 250 km in April and stations 250 km and 450 km in May/August (Fig. 2.4c).
Figure 2.4 (a) PON:POP, (b) POC:POP, and (c) POC:PON ratios (± SD) at four stations (7 km, 100 km, 250 km, and 450 km) along the offshore transect, measured at 7 m depth. Horizontal dashed lines represent the Redfield ratio of C:N:P = 106:16:1. For comparison, the horizontal grey band in (a) marks the transition between N and P limitation of phytoplankton growth according to Geider and La Roche (2002); PON:POP ratios below this band (<15) are indicative of N limitation and PON:POP ratios above this band (>30) of P limitation. Horizontal grey bands in (b) and (c) indicate the range of cellular C:P and C:N ratios reported under nutrient-replete conditions (Geider and La Roche, 2002). Bars that do not share the same letter are significantly different, as tested by two-way analysis of variance (Table 2.2) followed by post-hoc comparison of the means (Tukey's HSD test). The letters “n.d.” indicate that we have no data for May/August at station 100 km.
**Phytoplankton community**

The phytoplankton community varied according to month and location (Fig. 2.5). Total phytoplankton biomass was highest at the nearshore station 7 km in the spring months March–May and also reached high values at stations 70 km and 100 km in April and station 250 km in March and May (Fig. 2.5e–g), while total phytoplankton biomass was low at all five stations sampled in August (Fig. 2.5h). Numerically, picocyanobacteria and picoeukaryotes dominated the phytoplankton community throughout the entire transect, with co-dominance of *Phaeocystis* at stations 7 km and 450 km in April and at stations 7 km and 300 km in May (Fig. 2.5a–d). In terms of phytoplankton biomass, diatoms and dinoflagellates were the main contributors, with diatoms dominating at the majority of stations in March and April while diatoms and dinoflagellates were co-dominant at most stations in May and August (Fig. 2.5e–h). Furthermore, *Phaeocystis* and nanoflagellates contributed substantially to phytoplankton biomass at several stations in April and May (Fig. 2.5f,g). Among the dinoflagellates, seven of the nine identified genera consisted largely of species described as mixotrophs in the literature (Table S2.1). Likewise, the nanoflagellates were dominated by potential mixotrophs such as *Cryptomonas* and *Prymnesium* species (Table S2.2).

**Bioassay experiments**

Bioassay experiments were performed on-board at four stations along the transect to assess which nutrients limit phytoplankton growth. Monitoring of chlorophyll fluorescence indicated that the phytoplankton concentration either increased or remained constant during the bioassay experiments depending on the treatment and station (Fig. S2.3). An exception is the 100 km station in March, which showed a decrease in chlorophyll fluorescence during the first 48 h of the bioassay experiments (Fig. S2.3b). Growth responses based on phytoplankton biomass (Fig. 2.6) and Chl *a* concentration (Fig. S2.4) showed consistent results.

At the nearshore station 7 km, nutrient additions did not result in a significant increase of the phytoplankton growth rate in March (Fig. 2.6a). In April, both the +P and +Si treatment significantly increased the net growth rate of the total phytoplankton community when compared with the control, pointing at independent co-limitation of P and Si (Fig. 2.6e). The +N+P and +N+P+Si
treatments did not stimulate significantly more growth than the +P and +Si treatment. In May, growth of the phytoplankton community was also significantly enhanced by the +P and +Si treatments, indicating again independent co-limitation of P and Si (Fig. 2.6i). Furthermore, the growth rate was significantly higher in the +N+P+Si treatment than in the +P and +Si treatments, suggesting an interactive effect by the simultaneous addition of P and Si. The different phytoplankton groups responded differently to the bioassay experiments (Table 2.3). The net growth rates of diatoms at station 7 km were limited by Si in April and co-limited by Si and P in May, dinoflagellates and nanoflagellates were co-limited by N and P, *Phaeocystis* was limited by P and slightly by light, and picocyanobacteria were limited by P only (Table 2.3; see Figs. S2.5–S2.10 for details).

At station 100 km, total phytoplankton growth rates were negative and nutrient additions did not result in a significant increase in March (Fig. 2.6b). In April, however, the +P treatment and +N+P+Si treatment showed significantly higher growth rates of the total phytoplankton community than the control, while the +N+P treatment did not (Fig. 2.6f). This indicates serial co-limitation of P followed by Si as secondary limiting nutrient. Diatoms were independently co-limited by Si and P, nanoflagellates showed serial co-limitation by N and P with independent co-limitation by light, and *Phaeocystis* and picoeukaryotes were limited by P only (Table 2.3; Figs. S2.5–S2.10).

