UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

# INFLUENCE OF TEMPERATURE ON BIOMASS AND GROWTH RATE OF BENTHIC DIATOMS

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## Abstract

Temperature has a significant impact on structure and abundance of microalgal community as well as on physiology (Sheehan et al., 2020). Given the anticipated temperature fluctuations and likely rise in temperature during summer seasons in the greenhouse at Swedish Algae Factory (SAF), it is essential to comprehend the response of diatoms to temperature, in order to sustain growth and microalgal biomass production. In this study, the influence of three different temperature scenarios on biomass and growth rate of two species of benthic diatoms (*Nitzschia* sp. and *Diatoma* sp.) along with the algal community cultivated at SAF, were examined. Treatments were carried out in 100ml flasks, and nutrient levels, chlorophyll *a* fluorescence parameter ($F_v'/F_m'$), cell density, growth rates, and photosynthetic pigments (through HPLC) were measured. Pigments were calculated as concentrations (µg $L^{-1}$) and as ratios relative to chlorophyll *a*. The results showed an increase in biomass and growth rate at ambient temperature (11-16°C) and 20°C for the two species and the algal community, while a decline in biomass and growth rate was observed at 30°C. Moreover, results revealed that an increase in temperature to 30°C generated an increase in green algae and cyanobacteria within the algal community. Additionally, *Diatoma* sp. also exhibited green algae and cyanobacteria at 30°C, whereas *Nitzschia* sp. did not, even at high temperatures, indicating its suitability for SAF cultivations. Overall, these findings illustrate the variation in thermal performance among benthic diatoms and emphasize the importance of studying species-specific responses to temperature change.


Keywords: benthic, diatoms, cultivation, thermal performance, photosynthetic pigments

## Populärvetenskaplig sammanfattning

I min uppsats har jag undersökt hur olika temperaturscenarier påverkar biomassa och tillväxthastighet hos bottenlevande kiselalger. Kiselalger är mikroskopiska organismer i havet som, likt växter på land, tar upp koldioxid och vatten och omvandlar det till organiskt material medan de frigör syre. Swedish Algae Factory (SAF) är ett företag som utforskar användningen av kiselalger i industriella tillämpningar. Särskilt intresse riktas mot kiselalgernas skal (frustuler) och deras nanoporösa strukturer. För att uppnå en hållbar och miljövänlig produktion odlas algerna i ett växthus, vilket minskar behovet av uppvärmning och belysning genom naturligt ljusinsläpp. Dock kan temperaturvariationer uppstå i växthuset, och under de varma sommarmånaderna kommer temperaturen att öka. För att säkerställa en kontinuerlig produktion, är det därför viktigt att kiselalgerna kan tolerera olika temperaturer. I min studie använde jag huvudsakligen två metoder för att undersöka hur temperatur påverkar tillväxthastighet och biomassa hos kiselalger: cellräkning och analys av fotosyntetiska pigment, till exempel klorofyll *a*. Cellräkningen innebar att celler av kiselalger räknades under ett mikroskop för att bestämma en koncentration (celler per liter) och därmed mäta biomassan. Utifrån detta kunde sedan tillväxthastigheten beräknas. Pigmentanalysen användes också för att kvantifiera biomassan, genom att mäta koncentrationer av olika pigment. Mitt resultat visade att både tillväxthastighet och biomassa ökade vid temperaturerna 11–16°C samt i 20°C, men minskade vid 30°C. Dessutom, kunde grönalger och cyanobakterier urskiljas i 30°C, där de har en konkurrensfördel gentemot kiselalger. En av de undersökta arterna uppvisade inte några sådana inslag. Det indikerar att denna art kan vara lämplig att odla för SAF. Slutligen, vill jag betona vikten av att studera artspecifika reaktioner på temperaturförändringar, då det kan förekomma stora skillnader.

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# 1. Introduction

## *1.1. Aquaculture*

Over the last two decades, aquaculture has undergone a major transformation, growing from small-scale operations to large-scale commercial farming (Villar-Navarro et al., 2021). According to the Food and Agriculture Organization of the United Nations, global production of fish reached 179 million tons in 2018, of which almost 50% came from the aquaculture industry (FAO, 2022). Along with the increase in fish farming, the amount of aquaculture effluents generated also increases. Water originating from fish farming processes generally contains suspended solids, such as fish feed, feces, cleaning products, and possibly residues of medicine. Fish feed contains both nitrogen and phosphorus, and estimations show that only 20-30% of the nitrogen is assimilated or used up by the fish, while the rest is released into the water. Therefore, in addition to the suspended solids, a significant amount of nitrogen and phosphorus is often found in the aquaculture effluents (Hawrot-Paw et al., 2019). High nutrient content can stimulate excessive production of phytoplankton resulting in eutrophication of aquatic ecosystems which in turn has adverse effects, such as accumulation of organic carbon, reduction of light penetration, and oxygen depletion (Nicula et al., 2022; Saxena et al., 2022).

Moreover, there are different aquaculture techniques practiced today and the two main distinctions between different methods are open and closed systems. Recirculating aquaculture system (RAS) is an example of a closed system. By recirculating the water, aquaculture effluents are being discharged in lower volumes generating a decreased water use as well as carbon footprint (Villar-Navarro et al., 2021). However, the reuse of water in RAS results in aquaculture effluents containing much higher nutrient concentrations compared to open systems (Milhazes-Cunha & Otero, 2017). Thus, effective technologies for treatment of aquaculture effluents are highly needed to protect aquatic ecosystems (Saxena et al., 2022).

## *1.2. Microalgae as bioremediators*

Conventional ways of removing nutrients in RAS effluents, such as bacterial denitrification/ nitrification are being challenged by new and promising technologies based on microalgae and cyanobacteria (Araujo et al., 2021; Egloff et al., 2018). According to Hawrot-Paw et al. (2019), integrating the treatment of aquaculture wastewater with algal cultivation in RAS has the potential to bring both ecological and economic benefits. There is an interest in recovering phosphorus and ensuring denitrification to avoid accumulation of nitrogen in the system. Microalgal biotechnology is especially of interest since it removes nitrogen and phosphorus from water while generating valuable biomass (Villar-Navarro et al., 2021). Studies have shown that microalgae grown in wastewater from a fish farm were successful in removing both nitrogen and phosphorous from the water. The same studies also highlighted the importance of using new techniques such as microalgae instead of biofilters (Saxena et al., 2022; Tejido-Nuñez et al., 2019).

One type of microalgae that have been studied for its bioremediatory properties is diatoms. They have especially been subjects in heavy metal and toxic analyses but also for their ability to remove nutrients from aquaculture effluents and waste waters. Studies have shown that diatoms have a removal efficiency of ca 75% for total ammonium and up to 89% for phosphorus (Bhattacharjya et al., 2021; Chaib et al., 2021). In addition, diatoms have displayed removal efficiency between 42-60% of nitrate (Saxena et al., 2022).

*1.3. Diatoms in the marine ecosystem*

Diatoms are unicellular microalgae, universally distributed in all types of aquatic environments. They form the majority of pelagic and benthic microalgae in both marine and fresh waters (John, 2015) and are one of the most important food resources in marine and fresh-water ecosystems (Marsela Alikaj et al., 2019). While diatoms can thrive in tropical waters, they are more commonly outcompeted by other microalgal groups. However, diatoms are recognised for their ability to thrive in colder waters. In addition, benthic diatoms have been found to flourish in both very low and extremely high light conditions (Wulff et al., 2005; Zacher et al., 2007). Furthermore, studies on primary production estimates that almost 50% of all photosynthetic activity can be attributed to phytoplankton, of which the most common types are diatoms (D'Mello et al., 2022). Consequently, diatoms are major contributors to the global carbon and oxygen cycle. Globally, their photosynthesis yields over 40% of oceanic carbon fixation and 25% of all atmospheric oxygen (Baker et al., 2016; Sheehan et al., 2020). This makes them one of the most productive and diverse organism group on the planet, and their role in the environment is essential (Virta et al., 2019). Additionally, diatoms contain chlorophyll *a* and *c*, along with various carotenoids such as fucoxanthin, which gives them a brownish colour (Jeffrey et al., 1997).

In addition to being important primary producers, diatoms influence the biogeochemical cycling of elements by accumulating both macronutrients and trace metal such as N, P, Si and Fe. In order for diatoms to grow they require the uptake of nutrients and their conversion into biomass to form new cells (Sheehan et al., 2020). Since phytoplankton in the vast ocean are living in a very nutrient-diluted environment, they have evolved extremely efficient ways of assimilating nutrients (Olofsson, 2018). Furthermore, macronutrients are naturally occurring in the marine ecosystems (Sheehan et al., 2020). In seawater, nitrate ($NO_3$-) occurs in concentrations around 0-1 µmol/l (0-0.06 mg/l) during summer and around 10-20 µmol/l (0,6-1,24 mg/l) during winter in shallow waters (Bydén et al., 2003). Concentrations can be higher in coastal areas due to anthropogenic inputs (Olofsson, 2018). Moreover, ammonium ($NH_4^+$) generally occurs in lower concentrations, except in polluted areas (Olofsson, 2018). In seawater concentrations of ammonium are typically around 0-5 µmol/l (0-0,09 mg/l) (Bydén et al., 2003). It has been proposed that diatoms are strongly associated with the uptake of nitrate however they can also use ammonium (Andersen et al., 2020; Liu et al., 2022). Natural concentrations of phosphorous in coastal waters occurs around 1-10 µmol/l (0.03-0,3 mg/l) (Bydén et al., 2003).

Average phytoplankton community need for nutrients can be described by the relationship, between nitrogen and phosphorous – called the Redfield N:P ratio (Falkowski & Davis, 2004). In the formation of organic material, it is estimated that approximately 16 nitrogen atoms per every phosphorous atom is required (16:1) (Bydén et al., 2003). Furthermore, a deviation from the Redfield N:P ratio can indicate which nutrient that might be limiting (Kangro, 2010). If the N:P ratio is larger than 16, phosphorous is the limiting factor. However, if the ratio is smaller than 16, nitrogen is considered the limiting factor. Normally nitrogen is considered the limiting factor in the open sea, while phosphorous is considered limiting in coastal waters (Bydén et al., 2003).

Aside from nutrients, trace metals such as silica (Si) are also occurring naturally in the marine ecosystems (Sheehan et al., 2020). Silica is used by diatoms in the formation of their frustule (siliceous cell wall). When diatoms bloom, they can at times use up all available silica in the surface waters, making silica the limiting factor of the primary production. Silica never occurs in free form and are always bound to oxygen. Natural concentrations of silica in seawater varies between 0-200 µmol/l (0-12 mg/l) (Bydén et al., 2003). Furthermore, the frustule is considered to be the most

obvious feature of diatoms. The frustule is made up out of two halves, one upper lid (epitheca) and one lower lid (hypotheca). In addition, the frustule has a species-specific pattern of nanopores (Round et al., 1990) Based on morphology, diatoms are traditionally divided into two subclasses: pennate (biliteral symmetry) and centric (radial symmetry). Typically, centric diatoms are dominating the pelagic environments, while the benthic habitats are inhabited by pennate diatoms (Leynaert et al., 2018). The term benthos refers to the assemblage of organisms living and undergoing all or most of their life cycles associated with sediment (Round, 1971) Lastly, benthic diatoms are the most abundant, widely distributed and species rich benthic algae (Virta et al., 2019).

### *1.4. Microalgae and $CO_2$*

As a result of the high diversity and productivity of diatoms, their ability to sequester $CO_2$ is also high (D'Mello et al., 2022). Natural systems alone could potentially in the next decade mitigate some 37% of the greenhouse gas emissions necessary to limit global warming to 2°C (Griscom et al., 2017). Large emissions of $CO_2$ as a result of burning fossil fuels are recognised as one of the major contributors to global warming and even though emissions decreased slightly due to the COVID-19 pandemic in 2020, it has been estimated that emissions are once again increasing (He et al., 2023). Using microalgae for carbon capture, can potentially be seen as an eco-friendly carbon mitigation strategy and presents different advantages in comparison to the use of other photosynthetic organisms. For example, cultivating microalgae is not dependent on access to arable land and is therefore not competing with the food production industry (Bhattacharjya et al., 2021). In addition, in comparison to terrestrial plants, microalgae obtain higher growth rates and $CO_2$ fixation capacities. High $CO_2$ levels stimulate the growth of microalgae as well as it reduces biomanufacturing costs, which makes algae farming a long-term $CO_2$ emission reduction solution (He et al., 2023).

