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INSTITUTIONEN FÖR BIOLOGI OCH MILJÖVETENSKAP

Temperature Optimum for Biomass Production of two Species of Benthic Diatoms

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Abstract

Diatoms and other algae can be cultivated and beneficially used in several sectors with applications ranging from nutrient recycling to production of high-valued biomaterials used by the green energy industries, in pharmaceutical products or as food supplements. In this experimental study, the temperature optimum for biomass production was tested for two species of benthic diatoms, *Diatoma* sp. and *Nitzschia* sp., by measuring and relatively comparing parameters for biomass production and productivity between cultures grown at three different temperatures (13°C, 19°C, and 27°C). The parameters used as estimation for biomass and productivity to enable the comparison between the different treatments were cell density, concentrations of photosynthetic pigments, nutrient reduction, and growth rate. Photosynthetic activity was also measured to compare the influence of the different temperatures on the cell cultures and to enable for continuous supervision during the 16 days of the experiment.

The *Nitzschia* sp. yielded insufficient result, however, the *Diatoma* sp. was clearly found to grow in all temperatures tested, but with the results suggesting its temperature optimum range to include at least 13°C to 19°C, and its upper limit to be between 19°C and 27°C. Interestingly though, the results also indicate that the temperature optimum may vary depending on the intended use. Cultivating *Diatoma* sp. for its biomaterials, such as silica frustules or high-valued substances like for instance fucoxanthin, would not be recommended in temperatures above 19°C based on these results, although, if cultivated primarily for nutrient recycling, temperatures of up to at least 27°C seem to work just as well as temperatures of 13°C to 19°C.

Populärvetenskaplig sammanfattning

Kiselalger, liksom många andra typer av alger, kan odlas effektivt och har stor potential inom flera olika områden. De kan hjälpa oss att rena och återvinna näringsämnen från vatten men kan också användas till att producera värdefulla biomaterial och substanser till den gröna energiindustrin, läkemedel och till och med kosttillskott. I denna studie undersöktes hur tre olika temperaturer (13 °C, 19 °C och 27 °C) påverkade tillväxten och produktionen av biomassa hos två arter av bottenlevande kiselalger, *Diatoma* sp. och *Nitzschia* sp, med syfte att undersöka de olika arternas temperaturoptimum. De variabler som användes för att jämföra de olika temperaturernas inverkan var: celldensitet, koncentrationer av olika fotosyntetiskt aktiva pigment, näringsupptag och tillväxthastighet. Även fotosyntetisk aktivitet mättes och jämfördes mellan de olika temperaturerna samt för att möjliggöra kontinuerlig översyn av experimentet som varade i drygt två veckor.

Nitzschia sp. gav bristfälliga och icke tillförlitliga resultat medan *Diatoma* sp. visade sig växa väl i alla tre temperaturer men med ett tydligt optimum vid 13 °C och 19 °C. Resultatet tyder på att temperaturoptimumet för *Diatoma* sp. tycks ligga mellan 13 °C och 19 °C, med en övre gräns mellan 19 °C och 27 °C. Intressant nog skiljde sig resultatet för näringsupptag mot resterande parametrar genom att förbli relativt konstant i alla tre temperaturerna. Detta betyder därmed att temperaturoptimumet skiljer sig åt beroende på vilket det tänkta användnings-område för kiselalgen är. Om huvudsyftet är att framställa stora volymer av biomaterial, exempelvis kiselskal eller värdefulla substanser som fucoxantin, bör odling undvikas i temperaturer över 19 °C. Men om det primära målet är näringsåtervinning eller rening av vatten tycks temperaturer upp till minst 27 °C fungera lika bra som temperaturer mellan 13 °C och 19 °C.

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1 Introduction

According to The Food and Agriculture Organization of the United Nations (FAO), per capita consumption of aquatic animals has increased 1.4 % annually between 1961 and 2019 to a total average consumption of 20.5 kg per person per year, and the aquaculture sector has started becoming increasingly recognized for its role in global food production. The annual production volume coming from aquacultures increased with 609 % between 1990 and 2020 and now accounts for 88 million tonnes, corresponding to 49 % of the total aquatic animal production from fisheries and aquacultures combined (FAO, 2022b). Increasing the consumption of food from the aquatic environment, both harvested and cultivated, is seen as one of the important strategies in reducing world hunger and malnutrition, especially as the world is becoming affected by climate change. FAO therefore targets a further growth of at least 35 % in global sustainable aquaculture production until 2030 in their Blue Transformation roadmap for 2022-2030 (FAO, 2022a).

Aquaculture systems can be classified into three different main categories; flow-through systems (cages, pens, and raceways), semi-flow-through systems (ponds), and recirculating systems (RAS). They all differ in the amount of water flowing through the system and thereby also the amount of feed needed (due to generally more feed flowing out of the system with the through-flowing water than if the water is recirculated), and amount of wastewater produced. The recirculating systems are more energy demanding due to the heavy reliability of pumps to circulate the water (Ramli *et al*., 2020). However, they have the advantage of both reducing the water needed as well as minimizing nutrient pollution to the local environment, generally making for more sustainable aquaculture (Naylor *et al*., 2021; Ramli *et al*., 2020). In RAS, the water is usually treated both mechanically and biologically. The mechanical treatment removes larger particles whilst the biological treatment transforms nitrous waste products into inorganic nitrous gas using nitrifying and denitrifying bacteria, or makes ammonia assimilate in algae or bacteria (Ramli *et al*., 2020). Phosphorous assimilate in algae or macrophyte biomass and can thereby be removed (Adey *et al*., 1993). Controlled algae production can be effective in wastewater treatment, with nutrient removal rates calculated to be 100-250 times the uptakes by large wet-land areas used for natural wastewater treatment (Adey *et al*., 1993). In nature, diatomic algae are highly responsible for the cycling of both nutrients and carbon between the upper and bottom layers of our oceans (Raven & Falowski, 1999). The diatoms sequester carbon dioxide through photosynthesis and as the senescencing algae form aggregates, they sink which pulls the carbon and nutrients down into the ocean depths (Ragueneau *et al*., 2006; Raven & Falkowski, 1999). Implementing diatom cultivation in aquaculture wastewater likewise enhances nutrient recycling and thereby promotes better circularity in the bioeconomy (Bhattacharjya *et al*., 2021).

The algae biomass produced can be valuable itself as algae materials are highly sought after in several industries such as in the pharmaceutic and the energy industries as well as for supplements in food production (Marella *et al*., 2020; Ramli *et al*., 2020), with the economic value of the high-valued substances produced by the algae ranging from $310 to $10,000 per kilogram (Brennan & Owende, 2010). Caratenoids (primarily fucoxanthin) and lipids have pharmaceutical applications (Sahin *et al*., 2019), and due to the high content of polyunsaturated fatty acids, which can make up around 25 % of the total biomass, diatoms could be a potential source for biodiesel production (Ramachandra, 2009). Their silica-rich skeletons, so-called frustules, can be used in nanobiotechnology for implementation in solar cell panels and batteries and thereby also be important for the transition to renewable energy (Jeffryes *et al*., 2011).

From a previous experimental study comparing several microalgae species for freshwater RAS implementation in effluent water from a Swedish fish farm cultivating tropical fish, the benthic diatom *Nitzschia pusilla* was found to be a promising candidate (Karlsson, 2022). *Nitzschia pusilla* is a cosmopolitan species (Prelle *et al*., 2019) predominantly found in freshwater (Grunow, 1862, s. 71). A previous study on photosynthesis and respiration of Baltic Sea benthic diatoms by Prelle *et al*., (2019) indicated that *Nitzschia pusilla* seems to have a photosynthetic optimum at 30°C (measured through oxygen production). The *Nitzschia* genus is furthermore known to accumulate economically interesting lipids when subjected to stress (Cointet *et al*., 2019), but also to produce high volumes of lipids all together when grown in nitrogen excess combined with elevated iron concentrations (Sahin *et al*., 2019). However, apart from these studies (Karlsson, 2022; Cointet *et al.*, 2019; Sahin *et al*., 2019), there are to my knowledge limited studies conducted on the effectiveness of biomass production for this species, particularly in relation to aquaculture (e.g., Xing *et al*., 2018). Another genus that potentially will be cultivated in effluent water from aquaculture is the benthic diatoms of the genus *Diatoma.* There are eight known species of *Diatoma* in Sweden of which six are marine (SLU Artdatabanken, 2023), including the west coast native *Diatoma* sp. (Prof. A.Y. Al-Handal, 2019) that potentially will be commercially cultivated for silica-rich biomaterials originating from its frustules (www.swedishalgaefactory.com). Previous research by Hansson (2023) has examined the influence of temperature on the biomass production of this benthic diatom, suggesting optimal temperature to include 11-20°C with the upper limit somewhere between 20°C and 30°C. However, contamination by green algae resulted in somewhat inconclusive findings. This study aims to further investigate the influence of temperature on the effectiveness in biomass production of *Nitzschia* sp. and *Diatoma* sp., with the results possibly being of significance for both the aquaculture industry and the algae biomaterial industry.

