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# Paradox of relatively more phospholipids in phytoplankton in phosphorus limited sea

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## *Abstract*

Marine life is threatened by global warming and its indirect consequences, which include, among others, increased stratification leading to phosphorus (P) and nitrogen depletion in the upper water column. Phosphorus plays a key role in all biochemical systems; storage of metabolic energy, formation of genetic material, and subcellular compartmentalization. Our multi-year study of lipid biogeochemistry in the northern Adriatic (Mediterranean), which is becoming warmer and nutrient-poorer, particularly regarding P, has shown that under conditions of P scarcity, phospholipids are relatively more abundant and smaller plankton cells dominate. Consistent with the field data, experiments with seven phytoplankton monocultures, comprising microplankton, nanoplankton, and picoplankton, confirmed a relatively higher phospholipid content in the smaller phytoplankton species and, in particular, an increase in those grown under stress conditions in general, including, unexpectedly, P-limitation. We suggest two reasons for the observed "P paradox" of P-limited phytoplankton: (1) cell geometry: volume of the plasma membrane relative to the volume of the entire cell is greater in smaller cells and, therefore, the proportion of plasma membrane phospholipids to intracellular lipids is greater in smaller cells, (2) higher proportion of densely packed saturated fatty acids found in stressful conditions, including P oligotrophy, additionally increase the proportion of membrane phospholipids relative to intracellular lipids. Our findings contribute to the understanding of P cycling in the sea. In addition, our data suggest that higher phospholipid export to deep waters is possible by smaller plankton.

For the life and growth of all organisms on Earth, energy, space, and materials are required. These materials include carbon, water, and nutrients such as nitrogen and other elements from rocks, including phosphorus (Chapin et al. 2011). Phosphorus is one of the key nutrients that is assimilated into ATP, nucleic acids, complex carbohydrates, and phospholipids, all of these with essential roles in the cellular apparatus (Lin et al. 2016). Phospholipids are engaged in (1) establishing the permeability barrier for cells and cell organelles, (2) providing the matrix for the assembly and function of a wide variety of catalytic processes, (3) acting as P donors in the synthesis of macromolecules, and (4) actively influencing the functional properties of membrane-associated processes (Dowhan, 1997; Dowhan et al. 2008).

Due to climate change, marine phytoplankton often grows under stressful conditions (Boyd et al. 2010), including warming (Vrana et al. 2023), salinity change (D'ors et al. 2016), and lack of inorganic nutrients (Bristow et al. 2017). Phosphorus is the limiting nutrient in large parts of the ocean (Wu et al. 2000; Yoshimura et al. 2007) as well as in the coastal seas (Thingstad et al. 2005; Ivančić et al. 2016). The total inventory of P in the oceans is regulated by

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Additional Supporting Information may be found in the online version of this article.

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continental inputs, ultimately originating from the weathering of phosphate rocks, and the burial flux at the seafloor (Monteiro et al. 2012; Kemena et al. 2019). There are indications that the P budget of the ocean is unbalanced, since the accumulation of P in marine sediments exceeds the continental input of particulate and dissolved P (Wallmann 2010). These low and unbalanced P concentrations limit the transport toward the cellular P pool (quota) and consequently affect the cell growth rate (Moore et al. 2013). Phosphorus quotas in cells vary over ecologically relevant temporal and spatial scales (Núñez-Milland et al. 2010).

Phytoplankton employ a variety of strategies to deal with fluctuating P supply, including physiological adaptations and/or community response (Dyhrman et al. 2002; Brembu et al. 2017; Mousing et al. 2018). In the presence of low orthophosphate ($PO_4^{3-}$) concentrations, phytoplankton and other microbes can induce extracellular enzyme alkaline phosphatase, which enables them to use organic P esters as a P source in order to augment their P supply (Dyhrman and Ruttenberg 2006; Davis and Mahaffey 2017; Su et al. 2023). They can also reduce their cellular P requirement by exchanging part of the membrane phospholipids for sulfolipids (Van Mooy et al. 2006, 2009). Many studies on acclimation to P-limitation point to the replacement of phospholipids with glycolipids (e.g., Gašparović et al. 2013; Mühlroth et al. 2017) and by betaine lipids (e.g., Mühlroth et al. 2017; Murakami et al. 2018). Abida et al. (2015) observed that in the diatom *Phaeodactylum tricornutum* P-deprivation causes the replacement of phosphatidylglycerol by sulfolipid and of phosphatidycholine by betaine lipid. Additionally, some species regulate their P uptake kinetics in response to P limitation by significantly increasing maximum activity and specific affinity for both inorganic and organic P (Krumhardt et al. 2013; Jiang et al. 2019; Gao et al. 2022). Apart from these physiological adaptations there are even species-specific strategies identified within the same phytoplankton community (Ivančić et al. 2016) for overcoming the P stress.

In this study, we focused on the cellular response of phytoplankton to stress including low P supply, high temperature, and low salinity in terms of their phospholipid content, considering that the main producer of lipids in aquatic environments is eukaryotic phytoplankton (Gašparović et al. 2014). In the northern Adriatic Sea samples, we measured the particulate phospholipid concentration and their contribution to the cellular lipid pool, taking into account the abundance (as chlorophyll *a*, Chl *a*) and the composition of the phytoplankton community (as size-defined microphytoplankton and nanophytoplankton). The northern Adriatic is a prime location for such study as there exists a steep trophic gradient: from mesotrophic western side (higher P supply) to oligotrophic eastern side (lower P supply; Mozetič et al. 2010) in the short distance of 46 nautical miles (85 km) thus facilitating frequent sampling. In addition, a significant warming of the northern Adriatic has been recorded since 2003 (Vrana et al. 2023). On the basis of field observations, we laid two hypotheses: (1) smaller phytoplankton species have relatively higher phospholipid content, and (2) stress conditions lead to increase in phospholipid content. We have thus analyzed the lipids (including phospholipids) in cultured phytoplankton of three different size classes (micro, nano, and pico) under favorable and stress conditions.

In recent decades, the phytoplankton scientific community has defined several model species (i.e., *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and *Emiliania huxleyi*) that have led to important breakthroughs in understanding the physiological mechanisms that govern phytoplankton biology (Read et al. 2013; Falciatore et al. 2019). However, in our experimental work, we have chosen to take a broader approach, working on "atypical" models with the goal of understanding and incorporating unique organisms to advance our knowledge of biological questions in general (Peter et al. 2017). We have selected the following taxa: (1) microphytoplankton, diatoms *Chaetoceros pseudocurvisetus* Mangin 1910 and *Asterionella glacialis* Castracane, 1886, (2) nanophytoplankton, coccolithophores *Coccolithus pelagicus* subsp. *braarudii* (Gaarder 1962) Geisen et al. 2002 (from here onwards shortened as *C. braarudii*), and *Calcidiscus leptoporus* (Murray & Blackman 1898) Loeblich & Tappan, 1978 and a green alga *Dunaliella tertiolecta* Butcher, 1959, and (3) picophytoplankton, a green alga *Picochlorum* sp. and a cyanobacterium *Synechococcus* sp.; to study the cellular response of phytoplankton to stress.

