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Grosse, J.; Brussaard, C.P.D. & Boschker, H.T.S. (2019). Nutrient limitation driven dynamics of amino acids and fatty acids in coastal phytoplankton. *Limnology and Oceanography*, 64, 302-316

Published version: https://dx.doi.org/10.1002/lno.11040

NIOZ Repository: http://www.vliz.be/nl/imis?module=ref&refid=303624

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

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Keywords: nutrient limitation, fatty acids, amino acids, food quality, phytoplankton

Running head: Compound synthesis under N and P limitation

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 show how these were affected by different nutrient limitations in natural coastal phytoplankton communities. Nitrogen limited communities exhibited 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 not limiting conditions. On the contrary, phosphorus limited communities showed no such shift in amino acids. Both N and P limitation induced a shift from structural to storage fatty acids with a concurrent decrease in the synthesis of poly-unsaturated fatty acids. Reversed effects in fatty acid synthesis after N or P addition were only apparent after 72 h, when they could be found in both fatty acid biosynthesis and concentrations. The different strategies of qualitative and quantitative regulation of different biomolecule synthesis under nutrient scarcity may have far-reaching consequences for the phytoplankton's nutritional value. Higher trophic levels may have to cope with the loss of essential amino acids and poly-unsaturated fatty acids in nutrient limited phytoplankton, which could induce changes in the structure of food webs.

Introduction

Phytoplankton are main contributors to global primary production (Field et al., 1998), are the foundation of many marine food webs and the source of essential compounds for higher trophic levels (Arts and Kohler, 2009). Long-term perturbations of nutrient deliveries from land, however, led to eutrophication in many coastal seas, impacting phytoplankton community structure and productivity in unfavorable ways (e.g. Smith et al., 1999; Philippart et al., 2000). In order to counteract repercussions of high nutrient loads the neighboring countries of coastal seas started to reduce nutrient loads in river systems. For example, members of the OSPAR Convention (Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic) agreed to reduce inputs of riverine nitrogen (N) and phosphorus (P) to the North Sea by at least 50% compared to the year 1985 (OSPAR, 1988). Overall, nutrient reduction efforts were more effective for P removal compared to N removal (OSPAR, 2010; Troost et al., 2014) and resulted in drastic changes in nutrient availabilities, both of concentrations and relative ratios of dissolved inorganic N and P (DIN:DIP). Growing evidence suggests that P limitation is becoming more and more dominant in river influenced coastal areas such as the North Sea, the Gulf of Mexico or the South China Sea (Sylvan et al., 2007; Xu et al., 2008; Burson et al., 2016), thereby challenging the classical view, which considers N to be limiting in marine systems (Blomqvist et al., 2004; Howarth and Marino, 2006).

Changes in nutrient availability translate into changes in C:N:P ratios of phytoplankton biomass (Vrede et al., 2004; Diez et al., 2013), which can also be used as a tool to evaluate the nutritional quality of phytoplankton for higher trophic levels (Sterner and Elser, 2002). Looking closer, changes in C:N:P ratios illustrate shifts in the biomolecule composition. C:N:P requirements of major cellular biomolecules differ (Klausmeier et al., 2004); carbohydrates (CH) only contain C while amino acid (AA) synthesis requires N and RNA/DNA synthesis requires both N and P. Fatty

acids (FA), like carbohydrates, only contain C but they are found in complex membrane lipids that often require P and/or N (Van Mooy et al., 2009).

Phytoplankton can adapt growth strategies to nutrient scarcity and increase or decrease synthesis of different biomolecule groups. For example, synthesis of carbon rich storage compounds, such as glucose or storage lipids, increases during nutrient limitation and leads to increased C:N and C:P ratios, which concurrently decreases food quality (Plath and Boersma, 2001; Borsheim et al., 2005). N:P ratios are affected as well (Geider and LaRoche, 2002; Quigg et al., 2003), however, the specific underlying dynamics in the synthesis of ribosomal RNA, pigments and proteins are difficult to investigate in phytoplankton cells and have so far mainly been demonstrated in computational model runs (Falkowski, 2000; Klausmeier et al., 2004; Arrigo, 2005).

However, technological advances, especially in liquid chromatography-isotopic ratio mass spectrometry, make it now possible to obtain specific isotope information of a wide range of compounds and conduct synthesis rate measurements. Grosse et al. (2017) investigated the seasonal and spatial dynamics of concentrations and biosynthesis rates of total amino acids, storage fatty acids and carbohydrates as well as structural fatty acids and carbohydrates in North Sea phytoplankton communities. Results showed that nutrient limitation and season had large effects on the quantity of all biochemical classes, affecting both concentration and biosynthesis rates, e.g., AA concentration varied fourfold in concentration and up to eightfold in biosynthesis rates. The addition of the growth-limiting nutrient revealed an overall response in which storage compound synthesis decreased and AA synthesis was stimulated concurrently. Yet, the magnitude of response differed between limiting nutrients. While AA synthesis increased up to fivefold within 24 h upon the relief of N limitation, AA synthesis in P limited communities responded delayed and at a lower magnitude. And although separation of total FA and carbohydrates into

storage and structural groups occurred in this study (based on polarity and glucose vs. all others for FA and CH, respectively) a specific evaluation is missing.

A compound specific evaluation would allow for a much more detailed assessment of food quality since several AA and FA are considered essential to higher trophic levels (Raubenheimer et al., 2009) and the availability of a single compound can affect zooplankton growth and reproduction (Müller-Navarra, 1995; Burns et al., 2011). Earlier studies show the effects of nutrient supply, light and temperature on FA dynamics in a wide range of phytoplankton groups (Renaud et al., 2002; Xin et al., 2010; Piepho et al., 2012). In general the limitation by nutrients causes an increase in triglycerides (non-polar/storage FA), and a concurrent decrease in membrane lipids (polar/ structural FA) especially under P-limitation (Fidalgo et al., 1998; Lynn et al., 2000). Additionally, the degree of FA saturation is also affected by nutrient shortage, causing a shift from poly-unsaturated FA (PUFA, which include essential FA) towards saturated FA (SFA; Siron et al., 1989; Reitan et al., 1994; Breteler et al., 2005).

Little is known about dynamics of individual AA in nutrient stressed phytoplankton, but it is generally believed that the composition is relatively constant, an assumption mainly based on the geochemical composition in particulate organic carbon in the water column or from detrital matter in surface sediments (Dauwe and Middelburg, 1998; Dauwe et al., 1999). This would suggest that essential AA (EAA), which include amongst others phenylalanine (Phe), leucine (Leu), valine (Val), and arginine (Arg, [see Fig. 1 for full list]) (Claybrook, 1983; Lin et al., 2015) are supplied at a constant ratio to non-essential AA (NEAA). EAA are, however, generally synthesized from non-essential pre-cursor AA and their synthesis require additional steps and enzyme reactions (Fig. 1). Therefore, 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), which consequently could lead to the synthesis of different sets of

proteins, with a possibly different composition of individual AA, and affect the nutritional quality of phytoplankton.

Here we characterize nutrient driven dynamics in concentrations and biosynthesis rates of individual AA and several FA groups (based on their degree of saturation and nutritional value to higher trophic levels) obtained from the experiments by Grosse et al. (2017). We hypothesize that N and P availability or limitation affects the quality of AA and FA composition in phytoplankton. Hence the short-term response to the relief of N and P limitation has different effects on individual biomolecule synthesis rates. To address this hypothesis we evaluated the dynamics of individual compounds in phytoplankton field populations during pre-bloom, spring bloom and post-bloom conditions. Furthermore, we identified shifts in individual compound dynamics after the relief of nutrient limitation in nutrient addition experiments (24 – 72 h) in order to determine nutrient-specific and phytoplankton community-specific shifts in compound quality.

Material and Methods

Sampling transect. A total of five cruises spread over three consecutive years were 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. 2) with different nutrient settings were investigated. The coastal zone station (CZ) is only 7 km offshore and influenced by high nutrient riverine run-off and its shallowness (8 m) assures a completely mixed water column. The Oyster Ground (OG) station is located around 100 km offshore and 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 and Peperzak, 1990). The Dogger Bank station (DB, 250 km off-shore) shows low nutrient availability throughout the year and due to its shallowness does not display stratification. The central North Sea (CNS, 450 km off-shore) shows the highest influence of

Atlantic Ocean water, which is low in nutrients and induces thermal stratification during summer. The cruises took place 25 − 31 May 2011, 15 − 30 August 2011, 8 − 12 May 2012, 15 − 25 March 2013, and 24 April - 4 May 2013. The timing of the cruises coincided with pre-bloom conditions (March 2013), the peak (April 2013) and the decline (Mid May 2012) of the spring-bloom as well as post-bloom conditions (End May 2011) and the late summer period (August 2011).