### *1.5. Physiological and biochemical responses to temperature*

Coastal waters are expected to experience continued warming in the coming decades. However, the knowledge about how diatoms, the dominant primary producers in these habitats, will cope with these changes is scarce (Stock et al., 2019). Temperature is crucial to microalgal community structure, distribution, and abundance and has a strong influence on physiology of microalgae, regulating nutrient uptake, cell volume, and cell metabolism amongst others (Sheehan et al., 2020). Furthermore, growth rate of microalgae in response to temperature is often illustrated as a bell-shaped curve (Boyd et al., 2013). An increase in temperature has a positive effect on growth rates, where organisms exposed to sub-optimal temperatures emerge as they are being warmed towards their optima. However, as the temperature optimum are being exceeded, microalgae are experiencing heat stress, which negatively impacts enzyme function and proteins in photosynthesis, causing growth rates to decrease (Akimov & Solomonova, 2019; Sheehan et al., 2020).

Several studies have investigated the thermal optimum for growth rate in different species and strains of benthic diatoms. For example, Stock et al. (2019) found that *Cylindrotheca Closterium* (*C. Closterium*) had an optimal growth rate at 20°C. Similarly, Admiraal (1977) observed the highest growth rate in *Navicula arenaria* (*N. arenaria*) between 16 to 20°C. In the same study however, it was found that three other benthic diatoms species (*Nitzschia dissipata*, *Nitzschia sigma* and *Amphiprora paludosa*) had a growth rate peaking at 25°C. In addition, Adenan et al. (2013) reported that the marine tropical diatom *Chaetoceros calcitrans* (*C. calcitrans*) exhibited optimal growth rates at 30°C, suggesting that tolerance and acclimation to temperature change varies between species and strains. In contrast, Scholz & Liebezeit (2012) found that variations in temperatures from 10 to 30°C did not significantly affect growth rates in benthic diatoms, but that

there was a significant decrease in growth rate at more extreme temperatures of 4 and 40°C. The temperature range necessary for growth generally aligns with the biogeographical origin of strains, and a comparison of (sub)tropical strains to polar strains suggests that the latter has a much narrower thermal performance range (Stock et al., 2019).

Moreover, effects of temperature on microalgae extends beyond growth and photosynthesis as it encompasses short-term nutrient uptake, and in particular, nitrogen metabolism. Uptake of nitrate in diatoms are proposed to be strongly limited by temperature (Berges et al., 2002). Furthermore, Aranguren-Gassis et al. (2019) suggests that limitations in nitrogen hinders thermal adaptation, leaving diatoms vulnerable to high temperatures. In order for diatoms to grow they require the uptake of nutrients and their conversion into biomass to form new cells. The rate in these biochemical processes is influenced by temperature since it regulates cell metabolism. In addition, the effects of temperature on biochemical responses in diatoms have been shown to be species specific (Sheehan et al., 2020).

### *1.6. Swedish Algae Factory*

SAF was founded in 2016 with the aim to further explore the potential use of microalgae and more specifically diatoms, in industrial applications. Benthic diatoms grow in biofilms, thus, they grow efficiently in shallow waters, and compared to pelagic diatoms, less water volumes are needed for commercial cultivation. Their fatty acid profile is ideal for biodiesel and, in addition, promising as fish feed or food supplements for humans. They contain both polyunsaturated fatty acids ("omega-3") and antioxidants (e.g. photosynthetic pigments such as fucoxanthin) (Sharma et al., 2021). Diatoms can survive in harsh conditions and the nanopores make benthic diatoms of particular interest for a number of industrial applications (Sharma et al., 2021). At SAF, whole cells are being tested for supplements in fish feed, as well as the frustules are currently explored for their use in skin care products and enhancing the efficiency of solar cell panels. Furthermore, early on, SAF established a collaboration with a fish farming company located nearby called Smögenlax, with the purpose of cleaning their aquaculture effluents and simultaneously using it as their cultivation medium. However, Smögenlax has not been successful in receiving their environmental permit, which has resulted in a delay in their collaboration.

The trial concerning the environmental permit for Smögenlax has caught large attention. The established fish farm was in many ways in line with the ideal fish farming business with its extensive production capacity, new modern technology limiting nutrient leakage, integration with other companies in the immediate area as well as a location allowing continued conservation and development of coastal communities (Kyrönviita, 2022). Despite this, the application was rejected because it could not sufficiently ensure that the water quality was not compromised and thus it was not unanimous with the Swedish Water Framework. After appealing, the decision was uplifted and Smögenlax was granted a permit for a severely constrained business. The outcome has debated whether the framework is too strict, since it in fact has led to the disappearing of many fish farms from the coast. Instead, the fish farms are being established further into the country, in connection to water bodies that are naturally or anthropogenically lacking nutrients (Kyrönviita, 2022).

As a result of the delay, SAF is currently using a self-produced and artificially nutrient rich cultivation medium. The diatoms are grown in biofilms on large cultivation lanes. When reaching a sufficient biomass, they are extracted to produce *Algica*, which is a nanoporous silica material (the frustules) extracted from the diatoms (Fig. 1). In the production of 1 kg of Algica, a minimum of 8 kg of $CO_2$, 1 kg of nitrogen and 0,1 kg of phosphorous is captured

(https://www.swedishalgaefactory.com/). Furthermore, the algae cultivations are currently conducted in a large greenhouse. From a sustainability perspective using a greenhouse, with its natural lighting, is beneficial since it means that no external use of energy for light and heating is needed. However, this also means that temperatures can fluctuate depending on the weather conditions and it is therefore of importance that the cultivations can cope with these temperature variations.

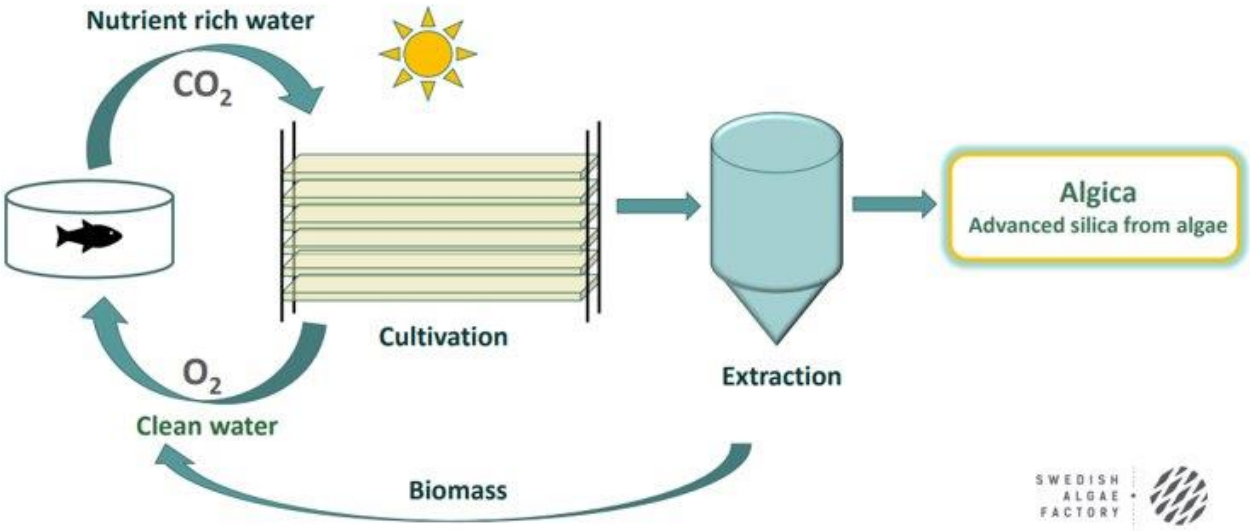



**Fig. 1** Production process at Swedish Algae Factory.

*1.7. Aim*

For SAF to be sustainable and efficient in their production, a minimum of energy input is required. Therefore, the diatom strains used should tolerate a wide range of temperatures. Consequently, this study aims to examine biomass and growth rate of benthic diatoms under three different temperature scenarios.

Hypothesis: The benthic diatoms will grow better in colder water temperature in comparison to warmer water temperatures.

## 2. Material and Methods

### *2.1. Culture conditions and experimental set-up*

A total of two different species of benthic diatoms (*Diatoma* sp. and *Nitzschia* sp.) as well as a sample from the algal community (dominated by pennate diatoms) cultivated at Swedish Algae Factory (SAF) were selected, based on recommendations from SAF, to investigate how particularly biomass and growth rate are influenced of three different temperatures. Prior to the experiment, *Diatoma* sp. and *Nitzschia* sp. were transferred to cultivation medium procured from SAF, containing f/2 medium (Guillard & Ryther, 1962) with addition of silica supplements. Detailed chemical analysis of the cultivation medium was performed by LMI AB, Helsingborg, Sweden. The chemical composition of the medium is shown in Table 1. Furthermore, the microalgae were acclimated at 15°C and photosynthetic active radiation of ca 91 µmol photons $m^{-2}$ $s^{-1}$ in a climate chamber with a light:dark interval of 16:8.

*Diatoma* sp. and *Nitzschia* sp. were continuously cultivated until sufficient cell concentrations had been achieved to start the experiment. The two species obtained varying initial cell densities because the algal stocks exhibited different growth rates. Once the two diatom stocks reached sufficient cell concentration they were transferred into a larger beaker and diluted with modified f/2 medium retrieved from SAF. The final volume was 330ml, prior to the division between the experimental treatments and replicates. Samples from the algal community were procured at sufficient cell concentration and was transferred into a larger beaker and thereafter diluted until it reached a final volume of 330ml. Before dilution, the community was put into an ultrasonication bath to avoid clothing and sedimentation.

**Table 1** Characteristics of the cultivation medium

| Elements | Chemical compounds | Concentrations |
|---|---|---|
| pH | | 8,3 |
| Conductivity mS/cm | | 37 |
| Nitrate mg/l | $NO_3$- | 180 |
| Ammonium mg/l | $NH_4^+$ | < 0,10 |
| Phosphorus mg/l | P | 38 |
| Potassium mg/l | K | 140 |
| Magnesium mg/l | Mg | 84 |
| Sulfur mg/l | S | 130 |
| Calcium mg/l | Ca | 75 |
| Mangan mg/l | Mn | 0,5 |
| Bor mg/l | B | 0,9 |
| Copper mg/l | Cu | < 0,029 |
| Iron mg/l | Fe | 0,27 |
| Zink mg/l | Zn | 0,09 |
| Molybden mg/l | Mo | < 0,016 |
| Silica mg/l | $SiO_2$ | 7 |
| Water hardness dH | | 22 |
| Sodium mg/l | Na | 4500 |
| Aluminum mg/l | Al | < 0,17 |

The experiment was conducted at the facilities of SAF, located on the west coast of Sweden in Kungshamn (Fig. 2) in a greenhouse, where the company is currently conducting their algae cultivations.

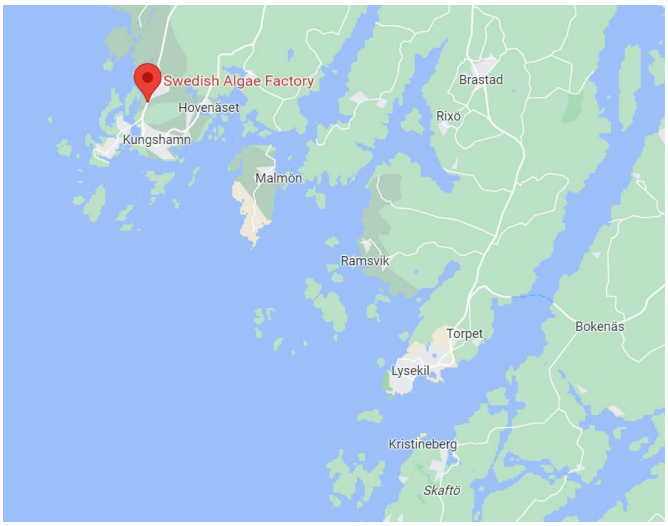



**Fig. 2** Map showing the location of Swedish Algae Factory on the west coast of Sweden.

The algal stocks were cultured in 100 ml glass flasks (Schott Duran and Pyrex®). The treatments consisted of water baths with 3 different temperature scenarios with the aim to study how growth rate and biomass of the diatoms were affected. Treatment 1 followed ambient temperature (11-16°C) in the greenhouse, treatment 2 was set to 20°C and treatment 3 was set to 30°C (Table 1, appendix for mean values and SD). Treatment 2 and 3 were chosen to mimic summer scenarios in the greenhouse, a critical time period for growing benthic diatoms. Water baths consisted of three plastic boxes containing ca 40 litres of water, completely covering the volume in the flasks (Fig. 3). A metal stand was placed in the water baths in order to increase the height of the flasks. Moreover, with the use of circulators (sous-vide circulator) water temperatures were regulated and kept homogenous. In addition, temperature loggers (HOBO® Pendant® Temperature/Light Data Logger) were placed in the water baths to log the temperature every 30 minute. 15 ml of respective algal stock and 55 ml of f/2 medium was added to the replicates in each experimental treatment, making the final volume 70 ml. The flasks were placed standing in the water baths.

