Single cell carbon and nitrogen dynamics in chain forming diatoms, including their resting stage

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Abstract

The oceans are a fundamental part of all life on earth, accounting for more than half of Earth's oxygen production. The ocean is also key to long term carbon dioxide sequestration. Diatoms are a group of phytoplankton differentiated by their silica shell/frustule and account for ~20% of global primary production. Some of these diatoms form colonies/chains which are viewed as a way to reduce grazing pressure, but also effect aggregation, sedimentation, and nutrient assimilation. Exponential growth conditions during algae blooms have been well studied. Less is known about how they survive between blooms when conditions are no longer optimal. In nature, high nutrient availability in the photic zone and subsequent blooms only last a few weeks. Growth limited conditions persist for most of the year.

In **chapter I** and **II**, shifts in carbon and nitrogen assimilation dynamics were investigated in two chain forming diatoms: *Chaetoceros affinis* (**I**) and *Skeletonema marinoi* (**II**) at the beginning of nitrate limitation. *C. affnis* which are often larger than *S. marinoi* and are relatively more abundant during summer conditions with low nitrate availability. *Skeletonema* produce smaller cells and dominate at the beginning of blooms, where they thrive and assimilate excess nitrogen. *C. affinis* produced exudates of sugars and was colonized by attached bacteria that assimilated both carbon and nitrogen derived from their host. Later, diatoms were remineralised by bacteria, releasing ammonium. This ammonium could balance carbon assimilation for active diatoms. I speculate that cells in chains could benefit from remineralization of other cells in the chain and supply active cells with ammonium. *S. marinoi* showed no difference in carbon and nitrogen assimilation depending on chain length. The cells assimilated nitrate at a rate 25-65 times lower than the diffusive supply could provide, when compared to modelled diffusive supply from the ambient water. This indicated that cells were limited by biological uptake rates rather than diffusive supply. In **chapter I** and **II**, I demonstrated that *C. affinis* and *S. marinoi* had different ways of dealing with nitrate limitation, corresponding to the niches they fill. *C. affinis* recirculate the nitrogen with help of bacteria, which would allow them to keep a standing population in low nitrogen availability between blooms. *S. marinoi* on the other hand assimilated an excess of nitrogen during high availability, where they usually dominate.

In addition to playing a key role in primary production and nutrient turnover, diatoms also contribute to particle transport from the photic zone to the sediment. Diatoms form resting stages which can survive decades to centuries in dark and anoxic sediments. Mechanism of survival is unknown, and they have previously been considered as "dormant" in the sediment. Basic mechanisms for cell maintenance in resting stages of *S. marinoi* were investigated in **chapter III**. I showed that they were able to assimilate both nitrate and ammonium in dark and anoxic conditions. The nitrogen specific generation time varied between 23-500 years which may be enough to maintain viable cells, but not for growth. In **chapter IV**, I investigated if resting stages could use nitrate as an electron acceptor and assimilate organic molecules available in the sediment (acetate and urea). The resting stages performed dissimilatory nitrate reduction to ammonium (DNRA) and assimilated N from urea. They could not assimilate carbon from urea but assimilated carbon from acetate. Hence, the sediment provides resting stages with both carbon and nitrogen for assimilation and respiration. I have shown that diatom resting stages are not as dormant as previously assumed. I also showed that two common chain forming diatoms have different mechanisms of circumventing reliance on nitrate diffusion from the ambient water. The next step is to connect this to marine monitoring and prediction models taking chain formation into account. This thesis has only scratched the surface on chain forming diatoms responses to adverse conditions. Considering the large diversity of chain forming diatoms, the responses to such conditions may be equally diverse.

Keywords: Diatoms, resting stages, N cycling, chain formation, and secondary ion mass spectrometry (SIMS)

Populärvetenskaplig sammanfattning

Havet är en fundamental del av allt liv på jorden, mer än hälften av allt syre produceras där genom fotosyntes. Havet spelar även en stor roll för långsiktig begravning av koldioxid från atmosfären. Kiselalger eller diatoméer är en grupp av fytoplankton som karakteriseras av sitt kiselskal och bidrar till 20% av jordens primärproduktion. Vissa av dessa diatoméer växer tillsammans i långa kolonier/kedjor. Dessa kedjor har historiskt setts som ett sätt för algerna att minska betningstrycket från zooplankton, men kedjebildning verkar också påverka aggregation, sedimentation och näringsupptag. Hur kedjebildande kiselalger växer i perfekta förhållanden som under en algblomning är väl studerat. Mindre är känt om hur dessa alger överlever mellan blomningarna när förhållandena inte är optimala. I naturen sker dessa blomningar ofta bara några veckor varje år, resten av året är algerna tillväxbegränsade.

I **kapitel I** och **II** undersökte jag hur kol och kväve upptag förändrades i slutet av en konstgjord algblomning för två olika kedjebildande kiselalger *Chaetoceros affinis* (**I**) och *Skeletonema marinoi* (**II**). *C. affinis* som generellt har större celler än S. marinoi och är som vanligast efter blomningar när det finns lite nitrat i vattnet. S. marinoi producerar generellt minder celler och dominerar början av algblomningar där de växter snabbt och tar upp extra kväve som de kan använda senare när kvävet i vattnet tar slut. *C. affinis* utsöndrade ”sockerföreningar” i vattnet, de blev koloniserade av bakterier som fäste sig direkt på kiselskalet och assimilerad kol och kväve utsöndrade från algerna. Nästan hälften av alla kiselalger blev inaktiva över den 21 daga långa inkubationen. Bakterier remineraliserade kiselalger och producerade ammonium. De kvarvarande aktiva diatoméerna assimilerade tillräckligt mycket ammonium för att balansera sitt kolupptag. Det fanns inga signifikanta skillnader i antal aktiva celler beroende på kedjelängd även om den genomsnittliga kedjelängden minskade över tid. Solitära aktiva celler hade högre kol och kväve specifik assimilering än aktiva celler i kedjor. Jag spekulerar att aktiva celler i kedjor kan dra nytta av att andra celler i kedjan reminerliseras och på så vis bidrar med ammonium. *S. marinoi* visade inga signifikanta trender i kol och kväve upptag beroende av kedjelängd och cellens specifika position i kedjan. De observerade nitrat upptagen var 25-65 gånger lägre än de modellerade nitrat transporterna från det omgivande vattnet genom diffusion till cellerna. Detta indikerade att cellernas nitratupptag var begränsade av biologiska faktorer och inte

diffusion vid nitratkoncentrationer lägre än 0.46 μM. Så i **kapitel I** och **II** visades att *C. affinis* och *S. marinoi* hanterade nitratbegränsning på väldigt olika sätt, dessa reflekterade deras nischer i naturen. *C. affinis* recirkulerade kvävet med hjälp av bakterier för att klara långa perioder utan nytt nitrat och *S. marinoi* tog upp så mycket nitrat som de kunde vid hög tillgång som vid början av en blomning där de oftast är bland de första att blomma. Detta är bara responsen från två av de vanligaste kedjeformande kiselalgerna.

Utöver sin roll som en av de viktigaste primärproducenterna på jorden så kan de tunga kiselalgerna bidra till transport av partiklar till sedimenten genom aggregatbildning som ökar sedimentations hastgeten för partiklar till bottnen. Kiselalger kan bilda vilostadier som kan överleva decennier till århundrande i anoxiska sediment utan ljus. Sen kan de blandas upp till ytan igen och starta en ny blomning. Överlevnad på så långa tidsskalor bör kräva någon typ av cellunderhåll, så som DNA reparationer. Potentiella mekanismer som kan hjälpa vilostadier av *S. marinoi* att underhålla cellerna undersöktes i **kapitel III**. Jag visade att vilostadierna kunde ta upp både nitrat och ammonium i syrefria miljöer utan ljus. Kväve assimilationen var snabb nog att dubbla cellens kvävemängd varje 23-500 år, vilket är tillräckligt för att underhålla cellen men inte tillräckligt för aktiv celldelning. I **kapitel IV** undersökte jag om dessa vilostadier kunde använda nitrat som en elektronacceptor och assimilera organiska föreningar (acetat och urea) vilka är vanliga i sediment. Vilostadierna kunde andas med hjälp av nitrat och genomförde dissimilatoriskt nitratreducering till ammonium (DNRA). Vilostadierna kunde assimilera kväve från urea med och utan nitrat tillgängligt som en elektronacceptor. De kunde inte assimilera kol från urea, i stället assimilerade de kol från acetat både med och utan nitrat närvarande.

Mer forskning behövs för att fullt undersöka diatoméers mekanismer för att överleva långa perioder i sedimenten. Jag har här visat att kiselalgers vilostadier inte är så inaktiva som tidigare antaget, nu behöver implikationerna av detta undersökas. Jag visade även att två vanliga kedjeformande kiselalger använder två olika mekanismer för att kringgå ett beroende av nitratdiffusion från vattenmassan. Nästa steg är att undersöka hur detta kan kopplas till marin övervakning och modellering av marina system. Den här avhandlingen har gett en första inblick i hur kedjeformande kiselalger hanterar nitratbegränsning, med tanke på hur ”diversa” kedjebildande kiselalger är så bör deras responser till kvävebegränsning vara lika mångfaldiga.

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I would like to thank two (people) towering above the crowd. For making the most difficult parts of the journey much easier and more enjoyable than what they would have been without them.

Olga Kourtchenko deserves special mentions for keeping the algae bank GUMACC and being the one wrangling the diatoms during and in between experiments. For showing me the ropes and letting me work in/with the algae bank, for giving the diatoms their antibiotics during the resting stage experiments and then waking them again in her MPN assays. She has played a vital role throughout the projects, none of them would be possible without her knowledge of diatom culturing and ability to keep structure in the labs.

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As controversial as it may sound, I would also like to thank the often-overlooked unnamed antagonists of this journey, the **Reviewers**. Your all-seeing eye was vital in making this journey meaningful, and the destination better. "No journey/story is better than its villain."

I would like to thank my family, my wonderful parents **Helen Stenow** and **Ola Stenow** that have encouraged me and endured many rants regarding my little diatoms. Also, my sister **Frida Stenow** for inspiring me with her own journeys.

I want to dedicate a sentence to the countless diatom resting stages who made the ultimate sacrifice for the: **"nitrate kick-starter incubation"** that did not make it to the final thesis.

Finally, I would like to thank **You** the reader for taking time to read this, I hope that you have as much fun reading the thesis that resulted from this journey as it was for me to embark on it. Now it is time for this journey to come to an end and for me to plan the next one.

