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Combined effects of temperature and salinity on fatty acid content and lipid damage in Antarctic phytoplankton

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ABSTRACT

We investigated the effects of ocean warming and glacial melting on phytoplankton assemblage composition and physiology in coastal Antarctica by exposing assemblages to a 4 °C increase in seawater temperature (T) and a 4 psu decrease in salinity (S) with respect to ambient values in a 6-day microcosm experiment. Seawater samples from Potter Cove in King George Island (Antarctica) were placed in outdoor microcosms and exposed to four treatments: ambient S-ambient T (S0T0, control), low S-ambient T (S-T0), ambient S-high T (S0T+), and low S-high T (S-T+). The relative abundance of unsaturated fatty acids (UFAs) 20:5ω3, 18:4ω3 and 16:1ω7 in relation to saturated FAs (14:0 and 16:0) significantly increased in all treatments at 24 h, compared to the control. At the same time, we detected a significant increase in the production of Thiobarbituric Acid Reactive Substances (TBARS), used as a proxy for lipid damage, in the S-T0 and the S0T+ treatments. In contrast, in S-T+, concentrations of TBARS remained significantly lower than in the control throughout the experiment. Although phytoplankton species composition did not change during the experiment, an increase in the relative abundance of diatoms (> 20 µm) was found in all treatments compared to the control at 24 h, with no further changes during the rest of the experiment. Furthermore, the relative abundance of small diatoms (10–20 µm) increased only in S0T+, and small prasinophytes decreased at S-T+ at the end of the incubation period.

Our results show a stable unsaturated to saturated FA ratio under the synergistic effects of high temperature and lower salinity, which may help protect phytoplankton cells from lipid damage. When phytoplankton assemblages were exposed to high temperature or low salinity, separately, the proportion of unsaturated FAs increased after 48 h. This increase in FAs resulted in greater lipid damage, which could be potentially avoided, as shown by previous studies, by antioxidant responses or changes in osmoregulatory proteins and FA synthesis by the activation or inactivation of desaturase enzymes. Variations in FA content due to changing environmental conditions can alter the quality of phytoplankton as a food source with potentially critical implications for the marine food web.

1. Introduction

The marine waters around the Western Antarctic Peninsula (WAP) have experienced the fastest rate of warming as well as the greatest sea ice loss on the planet (Vaughan et al., 2003). In view of the expected minimum global temperature rise of 1–4 °C over the next century (Turner et al., 2016), future trends in coastal Antarctic waters may include further widespread warming, sea ice retreat and runoff from glacial melting. Changes in seasonal sea ice dynamics and increased water column stratification from freshwater inputs will largely affect mixing regimes, nutrient supply, and light availability for Antarctic phytoplankton (Massom and Stammerjohn, 2010). Moreover, increasing winter convective storms (or mesocyclonic activity) in the atmosphere can result in intensified advection of moist warm air and

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more rain, which can lead to further melting of the ice cap (Falk and Sala, 2015) and increased inputs of glacial freshwater into coastal marine waters.

Meltwater input has already been shown to have an effect on Antarctic phytoplankton physiology and biomass. Hernando et al. (2015) showed that a few phytoplankton species from Potter Cove (King George Island or 25 de Mayo Island, Antarctica) exposed to hypotonic stress were able to grow at their natural rates while others showed decreased photosynthetic efficiency and inhibition of cell division. Although the composition of phytoplankton assemblage remained unchanged, low salinities affected the evenness of the phytoplankton assemblage and changed the relative contribution of diatoms (Hernando et al., 2015). Changes in composition and size of primary producers, especially diatoms, can ultimately affect higher trophic levels and thus the overall functioning of marine ecosystems. It is therefore essential to understand how the combined effects of warming and freshening of surface waters is affecting plankton composition and the size-structure of phytoplankton assemblages.

The energy flow in marine ecosystems can be quantified by the presence of trophic markers, such as fatty acids (FAs), lipid building blocks that are transferred from primary producers to consumers in the food web. In particular, FAs are important for the flow of energy between primary producers and primary consumers since polyunsaturated FAs (PUFAs, with two or more double bonds in the carbon chain) are almost exclusively synthesized by autotrophic organisms. However, a few PUFAs can be produced by animals also through elongation and desaturation of short-chain FAs (De Troch et al., 2012). PUFAs serve as precursors for important animal hormones and are also essential in animal's diets (Danielsdottir et al., 2007). In aquaculture studies, PUFAs were essential for maintaining high growth and reproduction rates and for the survival of marine organisms (Müller-Navarra et al., 2000). Moreover, FAs produced by microalgae are critical for the proper functioning of their own membranes, and provide the matrix for a wide variety of metabolites. As such, they play a crucial role in algal responses to environmental variability (Piepho et al., 2012). Under a sudden change in environmental conditions, de novo synthesis of unsaturated FAs cannot occur rapidly, but the desaturation of FAs may be adjusted by transferring specific acyl groups to other polar lipids and allowing rapid adaptive membrane reorganization (Makewicz et al., 1997; Khozin-Goldberg and Cohen, 2006). The properties of membranes are intimately related to the fluidity of the constituent lipids. In particular, salinity changes can induce elongation and desaturation of FAs chains to allow osmoregulation in microalgae (Azachi et al., 2002). Temperature stress can also induce changes in FAs in cell membranes. The most common regulatory strategy is a change in the lipid composition by increasing or decreasing the degree of FA unsaturation (homeoviscous adaptation, Hazel, 1995). Therefore, microalgae can survive in diverse and extreme conditions because of their ability to modify the type and quantity of their cellular lipids (Sato et al., 2000).

