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Declaration of independent work

I hereby declare that the work in this project has been performed independently and in accordance with the examination regulations of the Norwegian University of Science and Technology (NTNU).

Lotte M. B. Skolem

Preface

The present study was a continuation of the specialization project conducted at the Department of Materials Science and Engineering in autumn 2010. Both studies were a part of SOLBIOPTA - Biotechnological Production of Materials for Optimized Solar Cell Efficiency, a cooperation project between SINTEF Materials and Chemistry and the NTNU Departments Materials Science and Engineering, Biotechnology and Biology. SOLBIOPTA is funded by the Norwegian Research Council and runs from 2010 to 2013.

The cultivation experiments in this project were performed at the Department of Biotechnology, the ICP-MS assays were conducted at the Department of Chemistry, the TEM analysis was done at the Department of Physics and the feed solution preparations were performed at the Department of Materials Science and Engineering.

The present study was in many ways a pioneering work in the intercept of different scientific areas, and the project outline for this master project therefore had to be considered more as a starting point and guideline than a detailed plan to be followed. Some of the original objectives were abandoned when they turned out to be unnecessary or unrealistic, while others where added to complement the study. A comprehensive review of the analytical methods is not included in the Background and Theory section because they have not been the focus of the work, but a brief introduction to the different analytical methods is presented in the Materials and Methods section. This was done because of the multidisciplinary character of the study, in order to provide readers with different scientific backgrounds with enough information to understand how the results were produced.

I would like to thank Prof. Olav Vadstein and Prof. Mari-Ann Einarsrud for valuable advice and guidance, and Dr. Matilde S. Chauton and Dr. Lasse M. Olsen for assistance in the lab and for helping me navigate in the jungle of biotechnology literature. I would also like to thank Dr. Per Erik Vullum for helping me with the TEM analysis, Maren Skolem and Christian B. Skjelbred for kindly proofreading my texts and fellow Master student and friend Anne Kirsti Noren for rewarding discussions and for letting me use two of her images in my Master Thesis.

Finally, a special thank to Senior Engineer Syverin Lierhagen, who went out of his way to help me get my results when the ICP-MS instrument broke down.

Summary

The metabolic insertion of titanium into the biosilica frustules of the pennate diatom *Pinnularia* sp. and the centric diatom *Coscinodiscus wailesii* was explored in the present study. A total of five titanium incorporation experiments were conducted on *Pinnularia*, in addition to five control experiments. Titanium dissolved in HCl was co-delivered with silicate dissolved in NaOH to silicate replete and silicate deplete *Pinnularia* cultures over a 10 hour delivery period. Cell number, silicate and titanium concentration, Quantum Yield, Instantaneous Chlorophyll Fluorescence and pH was measured regularly throughout the experiments. The diatoms were left to consume the delivered silicate/titanium for 62 hours after delivery was completed before they were harvested and rinsed to remove the organic material. The resulting biosilica frustules were analyzed with (Scanning) Transmission Electron Microscopy to investigate the frustule structure, Inductively Coupled Plasma Mass Spectrometry to measure the elemental composition of the frustules and Energy Dispersive Spectroscopy to determine the location of the elements in the frustules.

Quantum Yield measurements were conducted to obtain information about the *Pinnularia* photo system condition, and high Quantum Yield values were interpreted as an indication of a good overall physiological state of the cell. It was found that a combination of high Quantum Yield values and silicate depleted cultures produced the highest titanium content in the *Pinnularia* frustules ($\sim$ 1.3 wt% relative to silicon). Furthermore, EDS analysis revealed that titanium was not evenly distributed throughout the biosilica frustule. The relative titanium content in the *Pinnularia* frustules was higher in the biosilica at the base of the large pores covering the frustule surface than in the biosilica between the pores. This was in accordance with findings reported in literature. The observed inhomogeneous distribution trend was found to apply also to phosphorous and iron present in the titanium-doped frustules. This observation has not been previously reported.

Cell aggregation of the *Pinnularia* cultures was observed upon silicate/titanium addition. Based on results from control experiments there was reason to believe that the aggregation was triggered by titanium, but the explanation for this cellular response as well as its effect on titanium uptake and incorporation was not determined.

Three titanium incorporation experiments were conducted in the proof on concept study on *Coscinodiscus* . Titanium dissolved in HCl and silicate dissolved in NaOH was co-delivered with a pipette once a day for three, five and seven days. On the day following the final addition, the diatoms were harvested and rinsed before they were analyzed in the same manner as *Pinnularia*. The experiments on *Coscinodiscus* revealed that metabolic insertion of titanium into the frustule biosilica was possible, but due to the small maximal titanium content achieved ($\sim$ 0.03 wt% relative to silicon) and the slow growth rate, *Coscinodiscus* was not considered an optimal choice of diatom species for titanium incorporation purposes.

It was not possible to rule out whether the frustule structure in any of the two species had been altered or impaired as a consequence of titanium exposure. Structural flaws

were observed in frustules with and without titanium incorporated. A faint irregular pattern on the nanoscale was observed in *Pinnularia* frustules containing titanium. No such pattern was observed in titanium free frustules.

The amount of boron measured in the frustule biosilica was higher than the regular dopant concentration in boron-doped silicon. The boron content in *Pinnularia* was found to be on average ∼0.1 wt% or ∼0.28 atomic % relative to Si, while the average in *Coscinodiscus* was nearly twice as high. Without further manipulation, this high boron natural boron concentration makes diatom frustules unusable for doped semiconductor purposes.

Future work should involve reproduction of the obtained results with more replicates, as well as larger culture volumes and, in the case of *Coscinodiscus* in particular, longer time scale. Titanium uptake and incorporation studies should if possible be performed on single *Coscinodiscus* cells.

Multiple continuous silicate starvation and titanium/silicate delivery cycles should be tested to determine if it is possible to incorporate titanium into a larger fraction of the culture population, as well as to increase the maximum amount of titanium incorporated into one frustule.

Aggregation of *Pinnularia* diatoms should be further investigated to determine reason for the observed reaction, the effects on titanium uptake/incorporation and possible strategies to prevent it from occurring.

Pinnularia frustules unexposed to titanium should be inspected in order to determine if the iron and phosphorous distribution pattern observed in the titanium-doped frustules is present in titanium-free frustules as well.

A completely artificial culture medium and plastic cultivation containers should be applied to explore how the boron content of diatom frustules can be tailored by controlled delivery of boron to the culture medium.

List of abbreviations

DF - Dark Field

dSi - Dissolved silicate

EDS - Energy Dispersive Spectroscopy

f/2 - Sea water medium designed for growing diatoms

Ft - Instantaneous Chlorophyll Fluorescence

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

SEM - Scanning Electron Microscopy

Si - Silicon

(S)TEM - (Scanning) Transmission Electron Microscopy

Ti - Titanium

QY - Quantum Yield

Contents

1 Introduction

Society today faces a number of challenges, such as climate changes, pollution, diminishing supplies of fossil fuels and an increasing world population requiring clean water, healthy food, medical care, high tech electronic devices and power to run it all. More advanced technology may help us handle many of these challenges, and in the search for new, smart solutions, looking to nature may prove helpful. Nature has been optimizing organisms for billions of years, producing highly effective constructions like the hard and strong, yet light and tough composite material bone, or the super hydrophobic micro- and nanostructured surface of a lotus flower. Scientist has been inspired by both to produce new, improved materials for human use. The diatom is another organism with interesting, possibly useful properties. With its ability to fixate carbon, produce carbohydrates and lipids, and utilize the silicic acid present in relatively large concentrations in waters all over the world to form beautifully patterned, nanostructured shells called "frustules" of biogenic silica, the diatom may provide a solution to more than one problem. In the following work, focus will be on the diatom frustules, but it is worth noting that it has been proposed to produce bio fuels, dietary supplements and animal fodder from the organic diatom cell body. The proposed applications for the highly ordered biosilica frustules are many, ranging from membrane applications, micro fluidic systems, energy storage, catalysis, sensors, and selective separation of nanoparticles to biological or bio mimetic production of nanostructured, functional materials and light harvesting. The size and structure of the pores covering the frustule surface varies with species, so in theory it might be possible to find a different species for every application. The alternative approach would be to focus on a couple of species and manipulate their properties to fit the requirements.

One way of altering the frustule properties would be to incorporate foreign elements into the biosilica. Depending on the element chosen, mechanical, structural or optical properties could possibly be tailored just by adding the element to the diatom feed medium. Silicon is widely applied in the solar cell industry today, and the semiconductor is usually doped with trace amounts of other elements such as boron. If boron could be incorporated into the silica frustule in controlled amounts before the silica was reduced to silicon, that would provide a cheap, environmentally friendly alternative route to producing boron doped silicon. Moreover, it has been proposed that diatom frustules enriched with titanium might be used to improve the efficiency of dye sensitized solar cells. Titanium incorporation into diatom frustules has previously been investigated by an American research group, but their results have not been reproduced and did not explored the correlation between the physiological condition of the diatom and the resulting frustules.

In general, diatoms have been studied from both a biological and a material science point of view, but the connection between the diatom cell biology and the frustule material properties has received little attention. More research therefore appears to be necessary in order to be able to utilize the full potential that may lay in the diatom frustule sometime in the future.

1.1 Aim of work

The main purpose of the present work was to investigate how the initial silicate concentration in the culture medium and the physiological condition of the pennate diatom *Pinnularia* sp. affected titanium uptake and incorporation into the biogenic silica frustule. Furthermore, the possibilities for titanium incorporation in the centric diatom *Coscinodiscus wailesii* was explored as a proof of concept study. The structure of frustules from both species was inspected for flaws and alterations, and the elemental composition of frustules produced in the titanium incorporation experiments was analyzed to determine the titanium, boron, iron and phosphorous content. The location of titanium, phosphorous and iron in the titanium doped *Pinnularia* frustules was examined.

2 Background and theory

2.1 Diatoms

Diatoms are eukaryotic, unicellular algae that live as single cells or in colonies, usually close to the water surface (the pelagic zone), but some species prefer to live close to the sea bottom (the benthic zone). All species that require sunlight to perform photosynthesis live in illuminated water layers (the photic zone). According to fossil records, the first diatoms occurred 185 million years ago [1]. Since then, they have spread out and become dominating in waters all over the world. Diatoms can be found in most marine and freshwater habitats, from oceans and lakes to humid air, ice and soil. The concentration of species is highest in tropical areas, but still many species can be found in polar regions, especially in ice-edge communities. "Diatom" is derived from Greek *diatomos*, which means "cut in half" [2]. The name refers to the shape of the biosilica shell, called frustule, that is characteristic for all diatoms. It is this biosilica frustule, with the general formula $[Si_nO_{2n-(nx/2)}(OH)_{nx}]$, x $\leq$4, that differentiate diatoms from other plankton [1]. Diatoms take up dissolved silicate (dSi) in the form of orthosilicic acid ($Si(OH)_4$) from the surrounding environment and use it to build the biosilica frustule. The frustule is covered with highly ordered two-dimensional micro and nano scaled arrays of chambers and pores of various sizes. The pore pattern is unique to every diatom species, and is frequently found to be hierarchical, with several layers of different sized pores stacked on top of each other (fig 1). The pattern is the criteria by which diatoms are classified into species, and it is estimated to be somewhere between 100 000 and 200 000 living diatom species in the world today, divided into more than 200 genera [4]. Because of this characteristic species-specific structure of diatom frustules, it is believed that frustule morphogenesis is genetically encoded [3].

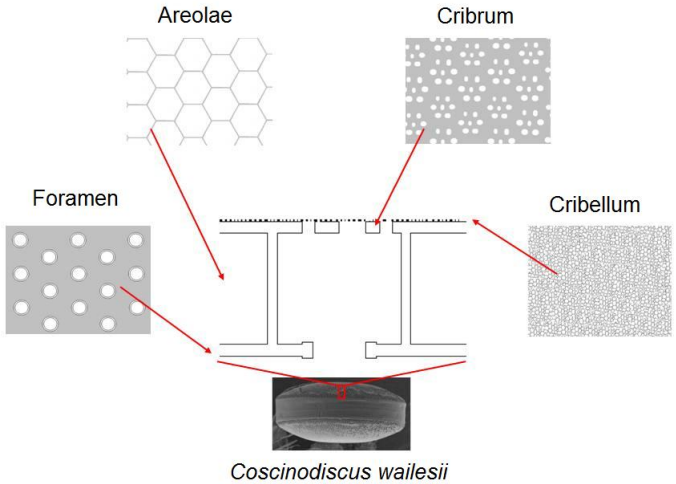


Figure 1: Frustule structure of *Coscinodiscus wailesii* [5]. The biosilica cage is buildt up of four distinct layers.

Diatoms consist of two asymmetric parts ("valves" or "theca"), with one valve slightly larger than the other. The largest valve (epitheca) overlaps the edge of the smaller valve (hypotheca), and the joint is lined by girdle bands, which are patterned silica bands that extend around the entire circumference of the diatom, providing mechanical support while still allowing the diatom to grow [6]. In addition to silica, the diatom frustule consists of organic material inside the silica and on the surface. This is why the frustule material is referred to as "biosilica" [4]. The purpose of the organic material is probably to improve the frustules mechanical properties and to prevent it from dissolving. Seawater is unsaturated in silicic acid, and its weak basic character (∼pH 8.2 [7]) has a corrosive effect on bare silica [1]. The surface charge of the diatom cell wall is determined by amino, carboxyl and silanol groups present in the polysaccharides and proteins coating the frustule surface [8]. Diatoms are known to display a strong affinity for a wide range of heavy metals and trace elements, and these compounds are trapped at the proton-binding sites of the organic coating [9]. When the organic coating of a dead diatom is broken down by bacteria, up to 90% of the trapped compounds are released to the environment. Diatoms therefore play a considerable role in the geographical transport of pollutants, and they also affect the uptake of heavy metals into the marine food chain. Many diatoms can protect themselves from ionic or loosely complexed metals by excreting organic compounds that effectively complex metals in solution, thereby reducing cellular uptake [10].

The main purpose of the diatom frustule is disputable. It is generally assumed that the frustule is primarily for mechanical support and protection, but in some species it may also be of aid in buoyancy control. Hale *et al.* proposed that the microtopographies of *Coscinodiscus* sp. diatom frustules controls the diffusion and advection of nanoparticles near the diatom surface [11]. This way, the frustule structure helps sorting out harmful bactria while concentrating smaller particles at the pore ridges, thereby reducing biofouling and enhancing nutrient uptake simultaneously. Hale *et al.* found that the drag exerted by living and dead *Coscinodiscus* frustules extended 5 μm above the surface. It has also been suggested that the frustules play some role in the diatom light harvesting process, directing light through the ornate pattern or maybe acting as a lens to focus the incoming light [12]. This theory is supported by the fact that the organelles responsible for absorbing sunlight and converting it to chemical energy through photosynthesis, the chloroplasts, in many species are located directly beneath the pores. However, the optical properties of the frustules are far from understood, and more research is required before their role in the photosynthetic process of diatoms can be determined.

Diatoms are conventionally divided into two orders, pennate and centric diatoms, based on the symmetry of their valves. Pennate diatoms are symmetric about a centerline (bilateral), but may otherwise have an oval shape in which one end is broader than the other. Centric diatoms have radial symmetry. The pennates and centrics can again be divided into radial and polar centrics, and pennates with and without raphes (fig 2). Most diatoms are between 2 and 200 μm in size, but some species can get as big as 2 mm.

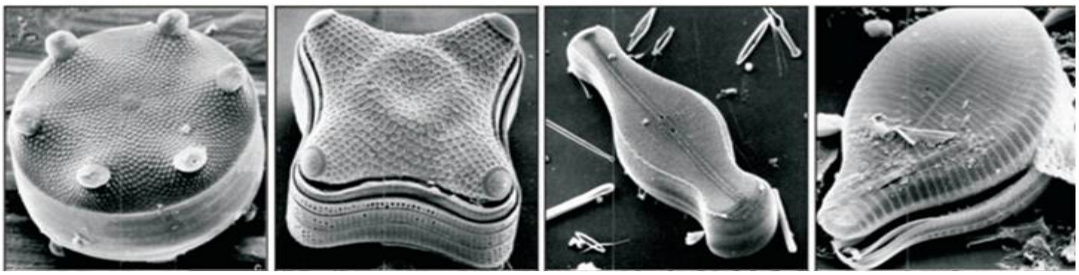


Figure 2: Examples of centric and pennate diatom species. Left to right: Radial centric, polar centric, raphid pennate and araphic pennate [3]

Diatoms play a vital part in the world's ecosystem. They alone account for ∼45% of the marine primary production, ∼20-25% of the world's total primary production and ∼20% of the total oxygen production [4]. Most diatoms are autotrophic (they require light in order to be able to produce complex organic compounds such as fat and carbohydrates from CO_2 through photosynthesis), but some are heterotrophic (not able to fixate CO_2 and therefore depend on access to organic carbon sources). Still some are autotrophic but can live heterotrophic in the absence of light, providing that there is sufficient organic carbon available in the surrounding environment.

2.1.1 Diatoms in the present study

Two types of diatoms, *Pinnularia* sp. and *Coscinodiscus wailesii*, henceforth only called *Pinnularia* and *Coscinodiscus*, were applied for the present study. Both species are autotrophic diatoms. *Pinnularia* was chosen because successful incorporation of titanium (Ti) in this diatom species was previously achieved by an American research group [13]. *Coscinodiscus* was chosen because it has a promising structure for optical applications.

Pinnularia is a relatively large (10-40 μm), pennant diatom that prefers to grow in colonies in still waters, forming long threads or thin sheets of multiple connected cells when the water turbulence is not too strong [14]. The size difference between two diatoms within a single culture can be quite large (illustrated in fig 3). The *Pinnularia* frustule is covered with a double porosity structure, consisting of nano-sized (50 nm) pores lining the circular base of sub-micro (200 nm) larger pores arranged in a highly ordered two-dimensional array. The "sp" in *"Pinnularia* sp" is short for "species", and indicates that it is not identified to species.

When the frustule structure and deposition process of *Pinnularia viridis* was investigated by Crawford *et al.* in 2009, it was found that the entire inner surface of the frustule was covered with a thin layer which displayed a considerable increased resistance to alkaline etching [15]. Crawford *et al.* proposed that this altered susceptibility to dissolution was due to different biosilica density and/or chemistry.

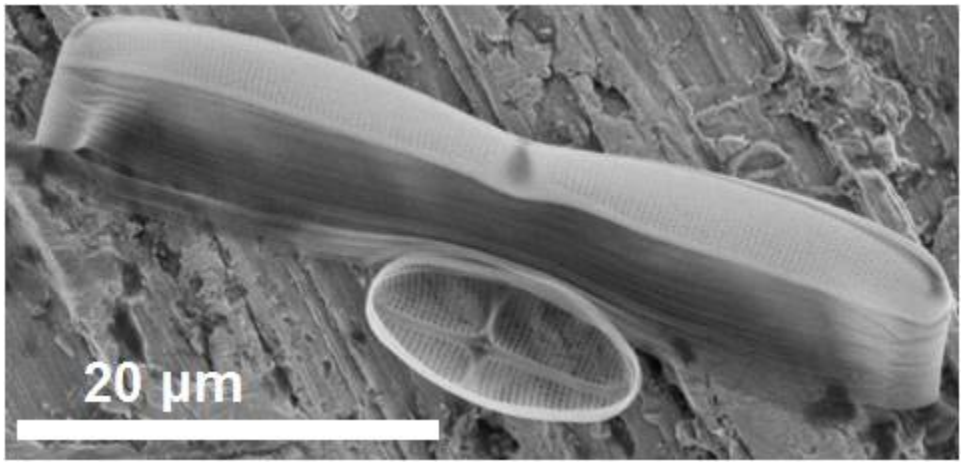


Figure 3: Scanning Electron Microscopy image of two *Pinnularia* diatoms. The largest diatom is oriented with the side up, girdle bands clearly visible. The smallest diatom is seen from directly above, and the raphe can be observed running down the long centerline [16].

Coscinodiscus is a centric diatom that is large enough to be seen with the naked eye (∼200 µm diameter). The size distribution in *Coscinodiscus* cultures is narrower than in *Pinnularia* cultures. Several studies have been published about the optical properties of *Coscinodiscus* frustules [17] [18]. The diatom has a high tolerance for heavy metals, alterations in temperature and salinity, and the large size makes it an unlikely target for planktonic herbivores [19][20]. It easily spreads to new regions where it replaces the indigenous species, and is therefore often described as a harmful species because it reduced biodiversity. However, research has shown that *Coscinodiscus* is displaced by smaller phytoplankton when the dSi concentration in the surrounding medium is low [21]. The large size makes *Coscinodiscus* grow slowly, and the cells sink quickly to the bottom if they are not kept in motion by water circulation.

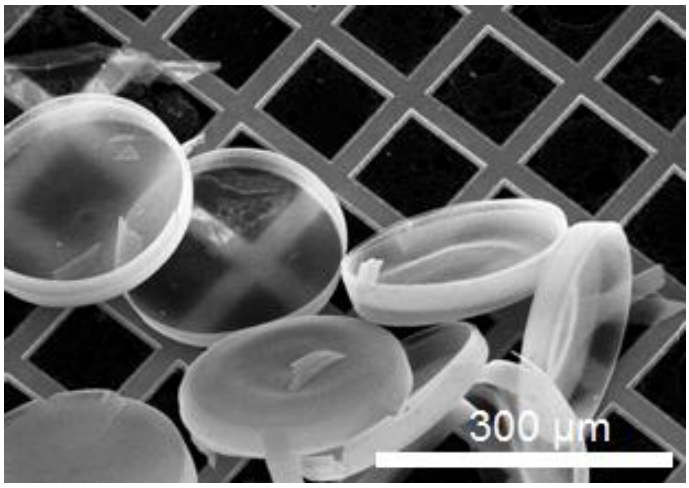


Figure 4: Scanning Tunneling Electron Microscopy (STEM) image of *Coscinodiscus* [5].

2.1.2 Biosilification and reproduction

The cell volume increases as the diatom absorbs nutrients from the surrounding environment. When the diatom has reached a certain size, cell division is initiated [3] . In the very centre of the cell body, two new valves start forming back to back. However, the diatom is confined in two dimensions by the solid biosilica frustule (fig 5A). The only way for the cell to compensate for the increasing volume is by expanding in the z-direction, allowing the epitheca and hypotheca to separate (fig 5B). The separation would have produced a gap running along the entire circumference of the diatom, leaving it open and vulnerable for large periods of the cell cycle. To prevent this, the diatom starts producing girdle bands as soon as the deposition of new valves is initiated. The bands are made up of the same biogenic silica as the rest of the frustule, but the pores are not necessarily of the same size and shape. New bands are produced continuously as the cell volume increases, and the bands are arranged next to each other in an overlapping manner.

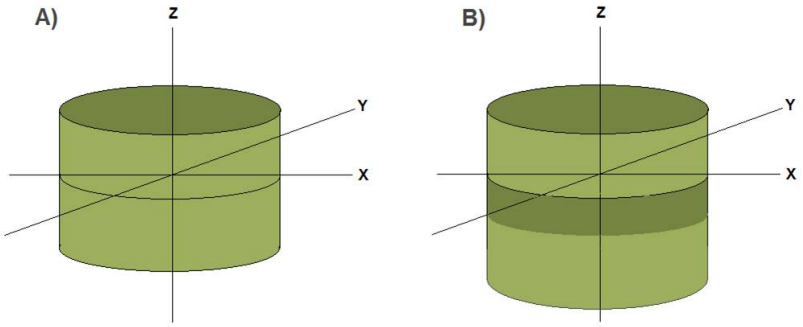


Figure 5: Diatom (A) confined in the X- and Y-direction by the solid silica frustule, and (B) expanding in the Z-direction, forming girdle bands between the two theca.