## Methods

### Study area and sample collection

The northern Adriatic Sea (Fig. 1) is the northernmost Mediterranean area. Its western part is the most productive part, while the eastern part is prevalently oligotrophic. The main factor influencing biogeochemical processes in the northern Adriatic is the Po River, on the western side, which dilutes the seawater and whose freshwater brings high loads of N-nutrients (128–202 $\mu$mol $L^{-1}$, median 168, $1^{st}$–$3^{rd}$ quartile) and $PO_4^{3-}$ (1.6–2.6 $\mu$mol $L^{-1}$, median 1.9, $1^{st}$–$3^{rd}$ quartile) nutrients (Cozzi et al. 2019). In addition, physical parameters such as the regular seasonal light cycle, the annual temperature cycle, and surface currents influence the distribution of biological productivity in the region (Solidoro et al. 2009). During winter, riverine waters are directed southwards in the narrow coastal belt due to the dominant Adriatic cyclonic circulation (Orlić et al. 1992). The cyclonic–anticyclonic gyre pairs often forms in the northern Adriatic (Lyons et al. 2007) enabling horizontal transport of freshened waters from the west towards the east supported by a well-stratified water column. Another P supply results from the regular autumn water column overturn that brings bottom-regenerated nutrients to the surface layer and is facilitated by the fact that the bottom depth of the region seldom exceeds 50 m. In general, P availability in the northern Adriatic is highly variable.

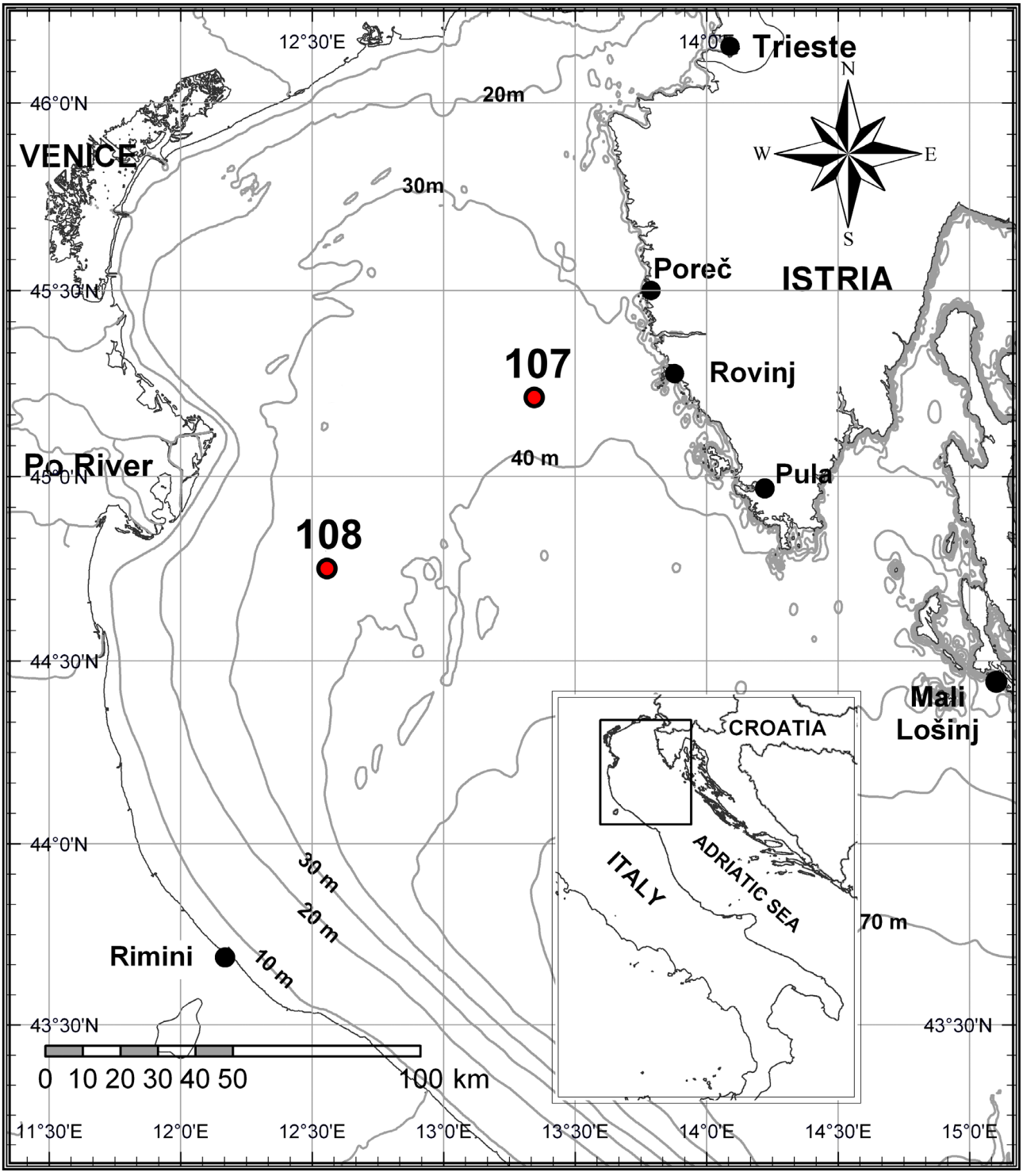


**Fig. 1.** Study area in the northern Adriatic Sea.

Surface samples (0.5 m depth) were collected with 5 L Niskin bottles in 2010, 2017, and 2021 at two stations in the northern Adriatic Sea, following the monthly sampling schedule of the time series conducted within the Croatian National Monitoring Program. The selected stations differ in $PO_4^{3-}$ supply: the mesotrophic Sta. 108 (44°45′24″N, 12°45′00″E) on the western side, which is mainly influenced by the Po River P supply, and the eastern oligotrophic Sta. 107 (45°02′53″N, 13°19′00″E) (Fig. 1).

Determination of nutrients was carried out on board immediately after sampling by spectrophotometric method, widely

used in oceanography (Parsons et al. 1984). Orthophosphate, $PO_4^{3-}$, was determined by the Molybdenum blue method (Murphy and Riley 1962). The ammonium ion, $NH_4^+$, was determined by the modified indophenol blue method (Ivančić and Degobbis 1984). Nitrite, $NO_2^-$, was determined as pink azo dye (Bendschneider and Robinson 1952), while nitrate, $NO_3^-$, was determined as nitrite after reduction on columns filed with metallic cadmium filings coated with copper (Wood et al. 1967). Total dissolved inorganic nitrogen (DIN) was calculated as the sum of $NO_3^-$, $NO_2^-$, and $NH_4^+$. The absorbance readings were made on Shimadzu UV-Mini 1240 spectrophotometer with 1, 5, and 10 cm quartz cuvettes, depending on the color intensity. Method accuracies for $PO_4^{3-}$, $NO_3^-$, $NO_2^-$, and $NH_4^+$ were ± 3%, ±3%, ±3%, and ± 5%, respectively, and detection limits were 0.02, 0.05, 0.01, and 0.1 $\mu$mol $L^{-1}$, respectively.

Following collection, the samples for the measurement of Chl *a* were prefiltered using a 290 $\mu$m Nybolt net and promptly filtered through Whatman GF/C filters (1 $\mu$m pore size) on board. The filters were kept at −20°C until the analyses, which were carried out fluorometrically in the inland facilities following a 3 h extraction in 90% acetone (in the dark, with grinding after addition of acetone). Method accuracy is 8%, and detection limit is 0.02 $\mu$g $L^{-1}$ and calculated according to equations of Parsons et al. (1984).

