**Effects of high temperatures and nitrogen availability on the growth and composition of the marine diatom *Chaetoceros pseudocurvisetus***

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**Running title**

Diatom *Chaetoceros pseudocurvisetus* and abiotic stress

**Highlight**

Environmental stresses such as increasing temperature and nitrogen deficiency affect different cell physiological and biochemical processes of the marine diatom *Chaetoceros pseudocurvisetus*, in addition to synergistic effects.

**Abstract**

The assimilation of inorganic nutrients by phytoplankton strongly depends on environmental conditions such as the availability of nitrogen (N) and temperature, especially warming. The acclimation or adaptation of different species to such changes remains poorly understood. Here, we used a multimethod approach to study the viability, and physiological and biochemical responses of the marine diatom *Chaetoceros pseudocurvisetus* to different temperatures (15, 25, and 30 °C) and different N: phosphorus (P) ratios (N-excess (N:P = 50:1), optimal (N:P = 16:1), and N-limiting (N:P = 2:1)). N limitation had a greater effect than high temperature on cell growth and reproduction, leading to a marked elongation of setae, decreased P assimilation, increased lipid accumulation and decreased protein synthesis. The elongation of setae observed under these conditions may serve to increase the surface area available for the uptake of inorganic and/or organic nitrogen. In contrast, high temperatures (30 °C) had a stronger effect than N deficiency on cell death, N assimilation, chlorophyll *a* accumulation, the cessation of setae formation and cell lipid remodelling. Significant changes in thylakoid lipids were observed in cells maintained at 30 °C, with increased levels of digalactosyldiacylglycerols (DGDG) and sulfoquinovosyldiacylglycerols (SQDG). These changes may be explained by the role of galactolipids in thylakoid membrane stabilization during heat stress.

**Keywords:** Global warming; nitrogen stress; diatom *Chaetoceros pseudocurvisetus*, physiological response; biochemical response; lipid remodeling

### **Abbreviations**

1,2DG - 1,2-diacylglycerols; 1,3DG - 1,3-diacylglycerols
ALC – Fatty alcohols; C – Carbon; Chl *a* – chlorophyll *a*; Cell C:Chl *a –* Cellular carbon-to-chlorophyll *a* ratio; CL – Cellular lipids; DGDG – Digalactosyldiacylglycerol; DIN – Dissolved inorganic nitrogen; DOC – Dissolved organic carbon; ER – Endoplasmic reticulum; FFA – Free fatty acids; GL – Glycolipids, HC – Hydrocarbons; KET – Fatty ketone; ME - Fatty acid methyl esters; MG – Monoacylglycerols; MGDG – Monogalactosyldiacylglycerol; MSGR – Maximum specific growth rate; N – Nitrogen; N+ - N-excess; N± - optimal; N- - N-limiting; OM – Organic matter; P – Phosphorus; PC – phosphatidylcholines; PE – phosphatidylethanolamines; PG – phosphatidylglycerols; PIG – Pigments; PL – phospholipids; POC – Particulate organic carbon; SE – Steryl esters; PSll – Photosystem ll;ST – Sterols; SQDG - Sulfoquinovosyldiacylglycerol; TG – triacylglycerols; TLC-FID - Thin-layer chromatography coupled with flame-ionization detection; TN – Total cellular nitrogen

**Introduction**

It is unequivocal that human influence has warmed the atmosphere, oceans, and land (IPCC, 2021). Warming of the global ocean, both surface temperature and heat content of the global ocean, has significant impacts on sea level rise, oxygen concentration, acidification, and nutrient cycling as well as primary production and carbon sinks (IPCC, 2021). Global sea surface temperature has increased by 0.88 °C since 1850-1900 to 2011-2020, with most of this warming mainly due to warming since 2003–2012, and the increase is projected to continue rapidly due to human activities (IPCC, 2021). However, the spatio-temporal trends of warming and the resulting changes differ significantly between different oceanic regions. The Mediterranean Sea is considered one of the regions most affected by climate change (Kim *et al.*, 2019). The average temperature in the Mediterranean Sea is 0.4 °C higher than the global average, with even more extreme seasonal variations (Nykjaer, 2009). The increased stratification of the seawater column caused by warming reduces the vertical nutrient supply from the deeper layers (oligotrophication of the surface layer), which has a direct negative impact on primary production. As a result, a decrease in Chl *a* concentration has been observed in the western Mediterranean Sea since the 2000s, which is strongly correlated with the increase in sea surface temperature (Kim et al., 2019). The increasing oligotrophication of the northern Adriatic Sea is explained by the reduced inflow of freshwater associated with the shortening of the snow cover period in the Italian Alps (Gašparović, 2012).

In contrast, coastal waters are exposed to anthropogenic nutrient inputs, such as Baltic Sea (Gustafsson *et al.,* 2012), that extend and accelerate the effects of eutrophication (Smil, 2004). Projections are dire and suggest that the problem will be exacerbated due to nutrient fluctuations in upwelling areas, extreme rainfall followed by runoff, and increased oxygen-depleted dead zones (Bindoff *et al.*, 2019). These physico-chemical changes will subsequently affect the diversity, abundance, and structure of marine communities.

Phytoplankton play an important role in the carbon cycle by photosynthetically fixing ~ 45 billion tons of carbon per year. In addition, phytoplankton supply organic matter (OM) to higher trophic levels in the food web (Falkowski and Woodhead, 1992) and sink towards the ocean floor (biological carbon pump), also playing an important role in regulating global CO2 on a longer time scale (Falkowski, 1997). In the era of global climate change, phytoplankton are adapting in terms of abundance, cellular composition of biomolecules (lipids, sugars, proteins), biochemical and morphological properties, surface features, and physiological activity of the cell (Novosel *et al*., 2021). Consequently, these changes are expected to influence the quantity and quality of organic matter exported to the deep ocean (a long-term sink for CO2) as well as to higher trophic levels.

Diatoms are one of the most important groups of phytoplankton (Malviya *et al.*, 2016), contributing 25 - 30% of ocean primary production (Uitz *et al*., 2010). When conditions are optimal for their growth, diatoms tend to dominate phytoplankton communities (Bopp *et al*., 2005). They are very efficient at transporting carbon to the ocean interior (Passow and Carlson, 2012), particularly due to their tendency to form large, rapidly sinking aggregates (Smetacek, 1985). As a result of surface warming and the associated increase in vertical stratification, unfavourable oligotrophic conditions for diatoms are occurring on a larger scale in the surface ocean (Bopp *et al*., 2005). These stressors not only alter the relative abundance and species structure of diatom communities, but also affect these microalgae on the physiological level. Studies have reported various cellular responses to nutrient limitation and temperature increases. Of particular interest are the effects of nitrogen depletion, as productivity in most of the ocean (~75%) is limited by the availability of inorganic nitrogen (Bristow *et al*., 2017). A variety of adaptations to nitrogen stress have been observed in diatoms: reorganization of the cell proteome, disruption of the photosynthetic apparatus, reduced growth, cell size, and net primary production (Berges *et al*., 1996; Jiang *et al*., 2012; Longworth *et al*., 2016; Ko *et al*., 2020). At the same time, temperature increases led to higher growth rates, a decrease in cell size and carbon to Chl *a* ratio, and carbon partitioning between major biochemicals (Anning *et al*., 2001; de Castro Araújo and Garcia, 2005; Liang *et al*., 2019). Both nitrogen depletion and sea surface warming have been found to trigger lipid remodeling in marine diatoms (Novak *et al*., 2019). Lipids are essential biomolecules that play an important role in supporting living cells by forming membranes, storage reserves and signalling pathways. Characterization of marine lipids at the molecular level enables their use as biogeochemical markers for identifying various sources and processes of OM in the ocean (Parrish *et al*.,1988; Gašparović *et al*., 2013).

Here we investigated the influence of the synergy of different temperatures (optimal 15 °C and 25 and 30 °C as temperature stress) and varying inorganic nitrogen availability, mimicking optimal, eutrophic, and oligotrophic conditions, on the cellular response of the model marine diatom *Chaetoceros pseudocurvisetus*. *Chaetoceros* taxa is the largest and most diverse diatom genus found in wide range of oceanic regions (Malviya *et al.,* 2016), and was consistently present in (Bosak *et al.,* 2016) and considerably contributing to phytoplankton community in the northern Adriatic Sea (Novak *et al.,* 2018; 2019). Experiments were conducted under non-axenic conditions (an intrinsic feature of seas and oceans) to allow circulation of nutrients by heterotrophs, which has been shown benefit autotrophs (Christie-Oleza *et al*., 2017). The two main questions we were interested in were: (i) Which of the stressors studied has a greater negative impact on specific physiological and biochemical processes in the cell? and (ii) Does the negative impact increase when the two stressors act synergistically? To answer these questions, we performed experiments with a monoclonal culture of *C. pseudocurvisetus* and performed nine cultivation treatments, including the combination of three temperatures with N-excess, optimal N:P ratio, and N-limiting conditions.

**Materials and methods**

**Microalgae cultures**

The chosen experimental organism, diatom *Chaetoceros pseudocurvisetus* Mangin 1910, was isolated from the norther Adriatic and was proven to be very amenable to experimental manipulation (Novak *et al.,* 2018; 2019). The monoclonal culture was established from *C. pseudocurvisetus* colony isolated from a sample collected in October 2014 in the northern Adriatic Sea. The culture was maintained in F/2 medium (Guillard, 1975) at 15°C, 4500 lx, and a 12:12 h light:dark photoperiod.

**Cultivation setup**

*C. pseudocurvisetus* was grown in triplicates in sterile Erlenmeyer flasks of 2000 mL capacity, with a culture volume of 1000 mL covered with a Petri dish, at three different temperatures (15, 25 and 30 °C). The Erlenmeyer flasks were chromosulfuric acid-cleaned and autoclaved before use for cell cultivation. The growth media were prepared according to Guillard (1975), adjusting the N and P concentrations and ratios. The experiment was designed to grow diatoms under the following conditions: N-excess N:P = 50:1, optimal N:P = 16:1, and N-limiting N:P = 2:1. The optimal condition was chosen based on empirical data on the amount of N and P consumed at 15 °C (Novak *et al.,* 2019). The dissolved inorganic nitrogen (DIN=NO3-+NO2-+NH4+) concentration of the N-excess, optimal, and N-limiting medium were 750.65 ± 2.62 μmol L-1, 240.65 ± 2.62 μmol L-1, and 30.65 ± 2.62 μmol L-1, respectively. The PO43- concentration, 15.05 μmol L-1, was the same in all described media. Control conditions were as follows: 15 °C, 240.65 ± 2.62 μmol L-1 DIN and 15.05 μmol L-1 PO43-.

The natural seawater used for the preparation of the media was obtained from a depth of 20 m in the Stončica Bay on the coast of the island of Vis in the middle Adriatic Sea (43° 3' 58.08" N, 16° 14' 33.22" E). Before further treatments, the collected seawater was stored in the dark for several months to ensure minimal organic matter content. Aged water was filtered through 0.7 μm Whatman GF/F filters and subjected to 24 h UV irradiation to remove any remaining bacteria and organic matter. Dissolved organic carbon concentration after UV-treatment was 0.30 ± 0.04 mg L-1.

The experiment was started with 105 cells L-1 in a thermostatic chamber (Inkolab, Croatia) with 12:12 h light/dark cycle under the illumination of ~ 4500 lux, with white LED light source. Inoculum was taken from preconditioned cultures in the exponential growth phase. Prior to inoculation, cells were maintained in F/2 replete media in 250 mL VWR® Tissue Culture Flasks (VWR, Radnor, Pennsylvania) under the temperature of the experiment. All cultures were grown in the same growth chamber to prevent possible influence of factors other than those being tested. Cultures were shaken manually twice a day. Growth was ended at the beginning of the stationary growth phase. However, the majority of data suggest that the growth of optimal culture (N±) at 25 °C was terminated too early (erroneously assuming the onset of stationary phase).

