**Cellular response of marine diatom *Chaetoceros pseudocurvistus* to warming and varying nitrogen availability**

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**Running title**

Diatom *Chaetoceros pseudocurvistus* 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 conversion of acquired inorganic nutrients into organic molecules needed for cell growth and division depends strongly on the environmental conditions of the phytoplankton. Nitrogen (N) excess and especially N scarcity, as well as warming, lead to a variety of responses in cellular metabolism aimed at acclimation or adaptation of species. Here, we used a multimethod approach to study the physiology and biochemical response of the marine diatom *Chaetoceros pseudocurvisetus* to different temperatures (15, 25, and 30 °C) and different N:P ratios (N-excess (N:P = 50:1), optimal (N:P = 16:1), and N- limiting (N:P = 2:1)). The studied stressors have different effects, with N-excess having the least effect. High temperature (30 °C) has a stronger effect than nitrogen deficiency on significant cell death, N assimilation, increased Chl *a* accumulation, cessation of setae formation, and cell lipid remodeling. The most significant change in cellular lipids at 30 °C was observed in thylakoid lipids. Increased levels of thylakoid lipids digalactosyldiacylglycerols (DGDG) and sulfoquinovosyldiacylglycerols (SQDG) are explained by the role of SQDG in achieving thermotolerance and the role of DGDG in balancing electrostatic repulsion between negatively charged SQDG. Nitrogen limitation has a greater effect than an increase in temperature on the retardation of *C. pseudocurvistus* growth and reproduction, a marked elongation of setae, decreased phosphorus assimilation, increased lipid accumulation, and decreased protein synthesis. The elongation of setae observed under conditions of N-limitation likely serves to increase the surface area available for uptake of inorganic and/or organic nitrogen. An optimal N:P ratio is important for cell health, as inferred from the highest protein-to-cell lipid ratio.

**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; SD – Standard deviation; SE – Steryl esters; PSll – Photosystem ll; SST – Sea surface temperature; ST – Sterols; SQDG - Sulfoquinovosyldiacylglycerol; TG – triacylglycerols; TLC-FID - Thin-layer chromatography coupled with flame-ionization detection; TN – Total cellular nitrogen

**Introduction**

The global ocean is subject to climate-driven changes in surface temperature, oxygen concentration, pH, and nutrient cycling (IPCC, 2019). Global sea surface temperature (SST) has increased by 0.79°C since the pre-industrial era and is projected to increase at a rate of 0.13°C per decade (IPCC, 2013). 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). Some projections suggest that the problem will be exacerbated due to increasing winds that promote upwelling of nutrient-rich waters, extreme rainfall followed by runoff, and increased hypolimnetic oxygen depletion (IPCC, 2007). 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, contributing to about 45% of oceanic and 20% of planetary primary production (Malviya et al., 2016). 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 (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*. 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 was the diatom *Chaetoceros pseudocurvisetus* Mangin 1910. 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. 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.

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. 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 mixed manually twice a day. Growth was ended at the beginning of the stationary growth 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) 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). For morphological analysis the length of at least 800 setae were measured.

**Chl *a* analysis**

Whatman GF/F filters (0.7 µm) remained after filtration of 50 mL culture media samples 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).

**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 burned at 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-burned 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 4.3 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 BSA 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. 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 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 S1). N-replete medium yielded highest cell abundances at all examined temperatures while cells were least abundant in N-limiting conditions. 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.



Fig. 1. Growth curves of *Chaetoceros 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.

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 |

We observed elongation of the setae in cultures grown in N-limiting medium compared to optimal and N-excess cultures (p < 0.001, one-way ANOVA) (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 7.15 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).



Fig. 2. Dependence of *C. pseudocurvisetus* setae length 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. Culture subsamples for imaging (as represented here) were harvested in early stationary phase preserved in formaldehyde solution. Photomicrographs were taken using the Zeiss Axio Observer 7 inverted microscope.

**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 (Fig. 3A, Supplementary Table S3). As shown in Fig. 3B, there was a decrease in the cellular carbon-to-Chl *a* ratio of the cells (Cell C:Chl *a*) with increasing temperature for all cultures (Supplementary Table S3). 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.



