Resource limitation and the biochemical composition of marine phytoplankton
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Chapter 4

Nutrient limitation driven dynamics of amino acids and fatty acids in coastal phytoplankton

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J.G. and H.T.S.B designed research; C.P.D.B. coordinated sampling campaigns; J.G. performed research; J.G. and H.T.S.B. analyzed data; J.G., C.P.D.B and H.T.S.B. wrote the paper.
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Abstract

Coastal seas like the North Sea have been subject to major changes in nutrient inputs over the last decades, resulting in shifts of limiting nutrients for phytoplankton communities. Here we investigated the seasonal and spatial distribution and synthesis patterns of individual amino acids and distinct fatty acid groups and how these were affected by different nutrient limitations in natural coastal phytoplankton communities. N-limited communities, dominating in late spring and summer, had substantially slower synthesis of essential amino acids compared to synthesis of non-essential amino acids. In short-term nutrient addition experiments this trend was reversed immediately after N addition to levels found under non-limiting conditions. P-limitation was mostly found near the coast in diatom dominating phytoplankton communities and did not show this shift. Both N and P-limitation caused shifts from structural to storage fatty acids with a decrease in the synthesis of poly-unsaturated fatty acids. Reversed effects in fatty acid synthesis after N or P addition were delayed and only apparent after 72 h, when they had also translated into fatty acid biomass. The different strategies of qualitative and quantitative regulation of amino acid and fatty acid synthesis under nutrient scarcity may have far-reaching consequences for the phytoplankton’s nutritional value and food web structure. In addition to a lower overall availability of amino acids and fatty acids per unit phytoplankton biomass higher trophic levels also have to cope with the loss of essential amino acids and fatty acids in nutrient limited phytoplankton.
**Introduction**

Phytoplankton is the main contributor to global primary production (Field et al. 1998) and forms the basis of most marine food webs. Anthropogenic activities resulted in extensive increases in nutrient inputs to coastal seas and consequent eutrophication had a major impact on phytoplankton community composition (Riegman et al. 1992; Philippart et al. 2000; Cadée & Hegeman 2002; Lancelot et al. 2007). In recent decades countries neighboring these coastal seas have raised much effort to drastically decrease riverine nutrient loads, however measures were more efficiently decreasing P than N and caused an increase in dissolved inorganic nitrogen to dissolved inorganic phosphorus (DIN:DIP) ratios (Justic et al. 1995; Turner et al. 2003).

Changes in the DIN:DIP ratio directly affect the C:N:P ratio of phytoplankton (Vrede et al. 2004; Diez et al. 2013), which is used as a tool to evaluate its nutritional value of phytoplankton for higher trophic levels (Sterner & Elser 2002). The increase in biosynthesis of carbon rich storage products such as glucose and storage lipids increase C:N and C:P ratios and consequently lower their quality as food (Sterner et al. 1993; Plath & Boersma 2001). Variations in the C:N:P ratio reflect variations in biomolecule composition, because C:N:P requirements of major cellular biomolecules differ and shifting DIN:DIP ratios can influence the cellular composition of phytoplankton (Klausmeier et al. 2004a). Carbohydrates (CH) do not contain N and P and their synthesis is mainly regulated by the availability of light. But the synthesis of all other major biomolecules relies on the availability of nutrients; for instance, amino acid (AA) synthesis requires N and RNA/DNA synthesis requires both N and P. And while fatty acids (FA) like carbohydrates do not contain N or P and are used for storage as triglycerides, they are also found in complex membrane lipids that often require P and/or N (Van Mooy et al. 2009).

Although studies focusing on shifts in biomolecule (sub-) class distribution in phytoplankton under different nutrient availabilities exist (Lynn et al. 2000; Mock & Kroon 2002b; Mock & Kroon 2002a; Pernet et al. 2003), they lack detailed information about individual compound dynamics. Recently developed methods in stable isotope analysis make it now possible to measure both concentrations and biosynthesis rates of individual compounds within the CH, AA and FA pools (Boschker 2004; McCullagh et al. 2006; Boschker et al. 2008; Grosse et al. 2009).
Thereby the contribution of detrital matter is excluded altogether and responses to nutrient availability in natural phytoplankton can be studied on short timescales.

Grosse et al. (in review/Chapter 3) directly measured seasonal and spatial dynamics of combined AA, FA and CH concentrations and their biosynthesis rates in North Sea phytoplankton. They demonstrated that nutrient limitation and season had large effects on composition and biosynthesis rates of these major classes of biochemicals. For instance, the contribution of protein derived amino acids varied up to 4-fold in concentration and up to 8-fold in biosynthesis rates. Under poor nutrient conditions, storage products such as carbohydrates and storage lipids are formed but nutrient addition assays revealed that AA biosynthesis increases immediately (within 24 h) after nutrient limitation is relieved, while storage compound synthesis decreased concurrently. As we detected major shifts in component classes (Chapter 3) it may also be interesting to look for compound specific effects in our field data, especially for AA and FA pools, which contain components that are essential for higher trophic levels.