***C. pseudocurvisetus* growth, cellular carbon and morphology**

To determine *C. pseudocurvisetus* growth rates in each of the conditions, subsamples of 2 mL were taken daily and cells were counted using Sedgewick Rafter Counting Chambers under an Olympus BX51-P polarizing microscope. Maximum specific growth rate was calculated from the slope of the linear portion of the exponential (logarithmic phase) part of the growth curve:

where Nt is the cell concentration (cell L-1) at time t; N0 is the cell concentration at the beginning of the logarithmic growth phase, and t indicates the duration of the logarithmic growth phase.

Additional subsamples (2 mL) from the early stationary growth phase were collected for cellular carbon calculation and visualization of morphological changes, and stored in formaldehyde solution until processing within two weeks. Images were acquired using Zeiss Axio Observer 7 inverted microscope. Zen 3.3 software was used for image analysis. Cell width and length were measured and assuming their cylindrical shape, the cell volume was calculated. The carbon content of the cells was determined according to the calculations of Menden-Deuer and Lessard (2000). The share of cellular carbon in POC was calculated as:
Cell C in POC (%) = ((Cell C \* 100) × cell abundance) / POC. For morphological analysis the length of at least 800 setae were measured.

**Chl *a* analysis**

For Chl *a* analysis, 50 mL of the culture medium was filtered through pre-combusted (450 °C/5 h) Whatman GF/F filters (0.7 µm) and they were stored at -20 °C until extraction. Filters were immersed in 100% ethanol, incubated at 60 °C for 1 h, extracted at 4 °C in the dark for 24 h, and filtered (ISO 10260, 1992). Chl *a* concentrations were determined spectrophotometrically using Shimadzu UV-1280 spectrophotometer (Shimadzu, Japan).

Chl *a* concentration is calculated as follows:

Chl *a* (µg L-1) = 29.6 \* (A1 – A2) \* (Ve / (V \* d))

where A1 = (A665 – AB665) - (A750 – AB750) is absorbance before acidification at 665 nm, corrected by turbidity adjustment at 750 nm (A is for sample and AB is for blank); A2 = (AK665 – ABK665) - (AK750 – ABK750) is absorbance after acidification at 665 nm, corrected by turbidity adjustment at 750 nm (AK is for sample and ABK is for blank); Ve is volume of extract, V is total volume of the filtered sample, d is path length of the cuvette; 29.6 is constant calculated from the maximum acid ratio (1.7) and the specific absorption coefficient of Chl *a* in ethanol (82 g L-1·cm) (ISO 10260, 1992).

**Nutrient analysis**

Subsamples for nutrient analysis were filtered through 0.7 μm Whatman GF/F filters and filtrates (180 mL) were collected and stored at -20 °C until analysis. Spectrophotometric determination of the concentration of NO3-, NO2-, NH4+ and PO43- was performed using Shimadzu UV-1280 spectrophotometer (Shimadzu, Japan) with 1 cm quartz cuvettes (Strickland and Parsons, 1968).

**Particulate and dissolved organic carbon analysis**

For particulate organic carbon (POC) analysis, 40 mL of the culture medium corresponding to the early stationary phase was filtered through 0.7 μm Whatman GF/F filters, which were pre-combustedat 450 °C for 5 h. The filters were stored at -60 °C until analysis. Measurements were performed using the high-temperature catalytic oxidation method using the solid sample module SSM-5000A connected to TOC-VCPH carbon analyser (Shimadzu, Japan), calibrated with glucose (Sugimura and Suzuki, 1988). POC concentrations were corrected using blank filter measurements. The average filter blank value with the instrument blank corresponds to 0.005 mg C L-1. The reproducibility for the glucose standard was 2%.

For dissolved organic carbon (DOC) analysis, the dissolved fraction, remaining after filtration of the culture medium for lipid analysis, was collected in triplicate in the 22 mL glass vials combusted at 450 °C for 4 h. Samples were preserved with 100 µL mercury chloride (10 mg L-1) and stored in the dark at 4 °C until analysis. A carbon analyser model TOC–VCPH (Shimadzu, Japan) with a Pt/Si catalyst and a non–dispersive infrared detector for CO2 measurements was used for DOC measurements and calibrated with potassium hydrogen phthalate. Concentration was calculated as an average of the three replicates. The average instrument and ultrapure water blank correspond to 30 μg C L-1 with high reproducibility (1.5%).

**Lipid analysis**

For lipid analysis, subsamples (100 mL) were filtered through 0.7 μm combusted (450°C/5h) Whatman GF/F filters, which were then stored at – 60 °C until extraction. Particulate lipids were extracted along with added internal standard (stearic acid methyl ester), following the modified procedure (Bligh and Dyer, 1959). Internal standard was added to each sample to estimate the recoveries in the subsequent steps of sample analysis. Extracts were evaporated to dryness under nitrogen flow and dissolved in 20 to 40 μL of dichloromethane (Merck, USA) before analysis.Separation and identification of lipid classes was performed by Iatroscan thin-layer chromatography coupled with flame-ionization detection (TLC-FID) (Iatroscan MK-VI, Iatron, Japan). The Iatroscan was operated at a hydrogen flow rate 160 mL min-1 and an air flow of 2000 mL min-1. The analyzed lipid classes, separated on Chromarods III, were composed of: hydrocarbons (HC), steryl esters (SE), fatty acid methyl esters (ME), fatty ketone (KET), triacylglycerols (TG), free fatty acids (FFA), fatty alcohols (ALC), 1,3-diacylglycerols (1,3DG), sterols (ST), 1,2-diacylglycerols (1,2DG), pigments (PIG), monoacylglycerols (MG), glycolipids (GL) including monogalactosyl-, digalactosyl-, and sulfoquinovosyl- diacylglycerol (MGDG, DGDG, and SQDG, respectively), and phospholipids (PL) including phosphatidylglycerols (PG), phosphatidylethanolamines (PE), and phosphatidylcholines (PC). Total lipid concentration was calculated as the sum of all detected lipid classes. Detailed procedure is described in Gašparović et al. (2015; 2017).

**Protein analysis**

Samples for protein determination were prepared by a modified Lowry method (Lowry *et al*., 1951; Price, 1965). Lowry reagents A, B, C and D were prepared according to Slocombe et al. (2013). Subsample (10 mL) was filtered through 0.7 μm Whatman GF/F filters, which were pre-combusted at 450°C for 5 h. The filters were stored at -60 °C until extraction. Prior to extraction, the filters were cut into small pieces and 3 mL of Lowry reagents D was added, mixed, and incubated in centrifuge tubes (15 mL) with screw caps in a thermoblock at 55°C for 1 h. After incubation, samples were cooled to room temperature and centrifuged at 4300 x g for 35 min (Rotofix 32A, Germany). The protein extract (200 µL) was placed in 96-well flat-bottom microplate (NUNC, Roskilde, Denmark) and shaken well. Then, 20 µL of diluted Folin-Ciocalteu phenol reagent (2 N Folin-Ciocalteu phenol reagent: ultra-pure water = 1:1) was added, shaken well and after 30 min at room temperature in the dark, the plate was read at 600 nm (Tecan Infinite M200, Austria). The estimated protein content was determined by comparison with a standard protein bovine serum albumin calibration curve.

**Data analysis**

Data in this study were reported as mean values from triplicate analyses, plus/minus their standard deviation (± SD). ANOVA and post-hoc Tukey’s test, Pearson’s Correlation Coefficient, Shapiro-Wilk test were performed at a significance level of *p* < 0.05 (unless stated otherwise)) in SPSS statistical software. Two-way ANOVA followed by post-hoc Tukey’s test was performed in Origin 7 software. Curve fitting was performed in OriginPro 9 assuming sigmoidal growth of batch cultures.

**Results**

**Effect of warming and N availability on *C. pseudocurvisetus* physiology and morphology**

Cultures of *C. pseudocurvisetus* were grown at three different temperatures (15, 25, and 30 °C) and three different nutrient conditions (N-excess (N+), optimal (N±), and N-limiting (N-)) to investigate their physiological responses to stresses of warming and variations in N availability. We observed the same growth patterns at all temperatures, with the highest cell abundance in N-excess and the lowest in N-limiting conditions (Fig. 1). The cell abundances were significantly different in respect to varying nitrogen and temperature treatments (p < 0.05, post-hoc Tukey test, Supplementary Table S1).

The highest abundance was recorded for cultures grown at 15 °C in N+ medium, 8.58 ± 0.11 x 107 cell L-1 (Fig. 1A, Supplementary Table S2). N-replete medium yielded highest cell abundances at all examined temperatures while cells were least abundant in N-limiting conditions (p < 0.05, post-hoc Tukey test, Supplementary Table S1a). However, the highest maximum specific growth rates were observed at 25°C (Table 1), while the lowest were observed at 15 °C for all conditions (p < 0.05, post-hoc Tukey test, Supplementary Table S1b).

We observed elongation of the setae in cultures grown in N-limiting medium compared to optimal and N-excess cultures (p < 0.05, post-hoc Tukey test, Supplementary Table S1d ) (Fig. 2). At 15 °C, the setae in the N-limiting culture were on average 3.1 and 3.6 μm longer than those of the optimal and N-excess cultures, respectively. At 25 °C, the mean differences were 7.3 and 7.2 μm, respectively. Setae lengths did not differ significantly between optimal and N-excess media (Fig. 2D). Data obtained for cultivation at 30 °C were insufficient for analysis due to the low abundance and even absence of setae (Fig. 2C).

**Effect of warming and N availability on *C. pseudocurvisetus* metabolism**

**Chl *a* biosynthesis**

Chl *a* concentration, expressed per cell (pg cell-1), increased the most with temperature increase (p < 0.05, post-hoc Tukey test, Supplementary Table S1e) (Fig. 3A, Supplementary Table S4). As shown in Fig. 3B, there was a statistically significant decrease in the cellular carbon-to-Chl *a* ratio of the cells (Cell C:Chl *a*) with temperature increasing from 15 °C to 25 °C for all cultures (p < 0.05, post-hoc Tukey test, Supplementary Table S1f; Supplementary Table S4). The difference in Cell C:Chl *a* ratio between N-excess, optimal, and N-limiting cultures was greatest at 15 °C and it decreased with temperature, finally converging to ~ 54 at 30 °C. This trend was caused with both the increase of Chl *a* and the decrease in cellular carbon content with increasing temperature (p < 0.05, post-hoc Tukey test, Supplementary Table S1c).

**Nutrient uptake**

*C. pseudocurvisetus* assimilated varying amounts of DIN and PO43- depending on growth conditions. The general trend is that a greater proportion of the total amount of DIN is taken up by the cells exposed to higher initial concentration of DIN (Table 2 and Supplementary Table S5). The effect of temperature on the amount of DIN taken up is observed for N+ and N± cultures, for which the decrease in N uptake parallels the increase in temperature (p < 0.05, post-hoc Tukey test, Supplementary Table S1g). The uptake rate of DIN was highest at 25 °C for N-excess and optimal cultures, and at 30 °C for N-limiting cultures. Nearly constant PO43-uptake rate of 0.01 - 0.02 pmol cell-1 d-1 was observed for all cultivation treatments (Table 2).

The percentage of DIN and PO43- taken up by *C. pseudocurvisetus* also depended on growth temperature (Fig. 4A and B). With increasing temperature there was a statistically significant decrease in DIN uptake from 76.2 ± 10.6 to 27.1 ± 9.3% in N-excess cultures and from 99.2 ± 0.2 to 56.8 ± 4.8% in N-optimal cultures (p < 0.05, post-hoc Tukey test, Supplementary Table S1g). The cells from the N-excess medium were unable to utilize very high DIN quantity. Because of the very low initial concentrations of DIN in N-limiting cultures, the cells consumed almost all of DIN, even at 30 °C. Cells grown in N+ medium utilized the most PO43-, but with a decreasing trend with increasing temperature, from 85.8 ± 3.4 at 15°C to 43.4 ± 3.0 % at 30 °C (Fig. 4B, p < 0.05, post-hoc Tukey test, Supplementary Table S1h). There was a decrease in PO43- uptake with increasing temperature also for N-excess and N-optimal cultures. The least PO43- was taken up by cells in N-limiting medium, 17.9 ± 7.5, 15.0 ± 2.5, and 7.0 ± 3.5 % at 15, 25, and 30 °C, respectively.