2 Aim and hypothesis

Two species of diatoms, *Nitzschia* sp. and *Diatoma* sp., were tested for biomass in three different temperature treatments (13°C, 19°C, and 27°C), with the aim to establish which temperatures that are optimal for each species. Both species were tested for two weeks with three true replicates per treatment. *Nitzschia* sp. was hypothesized to produce most biomass in the higher temperature, at 27°C, whilst *Diatoma* sp. was hypothesized to produce the most at the lower temperatures of either 13°C or 19°C.

3 Methods

3.1 Growth Medium

Since one of the diatoms used in this study was adapted to freshwater (*Nitzschia* sp.) whilst the other one was marine (*Diatoma* sp.), two different growth mediums had to be used. For the marine *Diatoma* sp., artificial nutrient-rich seawater was used as growth medium. This was obtained from the Swedish Algae Factory AB in Kungshamn (SAFAB, n.d.). Swedish Algae Factory is a startup that produces algae-derived biomaterial from diatom frustules and shortly will be using RAS effluent water from a salmon farm nearby (SmögenLax) as growth medium for their algae while at the same time helping in cleaning the effluent water from the fish farm.

The growth medium used for the limnic *Nitzschia* sp. was effluent water obtained from the Swedish fish farm Gårdsfisk (Gårdsfisk (Scandinavian Aquasystems AB), 2021) in 2022.

Gårdsfisk cultivates tropical (27–28 °C) freshwater fish (*Oreochromis niloticus* and *Clarias gariepinus*) for human food consumption in a land-based recirculated system. At the time when the effluent water was obtained, their system had a total volume of 180 m^3 and a fish density of 150 kg m^3 (Samuel, 2022). All effluent water used in the study was filtered to remove particles (Sarstedt Filtropur S 0.2 mm).

3.2 Algae Cultivation and Experimental Design

Prior to the start of the experiment, diatom cultures of *Nitzschia* sp. and *Diatoma* sp. were cultivated in 50 mL cell culture flasks, with artificial seawater from SAFAB as growth medium for the *Diatoma* sp., and freshwater L1 growth medium (Hallegraeff *et al.*, 2004) mixed with filtered water from Gårdsfisk (approximately 10:1) for the *Nitzschia* sp. The algae were cultivated in 13°C with a 16:8 light-darkness ratio. Once the tests were going to start, the algae concentrations were determined using a gridded Rafter Sedgewick counting chamber and an inverted light microscope (Nikon Eclipse Ts2). To ensure even cell densities, the flasks were sonicated in an ultrasound bath for three minutes each to loosen adhered cells.

Both species of diatoms were diluted with medium to lower their concentrations in two new flasks. This was done to enable a larger volume to be transferred to the treatment flasks and thereby lower the margin of error. From these new flasks, 10 mL of diluted diatoms were transferred to each of the 120 mL treatment flasks after 115 mL of either artificial seawater from SAFAB or effluent water from Gårdsfisk mixed with L1 medium (2:1) had been added. A total of 21 treatment flasks were initially used for each species, resulting in three true replicates for initial sampling, mid-time sampling and final sampling in each temperature treatment. Later on, three extra flasks were added, one for final sampling in each treatment, with *Diatoma* sp. to which no extra nutrients were added during the experiment.

The three different temperature treatments (13°C, 19°C, and 27°C) were set up using thermostat regulated heaters (Sous-Vide, Menuett)) in three, water filled, 15 L transparent plastic boxes placed upon Styrofoam boards to prevent heat loss and maintain more even temperatures. Plastic test tube racks, wrapped in aluminum foil to reflect light, were placed on the bottom of the boxes to elevate the treatment flasks, and thereby enable for a larger volume of water to be used in each treatment box without completely submersing the flasks. A temperature logger (HOBO) was put in each treatment box to log the water temperature hourly during the entire experiment time (13°C, 19°C, and 27°C ± 0.9°C). The light-darkness ratio used was 16:8 and daylight T8 light bulbs were used to generate an average light intensity of 113 ± 7 µmol photons m^{-2} s^{-1}, differing less than 10 percent between each treatment box (measured approximately 1 cm above the water surface using a LI-COR LI-1400 handheld probe at 45° angle). To lower the light intensity at the start of the experiment, grey nets were placed above the flasks for the first days, reducing the average light intensity to 70 ± 5 µmol photons m^{-2} s^{-1}. It was later decided that the shading would be kept in place during the entire experiment.

To minimize the potential contamination risk arising from capsizing, the flask lids remained tightly sealed throughout the experiment except from during analysis (every second to third day) or when nutrients were added to avoid nutrient depletion to skew the results. Addition of nutrients was decided only necessary for *Diatoma* sp. (determined through visual observation and the progression of the photosynthetic activity (the $F_{v'}/F_{m'}$-values)). Bioavailable silica was added before the mid-time sampling (final concentration approx. 5 times the concentration of F/2 medium (Guillard, 1975)) and nitrate and phosphate besides other F/2 nutrients were added

at two times after the mid-time sampling (final concentration approx. 5 and 10 times the concentration of F/2 medium (Guillard, 1975)).

3.3 Analysis

The samples were analyzed for cell density, growth rate, photosynthetic activity, and nutrient use up, all serving as relative parameters for biomass production or productivity to then be compared between the three different temperatures.

3.3.1 Cell Density and Growth Rate

Cell density was measured for initial and final samples using a gridded Rafter Sedgewick counting chamber under an inverted light microscope. To ensure adequate cell density, a minimum of 300 cells were counted across at least 10 squares. If the cell density was too low to reach 300 cells in 10 squares, additional squares were counted until 300 cells or 100 squares had been counted, whichever came first. The observed concentration of cells was then used to calculate the daily specific growth rate using equation [1].

$$[1] \qquad \mu = \frac{\ln\left(\frac{N_1}{N_0}\right)}{t_1 - t_0}$$

In mentioned equation [1], growth rate (μ) is given by dividing the cell density of the final sampling (N_1) with the initial cell density (N_0) and then dividing by the number of days between the day of the measurement (t_1) and the initial day of the experiment (t_0).

3.3.2 Photosynthetic Pigment Concentrations

The photosynthetic pigments chlorophyll-a, fucoxanthin, diadinoxanthin, and β-carotene were all measured for initial, mid-time and final samples using HPLC-analysis. The cells were taken from the pellet of centrifuged samples (50 mL except for the initial *Diatoma* sp. samples where 100 mL was used to increase the probability of measurements being above the detection limit) at 4000 RPM for 10 minutes and then extracted in 2 mL of acetone/methanol (80:20) while being sonicated to break up the cells. The HPLC-analysis was performed according to the methods by Wright & Jeffrey (1997) and the pigments were identified by their retention time and absorbance spectra, primarily guided by (Jeffrey *et al*., 1997). The concentration was then computed from the peak integral of the pigment, with a minimum level for measuring set to 5000 a.u., correlating to concentrations of approximately 0.3 $\mu g L^{-1}$. Pigments of interest were expressed as both the total concentrations ($\mu g\ L^{-1}$) and the cellular concentrations ($pg\ cell^{-1}$), where possible.

3.3.3 Effective Photochemical Yield ($F_{v'}/F_{m'}$)

The effective photochemical yield ($F_{v'}/F_{m'}$) was measured as a momentarily indicator for photosynthetic activity of photosystem II. This was measured every second to third day for all samples by subjecting them to a saturating light pulse (4000 μmol photons $m^{-2}s^{-1}$) to briefly suppress the photochemical yield to zero and thereafter induce maximum fluorescence yield. The analysis was conducted using a water-PAM (Pulse Amplitude Modulation) chlorophyll fluorometer (WATER-ED Emitter-Detector Unit, Walz GmbH, Effeltrich, Germany) connected to a computer with WinControl software (Walz GmbH). The effective photo-chemical yield was then given by equation [3].

[3] $$F_{v'}/F_{m'} = (F_{m'} - F_{0'})/F_{m'}$$

In the equation, $F_{m'}$ corresponds to maximum fluorescent yield and $F_{0'}$ to the fluorescent yield prior to the light pulse.

3.3.4 Nutrient Reduction

50 mL of sample from each treatment were filtered using a 0.2 µm syringe filter and sent to external labs (Kristineberg Center for Marine Research and Innovation & LMI AB) for water nutrient analysis. All initial, mid-time, and final samples were filtered and stored in refrigerator (mid-time samples were frozen) until delivered to the external labs in 50 mL plastic centrifuge tubes. The received nutrient analysis results were then examined for percentual nutrient reduction as shown in equation [4], and Redfield's nitrogen:phosphorous (N:P) uptake ratios (Falkowski *et al.*, 2004).

[4] $$\text{Percentual reduction} = 1 - \frac{C_1}{C_0} \times 100\ \%$$

In the equation, C_1 is the concentration of a specific nutrient at final or mid-time sampling whereas C_0 is the concentration of the same nutrient at initial or mid-time sampling. Nutrients added to the samples to prevent nutrient depletion were taken into account when performing equation [4] by adding the amount of added nutrients to a hypothetical start value to be able to get the total percentual reduction of each nutrient.