The two new theca each become hypotheca while the two old theca become epitheca in the two new diatoms produced from the cell division of the original diatom. It has not been completely determined whether or not more silica can be incorporated into a valve after it is fully formed, but it is considered unlikely [22]. Because the two new valves are always smaller than the original ones, each cell division results in two diatoms with marginally smaller average diameter. As cell division continues, the size of the new diatoms decrease until their diameter is $\sim$30% of the original value. When the diatom reaches this critical size, the reproduction mechanism is altered. Instead of the regular vegetative cell division, the diatom pair up with another cell to perform sexual reproduction. The diatoms undergo meiotic cell division from which two gametes without frustules are formed. The gametes fuse together and the resulting zygote is called an auxospore (fig 6). The only protection surrounding the auxospore is a soft cell wall. The absence of a solid silica frustule makes it vulnerable, but also allows it to expand dramatically without restrictions. During a short period of time, ranging from a couple of hours to a couple of days, the cell volume is multiplied many times, and

when the final size is reached, biosilification is initiated inside the spore. At the first cell division two large new silica frustules are deposited, and a new reproduction cycle begins.

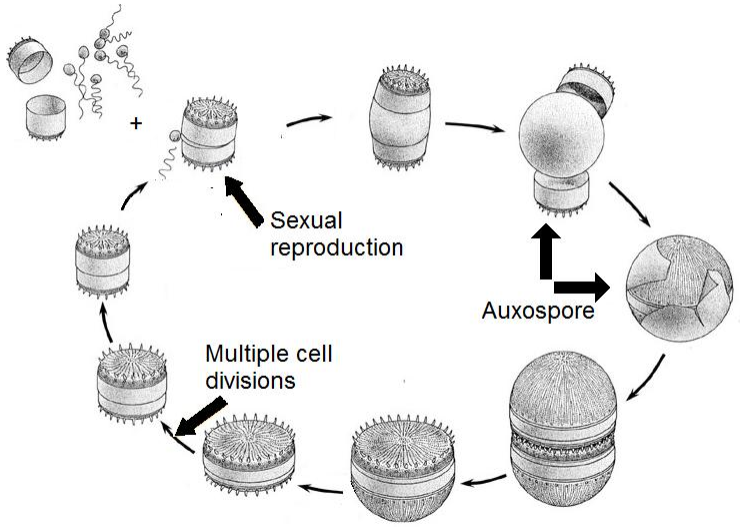


Figure 6: Reproducyion cycle of a centric diatom, modified from [23]

Numerous efforts have been put into understanding how diatoms are able to utilize the dSi in seawater to produce their highly structured solid biosilica frustules [22, 24, 25, 26]. Still, parts of the uptake and incorporation process are poorly understood. The large difference in dSi concentration in natural seawater (<1 to 10µM) and inside the diatom cell body (19 to 340µM) makes passive diffusion an unlikely uptake mechanism [27]. Instead, dSi is transported against a steep concentration gradient into the cell cytoplasm by silicon transfer proteins (SITs) [28, 29]. This is a carrier-mediated process, and dSi is taken up in the form of orthosilicic acid ($Si(OH)_4$), which is the most common form of soluble silicon found in seawater. A detailed description of the process is presented elsewhere [25].

The solubility of Si is approximately 2 mM at pH below 9 [1]. However, it increases at higher pH values due to the presence of H_3SiO_4- in addition to $Si(OH)_4$ in solution [30]. Most diatoms maintain intracellular pools of silicic acid at concentrations exceeding the solubility limit of Si. Some diatoms take up silicic acid independent of incorporation, and therefore require relatively large pools to store the silicic acid before it is used. In other species, uptake and incorporation is performed almost simultaneously, reducing the need for storage capacity and thereby pool size. No matter the size of the pool, diatoms have to take up large amounts of silicic acid from the environment in order to construct the silica frustule, but uncontrolled autopolymerization inside the cell cytoplasm has to be avoided. There are alternative theories as to how the diatoms handle this challenge. In the first theory, prevailing by most experts [1], it is assumed that the silicic acid is transported from the cytoplasm to an acidic compartment called

the silica deposition vesicle (SDV), bound by a membrane called the silicalemma. Complexation with organic molecules prevents the silicic acid from autoprecipitating inside the SDV. The transportation may occur by molecular diffusion through the cytoplasm, or it may be facilitated by SITs [31]. Inside the SDV, the silicic acid is believed to precipitate with the help of silaffins, phosphorylated polypeptides that are covalently linked to long-chain polyamines. Silaffins have a strong zwitterionic character due to the negative charges from the phosphates and the positive charges from the polyamines, and therefore easily self-assemble into large aggregates. When the silaffin molecules co-precipitate with the silicic acid, the composite material that makes up the diatom frustule is formed [22][32]. In the second theory it is assumed that silicon is present inside the cell as solid silica particles. These particles are formed in a clathrin mediated process at the cell membrane and transported to the SDV by silicon transfer vesicles (STVs) guided by motor molecules along microtubules spanning the distance between the plasma membrane and the SDV [4]. Controlled polymerization of silicic acid is believed to be performed at particular sites within the SDV.

Three different modes for dSi uptake are possible in most diatoms: surge uptake, internally controlled uptake and externally controlled uptake [33]. The mode displayed depends on the dSi concentration in the extracellular and intracellular medium. dSi replenishment in the extracellular medium to dSi-starved diatoms will induce a surge uptake, resulting in maximum concentration gradient and uptake rate (fig 7A). High concentrations of dSi in both the extracellular and intracellular medium will in most diatoms lead to a coupling between the uptake rate and the incorporation rate, resulting in an internally controlled uptake. A feedback mechanism controls the uptake rate to match the incorporation rate in the SDV (fig 7B). Finally, when the dSi concentration in the extracellular medium is low, the uptake rate will depend directly on the amount of dSi available for uptake, i. e. the uptake is externally controlled (fig 7C).

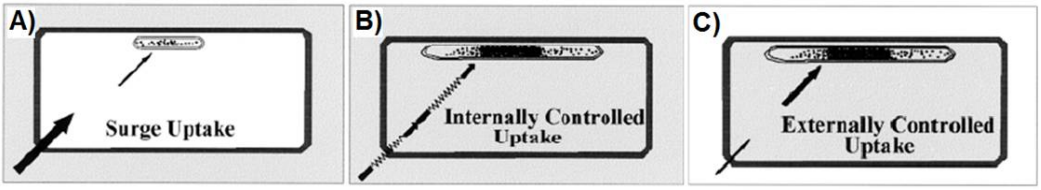


Figure 7: Three different modes of silicic acid uptake, (A) surge uptake, (B) internally controlled uptake, (C) externally controlled uptake, in diatoms, depending on dSi concentration in the surrounding environment (outer box) and inside the cell (middle box). The SDV is illustrated by the oblong box close to the edge of the middle box. Shaded area illustrates high dSi concentration, arrows represent concentration gradient and uptake rate [1].

It is believed that silaffins are responsible for organizing condensed silica into the complex structures that is characteristic for diatoms. The role of the silaffins in *Coscinodiscus* has been investigated using bio molecules mimicking silaffins to assist precipitation of silicic acid *in vitro* [32]. It was found that the pattern structure depended

on the size of the initial silica aggregates, and the size of the aggregates depended on the length and charge of the biomolecules assisting in the precipitation. It is therefore likely that the frustule pore pattern can be altered by manipulation silaffins.

A peptide sequence of a silaffin molecule has also been shown to effectively promote formation of micro- and nanostructured Ti from precursors containing Ti *in vitro* [34]. It was suggested that this chemical ability to react with Ti in solution may explain why diatoms are able to absorb and incorporate Ti into the biogenic silica frustule.

2.1.3 Requirements for growth and effects of starvation

Like most other living organisms, diatoms require nitrogen (N), phosphorous (P) and carbon (C) as well as a number of vitamins and trace metals to sustain life. Autotrophic diatoms also need sunlight in order to be able to fixate CO_2. pH-values in the range 8-9 is required for optimal growth conditions. Diatoms compete with numerous different organisms such as bacteria and other phytoplankton for many of the same nutrients. But unlike their competitors, diatoms utilize silicon (Si) to synthesize their cell wall. Si is the second most abundant element in the earth's crust, and the polymerization of silicic acid is a thermodynamically favourable process. Less energy is required to form a cell wall based on Si, compared to lignin and polysaccarides, and it may be the vast availability of silicon and the energy gain from the cell wall synthesis that have made diatoms able to outperform many other competing species [1].

Light, temperature and concentration of nutrients have implications on the diatom growth rate [35]. C and N metabolism, as well as photosynthesis, has been the main focus of studies investigating mechanisms that regulate cell growth in microalgae. However, Si metabolism and availability also have a large impact on diatom growth. The effect of dSi starvation on diatoms has received more attention during the last decade. One of the reasons for this is that water containment and straightening of water ways has lead to reduced supply of dSi to the world oceans, and combined with anthropogenic loadings of P and N this has produced a shift in the N:Si and P:Si ratios to higher values [36]. Diatoms can access N and P from storage pools inside the cell body or from breaking down internal compounds containing these elements under limiting conditions, but they can not break down compounds to make up for lack of dSi available for cell processes or frustule deposition. dSi starvation therefore inhibit cell growth faster than P and N starvation. Mineralization is usually inversely correlated to growth rate at non limiting dSi conditions. This means that if the growth rate is increased due to external factors such as higher temperature or photon concentration, the resulting frustules will contain less Si than if they had been deposited at lower growth rates. At dSi limited conditions, growth rate may be maintained near maximum while uptake is diminished, leading to thinner frustules.

In an experiment based on time series measurements of dSi concentration and biomass, Spilling *et al.* observed that growth in all cultures, even those with excess P and N, ceased before all the dSi in the culture medium was depleted [36]. This observation has also been reported in other studies by Paasche [37]. The reported con-

centration in the medium when the diatoms stopped to divide spanned from 0.5 to 5.6 µM. A possible explanation for this stable minimum concentration may be dissolution and remineralisation of dead diatoms. However, the dissolution rate for biosilica is probably too slow to account for the relatively high concentration of unused dSi [36]. Paasche proposed that the remaining dSi represented non-reactive dSi, while Spilling *et al.* proposed that some unexplained factor shut down the dSi uptake as the concentration values reached a critical level.

The effect of varying dSi concentration on photosynthetic performance in batch and chemostat cultures of the diatom *Thalassiosira weissflogii* was investigated by Lippemeier *et al.* [38]. The experiments showed that the photochemical efficiency of the reaction centres decreased steadily from the moment the diatoms started to experience dSi depletion. 20 min after the dSi concentration in the culture medium was raised artificially by adding Na_2SiF_6, a marked increase in photochemical efficiency was observed. However, the cell number remained constant for 15.6 hours after dSi addition. On average, 18 hours was needed after dSi was added to restore the physiological maximum value that was measured before dSi limitation occurred, but this time depended strongly of limitation duration and growth rate. It is a known fact that dSi limitation causes a strong decrease in protein synthesis [36]. The time required for full recovery may depend on the time necessary for protein synthesis to be restored. The marked lag between the increase in fluorescence values and the increase in cell number showed that the photosynthesis response to changes in dSi concentration is faster than the cell number. Parslow *et al.* observed that when the diatom *Thalassiosira pseudonana* had been dSi starved for more than 24 hours, the specific uptake rate declined in the first hour following dSi addition before the trend was reversed and the uptake rate increased in the second hour [39]. Parslow *et al.* also observed an apparent decline in cell number after 48 hours of dSi starvation in batch cultures of *T. pseudonana*. They attributed the decline to cell clumping and onset of sexual reproduction.

Diatoms cultured in laboratories have to be kept in a similar environment to their natural habitat. There have been developed many recipes for artificial or enriched seawater medium throughout the history of diatom research, and one of these was "f-medium", composed by Guillard and Ryther in 1962 [40]. The f-medium was based on filtered natural seawater into which N, P and dSi was added, along with trace metals and vitamins. Later, in 1975, Guillard reduced the concentration of the medium by half and added a ferric sequestrene [41]. The new medium was called "f/2 medium", and is the most common enriched seawater medium applied for growing diatoms today. The medium does not contain a carbon source, but CO_2 can be introduced by bubbling regular or carbonated air through the diatom culture.

2.1.4 Frustule elemental composition and incorporation of foreign elements

Diatoms are known to accumulate trace amounts of foreign elements [42, 43]. Martin *et al.* investigated the elemental composition of both the organic fraction and the biosilica frustule of marine diatoms, and found that trace amounts of Ti and aluminium (Al)

were nearly always present in the biosilica frustules even if the elements were not found in the organic fraction [43]. The maximum Ti content reported was 0.13 wt%, but the average value was ∼0.01 wt%. They also found a correlation between the amount of Fe and the amount of Ti present in the biosilica. Any detection of boron (B) was not reported. Martin *et al.* proposed that uptake of foreign elements increased in diatoms in which active growth had stopped, because the slower turnover rates provided more time for these elements to be taken up and incorporated.

Metabolic incorporation of foreign elements such as germanium (Ge), gallium (Ga) and Ti has expanded the range of possible applications for diatoms frustules. Foreign elements have been successfully incorporated into diatom frustules through the metabolic pathway in several independent studies [13, 16, 44-49]. The effect of exposing diatoms to Ge was investigated by Lewin already in 1966. She wanted to inhibit the growth of diatoms infecting her algae cultures, and proposed that adding Ge to the culture medium would affect the diatoms more than her own algae. The chemical and physical resemblance between Si and Ge caused Ge to act as a competitive inhibitor, displacing Si in metabolic reactions and valve formation. In later studies it was discovered that different Ge/Si ratios produced different inhibitory effects. Low ratios impaired the deposition of silica frustules and cell mitosis, while higher ratios in addition inhibited DNA synthesis [26, 47].

Several studies investigating the effect of Ge incorporation on frustule pore structure have been performed. In 2007, Jeffryes *et al.* conducted an experiment in which Ge was metabolically inserted into the frustule of *Pinnularia* sp. [45]. The diatom cultures were grown to dSi depletion before dSi and Ge was added simultaneously. The diatoms were left to consume it all before they were harvested and analyzed. The resulting frustules contained on average 0.96 wt% Ge evenly distributed throughout the biosilica. Inspection with Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Energy Dispersive Spectroscopy (EDS) revealed no apparent change in the overall frustule size, but the raphe and ribs had become thicker and many of the pores were partly filled with silica. Jeffryes *et al.* concluded that morphological changes in diatom frustules can be induced by addition of Ge to the feed medium.

A similar experiment was conducted by the same group on a different diatom species, the pennate diatom *Nitzschia frustulum* [48]. The structural alteration in the resulting frustules was even more pronounced than what was observed in *Pinnularia.* Large areas of silica was missing where the pores had fused to nanoslits, and the entire pore array had been transformed into a double-sided comb-like structure that possessed blue photoluminescence (fig 8). The group concluded that, in addition to morphological properties, optical properties can also be influenced by Ge incorporation.

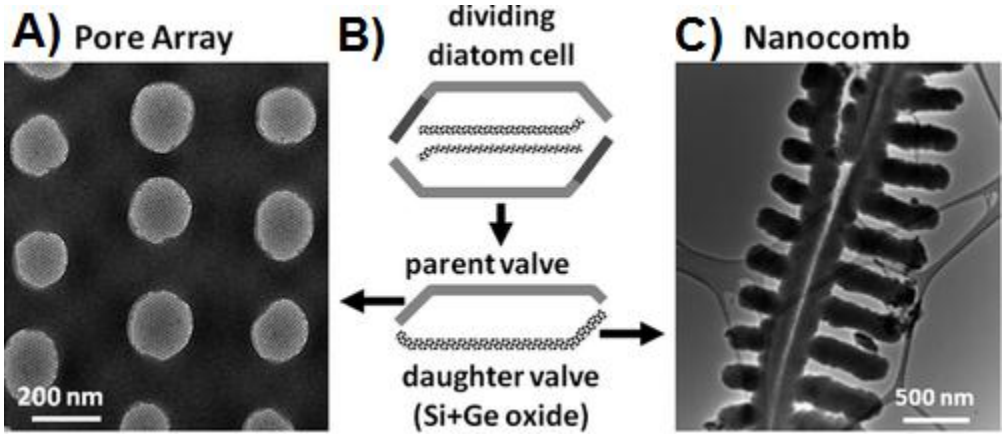


Figure 8: (A) SEM image of the normal frustule structure in *Nitzschia frustulum,* (B) illustration of cell division process upon addition of Ge, (C) SEM image of resulting frustule structure upon addition of Ge [48]

In a study performed by Ali *et al.* on the pennate diatom *Stauroneis sp.*, it was observed that increasing Ge/dSi-ratios resulted in more significant structural aberration [49]. They proposed that the impaired frustule structure was due to differences in bond length between silicon oxide and germanium oxide.

In addition to experimenting with Ge, Jeffryes *et al.* have studied the effect of adding Ti to *Pinnularia* diatoms [13]. Ti is present in seawater primarily as $Ti(OH)_4$, and as Si is taken up in diatoms as $Si(OH)_4$, Jeffryes *et al.* proposed that Ti could be taken up through the same pathway. Because of the extremely low solubility of Ti in water at pH $\sim$8, Ti had to be dissolved in hydrochloric acid (HCl) to achieve satisfactory concentration values in the feed solution. Ti dissolved in HCl was co-delivered with dSi dissolved in sodium hydroxide (NaOH) to the diatom culture over a time period of 10 hours. At the end of the experiments, the diatoms were cleaned with either hydrogen peroxide (H_2O_2) or sodium dodecyl sulfate (SDS) dissolved in ethylene diamine tetraacetic acid (EDTA). The total amount of Ti was higher in the frustules cleaned with SDS/EDTA (2.3 g Ti/100 g SiO_2) than in the ones cleaned with H_2O_2 (1.9 g Ti/100 g SiO_2). The proposed explanation for this difference was that Ti incorporated close to the surface of the frustules was etched by the H_2O_2. It was found that the relative concentration of Ti was many times higher in the thin silica layers at the base of the large pores than in the bulk frustule. It was suggested that the slow condensation rate of TiO_2 was the reason why the relative amount of Ti was remarkably higher in these regions, as deposition of the silica at the base of the large pores is one of the last steps in the frustule formation process.

Metabolic insertion of Ti into *Pinnularia* frustules was investigated by Skolem [16], using the same experimental setup as Jeffryes *et al.* Ti incorporation was achieved by co-delivery of Ti and dSi, but the amount of Ti incorporated was not quantified and the location of Ti in the frustule biosilica was not determined.

2.1.5 Possible applications for diatom frustules

Filters It has been proposed to utilize diatom frustules for selective separation of nanoparticles and other small molecules [4]. A strict pore size control is imperative for this application, and the required pore size will depend on the molecules to be filtered. As the diatom species are so numerous, one strategy could be to invest in a vast selection of diatoms and apply different species for separating different molecules. An alternative approach would be to focus on just a few species, and actively tailor their pores to the required size. Losic *et al.* managed to regulate the pore size of frustules from the diatom species *Coscinodiscus* sp. and *Thalassiosira eccentrica* by depositing thin layers of TiO_2 on the frustules using atomic layer deposition (ALD) [50]. The technique made it possible to reduce the pore size from the original 40 nm diameter size down to 5 nm, while still preserving the original three dimensional structure. Pore size may also be possible to custumize by incorporation of foreign elements such as Ge, as described in section 2.1.4.

Dye sensitized solar cells (DSSC) Another possible application for diatom frustules may be in dye sensitized solar cells (DSSCs) [44]. DSSCs have been developed as a cheaper alternative to the traditional ultra pure Si crystal based solar cells. In classic solid state junction devices, the same material is responsible for both light absorption and charge carrier transport. In a DSSC, a wide band gap semiconductor is applied to transport the charge carriers that are created when a sensitizer ("dye") absorbs sunlight [51]. The dye is covered by a conducting liquid or gel, acting as an electrolyte, while the semiconductor is connected to an anode. When an electron is excited in the dye and escapes into the semiconductor, it is replaced by an electron provided by the electrolyte which is again in connection with the cathode. The process is illustrated in fig 9. The materials applied for DSSCs today are cheaper and easier to produce than the ultra pure Si crystals. In addition, DSSCs can be made flexible, thereby opening up for a wide range of applications not suitable for the heavy and brittle traditional photovoltaic panels, such as portable installations. DSSCs can be made to absorb a wide spectrum of wave lengths, and the transportation of charge carriers in another material than where the absorption takes place reduces the risk of charge carrier recombination. However, when it comes to current conversion efficiency, the conventional devices are still superior to the DSSCs. But through intense research and new discoveries, the gap is decreasing.

Diatom frustules may be able to improve the efficiency of DSSCs in more than one way. The nanostructured pore pattern is believed to be able to provide resonances in the visible spectral range [17], thereby increasing the probability of an electron being excited by the incoming lights. Further on, the high density of hierarchical pores at the frustule surface increases the surface area of the diatom frustule considerably. DSSCs of today already apply a nanostructured surface on which TiO_2 is deposited in order to achieve maximum surface area for the dye to absorb as much light as possible [52]. Diatom frustules could replace this artificial nanostructured surface.

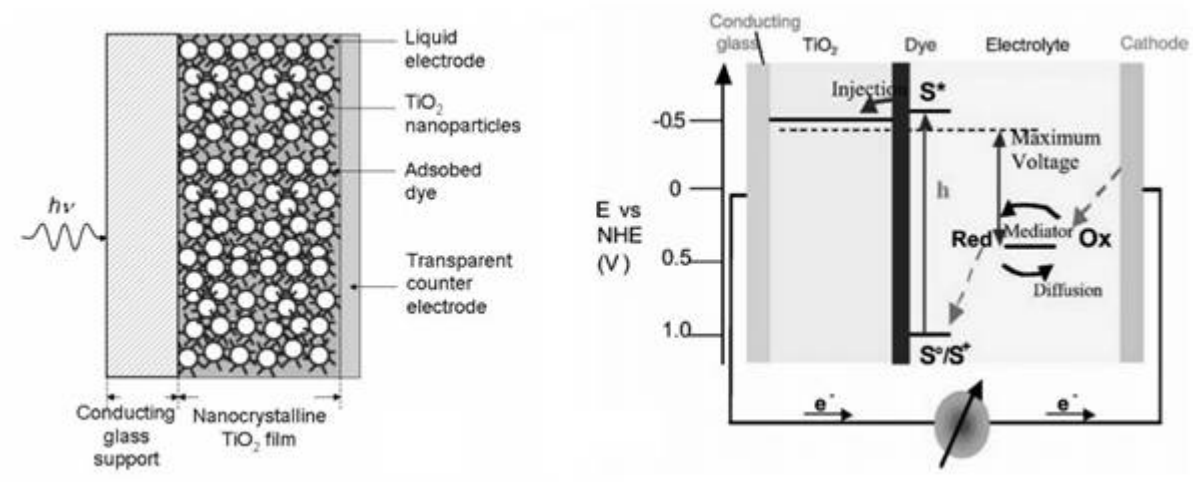


Figure 9: Schematic illustration of a dye sensitized solar cell (DSSC). (Left) Structure, (Right) Principle of operation of a DSSC. "S" denotes the sensitizer (dye) and NHE means that potentials are referred to the normal hydrogen electrode [52].