### Phytoplankton from the northern Adriatic

Samples (200 mL) for phytoplankton analysis were preserved in 2% (final concentration) formaldehyde neutralized with disodium tetraborate decahydrate and analyzed within 1 month of sampling. The subsamples were sedimented for a maximum of 30 h and the cells were counted using a Zeiss Axiovert 200 (Zeiss, Jena, Germany) according to the inverted microscope quantification method (Utermöhl 1958; Hasle 1978). The analyzed volume (10, 25, or 50 mL) was determined according to the cell concentration following recommendation of Lund et al. (1958) for a 95% confidence interval and an accuracy of ± 10%. Cells were determined to the highest possible taxonomic rank following Tomas (1997). Total phytoplankton abundances include all cells counted in the microphytoplankton (20–200 $\mu$m) and nanophytoplankton (2–20 $\mu$m) groups (Sieburth et al. 1978).

### Phytoplankton culture experiments

To test our hypotheses about phospholipid content, selected phytoplankton species from three size classes were grown under optimal and under stress growth conditions in terms of nutrient availability, temperature, and salinity. Monoculture experiments that included P-limitation, but also other limitations, were set up and maintained to ensure that only one stressor became limiting, while all other nutrients and growing conditions remained the same for the favorable and stressed cultivations. We used phytoplankton strains isolated from the Adriatic Sea and from the Roscoff Culture Collection (RCC) and the National Center for Marine Algae and Microbiota at the Bigelow Laboratory for Ocean Sciences (CCMP): (1) microphytoplankton *C. pseudocurvisetus* and *A. glacialis* (both isolated from the Adriatic), (2) the nanophytoplankton coccolithophores *C. braarudii* (RCC1197) and *C. leptoporus* (RCC1135), a green alga *D. tertiolecta* (strain CCMP 1320), and (3) the picophytoplankton a green alga *Picochlorum* sp. (RCC6905) and a cyanobacterium *Synechococcus* sp. (RCC2523).

Strains were grown in a thermostatic chamber (Inkolab, Zagreb, Croatia) with 12 : 12 h light/dark cycle under the illumination of ~ 4500 lx, with white LED light source. The cultured strains were pre-conditioned in the experimental media for 10 days before inoculation of the experiment. The experimental media were prepared using aged Adriatic seawater (salinity 38). After preconditioning, strains were inoculated with initial concentrations of $1.0 \times 10^5$ cell $L^{-1}$ for *C. pseudocurvisetus,* and *A. glacialis*; $1.3 \times 10^6$ cell $L^{-1}$ for *C. braarudii*; $1.2 \times 10^5$ cell $L^{-1}$ for *C. leptoporus*; $4.0 \times 10^7$ cells $L^{-1}$ for *D. tertiolecta*; $1.0 \times 10^6$ cell $L^{-1}$ for *Picochlorum* sp.; and $2.7 \times 10^6$ cell $L^{-1}$ for *Synechococcus* sp.

#### *Microphytoplankton experimental setup*

*C. pseudocurvisetus* (CP1) was grown under favorable conditions (CP1_F) at 15°C in a nutrient replete modified F/2 medium Guillard (1975) (240.65 $\mu$mol $L^{-1}$ $NO_3^-$ and 15 $\mu$mol $L^{-1}$ $PO_4^3$). Growth of *C. pseudocurvisetus* under stress was performed in conditions at 15°C without N (CP1_-N), high temperature stress at 30°C (CP1_HT) and double stress, without N and temperature stress at 30°C (CP1_-N_HT). Additionally, *C. pseudocurvisetus* (CP2) and *A. glacialis* (AG) were grown under favorable conditions (CP2_F and AG_F, respectively) at 15°C in a nutrient replete modified F/2 medium (882 $\mu$mol $L^{-1}$ $NO_3^-$ and 36.2 $\mu$mol $L^{-1}$ $PO_4^3$). These two cultures were also grown under stress without P addition (CP2_-P and AG_-P, respectively).

#### *Nanophytoplankton experimental setup*

Coccolithophores *C. braarudii* and *C. leptoporus* were grown at 16°C. *C. braarudii* (CB), was grown in modified F/2 medium with $NO_3^-$ and $PO_4^{3-}$ concentrations adjusted to 35 and 3.6 $\mu$mol $L^{-1}$, respectively. Samples for lipid analysis were taken on day 5 under favorable conditions (CB_F), and on day 8 when P and N had already decreased significantly and cells were under stress (CB_-P-N). *C. leptoporus* (CL) was grown under favorable conditions (CL_F) in a modified K medium (Keller et al. 1987) with four-fold lower concentrations of all nutrients (K/4 medium) and salinity adjusted to 35 with deionized water. *C. leptoporus* was also grown under stress without P (CL_-P). *D. tertiolecta*, DT, was grown under favorable conditions in an F/2 medium (DT_F) and under hyposalinity stress with salinity adjusted to 3 using deionized water (DT_-S) (details in Vrana et al. 2022).

### *Picophytoplankton experimental setup*

*Picochlorum* sp. (Pc) was grown under favorable conditions at 16°C in F/2 medium (Pc_F) and under stress without P added (Pc_-P). In P-stressed *Picochlorum* sp., enzyme alkaline phosphatase was expressed indicating P-limitation (e.g., Ivančić et al. 2016). The data for alkaline phosphatase can be found in the open data source: https://urn.nsk.hr/urn:nbn:hr:241:004757. *Synechococcus* sp. (Syn) was cultured only under favorable conditions at 23°C and nutrient concentrations of 52 $\mu$mol $L^{-1}$ DIN and 3 $\mu$mol $L^{-1}$ $PO_4^{3-}$ (Syn_F).

Experiments were performed in triplicate with two exceptions (*C. pseudocurvisetus*, Fig. 6. CP2_F and CP2_-P, data available at urn:nbn:hr:241:004757). Cultures were monitored throughout the course of the experiment, and cell concentrations were analyzed using Sedgewick-Rafter counting chambers (Graticules Optics Ltd, Tonbridge, UK) on a Zeiss Axiovert 200 microscope (Carl Zeiss AG, Jena, Germany).

### Lipid classes analysis

For the seawater lipid classes determination, 2 L of seawater were collected in glass containers and passed through a 200 $\mu$m stainless steel screen to remove zooplankton and larger particles. Immediately after sampling, seawater was filtered through 0.7 $\mu$m Whatman GF/F filters preburned at 450°C for 5 h. To determine the lipid composition of monocultures triplicates of 100 mL culture were filtered on precombusted 0.7 $\mu$m Whatman GF/F filters. The filters were stored in at −80°C for later lipid extraction and analysis. Particulate lipids were extracted by a modified one-phase solvent mixture of dichloromethane–methanol–water (Bligh and Dyer 1959). 2-Nonadecanone was added as internal standard to each sample to estimate the recoveries in the subsequent steps of the sample analysis. The extracts were evaporated to dryness under nitrogen atmosphere and redissolved in 18 or 20 $\mu$L dichloromethane depending on the sample concentration.