At station 250 km, nutrient additions again did not enhance total phytoplankton growth rates in March (Fig. 2.6c). In April, single nutrient additions did not significantly increase the growth rate of the total phytoplankton community, but the combined +N+P and +N+P+Si treatments enhanced phytoplankton growth, indicating simultaneous N and P co-limitation (Fig. 2.6g). In August, +N addition increased total phytoplankton growth, while the +N+P and +N+P+Si treatments led to an even higher growth rate, which points to serial co-limitation with N as primary limiting nutrient followed by P as secondary limiting nutrient (Fig. 2.6k). Diatoms, dinoflagellates, nanoflagellates, *Phaeocystis*, picoeukaryotes and picocyanobacteria were all co-limited by N and P during one or two of the months, while nanoflagellates were limited by N and picoeukaryotes by P in April (Table 2.3; Figs. S2.5–S2.10).
Figure 2.5 Phytoplankton community composition, quantified as (a–d) cell abundances and (e–h) phytoplankton biomass (expressed in terms of C) along the offshore transect, during the research cruises in (a, e) March 2013, (b, f) April 2013, (c, g) May 2012, and (d, h) August 2011. n.d., no data.
Figure 2.6 Growth rates (± SD) of total phytoplankton in the nutrient bioassay experiments, based on changes in phytoplankton biomass. The experiments were performed at four stations (7 km, 100 km, 250 km, and 450 km) along the offshore transect, during the (a–d) March 2013 cruise, (e–h) April 2013 cruise, (i) May 2012 cruise, and (k, l) August 2011 cruise. Treatments included the control (C) and additions of nitrate (N), phosphate (P), silicate (Si) and light (Table 1), with \( n = 4 \) replicates per treatment in March and April and \( n = 5 \) replicates per treatment in May and August. Within each panel, bars with different letters were significantly different, as tested by one-way analysis of variance followed by post-hoc comparison of the means (Tukey’s HSD test).
Table 2.3 Nutrient limitation of the phytoplankton community along the offshore transect, based on the bioassay experiments.

<table>
<thead>
<tr>
<th>Species group</th>
<th>Station</th>
<th>7km</th>
<th>100km</th>
<th>250km</th>
<th>450km</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total phytoplankton</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- March</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- April</td>
<td>P+Si(^a)</td>
<td>P+Si(^c)</td>
<td>N+P(^b)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>- May/August</td>
<td>P+Si(^a)</td>
<td>no data</td>
<td>N+P(^c)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><strong>Diatoms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- March</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- April</td>
<td>Si</td>
<td>Si+P(^a)</td>
<td>N+P(^b)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>- May/August</td>
<td>Si+P(^a)</td>
<td>no data</td>
<td>N+P(^c)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td><strong>Nanoflagellates</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>- March</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- April</td>
<td>N+P(^b)</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- May/August</td>
<td>N+P(^b)</td>
<td>no data</td>
<td>N+P(^b)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td><strong>Picoeukaryotes</strong></td>
<td></td>
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<td></td>
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<tr>
<td>- March</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- April</td>
<td>P</td>
<td>n.s.</td>
<td>P</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- May/August</td>
<td>P</td>
<td>no data</td>
<td>N+P+light(^a)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td><strong>Picocyanobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- March</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- April</td>
<td>P</td>
<td>n.s.</td>
<td>N+P(^b)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>- May/August</td>
<td>P</td>
<td>no data</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

The table summarizes the growth responses of the total phytoplankton community (Fig. 2.6) and of the different phytoplankton groups (Figs. S2.5-S2.10). Nutrients or light were considered limiting if their addition significantly enhanced phytoplankton growth rate in comparison to the control. \(^a\)=independent co-limitation; \(^b\)=simultaneous co-limitation; \(^c\)=serial co-limitation; n.s.=no significant response; the nutrient that gave the strongest growth response is mentioned first, in case of independent or serial co-limitation. See the Supporting Information for further details.

At the outer-shore station 450 km, the +N, +N+P and +N+P+S treatments stimulated significantly higher growth rates of the total phytoplankton community than the control in both April and August (Fig. 2.6h, l). However, the +N treatment did not differ significantly from the +N+P and +N+P+Si
treatments. These results show that N was the key limiting nutrient for phytoplankton growth at station 450 km. The growth rates of diatoms, dinoflagellates and nanoflagellates were limited by N, while Phaeocystis was co-limited by N and P and/or limited by light (Table 2.3; Figs. S2.5–S2.10).

Discussion

Increasing P limitation

Our results show phosphorus limitation of phytoplankton growth in the coastal waters of the North Sea. It is useful to interpret these results in a long-term context. Historical data on nutrient concentrations measured in our study area were obtained from Rijkswaterstaat, a branch of the Dutch Ministry for Infrastructure and the Environment, for their station Terschelling 4 km. This station is only 3 km south of our station 7 km, in the same transect. Both stations have a comparable depth of 6–8 m and are within the ROFI region with a salinity varying between 30 and 34. The data show a distinct decline in DIP concentration in the early 1990s (Fig. 2.7a), but only a slight decrease in DIN (Fig. 2.7b) and no evident change in the DSi concentration (Fig. 2.7c). The strongly reduced DIP concentration from the early 1990s onward was not accompanied by a similar decrease of the chlorophyll concentration, although there was a temporary reduction in chlorophyll levels during the years 1990–1995 (Fig. 2.7d). Rijkswaterstaat ended their measurements at station 4 km in 2007. The chlorophyll concentrations that we measured at station 7 km during our cruises (Fig. 2.7d) and estimated from satellite data (Fig. S2.1) in 2011–2013 seem somewhat lower, although the number of datapoints is too limited to draw firm conclusions. Hence, although overall reduction of phytoplankton biomass was one of the primary goals of the nutrient reduction policy, in terms of chlorophyll concentrations this policy has not yet proven successful, except perhaps for a decrease of the chlorophyll concentration during recent years.