The uptake ratio DIN:PO43- varies greatly depending on the culture medium (Fig. 4C, Supplementary Table S5). In N-optimal cultures it was almost equal for all cultivation temperatures, and varied around 20.7, slightly higher than the Redfield ratio (16:1). Cells from N-excess media grown at 15 °C had the highest possibility of DIN uptake compared to PO43- (DIN:PO43- = 44.3 ± 6.0) (p < 0.05, post-hoc Tukey test, Supplementary Table S1i). The uptake ratio DIN:PO43- for N-excess conditions dropped to 25.2 ± 1.4 at 30 °C. As expected, the lowest uptake ratio of DIN:PO43-was observed for cells grown under N-limiting conditions at 15 and 25 °C, while the ratio increased to 24.7 at 30 °C. It appears that at 30 °C the uptake ratio of DIN:PO43-converges to the same value for all three cultivations (~ 25).

**Total organic carbon production**

Total organic carbon produced by *C. pseudocurvisetus* cultures consists of particulate and dissolved fraction. The fact that production of both particulate and dissolved carbon in N-excess and optimal cultures was very similar at 15 and 30°C, but differed greatly at 25 °C, suggests that the growth of optimal culture was terminated too early (cf. Fig. 1B). Bulk POC concentration generally decreased with increasing temperature (p < 0.05, post-hoc Tukey test, Supplementary Table S1n), whereas opposite trends were observed for DOC production, but not statistically proven (post-hoc Tukey test, Supplementary Table S1o) (Figs. 5A and 5B, Supplementary Table S6).

POC normalized to cell concentration ranged from 205.9 ± 9.4 to 211.4 ± 23.1 pg C cell-1 for the N-excess, 213.7 ± 27.3 to 252.0 ± 36.9 pg C cell-1 for optimal, and 223.7 ± 7.4 to 271 ± 46.5 pg C cell-1 for the N-limiting culture (Fig. 5C, Supplementary Table S6). The share of cellular carbon (Table 1) in POC (Cell C in POC (%)) was calculated to determine a proportion of carbon in the particulate fraction that belongs to the active biomass, since a certain amount of POC content corresponds to dead cells (non-living POC) and particular organic matter that leaked out of the dead cells. Cellular carbon in POC (%) (Fig. 5D, Supplementary Table S6) decreased with increasing temperature (p < 0.05, post-hoc Tukey test, Supplementary Table S1r) and was lowest for the N- cultures. Of the two stressors, the increase in temperature had the greatest effect on the increase in the percentage of non-living POC, indicating high cell mortality. Cellular carbon in POC (%) decreased from 88.7 ± 12.1, 97.0 ± 10.4, and 72.8 ± 14.1 at 15 °C to 46.0 ± 3.7, 37.5 ± 9.2, and 35.7 ± 4.4 at 30 °C for N-excess, optimal, and N-limiting cultures, respectively.

Because of the much higher microbial degradation rates of POC compared to DOC degradation (Attermeyer et al., 2018), the DOC content per cell increased in parallel with an increased content of non-living POC (p < 0.05 for N+ and N± ANOVA, while for N- there is a positive trend but it is not statistically proven) (Fig. 5E, Supplementary Table S6). The highest value of DOC, 97.4 ± 26.1 pg C cell-1, was observed for the N-limiting condition at 30°C (p < 0.05, Post-hoc Tukey test, Supplementary Table S1s).

**Lipid production**

We processed data for cellular lipids (CL) produced by the *C. pseudocurvisetus* cultures that include membrane (PL, GL, and ST) and storage (TG and SE) lipids (Figs. 6). Bulk concentrations of cellular lipids decreased with increasing temperature and with decreasing N availability in the culture medium (p < 0.05, two-way ANOVA, Supplementary Tables S1t) (Fig. 6A). The highest CL concentration was detected at 15 °C for N-excess cultures (1.33 ± 1.5 mg L-1), and the lowest for N-limiting cultures at 30 °C (0.44 ± 0.05 mg L-1) (Supplementary Table S7). The CL content per cell (pg cell-1) increased with temperature (p < 0.05, two-way ANOVA, Supplementary Tables S1t), and was highest for N-limiting cultures at all three temperatures (ranging from 22.6 ± 2.4 to 29.2 ± 1.5 pg cell-1). Despite the observed trend, it is not statistically proven (post-hoc Tukey test, Supplementary Tables S1u) (Fig. 6B).

The relative quantities of CL classes changed as a function of increasing temperature and N availability in the medium (Fig. 6C-E, Supplementary Table S8). The most abundant were GL (MGDG+DGDG+SQDG), which accounted for 34 – 52% of cellular lipids. The relative proportions of lipid classes (%) of *C. pseudocurvisetus* grown at 15 °C were not statistically significantly different between N-excess, optimal, and N-limiting cultures. PC and TG differed significantly in respect to N availability at 25 °C, while MGDG, DGDG, SQDG, PE, and TG differed significantly at 30 °C. The proportion of MGDG and DGDG in cellular lipids decreased from N-excess to N-limiting media at 30 °C, while the opposite trend was observed for SQDG and TG. The MGDG contribution to CL decreased almost twofold from 26.07 ± 6.26 % at 15 °C to 14.11 ± 2.45 % at 30 °C in N-limiting medium.

The thylakoid lipids SQDG and DGDG were positively correlated with temperature, whereas the storage lipids TG and SE exhibited a negative correlation with the initial DIN concentration in the medium. Thus, the percentage of TG in cellular lipids was highest in N-limiting, amounting 10.17 ± 0.69% irrespective of growth temperature. The contribution of SE to cellular lipids was mainly less than 1%, except in the N-limiting cultures at 30 °C (1.1 ± 0.6%). Relevant statistical results are presented in the Supplementary Tables S9 and S10.

**Total protein production**

The effects of warming and N stresses on *C. pseudocurvisetus* growth was reflected in the decrease in bulk protein concentration (Fig. 7A, Supplementary Tables S11 and S12). This is statistically proven for N-optimal and N-excess cultures (p < 0.05, post-hoc Tukey test, Supplementary Table S1v). The highest bulk protein concentrations were found for the N-excess cultures, 9.84 ± 0.91 mg L-1, 8.27 ± 0.07 mg L-1, and 5.90 ± 0.59 mg L-1 at 15, 25, and 30 °C, respectively. Bulk protein concentrations were lowest for the N-limiting cultures, which did not differ among the three temperature cultivations, ~ 2.10 mg L-1. Data on the cellular protein content of *C. pseudocurvisetus* are shown in Fig. 7B and Supplementary Table S11. The highest content observed at 30 °C should be taken with caution, since undecomposed particulate proteins of dead cells probably contributed to the total proteins. One may also consider the fact that the cells were dividing less frequently at 30 °C and that they retained their cellular protein content. Statistically significant differences in cellular content of proteins are detected for N-limiting conditions (p < 0.05, post-hoc Tukey test, Supplementary Table S1w). The highest protein:cellular lipid ratio was detected for the *C. pseudocurvisetus* growing in optimal medium at 15 °C (9.49 ± 1.48), statistically confirmed between 15 and 25 °C (p < 0.05, post-hoc Tukey test, Supplementary Table S1x). We observed no statistical difference for the protein:cellular lipid ratio for the cultivations at 30 °C and three different N amendments (Supplementary Table S1x and S12). The ratios were 5.49 ± 1.64, 5.94 ± 0.90 and 4.55 ± 0.48 for the N-excess, optimal, and N-limiting cultivations, respectively. Interesting observation is that for the N-limiting cultures, on average highest protein:cellular lipid ratio is observed for the cultivation at 30 °C. It seems that protein:cellular lipid ratio at 30 °C converge to the same value of ~ 5.3.

**Discussion**

Physical and chemical conditions in the marine environment have a major influence on the physiology of phytoplankton and the distribution of cellular resources. Physiological and biochemical changes in phytoplankton can lead to shifts in community structure and trophic interactions that affect entire marine ecosystems. The Anthropocene era has already profoundly altered the global ocean. There is indisputable evidence of a variety of changes in the marine ecosystem due to global changes such as warming, oligo- and eutrophication. Temperatures of the Mediterranean Sea are continuously rising (Lejeusne *et al*., 2010). Summer temperatures in the surface waters of the Adriatic, for example, frequently approach and even exceed 30 °C (Gašparović, 2012). The Mediterranean Basin suffers more frequently from heat waves of increasing duration (Cramer *et al*., 2018). The nutrient budget of the Mediterranean basin is expected to decrease in the northwestern basin and the Adriatic Sea, while it could increase sharply in the basins of the southern and northern Levantine Seas (Ludwig *et al.,* 2010). Recently, nitrogen limitation has been observed more frequently in the northern Adriatic Sea (Penezić *et al*., 2022). Against this background, in the present study, we investigated the whole-cell response of the diatom *C. pseudocurvisetus* to warming and variable nitrogen supply. A summary of the main observed morphological, physiological, and biochemical responses of *C. pseudocurvisetus* to high temperature and availability of inorganic nitrogen is shown in Fig. 8.

***C. pseudocurvisetus* physiological and morphological acclimation**

Abundance and growth dynamics of *C. pseudocurvisetus* were influenced by both warming and nitrogen availability. However, nitrogen depletion has a stronger negative effect on total abundance than warming (Fig. 1, Supplementary Tables S1a and S2). Aranguren-Gassis et al. (2019) found that marine diatom *Chaetoceros simplex* survives high temperatures in abundance of nitrogen, while N limitation inhibits its adaptation to high temperatures. Optimal growth temperature allows cells to undergo photosynthesis without modifying inherent biochemical or physiological properties (Ras *et al*., 2013) and thereby produce large amount of biomass (Sharma *et al*., 2012). *C. pseudocurvisetus* cultures growing at sufficient DIN concentration (N-excess and N-optimal) and at 15 °C, which is considered as the optimal temperature for *C. pseudocurvisetus* (Novak *et al.,* 2018) reached their maximum abundance. An increase in temperature (below the sustainable maximum growth temperature) has a positive effect on photosynthesis and growth rates, mainly through increased enzymatic activities (Falkowski, 1980). Excessive temperature (30 °C) leads to high cell mortality (Fig. 5D). Here, the specific growth rate increased until 25 °C and decreased at 30 °C, suggesting that upper thermal limit for this species is between 25 and 30 °C (Fig. 1, Supplementary Table S1). This suggests that *C. pseudocurvisetus* would adapt well to conditions of increasingly warmer Mediterranean Sea, of course until the upper thermal limit is reached. When the temperature threshold was exceeded, the growth rate of microalgae decreased due to heat stress, which likely triggers the denaturation of enzymes and proteins involved in important metabolic processes (Chen, 2015). *C. pseudocurvisetus* isolated from the Sagami Bay (*in situ* temperature 25 °C) survive in a narrow temperature range (20 – 30 °C) (Suzuki and Takahashi, 1995). In contrast, *C. pseudocurvisetus* isolated from the Adriatic Sea appears to survive in a wider temperature range, as it survives and reproduces from at least 10 to 30 °C. These varying findings suggest a high evolutionary potential of this species, reflected in strong local adaptations.