Oxidative stress by free oxygen radicals (ROS) have been linked to changes in FA content and composition. The capacity of algae for environmental adaptation can also be determined by their responses to oxidative stress. ROS are a variety of molecules (chemical species with one unpaired electron) physiologically generated from the metabolism of molecular oxygen (González et al., 2015). Oxidative stress has been linked to a number of toxic cellular processes, including damage to proteins (Prasad, 1995), membrane lipid peroxidation, enzyme inactivation and DNA breakage (Halliwell and Gutteridge, 2007). A cell experiences oxidative damage when the production rate of ROS is higher than the rate of antioxidant activity that protects it (Halliwell, 2006).

The lipid peroxides produced by ROS can damage FAs, resulting in the formation of Thiobarbituric Acid Reactive Substances (TBARS), including malondialdehyde (MDA) (González et al., 2015). An increase in TBARS is therefore expected as ROS production increases. A decrease in PUFA content has been observed to coincide with increased levels of MDA under osmotic stress (Singh et al., 2002). These responses, which are temporarily associated with an increase in electrolyte leakage, suggest that osmotic stress induces cellular membrane damage via lipid peroxidation (Aziz and Larher, 1998). Cellular membranes, made up of large amounts of PUFAs, are highly susceptible to attack by ROS and consequently experience changes in membrane fluidity, permeability, and cellular metabolic functions (Bandyopadhyay et al., 1999; Schuhmann et al., 2011).

The objective of this study was to determine the effects of ocean warming and glacial melting on the composition of phytoplankton assemblages and phytoplankton physiology in coastal Antarctica. We exposed coastal seawater samples to a 4 °C increase in temperature (T) and a 4 psu decrease in salinity (S) with respect to ambient values in a 6-day microcosm experiment. We evaluated both the combined and separate effect of T and S on phytoplankton responses. Our ultimate goal was to test the hypothesis that an increase in seawater T and a decrease in S result in the production of ROS in phytoplankton assemblage, which in turn affect the phytoplankton FA composition.

2. Materials and methods

2.1. Sampling and experimental design

Microcosm experiments were conducted at the Carlini Station located on the shores of Potter Cove in King George Island, South Shetland Archipelago, Antarctica (62°14′S, 58°38′ W) between January 16th and 22th of 2014. The experimental set-up consisted of twelve 100-L plastic tanks (or microcosms) that were pre-cleaned with ~10% HCl and thoroughly rinsed with distilled water. Seawater was collected just outside the entrance to Potter Cove (Fig. 1) from 5 m depth with Niskin bottles, and was pre-filtered through a 300 μm Nitex screen to exclude mesozooplankton. The mesh pore size was selected according to a preliminary analysis of the composition of the zooplankton assemblage and to avoid removing long chains of diatoms. All treatments and controls were incubated for 6 days exposed to natural sunlight and without the addition of nutrients.

The experimental design consisted of four combinations of salinity and temperature (treatments), with three replicates per treatment: (1) ambient salinity (34 psu) and ambient temperature (1–2 °C) (S0T0), (2) low salinity (30 psu) and ambient temperature (S-T0), (3) ambient salinity and high temperature (5–6 °C) (S0T+), and (4) low salinity and high temperature (S-T+). For the low salinity treatments, the target salinity of 30 psu was obtained by adding 10 L of distilled water to the S- microcosms. Similarly, 10 L of seawater (filtered through a 0.7 μm

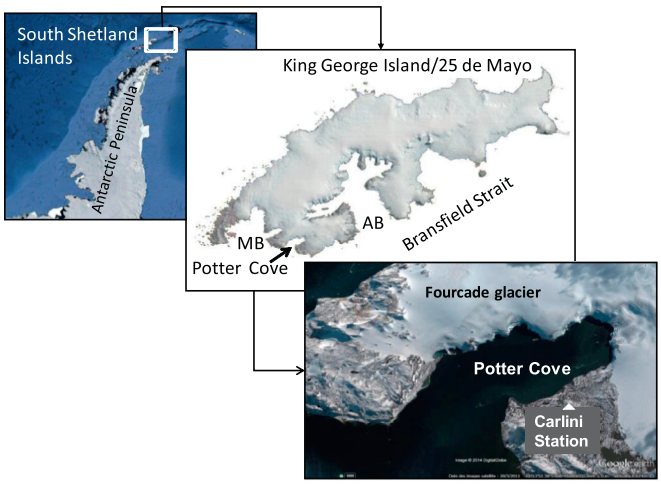

Fig. 1. Location of Potter Cove (62°14′S, 58°38′W) in King George (or 25 de Mayo) Island on the South Shetland Archipelago of the Antarctic Peninsula. AB: Admiralty Bay; MB: Maxwell Bay.

glass-fiber filter) were added to each replicate of the S0 treatments to maintain a constant water volume in all microscosms (and thus cell concentration per unit of volume). The microcosms for the T0 treatments were placed within a water bath connected to a stainless-steel sea-water pump (Lowara, Italy) that continuously circulated coastal surface seawater. For the T+ treatments, an automatic heating system (Recal Industry, Argentina) maintained a constant temperature of +4 °C with respect to ambient values for the duration of the experiment. Salinity and temperature were monitored in all microcosms every 12 h using a Horiba U-10 conductimeter. The heating system software allowed for temperature adjustments to be made within a period of not > 5 min. It was not necessary to adjust the salinity in any of the microcosms as it remained constant within their respective values throughout the experimental period. Seawater was collected from each microcosm on days: 0, 1, 2, 4 and 6 at 9 a.m. local time for the measurements of particulate carbon content, phytoplankton identification, TBARS and FA concentrations.