Effects of nutrient supply, light and temperature on FA dynamics in phytoplankton have been studied intensely covering a wide range of phytoplankton groups (Renaud et al. 2002; Pernet et al. 2003; Xin et al. 2010; Piepho et al. 2012). In general, the limitation by nutrients causes an increase in triglycerides (storage FA) and a concurrent decrease in membrane lipids (structural FA) especially under P-limitation (Fidalgo et al. 1998; Lynn et al. 2000). Additionally, the pattern of individual FA is also affected by nutrient shortage, causing a shift from poly-unsaturated FA (PUFA) towards saturated FA (SFA) (Siron et al. 1989; Reitan et al. 1994; Breteler et al. 2005). Several long chain PUFA, such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), are essential FA for higher trophic levels, play an important role in zooplankton growth and reproduction (Müller-Navarra 1995; Burns et al. 2011), and may become limiting compounds for consumers when phytoplankton becomes nutrient limited (Gulati & DeMott 1997).

Not much is known about dynamics of individual AA in phytoplankton, but it is assumed that the composition is more or less constant. This assumption is mainly based on the geochemical composition of particulate organic carbon in the water column or of detrital matter in surface sediments (Dauwe & Middelburg 2015/Chapter 2).
However, nutrient limitation can induce shifts in organelle composition (Arrigo 2005), and the up- or down-regulation of specific biomolecule pathways and their enzymes (Morey et al. 2011; Yang et al. 2011), could consequently lead to the synthesis of different sets of proteins and possibly a shift AA composition. In turn, this could have consequences for the nutritional value of phytoplankton, because several AA are considered to be essential for copepods and higher trophic levels. These essential AA cannot be synthesized de-novo by animals and have to be obtained through their diet and include amongst others phenylalanine (Phe), leucine (Leu), valine (Val), and arginine (Arg, [see Fig. 4.1 for full list]; Claybrook 1983). There are also differences in the biosynthesis patterns of essential and non-essential AA. The former are in general synthesized from non-essential pre-cursor AA and their synthesis requires additional steps and enzyme reactions (Fig. 4.1).

In this study we measured concentrations and biosynthesis rates of individual AA and several FA groups (based on their degree of saturation and nutritional value to higher trophic levels) and we show substantial seasonal and spatial variation in phytoplankton of the North Sea in response to nutrient limitation. Phytoplankton community composition and prevailing nutrient limitations are based on findings reported by Grosse et al. (in review/Chapter 3 [Tab. 4.1]). Data from nutrient addition experiments was used here to investigate nutrient specific changes within the AA and FA in order to determine strategies used by different phytoplankton communities to cope with changes in nutrient availability on the biomolecular level. We indeed detected major changes in the AA composition in response to N-limitation, while FA composition changed in response to both N and P-limitation. The different strategies used by phytoplankton to cope with nutrient stress and the possible consequences for their nutritional value for the food web will be discussed.
Figure 4.1: Schematic diagram of AA synthesis in phytoplankton. Amino acids essential for higher trophic levels were underlined. 3-PGA = 3 phosphoglyceric acid, Ser = serine, Gly = glycine, Cys = cysteine, PEP = phosphoenolpyruvate, Try = tryptophane, Tyr = tyrosine, Phe = phenylalanine, Ala = alanine, Leu = leucine, Val = valine, TCA = tricarboxylic acid cycle, Aspx = combined pools of aspartate/asparagine, Met = methinone, Thr = threonine, Ile = isoleucine, Lys = lysine, Glux = combined pools of glutamate/glutamine, His = histidine, Pro = proline, Arg = arginine. Met, Cys and Try were not detected in this study.
Table 4.1: Overview of community structure, nutrient limitations (identified by increase in total AA synthesis rates in nutrient addition treatments, Chapter 3) and nutrient concentrations at each station. Station # can be used to identify stations in PCA plots. Phytoplankton community identification is based on PCA results of structural FA (Fig. S3). Prevailing nutrient limitation was identified in Chapter 3 (Grosse et al. in review) based on response in AA biosynthesis after nutrient addition. DIN = nitrate + nitrite + ammonia. <DL = below detection limit, for Si(OH)₄ limit is 0.03 μmol L⁻¹.

<table>
<thead>
<tr>
<th>Month (Stn. #)</th>
<th>Phytoplankton community</th>
<th>Nutrient limitation</th>
<th>DIN [μmol L⁻¹]</th>
<th>PO₄³⁻ [μmol L⁻¹]</th>
<th>Si(OH)₄ [μmol L⁻¹]</th>
<th>DIN:P</th>
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<td>38.5</td>
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<td>16.6</td>
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<td>April (2)</td>
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<td>End May (4)</td>
<td>Diatom</td>
<td>P/Si</td>
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<td>0.09</td>
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<tr>
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<td>0.02</td>
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<td>9.90</td>
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<td>5</td>
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</table>
CHAPTER 4

Material and Methods

Field sampling

A total of five cruises over three consecutive years was conducted onboard the Dutch research vessel RV Pelagia, sampling a transect from the Dutch coast towards the center of the North Sea. Four stations (Fig. S4.1) were revisited before the spring bloom (15 – 25 March 2013), during the spring bloom (24 April - 4 May 2013), at the end of the spring bloom or just afterwards (8 – 12 May 2012 and 25 – 31 May 2011, respectively) and during the summer (15 – 30 August 2011). The seasonal study followed the succession of nutrient and phytoplankton dynamics, while the spatial component allowed investigation of different nutrient settings. The coastal zone station (CZ) is very shallow (8m) and therefore completely mixed and its close proximity to shore (7 km) makes it the station most influenced by high nutrient riverine run-off. The Oyster Ground (OG) can be affected by either mixed coastal or stratified waters, depending on the exact location of the Frisian Front, which is situated in the broad zone around 54°N (Peeters & Peperzak 1990). The Dogger Bank (DB) shows low nutrient availability throughout the year and due to its shallowness does not display stratification. The central North Sea (CNS) shows the highest influence of Atlantic Ocean water, which is low in nutrients and thermally induced stratification occurs during summer.

