**Global warming and oligotrophication lead to increased lipid production in marine phytoplankton**

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# ABSTRACT

Earth temperature is rising and oligotrophication is becoming apparent even in coastal seas. In this changing environment, phytoplankton use carbon and nutrients to form important biomolecules, including lipids. However, the link between lipid production and changing environment is still unexplored. Therefore, we investigated the phytoplankton lipid production in the diatom *Chaetoceros pseudocurvisetus* cultures under controlled temperatures ranging from 10 to 30 °C and nutrient regimes mimicking oligotrophic and eutrophic conditions. Results were compared to plankton community’s lipid production in the northern Adriatic at two stations considered as oligotrophic and mesotrophic during an annual monthly sampling. In order to gain detailed information on the investigated system, we supplemented lipid data with chlorophyll *a* concentrations, phytoplankton taxonomy, cell abundances and nutrient concentration along with hydrographic parameters. We found enhanced particulate lipid production at higher temperatures, and substantially higher lipid production in oligotrophic conditions. Enhanced lipid production has two opposing roles in carbon sequestration; it can act as a retainer or a sinker. Lipid remodeling, including change in ratio of phospholipids and glycolipids, is more affected by the nutrient status, than the temperature increase. Triacylglycerol accumulation was observed under the nitrogen starvation.

*Keywords:* marine lipids, temperature, nutrients*,* Northern Adriatic Sea, *Chaetoceros pseudocurvisetus*, diatoms

# Introduction

Global warming is defined as an increase in combined air and sea surface temperatures (T) averaged over the globe and over a 30-year period (IPCC, 2018). If the warming continues at this rate, it is likely to reach an increase above 1.5 °C between 2030 and 2052, in respect to pre-industrial levels (IPCC, 2018). Increase in T leads to the variable acceleration of both, chemical as well as biological reactions. For example, biologically important processes such as photosynthesis and respiration react differently to ambient T changes. The T rise strongly favors respiration leading to conversion of organic carbon back to CO2 (Allen et al., 2005).  Global warming might also induce a series of cascading feedbacks in both terrestrial and marine carbon cycling processes (Boscolo-Galazzo et al., 2018). Many uncertainties exist in understanding (i) the quality and quantity of produced organic matter (OM) in surface productive layer, (ii) factors influencing the increased/decreased OM production, and (iii) the OM transport into deep ocean layers. These are important factors influencing the vertical redistribution of bioactive elements within the ocean, also impacting carbon sequestration and consequently climate change.

Oligotrophication across the world oceans is evidenced by measured decrease in chlorophyll *a* concentrations (Agusti et al., 2017) and reduced primary production (Behrenfeld et al., 2006). In Europe, the air T increased for 0.9 °C from 1901 to 2005, and consequently decreased precipitation (35-40%) was noted. This resulted in decreased river nutrient inputs, which were identified as one of the main causes of oligotrophication. Oligotrophicaton was particularly noted in the southern Europe, including the northern Adriatic watershed (Cozzi and Giani, 2011). Increased T also leads to greater stratification of the water column and reduced diffusion of the vertical nutrient supply (Agusti et al., 2017).

Phytoplankton are responsible for almost half of the total global primary production (Field et al., 1998). Within phytoplankton, diatoms are the most abundant and ecologically most successful group (Malviya et al., 2016), playing a significant role in OM production and carbon cycling in the marine ecosystem (Obata et al., 2013). By fixing CO2 and consuming nutrients they synthesize carbohydrates, proteins, and lipids (Zulu et al., 2018). Lipids are an important component of marine productivity and an integral part of particulate organic matter (Parrish, 1988). As high-energy components rich in carbon, they are metabolic fuel as well as membrane and signal molecules for all organisms (Arts et al., 2009). Phytoplankton’s ability to adapt to the changing environment is reflected in the production of variety of lipids (Thompson, 1996). Characterization of marine lipids on the molecular level enables their use as biogeochemical markers. They are useful in identification of sources and cycling of OM, as well as phytoplankton adaptation to the environmental stress (Guschina and Harwood, 2009). The distribution between different lipid classes and quantity of lipids depend on environmental factors and the stage of the cell cycle of primary producers (Zhukova and Aizdaicher, 2001).

Lipids are hydrophobic molecules structured mostly of hydrophobic fatty acid chains. In addition, many lipids have a hydrophilic head (phospholipids, glicolipids, betaine lipids). The hydrophobic character of lipids influences their accumulation on hydrophobic phase boundaries, such as the sea surface where they can be readily transformed by rich microbial community and ultraviolet radiation (UV) (Cunliffe et al., 2013). Hydrophilic parts of the molecule enable their adsorption onto mineral particles and larger high molecular weight organic particles (Morris and Eglinton, 1977). Futhermore, they interact with bulk carbohydrates via hydrogen bonding, nonpolar and electrostatic interactions (Kozarac et al., 2000). These processes might be responsible for fast sedimentation of particle associated lipids to sea or ocean floor. Therefore, lipids may have potential to sequester carbon from the upper ocean when associated with sinking particles. It was found that lipid-like material is a significant source of the uncharacterized organic carbon in the ocean (Hwang and Druffel, 2003). Recent studies highlighted the importance of lipid saturation for the carbon export from the surface to the deep ocean (Gašparović et al., 2016; 2017b; 2018a). Moreover, highly unsaturated novel phospholipids, possibly formed by cross-linking of unsaturated compounds in oceanic depths, are selectively preserved in the ocean. That made them both phosphorus and carbon carriers to the ocean deepths (Gašparović et al., 2018b).