Finally, diatom frustules could be optimized by tailoring the pore size or incorporating foreign elements such as Ti into the biogenic silica frustules. TiO_2 is already widely used in the DSSC industry, and it has been shown that nanostructured mesoporous TiO_2 films show enhanced solar conversion efficiency compared to traditional films of the same thickness made of randomly ordered TiO_2 crystals [52]. One research group has attempted to incorporate diatoms frustules into a DSSC. The results were presented on a symposium in 2009, but correspondence with the responsible researcher confirmed that the final article on the experiment has not yet been published. It was stated in the symposium abstract that *Pinnularia* diatoms were placed on a TiO_2 anatase crystal and covered with a thin film of TiO_2 nanoparticles (20 nm) [53]. When the device was compared to a similar device without diatom frustules, the efficiency of the device modified with frustules was nearly tripled. As the thin TiO_2 layer on the frustules only constituted about 3% of the total TiO_2 amount in the cell, the group would not assign the increased efficiency to the increase in total amount of TiO_2 alone. The high dielectric contrast and the periodic structure of the frustules was assumed to increase light trapping efficiency, thereby increasing the overall current conversion efficiency of the device.

Photonic crystals The highly orders pore structure covering the diatom silica frustule make diatoms likely candidates for optical applications [18]. One of the many proposed applications for diatom frustules is as photonic crystals. The repeating pattern of regions with high and low dielectric constant in an optical crystal allows for light of certain wavelengths to be guided through the structure with low energy loss, while other wavelengths are prohibited from propagating [54]. In the wavelength interval where no light is allowed to pass, a photonic band gap is formed [55]. Many

optical phenomenon observed in nature is the result of Bragg scattering in periodic nanostructures [17]. Due to the small difference in refractive index in water and silica (n=1.33 and n=1.46, respectively), efficient Bragg scattering can not be expected in diatoms. However, diatom frustules can be regarded as photonic crystals composed of nanostructured materials with periodic refractive index. Yamanaka *et al.* observed frustules from the fresh water diatom *Melosira variance* with Field Emission Scanning Electron Microscopy (FE SEM) and TEM, and performed calculations based on the observed pore pattern do determine the absorption properties of the frustule. In addition, they investigated the actual absorption properties through ultrastructural studies of the frustules in water and in air. They observed that the frustules absorbed light mainly in the blue wavelength region in water, and this observation was supported by their calculations (fig 10). The absorption was even stronger in air due to the larger difference in refractive index (n=1 for air). The group proposed that this absorption may help reduce excess blue light and improve the efficiency of photosynthesis.

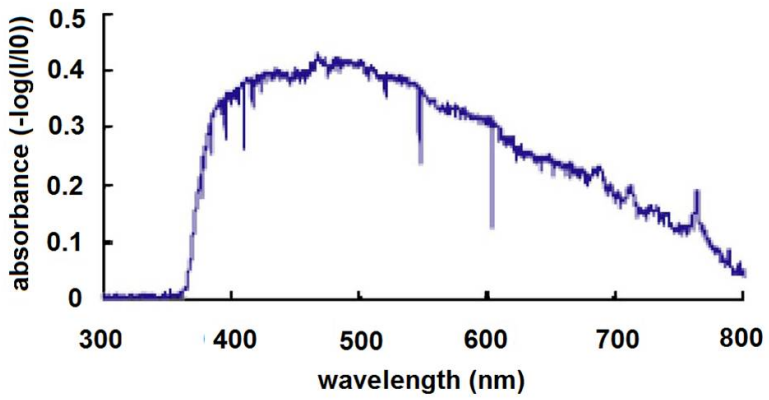


Figure 10: Absorption spectrum for the silica frustule of the marine diatom *Melosira variance*[17]

Optical analysis has also been performed on the marine diatom *Coscinodiscus granii* by Fuhrman *et al.* [12]. Due to its large size and circular symmetry, the diatom was used as a model for all centric diatoms. The pore pattern of *Coscinodiscus granii* frustules varied in both size and structure depending on where on the frustule it was located. The lattice constant in the pattern found on the top or bottom of the valve was larger than the corresponding constant in the girdle band, and hence light with long wavelengths was more suitable for coupling into waveguides in the valve, whereas shorter wavelengths were required for coupling into the girdle. A critical parameter for light propagation in a photonic crystal is the lattice constant. Because the average size of a diatom decreases as it proceeds in its reproduction cycle (section 2.1.2), it would be expected that the size and spacing between the pores decreases correspondingly. This would make diatom frustuls practically useless for optical crystal purposes. However, Fuhrman *et al.* found that even though the area available for pore formation decreased

with diatom size, the pore pattern remained virtually unchanged. The only parameter that decreased was the number of pores on the frustule surface.

Most diatoms possess the long ranged periodicity that is required for guiding light, and their ability to form nanostructured silica in large amounts with low energy requirements at ambient temperature make them a promising alternative for environmentally friendly and economically propitious production of photonic crystals.

Doped semiconductors The electronic properties of semiconductors can be modified by introducing dopants, trace impurity elements, into the crystal structure of the semiconductor in very small concentrations (10^{10}- 10^{18} atoms/cm^3) [56]. A semiconductor normally has $\sim 10^{22}$atoms/cm^3. The dopant has a different number of valence electron than the semiconductor, thereby forming majority charge carriers upon incorporation into the crystal lattice. Semiconductor doping can be conducted by either thermal diffusion or ion implantation, requiring high temperature and high voltage, respectively. Si is often doped with B. It can be imagined that if B could be metabolically inserted into the diatom silica frustule before the silica could be reduced to Si, nanostructured B-doped Si could be manufactured at ambient temperatures in an environmentally friendly way.

2.2 Titanium solubility

The solubility of Ti in water is extremely low at ambient temperatures and pH values. Some of the results presented in studies on Ti solubility are contradictory and the reported values for Ti solubility at pH 8 range from nanomolar [57] to micromolar [58][13]. However, the increased solubility of freshly precipitated titanium oxide is well known [59]. The solubility values for freshly precipitated titanium oxide was applied in the present study, as freshly precipitated titanium oxide was used to produce the Ti feed solutions. The solubility of this form of Ti in water as a function of pH is illustrated in fig 11. The Ti solubility in the diatom culture medium may deviate somewhat from the values presented in the figure because of the presence of numerous ions available for complexation with Ti in seawater.

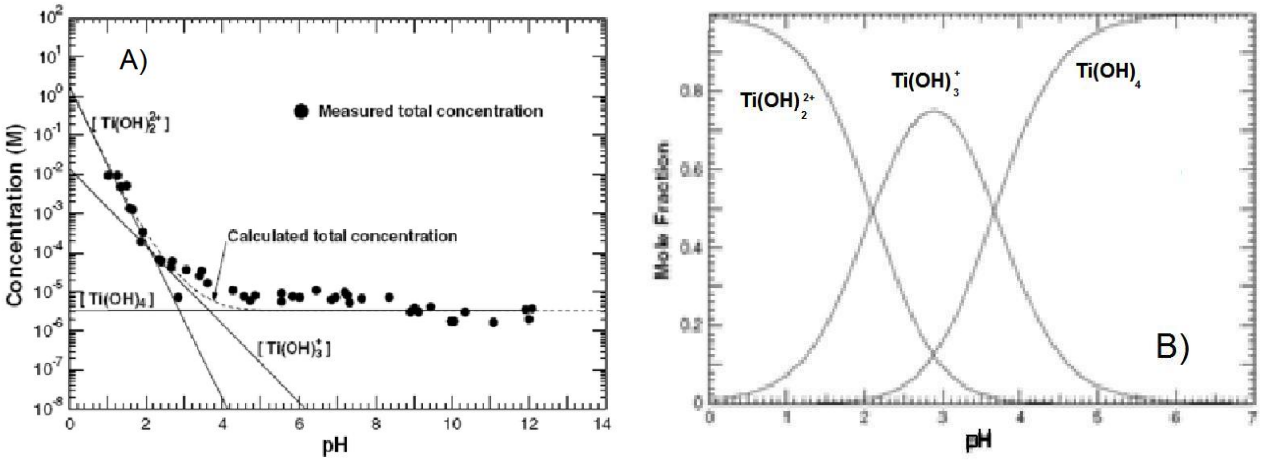


Figure 11: (A) Ti solubility in water and (B) mole fractions of $Ti(OH)_2{}^{2+}$, $Ti(OH)_3{}^{+}$ and $Ti(OH)_4$ as a function of pH [58].

At seawater pH (∼8.2 [7]) and down to ∼pH 3, the dominating form of Ti is the neutral complex $Ti(OH)_4$ [58]. At pH 1 or less, Ti is found mainly as the complex $Ti(OH)_2{}^{2+}$. The solubility of hydrous TiO_2 decreases with increasing pH, but is practically constant above pH 4, with a value of ∼2 x 10^{-6} M at at 25°C and ionic strength 0.1. At pH 1, the solubility of $Ti(OH)_2{}^{2+}$ in water is 3-8 x 10^{-2} M.

3 Materials and methods

3.1 Photo bioreactor setup

3.1.1 Setup for stem culture development

The diatoms were inoculated in rectangular plastic culture flasks (1000 ml). The normal cap was removed and replaced with a silicone stopper lid in which two holes were drilled to allow for silicone tubes to pass through. The air was led into the culture flask through a ventilation tube, while an outlet tube terminating in an air filter. Pressurized air was bubbled continuously through the culture to promote motion, keep cells in suspension and provide a carbon source. The air was provided by a regular aquarium pump. The setup was stored inside a climate chamber lined with fluorescent tubes, providing a constant 20°C temperature, 130 micromoles photons m^{-2} s^{-1} and 16:8 hours light/dark cycles.

3.1.2 Setup for continuous feed solution delivery

The cell cultures were contained in borosilicate culture vessels (1500 ml). The vessels terminated in a rounded end at the bottom and a silicon stopper lid on top. Pressurized air was bubbled continuously through the culture to promote motion, keep cells in suspension and provide a carbon source. Pressurized air provided by a regular aquarium pump was applied for culture aeration. Four holes were drilled in the silicone lid, and narrow glass pipettes were inserted. One pipette was connected to the aquarium pump through a ventilation tube, another to an air filter through an outlet tube. The two remaining pipettes were connected to syringes filled with feed solution through narrow silicone tubes. The glass pipettes terminated in pointed tips, requiring only very small volumes to pass through the pipettes before minute droplets fell of the end. This shape was chosen to make the delivery as continuous as possible. The feed solution syringes were mounted on syringe pumps (Cole-Parmer 94900 Syringe Pump). To avoid a siphon effect, the syringes were kept on a level below the container. The entire setup was stored inside the climate chamber described in section 3.1.1. The maximum space capacity inside the climate chamber was two complete experimental setups, as each vessel required two corresponding syringe pumps. The setup is displayed in fig 12).

Figure 12: Setup for continuous feed solution delivery.

3.1.3 Closed batch setup

The diatoms were inoculated in small (250 ml) or large (1000 ml) rectangular plastic culture flasks (fig 13). A small air filter in the plastic cap allowed for some ventilation, but no tubes were applied for active aeration. The flasks were rarely more than half full and the cap was removed at least once a day. It was assumed that the moderate ventilation would would be sufficient to sustain diatom growth. The flasks were stored inside the climate chamber described in section 3.1.1.

Figure 13: Plastic culture flasks applied for stem culture development and closed batch experiments.

3.2 Medium preparations

3.2.1 Preparation of culture medium

The diatom cultivation medium (f/2 medium) was prepared as described in the recipe by Guillard from 1975 [41]. All of the components were sterile filtered before they were added to filtered (0.20 µm) and autoclaved (120°C, 60 min) seawater from Trondheimsfjorden. In addition, dSi reduced f/2 medium was prepared in the same way as the regular f/2 medium, except that no dSi was added. A small amount of dSi was still present because natural seawater contains dSi (0.0066 mmol/l). A detailed overview of the components is presented in the table below.

Table 1: Composition of f/2 medium

Component	**Type of nutrient**	**Conc. in final f/2 medium (M)**
$NaNO_3$	Macronutrient	8.82 x 10^{-4}
$NaH_2PO_4 \cdot H_2O$	Macronutrient	3.62 x 10^{-5}
$Na_2SiO_3 \cdot 9\ H_2O$	Macronutrient	1.06 x 10^{-4}
$FeCl_3 \cdot 6H_2O$	Trace metal	1.17 x 10^{-5}
$Na_2EDTA \cdot 2H_2O$	Trace metal	1.17 x 10^{-5}
$MnCl_2 \cdot 4H_2O$	Trace metal	9.10 x 10^{-7}
$ZnSO_4 \cdot 7H_2O$	Trace metal	7.65 x 10^{-8}
$CoCl_2 \cdot 6H_2O$	Trace metal	4.20 x 10^{-8}
$CuSO_4 \cdot 5H_2O$	Trace metal	3.93 x 10^{-8}
$Na_2MoO_4 \cdot 2H_2O$	Trace metal	2.60 x 10^{-8}
Thiamine $\cdot$ HCl (vitamin B_1)	Vitamin	2.96 x 10^{-7}
Biotin (vitamin H)	Vitamin	2.05 x 10^{-9}
Cyanocobalamin (vitamin B_{12})	Vitamin	3.69 x 10^{-10}

3.2.1 Preparation of Ti feed solution

The preparation procedure followed the description reported by Jeffryes *et al.* [13]. Solid $Ti(OH)_4$ was precipitated from a titanium (IV) oxysulfate solution (15 wt%, Sigma-Aldrich) by cooling the solution to 0°C simultaneously as NaOH (4 M) was added drop wise until pH 12. The cooling effect was achieved by placing a beaker with the solution in a larger container filled with ice. A pH-meter was applied to monitor temperature and change in pH. When the procedure was completed, the solution had become highly viscous and milky white. A precipitate was isolated upon centrifugation (Biofuge Stratos centrifuge, 14 000 RPM,10 min), before it was resuspended in deionised water and centrifuged again. The washing procedure was repeated two more times to remove any remains of the precursor solutions. Finally, the solid white precipitate was dried at 100°C for 3 hours. The resulting powder displayed a faint green colour (fig 14).

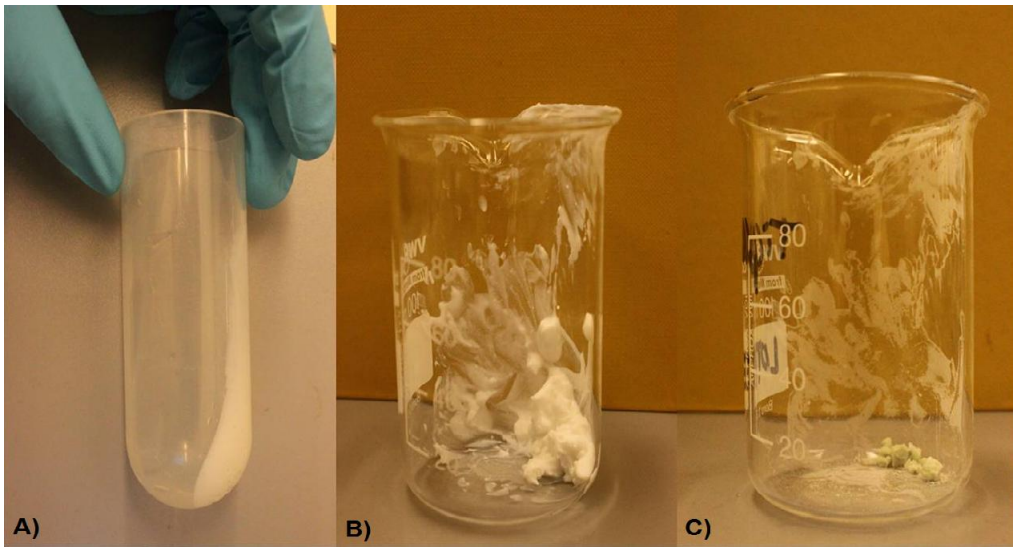


Figure 14: $Ti(OH)_4$ precipitate (A) after centrifugation, (B) prior to drying, (C) after drying

The Ti feed solution was prepared by dissolving the solid $Ti(OH)_4$ in concentrated HCl (37 wt%) upon magnetic stirring and heating on a hot plate (80°C). When the precipitate was completely dissolved, the solution was diluted with heated (80°C) deionised water until a final concentration of 500 mM HCl/ 0.36-0.62 mM Ti was obtained. The solution was stored on the hot plate under constant heating and stirring.

3.2.2 Preparation of dSi feed solution

The dSi feed solution was prepared by dissolving solid $Na_2SiO_3 \cdot 9H_2O$ in NaOH . The final concentration was 500 mM NaOH/ 3.6-8.9 mM dSi. The high NaOH concentration was necessary for the dSi feed solution to neutralize equal volumes of the acidic Ti feed solution upon addition to the cell culture. The solution was stored in a closed plastic container at room temperature.

3.2.3 Preparation of dSi feed solution without NaOH

The aqueous dSi feed solution was prepared by dissolving solid $Na_2SiO_3 \cdot 9H_2O$ in filtered deionised water (MilliQ) to a final concentration of 3 mM dSi. The solution was stored in a closed plastic container at room temperature.

3.3 Analytical methods

Cell number The cell number (cells/ml) in the *Pinnularia* culture samples was measured with flow cytometry. The technique counts particles in solution by sending a thin stream of entrainment fluid (10-20 µm wide) past a one or more focused lasers. Each time a particle passes, the laser light is scattered. The scattered light is detected by a photodiode and one event is registered [60]. Depending on the way the light is

scattered, information about the size, shape, autofluorescence and surface properties of the particle can be obtained. Cell enumeration by flow cytometry is less time consuming than manual counting by optical microscopy, and it also makes it easier to detect if there are large amounts of bacteria present in the sample.

The flow cytometer applied was a Becton Dickinson FACScan. The culture samples (4 ml) were collected and immediately fixated with glutaraldehyde (150 µl). The fixation stopped the cell cycle and helped preserve the intact diatoms and prevent them from decomposing. A bead solution contained inert uniform beads (1µm, spherical) that were used as a reference. The signal from the beads should be localized to a the same, limited area and be unchanged in intensity from run to run. A blurred, weak bead signal would be an indication that something was wrong with the flow. A volume of the culture sample (995 µl) was added bead solution (5 µl), placed in a flow cytometry tube and vortexed (5 seconds) before the mixture was run through the instrument (2 min, 70.3 µl/min flow rate). The event number obtained from the flow cytometry measurement was converted to cell number by using the following equation:

$$C_{pop} = (N_{pop} x \frac{\frac{V_{tot}}{V_{sample}}}{RxT})$$

where C_{pop} is the cell number (μl^{-1}), N_{pop} is number of registered events in 2 min, V_{tot} is the total volume of the sample (ml), V_{sample} is the sample volume (ml), R is the flow rate (µl/min) and T is the counting time (min).

During the experimental periods, the samples were analyzed the same day as they were collected. During the preparation periods, samples were stored for up to two weeks before they were analyzed.

Coscinodiscus cells were manually enumerated with an inverted microscope (Zeiss Axio Scope). Culture samples (6 ml) were conserved with Lugol's solution (20 µl) and counted the same day or the day after they were collected.

Instantaneous Chlorophyll Fluorescence (Ft) Damage to the photo system is often the first sign of stress or unsatisfactory conditions in a diatom [61]. Fluorescence measurements provide information about the photosynthetic performance of the cell. Instantaneous Chlorophyll Fluorescence (Ft) measurements give an indication of the amount of chlorophyll in a culture. The measured values can not be used to compare between different species, but it is a relative measurement and can be used to monitor the development in a culture.

Ft was measured with a chlorophyll fluorometer (AquaPen AP 100 Photon Systems Instrument fluorometer). The instrument measures photosynthetic parameters using an optical probe and blue light [62]. A plastic cuvette (10 mm x 10 mm) was filled with unfiltered sample (4 ml) and inserted into the fluorometer. The value was read after 1 min. The measurements were performed in duplicate.

Quantum Yield (QY) When a plant cell is exposed to light, there are three alternative outcomes: the light may be used to drive photosynthesis, it may be re-emitted

as light with a longer wavelength or it may be dissipated as heat [63]. QY is a measure of how much of the incoming light is absorbed by chlorophyll in the cell, i. e. how well the cell is able to utilize incoming light for energy production through photochemistry. A comprehensive description of the process has been presented elsewhere [63, 61]. QY measurements return ratio values between 0 and 1, 1 indicating that the cell uses all the incoming light for energy production (extremely good photo system condition) and 0 indicating that none of the light is converted to chemical energy (extremely poor photo system condition).

The QY measurement procedure was identical to the Ft procedure, except for the measurement mode on the fluorometer, which was tuned to "QY measurements".

Macronutrient concentration dSi, N and P concentration in the culture and feed media was measured using MERCK Spectroquant test kits and a spectrophotometer (Spectroquant Pharo 100 MERCK). The Spectroquant method is based on the principles for traditional manual analysis of compounds in seawater [64]. The chemical reaction between the components in the sample solution and the reagents from the kit produces a colour change (fig 15) that can be quantified by measuring absorbance using a photodiode in a spectrophotometer. A beam of monochromatic light is sent through the sample and the intensity of the transmitted light is compared with the intensity of light transmitted through a reference sample.

The kit reagents were added to a filtered (0.20 µm) culture sample. The kit instructions were followed and the correct analyzing wavelength was set by inserting a barcoded chip from the test kits into the instrument. The lower detection limit was 0.0002 mmol/l for dSi, 0.006 mmol/l for N and 0.0005 mmol/l for P.

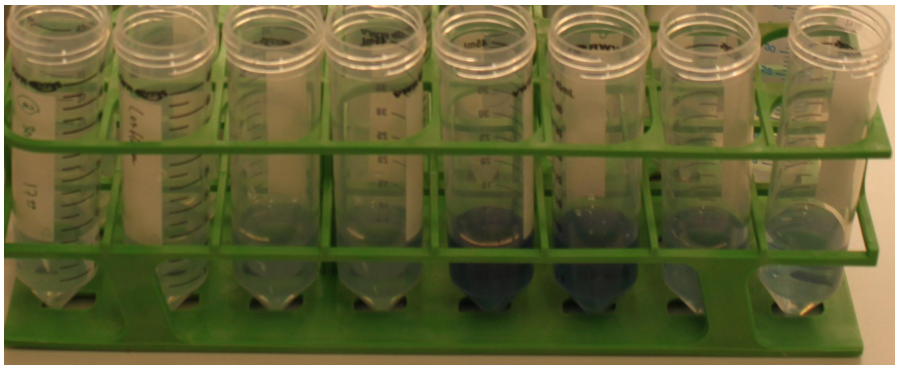
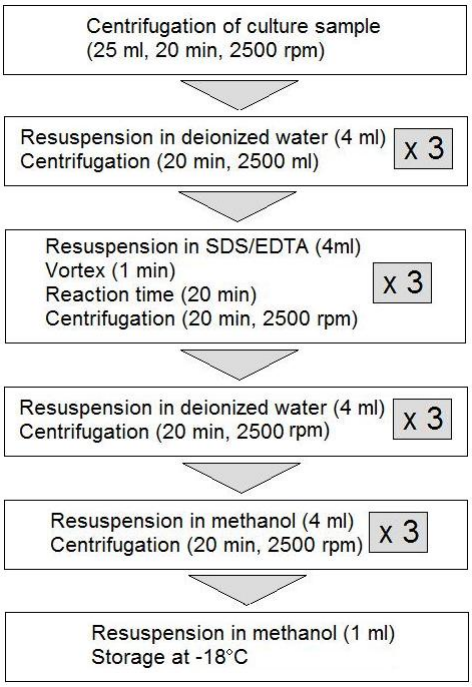


Figure 15: Culture medium samples added kit reagents to produce a color change.

