Carbon and nitrogen fluxes associated to marine and estuarine phytoplankton

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Till mina nära och kära

Abstract

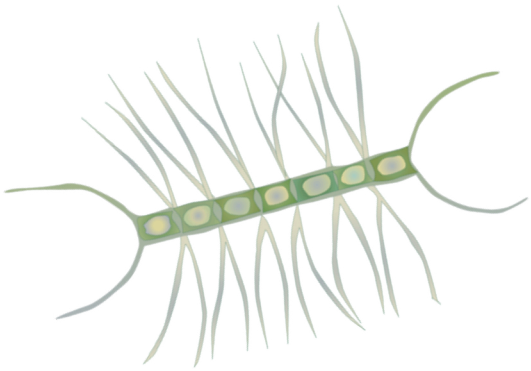
Globally, mainly nitrogen or phosphorus is limiting the primary production. New nitrogen can enter estuarine ecosystems as nitrate from upwelling events, from river runoff, atmospheric deposition, or by nitrogen fixation. Primary production driven by new sources of nitrogen is generally referred to as new production and suggested to equal the size of export production. Nitrate-based new production has been reported to range between 8 % and 40 % in tropical and temperate regions. Regenerated sources of nitrogen can be either ammonium or urea, recycled within the euphotic zone. Biological available phosphorus usually occurs as orthophosphate, entering the euphotic zone from river runoff, upwelling events or recycled within the pelagic zone. Carbon is biologically available mainly as dissolved carbon dioxide or as bicarbonate, and is usually not limiting in the euphotic zone. By using a combination of stable isotopic tracers, secondary ion mass spectrometry (SIMS) and elemental analysis isotope ratio mass spectrometry (EA-IRMS), we determined species-specific contributions to the total carbon and nitrogen assimilation rates and, thus, linked small- and large-scale fluxes within phytoplankton communities under varying abiotic conditions.

Every summer, extensive blooms of filamentous cyanobacteria occur in the Baltic Sea. We revealed that *Aphanizomenon* sp. with its long growth season and high biomass contributed with up to 80 % to the overall nitrogen fixation, even though *Nodularia spumigena* and *Dolichospermum* spp. had higher specific nitrogen fixation rates. The cyanobacteria contributed to the overall carbon fixation by 20 %, i.e. the new production in the area during summer. With lower fixation rates at the offshore station as compared to the coastal, we suggest phosphorus-limitation. In a laboratory study using natural Baltic Sea water, we demonstrated that the toxic cyanobacterial species *N. spumigena* and total nitrogen fixation increased exponentially when amended with small pulses of phosphate (1 μM). Differences in phosphorus storage capacity and affinity for ammonium were observed between strains.

Diatoms represent another functional phytoplankton type, which is key in nitrate-based new production in the pelagic ecosystem, producing 20 % of the oxygen on the planet. They can use either nitrate or ammonium as nitrogen source. During late summer on the Swedish west coast, nitrate-based new production ranged between 12 % and 27 %. The large chain-forming diatoms comprised 7 % of the carbon biomass, but assimilated 54 % of the available nitrate and 30 % of the ammonium. Their ammonium assimilation exceeded the diffusion-limited supply by 4.4 times, suggesting microbial interaction within the phycosphere to facilitate ammonium uptake. In a phytoplankton community in the tropical Mozambique, the nitrate-based

new production was 10 % of the total primary production and varied largely between tides. In order to address diversity in nutrient demands in the diatom *Skeletonema marinoi* across a century of increased eutrophication, we revived 80 and 15 yrs old resting stages. The carbon and nitrate assimilation correlated significantly within strains, but with a very large diversity at single cell level within and between strains independent of age. We suggest this diversity as a key to the large success by *S. marinoi* when spreading into new areas and being resistant to environmental changes. This thesis will contribute to the quantitative understanding of how tidal mixing, eutrophication, nitrogen fixation, and nitrate- and phosphate-limitation impact primary production in various estuarine ecosystems.

Keywords: Nutrient assimilation; Stable isotopes; Single cell diversity; Phytoplankton



Populärvetenskaplig sammanfattning

I min avhandling har jag och mina medförfattare studerat kol- och kväveflöden förknippade med vattenlevande växtplankton. Växtplankton är mikroskopiska små organismer i havet som precis som växter på land tar upp koldioxid och vatten och omvandlar det till organiskt material medan de släpper ut syre. Globalt sett är det framförallt näringsämnena kväve och fosfor som begränsar deras tillväxt. De behöver även oorganiskt kol i form av löst koldioxid eller bikarbonat för att växa, men det är sällan begränsande i havet. Kväve är tillgängligt i havet i form av nitrat och ammonium, och för vissa arter av cyanobakterier även som löst kvävgas, genom kvävefixering. Förhållandet mellan primärproduktionen baserad på nitrat eller löst kvävgas gentemot ammonium kallas för ny produktion, och den brukar kopplas till mängden organiskt material som transporteras ned i djuphaven. Studierna i denna avhandling har alla inkluderat mätningar av ny produktion i form av nitratupptag eller kvävefixering.

För att följa kol- och kväveupptaget hos växtplankton har vi använt oss av något som kallas stabila isotoper. Stabila isotoper är varianter av grundämnen med olika antal neutroner, där stabila former är de som inte sönderfaller (så kallade radioaktiva). Genom att tillsätta stabila isotoper av kol och kväve (i form av löst kvävgas, ammonium och nitrat) och sedan spåra dessa har vi kunnat mäta näringsupptaget hos växtplankton i våra studier. Vi har gjort mätningar på samhällsnivå, klon-nivå, och även individuell cellnivå med hjälp av dessa metoder. Att kunna mäta upptag i enstaka celler möjliggör att komma åt enskilda arter i ett blandat planktonsamhälle och därmed kunna mäta hur mycket de bidrar till den totala kolproduktionen och kväveupptaget. Därmed har vi även kunnat undersöka variationen i upptag mellan enskilda celler eller kloner för att visualisera den naturliga spridningen.

Under sommaren i Östersjön dominerar tre arter av filamentösa cyanobakterier, det är arter där cellerna sitter ihop som i långa kedjor, så kallade filament. Dessa har en fördel gentemot övriga växtplankton eftersom de kan fixera atmosfärens kvävgas (löst i havet). Sommartid kan dessa cyanobakterier växa till höga koncentrationer på grund av sin unika förmåga att fixera kvävgas samt genom gynnsamma förhållanden med relativt höga temperaturer och låga koncentrationer av tillgängligt kväve. Med en ökad tillgång till näringsämnen på grund av mänsklig aktivitet under det senaste århundrandet så har deras förekomst dessutom ökat kraftig. I media omtalas cyanobakterier ofta som ett problem i form av täckande mattor på badplatser eller som giftigt badvatten för hundar och barn. Det man inte skriver lika ofta är att de också är väldigt viktiga för Östersjöns ekosystem. Med sin unika möjlighet att ta upp sitt eget kväve, så släpper de också ut upp till 30 % av sin nyligen fixerade kvävgas i form av ammonium, vilket är biologiskt tillgängligt för många andra grupper av växtplankton.

Därmed så gynnar de även organismer uppåt i näringskedjan, till och med fiskproduktionen.

I en fältstudie undersökte vi hur de i Östersjön dominerande arterna av filamentösa cyanobakterier *Nodularia spumigena*, *Aphanizomenon* sp., och *Dolichospermum* spp. bidrog till det totala kväve- och kolupptaget från juni-augusti. Här såg vi att den art som totalt sätt bidrog till det mesta kväveupptaget var *Aphanizomenon* sp., som växer från tidig vår till slutet av sommaren. Vi kunde även påvisa att varken picocyanobakterier eller *Pseudoanabaena* utförde kvävefixering. Vi såg också att det var skillnad i upptagshastigheter av både kväve och kol när vi jämförde en lokal nära kusten med en lokal ute i öppet vatten. Denna skillnad tror vi berodde på tillgänglighet av fosfor och att detta var begränsande för kol- och kvävefixeringen ute på det öppna havet jämfört med vid den kustnära lokalen. I en uppföljning till fältstudien så undersökte vi under ett laborationsförsök hur den toxiska cyanobakteriearten *N. spumigena* påverkas av fosforbegränsning. Vi odlade två kloner av *N. spumigena* under fosforbegränsning (fosfor i form av fosfat) och undersökte hur deras kolupptag och kvävefixering påverkades. Vi använde fosfatkoncentrationer relevanta för sommarsituationen i Östersjön och såg att trots att vi bara tillsatte väldigt lite (upp till 1 μM) så kunde de ändå effektivt ta upp och använda fosfatet för exponentiell tillväxt. Vi såg även att det var en stor skillnad mellan klonerna när det kom till upptag av ammonium och kapacitet att lagra fosfat, vilket poängterar hur viktigt det är att använda mer än en klon i laborationsförsök.

Globalt sätt så är kiselalger en väldigt viktig grupp av växtplankton då de producerar upp till 20 % av syret på jorden. De är även viktiga då de med sina tunga skal av kisel sjunker och därmed transporterar organiskt material ner i djuphaven, den så kallade biologiska kolpumpen. Kiselalger kan använda både ammonium och nitrat som källa till kväve. För att studera storleken på ny produktion i en tropisk miljö, så utförde vi mätningar i fält av kol- och nitratupptag under fyra tidvattencykler i Maputobukten, tillhörande Mocambique. I kombination med upptagshastigheterna så identifierade vi även med hjälp av mikroskop de arter av växt- och djurplankton som fanns i vattnet under dessa mätningar. Mätningarna utfördes i februari, precis innan den årliga maximala tillväxten av växtplankton i denna bukt. Studier av detta slag är ytterst få i området, och därmed bidrar denna studie med ny information kring detta system. Vi fann även att proportionerna av så kallad ny produktion låg runt 10 %, vilket är liknande andra tropiska områden. Detta innebär att stor del av primärproduktionen drivs av återvunna kvävekällor, till exempel ammonium, som har en snabb omsättning i ytvattnet.

Flera arter av kiselalger bildar så kallade vilostadier mot slutet av en blomning när förhållandena i vattnet blir ogynnsamma, till exempel vid låg näring eller begränsning på ljus. Dessa viloceller bevaras sen i sedimenten tills förhållandena i omgivningen

blivit gynnsamma igen, och en ny blomning kan starta. Dessa viloceller går att isolera från sedimentkärnor och väcka till växande kiselalger på laboratoriet igen, och genom att datera sedimentet har man sett att de kan vara upp till hundra år gamla. I ett laborationsförsök använde vi oss av den vanligt förekommande kedjebildande kiselalgen *Skeletonema marinoi*. Vi isolerade totalt åtta kloner av 80 och 15 år gamla vilostadier från den danska Mariagerfjorden. Genom att kläcka dessa gamla vilostadier parallellt med vilostadier från ytligare lager kunde vi jämföra vad som hänt under nästan ett sekel. I fjorden har det under detta sekel varit en pågående övergödning. Syftet med denna studie var att mäta skillnader mellan dessa tidpunkter i form av näringsupptag och tillväxt hos kiselalgerna. Vi fann bara små skillnader mellan före och under den pågående övergödningen. Däremot hittade vi en omfattande diversitet mellan både kloner och individuella celler när det kommer till upptag av kol och nitrat. Denna stora diversitet i näringsbehov mellan individuella celler under seklet tror vi kan vara till en stor fördel hos kiselalgerna, då de med stor flexibilitet kan sprida sig till nya områden, samt vara motståndskraftiga mot förändringar i sin miljö.

I en ytterligare fältstudie undersökte vi ett blandat planktonsamhälle på den svenska västkusten under sensommaren. Samhället dominerades av kiselalger och dinoflagellater. Dinoflagellater är stora och olikformade växtplankton med två flageller som gör att de kan röra sig i vattnet. Många arter är även mixotrofa, i de arterna som vi fokuserat på så innebär det att de både utför fotosyntes (tar upp oorganiskt kol från vattnet och producerar syre), men även kan ta upp organiskt material genom att äta mindre organismer. Mätningen av kol- och kväveupptag (nitrat och ammonium) gjordes på individuell cellnivå för att särskilja olika arters upptag och behov. I de kedjeformande kiselalgerna såg vi att trots att de bara stod för 6 % av biomassan (kol), så bidrog de med 20 % av det totala kolupptaget och 54 % av det totala nitratupptaget. För en dominerande grupp dinoflagellater observerade vi det omvända mönstret, det vill säga stor del av biomassan men med ett litet upptag. Vi räknade även ut de olika dominerande arternas diffusions-begränsning, det vill säga den fysiska transport av näringsämnen som når cellerna. För nitratupptag var det faktiska och den beräknade upptaget ganska balanserat, medan för ammonium så tog de kedjeformande kiselalgerna upp 4.4 gånger mer än de beräknades kunna. Här föreslår vi att bakterier som lever i nära anslutning till de större organismerna kan ha en positiv inverkan genom att öka tillgängligheten av kväve. Att mäta upptag i ett blandat växtplanktonsamhälle på detta vis är något som ger unika möjligheter. Det gör att det går att mäta aktivitet hos arter som inte går att odla i kulturer, samt utföra studier i arters naturliga miljö och därmed observera deras naturliga behov och beteende.

Sammanfattningsvis så kan min avhandling bidra med viktig kunskap kring växtplankton och deras roll i naturliga planktonsamhällen, både runt Sverige och i tropiska vatten. Resultaten från våra artiklar visar på växtplanktons enorma variation

från artnivå ner till individuell cellnivå, och hur denna diversitet delvis kan förklara att vissa arter är så framgångsrika i att överleva och sprida sig till nya områden. Vi visar också att många arter är extremt effektiva på att ta upp näring, till exempel många kiselalger, och att andra är väldigt viktiga för omgivande arter genom att dela med sig av näring, exempelvis många filamentösa cyanobakterier. Genom att ha utfört studier i temperaturer mellan 13-32°C, så ser vi att det framförallt är näring, snarare än temperatur som styr tillväxthastigheten hos de växtplankton vi har undersökt.