The observed elongation of *C. pseudocurvisetus* setae (Fig. 2D, Supplementary Tables S1d and S3) suggests a previously unobserved morphological plasticity in response to nitrogen limitation. Smodlaka Tanković *et al*. (2018) reported a remarkable morphological strategy in response to nutrient limitation in *Chaetoceros peruvianus*, namely the thickening and elongation of setae belonging to cells grown in a phosphorus (P) limited environment. They showed increased extracellular alkaline phosphatase activity upon P-limitation and, interestingly, alkaline phosphatase was exclusively localized in the setae. This morphological change increased the surface area for alkaline phosphatase and enhance the ability of the species to utilize dissolved organic phosphorus when environmental PO43- concentrations are low. In contrast to the thick setae of *C. peruvianus*, the weakly silicified setae of *C. pseudocurvisetus* are thin (diameter 0.7 – 1.4 µm, Lee *at al*., 2014). The presence of extracellular enzymes on the setae as a mechanism to enhance the nitrogen catchment could explain the elongation of the setae. Phytoplankton can produce cell-surface attached amino acid oxidase and amine oxidase to use different forms of organic nitrogen (Palenik *et al.,* 1989). Setae are assumed to increase the absorptive surface of the cell (Margalef, 1978). Thus, the setae elongation of smaller cells growing under N-limited conditions could represent a strategy to increase surface area for inorganic nitrogen uptake, as an energy cost alternative to cell growth and reproduction. The growth and elongation of the setae is massively reduced at 30 °C (Fig. 2C). We assume that setae growth is suppressed as the resources are redirected to support essential metabolic processes and to extend survival. This phenomenon is likely to encompass consequences for the diatom in a real ecosystem. Setae provide (i) buoyancy and positioning of the diatoms in the water column (Gómez, 2020) and (ii) mechanical protection from grazers (Hamm *et al.,* 2003). Thus, the “setaeless” condition in warm waters leads to greater ecological vulnerability of diatoms as they are at risk from enhanced sinking and grazing. The former could facilitate downward export of organic matter, while the latter, in contrast, may enhance remineralization in the upper mixed layer. However, both may lead to declining abundance of *C. pseudocurvisetus* and analogous species functioning at high sea surface temperature such as 30 °C.

***C. pseudocurvisetus* biochemical acclimation**

The observed increase in Chl *a* content and decrease in C:Chl *a* ratio that follow the increase in temperature (Fig. 3, Supplementary Tables S1e and f, and S4) could be explained by the fact that cells respond by investing in Chl *a* synthesis to obtain energy for the physiological processes necessary for survival. Since photosynthesis produces carbohydrates that have obviously not been used to increase the carbon content of the cell (low cell C:Chl *a*), they are probably excreted to the dissolved fraction of primary production. Indeed, we observed a sharp increase in DOC with increasing temperature (Figs. 5B and E, Supplementary Tables S1o and S6). Nutrient availability appeared to be less influential on cellular carbon and Chl *a* content than high temperature (Supplementary Tables S1 c and e). Nitrogen limitation is reported to elevate phytoplankton C:Chl *a* ratio (Geider, 1987). In contrast, we found an inconsistent effect of N-deficiency on C:Chl *a* ratio (Fig. 3B, Supplementary Table S4). Growth conditions largely reflect on internal biochemistry. Temperature has a major influence on the rate of photosynthesis (Davidson, 1991) and thus, it is an important driver of the production and transformation of key macromolecules in phytoplankton (Lee *et al*., 2016). However, Chl *a* and primary production are either limited or stimulated in the optimal temperature range by nutrient availability in the environment (Davey *et al*., 2008).

Nutrient (DIN and PO43-) cell uptake greatly depends on the DIN concentration in the medium and it increases with the increasing initial N concentration (N-excess > N-optimal > N-limiting) (Table 2). This is consistent with the results from a study by Lomas and Gilbert (2000) in which all diatom species studied had the capability to continue taking up NO3- at increasing rates with NO3- concentration increase. While total acquired nitrogen relative to the initial concentration decreased with increasing temperature, as main negative influential factor (Fig. 4A and B), the response of DIN uptake rate to temperature rise in N-excess and optimal cultures showed a pattern similar to that of growth rate i.e. uptake rate was highest at 25°C (Table 2). Highest DIN uptake rates at 30 °C of N-limiting cultures is likely due to the energy savings of less important cellular processes, such as detected cessation of setae formation (Fig. 2C). There is a seeming discrepancy between the results indicating (i) increased cellular uptake rates and (ii) a decreased amount of DIN taken up from media with increasing temperature. It appears that even as the uptake rate of DIN increases with increasing temperature, the maximum cell density decreases resulting in an overall decreasing consumption of DIN relative to its initial concentration in the medium.

Nitrogen deficiency limits P acquisition, which is why less than 20% of P is taken up by N-limiting cultures (Fig. 4B, Supplementary Table S5). The ratio of assimilated N:P differs from Redfield's 16:1. Our data suggest that a ratio of about 20:1 is the optimal N:P ratio for *C. pseudocurvisetus*, as determined for the optimal culture (N±) at all temperatures (Fig. 4C, Supplementary Table S5). Interestingly, when temperature was optimal and N was abundant (N-excess cultures, 15 °C), the incorporated ratio averaged 44.3. This indicates the formation of nitrogen reserves in the cell. In conditions of nitrogen surplus, diatoms form nitrogen storage composed of the protein, amino acids, and nitrate (Dortch, 1982). Our data on protein:cellular lipid ratio (Fig. 7C, Supplementary Table S11) suggest that *C. pseudocurvisetus* stores excessively assimilated nitrogen in the form of proteins. The uptake of PO43- is also inhibited with increasing temperature (Fig. 4B, Supplementary Tables S1h and S5). It seems to be even more suppressed compared to nitrogen uptake under low N conditions, which we inferred from the increase in the N:P ratio at 30 °C compared with 15 and 25 °C, on average from 13.4, as observed at 15 and 25 °C, to 24.7 at 30 °C.

Increasing stress (warming and N-limitation) is reflected in the lower POC concentration in the medium, which is accompanied by the reduced number of cells (Figs. 1, 5A and 5C, Supplementary Table S6). At the same time increasing stress is accompanied by an increase in DOC concentration (Figs. 5B and E). The decreased contribution of cellular carbon to POC at high temperature and in N-limiting cultures (Fig. 5D, Supplementary Table S6), coupled with reduced cell abundances, suggests substantial cell die-off under stressful conditions with high primary production directed toward the dissolved fraction. Our data suggest that unfavourable temperature has stronger effect on *C. pseudocurvisetus* cell death than lack of nitrogen.

Higher bulk lipid concentrations can be expected in cultures with higher cell abundance. While the bulk lipid concentration is lowest, the accumulation of lipids per cell is the greatest in cultures grown at 30 °C, especially in N-limiting cultures (Fig. 6A, Supplementary Tables S1u and S7). The individual effects of temperature increase and particularly N-limitation cause significant accumulation of cell lipids, while the combination of the two stressors results in the highest cellular lipid content.

Lipid composition varies depending on environmental conditions and represents mechanisms of acclimation and adaptation. The marine diatom *Thalassiosira pseudonana* replaces PC and betaine lipids and PG and SQDG depending on P availability (Martin *et al*., 2011). The composition of phospholipid fatty acids changes depending on the estuarine conditions (Vrana Špoljarić *et al*., 2021). Here observed statistically significant increase in the proportion of DGDG and SQDG in the cells with temperature increase (Supplementary Table S9) can be explained with their role in thylakoid membranes and the role in achieving thermotolerance (Chen *et al*., 2006; Mizusawa *et al*., 2009; Sato *et al*., 2003). Lipids of thylakoid membranes, where early processes of photosynthesis occur (Murata and Siegenthaler, 1998), play important roles in the folding and assembly of protein subunits in photosynthetic complexes (Kobayashi *et al*., 2016). Photosynthesis is very sensitive to high temperature stress. The primary sites of targets of high temperature stress are Photosystem II (PSII) and Rubisco (Mathur *et al*., 2014). Bilayer-forming DGDG lipids are particularly present in the PSII complex (Mizusawa *et al*., 2013), shaping the final structure of PSII (Boudière *et al*., 2014). SQDGs are dominantly localized in the PSII core complex and PSII light harvesting complex (Sato *et al*., 1995). The observed increase in both DGDG and SQDG at 30 °C (Fig. 6E) could be explained by the role of DGDG in balancing the electrostatic repulsion between negatively charged SQDG as observed by Demé *et al.* (2014) that performed in vitro experiments on reconstituted thylakoid lipid extracts. This should serve to achieve thermotolerance. The observed reduction of non-bilayer-forming MGDGs at high temperature and in N deficiency (Fig. 6E) may be explained by the role of DGDG:MGDG ratio in stabilizing the newly adjusted thylakoid membrane, as observed for *Arabidopsis* (Gaude *et al*., 2007) and thermotolerant reef coral symbiont dinoflagellate *Durusdinium trenchii* (Rosset et al, 2019). Finally, these results clearly suggest reorganization of the photosynthetic apparatus under the influence of stressors. In particular, high temperature significantly affects lipid remodeling.

The intracellular nitrogen is distributed among proteins and amino acids, chlorophylls, RNA, DNA, and inorganic nitrogen. The distribution is strongly influenced by growth conditions. Proteins are the most important N-containing substances (59.3 to 96.8% of total cellular nitrogen (TN)), followed by inorganic nitrogen (0.4 – 30.4% of TN), nucleic acids (0.3 – 12.2% of TN), and chlorophylls (0.1 – 1.8% of TN) (Lourenço *et al*., 2004). Considering that proteins are particularly sensitive to temperature stress, the observed increase of cellular protein content in optimal and N-limited cultures at 30 °C is unexpected. However, under warmer temperatures, *Chaetoceros sp.* invest chaperones and folding catalysts that help to reduce endoplasmic reticulum (ER) stress, and the investment of peptidases and proteasomes can promote the removal of misfolded proteins in ER and support faster protein turnover (Liang *et al.,* 2019). Furthermore, Sheehan et al. (2020) found that the diatom *T. pseudonana* exhibit increased protein content over the temperature range of 14 to 28 °C. Temperature adaptation is based on changes in the expression of targeted genes and pathways to maintain normal cellular functions (Liang *et al.,* 2019). Photosynthetic complexes are highly dependent on the balanced cooperation of thylakoid lipids with embedded protein moieties. Sato *et al*. (2003) suggested that photosynthetic activity remains functional at high temperatures not by stabilizing PSII activity, but rather by reactivating PSII after damage. Its recovery is closely related to the *de novo* synthesis of proteins encoded by the nuclear and chloroplast genomes (Sato *et al*., 2003). We propose that at the supraoptimal temperature of 30 °C, proteins involved in repair of heat-induced damage(s) in PSII increase in parallel with the increase in lipids SQDG and DGDG involved in recovery.

Consideration of the protein:cellular lipid ratio gave us a good insight into cell biochemistry. Under optimal conditions regarding the N:P ratio (16:1), cells have the highest protein:cellular lipid ratio, indicating that an optimal N:P ratio in the growth medium is important for cell health, regardless of growth temperature. Smith et al. (1997) showed that lipid synthesis is favored over protein or carbohydrate synthesis in conditions of low nutrient availability. Protein:cellular lipid ratio converges to the same value of 5.3 at 30°C, regardless of the N availability. Since we observed the same ratios for all three different N amendments at 30°C for DIN:PO43- ratio (~ 25, Fig. 4C, Supplementary Table 4) and cell C:Chl *a* ratio (~ 54, Fig. 3B, Supplementary Table S4), we suggest that cell biochemistry is most strongly affected by excessively high temperature.

**Conclusions**

Understanding phytoplankton acclimation and adaptation is critically needed for predicting future ocean/sea responses to global change stressors. Different phytoplankton species respond differently, among which there will be „global winners“ that will adapt to climate change. Conclusively, species-level research is of great importance.