In this study, we aimed to investigate an impact of two environmental factors, T and nutrient availability, on lipid production by phytoplankton. We intended to determine which of these two factors could have more impact and if there is synergetic effect of the high T and nutrient scarcity. We first preformed experiments on phytoplankton monocultures in the laboratory and then proceeded to the *in situ* survey of the northern Adriatic ecosystem. As a model phytoplankton, we have chosen a diatom from the *Chaetoceros* family, common in the northern Adriatic (Bosak et al., 2016). We performed microcosm experiments in nutrient replete and depleted conditions, covering the recently registered data of the northern Adriatic annual T and nutrient variations. The northern Adriatic (Fig. 1) is characterized by a gradient of trophic conditions, from meso- and eutrophic (western) to oligotrophic (eastern) parts (Justić et al., 1995). Additionally, the northern Adriatic has large annual T variations (8 – 30 °C), especially in the upper water column (Gašparović, 2012). Our study provides an important contribution to projecting phytoplankton responses to future anthropogenic drivers.

# Methods

## Laboratory experiments

### Monoclonal culture establishment

For the study of T influence on the cell growth and lipid production, we selected a representative *Chaetoceros* taxon. *Chaetoceros* occurs in the northern Adriatic all year round indicating its possibility to accommodate to wide T range (Bosak et al., 2016). We manually isolated one chain of *Chaetoceros* from a mesh sample collected at the SJ101 station in October 2014 (Fig. 1), and identified it as *Chaetoceros pseudocurvisetus.* The successfully grown monoclonal culture was maintained in f/2 medium (Guillard, 1975) at 15 °C, 4500 lux, on 12:12 h light/dark photoperiod, and was sub-cultured every 2–3 weeks.

### DNA analysis

We performed a DNA analysis to confirm the microscopically preformed identification. We confirmed that the cultivated species was indeed *Chaetoceros pseudocurvisetus*. For the DNA extraction, 30 ml of the monoculture in exponential growth phase, was filtered through the 1.2 µm cellulose filter (Merck Millipore) and frozen at -80 ºC. Genomic DNA was extracted and amplified following methodology described in Smodlaka Tanković et al. (2018). The obtained sequences were first aligned and then analyzed using software Geneious 11.1.5 (Kearse et al., 2012). For search and comparison of data with NCBI GenBank database BLAST algorithm was used (Altschul et al., 1997; Benson et al., 2010). We used the large subunit 28S as DNA barcode. The Nucleotide BLAST similarity search found the 28S sequence in the Gene Bank database (Theriot et al., 2010), and it showed 100% pairwise identity (on 100/ query cover) with the sequences. The small and large subunit ribosomal RNA gene partial sequence was deposited in the GeneBank under accession numbers MG385841 FOR 18S DNA and MG385842 for 28S DNA.

### Chaetoceros pseudocurvisetus cultures

In the experiment, *C. pseudocurvisetus* was cultured in 850 ml sterile VWR® Tissue Culture Flasks (VWR, Radnor, Pennsylvania), under five T (10, 15, 20, 25 and 30 °C) representing the T range in the northern Adriatic (Gašparović et al., 2012). Light intensity was 4500 LUX, and the cultures were maintained in the 12:12 h light/dark photoperiod. We adjusted the optimum concentrations of nutrients according to nutrient replete f/2 medium (Guillard, 1975). In the replete medium, average of orthophosphate (PO4) was 36.33±6.56 µmol/l and dissolved inorganic nitrogen (DIN=NO3+NO2+NH4) of 1059±181.1 µmol/l. In phosphorous depleted (P-depleted) medium PO4 concentrations were 0.6±0.1 µmol/l. Concentrations of DIN in nitrogen depleted medium (N-depleted) were 7.6±1 µmol/l. Concentration of nutrients was measured before inoculation of cells. Corresponding N/P ratios were 29.4, 1765, and 0.2 for replete P-depleted and N-depleted media, respectively. Aseptic techniques in all culture manipulations were used, even though the cultures were monoclonal rather than axenic. To ensure a low organic content, seawater was taken during the winter on the oligotrophic side of the northern Adriatic, when phytoplankton activity was low. Collected water was pre-filtered through 0.7 µm Whatman GF/F filters and rested for 2 months in the dark. Rested water was filtered again through sterile 0.22 µm white plain filters (Merck Milipore ltd.), and boiled in microwave (Keller et al., 1988) before adding sterile media amendments.

We started the experiment by inoculating 105 cells into each 800 ml batch culture medium, in duplicates. Before inoculation, cells were kept in f/2 replete media in 250 ml VWR® Tissue Culture Flasks (VWR, Radnor, Pennsylvania) under T of experiment. To establish the grow rate of *C. pseudocurvisetus,* we preformed cell counts every two days with Fuchs-Rosenthal Chamber hemocytometer under an Olympus BX51-P polarizing microscope. We terminated the growth by filtering samples through 0.7 µm Whatman GF/F filters, at the beginning of the stationary phase. Filters cleaned of organic matter by pre-burning at 450°C for 4h were used. After filtration, filters were frozen at -80°C and stored until the lipid extraction.

In order to calculate DIN and PO4 uptake ratios, their concentrations were determined at the beginning and at the end of 15 °C batch culture experiments, using standard spectrophotometric methods (Parsons et al., 1984). DIN and PO4 uptake ratios were calculated as the difference between their initial and final concentrations.

Total particulate organic matter (POC) was measured using the high-temperature catalytic oxidation method. POC was analyzed by solid sample module SSM-5000A connected to Shimadzu TOC-VCPH carbon analyzer, calibrated with glucose (Sugimura and Suzuki, 1988). POC concentrations were corrected based on blank filter measurements. The average filter blank with the instrument blank corresponds to 0.005 mg/1. The reproducibility for the glucose standard was 3%.