Nyckelord: näringsupptag, stabila isotoper, diversitet, växtplankton

List of papers

The thesis is based on the following papers:

Paper I: Klawonn I, Nahar N, Walve J, Andersson B, **Olofsson M,** Svedén BJ, Littmann S, Whitehouse MJ, Kuypers MMM, Ploug H (2016) Cell-specific nitrogen- and carbon-fixation of cyanobacteria in a temperate marine system (Baltic Sea). Environ Microb 18(12): 4596-4609

Paper II: **Olofsson M**, Egardt J, Singh A, Ploug H (2016) Inorganic phosphorus enrichments in Baltic Sea water have large effects on growth, carbon fixation and N_2 fixation by *Nodularia spumigena*. Aquat Microb Ecol 77: 111-123

Paper III: **Olofsson M,** Karlberg M, Lage S, Ploug H (2017) Phytoplankton community composition and primary production in the tropical tidal ecosystem, Maputo Bay (the Indian Ocean). J Sea Res 125: 18-25

Paper IV: **Olofsson M**, Kourtchenko O, Zetsche E-M, Marchant HK, Whitehouse MJ, Godhe A, Ploug H. A century of evidence: Single cell diversity as a key for growth and success of a common coastal diatom in changing environments. *Under review.*

Paper V: **Olofsson M,** Robertson EK, Edler L, Whitehouse MJ, Ploug H. CO_2 sequestration can be mediated by a small, fast growing standing stock of chain-forming diatoms under nutrient limitation in the sea. *Manuscript.*

My contributions to the papers: (I) – Participating in fieldwork in June, July and August 2012, responsible for part of microscopy analyses, minor part in writing. (II, III, IV, V) – Main part in experimental design and implementation, main responsibility in data collection and analysis, and major part of writing.

Publications not included in the thesis:

Scientific papers

Olofsson M, Torstensson A, Karlberg M, Steinhoff FS, Dinasquet J, Riemann L, Chierici M, Wulff A. Limited response of cyanobacteria to elevated temperature and pCO_2 in an estuarine spring bloom scenario. *Under review.*

Wulff A, Karlberg M, **Olofsson M**, Torstensson A, Riemann L, Steinhoff FS, Mohlin M, Ekstrand N, Chierici M (2018) Ocean acidification and desalination: climate-driven change in a Baltic Sea summer microplanktonic community. Mar Biol 165: 63

Eriander L, Infantes E, **Olofsson M**, Olsen JL, Moksnes P-O (2016) Assessing methods for restoration of eelgrass (*Zostera marina* L.) in a cold temperate region. J Exp Mar Biol Ecol 479: 76-88

Olofsson M, Asplund M E, Karunasagar I, Rehnstam-Holm A-S, Godhe A (2013) *Prorocentrum micans* promote and *Skeletonema tropicum* disfavours persistence of the pathogenic bacteria *Vibrio parahaemolyticus*. Indian Journal of Geo-Marine Sciences 42(6): 729-733

Popular science paper

Olofsson M, Ploug H (2017) Nya metoder avslöjar cyanobakteriernas roll i Östersjön. Havsutsikt 1: 10-12

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Abbreviations

ATP	Adenosine Triphosphate
cf.	compare (latin *confere*)
CCM	Carbon Concentrating Mechanisms
d	day/days
DIC	dissolved inorganic carbon
DIN	dissolved inorganic nitrogen
EA-IRMS	Elemental Analysis - Isotope Ratio Mass Spectrometry
f-ratio	Use of new nitrogen in relation to total nitrogen assimilation
GC-IRMS	Gas Chromatography - Isotope Ratio Mass Spectrometry
MIMS	Membrane Inlet Mass Spectrometry
g	gram
h	hour/hours
m	meter
mm	millimetre
nm	nanometer
N. spumigena	*Nodularia spumigena*
nifH	nitrogenase gene
POC	particulate organic carbon
PON	particulate organic nitrogen
POP	particulate organic phosphorus
RUBISCO	Ribulose bisphosphate carboxylase/oxygenase
S. marinoi	*Skeletonema marinoi*
SIMS	Secondary Ion Mass Spectrometry
sp./spp.	species
yr/yrs	year/years
µm	micrometer

1. The aims of the thesis

About half of the oxygen on the planet is derived from primary production in aquatic environments. Nitrogen is globally the growth-limting nutrient in marine ecosystems, and bioavailable sources are nitrate and ammonium. Also phosphorus can locally limit the production, and the nutrient fluxes are, thus, a major key for phytoplankton dynamics. In this thesis, the aims are to track fluxes of nitrogen and carbon in order to determine surface dominating processes and limiting factors for the primary production. Using incubations with stable isotopic tracers throughout the thesis, carbon and nitrogen assimilation and fixation have been quantified on community, species, strains, and single cell level under variable conditions, and across different functional groups of phytoplankton. The specific aims of each paper were:

P**aper I**: The purpose was to reveal species-specific carbon and nitrogen fixation rates of Baltic Sea filamentous cyanobacteria, and their relative contribution to total carbon and nitrogen fixation within the phytoplankton community. We quantified cell-specific carbon and nitrogen fixation rates of the dominating species *Nodularia spumigena*, *Aphanizomenon* sp. and *Dolichospermum* spp., in addition to potential nitrogen fixation by the picocyanobacteria and *Pseudoanabaena* sp. In order to reveal potential regional and seasonal differences, this assay was performed at a coastal and offshore station during two summer seasons.

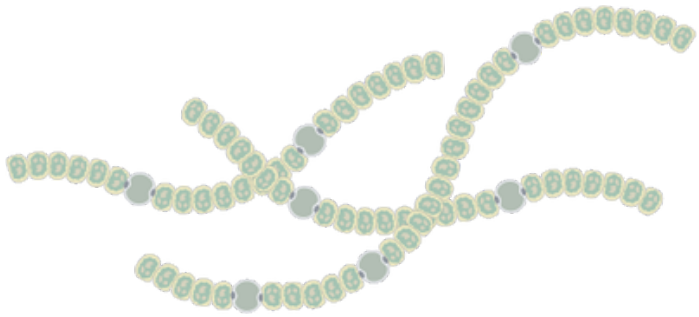
P**aper II**: The purpose was to quantify strain-specific differences in carbon and nitrogen fixation rates of *N. spumigena* under phosphorus-limitation. Further, the effects by phosphorus-limitation on heterocyst frequency and its correlation to nitrogen fixation, as well as the cellular carbon to nitrogen ratios were studied. In order to mimic natural conditions, the strains were inoculated into un-amended Baltic Sea water with small enrichments of phosphorus (up to 1 μM).

P**aper III**: The purpose was to quantify nitrate-based new primary production in a tropical ecosystem of Mozambique. We compared the phytoplankton community composition and the carbon and nitrate assimilation rates between spring-high and neap-low tide, during day and night, rough and calm conditions.

P**aper IV**: The purpose was to quantify the intraspecific diversity in recently revived resting stages of *Skeletonema marinoi* from young (15 yrs) and old (80 yrs) sediment layers. By comparing strain-specific and cell-specific carbon and nitrate assimilation rates during nitrate-limited and nitrate-replete conditions, the diversity in nutrient

demands across a century of increased eutrophication was revealed. Also, the dynamics of chain length were examined by comparing non-limited and limited nutrient conditions, hypothesizing that a decreased availability of nutrients may shorten the chains and, thus, increase their surface to volume ratio.

P**aper V**: The purpose was to quantify species-specific carbon, nitrate and ammonium assimilation rates. Thus, reveal the relative contribution to the total carbon and nitrogen assimilation in the mixed phytoplankton community during late summer in a temperate region. We quantifed nitrate-based new primary production, and the proportion to regenerated production, i.e. ammonium assimilation, in the diatom and dinoflagellate dominated community. We applied mass transfer theory, in order to reveal diffusion-limitation in large phytoplankton and potential microbial interactions in the mixed phytoplankton community.



2. Introduction

2.1 Nitrogen cycling and new production

Nitrogen is essential for all living organisms, and bioavailable forms are globally limiting the primary production (Moore et al. 2013, Kuypers et al. 2018). In photosynthetic organisms, nitrogen is needed in proteins, amino acids, and chlorophyll etc. Most photosynthetic organisms can take up inorganic nitrogen either as nitrate, i.e. new, or ammonium, i.e. re-generated sources. Ammonium is compared to nitrate less energetically costly for the organism, and its turnover rate is usually very high in the pelagial (Glibert & Goldman 1981, Adam et al. 2016, Bergkvist et al. accepted). As phytoplankton transform inorganic sources of nitrogen into organic, bacteria may recycle the organic matter within the euphotic zone into inorganic forms, i.e. ammonium, and, thus, available for phytoplankton again (Buchan et al. 2014). In contrast, bacteria may also remove bioavailable nitrogen from the ocean by denitrification, where nitrate is turned into nitrogen gas, and annamox, where nitrogen gas is produced by oxidation of ammonium using nitrite (Dalsgaard et al. 2003).

Since phytoplankton in the vast ocean lives in a very nutrient-dilute environment, they have evolved extremely efficient ways of assimilating nutrients, e.g. by diffusion, active transport, or both. Nitrate occurs in concentrations from undetectable up to 50 μM in the open ocean (Gruber 2008) but can be higher in coastal areas affected by anthropogenic inputs. Winter concentrations, however, can be 3-5 μM in the Baltic Sea (Larsson et al. 2001) up to 90 μM in coastal regions including eutrophicated fjords, e.g. the Danish Mariager Fjord (Sildever et al. 2016). Ammonium generally occurs in low concentrations, from undetectable up to 2 μM, except in polluted areas (Collos & Berges 2003) but has a very high turnover rate (Glibert & Goldman 1981). The balance between nitrate and ammonium assimilation is species-specific, where high concentrations of ammonium may suppress the nitrate assimilation in some organisms (Glibert et al. 2016). In a future ocean, the global nitrogen cycle is predicted to undergo some changes, including increased nitrogen fixation and decreased availability of nitrate (Hutchins & Fu 2017). Also, the general trend of fertilizers is changing from oxidized forms of nitrogen, as nitrate, towards reduced forms, as ammonium and urea, which may affect phytoplankton mainly sustaining on nitrate as a nitrogen source negatively (Glibert et al. 2006, 2016).

New nitrogen can enter the euphotic zone, either as nitrate from upwelling events or river runoff, atmospheric deposition, or as nitrogen-gas dissolved in the water, and fixed and reduced to ammonium by mainly cyanobacteria (Figure 1, Sohm

et al. 2011). Primary production driven by new nitrogen might be referred to as new production and is suggested to be directly related to the size of export production. However, in some areas the amount of nitrification needs to be considered (Yool et al. 2007, Raes et al. 2015), as well as lateral transport (Plattner et al. 2005). The use of "new" sources of nitrogen in proportion to total production can be calculated as f-ratio (Dugdale & Goering 1967, Eppley & Peterson 1979). In general, high f-ratios are typical for ecosystems dominated by large eukaryotic phytoplankton such as diatoms grazed by zooplankton, and low f-ratios are generally associated to oligotrophic food webs, consisting of small prokaryotic phytoplankton (Laws et al. 2000, Dunne et al. 2005). The proportion of nitrate-based production in tropical and temperate regions mainly ranges between 8-40 % (Dugdale & Goering 1967). In areas with low inorganic nitrogen availability, nitrogen-fixing organisms are in favor and can bypass the limitation, in contrast to microorganisms being dependent on nitrogen forms as nitrate or ammonium. Thus, nitrogen fixation can be a substantial part of the new production (Karl et al. 2002), especially in tropical and subtropical areas (Capone et al. 2005).

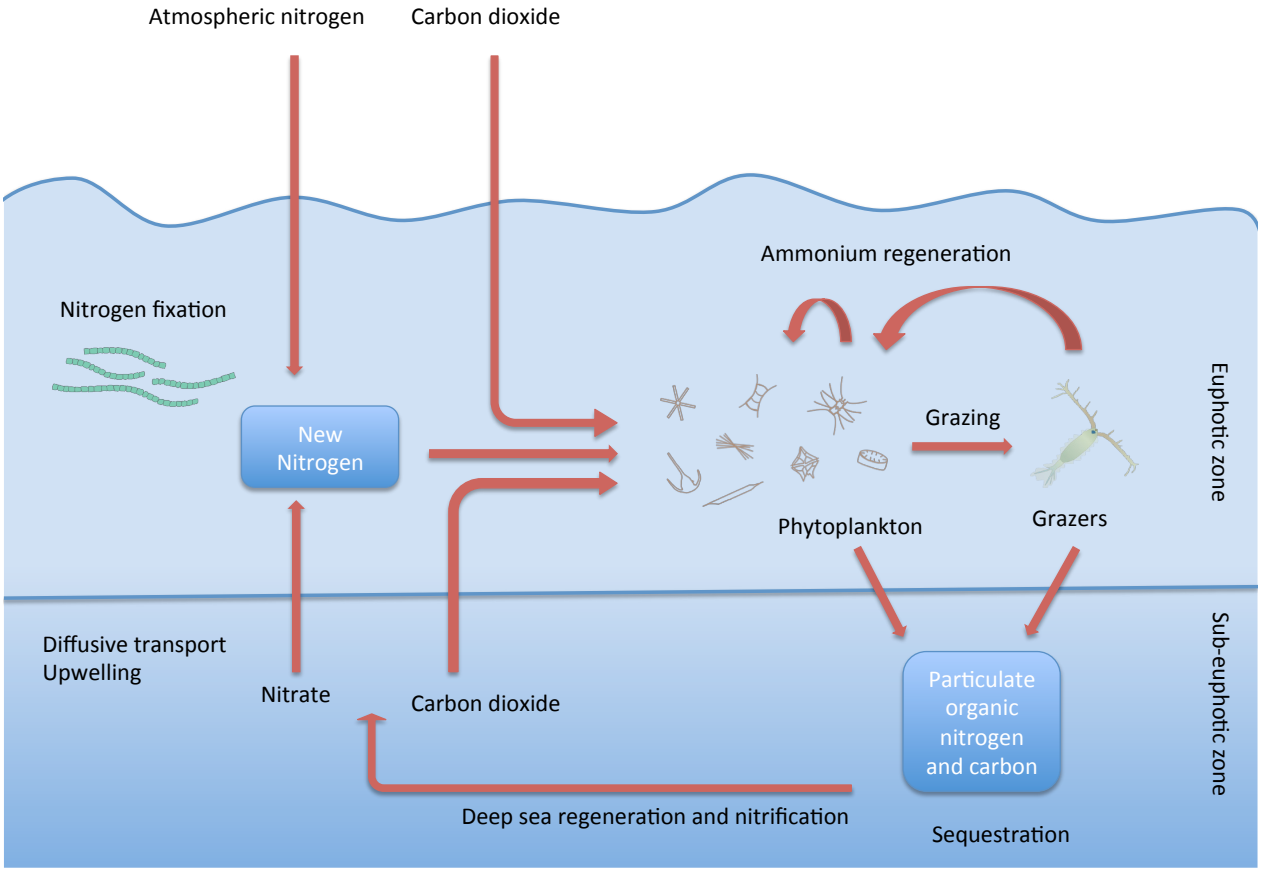


Figure 1. New nitrogen can enter the euphotic zone either as dissolved from the atmosphere, by nitrogen-fixing organisms, or as nitrate from upwelling events or river runoff. Regenerated sources of nitrogen are mainly by ammonium, or urea. Carbon dioxide is derived from the atmosphere or from respiration by primary producers, bacteria etc. Adapted from Sohm et al. 2011.