An overview of time and location, indicated by station number (#), dominating phytoplankton communities and prevailing nutrient limitations as they were determined in Chapter 3 (Grosse et al. in review) is given in Tab. 1. In short, structural FA composition (Fig. S4.2) revealed a distinction between diatom dominated and flagellate dominated communities, the latter including dinoflagellates, *Phaeocystis* spp. and nanoflagellates. Several stations could not clearly be identified and were referred to as “Mixed”. These communities contained either additional green algae biomarkers (OG and DB in March [Stns. # 6, 11]) or the identification was uncertain because the community was transitioning from diatom dominated to flagellate dominated (OG/Mid May, DB/April, DB/ Mid May, CNS/Mid May [Stns. #8, 12, 13, 17]). The shift from diatom dominated communities into flagellate community developed earlier in the year with increasing distance to the coast.
Water column distribution of salinity, temperature and photosynthetically available radiation were obtained using a Sea-Bird SBE911+ CTD sampler (Sea-Bird Electronics Inc., USA). Water for measurements and experiments was collected using a sampling rosette with 24 Niskin bottles (12 L).

**Nutrient addition experiments**

The experimental scheme was designed to determine both in-situ concentrations and biosynthesis rates of biomolecules in North Sea phytoplankton and how they respond to short-term changes in nutrient availability within 24 to 72 h. Sub-surface water (7 m) was collected shortly before sunrise and directly transferred into 10 L carboys and a control without added nutrients as well as the following three nutrient treatments were set up in duplicate: 

1. Control incubations without added nutrients
2. Addition of 80 µmol L\(^{-1}\) NaN\(_3\) (+N treatment)
3. Addition of 5 µmol L\(^{-1}\) K\(_2\)HPO\(_4\) (+P treatment)
4. Addition of 80 µmol L\(^{-1}\) NaNO\(_3\), 5 µmol L\(^{-1}\) K\(_2\)HPO\(_4\) and 80 µmol L\(^{-1}\) Si(OH)\(_4\) (+NPSi treatment)

All carboys were enriched with \(^{13}\)C-sodium bicarbonate (99% \(^{13}\)C) to a concentration of approximately 200 µmol L\(^{-1}\). Absolute \(^{13}\)C-DIC enrichment was measured in the laboratory (Grosse et al. 2015) and reached final labeling concentrations of 1.5 to 2% of ambient dissolved inorganic carbon (DIC) concentration. Initial, unlabeled samples for POC and biomolecules were also taken.

Carboys were placed in flow-through incubators on deck and continuously flushed with seawater to assure in-situ temperatures. The white carboys and additional shading on top of the incubators decreased light levels to similar levels as encountered at the sampling depth, without inducing changes in the available light spectrum. The incubation experiments lasted 24 h and were terminated by splitting each carboy’s content into 4 equal volumes and filtering these over pre-combusted GF/F filters (Whatman, 4 h at 450°C). Aliquot samples (0.3 and 2.0 L, depending on the phytoplankton density) were taken for particulate organic carbon (POC), neutral CH, AA and FA analysis. All filters were stored frozen at \(-80^\circ\)C until analysis (POC) or extraction of the biomolecules.

Nutrient additions lasting 72 h were also carried out at the CZ and DB during selected cruises to investigate whether short-term changes in biomolecule synthesis differ from long-term changes. Therefore, a second set of nutrient treatments was set-up (control, +N, +P, +NPSi, in duplicates) concurrently with
the above mentioned incubations, the $^{13}$C-sodium bicarbonate tracer was added after 48 h and the incubations were terminated after 72 h as described above.

**Analytical procedures**

POC concentrations and $^{13}$C labeling was measured by EA-IRMS. Frozen POC filters were lyophilized overnight, and a section of the filter (1/4 or less, depending on the amount of material on the filter) was acidified over fuming HCl to remove inorganic carbon, and subsequently packed into tin cups before analysis.

Detailed descriptions of extraction protocols for AA and FA, the LC- and GC/C-IRMS systems as well as compound separation protocols and conditions have been published by Grosse et al. (2015) and references therein. In short, AA samples were acid hydrolyzed and after an ion-exchange clean-up step analyzed by LC-IRMS using a Primsep A column, which separated a total of 17 individual AA (McCullagh et al. 2006). Due to the analytical procedures glutamate and glutamine (Glux) co-elute and form one peak. The same happened for aspartate and asparagine (Aspx). All AA with the exception of tryptophan can be measured with the method used, but cysteine and methionine were not included in our analyses because of their very low concentrations.