The decreasing DIP concentration has caused an increase in the DIN:DIP ratio, particularly in spring, from the late 1990s onward (Fig. 2.7e). Comparison with our 2011–2013 data of nearshore station 7 km indicates that the upward trend in DIN:DIP ratio has continued during the past decade. The DSi:DIP ratio shows a similar increase, from values that remained largely below the Redfield ratio of 15:1 throughout the year in the 1970s and 1980s to higher values exceeding Redfield every summer from the early 1990s onward (Fig. 2.7f). These
data indicate that nutrient limitation of these coastal waters has shifted from a balanced N+P co-limitation to severe P limitation for non-siliceous phytoplankton, and from Si limitation to Si+P co-limitation for diatoms.

Figure 2.7 Concentrations of (a) DIP, (b) DIN, (c) DSi, and (d) Chl a, and molar ratios of (e) DIN:DIP and (f) DSi : DIP in nearshore waters of the North Sea over the past 37 yr. The 1975–2006 data (circles) are from station 4 km of the monthly monitoring program along the Terschelling transect by Rijkswaterstaat. The 2011–2013 data (triangles) are from the nearshore station 7 km of this study.
Comparisons of ambient nutrient ratios against the Redfield ratio should be interpreted with caution, however, because ambient nutrient ratios do not necessarily reflect the flux rates of these nutrients and because of the known variability of the nutrient stoichiometry of phytoplankton (Geider and La Roche, 2002; Sterner and Elser, 2002; Klausmeier et al., 2004). We therefore performed on-board bioassays with sampled seawater to obtain a better understanding of the nutrient response of the phytoplankton community along the offshore gradient. The bioassay experiments show that at stations 7 km and 100 km growth rates of the total phytoplankton community and of the diatoms were co-limited by P and Si, dinoflagellates and nanoflagellates were co-limited by N and P, *Phaeocystis* was co-limited by P and light, and picoeukaryotes and picocyanobacteria were limited by P alone during and directly after the phytoplankton spring bloom (Table 2.3). Hence, the bioassay experiments confirm that P has become one of the key limiting nutrients in coastal waters of the North Sea.

Our findings provide firm experimental support for earlier indications of enhanced P limitation in the North Sea. Loebl et al. (2009) have alluded to an increasing P limitation potential along the North Sea coast since the 1990s. The authors used a Liebig’s Law-based model (Cloern, 1999) to investigate the potential limiting nutrient for phytoplankton growth in the North Sea. Based on a dataset of nutrient concentrations from 1990 to 2005 at several locations along the North Sea coast, they found indications for a general increase of the P limitation potential near-shore. Passy et al. (2013) and Troost et al. (2014) evaluated historical changes in riverine nutrient input and atmospheric nitrogen deposition into the North Sea using model analyses, and their model results also point at a recent shift toward P limitation. Ly et al. (2014) have performed bioassays on natural phytoplankton assemblages, which demonstrated P limitation of the spring bloom in the adjacent western Wadden Sea. All of these results converge to the conclusion that changes in riverine nutrient inputs have pushed the coastal waters of the North Sea toward severe P limitation.

**Offshore gradient from P to N limitation**

Whereas the nearshore waters have become P limited, the central North Sea is strongly influenced by exchanges with North Atlantic Ocean water which has a relatively low DIN:DIP ratio. Our results show that this has created an
offshore gradient from P limitation to N limitation of phytoplankton growth. This gradient is clearly seen in the DIN:DIP ratios and in the results of the nutrient bioassay experiments. The gradient was most evident in April, when nutrients were depleted by the phytoplankton spring bloom. During this time, the DIN:DIP ratio at the nearshore station was at the highest of the study (Fig. 2.3b), and P addition elicited significant growth in the nearshore bioassay experiments while N addition had no effect (Fig. 2.6e). The opposite result occurred at the most outer-shore station with a DIN:DIP ratio of 1:1, where N addition stimulated phytoplankton growth while P addition gave no response (Figs. 2.3b; 2.6h). Furthermore, the intermediate DIN:DIP ratios (Fig. 2.3b) and the results of the bioassay experiments at station 250 km (Table 2.3) point at a transitional zone between the coastal waters and the central North Sea with co-limitation of N and P.

