Hyposalinity induces significant polar lipid remodeling in the marine microalga *Dunaliella tertiolecta* (Chlorophyceae)

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Abstract

Marine microalgae often live in a fluctuating environment including a decrease in salinity caused by global warming induced sea ice melting and freshwater inflows. Under conditions of fluctuating salinity, microalgae have evolved a variety of survival mechanisms such as lipid accumulation and remodeling. The purpose of this study was to investigated the membrane lipid remodeling of the marine green microalga *Dunaliella tertiolecta* as a short-term acclimation mechanism in response to hyposalinity (20 and 3 PSU) with respect to growth at optimal salinity (38 PSU). We identified 34 lipid species belonging to seven polar lipid classes. *Dunaliella tertiolecta* accumulates cell lipids and remodels polar lipid classes and their fatty acids composition as response to hypoosmotic stress at 3 PSU. We found that the unsaturation of most polar lipids decreases overall, indicating decreased membrane fluidity and altered permeability, whereas shortening the length of fatty acids of polar lipids is not one of the strategies of *D. tertiolecta* to cope with the decrease in salinity. Increase in relative content (%) and unsaturation of monogalactosyldiacylglycerols (MGDG) and decrease in relative content (%) and unsaturation of phosphatidylglycerols (PG), suggesting changes in photosynthetic membranes of thylakoids at 20 and 3 PSU. At a very low salinity of 3 PSU, the relative content (%) of phosphatidylinositols (PI) increases, suggesting increased lipid trafficking and signaling in the cells. These changes are statistically significant and we hypothesize that *D. tertiolecta* is genetically adapted to withstand large salinity fluctuations through polar lipid composition.

Keywords Hyposaline · *Dunaliella tertiolecta* · Lipid remodeling · Polar lipids · Fatty acids

Introduction

Eukaryotic phytoplankton (microalgae) are photosynthetic organisms and the most important primary producers in aquatic ecosystems, responsible for photosynthetic fixation of about 50% of atmospheric carbon (Falkowski and Raven 2007). They possess remarkable plasticity and adaptability to abiotic stresses and can grow in a wide range of habitats (Shetty et al. 2019). Microalgae synthesize various fatty acids, but the most important are long chain polyunsaturated fatty acids (PUFA), which are necessary for healthy and balanced growth of higher trophic levels that have limited capacity to synthesize these fatty acids (Arts et al. 2001).

Ocean salinity has changed in the last 50 years; the Pacific and Southern Ocean are becoming fresher and the Atlantic and Indian Oceans are becoming saltier due to global climate change (Cheng et al. 2020). Salinity is highly variable in coastal regions, especially in estuaries and rock pools (Kirst 1990). Salinity fluctuation is one of the major abiotic stressors that induces an oxidative stress response in algae, affects external water potential, and disturbs turgor pressure, ion distribution and organic solutes in the cell, and inhibits cell division and growth rate (Richmond 1986; Kirst 1990; Mishra et al. 2008; Shetty et al. 2019). Microalgae respond and adapt differently to salt stress (Kirst 1990), including changes in photosynthesis, photorespiration, osmotic adjustment and compartmentalization, and amino acid and carbohydrate synthesis (Kirst 1990; Tammam et al. 2011; Neelam and Subramanyam 2013).

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Lipids (phospholipids, glycolipids and betaine lipids) in microalgae cells are important molecules that play vital roles in cell structure, signaling and trafficking and are essential for tolerance and acclimation to overcome stressful environmental conditions (Harwood and Guschina 2009; Du and Benning 2016). A major component of plasma membrane lipids in microalgae are the phospholipids phosphatidylcholines (PC), phosphatidylethanolamines (PE) and PG. Glycolipids play important roles in forming thylakoid lipid bilayers and protein assembly in photosynthetic complexes (Kobayashi 2016). Phosphatidylglycerol, the only phospholipid found in thylakoids, is essential for photosynthetic electron transport, the development of chloroplasts, and tolerance to chilling (Wada and Murata 2007). The phospholipid PI serves as a precursor for synthesizing other phospholipids (Harwood and Guschina 2009) and plays an important regulatory role in the cell physiology (Furt et al. 2011). For many years betaine lipids in microalgae were thought to serve as an adaptive strategy to cope with phosphate deficiency, but in certain algal species betaine lipids play a role other than of a mere substitute for phospholipids, due to their much greater unsaturation than in phospholipids (Murakami et al. 2018 and references therein). Regulation of lipid biosynthesis is one of the most important strategies in resistance physiology to salinity stress in microalgae (Guschina and Harwood 2006).

The genus *Dunaliella* is a unicellular photoautotrophic green microalgae with more than 20 marine and halophilic species (Borowitzka and Siva 2007) found in a wide range of waters, from marine areas to salinity variable brackish lagoons and estuaries (Borowitzka and Brown 1974; Gilmour et al. 1984; Ginzburg 1988; Borowitzka 2018a). *Dunaliella* also inhabits hypersaline environments, where it is responsible for most of the primary production (Oren 2005). In *Dunaliella* the cell wall is replaced by a thin, elastic plasma membrane and a glycocalyx surface coat that makes the cells morphologically more flexible (rapid changes in cell volume) to changes in salinity (Hamburger 1905; Oliveira et al. 1980; Svetličić et al. 2001; Oren 2005; Novosel and Ivošević DeNardis 2021). Under stressful environmental conditions, the cell can accumulate various economically valuable organic compounds such as vitamins, proteins and carotenoids (Ghoshal et al. 2002; Hadi et al. 2008; Borowitzka 2013).

Dunaliella tertiolecta is frequently exposed to hypoosmotic stress in its usual habitat (Gilmour et al. 1984). To date, there are only two papers on *D. tertiolecta* dealing with individual lipid classes and their remodeling during growth under stress conditions (light, temperature and nutrient N-deplete) (Kim et al. 2013, 2015). Several published papers on *D. tertiolecta* growth at different salinities focused on the content and composition of free fatty acid, total lipid content, glycerol synthesis/degradation, synthesis of antioxidant enzymes and β-carotene (Borowitzka and Brown 1974; Pick 2002; Jahnke and White 2003; Takagi et al. 2006; Tammam et al. 2011; Liang et al. 2017; Rizwan et al. 2020). The response of *D. tertiolecta* to hyposalinity includes an increase in cell size, a decrease in F_v/F_m (maximal photochemical efficienct of PS II) a decrease in ascorbate content, and an increase in the pools of α-tocopherol and total glutathione (Jahnke and White 2003). However, there is no report on individual lipid classes and lipid remodeling during growth of *D. tertiolecta* in hyposaline environment. Lipids play an essential biological role in microalgae cells as a defense response to stress conditions. Comprehension of the lipid remodeling will lead to a better understanding of microalgae acclimation/adaptation mechanisms to changes in salinity. Here, we investigate the acclimation of the marine green microalga *D. tertiolecta* to salinity decrease by analyzing changes in polar lipid composition. To elucidate lipid remodeling, lipidomics analysis based on hydrophilic interaction liquid chromatography coupled with electrospray ionization mass spectrometry (HILIC-ESI–MS) was used to identify and quantify lipid classes and their fatty acid composition. We demonstrate how *D. tertiolecta* remodels lipid content and fatty acids composition under hyposaline conditions. The statistically significant polar lipid remodeling is attributed to the fact that *D. tertiolecta* has already genetically adapted to a wide range of salinities through the composition of membrane lipids.