See you on the road,
Rickard Stenow

List of abbreviations

Abbreviation	
AB	Alcian Blue
ASW	Artificial sea water
C	Carbon
Cs	Cesium
DAPI	4′,6-diamidino-2-phenylindole
DBL	Diffusive boundary layer
DIC	Dissolved inorganic carbon
DNRA	Dissimilatory nitrate reduction to ammonium
EA-IRMS	Elemental analyzer coupled to an isotope ratio mass spectrometer
HCO_3^-	Bicarbonate
MPN	Most probable number assay
N	Nitrogen
nanoSIMS	Secondary ion mass spectrometer with nanometer scale resolution
NH_4^+	Ammonium
NO_3^-	Nitrate
NO_2^-	Nitrite
PFA	Paraformaldehyde
POC	Particulate organic carbon
POM	Particulate organic matter
PON	Particulate organic nitrogen
PSB	Phosphate-buffered saline
PSU	Practical salinity unit
Si	Silica
SIMS	Secondary ion mass spectrometry
TEP	Transparent exopolymer particles
XG	Xanthan gum

List of Chapters

I: Stenow, R., Robertson, K. E., Whitehouse, J. M., & Ploug, H. Single cell dynamics and nitrogen transformation in diatom chains and solitary cells. ISME Journal. [*In revision*]

II: Stenow, R., Olofsson, M., Whitehouse, J. M., & Ploug, H., Single cell carbon and nitrogen assimilation in the chain forming diatom *Skeletonema marinoi*. [*Manuscript in preparation for Limnology and Oceanography*]

III: Stenow, R., Olofsson, M., Robertson, K. E., Kourtchenko, O., Whitehouse, J. M., Ploug, H., & Godhe, A. (2020) Resting stages of *Skeletonema marinoi* assimilate nitrogen from the ambient environment under dark, anoxic conditions. Journal of Phycology, 56(3), 699-708. https://doi.org/10.1111/jpy.12975

IV: Stenow, R., Robertson, K. E., Kourtchenko, O., Whitehouse, J. M., & Ploug, H. Resting stages of *Skeletonema marinoi* assimilate organic compounds and respire using dissimilatory nitrate reduction to ammonium in dark, anoxic conditions. [*Manuscript in preparation for Environmental Microbiology*]

My contribution

I: Planned the study together with my supervisor. Implemented measurements of TEP, POC, PON, and assimilation of C and N at a single cell level, and DAPI for bacterial enumerations. Performed DIC analysis. Prepared all samples for SIMS and nanoSIMS analysis. Performed SIMS analysis at the NORDSIM facility at the Natural History Museum in Stockholm. Processed all raw isotopic data to final rates. Developed the classification record of individual cells in chains and individual cells to investigate C and N assimilation depending on if the cells where in a chain and if so if the position in the chain affected C and N assimilation. Performed daily tracking of cell abundance and manual cell counts of both diatom and attached bacteria. Measured chain length in Lugol fixed samples. Implemented nanoSIMS to quantify bacterial C and N cycling at the chemical imaging infrastructure (Astra Zenica, Mölndal, Sweden). Compiled and analysed the data, then produced all figures and tables. Conducted all statistical analyses. Wrote the first draft and was responsible for the main revisions during the review as the corresponding author.

II: Planned the study together with my supervisors. Performed SIMS analysis in the NORDSIM facility at the Natural History Museum, Stockholm. Processed all new isotopic raw data to assimilation rates. Developed the classification and record system of individual cells and cells in chains. This was done to investigate C and N assimilation depending on if the cells where in a chain and if so if the position in the chain affected C and N assimilation. Calculated potential diffusive supply to solitary cells and chains of various lengths. Arranged and analysed all new data, then combined them with the old datasets. Conducted all statistical analyses. Was responsible for the first draft of the manuscript and all figures/tables.

III: Contributed to development of the protocol used to induce the resting stage in vegetative cells and experimental setup. Prepared samples for POC, PON, MPN, and SIMS analysis. Performed SIMS analysis at the NORDSIM facility at the Natural History Museum in Stockholm. Processed all isotopic raw SIMS data to final turn over rates. Combined the datasets, performed data-analysis, and plotting. Inspected for bacterial presence using DAPI staining. Conducted all statistical analyses. Produced the main draft and all figures/tables. Responsible for the main revisions during the review process as the corresponding author.

IV: Planned the study together with my supervisors. Prepared samples for POC, PON, and SIMS analysis. Performed SIMS analysis at the NORDSIM facility at the Natural History Museum in Stockholm. Processed all isotopic raw SIMS data to final turn over rates. Organised, analysed, and plotted accumulated data. Conducted all statistical analyses. Wrote the first draft of the manuscript and produced all figures/tables, except the table showing the experimental setup.

Above each section I have added some scribbles for my own amusement with many quotes from lord of the rings. Please feel free to ignore them!

Background

Diatoms are one of the most important primary producers on Earth, responsible for 20% of all primary production and able to thrive in many different environments (Nelson et al. 1995, Armbrust 2009). Chain-forming diatoms such as *Skeletonema marinoi* and *Chaetoceros affinis* often dominate spring blooms in coastal waters where many key interactions with higher trophic levels take place (Godhe et al. 2016, Bergkvist et al. 2018). Theses blooms are usually short, and the diatoms spend most of their time in nutrient limited conditions or as resting stages buried in the sediment. The heavy silica frustule that characterizes diatoms increases the sedimentation rate in larger aggregates of diatoms and hence they can contribute much to vertical fluxes of C and N (Alldredge and Gotschalk 1988, Iversen et al. 2010). This in combination with the dormant resting stage that they can form to survive centuries in sediments, potentially makes them important for long time C sequestration.

The research in this thesis aims to investigate how diatoms survive between blooms and thus cover multiple steps of diatoms life cycle (in this thesis defined as the broad phases of a diatoms life in Figure 1). I start of by giving a background of diatoms, chain formation, and their cell cycle. Then I explain the conditions during a spring bloom, how diatoms become nutrient limited, and bacterial interactions. Finally, I describe how diatoms sediment out of the photic zone, the conditions in sediments and the diatoms' ability to form resting stages which can survive long periods there.

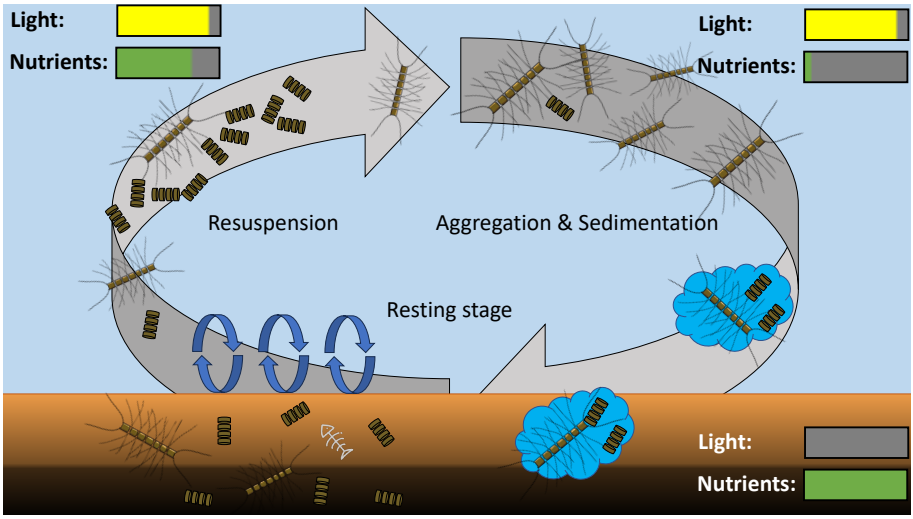

Figure 1. A schematic overview of diatoms life cycle. Upper left: early stage of a bloom. Upper right: late bloom conditions with aggregation and sedimentation. Bottom: Dark anoxic sediments. Blue arrows indicate water mixing. Heavily inspired by Figure 1 in (Ishii et al. 2022).

The travellers are chained together in a close knitted fellowship.

Diatoms cell cycle and chain formation

As diatoms grow, they mainly divide asexually. During division/mitosis, the diatoms produce one daughter cell of the original cell size and one slightly smaller, leading to a decrease in average cell size over multiple divisions. Cell volume can vary up to an order of magnitude between the largest and smallest cells of the same clonal strain depending on where they are in the cell cycle, see Figure 2 (Assmy et al. 2008). After an extended number of divisions, the frustule becomes too small to contain the cell. To regenerate larger cell sizes, the cells are enlarged, often sexually through the production of an auxospore which forms cells of the largest size and restarts the cycle. NH_4^+ has been shown to also trigger sexual reproduction and it have been speculated that germination from the resting stage might also trigger sexual reproduction (Moore et al. 2017).

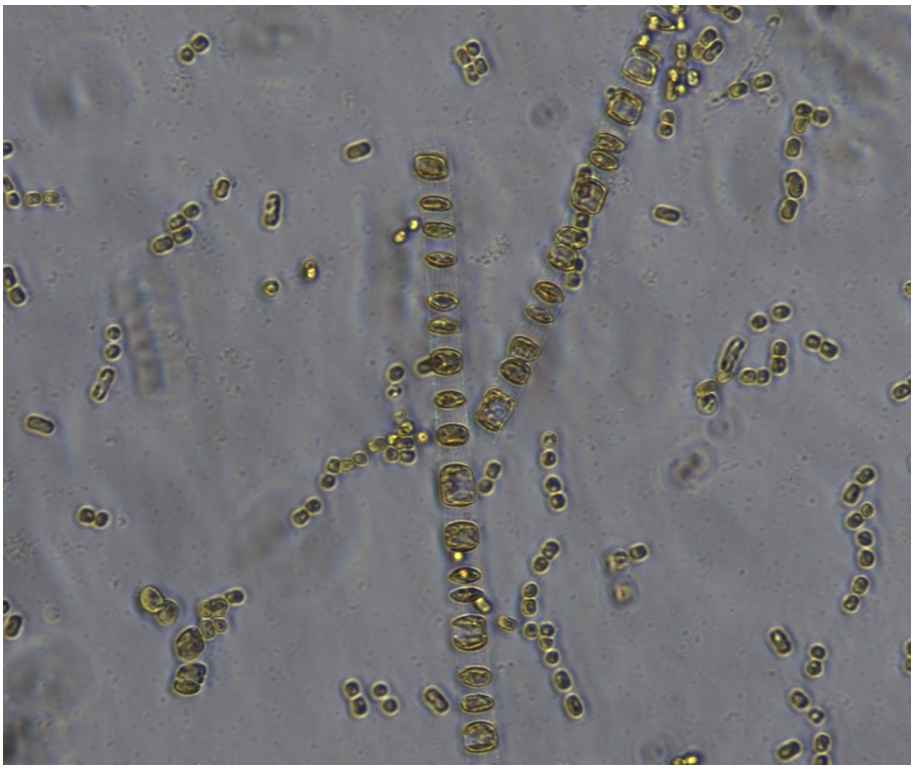

Figure 2. A monoclonal culture of Skeletonema marinoi, recently germinated from a resting stage incubation. Some cells recently underwent enlargement after germination. Notice the size difference between the large chains in the centre of the image compared to the smaller cells despite of them all being clones. The size difference is expressed by cells in different stages of the cell cycle. Photo: Olga Kurtchenko

Many diatom species form colonies with cells located next to each other in a single row, called chains. The diatoms within a colony can be attached to each other in different ways, in this thesis I focus on *C. affinis* and *S. marinoi* which are attached directly by the silica frustule. *C. affinis* and *S. marinoi* chains are formed during cell division, as the silica frustule between daughter cells are fused together as they form, resulting in monoclonal chains (Fryxell 1978). The mechanisms that regulate shortening of chain length and how the cells within a chain are attached varies between species. Generally, chains with cells attached by their frustule need to divide and create daughter cells no longer attached in order to break up chains. Chaetoceros produce daughter cells with terminal setae, which splits the chain during division (Gherardi et al. 2016, Amato et al. 2017). Chain formation has been considered a defensive mechanism against grazers (Pančić and Kiørboe 2018, Kenitz et al. 2020)

which can be regulated as a response to grazers, making smaller chains and thus harder to process by certain zooplankton (Bergkvist et al. 2012, BjÆrke et al. 2015, Rigby and Selander 2021). Other phytoplankton can both reduce or even maintain/increase colony size in order to avoid/reduce grazing pressure (Long et al. 2007). Chain formation have large implications for nutrient assimilation, please see the section called *"Implications of chain formation on diffusive fluxes"* for more details. Chains can also play a role in aggregation, please see *"Aggregation and sedimentation"*.

> The journey begins at the end of a large yearly celebration, with plenty of food and fireworks.

Phytoplankton blooms

Temperate regions often experience yearly spring phytoplankton blooms. During this time there is a near unlimited supply of nutrients and light for the phytoplankton trapped in the thin surface layer. This results in growth only limited by the phytoplankton's rate of exponential growth. Large portions of the annual production are produced in just a few days/weeks. During blooms, primary production is usually so high that grazers cannot balance the quick generation of new cells. Hence much organic matter can be transported to the sediment before being grazed/remineralized (see the section 'aggregation and sedimentation'). *S. marinoi* studied in chapter **II**, **III**, and **IV** often dominates the early blooms (Kiørboe et al. 1994, Tiselius and Kuylenstierna 1996). While *C. affinis* used in **chapter I** is relatively common in after blooms (Tiselius and Kuylenstierna 1996).