### Alkaline phosphatase activity

To determine the *C. pseudocurvisetus* alkaline phosphatase activity (APA), an additional experiment was performed. *C. pseudocurvisetus* (initial concentrations 1.65 x 105 cell/l) was inoculated in triplicate in the replete and P-depleted medium. Culture batches were incubated at 15 °C, with a light dark cycle of 12:12 h, in sterile 250 ml vented culture flasks (easy flasks, Nuclon, Denmark). The experiment lasted for 10 days. The APA was measured on days 3, 6, 8, and 10. The APA was measured by florogenic substrate, methylumbeliferyl phosphate (MUF-P), according to the procedure described in Hoppe (1983). The MUF-P was dissolved in 2-methoxyethanol and diluted in filtered (0.22 µm pore size) and autoclaved sea water prior to use. Fifty µL of substrate (concentration 250 µΜ) was added in 200 µL of live monoclonal cultures (final concentration 50 µM). The MUF-P hydrolysis product methylumbelliferone (MUF) fluorescence was measured by Tecan M200 Pro spectrofluorimeter (excitation at 365 nm and emission at 460 nm). The reaction was incubated at 16°C in the dark and fluorescence was measured at intervals of 0, 10, 30 and 60 minutes. The fluorescence increased linearly over the incubation time. The APA (nmol/lxs) was calculated as the difference between two measurements divided by the incubation time after calibration of instrument with the MUF. To produce a standard curve, a range of the MUF concentrations (0-1500 μM) was used. Cell-specific activity was calculated by dividing APA by the cell abundance.

## In situ measurements

### Site description, environmental measurements and sample collection

The northern Adriatic is a shallow basin, with an average depth of 30 m (Orlić et al., 1992). It is under eutrophic pressure by the Po River at its western part and oligotrophic influence from the middle Adriatic Sea at the eastern part (Djakovac et al., 2012). In warmer months (from April to October) anticyclonic circulation in the central and eastern part of the northern Adriatic induces a strong southeast current known as Istrian Coastal Countercurrent (ICCC) (Supić et al., 2003) (Fig.1). ICCC transports nutrient replete water toward eastern, oligotrophic part of the northern Adriatic, where it increases primary production (Giani et al., 2012).

We performed the sampling at two northern Adriatic stations with opposing trophic conditions. Station SJ101 is situated close to the Po River inflow and is usually considered eu-mesotrophic. Station RV001 is situated 1 nautical mile from the city of Rovinj, and is prevalently oligotrophic (Fig. 1). Surface water samples (0 m) were collected monthly, during research vessel Vila Velebita cruises from March 2013 to March 2014. In June, December, and February, sampling was not possible due to the bad weather conditions. Seawater T and salinity (S) were determined by CTD probe (SBE 25 Sea logger CTD, Sea-Bird Electronics, Inc., Bellevue, Washington, USA). Water samples for the analysis of nutrients, chlorophyll *a* (Chl *a*), lipids, and phytoplankton were collected with 5 l Niskin bottles.

For the lipid analysis, 3 l of water was pre-filtered through a 200 μm stainless steel screen and collected in glass bottles. Pre-filtration was preformed to remove larger particles including zooplankton. Immediately after, seawater sample was filtered on 0.7 µm pore size grade GF/F Whatman® glass microfiber filters, cleaned of organic matter by pre-burning at 450 °C for 4h. Filters were stored in cryotubes, first in liquid nitrogen, and then at -80 °C until further analysis.

Subsamples for nitrate (NO3), nitrite (NO2) and orthophosphate (PO4) determination were measured on board by standard spectrophotometric methods (Parsons et al., 1984). Ammonium (NH4) was analyzed using modified indo-phenol method (Ivančić and Degobbis, 1987).

For chlorophyll *a* (Chl *a*) determination, subsamples were filtered on GF/C Whatman® glass microfiber filters. Filters were grinded and Chl *a* was extracted in 90% acetone for 3h to 24 h (Arar and Collins, 1997) in the dark. Final Chl *a* concentrations were determined by turner TD-700 fluorimeter (Parsons et al., 1984).

### Quantitative and qualitative phytoplankton analysis

Samples (200 ml) for the identification and enumeration of phytoplankton cells were preserved in neutralized formaldehyde (2% final concentration) solution (Kemika d.d. Zagreb, Croatia) (Throndsen, 1978). Phytoplankton cells were enumerated (Utermöhl method) using an inverted microscope (Zeiss Axiovert 200; Zeiss GmbH, Oberkochen, Germany) equipped with phase contrast and differential interference contrast optics (Lund et al., 1958; Utermöhl, 1958). Phytoplankton were identified to the lowest possible taxonomic rank using determination keys listed in Godrijan et al. (2012).

## Lipid extraction and analysis

We have extracted particulate lipids by one–phase solvent mixture of dichloromethane–methanol–water (Blight and Dyer, 1959). As an internal standard, we added 10 µg n-hexadecanone into each sample. After extraction, samples were evaporated under nitrogen atmosphere to dryness and stored at -20 °C. Right before lipid separation, samples were re-dissolved in 20 – 40 µl dichloromethane. Eighteen lipid classes were separated on Chromarods SIII and quantified by an external calibration with standard lipid mixture and by thin–layer chromatography–flame ionization detection (TLC–FID) (Iatroscan MK–VI, Iatron, Japan), with a hydrogen flow of 160 ml/min and air flow of 2000 ml/min (Gašparović et al., 2015; 2017a). Each sample was analyzed in duplicate. Total lipid concentration is a sum of all lipid classes quantified by the flame ionization. Determined lipids include following classes: wax esters and sterol esters (WE/SE); fatty acid methyl esters (ME); fatty ketone hexadecanone (KET, internal standard); triacylglycerols (TG); free fatty acids (FFA); fatty alcohols (ALC); 1,3-diacylglycerols (1,3 DG); sterols (ST); 1,2-diacylglycerols (1,2 DG); pigments (PIG); monoacylglycerols (MG); mono- and di-galactosyldiacylglycerols (MGDG and DGDG); sulfoquinovosyldiacylglycerols (SQDG), phosphatidylglycerols (PG); phosphatidylethanolamines (PE); and phosphatidylcholine (PC). Concentration of glycolipids (GL) is expressed as a sum of MGDG, DGDG, and SQDG; and phospholipids (PL) as a sum of PG, PE, and PC. Analysis procedure was described by Gašparović et al. (2017) in detail. We would like to point out that in the monoculture experiment we discussed WE/SE TLC band as SE, while in the northern Adriatic samples the band was discussed as WE. Namely, WE are zooplankton storage lipids (Lee et al., 2006) and cannot be found in phytoplankton monocultures. Since the WE/SE TLC band contains only small amounts of sterol esters (Hudson et al., 2001) it is referred to as zooplankton wax esters for the northern Adriatic samples.