Materials and methods

Algal culture and growing conditions

Dunaliella tertiolecta (Chlorophyceae, CCMP 1320, Culture Collection Bigelow, Laboratory for Ocean Sciences, Bigelow, MN, USA) was used. Prior to the experiments, the microalga was grown (for two weeks) in seawater (salinity of 38 PSU (Practical Salinity Unit)) enriched with f/2 medium (Guillard and Ryther 1962; Guillard 1975) in 200 mL sterile VWR tissue culture flasks (VWR, USA). The different salinities of the enriched f/2 culture medium were prepared as follows: 1000 mL seawater (38 PSU), 480 mL seawater and 520 mL deionised water (20 PSU), and 80 mL seawater and 920 mL deionized water (3 PSU). The culture media were inoculated with 4×10^4 cells mL^{-1}. Cultures were gently stirred twice a day manually and counted every 2 to 3 days using Fuchs-Rosenthal counting chambers. All experiments were performed at 18 °C in a thermostatic chamber (Inkolab, Croatia) with a light/dark cycle of 12/12 h under an illumination of 72 μmol photons m^{-2} s^{-1}. Growth was terminated when the cells reached the third day of stationary phase. All experiments were performed in triplicate.

Chemicals

Lipid standards MGDG, digalactosyldiacylglycerols (DGDG), sulfoquinovosyldiacylglycerols (SQDG), PG, PC, PE, PI, phosphatidylserine (PS), phosphatidic acids (PA) and diacylglyceryl-N,N,N-trimethylhomoserines (DGTS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), while all other lipid standards were purchased from Sigma-Aldrich (Sigma-Aldrich Corporation, Saint Louis, MO, USA). The internal standards (Fmoc-glycine) and ammonium acetate were purchased from EMD Millipore (Merck Millipore, Burlington, MA, USA). Formic acid was purchased from Fluka (Honeywell International Inc, Charlotte, NC, USA). All solvents (acetonitrile, water, dichloromethane, methanol and isopropanol) were purchased from J.T.Baker (Phillipsburg, NJ, USA) and were HPLC-grade.

Lipid extraction

For lipid analysis, 100 mL of the culture was filtered through a 0.7 µm Whatman GF/F filter (pre-burned at 450 ºC for 5 h). The filters were then stored at – 80 °C prior to analysis. The lipid fraction was extracted according to the modified Bligh and Dyer method (Bligh and Dyer 1959). Extracts were evaporated to dryness under nitrogen and stored at – 20 °C until analysis. For HILIC-ESI–MS, dry lipid extracts were dissolved in dichloromethane:methanol (4:1 (v:v), 100 µL) and diluted with methanol:isopropanol (1:1 (v:v)) prior to analysis. An internal standard (Fmoc-glycine) was then added and samples were filtered through CHROMAFIL Xtra PTFE-20/13 filters (Macherey–Nagel, Germany). For thin-layer chromatographic analysis, dry lipid extracts were dissolved in 20 to 30 µL dichloromethane and an internal standard (n-nonadecanone) was added to each sample to estimate recovery.

Analysis of phospholipids, glycolipids and betaine lipids

Separation was performed with Waters Acquity HPLC system (Waters Corp, USA) equipped with a Hydrophilic Interaction Liquid Chromatography column (Acquity UPLC BEH HILIC 1.7 µm, 2.1 × 150 mm, Waters), using gradient elution. Mobile phase A was acetonitrile:water (95:5), and mobile phase B was water. Both phases contained 10 mM ammonium acetate and 0.1% formic acid. The gradient started at 100% A for 4 min, increased to 15% B over 8 min, and was held at 15% B for 1 min before returning to initial conditions after 1 min and held for 7 min. The flow rate was 0.3 mL min^{-1} and the injected

sample volume was 1 µL. The temperature of the column and sample was 40 and 8 °C, respectively.

A quadrupole time-of-flight electrospray ionization mass spectrometer (QqToF Premier, Waters, USA) was used to identify and quantify lipids. Lipid analysis was performed in positive and negative ion modes. The optimal conditions were as follows: capillary voltage 3 kV; sampling cone voltage 20 V; source temperature 100 °C; desolvation temperature 300 °C; collision gas argon. Accurate mass was maintained by introducing a lock-spray interface of leucine enkephalin (556.2771 [M + H]$^+$ or 554.2615 [M − H]$^-$). Data were acquired in the mass range (m/z) of 50 – 1200 Da with a scan duration of 0.2 s. The MS/MS experiments were performed with the following collision energies: SGDG, DGDG, MGDG and PI 20 eV, DGTS, PA and PE 25 eV and PG and PC 30 eV. All lipids were detected in both ionization modes (positive and negative), but quantification of individual lipid species was performed in one ionization mode, while the other was used as a conformation. In the negative mode, MGDG and PG species were analyzed as [M + HCOO]$^-$ and [M-H]$^-$, respectively. The remaining lipid classes were analyzed in positive mode; SQDG, DGDG and PI as [M + NH4]$^+$ and PC and DGTS as [M + H]$^+$. Instrument control and data analysis were performed using MassLynx 4.1 software (Waters). The concentration of each lipid was calculated from a calibration curve of known concentrations of the corresponding standards.

Lipid species are represented by the abbreviation of the lipid class (e.g. MGDG), while fatty acids are represented as $x_1:y_1/x_2:y_2$, where x_1 and x_2 are the number of carbon atoms in the first and second fatty acyl chain, and y_1 and y_2 are the number of double bonds in the first and second fatty acyl chain (e.g. MGDG 18:2/16:3).

Analysis of total lipids

Total cellular lipids were quantified by thin-layer chromatography coupled with flame-ionisation detection (Iatroscan MK-VI, Iatron, SES GmbH—Analytical Systems, Germany). The Iatroscan was operated at a hydrogen flow rate of 160 mL min^{-1} and an air flow rate of 2000 mL min^{-1}. Total lipid content was calculated as the sum of the ten lipid classes detected (MGDG, SQDG, DGDG, PG, PE, PC, pigments, sterols, steryl esters and triacylglycerols). The detailed procedure is described in Gašparović et al. (2015, 2017).

Data analysis

The growth rate was calculated using the following equation:

$$\mu = \frac{1}{(t_m - t_0)} \times \ln\left(\frac{N_m}{N_0}\right)$$

where N_m and N_o are the algal concentrations at the end (day t_m) and beginning (day t_o) of the exponential growth phase, and t is the time in days (Thompson et al. 1992).

Data are expressed as mean $\pm$ SD of three parallel measurements (three experiments for each salinity). A one-way ANOVA analysis (Microsoft Excel 2010 software) was used to examine the effect of salinity on lipid remodeling. A p-value < 0.05 is considered significant.

Results

Dunaliella tertiolecta growth

Growth curves of *D. tertiolecta* determined at salinities of 38, 20 and 3 PSU are shown in Fig. 1. A decrease in salinity has a significant effect on the growth of *D. tertiolecta* ($p < 0.05$). The highest cell density was observed at a salinity of 38 PSU ($2.76 \times 10^6 \pm 2.34 \times 10^5$ cells mL^{-1}), a 1.5-fold decrease in cell density was observed at salinity of 20 PSU ($1.89 \times 10^6 \pm 1.53 \times 10^5$ cells mL^{-1}), while the lowest cell density was observed at salinity of 3 PSU ($2.73 \times 10^5 \pm 2.94 \times 10^4$ cells mL^{-1}) (Fig. 1). The growth rate decreased ($p < 0.05$) from 0.217 ± 0.006 day^{-1} at 38 PSU to 0.181 ± 0.017 day^{-1} at 20 PSU and to 0.151 ± 0.026 day^{-1} at 3 PSU.