FA samples were extracted following the protocol of Bligh & Dyer (1959) and subsequently separated into storage lipids, glycolipids and phospholipids by silicate column chromatography. All three fractions were dried and glycolipid- and phospholipids were combined and are further referred to as structural lipids. After derivatization to fatty acid methyl esters (FAME), FAME from both storage and structural lipids were separated by GC/C-IRMS using the polar BPX-70 column. Fatty acids were notated A:BωC, where A is the number of carbon molecules in the fatty acid, B the number of double bonds and C the position of the first double bond relative to the aliphatic end. An additional ‘c’ indicates the cis-orientation of the double bond (e.g. 18:2₀₆c). Individual FA in both the structural and storage pools were further combined into saturated FA (SFA), mono-unsaturated FA (MUFA) and multiple poly-unsaturated FA (PUFA) fractions (see Results).
Calculation of $^{13}$C uptake rates

Carbon stable isotope ratios are expressed in the $\delta^{13}$C notation:

$$\delta^{13}C (\text{‰}) = \left( \frac{R_{\text{sample}}}{R_{\text{VPDB}}} - 1 \right) \times 1000,$$

where $R_{\text{sample}}$ and $R_{\text{VPDB}}$ denote the $^{13}$C/$^{12}$C ratio in the sample and the international standard, Vienna Pee Dee Belemnite ($R_{\text{VPDB}} = 0.0111802$), respectively. Incorporation of $^{13}$C into bulk carbon as well as individual compounds is reflected as excess (above background) $^{13}$C and is expressed as specific uptake after correction for enrichment of the DIC-pool (difference $\delta^{13}$C$_{\text{DICsample}}$ at end of incubation and $\delta^{13}$C$_{\text{DICbackground}}$ before addition of $^{13}$C-DIC), POC concentrations and incubation time in (nmol C (µmol POC)$^{-1}$ d$^{-1}$). More detailed information can be found in Grosse et al. (2015). Concentrations and biosynthesis rates were calculated for each individual compound per duplicate and added together to obtain values for each biomolecule groups (essential/non-essential AA, storage and structural SFA/MUFA/PUFA). Afterwards averages and standard deviations were calculated ($n=2$).

Statistical analysis

Relative AA and FA concentrations and biosynthesis rates were used to perform principle component analysis (PCA) using the package CRAN:factoMineR package in the open source software R to determine variations between different nutrient limitations and communities. PCA analyses was also used to visualize short-term effects (24 h) of nutrient addition on AA and FA biosynthesis.
Results

Nutrient concentrations showed a decrease from March towards August as well as from the coastal zone towards the central North Sea (Tab. 4.1). The phytoplankton community was dominated by diatoms at the CZ that were non-limited before the spring bloom in March, P/Si-(co)limited during the spring bloom and shifted towards N-limitation in summer. The other three stations showed mixed communities in early spring before they transitioned into N-limited flagellate dominated communities in summer. This succession occurred earlier in the year the farther away from shore (Tab. 4.1). Details were discussed in Burson et al. (2016) and Grosse et al. (in review/Chapter 3).

Individual amino acids

Relative concentration and biosynthesis of individual AA in the unamended control incubations varied substantially over seasonal and spatial scales (Fig. 4.2). Variations were larger in biosynthesis than in concentration, especially in the non-essential AA Aspx, Glux, Ala and Gly, while essential AA Val, His and Arg showed much smaller ranges in concentration and biosynthesis. Non-essential AA showed higher relative biosynthesis compared to their concentrations, while essential AA showed the opposite trend, leading to a relative over-synthesis in non-essential AA and a relative under-synthesis in essential AA in short-term (24 h) incubations.

Figure 4.2: Contribution of individual amino acids to total amino acids in C concentration (grey bars) and biosynthesis in the un-amended incubations (Control, white bars) from all cruises and stations separated into non-essential and essential (underlined) AA.
To study general trends, PCAs were performed with both biosynthesis and concentration data of individual AA (Fig. 4.3A, B). This revealed a separation of N-limited stations from all others. The PCA of AA synthesis (Fig. 4.3A) showed that the first two components explained 53% (38% + 15%) of the variance within the samples and separated two clusters of stations. Cluster I contained N-limited flagellate communities, which associated with all non-essential AA (except Pro), while Cluster II contained all other stations and associated with all essential AA and Pro. Stations #2 and 7 were an exception; they clustered with N-limited flagellates although they were identified as P and Si (co)-limited diatom dominated stations. But at both stations diatoms and flagellates contributed to the total community. However, diatoms were primarily Si limited, while flagellates were co-limited by N and P (Burson et al. 2016), which perhaps explained the contradictory grouping of those two stations.

PCA analysis of AA concentrations showed a similar clustering of stations with the first two components explaining 53% (32% + 21%) of the variance in the dataset. Again, a separation of N-limited communities from all others was observed along the first axis, with two exceptions. N-limited communities were associated especially with Aspx, Glux and the essential AA Thr, while all other communities associate with essential AA as well as non-essential Ala and Ser. Station #4 clustered with N-limited communities, even though it was P-limited, while station #16 behaved in the opposite way, clustering with non-limited communities even though it seemed to be N-limited and nutrient requirements of different phytoplankton groups within the community may have played a role as well. The separation along the second axis was mainly associated with Pro, Lys and Tyr. While nutrient availability seemed to affect AA distribution, intrinsic differences between phytoplankton groups and the contribution of detrital matter could also have an effect on the AA concentrations.
Figure 4.3: PCA biplot of relative contribution of individual AA to total AA concentration (A) and biosynthesis (B). Symbol shape refers to the dominating phytoplankton community and symbol color indicates limiting nutrients. Grey areas highlight clusters of similar stations. Panel C and D give examples for range of AA contributions for the stations CZ/End May (station #4, P-limited, diatom dominated) and CNS/August (station #18, N-limited, flagellate dominated). Shown are averages ± SD, n = 2. Essential AA were underlined.