Water for measurements and experiments was collected using a sampling rosette with 24 Niskin bottles (12 L), equipped with a Sea-Bird SBE911+ CTD sampler (Sea-Bird Electronics Inc., USA) to obtain water column distribution of salinity, temperature and photosynthetically available radiation.

Nutrient addition experiments. The experimental design was according to Grosse et al. (2017). In short, on-board nutrient addition experiments were performed to test for nutrient limitation. Concurrently, treatments were enriched with ^{13}C-DIC to trace photosynthetically fixed carbon into individual biomolecules. Sub-surface water (7 m) was collected shortly before sunrise and directly transferred into 10 L carboys. The following treatments were set up in duplicates: Control, +N (addition of 80 µM $NaNO_3$), +P (5 µM K_2HPO_4) and +NPSi (80 µM $NaNO_3$, 5 µM K_2HPO_4 and 80 µM $Si(OH)_4$). All carboys were enriched with ^{13}C-sodium bicarbonate (99% ^{13}C) to a final labeling concentration of 1.5 to 2 % of ambient DIC concentration (approximately 200 µM).

Carboys were incubated for 24 h in flow-through incubators on deck, which were continuously flushed with seawater to assure in-situ temperature and light condition. Aliquot samples were filtered over pre-combusted GF/F filters (Whatman, 4 h at 450°C) for POC, AA and FA analysis. , The filtered volume ranged from 0.3 - 2.0 L, depending on the phytoplankton density and POC filters were pre-weight to enable partial filter analysis later. All filters were stored frozen at −80°C until analysis.

During selected cruises, nutrient additions lasting 72 h were also carried out at the CZ and DB to investigate if short-term changes in biomolecule synthesis differ from long-term changes and if changes in biomolecule synthesis translate into shifts in relative biomolecule concentrations. 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-DIC was added after 48 h and the incubations were terminated after 72 h as described above.

Analytical procedures. Biomolecule extraction protocols, analytical procedures and calculation of ^{13}C uptake rates have been published in Grosse et al. (2015, 2017) 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 (Glx) co-elute and formed one peak, as do aspartate and asparagine (Asx). All AA with the exception of tryptophan can be measured with the method used, but because of very low concentrations of cysteine and methionine both were excluded from the data analysis.

FA samples were extracted following the protocol of Bligh and Dyer (1959) and subsequently separated into storage lipids, glycolipids and phospholipids by silicate column chromatography. All three fractions were dried down and the glycolipid- and phospholipid fraction were combined and are further referred to as structural lipids. After derivatization to fatty acid methyl esters (FAMEs), 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. In order to evaluate nutrient effects on the saturation of FA composition, FA of the structural and storage pools were further divided by their degree of saturation into saturated FA (SFA), mono-unsaturated FA (MUFA) and poly-unsaturated FA

(PUFA). In order to reflect the contribution of different phytoplankton groups, PUFAs were furthermore separated into PUFA containing 16 C-atoms (C16-PUFA; diatom specific) and PUFA containing 18 C-atoms (C18-PUFA; flagellate specific; Dijkman and Kromkamp, 2006). The PUFA 20:5ω3 and 22:6ω3 were evaluated individually because their ratio is indicative of the dominating phytoplankton group. 20:5ω3/22:6ω3 ratios ≥1 signal the dominance of flagellates, while a value <1 is suggestive of a greater contribution of diatoms (Budge and Parrish, 1998).

Biosynthesis rates of each individual compound were calculated from ^{13}C incorporation rates according to Grosse et al. (2015), and were added up in order to obtain values for each biomolecule group (essential and non-essential AA and storage and structural FA. Throughout this text, biomolecule concentrations and biosynthesis rates were reported relative to the total AA and total FA, respectively. Unless noted differently, all data is shown as average (± standard deviation), $n = 2$.

Statistical analysis. To explore differences in individual AA and FA composition under different nutrient limitations, principle component analysis (PCA) was performed with the relative contribution (%) of (*i*) individual AA and 12 FA group concentrations to total AA and FA concentrations (nmol C (µmol POC)$^{-1}$) and (*ii*) individual AA and 12 FA group synthesis rates to total AA and FA synthesis rates (nmol C (µmol POC)$^{-1}$ d^{-1}). The 12 FA groups comprise of 6 structural FA groups (SFA, MUFA, C16 PUFA, C18 PUFA, 20:5ω3, 22:6ω3) and 6 storage FA groups (**SFA**, **MUFA**, **C16 PUFA**, **C18 PUFA**, **20:5ω3**, **22:6ω3**). The package CRAN:factoMineR in the open source software R was used for the PCA analysis using a correlation matrix (Lê et al., 2008, R Core Team, 2013).

Results

General context of seasonal succession

Seasonal evolution of nutrients, resulting nutrient limitations and phytoplankton community composition have been discussed elsewhere (Burson et al., 2016; Grosse et al., 2017). In summary (Tab. 1), the development of inorganic nutrients showed a general decrease in concentrations over seasonal and special scales. The resulting DIN:DIP ratios suggested that near shore stations (CZ and OG) were P limited during bloom-cruises and shifted towards N limitation thereafter, while stations farther offshore were limited by N-limited during all sampling periods (Burson et al., 2016; Grosse et al., 2017). However, Grosse et al. (2017) used the response in total AA biosynthesis in nutrient addition assays (after 24h) to identify the prevailing nutrient limitation (summarized in Tab. 2), which concur with nutrient limitations derived from DIN:DIP ratios (see above).

They also used the structural FA composition to determine the dominating phytoplankton community to be either dominated by diatoms or flagellates (summarized in Tab. 2). A few stations could not be assigned clearly as either diatom or flagellate dominated and are therefore referred to as “Mixed”. The phytoplankton community composition showed the shift from diatom dominated communities to flagellate dominated communities that developed earlier in the year with increasing distance to the coast (Grosse et al., 2017).

Individual amino acid contributions

Field conditions

In un-amended incubations (n=18), nutrient and community dependent differences in AA biosynthesis and composition were investigated using PCA (Fig. 3a, b). The PCA of AA biosynthesis showed that the first two principal components explained 54% (39% + 15%) of variance in the dataset, while in PCA of AA concentration PC1 and PC2 explained 53% (32% + 21%) of the variance. For both biosynthesis and concentration a separation between not limited

mixed communities and N limited flagellate dominated communities occurred along the PC1 axis. P (co-) limited stations did not cluster together but mixed with the other two groups. Concurrently, there was a separation between essential AA (EAA) and non-essential AA (NEAA) in the biosynthesis data along PC1 (Fig. 3a). EAA and Pro associated with non-limited communities, while N-limited communities associated with all other NEAA. No such separation was observed within the concentration data (Fig. 3b). There N limited communities were associated especially with NEAA Asx, Glx and EAA Thr, while all other communities associate with EAAs as well as NEAA Ala and Ser. A separation along the PC2 axis occurred as well and was associated with Pro, Lys and Tyr. In order to demonstrate the variance between stations we 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. 3c, d), clearly illustrating discussed differences in AA contributions.

Across the whole dataset, there was substantial variation in both relative concentration and biosynthesis when normalized to total AA (Fig. S1a). Variations were greater in biosynthesis than in concentration, especially for NEAA such as Asx, Glx, Ala and Gly, while EAA Val, His and Arg showed much smaller ranges in concentration and biosynthesis. Non-essential AA showed higher relative biosynthesis compared to their concentration, while essential AA showed the opposite trend, leading to a relative "over-synthesis" of non-essential AA and a relative "under-synthesis" of essential AA in short-term (24 h) incubations.