Lipidomic analysis

Lipidomic analysis of *D. tertiolecta* samples from the stationary growth phase identified 33 polar lipid species (two
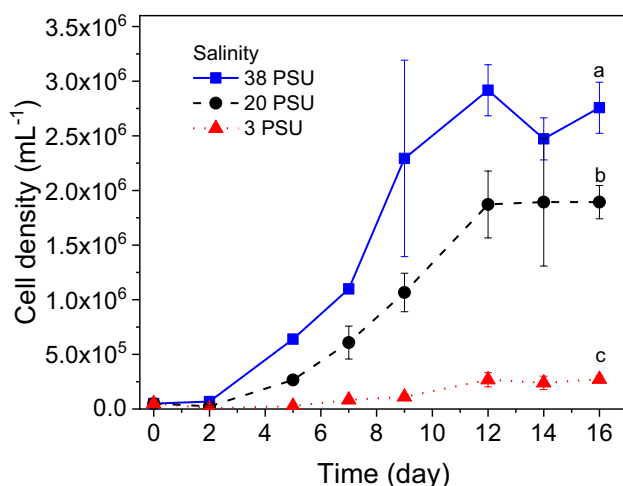

Fig. 1 Growth curves of *D. tertiolecta* at 38, 20 and 3 PSU. Data are mean $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences ($p < 0.05$) between salinity

MGDG, three SQDG, six DGDG, four PG, one PI, five PC and 12 DGTS species). In addition, at optimal salinity (38 PSU), one additional MGDG species was identified.

The hyposaline environment affected lipid production (Fig. 2a, Table S2). The highest amount (not statistically significant) of polar lipids (2.07 ± 0.18 pg cell^{-1}) was detected during growth at 3 PSU, while lower amounts of polar lipids were detected during growth at 20 and 38 PSU, 1.95 ± 0.08 and 1.77 ± 0.14 pg cell^{-1}, respectively. Total cellular lipid content (polar lipids plus triacylglycerols and sterols) (1.73 ± 0.32 pg cell^{-1}, 2.16 ± 0.37 pg cell^{-1}, and 3.63 ± 0.43 pg cell^{-1} at 38, 20 and 3 PSU, respectively) statistically differs for studied salinities ($p < 0.05$). With decreasing salinity, the cellular content of total glycolipids (MGDG, SQDG and DGDG) and betaine lipids (DGTS) increased, whereas the content of total phospholipids (PG, PI and PC) decreased (Fig. 2a, Table S2) ($p < 0.05$). Significant changes are observed in the lipid classes MGDG and PG at 20 PSU with respect to 38 PSU ($p < 0.05$) (MGDG $36.5 \pm 1.11\%$ and $42.38 \pm 1.78\%$ and PG $17.74 \pm 0.95\%$ and $14.54 \pm 0.62\%$ at 38 and 20 PSU, respectively). The MGDG/DGDG ratio increased with salinity decrease (2.1, 2.4, and 2.5 for salinities of 38, 20, and 3 PSU, respectively).

A statistically significant increase in MGDG, DGDG, PI, PC, and DGTS and a decrease in PG in total polar lipids were observed when salinity decreased from 38 to 3 PSU ($p < 0.05$; Fig. 2b, Table S3). The percentage of SQDG did not alter significantly in total polar lipid as salinity decrease.

The fatty acid composition of the identified lipid classes changed with salinity drop (Figs. 3–5, Table S4). The proportion of the most abundant MGDG 18:3/16:4 in total MGDG increased statistically significantly from $95.1 \pm 0.5\%$ at 38 PSU to $96.97 \pm 0.37\%$ at 20 PSU to $97.7 \pm 0.3\%$ at 3 PSU, while the proportion of MGDG 18:2/16:3 decreased from $4.70 \pm 0.4\%$ at 38 PSU to $3.03 \pm 0.37\%$ at 20 PSU and $2.3 \pm 0.3\%$ at 3 PSU ($p < 0.05$; Fig. 3a, Table S4).

The share of the most abundant SQDG 18:3/16:0 in the total SQDG decreased from $86.5 \pm 0.7\%$ at 38 PSU to $84.1 \pm 1.7\%$ at 3 PSU (not statistically significant), whereas SQDG 18:1/16:0 increased significantly ($p < 0.05$), while SQDG 16:0/16:0 remained statistically unchanged (Fig. 3b, Table S4). In parallel with the decrease in salinity from 38 to 3 PSU, the percentage of DGDG 18:3/16:3 decreased significantly ($p < 0.05$), while the relative proportions of DGDG 18:3/16:0, DGDG 18:2/16:0, DGDG 18:3/18:3 and DGDG 18:2/18:3 increased significantly ($p < 0.05$; Fig. 3c, Table S4) in total DGTS. The percentage of DGDG 18:1/16:0 in total DGDG remained unchanged.

The percentage of the most abundant PG 16:1/18:3 and PG 16:0/18:3 in the total PG decreased (p < 0.05) and the percentage of PG 16:0/18:2 increased ($p < 0.05$) when salinity decreased from 38 to 20 and 3 PSU, respectively (Fig. 4a, Table S4). The percentage of PG16:0/18:1 changed

Fig. 2 Lipid profile of *D. tertiolecta* at 38, 20, and 3 PSU. Cell lipid content (pg cell^{-1}) of glycolipids (GL), phospholipids (PL), betaine lipid DGTS (BET), total polar (Polar) and total cellular (CL) lipids (**a**) and composition of MGDG, SQDG, DGDG, PG, PI, PC and DGTS (expressed as percentage of total polar lipids—%) (**b**). Data are means ± SD ($n = 3$). Groups with different letters are statistically significantly different from each other ($p < 0.05$)
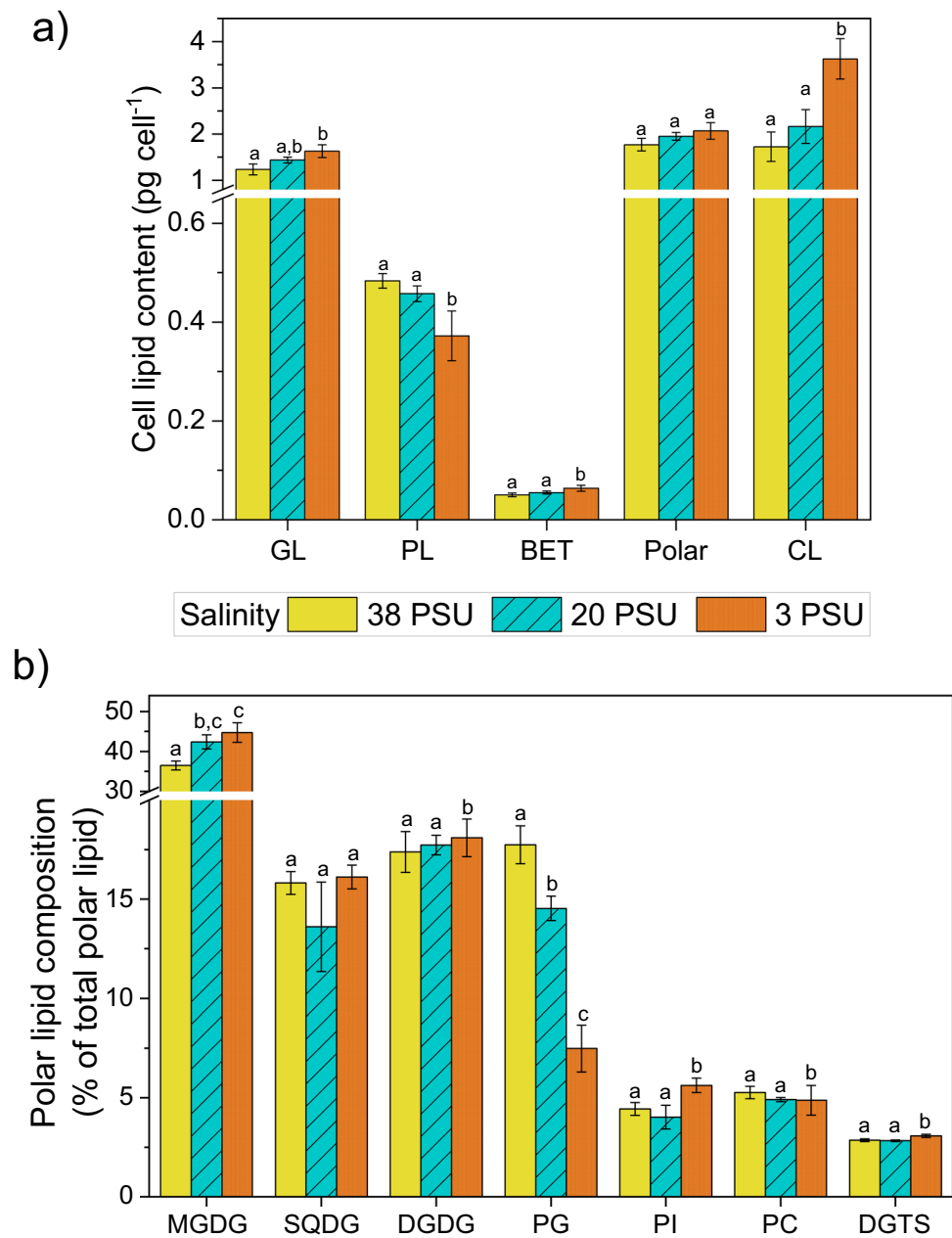
significantly between 38 and 20 PSU ($p < 0.05$). No remodeling occurred in PI, as only one PI18:1/16:0 species was identified at all three salinities. In the lipid class PC, no single species dominated at any salinity. The percentage of PC 18:3/18:2 in the total PC decreased between salinity of 38 and 3 PSU (Fig. 4b, Table S4). The percentage of PC 16:0/18:3 and PC 18:3/18:3 increased significantly ($p < 0.05$) in the total PC when salinity decreased from 38 to 20 PSU, and decreased significantly ($p < 0.05$) when salinity decreased from 20 to 3 PSU (Fig. 4b, Table S4). A small increase in PC 16:0/18:2 was observed when salinity decreased from 20 to 3 PSU ($p < 0.05$). The percentage of PC 18:3/18:1 decreased in the total PC when salinity