In order to demonstrate the variance between stations we also plotted individual AA concentration and biosynthesis of stations #4 and 18, representing a P-limited diatom community and a N-limited flagellate community, respectively (Fig. 4.3C, D), showing the discussed trends of the PCA analysis.

PCA was also performed on AA biosynthesis in nutrient addition treatments to reveal nutrient related short-term shifts (Fig. 4.4). Due to the separation of N-limited from P and non-limited communities in Fig.4.3 and the limited response in total AA biosynthesis in P- and Si-limited communities within the first 24 h after nutrient addition (Chapter 3), separate PCAs were run for (i) N-limited
communities and (ii) all others. The PCA of N-limited communities explained 59% of the variance in the data (similar to other PCAs) and a separation between treatments occurred. Again, separation along the first axis was mainly based on non-essential Aspx, Glux, and Ala, and to a lesser extent on Gly, versus all essential AA and Pro. The control and +P treatments associated with non-essential AA as these were mainly N-limited and when N-limitation was relieved in the +N and +NPSi treatments there was a clear shift towards synthesis of all essential AA and Pro. The +P treatments from four stations behaved differently and clustered with +N and +NPSi treatments. These were combined into the “Group X” and comprised of the +P treatments from the OG/End May, DB in April and End May, and CNS/Mid May (Stns. #9, 12, 14, 17). Although these stations were primarily N-limited they showed co-limitation by P or Si and the addition of P apparently also triggered a shift towards essential AA at these stations. In the PCA of non- and P-limited stations the two axes explained only 51% of the variance in the data and no differences between nutrient treatments were observed.

Figure 4.4: PCA biplot show biosynthesis of individual AA in nutrient addition treatments after 24 h. Phytoplankton was separated into N-limited flagellate and mixed communities (A) and not/P-limited communities plus the N-limited diatom community at Stn. #5 (B). Ellipses encircle distribution of the same treatment. Essential AA were underlined.
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Five longer-term incubations (72 h) were carried out and actual data are plotted in Fig. S4.3 as a PCA was not possible with this low number of samples. Effects of nutrient additions were mainly seen in the biosynthesis data and followed similar trends as in the 24 h incubations (Fig. S4.3). For the P and Si-limited CZ (Stn. #2) most non-essential AA decreased synthesis in the +P and +NPSi treatments, while synthesis of essential AA increased (Fig. S4.2C). At the N-limited DB (Stn. #12, 13), the shift in biosynthesis towards essential AA in the +N and +NPSi treatments persisted after 72 h and patterns were almost identical to those found after 24 h (Fig. S4.2G, I). Shifts in AA concentrations were less pronounced than in biosynthesis data and followed no distinct pattern.

**Fatty acid groups**

Composition of individual FA differed greatly between phytoplankton groups, especially within the structural FA fraction. For example, diatoms contain high amounts of 16:1ω7 and 20:5ω3 (Dalsgaard et al. 2003), while flagellates (including dinoflagellates, nano flagellates and the Prymnesiophyceae *Phaeocystis*) produce high amounts of 18:5ω3, 18:5ω5 and 22:6ω3 (Dijkman & Kromkamp 2006). Community composition and hence composition of individual structural FA varied substantially (Fig. S4.3) and in order to obtain a clearer picture we combined individual FA into specific groups of FA, both within the storage and the structural fraction. Saturated FA (SFA) and mono-saturated FA (MUFA) were combined, while poly-unsaturated FA (PUFA) were separated into PUFA containing 16 C-atoms (C16-PUFA) and PUFA containing 18 C-atoms (C18-PUFA). The PUFA 20:5ω3 and 22:6ω3 were evaluated individually, since they are considered essential dietary compounds for zooplankton and positively affect the growth rate of these organisms (Müller-Navarra 1995; Von Elert 2002).

Contribution of FA groups to total FA varied over seasonal and spatial scales (Fig. 4.5). Variations were larger in biosynthesis than in concentration of storage SFA and MUFA. These two groups also showed higher relative biosynthesis compared to their relative concentrations (over-synthesis), while structural FA groups showed the opposite trend (under-synthesis).
Figure 4.5: Contribution of fatty acid groups to total fatty acids in C concentration (grey bars) and the un-amended incubations (Control, white bars) separated by storage and structural (underlined) FA fractions. SFA = saturated FA, MUFA = mono-unsaturated FA, C16-PUFA/ C18-PUFA = poly-unsaturated FA containing 16 and 18 C atoms, respectively.