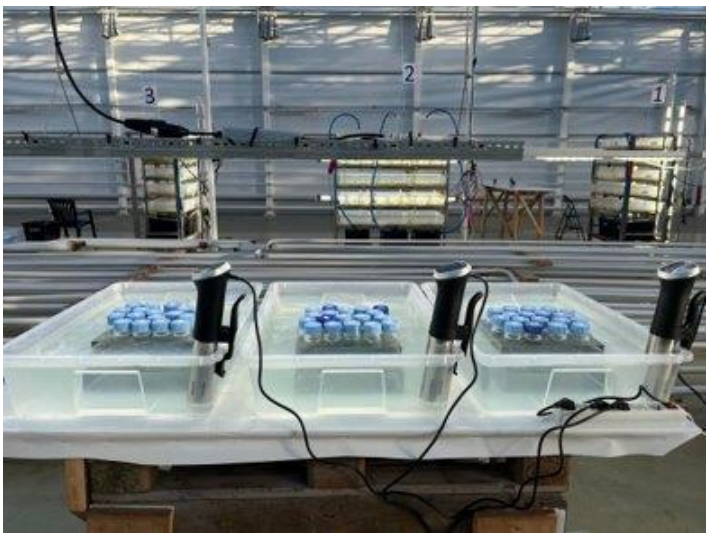



**Fig. 3** Experimental set up at Swedish Algae Factory.

Besides from ambient light, a stream of LED-lights was placed over the experimental site providing light for 12 hours (06.00-18.00) every day. Measurements of photosynthetically active radiation (PAR, 400-700 nm) in µmol photons $m^{-2}$ $s^{-1}$ were conducted using a spectrometer (LI-180 Spectrometer). PAR was measured to check differences between the three water baths. Measurements of PAR were carried out during both sunny and shady weather conditions and ranged from ca 130 - 400 µmol photons $m^{-2}$ $s^{-1}$ (Table 2, appendix for mean values and SD).

The experiment was running for 14 days with three true replicates for each treatment in the two species and the community. Three initial replicates represented start values for all treatments and in total 63 flasks were placed in the baths. Furthermore, sampling was carried out in the start, middle and end of the experiment and the flasks were discarded after each sampling. To avoid nutrient limitation, concentrated nutrients were added at day 5 and day 11. One nutrient "shot" (AlgaBoost$^{TM}$ f/2x200) containing 5ml was diluted in 10ml of f/2 medium. Volumes of 200 µl were thereafter added to all replicates.

### *2.2. Nutrient analysis*

At the start of the experiments (day = 0) sample of 105 ml of the f/2 medium were sent for analysis to LMI AB. In the middle (d = 7) and end (d = 14) samples of 35 ml was taken from each replicate, pooled (35 ml x 3 = 105 ml) and sent for inorganic nutrient analysis to LMI AB.

### *2.3. Microalgal cell counting*

Samples of 3 ml from each replicate were collected in 5 ml vials. The samples were fixed with 2-3 drops of Lugol's solution and thereafter stored dark and in room temperature (ca 20°C). Counting chambers (Sedgewick Rafter Chamber) were used for microalgal cell counts. Cells were counted in an inverted light microscope (Nikon ECLPSE Ts2). The specific growth rate for the two species and community in all treatment was calculated using the formula:

$$\mu = \frac{ln\frac{N_1}{N_0}}{t_1 - t_0}$$

Where, $N_1$ are the number of cells at the end (d = 14) of the experiment and $N_0$ the number of cells at the beginning (d = 0) divided by t, which is the duration of the experiment. Furthermore, a camera connected to the microscope was used to photograph each grid in the counting chamber for further analysis in the open software InkScape (InkScape 1.1.2). The camera was operated with the software IC Capture version 2.5 (The Imaging Source Europe GmbH, Bremen, Germany).

### *2.4. Photosynthetic pigments – HPLC analysis*

Samples of 40 ml for the initial flasks and 23 ml for middle and end flasks, from each replicate were preserved in 50 ml centrifuge vials and stored in - 40°C. Samples were later thawed and subsequently centrifuged for 15 minutes at 5000G (ca 5000rpm). The supernatant was carefully removed using a pipette. Algal pellets/cells were extracted in 2 ml acetone/methanol (80:20) whilst sonicated using a Vibra-cell sonicating probe operating at 80 W for 45 s. Consequently, the samples were filtered through a 0,2 µm PFTE syringe filter into brown glass vials. High performance liquid chromatographic (HPLC) analysis was performed according to Wright & Jeffrey (1997), using an absorbance diode array-based detector (Spectraphysics UV6000LP). AC18 column (Kinetex) was

used for separation. Furthermore, pigments were identified by their retention time and absorbance spectra (400-700 nm).

### *2.5. Pulse Amplitude Modulated Fluorometer*

By measuring the effective quantum yield ($F_v'/F_m'$) in the diatoms, photosynthetic activity in photosystem II (PSII) was estimated. The yield is a parameter that describes how well phytoplankton can assimilate light or photosynthesis and is an indicator of functional performance of algae (Akimov & Solomonova, 2019). This was done using a Pulse Amplitude Modulated fluorometer (PAM) (Water PAM, Walz GmbH, Effeltrich, Germany) in connection to a computer with the WinControl software (Walz GmbH). Samples of 2 ml were collected from each replicate and the measurements were done in the emitter-detector unit of the cuvette version. By applying a low level of light, minimum fluorescence ($F_0'$) was determined, while maximum fluorescence ($F_m'$) was determined by exposing the sample to a short saturation pulse of measuring light (>1500 µmol photons $m^{-2}$ $s^{-1}$ for 0.6 s). The yield was calculated according to $(F_m' - F_0')/F_m' = F_v'/F_m'$ and was determined for all samples. Moreover, additional measurements of $F_v'/F_m'$ were conducted five days after replicates from treatment 3 were moved to the water bath of treatment 1, with the aim to examine the recover capacity of the diatoms.

### *2.6. Statistical methods*

Temperature response in biomass and growth rates were tested using one-way ANOVA in Microsoft Excel Add-in package. Significance was tested on a level of $\alpha = 5\%$ and is indicated by using the words significant ($p < 0.05$) or non-significant ($p > 0.05$) in the results. The one-way ANOVA was used to assess differences in cell numbers, growth rates and photosynthetic pigments in *Diatoma* sp. and *Nitzschia* sp. as well as to assess photosynthetic pigments in the algal community, between the different temperature scenarios (treatment 1, 2 and 3). The experimental method was planned as a factorial design, where temperature is the main factor to be tested. Thus, time was excluded. In addition, since one-way ANOVA was carried out for three groups, pairwise comparisons were conducted as a post hoc test, to test for where statistical differences were found. Lastly, to check if data were normally distributed (homogeneity of variance) prior to conducting the one-way ANOVA, Cochran's test was used. For the algal community, the variance was heterogenous also after log transformation. Nevertheless, the ANOVA was run but the possible significant results should be carefully interpreted.

# 3. Results

## *3.1. Nutrient levels*

The primary objective of the present study is to examine how temperature influences the growth rates and biomass of benthic diatoms. However, if SAF intends to cultivate their algae using fish effluent water from Smögenlax, it is imperative to measure the removal efficiency and assess the influence of temperature on it. Nevertheless, it remains vital to determine the percentage change in macronutrients levels, including nitrogenous compounds (ammonium and nitrate), phosphorous and silica, to determine whether the diatoms experienced any nutrient limitations during the experimental phase.

### *3.1.1. Diatoma* sp.

In *Diatoma* sp., the levels of nitrate remained rather stable and high during the experimental period, with a percentual change in nutrient levels for nitrate at 6% (a decrease from 180 to 170 mg/l), for all treatments (Table 2). For phosphorous, the percentual change between day 0 and 14 was overall high in all treatments (86-96%). The initial concentration of phosphorus was 38 mg/l and by day 14, levels had decreased to under 6 mg/l in all treatments. Ammonium was generally under the level of detection. However, it can be noted that levels increased between start and end for all treatments. Lastly, the percentual change in silica levels were estimated to 36% in treatments 1 and 2 and 9% in treatment 3. The initial concentration of silica was 7 mg/l and by day 14 levels had decreased to 4,5 mg/l in treatment 1 and 2 and 6,4 mg/l in treatment 3.

**Table 2** Percentual change in nutrient levels of *Diatoma* sp. in mg/l at the start (d = 0) and end (d = 14) of the experimental period for all experimental treatments. Nutrients below the level of detection is depicted by < 0,1.

| ***Diatoma* sp.** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Nutrient level (mg/l) | | | | | | | | |
| **Element** | **Treat. 1 d = 0** | **Treat. 1 d = 14** | **Change %** | **Treat. 2 d = 0** | **Treat. 2 d = 14** | **Change %** | **Treat. 3 d = 0** | **Treat. 3 d = 14** | **Change %** |
| **Nitrate** | 180 | 170 | -6 | 180 | 170 | -6 | 180 | 170 | -6 |
| **Phosphorous** | 38 | 4,5 | -88 | 38 | 1.7 | -96 | 38 | 5.3 | -86 |
| **Ammonium** | < 0.1 | 0.2 | N/A | < 0,1 | 0.11 | N/A | < 0,1 | 0.1 | N/A |
| **Silica** | 7 | 4.5 | -36 | 7 | 4.5 | -36 | 7 | 6.4 | -9 |

### *3.1.2. Nitzschia* sp.

Between day 0 and 14, *Nitzschia* sp. in treatments 1 and 2 showed an 11% decrease in nitrate concentrations, while treatment 3 showed no percentual change (Table 3). Levels never decreased below 160 mg/l. Treatment 1 and 2 exhibited high percentual change in phosphorous (95 and 96% respectively), with concentrations declining from 38 mg/l to 1,5 and 2,1 mg/l respectively from day 0 to 14. However, the removal efficiency was substantially lower in treatment 3 at 27%, with a concentration of 27 mg/l at day 14. Treatment 2 had the highest percentual change in silica levels (41%) between day 0 and 14, compared to treatment 1 and 3 (21% and 14% respectively). The initial concentration of silica was 7 mg/l and levels at day 14 levels were 4,1 mg/l in treatment 2. Ammonium was not detected at day 0, and percentual change could therefore not be determined, although levels had increased at day 14.

**Table 3** Percentual change in nutrient levels of *Nitzschia* sp. in mg/l at the start (d = 0) and end (d = 14) of the experimental period for all experimental treatments. Nutrients below the level of detection is depicted by < 0,1.

| ***Nitzschia* sp.** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Nutrient level (mg/l) | | | | | | | | |
| **Element** | **Treat. 1 d = 0** | **Treat. 1 d = 14** | **Change %** | **Treat. 2 d = 0** | **Treat. 2 d = 14** | **Change %** | **Treat. 3 d = 0** | **Treat. 3 d = 14** | **Change %** |
| **Nitrate** | 180 | 160 | -11 | 180 | 160 | -11 | 180 | 180 | 0 |
| **Phosphorous** | 38 | 2.1 | -95 | 38 | 1,5 | -96 | 38 | 27 | -29 |
| **Ammonium** | < 0.1 | 0.13 | N/A | < 0.1 | 0.14 | N/A | < 0.1 | 0.72 | N/A |
| **Silica** | 7 | 5,5 | -21 | 7 | 4.1 | -41 | 7 | 6 | -14 |

*3.1.3. Algal community*

Percentual change in levels of nitrate was estimated to 6% for treatments 1 and 2 and 0% for treatment 3 (Table 4). Concentrations never dropped below 170 mg/l, which is almost the same as the initial concentration of 180 mg/l. Removal of phosphorous were generally high and percentual change between day 0 and 14 were over 90% for all treatments. Levels of phosphorous decreased from 38 mg/l to around 2-3 mg/l for all treatments. Ammonium was under the level of detection at day 0 for all treatments. However, levels had increased at day 14. Exact calculations of percentual change were not possible. Lastly, percentual change in silica was rather high in treatment 1 and 2 (50 and 57% respectively) and levels dropped from 7 mg/l at day 0 to between 3-3,5 mg/l at day 14. Concentration of silica in treatment 3 did not vary greatly between day 0 and 14.