The offshore gradient in nutrient limitation is also reflected in the elemental composition of particulate organic matter, with high PON:POP and POC:POP but low POC:PON ratios nearshore, intermediate values in the transitional zone, and the reverse pattern offshore (Fig. 2.4). Particulate organic matter in our samples consisted largely of phytoplankton, except for station 7 km in March, as verified by microscopic inspection and comparison against the C-biomass calculated from the phytoplankton counts. Hence, the wide range of PON:POP ratios, from 5–10 at the outershore stations to 175 at the nearshore station, signifies the response of the phytoplankton stoichiometry to changes in ambient nutrient conditions. The observed range spans the entire spectrum of cellular N:P ratios observed in laboratory experiments with marine phytoplankton. For instance, the classic lab experiments of Goldman et al. (1979) showed that cellular N:P ratios of the diatom *Thalassiosira pseudonana* and the chrysophyte *Monochrysis lutheri* varied with the growth conditions, ranging from 5 for N-limited growth to 110 for P-limited growth. Geider and La Roche (2002) summarized the literature by pointing out that the physiological range of cellular N:P ratios in phytoplankton experiments ranges from < 5 under severe N limitation to > 100 under severe P limitation, and Klausmeier et al. (2004) report phytoplankton N:P ratios ranging from 6 to 136. Hence, the wide span of PON:POP values in the North Sea emphasizes once more the striking offshore gradient from P-limited to N-limited conditions.
The steepness of the offshore gradient in nutrient limitation has a seasonal component, with the most pronounced P limitation during and after the phytoplankton spring bloom. In March, before the phytoplankton spring bloom, the DIN:DIP gradient was present but the nitrate and phosphate concentrations were not yet fully depleted. Thus, none of the added nutrients in the bioassays stimulated growth in March. In August, the strong offshore gradient in DIN:DIP ratio was absent, although the bioassays indicate that P remained a co-limiting nutrient nearshore. Colijn and Cadée (2003) suggest that the influence of freshwater riverine inputs tapers off in the late summer months allowing a higher influence of Atlantic water with a relatively low DIN:DIP ratio to the entire North Sea basin. However, we found that the salinity gradient persisted in the late summer (Fig. S2.2), and hence our data do not provide support for a reduced freshwater inflow that might explain the absence of the DIN:DIP gradient in August. An alternative explanation for the low DIN:DIP ratios in August might be regeneration of P from sediments triggered by higher water temperatures in summer (Jensen et al., 1995), which is consistent with the relatively high P concentrations near the sediment. Furthermore, Radach and Pätsch (2007) found a strong seasonal variation of N:P ratios within the riverine inputs to the North Sea. They found elevated N:P ratios during the spring months in the Elbe and Rhine rivers, but values much closer to Redfield occurred in the late summer. Therefore, the combined effects of P regeneration and closer-to-Redfield riverine inputs are likely drivers for the ubiquitous low DIN:DIP ratio in the late summer.

**Effects at the phytoplankton community level**

Eutrophication of the coastal North Sea from the 1960s to mid-1980s has led to major shifts in phytoplankton community composition. In particular, dinoflagellates, nanoflagellates and large diatom species such as *Rhizosolenia* and *Thalassiosira* spp. appeared to benefit from the high nutrient levels (Philippart et al., 2000). Recurrent blooms of the dinoflagellate *Dinophysis acuminata* in the coastal North Sea during the 1970s and early 1980s were related to several cases of diarrhetic shellfish poisoning (Kat, 1983). Raphidophytes of the red-tide genera *Chattonella* and *Fibrocapsa* were discovered for the first time in Dutch coastal waters in 1991, and in 1993 the potentially neurotoxic species *Chattonella marina* even developed a minor bloom at station 100 km (Vrieling et al., 1995). Furthermore, nuisance blooms of *Phaeocystis* markedly increased along the coast during this
eutrophication period (Cadée and Hegeman, 2002; Lancelot et al., 2007; Prins et al., 2012), causing extensive foam accumulation on the beaches (Lancelot, 1995; Blauw et al., 2010).

Thus, the OSPAR convention reasoned a reduction of nutrient loads may reverse these trends. However, as our results illustrate, at present *Phaeocystis*, dinoflagellates and nanoflagellates are still a major component of the phytoplankton community in the North Sea. For instance, during the spring bloom, *Phaeocystis* still comprised ~20% of the phytoplankton biomass at station 7 km while dinoflagellates and nanoflagellates together made up another 20% (Fig. 2.5f). *Phaeocystis* spp. appear to be strong competitors for phosphorus (Hegarty and Villareal, 1998; Schoemann et al., 2005; Gypens et al., 2007; but see Riegman et al., 1992) and can grow on organic P sources in low inorganic P environments (Veldhuis and Admiraal, 1987; Van Boekel, 1991). This may explain why, despite a 70% reduction of the P loads, *Phaeocystis* blooms are still a common phenomenon in many coastal areas of the North Sea (Lacroix et al., 2007; Blauw et al., 2010; Prins et al., 2012).

Our bioassay experiments revealed that the total phytoplankton community of the coastal waters (station 7 km) experienced independent co-limitation by P and Si during the spring months. A possible interpretation of independent co-limitation is that different members of the community are limited by different nutrients (Arrigo, 2004; Harpole et al., 2011). Indeed, the bioassays showed that the dominant phytoplankton groups differed in their growth response to the nutrient additions. Diatoms of coastal waters were limited by Si only in April and co-limited by Si and P in May, growth rates of *Phaeocystis*, picoeukaryotes and picocyanobacteria were limited by P, and dinoflagellates and nanoflagellates were co-limited by N and P (Table 2.3).

These results are in agreement with the recent model study of Passy et al. (2013), who reconstructed changes in riverine nutrient loads to the Belgian coastal zone of the North Sea during the years 1984–2007, and investigated the resulting effects on coastal *Phaeocystis* and diatom blooms. In essence, their model study showed that diatom growth in spring is largely controlled by Si, in agreement with our bioassay results, whereas the N and P left over by the diatoms is available for non-siliceous phytoplankton. In the 1980s, P was in excess and N was the major limiting nutrient for non-siliceous phytoplankton in the coastal zone (see also Riegman et al., 1992; Brussaard et al., 1996). However, from the
2000s onward, the annual biomass production of *Phaeocystis* was controlled by P loads, in agreement with P limitation of *Phaeocystis* reported in our bioassays. The model results indicate that, in the Belgian coastal zone, reduced P loads have led to a 50% decrease of both the magnitude and duration of *Phaeocystis* blooms from 1984 to 2007 (Passy et al., 2013). However, *Phaeocystis* blooms have not yet disappeared and annual peak abundances are still far above desired levels. Based on our bioassay experiments and the model predictions of Passy et al. (2013), a further reduction of the riverine P input is expected to further diminish the relative contribution of *Phaeocystis* to the coastal spring bloom.