decreased from 38 to 20 PSU and increased when salinity decreased from 20 to 3 PSU ($p < 0.05$; Fig. 4b, Table S4). Consequently, a small but significant ($p < 0.05$) decrease in total unsaturation was observed at 3 PSU compared to salinities 38 and 20 PSU (Fig. 6).

Betaine lipids were the most diverse lipid group, in which 12 different species were identified. When salinity decreased from 38 to 3 PSU, the relative amounts of DGTS 18:1/16:0, DGTS 18:3/18:4, DGTS 18:3/18:3, DGTS 18:3/18:2, and DGTS 18:3/18:1 decreased significantly ($p < 0.05$), whereas the relative amounts of DGTS 16:4/16:0, DGTS 18:2/16:0, DGTS 18:2/18:0, DGTS 18:3/16:0, and DGTS 18:3/18:0 significantly increased ($p < 0.05$) to the total DGTS (Fig. 5,

Fig. 3 The composition of glycolipid molecular species: MGDG (**a**), SQDG (**b**) and DGDG (**c**) of *D. tertiolecta* at 38, 20, and 3 PSU. Data are means ± SD ($n = 3$). Groups with different letters are statistically significantly different from each other ($p < 0.05$)
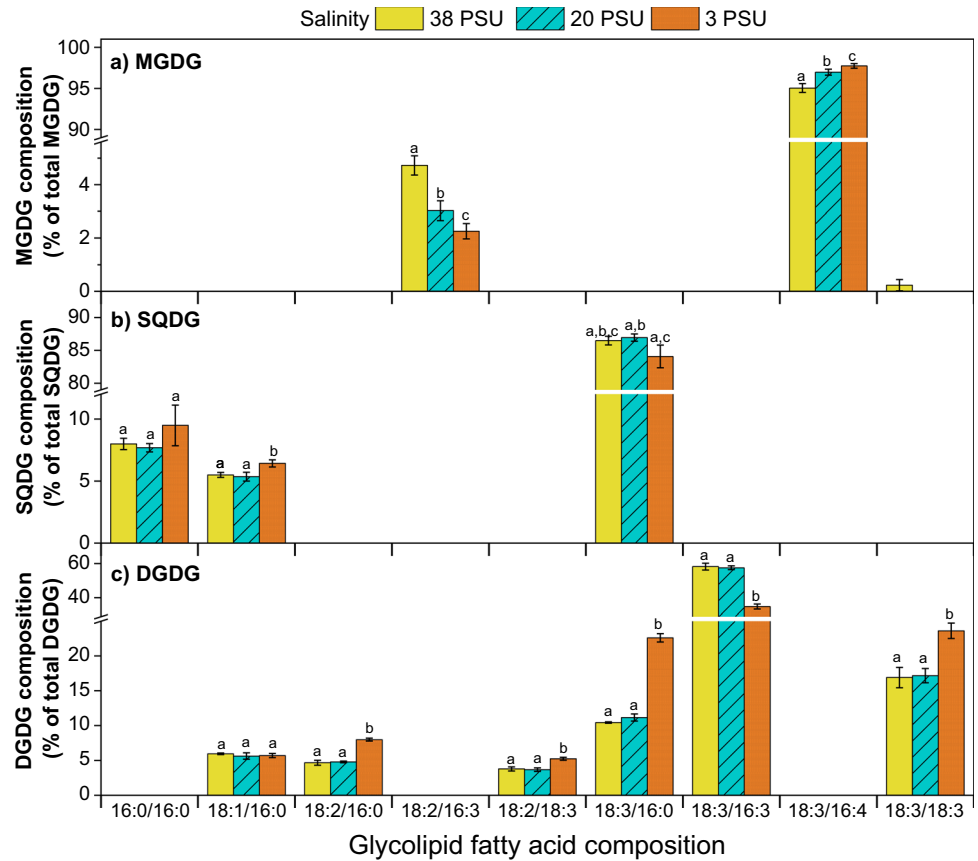

Table S4). The most significant change was observed for DGTS 18:3/18:4, where there was a ~14-fold decrease (Fig. 5, Table S4) when *D. tertiolecta* grew under hyposaline conditions. The proportion of lipid species DGTS 16:3/16:0 and DGTS 18:4/16:0 did not differ significantly between 38 and 3 PSU, whereas it increased significantly when salinity