Frustule isolation and removal of organic material The diatoms were isolated by centrifuging (20 min, 2500 rpm) a culture sample (25 ml) using a Beckman Coulter Allegra X-15R centrifuge. The supernatant was removed and the pellet was resuspended in filtered deionised water (MilliQ, 4 ml). The tube was vigorously shaken until the pellet had completely dissolved, before the centrifugation was repeated. A wash-

ing liquid was then prepared by dissolving sodium dodecyl sulphate (SDS, 50 g/l) in ethylene diamine tetraacetic acid (EDTA, 100 ml). The diatom pellet was dissolved in the washing liquid (4 ml), vortexed (1 min) and left to react (20 min). After this the diatoms were washed with MilliQ water and methanol (4 ml) before the clean frustules were finally resuspended in methanol (1 ml) and stored at -18°C. A detailed description is presented in the table below.

In the beginning of the procedure, the diatoms were clearly visible as a brown mass at the bottom of the centrifugation tube and the supernatant was coloured brown/green. After washing with SDS/EDTA three times, the pellet usually turned white and the supernatant was completely transparent. However, if the original cell culture was very dense (many cells per ml), a fourth round of washing with SDS/EDTA was sometimes required to remove the last remains of organic material. Towards the end of the washing process the pellet easily broke up, so extra care had to be taken to ensure that no frustules were removed together with the supernatant.



Ti concentration and frustule elemental composition Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was applied for determination of Ti content in the culture medium and in the cleansed diatom frustules. An ICP-MS instrument consists of an argon plasma source and a mass spectrometer [65]. The sample is usually introduced to the argon plasma as a gas, and the elements in the sample are first converted into gaseus atoms before they are ionized by the plasma. The beam of charged ions is subsequently collimated and focused to the entrance aperture of the mass spectrometer by a set of electrostatic lenses. Inside the mass spectometer, the ions are separated by their

mass-to-charge ratio. A comprehensive introduction to ICP-MS has been published by Amman [66].

To measure the Ti content in the culture medium, samples (2 ml) were filtered (0.2 µm) and conserved with nitric acid (HNO_3) to make the container wall positively charged, thereby prohibiting Ti-ions from adsorbing to the container. The samples were finally diluted with deionised water (x10). Due to limited resources, Ti concentration in the culture medium was not measured in every culture.

To measure the elemental composition of the biosilica frustules, most of the methanol in which the frustules were stored was removed before the sample and the container was rinsed with HNO_3 (1 g, grade ultrapure). The acid and frustule mixture was then transferred to 25 ml PFA-containers and added concentrated hydrofluoric acid (HF) (0.18 ml, grade supra pure). When the frustules had dissolved, the mixture was diluted to a total volume of 100 ml, with 0.1 M HNO_3 and 0.25% HF in the final analyzing solution (identical to the calibration solution).

pH A Mettler Toledo MP 220 pH-meter was applied to measure pH in unfiltered culture samples (5 ml). The measurements were performed at room temperature.

Frustule structure and elemental composition The frustule structure was examined with (Scanning) Transmission Electron Microscopy ((S)TEM). Both TEM and STEM are based on accelerating electrons towards a sample, using an electron gun/tungsten filament/lanthanum hexaboride (LaB6) source to generate the electrons and a high voltage source (typically 200 kV) to accelerate them [67]. The electron beam is collimated and focused by an electromagnetic lens system. The beam is transmitted through a very thin sample and produces a signal when it hits a fluorescent screen/detector system on the other side of the sample. Computer software is then used to interpret the signal and produce an image of the sample structure. The difference between STEM and TEM is that in STEM, the beam is scanned over the sample surface to produce an image. Due to the high energy (i. e. short wavelength) of the electrons, considerably higher resolution can be obtained with (S)TEM than with a traditional optical microscope. The simplest microscopy mode is bright field (BF) mode, in which the probe is transmitted through the sample and contrast is produced by absorption in denser/thicker areas of the sample. This mode is applied in normal optical microscopes. In (S)TEM, dark field (DF) mode can be applied as well. In DF mode, the electrons that are scattered with a large angle in reciprocal space is detected, while the electrons that follow a trajectory along the optical axis or are scattered in small angles are not registered by the detector. DF mode is also called "z-contrast mode" because heavy elements (high Z) have a higher probability of scattering electrons, thereby lighting up in DF images. The contrast in DF mode increases proportionally with sample thickness.

The elemental composition and distribution in the biosilica frustules was examined with Energy Dispersive Spectroscopy (EDS). EDS is based on detecting and interpreting the X-rays emitted by a sample when it is bombarded with a high-energy beam of charged particles, in this case electrons. The wavelengths of the emitted X-rays are

characteristic for every element due to their unique atomic structure, and the signal can therefore be used to determine the elemental composition of the sample. The accuracy is not as high as with ICP-MS, but in return, information about the relative distribution and location of elements in the sample can be obtained.

The (S)TEM samples were prepared by placing a small drop (30 µl) of cleansed frustules suspended in methanol on a copper TEM grid covered with a holey carbon film. This particular type of TEM grid was chosen to minimize charge build-up in the sample. A JEOL 2010 operating at 200 kV was applied for the analysis. The resolution in TEM mode was 0.21 nm, while the resolution in STEM mode was 0.7 nm due to the diameter of the probe (the electron beam) required for EDS-analysis.

3.4 Experimental design

3.4.1 *Pinnularia*

Experimental preparation The photobioreactor system was set up as described in section 3.1.2. A total of five Ti incorporation experiments were conducted on the *Pinnularia* diatoms, and different combinations of QY values and dSi starvation were tested. All of the experiments except from the very first were performed in duplicate.

All of the *Pinnularia* diatoms applied in the present study originated from the same stem culture. Small volumes (20 ml) of the original stem culture were used to inoculate larger "stem cultures", prepared and stored as described in section 3.1.1. These stem cultures were subsequently used to inoculate the experimental cultures (fig 16).

In the following sections, the cultures will be named according to experiment number, degree of dSi starvation and high or low QY values. For example, a dSi Deplete Low Quantum Yield culture from the second Ti incorporation experiment will be referred to as 2 dSi-Dep-LQY.

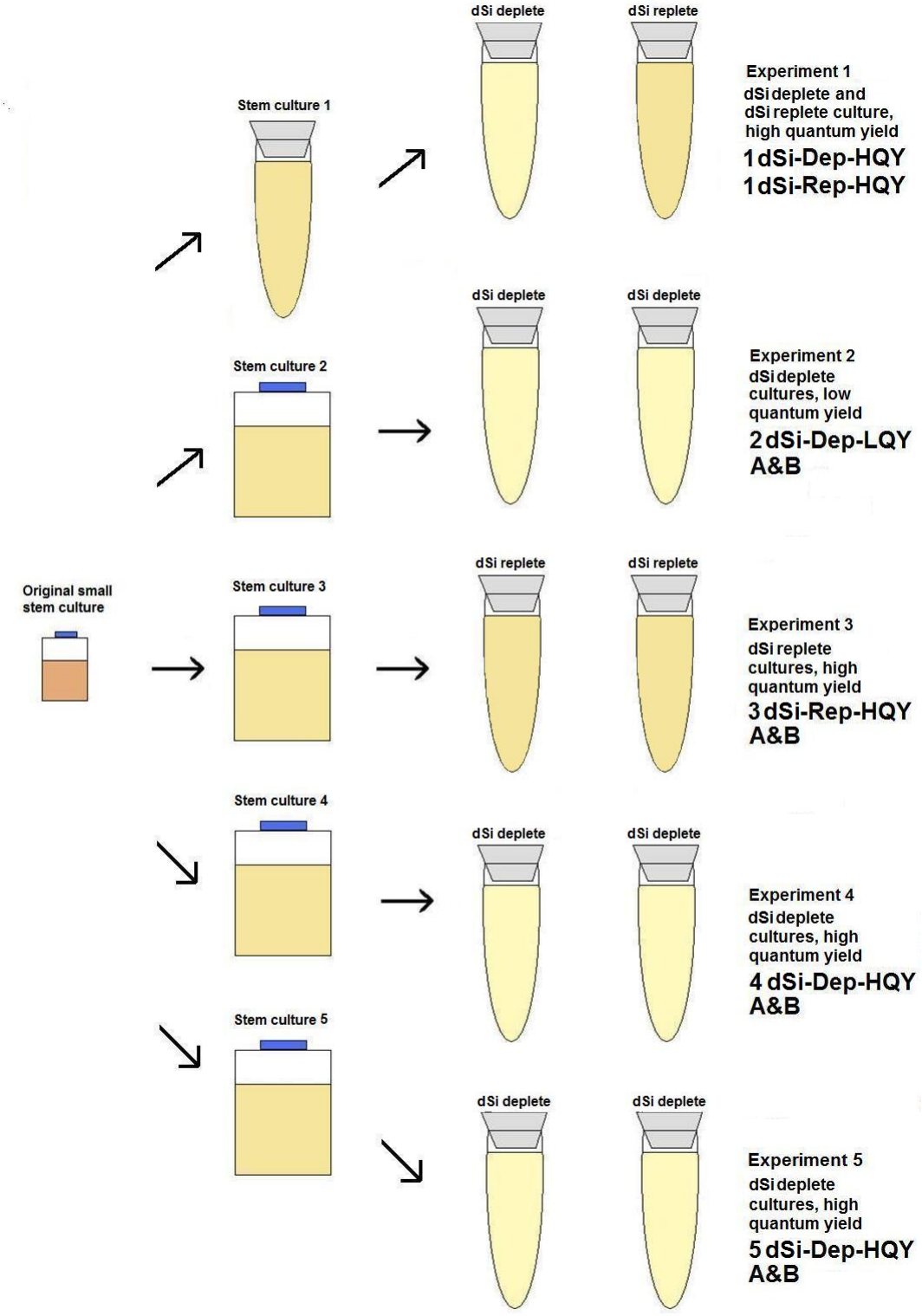


Figure 16: Overview of the different *Pinnularia* cultures applied for the Ti incorporation experiments.

As the average diatom size in a culture may vary considerably depending on their progression in the reproduction cycle, the average dSi consumption per diatom per cell division (dSi/cell) may vary between cultures of the same diatom species. In the days preceding the experiments, the dSi concentration and cell number development in the stem cultures was measured and used to calculate dSi/cell values for that particular culture. The resulting value was used to estimate how much dSi to add in order to make the entire diatom population in a culture divide once. It was assumed that the dSi/cell values calculated for the stem cultures would be transferable to the experimental cultures.

Ti incorporation experiments The amount Ti to be added was found from the calculated dSi value. Relatively low Si:Ti molar ratios (10:1 to 16:1) were applied. Theoretical concentration development was calculated for all the experiments to illustrate how the dSi and Ti concentration in a culture would have developed had there been no diatoms present. Corrections were made for alterations in culture volume as a result of feed solution addition and sampling. Other possible reasons for reductions in Ti and Si concentrations, such as precipitation and adsorption to the container wall, were not taken into consideration.

The cell number was determined on the morning of the experiment immediately prior to delivery initiation. The first 10 hours of the experiments, dSi and Ti feed solutions were co-delivered continuously to the diatom cultures. Every 2.5 hour of the delivery period, a small volume (20 ml) was removed for sampling. This was done by pausing the syringe pumps, removing the culture vessel from the climate chamber, replacing the perforated cap with a rubber cap and carefully shaking the tube until all the diatoms had come off the vessel wall and were evenly distributed in the culture medium. The sample volume was poured over in a 45 ml plastic centrifuge tube which was closed with a plastic lid and shaken vigorously before the sample volume was distributed into smaller tubes and cuvettes for further preparation and analysis. The samples were used to measure dSi and Ti concentration in the medium, Ft, QY, pH and cell number. N and P concentrations were measured at the beginning and end of the experiments to rule out N or P limitation. Sampling was performed less frequently after the delivery of feed solution was completed. Samples were removed for analyzation at 24, 48 and 72 hours after delivery initiation. When the last sample had been collected, the diatoms were harvested and cleaned. This ended the dSi/Ti uptake/incorporation process in the diatoms, as all dSi/Ti associated with the organic material in the diatoms was removed during the washing process.

The first experiment was the only Ti incorporation experiment performed on two different cultures, and the only experiment in which the stem culture was contained in a borosilicate vessel (fig 16). When the stem culture was dSi deplete it was divided and one culture was added dSi reduced f/2 medium (1 dSi-Dep-HQY A) while the other was added regular f/2 medium (1 dSi-Rep-HQY B). The preparation period was counted from the day of culture division. In this period, both cultures consumed all the dSi initially present before they were added a small volume of dSi reduced f/2 medium/

regular f/2 medium two days before the Ti incorporation experiment was initiated. At the morning of the experiment, 1 dSi-Dep-HQY A had been dSi depleted for 24 hours while 1 dSi-Rep-HQY B was dSi replete.

The stem culture applied for the second experiment was grown to complete dSi depletion and then kept in a dSi starved state for four days until the QY values had dropped considerably (∼0.1). On the day before the experiment was initialized, the culture volume was evenly divided into two borosilicate vessels and diluted with dSi reduced f/2 medium. At the morning of the experiment, the diatoms of 2 dSi-Dep-LQY had been dSi deplete for five days.

The stem culture applied for the third experiment was left to grow to dSi depletion, but as soon as all the dSi was consumed, the culture was divided evenly and diluted with regular f/2 medium before the QY values had time to drop significantly. 3 dSi-Rep-HQY was initialized two days after dilution.

The stem culture for the fourth experiment was grown to dSi depletion, divided and diluted with regular f/2 medium and left to grow to dSi depletion once more. The diatoms of 4 dSi-Dep-HQY were in a dSi starved state for 48 hours before delivery was initiated. In addition to the general analyses, a sample (5 ml) collected from 4 dSi-Dep-HQY A 10 hours after experiment initialization was examined with an optical microscope. Further on, an extra volume (25 ml) of the culture medium was collected when the diatoms were harvested. It was centrifuged, resuspended once in Milli-Q water and centrifuged again, but the resulting pellet was not further rinsed to remove the organic material. Instead, the pellet was dissolved directly in HF and analyzed with ICP-MS.

The stem culture for the fifth experiment was divided and diluted already at the first day of the preparation period due to time restrictions. 5 dSi-Dep-HQY was initiated 24 hours after the cultures had reached dSi depletion.

Control experiments Five different control experiments were performed. The experimental equipment for the first four control experiments was set up as described in section 3.1.2, and the experiments and sampling was conducted in the same manner as in the five *Pinnularia* Ti incorporation experiments.

The purpose of the first control experiment (1C-f/2) was to investigate how the dSi and Ti concentration would have developed in the culture medium if there were no diatoms present. Feed solution was delivered to culture vessels filled with regular f/2 medium instead of diatom cultures.

The second control experiment (2C-dSi) was conducted to explore how the cell number in duplicate dSi deplete high QY *Pinnularia* cultures changed if only dSi was added during the 10 hour delivery process. A dSi solution without NaOH was applied (section 3.2.3).

The third control experiment (3C-Ti/HCl/NaOH) was performed to investigate how the cell number was affected by the delivery of Ti without dSi. Due to the low Ti solubility in water, Ti still had to be dissolved in HCl. To compensate for the acid, pure 0.5 M NaOH was co-delivered with the Ti feed solution to duplicate dSi starved

Table 2: Cultivation parameters for the five *Pinnularia* Ti incorporation experiments

Culture	Initial volume (ml)	Initial cell number (ml^{-1})	Initial [dSi] (mmol/l)	Initial QY	Total dSi added (µmol)	Total Ti added (µmol)	[dSi] in feed sol. (mmol/l)	[Ti] in feed sol. (mmol/l)	Molar ratio Si:Ti
1 dSi-Dep-HQY A	1500	26000	0.0013	0.49	83.3	5.7	8.9	0.62	13:1
1 dSi-Rep-HQY B	1500	23500	0.011	0.51	75.3	5.2	8.9	0.62	13:1
2 dSi-Dep-LQY A	1200	16600	0.001	0.13	43.6	2.99	8.9	0.62	13:1
2 dSi-Dep-LQY B	1200	17000	0.001	0.13	43.6	2.99	8.9	0.62	13:1
3 dSi-Rep-HQY A	1120	18300	0.043	0.43	43.6	2.99	8.9	0.62	13:1
3 dSi-Rep-HQY B	1100	19000	0.043	0.43	44.5	3.05	8.9	0.62	13:1
4 dSi-Dep-HQY A	1210	41300	0.0003	0.43	59.5	3.45	6.2	0.36	16:1
4 dSi-Dep-HQY B	1095	40800	0.0004	0.41	53.2	3.10	6.2	0.36	16:1
5 dSi-Dep-HQY A	1150	35100	0.0003	0.38	48.3	4.97	3.6	0.36	10:1
5 dSi-Dep-HQY B	1190	33400	0.0003	0.36	47.6	4.90	3.6	0.36	10:1

high QY *Pinnularia* cultures.

The purpose of the fourth control experiment (4C-HCl/NaOH) was to investigate how addition of HCl and NaOH alone affected the cell number. Duplicate dSi starved high QY *Pinnularia* cultures were added 0.5 M HCl and NaOH in volumes equivalent to the volumes of feed solution that would have been added in a regular Ti incorporation experiment.

The fifth control experiment (5C-plastic) was conducted to determine how the elemental composition of the frustules was affected by the vessel the diatoms were cultured in. *Pinnularia* diatoms were inoculated with regular f/2 medium in large rectangular plastic flasks (see section 3.1.1) and left to grow undisturbed except from careful stirring once a day. When the cultures were dSi depleted, they were harvested and cleaned in the same manner as the diatoms from the Ti incorporation experiments.

In addition to the five control experiments, the development in Ft and QY values was measured every 2.5 hours for 10 hours in two dSi deplete and two dSi replete *Pinnularia* cultures. The measurements were performed at the same time of day as the Ti incorporation experiments were normally conducted. This was done to investigate the natural daily Ft and QY development in cultures that were not exposed to feed solution. No other parameters were measured in these cultures.

Table 3: Cultivation parameters for control experiments

Control experiment	Initial volume (ml)	Initial cell number (ml^{-1})	Initial [dSi] (mmol/l)	Total dSi added (µmol)	Total Ti added (µmol)
1C-f/2	1500	0	0.106	83.3	5.7
2C-dSi	1000	38000	0.0002	45.7	0
3C-Ti/HCl/NaOH	975	37500	0.0002	0	4.4
4C-HCl/NaOH	1200	37000	0.0003	0	0
5C-plastic	700	270	0.097	-	-

3.4.2 *Coscinodiscus*

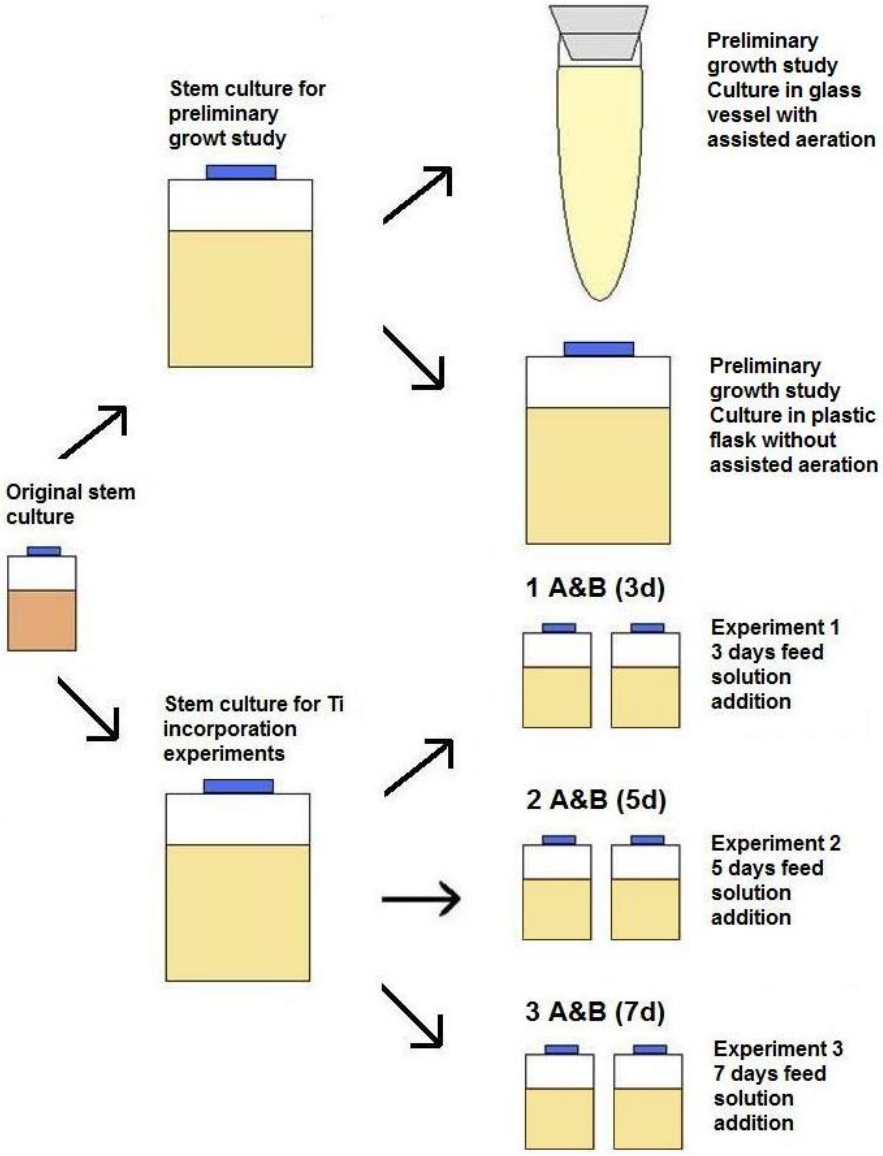


Figure 17: Overview of *Coscinodiscus* cultures for preliminary and Ti incorporation experiments.

Preliminary growth study Two *Coscinodiscus* cultures were inoculated with regular f/2 medium. One culture was stored in a large rectangular plastic culture flask (section 3.1.3) and one in a glass vessel (section 3.1.2). dSi concentration, pH and cell number was measured 5 and 13 days after inoculation and dSi/cell values were calculated. The diatoms were subsequently harvested, washed and analyzed with ICP-MS.

Ti incorporation experiments Three attempts to incorporate Ti into the frustules of *Coscinodiscus* diatoms were carried out. A large *Coscinodiscus* culture was inoculated from the original stem culture, and after a few days of acclimatization to the new medium, the culture was divided into 6 small rectangular plastic flasks. The reason why such small cultures were applied was that the growth rate was too slow for larger cultures to be practically feasible within the time frame of the study. Based on the calculated dSi/cell values from the preliminary growth study, the amount of dSi to be added to the new cultures was determined. The concentration of the dSi feed solution was 0.5 M NaOH/8.9 mM dSi and the Ti feed solution was 0.5 M HCl/0.62 mM Ti, resulting in a molar Si:Ti ratio 13:1. The 10 hour feed solution delivery process applied in the Ti incorporation experiments on *Pinnularia* could not be used because of the slow growth rate of *Coscinodiscus*. Instead, the feed solutions were added simultaneously by pipette once a day. For the first experiment (1 (3d)), the total volume to be added was divided into three deliveries performed in three following days. For the second experiment (2 (5d)), feed solution was added every day for five days. For the third experiment (3 (7d)), feed solution was added to the cultures every day for seven days. In all three experiments, the accumulated amount of Si added was estimated to be enough to make the entire population divide once. Considering the limited culture volume, sampling was reduced to an absolute minimum. For the first and second experiment, sampling was only performed immediately before the first feed solution addition and on the day after the last feed solution addition (four and six days after experiment initialization, respectively). For the third experiment, a third sampling was performed three days into the experiment. Samples were used to measure Si concentration, QY, cell number and pH.