**Table 4** Percentual change in nutrient levels of the algal community in mg/l at the start (d = 0) and end (d = 14) of the experimental period for all experimental treatments. Nutrients below the level of detection is depicted by < 0,1.

| **Algal community** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Nutrient level (mg/l) | | | | | | | | |
| **Element** | **Treat. 1 d = 0** | **Treat. 1 d = 14** | **Change %** | **Treat. 2 d = 0** | **Treat. 2 d = 14** | **Change %** | **Treat. 3 d = 0** | **Treat. 3 d = 14** | **Change %** |
| **Nitrate** | 180 | 170 | -6 | 180 | 170 | -6 | 180 | 180 | 0 |
| **Phosphorous** | 38 | 3.2 | -92 | 38 | 2,3 | -94 | 38 | 2.6 | -93 |
| **Ammonium** | < 0.1 | 0.41 | N/A | < 0.1 | 0.18 | N/A | < 0.1 | 0.16 | N/A |
| **Silica** | 7 | 3.5 | -50 | 7 | 3 | -57 | 7 | 6.3 | -10 |

*3.2. Photosynthetic activity*

*3.2.1. Diatoma* sp.

Temperature driven differences in effective quantum yield ($F_v'/F_m'$) were detected in *Diatoma* sp. The highest values were identified in treatment 2, followed by treatment 1 with a peak at day 7 in both treatments (Fig. 4). Interestingly there was a slight decline in $F_v'/F_m'$ in treatment 1 and 2 between day 7 and day 14. Throughout the experimental period the lowest values of $F_v'/F_m'$ could be observed in treatment 3. Overall values were never critically low in any of the treatments.

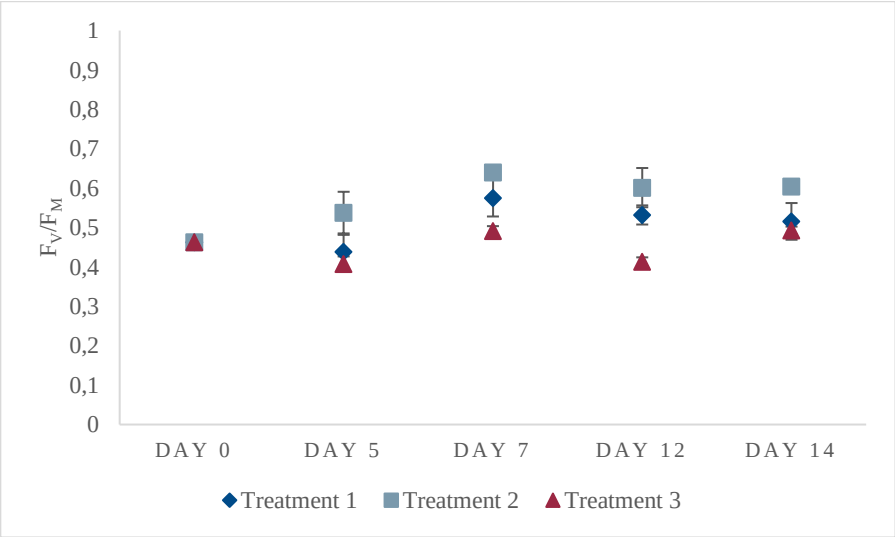



**Fig. 4** Scatter plot over effective quantum yield ($F_v'/F_m'$) for *Diatoma* sp. in all treatments over the experimental period. Data represents mean values (n = 3) ± SD.

*3.2.2. Nitzschia* sp.

Values of $F_v'/F_m'$ in treatment 2 were the highest as well as the most stable ones, with only minimal variations between day 0 and day 14 (Fig. 5). Furthermore, values in treatment 1 were slightly lower in comparison. In addition, values of photosynthetic activity dropped at day 12 in treatment 1. The lowest values of $F_v'/F_m'$ could be observed in treatment 3 and decreased over the experimental period.

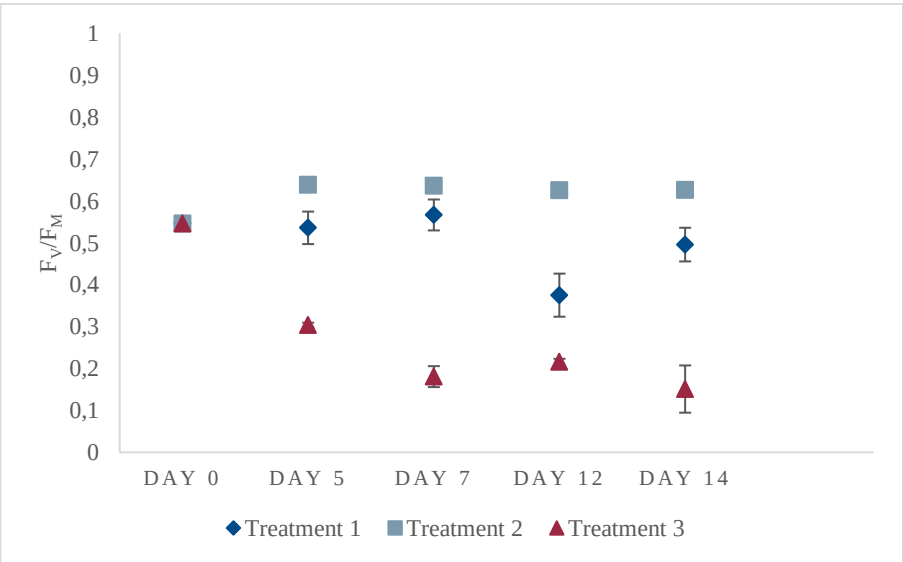



**Fig. 5** Scatter plot over effective quantum yield of PSII ($F_v'/F_m'$) for *Nitzschia* sp. in all treatments over the experimental period. Data represents mean values (n = 3) ± SD.

*3.2.3. Algal community*

For the algal community values of $F_v'/F_m'$ in treatment 2 were the highest (Fig. 6). However, measurements on day 7 and day 12 shows that values in treatment 1 were similar to values in treatment 2. In addition, values of photosynthetic activity dropped at day 12 in treatment 1 and 2. The lowest values of $F_v'/F_m'$ were found in treatment 3 and a decreasing trend could be observed over the experimental period, with a minor deviation at day 7.

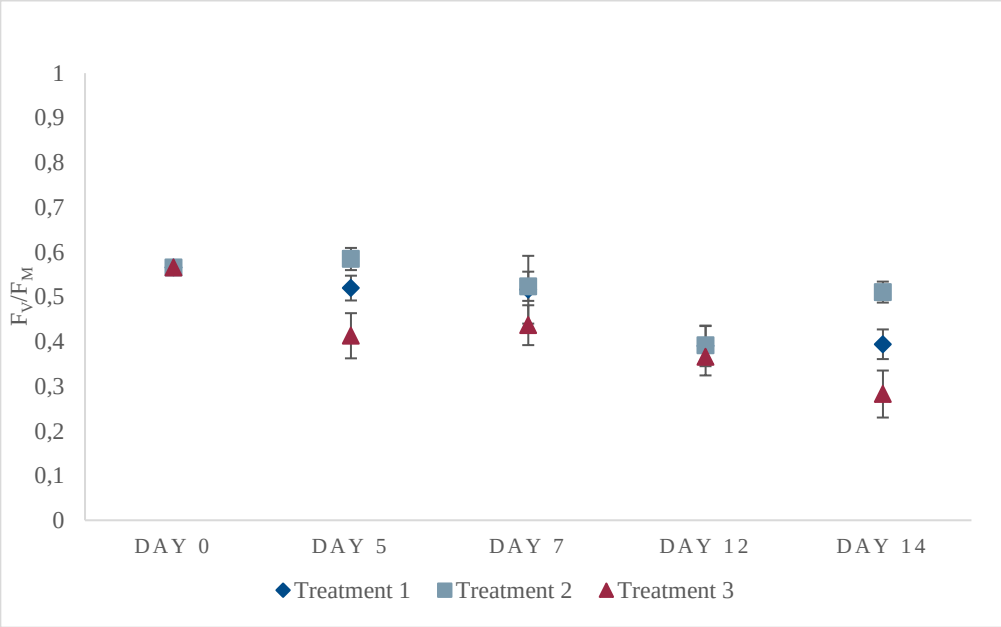



**Fig. 6** Scatter plot over effective quantum yield of PSII ($F_v'/F_m'$) for the *Algal community* in all treatments over the experimental period. Data represents mean values (n = 3) ± SD.

*3.3. Photosynthetic pigments*

Typical HPLC chromatograms are shown in Fig. 7a and 7b, and the peaks of interest for this study are indicated.

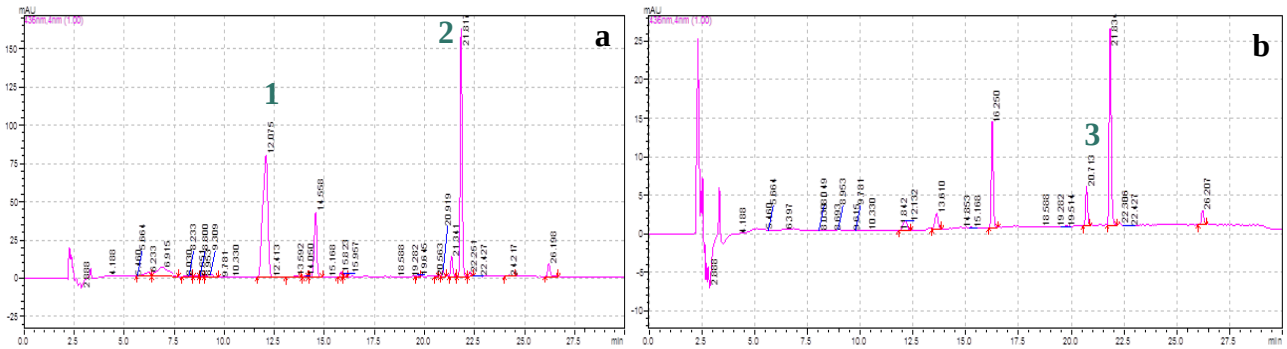



**Fig. 7** The figures illustrate chromatograms from the HPLC analysis of photosynthetic pigments, where the x-axis displays retention time (in minutes) and the y-axis show the milli-absorbance unit (mAU). The peaks of interest for this study are indicated (1) Fucoxanthin (12.08), (2) chlorophyll *a* (21.89) and (3) chlorophyll *b* (20.71).

*3.3.1. Diatoma* sp.

Clear temperature trends or changes in chlorophyll *a* could be identified for *Diatoma* sp. Throughout the experimental period, the concentration increased in all treatment, with treatment 2 (ca 500 µg/l) exhibiting a statistically significant higher concentration ($p < 0.05$) compared to treatments 1 and 3 at the end of the experiment (d = 14) (Fig. 8a). The concentration of chlorophyll *a* in treatment 3 were comparable to that in treatment 1, with both treatments showing concentrations of ca 260 µg/l and 270 µg/l, respectively at the end of the experiment (d = 14). Furthermore, fucoxanthin was present in all treatments (Fig. 8b). Concentration increased between treatments 1 and 2 ($p < 0.05$). In addition, concentration in treatment 3 were significantly lower ($p < 0.05$) in comparison to both treatment 1 and 2 at day 14. Lastly, chlorophyll *b* was only observed in treatment 3 (Fig. 8c).