Fig. 4 The composition of phospholipid molecular species: PG (**a**) and PC (**b**) of *D. tertiolecta* at 38, 20, and 3 PSU. Values represent the means ± SD ($n = 3$). Groups with different letters are statistically significantly different from each other ($p < 0.05$)
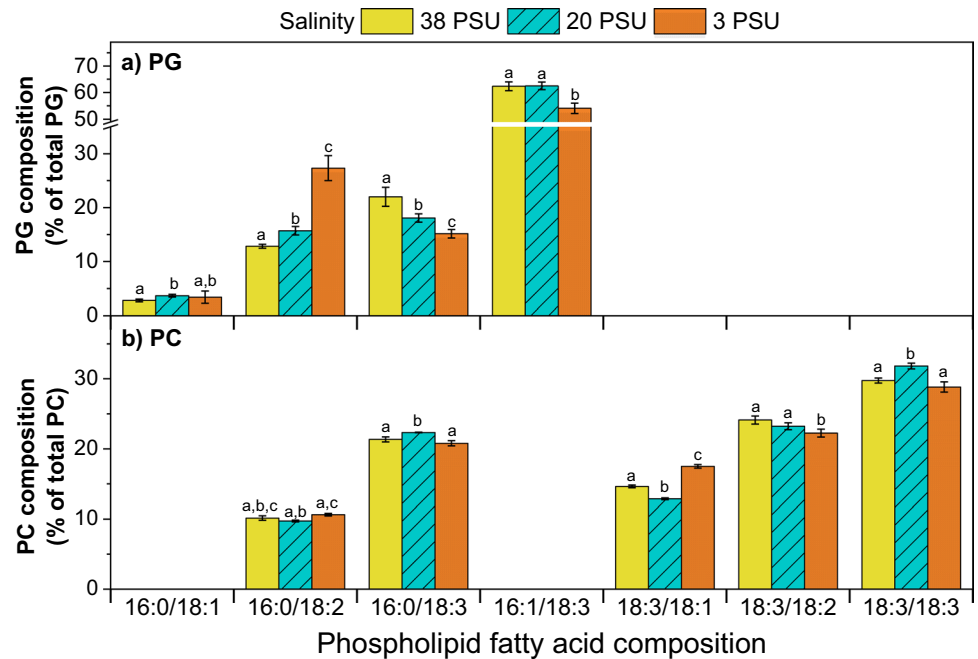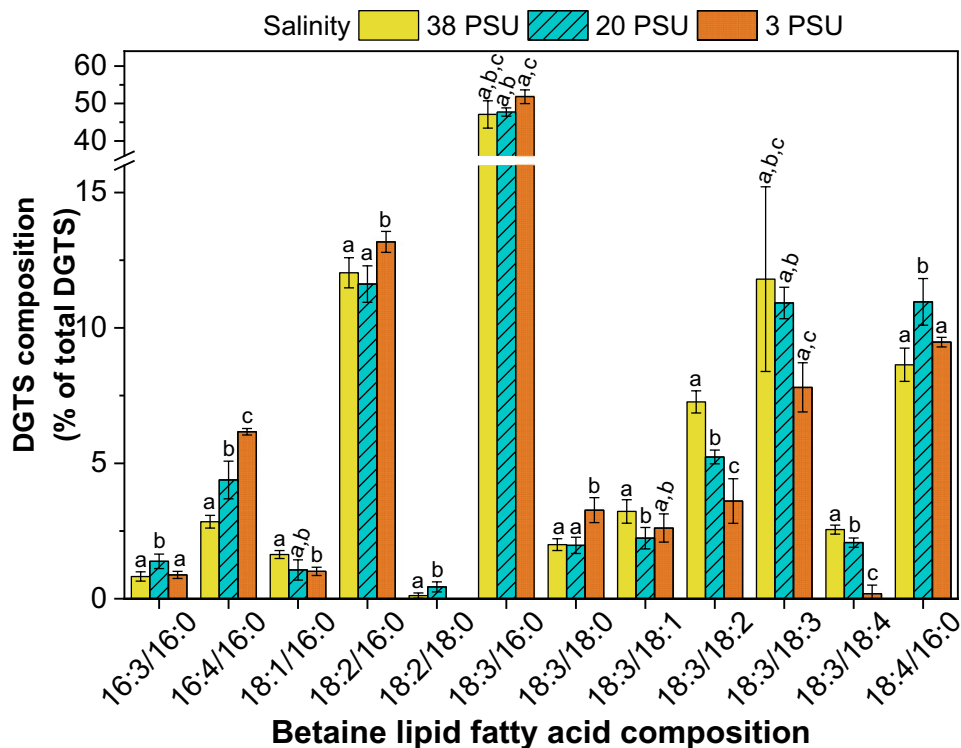
Fig. 5 The composition of betaine (DGTS) molecular species of *D. tertiolecta* at 38, 20, and 3 PSU. Values represent the means ± SD (*n* = 3). Groups with different letters are statistically significantly different from each other (*p* < 0.05)

decreased from 38 to 20 PSU (*p* < 0.05; Fig. 5, Table S4). A significant increase (*p* < 0.05) for DGDG 16:4/16:0 was observed for all salinities studied.

We identified only C16 and C18 fatty acids in all detected polar lipid species with different degrees of unsaturation (number of double bonds). A salinity drop resulted in changes in the distribution of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), PUFA, fatty acids C16, C18, C16:0, and C18:3 (Table 1.). All lipid classes were
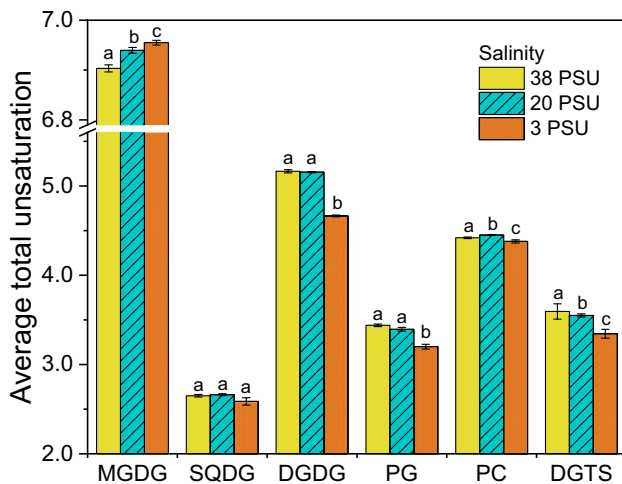

Fig. 6 Average unsaturation of polar lipid classes (sum of double bonds of the two fatty acids) of *D. tertiolecta* at 38, 20, and 3 PSU. Values represent the means ± SD (*n* = 3). Groups with different letters are statistically significantly different from each other (*p* < 0.05)

dominated by PUFA, with the exception of SQDG, in which similar proportions of SFA and PUFA were present. Hyposaline conditions (changes between 38 and 3 PSU) resulted in a decrease in the share of PUFA and a corresponding increase in the share of SFA for DGDG and DGTS (*p* < 0.05). Replacement of MUFA by SFA at 3 PSU was observed for PG (*p* < 0.05). Higher proportions of MUFA were observed in lipid classes SQDG and PC at 3 PSU. Significant changes (*p* < 0.05) in the proportion of MUFA were observed in the lipid classes PC and DGTS at all salinities studied.

The total unsaturation (sum of the double bonds of the two fatty acids) of the polar lipid classes is shown in Fig. 6. With the exception of SQDG, a statistically significant (*p* < 0.05) change in total unsaturation was observed for all other lipid classes at 3 PSU. Total unsaturation of MGDG was highest at salinity of 3 PSU, while it was lowest for lipid classes DGDG, PG, PC and DGTS. Significant (*p* < 0.05) changes in unsaturation were observed for lipid classes MGDG, PC and DGTS for salinity decrease from 38 to 20 PSU.

A change in fatty acid chain lengths with decreasing salinity was observed, i.e. replacement of C16 by C18 and vice versa (Table 1). In general, all lipids showed a similar distribution of C16 and C18, except PC, where C18 fatty acids dominated. A decrease to salinity of 3 PSU resulted in an elongation of fatty acid chains (C16 to C18) in DGDG and a shortening in DGTS (C18 to C16) (*p* < 0.05), whereas no significant changes were observed in the lipid classes MGDG, SQDG, PG and PC.