Based on nitrogen-fixing filamentous cyanobacteria, the new production in the Baltic Proper during the summer bsloom was quantified to ca. 20 % (**Paper I**). In the tropical Mozambique, however, the nitrate-based new production ranged between 2-10 %, (**Paper III**). In a mixed community during late summer on the Swedish west coast, the new production based on nitrate assimilation ranged between 12-27 % (**Paper V**).

An efficient way of tracking nitrogen fluxes in the euphotic zone is by using stable isotopes (See stable isotope section). By combining the conventional method with Secondary Ion Mass Spectrometry (SIMS), rates can be measured on cell-specific level and reveal relative contributions by organisms in a mixed field population (**Paper I** and **Paper V**). Here, nitrate and ammonium assimilation as well as nitrogen fixation rates can be quantified.

2.2 Carbon cycling

In the ocean, inorganic carbon is transferred from the atmosphere, dissolved into the water and transformed into organic matter by e.g. phytoplankton during photosynthesis. The organic matter can then either be grazed by zooplankton within the euphotic zone or recycled by bacteria and, thus, respired back into carbon dioxide. The zooplankton also produces fecal pellets that sinks, thus, transfer organic matter down to the deep ocean. Some of the organic matter produced during photosynthesis might aggregate and sink, where a large fraction is reminerelized into carbon dioxide by bacteria during the transport, and the remaining part that reach the sediment can be stored for a long time, known as carbon sequestration. This latter process is referred to as the biological pump (Figure 2). Photosynthesis is connected to respiration, where energy is released and can be used as fuel in the organism. The inorganic carbon assimilated into organic matter during photosynthesis is referred to as net-fixation, and together with the carbon released during respiration gross-fixation.

Respiration losses of carbon in phyoplankton have been reported to ca. 9 % of photosynthesis during exponential growth phase, and 22 % during stationary growth phase (López-Sandoval et al. 2014). With a large variation among taxa, respiration rates of 0.01-0.60 d^{-1} have been suggested, but commonly around ca. 0.20 d^{-1} under nutrient replete conditions (Geider & Osborne 1989), and to range between 10-15 % of gross photosynthetic rates (Raven & Beardall 2016). As organic matter sinks into the deep ocean, carbon-specific respiration rates in phytoplankton aggregates have been reported to 0.03 d^{-1} at 4°C (Iversen & Ploug 2013), and globally, bacterial respiration sometimes exceeds phytoplankton production (del Giorgio et al. 1997).

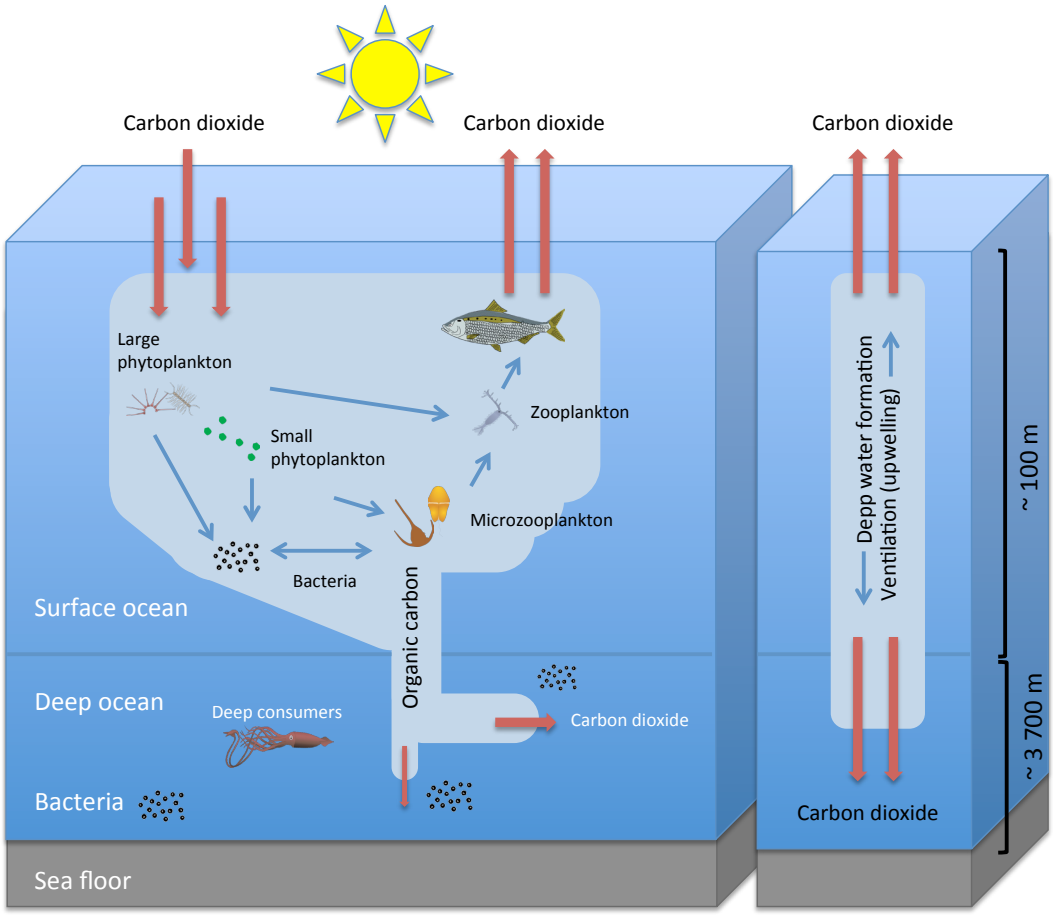


Figure 2. The biological pump (left panel) is driven by the photosynthetic based food web. Here, carbon dioxide is dissolved into the ocean and assimilated during photosynthesis and transformed into organic material. This organic carbon can either be grazed by zopplankton or recycled by bacteria within the photic zone, and respired back into carbon dioxide. Alternatively, it can be aggregated and transported down to the seafloor, where most of it is remineralized back into carbon dioxide by bacteria, and the residual fraction might be stored in the sediment for a long time, known as carbon sequestration. The solubility pump (right panel) is driven by chemical and physical processes, maintaining a gradient of carbon dioxide between the atmosphere and the deep oceans. Adapted from Chisholm 2000.

The ocean works as a large carbon sink and is central in the global carbon budgets when predicting future effects by the ongoing climate change. Due to the carbonate system, the ocean works as a large buffering system for the increased carbon dioxide levels, but if reaching too high concentrations it may have devastating effects on the life in the ocean. The most common source of bioavailable carbon in the ocean at pH around 8, is bicarbonate, followed by dissolved carbon dioxide. Inorganic carbon is rarely limiting in the euphotic zone in relation to nutrients like nitrogen and phosphorus. However, even with an increased level of carbon dioxide in the water, there is a predicted decrease in microbial photosynthesis and vertical transport due to

changes in community composition, temperature, nutrient availability and stratification (Hutchins & Fu 2017). Direct effects from decreased pH due to elevated carbon dioxide levels are species-specific and hard to differentiate from co-occurring climate related changes. Also, as the diel cycle of carbon dioxide and pH fluctuates with the photosynthesis during primary production, many organisms are acclimated to natural fluctuations (Wulff et al. 2018). Large fluctuations in pH have also been reported in aggregates of *Trichodesmium*, thus, potentially less affected by predicted changes in pH from elevated CO_2 levels (Eichner et al. 2017).

Even though inorganic carbon is rarely limiting in the ocean, the intracellular competition between carbon dioxide and oxygen has resulted in the evolution of different types of carbon concentrating mechanisms (CCMs) in many phytoplankton species (Giordano et al. 2005). These mechanisms may increase the concentration of carbon dioxide for the active sites of RUBISCO, and includes a large diversity of different types of CCMs in different organisms, where the modulation may be governed by environmental factors. The use of CCMs has also been discussed in terms of global climate change, with various predictions of the outcome depending on environmental factors and species examined (Kranz et al. 2011, Raven et al. 2011).

In all papers included in this thesis, the community, strain and/or single cell net carbon assimilation rates by the phytoplankton community has been quantified in various environments representing a vast range of conditions.

2.3 The study areas

This thesis includes field studies performed in various estuarine and marine environments. From the Baltic Sea on the east coast of Sweden (**Paper I**), to Maputo Bay in Mozambique (**Paper III**), and to the Gullmar Fjord on the west coast of Sweden (**Paper V**). In addition, it includes laboratory experiments using two strains of *Nodularia spumigena* isolated from the Baltic Sea (**Paper II**), and newly revived resting cells of *Skeletonema marinoi* hatched from sediment cores, collected in the Danish Mariager Fjord (**Paper IV**).

2.3.1 The Baltic Sea, Sweden

The Baltic Sea is one of the largest brackish water bodies in the world, together with the Black Sea and the Caspian Sea (Snoeijs-Lejonmalm & Andrén 2017). It consists of several basins and is connected to the Kattegat by the Danish straits in southwest, but with narrow and shallow inlets it has limited exchange with the outer North Sea. Also, the Baltic Sea has more than 200 rivers that discharge into it, and thus, diluting the

salinity. The Baltic Proper is located in the central Baltic Sea, at the latitude of Stockholm, and has a salinity of 5-6 (Figure 3). Around 85 million people live in the catchment area of the Baltic Sea, resulting in a large pressure on the environment, e.g. by eutrophication from human activities, fishing, pollution etc. During the last century there has been an increased eutrophication of the Baltic Sea. In addition, the extended nitrogen fixation performed by the filamentous cyanobacteria during the summer blooms, contributes with a yearly input of nitrogen up to the size of the entire riverine load (480 Gg N yr^{-1}), and twice the atmospheric load (about 200 Gg N yr^{-1}) (Larsson et al. 2001, Wasmund et al. 2001, Moisander et al. 2007). Thus, the Baltic Sea is still considered eutrophied. The yearly spring bloom of diatoms removes most nitrate and phosphate from the surface water (Larsson et al. 2001), providing a niche for the summer blooms of nitrogen-fixing filamentous cyanobacteria. Also, with large parts of the bottom water being anoxic (Conley et al. 2009, Snoeijs-Lejonmalm & Andrén 2017), the eutrophication is further enhanced by the release of phosphorus from the sediments when oxygen concentrations are low. This creates a negative spiral, where the system accelerates itself (Box 1). The effects by phosphorus-limitation were addressed both in **Paper I** and **II**, where the field survey in **Paper I** were performed in the Baltic Proper and the latter under laboratory conditions using strains isolated from the Baltic Sea.

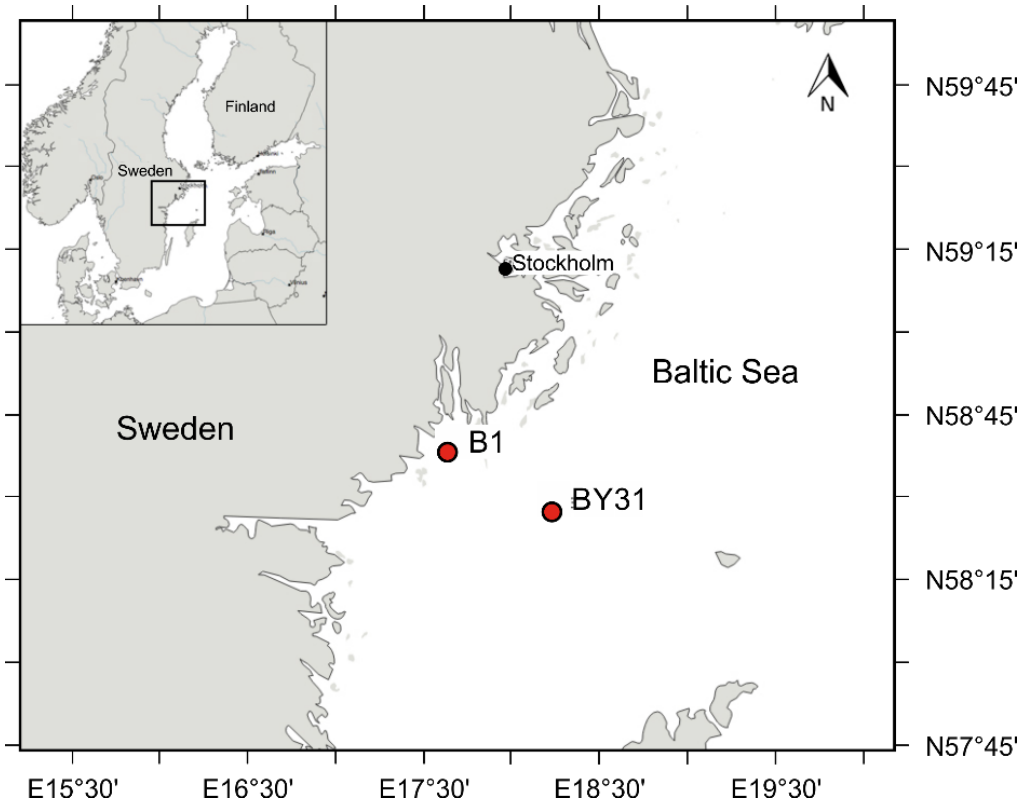


Figure 3. The insert shows the location of the Baltic Sea. The large map includes the Baltic Proper with the stations BY31 in the Landsort Deep, and B1, outside of Askö (map from **Paper I**).

2.3.2 Inhaca Island, Mozambique

The field survey of **Paper III** was performed in Maputo Bay outside Inhaca Island, located in southern Mozambique (Figure 4). The tidal differences in the bay between spring tide and neap tide are as high as 3 m and creates a large mixing (Canhanga & Dias 2005). In addition, salinity changes caused by freshwater input from the rivers increase the mixing in the bay (Markull et al. 2014). As a result, the visibility in the water is usually low as compared to the open ocean outside of the bay, due to re-suspended sediment in the water, especially at spring tide (de Boer et al. 2000).

The field study was performed in January and February, where the latter is regarded as the wettest and hottest month, and during the peak of the rain season (Raj et al. 2010). As a result of the rain season, all nutrients mobilized will enhance the highest phytoplankton concentrations during the year (Paula et al. 1998). *In situ* studies on primary production and phytoplankton community composition in the area are very scarce. Thus, **Paper III** was conducted during both high and low tide, to collect data with the aim to fill gaps on the primary production and phytoplankton species composition in the area during the biomass peak.