2.2. Particulate carbon analysis

Samples (~500 mL) from each microcosm were filtered through precombusted 0.7 μm glass-fiber filters. Filters were then dried at 60 °C for 48 h and kept in a desiccator until further analysis. Total particulate C content on the filters was analysed at the University of California Davis Stable Isotope Facility, using a PDZ Europa ANCA-GSL elemental analyzer.

2.3. Phytoplankton identification and enumeration

Samples (250 mL) from each microcosm were fixed with acidic lugol solution in plastic amber bottles and kept in cold and dark conditions until microscopic analyses. Cells were enumerated using a phase contrast Leica DMIL LED inverted microscope according to the procedures described by Utermöhl (1958). Aliquots of 25 or 50 mL (depending on cell density) were left to settle for 24 h in a sedimentation chamber. At least 100 cells of the dominant taxa were counted in one or more strips of the sedimentation chamber or in random fields at a magnification of 250× or 400×, depending on cell concentration and size. The entire chamber bottom was also scanned at 100× to quantify large and rare species.

2.4. Fatty acid analysis

Samples (~500 mL) from each microcosm were filtered through 0.7 μm glass-fiber filters and filters were then stored for less than two months at −80 °C before analyses. Hydrolysis of total lipid extracts and methylation to FAs methyl esters (FAME) in the particulate material collected on the filters were achieved by a modified one-step derivatisation method after Abdulkadir and Tsuchiya (2008) as in De Troch et al. (2012). This technique was modified by replacing the borontrifluoride-methanol reagent with a 2.5% H_2SO_4-methanol solution since BF_3-methanol can cause artefacts or loss of PUFAs (Eder, 1995). FAME were analysed using a gas chromatograph (HP 6890 N) coupled to a mass spectrometer (HP 5973). Samples were run at 250 °C using a HP88 column (Agilent J&W; Agilent Co., USA). The oven temperature was programmed at 50 °C for 2 min, followed by a ramp of 25 °C min^{-1} to 175 °C and then a final ramp of 2 °C min^{-1} to 230 °C with a 4 min hold. FAME were identified by comparison with retention times and mass spectra of authentic standards and mass spectral libraries (WILEY, NITS05), and analysed with the software MSD ChemStation (Agilent Technologies). Quantification of individual FAMEs was accomplished by the use of external standards (Supelco #47885, Sigma-Aldrich Inc., USA), and was obtained by linear regression of the chromatographic peak areas and known concentrations of the standards (ranging from 25 to 200 μg mL^{-1}).

Shorthand FA notations of the form A:BωX are used, where A represents the number of carbon atoms, B gives the number of double bonds and X gives the position of the double bond closest to the terminal methyl group (Guckert et al., 1985).

2.5. TBARS analysis

Samples (~500 mL) from each microcosm were filtered through 0.7 μm glass-fiber filters, which were stored for less than two months at −80 °C before analyses. Filters were submerged in 2 mL of both 120 mM potassium chloride and 50 mM potassium phosphate buffer (pH 7.0), sonicated, and then centrifuged at 600 g for 10 min. An aliquot (0.8 mL) of the supernatant was treated with 0.7 mL 30% (w/v) Trichloroacetic acid (TCA) and 50 mM potassium phosphate buffer (pH 7.0), and brought to a final volume of 2 mL before centrifugation. A volume of 0.2 mL of 3% (w/v) sodium dodecyl sulfate (SDS) and 0.05 mL of 4% (w/v) butylatedhydroxytoluene (BHT) in ethanol were added to 1 mL of the supernatant from the second centrifugation. After mixing, 2 mL of 0.1 N HCl, 0.3 mL of 10% (w/v) phosphotungstic acid and 1 mL of 0.7% (w/v) 2-thiobarbituric acid were added. The mixture was heated at 95 °C in a water bath for 45 min and 5 mL of n-butanol were added before the sample was vortexed, and centrifuged at 600 g for10 min. The fluorescence of the supernatant organic layer was measured at λ_{ex} = 515 nm and λ_{em} = 555 nm. The values were expressed as nmol TBARS (malondialdehyde equivalents) per particulate carbon units. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane (Malanga and Puntarulo, 1995).

2.6. Statistical analyses

We performed repeated analyses of variance (RMANOVA, Statistica, version 9) to determine the significance of the differences observed in TBARS concentrations, and the relative abundance of main phytoplankton groups among treatments. Normality was verified using a one-sample Kolmogorov–Smirnov test, whereas the sphericity assumption that concerns variance homogeneity was checked using the Mauchley's test. The main factors considered in the analysis were the number of days of exposure and the type of treatment. After the ANOVA analysis, a Tukey test was performed to determine differences between treatments. When the interaction was significant or the assumptions of sphericity were not met, even with log-linearized rates, a one-way ANOVA was performed to evaluate the effect of treatment at different days of exposure (Scheiner, 2001).

To test for the effect of incubation time, a multivariate analysis of the overall FA composition was conducted with a non-metric multidimensional scaling method (NMDS) based on Bray-Curtis similarity using Primer 6 software (Clarke and Gorley, 2006). A one-way analysis of similarity (ANOSIM) was performed to test for significant differences between the groups based on the experimental day. Similarly, a percentage analysis (SIMPER) was calculated to determine the main FAs contributing to the differences found.