Response to short-term nutrient addition

The effect of short-term nutrient addition (24 h) on AA biosynthesis was also studied using PCA analysis (Fig. 4). Since AA biosynthesis seems to be nutrient specific we ran separate PCAs for (*i*) N-limited communities at 9 stations and (*ii*) not and P limited communities at the other 9 stations (as identified in Tab.2). The PCA of N limited communities explained 59% (PC1: 44%,

PC2: 15%) of the variance in the data and a clear separation between treatments occurred (Fig. 4a). Separation along the PC1 axis occurred between the NEAA Asx, Glx, Ala, and Gly, and all EAA and Pro. The treatments where N limitation was relieved (+N and +NPSi) associated with all EAA and Pro, opposite to control and +P treatments, which continued to associated with NEAA thereby demonstrating clear and swift shifts in synthesis rates. The +P treatments of four stations (9, 12, 14, 17) formed an exception and also developed an association with EAA, comparable to the +N and +NPSi, hereafter referred to as "Group X". Although these stations were primarily N limited they showed co-limitation by P or Si and the addition of P also triggered a shift towards EAA at these stations. In the PCA of not and P limited stations (Fig. 4b) PC1 and PC 2 explained 36% and 21% of the variance, but differences in AA synthesis between different nutrient addition treatments were not found.

Fatty acid groups

Field conditions

PCA was performed with FA group biosynthesis and concentration data (Fig. 5, *n*=18) and revealed a separation between flagellate and diatom dominated communities with mixed communities in between. The first two axes explained 55% (PC1: 31%, PC2: 24%) and 62% (PC1: 34%, PC2: 28%) of the variance in the biosynthesis and concentration data, respectively. As expected, communities were associated with group specific FA (see Material & Methods). 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 clear effect of nutrient limitation could be observed. However, the Si limited diatoms at station 7 clearly separated from other stations in the biosynthesis and showed higher concentrations of structural MUFA.

Similar to individual amino acids, the contribution of the FA groups to total FA varied over seasonal and spatial scales (Fig. S1b). Storage SFA and MUFA showed highest contributions as well as highest variability in both concentration and biosynthesis. Compared to the relative concentration, storage SFA and MUFA showed higher relative biosynthesis leading to a relative "over-synthesis" of these compound groups, while structural FA groups showed the opposite trend ("under-synthesis").

Response to nutrient addition

PCA was also performed on FA biosynthesis in nutrient addition treatments but revealed no nutrient related short-term shifts (24 h; data not shown), 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. Nutrient specific responses were seen in both N and P limited station (9 stations and 4 stations, respectively, Fig.6). For N limited stations the first two PC axes explained 64% (PC1: 48% + PC2: 16%) of the variation. The +N and +NPSi treatments responded with an increase in structural FA groups, while decreased N:P ratios in the +P treatment (induced through PO_4 addition) caused synthesis of storage FA groups to intensify. The N limited diatom community (Stn. 5) showed a shift towards storage C16 PUFA and storage 20:5ω3 (Fig.6a).

For P limited stations the first two PC axes explained 66% (PC1: 39% + PC2: 27%) of the variation. P limited stations showed the same response to the relief of nutrient limitation; at the majority of stations the +P and +NPSi treatments showed a relative increase in all structural FA groups, while +N treatments (with increased N:P ratios during the incubation) increased synthesis of storage SFA and storage C16 and C18 PUFA (Fig. 6b).

Longer-term effects of nutrient addition

297 Five longer-term incubations (72 h) were carried out to detect qualitative changes in AA
298 synthesis and concentrations. Due to the low number of stations data evaluation using PCA was
299 not possible and we decided to depict individual AA concentration and biosynthesis of the most
300 severe N limited station (Stn. 13) because there the response to nutrient addition was expected to
301 be greatest (Fig. 7a,b). Overall, nutrient additions showed similar effects in biosynthesis patterns
302 as seen after 24 h for Stn.13. There was a persistent decrease of NEAA and increased EAA
303 biosynthesis in the +N and +NPSi treatments, identical with the pattern found after 24 h.
304 Responses at the P limited station (Stn.2) also occurred but only a few NEAAs decreased (e.g.
305 Glx, Gly) and a few EAAs increased (e.g. Ile, Leu) in the +P and +NPSi treatments (Fig. S2a, b).
306 A similar effect was not seen in Stn. 1 and 3 (data not shown). Furthermore, resulting relative
307 changes in AA concentrations were difficult to identify at any station.
308 After 72 h shifts could be identified both in biosynthesis and concentration of relative FA
309 distribution (Fig. 7c, d, S2c, d). The relief of N limitation (+N and +NPSi, Stn. 13) and P
310 limitation (+P and +NPSi, Stn 2) caused a relative increase in the synthesis of structural MUFA
311 and all three structural PUFA groups with a concurrent decrease in all storage FA groups. This
312 shift in biosynthesis was also translated into the relative distribution of FA concentration, with
313 increasing structural FA groups and decreasing storage FA groups. Consequently, quantitative and
314 qualitative changes of FA concentrations need several days to become detectable.
315

316 **Discussion**

317 **Response of phytoplankton to nutrient limitation**

318 Coastal seas experience large perturbations in nutrient inputs, which may have important effects
319 on the cellular composition of phytoplankton and its nutritional quality for higher trophic levels.
320 Here we show that both, individual AA and groups of FA, exhibit specific responses to different

nutrient limitations in biosynthesis as well as composition. Biosynthesis patterns of individual AA responded differently to N than to P limitation, and changes in relative AA contributions were visible within 24 h. Biosynthesis patterns in FA groups showed similar responses to N and P limitation and effects on relative FA group contribution were only clearly seen after 72 h.

Amino acid dynamics

Although re-supplying the limiting nutrient led to an increase in total AA synthesis (Grosse et al., 2017), only the N limited phytoplankton communities showed shifts in the distribution of individual AA after 24 h. Results from both un-amended and nutrient addition incubations showed that under N limitation the pools of NEAA increased, especially Asx, Glx and Ala, while synthesis of EAA and Pro decreased. The strong nutrient dependent separation of Glx and Asx from other AA in PCAs of concentration, biosynthesis and nutrient additions (Fig. 3, 4) highlights their function as pre-cursor in more complex pathways for essential AA (Fig. 1). The NEAA Pro stands opposite to Glx even though it only requires a one-step reaction to synthesize Pro directly Glx. However, Pro serves a special function in osmoregulation and in order to reduce 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). This causes Pro to be primarily synthesized under N replete conditions and therefore to cluster with essential AA. The conversion of NEAA to EAA relies on numerous additional enzymes, proteins themselves, and it may be beneficial to reduce the production of these enzymes under N limitation. The up-regulation of AA biosynthesis after P addition took much longer (several days) to be detected, compared to N limited stations (Grosse et al., 2017). 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 and Devol, 2008; Hessen et al., 2010). Furthermore, only a few selected AA changed their relative

contribution to biosynthesis and no clear shift was visible that affects all AA (Fig. S2a, b), causing a more uniform increase in the synthesis of individual AA. This would especially affect translation, regulating the synthesis of protein chains, instead of being driven by the availability of individual AA. 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).

Interestingly, several N/P co-limited stations also showed the shifts towards EAA after P addition (Group X, Fig. 4a). The response is similar to solely N limited stations and probably depicts the response of different groups of phytoplankton within complex communities. Co-incidentally, Burson et al. (2016) investigated phytoplankton group specific responses to nutrient addition on one of these stations (Stn. 12). They found that dinoflagellates were not nutrient limited, while nano-flagellates were limited by N, pico-eukaryotes were P limited, and Phaeocystis sp., diatoms and pico-cyanobacteria showed N/P co-limitation. Co-limited phytoplankton may be able to utilize remaining N concentrations after P addition (Harpole et al., 2011) causing AA biosynthesis patterns to become similar to those after N addition.