Lipid classes were determined by thin–layer chromatography–flame ionization detection (TLC–FID; Iatroscan MK–VI, Iatron, Japan). Eighteen lipid classes, which make up total lipids, may be detected by this technique including hydrocarbons, wax esters and steryl esters, fatty acid methyl esters, fatty ketones, triacylglycerols, free fatty acids, fatty alcohols, 1,3- and 1,2-diacylglycerols, sterols, pigments, monoacylglycerols, three glycolipids (monogalactosyldiacylglycerols, digalactosyldiacylglycerols, and sulfoquinovosyldiacylglycerols), and three phospholipids (phosphatidylglycerols, phosphatidylethanolamines, and phosphatidylcholines; Supporting Information Fig. S1). The lipid classes were separated on Chromarods SIII and quantified by an external calibration with a mixture of standards (Sigma-Aldrich, St. Louis, Missouri, United States) of all investigated lipids, with a hydrogen flow of 160 mL $min^{-1}$ and air flow of 2000 mL $min^{-1}$. The separation scheme includes seven elution steps using polarity-increasing solvent systems. Hydrocarbons, wax esters and steryl esters, fatty acid methyl esters and fatty ketones were separated for 28 min with *n*-hexane–diethyl ether–formic acid (97 : 3 : 0.2, v :v : v). Triacylglycerols and free fatty acids were separated during 30 min with *n*-hexane–diethyl ether–formic acid (80 : 20 : 0.2, v : v : v). Fatty alcohols, 1,3– and 1,2–diacylglycerols and sterols were separated with an additional 20 min in the previous solvent mixture. Pigments and monoacylglycerols were separated during 32 min with chloroform–acetone–formic acid (95 : 5 : 0.6, v : v : v). Monogalactosyldiacylglycerols and digalactosyldiacylglycerols were separated with chloroform–acetone (72 : 28, v : v) for 30 min. Sulfoquinovosyldiacylglycerols and phosphatidylglycerols were separated solvent mixture acetone–chloroform–methanol–formic acid (33 : 33 : 33 : 0.6, v : v : v : v) for 40 min and chloroform-methanol-ammonium hydroxide (50 : 50 : 5, v : v : v) during 40 min allowed separation of phosphatidylethanolamines, and phosphatidylcholines. Phospholipids were calculated as the sum of three classes: phosphatidylglycerols, phosphatidylethanolamines, and phosphatidylcholines. Cell lipids were calculated as the sum of phospholipids, monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulfoquinovosyldiacylglycerols, triacylglycerols, sterols, and pigments. The standard deviation determined from duplicate runs accounted for 0%–14% of the relative abundance of lipid classes. Detailed procedure is described in Gašparović et al. (2015, 2017).

### Statistical analysis

Principal-component analysis was used to statistically identify and visualize patterns and relationships between chemical and biological variables: phospholipids, phytoplankton, and nutrient data. Principal-component analysis was developed to reduce the complexity of the data by compressing them into fewer dimensions with the aim to provide pattern recognition in the data that allows visual representation of similarity between variables (Clarke and Warwick 2001). It was performed using Statistica Release 7 software (StatSoft, Tulsa, OK, USA) and followed a log $(1 + x)$ data transformation to reduce the impact of extreme values or outliers.

## *Results*

### The northern Adriatic Sea

We investigated the environmental conditions and phytoplankton and phospholipid concentrations in the 2010, 2017, and 2021 (Figs. 2–4; Table S1). While in Figs. 2–4 we present total DIN concentrations, the concentrations of nitrate, nitrite, and ammonia are added in the open data source: https://urn.nsk.hr/urn:nbn:hr:241:004757.

During 2010, the nutrient-richer Sta. 108 had higher $PO_4^{3-}$ concentrations than Sta. 107, ranging from 0.03 to 0.46 $\mu$mol $L^{-1}$, with the exception of July. At the same time $PO_4^{3-}$ concentrations at Sta. 107 ranged from bellow detection limit to 0.09 $\mu$mol $L^{-1}$ (Fig. 2a). The temporal distribution

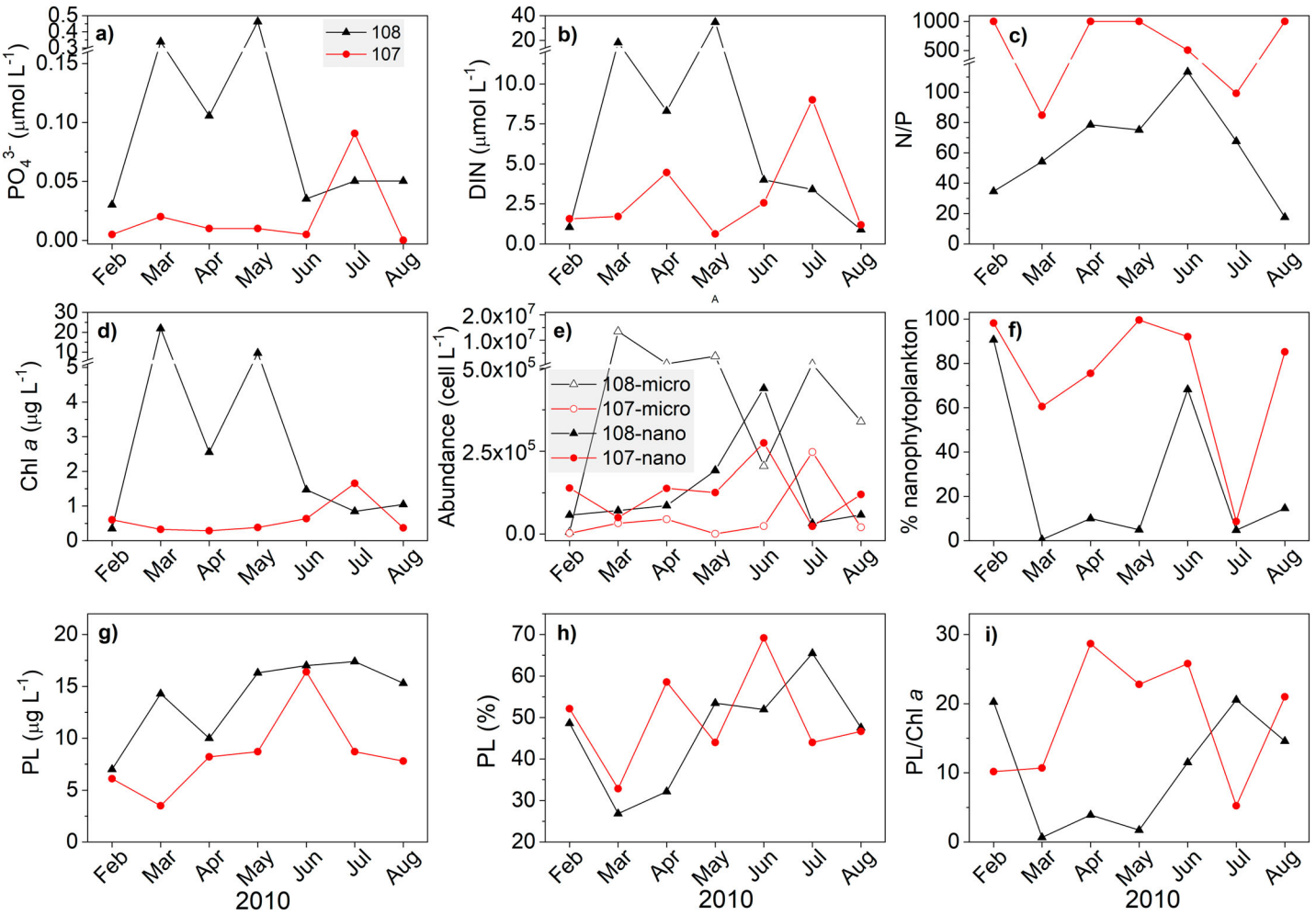


**Fig. 2.** Characteristics of the northern Adriatic surface waters in 2010 at mesotrophic Sta. 108 (triangles) and at oligotrophic Sta. 107 (circles). (**a**) Orthophosphate ($PO_4^{3-}$) and (**b**) DIN concentrations, (**c**) inorganic nitrogen to phosphorus ratios (N/P), (**d**) Chl *a* concentrations, (**e**) abundance of microphytoplankton and nanophytoplankton, (**f**) relative proportion of nanophytoplankton to the phytoplankton community (% nanophytoplankton), (**g**) phospholipid concentrations (PL), (**h**) relative proportion of phospholipids to cell lipids (PL (%)) and (**i**) phospholipids to Chl *a* ratio (PL/Chl *a*). The value for the $PO_4^{3-}$ concentration below the detection limit is plotted as zero. The N/P values are fictitiously set to 1000 in the case when the $PO_4^{3-}$ concentrations were below the detection limit.