PCA was performed with FA group’s biosynthesis and concentration (Fig. 4.6) and both PCAs revealed a separation between flagellate and diatom dominated communities (Cluster I and II) with mixed communities in between. The first two axes explained 55% and 62% of the variance on the dataset, respectively. Flagellate communities were associated with structural and storage C18-PUFA as well as structural 22:6ω3, while diatoms were associated with storage MUFA, storage and structural C16-PUFA and storage 20:5ω3. No effect of nutrient limitation was found. However, the Si-limited diatoms at station #7 separated from other stations in the FA biosynthesis and showed higher concentrations of structural MUFA. Lower temperatures in March may have also been a prominent factor that influences FA composition.
Figure 4.6: PCA biplot of relative contribution of FA groups to total FA biosynthesis (A) and C concentration (B). Symbol shape refers to the dominating group of the phytoplankton community and symbol color indicates prevailing nutrient-limitation. Gray areas highlight similar stations. FA groups are separated by structural and storage FA fractions. SFA = saturated FA, MUFA = mono-unsaturated FA, C16-PUFA/ C18-PUFA = poly-unsaturated FA containing 16 and 18 C atoms, respectively. Structural FA groups are underlined.

PCA was also performed on FA biosynthesis in nutrient addition treatments but did not reveal nutrient related short-term (24 h) shifts, suggesting shifts in FA biosynthesis occurred slower than shifts in AA. Therefore, we performed a PCA using the relative differences in FA biosynthesis between control and nutrient addition treatments. These showed effects of nutrient addition in both P and N-limited station (Fig. 4.7). At the majority of P-limited stations the +P and +NPSi treatments showed a relative increase in all structural FA groups, while +N treatments (with increased N:P ratios) increased synthesis of storage SFA, C16 and C18 storage PUFA. N-limited stations showed the same response to the relief of nutrient limitation. The +N and +NPSi treatments were associated with an increase in structural FA, while decreased N:P ratios in the +P treatment caused synthesis of storage FA to intensify. The only N-limited diatom community (Stn. #5) showed a shift towards C16 storage PUFA and storage 20:5ω3.
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Figure 4.7: PCA biplot show shifts in biosynthesis of FA groups in nutrient addition treatments relative to the Control treatment after 24 h. Phytoplankton was separated into N-limited (A) and not and P-limited communities (B). Gray areas highlight cluster of similar treatments. Structural FA groups are underlined.

Longer-term incubations (72 h) showed shifts in the relative distribution of FA groups both in concentration and biosynthesis (Fig. 4.8). The relief of N and P limitation caused a relative increase in synthesis of structural MUFA and PUFA with a concurrent decrease in all storage FA groups. This shift in biosynthesis was also translated into the relative distribution of FA concentration in a similar fashion. Apparently, these quantitative and qualitative changes in FA concentrations need several days to become detectable.

Discussion

The coastal ocean is experiencing major perturbations in nutrient cycling, which may have important effects on the cellular composition of phytoplankton and its food quality for the coastal food web. The aim of this study was to investigate the effect of changing nutrient availability on the biosynthesis and composition of individual AA and groups of FA in the phytoplankton of the North Sea. AA biosynthesis was mainly affected by nutrient availability and showed nutrient dependent behavior. N-limitation caused a decrease in the biosynthesis of essential AA from non-essential pre-cursor AA, while P and/or Si co-limitation did not cause this apparent shift in individual AA biosynthesis. The re-supply of N
led to an immediate increase in the conversion of non-essential to essential AA. The synthesis of FA groups was primarily determined by the community composition and probably by temperature. Minor nutrient effects were detected for FA within 24 h when the limiting nutrient was re-supplied and became clearly visible after 72 h. Overall, nutrient re-supply caused a shift from storage FA to structural membrane FA and an increase in essential PUFA that was independent of the prevailing nutrient limitation.

We chose an incubation period of 24 h to investigate one complete daily cycle in biomolecule synthesis, because the synthesis of major biomolecule groups can be separated between light and dark period (Behrenfeld et al. 2008). Within this time frame many compounds showed an over- or under-synthesis compared to their relative contribution in the POC concentration, a reflection of the length of synthesis pathways of individual compounds. $^{13}$C tracer was first accumulated in pre-cursor compounds and compounds with short synthesis pathways (e.g. Aspx or storage SFA) and showed a lower distribution in compounds with longer synthesis pathways (e.g. Leu or structural PUFA). However, the approach used is sensitive and allows for easy and fast quantification and identification of biomolecule synthesis (Dijkman et al. 2009; Ly et al. 2014).

While shifts in biosynthesis translated into FA concentrations after 72 h no such trend was seen in AA. The main reason may be that AA pools are much larger than FA pools, hence changes take longer to translate into AA concentrations. Also, we encountered only one N-limited station, with a mixed community where we would have expected to see a shift in AA concentrations after 72 h (Stn. #13) based on the prevailing N-limitation and the increase in total AA biosynthesis (>20%) and AA in biomass (13% and 17% for +N and +NPSi, respectively) between 24 h and 72h. However, only small shifts in AA concentrations were detected, for example in Glux and Ser (Fig. S4.3J). With average phytoplankton biomass turnover times of ~7 days (Chapter 3) even the 72 h incubation period may have been too short to detect significant changes and perhaps future studies can incorporate even longer incubation periods to investigate this issue further. A second reason can be that FA are part of either storage and structural pools and that depending on present nutrient regimes FA can flow between both pools without requiring de-novo synthesis.
Figure 4.8: Distribution of FA groups in C concentration (left panel) and biosynthesis (right panel) after 72 h at the Coastal Zone (A-F) and the Dogger Bank (H-J). Prevailing nutrient limitation is indicated in right upper corners of concentration graphs. Structural FA groups are underlined. SFA = saturated FA, MUFA = mono-unsaturated FA, C16-PUFA/ C18-PUFA = poly-unsaturated FA containing 16 and 18 C atoms, respectively.
Response of phytoplankton to nutrient limitation