 A combination of physiological, morphological and biochemical data gave us insight into the response of *C. pseudocurvisetus* to adverse environmental conditions, high temperatures as well as the excess of inorganic nitrogen load and shortage thereof. Under elevated temperatures, metabolic reactions and cell growth accelerate, until the thermal niche width of *C. pseudocurvisetus* species is exceeded (> 25 °C). Key research findings include:

* the morphological response to N-limitation is setae elongation (surface area increase), which most likely increases N uptake;
* cessation of setae formation at 30 °C appears to be a mechanism to divert resources to support essential metabolic processes and to prolong survival;
* N- limitation restricts P uptake;
* increasing temperature significantly directs the primary production to the dissolved fraction;
* lipid most sensitive to temperature rise are thylakoid lipids, which are significantly remodelled (altered share of DGDG and SQDG (increase) and MGDG (decrease));
* under optimal N:P ratio (16:1) and at 15 °C cells are the healthiest with respect to N-excess and N-limiting conditions, as indicated by the highest protein:cellular lipid ratio
* even in comparison to severe N-limitation, cell biochemistry is most affected by excessively high temperature.

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**Author contributions**

LF and BG: writing—original draft; LF: investigation, data collection, formal analysis; IV: investigation; ACK: investigation; JG: investigation; TN: investigation; AP: investigation; BG: conceptualization, supervision, resources. All authors: writing—revision & editing. All authors read and approved the submitted version.

**Data Availability**

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

**References**

**Anning T, Harris G, Geider R.**2001. Thermal acclimation in the marine diatom *Chaetoceros calcitrans* (Bacillariophyceae). European Journal of Phycology **36**, 233-241.

**Aranguren-Gassis M, Kremer CT, Klausmeier CA, Litchman E.** 2019. Nitrogen limitation inhibits marine diatom adaptation to high temperatures. Ecology Letters **22**, 1860-1869.

**Attermeyer K, Catalán N, Einarsdottir K, Freixa A, Groeneveld M, Hawkes JA, Jonas Bergquist J, Tranvik LJ.** 2018. Organic carbon processing during transport through boreal inland waters: Particles as important sites. Journal of Geophysical Research: Biogeosciences **123**, 2412–2428.

**Berges JA, Charlebois DO, Mauzerall DC, Falkowski PG.**1996. Differential effects of nitrogen limitation on photosynthetic efficiency of photosystems I and II in microalgae. Plant Physiology **110**, 689-696.

**Bindoff NL, Cheung WWL, Kairo JG** *et al*. 2019: Changing Ocean, Marine Ecosystems, and Dependent Communities. In: Pörtner H-O, Roberts DC, Masson-Delmotte V *et al*., eds. IPCC Special Report on the Ocean and Cryosphere in a Changing Climate.

**Bligh EG, Dyer WJ .**1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology **37**, 911-917.

**Bopp L, Aumont O, Cadule P, Alvain S, Gehlen M.**2005. Response of diatoms distribution to global warming and potential implications: A global model study. Geophysical Research Letters **32**, 1-4.

**Bosak S, Godrijan J, Šilović T.** 2016.Dynamics of the marine planktonic diatom family *Chaetocerotaceae* in a Mediterranean coastal zone. Estuarine, coastal and shelf science **180**, 69-81.

**Boudière L, Michaud M, Petroutsos D, Rébeillé F, Falconet D, Bastien O, Roy S, Finazzi G, Rolland N, Jouhet J, Block MA, Maréchal E.**2014. Glycerolipids in photosynthesis: composition, synthesis and trafficking. Biochimica et Biophysica Acta **1837**, 470-480.

**Bristow LA, Mohr W, Ahmerkamp S, Kuypers MMM.**2017. Nutrients that limit growth in the ocean. Current Biology 27, 474-478.

**Bromke MA, Sabir JS, Alfassi FA, Hajarah NH, Kabli SA, Al-Malki A, Ashworth MP, Méret M, Jansen RK, Willmitzer L.**2015. Metabolomic profiling of 13 diatom cultures and their adaptation to nitrate-limited growth conditions. PLOS ONE 10:e0138965.

**Chen B.**2015. Patterns of thermal limits of phytoplankton. Journal of Plankton Research **37**, 285-292.

**Chen J, Burke JJ, Xin Z, Xu C, Velten J.**2006. Characterization of the *Arabidopsis* thermosensitive mutant atts02 reveals an important role for galactolipids in thermotolerance. Plant, Cell and Environment**29**, 1437-1448.

**Christie-Oleza J, Sousoni D, Lloyd M, Armengaud J, Scanlan DJ.**2017. Nutrient recycling facilitates long-term stability of marine microbial phototroph–heterotroph interactions. Nature Microbiology 2017; 2:17100.

**Cramer W, Guiot J, Fader M, Garrabou J, Gattuso J.-P. et al.** 2018.Climate change and interconnected risks to sustainable development in the Mediterranean.” Nature Climate Change **8**, 972-980.

**Davey M, Tarran GA, Mills MM, Ridame C, Geider RJ, La Roche J.** 2008. Nutrient limitation of picophytoplankton photosynthesis and growth in the tropical North Atlantic, Limnology and Oceanography **53**, 1722–1733.

**Davison IR.**1991. Environmental effects on algal photosynthesis: temperature. Journal of Phycology**27**, 2-8.

**de Castro Araújo S, Garcia VMT.**2005. Growth and biochemical composition of the diatom *Chaetoceros cf.* *Wighamii Brightwell* under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids. Aquaculture **246**, 405-412.

**de Jesús-Campos D, López-Elías JA, Medina-Juarez LÁ, Carvallo-Ruiz G, Fimbres-Olivarria D, Hayano-Kanashiro C.**2020. Chemical composition, fatty acid profile and molecular changes derived from nitrogen stress in the diatom *Chaetoceros muelleri*. Aquaculture Reports16, 10281.

**Demé B, Cataye C, Block MA, Maréchal E, Jouhet J.**2014. Contribution of galactoglycerolipids to the 3-dimensional architecture of thylakoids. FASEB Journal: official publication of the Federation of American Societies for Experimental Biology **28**, 3373-3383.

**Dortch Q.**1982. Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids, and protein in three marine diatoms. Journal of Experimental Marine Biology and Ecology **61**, 243-264.

**Falkowski PG, Woodhead AD.**1992. Primary Productivity and Biogeochemical Cycles in the Sea. Environmental Science Research,Vol. 1: Springer, Boston, MA.

**Falkowski PG.** 1980.Light-Shade Adaptation in Marine Phytoplankton. In: Falkowski P.G. (eds) Primary Productivity in the Sea. Environmental Science Research, vol 19. Springer, Boston, MA, 99-119

**Falkowski PG.**1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO2 in the ocean. Nature**387**, 272-275.

**Field CB, Behrenfeld MJ, Randerson JT, Falkowski PG**. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science **281**, 237– 240.

**Gašparović B.**2012. Decreased production of surface-active organic substances as a consequence of the oligotrophication in the northern Adriatic Sea. Estuarine, Coastal and Shelf Science**115**, 33-39.

**Gašparović B, Godrijan J, Frka S, Tomažić I, Penezić A, Marić D, Djakovac T, Ivančić I, Paliaga P, Lyons D, Precali R, Tepić N.**2013. Adaptation of marine plankton to environmental stress by glycolipid accumulation. Marine Environmental Research**92**, 120-132.

**Gašparović B, Kazazić SP, Cvitešić A, Penezić A, Frka S.**2015. Improved separation and analysis of glycolipids by Iatroscan thin-layer chromatography–flame ionization detection. Journal of Chromatography A **1409**, 259-267.

**Gašparović B, Kazazić SP, Cvitešić A, Penezić A, Frka S.**2017. Corrigendum to “Improved separation and analysis of glycolipids by Iatroscan thin-layer chromatography–flame ionization detection” [J. Chromatogr. A 1409 (2015) 259–267]. Journal of Chromatography A **1521**, 168-169.

**Gaude N, Bréhélin C, Tischendorf G, Kessler F, Dörmann P.**2007. Nitrogen deficiency in Arabidopsis affects galactolipid composition and gene expression and results in accumulation of fatty acid phytyl esters. The Plant Journal **49**, 729-739.

**Geider RJ.**1987. Light and temperature dependence of the carbon to chlorophyll a ratio in microalgae and cyanobacteria: implications for physiology and growth of phytoplankton. The New Phytologist**106**, 1-34.

**Gómez F.** 2020. Symbioses of Ciliates (Ciliophora) and Diatoms (Bacillariophyceae): Taxonomy and Host–Symbiont Interactions. Oceans **1**, 133-155.

**Guillard RRL.**1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH, eds. Culture of marine invertebrate animals: Proceedings — 1st Conference on Culture of Marine Invertebrate Animals Greenport*,*Boston, MA: Springer US, 29-60.

**Gustafsson B, Schenk F, Blenckner T, Eilola K, Meier H, Müller-Karulis B, Neumann T, Ruoho-Airola T, Savchuk O, Zorita E.**2012. Reconstructing the development of Baltic Sea eutrophication 1850-2006. Ambio **41**, 534-548.

**Hamm, C, Merkel R, Springer O, Jurkojc P, Maier C, Prechtel K, Smetacek V.** 2003. Architecture and material properties of diatom shells provide effective mechanical protection. Nature **421**, 841–843.

**Kim G, Seo K, Chen D.**2019. Climate change over the Mediterranean and current destruction of marine ecosystem. Scientific Reports **9**, 18813.

**Intergovernmental Panel on Climate Change. 2021**. Climate Change 2021: The Physical Science Basis.

**International Standard ISO:10260**. 1992. Spectrometric determination of the chlorophyll-a concentration.

**Jiang Y, Yoshida T, Quigg A.**2012. Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae. Plant physiology and biochemistry **54**, 70-77.

**Ko E, Gorbunov MY, Jung J, Joo HM, Lee Y, Cho K, Yang EJ, Kang S, Park J.**2020. Effects of nitrogen limitation on phytoplankton physiology in the Western Arctic Ocean in summer. Journal of Geophysical Research: Oceans125(11), [e2020JC016501].

**Kobayashi K.** 2016. Role of membrane glycerolipids in photosynthesis, thylakoid biogenesis and chloroplast development. Journal of Plant Research**129**, 565-580,

**Lee J, Lee D, Kang J, Joo H, Lee JH, Lee H, Ahn SH, Lee S.**2017. The effects of different environmental factors on biochemical composition of particulate organic matters in Gwangyang Bay, South Korea. Biogeosciences14, 1903–1917.

**Lee SD, Joo H, Lee J.**2014. Critical criteria for identification of the genus Chaetoceros (Bacillariophyta) based on setae ultrastructure. II. Subgenus Hyalochaete. Phycologia **53**, 614-638.

**Lejeusne C, Chevaldonné P, Pergent-Martini C, Boudouresque CF, Pérez** T. 2010. Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. Trends in Ecology and Evolution **25**, 250-60.

**Liang Y, Koester JA, Liefer JD, Irwin AJ, Finkel ZV.**2019. Molecular mechanisms of temperature acclimation and adaptation in marine diatoms. The ISME Journal **13**, 2415-2425.

**Lomas MW, Glibert PM.**2000. Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. Journal of Phycology **36**, 903-913.

**Longworth J, Wu D, Huete-Ortega M, Wright PC, Vaidyanathan S.**2016. Proteome response of *Phaeodactylum tricornutum*, during lipid accumulation induced by nitrogen depletion. Algal research (Amsterdam) **18**, 213-224.

**Lourenço SO., Barbarino E, Lavín PL, Lanfer Marquez UM, Aidar E.**2004. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. European Journal of Phycology **39**, 17-32.

**Lowry OH, Rosebrough NJ, Farr AL, Randall RJ**. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry **193**, 265–275.

**Ludwig W, Bouwman AF, Dumont F, Lespinas F.** 2010. Water and nutrient fluxes from major Mediterranean and Black Sea rivers: Past and future trends and their implications for the basin- scale budgets. Global Biogeochemical Cycles, **24**, GB0A13.

**Malviya S, Scalco E, Audic S, Vincent F, Veluchamy A, Poulain J, Wincker P, Iudicone D, de Vargas C, Bittner L, Zingone A, Bowler C.** 2016. Insights into global diatom distribution and diversity in the world’s ocean. Proceedings of the National Academy of Sciences of the United States of America **113**, E1516-25.