While blooms are important for the coastal system, it is vital to consider that they only occur a few days each year and that the "standard conditions" experienced by phytoplankton in temperate regions are some combination of nutrient and/or light limitation. Most studies conducted on phytoplankton today are performed during unlimited conditions similar to those in blooms. While there is indeed merit to understanding these key events in the annual cycle of the plankton community, there is a distinct lack in our knowledge in how these phytoplankton maintain their populations between the blooms.

But what about a second breakfast?

Nutrient requirements and diffusive fluxes

To investigate nutrient limitation, we must first understand the nutrient requirements of diatoms. The most fundamental understanding we have of marine phytoplankton today is their average stoichiometry, the Redfield ratio 106:16:1 mole ratio of C:N:P, (Redfield 1934). Diatoms also need Si to form their frustule leading to: 106:16:16:1 C:N:Si:P. The ratio has been a key concept in marine science for almost 100 years. There is much variance around this average, depending on nutrient regimes and biological variance (Gruber and Deutsch 2014, Moreno and Martiny 2018). In marine environments, N is often the nutrient limiting further growth, but Si, P, and micro nutrients such as Fe may also limit phytoplankton growth in natural conditions (Moore et al. 2013). DIC is seldom limiting to phytoplankton growth, due to high availability and the different forms of DIC in the bicarbonate system in seawater (Millero 1979, Feely et al. 2004). In this thesis, I will focus on N limitation, as it is the most common limitation in coastal environments. Nutrient acquisition during limited availability is complicated at a μm scale, where mixing of the surrounding water is ineffective due to the high viscous forces. Hence a diffusive boundary layer (DBL) of unmixed water is formed around the cell/chain, around which nutrients are transported only through diffusion (Ploug et al. 1999, Bergkvist et al. 2018). Because of this limitation, the surface area of a cell determines how much of a certain nutrient they can assimilated during a limited availability. A larger surface area gives more space for ion transporters leading to larger uptake. Cells with a larger volume typically have a larger nutrient demand than their smaller counterparts. Therefore, the area:volume ratio is often considered a proxy for cells ability to acquire nutrients compared to their demand. The area:volume ratio varies with cell shape, generally the ratio decreases when the size of the cell increases. Hence the potential nutrient uptake compared to their demand favors smaller cells during low nutrient availability.

One primary producer to rule them all, one microscope to find them, one chain to bring them all, and in the darkness bind them.

Implications of chain formation on diffusive fluxes

Chain formation effectively reduces the area:volume ratio, due to clonal cells within the chains being so close that their DBL overlaps. Chain formation has therefore been considered a disadvantage during stagnant, nutrient limited conditions (Takabayashi et al. 2006). This has also been confirmed by

modelling diffusive fluxes to chains of different lengths (Pahlow et al. 1997). During turbulent conditions cells in the terminal end of non-flexible chains display higher assimilation rates as compared to cells in the middle (Karp-Boss et al. 1996, Pahlow et al. 1997, Musielak et al. 2009). This may be the reason for observations of longer diatom chains forming during exposure to increased turbulence (Dell'Aquila et al. 2017). The close proximity between cells within chains potentially allows for fast diffusion of nutrient and signaling substances between cells.

> The fellowship seek aid from a guide with a divided Jekyll and Hyde personality, that can both help and harm them on their journey.

Bacterial interactions

Bacteria are often found living in close proximity to diatoms in nature, many living directly attached to the diatom frustule and interacting with the host in a plethora of ways (Amin et al. 2012). Their small size and subsequent high surface area:volume ratio enables them to overcome nutrient limitation, having only small individual cells to maintain. The close association of bacteria and diatoms reduces the diffusion distance for chemical exchanges compared to free floating bacterial cells (Figure 3). There are a host of interactions between diatoms and bacteria, many involve exchange of nutrients and vitamins (Amin et al. 2012). The bacteria *Phaeobacter gallaeciensis* associated with the coccolithophore *Emiliania huxleyi* can both help and harm its host by synthesizing growth hormones and antibiotics to aid their host, then shifts over to harmful algaecide production as the algae host ages (Seyedsayamdost et al. 2011).

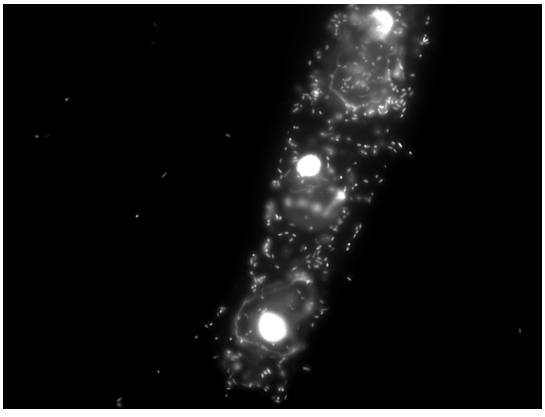

Figure 3. DAPI stained microscopy image at 1000X for C. affinis (large white areas with the nucleus highlighted) and its associated bacteria (small dots) in the late stationary phase (**Chapter I**). See the method section for more information of DAPI staining.

The water adjacent to a planktonic photosynthetic cell, where microbial interactions take place often contains exudates from the primary producer similar to the rhizosphere in terrestrial systems, is often

called the phycosphere (Bell and Mitchell 1972, Cole 1982). Since the term was coined, the significance of it has been discussed, but measurements of cell-specific transfers in this small size scale have not been possible until the recently. Recent advances have now permitted new methods to be employed in microbial ecology such as genomics and isotopic labeling in combination with SIMS and nanoscale secondary ion mass spectrometry (nanoSIMS). Such methods have become sophisticated enough to start mapping out these complex interactions in the phycosphere (Arandia-Gorostidi et al. 2017, 2022, Seymour et al. 2017, Arandia-Gorostidi et al. 2020). The main features of the phycosphere are high abundances of extracellular exudates released by the diatoms, containing high amounts of complex C molecules. Presence of associated bacteria has been shown to stimulate release of these extracellular exudates; for example, C fixation rates determined in diatoms are up to 98% higher with associated bacteria compared to without them. Bacteria also assimilated some of the diatom derived C and N (Arandia-Gorostidi et al. 2017, Samo et al. 2018). The presence of bacteria has been shown to extend the stationary growth phase before collapse in some cases for diatoms compared to cultures without bacteria (Grossart 1999). Thus, the associated bacteria seem to be important for diatom survival at the end of blooms. Further, bacterial presence has been shown to increase aggregation in the chain forming diatom *Thalassiosira weissflogii*, compared to axenic cultures without bacteria (Gärdes et al. 2011).

> It must be taken deep into the sediment and cast back into the anoxic chasm from whence it came.

Aggregation, sedimentation, and conditions in anoxic sediments

Sedimentation of solitary phytoplankton cells is generally very slow and depending on water depth, cells are often remineralized or grazed before reaching the bottom. Sedimentation rates for phytoplankton can be less than a meter per day (Wang et al. 2022). Diatoms usually have slightly higher rates thanks to their colony formation and the heavy silica frustule. Sedimentation rates of 15-30 m day^{-1} have been observed for Chaetoceros (Passow 1991). Plankton often form aggregates or marine snow during the end of blooms which rapidly transports them to the sediment (Riebesell 1991a, 1991b, Kiørboe et al. 1994). Sedimentation of marine snow sediment has been shown to vary between 50-200 m day^{-1} both in situ and in a laboratory setting (Alldredge and Gotschalk 1988, Ploug and Grossart 2000).

The factors controlling aggregation are numerous and depend on the system but simplified it is a function of particle encounter rate and the stickiness of said particles. Number of cell collisions, is facilitated by the concentration of cells in the water (Kiørboe et al. 1994). Different properties of cells can increase this even more, such as cell size and the shape of the cell, such as spines sticking out increasing the effective volume of the cell. Besides cell concentration, differences in sinking rate and mixing due to turbulence can also increases this encounter rate and aggregation (Alldredge and Silver 1988, Beauvais et al. 2006).

The main factor increasing cell stickiness is production of C rich organic compounds such as transparent exopolymeric particles (TEP) (Alldredge et al. 1993, Passow et al. 1994). Stickiness of TEP can vary with composition. These organic compounds can form particles or coat cells and thus make them stickier. Generally, TEP has a low density and thus sediments slowly unless aggregated with heavier particles. Chemical cues from copepods, a common grazer of diatoms can increase TEP stickiness and thus aggregation in multiple diatom species (Grønning and Kiørboe 2022). TEP production is considered to play a role in diatoms interactions with bacteria as bacteria can colonize it (Passow 2002). Marine snow can host a vast and varied bacterial community, bacterial remineralization can deplete O_2 in the center of these particels and create anoxic micro niches (Stief et al. 2016, Klawonn et al. 2019). Viral lysis of diatoms has also been suggested as a factor increasing aggregation formation in marine systems (Yamada et al. 2018). There are more factors contributing to aggregation, such as smaller copepods seem to increase aggregation by creating turbulence or mixing when they swim (Toullec et al. 2019). Larger copepods create more turbulence that can break already formed aggregates and thus reduce aggregation. Diatoms are often transported to the sediment while balasting marine snow particles, and even faecal pellets from zooplankton (Ploug et al. 2008). Therefore, they need to survive conditions in costal sediments so that they can return to the photic zone and regerminate.

These anoxic sediments are characterized by high bacterial remineralization of the organic matter that sediments down from the photic zone. Here, diatom cells are typically exposed to much higher nutrient availability than in the water column. In addition to inorganic nutrients such as NH_4^+ and NO_3^- there is also an abundance of organic material such as acetate and urea made available from remineralization (Lomstein et al. 1989, Thamdrup and Canfield 2000). The main factor prohibiting excess diatom growth is the lack of light.

> From the ashes a fire shall be woken. A light from the shadow shall spring.
> Renewed shall be chain that was broken. The crownless again shall be king.

Diatom resting stages

Diatoms are photosynthetic and use light to fix inorganic C and harvest energy from light. Diatoms generally form resting stages during adverse conditions. Some factors inducing the resting stages are: nutrient limitation, high cell concentrations, and virus infections (Pelusi et al. 2019, 2020, 2021). Aggregation and sedimentation might also induce the resting stage by the removal of light (Kiørboe et al. 1994, Agrawal 2009, Bergkvist et al. 2018). Sedimentation out of the photic zone implies that the cells have few opportunities of gaining energy, often the cells never resurface again. Much POC is remineralized during sedimentation before reaching the sediment-surface (De La Rocha and Passow 2007, Robinson et al. 2010, Devries et al. 2012). However, resting stages can be a large portion of the "surviving C" even if the direct effects are difficult to directly measure. Many diatoms have a mechanism that allows them to cope with long periods without nutrients, light and even anoxia, they form resting stages (Smetacek 1985, McQuoid and Hobson 1996, Ellegaard et al. 2018).

Diatoms produces 3 major forms of resting stages, according to (McQuoid and Hobson 1996):

1. **Resting spores** that have a distinct morphology form the vegetative cells, they are also suspected to be an important part of diatom life cycle connected to sexual reproduction (Davis et al. 1980). Typically, they have a thicker frustule than vegetative cells and can endure a range of hostile conditions. Resting spores can be formed inside the frustule of vegetative cells (Pelusi et al. 2019).
2. **Resting cells** that retain the same frustule as vegetative cells when they enter dormancy. Resting stages of *Chaetoceros pseudocurvisetus* have been less survival than resting spores during N limitation. Instead the resting stages resume rapid vegetative growth immediately after dormancy while resting spores experienced a lag period of around one day (Kuwata and Takahashi 1999).
3. **Winter spores**, a seasonal dormant stage with distinct morphology from vegetative cells like resting spores, but without larger internal stores and with significantly shorter survival times, which I will not investigate further in this thesis.