## Data analysis

We calculated the growth rate with the equation:

No and Nm are algal concentrations at the beginning and at the end of batch culture experiment, and *t* is time in days (Thompson et al., 1992).

To evaluate the relationship between different measured parameters Principal Component Analysis (PCA) in Statistica Release 7 software was used. The strength of a linear association between two variables was evaluated by Pearson correlation coefficientsusing Excel 2016 software.

# Results

## Chaetoceros pseudocurvisetus cultures

### *Chaetoceros pseudocurvisetus physiology*

We have studied the effect of T changes and nutrient availability on the growth rate and lipid production of diatom *C. pseudocurvisetus*. The cells were grown at 10, 15, 20, 25 and 30 °C, and in three opposing media: replete, P- depleted, and N-depleted. *C. pseudocurvisetus* cell counts, growth rates, and average carbon content are presented in Table 1. As a measure of the cell carbon content we used POC and normalized it to cell numbers. This was done because all POC in the experiment was produced during *C. pseudocurvisetus* growth, as it was zero at the beginning of the experiment. These data were used to calculate percentage of intracellular lipid carbon (carbon allocated to lipids).

Maximum cell abundance was detected at 15 °C for all batches, and minimum was detected at 10 °C for replete, at 20 °C for P-depleted and at 30 °C for N-depleted. There is a significant difference in growth rates depending on T and medium. The highest growth rate in replete and N-depleted growth was detected between 20 °C and 25 °C, and lowest at 10 °C. In P-depleted medium, the highest growth rate was detected at 15 °C, and lowest at 10 °C and 20 °C. The number of days for the cultures to reach the stationary phase were 14.2±1.6, 10.7±0.5, 7.2±0.2, 7.7±0.0, 8.7±0.5 for 10, 15, 20, 25 and 30 °C, respectively.

To investigate if *C*. *pseudocurvisetus* overcomes the PO4 deficiency by alkaline phosphatase (AP) activation, we measured APA of batch cultures grown in replete and P-depleted media at 15 °C (Fig. 2). *C. pseudocurvisetus* activate AP at both growing conditions. Cell-specific APA was much higher in cultures grown under P-limited conditions.

We tested the DIN/PO4 uptake for *C. pseudocurvisetus* grown at 15°C, what is considered as optimal T for their growth (Novak et al., 2018). N/P uptake ratio was 16.4±0.2 in replete medium which corresponds to the Redfield ratio (Redfield, 1934). More phosphorous was consumed (N/P=2.6±0.3) in N-depleted and more nitrogen (N/P=72.9±4.2) in P-depleted medium.

### Chaetoceros pseudocurvisetus lipid production

The T and nutrient availability (Fig. 3 and supplementary Table S1) affected lipid production. To get an insight into lipid production we normalized lipids to number of cells. Lipid classes are divided in two groups. First, the cell lipids with two subgroups: (i) lipids that are located predominantly in membranes (membrane lipids) (PL, GL, ST, and PIG) and (ii) intracellular reserve lipids (SE, inert storage forms of sterols, and TG being phytoplankton metabolic energy reserves). Second group are free fatty acids, alcohols, diacylglycerols, and monoacylglycerols that represent cell lipid degradation indices (DI). Membrane lipids have the highest contribution in cell lipids. *C*. *pseudocurvisetus* synthesized the lowest cell lipids quantity at 15 °C in replete < P-depleted < N-depleted order. The highest lipid content was detected at 30 °C in replete < N-depleted < P-depleted order. In the T range 20 to 30 °C, the cell lipid content increased more than twice under nutrient depleted conditions, compared to replete conditions. Phospholipid cell content increased in N-limited, as well as in P-limited conditions. TG cell content was the highest for N-depleted cultures. The highest contribution of lipid carbon to the total carbon was at 30 °C under all described growth conditions. The lowest contribution of lipid carbon to the total carbon was at 10 °C under N-depleted, 15 °C under P-depleted and 20 °C under the replete conditions. The highest content of cell membrane lipids was detected at 30 °C. Reserve lipids also have the highest content in total lipids at 30 °C, with an exception of P-depleted medium where the highest content was measured at 15 °C. DI content per cell was the highest at 30 °C, following the cell lipids trend.

The contribution of membrane lipid classes to cell lipids varies depending on T and growth medium. As calculated (Supplementary Table S1) GL contribution (%GL) was the highest at 15 °C in all batches (max. in replete 56.4%). The highest PL contribution (%PL) was detected at 30 °C in replete medium (45.3%), at 20 °C in P-depleted medium (47.7%), and at 25 °C in N-depleted medium (26.2%). PIG contributions (%PIG) peaked at 30 °C in replete medium (11.1%), at 25 °C in both N-depleted (25.6%) and P-depleted medium (7.7%). In general, ST contributions (%ST) exhibited a decreasing trend with T. TG contribution (%TG) tended to increase at high T for replete and P-depleted growth conditions. At N-depleted conditions %TG was high at all T and much higher in comparison to replete and P-depleted media (an average of 15.7±2.1%).

To evaluate the relationship between T and produced cell lipids distribution, which might suggest cell lipid remodeling, the principal component analysis (PCA) for each culture medium was preformed (replete, P-depleted, and N-depleted) (Fig. 4). PCA of nutrient replete conditions explained 82.8% total variability between seven variables. The first principal component (PC1) had the highest negative loadings for temperature T, %PL, and %PIG, whereas, positive loadings were observed for %GL and %ST. Such distribution of variables indicates increased PL and PIG contribution, and decreased GL and ST contribution with T rise. Reserve lipids TG and SE contributions were not correlated with the T rise.