of DIN concentrations followed distribution of $PO_4^{3-}$ concentrations (Fig. 2b). The concentration of DIN ranged from 0.89 to 34.81 $\mu$mol $L^{-1}$ and 0.62 to 9.00 $\mu$mol $L^{-1}$ at Sta. 108 and 107, respectively. Such N and P concentration unbalance lead to extremely high N/P ratios at Sta. 107 (84–1000), while lower N/P ratios were evaluated for Sta. 108 (18–113) (Fig. 2c). In addition, higher Chl *a* concentrations were measured at Sta. 108 (0.3–21.9 $\mu$g $L^{-1}$), except in February and July (Fig. 2d). The abundance of microphytoplankton at both stations followed the availability of nutrients (Fig. 2e). At Sta. 108 lower relative contribution of nanophytoplankton to the phytoplankton community (0.5–90.6%) (Fig. 2f) was observed throughout 2010, along with the higher phospholipid concentrations, ranging from 7.0 to 17.4 $\mu$g $L^{-1}$ (Fig. 2g). Mesotrophic Sta. 108 was characterized with a lower relative proportion of phospholipids to cell lipids (28.8–51.9%), except in May and July (53.5% and 65.5%, respectively) (Fig. 2h), and a lower phospholipids to Chl *a* ratio was recorded (0.7–14.6) (Fig. 2i), except in February and July (20.2 and 20.5, respectively).

Of the 6 months investigated in the year 2017, mesotrophic Sta. 108 had higher $PO_4^{3-}$ concentrations than oligotrophic Sta. 107 in 3 months: March, June, and July (0.12, 0.11, and 0.05 $\mu$mol $L^{-1}$, respectively) (Fig. 3a). As for these 3 months, DIN concentrations were higher in March and June (Fig. 3b), while the N/P ratio at Sta. 108 was lower that at Sta. 107 in June and July (Fig. 3c). In the same 3 months, higher Chl *a* concentrations (3.1, 2.4, and 0.6 $\mu$g $L^{-1}$, respectively; Fig. 3d) and higher microphytoplankton abundance (Fig. 3e) were also observed. With respect to these 3 months, Sta. 108

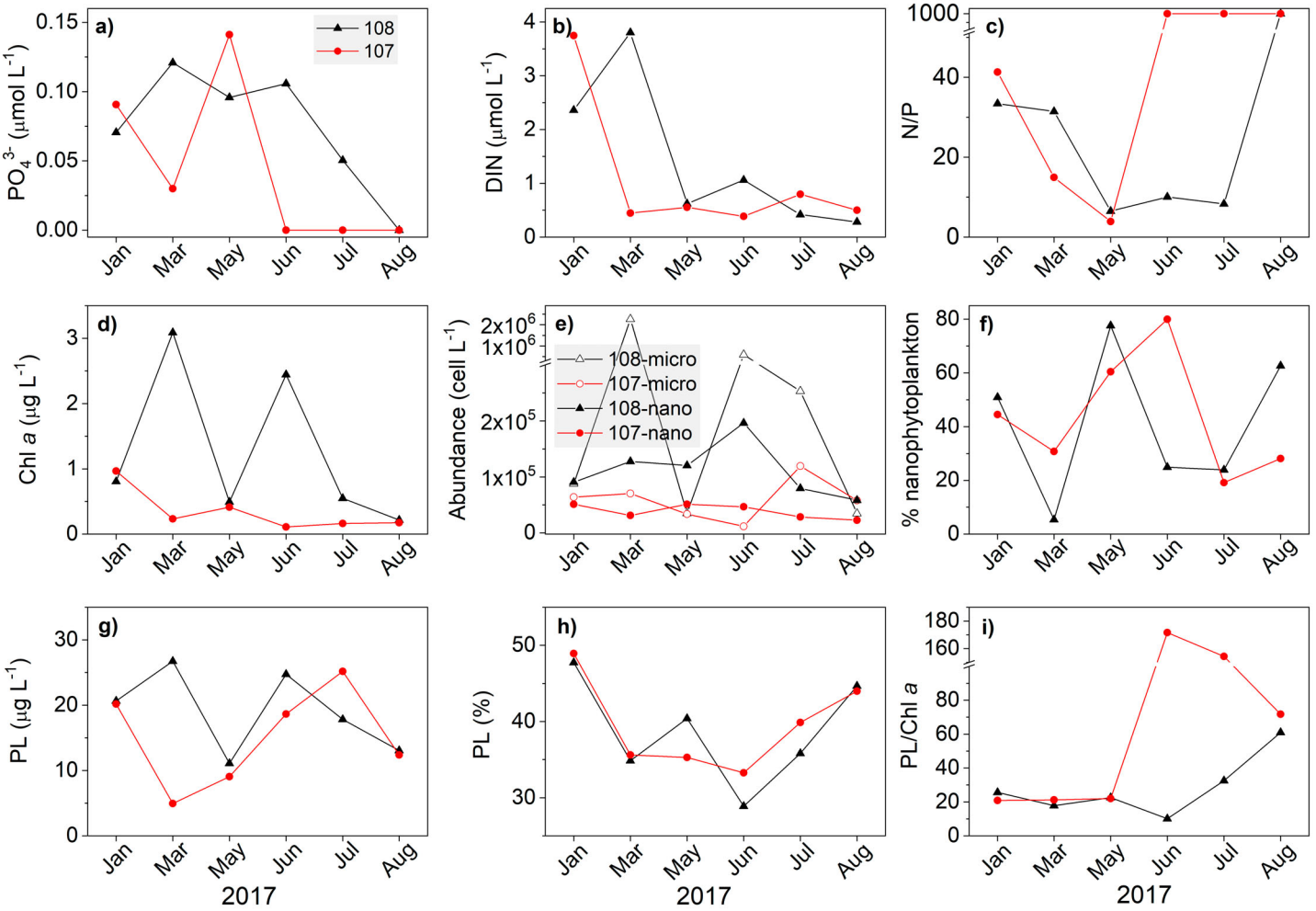


**Fig. 3.** Characteristics of the northern Adriatic surface waters in 2017 at mesotrophic Sta. 108 (triangles) and at oligotrophic Sta. 107 (circles). (**a**) Orthophosphate ($PO_4^{3-}$) and (**b**) DIN concentrations, (**c**) inorganic nitrogen to phosphorus ratios (N/P), (**d**) Chl *a* concentrations, (**e**) abundance of microphytoplankton and nanophytoplankton, (**f**) relative proportion of nanophytoplankton to the phytoplankton community (% nanophytoplankton), (**g**) phospholipid concentrations (PL), **(h)** relative proportion of phospholipids to cell lipids (PL, %), and (**i**) phospholipids to Chl *a* ratio (PL/Chl *a*). The value for the $PO_4^{3-}$ concentration below the detection limit is plotted as zero. The N/P value is fictitiously set to 1000 in the case when the $PO_4^{3-}$ concentration was below the detection limit.

was characterized by a lower relative contribution of nanophytoplankton to the phytoplankton community in March and June (5.3% and 24.9%, respectively; Fig. 3c), higher phospholipid concentrations in March and June (54.9 and 24.7 $\mu$g $L^{-1}$, respectively; Fig. 3d), a lower relative proportion of phospholipids to cell lipids in June and July (18.5% and 27.6%, respectively; Fig. 3e) and a lower phospholipids to Chl *a* ratio in the 3 months mentioned above (17.8, 10.1, and 32.6, respectively; Fig. 3f).