Fatty acid dynamics

FA distribution is affected by multiple factors. Firstly, phytoplankton group specific FA shape the FA composition of a community. On top of this, low nutrient availability regulates the degree of FA saturation and the magnitude of storage FA synthesis. These responses seem to be the same

under N and P limited conditions. This shift from polar structural lipids (including phospholipids and glycolipids) towards neutral storage lipids, has been described earlier by other authors (Weers et al., 1997; Lynn et al., 2000; Mock and Kroon, 2002), however, only for changes in FA or lipid concentrations. Shift from PUFA to MUFA could also be attributed to changes in synthesis pathways, when desaturases and elongases cannot be synthesized in required amounts anymore under nutrient limiting conditions (Flynn et al., 1992). In our study all structural FA groups showed increased biosynthesis after 72 h but at different degrees after nutrient limitation was alleviated: PUFA groups increased synthesis up to sixfold, while increase of SFA and MUFA were about twofold or less (Fig. 7c,d). The delayed response in the FA fractions may be contributed to the fact that depending on present nutrient regimes FA can flow between storage and structural pools without requiring de-novo synthesis. Already synthesized storage FA may have been used to increase amounts of structural FA within 24 h, but since they were not labeled by ^{13}C tracers this synthesis would have been undetected. This is supported by finding from Bender et al., (2014), who showed that genes involved in FA metabolism are also immediately regulated after changes in nutrient status.

To complete the list of parameters that affect biomolecule composition environmental parameters such as light availability and temperature must be mentioned briefly (Mortensen et al., 1988). With regards to FA, water temperatures impact membrane fluidity, causing phytoplankton to adapt by decreasing the degree of saturation in colder waters, e.g. decrease PUFA contributions (Tedesco and Duerr, 1989). This may have played a role in March when Mixed communities showed higher relative concentrations of structural MUFA and SFA.

Several groups of phytoplankton, including diatoms, have an additional way to cope with P-scarcity. They are able to substitute P containing membrane lipids 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; Maat et al., 2016).This mechanism may have played a role at the Coastal Zone station (Stn. 2-4), however, under the high DIN:DIP ratios encountered (up to 333) in April this mechanisms may not have been effective to overcome long-term P deficiencies and consequently also resulted in increased storage FA concentrations and loss of PUFA during the peak of the spring bloom. This resulted in a lower food quality of coastal phytoplankton groups as early as April during the peak of the spring bloom (see below).

Biomolecule distribution in natural phytoplankton communities could be used to identify prevailing nutrient limitations. Biosynthesis of individual AA in control incubations can be used to distinguish between N limited and not/P limited stations as they cluster with NEAA and EAA respectively (Fig. 3a). However, after nutrient addition long-term changes in AA concentrations are only informative when considering total AA (Grosse et al., 2017), not individual AA (this study). The main reason may be that AA concentrations were much higher than FA concentrations (Grosse et al., 2017), hence changes in relative AA biosynthesis may take longer to translate into relative AA concentrations. With average phytoplankton biomass turnover times of ~7 days (Grosse et al., 2017) even the 72 h incubation period may have been too short to detect significant shifts in AA composition. Therefore, future studies should incubate even longer to investigate this issue further.

Contrary, nutrient-limitation was difficult to determine when only considering FA group biosynthesis and concentration in control incubations (Fig. 5). This was due to the very diverse FA composition between phytoplankton groups. Therefore, we suggest only considering the results of the nutrient addition to identify nutrient specific effects, as species composition remained constant

during the 24-72 h incubations. In contrast to AA, changes in FA concentrations can be used to determine the limiting nutrient in long-term nutrient addition assays. (this study).

Consequences for food quality

Biomolecule composition of phytoplankton has a direct effect on nutritional value for consumers. Previous research has shown that EAA and PUFA are important determinants for zooplankton growth (Müller-Navarra, 1995; Weers et al., 1997; Fink et al., 2011). In our study, both pools were affected by nutrient limitations in diatom as well as photoautotrophic flagellate communities, indicating that phytoplankton food quality varied substantially on both temporal and spatial scales in the North Sea due to shifts in nutrient availability. It should be mentioned that light intensity also modulates food quality, when increased light intensities result in higher relative carbohydrate contribution, thus increasing C:N and C:P ratios and thereby decrease food quality (van Oijen et al., 2007; Walter et al., 2015). However, light levels during incubations were chosen in such a way that we exclude light stress to be a modulator of compound biosynthesis and concentrations in our incubations.

Both diatoms and several autotrophic dinoflagellates are considered to have good food qualities for grazers (Ianora et al., 1999; Turner et al., 2001; Turner et al., 2002). We found diatoms dominating in early spring as well as throughout most of the year at the coastal zone (Grosse et al., 2017), typically dominating spring bloom events in temperate zones (Smetacek, 1999; McQuatters-Gollop et al., 2007). These communities were either not nutrient limited or experienced P-limitation, which caused a decrease in AA quantity (Grosse et al., 2017) but did not affect AA quality (this study, Fig. 4b). On the other hand, flagellates (including both dinoflagellates and *Phaeocystis* sp., Burson et al., 2016; Grosse et al., 2017), which were dominant at the three stations farther offshore showed strong N limitation in late spring and summer. The decrease in AA quantity under N limitation (Grosse et al., 2017) was accompanied by a qualitative

shift towards NEAA (this study, Fig. 4a). Both low quality and quantity of AA negatively affect consumer's growth (Guisande et al., 2000) and a lack of certain EAA was found to restrict the reproduction of *Daphnia* and lead to changes in their life cycles (Fink et al., 2011; Koch et al., 2011). Nutrient limitation affected FA synthesis in several ways. Relative FA synthesis was shifted (*i*) away from structural FA towards storage FA and (*ii*) away from PUFA towards SFA, especially under P limitation (Fig. 6b). Among the PUFA groups, the highly unsaturated FA 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). Consequently, trophic transfer efficiency and food web structure will be affected too (Brett and Müller-Navarra, 1997). Therefore, these tow FA are considered to be good indicators 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 essential compounds.

Overall, the phytoplankton response to nutrient addition is rather general resulting with an overall increase in AA synthesis before the increase of other structural compounds (Grosse et al., 2017). However, the restricted pathways differ, i.e. N limitation inhibits protein synthesis and P limitation inhibits synthesis of RNA (Loladze and Elser, 2011; Alipanah et al., 2015). The release of nutrient limitation may occur in proximity to river plumes, upwelling regions, or through input of deep water upon mixing (breakdown of vertical stratification in the water column). Even though these inputs may be brief, we show that the phytoplankton community responds within 24 – 72h. Subsequent effects on zooplankton may also be observed on relative short time scales, especially in micro-zooplankton population dynamics with short generation times (17 – 30 days, Halsband-Lenk et al., 2002; Bonnet et al., 2005). Anthropogenically induced changes in nutrient inputs from land push coastal seas more and more from N limited into P limited systems (Thingstad et al.,

1998; Philippart et al. 2000; Burson et al., 2016). Because zooplankton appears to be more sensitive to P than to N limitation (Breteler et al., 2005), we recommend that future research also considers other intracellular P-pools such as phospholipids, RNA and DNA.

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Acknowledgments

The authors thank the captain and crew of the R/V Pelagia for their help and support during the cruises, and Amanda Burson, Ruben van Drie and Elodie Burrillon for their skilled assistance with experimental work onboard. Special thanks to the Nutrient Lab at NIOZ-Texel for providing nutrient data, the Analytical Lab at NIOZ-Yerseke for the analysis of pigment samples and the reviewers for their constructive comments. This research is part of the CHARLET project and was supported by the Netherlands Organization for Scientific Research (NWO) to HTSB (grant ZKO 839.10.511).

Figure legends

Fig. 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, Asx = combined pools of aspartate/asparagine, Met = methinone, Thr = threonine, Ile = isoleucine, Lys = lysine, Glx = combined pools of glutamate/glutamine, His = histidine, Pro = proline, Arg = arginine. Met, Cys and Try were not detected in this study.

Fig. 2: Map of stations revisited during five cruises between 2011-2013

Fig. 3: PCA biplot of relative contribution of individual AA to total AA biosynthesis (a) and concentration (b). Symbol shape refers to the dominating phytoplankton community and symbol color indicates limiting nutrients. 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.