Table 4: Cultivation parameters for Ti incorporation experiments on *Coscinodiscus*

Culture	Initial vol. (ml)	Initial cell number (ml^{-1})	Initial [dSi] (mmol/l)	Initial QY value	Total dSi added (µmol)	Total Ti added (µmol)	dSi/Ti solution delivery rate (µl/day)
1 (3d) A	150	18.5	0.0017	0.48	5.4	0.38	200
1 (3d) B	150	18.8	0.0016	0.49	5.5	0.39	206
2 (5d) A	150	14.8	0.0058	0.58	4.33	0.3	97.2
2 (5d) B	150	12.3	0.0058	0.56	3.6	0.25	80.1
3 (7d) A	130	11.9	0.001	0.45	3.02	0.24	48.5
3 (7d) B	130	10.7	0.0009	0.43	2.73	0.22	43.8

4 Results

4.1 Culture development

4.1.1 *Pinnularia*

Based on the amount of dSi added, a doubling of the cell number was expected during the first light cycle in all of the five Ti incorporation experiments. Instead, a rapid drop in cell number was observed during the first 10 hours of the experimental period. Possible reasons for this will be discussed later, but as a result of this unexpected development, the measured data had to be presented in a different way than what was originally intended. The number of actual diatoms corresponding to one event counted in the flow cytometer may have differed depending on when in the experimental period the sample was collected. In order to avoid misleading dSi/cell values, dSi/cell calculations were based on the initial cell number, i. e. the number of cells in the culture just before feed solution delivery was initiated. This way it would still be possible to compare the values from one experiment with the values from the other experiments, as well as with values from literature.

The measured dSi concentration in the diatom cultures was never below 0.3 µM. Cultures with dSi concentrations of this order are referred to as "dSi depleted" in the following text, as cell division ceased to occur at this concentration level.

The development in the experimental cultures in the days prior to experiment initiation is illustrated in the "preparation culture" plots in the following section. As all of the duplicate cultures in experiment 2-4 were made by diluting a preparation culture just before the experiment was initiated, only one graph for each parameter is displayed in these plots. The last day of the preparation period equals day 0 of the experimental period.

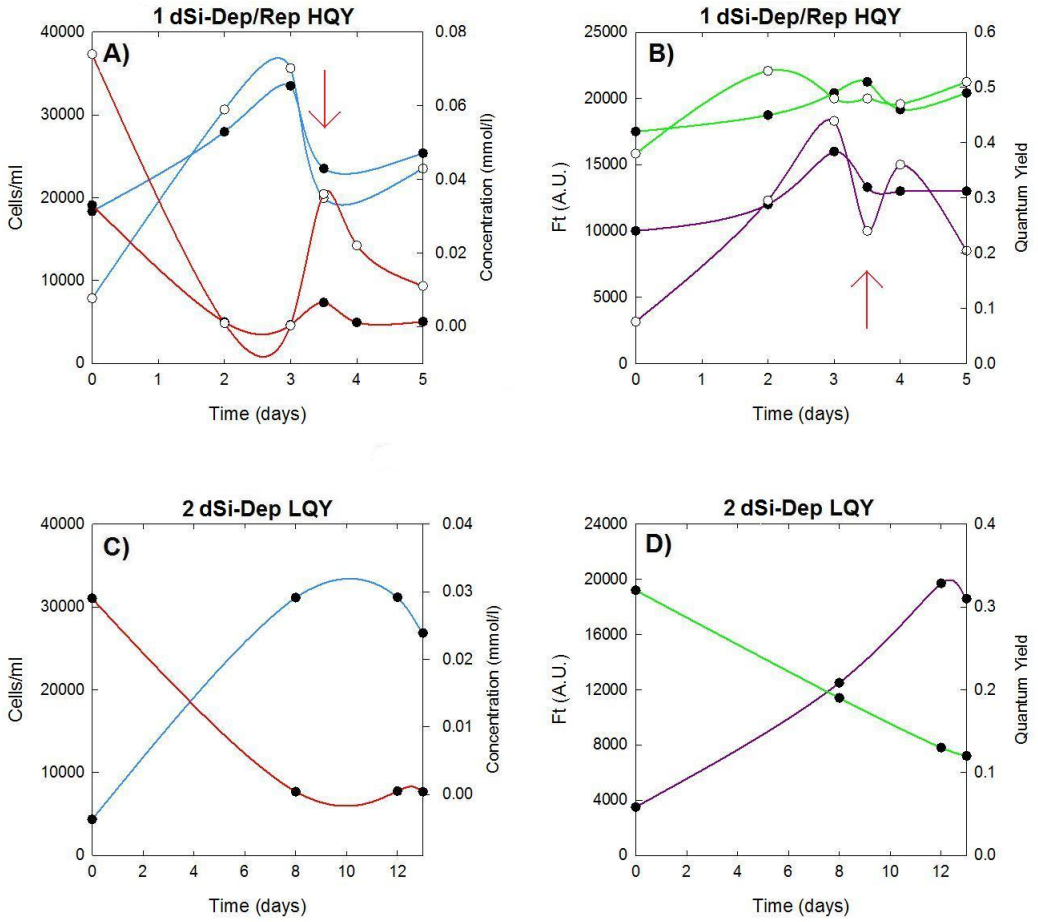


Figure 18: Development in (A, C) cell number (blue lines, left axis), dSi concentration (red lines, right axis), (B, D) Ft (purple lines, left axis) and QY (green lines, right axis) values in the preparation cultures for 1 dSi-Dep/Rep-HQY and 2 dSi-Dep-LQY. In (A, B), 1 dSi-Dep-HQY is denoted with closed circles and 1 dSi-Rep-HQY with open circles. Red arrows indicate time of dilution.

Development in the preparation cultures A steady increase in cell number and Ft values along with a decrease in dSi concentration was initially observed in all of the preparation cultures (figs 18, 19). A sudden decrease in cell number and Ft values simultaneously with an increase in dSi concentration was observed upon dilution. QY values were high/increasing as long as dSi was in excess in the culture medium, but as soon as the diatoms started to experience dSi depletion, the QY curve levelled off and the values started to drop.

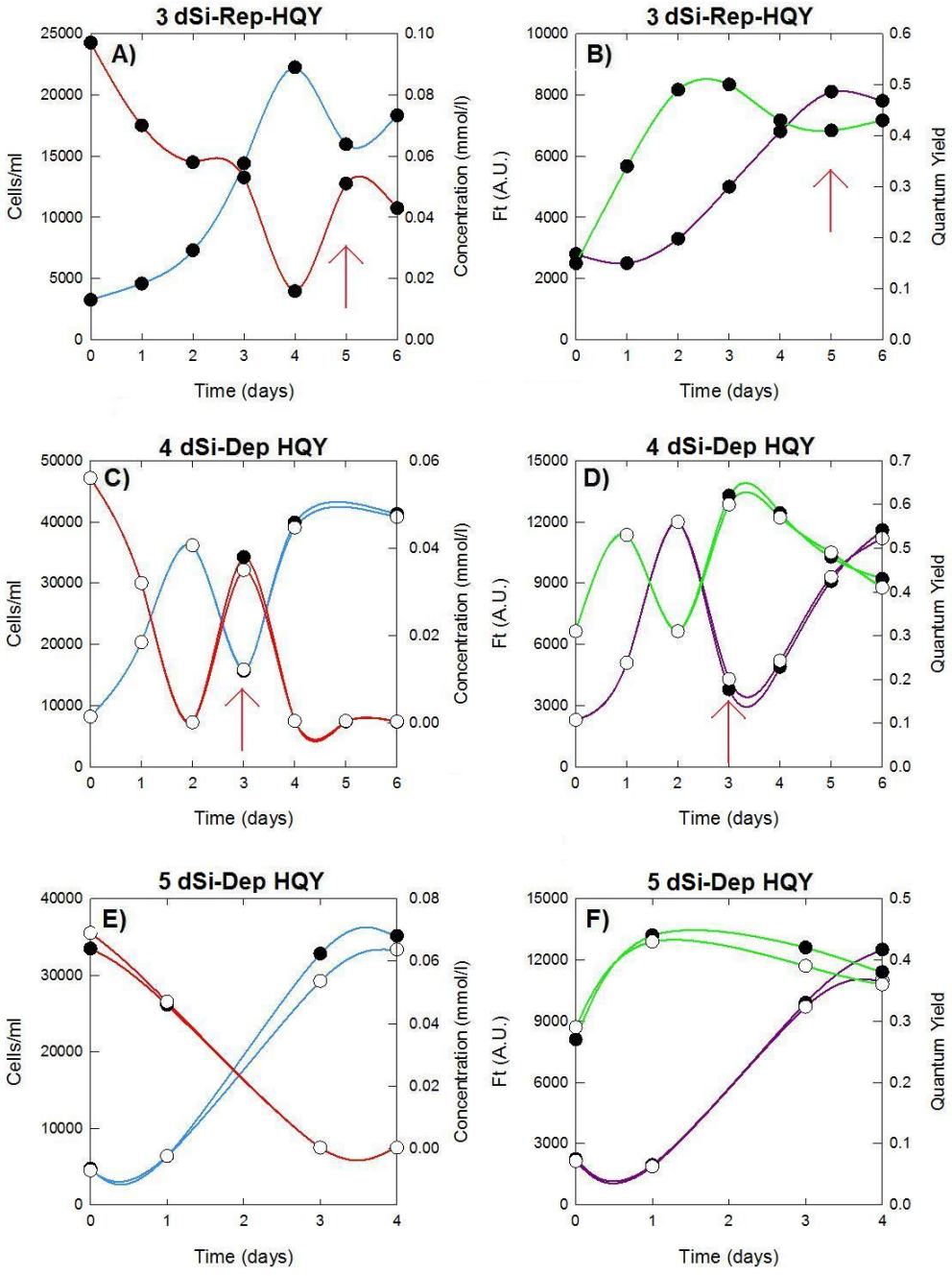


Figure 19: Development in (A, C, E) cell number (blue lines, left axis), dSi concentration (red lines, right axis), (B, D, F) Ft (purple lines, left axis) and QY (green lines, right axis) values in the preparation cultures for 3 dSi-Rep-HQY, 4 dSi-Dep-HQY and 5 dSi-Dep-HQY. Red arrows indicate time of dilution.

Development in the experimental cultures

Ft and QY The Ft and QY values developed markedly different in 1 dSi-Dep-HQY A compared to 1 dSi-Rep-HQY B (fig 20 A), and the differences were representative for the other dSi deplete/replete cultures. A pronounced drop in both Ft and QY values during the first 5-7.5 hours before the declining trend was reversed was observed in all of the dSi deplete cultures (1 dSi-Dep-HQY A, 2 dSi-Dep-LQY, 4 dSi-Dep-HQY and 5 dSi-Dep-HQY) while an increase in Ft values and only a modest reduction in QY values was observed in the dSi replete cultures (1 dSi-Rep-HQY B, 3 dSi-Rep-HQY) in the same time period (fig 20).

The QY values in 2 dSi-Dep-LQY increased somewhat after the 10 hour delivery process was completed, but never exceeded 0.2 and the values were therefore classified as low for the entire experiment (fig 20B).

The development in 5 dSi-Dep-HQY A diverged from the development in 5 dSi-Dep-HQY B 7.5 hours into the delivery period. At this point, the declining trend observed in the other dSi deplete cultures had been reversed, but the Ft and QY values in 5 dSi-Dep-HQY A continued to decrease.

The Ft development in the cultures that had not been exposed to feed solution was similar in the dSi deplete and dSi replete cultures, increasing from morning to evening (fig 20F) . The QY values developed differently depending on dSi concentration in the culture medium. In the dSi deplete cultures, QY values decreased from morning to evening, while the opposite development was observed in the dSi replete cultures.

pH pH was stable between 8 and 8.35 throughout all of the *Pinnularia* experiments except 5 dSi-Dep-HQY A, where a temporary stop in the Ti feed solution syringe pump shifted pH to 9.14 before it was corrected.

Cell number A pronounced drop in cell number was observed in all of the five Ti incorporation experiments (fig 21), regardless of the initial dSi concentration in the cultures. The cell number measured at the end of the delivery period spanned from $\sim$60% of the original value in 2 dSi-Dep-LQY to $\sim$15% of the original value in 5 dSi-Dep-HQY. The cell number increased somewhat towards the end of the experimental periods, but only in 1 dSi-Dep/Rep-HQY was the final cell number higher than the initial value.

In the control experiments, the cell number was nearly doubled after only 24 hours when only dSi was added to the culture medium (2C-dSi) (fig 22A). When pure HCl and NaOH was added (4C-HCl/NaOH), the cell number remained constant throughout the 24 hour experimental period (fig 22C). A marked decrease in cell number was observed when Ti feed solution was co-delivered with pure NaOH (3C-Ti/HCl/NaOH). By the end of the delivery period, cell number was reduced to $\sim$25% of the original value (fig 22B).

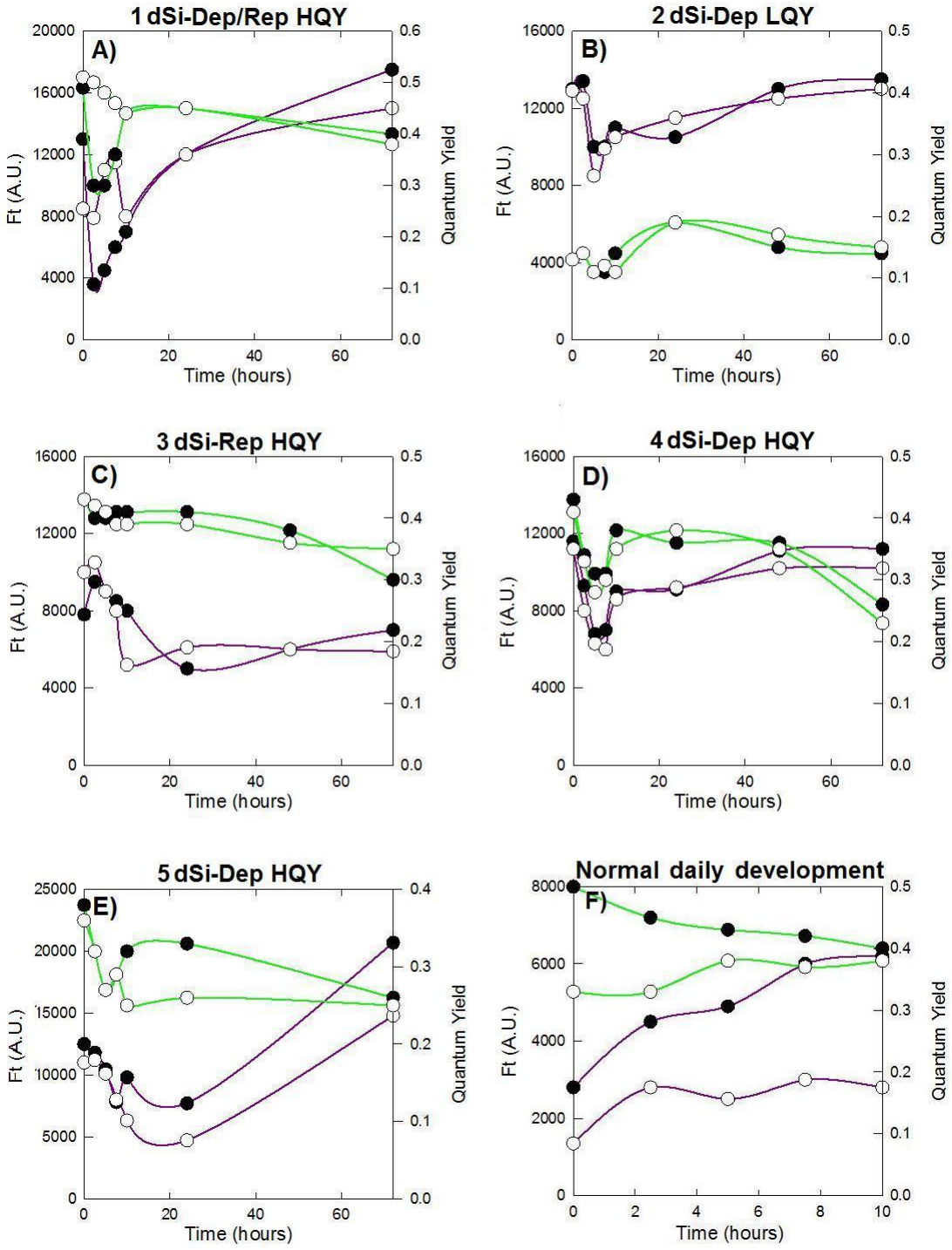


Figure 20: Development in Ft (purple, left axis) and QY (green, right axis) values during (A-E) the 72 hours of the five *Pinnularia* Ti incorporation experiments and (F) a 10 hour control period (morning to evening) of duplicate dSi deplete (open circles) and dSi replete (closed circles) *Pinnularia* cultures. In (A), 1 dSi-Dep-HQY is denoted with closed circles and 1 dSi-Rep-HQY with open circles.

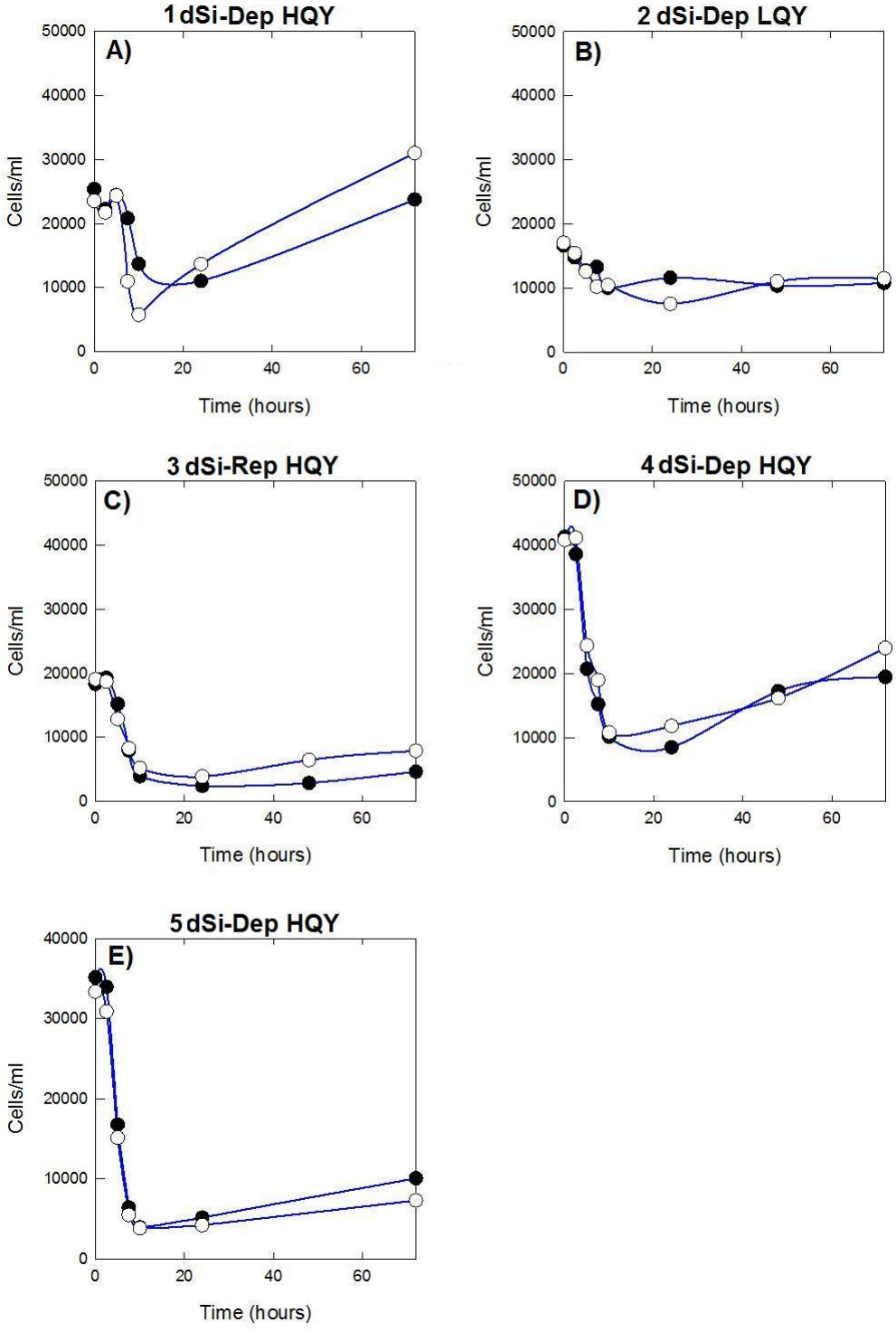


Figure 21: Cell number development during the 72 hours of the five *Pinnularia* Ti incorporation experiments. In (A), 1 dSi-Dep-HQY is denoted with closed circles and 1 dSi-Rep-HQY with open circles.

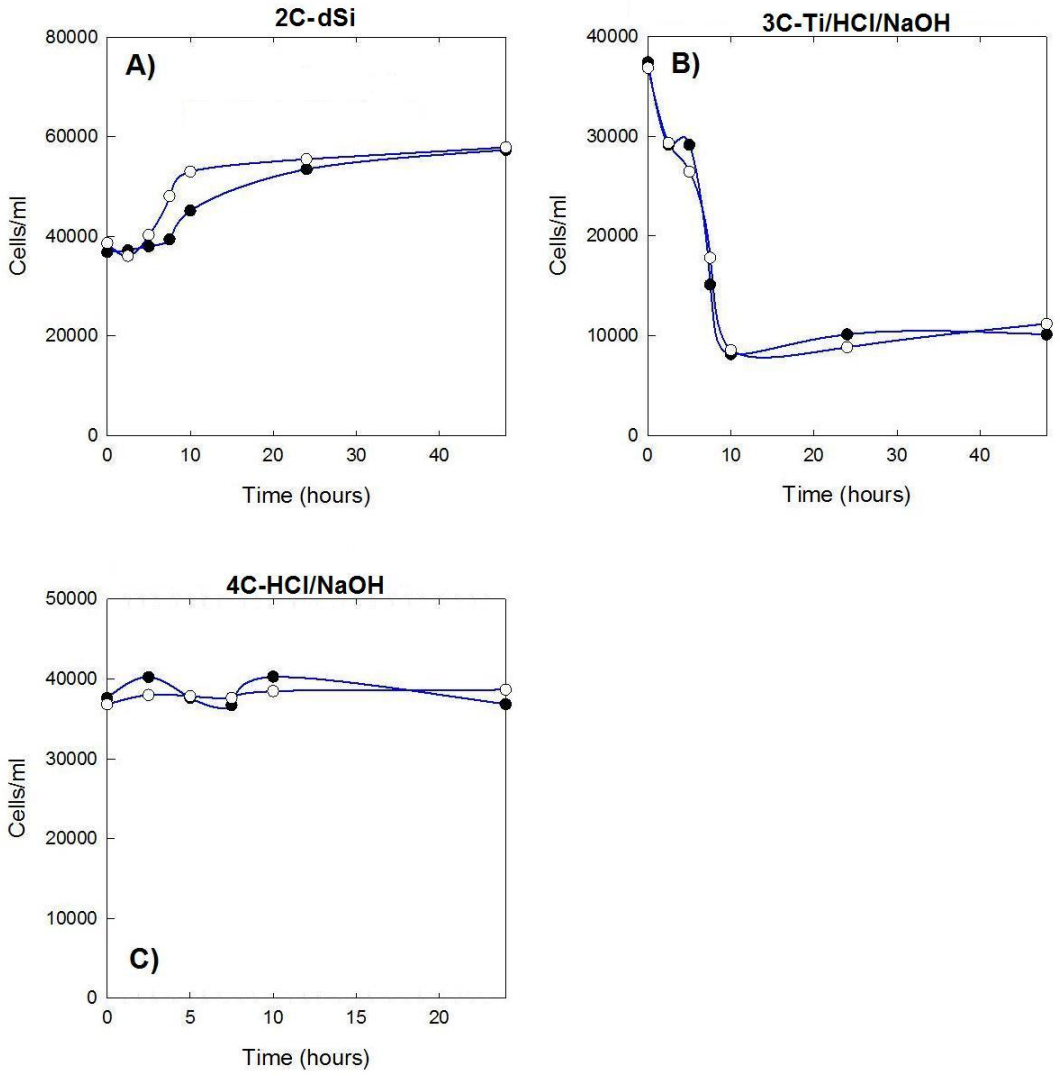


Figure 22: Cell number development in control experiments, where dSi starved *Pinnularia* cultures were added (A) dSi, (B) Ti, HCl and NaOH, and C) HCl and NaOH. Notice different time periods on x-axis.