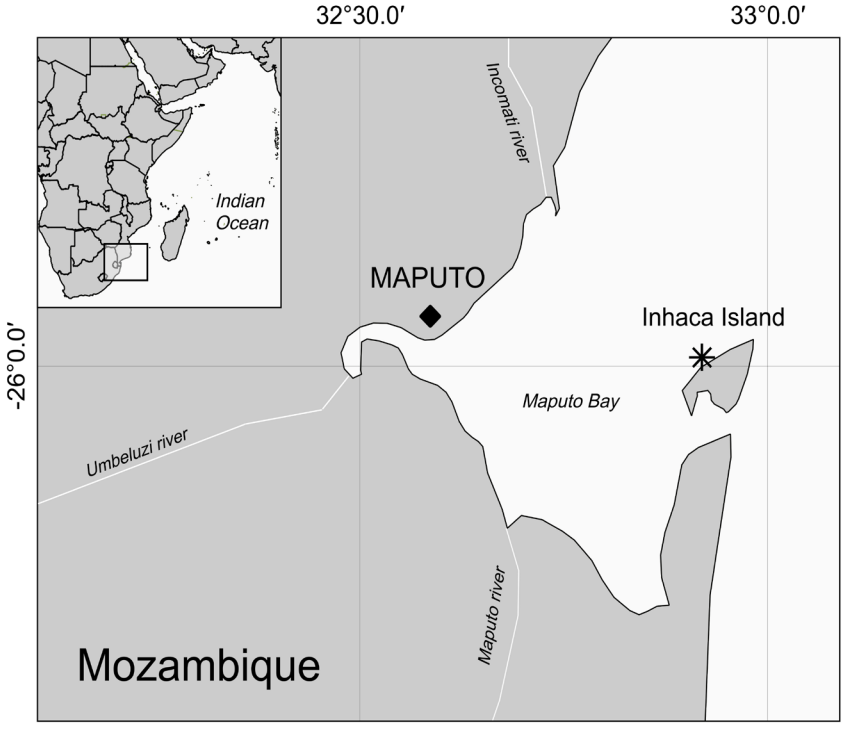


Figure 4. Map of Maputo Bay, located in southern Mozambique. Maputo city is placed on the left and Inhaca Island to the right of Maputo Bay. The Biological Station where the study of **Paper III** was performed is marked with a star, located on Inhaca Island, facing the bay.

2.3.3 Mariager Fjord, Denmark

The Danish Mariager Fjord (Figure 5) has experienced an increased load of anthropogenic nitrogen and phosphorus during the last century, and especially since the 1950s (Clarke et al. 2006). With nitrogen to phosphorus ratios of 30, wintertime nitrate concentrations in the fjord has been quantified to 90 μM (Sildever et al. 2016). Several coastal areas, including the Mariager Fjord, experienced extensive oxygen depletion during the 1980s, followed by legislative actions aiming to reduce the nutrient loading (Diaz & Rosenberg 2008, Fallesen et al. 2000). With oxygen depletion, the sediment has been preserved without any bioturbation, leaving resting cells/stages of phytoplankton stored, and can, thus, be isolated from distinct layers and hatched (Härnström et al. 2011). In the laboratory study of **Paper IV**, eight strains of the common diatom *Skeletonema marinoi* were revived from isotope-dated sediment cores, whereof four from old (80 yrs) and four from recent (15 yrs) layers, before and during the ongoing eutrophication.

Figure 5. Map of Denmark showing the location of the Mariager Fjord, with its entrance to the Kattegat Sea (**Paper IV**). Also, the Gullmar Fjord on the west coast of Sweden is indicated with a ring, with its entrance to the Skagerrak (**Paper V**).

2.3.4 The west coast of Sweden

In August and September 2017, two field surveys were performed where natural communities of phytoplankton were examined (**Paper V**). They were conducted in the Gullmar Fjord outside of Sven Lovén center for Marine Sciences, Kristineberg (Figure 5). The seawater including natural communities of phytoplankton were collected in the fjord near the coastal monitoring buoy 'Gullmarn' (N58°25'63, E11°45'14). The Gullmar Fjord is Sweden's only real fjord and is monitored by the Swedish Meteorological and Hydrological institute (SMHI) and the Sven Lovén center during most of the year. The Swedish west coast is influenced by surface currents from the Baltic Sea, decreasing the salinity, as well as by cold salty water from the North Sea increasing the salinity, resulting in an average of around 25 in the surface water. The fjord inhabits a diverse phytoplankton community, which during late summer and autumn often is a mix of dinoflagellates and diatoms (Tiselius et al. 2015).

2.4 The study organisms

With a focus on aquatic phytoplankton, this thesis includes a wide range of different taxa. **Paper I** includes a mixed natural population of nitrogen-fixing filamentous cyanobacteria, followed by two strains of the filamentous cyanobacteria species *Nodularia spumigena* in **Paper II**. Both **Paper III** and **V** include a mixed population of diatoms and dinoflagellates. In the laboratory study of **Paper IV**, the common diatom *Skeletonema marinoi* was in focus.

2.4.1 Phytoplankton functional types

During the last century, a puzzling question among aquatic ecologists has been referred to as the "plankton paradox" (Hutchinson 1961). The puzzling phenomenon many want to untangle is how diverse communities of phytoplankton can include a vast amount of coexisting species under nutrient limited conditions without going extinct. This perplex question has been widely speculated since it was first addressed, and with many suggested explanations. Hutchinson (1961) described several suggested factors resolving this paradox, e.g. vertical gradients, turbulence, light or symbiosis between organisms. In addition, one possible explanation is the "killing the winner" hypothesis, where species-specific grazing keeps the number of fast-growing species down. A recent modeling study that managed to keep a high diversity within a phytoplankton community included the species as individual cells, and also, enabled evolution within their test-community in order to succeed (Xue & Goldenfeld 2017).

Locally, there might be factors affecting the number of species. In the Baltic Sea, for example, the number of phytoplankton species is much lower as compared to the west coast of Sweden, and that is also true for larger organisms. This might be related to the salinity gradient in the Baltic Sea, complicating the life of many organisms. With the ability of nitrogen fixation and also being less grazed as compared to e.g. diatoms, a few species of the filamentous cyanobacteria species dominates the carbon biomass during summer in the Baltic Sea.

Generally, planktonic organisms have been divided into functional types based on a common trait, i.e. nitrogen-fixers, denitrifiers, nitrifiers, calcifiers, silicifiers etc. These groupings are often based on functionality, such as export of organic carbon or local recycling. However, several of these groupings are today outdated, as some organisms may perform more then one process e.g. nitrogen fixation and denitrification simultaneously (Kuypers et al. 2018 and references within). Even though problematic, it might sometimes be of interest to group organisms in some way or another, and several approaches have been applied over the years.

Whereas diatoms generally dominate phytoplankton communities with high nutrient concentrations in periods of mixing (commonly defined as r-strategists), dinoflagellates are commonly thrived by the opposite; oligotrophic conditions and stratified waters (commonly defined as K-strategists) (Margalef 1978). Also, cyanobacteria generally prefer stratified waters and high temperatures, and species that fix their own nitrogen are stimulated by low nitrogen concentrations. Further, this concept has recently been divided into three levels, based on Reynolds (1988). These levels constrain R (ruderals) with most diatoms and some species of dinoflagellates, e.g. *Alexandrium catenella*, *Ceratium fusus*, *Ceratium lineatum* and *Ceratium pentagonum*, and C-strategists (Colonist-invasives) with the rest of the diatoms and additional dinoflagellates, e.g. *Heterocapsa triquetra*, *Scrippsiella trochoidea* and *Gymnodinium* sp., and finally S-strategists (Stress-tolerant) including a mix of tolerant species, e.g. *Dinophysis acuminata*, *Dinophysis acuta* and *Coscinodiscus* sp. (Alves-de-Souza et al. 2008). However, at all three levels there are groups who uses some kind of mixotrophy, i.e. using different way of acquiring carbon and energy, complicating the way we commonly look at food web structures (Flynn et al. 2013, Stoecker et al. 2017).

In **Paper III** and **Paper V**, species from all of the three levels mentioned above were present, mostly dominated by R-strategists in the latter. The most common mixotrophs in **Paper V** were several species of the dinoflagellate genera *Tripos*/*Ceratium*. Most of the species from the genus *Ceratium* were recently moved to *Tripos* (Gómez et al. 2010, Gómez 2013). This genus may combine photosynthesis and the assimilation of dissolved inorganic carbon (DIC) with the possibility to ingest pray, thus, has an advantage in oligotrophic areas. In addition, as nutrient assimilation by large organisms is diffusion-limited due to their size (**Paper V**), they can by being

mixotrophic bypass diffusion-limitation by ingesting nutrients from prey. This was suggested in **Paper V**, as the large dinoflagellates assimilated 30 % more ammonium as compared to their size-dependent diffusion-limitation. Mixotrophy has been shown to boost primary production by up to 50 % in oligotrophic areas by transferring carbon up in the food web, but also, it may possibly decrease the overall primary production in eutrophic areas due to higher abundance of possible grazers (Stoecker et al. 2017).

Another way of dividing phytoplankton into groups has also been suggested according to difference in growth strategies based on their resulting cellular nitrogen to phosphorus ratios (Arrigo 2005). Here, the subgroups are defined as the survivalist (nitrogen to phosphorus ratio above 30), the bloomer (nitrogen to phosphorus ratio below 10) and the generalist (nitrogen to phosphorus ratio near Redfield), where pigment, enzymes and proteins have a high nitrogen to phosphorus ratio, and ribosomal RNA has a low nitrogen to phosphorus ratio. Many nitrogen-fixing organisms have unusually high nitrogen to phosphorus ratios (sometimes above 40), where the light-harvesting machinery, which drives the nitrogen fixation, is poor in phosphorus (Arrigo 2005).

2.4.2 Phenotypic plasticity and single cell diversity

A phenotype of an organism is a trait that can be either observed or quantified, e.g. as size, color or growth rate, while a genotype is a difference in the genetic code. When an organism is experiencing a change in its environment, it might change its phenotype, growing faster or become smaller, as it is acclimating. However, if the population of that organism stay in the new environment for a long time and over generations, it might adapt to the new conditions, with a permanent change in the genetic code, i.e. the genotype is changed. Thus, phenotypic adaptation is a permanent change in the genes, like a higher growth rate or faster assimilation of nutrients under a set condition.

Within species there might also be cellular plasticity, where individual cells and strains can vary in e.g. nutrient assimilation rates. Species can also be plastic by going from colony-formation to being solitary. Chain-forming diatoms have also shown plasticity between cells. Chain lengths may change due to grazing (Bergkvist et al. 2012) or nutrient availability (Takabayashi et al. 2006). Phenotypic plasticity may help phytoplankton to cope with environmental changes (Litchman et al. 2012).

In this thesis, different phenotypical traits were studied, e.g. growth rate, nutrient uptake rates, chain length etc. Both *N. spumigena* (**Paper II**) and *S. marinoi* (**Paper IV**) showed a large intraspecific variation. The two strains of *N. spumigena* revealed a large variation with regards to response in phosphorus storage capacity and affinity for

ammonium. Bertos-Fortis (2016) also observed plasticity between strains of *N. spumigena*, related to different salinities, and Wulff et al. (2007) in terms of UV-B radiation tolerance. In **Paper IV**, we demonstrate a large intraspecific variation in *S. marinoi*, where shorter chains were detected during later growth phases when the nutrient availability was limited as compared to under nutrient-replete conditions. Also, a large diversity within strains, between cells, was detected in terms of difference in nutrient assimilation rates. A large intraspecific variation helps species to cope with changes in the environment (Godhe & Rynearson 2017), and by having variable nutrient demands the species might be more resistant to natural fluctuations and thus, an ecological advantage when spreading into new areas and living in a wide range of various conditions. Strains arriving first to a new environment may be enhanced over later arrivals, when e.g. re-seeding from the sediment (Sefbom et al. 2015).

2.4.3 Filamentous cyanobacteria

In the Baltic Sea during summer, the mixed community of filamentous cyanobacteria is dominated by *N. spumigena*, *Aphanizomenon* sp. and *Dolichospermum* spp. (Lehtimäki et al. 1997, Bianchi et al. 2000, Hajdu et al. 2007, Figure 6). With their ability to fix nitrogen dissolved in the water from atmospheric nitrogen gas, these cyanobacteria have an advantage over surrounding phytoplankton, and can grow to high abundances during the nitrogen-limited summertime (Granéli et al. 1990). They are known to release up to 30 % of its newly fixed nitrogen as ammonium (Ploug et al. 2010, 2011), thus, the filamentous cyanobacteria stimulate the summer production all the way up to fish (Karlsson et al. 2015, Svedén et al. 2016). In contrast, the cyanobacteria only assimilate ammonium at very low rates or not at all (Adam et al. 2016). Also, some species of cyanobacteria are considered toxic, where *N. spumigena* is the only one being toxic out of the dominating filamentous species in the Baltic Sea (Edler et al. 1985). In the seasonal study of **Paper I**, the cyanobacterial community was in focus, but no nitrogen fixation was detected by picocyanobacteria or *Pseudoananbaena* sp., even though abundant during the summer in the Baltic Sea. As they are not able to fix nitrogen, they are instead competing for the available ammonium released from the filamentous cyanobacteria or produced by remineralization.

The filamentous cyanobacteria species in the Baltic Sea all have differentiated cells called heterocysts where the nitrogen fixation is performed. These cells are lacking oxygen production, but have a high respiration rate, in order to keep the enzyme nitrogenase in an anaerobic environment (Adams & Duggan 1999). The number of heterocysts per vegetative cell in *Aphanizomenon* sp. is 1-3 %, and for *N. spumigena* it is 5-10 % (Walve & Larsson 2007, Ploug et al. 2010, Mohlin et al. 2012). The heterocyst frequency in *Aphanizomenon* sp. varies over the season, with a higher

frequency during spring at 10°C, to compensate for the low temperature, as compared to later during the season when the frequency decreases (Zakrisson & Larsson 2014, Svedén et al. 2015). The heterocyst frequency was also studied in **Paper II**, where no correlation was found with nitrogen fixation. As heterocysts may be present without activity in the NifH gene, this result suggests that heterocyst frequency is a non-reliable proxy for nitrogen fixation. With a large number of heterocysts, the cyanobacteria may quickly upregulate nitrogen fixation under nitrogen-limited conditions (Vintila & El-Shehawy 2007, Vintila et al. 2010).

At the end of a bloom, some cyanobacteria may form akinetes, spore-like resting cells. In *Dolichospermum* spp. (former *Anabaena* spp.) and also partly *N. spumigena*, the akinetes are suggested to germinate at the initiation of a bloom (Suikkanen et al. 2010). It was recently suggested that *N. spumigena* cannot initate a bloom with only akinetes, but is also depending on the overwintering filaments (Wasmund 2017). Further studies are needed to fully understand the bloom dynamics of the filamentous cyanobacteria in the Baltic Sea.