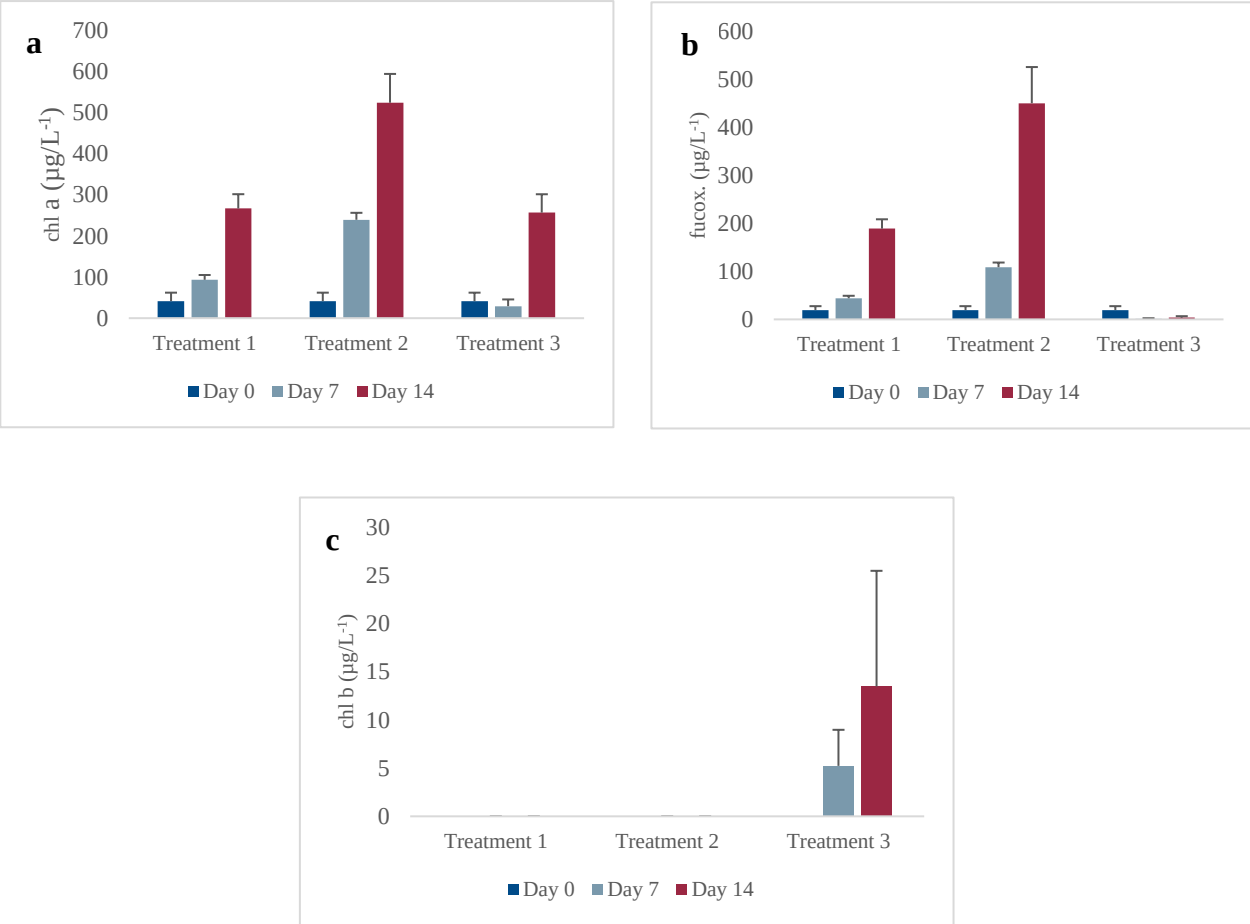



**Fig. 8** Bar plots over photosynthetic pigments (**a**) chlorophyll *a*, (**b**) fucoxanthin and (**c**) chlorophyll *b* in µg/l for *Diatoma* sp. for all treatments at day = 0, day = 7 and day = 14. Data represents mean values (n = 3) + SD.

*3.3.2. Nitzschia* sp.

Variations in chlorophyll *a* were noted for *Nitzschia* sp., across the different treatments (Fig. 9a). A significant increase in concentration was observed between treatments 1 and 2 ($p < 0.05$), with treatment 2 exhibiting the highest at around 400 µg/l in day 14. Conversely, chlorophyll *a* concentration in treatment 3 was significantly lower ($p < 0.05$) compared to both treatment 1 and 2. Furthermore, all treatments were found to contain fucoxanthin (Fig. 9b), with treatment 3 showing a concentration of 4 µg/l by day 14, significantly lower ($p < 0.05$) compared to both treatment 1 and 2. In addition, the concentration of fucoxanthin increased between treatment 1 and

2 by day 14 ($p < 0.05$), peaking at ca 750 µg/l. Interestingly, chlorophyll *b* was not detected in *Nitzschia* sp. in any of the treatments.

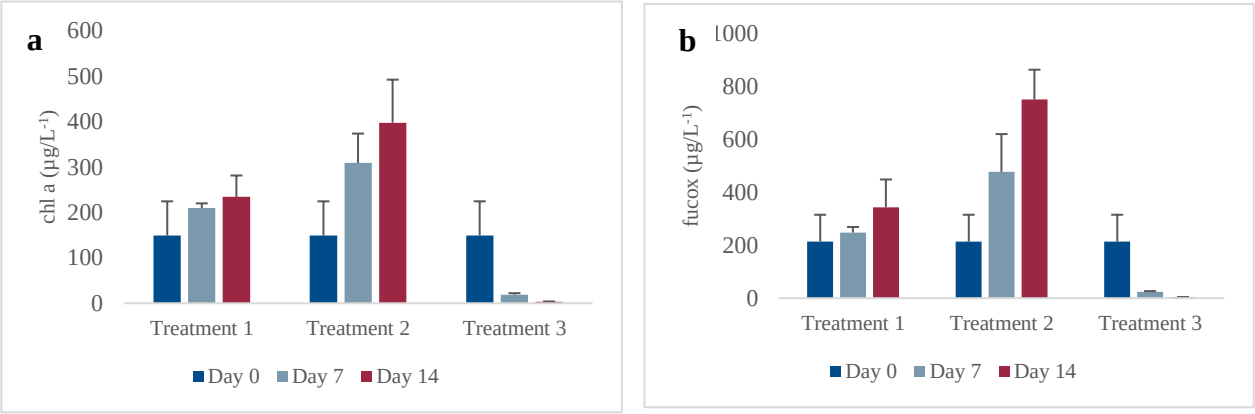
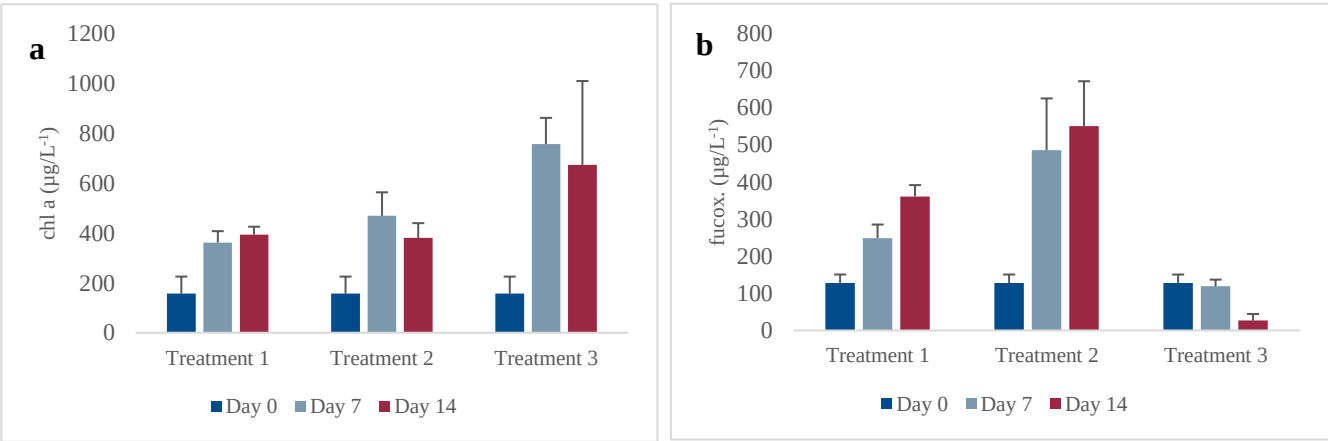



**Fig. 9** Bar plots over photosynthetic pigments (**a**) chlorophyll *a*, (**b**) fucoxanthin in µg/l for *Nitzschia* sp. for all treatments at day = 0, day = 7 and day = 14. Data represents mean values (n = 3) + SD.

### *3.3.3. Algal community*

Temperature driven differences in chlorophyll *a* were observed between treatments in the algal community (Fig. 10a). The highest concentration of chlorophyll *a* was detected in treatment 3, yet in comparison to treatments 1 and 2, no statistically significant differences were detected ($p > 0.05$). Furthermore, the concentration of chlorophyll *a* was lower in treatment 1 compared to treatment 2, but the difference was not statistically significant ($p > 0.05$). Moreover, fucoxanthin was identified in all treatments (Fig. 10b). Concentrations had increased between treatment 1 and 2 by day 14, but not significantly ($p > 0.05$). In treatment 3, however, concentrations of fucoxanthin were significantly lower ($p < 0.05$) compared to both treatment 1 and 2. Chlorophyll *b* was observed in all treatments, with the highest values observed in treatment 3 (Fig. 10c). Concentrations were lower in treatments 1 and 2. However, observed differences between the treatments were never statistically significant ($p > 0.05$).



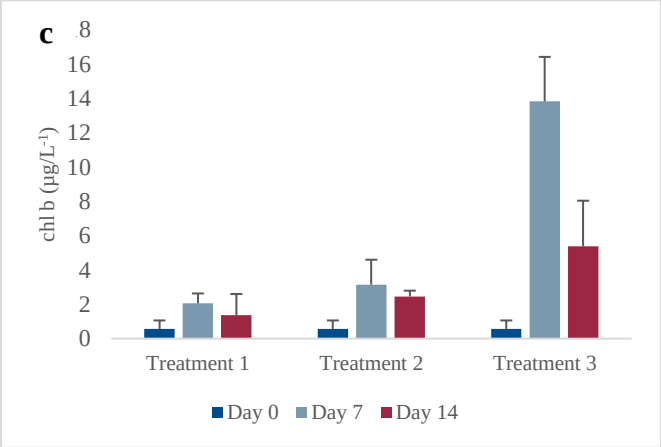



**Fig. 10** Bar plots over photosynthetic pigments (**a**) chlorophyll *a*, (**b**) fucoxanthin and (**c**) chlorophyll *b* in µg/l for the algal community for all treatments at day = 0, day = 7 and day = 14. Data represents mean values (n = 3) + SD.

*3.3.4. Pigment ratios*

In *Diatoma* sp., the pigment ratio of fucoxanthin to chlorophyll *a*. (Fig. 11a) showed an increase between treatment 1 and 2 by day 14. Conversely, the ratio had declined in treatment 3 by day 14. Furthermore, the ratio between chlorophyll *b* and *a* in *Diatoma* sp. (Fig. 11b) could not be determined in treatment 1 and 2 due to the absence of chlorophyll *b*. However, in treatment 3, the ratio decreased from day 7 to 14. In *Nitzschia* sp., the ratio between fucoxanthin and chlorophyll *a* (Fig. 12) was highest in treatment 2 and lowest in treatment 3 by day 14. Moreover, since chlorophyll *b* was not detected in *Nitzschia* sp. no ratio was calculated. The algal community exhibited an increase in the fucoxanthin and chlorophyll *a* ratio (Fig. 13a) between treatments 1 and 2. Furthermore, in treatment 3, the trend was observed to be decreasing, with the lowest ratio observed at day 14. The ratio between chlorophyll *b* and *a* (Fig. 13b) was highest in treatment 3, with a peak on day 7. Ratios in treatments 1 and 2 were rather similar.

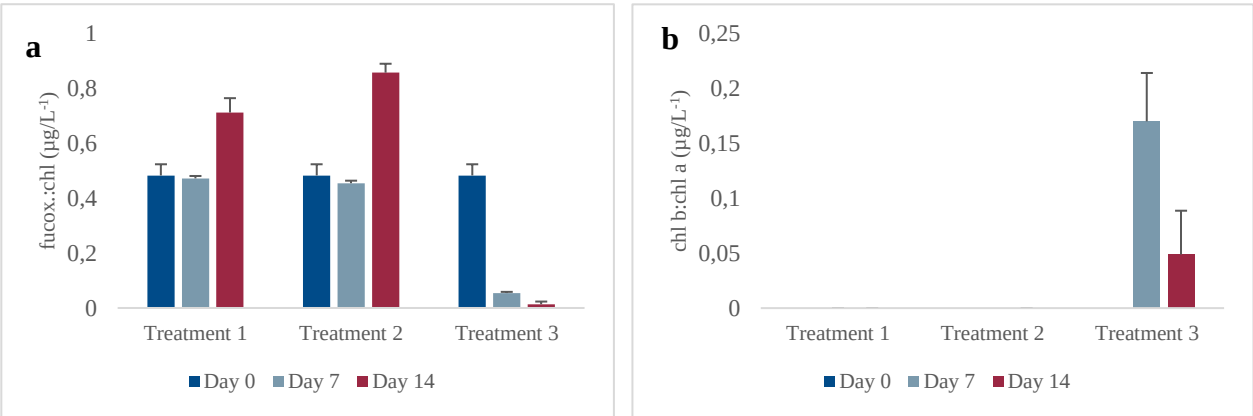



**Fig. 11** Bar plots with photosynthetic pigment ratios for (**a**) fucoxanthin/chlorophyll *a* and (**b**) chlorophyll *b*/chlorophyll *a* for *Diatoma* sp. Data represents mean values (n = 3) + SD.