The resting stages can survive extended periods of time in darkness and anoxia, in a time scale of decades (Lundholm et al. 2011). Diatom resting stages are likely to survive better in anoxia than oxygenated conditions during

extended periods of dormancy (Lundholm et al. 2011). These resting stages have implications for the population structure and can potentially anchor a population of these pelagic plankton to a location by having a large population of resting stages in the sediment working as a "gene bank" keeping old traits, that can get resuspended and reintegrated into the active population in the photic zone (Itakura et al. 1997, Godhe et al. 2013, Sundqvist et al. 2018). Resuspension is presumed, but the exact methods are not known, bioturbation and vertical water mixing might factor in. Even if the portion of resuspended cells is low, there are many resting stages in sediments. Reports of over 10^6 viable diatom resting stages g^{-1} (wet weight) sediment are not uncommon at the sediment surface (Zhang et al. 2010, Fukai et al. 2019, 2021, Andersson et al. 2022). The nature of exponential growth and the diatoms capability of sexual reproduction means that only a few resuspended cells might have a large impact on the active population in the photic zone. A recent study have shown that the size of a vegetative population in the photic zone did not always correlate with the emergence flux from the sediment (Ishii et al. 2022). The potential to start a new bloom of these resting stages is assumed and the impacts have been seen in structures of populations. The resting stage have been suspected of being a driving force in retaining local population and thus creating local populations even in widespread spices such as *Skeletonema marinoi* (Godhe and Härnström 2010, Godhe et al. 2013). (Sundqvist et al. 2018) It has been shown that cells deeper in the sediment/cells that have been dormant for a longer period of time need longer time to germinate and start growing (Sicko-Goad et al. 1986). Others report little difference in germination time after 6 months of dormancy or even centuries (Pelusi et al. 2019, Sanyal et al. 2022).

As resting stages seem to survive better in anoxic sediments, one or multiped alternative electron acceptors to O_2 would be required for ATP generation (Lundholm et al. 2011). Anoxic marine sediments contain a host of alternative electron acceptors that can be used in the absence of O_2. Most efficient acceptors get depleted high up in the sediment then the next is utilized progressively deeper in the sediment, most of this respiration is performed by the bacterial community. The order of electron acceptors in costal marine sediments generally are: O_2, NO_3^-, Mn_4^+, Fe_3^+, SO_4^{2-} and CO_2 (Stumm and Morgan 1996, Burdige 2006). NO_3^- is one of the most efficient electron acceptors readily available in the surface layers of anoxic sediments, which I will focus on.

Multiple species of pelagic diatoms have been shown to use internal NO_3^- stores acquired during light oxic conditions to perform dissimilarly nitrate reduction to ammonium (DNRA) at rates that would deplete these stores at

the time scale of days (Kamp et al. 2011, 2013, 2016). It is believed that this would be used by the diatoms to survive short periods of anoxia. Such conditions could be experienced by pelagic diatoms in oxygen minimum zones often found underneath blooms during remineralization. DNRA has also been observed in benthic diatoms (Merz et al. 2021). They assimilate excess NO_3^- during light at the surface of a sediment/biofilm. During darkness they migrate down below the oxygenated zone and rely on the NO_3^- stores while preforming DNRA. Diatoms have been shown to perform DNRA on short time scales using internal stores therefore it might be worth to investigate if they can use external NO_3^- sources for longer time scales of anoxia.

Diatom resting stages are not well studied and we don't know how they survive dark anoxic sediments or how they later can reappear in the photic zone and initiate new blooms. The studies available today all indicates that the resisting stage is a vital part of pelagic diatoms life cycle. In this thesis, I start to disentangle processes that the resting stages can perform in the dark half of their life cycle. In **chapter III** and **IV**, I investigate active processes in dormant *S. marinoi* cells, who form resting cells with the same frustule as the vegetative cells and do not form morphological distinct resting spores as documented in for example *C. socialis* (McQuoid and Hobson 1996, Pelusi et al. 2019).

Knowledge gaps and thesis aims

Exponential growth conditions during algae blooms have been well studied. Many unanswered questions regarding how pelagic chain forming diatoms survive the unfavourable conditions between blooms remains. Blooms only lats a few weeks each year while growth limited conditions persist for most of the year. Despite this, diatoms persist in the photic zone between blooms in reduced numbers. Likely they have ways of coping with these adverse conditions. The importance of diatom-bacteria interaction is considered vital for both diatoms and their associated bacteria during these conditions. Interactions between chain forming diatoms and their associated bacteria during nutrient limitation is not well studied because the difficulties involved in measuring fluxes on this small scale. However, developments of new methods have become sophisticated to quantify some of these small-scale fluxes. Diffusive fluxes of N have been modelled for both solitary cells and cells within chains during N limitation. Here I will directly measure of N fluxes on a single cell level between diatoms and their associated bacteria as well variance of C and N assimilation within diatom chains. This might shed some light on how the diatoms can persist in the photic zone between blooms.

Diatoms can form resting stages to completely the nutrient limited conditions in the photic zone. The trade of is that survival and resuspension, probably is very rare. The mechanisms for diatoms survival in the sediments and regermination are even more enigmatic than in the photic zone. Diatom resting stages have been considered more or less completely dormant, but there has been no investigation of processes that they perform during long periods of darkness and anoxia. Resting stages would presumably need an alternative electron acceptor in the absence of O_2. *S. marinoi* have been shown to perform DNRA using internal NO_3^- stores. These stores have been estimated to be depleted on a time scale of days. It has not been investigated if the resting stages could perform DNRA using external NO_3^-. Presumably the resting stages would need to perform some form of cell maintenance during centuries of dormancy. Such maintenance probably require incorporation of both C and N during dark, anoxic conditions. Therefore, I investigated the potential for DNRA as well as assimilation of C and N in resting stages of *S. marinoi.*

The overall goal in this thesis is to gain a better understanding of how diatoms survive outside of optimal bloom conditions.

Thesis aims/research questions

Chapter I Investigate how C and N cycling change during the stationary growth phase in a chain forming diatoms and its attached bacteria.

Chapter II Measure how chain length and cell position in the chain affect C and N cycling during NO_3^- limiting conditions in two different strains of *S. marinoi*.

Chapter III Are diatom resting stages entirely dormant in sediments or do they assimilate C and N at slow rates in order to survive centuries in the sediment?

Chapter IV If not, what electron acceptors and donors can the resting stage utilize and what forms of C and N can they assimilate?

Methods

The algae bank and diatom strains used

Two spices of chain forming marine diatoms from the Gothenburg university marine algae culture collection (GUMACC) were used in this thesis (Figure 4): *C. affini*s and *S. marinoi*. *C. affinis* CCAP 1010/27, used in **chapter I**, isolated from Naples Italy 2008. This chain forming diatom is characterized by spines or chaeta protruding from its frustule, which may both protect from grazing and increase aggregation. *C. affinis* specie is usually found in relatively high abundance between blooms (Kiørboe et al. 1994, Tiselius and Kuylenstierna 1996).

Figure 4. Gothenburg university marine algae culture collection.

S. marinoi is a cosmopolitan chain forming diatom that often dominates the early spring bloom (Godhe et al. 2016). High division rates and excess N assimilation allows *S. marinoi* to growth before other species can utilize the favorable conditions with up to almost 3 cell divisions per day (Anderson and Rynearson 2020). After witch they can induce programmed cell death (Gallo et al. 2017). *S. marinoi* have until 2005 been considered a part of *Skeletonema costatum* (Sarno et al. 2005). In **chapter II** two strains of *S. marinoi* recently germinated from sediment cores in Danish Mariager Fjord were investigated. These diatoms had been dormant in the sediment for ~15 years (Olofsson et

al. 2019a). Three strains of cultured *S. marinoi* are used in the resting stage experiments (**chapter III** & **IV**):

1. R05AC (sometimes referred to as R05) that was isolated April 2010, at Öresund, Sweden at 14 m depth. The strain and its associated bacteria are used various genome studies eg. (Töpel et al. 2019a, 2019b). This strain is used as a genome reference strain for *S. marinoi*. C**hapter III** and **IV**.
2. GF04 isolated the 18 February 2004, at Gullmar Fjord, Sweden during a spring bloom (Taylor et al. 2009). **Chapter III** and **IV**.
3. HakH isolated in Hakefjorden, only used in **chapter III** due to poor survival rate of resting stages.

Stable isotope incubations

Stable isotopes of C and N were used to trace assimilation of C and N into diatom/bacterial biomass as well as dissimilatory processes such as DNRA and denitrification. ^{13}C and ^{15}N occurs naturally as rare stable isotopes. These isotopes do not decay or produce radioactivity in the form of alpha beta or gamma rays, as radioisotopes do. The stable isotopes used in this thesis are one atom weight unit heavier than the most abundant natural isotopes, ^{12}C and ^{14}N. The stable isotopes appear roughly in the following ratios in sea water, ^{13}C:^{12}C, 0.011 and ^{15}N:^{14}N, 0.00367. Nutrients (^{15}N-NH_4^+, ^{15}N-NO_3^-, ^{13}C-HCO_3^-, ^{13}C-acetate and ^{13}C-&^{15}N-urea) with higher ratios of the heavier stable isotopes than the natural background were added in every chapter. The enrichment of said isotopes were then measured in the diatoms, bacteria or media-water and compared to the natural ratio. Around 5% ^{13}C and ^{15}N was added in **chapter I** and **II**. Thus, there was no significant increase in C and N availability, instead the pre-existing C and N got labeled. In contrast the C and N incubations in **chapter III** and **IV** consisted to ~99% of ^{13}C and ^{15}N, this was done in order to detect the much lower C and N fluxes in resting stages compared to vegetative cells. Using the incubation time, the enrichment rate of the available media, the natural isotopic ratio, and the standing biomass I calculated uptake rate for individual cells and the total populations during the experiments. The dissolved concentrations of the stable isotopes were also measured in the ambient water to determine labeling percentages and potential dissimilatory processes such as DNRA and denitrification.

Isotopic calculations

C and N assimilation was calculated from isotopic ratios compared to isotopic controls. C-specific C or N-specific N assimilation rates k (h^{-1}) were calculated:

$$k(h^{-1}) = \frac{IR_{t1} - IR_{t0}}{F_{ambient}(t_1 - t_0)} \quad (1)$$

where $F_{ambient}$ was the excess % of either the ^{13}C or ^{15}N in the ambient water and IR the change in isotopic ratio within the bacteria, diatom, or POM during the incubation time t_1. These specific rates were then used calculate uptake over a time interval *a* (g):

$$a = k \times t \times M \quad (2)$$

where M was the C or N mass per cell (g). The C and N content for diatoms was estimated by calculate the cell volume from average size measurements according to (Sun 2003) these volumes were then used to estimate C content per cell according to (Menden-Deuer and Lessard 2000). N content of the diatoms were then estimated using the redfield ratio (Redfield 1934). Bacterial cell volume was estimated assuming a rod-shaped cylinder with a half sphere at each end of the cylinder. C and N content were then estimated according to (Khachikyan et al. 2019). See the method in **chapter I** for the more details of the mass calculations. Assimilation rates for diatoms were calculated linearly over all chapters because the cells where either in the stationary growth phase or resting stages with no divisions. The bacterial growth rates were assumed to be exponential in **chapter I** which corresponded well to the observed growth using DAPI observations. The exponential bacterial C and N specific growth rates were calculated $GR_{bacteria}$ (h^{-1}):

$$GR_{bacteria} = \frac{k_{bacteria}}{\ln(2)} \quad (3)$$

Particulate C and N analysis

Particulate organic C (POC) and particulate organic N (PON) was analysed for all chapters. Total abundance of standing POC and PON was measured and normalized per mL or per cell. Isotopic enrichment was also measured for calculation of C and N assimilation during the incubation. This allowed me to calculate C & N content and assimilation per cell using diatom abundance from manual cell counts. These rates were averages of 300 000 – 9 000 000 cells depending on chapter. Even if valuable data it did not capture the diversity of C and N assimilation that SIMS and nanoSIMS are capable of.