For 4 months of the year 2021, shown in Fig. 4, higher $PO_4^{3-}$ concentrations were found at Sta. 108 in June, July, and September (0.03–0.06 $\mu$mol $L^{-1}$) if compared to Sta. 107 (Fig. 4a). DIN concentrations were higher in the four months studied (0.48–5.75 $\mu$mol $L^{-1}$; Fig. 4b). The N/P ratio was lower in June and July (Fig. 4c). The abundances of microphytoplankton and nanophytoplankton were generally higher at Sta. 108 (Fig. 4e). The months in which higher Chl *a* concentrations (0.5–2.0 $\mu$g $L^{-1}$; Fig. 4d) were recorded there was a lower relative proportion of nanophytoplankton to the phytoplankton community in June and July (37.0% and 7.0%, respectively; Fig. 4f). Higher phospholipid concentrations were found in all 4 months (21.6–29.0 $\mu$g $L^{-1}$; Fig. 4g). A lower relative proportion of phospholipids to cell lipids in June and September (35.3% and 44.1%, respectively; Fig. 4h) and a lower phospholipids to Chl *a* ratio in June and September (14.5 and 52.9, respectively; Fig. 4i) were recorded.

Principal component analysis (PCA) was performed to assess the correlations between the concentrations of $PO_4^{3-}$, DIN, Chl *a* and phospholipids, N/P ratio, relative proportion of nanophytoplankton in the phytoplankton community,

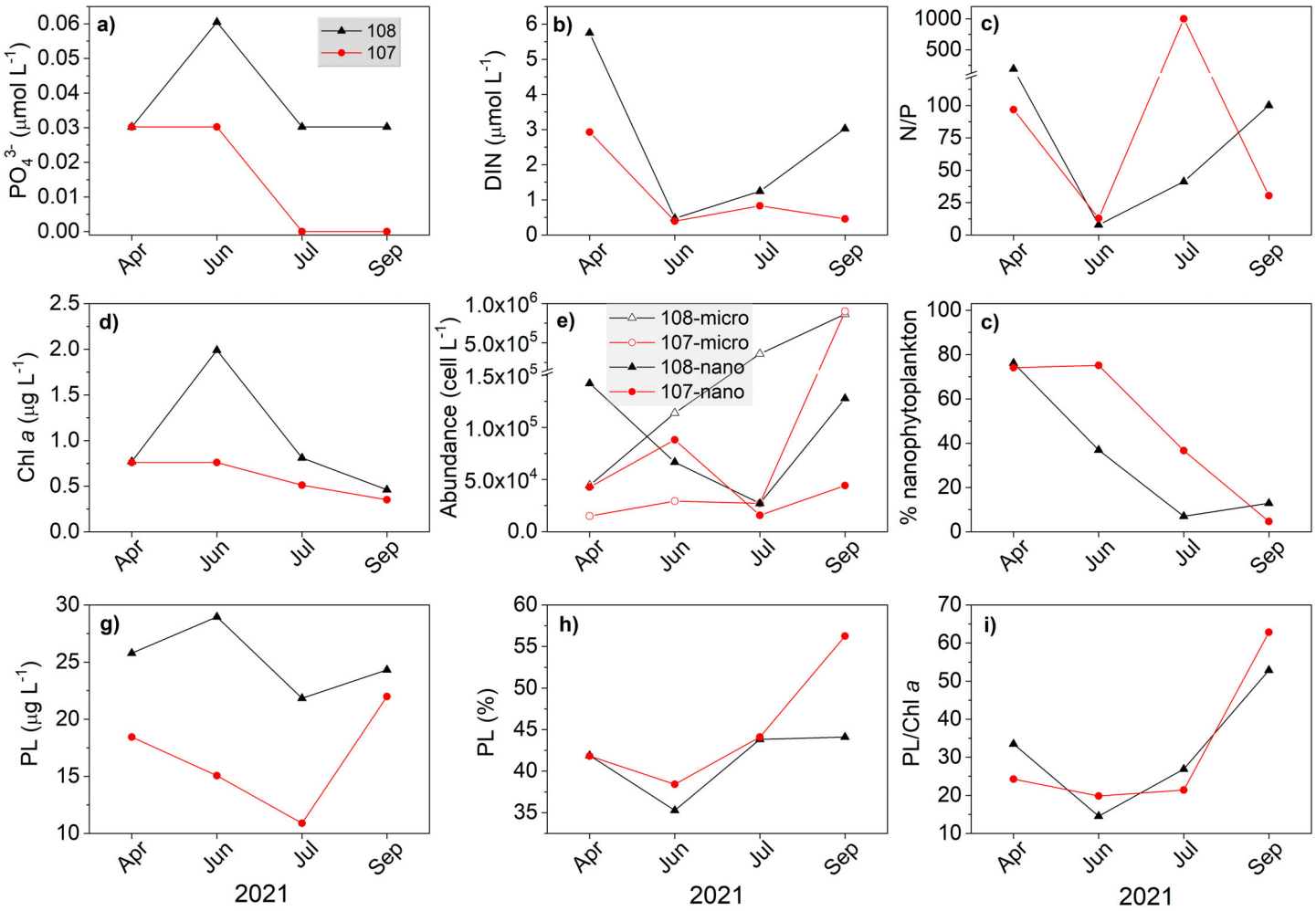


**Fig. 4.** Characteristics of the northern Adriatic surface waters in 2021 at mesotrophic Sta. 108 (triangles) and at oligotrophic Sta. 107 (circles). (**a**) Orthophosphate ($PO_4^{3-}$) and (**b**) DIN concentrations, (**c**) inorganic nitrogen to phosphorus ratios (N/P), (**d**) Chl *a* concentrations, (**e**) abundance of microphytoplankton and nanophytoplankton, (**f**) relative proportion of nanophytoplankton to the phytoplankton community (% nanophytoplankton), (**g**) phospholipid concentrations (PL), (**h**) relative proportion of phospholipids to cell lipids (PL, %), and (**i**) phospholipids to Chl *a* ratio (PL/Chl *a*). The value for the $PO_4^{3-}$ concentration below the detection limit is plotted as zero. The N/P value is fictitiously set to 1000 in the case when the $PO_4^{3-}$ concentration was below the detection limit.

relative proportion of phospholipids in cell lipids, and phospholipids to Chl *a* ratios (Fig. 5; Table S2). The first (PC1) and second (PC2) principal components explained a total of 68.25% of the data variance. PC1 had the highest negative loadings for Chl *a*, $PO_4^{3-}$ and DIN concentrations. The concentrations of Chl *a*, $PO_4^{3-}$ and DIN were inversely related to the relative proportion of nanophytoplankton to the phytoplankton community, phospholipids/Chl *a* and the N/P ratios. PC2 had the highest positive loadings for the relative proportion of phospholipids in cell lipids, N/P ratio, and DIN.

Overall, the general trend is that at higher $PO_4^{3-}$, DIN, and Chl *a* concentrations, the relative contribution of nanophytoplankton to the phytoplankton community, and the ratio of phospholipids to Chl *a* are lower. This situation is mainly found at mesotrophic Sta. 108, in contrast to oligotrophic Sta. 107. At high N/P ratios there is high relative proportion of phospholipids in cell lipids (Figs. 2–4; Table S1).