PCAs of P-depleted conditions explained 77.8% of the total variability between seven variables. The T dominated at positive value of PC2 and correlated only with %TG. Other variables did not correlate to T. Such distribution indicates that P depletion is more important for the lipid classes’ distribution than T rise. PCAs of the N-depleted conditions explained 87.1% of total variability between seven variables. The T dominated at the positive value of PC2 and correlated only with %SE. Other variables did not correlate to T. Therefore, we assume that nitrogen depletion is more important for the lipid classes’ distribution than T rise.

The strength of a linear association between two variables from the PCA analysis for all growth conditions are evaluated by Pearson correlation coefficients, *r* (Supplementary Table S2). Although there are multiple influences on a particular lipid parameter, Pearson correlation coefficients are significant for those parameters that appeared significantly correlated in the PCA analysis.

## Northern Adriatic

### Environmental conditions

We investigated the influence of T and nutrient availability on lipid production at two northern Adriatic stations, SJ101 and RV001, with contrasting trophic status. T (Fig. 5a) at both stations revealed sinusoidal annual curves with maxima in July and August, and a minimum in March 2013. Station SJ101 had higher nutrient concentrations (Figs. 5c and d) than station RV001 that correlated with S variations (Fig. 5b). Average surface PO4 and DIN concentrations were 0.12 µmol/l and 15.33 µmol/l, respectively, for station SJ101, and 0.05 µmol/l and 2.22 µmol/l, respectively, for station RV001. Exact values for described parameters are given in the Supplementary Table S3.

Chl *a* distribution (Fig. 6a) followed the distribution of nutrients, with higher concentrations observed at station SJ101 (0.09-10.02 µg/l). The highest peak was detected in March at station SJ101 (10.02 µg/l). Concentrations of Chl *a* were much lower at RV001 (0.16-0.76 µg/l) with maximum in November, in accordance to regular autumn water column mixing.

Phytoplankton abundances and taxonomy (nano and micro fraction) were determined for both stations. The contribution of *Chaetoceros* taxa in the phytoplankton community is given in Fig. 6b. Higher dominance of *Chaetoceros taxa* was determined in March 2013 at station SJ101, with the highest contribution in total phytoplankton community (50%). All sets of values for Chl *a* and the contributions of *Chaetoceros* taxa, from Fig. 6, are given in the Supplementary Table S3.

### *Lipid production and composition*

Starting from the fact that phytoplankton is the main lipid producer in seas and oceans (Gašparović et al., 2014), we normalized lipid concentration to Chl *a* (Fig. 7, Supplementary Table S3). The highest cell lipid/Chl *a* values were calculated for July at both stations, when T was 24.67 °C for station SJ101 and 23.41 °C for station RV001. The cell lipid/Chl *a* ratio was more or less uniform within T range from 10 to 20 °C, while there was a rise from 20°C upward at both stations. Although, lipid production at station RV001 was much lower than at SJ101, there was also lower phytoplankton biomass (measured as Chl *a*, Fig. 6a). Consequently, there was a higher average ratio of cell lipid/Chl *a* for station RV001. The average values were 48.4 and 56.1 for stations SJ101 and RV001, respectively. The highest PL cellular content was measured in July at station SJ101, and the lowest was in March with measured temperatures: 24.67°C and 14.33 °C, respectively. In general, higher content of PL/Chl *a* was detected at the nutrient poorer station RV001 than at the nutrient richer station SJ101. The highest and lowest GL cellular contents were measured at station SJ101 in September (T = 21.8 °C) and March (T = 14.33 °C), respectively. The trend of lipid content degradation indices (DI) per Chl *a* followed the trend of total cell lipids content, with the highest values observed during the warmer season.

As can be calculated from the lipid data and the measured temperature (Supplementary Table S3), the lipid classes contribution to the cell lipids decrease in the following order: membrane lipids > degradation indices > reserve lipids. The highest contribution of membrane lipids to the total lipids was measured in September at station SJ101 (91.64%) at T 21.82 °C, and the lowest contribution in August at station SJ101 (51.4%) at low T 24.07°C. The membrane lipids contribution was on average 66.0±10.1% at SJ101, and 65.5±8.4% at RV001. GL at station SJ101 (average 42.9±9.2 %) and PL at station RV001 (average 46.8±6.5 %) were among the highest contributors to the membrane lipids. ST and PIG had a low contribution to membrane lipids: on average ST and PIG contributed 15.4±6.9%, and 3.5±2.8%, respectively to the total membrane lipids at station SJ101; and at station RV001 9.7±3.5%, and 5.0±2.7%, respectively. Reserve lipids (TG and WE) had a small contribution to the total lipids. Based on the results presented in the Supplementary Table S3, average contribution of TG to the total lipids was calculated to be 4.0±3.5% at station SJ101, and 4.3±3.1% at station RV001. Months with higher dissolved inorganic nitrogen (DIN) inputs (>10 µmol/l) had lower TG contribution (2.7±1%). The average contributions of WE to the total lipids were 2.5±1.2%, and 4.8±2.5% at stations SJ101 and RV001, respectively.