**Margalef R.** 1978. Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanologica Acta **1**, 493-509.

**Martin P, Van Mooy BAS, Heithoff A, Dyhrman ST.**2011. Phosphorus supply drives rapid turnover of membrane phospholipids in the diatom *Thalassiosira pseudonana*. The ISME Journal **5**, 1057-1060.

**Mathur S, Agrawal D, Jajoo A.**2014. Photosynthesis: Response to high temperature stress. Journal of Photochemistry and Photobiology B: Biology**137**, 116-126.

**Menden-Deuer S, Lessard EJ.**2000. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. Limnology and Oceanography**45**, 569-579.

**Mizusawa N, Sakata S, Sakurai I, Sato N, Wada H.** 2009. Involvement of digalactosyldiacylglycerol in cellular thermotolerance in *Synechocystis* sp. PCC 6803. Archives of microbiology **191**, 595-601.

**Mizusawa N, Sakata S, Sakurai I, Kubota H, Sato N, Wada H.**2013. Essential role of digalactosyldiacylglycerol for photosynthetic growth in Synechocystis sp. PCC 6803 under high-temperature stress. In: Photosynthesis Research for Food, Fuel and the Future. Advanced Topics in Science and Technology in China. Springer, Berlin, Heidelberg.

**Murata N, Siegenthaler** P. 1998. Lipids in photosynthesis: an overview. In: Siegenthaler P, Murata, N, eds. Lipids in Photosynthesis: Structure, Function and Genetics. N. pp. 1–20. Kluwer Academic Publishers, Dordrecht.

**Novak T, Godrijan J, Pfannkuchen DM, Djakovac T, Medić N, Ivančić I, Mlakar M, Gašparović B.**2019. Global warming and oligotrophication lead to increased lipid production in marine phytoplankton. The Science of the Total Environment**668**, 171-183.

**Novak T, Godrijan J, Pfannkuchen DM, Djakovac T, Mlakar M, Baricevic A, Tanković MS, Gašparović B.**2018. Enhanced dissolved lipid production as a response to the sea surface warming. Journal of Marine Systems**180**, 289-298.

**Novosel N, Mišić Radić T, Zemla J, Lekka M, Čačković A, Kasum D, Legović T, Žutinić P, Gligora Udovič M, Ivošević DeNardis N**. 2021. Temperature-induced response in algal cell surface properties and behavior. Journal of Applied Phycology, 10.1007/s10811-021-02591-0.

**Nykjaer L.**2009. Mediterranean Sea surface warming 1985–2006. Climate Research**39**, 11-17.

**Palenik B, Kieber DJ, Morel FMM** 1989. Dissolved organic nitrogen use by phytoplankton: The role of cell-surface enzymes. Biological Oceanography **6**, 347-354.

**Parrish CC.**1988. Dissolved and particulate marine lipid classes: a review. Marine Chemistry**23**, 17-40.

**Parrish CC, Wangersky PJ.**1987. Particulate and dissolved lipid classes in cultures of *Phaeodactylum tricornutum* grown in cage culture turbidostats with a range of nitrogen supply rates. Marine Ecology Progress Series **35**, 119-128.

**Passow U, Carlson CA.**2012. The biological pump in a high CO₂ world. Marine Ecology Progress Series **470**, 249-272.

**Penezić A, Gašparović B, Cuculić V, Strmečki S, Djakovac T, Mlakar M.** 2022. Dissolved trace metals and organic matter distribution in the northern Adriatic, an increasingly oligotrophic shallow sea. Water **14**, 349.

**Price CA**. 1965. A membrane method for determination of total protein in dilute algal suspensions. Analytical Biochemistry **12**, 213–218.

**Ras M, Steyer J, Bernard O.**2013. Temperature effect on microalgae: a crucial factor for outdoor production. Reviews in Environmental Science and Bio/Technology **12**, 153.

**Rosset S, Koster G, Brandsma J, Hunt AN, Postle AD, D’Angelo C.** 2019. Lipidome analysis of Symbiodiniaceae reveals possible mechanisms of heat stress tolerance in reef coral symbionts. Coral Reefs **38**, 1241–1253.

**Sato N, Aoki M, Maru Y, Sonoike K, Minoda A, Tsuzuki M.**2003. Involvement of sulfoquinovosyl diacylglycerol in the structural integrity and heat-tolerance of photosystem II. Planta **217**, 245-251.

**Sato N, Sonoike K, Tsuzuk M, Kawaguchi A.**1995. Impaired Photosystem II in a mutant of *Chlamydomonas Reinhardtii* defective in sulfoquinovosyl diacylglycerol. European Journal of Biochemistry **234**, 16-23.

**Sharma KK, Schuhmann H, Schenk PM.**2012. High lipid induction in microalgae for biodiesel production. Energies (Basel) **5**, 1532-1553.

**Sheehan C., Baker KG, Nielsen DA, Petrou K.** 2020. Temperatures above thermal optimum reduce cell growth and silica production while increasing cell volume and protein content in the diatom *Thalassiosira pseudonana*. Hydrobiologia **847**, 4233–4248.

**Slocombe SP, Ross M, Thomas N, McNeill S, Stanley MS**. 2013. A rapid and general method for measurement of protein in micro-algal biomass. Bioresource Technology **129**, 51–57.

**Smetacek VS.** 1985. Role of sinking in diatom life-history cycles: ecological, evolutionary and geological significance. Marine Biology**84**, 239-251.

**Smith REH, Gosselin M, Kattner G, Legendre L, Pesant S.** 1997. Biosynthesis of macromolecular and lipid classes by phytoplankton in the Northeast Water Polynya. Marine Ecology Progress Series **147**, 231–242.

**Smodlaka Tanković M, Baričević A, Ivančić I, Kužat N, Medić N, Pustijanac E, Novak T, Gašparović B, Marić Pfannkuchen D, Pfannkuchen MM.**2018. Insights into the life strategy of the common marine diatom *Chaetoceros peruvianus* Brightwell. *PLOS ONE*13(9): e0203634.

**Strickland JDH, Parsons TR** 1968. Determination of reactive phosphorus. In: A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, Bulletin **167**, 49-56.

**Sugimura Y, Suzuki Y.**1988. A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. Marine Chemistry **24**, 105-131.

**Suzuki Y, Takahashi M.**1995. Growth responses of several diatom species isolated from various environments to temperature. Journal of Phycology **31**, 880-888.

**Smil V.**2004. Enriching the Earth: Fritz Haber, Carl Bosch, and the Transformation of World Food Production. Cambridge, United States: MIT Press Ltd.

**Uitz J, Claustre H, Gentili B, Stramski D.** 2010. Phytoplankton class-specific primary production in the world's oceans: Seasonal and interannual variability from satellite observations. Global Biogeochemical Cycles **24**, GB3016.

**Vrana Špoljarić I, Novak T, Gašparović B, Kazazić SP, Čanković M, Ljubešić Z, Hrustić E, Mlakar M, Du J, Zhang R, Zhu Z.**2021. Impact of environmental conditions on phospholipid fatty acid composition: implications from two contrasting estuaries. Aquatic Ecology**55**, 1-20.

**Tables**

Table 1. Physiological characteristics of early stationary growth phase of *Chaetoceros pseudocurvisetus*: maximum specific growth rate (MSGR), cell volume, and cellular carbon content for growth in N-excess (N+), optimal (N±), and N-limiting (N-) media at 15, 25, and 30 °C.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Medium type | Temperature | MSGR | Cell volume | Cell carbon content |
|  | °C | d-1 | µm3 | pg C cell-1 |
| N+ | 15 | 0.63 ± 0.01 | 2867 ± 546 | 183.0 ± 28.6 |
| 25 | 1.02 ± 0.02 | 1550 ± 346 | 111.1 ± 20.0 |
| 30 | 0.66 ± 0.02 | 1306 ± 143 | 96.9 ± 8.6 |
| N± | 15 | 0.62 ± 0.00 | 3522 ± 381 | 216.6 ± 19.1 |
| 25 | 1.02 ± 0.00 | 1150 ± 22 | 87.4 ± 1.3 |
| 30 | 0.65 ± 0.01 | 1613 ± 180 | 115.0 ± 10.4 |
| N- | 15 | 0.59 ± 0.03 | 2285 ± 239 | 152.5 ± 13.0 |
| 25 | 1.03 ± 0.03 | 1364 ± 385 | 100.0 ± 22.7 |
| 30 | 0.62 ± 0.03 | 1209 ± 352 | 90.6 ± 21.6 |

Table 2. Total DIN and PO43-taken upand their uptake rates at early stationary growth phase of *C. pseudocurvisetus* for N-excess (N+), optimal (N±), and N-limiting (N-) conditions and at 15, 25 and 30 °C. Results on two-way ANOVA tests are given in Supplementary Tables S1j, k, l and m.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | Uptake | Uptake rate |
|  | T | DIN | SD | PO43- | SD | Culture age | DIN | PO43- |
|  | °C | pmol cell-1 | d | pmol cell-1 d-1 |
| N+ | 15 | 6.79 | ±0.99 | 0.15 | ±0.00 | 16 | 0.42 | 0.01 |
| 25 | 5.87 | ±1.28 | 0.18 | ±0.01 | 10 | 0.59 | 0.02 |
| 30 | 3.46 | ±1.52 | 0.14 | ±0.01 | 13 | 0.27 | 0.01 |
| N± | 15 | 3.23 | ±0.12 | 0.17 | ±0.01 | 16 | 0.20 | 0.01 |
| 25 | 3.87 | ±0.99 | 0.18 | ±0.05 | 8 | 0.48 | 0.02 |
| 30 | 3.70 | ±0.47 | 0.18 | ±0.03 | 13 | 0.28 | 0.01 |
| N- | 15 | 1.32 | ±0.38 | 0.12 | ±0.06 | 14 | 0.09 | 0.01 |
| 25 | 0.81 | ±0.05 | 0.06 | ±0.01 | 9 | 0.09 | 0.01 |
| 30 | 2.24 | ±0.34 | 0.07 | ±0.03 | 10 | 0.22 | 0.01 |

**Figure legends**Fig. 1. Growth curves of *C. pseudocurvisetus* at (A) 15°C, (B) 25°C and (C) 30°C in N-excess (N+), optimal (N±), and N-limiting (N-) medium. Growth curves were obtained by fitting a sigmoidal curve based on plotted averaged cell counts of triplicates with error bars representing SD. Probability values refer to two-way ANOVA results for interaction of temperature and N availability influences on cell abundance. Probability values (p-values) for all temperature and N availability (N+, N± and N-) combinations relating differences in cell abundances (post hoc Tukey test) are given in Supplementary Table S1a.

Fig. 2. Dependence of *C. pseudocurvisetus* setae length (µm) on growth conditions. Photomicrographs of cells and belonging setae grown in optimal (A) and N-limiting (B) conditions at 25 °C and cells lacking setae at 30 °C (C). Measured setae length in dependence on N availability at 15 °C and 25 °C (D). Values presented are averages of triplicates with SD as error bars. Probability values refer to two-way ANOVA results for interaction of temperature and N availability influences on cell setae length. Probability values (p-values) for all temperature and N availability (N+, N± and N-) combinations relating differences in cell saetae length (post hoc Tukey test) are given in Supplementary Table S1d.

Fig. 3. Changes in Chl *a* cellular concentration of *C. pseudocurvisetus*. Cellular concentrations of Chl a (pg cell-1) (A) and cellular carbon-to-Chl *a* ratio (B) for N-excess (N+), optimal (N±), and N-limiting (N-) cultures of *C. pseudocurvisetus* cultures at 15, 25 and 30 °C. Values presented are averages of triplicates with SD as error bars. Probability values refer to two-way ANOVA results for interaction of temperature and N availability influences on cell content of Chl *a* and of cellular carbon-to-Chl *a* ratio. Probability values (p-values) for all temperature and N availability (N+, N± and N-) combinations relating differences in cell content of Chl *a* and of cellular carbon-to-Chl *a* ratio (post hoc Tukey test) are given in Supplementary Tables S1e and f.