Re-supplying the limiting nutrient led to an increase in total AA synthesis (Grosse et al. in review, Chapter 3) but only N-limited phytoplankton showed shifts in the distribution of individual AA after 24 h. Results from both unamended and nutrient addition incubations showed that under N-limitation the pools of non-essential AA increased, especially Aspx, Glux and Ala, while synthesis of essential AA and Pro decreased. The strong nutrient dependent separation of Glux and Aspx from other AA in PCAs of concentration, biosynthesis and nutrient additions highlights their function as pre-cursor in more complex pathways for essential AA (Fig. 4.1). The non-essential AA Pro stands opposite to Glux, for example in Fig. 4.3A, even though it only requires a one step reaction to synthesize Pro directly from Glux. However, Pro serves a special function in osmoregulation and in order to decrease N-requirements several phytoplankton groups are capable of substituting Pro with other osmolytes under N-limiting conditions (Bromke et al. 2013; Xiao et al. 2013) causing it to be primarily synthesized under N-replete conditions and cluster with essential AA. The conversion of non-essential to essential AA relies on numerous additional enzymes, proteins themselves, and it may be beneficial to decrease the production of these enzymes under N-limitation. Interestingly, several N/P co-limited stations also showed the shifts towards essential AA after P-addition (Group X, Fig. 4.4A) similar to solely N-limited stations, while no such shift in individual AA contributions could be identified at P (Si co-) limited stations after the addition of P, not even after 72 h. At this point, total AA biosynthesis rates were significantly increased (Grosse et al. in review/Chapter 3), which implies that P-limitation caused a uniform decrease in the synthesis of individual AA. The up-regulation of AA biosynthesis after P-addition took much longer (several days) to be detected, compared to N-limited stations (Chapter 3). It was suggested that rRNA and ribosome synthesis had to precede the up-regulation of AA (protein) synthesis, as they are main P-containing compounds in phytoplankton and their content is reduced under P-scarcity (Elser et al. 2000; Van Mooy & Devol 2008; Hessen et al. 2010). This supports the hypothesis of a uniform decrease since translation would be regulated on the protein synthesis level not on the AA supply level. Moreover, regulation at the gene-level is nutrient dependent as well. The lack and the re-supply of N and P strongly affect gene expression patterns in a similar timely manner (Morey, et al. 2011, Yang, et al. 2011). Genes related to ribosomes, carbohydrate metabolism, FA metabolism as well as carbon fixation are both
down- and up-regulated at different magnitudes indicating changes in metabolic pathways and therefore highly affect protein composition hence AA composition within cells (Morey et al. 2011; Yang et al. 2011; Bender et al. 2014). Silicon starvation and replenishment in diatoms have similar effects on a large number of genes that encode for many yet unknown proteins (Mock et al. 2008; Shrestha et al. 2012) and even closely related species show large dissimilarities in gene-expression patterns (Bender et al. 2014). Thus, both nutrient and species specific mechanisms may shape the AA landscape.

Low nutrient availability affects FA synthesis in two ways. A shift from polar lipids (including phospholipids and glycolipids) towards neutral lipids under both P and N-limitation has been described by several authors (Weers et al. 1997; Lynn et al. 2000; Mock & Kroon 2002b) and similar changes occurred during our investigation as reported in Chapter 3 (Grosse et al. in review), showing a decrease in storage FA biosynthesis after addition of the limiting nutrient, especially in flagellate dominated communities. The profiles of individual FA can also be affected by the shortage of nutrients caused by changes in synthesis pathways, when desaturases and elongases cannot be synthesized in required amounts anymore, which leads to a shift from PUFA to MUFA (Flynn et al. 1992). And although all structural FA groups show increased biosynthesis after 72 h, PUFA groups increased synthesis up to six-fold, while increase of SFA and MUFA were about twofold or less (Fig. 4.8). Shorter-term changes after nutrient addition were hardly seen, but may have not been detectable if already synthesized storage FA were used to increase amounts of structural FA, since they were not labeled by $^{13}$C tracers.

P containing membrane lipids can be substituted for N or sulfur containing lipids thereby saving enough P to keep growth rates constant for several more cell divisions without affecting FA composition (Van Mooy et al. 2009; Martin et al. 2011). However, under the high DIN:P ratios encountered (up to 333) in coastal waters this mechanism may not have been effective to overcome long-term P-deficiencies and consequently also resulted in increased storage FA concentrations and loss of PUFA, causing a lower food quality of coastal phytoplankton groups as early as April during the peak of the spring bloom (see below). FA pools take several days to show quantitative and qualitative changes indicating that changes in AA metabolism have priority over FA, even though expression of genes
involved in FA metabolism immediately moderated after changes in nutrient status (Bender et al. 2014).

Biosynthesis patterns reflected a community specific response to the imminent nutrient limitation at this point in time, indicated by quick shifts in biosynthesis after nutrient addition. On the contrary, the distribution of individual compounds in the POC concentrations relates to the long-term history of nutrient limitation experienced by the cell over several days (biomass turnover time 3 – 12 days) and is furthermore affected by community specific differences in biomolecule composition. Additionally, with age the AA distribution in detritus shifts and has to be considered as well (Dauwe & Middelburg 1998; Dauwe et al. 1999). When communities transitioned from one dominating phytoplankton group into another, as seen at several stations in this study, it can occur that the declining population that determines the concentration is limited by one nutrient whereas the upcoming population determining photosynthesis is either non-limited or limited by another set of nutrients causing clear shifts in compounds biosynthesis.