We preformed PCA to evaluate the relationship between T, contribution of lipid classes to the total lipids (indicating lipid remodeling), and available nutrients for both northern Adriatic stations. Two PCAs of northern Adriatic stations SJ101 and RV001 (Figs. 8a and b), explained 56.5% and 62.7% of the total variability among 9 variables. Temperature, %TG, and %PIG were not correlated in the plane defined by PC1 and PC2 at station SJ101 (Fig. 8a). PC1 had the highest positive value for DIN, PO4, %ST, and %GL and the highest negative value for %PL and %WE. The strength of a linear association between two variables from PCA were evaluated by Pearson correlation coefficients, *r* (Supplementary Table S5). Although, there are multiple influences on a particular lipid parameter, Pearson correlation coefficients were mainly significant for those parameters that appeared significantly correlated in the PCA. Results indicate that at the nutrient richer station, nutrient availability is more important for the lipid classes’ distribution than T. The PCA at station RV001 (Fig. 8b) shows that T had the greatest negative loading of PC2 together with %GL, while the highest positive loadings of PC2 were for %TG and %WE. This suggests that the GL contribution increases with T rise. The greatest positive PC1 loadings were evident for DIN, %PL, and %PIG. The greatest negative PC1 loadings were for %ST. The PO4 variable was not correlated in the plane defined by PC1 and PC2. The %PL and %GL were inversely correlated for both stations, indicating their interchange depending on the environmental conditions. Pearson correlation coefficients were predominantly significant for those parameters that appeared significantly correlated in the PCA analysis (Supplementary Table S5).

We aimed to evaluate the influence of nutrient availability on cell lipid distribution under both the optimal and high T scenarios. We preformed a PCA for the T range 15-20 °C, which is considered an optimal T range for phytoplankton growth. This was compared to PCA for the T range from 20 to 25 °C considered as the high T range for phytoplankton growth (Figs. 9a and b). For the optimal T range the PC1 had the greatest positive loadings for DIN and %ST (Fig. 9a), while the greatest negative PC1 loadings were noted for %TG, %WE, and %PIG. The greatest positive PC2 loadings were for PO4 and %PL, while the greatest negative loading on PC2 was for %GL. This indicates that at the optimal T range northern Adriatic lipid class distribution is „by the book“. This includes more PL and less GL at higher PO4 concentrations (Van Mooy et al., 2006), as well as an increase in TG contribution which coincide with a decrease in DIN availability (Bourguet et al., 2009; Parrish and Wangersky, 1987). Pearson correlation coefficients were mostly significant for those parameters that appeared significantly correlated in the PCA analysis (Supplementary Table S6).

For the higher T range the greatest positive PC1 loadings were for %PL, %PIG, %TG, and %WE (Fig. 9b), while %GL and DIN had the greatest negative effect on PC1. Variables PO4 and %ST were explained by PC2 with negative loading values. The PO4 increase does not result in greater PL contribution to total lipids. PCA shows that the relationship between nutrients and lipid classes is more complex at higher T than at the optimal T range, indicating the important role of higher T for the lipid biochemistry. Pearson correlation coefficients were mostly significant for those parameters that appeared significantly correlated in the PCA analysis (Supplementary Table S6).

# Discussion

Phytoplankton response to T increase and nutrient availability reduction was the focus of our study. We investigated the possible cascade effect on lipid production in response to T rise as the primary, and nutrient depletion as the secondary, ecosystem influence. We compared the experimental results of the model diatom *Chaetoceros* with a one-year monthly sampling of the complex northern Adriatic system, a characteristic coastal sea ecosystem highly influenced by the global change processes. Prior studies noted the importance of T influence on phytoplankton metabolism referring to lipid production (e.g. Opute, 1974; Toseland et al., 2013), but they rarely took into account both laboratory and *in situ* experiments. During our one-year sampling period (2013-2014) T higher than 25 °C were not observed at RV001 and SJ101, even though there were events of surface water T up to 30 °C in northern Adriatic (e.g. Novak et al., 2018).

Each phytoplankton species has an optimal T range for growth. An optimum T for *C. pseudocurvisetus* growth is 15 °C. At this T, the highest cell abundance and the lowest lipid cell content were measured, together with the lowest primary production directed to dissolved fraction (Novak et al., 2018). The highest T used in the *C. pseudocurvisetus* cultivation (30 °C) lead to the cell abundance stagnation and significant cell lipid accumulation. Increased cellular content of lipids at the highest growth T parallels *C. pseudocurvisetus* reduced reproduction. We assume that reproduction slowed down due to thermo-sensitivity of enzymes involved while accumulation of lipids continued. However, at the lowest T growth (10°C) *C. pseudocurvisetus* showed high lipid cellular content as well. Sharma et al. (2012) have shown that under optimal growth conditions, also regarding T range, large amount of phytoplankton biomass is produced, with relatively low lipid content. Growing in environmentally unfavorable conditions, many phytoplankton species alter their lipid biosynthetic pathways towards the formation and accumulation of neutral ones (Sharma et al., 2012). This is observed for *Ochromonas danica* in T range 15 to 30 °C (Aaronson, 1973), three diatoms *N. paleacea*  in T range 15-25 °C *N. closterium* and *Isochrysis* sp. (PS 11) in T range 20-30 °C (Renaud et al., 1995). However, opposing to our and referenced findings two *Chaetoceros* species, *C. cymplex* and *C. gracilis,* showed a decline in lipid per cell in T range from 10 to 25 °C (Thompson et al., 1992). Other six investigated microalgae species showed inconsistent relationship between T and lipid content (Thompson et al., 1992). Nonetheless, at the extreme high or low growth T a decrease has been observed in the microalgae lipid production (Aaronson, 1973; Opute, 1974). Opute (1974) suggested that this effect was caused by discontinuation of growth due to irreversible damage on enzymes. Our experiment was based on T range typically occurring in the NA, and major extremes (below 10 and above 30 °C), were not taken into account. Within this T range *C. pseudocurvisetus* was able to grow and reproduce, and no discontinuation of growth was observed. However, we unsuccessfully tried to grow *C. pseudocurvisetus* at 7 °C since cells did not divide.

In addition to T, increased lipid production in *C. pseudocurvisetus* is also affected by lack of nutrients. Phosphorus scarcity appeared to have a greater role in lipid accumulation at higher T. Oppositely; nitrogen deprivation has a higher influence on enhanced lipid cell accumulation at lower T. As an example, N starvation resulted in increased total lipid content of cell for some species up to 50% (Schuhmann et al., 2012).