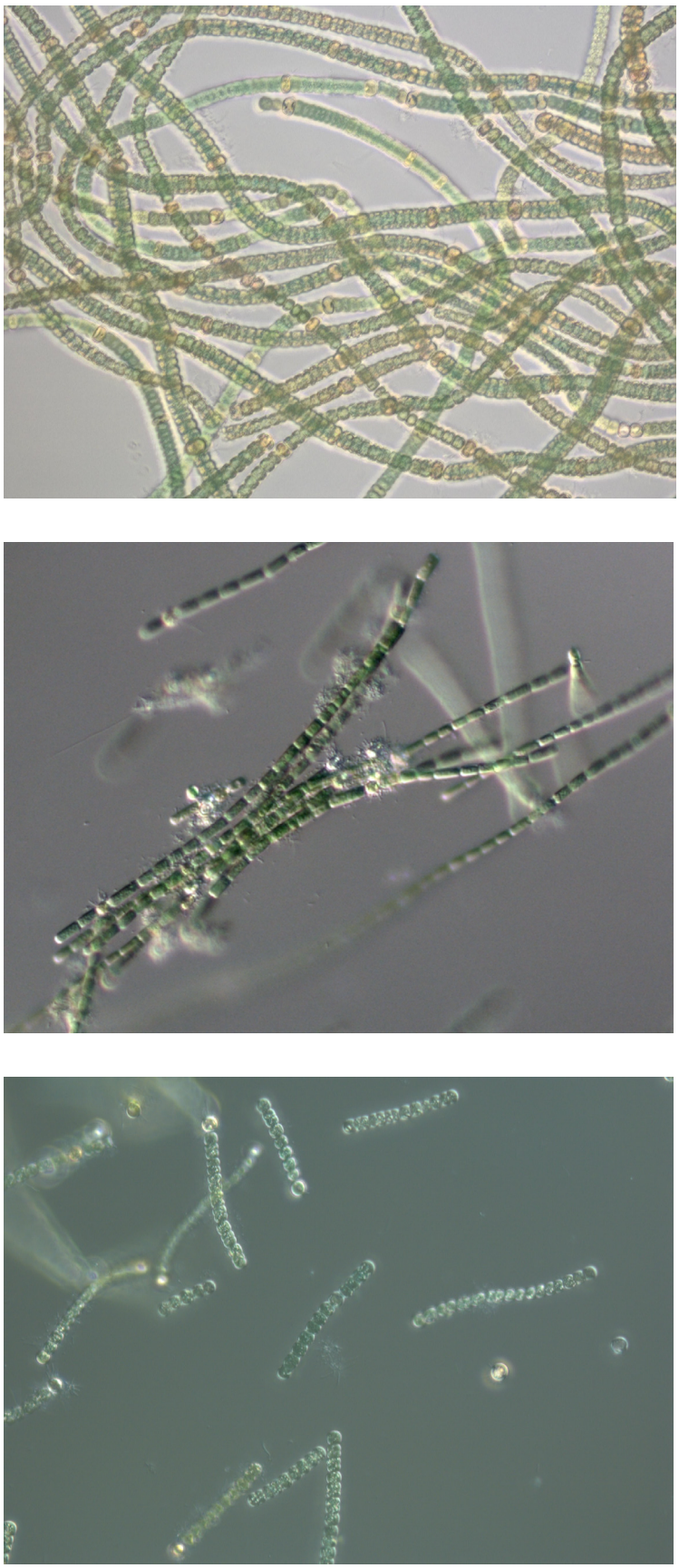


Figure 6. The filamentous cyanobacteria *Nodularia spumigena*, *Aphanizomenon* sp. and *Dolichospermum* sp. Photo: Malin Olofsson & Malin Mohlin

Phosphorus is the most important growth-limiting nutrient for the filamentous cyanobacteria in the Baltic Sea (Stal et al. 1999, Moisander et al. 2003, 2007, Rahm & Danielsson 2007). Species-specific phosphate storage capacities and availability have been suggested to determine the spatiotemporal distributions of nitrogen-fixing cyanobacteria in the Baltic Sea (Grönlund et al. 1996, Walve & Larsson 2007, Mohlin 2010). The effects of phosphorus-limitation were addressed under natural conditions in **Paper I** and under laboratory conditions in **Paper II**, using two strains of *N. spumigena*. In **Paper I**, the phosphate-amended conditions received low enrichments of phosphate (up to 1 μM), into otherwise un-amended Baltic Sea water, whereas phosphate-limited conditions were without excess phosphate during the three weeks of the experiment.

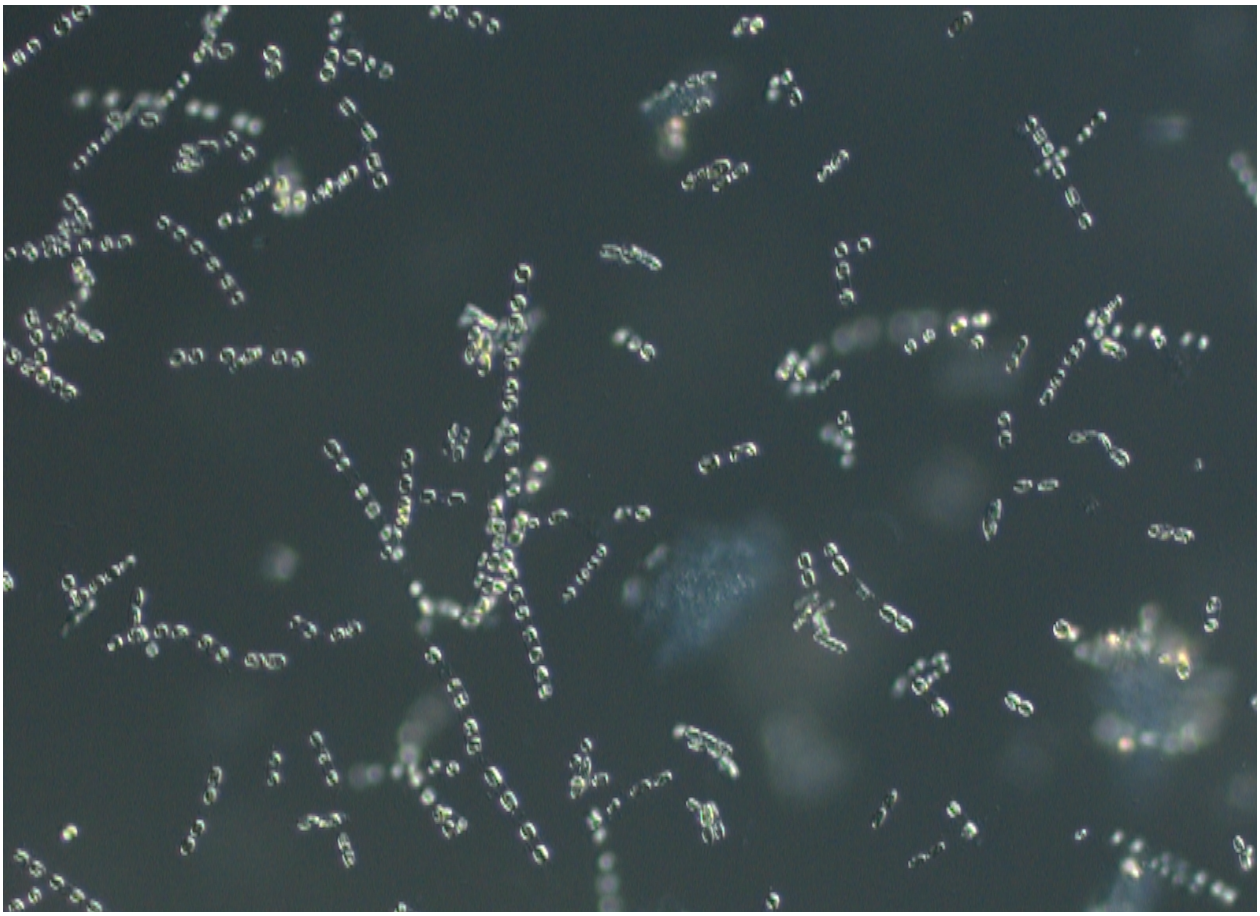


Figure 7. The chain-forming diatom *Skeletonema marinoi*. Photo: Malin Mohlin

2.4.4 Diatoms

Diatoms are key players in the ocean food web, and they produce about 20 % of the oxygen on the planet. They can use both nitrate and ammonium as a nitrogen source and represent a significant phytoplankton functional type especially during spring

blooms. They also comprise a large fraction of the vertical carbon fluxes to the deep-sea, i.e., the biological carbon pump, by acting as biogenic ballast with their heavy silica frustules (Alldredge & Gotschalk 1988, Iversen & Ploug 2010). These silica frustules show a vast diversity in forms, where one is slightly larger and allows the other to fit inside its edge. Diatoms are either centric or pennate in their shape, where pennate diatoms generally are benthic and centric more often pelagic, and where many form chains, e.g. *Skeletonema* spp., *Chaetoceros* spp. etc. Many chain-forming diatoms form fast-sinking aggregates and sink down to the bottom and form resting stages or resting cells when growth conditions are non-favorable in the euphotic zone. The resting stages/cells can stay in the sediment for a long time and wake up when conditions are favorable (Härnström et al. 2011).

In **Paper III** and **V**, diatoms have been in focus, along with dinoflagellates, including a phytoplankton community in Mozambique and from the Swedish west coast, respectively. In **Paper IV**, eight resting stages of *Skeletonema marinoi* were revived from recent (15 yrs) and old (80 yrs) sediment layers (Figure 7), collected in the eutrophied Danish Mariager Fjord. The carbon and nitrate assimilation rates were quantified during nutrient-replete and nutrient-limited conditions, both on strain-specific and cell-specific level during isotope tracer experiments.

2.4.5 Dinoflagellates

There are approximately 2000 known living species of dinoflagellates, where about half of them feed only on organic matter, i.e. heterotrophs, and the other half is strictly phototrophic or mixotrophic, which the latter is a mix of different trophic modes. However, the number of potential mixotrophic species is still increasing (Jeong et al. 2010). When mixotrophy by bacterioplankton is common in oligotrophic areas, it may enhance the carbon transfer in the food web, while when mixotrophy is common in eutrophic areas it may instead decrease the transfer by increased grazing (Stoecker et al. 2017). Further *in situ* studies on the activity of the mixotrophic organisms have been addressed, since most studies so far has been conducted under laboratory conditions using cultures (Smalley et al. 2003, Baek et al. 2008).

Dinoflagellates show a large variation in sizes and forms depending on their way of life (Figure 8). They are motile at some life stage and may be armored and create cysts. Several species are toxin-producing, e.g. *Dinophysis* spp. producing diarrheic shellfish poisoning (Suthers & Rissik 2009). In **Paper III** and **V**, dinoflagellates were in focus along with diatoms, first in a tropical ecosystem of Mozambique, and in a mixed community on the west coast of Sweden. By using stable isotope tracers combined with SIMS, it is now possible to perform *in situ* measurements of species-specific inorganic carbon and nitrogen assimilation in field populations (**Paper V**).

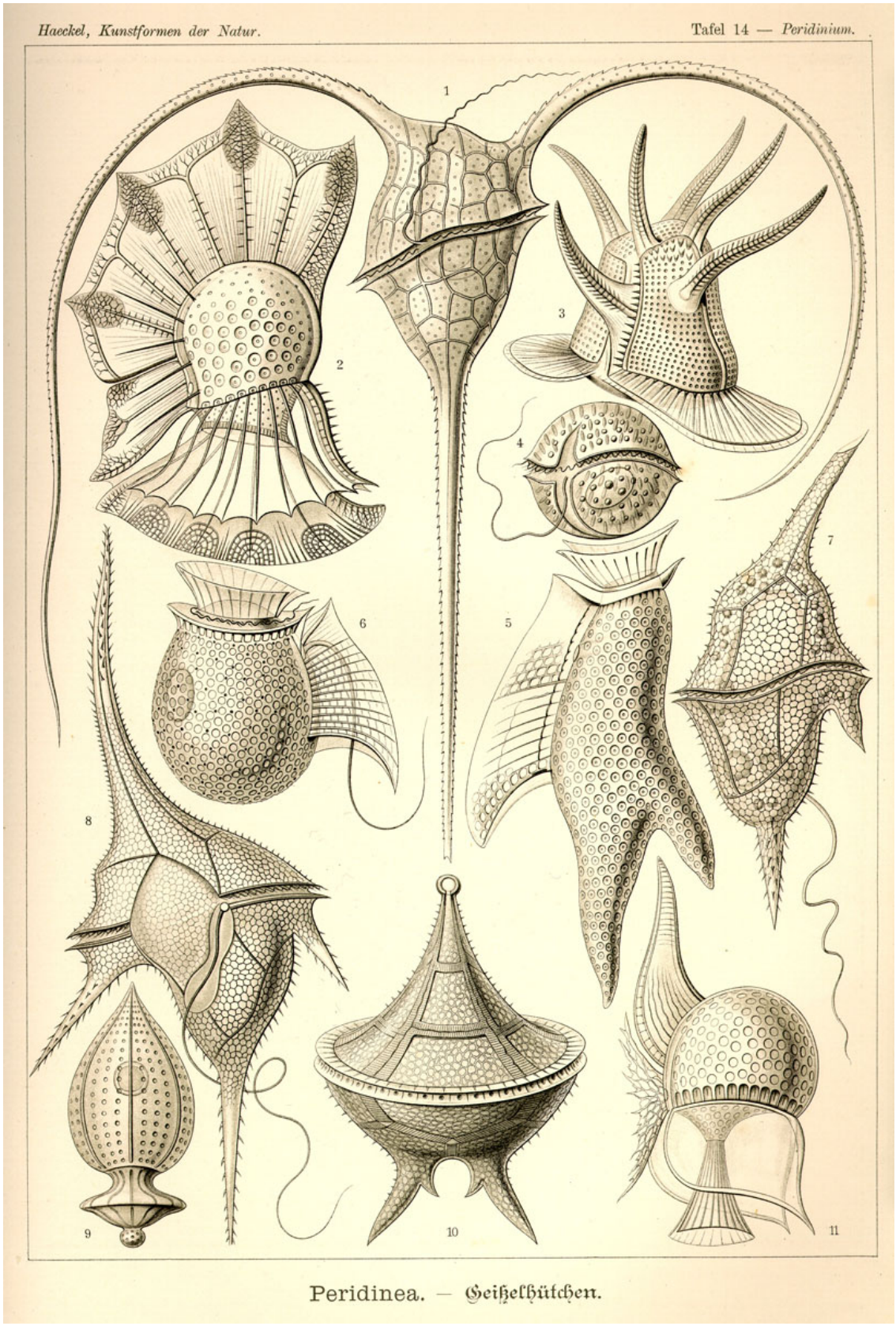


Figure 8. The diversity of dinoflagellates, Ernst Haeckel ca. 1900.