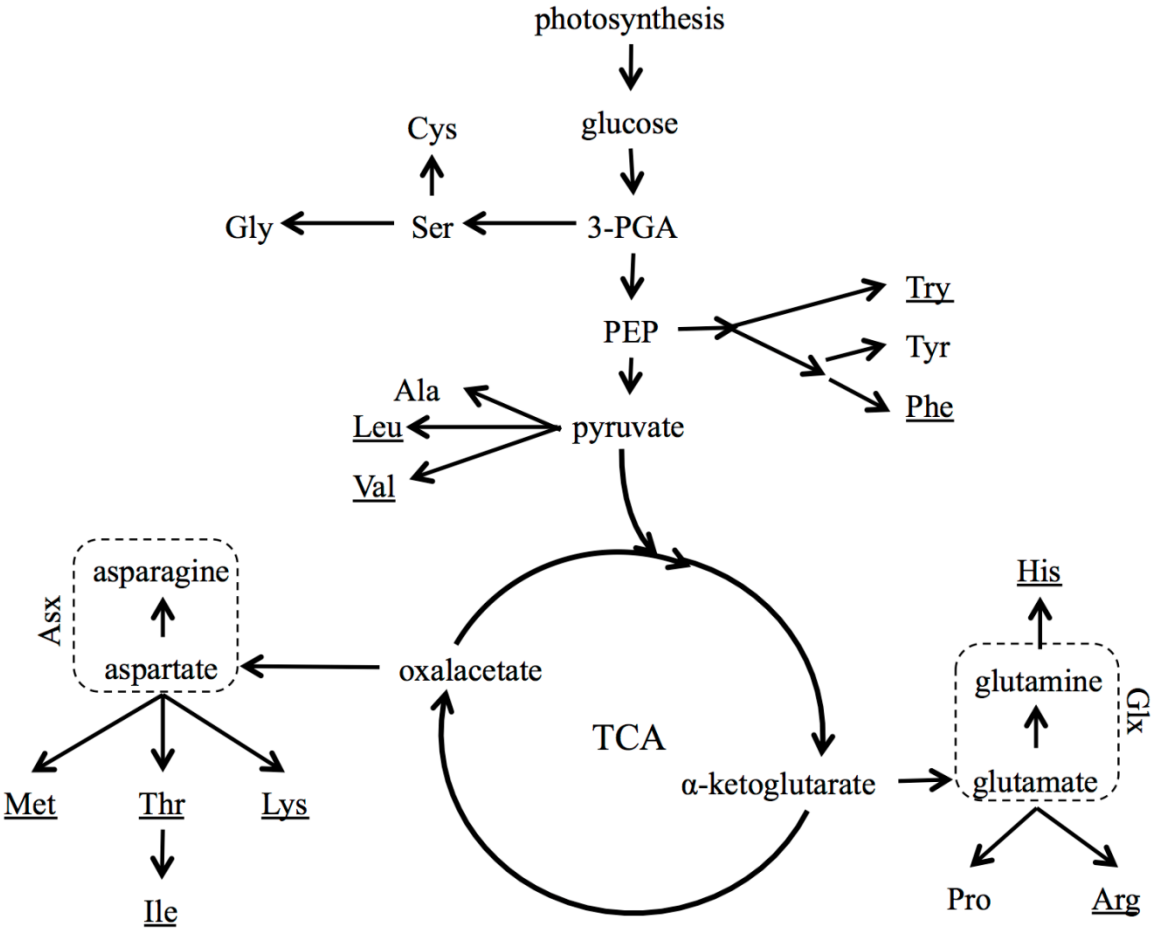
Fig. 4: PCA biplot show biosynthesis of individual AA in nutrient addition treatments after 24 h. Phytoplankton was separated into N-limited flagellate and mixed communities (a; 9 stations, n=36) and not/P limited communities (b; 9 stations, n=36). Ellipses encircle distribution of the same treatment and Group X (black), respectively. Essential AA were underlined.

Fig. 5: 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. FA groups are separated into structural FA (underlined) and storage FA (**bold**) groups. SFA = saturated FA, MUFA = mono-unsaturated FA, C16-PUFA/ C18-PUFA = poly-unsaturated FA containing 16 and 18 C atoms, respectively.

Fig. 6: 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 communities (a; 9 stations, *n*=27) and P limited (b; 4 stations, *n*=12). Structural FA groups are underlined, storage FA groups are shown in **bold**.

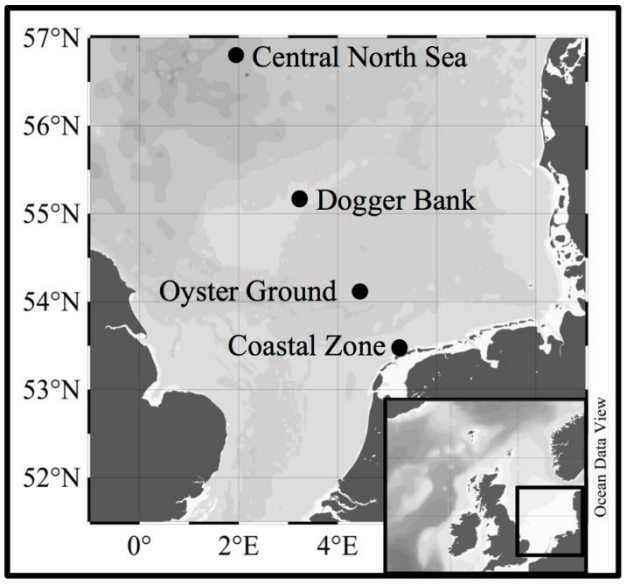
Fig. 7: Distribution of individual AA and FA groups in concentrations (open bars) and biosynthesis (striped bars) after 72h at the Dogger Bank in Mid May (N limited, mixed community).