The northern Adriatic phytoplankton community followed the tendency of *C. pseudocurvisetus* monoculture experiment. The highest lipid content per Chl *a* was observed in winter and particularly in warmest summer months. Together with significant increase in cell lipids, lipid degradation indices content increased with rising T, both, for *C. pseudocurvisetus* cultures as well as the northern Adriatic phytoplankton community. This suggests that the enhanced lipid production and lipid degradation processes take place at high T. P-depleted conditions influence even greater lipid accumulation as observed at P-depleted station RV001. This indicates that nutrient scarcity has an additional effect on phytoplankton lipid accumulation alongside the high T, as shown in the example of the northern Adriatic.

The T rise and nutrient shortage influence higher production of all lipid classes, as observed in monoculture experiments and for the northern Adriatic. There are two major membrane lipids PL and GL. PL mainly reside in the plasma membrane and many endoplasmic membrane systems, while GL are enriched in the chloroplast (Guschina and Harwood, 2009). We detected higher PL content per Chl *a* for the nutrient poorer station RV001. The same increased PL content per cell was observed for *C. pseudocurvisetus* grown in both P and N scarcity. We propose that PL quota for cells living in replete conditions is lower in comparison to depleted conditions. This could be due to PL dilution during higher rate of cell divisions in replete condition. Also, cell that are in nutrient stress, and cannot successfully divide, might store P in phospholipids (Abida et al., 2015). In case of favorable environmental conditions, P from PL could be re-allocated to any P containing molecule important for vital function(s) (e.g. DNA and RNA). Additional forms of P reserves for PL synthesis in cells are polyphosphates (Martin et al., 2014). Polyphosphates might be formed in cells that were pre-grown in nutrient replete conditions, and used during cell growth under investigated nutrient depleted conditions. GL accumulation was observed at temperatures higher than 19 °C in the northern Adriatic. This accumulation was explained as a mechanism to achieve thermal stability (Gašparović et al., 2013).

Scarcity of N is reflected in the increased content of TG per cell or per Chl *a*. *C. pseudocurvisetus* accumulates lipids in N-depleted medium, mostly due to significant increase of TG, approximately two times more than in other conditions. *In situ* samples respond in the same pattern, the oligotrophic northern Adriatic station RV001 showed higher TG production when compared to mesotrophic station SJ101. Increased TG/Chl *a* was observed at both stations for months when the lowest DIN concentrations were measured. Nonetheless, N-limitation can be overcome by nitrogen fixation by marine diazotrophs in oceans and seas (Capone, 2001). Diatoms are found to have N fixation (Poulton et al., 2009), N storage (Lomas and Glibert, 2000) and direct utilization of organic N (Morando and Capone, 2018) capabilities. A general trend of TG accumulation as a response to N-depletion has been observed in numerous taxa (Sharma et al., 2012; Thompson, 1996).

There is a synergetic impact of T rise and nutrient scarcity on lipid remodeling. The nutrient status appeared to be more important for lipid composition remodeling than T, at least within T range covered by our investigation. Lipid remodeling occurs in higher T range, in particular phospholipid remodeling, indicating that increased T interferes with PL biosynthesis. The contribution of PL and GL to the cell lipids interchange depends on T and nutrient availability. Decreased PL contribution to cell lipids, at the expense of GL, was promoted by P scarcity, this was observed for both the P depleted station RV001 and in P-depleted *C. pseudocurvisetus* cultures. At optimal T range (15-20 °C) lipid classes' distribution followed published trends. This entailed enhanced PL contribution in P favorable conditions in parallel with decline in GL (Van Mooy et al., 2006), and accumulation of TG that followed DIN depletion (Bourguet et al., 2009; Parrish and Wangersky, 1987).

*C. pseudocurvisetus* metabolism changed in dependence to nutrient availability. *C. pseudocurvisetus* used N and P in Redfield ratio 16.4±0.2 indicating a balanced metabolism in the nutrient favorable medium. The metabolism was directed towards the synthesis of N-rich molecules in the P-depleted medium (Grosse et al., 2017), indicated by the high ratio of N/P uptake (72.9±4.2). Under these conditions cells have several mechanisms to overcome P-deficiency by itself, including alkaline phosphatase (AP) activation (Hoppe, 2003) (also employed by *C. pseudocurvisetus*) and PL substitution with GL: SQDG (Van Mooy et al., 2006; 2009; Martin et al., 2011), betaine lipid (Van Mooy et al., 2009; Martin et al., 2011), and DGDG (Hartel et al., 2000). While N-limitation with DIN concentration <1 µmol/L (Justić et al., 1995) was occasionally noticed in the northern Adriatic (Gašparović et al., 2013), the northern Adriatic is a P-limited sea (Ivančić et al., 2016). Phytoplankton can form P reserves in the form of polyphosphates when P favorable conditions occur (Martin et al., 2014; Romans et al., 1994). The northern Adriatic phytoplankton community, especially diatoms, under P deficiency, induces great APA in order to obtain P from dissolved organic matter (Ivančić et al., 2016; 2012). *C. pseudocurvisetus* N/P uptake (2.6±0.3) was low in N-depleted medium, indicating lower N-containing molecules synthesis (enzymes, proteins). This leads to TG accumulation, molecules containing only carbon, hydrogen and oxygen.

High T and oligotrophication influence cell carbon allocation toward the synthesis of carbon rich molecules, including lipids. This will be reflected in the modification of the carbon pump, as different biomolecules have different decay constants. The highest decay constant have carbohydrates and it further decreases from proteins to lipids (Benner and Amon, 2015). The efficiency of lipids in carbon sequestration might be antagonistic. Lipid buoyancy very likely enhances their retention in the surface layer of the ocean, where remineralization processes take place. However, their adsorption to particles (Morris and Eglington, 1977) influences lipid removal to deeper ocean/sea layers via the sedimentation process. Accumulation of GL, as observed in nutrient poorer station RV001, may assist more successful lipid sedimentation. Namely, GL are less attractive substrates for microbial degradation, due to their molecular composition (carbon, hydrogen, sulphur, and oxygen), without the essential P and N.