Table 1 Fatty acid profiles of glycolipids (MGDG, SQDG and DGDG), phospholipids (PG and PC) and betaine lipid (DGTS) of *D. tertiolecta* at 38, 20, and 3 PSU

Salinity (PSU)	SFA	MUFA	PUFA	C16	C18	C16:0	C18:3
MGDG (monogalactosyldiacylglycerols)							
38			100 ± 0.00^a	49.89 ± 0.09^a	50.12 ± 0.09^a		47.75 ± 0.11^a
20			100 ± 0.00^a	50.00 ± 0.00^a	50.00 ± 0.00^a		48.49 ± 0.15^b
3			100 ± 0.00^a	50.00 ± 0.00^a	50.00 ± 0.00^a		48.87 ± 0.12^c
SQDG (sulfoquinovosyldiacylglycerols)							
38	54.00 ± 0.19^a	2.75 ± 0.08^a	43.25 ± 0.27^a	54.00 ± 0.19^a	46.00 ± 0.19^a	54.00 ± 0.19^a	43.25 ± 0.27^a
20	53.84 ± 0.14^a	2.68 ± 0.15^a	43.48 ± 0.22^a	53.84 ± 0.14^a	46.16 ± 0.14^a	53.84 ± 0.14^a	43.48 ± 0.22^a
3	54.75 ± 0.67^a	3.21 ± 0.12^b	42.04 ± 0.70^b	54.75 ± 0.67^a	45.26 ± 0.67^a	54.74 ± 0.67^a	42.04 ± 0.70^b
DGDG (digalactosyldiacylglycerols)							
38	10.53 ± 0.19^a	2.98 ± 0.05^a	86.49 ± 0.24^a	39.66 ± 0.72^a	60.34 ± 0.72^a	10.53 ± 0.19^a	53.14 ± 0.59^a
20	10.79 ± 0.10^a	2.82 ± 0.19^a	86.39 ± 0.19^a	39.57 ± 0.52^a	60.43 ± 0.52^a	10.79 ± 0.10^a	53.38 ± 0.58^a
3	18.15 ± 0.11^b	2.85 ± 0.12^a	79.00 ± 0.16^b	35.57 ± 0.53^b	64.43 ± 0.53^b	18.15 ± 0.11^b	54.96 ± 0.65^b
PG (phosphatidylglycerols)							
38	18.82 ± 0.68^a	32.56 ± 0.61^a	48.60 ± 0.10^a	50.00 ± 0.00^a	50.00 ± 0.00^a	18.82 ± 0.68^a	42.18 ± 0.05^a
20	18.74 ± 0.59^a	33.11 ± 0.49^a	48.16 ± 0.11^a	50.00 ± 0.00^a	50.00 ± 0.00^a	18.74 ± 0.59^b	40.30 ± 0.42^b
3	22.95 ± 0.78^b	28.76 ± 0.94^b	48.30 ± 0.46^a	50.00 ± 0.00^a	50.00 ± 0.00^a	22.95 ± 0.78^c	34.64 ± 0.64^c
PC (phosphatidylcholines)							
38	15.75 ± 0.22^a	7.31 ± 0.08^a	76.93 ± 0.18^a	15.75 ± 0.22^a	84.25 ± 0.22^a	15.75 ± 0.22^a	59.80 ± 0.06^a
20	16.04 ± 0.05^a	6.43 ± 0.05^b	77.53 ± 0.10^b	16.04 ± 0.05^a	83.96 ± 0.05^a	16.04 ± 0.05^a	61.04 ± 0.17^b
3	15.71 ± 0.22^a	8.74 ± 0.10^c	75.54 ± 0.32^c	15.71 ± 0.22^a	84.29 ± 0.22^a	15.71 ± 0.22^a	59.10 ± 0.35^c
DGTS (diacylglyceryl-N,N,N-trimethylhomoserines)							
38	37.58 ± 1.58^a	2.43 ± 0.19^a	60.00 ± 1.40^a	38.36 ± 1.60^a	61.64 ± 1.60^a	36.52 ± 1.61^a	42.86 ± 1.48^a
20	39.77 ± 0.52^a	1.65 ± 0.31^b	58.58 ± 0.25^a	41.45 ± 0.71^a	58.55 ± 0.71^a	38.57 ± 0.34^a	40.54 ± 1.08^a
3	42.90 ± 0.99^b	1.81 ± 0.25^c	55.29 ± 0.77^b	44.79 ± 0.79^b	55.21 ± 0.79^b	41.26 ± 0.81^b	38.55 ± 0.48^b

All values are expressed as % of total fatty acids. Data are expressed as means $\pm$ SD ($n = 3$). Values in the same column for a given lipid class with different letter are statistically significantly different from each other ($p < 0.05$)

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *C16* fatty acid with 16 carbon atoms, *C18* fatty acid with 18 carbon atoms, *C16:0* palmitic acid, *C18:3* α-linolenic acid

Omega-3 fatty acid, α-linolenic acid (18:3) type, was the most abundant PUFA in all lipid classes (Table 1). Its percentage decreased with decreasing salinity in all lipid classes, except in MGDG and DGDG, where it increased ($p < 0.05$). Significant changes ($p < 0.05$) were observed in the lipid classes MGDG, PG and PC for all salinities studied. Another abundant fatty acid was palmitic acid (16:0). Its share significantly ($p < 0.05$) increased under hyposaline conditions for DGDG, PG and DGTS lipid classes (Table 1) and significantly changed ($p < 0.05$) for PG at all salinities studied.

Discussion

Cell growth in response to salinity decrease

Dunaliella tertiolecta growth and reproduction are little affected when salinity decreases from an optimal salinity of 38 to 20 PSU, suggesting a very good physiological acclimation to such a significant salinity drop (Fig. 1, Table S1). The prolonged lag period (five days) of *D. tertiolecta* growth at 3 PSU compared to 38 and 20 PSU (two days), together with substantial retardation of growth and reproduction, suggest that the cells are under severe stress (Fig. 1, Table S1). Jahnke and White (2003) demonstrated that while different *Dunaliella* species can grow in a wide range of salinities (2.9 – 175 PSU) only *D. tertiolecta* can grow in salinity lower than 3 PSU, as we also observed here. A decrease in *D. tertiolecta* growth under hyposalinity was previously observed (Jahnke and White 2003; Rizwan et al. 2017). In addition, hyposalinity was found to lead to an increase in *D. tertiolecta* cell size, which had reduced F_v/F_m levels and a smaller ascorbate pool along with an increased pool of α-tocopherol and total glutathione (Jahnke and White 2003). It was also found that the rate of nutrient uptake by *D. tertiolecta* depends on the NaCl concentration (Hellebust 1985). All of these findings may explain here observed decrease in

cell density after two days of growth at 3 PSU. Although, cell density decreases within two days, *D. tertiolecta* was able to adapt to hyposalinity stress, but with a longer lag phase and lower cell density after 16 days of growth compared to growth at 38 PSU. As previously noted, cell growth cannot resume after hyposalinity shock until the osmotic equilibrium of the cell is restored (Borowitzka 2018b).

Lipid accumulation in response to salinity decrease

As observed earlier, the influence of various stressful growth conditions (eg warming, nutrient depletion) leads to lipid accumulation in microalgae (Novak et al. 2019; Rizwan et al. 2020; Xiang et al. 2021). Growth of *D. tertiolecta* under hyposalinity also leads to accumulation of total cellular lipids (Fig. 2a, Table S2). This suggests that its complex intracellular biochemical processes are more focused on lipid synthesis under hyposaline conditions than under optimal salinity. Recently, reactive oxygen species formed in response to biotic or abiotic stressors were found to play a role in redirecting the cellular metabolism of *Chlorella protothecoides* (renamed *Auxenochlorella protothecoides*) to the accumulation of lipids instead of growth and storage of carbohydrates (Wang et al. 2016). The higher content of total cellular (pg cell^{-1}) than total polar (pg cell^{-1}) lipids (Fig. 2a, Table S2) is the result of the accumulation of non-polar triacylglycerols. Non-polar lipids in microalgae cells are energy-rich storage substances and are often synthesized under unfavorable environmental conditions, especially in response to nutrient deficiencies (Fields et al. 2014; Novak et al. 2019). The accumulation of non-polar lipids in hyposalinity could be a sign of cell enlargement and slowing of cell division in a hypoosmotic conditions, as has been proposed for *Dunaliella salina* (Yao et al. 2016).