3.4 Statistical Analysis

Obtained data for cell density, growth rate, concentration of photosynthetic pigments (per liter and per cell), effective photosynthetic yield ($F_{v'}/F_{m'}$), nutrient reduction, and Redfield's N:P uptake ratios were expressed as means ± standard deviations for each temperature treatment. The results (except from effective photosynthetic yield ($F_{v'}/F_{m'}$)) were then tested for homogeneity of variance and compared to appropriate value from Cochran's table. Variables with homogeneity of variance above the accepted level (0.87) were also tested as log-transformed (ln(x+1)) and as square root-transformed (sq(x)), resulting in only the final fucoxanthin concentration (0.94), final cellular fucoxanthin concentration (0.98), final cellular diadinoxanthin concentration (0.94), mid-time percentual ammonia reduction (0.88), final percentual nitrate reduction (0.93), final percentual phosphate reduction (0.99), and final N:P ratio (0.89) being above the acceptance level. However, since the statistical tests to be conducted were one-way ANOVA:s, known for being robust (Underwood, 1997) and all deviations from the acceptance level of homogeneity of variance were small (<0.12), it was decided tolerable to run the ANOVA:s for all parameters. The one-way ANOVA:s were combined with Tukey's post hoc significance test with a chosen critical p-value < 0.05. The statistical analyses and the graphs were made in the software Microsoft Excel (ver. 16.72) and RStudio (ver. 2022.07.1).

4 Results

4.1 *Diatoma* sp.

4.1.1 Cell Density and Growth Rate

The cell density of *Diatoma* sp. was significantly different between treatment groups. It was significantly different between 13°C and 27°C, and 19°C and 27°C, however the difference was not significant between the 13°C and 19°C treatment groups (Figure 1). The growth rate was also found to be significantly different between treatments, with significant difference between 13°C and 27°C as well as between 19°C and 27°C (Figure 2). A higher proportion of smaller cells was found in the final samples in the 13°C and 19°C treatments compared to the 27°C treatment, as can be noted in the example photos of the different temperature treatments from the final cell counting (Figure 3-5).

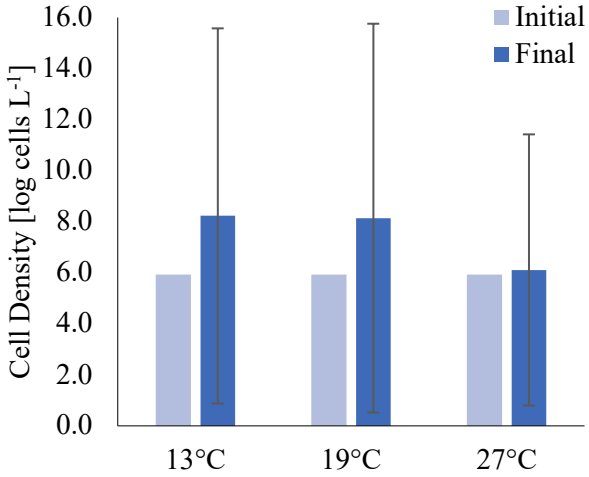

Figure 1. Cell density expressed as means ± standard deviations for all initial and final samples in the three different temperature treatments for *Diatoma* sp. Note that the y-axis has been log-transformed.

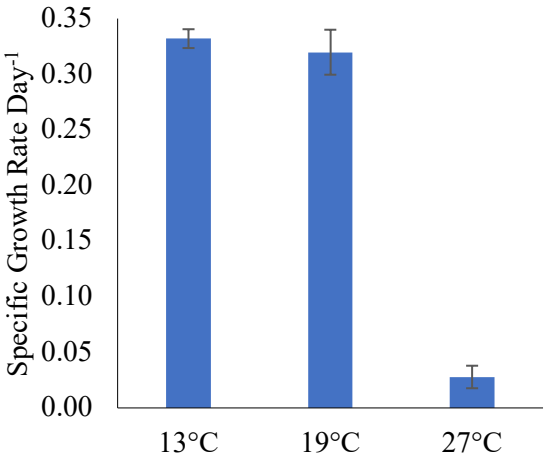

Figure 2. Average growth rate for the total experiment period expressed as means ± standard deviations for *Diatoma* sp. in all temperature treatments.

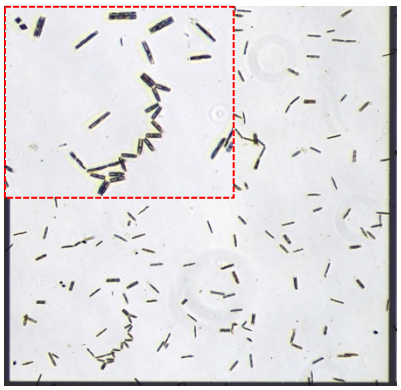

Figure 3. Photograph of the final cell density in 13°C for *Diatoma* sp. with enlargement in the upper left corner. The photo is approximately equal to 1 µL.

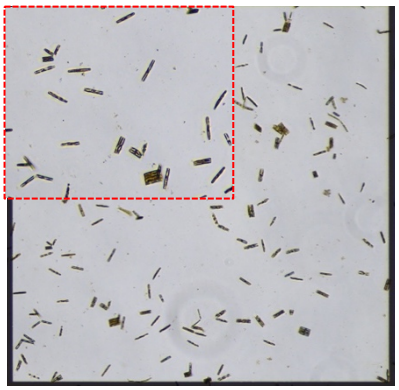

Figure 4. Photograph of the final cell density in 19°C for *Diatoma* sp. with enlargement in the upper left corner. The photo is approximately equal to 1 µL.

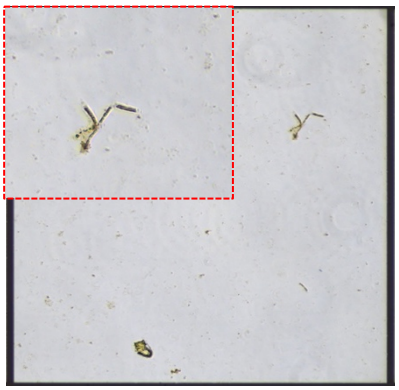

Figure 5. Photograph of the final cell density in 27°C for *Diatoma* sp. with enlargement in the upper left corner. The photo is approximately equal to 1 µL.

4.1.2 Photosynthetic Pigments

A chromatogram obtained from the HPLC is shown as an example in Figure 6, with the four analyzed photosynthetic pigments present (fucoxanthin at 12.183 min, diadinoxanthin at 14.604 min, chlorophyll-a at 21.810 min, and β-carotene at 26.186 min).

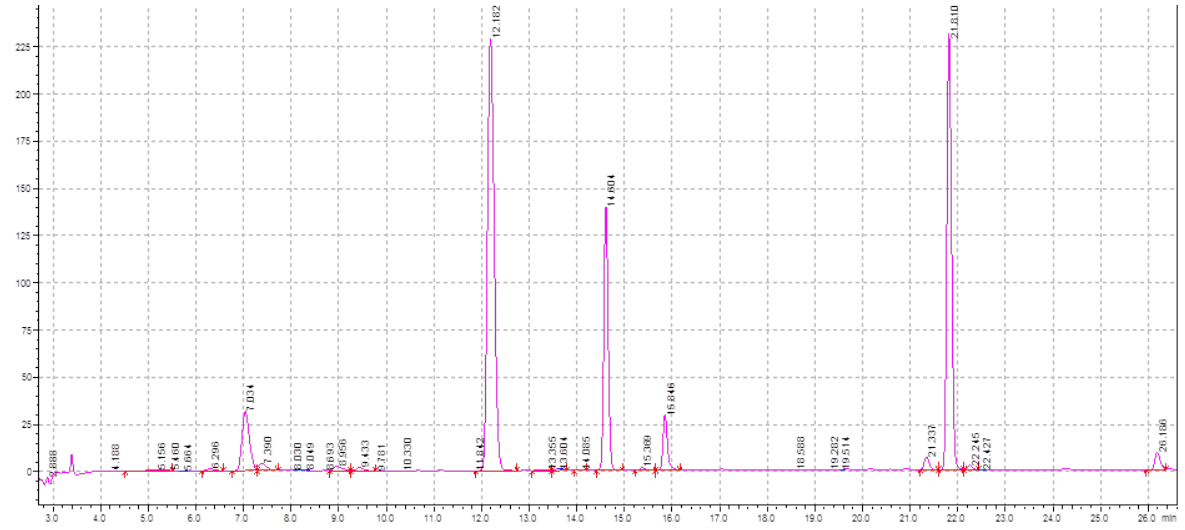

Figure 6. Example of a chromatogram (*Diatoma* sp. in 19°C) attained from the HPLC analysis.

The photosynthetic pigment concentrations were significantly different between treatments. For chlorophyll-a, the difference between treatment groups was only significant between 19°C and 27°C (both mid-time and final), and close to significant for the mid-time concentration difference between 13°C and 27°C ($p = 0.07$) (Figure 7). The concentrations of fucoxanthin were significantly different between treatment groups at mid-time and final sampling and differed significantly between all treatment groups except for between 13°C and 19°C at the final sampling (Figure 8). Diadinoxanthin did also differ significantly between groups, with significance between 13°C and 27°C as well as 19°C and 27°C for both mid-time and final samples, close to significant difference between 13°C and 19°C at the mid-time sampling ($p = 0.05$), but non-significant difference between the same treatments at final sampling (Figure 9). The concentrations of β-carotene were significantly different between treatments at mid-time and final sampling, but significance was only found between the 19°C and 27°C treatment groups (Figure 10).