Interestingly, our bioassay experiments indicate that dinoflagellates and nanoflagellates (other than *Phaeocystis*) were co-limited by N and P in the coastal zone (Table 2.3), despite the extremely high DIN:DIP ratios of these coastal waters. We note that the dinoflagellates and nanoflagellates were dominated by potentially mixotrophic species (Tables S2.1, S2.2), which can obtain their nutrients not only through uptake of inorganic nutrients but also from dissolved organic substances or through predation on other species. In several of these mixotrophic species, inorganic nutrient limitation stimulates grazing (Li et al., 2000; Legrand et al., 2001; Smalley et al., 2003; but see Skovgaard et al., 2003). Their ability to access alternative P sources may explain why dinoflagellates and nanoflagellates in the coastal zone were co-limited by N and P rather than by P alone, and may also explain the competitive success of these mixotrophic species when inorganic P is depleted (Nygaard and Tobiesen, 1993; Lagus et al., 2004). We may thus hypothesize that mixotrophic dinoflagellates and nanoflagellates, including several harmful algal species (e.g., Burkholder et al., 2008), are likely to benefit from reduced P inputs into the coastal zone.

It will be interesting to continue monitoring of the coastal phytoplankton in the North Sea to establish how the species composition, and in particular *Phaeocystis* and potentially toxic dinoflagellate and nanoflagellate species, will develop in the coming years.

**Higher trophic levels**

Changes in the nutrient stoichiometry of phytoplankton may also affect the marine food web. Our results show high POC:POP ratios at the nearshore station, indicative of P deficiency of coastal phytoplankton. In particular, during the spring bloom in April we found POC:POP levels of ~550:1, which is similar to
POC:POP ratios observed in severely P-limited freshwater ecosystems (Hecky et al., 1993; Hassett et al., 1997). Phytoplankton with such low P content may offer food of a poor nutritional quality for herbivorous zooplankton and benthic filter feeders (Sterner and Elser, 2002). Several studies have shown that P-deficient phytoplankton may cause lower growth rates in marine zooplankton (Malzahn et al., 2007; Malzahn and Boersma, 2012; Schoo et al., 2013). Furthermore, herbivores feeding on high C:P phytoplankton have to get rid of the excess carbon, for instance by enhanced respiration or DOC excretion. This waste of photosynthetically fixed carbon results in a decreased efficiency of the food chain (Sterner et al., 1998; Hessen et al., 2004; Boersma et al., 2009) while it may exacerbate P deficiency of DOC-processing bacteria in the microbial loop (Hessen and Anderson, 2008; Li et al., 2014). The decreasing P availability in coastal waters of the North Sea coincides with rising atmospheric CO₂ levels, which may contribute to further increases of the C:P ratios of primary producers (Maat et al., 2014; Verspagen et al., 2014), and hence further deterioration of their nutritional quality (Van de Waal et al., 2010a; Schoo et al., 2013).

Changes in the nutritional quality of phytoplankton may also affect trophic levels beyond the primary consumers. Elevated C:P ratios of copepod grazers fed on P-deficient phytoplankton can have detrimental effects on larval growth of economically valuable species such as herring (Malzahn et al., 2007) and European lobster (Schoo et al., 2014). In contrast, elevated C:P ratios of the same copepods had no significant effects on the biomass growth and reproductive rates of gelatinous predators such as ctenophores and jellyfish (Malzahn et al., 2010; Schoo et al., 2010). This disparity in response to P-deficient phytoplankton may allow gelatinous species to dominate over larval fish in their competition for shared copepod prey in P-limited systems. Dominance by gelatinous organisms can have strong impacts on the structure and functioning of marine food webs (Condon et al., 2011; Dinasquet et al., 2012) with potentially negative impacts on fish productions (e.g., Purcell and Arai, 2001; Acuña et al., 2011). Furthermore, Philippart et al. (2007) showed that reduced P loading of the Wadden Sea from the mid-1980s onward was associated with changes in the species composition of the phytoplankton community, of the macrozoobenthic community feeding on the phytoplankton, and of the bird community feeding on the macrozoobenthos.
Challenges of de-eutrophication efforts

There is considerable debate about the question of whether single nutrient reductions are adequate or if de-eutrophication efforts should implement dual nutrient reductions of both N and P (Bryhn and Håkanson, 2009; Conley et al., 2009; Schelske, 2009; Schindler and Hecky, 2009). Our results demonstrate that a strong emphasis on P reduction in lakes and rivers on the European mainland has caused a shift to P limitation in the downstream coastal waters of the North Sea. This phenomenon is most likely not unique to the North Sea. Many studies have shown a steady increase of N:P ratios in coastal areas, for example in the Baltic and Mediterranean seas (Granéli et al., 1990; Krom et al., 2004; Nausch et al., 2004), the highly eutrophied Mississippi River plume in the northern Gulf of Mexico (Sylvan et al., 2006), the Chesapeake Bay estuary (Fisher et al., 1999) and the estuary of the Pearl River in Hong Kong (Xu et al., 2008).