Figure 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.

**Nutrients' 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 S4). 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. 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 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. 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). 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 S4). 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). 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).



Fig. 4. *Chaetoceros pseudocurvisetus* nutrients' 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 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.

Table 2. Total DIN and PO43-taken upand their uptake rates for N-excess (N+), optimal (N±), and N-limiting (N-) conditions and at 15, 25 and 30 °C.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | 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 |

**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 (erroneously assuming the onset of stationary phase) (cf. Fig. 1B). Bulk POC concentration generally decreased with increasing temperature and with decreasing N availability, whereas opposite trends were observed for DOC production (Figs. 5A and 5B, Supplementary Table S5).

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 S5). 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). Cellular carbon in POC (%) (Fig. 5D, Supplementary Table S5) decreased with increasing temperature 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 high reactivity of non-living POC (Harvey et al., 1995), the DOC content per cell increased in parallel with an increased proportion of non-living POC (Fig. 5E, Supplementary Table S5). The highest value of DOC, 97.4 ± 26.1 pg C cell-1, was observed for the N-limiting condition at 30°C.



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.

**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 (Fig. 6A). The highest CL concentration was detected at 15 °C for N-excess cultures (1.37 ± 1.5 mg L-1), and the lowest for N-limiting cultures at 30 °C (0.44 ± 0.05 mg L-1) (Supplementary Table S6). The CL content per cell (pg cell-1) increased with temperature in all media types and was highest for N-limiting cultures at all three temperatures for which it ranged from 22.6 ± 2.4 to 29.2 ± 1.5 pg cell-1 (Fig. 6B). The minimum content of cellular lipids was found at 15°C for N-optimal cultures (14.2 ± 2.9 pg cell-1).

The relative quantities of CL classes changed as a function of increasing temperature and N availability in the medium (Fig. 6C-E, Supplementary Table S7). 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 S8 and S9.



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). Values presented are averages of triplicates with SD as error bars.

**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 S10 and S11). 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 S10. 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. The highest protein:cellular lipid ratio was detected for the *C. pseudocurvisetus* growing in optimal medium at 15 °C (9.49 ± 1.48). We observed no statistical difference for the protein:cellular lipid ratio for the cultivations at 30 °C and three different N amendments (Supplementary Table S11). 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.



Fig. 7. *C. pseudocurvisetus* protein production. Bulk (A) and protein concentrations normalized to cell (B), and (C) protein to cellular lipid ratio. 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.

**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. Against this background, in the present study, we investigated the whole-cell response of the diatom *C. pseudocurvisetus* to warming and variable nitrogen supply.

***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 Table S1). 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 with sufficient DIN concentration (N-excess and optimal) were able to grow undisrupted and reach their maximum abundance at 15 °C, which is considered as the optimal temperature for *C. pseudocurvisetus* (Novak et al., 2018). No significant difference between maximum abundance under N-excess and optimal conditions, suggests that the very dense phytoplankton population that characterizes eutrophic seas (e.g. the Baltic sea (Gustafsson et al., 2012) may be a consequence of continuous blooms of many different species, rather than a “superbloom” of one species. 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 Table S2) 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 *C. 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). Also, setae elongation of smaller cells growing under N-limited conditions probably represents an energy saving mechanism on expenditure of cell size to increase surface area for inorganic nitrogen uptake (nitrogen transport systems) while increasing and maintaining buoyancy. 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 and (ii) mechanical protection from grazers (Bosak et al., 2016). 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 Table S3) 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 Table S5). Nutrient availability appeared to be less influential on cellular carbon and Chl *a* content than high temperature. 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 S3). 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 > 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 S4). 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 S4). 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 S10) 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 Table S4). 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 S5). 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 S5), 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 Table S6). 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. Marine diatoms *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 S8) can be explained with their role in thylakoid membranes and the role in achieving thermotolerance (Chen *et al*., 2006; 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 (Demé *et al.,* 2014), serving 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 sea-ice diatoms (Mock and Kroon, 2002). 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 N-referent 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 3), 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.

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