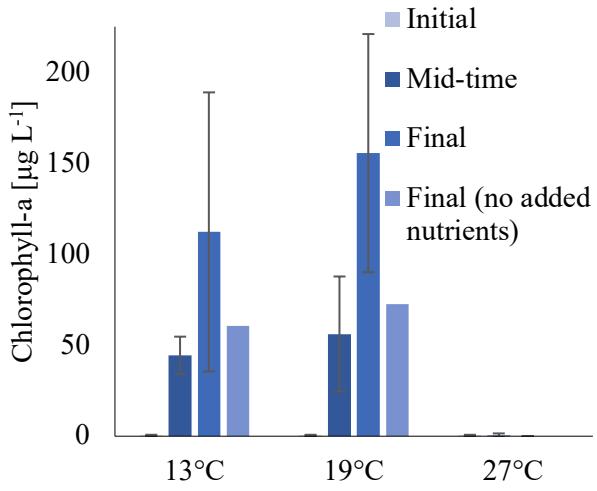

Figure 7. Concentration of chlorophyll-a per liter, expressed as means ± standard deviations for each treatment and sampling time for *Diatoma* sp. Note, n=1 in "Final (no added nutrients)".

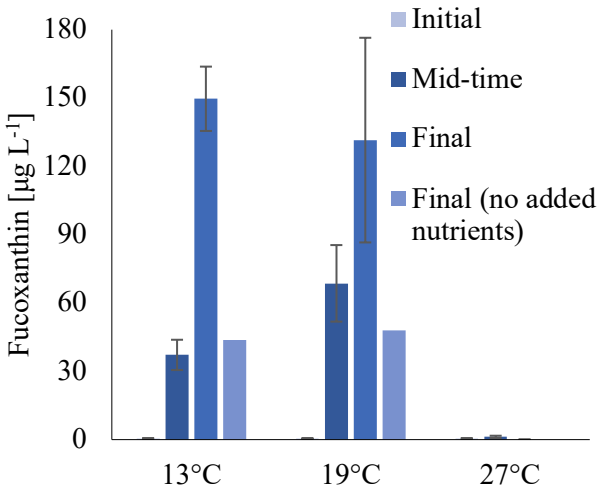

Figure 8. Concentration of fucoxanthin per liter, expressed as means ± standard deviations for each treatment and sampling time for *Diatoma* sp. Note, n=1 in "Final (no added nutrients)".

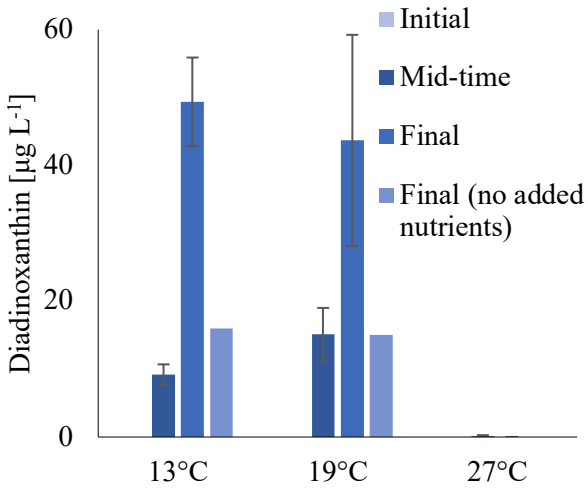

Figure 9. Concentration of diadinoxanthin per liter, expressed as means ± standard deviations for each treatment and sampling time for *Diatoma* sp. Note, n=1 in "Final (no added nutrients)".

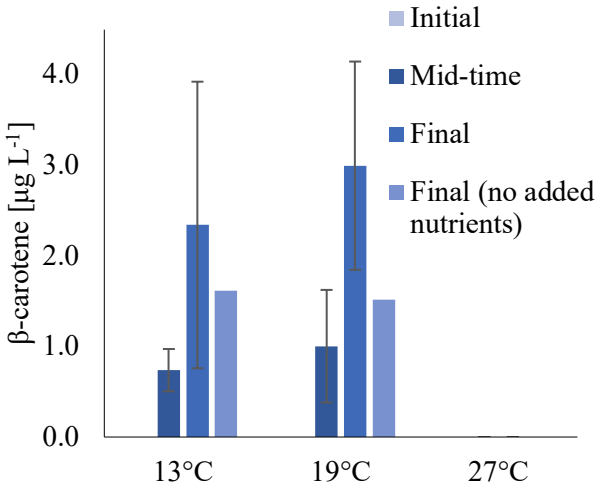

Figure 10. Concentration of β-carotene per liter, expressed as means ± standard deviations for each treatment and sampling time for *Diatoma* sp. Note, n=1 in "Final (no added nutrients)".

The cellular concentration of chlorophyll-a was close to significantly different due to treatment ($p = 0.06$) with the difference between 19°C and 27°C closest to being significant ($p = 0.05$) (Figure 11). The cellular concentrations of fucoxanthin, diadinoxanthin, and β-carotene, however, were significantly different between treatments, but significant different concentrations were only found between the 19°C and 27°C treatments, while being close to significant between 13°C and 27°C for fucoxanthin ($p = 0.06$) (Figure 11) and diadinoxanthin ($p = 0.05$) (Figure 12).

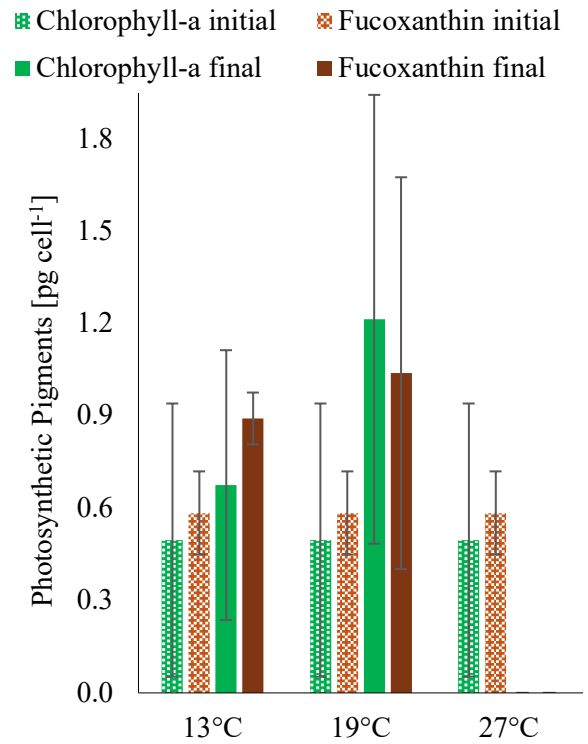

Figure 11. Initial and final cellular concentration of chlorophyll-a and fucoxanthin, expressed as means ± standard deviations, for each treatment for *Diatoma* sp.

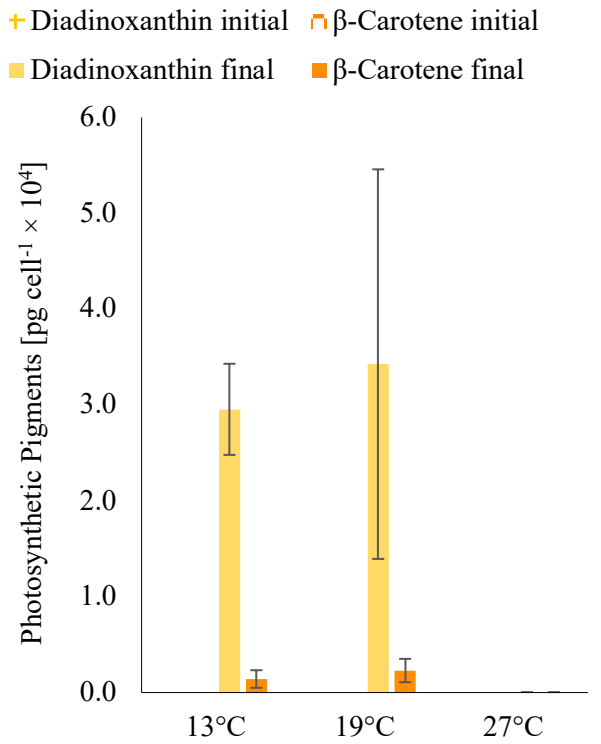

Figure 12. Initial and final cellular concentration of diadinoxanthin and β-carotene, expressed as means ± standard deviations, for each treatment for *Diatoma* sp.

4.1.3 Effective Photosynthetic Yield, $F_{v'}/F_{m'}$

The effective photosynthetic yield ($F_{v'}/F_{m'}$) of *Diatoma* sp. first increased for all treatments but stabilized at approximately 0.55 in 13°C and 19°C in contrast to 27°C where the effective photosynthetic yield decreased after the early increase down to approximately 0.1, as can be seen in Figure 13. Recovery was measured in 13°C for all treatments 14 days after the end of the experiment, showing an increase in effective photosynthetic yield for the 27°C treatment, however, variability was large as displayed by the standard deviations. Additionally, an approximate background noise level of 0.015 (Prof. A. Wulff, personal communication, May 2023) has been included in the graph (Figure 13).