2.5 Nutrient limitation

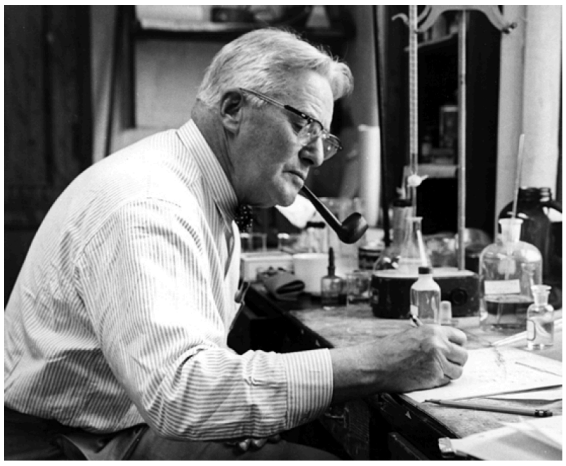


Figure 9. Alfred C. Redfield at his work, in 1955. Courtesy of Woods Hole Oceanographic institution archives.

Globally, mainly nitrogen is limiting primary production, however, it varies between areas, and also phosphorus and iron might be key limiting nutrients. Nitrogen tends to limit production in lower latitude, while iron can be limiting where subsurface nutrient supply is enhanced (Moore et al. 2013). Nitrogen is essential in organic material, DNA etc. (see Nitrogen cycling). In addition, phosphorus is essential as an energy currency within microalgal and cyanobacterial cells as ATP, and is also found in lipids, RNA and DNA etc. Phosphorus is mainly present as phosphates in the ocean, but also phosphite and organic forms are present, but not available to all organisms (Karl 2014). Phosphorus is the main limiting nutrient for nitrogen-fixing cyanobacteria in the Baltic Sea (Moisander et al. 2003, 2007, Rahm & Danielsson 2007), while globally iron may also be limiting for nitrogen-fixing cyanobacteria (Stal et al. 1999).

Since almost a century ago, a concept known as the Redfield ratio has been applied for phytoplankton when defining nutrient limitation, named after Alfred C. Redfield (1934, 1954, Figure 9). However, the classical molar ratio of 106:16:1 (carbon, nitrogen and phosphorus) has lately been revised due to observed fluctuations in the ratio, especially in algae and cyanobacteria under culture conditions with variable nutrient conditions (Geider & LaRoche 2002, Gruber & Deutch 2014), but also observed under *in situ* conditions (Singh et al. 2013). However, the stoichiometry of algal cells varies between species, environmental conditions and status of the cells (Finkel et al. 2010, Moreno & Martiny 2018). These ratios may be affected by a change in the global nitrogen cycle to climate change, ultimately decreasing the available carbon to nitrogen ratio of the oceans (Arrigo 2005, Hutchins & Fu 2017). Since the introduction of fertilizers during the last century, there is also an increase of available nitrogen in coastal areas (Howarth et al. 1996, Clarke et al. 2006). Also deep-sea trends in oceanic Redfield ratios indicate an increase of nitrogen (Pahlow & Riebesell 2000).

This thesis includes several studies with a large diversity and flexibility in stoichiometric ratios. The large variation indicates that the cells have flexibility in their cellular ratios when under nutrient-limiting condition. In **Paper IV** we tested if this variation in ratios has changed across a century, since the time of Redfield. There are several methods used to measure nutrient limitation, from conventional methods using assimilation rates and associated cell quota to more recent introduction of

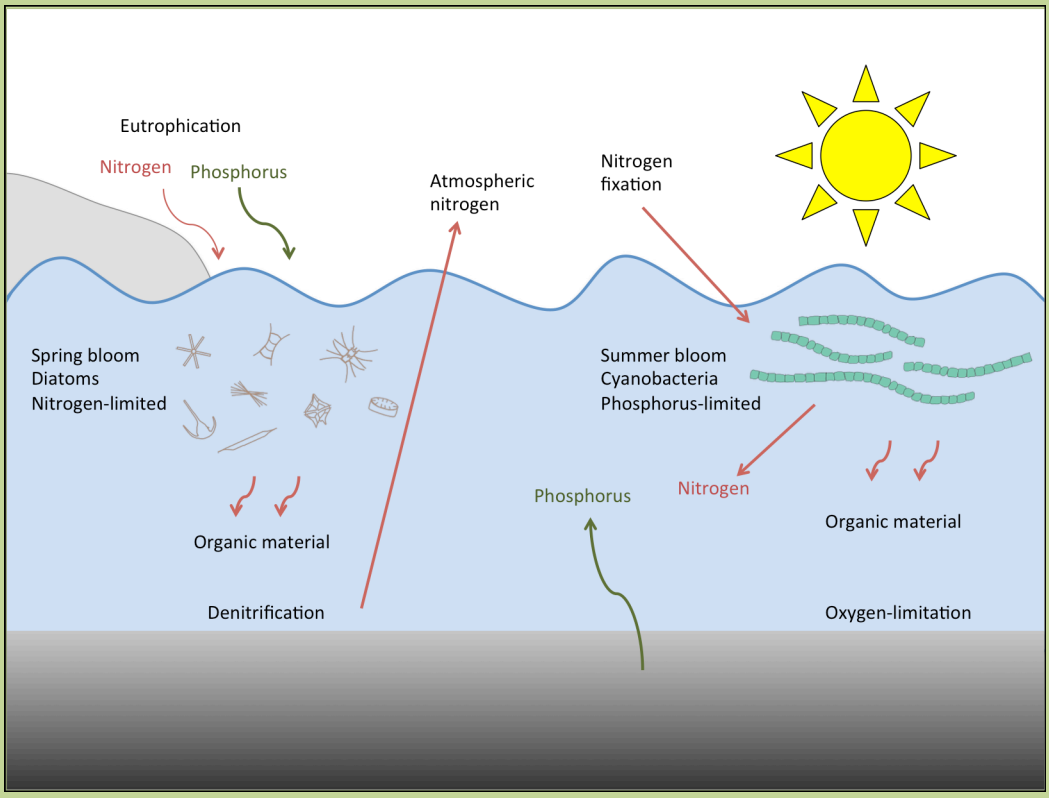
spectroscopy techniques (Beardall et al. 2001a, 2001b). Generally, based on Redfield ratios, phytoplankton with cellular carbon to nitrogen ratios above 6.6 are nitrogen-limited and with ratios below 6.6 has sufficient concentrations of nitrogen. For diazotrophic cyanobacteria with the ability to fix their own nitrogen, their carbon to nitrogen fixation ratios are mainly close to Redfield ratios in natural environments (Ploug et al. 2010, 2011, Martínez-Pérez et al. 2016). For cyanobacteria with carbon to nitrogen fixation ratios above their cellular carbon to nitrogen ratio, the cells are possibly using other sources of nitrogen than dissolved nitrogen gas to cover their nitrogen demand, i.e. ammonium. When cellular ratios are close to Redfield and their fixation ratios are far below, they are potentially releasing excess nitrogen as ammonium, which can be assimilated by the surrounding community. By using stable isotopes to measure nitrogen fixation or nitrate assimilation in addition to determine cellular carbon to nitrogen ratios, it might reveal what sources of nitrogen that was mainly used. In **Paper I**, the fixation ratios were above the cellular ratios at the offshore and below at the coastal station, indicating a deficit and excess in nitrogen fixation, respectively. Dinoflagellates are suggested to have carbon to nitrogen ratios between ca. 3-7 under nutrient-replete conditions, with less carbon per cell, but it varies with the size of the species, where both carbon and nitrogen density decreased with cell size (Menden-Deuer & Lessard 2000). Thus, this change with size might underestimate the content in small size cells and over-estimate in large size cells of dinoflagellates.

The cellular carbon to phosphorus ratio in cyanobacterial species may vary largely with the availability of phosphorus. In the Baltic Sea, the cellular carbon to phosphorus ratios of filamentous cyanobacteria varies over the season. From low ratios in the spring, with suggested internal storage, whereafter it increases until the biomass peak in August, with carbon to nitrogen ratios of almost Redfield ratios, but carbon to phosphorus ratios up to four times Redfield ratios (Larsson et al. 2001). In **Paper II**, phosphorus-limitation of growth, carbon and nitrogen fixation was studied in two different strains of *N. spumigena*, isolated from the Baltic Sea. Overall, the open Baltic Proper is generally nitrogen limited, while Gulf of Bothnia is rather phosphorus limited (Granéli et al. 1990). Lately, however, it alters towards more nitrogen-limitation due to phosphorus-rich bottom waters derived from the northern Baltic Proper (Rolff & Elfwing 2015). The primary production in the Gulf of Bothnia is also limited by light, as a large quantity of dissolved organic carbon is released from the rivers (Andersson et al. 2018). The balance between nitrogen- and phosphorus-limitation varies over the year, with possible release of phosphorus from the sediments under anoxic conditions, which may accelerate the system itself (Box 1). **Paper II** showed that *N. spumigena* possess a large, but strain-specific, phosphorus-storage capacity.

When conducting nutrient-limited studies, a large storage capacity may increase the risk of underestimating the actual effects of nutrient limitation.

Box 1. The negative spiral of the Baltic Sea

At the end of the Baltic Sea spring bloom, the availability of nitrogen is limited. These conditions are in favor of the filamentous cyanobacteria with the ability of performing nitrogen fixation. The cyanobacteria are instead phosphorus-limited. Therefore, they depend on internal storages of phosphorus and fluxes from riverine runoff or release from the sediment in order to increase in biomass. After almost a century of increased eutrophication, there is now a large internal storage of phosphorus bound to the sediment. In the Baltic Proper, this phosphorus might be released under anoxic conditions. During years with extensive blooms of cyanobacteria, an increased amount of organic matter is transported down to the sediment. This increased amount of organic material in the bottom waters increases the oxygen-limitation, thus creates and accelerates a negative spiral.



The nitrogen to phosphorus ratio can be very plastic in phytoplankon, ranging from below 5 when phosphorus is relatively more abundant as compared to nitrogen, up to above 100 when inorganic nitrogen is present greatly in excess of phosphorus (Geider & LaRoche 2002). This flexibility was also demonstrated in **Paper IV**, where nitrogen to phosphorus ratios around 6 was observed under nitrate-limiting conditions and of 25 when nitrate was present in excess of phosphate.

Diatoms can assimilate nutrients in proportions deviating from Redfield, depending on the availability. Diatoms need silica for their frustule, and an uptake ratio of 1:1 (mol:mol) between nitrogen to silicate is suggested the optimum under nutrient-replete conditions (Brzezinski 1985), as also shown in **Paper IV** for *S. marinoi*. However, they can grow under low silicate conditions, but with a lower growth rate in some species (Martin-Jézéquel et al. 2000, Gilipin et al. 2014). Many diatoms can store nitrate in their vacuoles, so their actual use of nitrate may not be directly reflected in a high carbon to nitrogen assimilation ratio (Dortch et al. 1985, Kamp et al. 2015). A high assimilation of nitrate might be referred to as a luxury uptake, ensuring availability. Collos et al. (1992) showed that *Skeletonema costatum* increase the uptake of nitrate when external concentrations are above 50-100 μM, supposedly using an uptake system adapted to high concentrations. Consistently, an early luxuary uptake of nitrate was observed in some strains in **Paper IV**, where carbon growth was delayed and continued after nitrate was depleted. Luxury uptake can also include assimilation of phosphate when available at high concentrations, which is then stored as polyphosphates (Dyhrman 2016).

Paper I, **III** and **V** all involve natural nutrient conditions but at different locations. During all studies, natural phosphate concentrations were low, possibly limiting the primary production rates. We investigated the primary production in terms of new and regenerated nitrogen driven production, in the summer time Baltic Sea, phytoplankton biomass peak in Mozambique, and during late summer on the Swedish west coast. In all studies, cellular stoichiometric quota and assimilation ratios were quantified, in order to address limitation, variability and demands.

3. Methods

3.1 Stable isotope labeling

Similar methods have been used in all papers of this thesis. In order to measure carbon and nitrogen fixation, and nitrate and ammonium assimilation, stable isotope labeling was used (Montoya et al. 1996, Klawonn et al. 2015). Stable isotopes are occurring naturally in the environment, and nitrogen (N) has two, with atomic masses of 14 and 15. The isotope ^{14}N is the most common (99.64 %), while the heavier ^{15}N is less common (0.36 %), and this large difference in occurrence makes it ideal to use as a tracer for nitrogen cycling. Carbon (C) has a similar pattern, with the stable isotopes ^{12}C and ^{13}C, which can be used for tracing when measuring carbon fixation, with ^{13}C as a tracer due to its low abundance (1.11 %).

When quantifying nitrogen fixation, there has until recently been a commonly detected underestimation of fixation rates due to injections of nitrogen gas as a bubble directly into the incubation bottles (White 2012). In order to reduce previous underestimation, we applied an improved method where the gas was dissolved in a separate bottle before injection (Klawonn et al. 2015, Mohr et al. 2010). Therefore when measuring nitrogen fixation in **Paper I** and **II**, $^{15}N_2$-gas was dissolved into pre-filtered (0.2 μm) degased seawater in gas tight bottles. Thereafter, the water containing the dissolved ^{15}N was added into the sample water in gas tight bottles, and then top filled. In **Paper I**, *in situ* incubations were performed for 12 h day (9 am to 9 pm) and 12 h night (9 pm to 9 am). In **Paper III** and **V**, *in situ* incubations were performed for 12 and 24 h (7 am to 7 pm and 7 am), with additional incubations for 2 and 5 h during light (7 am to 9 and 12 am) and dark (7 pm to 9 pm and 12 pm) in **Paper V**. Laboratory incubations were performed for 6 h in **Paper II** (9 am to 3 pm) and 24 h in **Paper IV** (9 am to 9 am).

In order to measure ^{13}C-carbon (**all papers**), ^{15}N-nitrate (**Paper III**, **IV** and **V**), and ^{15}N-ammonium (**Paper V**) assimilation, the stable isotopes were dissolved into MilliQ in gas tight exetainers, and then added into the incubation bottles. All incubations were terminated by filtration onto pre-combusted (450°C) GF/F glass microfiber filters (0.7 μm pore size). The filters were dehydrated at 60°C for 8 h and de-calcified by HCl smoke in a desiccator overnight. For analysis, the filters were packed into tin cups, and sent to UC Davis stable isotope laboratory for analysis (California) in **Paper I**, **II** and **III**, and to GVC at Gothenburg University for **Paper IV** and **V**. The incorporation of ^{15}N and ^{13}C into organic matter at the community or strain-specific level was quantified by Elemental Analysis Isotope Ratio Mass Spectrometry (EA-IRMS).

For **Paper I** and **II**, the labelling% of ^{15}N to ^{14}N was measured by Membrane Inlet Mass Spectrometry (MIMS), while ^{13}C to ^{12}C was measured by trace gas-IRMS, which was also performed in **Paper III**. For **Paper IV** the ^{15}N to ^{14}N in nitrate and ammonium was analysed by Gas Chromatography (GC)-IRMS after conversion to $^{15}N_2$ gas (Warembourg 1993) in Bremen. The ^{13}C to ^{12}C labelling% was analysed in Bremen by Picarro after conversion of bicarbonate to CO_2 (Cavity ring down Spectrometer G2201-I coupled to an Isotopic CO_2/CH_4 IRMS, Liaison A0301). In **Paper V** both the ^{15}N to ^{14}N and the ^{13}C to ^{12}C labelling% was determined by GC-IRMS in Odense.