SIMS and nano-SIMS

Since the days of Redfield, classic phytoplankton ecology has involved the filtration of millions of individual cells after an experiment has been conducted. Then average bulk cell content and assimilation rates have been calculated for these cells, without accounting for individual variance. The

introduction of SIMS (Secondary ion mass spectrometry) and nanoSIMS allows its users to determine assimilation rates for individual cells, which gives a better understanding of how C and N assimilation is distributed within a population. SIMS is a mass spectrometer connected to a high-resolution ion beam. This allows it to produce images of a sample with high spatial resolution (> 1 μm). The resulting images contains abundances of different ions, which can be used to determine the location of cells and calculate isotopic ^{13}C:^{12}C and ^{15}N:^{14}N ratios. SIMS has traditionally been used in geological and astrophysical material studies. Over the last decades it has been implemented in bioscience. Advances in the technology have allowed the machines to resolve higher spatial resolutions than SIMS and nanoSIMS was developed. Compared to SIMS, nanoSIMS operates on a spatial resolution around 50 nm (see Figure 5). This has opened up for even small samples to be analyzed such as individual bacterial cells.

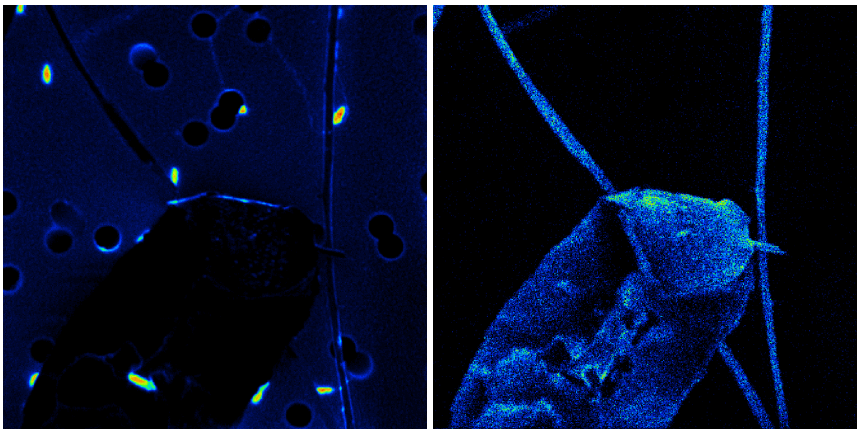

Figure 5. Chaetoceros affinis and its associated bacteria, captured using nanoSIMS, ^{12}C ^{14}N Cts, a proxy for biomass (left) and ^{28}Si Cts, the extent of the diatoms Si frustule (right). Both images show the same area, best demonstrated by the spines which can be seen in both images.

A drawback of using the higher resolution is that sampling time per area is increased. This means that while it is possible to use nanoSIMS to sample diatoms it would take a very long time to sample a significant number of diatoms using the same settings that I used for bacterial analysis. Not only does SIMS/nanoSIMS provide variance for individual cells, it also generates an image that can be used to provide spatial information about the cells, such as: whether they are solitary or within a colony, where in a colony they are located from the end, and even if they are damaged or not. Previous studies have incubated mixed field populations together and different species contribution to C and N assimilation disentangled within the natural

population. Bacteria can be grouped into cells attached directly on diatoms or as free-living as in **chapter I,** the works of Arandia-Gorostidi (Arandia-Gorostidi et al. 2017, 2022, Arandia-Gorostidi et al. 2020) and Rachel Foster (Foster et al. 2011, 2022). Despite the advantages that SIMS and nanoSIMS provide, it is not a common practice today. The main reasons being the costs of operation and the time required to analyze thousands of cells individually instead of just using averages. Another disadvantage compared to EA-IRMS is that SIMS/nanoSIMS measure the isotopic ratios, not the standing amount of POC and PON as EA-IRMS. SIMS/nanoSIMS therefore need to be combined with C and N measurements per cell or estimates thereof to produce assimilation rates in mole or gram per cell. Because of these reasons, SIMS/nanoSIMS is mainly applied in plankton ecology when the research question requires observations of individual variance and in combination with other methods that can provide an estimate of the cell's C/N content.

Sample preparation and analysis

Samples for all chapters were fixed in 1-2% paraformaldehyde (PFA) at 4 °C overnight, then filtered onto a TTTP filter 2 μm pore size, rinsed with PBF buffer to wash away PFA and prevent salt crystal formation on the filters. After drying, the filters were stored in Petri dishes at room temperature until analysis (Figure 6). Then samples were mounted on glass plates with double sided tape. Filters were then sputtered with a thin (5 nm) layer of Au, then analyzed using a large-geometry Secondary Ion Mass Spectrometer IMS 1280 (CAMECA, Gennevillers, France) at the NordSIMS facility (Natural History Museum, Stockholm, Sweden).

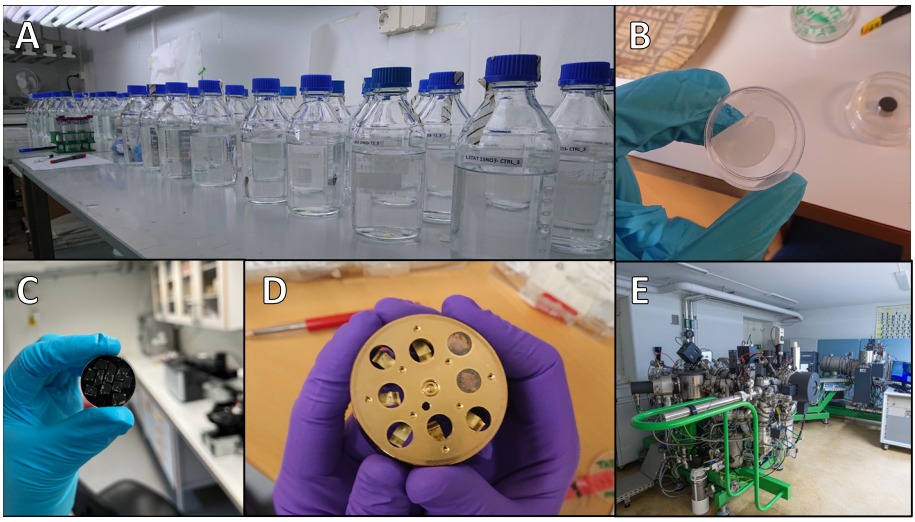

Figure 6. A: The stable isotope incubation performed in **chapter I**. B: An 2µm pore size filter in a petri dish after sampling, rinsing, and drying. C: Filter pieces from multiple replicates mounted on a glass disc using black double-sided tape prior to Au sputtering and sampling. D: Au sputterd filters mounted on smaller 1 cm holders. E: The large-geometry Secondary Ion Mass Spectrometer IMS 1280 (CAMECA, Gennevillers, France) at the NordSIMS facility (Natural History Museum, Stockholm, Sweden) utilized in all chapters.

Both the SIMS and nanoSIMS place the sample in a strong vacuum (this takes hours to cycle/establish) and then "pre-sputters" or implant the sample with a powerful $^{133}Cs^{+}$ ion beam to saturate the surface with these ions before sampling. When sampling diatoms, I used this beam for an extended time to remove the silica frustule of the diatoms and allow for sampling of the organic compounds inside. Areas containing diatoms (80 × 80 µm) were pre-sputtered for 60 s for *S. marinoi* and 180 s for *C. affinis*. In these studies, a $^{133}Cs^{+}$ beam was used for analyzing electronegative ions but other ions such as $^{16}O^{-}$ can be used for electropositive ions. During sampling, the primary $^{133}Cs^{+}$ beam a was set to lower power, 50 pA, with a spatial resolution of 0.5 µm. This was rastered over a 70 × 70 µm area. The machine fires a burst of these primary ions against the sample that dislodges a small portion of the sample (similar to the white ball in a game of pool/snooker). The dislodged ions are referred to as secondary ion (similar to the colored balls in pool/snooker). These secondary ions are directed into a mass spectrometer, where they are separated by weight and charge with the help of magnets. Secondary ions were analyzed with a mass resolving power of 12 000 M/ΔM. Which allowed for separation between CN^{-} species from potential interference such as $^{11}B^{16}O^{-}$ at nominal mass 27. This process was then repeated for each pixel in the image.

To lower uncertainty, I generate the entire image multiple times (in multiple cycles) to analyze more ions and obtain lower variance. I typically ran 30-100 cycles depending on ion abundance. This is a destructive process, and each cycle digs deeper into the sample. Therefore, each cycle was manually inspected for burn-through. Both SIMS and nano-SIMS only measure C and N in organic compounds (molecules containing both C and N atoms). This means that SIMS and nanoSIMS cannot measure inorganic matter inside cells, such as NO_3^- stores in diatom vacuoles.

NanoSIMS was deployed similar to SIMS, but on a smaller nanometer scale. Samples were prepared the same way as for SIMS analysis and measured using a NanoSIMS 50L (CAMECA, Gennevillers, France) at the chemical imaging infrastructure (Astra Zeneca, Mölndal, Sweden). The filters were mounted on 1 cm diameter metal discs (one per filter) instead of the lager glass discs used for SIMS. The sampling area was pre-sputerd with a 150 pA Cs^+ beam for 75 s over an area of 30 × 30 μm that was then analyzed using a 10 nA Cs^+ beam for 15 cycles. The nanoSIMS collected $^{12}C^{13}C^-$ and $^{12}C^{12}C^-$ ions instead of $^{13}C^{14}N^-$ ions, which was corrected for in the calculations according to (Pett-Ridge and Weber 2012). See the method in **chapter I** for the correction and an in-depth description of the settings used for nanoSIMS analysis.

Analysis of SIMS/nanoSIMS data

The resulting data were analysed using WinImage (developed by CAMECA). All collected cycles (layers) was manually checked for burn trough of the cells and/or oversaturation of the collection bins. Layers not passing the manual quality control were discarded and the remaining cycles were added together (aggregated) to create one layer for each ion observed with all observation for that location. Then cells were selected by hand from high abundance in the aggregated figure for $^{12}C^{14}N^-$ which can be used as a proxy for biomass (Figure 7 A). The ^{13}C:^{12}C and ^{15}N:^{14}N ratios were calculated for each selected cell, including on pixel correction for the detector deadtime (Figure 7 B & C). The natural background ratios of ^{13}C:^{12}C and ^{15}N:^{14}N were determined from the average ratios in non-labeled controls and used to calculate assimilation rates in enriched samples. Multiple images were required to fully capture longer chains in **chapter I** and **II**. These were taken with ~15 μm overlap and was then compared to lower magnification, light microscopy images to determine the exact positions of individual cells in chains and how to connect the overlap of different images. Each chain was numbered. The location of each cell relative to one end was recorded together with total chain length. Further each cell was classified as either: a solitary cell, a cell in a two-cell chain, a cell at the end of a chain and if the cell was located at the end of a chain. The

combined information could then be used to determine the location of a cell from both ends of a chain.

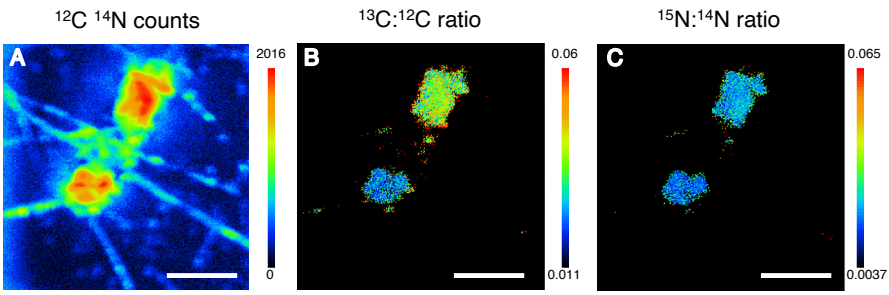

Figure 7. DIC and NO_3^- assimilation in C. affinis chains, captured using SIMS. A: $^{12}C^{14}N$ ion counts per pixel, a proxy for diatom biomass used to determine the location of each cell. B: ^{13}C:^{12}C ratio, a proxy for DIC assimilation. C: ^{15}N:^{14}N ratio, a proxy for NO_3^- assimilation. The color scale varies between subplots. Scalebars represents 20 µm.