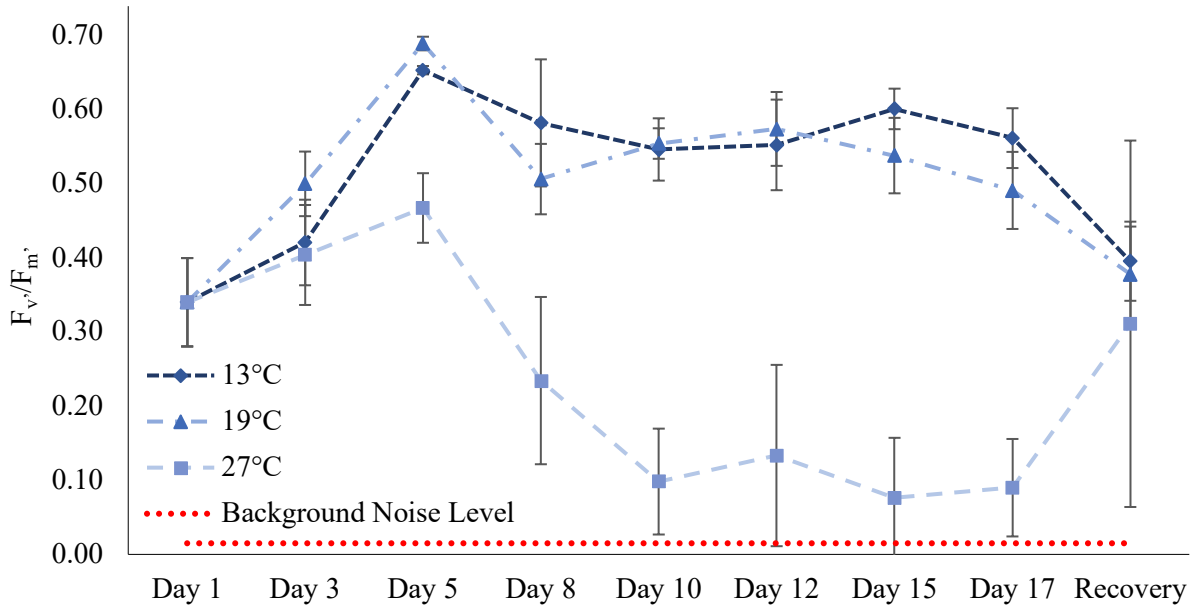

Figure 13. Progression of effective photosynthetic yield expressed as means ± standard deviations for three temperature treatments for *Diatoma* sp. The measurement labeled as "Recovery" equals day 31 and back in 13°C ambient temperature for all treatments. The background noise level is shown as an approximate level under which measurements cannot be distinguished from the noise levels of the equipment. n = 7.

4.1.4 Nutrient Reduction

The reductions in phosphate (PO_4^{2-}) and silica (SiO_2) concentrations were found to significantly differ between the temperature treatments. The phosphate reductions were significantly different between all treatments except from between 13°C and 19°C (Figure 14). The silica reductions were significantly different between 19°C and 27°C at the mid-time and final sampling, as well as between 13°C and 19°C at the final sampling (Figure 15). The reduction in nitrate was not significantly different between treatments, with the difference only close to significant at the mid-time sampling ($p = 0.11$) and final sampling ($p = 0.13$), and with the difference between 13°C and 27°C being closest to significant ($p = 0.09$ and $p = 0.13$) (Figure 16). None of the Redfield's N:P uptake ratios were significantly different between the temperature treatments (Figure 17).

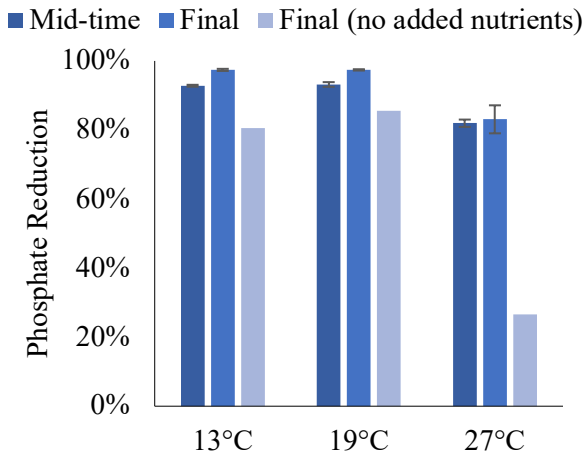

Figure 14. Percentual reduction of phosphate from the water, measured for mid-time, final and final with no added nutrients in all three temperature treatments for *Diatoma* sp.

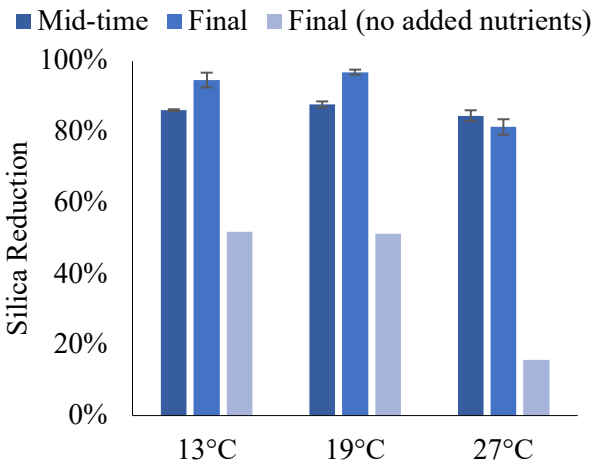

Figure 15. Percentual reduction of silica from the water, measured for mid-time, final and final with no added nutrients in all three temperature treatments for *Diatoma* sp.

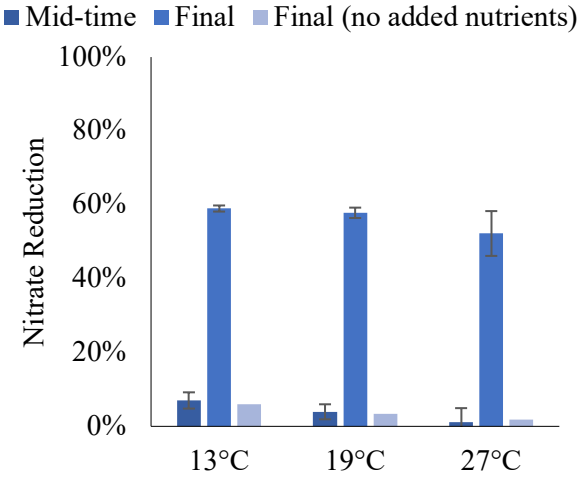

Figure 16. Percentual reduction of nitrate from the water, measured for mid-time, final and final with no added nutrients in all three temperature treatments for *Diatoma* sp.

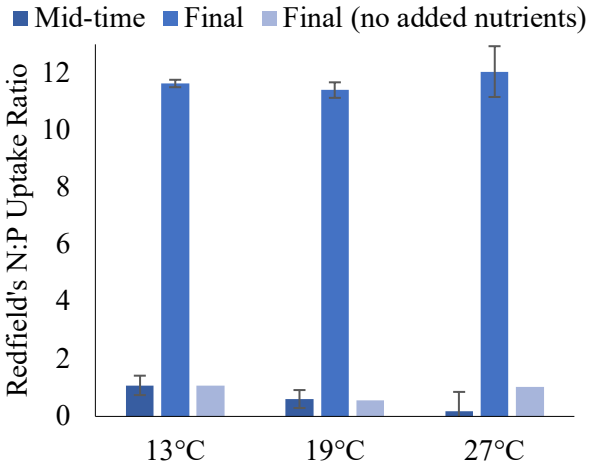

Figure 17. Calculated Redfield' nitrogen-phosphorous ratios of nutrient reduction/uptakes, measured for mid-time, final and final with no added nutrients in all three temperature treatments for *Diatoma* sp.

4.2 *Nitzschia* sp.

4.2.1 Cell Density and Growth Rate

Cell density was only measured for one replicate per treatment and could therefore not be statistically tested (Figure 18). The growth rate was calculated to be 0.10 cells L^{-1} day^{-1} in 13°C, 0.081 cells L^{-1} day^{-1} in 19°C, and 0.067 in 27°C. Photos displaying the cells prior to the experiment started (Figure 19), the cells at final sampling (Figure 20), and a typical ciliate found (Figure 21), are also presented.

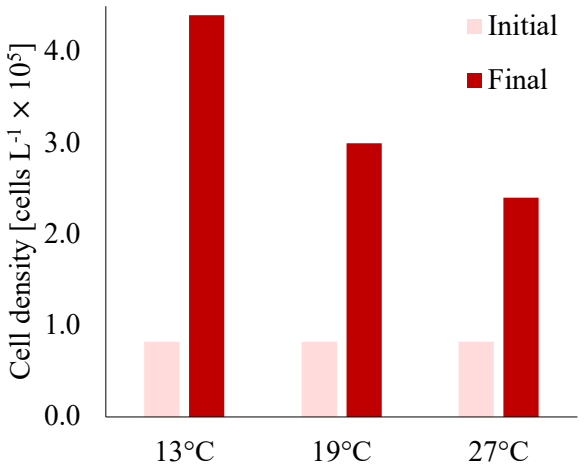

Figure 18. Cell density in 13°C, 19°C and 26°C for *Nitzschia* sp. n = 1.