Visual appearance At the beginning of the experiments, all of the cultures displayed an even, light brown colour. Particulates were barely visible to the naked eye from very short observation distance. After 5 hours of feed solution delivery, the cultures had become more transparent, and a dark brown mass could be observed at the bottom of the vessel, as well as along the aeration tube. 10 hours after delivery initiation, the colour loss was even more pronounced, and large particulates were floating through the culture medium as the vessel was stirred. Most of the particulates settled at the bottom of the vessel as soon as the stirring ceased (fig 24), but some them were observed just beneath the culture surface (fig 23). Figs (23,24) display particulates observed in 4 dSi-Dep-HQY, but they are representative for all of the *Pinnularia* cultures exposed to Ti feed solution.

Figure 23: Dark particulates were observed floating about just beneath the culture surface 7.5 hours after delivery initiation in 4 dSi-Dep-HQY A.

Figure 24: Dark particulates floating about in solution and sinking to the container floor was observed after feed delivery was completed in 4 dSi-Dep-HQY A.

Flow cytometry results During the first 10 hours of the Ti incorporation experiments, the number of events registered with flow cytometry decreased with each sample counted. Fig 25 shows the development in 4 dSi-Dep-HQY A, but is illustrative for the trend observed in all of the *Pinnularia* cultures exposed to Ti feed solution. Apart from the reduction in registered cell events (red dots in fig 25) no other changes were observed in the flow cytometry plots. The number of control fluorescent beads (green dots in fig 25) was constant in all samples.

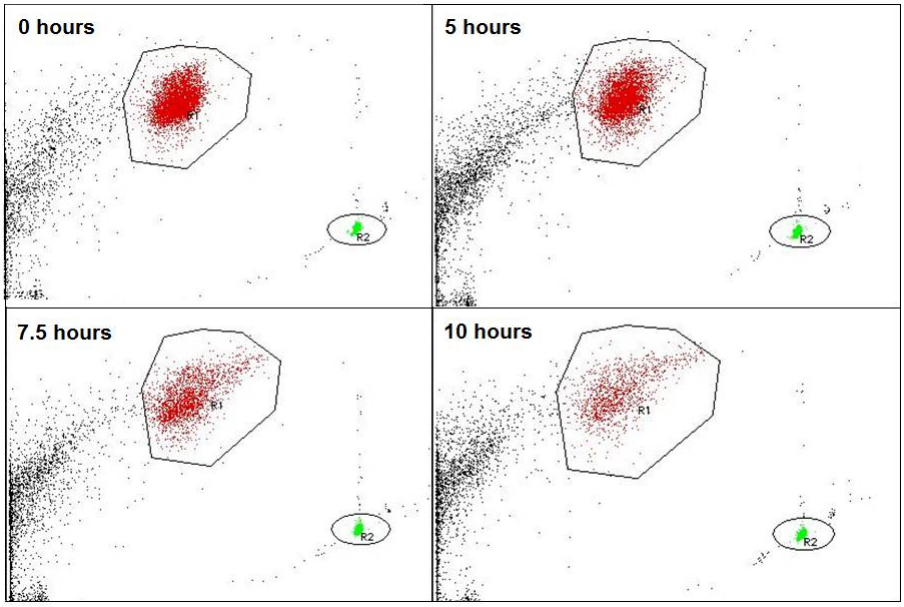


Figure 25: Flow cytometry spectra from the 10 first hours of 4 dSi-Dep-HQY A. Diatoms are illustrated by red dots, (area R1) and control beads are illustrated with green dots (area R2). Black dots are organic particulates, bacteria and background noise.

Examination with optical microscope The inspection of a culture sample from 4 dSi-Dep-HQY A with optical microscopy revealed that many of the diatoms were clustered together in large aggregates, while some were single or in pairs. The aggregate in fig 26 consisted of approximately 200 cells and was about 350 µm in diameter.

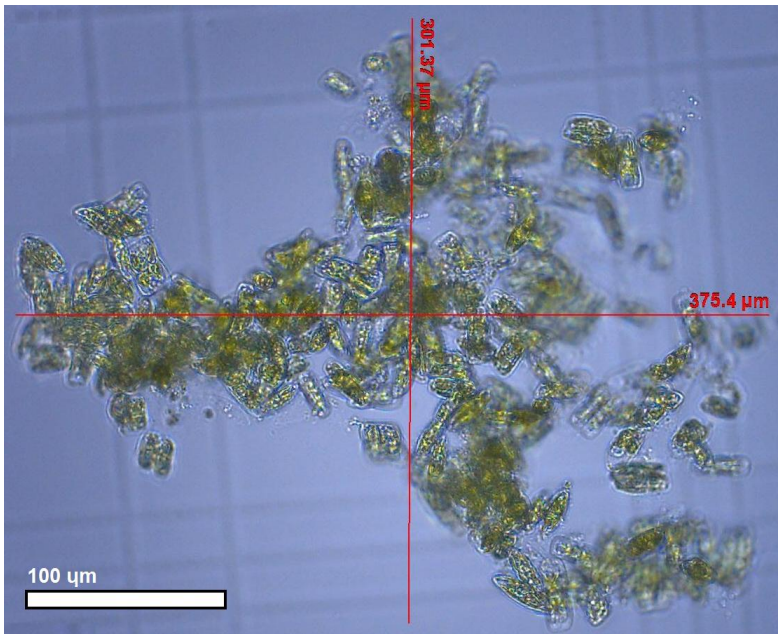


Figure 26: Optical microscopy image of diatom aggregate from 4 dSi-Dep-HQY A.

dSi concentration At the end of 1 dSi-Dep/Rep-HQY, both of the cultures were dSi depleted (fig 27A). 1 dSi-Dep-HQY A was dSi depleted already after 48 hours.

None of the cultures in 2 dSi-Dep-LQY were dSi depleted 48 hours after delivery initiation, even though both were dSi starved at the beginning of the experiment (fig 27B).

Most of the gap between the measured and estimated dSi concentration in 3 dSi-Rep-HQY originated from the first five hours of the delivery period (fig 27C). After this, the dSi concentration remained nearly constant for 48 hours before a marked decrease was observed. At the end of the experimental period, none of the cultures were dSi depleted.

The dSi concentration initially increased rapidly in 4 dSi-Dep-HQY, but declined equally fast after delivery was completed. 48 hours after initiation both of the cultures were dSi depleted.

The dSi concentration development in 5 dSi-Dep-HQY was similar to 4 dSi-Dep-HQY. Both culture were dSi deplete after 48 hours (fig 27E).

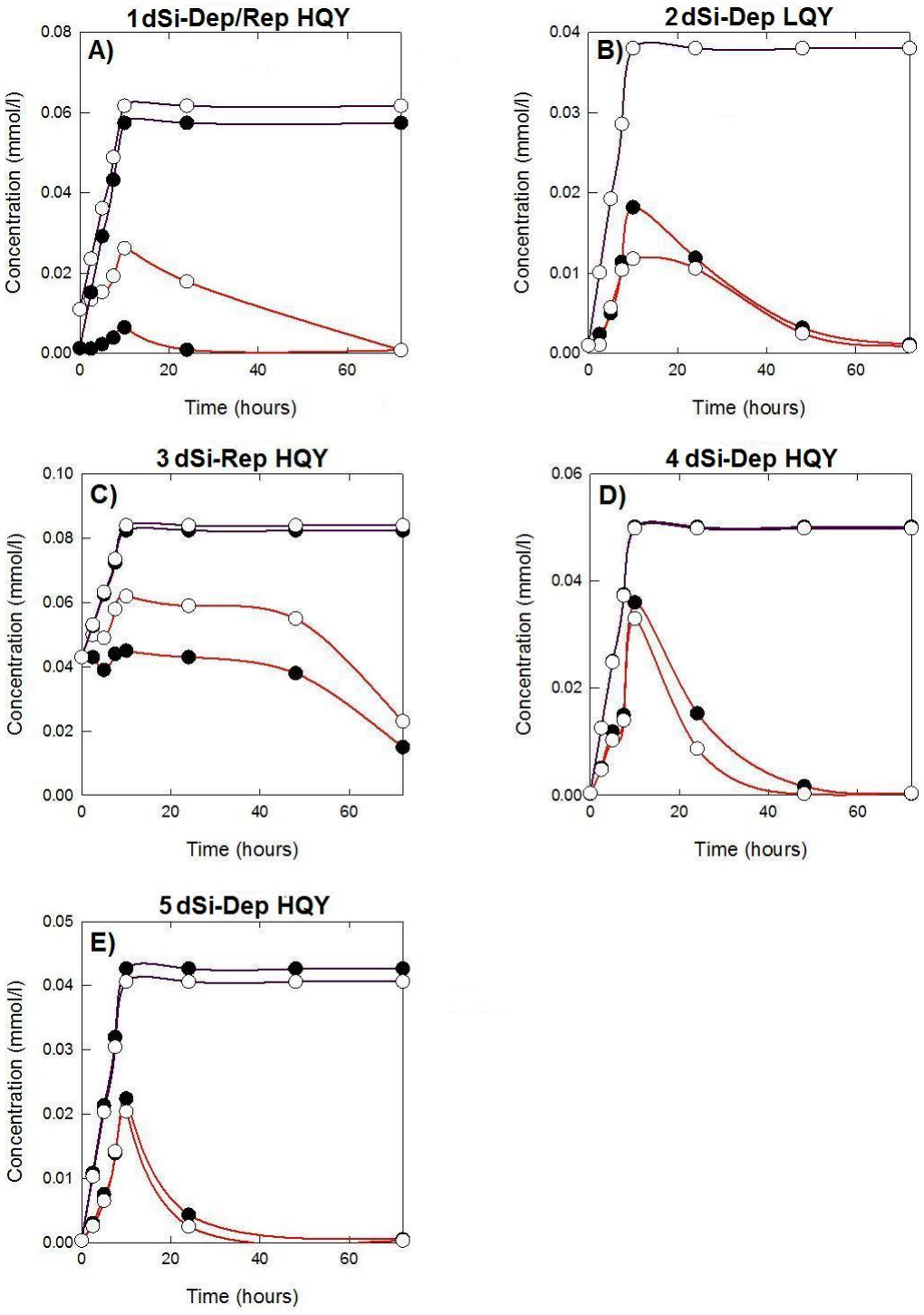


Figure 27: Changes in measured (red lines) and estimated (dark purple) dSi concentration during the 72 hours of the five *Pinnularia* Ti incorporation experiments.In (A), 1 dSi-Dep-HQY is denoted with closed circles and 1 dSi-Rep-HQY with open circles.

Ti concentration The development in Ti concentration was similar in all of the five Ti incorporation experiments (fig 28). The measured concentration did not follow the steep line illustrating the estimated theoretical concentration, but instead the measured concentrations increased slowly until they reached a maximum value far below the estimated values. After delivery was completed, the measured concentration decreased even slower during the remaining 62 hours of the experiments. The maximum Ti concentration was measured in 2 dSi-Dep-LQY (0.27 µmol/l).

The maximum Ti concentration in the control experiment C1-f/2 was measured to be more than ten times the maximum concentration in the Ti incorporation experiments (2.98 µmol/l). The measured concentration in C1-f/2 developed nearly as estimated, increasing rapidly initially before levelling out just before 3 µM.

Due to a temporal stop in the syringe pump delivering Ti feed solution to 5 dSi-Dep-HQY, the concentration development in this culture probably deviated somewhat from the other cultures, but the Ti concentration in the medium from this culture was not measured with ICP-MS.

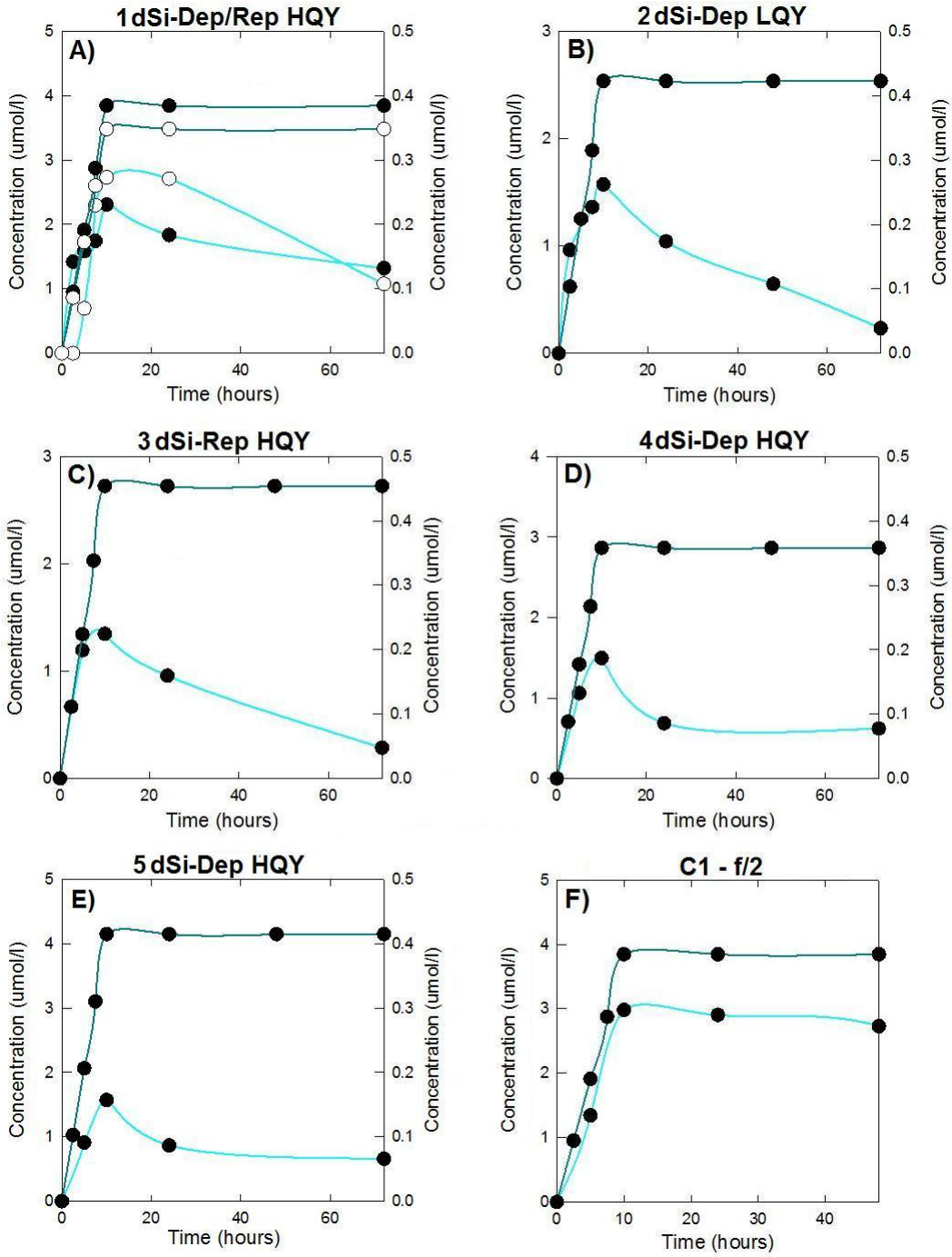


Figure 28: Changes in measured (light blue lines, right axis) and estimated (dark blue lines, left axis) Ti concentration during (A-E) the 72 hours of the five *Pinnularia* Ti incorporation experiments, and (F) the 48 hours of the control experiment in which Ti feed solution was added to pure f/2 medium (C1-f/2). In (A) 1 dSi-Dep-HQY A is denoted with closed circles and 1 dSi-Rep-HQY B with open circles. Notice that units on the left and right axis are the same (µmol/l), but values are higher on the left axis. In (F) there is only a left axis.

Table 5: Results from the five Ti incorporation experiments on *Pinnularia*

Culture	Final cell number (ml^{-1})	Final QY	[dSi] in culture medium after 10 hours (µmol)	[dSi] in culture medium after 72 hours (µmol)	[Ti] in culture medium after 10 hours (µmol)	[Ti] in culture medium after 72 hours (µmol)	Estimated dSi (pmol)	Si/dSi/cell after 72 hours (pmol)
1 dSi-Dep-HQY A	23750	0.40	6.5	0.8	0.23	0.13	2.13	2.05
1 dSi-Rep-HQY B	30970	0.38	26.2	0.8	0.26	0.11	2.13	2.41
2 dSi-Dep-HQY A	10760	0.14	18.2	1.1	0.27	0.11	2.14	1.93
2 dSi-Dep-HQY B	11480	0.15	11.8	0.9	*	*	2.14	1.92
3 dSi-Rep-HQY A	4620	0.30	45	15	0.22	0.05	2.13	3.25
3 dSi-Rep-HQY B	7880	0.35	62	23	*	*	2.13	2.82
4 dSi-Dep-HQY A	19450	0.26	36	0.3	0.19	0.07	1.19	1.08
4 dSi-Dep-HQY B	23950	0.23	33	0.4	*	*	1.19	1.07
5 dSi-Dep-HQY A	7290	0.26	22.4	0.5	*	*	1.20	1.08
5 dSi-Dep-HQY B	10050	0.25	20.4	0.3	0.25	0.07	1.20	1.09

*Not measured

4.1.2 *Coscinodiscus*

Cell division and dSi uptake in preliminary growth study The culture inoculated in the borosilicate vessel with assisted aeration did not grow. Inspection with optical microscopy 14 days after inoculation revealed many dead cells (empty frustules), and the cell number had decreased from 11.4 ml^{-1} to 5.1 ml^{-1}. It was decided not to continue with this experimental setup for *Coscinodiscus*.

The cell number in the culture grown in a plastic flask with no assisted aeration had more than doubled in 8 days, from 11.7 ml^{-1} to 26.3 ml^{-1}. In the same time period, dSi concentration had decreased from 0.055 mM to 0.0264 mM and QY values had decreased slightly from 0.58 to 0.55. Based on this cursory survey, a growth rate of 0.14 day^{-1} and dSi/cell of 1.95 nmol was estimated.

Development in experimental cultures The *Coscinodiscus* cultures were not dense enough for Ft measurements to be feasible. QY measurements returned either an error message saying "low value", or a relatively high value (>0.4). pH fluctuated moderately between 8.1 and 8.35.

In neither of the three Ti incorporation experiments were the diatoms able to take up dSi at the same rate at is was added, which resulted in an increase in dSi concentration in the culture media over time (fig 29). Cell enumeration with optical microscopy revealed an increasing number of empty frustules with time.

Table 6: Results from Ti incorporation experiments on *Coscinodiscus*

Culture	Final [dSi] (mmol/l)	Final cell number (ml^{-1})	Final QY value	Total number of cells formed	% cells divided	dSi consumed (µmol)
1 (3d) A	0.0071	27.9	0.47	1410	50.8	4.59
1 (3d) B	0.0071	30.6	0.49	1770	62.8	4.69
2 (5d) A	0.0121	39.1	0.54	3645	164.2	3.39
2 (5d) B	0.0113	30.6	0.54	2745	148.8	2.66
3 (7d) A	0.0099	19.2	0.43	949	61.3	1.86
3 (7d) B	0.0101	16.3	0.42	728	52.3	1.57

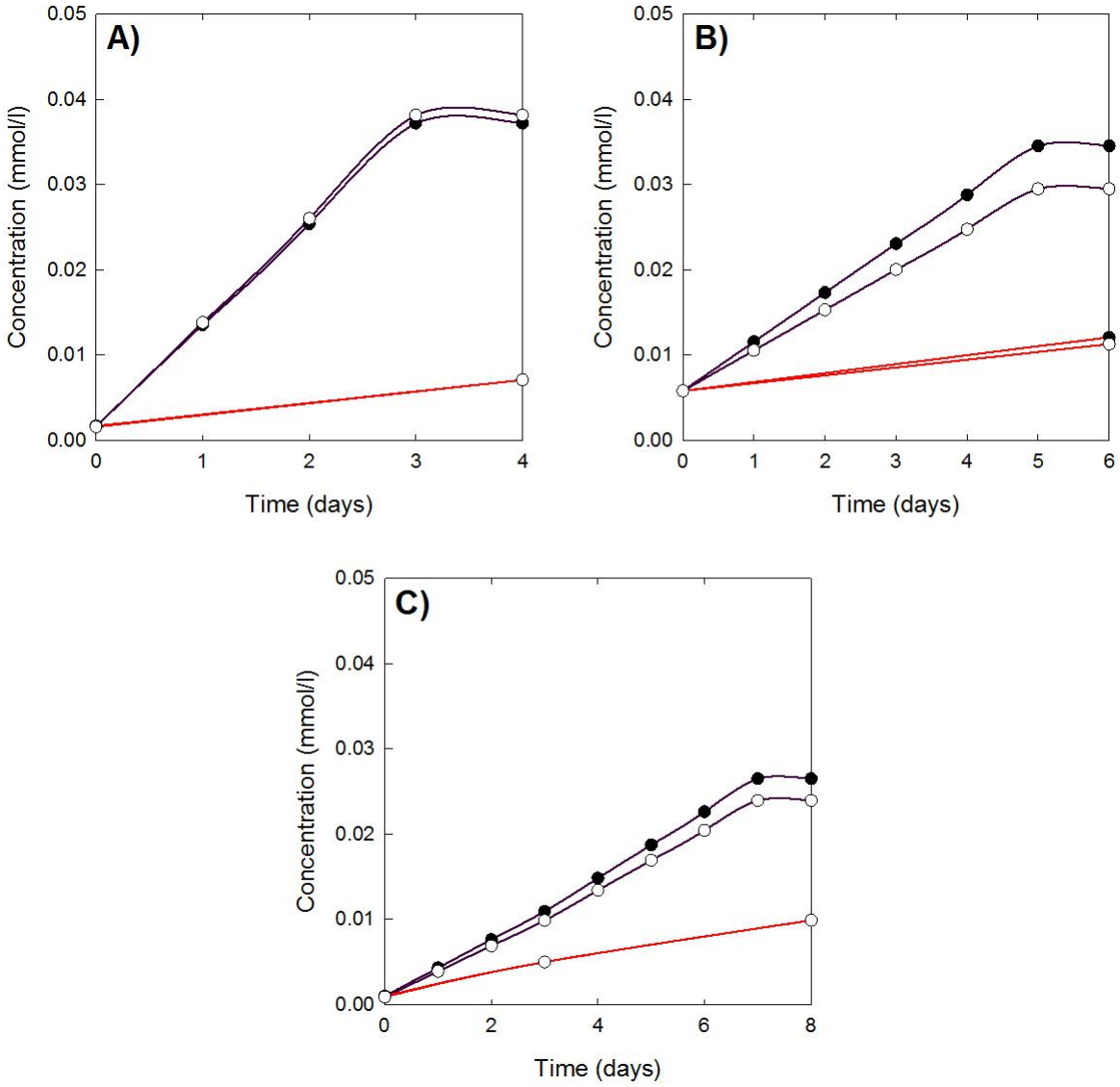


Figure 29: Change in measured (red) and estimated (dark purple) dSi concentration in the *Coscinodiscus* culture medium during the Ti incorporation experiments (A) 1 (3d), (B) 2 (5d) and (C) 3 (7d).