Polar lipid classes remodeling in response to salinity decrease

Polar lipids play irreplaceable roles in microalgae cells. They are the major molecules that form cell and inner membranes and play an important role in cell signaling and trafficking (Harwood and Guschina 2009). Without the ability to remodel polar lipids under stressful conditions, microalgae cells cannot adapt/acclimatize and survive.

An increase in the cellular content of glycolipids (Fig. 2a, Table S2) and an increase in the relative abundance (% of total polar lipids) of especially nonionic MGDG and DGDG (Fig. 2b, Table S3) in parallel with a decrease in salinity, indicate thylakoid membranes remodeling. The observed increased ratio of non-bilayer to bilayer forming lipid, MGDG/DGDG, with decreasing salinity is likely related to the role of MGDG in photosynthesis. It is hypothesized that MGDG controls light harvesting of the light harvesting

complex II by modulating the hydrostatic lateral membrane pressure sensed by the LHCII-bound peripheral pigments (Tietz et al. 2020). These simultaneously suggest changes in photosynthesis, as photosynthetic reactions depend mainly on the glycolipid-supported architecture of the photosynthetic machinery (Garab 2014). An increase in the relative abundance of bilayer-destabilizing glycolipids MGDG may adversely affect membrane stability and structural organization that consequently affect fluidity and permeability of the thylakoids (Pal et al. 2013). A significant increase in MGDG detected at 20 and 3 PSU (Fig. 2b, Table S3), suggests that MGDG remodeling is one of the first signals that cells are in an unfavorable hyposaline conditions. Acclimation of microalgae to stress may involve synthesis of new lipid species (Lu et al. 2012b), no fatty acid remodeling (Kim et al. 2013, 2015), as well as the absence of specific lipid species, as observed here for MGDG 18:3/18:3 at 20 and 3 PSU. Our data suggest that DGDG and especially MGDG are important in acclimation to salinity decrease.

The higher diversity of phospholipids (four PG, five PC and one PI) we identified compared to Kim et al. (2013) (two PG and one PI) may indicate that the two *D. tertiolecta* species are isolated from different regions.

The anionic lipids SQDG and PG may substitute each other to ensure a constant proportion of anionic lipids in thylakoids (Yu and Benning 2003). The significant decrease (60%) in the proportion of PG at 3 PSU in comparison to 38 PSU and no statistically significant changes in the proportion of SQDG in total polar lipids observed here (Fig. 2b, Table S3) indicate seriously compromised thylakoid membrane composition and probably function at 3 PSU. Liu et al. (2012) found significant inhibition of PSII efficiency in *D. salina* grown under hyposaline conditions. Phospholipid PG plays an essential role in photosynthetic activity (the maintenance and stability of the photosystem II) and cell growth (Lu et al. 2012a). Noh et al. (2020) considered that PG is involved in the cyanobacteria protection from high salinity, as a higher amount of PG was found in marine than in freshwater *Synechocystis* strains. The decrease in the proportion of PG in total lipids under hyposalinity (Fig. 2a, Table S1) indicate that growth at 20 and 3 PSU compromises PG synthesis. A reduced level of PG suggests impaired photosynthetic activity as reflected by the retardation of cell growth (Fig. 1) and growth rate at 20 and 3 PSU.

Our data suggest that extreme hyposalinity (3 PSU) causes replacement of PC (decrease in relative proportion) by DGTS (increase in relative proportion) (Fig. 2b, Table S3). Because both are zwitterionic lipids that have similar chemical and physical properties (Sato and Furuya 1985; Sato 1992), the assumption is reasonable. Betaine lipid DGTS (Eichenberger 1993) and PC (Moore 1982) are primarily localized in non-plastidial membranes. We may hypothesize that this replacement plays a role in altering

plasma membrane permeability, as suggested for salt tolerance in plants (Mansour 2012). Betaine lipid DGTS is not present in all algal species (Sato 1992). In some algae, such as *Chlamydomonas reinhardtii*, PC is completely replaced by the betaine lipid DGTS. In others, such as *C. minutissima*, high amounts of DGTS and PC were detected, while in *Chlorella vulgaris* DGTS could not be detected (Giroud et al. 1988; Sato 1992; Haigh et al. 1996). Different eukaryotic phytoplankton species respond differently to salt stress in terms of DGTS content. Unlike in this study, where we observed a slight increase in the relative proportion of DGTS content with a decrease in salinity, the haptophyte *Isochrysis galbana* preserves the relative proportion of DGTS over a wide range of salinity (Cañavate et al. 2020). The fact that DGTS has the greatest diversity of molecular species indicates the importance of this lipid class to *D. tertiolecta* (Fig. 5, Table S4). The localization of DGTS within the cell has not yet been fully elucidated. Experiments with the green alga *C. reinhardtii* (Janero and Barrnett 1982) have shown that ~ 40% of cellular DGTS is localized in the thylakoid membrane. In contrast, Sheffer et al. (1986) suggested that DGTS is not present in significant amounts in the thylakoid membrane but is a major component of the plasma-membrane (23.5%) of the green alga *D. salina*. A similar result was reported for *D. salina* grown at different salinities, where DGTS accounted for 19.4, 16.0 and 26.8% of the total lipids in the plasma membrane at salinities ~ 50, 100, and 200 PSU, respectively (Peeler et al. 1989). The high molecular diversity of DGTS that we identified may indicate its localization in membranes of different cell organelles. However, the proposed replacements of PC by DGTS suggest a major localization in the plasma membrane.

An increase in phospholipid PI with decreasing salinity can be explained by its role in controlling membrane trafficking and lipid signaling (Peeler et al. 1989; De Craene et al. 2017). We assume that in a hyposaline environment, synthesis of PI is stimulated to allow *D. tertiolecta* to acclimate to salinity decrease and that one molecular species (PI 18:1/16:0) appears to be important for this alga. The same PI species was detected in *D. tertiolecta* grown under different stress conditions (Kim et al. 2013, 2015).

Fatty acid remodeling of polar lipid species in response to salinity decrease

The fatty acid composition of the polar lipids of *D. tertiolecta* is consistent with the reports of Evans and Kates (1984). The redistribution of fatty acyl moieties within a single lipid class is reflected in the changes in the degree of unsaturation as salinity decreases from 38 to 3 PSU, and for MGDG, PC and DGTS these changes are already present at 20 PSU (Fig. 6). The observed high MGDG unsaturation appears to be essential for proper functioning of the

thylakoid membranes in *D. tertiolecta*, and this becomes even more important with decreasing salinity. The most abundant molecular species MGDG 18:3/16:4 increases with decreasing salinity at the expense of MGDG 18:2/16:3 (Fig. 3a, Table S4). We can conclude that MGDG desaturase acts on MGDG 18:2/16:3 in response to the downward shift in salinity. Unlike in the case of hyposalinity, no MGDG remodeling was detected in *D. tertiolecta* growing under low-nitrate conditions (Kim et al. 2013).