Univariate analyses of the effect of the treatments on FAs with a contribution of > 15% on all sampling days (i.e. FAs 14:0, 16:0, 18:0, 16:1ω7, 18:4ω3 and 20:5ω3) were performed on non-transformed absolute FA concentrations (standardized by carbon content). A two-way multivariate PERMANOVA with repeated measures was performed to determine whether these FAs differed between the treatments during the incubation time (fixed factors were salinity and temperature, and the random factor was incubation time). All these analyses (NMDS, ANOSIM, SIMPER and PERMANOVA) were performed with Primer 6.1.11 software (Clarke and Gorley, 2006) with PERMANOVA add-on software (Anderson et al., 2008).

Changes in the relative amount of unsaturated FAs in relation to incubation time was further evaluated by RMANOVA analyses based on the concentration ratio of PUFAs/saturated FAs (PUFAs/SFAs). The same FAs as in the PERMANOVA were used based on the same > 15% threshold: 20:5ω3 (eicosapentaenoic acid or EPA), 18:4ω3 and 16:1ω7

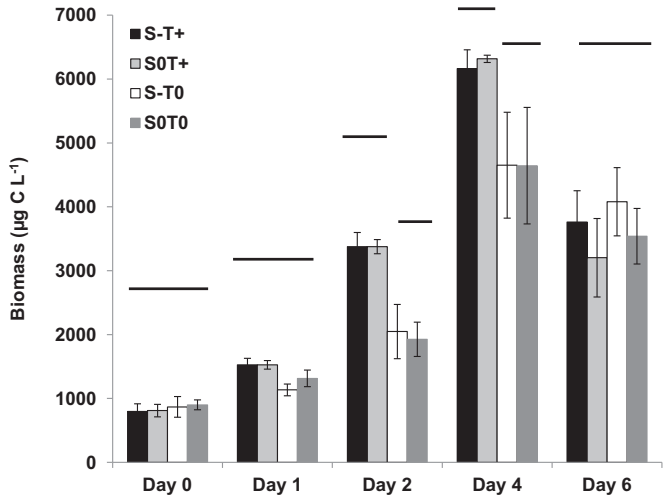

Fig. 2. Carbon biomass of the plankton community as a function of incubation time for four experimental treatments: low salinity and high temperature (S-T+); ambient salinity and high temperature (S0T+); low salinity and ambient temperature (S-T0) and ambient salinity and ambient temperature (S0T0). Bar height represent the average of 3 replicates ± S.D. Horizontal lines at the same height indicate that there are no significant differences (Tukey Test) between treatments for each day.

as unsaturated FAs and 14:0 and 16:0 for SFAs·The SFAs used for such index were 14:0 plus 16:0 considering that both of them showed the same increasing trends in their concentrations as a function of incubation time ($R = 0.96$). The other saturated FA found (18:0) decreased to 1% of the initial concentration after day 1.

3. Results

3.1. Carbon content

Total phytoplankton biomass, measured as carbon content, increased significantly with time in all treatments until day 4 ($P < 0.05$). By the end of the experiment on day 6, the carbon content decreased significantly in T+ treatments but remained without significant changes (relative to day 4) on T0 treatments ($P = 0.27$). On days 2 and 4, the carbon concentration was significantly higher in both T+ treatments relative to T0 treatments ($P < 0.01$). However, there were no significant differences between both high temperature (S-T+ and S0T+) ($P = 0.99$) or between both ambient temperature (S-T0 and S0 T0) treatments ($P = 0.12$) (Fig. 2). On day 6, there were no significant differences in carbon biomass among any of the treatments ($P = 0.29$).

3.2. Composition of phytoplankton assemblages

The initial (Day 0) phytoplankton assemblage was characterized by large numbers of diatoms dominated by *Porosira glacialis* and in less proportion by *Thalassiosira antarctica*. These two relatively large species (20–50 µm) accounted for about 20% of the total phytoplankton abundance (in cells L^{-1}) on day 0 (Table 1). Such percentage is an average of low and ambient salinity replicates considering that there were no significant differences between them, $P = 0.8$. In addition to diatoms, other phytoplankton groups (prasinophytes, cryptophytes, prymnesiophytes and other < 5 µm unidentified phytoflagellates) represented ~65% of the total phytoplankton abundance. The relative abundance of large centric diatoms (20–50 µm) showed a significant increase from day 1 until the end of the experiment ($P < 0.01$) in all treatments, in spite of their increased relative abundance in the control. Small (10–20 µm) centric diatoms increased significantly under S0T+ conditions ($P < 0.01$) from day 1 to day 6. In both T+ treatments (S-T+ and S0T+), there was a significant decrease in prasinophytes

Table 1
Average relative abundance (%) ± S.D. of the main phytoplankton groups between initial (Day 0), first (Day 1), second (Day 2) and last day (Day 6) of the experiment for all treatments: low salinity and high temperature (S-T+); ambient salinity and high temperature (S0T+); low salinity and ambient temperature (S-T0) and ambient salinity and ambient temperature (S0 T0). Bold numbers indicate statistically significant differences ($P < 0.01$) with the initial conditions in the control treatment (S0 T0).