One important lesson from our work is that large-scale de-eutrophication measures must be approached with a long-term view and, if implemented, the consequences of future shifts in nutrient limitation should be considered at the landscape level (cf. Granéli et al., 1990). Banning of phosphates from laundry detergents and increased P removal from domestic and industrial wastewater have led to a pronounced decrease of the riverine P inputs into the North Sea during the past 25 yr (Grizzetti et al., 2012). In contrast, despite more effective N removal from wastewater, the N load of European rivers has been only marginally reduced due to further intensification of agriculture and extensive use of N-rich fertilizers (Lenhart et al., 2010; Passy et al., 2013). This imbalance in the reduction of P when compared with N inputs has resulted in severely P-limited conditions in the coastal zone, particularly in spring.

The goals of the OSPAR convention included the reduction of eutrophication with hopes of ultimately eliminating extensive blooms by nuisance species. Our results show that responses to reduced nutrient inputs differ among phytoplankton groups, and vary in space and time. Although *Phaeocystis* blooms are still a common phenomenon, our field results and earlier model studies (Passy et al., 2013) show that the growth of *Phaeocystis* in the coastal zone is currently P controlled. Further reductions of the P loads are therefore likely to further reduce colonial *Phaeocystis* blooms. Our results also show that dinoflagellates and nanoflagellates are co-limited by
N and P in the coastal zone, and consist largely of potentially mixotrophic species that can access alternative P sources. Hence, further reduction of P loads without concomitant reduction of the N loads may be less effective in diminishing the risk of harmful algal blooms by potentially toxic nano- and dinoflagellates. Furthermore, and possibly more disconcerting, our results illustrate that P limitation can lead to a very high C:P stoichiometry of the phytoplankton, which may reduce their nutritional quality as food for zooplankton and benthic filter feeders. Therefore, P reduction in concert with N reduction, leading to a more balanced N:P ratio, is likely to be more beneficial to coastal zones than further P-dominated reduction efforts alone.

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Supporting Information

Figure S2.1 Seasonal variation in surface chlorophyll concentrations at the different stations in (a) 2011, (b) 2012, and (c) 2013. The surface chlorophyll concentrations are 8-day image composites derived from the Moderate Resolution Imaging Spectroradiometer (MODIS) sensor on NASA's Aqua satellite. The data were downloaded from the National Oceanic and Atmospheric Administration (NOAA) Coast Watch Program (http://coastwatch.pfeg.noaa.gov). Gray bars indicate the cruise periods.
Figure S2.2 (a-d) Temperature, and (e-h) salinity along the offshore transect, during research cruises in March 2013, April 2013, May 2012 and August 2011. Dots indicate depths from which samples were collected. In (a-d), salinity of 34 psu is indicated by the black isocline. Graphs were generated using Ocean Data View software (http://odv.awi.de).
Figure S2.3 Development of the phytoplankton concentration (expressed as chlorophyll fluorescence; ± SD) during the bioassay experiments. The experiments were performed at four stations (7km, 100km, 250km and 450km) along the offshore transect, during the (a-d) March 2013 cruise, (e-h) April 2013 cruise, (i) May 2012 cruise, and (k, l) August 2011 cruise. Treatments included the control (C) and additions of nitrate (N), phosphate (P), silicate (Si) and light (Table 2.1). The bioassay experiments were harvested for further analysis after 96-144 hours, depending on the cruise length.
Figure S2.4 Growth rates (± SD) of total phytoplankton in the nutrient bioassay experiments, based on changes in chlorophyll a concentration. The experiments were performed at four stations (7km, 100km, 250km and 450km) along the offshore transect, during the (a-d) March 2013 cruise, (e-h) April 2013 cruise, (i) May 2012 cruise, and (k, l) August 2011 cruise. Treatments included the control (C) and additions of nitrate (N), phosphate (P), silicate (Si) and light (Table 1). Within each panel, bars with different letters were significantly different, as tested by one-way analysis of variance followed by post hoc comparison of the means (Tukey’s HSD test).
Figure S2.5 Growth rates (± SD) of diatoms in the nutrient bioassay experiments, based on changes in their C-biomass. For further details, see the legend of Fig. S2.4.
Figure S2.6 Growth rates (± SD) of dinoflagellates in the nutrient bioassay experiments, based on changes in their C-biomass. For further details, see the legend of Fig. S2.4.
**Figure S2.7** Growth rates (± SD) of nanoflagellates in the nutrient bioassay experiments, based on changes in their C-biomass. For further details, see the legend of Fig. S2.4.
Figure S2.8 Growth rates (± SD) of Phaeocystis spp. in the nutrient bioassay experiments, based on changes in their C-biomass. For further details, see the legend of Fig. S2.4.
Figure S2.9 Growth rates (± SD) of picoeukaryotes in the nutrient bioassay experiments, based on changes in their C-biomass. For further details, see the legend of Fig. S2.4.
Figure S2.10 Growth rates (± SD) of picocyanobacteria in the nutrient bioassay experiments, based on changes in their C-biomass. For further details, see the legend of Fig. S2.4.
Nutrient limitation of the different phytoplankton groups

Below we summarize, for the total phytoplankton community and for each individual phytoplankton group, the nutrient limitation patterns deduced from the bioassay experiments. One-way analysis of variance with post-hoc comparison of the means (Tukey’s HSD test) was used to assess significant differences in growth rates among the treatments, using a significance level $\alpha = 0.05$. Nutrients (or light) were considered limiting if their addition significantly enhanced phytoplankton growth rate in comparison to the control. We distinguished between three different types of co-limitation (Harpole et al., 2011). ‘Independent co-limitation’ was defined as the significant stimulation of phytoplankton growth by two or more single nutrient treatments (e.g., +N only and +P only) as well as by the combined nutrient treatment (+N+P). ‘Simultaneous co-limitation’ was defined as the significant stimulation of phytoplankton growth by the combined nutrient treatment, but not by the single nutrient treatments. Finally, ‘serial colimitation’ was defined as the significant stimulation of growth by only one of the single nutrient treatments (e.g., +N only, but not +P only) and a significantly stronger stimulation by the combined nutrient treatment (+N+P). In case of independent or serial co-limitation, the nutrient that gave the strongest growth response is mentioned first.