Fig. 4. *Chaetoceros pseudocurvisetus* nutrient uptake. Percentage of DIN (A) and PO43- (B) uptake based on the amount of nutrients that have been depleted from the media from the beginning until the early stationary growth phase in each experimental batch, and DIN:PO43- uptake per cell (C) for growth at 15, 25, and 30 °C and in N-excess (N+), optimal (N±), and N-limiting (N-) conditions. Values presented are averages of triplicates with SD as error bars. Probability values refer to two-way ANOVA results for interaction of temperature and N availability influences on DIN, PO43- and DIN: PO43- uptake. Probability values (p-values) for all temperature and N availability (N+, N± and N-) combinations relating differences in nutrient uptake data (post hoc Tukey test) are given in Supplementary Tables S1g, h and i.

Fig. 5. Organic carbon production as a function of N availability and temperature. Particulate organic carbon (POC) (mg C L-1) (A), dissolved organic carbon (DOC) (mg C L-1) (B), POC normalized to cell (pg C cell-1) (C), cellular carbon in POC (%) (D), and DOC normalized to cell (pg C cell-1) (E). Values presented are averages of triplicates with SD as error bars. Probability values refer to two-way ANOVA results for interaction of temperature and N availability influences on organic carbon data. Probability values (p-values) for all temperature and N availability (N+, N± and N-) combinations relating differences in organic carbon data (post hoc Tukey test) are given in Supplementary Tables S1n, o, p and r.

Fig. 6. *Chaetoceros pseudocurvisetus* lipid production. Bulk cellular lipids (CL) (mg L-1) (A), cellular lipids normalized to cell (pg cell-1) (B), proportions of lipid classes to cell lipids (%) for the N-excess (N+), optimal (N±), and N-limiting (N-) cultivations carried out at 15 °C (C), 25 °C (D) and 30 °C (E). Lipid classes: monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), sulfoquinovosyldiacylglycerols (SQDG), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidylcholines (PC), sterols (ST), triacylglycerols (TG) and steryl esters (SE). Values presented are averages of triplicates with SD as error bars.

Fig. 7. *C. pseudocurvisetus* protein production. Bulk (A) and protein concentrations normalized to cell (B), and protein to cellular lipid ratio (C). Data are presented for the cultures grown at 15, 25, and 30 °C and in N-excess (N+), optimal (N±), and N-limiting (N-) medium. Values presented are averages of triplicates with SD as error bars. Probability values refer to two-way ANOVA results for interaction of temperature and N availability influences on cellular protein content and protein:cellular lipid ratio. Probability values (p-values) for all temperature and N availability (N+, N± and N-) combinations relating differences in cellular protein content and protein:cellular lipid ratio (post hoc Tukey test) are given in Supplementary Table S1w and x.

Fig. 8. A diagrammatic representation highlighting the most important findings.

**The effects of high temperatures and nitrogen availability on the growth and composition of the marine diatom *Chaetoceros pseudocurvisetus***

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Table S2. Response of *C. pseudocurvisetus* growth to nitrogen availability and increasing temperature.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Culture age | **N +** | SD | **N ±** | SD | **N -** | SD |
| day | Cell L-1 |
|  | 15 °C |
| 0 | 1.00E+05 | 0.00E+00 | 1.00E+05 | 0.00E+00 | 1.00E+05 | 0.00E+00 |
| 3 | 1.90E+05 | 9.18E+04 | 3.15E+05 | 3.23E+04 | 2.45E+05 | 5.56E+04 |
| 4 | 3.10E+05 | 9.08E+04 | 5.31E+05 | 1.35E+05 | 3.97E+05 | 1.15E+05 |
| 6 | 1.33E+06 | 5.00E+04 | 1.97E+06 | 1.77E+05 | 1.11E+06 | 3.88E+05 |
| 7 | 4.58E+06 | 1.13E+05 | 5.29E+06 | 6.28E+05 | 4.41E+06 | 6.45E+05 |
| 8 | 9.09E+06 | 2.93E+06 | 1.18E+07 | 1.09E+06 | 9.23E+06 | 9.70E+05 |
| 9 | 1.95E+07 | 2.80E+06 | 1.97E+07 | 1.13E+06 | 1.41E+07 | 3.63E+06 |
| 10 | 2.79E+07 | 1.71E+06 | 3.00E+07 | 7.11E+05 | 2.23E+07 | 1.21E+06 |
| 11 | 4.08E+07 | 2.44E+06 | 3.89E+07 | 5.58E+06 | 2.69E+07 | 2.94E+06 |
| 12 | 6.05E+07 | 1.83E+06 | 5.30E+07 | 2.97E+06 | 2.79E+07 | 3.55E+06 |
| 13 | 6.91E+07 | 4.71E+06 | 5.55E+07 | 4.67E+06 | 2.57E+07 | 2.35E+06 |
| 14 | 7.37E+07 | 5.09E+06 | 7.15E+07 | 2.61E+06 | 2.41E+07 | 7.81E+06 |
| 15 | 8.58E+07 | 1.13E+06 | 7.94E+07 | 4.79E+06 |  |  |
| 16 | 8.43E+07 | 1.21E+06 | 7.40E+07 | 2.76E+06 |  |  |
|  | 25 °C |
| 0 | 1.00E+05 | 0.00E+00 | 1.00E+05 | 0.00E+00 | 1.00E+05 | 0.00E+00 |
| 2 | 6.62E+05 | 8.72E+04 | 5.39E+05 | 7.19E+04 | 5.89E+05 | 1.51E+05 |
| 3 | 1.68E+06 | 1.45E+05 | 1.88E+06 | 1.54E+05 | 2.45E+06 | 1.93E+05 |
| 4 | 7.46E+06 | 1.22E+06 | 6.39E+06 | 1.06E+06 | 7.82E+06 | 6.17E+05 |
| 5 | 2.10E+07 | 2.36E+06 | 2.09E+07 | 5.01E+05 | 2.34E+07 | 6.02E+05 |
| 6 | 3.45E+07 | 2.70E+06 | 3.33E+07 | 3.73E+06 | 3.21E+07 | 9.88E+05 |
| 7 | 4.91E+07 | 8.00E+06 | 5.43E+07 | 4.25E+06 | 4.30E+07 | 3.51E+06 |
| 8 | 5.71E+07 | 4.92E+06 | 4.09E+07 | 7.62E+06 | 3.98E+07 | 8.75E+05 |
| 8.5 | 5.71E+07 | 4.92E+06 | 4.09E+07 | 7.62E+06 | 3.95E+07 | 8.00E+05 |
| 9 | 7.00E+07 | 9.50E+06 |  |  | 3.74E+07 | 2.20E+06 |
| 10 | 6.84E+07 | 9.45E+06 |  |  |  |  |
|  | 30 °C |
| 0 | 1.00E+05 | 0.00E+00 | 1.00E+05 | 0.00E+00 | 1.00E+05 | 0.00E+00 |
| 1 | 8.87E+04 | 1.17E+04 | 7.23E+04 | 2.39E+04 | 1.06E+05 | 3.38E+04 |
| 2 | 2.58E+05 | 1.71E+04 | 2.36E+05 | 2.40E+04 | 2.37E+05 | 9.54E+04 |
| 3 | 6.50E+05 | 1.89E+05 | 7.08E+05 | 7.64E+04 | 7.00E+05 | 2.14E+05 |
| 4 | 1.20E+06 | 3.25E+05 | 9.92E+05 | 7.22E+04 | 1.03E+06 | 2.14E+05 |
| 5 | 1.78E+06 | 2.27E+05 | 2.28E+06 | 2.75E+05 | 2.18E+06 | 5.66E+05 |
| 6 | 4.73E+06 | 8.36E+05 | 3.40E+06 | 5.43E+05 | 2.94E+06 | 2.47E+05 |
| 7 | 7.80E+06 | 8.66E+05 | 6.99E+06 | 7.63E+05 | 7.22E+06 | 1.03E+06 |
| 8 | 1.25E+07 | 3.22E+06 | 1.06E+07 | 1.05E+06 | 9.33E+06 | 3.66E+05 |
| 9 | 2.00E+07 | 2.79E+06 | 1.93E+07 | 2.29E+06 | 1.71E+07 | 3.18E+06 |
| 10 | 2.78E+07 | 5.98E+06 | 2.24E+07 | 2.87E+06 | 1.53E+07 | 2.10E+06 |
| 11 | 3.11E+07 | 8.42E+06 | 3.31E+07 | 3.50E+06 |  |  |
| 12 | 4.88E+07 | 9.87E+05 | 4.30E+07 | 5.78E+06 |  |  |
| 13 | 4,75E+07 | 3,47E+06 | 3,82E+07 | 7,52E+06 |  |  |

Table S3. Average length and abundance of measured setae for cells grown in N-replete (N+), referent (N±) and N-limited (N-) at 15 and 25 °C. Cells grown at 30 °C had insufficient number of setae for relevant statistical analysis. Differences in setae length between cultures are either not significant (ns) or are significant at the 0.001 level (\*\*).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | Measured setae |  |  | Difference in setae length |
| T |  | Abundance | Length | SD |  |  |  |  |
| °C |  |  | µm |  |  | N+ | N± |
| 15 | N+ | 154 | 22.31 | 0.83 |  | N+ | - | ns |
| N± | 179 | 22.84 | 0.44 |  | N± | ns | - |
| N- | 152 | 25.94 | 0.79 |  | N- | \*\* | \*\* |
|  |  |  |  |  |  |  |  |  |
| 25 | N+ | 98 | 22.62 | 1.29 |  | N+ | - | ns |
| N± | 133 | 22.44 | 1.73 |  | N± | ns | - |
| N- | 158 | 29.75 | 1.68 |  | N- | \*\* | \*\* |

Table S4. Changes in *C. pseudocurvisetus* cellular pigment concentration.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| T |  | Chl *a* | SD | C:Chl *a* | SD |
| °C |  | pg cell-1 |  |  |
| 15 | N+ | 0.47 | 0.03 | 390.62 | 53.93 |
| N± | 0.51 | 0.02 | 422.47 | 28.78 |
| N- | 0.54 | 0.12 | 289.28 | 46.35 |
|  |  |  |  |  |  |
| 25 | N+ | 2.46 | 0.34 | 45.49 | 7.76 |
| N± | 1.66 | 0.10 | 52.81 | 2.33 |
| N- | 1.02 | 0.23 | 105.04 | 47.80 |
|  |  |  |  |  |  |
| 30 | N+ | 1.84 | 0.24 | 53.12 | 6.52 |
| N± | 2.13 | 0.51 | 56.08 | 14.71 |
| N- | 1.77 | 0.07 | 51.10 | 10.65 |

Table S5. *C. pseudocurvisetus* nutrients' uptake.

|  |  |  |
| --- | --- | --- |
|  |  | Consumed |
| T |  | DIN | SD | PO4 | SD | DIN:PO4 | SD |
| °C |  | % |  |  |
| 15 | N+ | 76.16 | 10.62 | 85.78 | 3.45 | 44.28 | 5.96 |
| N± | 99.17 | 0.17 | 81.41 | 1.05 | 19.48 | 0.26 |
| N- | 97.49 | 0.51 | 17.85 | 7.54 | 13.18 | 7.32 |
|  |  |  |  |  |  |  |  |
| 25 | N+ | 52.34 | 3.33 | 82.88 | 8.39 | 31.85 | 5.34 |
| N± | 63.77 | 5.41 | 47.44 | 2.20 | 21.48 | 1.16 |
| N- | 98.90 | 0.52 | 15.01 | 2.53 | 13.70 | 2.49 |
|  |  |  |  |  |  |  |  |
| 30 | N+ | 27.10 | 9.29 | 43.37 | 2.97 | 25.21 | 1.42 |
| N± | 56.78 | 4.80 | 43.71 | 2.96 | 21.07 | 0.38 |
| N- | 98.02 | 0.14 | 7.02 | 3.52 | 24.73 | 0.05 |