Phytoplankton group	S-T+				S0T+				S-T0			S0T0		
	Day 0	Day 1	Day 2	Day 6	Day 0	Day 1	Day 2	Day 6	Day 0	Day 1	Day 2	Day 6	Day 0	Day 1	Day 2	Day 6
Centric Diatoms 20–50 µm	26 ± 11	**44 ± 2**	**62 ± 7**	**58 ± 2**	14 ± 5	**43 ± 8**	**57 ± 4**	**46 ± 1**	26 ± 11	**40 ± 6**	**56 ± 2**	**54 ± 1**	14 ± 5	**30 ± 6**	**40 ± 5**	**36 ± 3.7**
Centric Diatoms 10–20 µm	1 ± 0.1	4.4 ± 1	2.6 ± 1	11 ± 5	1 ± 0.1	4.7 ± 1	6.9 ± 4	**25 ± 1**	1 ± 0.1	4 ± 1	4 ± 2	11 ± 1	1 ± 0.1	2 ± 1	5 ± 0.7	**13 ± 1.8**
Prasinophytes	1 ± 0.1	3 ± 2	6.7 ± 3	**1.6 ± 1**	1 ± 0.2	5 ± 2	4.8 ± 2	**2 ± 1**	1 ± 0.1	7 ± 2	7.6 ± 0.9	14 ± 1	1 ± 0.2	7 ± 2	9 ± 2	**14 ± 2.8**
Phytoflagellates < 5 µm	62 ± 18	40 ± 1	23 ± 0.6	24 ± 6	69 ± 14	40 ± 2	25 ± 3	22 ± 1	63 ± 19	40 ± 7	35 ± 10	20 ± 3	69 ± 14	45 ± 8	35 ± 5	28 ± 7
Cryptophytes	0.8 ± 0.6	0.8 ± 0.5	0.6 ± 1	0.5 ± 1	0.3 ± 0.2	0.4 ± 1	0.5 ± 0.8	3 ± 1	0.8 ± 0.6	1 ± 1	1 ± 0.5	1.5 ± 1	0.3 ± 0.2	0.4 ± 1	0.5 ± 1	4 ± 2

Table 2
Average (± S.D.) total concentrations of fatty acids (UFAs, PUFAs and SFAs) (μg L^{-1}) at (A) low salinity (S-) and high temperature (T+) or ambient temperature (T0), and (B) ambient salinity (S0) and high temperature (T+) or ambient temperature (T0). "ND" means no data.