769

770 Fig. 1

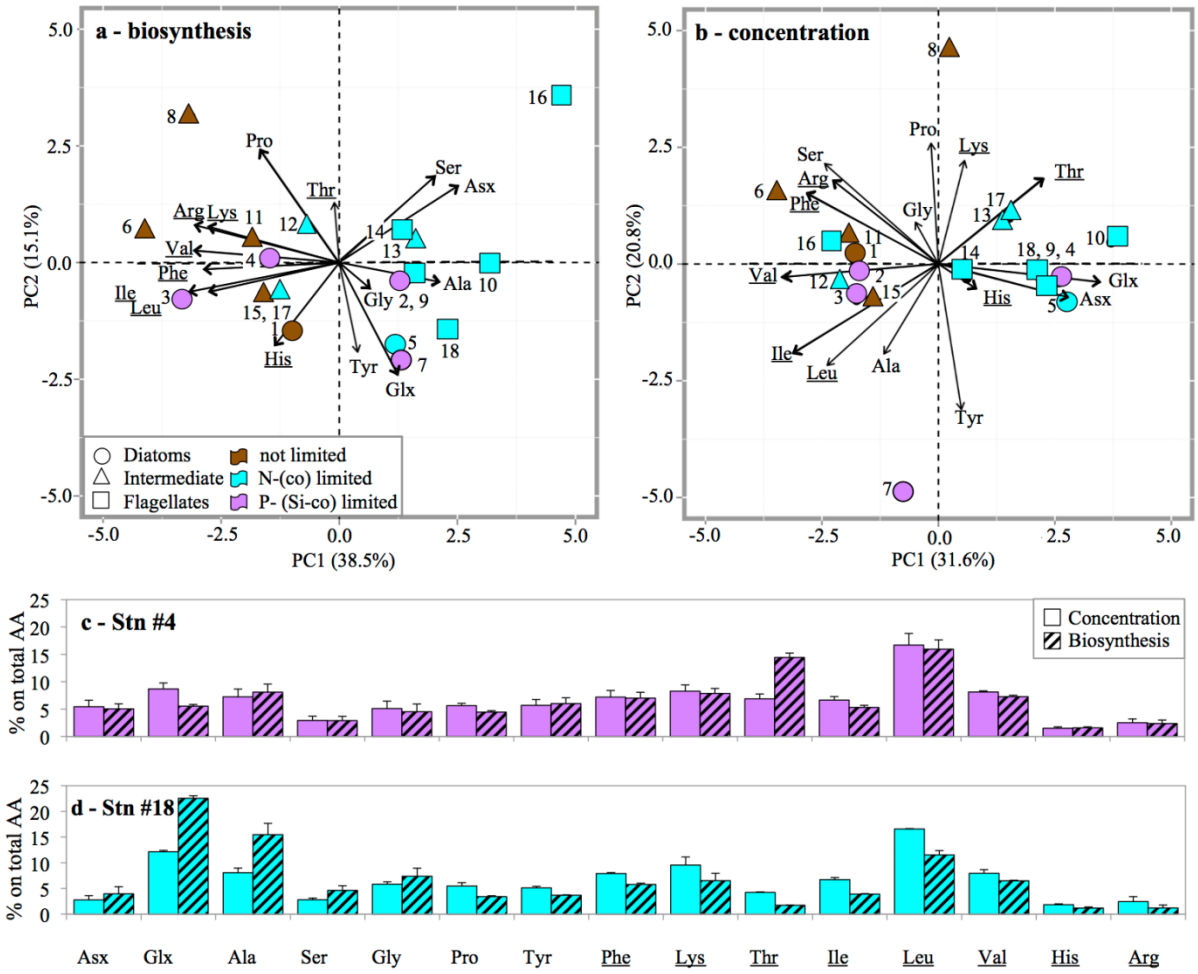
771



772

773 Fig.2

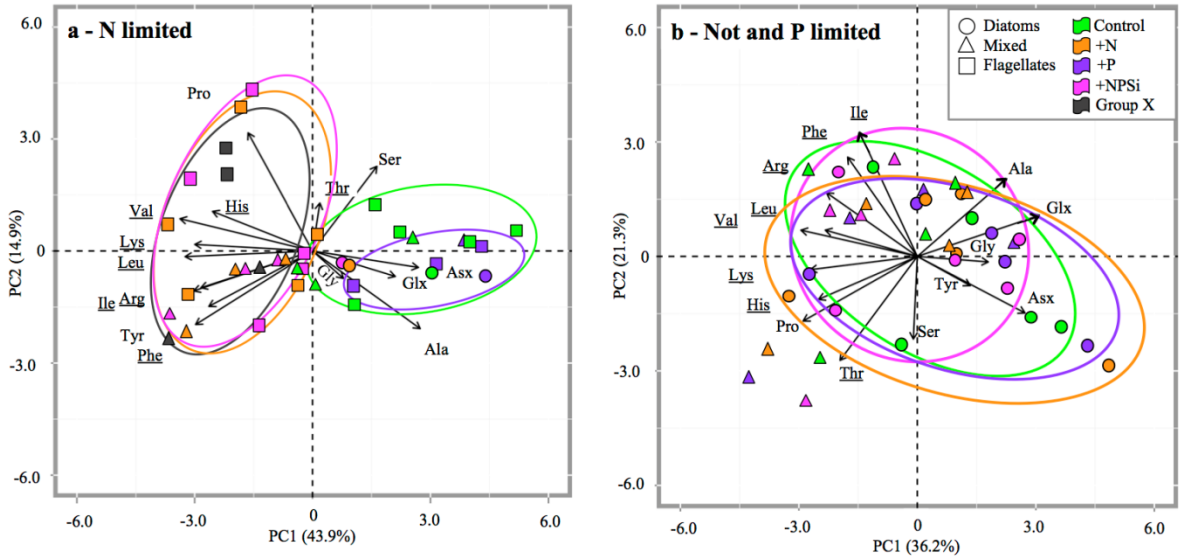
774



775

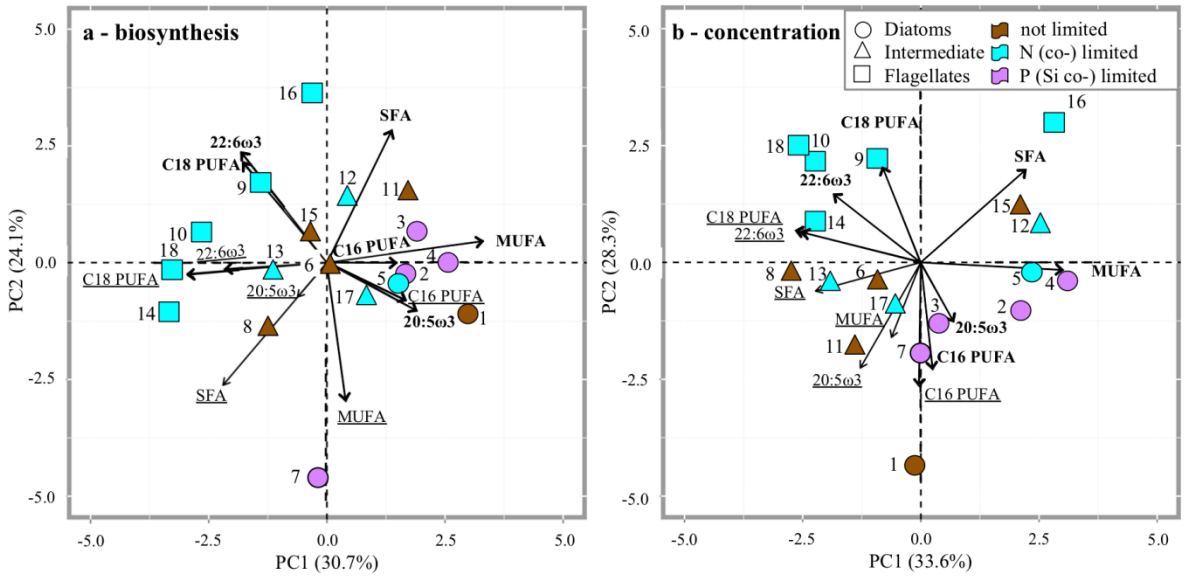
776 Fig.3

777


a - N limited
b - Not and P limited
Diatoms
Mixed
Flagellates
Control
+N
+P
+NPSi
Group X
PC1 (43.9%)
PC2 (14.9%)
PC1 (36.2%)
PC2 (21.3%)
Pro
Ser
Thr
His
Val
Lys
Leu
Gly
Glx
Asx
Ile
Arg
Tyr
Phe
Ala

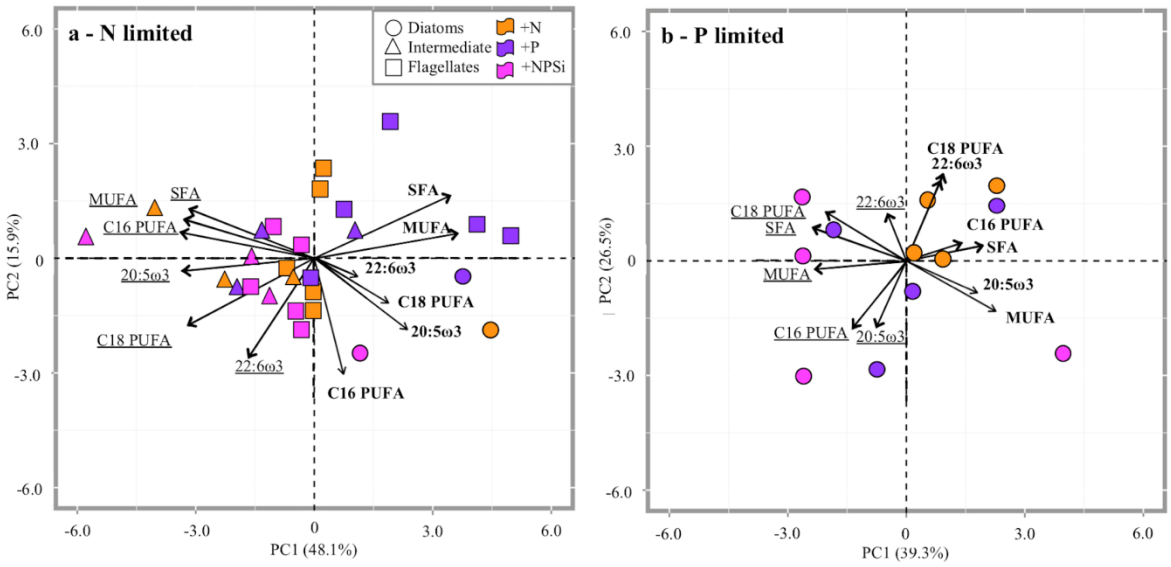
778

779 Fig.4


a - biosynthesis
b - concentration
Diatoms
Intermediate
Flagellates
not limited
N (co-) limited
P (Si co-) limited
PC1 (30.7%)
PC2 (24.1%)
PC1 (33.6%)
PC2 (28.3%)
SFA
MUFA
C16 PUFA
C18 PUFA
22:6ω3
20:5ω3