A decrease in salinity leads to a statistically significant remodeling of SQDG fatty acids (Fig. 3b), whereas the overall unsaturation (Fig. 6) and the fatty acid chain lengths (Table 1) do not change. One function of the glycolipid SQDG is to stabilize Photosystem II protein complexes in the chloroplast membranes of the green algae *Chlamydomonas* (Minoda et al. 2003). Noh et al. (2020) reported that the marine *Synechocystis* strain contains relatively longer fatty acyl chains in SQDG than the freshwater *Synechocystis* strain. Growth of *D. tertiolecta* under high light and nitrogen starvation did not cause significant changes in SQDG (Kim et al. 2013), nor under nitrate-replete and nitrate-depleted conditions (Kim et al. 2015).Thus, we assume that the stable fatty acid composition (total unsaturation and chain length) of SQDG is important for the acclimation and survival of *D. tertiolecta*.

The response of *D. tertiolecta* to a decrease in salinity also includes a remodeling of DGDG fatty acids (Fig. 3c, Table S4) that includes decreased unsaturation (Fig. 6), an increasing proportion of SFA with a decreasing PUFA proportion, and an increase in fatty acid chain length from C16 to C18 (Table 1). An extensive search of the published literature did not reveal an explanation for the decrease in DGDG unsaturation in response to a decrease in salinity.

The major PG species of *D. tertiolecta* is PG 16:1/18:3 (Fig. 4a, Table S4). Salinity decrease caused a decrease in the unsaturation of PG fatty acids (Fig. 6), the replacement of PG 16:1/18:3 and PG 16:0/18:3 by PG 16:0/18:2 (Fig. 4a, Table S4), the replacement of MUFA by SFA, whereas the PUFA content did not change (Table 1). This restructuring did not change the ratio of C16 and C18 fatty acids. Furthermore, PG remodeling was not detected in nitrate-deficiency condition (Kim et al. 2013, 2015). The reduction in the unsaturation of PG and DGDG fatty acids observed here in response to a decrease in salinity suggests a lower nutritional value of *D. tertiolecta* for higher trophic levels.

Unlike other detected polar lipids, PC is not characterized by the dominance of a single molecular species. The response of PC to a decrease in salinity, including fatty acid remodeling (Fig. 4b, Table S4), decrease in unsaturation (Fig. 6), and increased proportion of MUFA on expense of PUFA (Table 1) are small, but statistically significant. The relative invariability of PC total unsaturation and fatty acid chain lengths was found over a wide range of salinity in

two estuaries studied and was explained by the maintaining the estuarine plankton cell integrity and preserving the physicochemical properties of the membranes through low variability in PC unsaturation and fatty acid chain length (Vrana Špoljarić et al. 2021, 2020). We assume that the same conclusion applies to the growth of *D. tertiolecta* at different salinities.

Acclimations to lower salinity include complex remodeling of DGTS (Fig. 6, Table 1), including reduced unsaturation and fatty acids shortening. These indicate decreased membrane fluidity involving DGTS. Similarly, intense DGTS remodeling occurred during growth of *D. tertiolecta* in nitrate-deficient conditions (Kim et al. 2015).

The observed decrease in PUFA (α-linolenic acid) in PG, DGTS, SQDG, and PC with decreasing salinity, suggests that the decrease in salinity reduces/inactives membrane-bound enzymes that synthesize PUFA via the aerobic or anaerobic pathway that are involved in α-linolenic acid biosynthesis (Certik and Shimizu 1999). In microorganisms, most of the PUFA is stored in the form of triacylglycerol. The fatty acyl chains are incorporated into the phospholipid PA before they are available for the biosynthesis of diacylglycerol and triacylglycerol (Frentzen 1993). The diacylglycerols are further used for the synthesis of glycolipids (Kalisch et al. 2016). We can conclude that acclimation to salinity decrease in *D. tertiolecta* diverts synthesis of α-linolenic acid to incorporation into the glycolipids DGDG and MGDG to maintain membrane integrity at the expense of decreasing α-linolenic acid in other lipid classes. However, salinity decrease in *D. tertiolecta* has a negative effect on total α-linolenic acid in the polar lipids. Since α-linolenic acid is the most abundant omega-3 fatty acid, if not the only PUFA, in the polar cell lipids, the reduction of this fatty acid will have negative impact on the higher trophic levels as *D. tertiolecta* will be less nutritious food for them.

One of the possible strategies of microalgae to cope with stress is to alter the fatty acid chain length (Goss and Wihelm 2009; Kim et al. 2013). It seems that this acclimation mechanism does not play a major role in the *D. tertiolecta* hypoosmotic acclimation, as we only observed an increase in C18 fatty acids in DGDG and their decrease in DGTS.

In addition, lipid remodeling could also affect cell surface properties and thus functional cell behavior at interfaces (Novosel et al. 2022b). The cells of *D. tertiolecta* change their mechanical properties from stiffer and hydrophobic in the exponential phase to less stiff and hydrophilic in the stationary phase of growth (Pillet et al. 2019). Also, the stiffness of *D. tertiolecta* cells decreases and hydrophilicity increases as the growth temperature increases (Novosel et al. 2021, 2022a). The observed mechanical and chemical changes in *D. tertiolecta* cells may indicate molecular modification of the cell envelope as well as changes in the lipid composition of cell membranes. Thus, the interplay between lipid composition and cell mechanics would allow us to better understand the acclimation/adaptation mechanism of microalgae cells to hyposaline conditions.

Conclusion

Dunaliella tertiolecta acclimates to growth under hypoosmotic conditions (3 PSU) with a significant retardation of growth and reproduction. Acclimation to salinity of 3 PSU involves statistically significant cellular lipid accumulation, remodeling of polar lipid classes and fatty acid composition. The major lipid responses, seen even at 20 PSU, include increase in MGDG and decrease in PG in the total polar lipids. These suggest that these two polar lipids play the most important role in *D. tertiolecta* acclimation to hyposaline environment. So, we hypothesize that this microalga is genetically adapted through membrane lipid composition to ensure survival under large salinity fluctuations. The observed decrease in unsaturated fatty acids in most polar lipids of *D. tertiolecta* with decreasing salinity and the replacement of PC by DGTS likely plays a role in reducing plasma membrane fluidity and altering permeability to prevent unfavorable inflow and outflow of water and ions into and out of cells. The high molecular diversity of DGTS, the relatively uniform representation of PC species, PI 18:1/16:0 and the dominance of one species among MGDG, SQDG, DGDG, PG, and DGTS characterize the polar lipid composition in a broad salinity range between 38 and 3 PSU. The polar lipid fraction of *D. tertiolecta* is characterized exclusively by C16 and C18 fatty acids. The change in the fatty acid chain length is not a mechanism of *D. tertiolecta* to cope with the decrease in salinity. Hyposalinity also causes replacement of the omega-3 (linolenic) fatty acid by saturated palmitic acid in PG and DGTS and causes the absence of MGDG 18:3/18:3 lipid species.

Studies on lipid remodeling in microalgae cells in response to hypoosmotic stress are rarely performed. Therefore, the results of our study are important as they provide a new aspect to the fundamental understanding of how *D. tertiolecta* cells achieve equilibrium in hyposaline conditions with respect to optimal salinity. As the global climate change and extreme precipitation becomes more frequent, it is important to understand the mechanisms that act in the acclimation and adaptation of microalgae to a sudden drop in salinity. Further lipidomics research should be extended to other microalgae species. From a biotechnological point of view, the result of increased content of non-polar lipids in *D. tertiolecta* cells under hyposalinity suggests that *D. tertiolecta* may be a good candidate for biodiesel production. Therefore, lipidomic studies should be performed to investigate the remodeling of non-polar lipids of *D. tertiolecta* under hyposaline conditions.

Data availability All datasets generated during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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