Type of FA	Day 0		Day 1		Day 2		Day 4		Day 6	
	T+	T0	T+	T0	T+	T0	T+	T0	T+	T0
(A) Low salinity (S-)										
14:0	5.6 ± 1.4	5.6 ± 1.4	3.1 ± 2	5.9 ± 0.7	3.1 ± 0.2	5.7 ± 1.2	4.7 ± 0.1	6.3 ± 0.2	7.4 ± 1.6	5.7 ± 1.3
15:0	0.6 ± 0.1	0.6 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
16:0	10 ± 2.5	10 ± 2.5	5.5 ± 2.2	9 ± 1.4	3.2 ± 0.2	7.8 ± 2.7	4.7 ± 0.4	8.7 ± 0.6	8.8 ± 2.9	10 ± 2.3
16:1ω9	1.7 ± 0.4	1.7 ± 0.4	1.2 ± 0.9	2.1 ± 0.1	0.6 ± 0.01	19 ± 0.6	0.3 ± 0.01	0.7 ± 0.01	0.4 ± 0.1	0.6 ± 0.2
16:1ω7	3 ± 1.6	3 ± 1.6	0.6 ± 0.5	7.1 ± 1.8	4.9 ± 0.5	12.3 ± 3.6	13 ± 0.01	23.2 ± 2.7	19 ± 0.9	24.5 ± 5
16:1ω5	0.6 ± 0.6	0.6 ± 0.6	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.01	0.4 ± 0.1	0.4 ± 0.1
16:2ω6	2 ± 1.6	2 ± 1.6	0.9 ± 1	1.1 ± 1	1.3 ± 0.2	2.3 ± 2	1.1 ± 0.01	2.5 ± 0.01	1.4 ± 1.2	3 ± 1.9
16:3ω3	3.8 ± 3.5	3.8 ± 3.5	0.7 ± 1	1.1 ± 0.9	1.8 ± 0.2	2.1 ± 1.7	0.7 ± 0.01	1.6 ± 0.1	0.5 ± 0.6	1.7 ± 0.8
18:0	3 ± 1.6	3 ± 1.6	2.2 ± 1.6	1.4 ± 0.3	0.4 ± 0.01	0.9 ± 0.9	0.5 ± 0.4	0.6 ± 0.3	0.5 ± 0.2	0.4 ± 0.1
18:1ω9	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.5	1 ± 0.3	0.3 ± 0.01	1 ± 0.6	0.7 ± 0.01	1 ± 0.7	1.2 ± 0.2	1.2 ± 0.7
18:1ω7	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.01	0.4 ± 0.3	0.2 ± 0.01	0.5 ± 0.3	0.5 ± 0.1	0.4 ± 0.1
18:2ω6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.01	0.3 ± 0.2	0.2 ± 0.2	0.5 ± 0.01	0.7 ± 0.2	0.7 ± 0.2
18:3ω3	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.1	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.1	ND	ND	0.2 ± 0.2	0.2 ± 0.2
18:4ω3	2.7 ± 1.8	2.7 ± 1.8	3.2 ± 2.9	5.5 ± 2.4	2.1 ± 2	12.8 ± 2.9	8.8 ± 0.1	15.9 ± 2.3	13 ± 2.2	17 ± 4.4
ARA	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0.1	0.2 ± 0.01	0.2 ± 0.2	0.2 ± 0.1	0.4 ± 0.1
EPA	2.7 ± 1.9	2.7 ± 1.9	3 ± 3	6.5 ± 1.6	4.8 ± 1	8.7 ± 6	5.9 ± 0.01	11.4 ± 0.4	7.6 ± 0.9	10 ± 3.8
DHA	2.7 ± 1	2.7 ± 1	0.6 ± 0.9	1.7 ± 0.6	1.1 ± 0.	2.6 ± 2	1 ± 0.01	2.2 ± 0.2	1 ± 0.01	1.9 ± 0.9
(B) Ambient salinity (S0)										
14:0	4.4 ± 1.9	4.4 ± 1.9	6.1 ± 1.8	2.7 ± 0.7	4.7 ± 0.4	4.2 ± 0.9	7 ± 0.5	5.8 ± 0.5	8.8 ± 0.4	4 ± 2.6
15:0	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
16:0	9 ± 1.2	9 ± 1.2	7.3 ± 0.1	5.9 ± 1.2	4.1 ± 0.5	6.2 ± 2.5	7 ± 0.2	7.6 ± 0.9	11 ± 0.9	8.9 ± 0.2
16:1ω9	1.4 ± 0.7	1.4 ± 0.7	2 ± 0.4	0.7 ± 0.2	11 ± 0.01	12 ± 0.3	0.4 ± 0.01	0.5 ± 0.3	0.5 ± 0.1	0.4 ± 0.3
16:1ω7	6.2 ± 3.5	6.2 ± 3.5	9.2 ± 5.4	1.1 ± 0.9	7.9 ± 1.4	7.9 ± 2.6	22.8 ± 1.6	19.3 ± 1.6	26.2 ± 1.9	23 ± 4
16:1ω5	0.3 ± 0.01	0.3 ± 0.01	0.2 ± 0.1	0.1 ± 0.01	0.3 ± 0.2	0.2 ± 0.01	0.2 ± 0.2	0.1 ± 0.1	0.4 ± 0.01	0.2 ± 0.1
16:2ω6	2 ± 1.3	2 ± 1.3	2.9 ± 1.8	0.7 ± 1	2.7 ± 0.4	2.1 ± 0.7	1.9 ± 0.01	2.3 ± 0.3	2 ± 1.7	1.5 ± 1
16:3ω3	1.4 ± 1	1.4 ± 1	3.6 ± 1.9	0.3 ± 0.4	4.2 ± 0.8	2.2 ± 0.7	1.6 ± 0.1	1.6 ± 0.01	1 ± 0.8	0.6 ± 0.3
18:0	4.9 ± 0.8	4.9 ± 0.8	1.7 ± 0.2	1.4 ± 0.4	0.4 ± 0.1	1.7 ± 1.1	0.8 ± 0.7	1.1 ± 1.1	0.6 ± 0.1	0.4 ± 0.3
18:1ω9	1 ± 0.4	1 ± 0.4	0.9 ± 0.01	1.7 ± 1	0.3 ± 0.2	0.7 ± 0.1	1.3 ± 0.1	0.5 ± 0.4	1.7 ± 0.1	0.9 ± 0.6
18:1ω7	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.1 ± 0.01	0.4 ± 0.01	0.4 ± 0.1	0.3 ± 0.01	0.1 ± 0.01	0.6 ± 0.1	0.2 ± 0.1
18:2ω6	0.6 ± 0.3	0.6 ± 0.3	0.4 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.01	0.5 ± 0.01	0.9 ± 0.5	0.5 ± 0.3
18:3ω3	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0.01	0.05 ± 0	0.1 ± 0.01	0.1 ± 0.01	ND	ND	0.2 ± 0.01	0.2 ± 0.2
18:4ω3	5.6 ± 3.7	5.6 ± 3.7	10.6 ± 7	0.8 ± 0.8	8.9 ± 1.5	8 ± 2.2	15.5 ± 1.4	12 ± 1.5	17 ± 1.3	10 ± 6.7
ARA	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0 ± 0	0.2 ± 0.01	0.1 ± 0.01	0.4 ± 0	0.2 ± 0.2	0.4 ± 0.01	0 ± 0
EPA	5.5 ± 3.7	5.5 ± 3.7	9.7 ± 6	0.8 ± 1	9 ± 1.3	5.4 ± 4	10 ± 1	9.9 ± 0.5	10.4 ± 0.8	6.8 ± 4.7
DHA	1.5 ± 1	1.5 ± 1	2.6 ± 1.7	0.1 ± 0.1	2.2 ± 0.3	2 ± 0.8	1.6 ± 0.4	1.5 ± 0.1	1.6 ± 0.3	1 ± 0.7

($P < 0.01$) on day 6 relative to day 1, however no significant changes ($P = 0.81$) were observed relative to day 0. No significant differences were found in the relative abundance of small centric diatoms or prasinophytes between days 1 and 2 in comparison with the differences observed in the control for each treatment ($P = 0.96$) (Table 1).The relative abundance of phytoflagellates (< 5 μm) was significantly lower ($P < 0.05$) at the end of the experiment in every treatment compared with the initial phytoplankton assemblage. No significant differences were found in the relative abundance of cryptophytes with time or treatment ($P = 0.78$ and $P = 0.96$ respectively).