3.2 Secondary Ion Mass Spectrometry (SIMS)

In **Paper I**, **IV** and **V**, Secondary Ion Mass Spectrometry (SIMS) was used in order to quantify assimilation rates on a single cell level within strains or a mixed community. SIMS was recently introduced into biological oceanography and microbiology by which we can gain insight into life at a single-cell level in mixed field populations in the ocean (Musat et al. 2008, Ploug et al. 2010, 2011, Adam et al. 2016). SIMS combines the qualities of a microscope with those of a mass spectrometer and reveals elemental and isotopic compositions at a spatial resolution of 1 μm (SIMS) or even 50 nm (nanoSIMS), i.e. at the size scale of single cells of phytoplankton and bacteria, respectively (Musat et al. 2012). The measuring principle is high-resolution mass spectrometry of secondary ions, e.g. CN^-, sputtered from a solid sample being bombarded by primary ions (Cs^+). The method allows high lateral resolution and visualization of the isotopic and elemental composition of a solid sample, including single cells of microbial organisms.

We applied SIMS measurements after incubation experiments using stable isotope labeling (See above). The incubations were terminated by preserving sub-samples from the incubations with paraformaldehyde (1-2 %), stored at 4°C for up to 24 h in the dark. The preserved samples where thereafter filtered onto GTTP/TTTP (0.2/2.0 μm pore size) polycarbonate filters. In **Paper IV** and **V**, the filters were also washed with phosphate buffered saline (PBS, 10X, pH 7.4) to remove salt particles. The filters were stored dark at room temperature until analyzed on the IMS 1280 at the NordSIM facility situated at the Natural History Museum, Stockholm. The filters were prepared for analysis by cutting out small squares of interest (ca. 4 x 4 mm), which were glued onto glass-slides and covered with a thin layer of gold (ca. 5 nm). The thin gold layer is used as support for the microbial cells during the analysis, and to prevent charging when the sample is sputtered with primary ions, keeping the sample on the original potential.

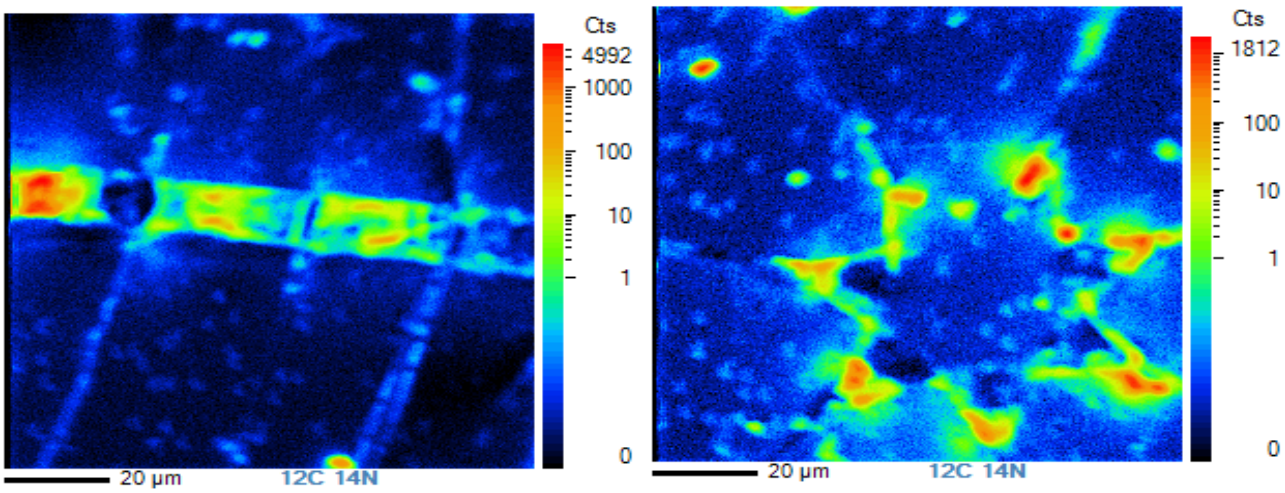


Figure 10. Secondary ion mass spectrometry images of *Chaetoceros* sp. (a) and *Asterionellopsis glacialis* (b) from image analysis, where the $^{12}C^{14}N$ counts (Cts) indicate biomass distribution.

When initiating the analysis, an area of interest to be imaged was decided based on the size of the target organisms. A raster size of 90 x 90 µm was used in **Paper I**, of 80 x 80 µm in **Paper IV**, and of 100 x 100 and 140 x 140 µm in **Paper V**. In order to remove the gold layer and also to remove the cell wall to get into the cells, the filamentous cyanobacteria were pre-sputtered with a primary caesium-ion beam ($^{133}Cs^{+}$) of 3 nA for 100 sec (**Paper I**), and for 240 sec for the dinoflagellates (**Paper V**). The diatoms (**Paper IV** and **V**) were pre-sputtered with a beam of 10 nA for 300 sec to remove the gold layer and the diatom frustules. Analyses were automated after test runs to determine the accurate pre-sputtering needed to reach the interior of cells with high carbon and nitrogen content. The pre-sputtered area was larger than the imaged area in order to eliminate possible slight offsets between the sputter and analytical beams, and edge effects. During the analysis, the primary beam moves in steps across the sample surface, and dwells on each pixel within the area of interest, and the secondary ions sputtered from each pixel arrive to the detectors and are counted simultaneously. The cyanobacteria in **Paper I** were imaged using a 40-60 pA primary beam with a spatial resolution of 1 µm for 100 cycles. The diatoms in **Paper IV** were imaged using a primary beam of 50 pA for 80 cycles, and in **Paper V**, for 60 cycles using a 100 pA beam. For all cells, we recorded secondary ion (SIMS) images (265 x 265 pixel) of $^{12}C^{15}N^{-}$, $^{13}C^{14}N^{-}$ and $^{12}C^{14}N^{-}$, using a peak-switching routine at a mass resolution of 6 000 in **Paper I** and 12 000 (M/ΔM) in **Paper IV** and **V**. Processing of the images was done using the Cameca WinImage2 software, where individual cells were defined as reigions of interest (ROIs), from which the cell-specific isotope-ratios (^{15}N to ^{14}N and ^{13}C to ^{12}C) were measured and cell-specific carbon and nitrogen assimilation rates were calculated.

In a mixed natural phytoplankton community, SIMS is a very powerfull tool to quantify cell-specific uptake rates by individual taxa. The individual cells are manually marked during image analysis, and thereafter the assimilation rates can be calculated based on the excess isotope ratios (example of images in Figure 10). In **Paper I**, we show that nitrogen fixation measured by EA-IRMS and SIMS data correlates. Therefore, they can effectively be used in a combination to reveal bulk assimilation in addition to species-specific contributions in a mixed phytoplankton community (**Paper I** and **V)**. The combination can also successfully be used to determine differences within a species on a strain-specific and cell-specific level, as in **Paper IV**.

3.3 Calculation of assimilation rates

Assimilation rates were calculated from the particulate organic carbon and particulate organic nitrogen measured by EA-IRMS and SIMS. For carbon and nitrate assimilation, the initial labeling% was used to calculate changes in isotope composition over the time of inoculations. For ammonium assimilation (**Paper V**), the excess labeling% was calculated based on an exponential decrease in ^{15}N to ^{14}N ratio over the time of the incubations, due to regenerated production of ^{14}N-ammonium decreasing the ^{15}N:^{14}N ratio by production of ^{14}ammonium (Glibert et al. 1982).

The ratio of dinitrogen or nitrate to ammonium assimilation was used to calculate the proportion of new production (as nitrogen fixation or nitrate assimilation) to total nitrogen-based production, and to reveal possible limitations or excess fixation/assimilation. However, when ammonium assimilation was not measured, Redfield ratio (C:N = 6.6) was used to calculate the proportion of carbon assimilation driven by new or regenerated sources.

3.3 Microscopy analysis

Microscopy analysis was included in all papers in the thesis. A natural phytoplankton community in **Paper I** (Baltic Sea), **III** (Mozambique) and **V** (west coast of Sweden), and cultures of *N. spumigena* in **Paper II**, and isolates of *S. marinoi* in **Paper IV**. The guidelines of HELCOM (Olenina et al. 2006) were applied for the microscopy analyses in **Paper I-IV**, and guidelines of Edler & Elbrächter (2010) for **Paper V**. The cells were fixed with Lugol´s solution, where different kinds (alkaline, acidified and neutral) can be stored for different time without bias in the samples and varying depending on the expected organisms in the samples (Williams et al. 2016). Samples were counted until a stable mean of number of cells or length of filaments were

established between squares in the Sedgewick rafter chamber. For natural communities, Utermöhl sedimentation chambers were used, where transects or views were used depending on abundance and size of the organisms. In **Paper IV**, the growth was also recorded by daily measurements of relative fluorescence (Gross et al. 2017).

3.4 Dissolved inorganic nutrients

For **paper I**, the Swedish Meteorological and Hydrological Institute (SMHI) provided concentrations of dissolved inorganic nutrients (phosphate, ammonium, nitrite, and nitrate). In **Paper II**, the phosphate (Strickland & Parsons 1972) and ammonium concentrations (Holmes et al. 1999) were measured by the authors using a Turner Trilogy fluorometer. This method was also used in **Paper IV** to determine total ammonium concentrations.

For **Paper III**, samples were fixed with 20 µl formalin in Mozambique and the concentrations of inorganic nutrients (ammonium, nitrite, nitrate, silicate and phosphate) were analyzed according to Grasshoff et al. (1999) at Sven Lovén Centre for Marine Sciences, Kristineberg. For **Paper IV** and **V** bulk samples were collected during the experiments by using a syringe with a filter attached (0.2 µm), and frozen (-20°C) until analyzed for inorganic nutrient concentrations (ammonium, nitrite, nitrate, silicate and phosphate) at Sven Lovén Centre for Marine Sciences, Kristineberg.

3.5 Particulate organic nutrients

The samples prepared for isotope analysis were also used for analysis of particulate organic carbon, nitrogen and phosphorus. A set volume of water was filtered onto pre-combusted GF/F filters, dried overnight and put in a desiccator with HCl smoke overnight. In all papers, the filters were analyzed for particulate organic carbon and nitrogen by elemental analysis isotope ratio mass spectrometry (EA-IRMS) either at UC Davis stable isotope laboratory (California) for **Paper I**, **II** and **III**, or in the Geological sciences building (GVC) in Gothenburg for **Paper IV** and **V**. In addition, for **Paper IV**, particulate organic phosphorus was analysed on GF/F filters by using ICP Emission spectroscopy on an iCAP 6500 at Royal Netherlands Institute for Sea Research. Thereafter, particulate organic nutrient ratios were calculated in order to reveal potential nutrient limitations.

4. Main results and discussion

4.1 Paper I

Nitrogen fixation in the Baltic Sea has so far only been detected by the filamentous cyanobacteria *Nodularia spumigena*, *Aphanizomenon* sp. and *Dolichospermum* spp. Consistently, nitrogen fixation was absent in the cyanobacteria *Pseduoanabaena* sp., and the unicellular and colonial picocyanobacteria analyzed in this paper. Overall, the nitrogen-fixing cyanobacteria contributed with ca. 20 % of the total carbon fixation by the phytoplankton community, both at a coastal and an offshore station. Nitrogen fixation rates were 8-fold higher at the coastal station as compared to the offshore station over the season during 2012 and 2013, presumably due to higher phosphorus concentrations at the coastal as compared to the offshore station.

With its high biomass during summers in the Baltic Sea, *Aphanizomenon* sp. contributed with up to 79 % of the overall nitrogen fixation. However, the specific nitrogen fixation rates were slightly higher for *N. spumigena* and *Dolichospermum* spp. as compared to *Aphanizomenon* sp. The specific nitrogen fixation for *Aphanizomenon* sp. and *Dolichospermum* spp. were highest in June when the temperature was ≤14°C, presumably due to a sufficient availability of phosphate and at least for *Aphanizomenon* sp. a higher heterocyst frequency at lower temperatures (Svedén et al. 2015). Bulk measurement by EA-IRMS correlated well with the measurements at a single-cell level by SIMS, and supported the combination of EAIRMS and SIMS for *in situ* studies of mixed communities to reveal species-specific contributions.

Based on the proportion of carbon fixation by the nitrogen-fixing community, the new production in the Baltic Sea was about 20 % during the summer. New production is suggested to be directly related to export production and may be referred to as an f-ratio when related to total nitrogen-based production.

Highlights

- Ca. 20 % new production based on nitrogen fixation during summer time
- *Aphanizomenon* sp. contributed with 79 % of the total nitrogen fixation
- Difference in fixation rates between coastal and offshore station
- No nitrogen fixation by *Pseudoanabaena* sp. or the picocyanobacteria
- High correlation between EA-IRMS and SIMS

4.2 Paper II

By using two different strains of *N. spumigena,* this study showed that even small pulses of inorganic phosphorus (final concentration of 1 μM) stimulated exponential growth, total carbon fixation and total nitrogen fixation. At the end of the 21 d long laboratory experiment, the growth rate and average carbon-specific carbon fixation were significantly higher under phosphorus-enriched conditions. The phosphorus-limited filaments were orange and pale as compared to the phosphorus-enriched filaments, which was green and supposedly rich in chlorophyll. The total nitrogen fixation was significantly higher under phosphorus-enriched conditions at day 21. The average nitrogen fixation during the experiment was significantly higher for one strain as compared to the other, independent of treatment.

Strain-specific differences were also found with regards to phosphorus storage capacity and affinity for ammonium. When ammonium concentration in the surrounding water was high during the first 7 d of the experiment, a significantly higher nitrogen fixation rate was found for one of the strains as compared to the other. After 7 d, when the ammonium concentration decreased, the nitrogen fixation rate increased for both strains, while carbon fixation decreased, supposedly due to reallocation of energy. This was also reflected by a decrese in carbon to nitrogen assimilation ratios. Further, there was no correlation between nitrogen fixation and heterocyst frequency. Thus, heterocyst frequency is not a good proxy for nitrogen fixation rates, as heterocysts can be present without activity in the NifH gene.

This study demonstrates the importance of using more than one strain in culture experiments. As strains may act differently, and thus one single strain does not reflect a natural composition of nutrient demands and diversities. During summers with strong stratification and low influx of phosphorus, *N. spumigena* in a coastal environment may be stimulated by small pulses of phosphorus from anoxic sediments, due to its efficient storage capacity and high affinity for phosphorus.