Bacteria were also selected using the abundance of $^{12}C^{14}N^-$ ions. The pre-sputtering used in the nanoSIMS was too weak to penetrate the Si frustule of the diatom, so the diatoms appeared as areas without any ion reads (Figure 5). Therefor ^{28}Si was sampled and used to determine if the bacterial cells were attached directly to the diatoms or in the open water.

Diatom abundance and chain-length

Diatoms were fixed and stained by acidified Lugol's solution for long time storage. Cells and chain length was counted using a light microscope and a Sedgewick Rafter counting chamber until a stable mean where established. In **chapter I** and **II**, cell abundance was estimated using autofluorescence measured with a Varioskan Flash multimode fluorescence plate-reader (ThermoScientific Waltham, MA USA) using 425 nm excitation and 680 nm detection wavelength (Gross et al. 2018). These readings where calibrated against manual cell count during the different growth phases to account for changes in fluorescence per cell.

Bacterial abundance

Samples were fixated in PFA 1-2% to stop biological activity and stored in fridge until analysis. Staining was conducted according to (Porter and Feig 1980). The sample was filtered onto a black 0.4 µm pore filter using a GF/F filter underneath as support and then rinsed using phosphate buffer solution (PBS). The dissolved staining solution 4',6-diamidino-2-phenylindole (DAPI) ~5 µg mL^{-1}, is light sensitive and therefore all work involving it is performed

in the dark. The filter was stained for at least 1 h before being analysed using a florescent microscope. Diatom abundance was quantified either per volume (bacteria mL^{-1}) or the number of bacteria attached per diatom (bacteria $diatom^{-1}$) by counting cells over all focus layers until a stable mean and standard error was established.

Mass transfer theory

NO_3^- transfer to diatom chains of different lengths were modelled to chains of different lengths in **chapter II**. I assumed a linear correlation between chain length and the number of cells within each chain. Then the diffusive Q_t flux to each chain was calculated according to (Clift et al. 1978):

$$Q_t = \left[8 + 6.95 \times \left(\frac{l}{d}\right)^{0.76}\right] r_0 \times D(C_\infty - C_0) \quad (4)$$

Where l was the length of the chain, d the diameter of the chain, and r_0 the radius of the Chain. C_0 was the NO_3^- concentration at the cell surface and C_∞ the concentration in the bulk water during still conditions. The diffusion coefficient D, was set to was set to 1.35×10^{-5} cm^2 s^{-1} (at 12 °C, 26 PSU and 1 atm) using the marelac r package (Soetaert and Petzoldt 2020) based of Chapter 4 in (Boudreau 1997). This equation is accurate for chains with a length:diameter ratio < 8.

Diffusive flux to longer cylindrical chains (length:diameter ratio < 100) compared to spherical cells was estimated according to (Jumars 2019).

$$\frac{Cylinder\ flux}{Sphere\ flux} = \frac{2}{\pi} + 0.748 \times \frac{l}{d} + 0.493 \times \left(\frac{l}{d}\right)^{0.694} \quad (5)$$

the total area-integrated sphere flux Q_t (diffusive supply) was expressed as:

$$Q_t = 4 \times \pi \times r_0 \times D(C_\infty - C_0) \quad (6)$$

Inducing the resting stage

In **chapter III** and **IV** the resting stage was induced monoclonal cultures of *S. marinoi*. Most other studies of diatom resting stages uses resting stages freshly germinated from sediment cores as in **chapter III**. Instead, I choose to induce the resting stage in monoclonal strains of *S. marinoi* and to avoid many of the draw backs of using resting stages from sediment cores (Andersson et al. 2022). Such as the unpredictability of natural populations, patchiness, and effects from the sediment itself. Sediments host a diverse community of microbes that would greatly influence the C and N cycling measured here. The resting stage incubations here was performed using monoclonal strains, one of which have been sequenced, annotated, (Töpel et

al., in prep.) and proposed as a model organism for chain forming diatoms (Johansson et al. 2019). Recently there the possibility of "diatom" smearing from the top to deeper layers have been suggested by (Andersson et al. 2022).

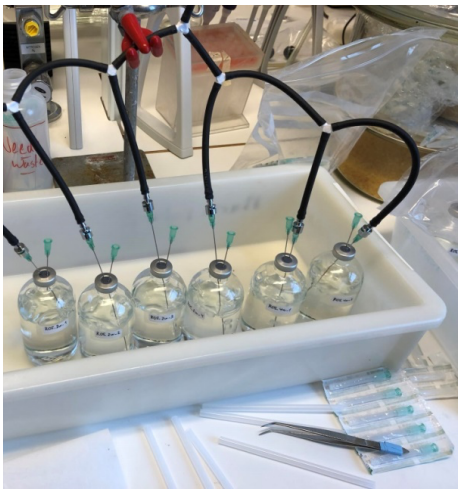

Figure 8. Degassing of serum vials for resting stages in **chapter IV**. Photo: Olga Kourtchenko

The resting stage was induced in *S. marinoi* by removing the cells form standard culture conditions after reaching the stationary phase: 10 °C, light to dark cycle of 12:12 h light:dark, at 50 µmol photons m^{-2} s^{-1}, in F/2 medium enriched with silicate (Guillard 1975) in 0.2 µm filtered seawater (26 PSU). The cultures were left to sediment, then the top half of the medium was discarded to concentrate the cells. Cells were then centrifuged. The supernatant was discarded, and then N free artificial seawater (ASW) was added. This rinsing was repeated once more to wash the cells from any remaining growth media containing N. The cells where then diluted to desired concentration in more N free ASW with nutrients corresponding to F/16 medium without N additions. A sample was fixed using Lugol's solution and counted using light microscopy and a Sedgewick Rafter counting chamber to determine the initial number of cells per mL. Sterile, N-free F/16 ASW was added to 120 mL serum vials (100 mL ASW, 20 mL head space) which were crimp-sealed. Before culture addition, water was degassed using Helium gas for 10 minutes (Figure 8). During the final minute of degassing, 1 mL of the concentrated diatom culture was added to vials and immediately put in darkness at 4 °C for ~30 days. The stable isotopic tracers were degassed and injected to the vessel through the rubber stopper after the 30 days. All tracer additions were done under a far-red light (745 nm) so as not to cause initiation of photosynthesis during handling and sampling.

MPN

A most probable number analysis was performed in all resting stage experiments to estimate the surviving number of cells for the survival rate according to (Briones and Reichardt 1999). Not all cells survived the treatment, and I could not differentiate live and dead diatoms using the microscopes available. Therefore, these dilution series was conducted to

estimate the number of germinating cells in each resting stage incubation (**chapter III** and **IV**). Each sample was diluted in 6 steps. The exact concentrations used varied between experiments but the difference between each dilution was to the power of ten, e.g., $0.5{:}10^0$, $0.5{:}10^1$, $0.5{:}10^2$, $0.5{:}10^3$, $0.5{:}10^4$, and $0.5{:}10^5$ sample per well. Each well was filled with the same F/2 growth medium used for cultivation in ~4 replicates (Guillard 1975). The wells were checked for germination using light microscopy for at least 2 weeks to determine the original concentration of cells. The calculations was performed using the Excel template provided by (Jarvis et al. 2010). The conversion from binary observations (germination/no germination) to an abundance introduces a lot of uncertainty and there are other methods of quantifying diatoms, but none other takes the differences in diatom resting stage germination time into account. The result is just an estimate, hence the name "most probable number analysis".

Summary of papers

Chapter I

Chain formation in marine diatoms has long been considered a disadvantage during nutrient limitation because the cells within the colony presumably would compete for resources provided by diffusive flux (Pahlow et al. 1997, Ploug et al. 1999, Van Oostende et al. 2018). In this chapter, the C and N dynamics of the stationary growth phase in *C. affinis* and its attached bacteria were investigated under NO_3^- limited conditions. This experiment was carried out to simulate the environmental conditions at the end of spring blooms where *C. affinis* often dominate. Nutrients was added to incubations, like those observed in nature, C and N dynamics was over 21 days in the chain forming diatom *C. affinis* and its attached bacteria as they deplete nutrients. C assimilation and NO_3^- assimilation varied very little within chains during the early stationary growth phase. Diatoms performed "microbial gardening" during the early stationary growth phase, with up to 5% of freshly diatom assimilated ^{13}C and ^{15}N being detected in attached bacteria within 12 h. The number of diatom-attached bacteria increased from 10 ±0.4 bacteria diatom^{-1} to 36 ±4 bacteria diatom^{-1}, and 86 ±4 bacteria diatom^{-1} in the early, mid, and late stationary phase, respectively. No extra NH_4^+ was added to cultures, but the dissolved NH_4^+ increased to 0.4 μmol L^{-1} and 1.6 μM μmol L^{-1} in the mid and late stationary growth phase (Figure 9). During the late stationary phase NH_4^+ contributed to 96% of total diatom DIN assimilation despite only making up 5.4% of the available DIN pool.

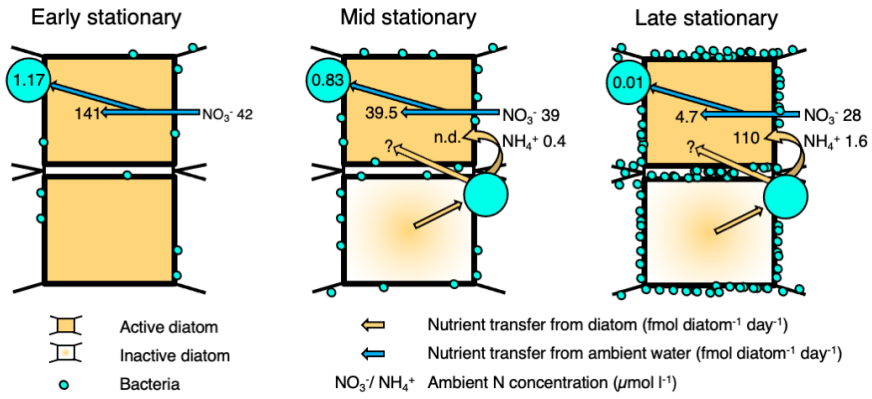

Figure 9. Measured N transport between active diatoms, diatom attached bacteria, and inactive diatoms within a chain, during the early, mid and late stationary phase. Note how N assimilation shifts from NO_3^- in the ambient water to NH_4^+ originating from bacterially remineralized neighbouring diatoms between the growth phases. The question marks (?) represent the 'masked' flux of N from a bacterially remineralized diatom directly to the neighbour, before mixing with the ambient ^{15}N-NH_4^+ enriched water. See Table S1 & S2 in **chapter I** for more details and SE.

Chain length was reduced over the growth phases as expected from other studies (Takabayashi et al. 2006, Olofsson et al. 2019a). Interestingly a relatively small portion of cells were solitary, as opposed being in a chain. Diffusion theory suggests that solitary cells would have a higher potential diffusive flux compared to cells in a chain that would have to compete for available recourses. Likewise, cells at the end of a chain would potentially have higher diffusive fluxes available compared to cells in the middle of chains. In the mid stationary growth phase, a portion of inactive cells was observed while the rest were active and assimilating both C and N. Inactive cells were determined by visual inspection for bacterial remineralization or as having as not being significantly enriched in ^{13}C (Figure 10). When excluding these inactive diatoms, solitary diatoms had significantly higher C and NO_3^- assimilation in the mid stationary growth phase.