3.3. Fatty acid profiling and lipid damage

The abundance of PUFA relative to total FAs was high in all treatments, with an average of 50–60% at the beginning of the experiment (Table 2A–B). The ANOSIM analysis showed that all FAs concentrations were significantly different (global $R = 0.7$) between the time intervals (days) and that all pairwise comparisons between days (except for day 0-day 1) yielded $R > 0.4$. The NMDS analysis showed clear differences in FA composition as a function of incubation time (Fig. 3). In particular, the initial days of incubation (day 0-day 2) differed considerably from the later phases (day 4–day 6) with $R = 0.8$–1.0. All possible combinations of incubation times (days) showed strong differences ($R > 0.8$) except for day 0–day 1, and day 1–day 2. There was on average low similarity (51.8%) in the FA profiles between the 12 microcosms on day 1, which was followed by changes in FA composition on day 2 (Table 3). On the other hand, the NMDS analysis showed that

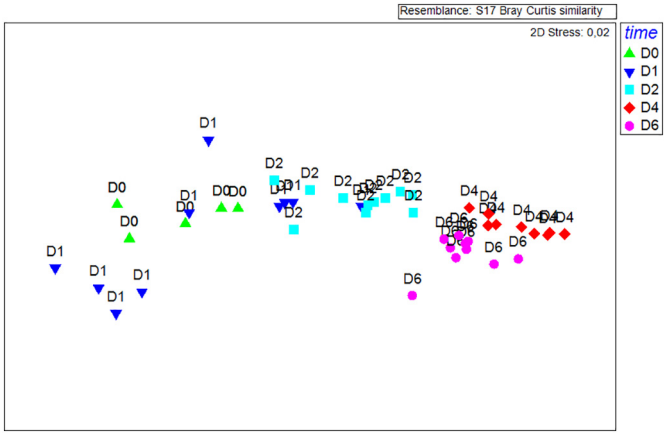

Fig. 3. Multidimensional scale plot (NMDS) based on Bray-Curtis similarity between total fatty acid (FAs) composition of the phytoplankton assemblage of all experimental treatments at different incubation time (time factor). Data is presented for 17 FAs.

salinity and temperature (tested separately) had no significant effects on the absolute concentrations of individual FAs (Table 2A–B). Only incubation time yielded a clear separation in the NMDS plot (2D stress = 0.02) (Fig. 3, Simper overall $R = 0.73$). The PERMANOVA test with repeated measures on the absolute FA concentration of selected FAs showed the same outcome: only a significant effect of incubation time was found (p (perm) = 0.001). However, changes in the relative

Table 3
SIMPER results for the 'time' factor, i.e. samples grouped per sampling day. Average similarities of fatty acids within each group are listed as well as the FAs contributing the most to the similarity within the samples. For each FA, its percent contribution to the within-group similarity is indicated between brackets.

	Day 0	Day 1	Day 2	Day 4	Day 6
Average similarity	75.4	51.8	76	82.4	82.4
FA	16:0 (27.9)	16:0 (32.8)	16:1ω (18.5)	16:1ω7 (25.6)	16:1ω7 (26.4)
FA	14:0 (41.8)	14:0 (19.3)	18:4ω3 (18.4)	18:4ω3 (16.8)	18:4ω3 (23)
FA	18:0 (55.3)	18:0 (9.3)	20:5ω3 (15.1)	18:3ω3 (16.8)	16:0 (15)

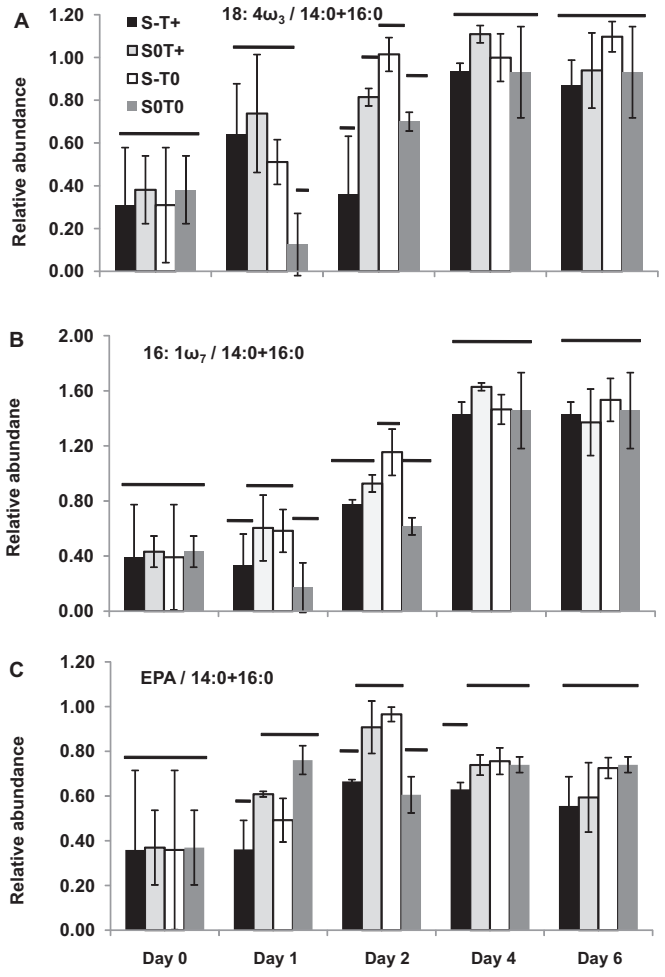

Fig. 4. Relative abundance of the main unsaturated FAs (20:5ω3, 18:4ω3 and 16:1ω7) in relation to saturated FAs (14.0 + 16.0) as a function of incubation time. The height of each bar represents the mean of 3 replicates ± S.D. Horizontal lines at the same height indicate that there are no significant differences(Tukey Test) between treatments for each day. Ratio between total concentration of unsaturated/(14:0 + 16:0) for (A) 18:4ω3, (B) 16:1ω7 and (C) 20:5ω3 (EPA). Note the different scales used for the y-axis for B.