Highlights

- Small pulses of phosphorus stimulated exponential growth
- Variable storage capacity of phosphorus between strains
- Strain-specific differences in affinity for ammonium
- Correlation between total carbon to nitrogen assimilation
- Heterocyst frequency is not a good proxy for nitrogen fixation

4.3 Paper III

This field survey was performed in Mozambique during January and February 2014. With a more diatom dominated phytoplankton community present at high tide as compared to low tide and dinoflagellate dominating community in a reverse pattern, the phytoplankton composition was diverse and mixed. The total biovolume of phytoplankton was higher at spring-high tide as compared to neap-low, as presumably more mixing with ocean surface water occurred during the former. We quantified bulk assimilation of nitrate and carbon during spring-high and neap-low tide. However, the nitrate-based carbon assimilation was below 10 % of total community primary production, and the residual part was supposedly driven by regenerated sources of nitrogen, e.g. ammonium. The difference in community composition might be related to that dinoflagellates are generally more sensitive to mixing compared to diatoms. Some dinoflagellates are grouped together with diatoms as R-strategists, while some as C-strategists and some as S-strategists (Alves-de-Souza et al. 2008). In this survey, species from all three groups were present.

Rough winds during the first two incubations, one at low and one at high tide, resulted in more particles in the water and relatively low carbon-specific carbon assimilation rate. At calm conditions and spring high tide, the highest surface f-ratio was found, with relatively more nitrate-based primary production as compared to the other incubations. However, low surface f-ratios were measured in all incubations. The organic matter carbon to nitrogen ratio and carbon to nitrogen assimilation ratio were highest under rough weather conditions, when more organic matter was present in the water. As indicated by a high proportion of particulate organic matter not identified in the microscopy analysis, a large part of the community supposedly consisted of picoplankton.

The carbon assimilation rates were similar to those measured in Sweden at 10°C (Svedén et al. 2015) despite temperatures in Mozambique above 30°C. Hence, primary production was presumably limited by nutrients rather than by temperature in both systems. Compared to studies in the Arabian Sea and along the Indian coast, the total carbon assimilation rates in Maputo Bay were up to 300 times higher.

Highlights

- Difference in phytoplankton community composition between the tides
- Primary production mainly driven by re-generated sources of nitrogen
- Comparable assimilation rates to the much colder Baltic Sea
- High carbon assimilation rates compared to Arabian Sea and Indian coast

4.4 Paper IV

In a study using the common diatom species *Skeletonema marinoi*, four strains revived from old resting stages (80 yrs) and four from more recently formed resting stages (15 yrs) were used. They were hatched from sediment cores collected in the eutrophic Danish Mariager Fjord. Using the newly hatched strains, stable isotope incubation experiments were performed at nutrient replete (exponential) and nutrient limited (early stationary and late stationary) growth phases, under replete (200 μM) and limited (50 μM) nitrate concentrations. The carbon and nitrate assimilation rates were compared between and within old and young strains. Thus, the diversity in nutrient demands between ages, between strains within ages and between cells within strains were revealed. During the exponential growth phase, the carbon and nitrate assimilation correlated within each strain, but with a large diversity in ratios between strains, independent of ages. Also, the magnitude of rates was large within each strain among individual cells.

All strains had longer chains (up to 14 cells per chain) during exponential growth phase as compared to during stationary (ca. 2-3 cells per chain), presumably due to nutrient limitation in the latter phase. A decrease in chain length will increase the surface to volume ratio and, thus, the availability of nutrients.

We observed an early nitrate assimilation in some strains, which was also independent of age. This early assimilation might be refered to as a luxury uptake, were cells are ensuring nutrient availability. Following this early assimilation of nitrate, there was a delay in carbon-based growth, and a temporal uncoupling with up to several days in all experiments. This uncoupling as well as the large diversity on a single cell level might both be efficient strategies in a changing environment across the last century.

Highlights

- Very large diversity between single cells within strains, independent of age
- Large diversity between strains in terms of nutrient demands
- Shorter chain lengths as the nutrient availability decreased
- Luxurious uptake detected in some strains
- Temporal uncoupling between carbon and nitrate assimilation
- High adaptation potential to eutrophication in *Skeletonema marinoi* populations due to a high single cell variability of nitrogen demands

4.5 Paper V

This field survey was performed on the Swedish west coast. By quantifying cell-specific assimilation rates of nitrate and ammonium during 2, 5, 12 and 24 h incubation experiments we describe a late summer community and the relative contribution by the dominating phytoplankton organisms. The species diversity was high, with more than 117 identified taxa, and mostly R-strategists (Alves-de-Souza et al. 2008). Diatoms and dinoflagellates dominated the phytoplankton communities, including *Chaetoceros* spp., *Asterionellopsis glacialis* and other chain-forming diatoms as well as species belonging to the mixotrophic genera *Tripos*/*Ceratium* spp.

The measured nutrient concentration was low, but with a high turnover time of ammonium (2-5 h). The high turnover rate of ammonium was similar to a Baltic Sea summer bloom (Adam et al. 2016). The primary production was mainly driven by regenerated sources of nitrogen, e.g. ammonium (73-88 %), and the nitrate-driven new production in the area equalled the remaining part of the primary production. Even though the chain-forming diatoms only comprised 6 % of the total particulate organic carbon, they contributed by 20 % to the total primary production and assimilated 54 % of the available nitrate, and 32 % of the ammonium. In contrast, the dinoflagellates comprised 11 % of the particulate organic carbon, and contributed by 14 %, to the total carbon assimilation and 4-9 %, only, to the nitrogen assimilation.

By calculating the diffusion-limited nutrient supply, we coupled mass transfer theory with empirical measurements on a single cell level. The measured assimilation rates for nitrate were close to those predicted by diffusion limitation in *Chaetoceros* spp. and large dinoflagellates. The measured assimilation rate of ammonium was close to the theoretical value in large dinoflagellates, but the ammonium assimilation was 4.4-fold higher than diffusion-limited fluxes in *Chaetoceros* spp., suggesting microbial interactions to occur in the phycopshere. No nitrification was detected, thus, no regeneration of nitrate, it was only derived from new sources. The growth rates in large dinoflagellates were diffusion-limited by low nutrient concentrations in the ambient water and higher growth rates must be supported by mixotrophy.

Highlights

- Net CO_2 sequestration (new production) can be high although mediated by the least abundant organisms within the phytoplankton community
- Disproportion in particulate organic carbon and assimilation rates in diatoms and large dinoflagellates
- Diffusion-limited nutrient supply was compared with measured assimilation rates in field populations of large phytoplankton
- High turnover rates of ammonium
- No nitrification detected

5. Synthesis and outlook

In a future ocean, the global nitrogen cycle is predicted to undergo some changes, partly as a result from the elevated use of fertilizers during the last century. The prediction includes enhanced nitrogen fixation and denitrification, resulting in less nitrate available and more of the reduced forms, e.g. ammonium (Glibert et al. 2006, 2016). However, the direct effects of climate change conditions are hard to predict, due to complex interaction effects in nutrient availability, temperatures, increased stratification, and elevated carbon dioxide levels (Hutchins & Fu 2017). With decrease in the nitrate availability in addition to an increase in nitrogen fixation and release of ammonium (Mulholland & Capone 2001, Mulholland 2007, Adam et al. 2016), surface-dominating processes will become increasingly based on ammonium.

The global carbon cycle in a future ocean may result in an increased uptake of atmospheric carbon dioxide through ocean acidification, but a decrease in the vertical flux and primary production (Hutchins & Fu 2017). The effect from an elevated carbon dioxide (ocean acidification) is species-specific and also interacting with changes in nutrient availability, temperature etc. In the Baltic Sea, there have been studies indicating limited effects on the cyanbacterial community by temperature and elevated carbon dioxide (Wulff et al. 2018, Olofsson et al. under review). Even though cyanobacteria generally grow better at higher temperatures as compared to low (Paerl & Huisman 2008), the availability of nutrients is often an even more important factor (**Paper II**). A secondary effect from global warming is a change in air-sea temperature gradients and consequently wind speed and turbulence in the ocean. Increased turbulence stimulates both small- and large-scale carbon dioxide sequestrations in diffusion-limited large diatoms (Bergkvist et al. accepted).

In this thesis, new production based on both nitrate assimilation and nitrogen fixation has been quantified in several areas ranging from the brackish Baltic Sea, to the more saline Swedish west coast and also to the saline tropical coastline of Mozambique. Including a large variation of phytoplankton functional types and conditions, the carbon-specific carbon assimilation rates (carbon growth rates) per day ranged from 0.07-0.15 in large phytoplankton (diatoms and dinoflagellates) during late summer on the Swedish west coast to 0.11-0.19 in tropical Mozambique, including a similar phytoplankton community. Further, we measured slightly higher specific carbon assimilation rates of 0.12-0.52 during summer in the Baltic Sea, and the overall highest rates of 0.20-0.81 in the laboratory under nutrient-replete conditions. Overall, the carbon assimilation rates did not correlate with temperatures ranging from 11°C to 32°C. The nutrient availability was presumably the most important driver of carbon assimilation rates, rather than temperature. Also the light conditions varied, even

though the highest carbon fixation rates were observed when nutrients were replete, and not under the highest light levels. Light levels (photosynthetically active radiation, PAR) were measured using a Sea-Bird irradiance sensor in **Paper I**, and a LiCor irradiance sensor in **Paper II** and **IV**, while crude measurements using HOBO loggers recording Lux were used in **Paper III** and **V**. The importance of light was demonstrated in **Paper I**, when both the light and the carbon assimilation decreased with depth, from a maximum of ca. 600-1100 μmol photons m^{-2} s^{-1} at 1 m depth. While there was approx. twice the amount of light in Mozambique (**Paper III**) as compared to the Baltic Sea during summer (**Paper I**), the overall lowest light levels were detected during the fall on the Swedish west coast (**Paper V**). However, photosynthesis in most species is light saturated above 100-200 μmol photons m^{-2} s^{-1}, with variation between species (Edwards et al. 2016). Whereas light was possibly a limiting factor for the carbon fixation during fall on the Swedish west coast, the only of the papers where light conditions below the suggested saturation-level of most species, nutrients were more important at light levels above saturation.

With high rates of carbon assimilation in Maputo Bay, but low nitrate-based and nitrogen fixation-based production, the net carbon assimilation was mainly fueled by regenerated sources of nitrogen (ca. 90 %), e.g. ammonium (**Paper III**). Even though the measured ammonium concentration was very low, ammonium is known to be produced and consumed at high rates resulting in a fast turnover rate in the euphotic zone (Glibert & Goldman 1981). In the Baltic Sea, the turnover rate of ammonium is ca. 5 h (Adam et al. 2016), and ca. 20 % of the primary production was driven by nitrogen fixation, i.e. new production during our seasonal study in **Paper I**. On the Swedish west coast, the ammonium turnover time was ca. 2-5 h, fuelling 73-88 % of the primary production (**Paper V**). Thus, higher proportion of new compared to regenerated production occurred on the west coast of Sweden as well as the Baltic Sea as compare to Maputo Bay in Mozambique. Ammonium production and consumption is often overlooked due to its high turnover rate. Thus, the measured concentration in the water usually not reflects the availability. In **Paper V**, high rates of both ammonium and nitrate assimilation were determined, but with a disproportion to the carbon biomass. We also found the large dinoflagellates to assimilate both ammonium and nitrate, being flexible to changes in availability. However, these organisms were diffusion-limited and slow-growing due to their large size. Consequently, mixotrophy is an alternative strategy to gain extra nutrients at higher growth rates.

With predicted elevated temperatures and freshening of the seawater, a stronger stratification may appear in a future ocean. This situation would result in less nitrate being transported across the thermocline from the deeper ocean, and thus in favor of small phytoplankton which are not diffusion-limited as well as mixotrophic organisms which can cover their nutrient demand partly by prey. Therefore, further knowledge

on their *in situ* behavior is highly needed in order to include them in ecological models for future predictions (Stoecker et al. 2017). Also relatively smaller sizes of phytoplankton are suggested to increase in abundance relative to larger ones in a future ocean due to size-dependent diffusion-limitation at low ambient concentrations of nutrients (Hutchins & Fu 2017). In a recent study, common groups of picocyanobacteria were determined as mixotrophs, at high temperatures and low nutrient availability (Duhamel et al. 2018). By combining the use of secondary ion mass spectrometry (SIMS) for single cell analysis with isotope labeled inorganic and organic carbon, mixotrophy would be possible to study further. One example would be to feed bacteria with glucose (^{13}C-glucose), whereafter they are fed to small ciliates and then those are fed to mixotrophic organisms *in situ*. This feeding experiment should be run in parallel to tracer experiments using inorganic labeled carbon (^{13}C-bicarbonate). This setup would reveal mixotrophic activities *in situ*, yet lacking in ecological models.

This thesis provides new knowledge on how phosphorus-limitation affects nitrogen-fixation (**Paper II**) which may also be important in field populations, where nitrogen was fixed in excess at the coastal station with higher phosphate concentrations as compared to offshore station (**Paper I**). However, there is a need to develop other methods to study phosphorus-limitation on a single cell level. By reviving resting stages of diatoms in **Paper IV** we were able to reveal nutrient demands across a century. Also some species of filamentous cyanobacteria form resting cells, known as akinetes. Yet, the survival time of the akinetes in the sediment is unknown, but if stored for as long as the diatoms, they would be possible to hatch for e.g. the toxic cyanobacteria *Nodularia spumigena*, and reveal nutrient demands and diversity across the last century of ongoing eutrophication in the Baltic Sea. Interestingly, a recent study revealed *N. spumigena* to grow even at temperatures below 4°C (Olofsson et al. under review). In terms of nitrogen fluxes in the Baltic Sea, the winter activity and nutrient demands would be of interest in order to understand its complexity and seasonal dynamics.

The advantages of using SIMS are large, as it enables to study species of phytoplankton and bacteria under their natural conditions. By quantifying nutrient assimilation rates and activities *in situ*, it may include species that are non-culturable, and also, down to a single cell level. In **Paper V**, we take this application one step further and couple single cell measurements to diffusion-limitation theory. This would not have been possible before the introduction of SIMS into ecological studies. We show that theoretical calculations on diffusion-limitations and *in situ* measurements agree well and suggest possible interactions within the phycosphere. Diffusion-limited nutrient assimilation in large phytoplankton also implies that these are highly sensitive to turbulent shear in the environment (Bergkvist et al. accepted).

Although the future will include ocean acidification and warming in the global ocean, several studies have shown microalgae to be highly resilient to changes in pH and flexibile in terms of stoichiometric ratios. By applying single cell measurements (SIMS) to natural populations we will continue to learn more about the complexity of the pelagic system and on how ecosystems are responding. Even though I am also worried about what we as humans are doing to the planet, there is also curiousity of what the future will bring in terms of new techniques and possibilities. Anyhow, I am convinced that microorganisms in the aquatic environments will find ways to cope with our changing world, and hopefully we will find even better tools to follow their success.

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