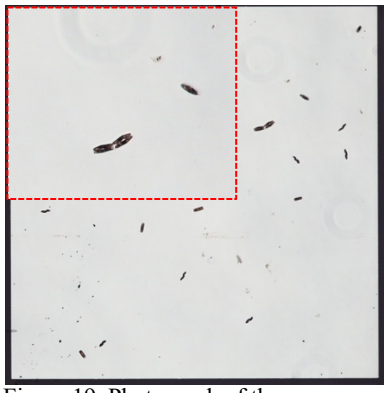

Figure 19. Photograph of the pre-initial cell density for *Nitzschia* sp., prior to being diluted and transferred to the experiment flasks. The box in the upper left corner shows an enlargement. The photo is approximately equal to 1 µL.

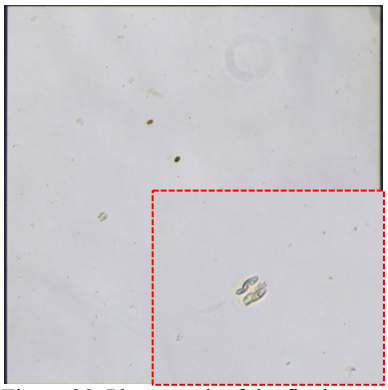

Figure 20. Photograph of the final cell density for *Nitzschia* sp. with an enlargement in the lower right corner. The photo is approximately equal to 1 µL.

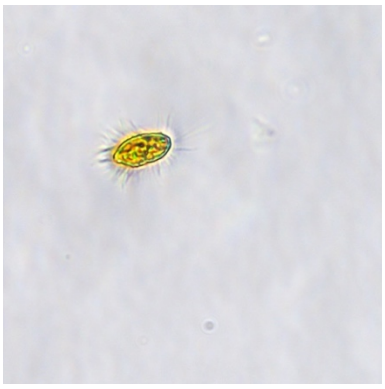

Figure 21. Photograph of ciliate found in the final samples for *Nitzschia* sp.

4.2.2 Photosynthetic Pigments

The concentrations of photosynthetic pigments in *Nitzschia* sp. were too low to be detectable for all pigments and treatments.

4.2.3 Effective Photosynthetic Yield, F_v'/F_m'

The measured effective photosynthetic yield F_v'/F_m' decreased rapidly for all treatments, stabilizing below the approximate level for background noise (0.015 (Prof. A. Wulff, personal communication, May 2023)), see Figure 22. Recovery was measured in 13°C for all treatments 14 days after the end of the experiment.

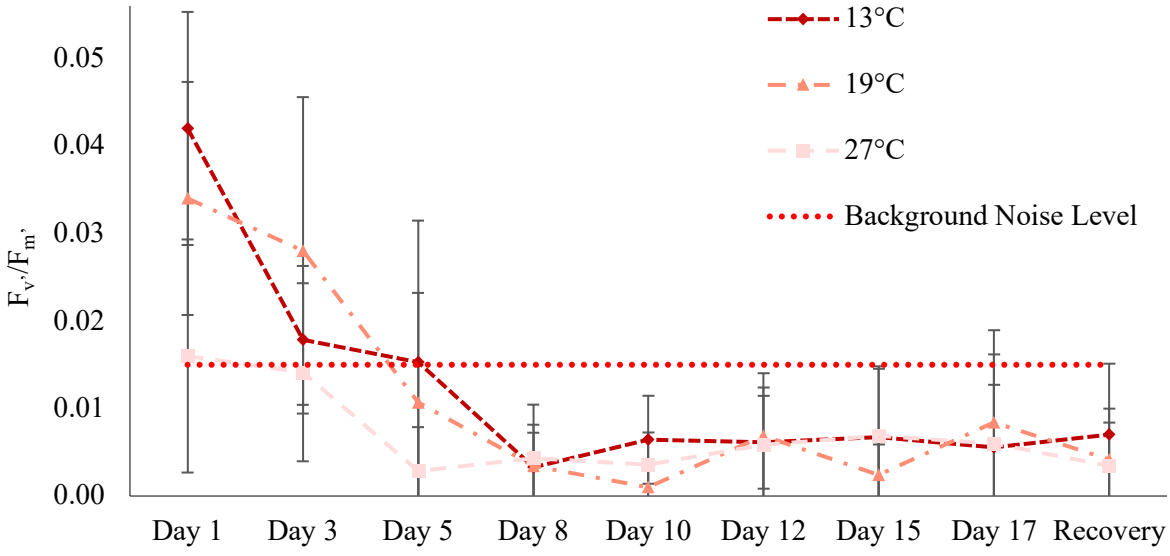

Figure 22. Progression of effective photosynthetic yield expressed as means ± standard deviations for the three temperature treatments for *Nitzschia* sp. The measurement labeled as "Recovery" equals day 31 and back in 13°C ambient temperature for all treatments. The background noise level is shown as an approximate level under which measurements cannot be distinguished from the noise levels of the equipment. n = 7.

4.2.4 Nutrient Reduction

The growth medium in which the *Nitzschia* sp. was cultivated was sent for water analysis to ensure its quality and identify any potential issues, but no nutrient analysis was conducted for *Nitzschia* sp. due to the lack of growth and biomass production observed through the rest of the parameters.

5 Discussion

5.1 *Diatoma* sp.

The *Diatoma* sp. produced more biomass (estimated as cell density and photosynthetic pigment concentrations) in all temperature treatments throughout the experiment. The highest cell densities were obtained in the 13°C and 19°C temperature treatments, with variation between the two being lower than between similar temperatures for the same species reported on by Hansson (2023), and growth rates substantially higher (highest growth rate being 0.33 as compared to 0.094 by Hansson (2023). In this study, the cell densities obtained were approximately 100 times higher in the 13°C and 19°C treatment than in the 27°C temperature treatment, and growth rate being approximately ten times higher than in the 27°C temperature treatment. An equal pattern was seen when comparing the concentrations of photosynthetic pigments between the different temperatures, with final concentrations in the 27°C treatment even as low as zero for all four pigments. However, the decreases in pigment concentrations in the 27°C treatment don't necessarily have to be as drastic as it looks based on the graphs since the initial concentrations that could be measured were just above the minimum level set for measurement (≈ 0.3 µg/L) and even the smallest decrease would thereof result in concentrations stated as zero µg/L. This also resulted in cellular concentrations being infeasible to calculate, but because the cell density in 27°C had increased with 50 % until the final sampling, one can establish that the cellular concentration of chlorophyll-a, fucoxanthin, diadinoxanthin and β-carotene decreased in this temperature treatment. In contrast, the total concentrations, as well as the cellular concentrations, increased in the 13°C and 19°C treatments indicating an increase in biomass but also in the density of photosynthetic pigments in the cells, possibly as an adaptation to more favorable nutrient conditions, as the opposite is known to repress genes associated with photosynthetic activity and biosynthesis of the pigments chlorophyll-a and fucoxanthin (Alipanah *et al.*, 2015), resulting in decreasing cellular concentration of chlorophyll-a as a response to nutrient (especially nitrogen) limitation (Beardall *et al*., 2001). However, cellular pigment concentrations are also known to increase as a response to low light conditions (Cointet *et al*., 2019), but since the light intensity was higher during the experiment than before, this was probably not the reason for the observed increase in cellular photosynthetic pigment concentrations. The increase in cellular pigment concentration would probably have been even larger if taken into account that the proportion of smaller cells were considerably higher in the 13°C and 19°C treatments at the final sampling compared to the 27°C treatment. This observed higher cellular concentrations of photosynthetic pigments align with the measurements of photosynthesis from measured effective photochemical yield ($F_{v'}/F_{m'}$), as these findings indicate a significantly reduced photosynthetic activity in the 27°C treatment compared to the 13°C and 19°C treatments. Yet, when recovery was measured about two weeks after the temperature had been reduced back to 13°C, the measurement of effective photochemical yield showed a significant increase. Although there was larger variability among the flasks, this suggests that at least some of the cells were able to greatly recover once back in cooler temperature. In contrast, the 13°C and 19°C treatments showed a decrease in effective photochemical yield when recovery (in 13°C) was measured but because their productivity during the experiment had been higher, there's a major possibility that they had started to get nutrient limited as no

more nutrients were added after day 15, approximately two weeks prior to the recovery measurement. This theory is consistent with the decrease in maximum photochemical efficiency (F_v/F_m) in diatoms due to nutrient depletion as researched by Tan *et al*. (2019).