780

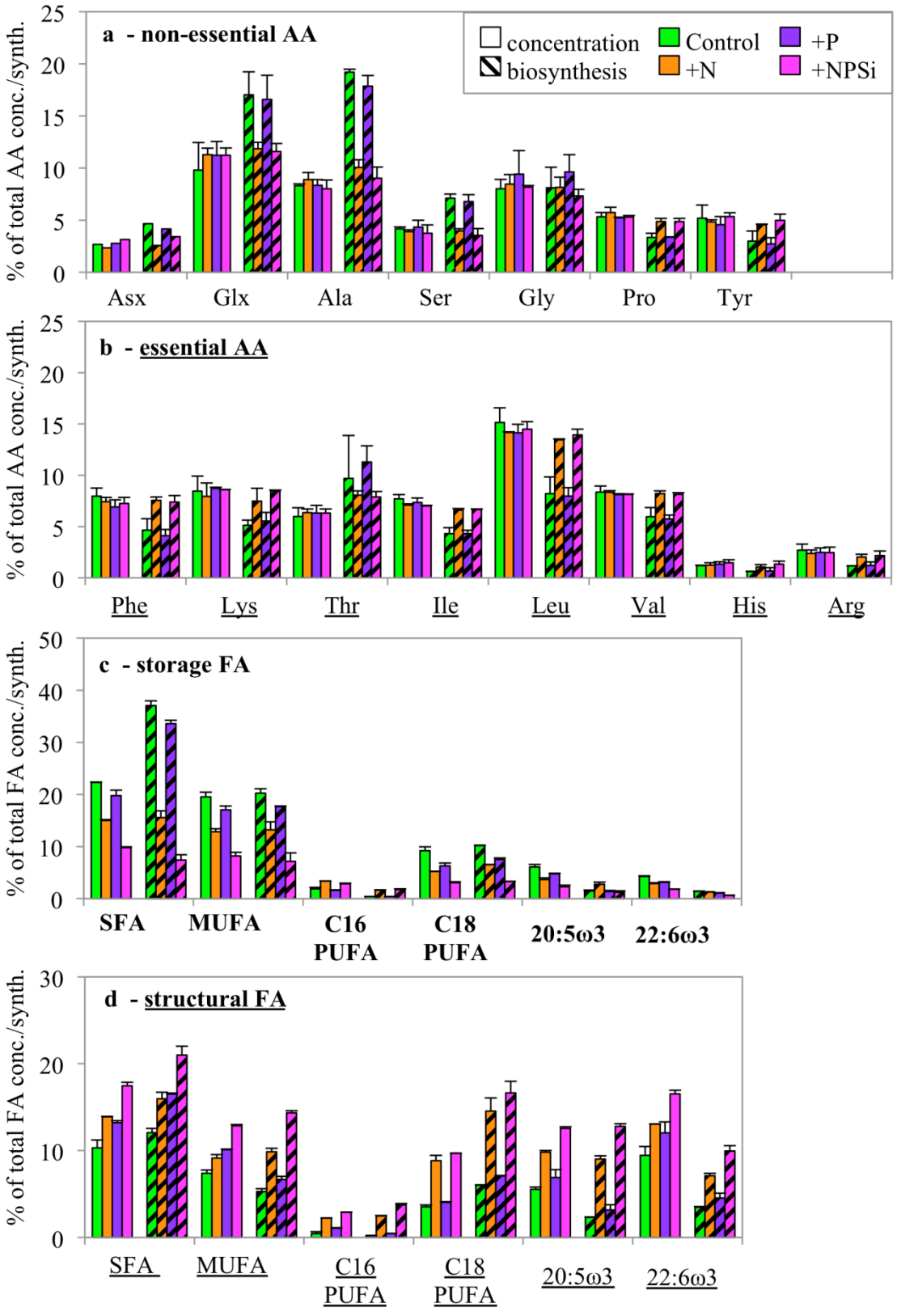
781 Fig. 5



782

783 Fig. 6

784


a - non-essential AA
concentration
biosynthesis
Control
+N
+P
+NPSi
% of total AA conc./synth.
Asx
Glx
Ala
Ser
Gly
Pro
Tyr
b - essential AA
Phe
Lys
Thr
Ile
Leu
Val
His
Arg
c - storage FA
% of total FA conc./synth.
SFA
MUFA
C16 PUFA
C18 PUFA
20:5ω3
22:6ω3
d - structural FA

785

786 Fig. 7

Table 1: Overview of nutrient concentrations and resulting DIN:DIP ratios at each station as determined in Grosse et al. (2017). Station # can be used to identify stations in PCA plots. DIN = nitrate + nitrite + ammonia. <DL = below detection limit, for $Si(OH)_4$ limit is 0.03 µM.

Month (Stn. #)	DIN [µM]	PO_4 [µM]	$Si(OH)_4$ [µM]	DIN:DIP
Coastal Zone (CZ; 5.150° E/ 53.401°N)				
March (1)	38.5	0.71	16.6	54
April (2)	16.3	0.05	0.13	325
Mid May (3)	9.56	0.05	0.37	191
End May (4)	5.64	0.09	1.1	63
August (5)	0.39	0.02	0.37	20
Oyster Ground (OG; 4.330° E/ 54.130°N)				
March (6)	9.90	0.54	6.3	18
April (7)	1.09	0.04	< DL	27
Mid May (8)	1.28	0.16	1.4	8
End May (9)	0.48	0.03	0.77	16
August (10)	0.12	0.13	2.2	1
Dogger Bank (DB; 3.150° E/ 55.170°N)				
March (11)	5.32	0.54	4.7	10
April (12)	0.27	0.14	0.03	2
Mid May (13)	0.21	0.06	0.09	4
End May (14)	0.10	0.09	0.44	1
Central North Sea (CNS; 2.165° E/ 56.579°N)				
March (15)	6.51	0.53	2.5	12
April (16)	0.24	0.16	0.28	2
Mid May (17)	1.28	0.17	0.84	8
August (18)	0.10	0.02	0.76	5

795 **Table 2:** The table summarizes treatments that responded to nutrient addition by increasing
796 relative biosynthesis of amino acids as shown in Grosse et al. 2017. Listed first is the treatment
797 with the highest increase in AA biosynthesis (compared to un-amended control), followed by
798 treatments with equal increase (=) of lower increase (>). Additionally, listed are the derived
799 nutrient limitations prevailing at each station and the phytoplankton community structure which
800 has been determined in Grosse et al. (2017) using individual structural FA.
801 Data included in different PCA datasets:
802 [*]Included in Fig. 4a, Control, +N, +P, +NPSi treatment
803 [†]Included in Fig. 4b, Control, +N, +P, +NPSi treatment
804 [‡]Included in Fig. 6a; differences Control to +N, Control to +P, Control to +NPSi treatment
805 [$]Included in Fig. 6b; differences Control to +N, Control to +P, Control to +NPSi treatment
806

Month (Stn. #)	Increase in relative AA biosynthesis	Inferred nutrient limitation	Phytoplankton community
Coastal Zone (CZ)			
March (1) [†]	---	---	Diatom
April (2) [†$]	+NPSi > +P (after 72h)	P/Si	Diatom
Mid May (3) [†$]	+NPSi > +P (after 72h)	P/Si	Diatom
End May (4) [†$]	+NPSi> +P	P/Si	Diatom
August (5) [*‡]	+N = +NPSi	N	Diatom
Oyster Ground (OG)			
March (6) [†]	---	---	Mixed
April (7) [†$]	+NPSi > +N = +P	Si/N/P	Diatom
Mid May (8) [†]	---	---	Mixed
End May (9) [*‡]	NPSi > +N	N/Si	Flagellate
August (10) [*‡]	+N = +NPSi	N	Flagellate
Dogger Bank (DB)			
March (11) [†]	---	---	Mixed
April (12) [*‡]	+NPSi > +N	N/Si	Mixed
Mid May (13) [*‡]	+N = +NPSi	N	Flagellate
End May (14) [*‡]	+N = +NPSi	N	Flagellate
Central North Sea (CNS)			
March (15) [†]	---	---	Mixed
April (16) [*‡]	+N = +NPSi	N	Flagellate
Mid May (17) [*‡]	+N = +NPSi > +P	N/P	Mixed
August (18) [*‡]	+N = +NPSi	N/Si	Flagellate