## Phytoplankton cultivations

To test the hypotheses that smaller phytoplankton species and growth in stress conditions lead to elevated phospolipid content we performed cultivation experiments. Figure 6. shows the relative proportion of phospholipids in cell lipids (%) for two micro diatoms (cultures *C. pseudocurvisetus* and *A. glacialis*), two nano coccolithophores and nano green algae (cultures *C. braarudii*, *C. leptoporus* and *D. tertiolecta*), and pico green algae and pico cyanobacteria (cultures *Picochlorum* sp. and *Synechococcus* sp.). The data are presented in two rows. The first row shows cultures grown under favorable

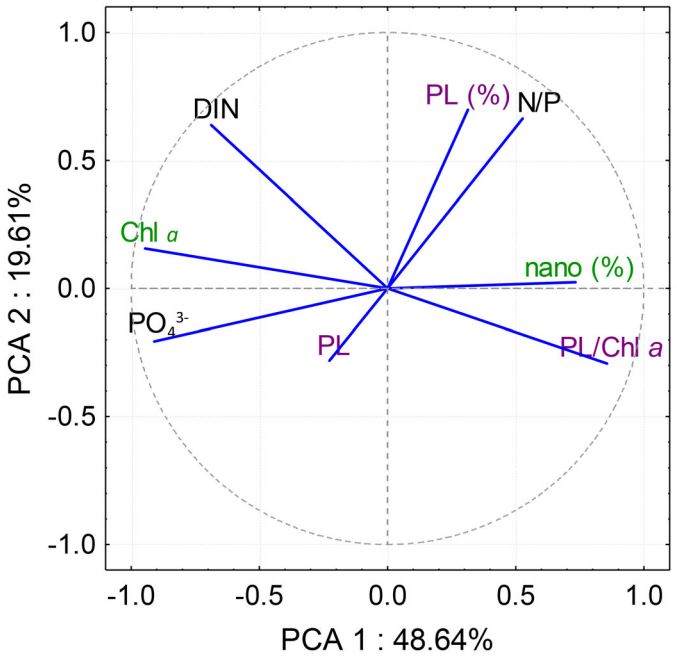


**Fig. 5.** PCA of the northern Adriatic data. Relationship between concentrations of orthophosphate ($PO_4^{3-}$), DIN, inorganic nitrogen to phosphorus ratios (N/P), Chl *a*, phospholipids (PL), relative proportion of nanophytoplankton in the phytoplankton community (nano (%)), relative proportion of phospholipids in cell lipids (PL (%)), and phospholipids to Chl *a* (PL/Chl *a*) ratios for the northern Adriatic Sta. 108 and Sta. 107 and for the periods shown in Figs. 2–4.

conditions, while the second row shows cultures grown under stress (low nutrient availability, high temperature, and low salinity).

The general trend for cultures grown under favorable conditions includes an average increased proportion of phospholipids with decreasing cell size from micro to pico (Fig. 6, first row). This is confirmed by statistical two sample *t*-test for the majority of size classes (Table S3). Growth under stress conditions (Fig. 6, second row), including P-deficiency (Fig. 6. _-P cultures: *C. pseudocurvisetus*, *A. glacialis*, *C. leptoporus*, and *Picochlorum* sp.) results in an increased proportion of phospholipids in the cell. Although the trends are obvious, it is statistically confirmed for the cultures of *C. pseudocurvisetus*, *D. tertiolecta* and *Picochlorum* sp. (Table S3). Cultivation of *C. pseudocurvisetus* has shown that with increasing stress, the phospholipid content in cell lipids increases (compare *C. pseudocurvisetus* cultures in nitrogen stress, high temperature stress and combination of the two in Fig. 6.).

## Discussion

In general, the range of cell size within the phytoplankton community is related to the availability of nutrients. In conditions of low nutrient levels (oligotrophic), smaller phytoplankton cells tend to dominate, while in conditions of high nutrient levels (eutrophic), larger phytoplankton cells tend to dominate (Irwin et al. 2006). Consistent with this, our data from the northern Adriatic showed that the smaller plankton fraction, that is, nanophytoplankton, contributed more at the P poorer Sta. 107 than at the P richer Sta. 108, where microphytoplankton bloomed more frequently and which was characterized by higher Chl *a* concentrations (Figs. 2–4 panels d–f; Table S1).

Here, we showed that the proportion of phospholipids in marine phytoplankton is higher in smaller cells (nanophytoplankton vs. microphytoplankton) and those growing in any stress conditions. However, one might expect that the proportion of phospholipids in marine phytoplankton would be lower under P-limited growth conditions. We have shown that under P-oligotrophy in the natural phytoplankton community of the northern Adriatic, the relative proportion of phospholipids to total cellular lipids increased, along with a higher content of phospholipids per biomass, as determined by Chl *a* (PL/Chl *a*, Figs. 2–4). These changes were consistent with the observed phytoplankton community shift towards smaller cells. Cultivation experiments with phytoplankton from three size classes grown under favorable conditions (Fig. 6, first row) have shown that the content of phospholipids is relatively higher in smaller than in larger phytoplankton. Phosphorus deficiency causes an increase in phospholipids in relation to total cellular lipids (Fig. 6, second row, _-P cultures of *C. pseudocurvisetus*, *A. glacialis*, *C. leptoporus*, and *Picochlorum* sp.). In the following discussion, we offer explanations for these findings.

We had several hypotheses to explain higher percentages of phospholipids to cell lipids that were observed in oligotrophic P-limiting conditions, particularly at oligotrophic Sta. 107. One of our initial hypotheses was that under P-limiting conditions, where phytoplankton synthesize the enzyme alkaline phosphatase to obtain P from P-containing organic molecules, the increase in phospholipids is a consequence of the increased phospholipid phosphatidylinositol synthesis. This is because phosphatidylinositol serves as an anchor for this enzyme at the cell surface. Attachment of alkaline phosphatase to membrane is enabled by covalent linkage between phosphatidylinositol and an ethanolamine phosphate part of the enzyme (Sprong et al. 2001). However, this assumption was rejected as P-limitation, resulting in the production of alkaline phosphatase and consequently phosphatidylinositol, can occur even under mesotrophic conditions. Specifically, when the high N and P concentrations enter the northwestern Adriatic through the Po River (Sta. 108) they allow for a strong bloom(s) to develop, particularly dominated by diatoms (Godrijan et al. 2023). However, due to the high riverine imbalance of N and P ($DIN/PO_4^{3-} = 37–418$; Cozzi and Giani 2011), $PO_4^{3-}$ is used up while plenty of DIN remains. In such mesotrophic conditions (Sta. 108) plankton synthesize high concentrations of alkaline phosphatase, even higher to those recorded for oligotrophic side of the northern Adriatic (Sta. 107) as indicated by high alkaline phosphatase activity in