The nutrient analysis displayed a much smaller variation between the treatments than the other variables did, thereby resulting in less significant results. The differences in percentual nutrient reduction for the 13°C and the 19°C treatments compared to the 27°C treatment was less than 20 % for NO_3, PO_4, and SiO_2. In contrast, the difference in biomass between the same treatments was approximately 100 %, i.e., five times higher. However, this doesn't necessarily mean that the results are contradictive. Both parameters were measured to assess the variation in biomass between different treatments, but cell density measures the number of cells whereas the nutrient reduction rather correlates to the mass of the cells, as a larger cell generally needs more nutrients than a smaller one (Lindemann *et al*., 2016). From applying this rational, in addition to the observed high proportion of smaller cells in the 13°C and 19°C treatments, one can argue that this is further evidence for the preferred temperature of *Diatoma* sp. being closer to the two lower temperature treatments than the higher one as diatoms are known to duplicate faster during the vegetative phase of wild populations when environment conditions are favorable (Potapova & Snoeijs, 1997). Diatoms are also known to accumulate storage products (i.e., lipids and carbohydrates) when subjected to stressful conditions such as high light intensity and low nitrogen availability (Cointet *et al*., 2019), and as the cells in the 27°C treatment possibly were subjected to heat stress additionally, this may explain the lower variation in nutrient reduction between treatments to some extent. However, this was also the reason why the shading was used, as stress from other environment conditions than temperature was tried to be avoided. More likely, this lower amount of variability can be explained by the nonlinear relationship between nutrient uptake and cell radius due to the higher investment costs for smaller cells to fully profit from the uptake as described by Lindemann *et al*. (2016).

The NO_3 reduction was above 50 % whilst PO_4 and Si reduction were above 80 % at both mid and final sampling across all treatments suggesting the possibility that the algae could have been starting to get nutrient limited by either phosphate or silica, however, the percentual reduction of each nutrient were never higher in the samples without added nutrients (no replicates) although these likely would have encountered nutrient depletion and thereby also nutrient limitation earlier than samples with added nutrients. These results seem to indicate that the diatoms weren't nutrient limited, and this can further be argued for when assessing the Redfield's N:P ratios. The N:P ratios didn't differ between treatments but were on the other hand very different within treatments with final ratios of almost 12:1, but mid-time ratios and ratios of samples without added nutrients of close to 1:1. When the mid-time samples were sampled for nutrient analysis they had only had bioavailable silica added to them but no other nutrients, whereas the final samples had had both silica and F/2 nutrients added to them. The results therefore indicates that silica haven't been the limiting substance as there then would have been a difference between the ratios in the graph for the mid-time samples and the samples with no added nutrients. Based on the substantial disparity between these two and the N:P ratio of the final samples, much points to some of the nutrients in the F/2 mix being limiting growth and biomass production in the samples where no F/2 mix were added. Natural ratios are usually stable around 16:1 (Bydén *et al*., 2003), but can be altered towards 6:1 during nitrogen limitation or towards 60:1 during phosphate limitation, suggesting that the *Diatoma* were nitrogen limited. However, the percentual nitrogen use up in the final samples were well below 60%, and not closer to 100% as one could speculate them to be to be able to inflict nutrient limitation on the cells. The shifting of the N:P uptake ratio towards showing signs of nitrate limitation (where a lot of nutrients had been added) could also possibly be explained by the "luxury phosphate consumption" as shown by Cade-Menun & Paytan (2010) when algae are being cultivated in phosphate excessive conditions, as this then also would explain the higher phosphate reduction in the samples with extra nutrients added to them compared to those without.

From the overall assessment of the results of the different measured parameters, *Diatoma* sp. seems to have a temperature optimum that covers the two lower temperatures tested in this study, 13°C and 19°C, but not the higher temperature of 27°C, proving the initial hypothesis. This result is in line with previous suggested temperature optimum of 11-20°C by Hansson (2023), while narrowing down the upper limit of the preferred temperature to somewhere between 20°C and 27°C. *Diatoma* sp. seems to have a broad temperature tolerance, but the result from this study opposes the general temperature tolerance of 13°C to 30°C for marine benthic diatoms native to northern European seas established by Prelle *et al*. (2019) and Scholz & Liebezeit (2012), suggesting the upper limit for at least *Diatoma* sp. to be lower than 27°C.

This study also shows that if *Diatoma* sp. should be cultivated for biomaterials, such as their silica frustules, or valuable substances, such as fucoxanthin, this should not be done in warmer temperatures such as 27°C. However, if only the nutrient removal capacity is of interest, such as in the aquaculture industry, all tested temperatures allow for efficient nitrate and phosphate reduction with minimal or insignificant differences between 13°C, 19°C, and 27°C.

5.2 *Nitzschia* sp.

The cell density of *Nitzschia* sp. appears to have increased during the experiment for all treatments, approximately three to five times, with highest biomass obtained in 13°C, followed by 19°C. However, due to the clear decrease in photosynthetic activity ($F_{v'}/F_{m'}$) to levels even lower than those inferred by background noise, no replicates were analyzed for cell density and growth rate and therefore no statistical analysis was conducted. The growth rates were 50-75% of the growth rate for the same species and growth medium found by Karlsson in 2022, but once again these results may not be accurate as stated before. There was also a clear visible change in morphology (i.e., cell deformation and less dense cytoplasm) between the cells from the final sampling and the cells in the pre-initial sample before the effective photochemical yield measurements started to show a clear decline. Because of this, deciding whether cells were alive or not became much more difficult, resulting in possibly more cells being counted than should have been and thus resulting in growth rates higher than what was true.

The concentrations of photosynthetic pigments were constantly under the minimum level for measurement (three replicates per treatment and sampling time) which indicate the poor health and better verifies the low biomass production of the *Nitzschia* sp. Ciliates were found in concentrations comparable with the algae cells but since the diatom cells found looked unwell and the effective photochemical yield showed no photosynthetic activity of the still existing cells, the ciliates were probably not to blame for the lack of strong evidence for biomass production in *Nitzschia* sp., except for the cell density measured with only one replicate. Finally, to determine that the cells weren't just temporarily affected by changed environment conditions (growth medium, light intensity, etc.), the recovery was measured for effective photochemical yield but since there was no recovery seen it was clear that something else was causing the complications. An extensive troubleshooting was conducted but no clear cause was found, and the hypothesis could therefore neither be proven or disproven.

5.3 Methodological Considerations

Biomass production and productivity are parameters for which several variables can be used for quantification and/or relative comparison. The different variables complement each other and by measuring more than one such variable, the certainty in the conclusions drawn from the results can be increased significantly (Underwood, 1997). If you were to only e.g., count cells to determine the biomass as cell density then there is a risk that large cell clumps either are missed or overrepresented. But if you also measure the concentrations of photosynthetic pigment (e.g., chlorophyll-a) to relatively compare between treatments, you will both be able

to detect the large cell clumps and avoid the risk of overrepresentation. If also nutrient reduction, dry weight, or any other variable is measured the level of certainty in the conclusions you are able to draw will increase further. In this study, the analyses were not meant to be absolute quantitative, but merely act as means for allowing relative comparison between the three different temperature treatments. They were also chosen so that other factors that potentially could be affecting the results should be possible to distinguish and take account for when making inferences based on the results.

Some changes from the initial plan were made during the study, both intentionally and unintentionally. The number of replicates was originally planned to be five but was later reduced to three plus the one extra *Diatoma* replicate per treatment without added nutrients. This lowered the statistical power and the initial plan of five replicates would possibly have resulted in fewer nonsignificant results where differences between the treatments still were rather evident in the graphs.

The actual temperatures were unintentionally changed to the initial plan as both the heat-regulators systematically held a temperature of +1°C from the set temperature as well as the ambient temperature in the room where the algae were grown fluctuated over the day due to heat generated from the high number of active lightbulbs in the daytime. The original temperatures were set to be 10°C, 18°C, and 26°C and this clearly demonstrates the importance to use temperature loggers as this enabled for post correction of the actual temperatures.

The light intensity differed within the treatments which might have increased the variability within each temperature treatment (even though the flasks were moved around to address this), however, the light intensity was constant between treatments and shall therefore not have induced errors affecting the actual comparisons in other ways than by possibly lowering the significance. Other factors that might have induced errors are that the algae cultures were non-axenic, and bacteria therefore might have affected the nutrient analysis, and also that the pigment concentration in the final HPLC samples might have been too high for producing accurate results with the method used and thereby induced more variability within the treatments as can be seen in the large standard deviations in all figures of pigment concentrations. Even larger variability was seen in the analysis of ammonia concentration (>50% in growth medium samples from the same flask), however, in this case the analysis was deemed completely unreliable, and the statistical analysis and graph were therefore excluded.

6 Conclusions

Based upon the findings of this study, the overall temperature optimum for *Diatoma* sp. is believed to include the temperature span between 13°C and 19°C, and have an upper limit between 19°C and 27°C. However, no lower limit can be suggested based on these findings. Furthermore, this study shows that, for industrial purposes, the temperature optimum may vary depending on indented use, with the upper limit for *Diatoma* sp. being higher for aquaculture implementations aimed at nutrient recycling than for industrial implication for production of biomaterial and/or high-value substances.