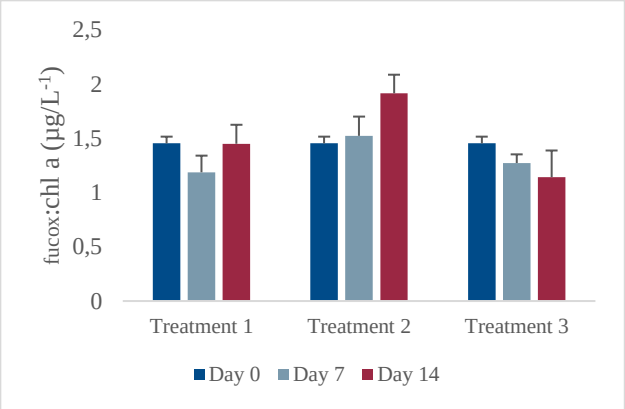



**Fig. 12** Bar plots with photosynthetic pigment ratios for fucoxanthin/chlorophyll *a* for *Nitzschia* sp. Data represents mean values (n = 3) + SD.

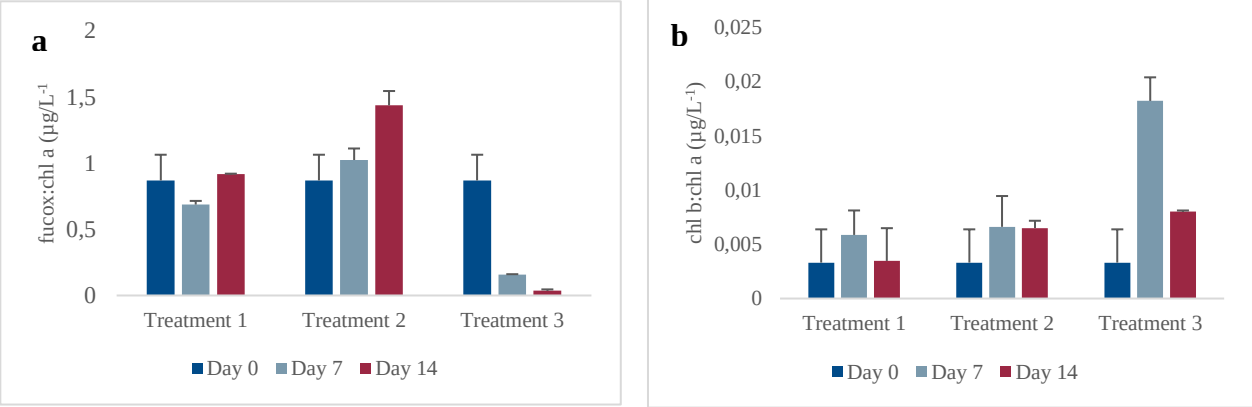



**Fig. 13** Bar plots with photosynthetic pigment ratios for (**a**) fucoxanthin/chlorophyll *a* and (**b**) chlorophyll *b*/chlorophyll *a* for the algal community. Data represents mean values (n = 3) + SD.

### *3.4. Cell density*

Table 5 displays the initial and final cell density, as well as the percentage change for *Diatoma* sp. and *Nitzschia* sp. at the start (d = 0) and the end (d = 14) of the experiment. Cells per litre of *Diatoma* sp. had increased by day 14 in treatment 1 and 2, by 274% and 105% respectively. Conversely, cells per litre had decreased by 98% in treatment 3. Furthermore, *Nitzschia* sp. showed an increase in cells per litre in treatment 1 and 2, with 81% and 212% respectively. However, treatment 3 did not reveal any *Nitzschia* sp. cells, resulting in a decrease in cells per litre by the end of the experiment (d = 14).

**Table 5** Cell density in cells per litre at the start (d = 0) and the end (d = 14) of the experiment, including percentual change. Data represent mean values (n = 3).

| Cells/L | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Treatment 1** | | | **Treatment 2** | | | **Treatment 3** | | |
| | d = 0 | d = 14 | Change % | d = 0 | d = 14 | Change % | d = 0 | d = 14 | Change % |
| ***Diatoma* sp.** | $38.6*10^6$ | $144.3*10^6$ | 274 | $38.6*10^6$ | $79*10^6$ | 105 | $38.6*10^6$ | $0.51*10^6$ | -98 |
| ***Nitzschia* sp.** | $398.7*10^6$ | $722*10^6$ | 81 | $398.7*10^6$ | $1245*10^6$ | 212 | $398.7*10^6$ | 0 | -100 |

When analysing the data presented in Fig. 14a it is evident that *Diatoma* sp. showed significantly ($p < 0.05$) higher cell numbers in treatment 1, compared to treatment 2. In addition, treatment 3 presents significantly lower ($p < 0.05$) cell numbers compared to both treatment 1 and 2. In *Nitzschia* sp. cell density was significantly higher ($p < 0.05$) in treatment 2 compared to treatment 1 (Fig. 14b). As no cells were detected in treatment 3, statistical differences in cell numbers were not compared to the other treatments.

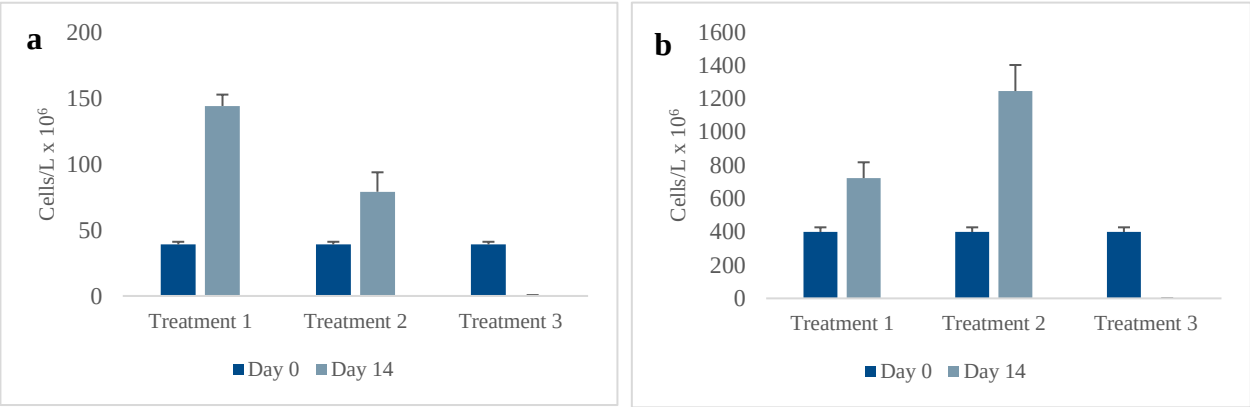



**Fig. 14** Bar plots displaying cells per litre for (**a**) *Diatoma* sp. and (**b**) *Nitzschia* sp. measured at the start (d = 0) and the end (d = 14) of the experiment. The bar plot depicts the two species grown in treatment 1, 2 and 3. Data represents mean values (n = 3) + SD.

### *3.5. Growth rates*

In *Diatoma* sp. treatment 1 displayed a significantly ($p < 0.05$) higher growth rate when compared to treatment 2 (Table 6). Additionally, treatment 3 exhibited a negative growth rate, indicating a decrease in the number of cells during the experimental period, and the growth rate were thus significantly different ($p < 0.05$) compared to treatments 1 and 2. In the case of *Nitzschia* sp. treatment 2 demonstrated a significantly higher growth rate ($p < 0.05$) compared to treatment 1. However, as no cells were detected in treatment 3 during the cell counting process, the growth rate is not statistically comparable to the other treatments.

**Table 6** Growth rates ($d^{-1}$) in *Diatoma* sp. and *Nitzschia* sp. in all treatments. Data represents mean values (n = 3) ± SD.

| | **Growth rate ($d^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | Treatment 1 | | Treatment 2 | | Treatment 3 | |
| ***Diatoma* sp.** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| | 0.094 | ± 0.004 | 0.05 | ± 0.014 | -0.31 | ± 0.019 |
| ***Nitzschia* sp.** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| | 0.042 | ± 0.009 | 0.081 | ± 0.009 | 0 | ± 0 |

*3.6. Visual observations*

Visual observation indicated the presence of green algae or cyanobacteria in *Diatoma* sp. treatment 3, both in the sample during cell counts and, in the flasks (Fig. 15).

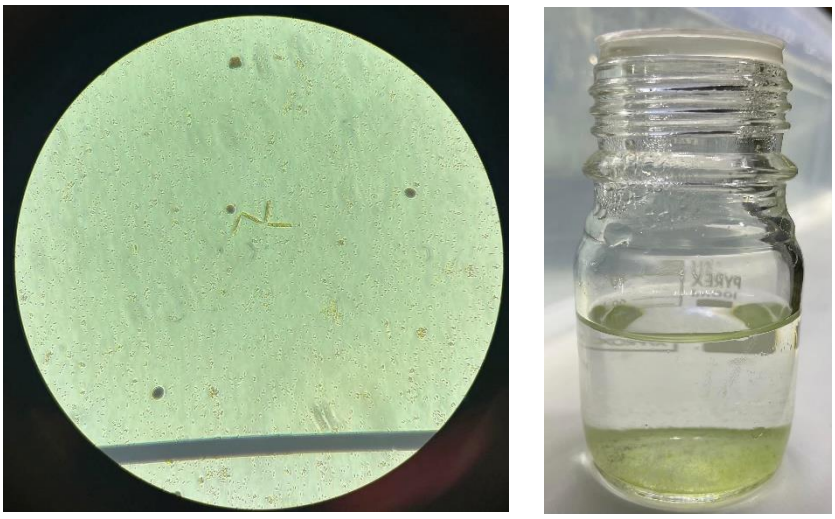


**Fig. 15** Visual observation of *Diatoma* sp. treatment 3 at the end of the experiment (d = 14), showing indications of the presence of green algae or cyanobacteria.

Microscope images of the algal community in treatment 1, 2 and 3 at the end of the experiment (d = 14), indicating the presence of both green algae and cyanobacteria gradually increasing as temperatures are elevated (Fig. 16).

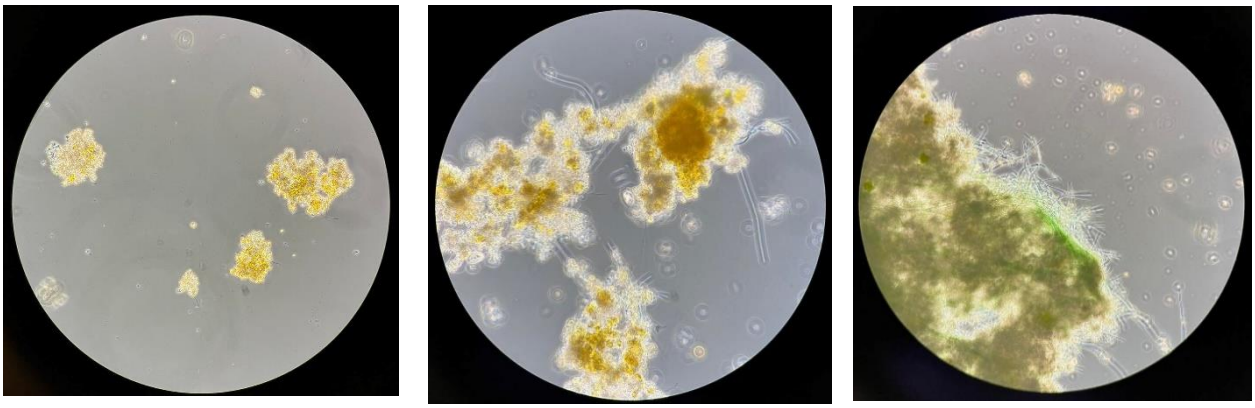


**Fig. 16** Microscope images of the algal community in treatment 1, 2 and 3 at the end of the experiment (d = 14), indicating a gradual increase in green algae and cyanobacteria as temperature increases.

*3.7. Recovery capacity*

Additional measurements of *Nitzschia* sp. showed an increased yield after five days of recovery. The mean value of $F_v'/F_m'$ for *Nitzschia* sp. in treatment 3 on day 14 was 0.151, which increased to a mean value of 0.419 after five days, suggesting a recovery of photosynthetic activity (Fig. 17).