807
808

Supplemental Information

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

Julia Grosse, Corina P.D. Brussaard and Henricus T. S. Boschker

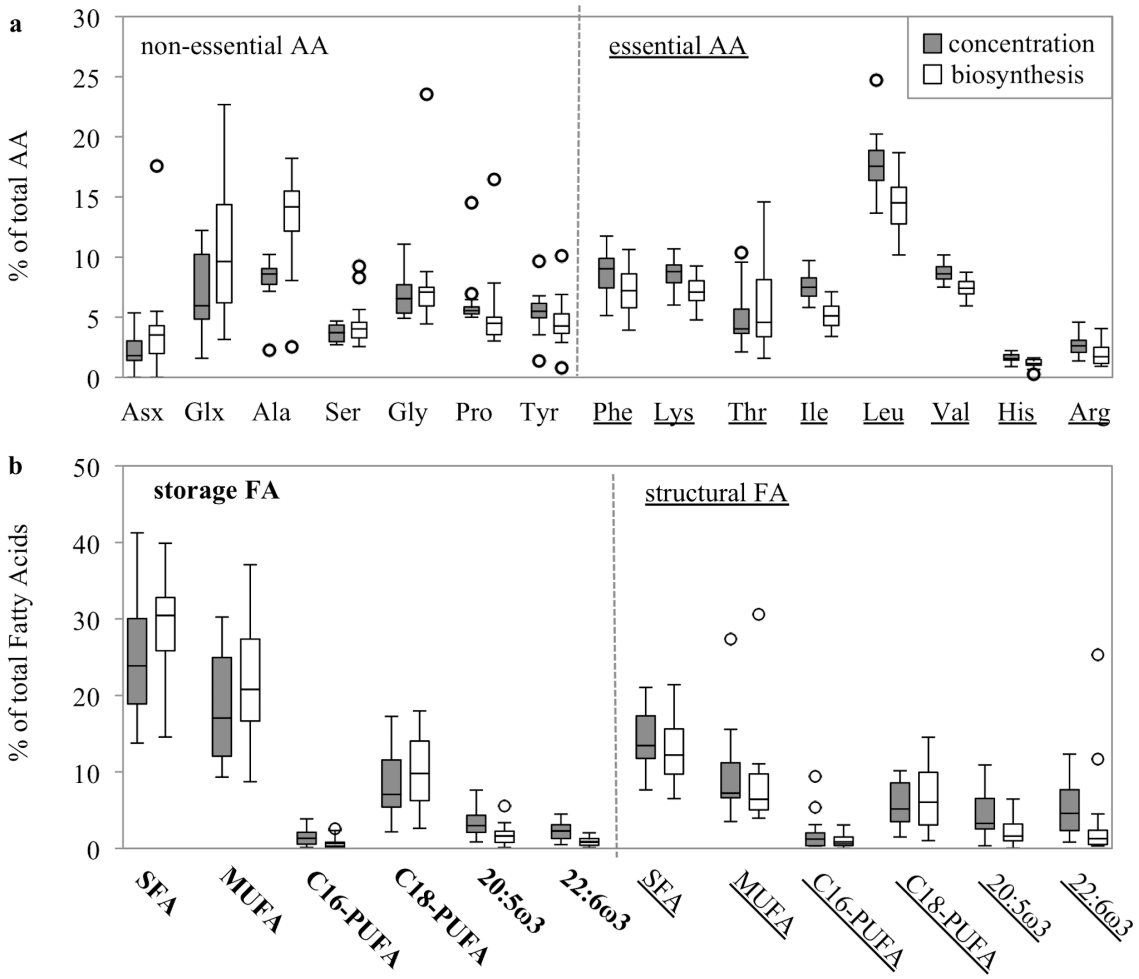


Fig. S1: Box-plot showing the contribution of individual amino acids to total amino acids in C (a) contribution of fatty acid groups to total fatty acids in C (b). Grey bars represent concentration data and white bars the biosynthesis data for un-amended incubations (Control) from all cruises and stations (n = 36, including duplicates). Horizontal lines are medians, boxes show the interquartile range (IQR), error bars show the full range excluding outliers (circles) defined as being more than ± 1.5 IQR outside the box. Essential AA and structural FA were underlined, storage FA are shown in bold. SFA = saturated FA, MUFA = mono-unsaturated FA, C16-PUFA/ C18-PUFA = poly-unsaturated FA containing 16 and 18 C atoms, respectively. Circles represent outliers.

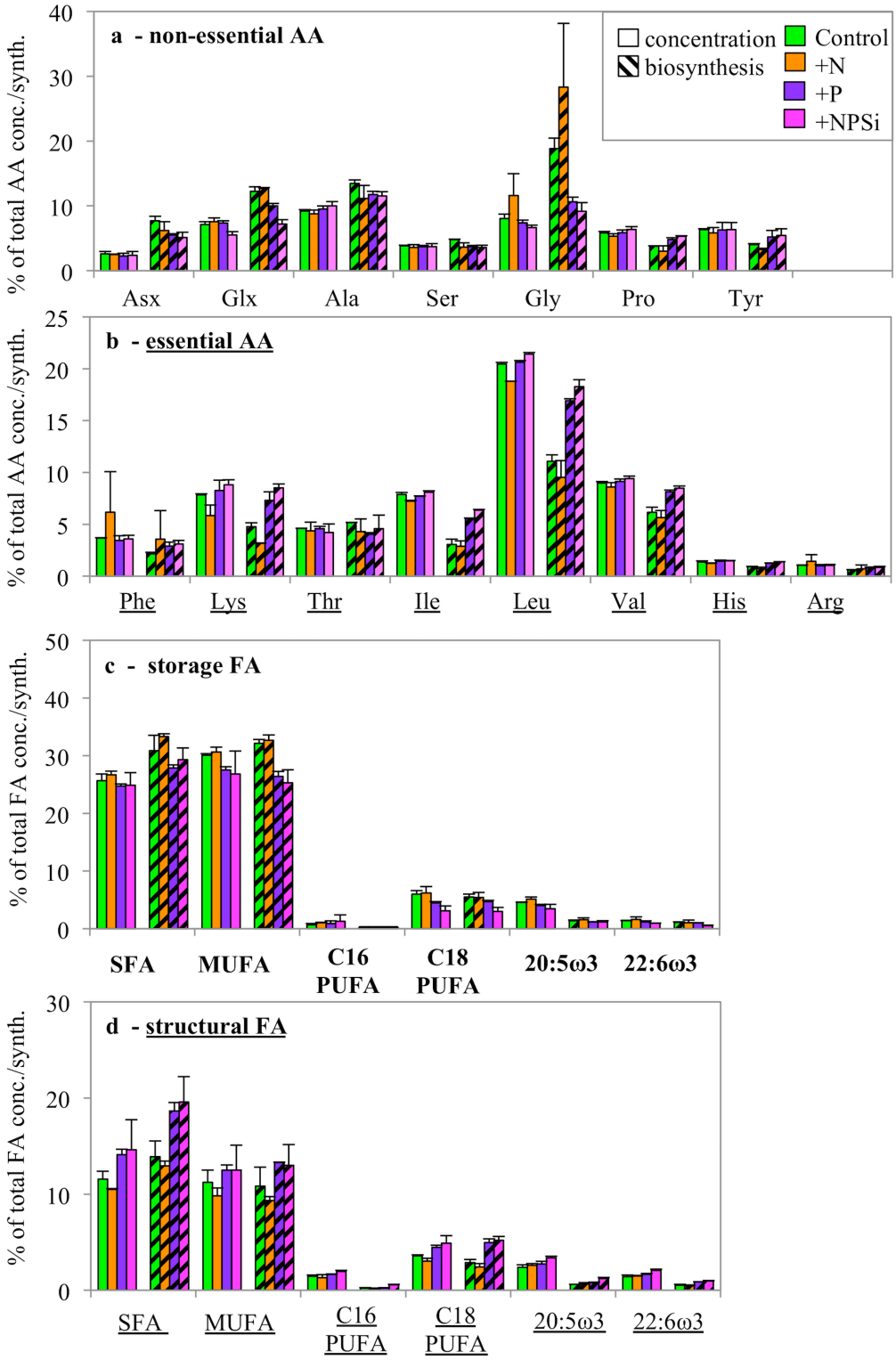


Fig. S2: Distribution of individual AA and FA groups in concentrations (open bars) and biosynthesis (striped bars) after 72h at the Coastal Station in April (Stn. 2, P/Si limited, diatom dominated). Shown are averages ± standard deviation, $n = 2$.