Total phytoplankton community (based on phytoplankton biomass)

March 7km, 100km, 250km and 450km (Fig. 2.6a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. 2.6e): Growth rates of the +P, +Si, +N+P and +N+P+Si treatments were significantly higher than the control; the +P, +Si, +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: independent P+Si co-limitation.

April 100km (Fig. 2.6f): Growth rates of the +P and +N+P+Si treatments were significantly higher than the control; the growth rate of the +N+P+Si treatment was significantly higher than the +P treatment. Conclusion: serial P+Si co-limitation (first P, then Si).

April 250 km (Fig. 2.6g): Growth rates of the +N+P and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other, and +N and +P
alone did not stimulate significantly higher growth rates than the control. Conclusion: simultaneous N+P co-limitation.

April 450km (Fig. 2.6h): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N, +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: N limitation.

May 7km (Fig. 2.6i): Growth rates of the +P, +Si, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N+P+Si treatment was significantly higher than the +P and +Si treatments, but the +N+P was not significantly higher than the +P treatment. Conclusion: independent P+Si co-limitation.

August 250km (Fig. 2.6k): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments were significantly greater than the +N but did not differ significantly from each other. Conclusion: serial N+P co-limitation (first N, then P).

August 450km (Fig. 2.6l): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N, +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: N limitation.

Total phytoplankton community (based on chlorophyll a concentration)

March 7km, 100km, 250km and 450km (Fig. S2.4a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. S2.4e): Growth rates of the +P, +Si, +N+P and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: independent P+Si co-limitation.

April 100km (Fig. S2.4f): Growth rates of the +Si and +N+P+Si treatments were significantly higher than the control; the growth rate of the +N+P+Si treatment was significantly higher than the +Si treatment. Conclusion: serial N+P+Si co-limitation (first Si, then N or P).

April 250 km (Fig. S2.4g): Growth rates of the +N, +P, +N+P and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: independent N+P co-limitation.
April 450km (Fig. S2.4h): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N, +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: N limitation.

May 7km (Fig. S2.4i): Growth rates of the +P, +N+P, and +N+P+Si treatments were significantly higher than the control; the growth rate of the +N+P+Si treatment was significantly higher than the +P and +N+P treatments which did not differ significantly. Conclusion: serial P+Si co-limitation (first P, then Si).

August 250km (Fig. S2.4k): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N, +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: N limitation.

August 450km (Fig. S2.24l): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments were significantly higher than the +N but did not differ significantly from each other. Conclusion: serial N+P co-limitation (first N, then P).

Diatoms

March 7km, 100km, 250km and 450km (Fig. S2.5a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. S2.5e): Growth rates of the +Si and +N+P+Si treatments were significantly higher than the control; the +Si and +N+P+Si treatments did not differ significantly from each other. Conclusion: Si limitation.

April 100km (Fig. S2.5f): Growth rates of the +P, +Si, and +N+P+Si treatments were significantly higher than the control; the growth rate of the +N+P+Si treatment was significantly higher than the +P and +Si treatments. Conclusion: independent Si+P co-limitation.

April 250 km (Fig. S2.5g): Growth rates of the +N+P and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other, and +N and +P alone did not stimulate significantly higher growth rates than the control. Conclusion: simultaneous N+P co-limitation.
April 450km (Fig. S2.5h): Growth rates of the +N treatment were significantly higher than the control; the +N, +N+P and +N+P+Si treatments did not differ significantly. Conclusion: N limitation.

May 7km (Fig. S2.5i): Growth rates of the +P, +Si, +N+P, and +N+P+Si treatments were significantly higher than the control; the +P and +N+P treatments did not differ significantly from each other. Conclusion: independent Si+P co-limitation.

August 250km (Fig. S2.5k): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other. +N+P was significantly higher than +N. Conclusion: serial N+P co-limitation (first N, then P).

August 450km (Fig. S2.5l): None of the treatments gave a significantly higher growth rates than the control. Conclusion: n.s.

Dinoflagellates

March 7km, 100km, 250km and 450 km (Fig. S2.6a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. S2.6e): Growth rates of the +N+P treatment were significantly higher than the control; +N and +P alone did not significantly stimulate higher growth than the control. Conclusion: simultaneous N+P co-limitation.

April 100km, 250km, and 450km (Fig. S2.6f-h): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

May 7km (Fig. S2.6i): Growth rates of the +N+P and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other; +N and +P alone did not significantly stimulate higher growth than the control. Conclusion: simultaneous N+P co-limitation.

August 250km (Fig. S2.6k): Growth rates of the +N+P treatment were significantly higher than the control; the single +N and +P treatments did not significantly stimulate higher growth than the control. Conclusion: simultaneous N+P co-limitation.

August 450km (Fig. S2.6l): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N, +N+P and
+N+P+Si treatments not differ significantly from each other. Conclusion: N limitation.