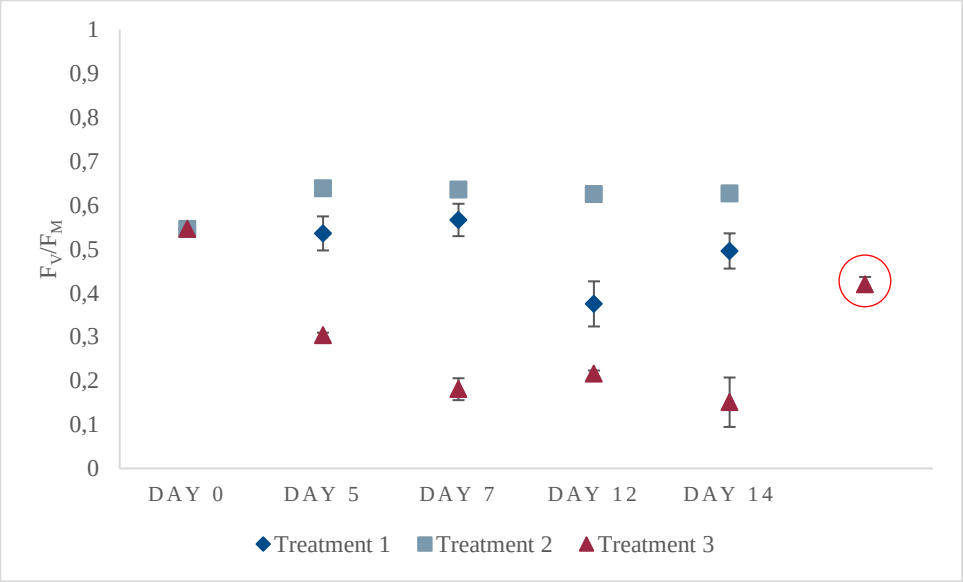



**Fig. 17** Scatter plot over effective quantum yield of PSII ($F_v'/F_m'$) for *Nitzschia* sp. in all treatments over the experimental period with an additional measurement value after five days of recovery (circled in red). Data represents mean values (n = 3) ± SD.

# 4. Discussion

In this section, an analysis is presented regarding the primary findings, with a specific emphasis on the influence of temperature on biomass and growth rates of *Diatoma* sp., *Nitzschia* sp., and the algal community. Furthermore, a discourse is provided regarding nutrient levels in relation to temperature along with the inclusion of methodological considerations.

## *4.1. Nutrient levels*

### *4.1.1. Levels of nitrogenous compounds*

Studies suggest that diatoms are strongly associated with nitrate uptake, although they can also utilize ammonium as a nutrient source (Andersen et al., 2020; Liu et al., 2022). In general, ammonium levels ranged between 0,1-0,2 mg/l, which is higher compared to natural concentrations in seawater (Bydén et al., 2003). In addition, as a result of supplementary nutrients added during the experimental period, nitrate levels in all treatments for the two species and the community never fell below 160 mg/l, which is higher than natural concentrations found in seawater (Bydén et al., 2003). Moreover, diatoms are known for their high removal rates of nitrate, with some studies reporting removal efficiency of up to 60% (Saxena et al., 2022). However, temperature is proposed to be a significant limiting factor for nitrate uptake in diatoms (Berges et al., 2002). Therefore, if SAF intends to utilize fish effluent water as their cultivation medium, examining the nitrate removal efficiency under varying temperature conditions would be of great interest. In addition, nitrogen limitations may hinder thermal adaptation in diatoms, leaving them vulnerable to high temperatures (Aranguren-Gassis et al., 2019), This is another important consideration for SAF, particularly during the summer months when temperatures may rise.

### *4.1.2. Levels of phosphorous*

Furthermore, removal of phosphorous was high in all treatments in the two species and the community. Compared to nitrate, removal of phosphorous was evidently higher which may be attributed to a capacity to store phosphate in the diatoms. Percentual change was between 88-96% in treatment 1 and 2, which is comparable to previous studies on removal efficiency of phosphorous in diatoms (Bhattacharjya et al., 2021). Despite the high removal rates, levels of phosphorous were still sufficient in all treatments in the two species and the community, when compared to natural concentrations in the marine ecosystem (Bydén et al., 2003). Moreover, in *Nitzschia* sp. treatment 3, removal efficiency was substantially lower, which can potentially be explained by the higher temperatures causing a decrease in photosynthetic activity in the diatoms, which in turn leads to a reduced demand for nutrients to stimulate cell division and growth (Sheehan et al., 2020). However, removal efficiency for *Diatoma* sp. and the algal community in treatment 3 were still high (86 and 93% respectively) despite the warm temperature. A possible explanation for this might be that other organisms present in these samples continued to thrive in the warmer temperature and assimilate phosphorous, even though the diatoms were declining.

### *4.1.3. Levels of silica*

Since diatoms depend on silica for the formation of their frustules (Bydén et al., 2003), ensuring an adequate supply of silica is crucial. The percentual change in levels of silica varied among treatments, with treatment 2 showing the highest total removal for the two species and the community. The initial concentration of silica was 7 mg/l for all treatments and by day 14, the lowest measured concentration had more than halved to 3 mg/l. Determining whether the diatoms have experienced limited access to silica is challenging, as natural concentrations in seawater can vary widely between 0-12 mg/l (Bydén et al., 2003). Moreover, studies have shown that frustule silicification in diatoms decreases linearly with increasing temperature, as observed by (Baker et

al., 2016) and consistent with the results of the present study. Specifically, removal of silica was lowest in treatment 3 for the two species and the community, which may have implications on diatom growth rates and biomass, as silica is an essential component in their frustule production. Additionally, diatoms can generate organic material with silica constituting up to 50% of their dry weight (Bydén et al., 2003). Considering that silica is a key component in the production of Algica at SAF, it is of importance to consider how temperature may impact silica availability and uptake during cultivation.

### *4.2. Photosynthesis, biomass, and growth rates*

Temperature plays a crucial role in determining the structure and abundance of microalgal community. It also has a significant impact on the physiology of microalgae (Sheehan et al., 2020). An increase in temperature below optimum have been shown to have a positive effect on growth rates. However, if the temperature optimum is being exceeded, microalgae are at risk of experiencing heat stress, which can cause growth rates to decrease (Akimov & Solomonova, 2019; Sheehan et al., 2020). The effects of increasing temperatures during summer will be noticeable in water temperatures in the SAF greenhouse. It is therefore crucial to understand how diatoms will respond to rising temperatures, to sustain growth and microalgal biomass production.

Moreover, in the present study, two commonly used methods were selected to quantify microalgal biomass: cell counting and analysing photosynthetic pigments. The occurrence of photosynthetic pigments varies among photosynthetic organisms. For example, diatoms contain chlorophyll *a* and *c*, as well as various carotenoids like fucoxanthin, while phytoplankton, like green algae, possesses chlorophyll *a* and *b* (Jeffrey et al., 1997). Therefore, in this study, measurements of different photosynthetic pigments serve not only as a means to quantify biomass, in addition to cell numbers, but also as an indicator of the type of microalgae that are present in the replicates.

Furthermore, values of $F_v'/F_m'$ were considered for the two species and the algal community in all treatments, with the aim to evaluate photosynthetic activity in the diatoms (i.e., used as a crude measurement of the health of the diatoms) during the experimental period. Furthermore, the values of $F_v'/F_m'$ may be influenced by various environmental factors (Gan et al., 2019). Nutrient limitation in the diatoms could for instance result in a lower $F_v'/F_m'$ and can thus be used as an indicator for nutrient limitation (Tan et al., 2019). In addition, excessive exposure to light may result in dynamic photoinhibition of diatoms, which may also impact values of $F_v'/F_m'$ (A. Wulff personal communication, March 15$^{th}$ 2023). Nonetheless, $F_v'/F_m'$ values do not provide any information regarding the carbon incorporation process. Considerations of high respective low values of $F_v'/F_m'$ are done in relation to "standard" maximum values, where Tan et al. (2019) found that 14 species of algae in sufficient nutrients conditions displayed a maximum in $F_v'/F_m'$ ranging from 0.43 to 0.72 (with an average of 0.60 for 5 diatom species). This demonstrates that these "maximal" values differ across various groups, such as diatoms, green algae, and cyanobacteria (Tan et al., 2019).

#### *4.2.1. Diatoma* sp.

Results of chlorophyll *a* in *Diatoma* sp., displayed that an increase in temperature from treatment 1 to 2 led to an increase in chlorophyll *a*. However, in treatment 3 where temperatures were elevated further, the chlorophyll *a* decreased compared to treatment 2. Moreover, treatment 1 and 2 displayed increasing growth rates from the start (d = 0) to the end of the experiment (d = 14), while treatment 3 displayed a decline in growth rate. These results suggests that the thermal tolerance level for *Diatoma* sp. is found below 30°C. Previous studies have shown that thermal optimum for

growth rate in benthic diatoms is around 20°C (e.g. Admiraal, 1977; Stock et al., 2019), which could potentially be the case in the present study. However, it should be noted that the thermal optimum differs between species and strains of diatoms. For instance, Adenan et al. (2013) found that the tropical benthic diatom *C. calcitrans* achieved optimal growth rates at 30°C, which is not consistent with the findings in the present study. In addition, some studies have even found that there are no observed effects on growth rates within a temperature range of 10 to 30°C, and that significant responses are only observed at more extreme temperatures of 4 or 40°C (Scholz & Liebezeit, 2012). Since growth generally aligns with biogeographical origin of strains (Stock et al., 2019), it is not surprising that the optimal temperature varies between different studies.

Furthermore, the analysis of cell density and growth rates in *Diatoma* sp., showed that treatment 1 had a higher growth rate and cell numbers compared to treatment 2. This opposes the outcome of the analysis of photosynthetic pigments (chlorophyll *a* and fucoxanthin) which indicated higher levels in treatment 2 than in treatment 1. This inconsistency can be explained by the abundant presence of another diatom species, thus also containing chlorophyll *a* and fucoxanthin, which was visually identified during cell counting. Therefore, although *Diatoma* sp. displayed a lower growth rate and cell numbers in treatment 2 than in treatment 1, the values of chlorophyll *a* and fucoxanthin were still higher in treatment 2 due to the presence of this other diatom species. Moreover, the results obtained for $F_v'/F_m'$ in *Diatoma* sp., show that the highest values, indicating the highest photosynthetic activity, were observed in treatment 2. However, it is worth noting that measurements of $F_v'/F_m'$ are merely snapshots in time and can rapidly change depending on external factors, such as nutrient availability and light exposure (Tan et al., 2019). Nevertheless, since measurements of biomass and growth rates also indicate high values in treatment 2, it is probable that *Diatoma* sp. were not nutrient limited in this treatment.

Moreover, when comparing the results of chlorophyll *a* with the concentration of chlorophyll *b*, it can be concluded that treatment 1 and 2 exhibited a complete absence of chlorophyll *b*, indicating that the concentration of chlorophyll *a* is likely derived solely from diatoms. However, visual observations in treatment 3, indicated the likely presence of green algae early in the experiment, and when examining the results of chlorophyll *b* in this treatment, concentrations were indeed detected. This observation raises the possibility that the chlorophyll *a* concentration in treatment 3 by day 14, may not solely be attributed to the continued diatom growth, but rather could be indicative of green algae. The findings related to fucoxanthin further supports this observation. The results indicate that treatment 1 and 2 had comparatively higher concentrations of fucoxanthin, while treatment 3 had significantly lower concentrations. Since fucoxanthin are found in diatoms and not in green algae, it aligns with the possibility of green algae being present in treatment 3. Nevertheless, it is worth noting that the analysis of photosynthetic pigments by the HPLC are influenced by cell size, generally resulting in lower pigment concentrations if the cells are smaller. During the cell counting procedure, visual observations indicate that the cell size of *Diatoma* sp. in treatment 3 was considerably smaller than the cells counted in treatment 1 and 2, which could potentially also contribute to the low concentrations of fucoxanthin in treatment 3. However, within a diatom population, mean cell size usually decreases with each cell division, and when the cells reach a minimum size, sexual reproduction is generally induced (Round et al., 1990). No sexual reproduction was observed for *Diatoma* sp., but indeed a large difference in cell size was found. The presence of auxospores as a result of sexual reproduction was later confirmed in the stock culture at SAF (A. Wulff personal communication, May 11$^{th}$ 2023).