# Conclusions

With this study, we can conclude that rising sea T caused enhanced lipid accumulation for the *Chaetoceros pseudocurvisetus* and northern Adriatic phytoplankton population. This effect was more pronounced in conditions of nutrient scarcity. Taking into account monoculture experiments, P scarcity enhanced lipid accumulation at higher T more than N depletion, while it was opposite for lower T. Between the two influential parameters on lipid synthesis, lack of nutrients had a greater role. We assume that oligotrophic seas and oceans, like tropical oceans and the eastern Mediterranean Sea, would be areas of higher lipid accumulation. Consequently, this change in carbon allocation will be reflected in the carbon pump alteration as different biomolecules have different biogeochemistry in water column.

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# Figure captions

Fig. 1. Map of sampling stations SJ101 and RV001 in the northern Adriatic Sea with the scheme of winter prevailing Eastern Adriatic Current (EAC), Western Adriatic Current (WAC) and summer prevailing Istrian Coastal Countercurrent (ICCC) (after Supić et al. (2003)).

Fig. 2. Cell-specific alkaline phosphatase activity (sAPA) of *C. pseudocurvisetus* for growth in replete (triangles) and P-depleted (circles) medium.

Fig. 3. Main parameters of lipid production normalized to *C. pseudocurvisetus*cell abundance in three different batch cultures, replete (left pannel), P- (middle pannel) and N-depleted (right pannel) at five temperatures (10, 15, 20, 25 and 30 °C) at the end of the experiments. Abbreviations: sterol esters (SE), triacylglycerols (TG), sterols (ST), pigments (PIG), phospholipids (PL), glycolipids (GL), and degradation indices (DI).

Fig. 4. Principal component analysis (PCA) for the variables; temperature (T), contribution of different lipid classes to total cell lipids: triacylglycerol (%TG), sterol ester (%SE), sterol (%ST), pigment (%PIG), phospholipid (%PL), and glycolipid (%GL) at replete (a), P-depleted (b) and N-depleted (c) growth conditions.

Fig. 5. The northern Adriatic environmental parameters: temperature (a), salinity (b), orthophosphate (c) and dissolved inorganic nitrogen (d) at the mesotrophic station SJ101 (triangles) and oligotrophic station RV001 (circles) during the investigation period in 2013-2014.

Fig. 6. The northern Adriatic phytoplankton status. Chlorophyll *a* (a) and contribution of *Chaetoceros* taxa abundance to phytoplankton community abundance (b) at the mesotrophic station SJ101 (triangles) and the oligotrophic station RV001 (circles) during the investigation period in 2013-2014.

Fig. 7. Lipid production measured at stations SJ101 (left pannel) and RV001 (right pannel) of the northern Adriatic in the period from March 2013 to March 2014. All lipid values are given based on Chl *a*. Abbreviations: sterol esters (SE), triacylglycerols (TG), sterols (ST), pigments (PIG), phospholipids (PL), glycolipids (GL), and degradation indices (DI).

Fig. 8. Principal component Analysis (PCA) for variables; temperature (T), dissolved inorganic nitrogen (DIN), orthophosphate (PO4), contribution of cell lipid classes: triacylglycerol (%TG), wax esters (%WE), sterols (%ST), pigments (%PIG), phospholipids (%PL), glycolipids (%GL) measured at two northern Adriatic stations SJ101 (a) and RV001 (b).

Fig. 9. Principal component Analysis for variables; temperature (T), dissolved inorganic nitrogen (DIN), orthophosphate (PO4), contribution of cell lipid classes: triacylglycerol (%TG), wax esters (%WE), sterols (%ST), pigments (%PIG), phospholipids (%PL), glycolipids (%GL) measured for the both northern Adriatic stations for two temperature ranges, 15-20 °C (a) and 20-25 °C (b).

# Table captions

Table 1. Cell number counted at stationary growth phase, growth rate and average carbon content for *Chaetoceros pseudocurvisetus* grown at five different temperatures and different media (replete, P- and N-depleted)

Figures:



Fig. 1. Map of the locations of sampling stations SJ101 and RV001 in the northern Adriatic Sea with the scheme of winter prevailing Eastern Adriatic Current (EAC), Western Adriatic Current (WAC) and summer prevailing Istrian Coastal Countercurrent (ICCC) (after Supić et al. (2003)).



Fig. 2. Alkaline phosphatase activity (APA) of *C. pseudocurvisetus* for growth in replete (triangles) and P-depleted (circles) medium.



Fig. 3. Main parameters of lipid production normalized to cell abundance in three different batch cultures (replete, P- and N-depleted) at five temperatures (10, 15, 20, 25 and 30 °C): total cell lipids (a), reserve lipids (b), membrane lipids (c), and degradation indices (d) at the end of the experiments.

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Fig. 4. Principal component analysis (PCA) for the variables; temperature (T), contribution of different lipid classes to total cell lipids: triacylglycerol (%TG), sterol ester (%SE), sterol (%ST), pigment (%PIG), phospholipid (%PL), and glycolipid (%GL) at replete (a), P-depleted (b) and N-depleted (c) growth conditions.



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Fig. 8. Principal component Analysis (PCA) for variables; temperature (T), dissolved inorganic nitrogen (DIN), orthophosphate (PO4), contribution of cell lipid classes: triacylglycerol (%TG), wax esters (%WE), sterols (%ST), pigments (%PIG), phospholipids (%PL), glycolipids (%GL) measured at two northern Adriatic stations SJ101 (a) and RV001 (b).

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Fig. 9. Principal component Analysis for variables; temperature (T), dissolved inorganic nitrogen (DIN), orthophosphate (PO4), contribution of cell lipid classes: triacylglycerol (%TG), wax esters (%WE), sterols (%ST), pigments (%PIG), phospholipids (%PL), glycolipids (%GL) measured for the both northern Adriatic stations for two temperature ranges, 15-20 °C (a) and 20-25 °C (b).