Consequences for food quality

Biomolecule composition of phytoplankton has a direct effect on its nutritional value to consumers, primarily zooplankton. Previous research has shown that essential AA and PUFA are important determinants for zooplankton growth (Claybrook 1983; Müller-Navarra 1995; Weers et al. 1997). In our study, both these pools were affected by nutrient limitations in both diatom and flagellate communities suggesting that phytoplankton food quality varied substantially on both temporal and spatial scales in the North Sea due to shifts in nutrient availability.

Especially diatoms, but also several dinoflagellates are considered to have good food qualities (Ianora et al. 1999; Turner et al. 2001; Turner et al. 2002). Here we found diatoms dominating in early spring and throughout most of the year at the CZ. They were mainly encountered during P-limitation during spring, while flagellates (including both dinoflagellates and Phaeocystis sp. [Grosse et al. in review/Chapter 3; Burson, et al. 2016]) were dominant at the three stations farther offshore showing strong N-limitation in late spring and summer. Under resource limitation, phytoplankton shows a general response in the decrease of total AA synthesis (Grosse et al. in review/ Chapter 3) and we show here that during N-
limitation synthesis is shifted towards non-essential AA. Both low quality and quantity of AA negatively affect consumer’s growth (Guisande et al. 2000). Furthermore, a lack of individual essential AA was found to restrict the reproduction of Daphnia and lead to changes in their life cycles (Fink et al. 2011; Koch et al. 2011). Finally, under nutrient limitation FA synthesis was shifted from structural towards storage FA as well as from PUFA to SFA, especially under P-limitation. Although C16 and C18 PUFAs decreased as well, the highly unsaturated FA such as 20:5ω3 and 22:6ω3 are crucial for zooplankton survival and the maintenance of high growth and reproductive rates (Müller-Navarra 1995; Burns et al. 2011). They furthermore affect trophic transfer efficiency and food web structure (Brett and Müller-Navarra 1997), and are therefore considered to be an indicator for food quality (Park, et al. 2002). In conclusion, food quality in terms of AA and FA are both negatively and concurrently affected by nutrient limitation that will cause a decline in quantity and quality of individual compounds.

The phytoplankton response to nutrient addition is rather general resulting in an overall increase in AA synthesis first before the increase of other structural compounds (Grosse et al. in review/ Chapter 3). However, different metabolic pathways are affected when N or P is lacking. A lack of N can be directly linked to AA molecule structure because of for instance differences in C:N ratios between AA and but a lack of P decreases the translation of proteins due to the effect on the molecular structure of ribosomal RNA and the consequent decrease of the overall requirements of ribosomes in the cells. Other intracellular P-pools, such as phospholipids, RNA and DNA should be considered as well, because zooplankton appears to be more sensitive to P- than to N-limitation (Breteler et al. 2005). Although the majority of research has been conducted on the freshwater inhabiting cladocera Daphnia (Boersma 2000; Plath & Boersma 2001), and lakes are mostly considered P-limiting opposite to N-limiting marine waters (Hecky & Kilham 1988; Howarth & Marino 2006), anthropogenically induced changes in nutrient inputs from land push coastal seas more and more from N-limited into P-limited systems.

In contrast to other studies, we did not investigate changes in compounds when N or P limitation is induced (Flynn et al. 1992; Mock & Kroon 2002b) but after a sudden repletion of nutrients. This scenario is comparable in proximity to river plumes, upwelling regions, or though input of deep-water nutrients after intense
mixing and the breakdown of stratification in the water column. Even though these sudden inputs may be brief, the phytoplankton community responded quickly. Subsequent effects on zooplankton may also be observed on relative short time scales, especially in the micro- and meso-fraction since generation times are short for example 30 and 17 days for Calanus helgolandicus and C. typicus, respectively (Halsband-Lenk et al. 2002; Bonnet et al. 2005). With continuing alterations in riverine DIN and DIP supply it is likely that smaller zooplankton species could replace larger ones (Fransz et al. 1992) and thereby redirecting primary production to species with rapid turnover, such as protozooplankton and rotifers (Viitasalo 1992) and induce changes in the structure of the entire food web.

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Supplemental Information

Figure S4.1: Map of stations revisited during five cruises between 2011-2013.

Figure S4.2: PCA biplot of relative contribution of individual FA to structural FA in POC concentration (A) and biosynthesis (B). Symbol shape refers to the dominating group of the phytoplankton community and symbol color indicates prevailing nutrient-limitation (phytoplankton group and nutrient limitation were identified in Chapter 3).

Figure S4.3 (next page): Distribution of individual AA in C-fixation (A, C, E, G, I) and biomass (B, D, F, H, J) in different nutrient addition treatments after 72 h at the Coastal Zone in March, April, Mid May (Station #1, 2, 3) and the Dogger Bank in April and Mid May (Station #12, 13). Essential AA were underlined and prevailing nutrient limitation was indicated in right upper corners of biosynthesis graphs for each station.