Furthermore, considering pigment ratios, *Diatoma* sp. exhibited a relatively low pigment ratio between chlorophyll *a* and *b* in treatment 3, due to rather low values of chlorophyll *b* relative to chlorophyll *a* by the end of the experiment (d = 14). This suggests that although green algae may be present in the treatment, it is not a significant contributor to the overall biomass. In addition, the reduction in the fucoxanthin to chlorophyll *a* ratio towards the end of the experiment implies that diatoms may not be the dominant species in this treatment either. Moreover, visual observations indicate the presence of smaller organisms, possibly cyanobacteria, in treatment 3. Given that cyanobacteria generally thrive in warmer temperatures and contain chlorophyll *a* (Walls et al., 2018), there presence is likely. This assumption is reinforced by the visual confirmation of cyanobacteria in treatment 3 of the algal community, further supporting their potential presence in *Diatoma* sp. treatment 3. Furthermore, the presence of cyanobacteria could have been determined by looking at zeaxanthin in the pigment analysis. However, this pigment is also present in green algae.

*4.2.2. Nitzschia* sp.

The results of the study revealed that chlorophyll *a* increased in treatments 1 and 2 over the experimental period. In addition, chlorophyll *a* was higher in treatment 2 than in treatment 1, suggesting a positive correlation between temperature and chlorophyll *a*. However, this correlation appears to be valid only up to a certain threshold, as evidenced by the decrease in chlorophyll *a* in treatment 3 when the temperature was further increased. In addition, the analysis of fucoxanthin, demonstrated higher concentrations in treatment 1 and 2, reaching a peak in treatment 2, similarly to levels of chlorophyll *a*. Treatment 3 on the other hand, exhibited significantly lower levels of fucoxanthin, close to 0 µg/l by day 14, indicating a rapid decline in diatom biomass in this treatment. This is further supported by the results of $F_v'/F_m'$, which showed a negative correlation between temperature and photosynthetic activity, as values dropped in treatment 3, indicating a decline in photosynthetic activity in this treatment. This is likely not attributed to nutrient limitation or dynamic photoinhibition since values of $F_v'/F_m'$ in treatments 1 and 2 were maintained high, compared to standard maximum values (Tan et al., 2019).

Additionally, an increase in cell density and growth rates were observed as temperature was raised from treatment 1 to 2, which is consistent with previous studies indicating that an increase in temperature below optimum can stimulate growth rates (Sheehan et al., 2020). However, surpassing the optimal temperature range can cause thermal stress and ultimately lead to decreased growth rates (Akimov & Solomonova, 2019), which was observed in *Nitzschia* sp. treatment 3, where no cells were detected during cell counts. This suggests that the thermal tolerance level for *Nitzschia* sp. lies below 30°C, similar to that of *Diatoma* sp. However, in order to establish a more accurate tolerance level, a broader range of temperatures would need to be examined. Furthermore, none of the treatments contained chlorophyll *b* and the presence of green algae can therefore be ruled out. However, visual observations during cell counts show the presence of other types of organisms in treatment 3. It is possible that cyanobacteria could be present in this treatment as well, given that they have been observed in other species and treatments and are known to thrive in warm temperatures (Walls et al., 2018). Nevertheless, values of $F_v'/F_m'$ were low at the end of the experiment in *Nitzschia* sp., treatment 3, in comparison to standard maximum values (Tan et al., 2019), which speaks against the presence or at least the abundance of cyanobacteria in this treatment since these organisms are photosynthesising. Worth noting is that different algal groups have different optimum in $F_v'/F_m'$ (using Water PAM), where cyanobacteria usually display lower values of $F_v'/F_m'$ compared to diatoms (A. Wulff personal communication, May 11$^{th}$ 2023).

*4.2.3. Algal community*

The algal community have a diverse range of organism besides diatoms, such as ciliates, grazers, and other micro-fauna, which posed a challenge in performing cell counts to determine biomass and growth rates. Nevertheless, measurements of photosynthetic pigments were feasible, where the outcomes can be used as a valuable means of quantifying biomass as well as determining the composition of photosynthesising organisms. Moreover, an increase in chlorophyll *a* concentration was observed in the algal community as temperatures were raised over the treatments, with a peak in treatment 3. This indicates a positive correlation between chlorophyll *a* and temperature. Upon examining the results for fucoxanthin however, it was observed that concentrations were low in treatment 3, particularly towards the end of the experiment (d = 14). When also taking the pigment ratio between fucoxanthin and chlorophyll *a* into account, it may be suggested that the high levels of chlorophyll *a* detected in treatment 3 were not generated from diatoms. This indicates that when temperatures rise as high as 30°C there is a decline in diatom biomass, also observed in *Nitzschia* sp. and *Diatoma* sp. Nonetheless, values of fucoxanthin in treatment 1 and 2 were still high, which indicates that diatom biomass was high in these two treatments.

Furthermore, when analysing the concentrations of chlorophyll *b*, it was found that levels were low in treatment 1 and 2, but significantly higher in treatment 3, especially on day 7. Considering that chlorophyll *b* is a pigment found in green algae, it is highly likely that green algae were present in all three treatments, with the highest concentration in treatment 3. This suggests that as temperature increased beyond a certain point, the algal community underwent a shift in species composition, resulting in the dominance of other microalgae, such as green algae, rather than diatoms. This is also supported by visual observations, indicating the presence of green algae as well as filaments of cyanobacteria, and in addition displaying how these elements increased as the temperature was elevated. In addition, even though there was a decline in diatom biomass in treatment 3, values of $F_v'/F_m'$ were kept relatively high compared to standard maximum values (Tan et al., 2019), likely due to the presence of other photosynthesising organisms, such as cyanobacteria and green algae.

*4.3. Recovery capacity*

Results from the additional measurements of $F_v'/F_m'$, showed that *Nitzschia* sp. did indeed display higher photosynthetic activity after five days, indicating a capacity to recover after the evident decline in biomass in treatment 3. However, the presence of other photosynthesizing organisms in *Diatoma* sp. and the algal community in treatment 3, resulted in $F_v'/F_m'$ values not decreasing as drastically as observed in *Nitzschia* sp. As a result, it was challenging to interpret the additional measurements of $F_v'/F_m'$ to identify indications of recovery. To detect indications of recovery in *Diatoma* sp. and the algal community, measuring photosynthetic pigments to determine whether chlorophyll *b* decreased again after the recovery period could have been possible. Additionally, visual observations could have been made to detect an increase in diatom cells or a decrease in green algae/cyanobacteria, and for *Diatoma* sp., additional cell counts could have been performed. Moreover, *Diatoma* sp. exhibited a greater increase in cell density and chlorophyll *a* between day 7 and 14, compared to the increase observed between day 0 and 7. This suggests the presence of a lag phase in the flasks, during which the diatoms needed time to adjust to the culture conditions. From these findings, it can be speculated that given a longer duration of the experiment, the diatoms might have eventually acclimated to the highest temperature. Nevertheless, the results indicating a capacity to recovery in *Nitzschia* sp. may be of value for SAF when considering microalgal community structure in their cultivations.

### *4.4. Methodological considerations*

The purpose of the present study was to examine the influence of temperature on growth rates and biomass of benthic diatoms. To achieve this, three different temperature scenarios were chosen, with treatment 2 and 3 mimicking potential summer scenarios, with higher temperatures. It should be emphasised that the temperatures were kept constant throughout the experimental period in the present study, while in actual summer conditions, temperature fluctuations are expected due to weather and diurnal changes. Nonetheless, despite the potential for less consistency in real-world conditions, conducting experiments under constant temperatures can still offer valuable insights into how growth rates and biomass may be affected by summer temperatures. Moreover, the initial cell densities of *Diatoma* sp. and *Nitzschia* sp. varied due to their distinctive growth rates. However, in the current study, this discrepancy had no significant impact on the experiment since no comparisons were made between the two species in terms of biomass and growth rates. Nevertheless, it could be argued that the different cell densities may have led to different crowding effects, potentially causing *Nitzschia* sp. to experience greater light exposure compared to *Diatoma* sp., due to a reduced cell shading. In turn, this could have influenced growth rates and the measured values of photosynthetic activity.

Moreover, there are two commonly used methods, chosen in the present study, for measuring microalgal biomass: analysing photosynthetic pigments and counting cells. While each approach has its strengths and weaknesses, it is important to examine their accuracy. When counting cells, one frequently encountered issue is cluster of diatoms cells. The clusters make it challenging to obtain an exact number of cells, and estimations of cell numbers must sometimes be made, resulting in less precise measurements. In this case, analysing photosynthetic pigments may provide a more precise measurement of biomass, since chlorophyll *a* can be measured despite the presence of clusters. In addition, a larger volume was used when conducting the HPLC analysis, compared to the cell counts, which minimises the risk of outliers in the data. Nevertheless, using chlorophyll *a* as a measurement of biomass is accompanied by uncertainties. Microalgae can for instance produce more chlorophyll *a* as a coping mechanism to changes in environmental conditions. An illustration of this can be seen in the study conducted by Ratomski & Hawrot-Paw (2021), where the researchers observed a decline in chlorophyll *a* content in microalgal cells as nitrogen levels in the cultivation medium increased. However, the effects of nutrient availability on chlorophyll *a* content appears to vary depending on the species. To establish such trends in the present study, measurement of chlorophyll *a* per cell would need to be conducted. Another potential weakness of using chlorophyll *a* as a quantification of biomass, is the fact that is can be broken down into biproducts, such as phaeophytin's, which was identified in the chromatogram from the HPLC analysis. Although, this could be avoided by freezing samples in liquid $N_2$ and consequently store them at -80°C until analysis. Finally, the HPLC analysis can only provide concentrations of various photosynthetic pigments present in the sample, whereas when conducting cell counts the composition of organisms present in the sample can be achieved through visual observations in the microscope. This shows the importance of combining different methods to increase the accuracy in the results.

Furthermore, sampling the algal community in treatment 3 posed a challenge, due to large clusters of green algae and cyanobacteria that were resistant to dissolution. Attempts to resolve this issue by using an ultrasonicating bath as well as magnet stirrers, proved unsuccessful. This likely contributed to deviations in data, such as the high standard deviation observed in the chlorophyll *a* measurement in treatment 3 for the algal community, thereby impacting the statistical differences between this and the other treatments. To improve overall certainty, increasing the final volume in the replicates or conducting more replicates, could minimise the occurrence of outliers in the data.

Moreover, the accuracy of the nutrient analysis in the cultivation medium is a subject that requires attention. To ensure sufficient sample volume for the nutrient analysis, the replicates in each treatment were pooled into a single homogenous sample. This methodology made it challenging to detect differences between replicates within a treatment. Consequently, drawing conclusions about nutrient concentrations and ratios within a particular treatment was difficult.

## 5. Conclusions

Due to the expected increase in temperatures in the SAF greenhouse during summer, it is crucial to understand how diatoms will respond to sustain growth and microalgal biomass production. Therefore, the present study aimed to examine the influence of temperature on biomass and growth rates in benthic diatoms *Nitzschia* sp. and *Diatoma* sp. as well as the algal community, cultivated at SAF. In conclusion, the results showed an increase in biomass and growth rate at ambient temperatures (11-16°C) and 20°C for the two species and the algal community, while a decline in biomass and growth rates was observed at 30°C. However, to determine a more precise thermal tolerance level, a more comprehensive range of temperatures needs to be examined. Moreover, the results showed that an increase in temperature to 30°C, resulted in an increase in green algae and cyanobacteria within the algal community. Additionally, green algae and cyanobacteria were also detected in *Diatoma* sp. at 30°C. However, *Nitzschia* sp. never showed any indications of green algae, even at high temperatures, indicating its suitability for SAF cultivations. Lastly, these findings demonstrate the variability in thermal performance among benthic diatoms and emphasize the importance of studying species-specific responses to temperature change.

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# Appendices

**Table 1** Temperature in the water baths for treatment 1, 2 and 3. Data represents mean values ± SD.

| **Temperature in °C** | | | | | |
|---|---|---|---|---|---|
| Treatment 1 | | Treatment 2 | | Treatment 3 | |
| **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| 12.19 | ± 1.21 | 19.95 | ± 2.27 | 30.17 | ± 0.36 |

**Table 2** Photosynthetically active radiation (PAR, 400-700nm) measured in µmol photons $m^{-2}$ $s^{-1}$, over the water baths under two different weather conditions (shady conditions/sunny conditions). Data represents mean values (n = 6) ± SD.

| | **PAR (µmol photons $m^{-2}$ $s^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | Treatment 1 | | Treatment 2 | | Treatment 3 | |
| **Shady conditions** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| | 136 | ± 5.5 | 131 | ± 4.3 | 138 | ± 2.9 |
| **Sunny conditions** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| | 412 | ± 37.8 | 400 | ± 24.2 | 432 | ± 13.7 |