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8 References

Adey, W., Luckett, C., & Jensen, K. (1993). Phosphorus Removal from Natural Waters Using Controlled Algal Production. *Restoration Ecology*, *1*(1), 29-39. https://doi-org.ezproxy.ub.gu.se/10.1111/j.1526-100X.1993.tb00006.x

Alipanah, L., Rohloff, J., Winge, P., Bones, A., & Brembu, T. (2015). Whole-cell response to nitrogen deprivation in the diatom Phaeodactylum tricornutum. *Journal of Experimental Botany*, *66*(20), 6281-6296. https://doi.org/10.1093/jxb/erv340

Beardall, J., Young, E., & Roberts, S. (2001). Approaches for determining phytoplankton nutrient limitation. *Aquatic Sciences*, *63*(1), 44-69. https://doi-org.ezproxy.ub.gu.se/10.1007/PL00001344

Bhattacharjya, R., Singh, P., & Tiwari, A. (2021). Aquaculture water as a source of sustainable growth medium for diatom cultivation and its nutritive suitability as a potential aqua feed. *Environmental Technology & Innovation*, *24*. https://doi.org/10.1016/j.eti.2021.101987

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable & Sustainable Energy Reviews*, *14*(2), 557-577. https://doi.org/10.1016/j.rser.2009.10.009

Bydén, S., Larsson, A.-M., & Olsson, M. (2003). *Mäta vatten – undersökningar av sött och salt*. Avdelningen för tillämpad miljövetenskap och avdelningen för oceanografi, Göteborgs Universitet.

Cade-Menun, B., & Paytan, A. (2010). Nutrient temperature and light stress alter phosphorus and carbon forms in culture-grown algae. *Marine Chemistry*, *121*(1), 27-36. https://doi.org/10.1016/j.marchem.2010.03.002

Falkowski, P. G., & Davis, C. S. (2004). Natural proportions. *Nature*, *431*, 131. https://doi.org/10.1038/431131a

FAO. (2022a). *Blue Transformation - Roadmap 2022–2030: A vision for FAO's work on aquatic food systems*. Rome. https://doi.org/10.4060/cc0459en

FAO. (2022b). *The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation.* Rome, FAO. https://doi.org/10.4060/cc0461en

Grunow, A. (1862). Die österreichischen Diatomaceen nebst Anschluss einiger neuen Arten von anderen Lokalitäten und einer kritischen Übersicht der bisher bekannten Gattungen und Arten. *Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 12*. 315-472

Guillard, R.R.L. (1975). Culture of Phytoplankton for Feeding Marine Invertebrates. In: Smith, W.L., Chanley, M.H. (eds) Culture of Marine Invertebrate Animals. Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-8714-9_3

Gårdsfisk, Scandinavian Aquasystems AB. (2021). *Om oss*. Gårdsfisk. Retrieved 2023-03-25 from https://www.gardsfisk.se/om-oss

Hallegraeff, G. M., Anderson, D. M., Cembella, A. D., & Enevoldsen, H. O. (2004). *Manual on harmful marine microalgae*. 2nd ed. UNESCO.

Hansson, E., (2023). [Master's thesis, University of Gothenburg / Department of Biological Environmental Sciences]. *Influence of temperature on biomass and growth rate of benthic diatoms.* Gothenburg University Publications Electronic Archive.

Heinz Walz GmbH. (2013). *Instrument Description and Information for Users 2.137 / 11.00.* 2nd ed. https://www.walz.com/files/downloads/manuals/water-pam/waterp3e.pdf

Jeffrey, S.W., Mantoura, R.F.C., & Wright, S.W. (Eds.). (1997). *Phytoplankton pigments in oceanography: Guidelines to modern methods*. Paris: Unesco Publishing.

Jeffryes, C., Campbell, J., Li, H., Jiao, J., & Rorrer, G. (2011). The potential of diatom nanobiotechnology for applications in solar cells, batteries, and electroluminescent devices. *Energy & Environmental Science*, *4*(10), 3930-3941. https://doi-org.ezproxy.ub.gu.se/10.1039/C0EE00306A

Karlsson, S., (2022). [Master's thesis, University of Gothenburg / Department of Biological Environmental Sciences]. *Using microalgae for removing nutrients from RAS aquaculture effluents - a viable strategy? A case study*. Gothenburg University Publications Electronic Archive. https://hdl.handle.net/2077/72713

Lindemann, C., Fiksen, &., Andersen, K., & Aksnes, D. (2016). Scaling laws in phytoplankton nutrient uptake affinity. *Frontiers in Marine Science*, *3*. https://doi.org/10.3389/fmars.2016.00026

Marella, T. K., López-Pacheco, I. Y., Parra-Saldívar, R., Dixit, S., & Tiwari, A. (2020). Wealth from waste: Diatoms as tools for phycoremediation of wastewater and for obtaining value from the biomass. *Science of the Total Environment*, *724*. https://doi.org/10.1016/j.scitotenv.2020.137960

Naylor, R., Hardy, R., Buschmann, A., Bush, S., Cao, L., Klinger, D., Little, D., Lubchenco, J., Shumway, S., Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature*, *591*, 551-563. https://doi-org.ezproxy.ub.gu.se/10.1038/s41586-021-03308-6

Potapova, M. & Snoeijs, P. (1997). The natural life cycle in wild populations of Diatoma moniliformis (Bacillariophycea) and its disruption in an aberrant environment. *Journal of Phycology*, *33*, 924-937. https://doi-org.ezproxy.ub.gu.se/10.1111/j.0022-3646.1997.00924.x

Prelle, L., Graiff, A., Gründling-Pfaff, S., Sommer, V., Kuriyama, K., & Karsten, U. (2019). Photosynthesis and respiration of baltic sea benthic diatoms to changing environmental conditions and growth responses of selected species as affected by an adjacent peatland (Hütelmoor). *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01500

Ramachandra, T., Mahapatra, D., B, K., & Gordon, R. (2009). Milking Diatoms for Sustainable Energy: Biochemical Engineering versus Gasoline-Secreting Diatom Solar Panels. Industrial & Engineering *Chemistry Research*, *48*(19), 8769-8788. https://doi-org.ezproxy.ub.gu.se/10.1021/ie900044j

Ramli, N., Verreth, J., Yusoff, F., Nurulhuda, K., Nagao, N., & Verdegem, M. (2020). Integration of Algae to Improve Nitrogenous Waste Management in Recirculating Aquaculture Systems: A Review. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.01004

Swedish Algae Factory AB. (n.d.). *Circularity*. Swedish Algae Factory. Retrieved 2023-04-28 from https://www.swedishalgaefactory.com/circularity/circularity-in-practice

Scholz, B., & Liebezeit, G. (2012). Growth responses of 25 benthic marine Wadden Sea diatoms isolated from the Solthörn tidal flat (southern North Sea) in relation to varying culture conditions. *Diatom Research*, *27*(1), 65-73. https://doi.org/10.1080/0269249X.2012.660875

Tan, L., Xu, W., He, X., & Wang, J. (2019). The feasibility of Fv/Fm on judging nutrient limitation of marine algae through indoor simulation and in situ experiment. *Estuarine, Coastal and Shelf Science*, *229*. https://doi.org/10.1016/j.ecss.2019.106411

Underwood, A.J. (1997). *Experiments in ecology*. Cambridge University Press, Cambridge.

Xing, R-L, Ma, W-W, Shao, Y-W, et al. (2018). Growth and potential purification ability of *Nitzschia* sp. benthic diatoms in sea cucumber aquaculture wastewater. *Aquac Res*. *2018*(49), 2644-2652. https://doi.org/10.1111/are.13722

Appendix

Initially, the study was going to be conducted on solely *Nitzschia pusilla* in effluent water from Gårdsfisk. In a similar set-up but with five true replicates instead of three and the temperatures 12 °C, 20 °C, and 28 °C. The diatoms were cultivated in two 250 mL flasks (one primary and one backup) using freshwater f2 medium mixed with 0.2 mm filtered water from Gårdsfisk in 12 °C and 16:8 light-darkness ratio. Nutrients were added during the cultivation phase by adding 20 mL of AlgalBoost f2 (AusAqua Pty Ltd) and bioavailable silica diluted in MilliQ-water. Once the tests were going to start, the concentration of algae were determined using a counting chamber and a light microscope. All treatment flasks were prepared by adding 140 mL of filtered effluent water before adding approximately 10 mL of diluted algae culture so that an approximate start concentration of 5×10^5 cells L^{-1} was obtained in the treatment flasks. All flasks were then loosely sealed with lids and ten put in each treatment group; 12 °C, 20 °C, and 28 °C. The remaining five flasks were analyzed directly following the procedure for each analysis and then kept as back-up flasks.

However, during the next couple of days, the analyses showed growth but almost no photosynthetic activity at all. After a lot of unsuccessful troubleshooting, it was therefore decided for the experiment to be canceled and restarted with new algae cultures.

Due to the time being limited and the urgency for high cell concentrations in the algae cultures, SAFAB was contacted and thankfully they were able to assist with preparing cell culture flasks with higher cell densities of their cultivated *Diatom sp.* These, and 4 L of their artificial sea-water medium, were then collected in Kungshamn. A new promising cell culture flask with *Nitzschia* sp. from Gothenburg university's algae bank were also cultivated to be tested along-side the *Diatoma*.