*Nanoflagellates*

March 7km, 100km, 250km and 450km (Fig. S2.7a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. S2.7e): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 100km (Fig. S2.7f): Growth rates of the +N, +N+P, +N+P+Si and +light treatments were significantly higher than the control; the growth rate of the +N+P treatment was significantly higher than the +N treatment and did not differ significantly from the +N+P+Si treatment. Conclusion: serial N+P co-limitation (first N, then P), and light limitation.

April 250 km (Fig. S2.7g): Growth rates of the +N treatment were significantly higher than the control. Conclusion: N limitation.

April 450km (Fig. S2.7h): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

May 7km (Fig. S2.7i): Growth rates of the +N+P treatment were significantly higher than the control; the single +N and +P treatments did not significantly stimulate higher growth than the control. Conclusion: simultaneous N+P co-limitation.

August 250km (Fig. S2.7k): Growth rates of the +N+P, and +N+P+Si treatments were significantly higher than the control; the +N+P and +N+P+Si treatments did not differ significantly from each other. The single +N and +P treatments did not significantly stimulate higher growth than the control. Conclusion: simultaneous N+P co-limitation.

August 450km (Fig. S2.7l): Growth rates of the +N, +N+P, and +N+P+Si treatments were significantly higher than the control; the +N, +N+P and +N+P+Si treatments not differ significantly from each other. Conclusion: N limitation.

*Phaeocystis spp.*

March 7km, 100km, and 250km (Fig. S2.8a-c): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

March 450km (Fig. S2.8d): Growth rates of the +light treatment were significantly higher than the control. Conclusion: light limitation.
April 7km (Fig. S2.8e): Growth rates of the +P, +N+P, +N+P+Si and +light treatments were significantly higher than the control; growth rates of the +N+P and +N+P+Si treatments were not significantly higher than the +P treatment. Conclusion: independent P+light colimitation.

April 100km (Fig. S2.8f): Growth rates of the +P, +N+P, and +N+P+Si treatments were significantly higher than the control; the growth rates of the +P, +N+P and +N+P+Si did not differ significantly. Conclusion: P limitation.

April 250 km (Fig. S2.8g): Growth rates of the +N, +P, +N+P, and +N+P+Si treatments were significantly higher than the control; growth rates of the +N+P treatment were significantly higher than the +N and +P treatments; the +N+P and +N+P+Si treatments not differ significantly from each other. Conclusion: independent N+P co-limitation.

April 450km (Fig. S2.8h): Growth rates of the +N+P and +N+P+Si treatments were significantly higher than the control; the single +N and +P treatments did not significantly stimulate higher growth than the control and the +N+P and +N+P+Si treatments did not differ significantly. Conclusion: simultaneous N+P co-limitation.

May 7km (Fig. S2.8i): Growth rates of the +P, +N+P and +N+P+Si treatments were significantly higher than the control; the +P, +N+P and +N+P+Si treatments did not significantly differ. Conclusion: P limitation.

August 250km (Fig. S2.8k): Growth rates of the +N and +P treatments were significantly higher than the control. Conclusion: independent N+P co-limitation.

August 450km (Fig. S2.8l): Growth rates of the +N, +P, +N+P, +N+P+Si and light treatments were significantly higher than the control; the +N, +P, +N+P and +N+P+Si treatments not differ significantly from each other. Conclusion: independent N+P+light colimitation.

Picoeukaryotes

March 7km, 100km, 250km and 450km (Fig. S2.9a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. S2.9e): Growth rates of all treatments were significantly higher than the control; however, it is more likely an error of the control treatment than a real growth response of picoeukaryotes to all other treatments. Conclusion: n.s.
April 100km (Fig. S2.9f): Growth rates of the +P, +N+P, and +N+P+Si treatments were significantly higher than the control; the growth rates of the +P, +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: P limitation.

April 250 km (Fig. S2.9g): Growth rates of the +P treatment were significantly higher than the control. Conclusion: P limitation.

April 450km (Fig. S2.9h): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

May 7km (Fig. S2.9i): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

August 250km (Fig. S2.9k): Growth rates of the +N+P, +N+P+Si and +light treatments were significantly higher than the control; the single +N and +P treatments did not significantly stimulate higher growth than the control, and the +N+P and +N+P+Si treatments did not differ significantly from each other. Conclusion: simultaneous N+P co-limitation with independent light limitation.

August 450km (Fig. S2.9l): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

Picocyanobacteria
March 7km, 100km, 250km and 450km (Fig. S2.10a-d): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 7km (Fig. S2.10e): Growth rates of the +N+P+Si treatment were significantly higher than the control; growth rates of the +N+P+Si treatment did not differ significantly from the +P and +N+P treatments, even though the latter two treatments were not significantly greater than the control; growth rates of the +P treatment were significantly higher than the +N, the +Si and the +light treatments. Conclusion: Most likely P limitation rather than simultaneous N+P+Si co-limitation.

April 100km (Fig. S2.10f): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

April 250 km (Fig. S2.10g): Growth rates of the +N+P treatment were significantly higher than the control; the single +N and +P treatments did not significantly stimulate higher growth than the control. Conclusion: simultaneous N+P co-limitation.
April 450km (Fig. S2.10h): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

May 7km (Fig. S2.10i): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.

August 250km and 450km (Fig. S2.10k,l): None of the treatments gave significantly higher growth rates than the control. Conclusion: n.s.