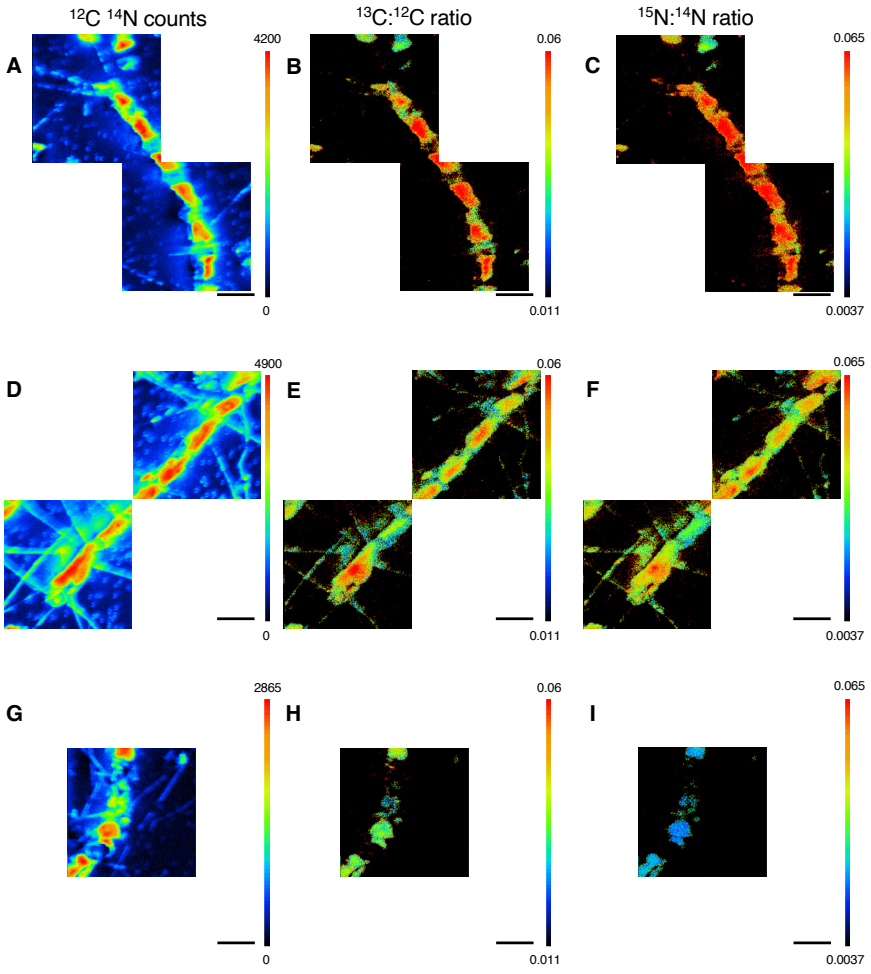

Figure 10. DIC and NO_3^- assimilation in C. affinis chains, captured using SIMS. A-C: fully active chain in the early stationary growth phase, D-F: an active chain with one inactive cell (the second from the bottom left) during the early stationary growth phase, G-H: a bacterially remineralized cell in the late stationary growth phase (the second from the top), A, D, & G: $^{12}C^{14}N$ ion counts per pixel, a proxy for diatom biomass used to determine the location of each cell. B, E, & H: ^{13}C:^{12}C ratio, a proxy for DIC assimilation, note the higher variance between cells in subfigure E compared to B. C, F, & I: ^{15}N:^{14}N ratio, a proxy for NO_3^- assimilation. The color scale varies between subplots. Scalebars represents 20 μm.

Diffusion time from a source increases with the square of the distance (Berg, Howard 1993). The short diffusion distance between cells in a chain suggests that nutrient transports from remineralized cells would be more efficient than

between solitary cells with longer and varying distance between them. I did not observe any autotropic bacteria in this experiment and therefore assume that NH_4^+ was produced as result of bacterial remineralization of inactive diatoms and not N fixation. The recycling of N might be one reason for cells not completely breaking the chains into solitary cells during this experiment. Another reason for the low abundance of solitary cells might be that end cells in chains need to divide in order to produce solitary cells. Here it was shown how one strain of chain forming diatoms respond to NO_3^- limitation. Other studies have shown that diatom-bacteria interaction within the phycosphere can lead to NH_4^+ production. Here I was demonstrated how a large chain forming diatom can survive in late bloom conditions that should be much more favourable for smaller plankton with higher area:volume ratios.

Main findings:

- Up to 5% of freshly diatom assimilated ^{13}C and ^{15}N was detected in attached bacteria within 12 h
- Up to 47% of the diatoms became inactive over the experiment
- Bacterial remineralization released ammonium into the ambient water
- During the late stationary phase, nitrate assimilation ceased in diatoms and ammonium assimilation balanced C fixation
- Cells in chains presumably had an advantage in NH_4^+ assimilation because they would have a shorter diffusion distance from the NH_4^+ source than solitary cells

Chapter II

The effects of NO_3^- limitation on C and N cycling was investigated on a large chain forming diatom most abundant after spring blooms in **chapter I**. In **chapter II**, it was investigated how another diatom species which typically dominates in early blooms respond to NO_3^- limitation. The two strains used in the present chapter were germinated from ~15 year old resting stages collected at the hyper-eutrophic Mariager fjord in Denmark (Tiselius et al. 2008, Lundholm et al. 2011). The fjord is considered eutrophic. The two strains used in this study were kept in laboratory cultures for less than half a year after germination before the incubations. This means that the stains used have had little time to adapt to the extremely high nutrient regimes used for long term cultivation of phytoplankton, many times higher than those found in nature.

Solitary cells were rare for both strains and did not exceed 5% of all cells during either the exponential or stationary growth phase. The observed cell specific NO_3^- assimilation were 25-65 times lower than those modelled using

mass transfer theory (Figure 11). The model has previously been used to accurately predict NO_3^- assimilation rates (Bergkvist et al. 2018, Olofsson et al. 2019b). When modelling the NO_3^- concentration at the cell surface, it was only ~3% lower than the NO_3^- concentration in the ambient water. Hence, I conclude that the cells here lacked the biological capabilities required to "fully" deplete NO_3^- at the cells' surface, which would allow for higher/maximal diffusive fluxes. This is not the first time that it has been suggested that Skeletonema might lack the biological ability to fully deplete the ambient NO_3^-, but to my knowledge this is the first time that it has been empirically quantified from assimilation rates on a single cell level. The cells were not limited by diffusive NO_3^- supply and the assimilation rates were not dependant on chain length. This suggests that the observed chain length reduction probably was controlled by another unknown factor.

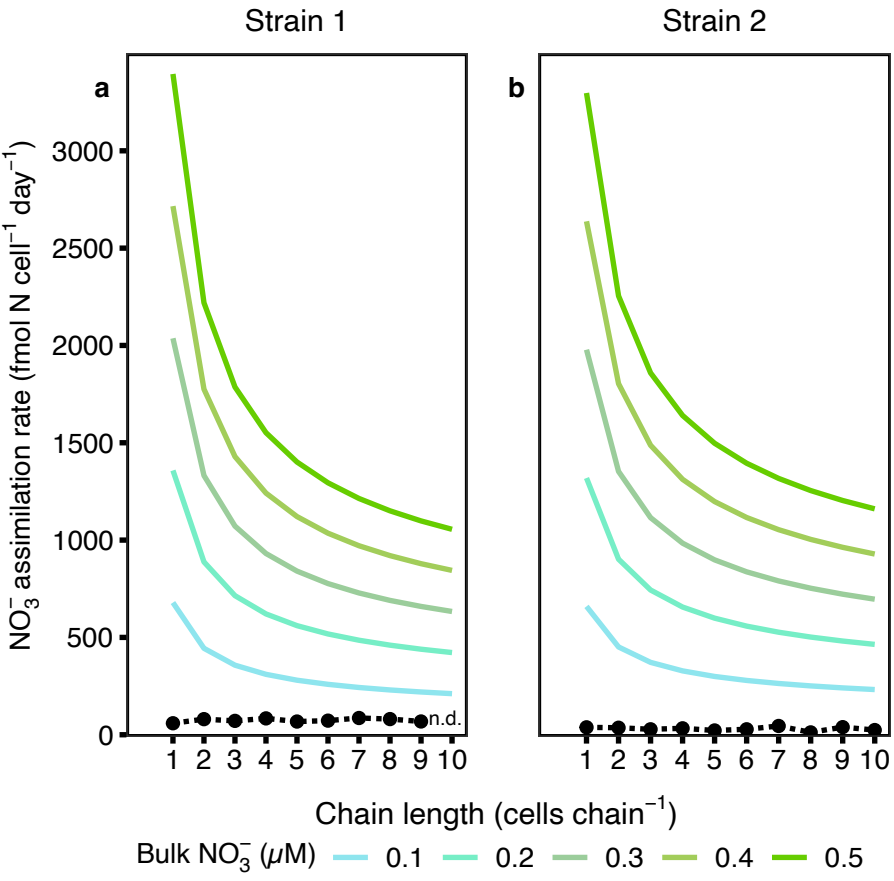

Figure 11. Observed and modelled NO_3^- depending on chain length at given ambient NO_3^- concentration. For strain 1 (a) and 2 (b). Blue and green lines represent the mass transfer model, black lines show observed values with standard error of the mean during the stationary phase, error bars are completely covered by the symbols. NO_3^- abundance during the stationary growth phase was 0.46 µM and 0.37 µM for strain 1 and 2 respectively. n.d. = no data, no chains 10 cells long were observed for strain 1.

The C:N assimilation ratio in *Skeletonema* was ~3 during the exponential phase, and it has demonstrated that they can store the surplus NO_3^- for later use during the stationary phase or anoxia (DeManche et al. 1979, Collos et al. 1992, Kamp et al. 2011, 2016). Hence the Skeletonema excels at gaining as much NO_3^- as possible during high availability and then rely on internal storages to shift over for more longer survival. *C. affinis*, studied in **chapter I** on the other hand recycled the available organic material between cells with the help of microbial interactions and managed to retain the standing biomass

over 21 days. In these two chapters it was demonstrated that *C. affinis* and *S. marinoi* have very different ways of dealing with nitrate limitation, corresponding to the different niches they fill. *C. affinis* recirculate N with the help of bacteria that allow them to survive in low N availability between blooms. *S. marinoi* on the other hand assimilated excess N during high availability in the beginning of a bloom.

Main findings:

- Little variance in C and N assimilation depending on chain length and the cells position within the chain
- Low proportion of single cells, up to 5% in the stationary growth phase
- Lower N assimilation than diffusive transport would allow
- This indicates that the cells were lacking low affinity NO_3^- transporters
- Skeletonema in this chapter and Chaetoceros in **chapter I** seem to have different "strategies" to cope with NO_3^- limitation
- This and the previous chapter have given a glimpse of potential NO_3^- limitation in chain forming diatoms, potentially there are a wide amount of other response in other species/strains left to be discovered

Chapter III

In **chapter III**, I investigated assimilation potential of NO_3^-, NH_4^+ and survival rate in resting stages of three *S. marinoi* strains (GF0410J, HakH, and R05AC). This also included developing a protocol to induce the resting stage in cultured monoclonal strains of *S. marinoi*. Both NO_3^- and NH_4^+ were assimilated by resting stage cells under dark, anoxic conditions.

Overall, there was a large variance in N assimilation rate between strains and the assimilation rate corresponded to a turnover time of 77-380 years. Even at such slow rates, it might be enough to maintain the cell during long term dormancy. Potentially associated bacteria could remineralize dead resting stages and release C in organic compounds such as urea and acetate that would be easy for the resting stages to assimilate. Survival rate also varied greatly between strains and treatments, there seemed to be a correlation between survival rate and ^{15}N enrichment (Figure 12). From this experiment I could not determine if the N assimilation led to higher survival rate or that the higher survival rate allowed for more N assimilation. However, the N assimilation observed might play a role in allowing the resting stages to survive extended periods of anoxia in dark conditions.