Table S6. *C. pseudocurvisetus* total organic matter production.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| T |  | **DOC** | SD | **DOC** | SD | **POC** | SD | **POC** | SD | **Non-living POC** | SD |
| °C |  | mg L-1 |  | pg cell-1 |  | mg L-1 |  | pg cell-1 |  | pg cell-1 |  |
| 15 | N+ | 1.30 | 0.10 | 15.47 | 1.04 | 17.36 | 0.90 | 205.95 | 9.38 | 22.9 | 24.0 |
| N± | 1.39 | 0.17 | 18.89 | 2.73 | 16.55 | 0.67 | 223.65 | 7.38 | 12.8 | 14.8 |
| N- | 0.69 | 0.01 | 30.46 | 8.31 | 5.23 | 2.24 | 213.14 | 27.29 | 60.6 | 36.3 |
|  |  |  |  |  |  |  |  |  |  |  |  |
| 25 | N+ | 1.97 | 0.23 | 35.93 | 10.15 | 14.99 | 1.05 | 220.93 | 17.39 | 109.8 | 28.94 |
| N± | 1.12 | 0.01 | 24.82 | 0.40 | 8.24 | 0.57 | 181.91 | 13.87 | 118.1 | 33.88 |
| N- | 0.85 | 0.09 | 22.92 | 3.29 | 8.59 | 0.28 | 230.26 | 7.13 | 130.3 | 24.49 |
|  |  |  |  |  |  |  |  |  |  |  |  |
| 30 | N+ | 1.80 | 0.05 | 38.06 | 3.11 | 9.98 | 0.44 | 211.39 | 23.12 | 114.5 | 18.05 |
| N± | 1.73 | 0.05 | 46.54 | 8.89 | 10.15 | 0.99 | 271.58 | 46.54 | 156.6 | 46.50 |
| N- | 1.45 | 0.16 | 97.45 | 26.14 | 3.79 | 0.15 | 251.97 | 36.90 | 161.4 | 18.26 |

Table S7. Quantitative data for cellular lipids of *C. pseudocurvisetus* grown in N-replete (N+), referent (N±) and N-depleted (N-) conditions at 15, 25 and 30 °C.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| T (°C) | Medium | Cell lipids (mg L-1) |  |  |
|  |  | **MGDG** | SD | **DGDG** | SD | **SQDG** | SD | **PG** | SD | **PE** | SD | **PC** | SD | **ST** | SD | **TG** | SD | **SE** | SD | Total cell lipids | SD |
| 15 | N+ | 0.389 | 0.236 | 0.022 | 0.021 | 0.191 | 0.086 | 0.298 | 0.115 | 0.238 | 0.193 | 0.018 | 0.021 | 0.101 | 0.039 | 0.065 | 0.020 | 0.002 | 0.004 | 1.326 | 0.435 |
| N± | 0.333 | 0.077 | 0.000 | 0.000 | 0.132 | 0.043 | 0.229 | 0.060 | 0.103 | 0.031 | 0.004 | 0.002 | 0.133 | 0.041 | 0.075 | 0.028 | 0.006 | 0.006 | 1.014 | 0.202 |
| N- | 0.144 | 0.065 | 0.006 | 0.010 | 0.089 | 0.016 | 0.138 | 0.024 | 0.054 | 0.018 | 0.003 | 0.000 | 0.038 | 0.022 | 0.056 | 0.025 | 0.004 | 0.004 | 0.532 | 0.170 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 25 | N+ | 0.307 | 0.057 | 0.014 | 0.015 | 0.154 | 0.017 | 0.320 | 0.075 | 0.245 | 0.026 | 0.029 | 0.025 | 0.136 | 0.019 | 0.121 | 0.028 | 0.006 | 0.005 | 1.332 | 0.119 |
| N± | 0.282 | 0.103 | 0.023 | 0.008 | 0.150 | 0.064 | 0.231 | 0.031 | 0.135 | 0.059 | 0.065 | 0.014 | 0.114 | 0.005 | 0.043 | 0.004 | 0.005 | 0.001 | 1.048 | 0.110 |
| N- | 0.225 | 0.057 | 0.015 | 0.004 | 0.107 | 0.056 | 0.184 | 0.084 | 0.147 | 0.108 | 0.022 | 0.013 | 0.074 | 0.008 | 0.093 | 0.015 | 0.007 | 0.005 | 0.874 | 0.193 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 30 | N+ | 0.294 | 0.047 | 0.049 | 0.003 | 0.187 | 0.032 | 0.248 | 0.064 | 0.079 | 0.018 | 0.014 | 0.003 | 0.064 | 0.006 | 0.062 | 0.010 | 0.003 | 0.003 | 1.001 | 0.163 |
| N± | 0.215 | 0.034 | 0.021 | 0.013 | 0.161 | 0.034 | 0.211 | 0.056 | 0.152 | 0.047 | 0.008 | 0.002 | 0.066 | 0.016 | 0.065 | 0.017 | 0.008 | 0.002 | 0.907 | 0.152 |
| N- | 0.062 | 0.010 | 0.009 | 0.002 | 0.103 | 0.008 | 0.123 | 0.016 | 0.062 | 0.023 | 0.005 | 0.002 | 0.022 | 0.001 | 0.042 | 0.008 | 0.005 | 0.003 | 0.433 | 0.050 |

Table S8. Quantitative data for contribution of lipid classes to cell lipids (%) for N-replete (N+), referent (N±) and N-depleted (N-) conditions at 15, 25 and 30 °C.

|  |  |  |
| --- | --- | --- |
| T(°C) | Medium | Cell lipids (%) |
|  |  | **MGDG** | SD | **DGDG** | SD | **SQDG** | SD | **PG** | SD | **PE** | SD | **PC** | SD | **ST** | SD | **TG** | SD | **SE** | SD |
| 15 | N+ | 28.65 | 12.83 | 1.37 | 1.28 | 13.41 | 2.45 | 21.41 | 2.67 | 15.99 | 10.44 | 1.14 | 1.25 | 8.76 | 6.61 | 5.30 | 2.94 | 0.26 | 0.45 |
| N± | 31.52 | 1.35 | 0.00 | 0.00 | 12.36 | 1.94 | 21.63 | 2.11 | 9.68 | 1.44 | 0.40 | 0.21 | 13.12 | 4.85 | 7.09 | 2.06 | 0.62 | 0.55 |
| N- | 26.07 | 6.25 | 0.75 | 1.29 | 16.89 | 2.66 | 26.47 | 5.50 | 10.02 | 1.12 | 0.60 | 0.20 | 6.60 | 1.60 | 10.12 | 1.66 | 0.64 | 0.55 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 25 | N+ | 22.56 | 2.99 | 0.97 | 0.98 | 11.46 | 2.07 | 23.45 | 3.97 | 18.05 | 0.71 | 2.21 | 1.95 | 10.14 | 1.96 | 8.89 | 1.41 | 0.44 | 0.39 |
| N± | 27.01 | 9.90 | 2.16 | 0.54 | 14.03 | 4.95 | 21.93 | 2.29 | 12.58 | 4.56 | 6.08 | 0.78 | 10.87 | 1.46 | 4.16 | 0.77 | 0.51 | 0.11 |
| N- | 26.79 | 10.39 | 1.73 | 0.56 | 11.59 | 3.40 | 20.15 | 4.73 | 15.48 | 7.94 | 2.72 | 1.80 | 8.49 | 1.34 | 10.88 | 3.30 | 0.87 | 0.70 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 30 | N+ | 28.79 | 1.10 | 4.91 | 0.79 | 18.34 | 0.42 | 24.10 | 2.25 | 7.71 | 0.75 | 1.32 | 0.14 | 6.37 | 1.08 | 6.11 | 1.12 | 0.35 | 0.36 |
| N± | 23.66 | 4.00 | 2.35 | 1.51 | 17.47 | 0.99 | 22.78 | 2.46 | 16.60 | 4.60 | 0.84 | 0.10 | 7.21 | 1.82 | 7.00 | 0.68 | 0.91 | 0.21 |
| N- | 14.11 | 2.45 | 2.02 | 0.40 | 23.26 | 0.84 | 27.90 | 2.63 | 13.82 | 3.76 | 1.14 | 0.39 | 4.93 | 0.52 | 9.50 | 1.08 | 1.08 | 0.60 |

Table S9 .Contribution of individual lipid classes to cell lipids (%) analyzed for mean differences (n = 9) in respect to N availability at each growth temperature (T). Significance level at: 0.05 (\*), 0.01 (\*\*), not significant (ns).

|  |  |
| --- | --- |
| T (°C) | Cell lipids (%) |
|  | **MGDG** | **DGDG** | **SQDG** | **PG** | **PE** | **PC** | **ST** | **TG** | **SE** |
| 15 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| 25 | ns | ns | ns | ns | ns | \* | ns | \* | ns |
| 30 | \* | \* | \*\* | \* | ns | ns | ns | \* | ns |

Table S10. Pearson correlation coefficient (r) among growth
temperature, DIN concentration and content of cellular lipid classes.
Significance level at: 0.05 (\*), 0.01 (\*\*), not significant (ns).

|  |  |  |  |
| --- | --- | --- | --- |
| Cell lipids (%) | Temperature | DIN concentration |  |
|  | r | p | r | p | n |
|  |  |  |  |  |  |
| MGDG | -0.35 | ns | 0.20 | ns | 27 |
| DGDG | 0.62 | \*\* | 0.28 | ns | 27 |
| SQDG | 0.42 | \* | -0.24 | ns | 27 |
| PG | 0.14 | ns | -0.15 | ns | 27 |
| PE | 0.11 | ns | 0.07 | ns | 27 |
| PC | 0.22 | ns | 0.04 | ns | 27 |
| ST | -0.34 | ns | 0.11 | ns | 27 |
| TG | 0.02 | ns | -0.41 | \* | 27 |
| SE | 0.23 | ns | -0.45 | \* | 27 |

Table S11. *C. pseudocurvisetus* protein production.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| T |  | Protein | SD | Protein | SD | Protein:Cell lipid | SD |
| °C |  | mg L-1 |  | pg cell-1 |  |  |  |
| 15 | N+ | 9.84 | 0.91 | 116.70 | 9.92 | 7.74 | 2.72 |
| N± | 9.78 | 0.67 | 132.07 | 7.82 | 9.49 | 1.48 |
| N- | 2.17 | 1.23 | 85.96 | 20.40 | 3.82 | 0.89 |
|  |  |  |  |  |  |  |  |
| 25 | N+ | 8.27 | 0.07 | 122.59 | 17.57 | 6.12 | 0.48 |
| N± | 3.91 | 0.76 | 86.25 | 17.44 | 3.78 | 0.49 |
| N- | 2.10 | 0.41 | 56.62 | 12.47 | 2.38 | 0.05 |
|  |  |  |  |  |  |  |  |
| 30 | N+ | 5.90 | 0.59 | 129.27 | 7.88 | 4.67 | 1.48 |
| N± | 5.42 | 0.94 | 142.54 | 7.60 | 5.94 | 0.90 |
| N- | 2.00 | 0.14 | 132.58 | 14.31 | 4.55 | 0.48 |

Table S12. Bulk (mg L-1) and normalized (pg cell-1) protein content and Protein:Cell lipid ratio analyzed for significance of the mean difference between N+, N± and N- culture at each growth temperature (T). Significance level at: 0.05 (\*), 0.01 (\*\*), not significant (ns).

|  |  |  |
| --- | --- | --- |
| T | Protein | Protein:Cell lipid |
| °C | mg L-1 | pg cell-1 |  |
| 15 | \* | ns | \* |
| 25 | \*\* | ns | \*\* |
| 30 | \*\* | \* | ns |