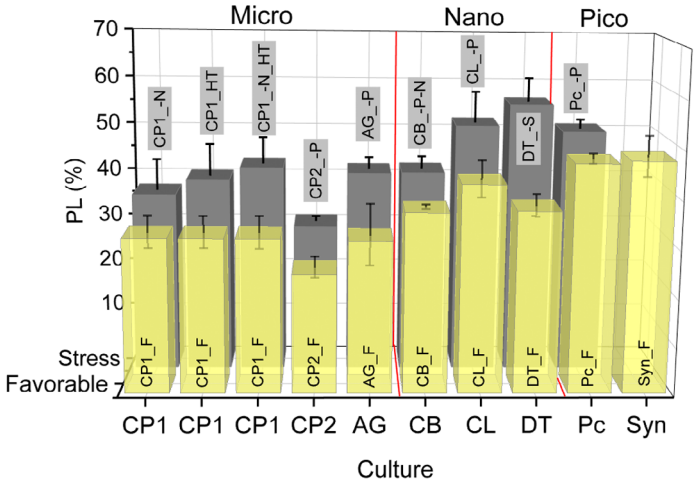


**Fig. 6.** The relative proportion of phospholipids to cell lipids (PL (%)) of the phytoplankton taxa studied (mean +/− SD). Cultures of studied species were grouped into microphytoplankton (Micro): "CP1" and "CP2"—*Chetoceros psudocurvisetus*; "AG"—*A. glacialis*; nanophytoplankton (Nano): "CB"—*Coccolithus braarudii*; "CL"—*Calcidiscus leptoporus*; "DT"—*Dunaliella tertiolecta*; and picophytoplankton (Pico): "Pc"—*Picochlorum* sp. and "Syn"—*Synechococcus* sp. The first row (in yellow) represents cultures grown under species specific favorable conditions (Favorable/_F) of availability of nutrients, temperature, and salinity. The second row (in gray) represents cultures grown under stressful conditions (Stress), including lack of nitrogen (_-N), lack of phosphorus (_-P), lack of phosphorus and nitrogen (_-P-N), high temperature (_HT), and low salinity (_-S).

2017 and 2021 (Table S4). Additionally, Labry et al. (2016) found that in phosphate replete waters of two estuaries, a high alkaline phosphatase activity results from bacterial activity on particulate organic phosphorus. This then indicates high proportion of phosphatidylinositol, contributing to phospholipid pool, in cell lipids of the phytoplankton from mesotrophic conditions (Sta. 108) and thus gives grounds for rejection of the initial hypothesis of higher percentage of phospholipids to cell lipids due to high concentration of phosphatidylinositol in oligotrophic P-limiting conditions. It is worth noting that Sta. 108, which was considered eutrophic in the 1980s and mesotrophic thereafter, becomes more oligotrophic (c.f. Table S1), with $PO_4^{3-}$ concentrations often below 0.1 $\mu$mol $L^{-1}$, the concentration considered indicative of P limitation (Justić et al. 1995).

The possible reason for the higher relative proportion of phospholipids in the P-limited sea and higher phospholipid to Chl *a* ratio (Sta. 107), where smaller phytoplankton survive, compared to the mesotrophic region, could be the fact that the proportion of phospholipids in the plasma membranes of small cells to lipids including also lipids inside the cell is higher than in larger cells. According to Finkel et al. (2010), phytoplankton can be classified based on cell size into three categories: picoplankton with a cell diameter of $< 2\ \mu$m, nanoplankton cells with a diameter ranging from 2 to 20 $\mu$m, and microplankton cells with a diameter of up to 20–200 $\mu$m. We performed a simple calculation: if picophytoplankton has a diameter of 1 $\mu$m, nanophytoplankton has a diameter of 10 $\mu$m and microphytoplankton has a diameter of 100 $\mu$m, and the plasma membrane thickness is 4 nm, the proportion of membrane volume to cell volume is 2.4% for picophytoplankton, 0.24% for nanophytoplankton and 100 and 10 times lower for microphytoplankton, 0.024% regarding picophytoplankton and nanophytoplankton, respectively. This suggests that relatively more lipids (namely phospholipids, which are the major lipids in the plasma membrane) are present in the plasma membranes of smaller cells.

In addition, the observed difference in phospholipids proportion between P-oligotrophy and P-mesotrophy could also be likely due to the difference in fatty acid composition of phospholipids synthetized by phytoplankton under different trophic condition. We hypothesized that more saturated fatty acids are synthesized under unfavorable growth conditions at Sta. 107. This is consistent with the findings of Shin et al. (2003), who studied fatty acid production in the East China Sea. They found that the production of saturated fatty acids increased (five-fold) from mesotrophic to oligotrophic stations at the expense of unsaturated fatty acids, while the contribution of diatoms decreased. The same was observed by Mayzaud et al. (2014). They found that the proportion of polyunsaturated fatty acids in the northern Atlantic was much higher under the prevailing mesotrophic conditions, while the proportion of polyunsaturated fatty acids decreased significantly under oligotrophic conditions. Since saturated fatty acid tails are straight, saturated lipids are more tightly packed in membranes than unsaturated lipids, resulting in a smaller surface area per lipid for lipids with saturated acyl chains (Niemelä et al. 2006). Thus, denser packing means more phospholipids per unit membrane area. Therefore, we assume that possibly more densely packed fatty acids in plasma bilayer of phytoplankton that grow in P-scarcity could additionally contribute to a higher proportion of plasma membrane phospholipids at oligotrophic Sta. 107 compared to intracellular lipids with respect to phytoplankton that grow in P favorable conditions as it is mesotrophic Sta. 108. This finally leads to a higher proportion of phospholipids to cell lipids of phytoplankton that grow in P oligotrophic conditions (such as Sta. 107), compared to P mesotrophic conditions (such as Sta. 108).

Our experiments with various phytoplankton strains indicate that exposure to stressful growth conditions leads to an increase in the proportion of phospholipids in the cell lipids (Fig. 6, second row). In a recent study, Vrana et al. (2023) demonstrated that *C. pseudocurvisetus*, when grown at higher than optimal temperatures, undergoes phospholipid remodeling by enhancing the production of saturated fatty acids relative to unsaturated fatty acids. This adaptation is thought to be necessary to prevent excessive membrane fluidity at high temperatures, as saturated fatty acids have a higher melting temperature and thus promote membrane stability. Similarly, Vrana et al. (2022) observed that *D. tertiolecta* undergoes fatty acid remodeling in response to hypoosmotic stress, leading to

an increase in phospholipid fatty acid saturation. The observed increase in saturated fatty acid content is thought to play a critical role in reducing plasma membrane fluidity and altering membrane permeability to prevent the adverse inflow and efflux of ions and water into and out of cells at low salinity.

The non-linear relationship observed between phospholipid content and stress level (compare *C. pseudocurvisetus* cultures in nitrogen stress, high temperature stress, and combination of the two in Fig. 6) can be attributed to the severity of the raising stress. In addition, it is worth noting that phytoplankton often accumulate storage lipids, such are triacylglycerols, during periods of stress (Morales et al. 2021), which decrease the phospholipid to cell lipid ratio and thus influence the relative phospholipids content in the cells.

In summary, our findings suggest that under stressful conditions, including oligotrophy, phytoplankton cells tend to preserve high P content in the phospholipids. This is indicative of the important role of phospholipids for phytoplankton in adapting to adverse environmental conditions that they are increasingly facing in the recent era of global change. Under such conditions, small/smaller phytoplankton cells are expected to dominate (Armstrong McKay et al. 2021). Smaller cells with a higher surface-to-volume ratio, which allows better diffusion towards the cell membrane, have a higher affinity for nutrient uptake and thus the ability to dominate in future oligotrophic marine conditions. Additionally, smaller cells tend to sink more slowly than larger cells. This may indicate that P from phospholipids of small/er cells can be recycled in the photic layer and used for the next phytoplankton bloom cycle. But, smaller cells and those growing under stress tend to have lipids with a higher proportion of saturated fatty acids (Vrana et al. 2022, 2023), which can make them less degradable (Gašparović et al. 2023) and thus more likely to be exported to the deep sea, resulting in P losses for phytoplankton in the sunlit zone.

**Data availability statement**

Source data used for figures are available publicly available through https://urn.nsk.hr/urn:nbn:hr:241:004757.

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## Conflict of Interest

Authors declare no conflict of interest.

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