4.1.3 Frustule elemental composition

Because the frustules were suspended in methanol when they were dissolved in HF, the ICP-MS results would not have been accurate for the oxygen content. Oxygen was therefore left out of the following overview. The values presented in the table below are given relative to the weight of Si in the same sample. Assuming that oxygen constitutes $\sim$ 50 wt% of the frustule biosilica, the wt% of an element relative to the total sample weight is approximately half the value of the wt% relative to Si. This assumption is not valid for the sample where the organic material was not removed prior to ICP-MS analysis.

Pinnularia All of the frustules contained B, even the control frustules from diatoms cultivated in a plastic container, unexposed to borosilicate and feed solutions. There was a marked difference between the Ti content in 1 dSi-Dep-HQY A and 1 dSi-Rep-HQY B. The frustules from the dSi deplete high QY cultures (1 dSi-Dep-HQY A, 4 dSi-Dep-HQY and 5 dSi-Dep-HQY) contained considerably more Ti than the frustules from the other experiments. The amount of P, Ti and Fe relative to Si was larger in the sample in which the organic material had not been removed than in any of the other samples. Neither Ti nor Fe was found in the frustules from the plastic flask culture, but B and P was present in small amounts.

Table 7: Wt% of elements relative to Si in the *Pinnularia* frustules

Culture	B	P	Ti	Fe
1 dSi-Dep-HQY A	0.45	0.47	0.50	0.37
1 dSi-Rep-HQY B	0.36	0.14	0.01	0.14
2 dSi-Dep-LQY A	0.11	0.23	0.31	0.59
2 dSi-Dep-LQY B	0.11	0.31	0.48	0.49
3 dSi-Rep-HQY A	0.05	0.13	0.32	0.23
3 dSi-Rep-HQY B	0.09	0.13	0.24	0.18
4 dSi-Dep-HQY A	0.06	0.59	1.31	0.55
4 dSi-Dep-HQY B	0.09	0.54	1.24	0.57
5 dSi-Dep-HQY A	0.02	0.21	0.59[1]	0.16
5 dSi-Dep-HQY B	0.02	0.40	1.18	0.36
4 dSi-Dep-HQY A, organic material not removed	0.03	4.77	2.86	9.56
***Pinnularia* cultured in plastic flask**	0.05	0.07	0	0

[1]Ti feed solution syringe pump stopped

Coscinodiscus The frustules from 2 (5d) had the highest Ti content relative to Si. The frustules from 1(3d) contained very small amounts of Ti, while the frustules from the preliminary growth study, untreated with feed solution, did not contain Ti at all. All of the frustules contained a relatively stable amount of B and P, with small differences in average value between the experiments. Fe was detected in some of the samples, but the results are not presented because the values were close to the instrument detection limit.

Table 8: Wt% of elements relative to Si in the *Coscinodiscus* frustules

Culture	B	P	Ti
1 (3d) A	0.212	0.161	0.003
1 (3d) B	0.232	0.137	0.007
2 (5d) A	0.160	0.159	0.031
2 (5d) B	0.228	0.113	0.037
3 (7d) A	0.124	0.173	0.014
3 (7d) B	0.119	0.149	0.024
Untreated *Coscinodiscus*	0.120	0.309	0

T-test A t-test was performed to investigate whether the Ti content results from the incorporation experiments were significantly different. The values from 1 dSi-Dep/Rep-HQY were omitted because the cultures were not replicates, and the values from 5 dSi-Dep-HQY were omitted because of the error in the syringe pump delivering Ti feed solution to 5 dSi-Dep-HQY A.

In the *Pinnularia* frustules, it was found that the Ti content in 4 dSi-Dep-HQY was significantly different from the content in 2 dSi-Dep-LQY (p=0.011) and 3 dSi-Rep-HQY (p=0.003). The Ti content in 2 dSi-Dep-LQY was not significantly different from the content in 3 dSi-Rep-HQY.

In the *Coscinodiscus* frustules, there was a significant difference between the Ti content in 1 (3d) and 2 (5d) (p=0.015). Neither 1 (3d) and 3 (7d) nor 2 (5d) and 3 (7d) were significantly different.

The results are summarized in the two tables below. P-values indicating significant differences (P<0.05) are written in italic.

Table 9: P-values from t-tests on *Pinnularia* frustules

	2 dSi-Dep-LQY	**3 dSi-Rep-HQY**	**4 dSi-Dep-HQY**
2 dSi-Dep-LQY	-	0.643	*0.011*
3 dSi-Rep-HQY	0.643	-	*0.003*
4 dSi-Dep-HQY	*0.011*	*0.003*	-

Table 10: P-values from t-tests on *Coscinodiscus* frustules

	1 (3d)	**2 (5d)**	**3 (7d)**
1 (3d)	-	*0.015*	0.122
2 (5d)	*0.015*	-	0.124
3 (7d)	0.122	0.124	-

4.1.4 Frustule structure and location of elements

It was not possible to detect any systematic alterations in the frustule structure of the diatoms that had been exposed to Ti. Structural flaws were observed in some frustules, but they were also found in Ti-free frustules.

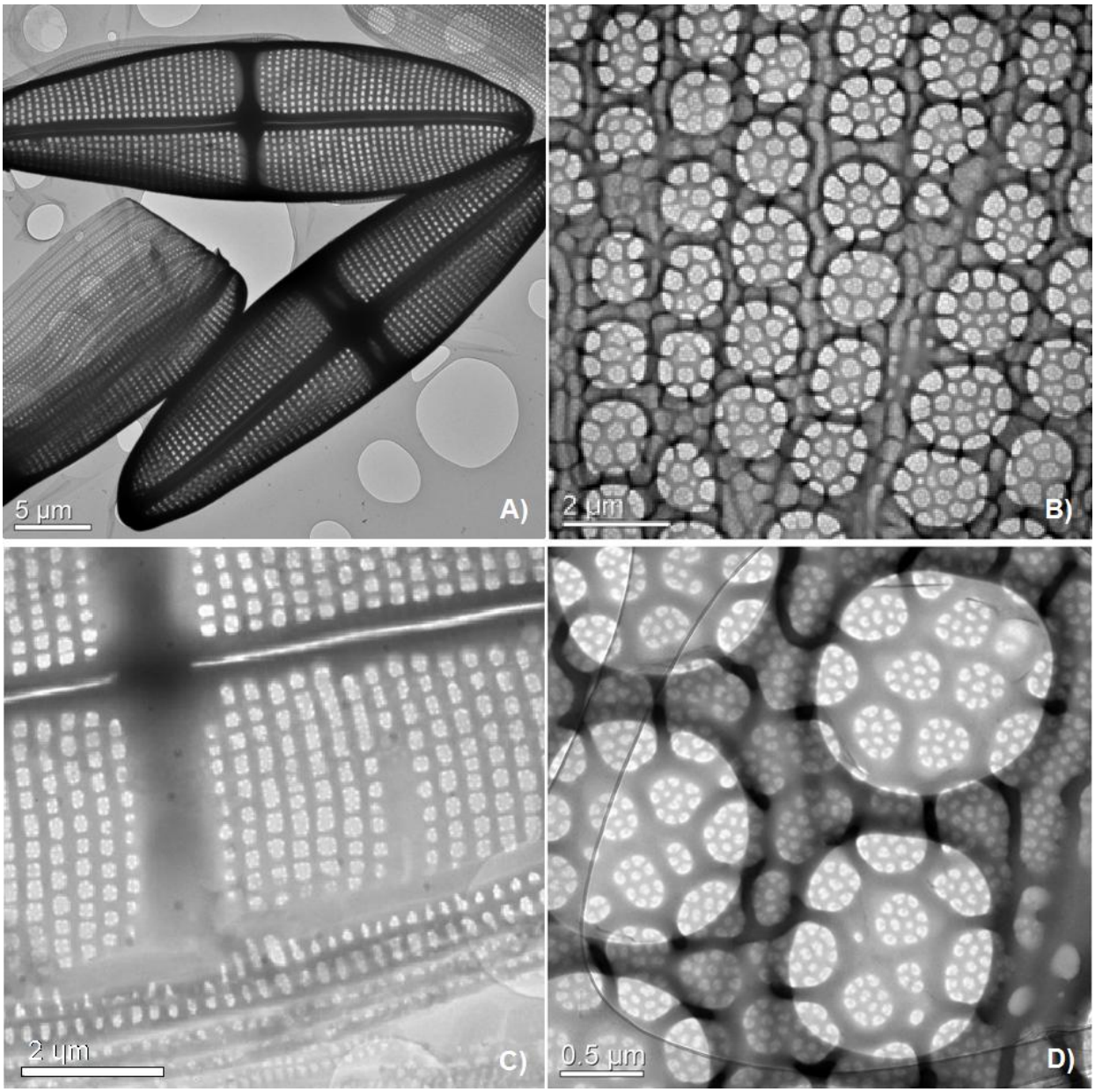


Figure 30: BF TEM images of (A, C) *Pinnularia* frustules from 4 dSi-Dep-HQY A and (B, D) *Coscinodiscus* frustules from 2 (5d) A. Fused pores and irregularities in both the valve and the girdle bands are clearly visible in (C).

A faint white pattern was observed in the DF STEM images of the frustules containing Ti (figs 31 A, B). The same pattern was not found in frustules without Ti (fig 31 C).

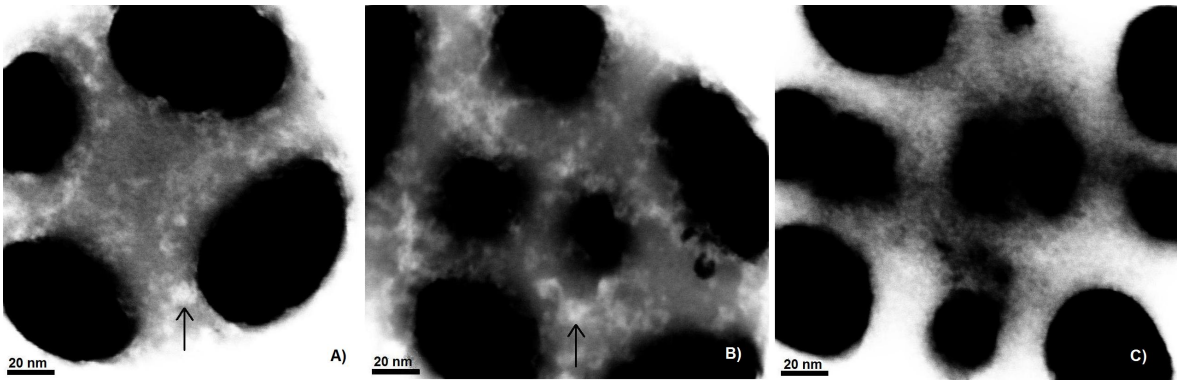


Figure 31: DF STEM images of the biosilica at the base of the large pores in *Pinnularia* frustules that (A, B) contained Ti and (C) did not contain Ti. All frustules were from 4 dSi-Dep-HQY A.

EDS scans of several frustules revealed that the relative distribution of Ti was not uniform throughout the biosilica. Furthermore, P and Fe was detected and also appeared to be unevenly distributed. Comparing scans of the thin silica layer located at the base of the large pores ("pore silica") (white circles in fig 33) with scans of the silica located between the large pores (black squares in fig 33) revealed that the amount of Ti, P and Fe relative to Si was higher in the pore silica than in the thicker silica layers between the pores.

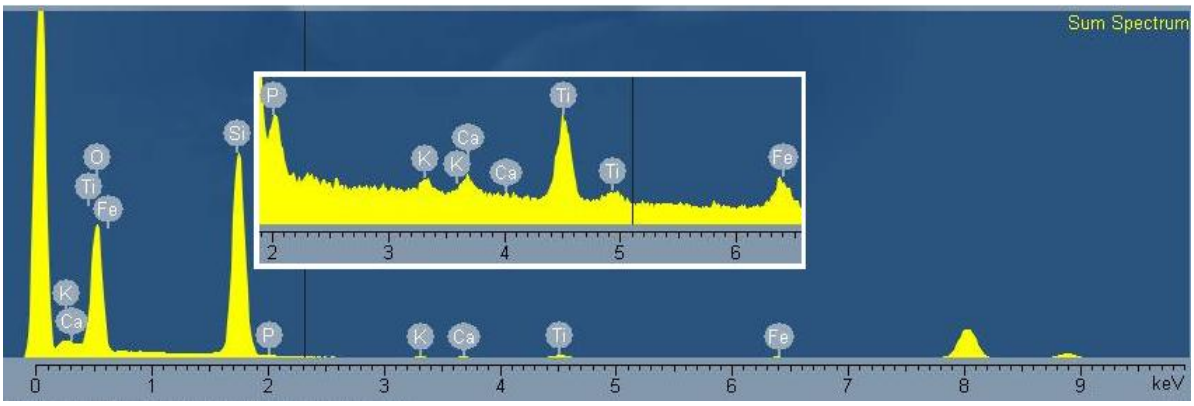


Figure 32: EDS spectrum of the entire area depicted in fig 33. Inset is a magnification of the range 1.9-6.6 keV.

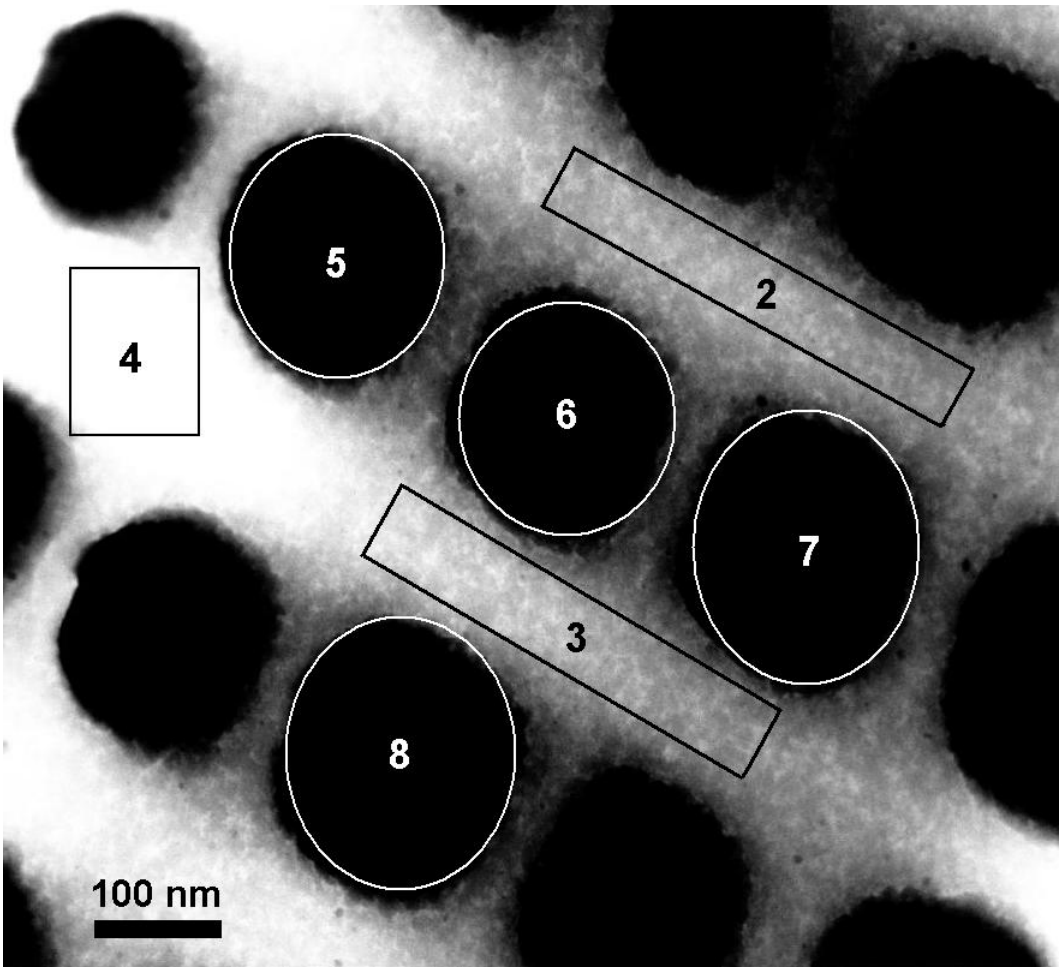


Figure 33: DF STEM image of area for EDS analysis of *Pinnularia* frustule from 4 dSi-Dep-HQY A. Black squares illustrate that EDS spectra were aquired from an area between the large pores. White circles illustrate that the EDS spectra were aquired from the thin layer of biosilica at the base of the pores (the differences in sample thickness make features at the base of the pores too dark to be distinguished).

Table 11: Elements (wt %) detected with EDS in the sample depicted in fig 33

Spectrum	O	Si	P	Ti	Fe
Entire area	**49.7**	**48.2**	**0.63**	**0.93**	**0.36**
Spectrum 2	49.0	50.0	0.22	0.83	0
Spectrum 3	49.9	48.5	0.57	0.72	0.20
Spectrum 4	45.8	52.7	0.52	0.84	0.14
Spectrum 5	47.4	48.8	1.22	1.92	0.71
Spectrum 6	48.9	47.9	1.14	1.64	0.31
Spectrum 7	51.9	44.7	0.99	1.52	0.87
Spectrum 8	49.5	47.0	1.05	1.74	0.72

5 Discussion

5.1 Diatom response to feed solution in *Pinnularia* cultures

5.1.1 Cell number

Based on experience from literature [28, 31, 48] and earlier studies [16], it was expected that the addition of dSi would produce a doubling of the cell number within the first light cycle. However, the cell number did not develop as expected during the delivery process in the *Pinnularia* Ti-incorporation experiments, and the reason for the observed decline was not immediately obvious. The diatoms could not simply have disappeared, because it takes more than a day for the organic material protecting the diatom to dissolve and for the biosilica to remineralize [1]. The diatoms would therefore still be registered in the flow cytometer even if the added chemicals had been harmful. Dissolution could have been accelerated by a very high or low pH, but the pH in the cultures was stable throughout all of the experiments except from a short period in 5 dSi-Dep-HQY A. And if the added chemicals had severely impaired the physiological condition of the diatoms, it would have been detected because QY values would have dropped to very low levels. All of this implies that the reason for the distinct decline in the observed cell number was not that the diatoms actually disappeared from the culture. It was therefore assumed that the initial diatoms, as well as potentially newly formed diatoms, were still present in the culture medium but not properly counted by the flow cytometer. Such a condition could be explained by cell aggregation, because the flow cytometer detects single events but has a poor size resolution. Visual observations of large particulates in the culture and aggregates detected with optical microscopy supports this theory (fig 24, 26).

However, there is no apparent reason why the diatoms would suddenly start sticking together upon addition of dSi. The control experiment in which dSi alone was added to the cultures (2C-dSi, fig 22A) combined with reports from literature and previous experiences indicate that it is unlikely that dSi triggered the cell aggregation. The results from 3C-Ti/HCl/NaOH and 4C-HCl/NaOH (figs 22 B, C) revealed that the addition of NaOH and HCl alone did not have any effect on the cell number, but Ti together with NaOH and HCl produced a dramatic drop. It is therefore reason to believe that the observed decline in cell number was at least partly due to the addition of Ti.

Sutcliffe *et al.* described how continuous aeration of filtered seawater in a water column produced organic particles that aggregated to larger particulates and settled out within an hour [68]. They suggested that the aggregates were formed when organic particles adsorbed to form a thin film on the air bubble surface and as the bubble collapsed, the film was folded into larger particulates or fibres. They suspected that a polar or surface-active molecule was involved in the adsorption of organic material. Zhou *et al.* proposed that bubble-adsorption of surface-active carbohydrates produced by diatoms is an important pathway to formation of organic particulates in seawater, and claimed that diatoms covered in such organic compounds were known to aggregate

towards the end of algal bloom periods [69]. This bubble-mechanism would explain how the *Pinnularia* aggregates were formed, but not why aggregation was not observed until Ti was added. The rapid response to feed solution addition gave reason to believe that the reaction was more chemical than biological. But if most of the Ti adsorbed on the cell surface, the diatoms would still be too big for electrostatic effects alone to make them stick together. And even if the diatoms had been smaller, such an electrostatic effect would probably be prevented by the large amount of ions already present in the culture, as high salt concentrations reduce the Debye-Huckel length for electrostatic force to the order of nanometres [11]. Another possibility may be that the added Ti triggered excretion of organic molecules, like the compounds described by Zhou *et al.*, for cell protection [10]. The purpose of the excretion may have been to remove molecular Ti from the culture medium by complexation, or to remove biosorbed Ti from the diatom surface. The organic compounds alone may have caused the cells to aggregate, or the clumping may have been a result of excretion combined with turbulence in the culture vessel produced by the aeration tube.

No decline in cell number was reported by Jeffryes *et al.* in the reports from their similar experiments [13][45], and neither was any drop in cell number observed in our previous work [16]. It is difficult to pinpoint the details that were different in the experiments conducted by Jeffryes *et al.* compared to the experiments conducted in the present study. The chemicals were acquired from the same producers and the process parameters were similar. Jeffryes *et al.* conducted the experiments with several different dSi/Ti feed solution ratios both larger and smaller than the ratios applied in the present study, but a decline in cell number was never reported. They applied larger vessels to contain the diatom cultures, but apart from that the setup was nearly identical. The same applies to our previous work on Ti addition. The only components that differed from the previous experiments were the feed solutions, which were freshly prepared for the present experiments but made from the exact same stock solutions as before, and the culture vessels, which were of the same shape but with a larger diameter than the vessels applied in the previous experiments. It may be possible that the new dimensions of the culture vessel combined with the air bubbles facilitated the formation of small aggregates, and that these small aggregates acted as nucleation seeds where other cells more easily attached. Another alternative may be that Ti itself did not cause the aggregation, but that the Ti stock solution had somehow been contaminated with a compound that triggered the excretion of organic compounds as a protective cell response.

Based on the experiments conducted in the present study, it is not possible to say if the apparent drop in cell number had any effect on Ti uptake and incorporation. If the aggregation occurred as a protective response to Ti in particular, it is likely that the uptake was to some extent reduced as a result of the aggregation. The maximum Ti content incorporated into the frustule biosilica in the present study was lower than the values reported by Jeffryes *et al.* As the experiments were otherwise similar but they did not report any observations of cell aggregation, it may be possible that the cell aggregation did in fact limit Ti incorporation. But the ICP-MS and EDS results

clearly show that uptake was still conducted to some degree. However, the unexpected cell number development made it more difficult to interpret the acquired results. The actual number of cells in the experiments may have been more than doubled, and in that case Ti should have been incorporated into more than 50% of the frustules. But if only 10% of the diatoms divided during the 72 hours the experiments lasted, then Ti should be found in ∼9% of the frustules. In the latter case, the few frustules that did in fact contain Ti would have a considerable higher average Ti content than what was measured with ICP-MS. Even if all of the diatoms had divided once, the ICP-MS values would still be 50% lower than the actual average Ti content in the Ti-doped frustules, due to the dilution with old biosilica frustules.