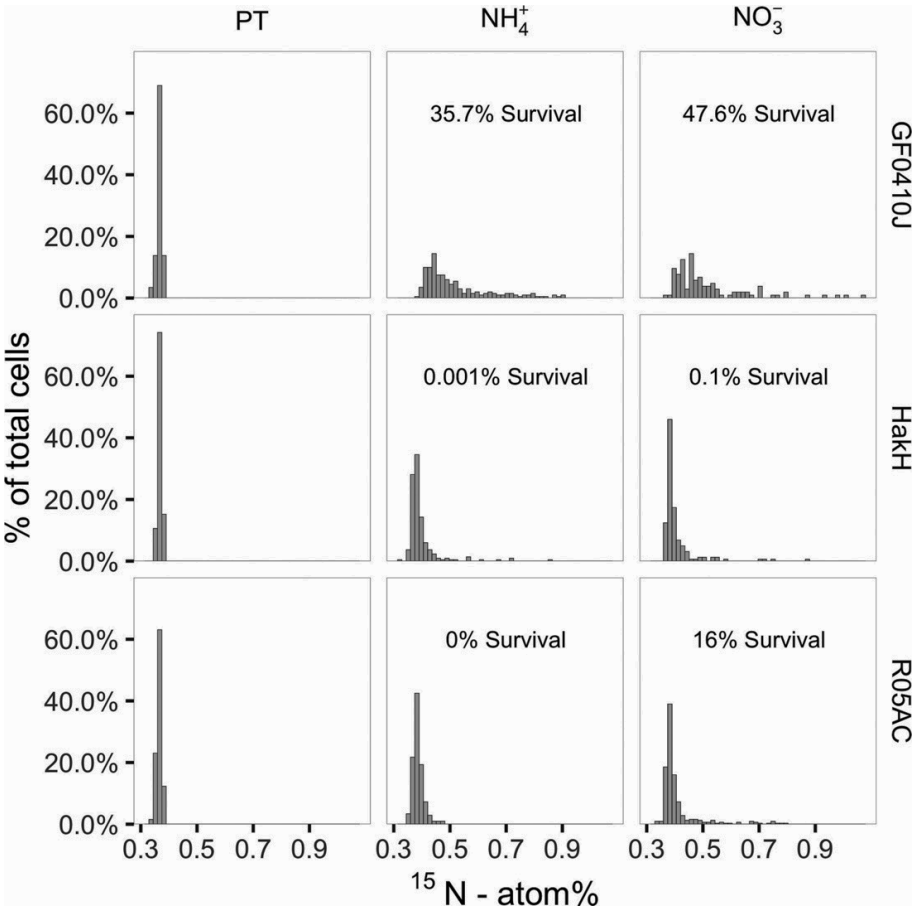

Figure 12. Histogram of ^{15}N atom%, a proxy of NH_4^+ and NO_3^- assimilation for each strain (GF0410J, HakH, and R05AC) and treatment (Pre-tracer samples, NH_4^+, NO_3^-). Pre-tracer samples showing the natural ^{15}N atom%, collected before addition of labeled N. The NH_4^+- and NO_3^-treated cells were sampled after 2 months incubation with labeled N during aphotic and anoxic conditions. The survival rate (%) was measured using cell counts in Sedgewick Rafter counting cambers during inoculation and MPN at the end of the incubation. Note that only surviving cells assimilate N and thereby show a more skewed normal distribution than the pre-tracer samples/dead strains.

Overall, the resting stages showed higher survival rates in the NO_3^- treatment over the NH_4^+ treatment. A potential reason for this is that NO_3^- can act as an electron acceptor in absence of O_2. The use of NO_3^- under anoxic conditions has been documented in culture studies for several pelagic diatom species. Internal NO_3^- stores were depleted in a timescale of days in conditions similar to those in oxygen minimum zones (Kamp et al. 2011, 2013, 2016). It has also

been shown that benthic diatoms can migrate below the oxygenated portion of sediments and preform DNRA using internal NO_3^- stores (Merz et al. 2021). The resting stage would presumably require respiration using another electron acceptor than NO_3^- or O_2 to generate ATP and assimilate NH_4^+. Two possible pathways for this, glycolysis and anaerobic fermentation have been found in the metabolic network of the model diatom *Phaeodactylum tricornutum* (Kim et al. 2016).

This was one of the first studies investigating potential C and N assimilation in individual diatom resting stages and raised important questions for the following chapter such as the role of associated bacteria, potential use of NO_3^- as an electron acceptor and potential assimilation of external organic C/N sources.

Main findings:

- Produced a protocol to induce the resting stage in culture strains of *S. marinoi*
- Cells survived and germinated after the experiment
- NH_4^+ and NO_3^- assimilation measured in individual cells
- Skewed distribution of the N assimilation, likely a result from cells perishing over time

Chapter IV

Experiments in this chapter were performed to answer some of the questions raised in **chapter III**. Such as, determining an organic C and N source that the resting stages can utilize, that also is common in sediments. The overall higher survival rate in cells treated with NO_3^- compared to NH_4^+ in **chapter III** could suggest that NO_3^- was used as an electron acceptor. Therefore, DNRA and denitrification were measured with and without antibiotics in this chapter to determine the diatoms capability of utilising either of the two pathways. The HakH strain was no longer used because of the low survival in **chapter III**. The experiment was performed on GF0410J (here shortened to GF04) and R05AC (here shortened to R05).

The first incubation was performed similarly to in **chapter III**. A extra treatment was added with cells exposed to an antibiotic cocktail prior to induction of the resting stage, as described by (Clarke 2017). DNRA was detected at similar rates in both strains with and without the addition of antibiotics (Figure 13). Denitrification was detected in the treatment without antibiotics but supressed in the antibiotic treatment. This indicated that bacteria and not the resting stages were performing denitrification.

During the second incubation in this chapter, all cells of the two strains were treated with the antibiotic cocktail. The resting stages of both strains were able to assimilate N from urea both with and without NO_3^- available as an electron acceptor. The resting stages were not enriched by the ^{13}C from the urea, either with or without NO_3^- available as an electron acceptor. C in urea is oxidised and would require additional energy to incorporate into the organic matter of the cells. C was assimilated from acetate, both with and without NO_3^- available for DNRA. Cells were checked for germination using MPN assays, all replicates had germinating cells during sampling. The DNRA rates correlated well with the initial number of diatoms, even between the two different incubations.

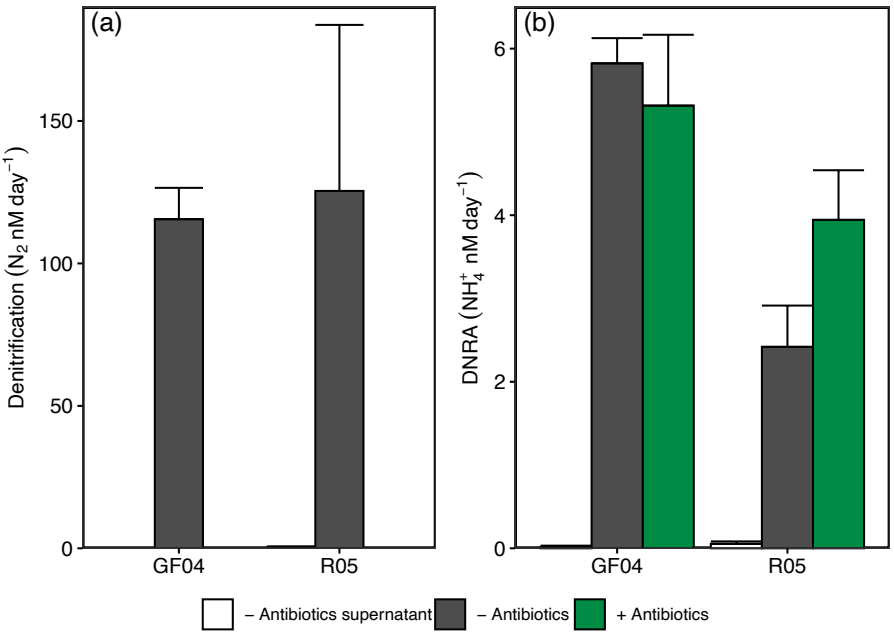

Figure 13. (a) Denitrification and (b) DNRA, (nM day^{-1}). For the strains GF04 and R05 in incubation 1. Supernatant without antibiotics (white), cells not treated with antibiotics (grey), and cells treated with antibiotics (green). The error bars represent SE. Note difference scales on y-axes.

In this chapter acetate and urea were also added to incubations as potential C and N sources for resting stages of these *S. marinoi* strains. Turnover times of C and N were as in **chapter III** on a scale of centuries which could likely be enough for cell maintenance, but not enough for active growth. Resting stages of *S. marinoi* were shown to perform DNRA in absence of light and O_2.

Considering the importance of the resting stage for the lifecycle of phytoplankton there likely exists a range of different resting stage "strategies" with varying amounts of active processes. It is however unlikely that any of the long enduring resting stages are completely dormant. There are many metabolic processes left to be discovered for different diatom resting stages such as resting spores. Finally, it needs to be considered that the resting stages in natural sediments encounter a wide range of electron acceptors and donors, in addition they are a part of a diverse and ever shifting microbial community.

Main findings:

- Denitrification was observed, but supressed with antibiotics
- The diatoms apparently performed DNRA regardless of antibiotic treatments
- Resting stages assimilating N from urea with and without NO_3^- available as an electron acceptor
- They assimilated C from acetate, but did not assimilate C from urea, likely because that C in urea was oxidized and thus would necessitate energy to incorporate
- The electron acceptor used to assimilate NH_4^+ in **chapter III** and urea & acetate without NO_3^- available remains unknown

Conclusion and further outlook

In **chapter I** and **II**, it was shown that *C. affinis* and *S. marinoi* have different ways of circumventing diffusion limited NO_3^- fluxes from the ambient water. The findings in this thesis suggests that diffusive supply of nutrients might not be influencing chain length reduction as much as previously assumed. In **chapter I** and **II**, I scratched the surface of diversity in marine chain forming diatoms responses to NO_3^- limitation. Now there is an abundance of other species/strategies to investigate, such as a flexible chain eg. *Thallasorisira* would be interesting investigate. Pennate chain forming diatoms such as *Fragilaria sp.* would potentially have larger differences between solitary cells and cells in chains. The longer sides of *Fragilaria sp.* face each other in chains, so potentially they would suffer greater diffusion limitation within the colony as compared in solitary cells. Another avenue of further research is to measure C and N assimilation in chain forming diatoms while accounting for individual cell size during biomass estimations. This would allow for estimation of C and N assimilations depending on size in a population in different steps of the cell cycle instead of assuming that they all are the same size.

In **chapter III** and **IV**, it was demonstrated that resting stages are not as passive as previously assumed. They can indeed assimilate both C and N in different forms at slow rates from the ambient environment. Then I showed that the resting stages can perform DNRA and assimilate C and N from small organic molecules which presumably can diffuse across the cell membrane. In sediment environments, there are a wide range of other potential electron acceptors and donors available with which resting stages may potentially use to generate energy for cell maintenance. Incubations have already been conducted by myself and colleagues to investigate the resting stages' potential capability to utilize H_2S and Fe^{2+} as an electron donors for NO_3^- reduction. In parallel, we have also investigated the potential of dark DIC assimilation by the *S. marinoi* resting stages. While it has now been shown that resting cells of *S. marinoi* perform multiple active processes, we don't know if the dedicated resting spores of other diatoms (such as those produced by *C. socialis*) have the same capabilities. This is a promising avenue for future studies. A further step would be to investigate if resting cells/spores of other phytoplankton classes, such as dinoflagellates have the same capabilities as demonstrated in diatom resting stages.

There still is a huge gap in our knowledge about diatom resting stages and how they can contribute to new blooms. Distinct populations can be found in different basins, which is suspected to be due to resting stages. Still, it is not known how the resting stages are resuspended back to the photic zone. It is very likely that processes such as bioturbation, different mixing events of the water column and even bubbles of methane gas from anoxic sediments contribute to this resuspension. Here we have a vital step of the life cycle of diatoms that we know nothing about. This presents an obvious avenue/opportunity for further research and might broaden our understanding of how diatoms pass through the yearly population bottleneck after each bloom and then seed new blooms.

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