The number of frustules inspected with STEM was not high enough to determine the percentage of Ti-doped frustules in a sample. About 50% of the frustules that were analyzed with EDS contained Ti, and the average Ti wt% relative to Si measured with EDS was only ∼50% higher than what was measured with ICP-MS. Both of these observations indicate that the true cell number was actually doubled, but it can not be confirmed based on the limited number of frustules examined.

If similar experiments are to be conducted in the future, another method should be considered for enumerating cells after feed solution delivery has been initiated. Flow cytometry may not be ideal for this particular species and other species with a tendency to aggregate, because it is difficult to interpret how many diatoms have actually divided without knowing the average number of diatoms in an aggregate and the probability of a diatom being present in an aggregate or as a single cell. However, this information could be obtained by manual inspection of culture samples with optical microscopy. It would be time consuming to examine sufficiently many cells to provide a foundation for statistical calculations, but it would make it possible to determine how many cells did in fact divide. The same information could be obtained by counting number of Ti-doped frustules in a sample with EDS, and compare it to the total number of frustules in that sample.

5.1.2 Ft and QY

The degree of dSi starvation in the *Pinnularia* cultures appeared to affect the development in Ft and QY values during the delivery period of the Ti incorporation experiments. All of the dSi deplete cultures experienced a pronounced drop in Ft and QY values, while a similar drop was not observed in any of the dSi replete cultures (fig 20). When compared to the development in cultures unexposed to feed solution (fig 20F), the observed development in the dSi replete cultures was not so different from the development that could be expected at the same time in the light cycle in a replete culture on an experiment-free day. The difference between the dSi deplete experimental cultures and the dSi deplete non-experimental cultures was more pronounced. Based on these observations, it appears that the addition of feed solution had a stronger impact on the dSi deplete cultures than the dSi replete cultures. A rapid response in the photochemical parameters to feed solution addition is in accordance with the observations

made by Lippemeier *et al.* [38]. The reason for the difference in fluorescence response between the dSi deplete and the dSi replete cultures may be related to the physiological state of the diatoms at different levels of dSi depletion. As long term dSi starvation causes dSi depleted cultures to alter cell processes to adapt to the starved condition [39], a sudden dSi replenishment may require some time for the cells to rearrange back to the original state. In this time period a temporal decrease in fluorescence efficiency could be expected. This reorganization would not be necessary in the dSi replete cultures, and may therefore explain why the observed development in Ft and QY values in the dSi replete experimental cultures deviates so little from the expected development.

5.1.3 Connection between QY, dSi concentration and Ti content

Based on the results from the five Ti incorporation experiments conducted in the present study, QY values and dSi depletion of the culture medium prior to feed delivery initiation appear to be significant for Ti incorporation efficiency in *Pinnularia* diatoms. As described in section 4.1.1, dSi depletion in this context is defined as the concentration at which cell division ceases and the diatoms are unable to take up more dSi from the medium. In the present study, the cultures were dSi depleted at 0.3-0.8µM. This corresponds well with values reported in literature [36, 37].

The dSi deplete cultures with high QY values (1 dSi-Dep-HQY, 4 dSi-Dep HQY and 5 dSi-Dep HQY) had the highest Ti content. The Ti content in the frustules from the dSi deplete low QY experiment (2 dSi-Dep-LQY) and the dSi replete high QY experiment (3 dSi-Rep HQY) was of the same magnitude, but both were significantly lower than the dSi deplete high QY cultures. However, it should be kept in mind that the t-test was performed on very limited data sets (n=2). The results indicate that both insufficient dSi starvation and poor physiological condition has a negative effect on Ti incorporation. The marked difference in Ti content between the dSi deplete and the dSi replete culture from the first experiment (1 dSi-Dep-HQY and 1 dSi-Rep-HQY) illustrates that dSi starvation is beneficial for Ti incorporation. The reason for this may be that dSi starved diatoms undergo a surge uptake upon dSi replenishment [1]. This uptake mechanism may not be as specified when it comes to distinguishing between $Si(OH)_4$ and $Ti(OH)_4$ as internally controlled uptake. Furthermore, dSi depleted diatoms have a smaller intracellular silicic acid pool to "dilute" the absorbed Ti. Most of the compounds used to deposit a new frustule in a dSi starved diatom would therefore come from the surge uptake instead of the intracellular storage pools.

In all of the *Pinnularia* experiments it was observed that QY values started to decline when dSi was no longer in excess in the culture medium. It has been reported that impaired cell condition as a result of dSi starvation reduces protein synthesis efficiency and dSi uptake rate [36, 39]. A low QY value may therefore be indicative of a less than optimal function in several cell processes, including dSi uptake. If the photo system condition (QY values) is representative for the total physiological condition of the cell, then a poor physiological condition appears to decrease the amount of Ti incorporated into the diatom frustule. A diminished immediate surge uptake as

a function of poor cell condition may have shifted the majority of the uptake to the internally controlled uptake mechanism. If this uptake mechanism is more sensitive to the differences between $Si(OH)_4$ and $Ti(OH)_4$, the shift may have resulted in a lower Ti uptake. Other explanations involving more complex cell processes may be possible, but it is at least worth noting that the highest maximum Ti-concentration in the culture medium of all the experimental cultures was measured in the low QY culture (2 dSi-Dep-LQY A, fig 28B), indicating that the Ti uptake in this culture may have been somewhat reduced.

If the observations made in the present study are representative for dSi deplete and dSi replete *Pinnularia* cultures in general, it appears to be a fairy small time window in the diatom cultivation process in which Ti addition will result in maximum incorporation. There will be a balance between initiating delivery too early, in which case the diatoms will not be properly dSi starved, and leaving the diatoms in a dSi starved state for too long, thereby causing the QY values to drop. Both scenarios would result in less than optimal Ti incorporation, as illustrated by the results from the dSi replete and low QY experiments respectively.

5.1.4 Deviations from the observed trends

Both 1 dSi-Rep-HQY and 1 dSi-Dep-HQY had a lower Ti content than what could be expected when compared to the other experiments with similar dSi concentrations and QY values (table 7). The low Ti content may be explained by the Ti concentration in the feed solution. It is possible that all of the solid Ti had not completely dissolved in the acid at the time of the first Ti incorporation experiment, as the solution was prepared only a day in advance. Ti in the form of nanoparticles would be unavailable for diatom uptake, but would still be measured with ICP-MS because the plasma dissolves particles up to 0.1 μm prior to analysis. Undissolved Ti was probably not a problem in the later experiments, as the feed solution was heated and stirred for over a week before the next experiment was conducted. However, this explanation may not be valid because the measured Ti concentration in the culture medium (fig 28 A) was of the same magnitude as in the other Ti incorporation experiments. Another possibility is that the exposure to higher doses of B in 1 dSi-Dep/Rep-HQY somehow affected the Ti uptake and/or incorporation. However, any correlation between B and Ti content in diatom frustules has not been reported in literature, so more research is necessary before this theory can be considered.

The low Ti content measured in the frustules from 5 dSi-Dep HQY A compared to the frustules from 5 dSi-Dep HQY B was most likely a result of an error in one of the syringe pumps that caused the delivery of Ti feed solution to stop for 2.5 hours during the delivery period. In addition to a smaller total amount of Ti delivered, the temporal increase in pH caused by the stop may also have affected the Ti uptake and/or incorporation.

Due to a measurement error in the preparation of Ti feed solution for 4 dSi-Dep-HQY, the Ti concentration in the final solution was not as high as intended. This

caused the molar Si:Ti ratio in the feed solutions applied for 4 dSi-Dep-HQY to be lower than in the first three Ti incorporation experiments (16:1 and 13:1, respectively). A lower Si:Ti ratio was therefore chosen for 5 dSi-Dep-HQY (10:1). This way, the cultures from the two last Ti incorporation experiments were both dSi deplete with high QY values, but the Si:Ti molar ratios in the feed solutions were different. Still, the Ti content values obtained in 4 dSi-Dep-HQY were reproduced in 5 dSi-Dep-HQY B, indicating that degree of dSi depletion and QY values had a larger impact on Ti uptake/incorporation than the molar ratio in the feed solutions.

5.1.5 Frustule structure, elemental composition and elemental distribution

Structural flaws were observed in frustules containing Ti, but similar observations were also made in Ti-free frustules. This made it difficult to determine whether or not Ti incorporation had a detrimental effect on the frustule structure. However, it was possible conclude that the frustule structure had not been dramatically altered, like the double comb structure observed upon addition of Ge in *Nitzschia frustulum* [48]. Unlike the Ge incorporation study, in which Ge was distributed evenly throughout the frustule, the relative amount of Ti in the frustules produced in the present study was considerably higher in the biosilica at the base of the large pores (pore silica) compared to the biosilica between the pores (fig 33 and table 11). This is in accordance with the observations reported by Jeffryes *et al.* from their Ti incorporation experiments [13] (section 2.1.4). However, the increased relative amount of P and Fe in the same regions was never reported in any of the previous incorporation studies. From the EDS analysis it appeared like the relative amounts of P and Fe followed the same pattern as Ti, and the analysis also indicated that the total amount of P and Fe present in the frustules was to some degree related to the Ti content. But it should be kept in mind that EDS is not the most accurate technique for detecting elements quantitatively. Also, only a small number of frustules were examined, and it remains to be determined whether the results are transferable to other frustules.

It has been suggested that Ti is incorporated in the biosilica frustule towards the end of the deposition process, either because the cell runs out of silicic acid or because the slow condensation rate of Ti does not allow for it to be incorporated any sooner [13]. Another possibility may be that the Ti is passively deposited as the cell transports compounds into the cell body through the pores. According to Hale *et al.*, the micromorphology of the diatom frustule directs the diffusion and advection of small molecules along the frustule surface [11]. A passive deposition process would explain why Fe and P are also found in higher concentrations in the same areas. However, it would not explain why physiological condition and degree of dSi starvation would have any effect on the amount of Ti incorporated, unless poor physiological condition reduces the general flow through the pores and dSi depletion reduces the production of organic compounds protecting the pore area from deposition. This passive deposition mechanism is unlikely because the Si-O bonds are strong [15] and loosely associated Ti would probably be washed away during the removal of the organic material. However,

the suggestion that Ti is found predominantly in the pore silica because of its slow condensation rate does not explain the increased relative amount of P and Fe in the same area.

Jeffryes *et al.* claimed that cleaning the frustules with H_2O_2 was not preferable because Ti incorporated in the biosilica layers close to the surface was etched away with this treatment [13]. Etching of the surface layers would not have had any effect on the Ti content relative to Si if Ti was uniformly distributed throughout the frustule biosilica, but etching of the pore silica would have decreased the relative Ti content. However, the volume of pore silica compared to the total frustule volume is probably not large enough to fully explain the reduced Ti content relative to Si observed after H_2O_2 treatment. A possible explanation may be that Ti is not deposited preferably just at the base of the large pores, but in an entire thin layer of silica covering most of the inner frustule surface. If this layer was etched it would explain the marked reduction in relative Ti content upon H_2O_2 treatment. Such a layer would also explain the larger relative amount of Ti in the pore silica compared to the bulk silica, as the biosilica is thicker in the areas between the large pores (evident from the DF STEM images where the pore silica layer is too thin to be visible, fig 33). The thick biosilica would dilute the relative Ti content in the areas between the large pores, making it look like the Ti content in these regions was lower than in the pore silica. This thin layer would explain the observed relative increase of P and Fe in the pore silica. Other elements than Si present in the cell body would not be actively transported to the site of deposition, but if they were to passively deposit they would also probably do so in a uniform layer close to the cell body. Crawford *et al.* reported that the inner silicic layer of the frustule had different material properties than the rest of the biosilica frustule, and speculated that this was due to a different chemical composition [15]. However, the layer described by Crawford *et al.* resisted alkaline etching, but for this proposed theory to hold, the layer should be possible to etch with H_2O_2 . If such a layer existed, the P and Fe distribution observed in the Ti-doped frustules should also be seen in Ti-free frustules.

5.2 Growth behaviour and Ti incorporation in *Coscinodiscus* cultures

The calculated dSi/cell values for *Coscinodiscus* based on the results from the preliminary growth study were approximately 1000 times higher than the dSi/cell values for *Pinnularia*. A rough estimate assuming *Pinnularia* is shaped like a rectangular box with dimensions length = 2 0 µm, width = 10 µm, height = 5 µm returns the volume of the frustule biosilica (without the inner cell body volume):

$$(2 \times 20 \times 10 \times 0.1)\,\mu m^3 + ((2 \times 20) + (2 \times 10)) \times 5 \times 0.1)\,\mu m^3 = 70\,\mu m^3$$

Correspondingly, the volume of the *Coscinodiscus* frustule biosilica can be estimated by assuming that *Coscinodiscus* is shaped like a petri dish with radius = 100 µm, height = 40 µm and frustule thickness = 0.5 µm

$$(2 \times \pi \times 100^2 \times 0.5)\,\mu m^3 + ((2\pi \times 100 \times 40 \times 0.5))\,\mu m^3 = 43960\,\mu m^3$$

Based on this this rough estimate, and considering that *Pinnularia* is more rounded off than a rectangular box, the dSi/cell values calculated for *Coscinodiscus* does not appear to be completely unreasonable.

As this was a proof of concept study and sampling was reduced to a minimum, the QY values were not interpreted in the same manner as in the *Pinnularia* experiments. The culture density was so low that QY measurements probably just provided information about the photo system condition in a few single cells. However, the stable high QY values measured in the *Coscinodiscus* experiments indicate that the fluorescence response to alterations in dSi concentration and addition of Ti is slower or less pronounced in *Coscinodiscus* than in *Pinnularia*. The explanation may be this particular diatom‘s high tolerance for heavy metals and alterations in salinity [20, 19].

A high Ti content in the frustule biosilica was not achieved in any of the three Ti incorporation experiments. The highest value was detected in the frustules from diatoms to which Ti had been added for 5 days (2 (5d)). These cultures also had the lowest dSi/cell values, the highest initial dSi concentration and the highest % of cells divided (table 6). On average, every cell in these cultures had divided more than once during the experimental period. It was expected that the diatoms that had been added Ti for seven days (3 (7d)) would produce the frustules with the highest Ti content, but a very slow growth was observed in these cultures and only ∼50 % of the cells had divided at the end of the experimental period. This means that the frustules containing Ti from 3 (7d) were diluted with more “old” frustules in the ICP-MS analysis than the frustules from 2 (5d). The Ti content in the few Ti-doped frustules from 3 (7d) must have been considerably higher than the measured average, and it was probably also higher than the Ti content of the frustules from 2 (3d). This would be in accordance with Martin *et al.,* who suggested that slow growth provides more time for incorporation of foreign elements [43]. The slow growth rate in 3 (7d) may be assigned to a combination of very low initial dSi concentration and small daily doses of feed solution added. If the inner

silicic acid storage pools were completely depleted at the beginning of the experiment, more dSi would have to be taken up by the cells before new frustules could be deposited. This fits well with the observation that Si/cell values were higher in 3 (7d) than in 2 (5d). The diatoms in 2 (5d) could apply some of the stored intracellular Si for frustule deposition, and therefore appeared to use less dSi per cell division.

The frustules from the 1 (3d) had a very low Ti content. This was expected, as the experimental period was shorter than the estimated time for cell division. The cultures from 1 (3d) had not been dSi depleted for as long as 3 (7d) prior to experimental initiation. Cell division was therefore probably already in progress, using mostly stored intercellular Si for frustule deposition. The new cells were therefore most likely a product of dSi consumed at an earlier stage, not the dSi and Ti taken up during the experimental period. From the high dSi/cell values it appears that the diatoms were able to take up parts of the relatively large dSi/Ti feed solution doses that were delivered, but they did not have time to divide after the uptake.

The proof of concept study on *Coscinodiscus* was conducted to get an indication of whether or not metabolic Ti incorporation can be achieved in *Coscinodiscus* frustules. For more quantitative studies, experiments with larger culture volumes, more replicates and longer time scales should be conducted. Alternatively, because of the large size of *Coscinodiscus*, it might be possible to perform a study on a single cell. However, the slow growth rate and low maximal Ti content does not make *Coscinodiscus* a promising candidate for Ti incorporation applications.

B content in *Pinnularia* and *Coscinodiscus* frustules *Coscinodiscus* is known to have a high tolerance to heave metals [20], but B appears to be easily incorporated. The higher average B content in the *Coscinodiscus* frustules as compared to *Pinnularia* frustules is in accordance with Martin *et al.,* who proposed that slower growth rate would allow for more foreign elements into the frustule biosilica [43].

The diatoms from the first *Pinnularia* experiment (1 dSi-Dep/Rep-HQY) were probably exposed to a larger dose of B than the other cultures because the stem culture for this experiment was contained in a borosilicate vessel. Because the inoculate volume was very small, the culture was contained in the glass vessel for a relatively long time period (15 days). The remaining *Pinnularia* stem cultures were contained in plastic flasks after the high B content in the frustules from the first experiment was observed. This was probably the reason for the high B content measured in 1 dSi-Dep/Rep-HQY compared to the other cultures. Likewise, the low B content in 5 dSi-Dep-HQY may be due to the short time period these diatoms spent in the borosilicate vessels (fig 19E, F). However, the fact that the *Pinnularia* diatoms cultured in a plastic flask contained more B than the diatoms in 5 dSi-Dep-HQY indicates that there may be other factors affecting B uptake that have to be considered as well.

The B content in *Pinnularia* was on average $\sim$0.1 wt% or $\sim$0.28 atomic % relative to Si, while the average in *Coscinodiscus* was nearly twice as high (tables 7,8). Thus, the B concentration in the diatoms frustules was on the order of 10x the concentration of highly doped semiconductors [56]. In principle, this makes diatom frustules unusable

for B-doped semiconductor purposes. However, it has been reported that diatoms can contain aluminium (Al) [43] and Al was also detected in the ICP-MS analysis in the present study (results are not shown because the obtained values were close to the detection limit of the instrument). Doping with Al would counteract the effect of B, thereby allowing for higher concentrations of both. A lower B content in the frustules could also possibly be achieved if the diatoms were cultivated in a completely artificial medium. Seawater contains B [70], but if the medium was not based on natural seawater and the culture container was not made of borosilicate, the amount of B delivered to the diatoms could be accurately controlled, and it is possible that the B content in the frustules could be tailored to the desired amount.

6 Conclusions

Titanium (Ti) was successfully incorporated into the biosilica frustules of the pennate diatom *Pinnularia* sp. and the centric diatom *Coscinodiscus wailesii* through the metabolic pathway.

Quantum Yield (QY) measurements were conducted on the experimental *Pinnularia* cultures to obtain information about the photo system condition, and high QY values were interpreted as an indication of a good overall physiological state of the cell. It was found that silicate (dSi) deplete high QY cultures produced the highest Ti content in the *Pinnularia* frustules, while both a high initial dSi concentration and low QY values in the diatom culture resulted in reduced amounts of Ti incorporated. It was observed that long term dSi starvation caused QY values to decline. A time window of $\sim$24 hours was identified, after dSi depletion has occurred but before QY values drop significantly, in which Ti/dSi delivery should be initiated in order to achieve optimal Ti incorporation in *Pinnularia* diatoms.

Cell aggregation was observed in the experimental *Pinnularia* cultures upon Ti/dSi delivery. There is reason to believe that the aggregation occurred as a cellular response to Ti. The explanation for this reaction to Ti is not known but it was proposed that secretion of organic compounds combined with turbulence in the culture vessel may have caused the cells to aggregate. It is not possible to say whether the cell aggregation had any consequences for the uptake and incorporation of Ti, but if it was indeed a defence mechanism then it is likely that Ti uptake was reduced to some degree by the cell aggregation.

A faint irregular pattern in the *Pinnularia* frustule biosilica was observed on the nanometer scale in Ti-doped frustules. Further on, a pattern in the relative distribution of Ti was observed. The amount of Ti relative to silicon (Si) was higher in the biosilica located at the base of the largest pores compared to the biosilica located between the pores. Surprisingly, this appeared to apply to iron (Fe) and phosphorous (P) as well. A correlation between Ti and Fe content in diatom frustules has been reported in literature, but similar distribution patterns have previously not been discovered.

The proof of concept study performed on *Coscinodiscus* revealed that smaller amounts of Ti relative to Si are incorporated into the biosilica of *Coscinodiscus* frustules as compared to *Pinnularia* frustules. This was as expected from the characteristics of *Coscinodiscus* reported in literature. A high tolerance to metals combined with slow growth rate makes *Coscinodiscus* an unlikely candidate for further Ti incorporation studies.

Structural flaws were observed upon inspection with TEM of frustules from both diatom species, with and without Ti incorporated. It was therefore not possible to rule out that Ti exposure had a detrimental effect on the frustule structure.

From measurements conducted with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) it was discovered that both *Pinnularia* and *Coscinodiscus* frustules contained boron (B) on the order of 0.1 wt% relative to Si. This implies that diatom frustules can not be applied for B-doped semiconductor purposes without active manipulation of the B content.

The connection between physiological parameters in diatoms and the structural and elemental composition of the biosilica frustules has not been a priority in earlier diatom research, neither from the biological nor from the material science point of view. However, the results from the present study indicate that knowledge about the biological processes in diatoms may be beneficial for optimal tailoring of diatom frustule material properties.

7 Future work

Future work should involve reproducing each of the *Pinnularia* Ti incorporation experiments with more replicates to obtain sufficient data to perform statistical analyses and correlation calculations.

Multiple continuous dSi starvation and Ti/dSi delivery cycles could be tested to explore if it is possible to incorporate Ti into a larger fraction of the culture population, as well as to increase the maximum amount of Ti incorporated into one frustule.

An alternative method for enumerating *Pinnularia* cells should be sought. A larger number of frustules could be scanned with EDS to determine the percentage of Ti-doped frustules in a sample, thereby enabling calculation of percentage of diatoms divided. This value van subsequently be used to calculate the average Ti content in the frustules that actually contain Ti from the ICP-MS results.

The reason for aggregation in the first place should also be investigated, and, if possible, prevented. A possible connection between aggregate size/ percentage of cells found in aggregates and amount of Ti added should be explored. A new Ti stock solution should be applied for Ti feed solution preparation to rule out the possibility of stock solution contamination.

Frustules from *Pinnularia* diatoms that have not been exposed to Ti should be scanned with EDS to determine whether the distribution pattern of P and Fe observed in the present study is found in Ti-free frustules as well.

It would be interesting to explore the amount of foreign elements associated with the organic cell body. This can be done by comparing samples of rinsed and natural diatoms from equal volumes of the same diatom culture. If the initial volume is large enough, the loss of frustules throughout the cleansing process will not have a large effect on the final result when the ICP-MS values are compared.

Further experiments on *Coscinodiscus* should be performed on larger culture volumes and the experiments should run over longer time periods. Sampling should be performed more frequently and sample volumes should be increased due to the low culture density of this diatom species. If possible, uptake experiments could be performed on single *Coscinodiscus* cells to obtain more accurate measurements and avoid cultures with considerable amounts of empty shells complicating the experiments.

It should be attempted to tailor the amount of B from the frustule biosilica by cultivating the diatoms in plastic containers using a 100% artificial medium. This way, the amount of B added to the cultures can be accurately controlled, and it can be explored how low B concentrations the diatoms can endure before cell processes are inhibited.

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