abundance of PUFAs (18:4ω3, 16:1ω7 and EPA) on exposure days 1 and 2 were evident in the different treatments (Fig. 4A–C). From day 0 to day 2, the ratio between PUFAs and SFAs increased, mainly due to increased PUFAs in both S-T+ and S0T+ (Fig. 4A–C). From day 2 to 4, the treatments had a significant effect on this ratio ($P < 0.01$). Moreover, the low PUFA vs. saturated FA ratio in S-T+ was due to a lower increment rate of unsaturated compared to saturated FAs. The relative concentration of 18:4ω3 and 16:1ω7 increased in all treatments but

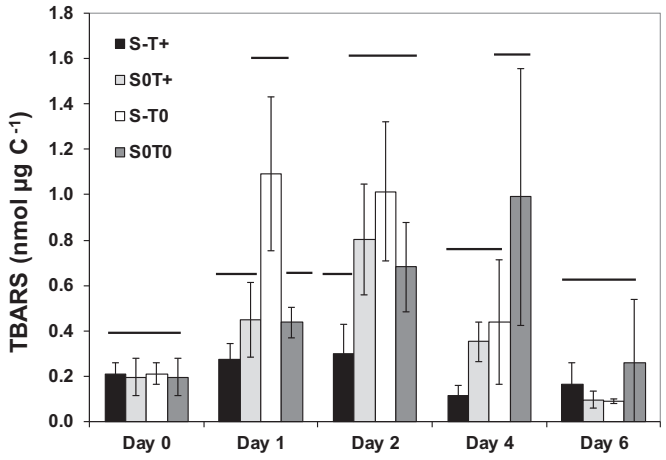

Fig. 5. Cell content of TBARS vs incubation time. The height of each bar represents the mean of 3 replicates ± S.D. Horizontal lines at the same height indicate that there are no significant differences (Tukey Test) between treatments for each day.

especially at elevated temperature and ambient salinity (S0T+; Fig. 4A and B). Although the proportion of PUFAs vs. SFAs changed during the first 2 days (Fig. 4A–C), the composition of phytoplankton assemblage did not (Table 1). Furthermore, the FA 16:1ω7, which is a good proxy for diatoms, continued to increase until day 4 in all treatments (Fig. 4B). Days 4 and 6 showed no significant difference in the ratio of PUFAs/saturated FAs ($P = 0.85$ and 0.97, respectively) in any treatment.

On day 1, a significant increase in TBARS concentration (indicative of lipid damage) was only measured in the S-T0 treatment ($P < 0.01$) (Fig. 5), which coincided with an increase in unsaturated FAs (Fig. 4A–C). Although an increase in TBARS was also observed in the other three treatments, there were no significant differences between S0T+ and S-T+ ($P = 0.7$) or between S0T+ and the control ($P = 0.9$) on day 1 (Fig. 5). On day 2, TBARS increased significantly in all treatments in comparison to Day 0 ($P < 0.05$), except in the S-T+ ($P = 0.3$). On day 4, there was a significant decrease in TBARS in all treatments when compared to the control ($P < 0.01$) (Fig. 5) in spite of the increase in unsaturated FAs (18:4ω3 and 16:1ω7) from days 2 to 4, in both S-T+ and S0T+ (Fig. 4B-C). The decrease in TBARS on day 4 coincided with an increased concentration of non-enzymatic antioxidants (data no shown). On day 6, there were no differences in TBARS content among treatments, returning to control levels measured on Day 0 (Fig. 5). The TBARS concentrations in S-T+ remained lower than the control throughout the experiment (Fig. 5) even at high concentrations of unsaturated FAs (Fig. 4A-B).

4. Discussion

Previous studies using individual phytoplankton species have shown an effect of temperature on FA composition, and specifically a decrease in ω3 PUFAs (EPA and DHA) with increasing temperature (Renaud et al., 2002; Guschina and Harwood, 2006). However, the combined effects of temperature and salinity on natural coastal Antarctic assemblages have not been investigated to date. Here, we show for the first time the effect of decreased salinity combined with increased temperature on the FA content in natural assemblages of coastal Antarctic phytoplankton. Our study revealed several relationships between phytoplankton FAs, the relative abundance of FAs and increasing water temperature in phytoplankton, which support the idea that phytoplankton modify their cell membrane FA composition in order to adapt to changes in ambient temperature (i.e., homeoviscous adaptation, Hazel, 1995). Previous studies have proposed that FAs in a phytoplankton assemblage are determined primarily by the taxonomic composition of such assemblage (e.g. Winder and Sommer, 2012). Given

that only minor changes in phytoplankton composition were observed throughout our experiment, the observed variation in FA composition seemed to have occurred within species due to changes in temperature and salinity (Chen et al., 2008; Jiang and Gao, 2004; Liang et al., 2006; Piepho et al., 2012). FA variations within individual species can have consequences for marine consumers that cannot synthesize long-chain PUFAs in sufficient quantities for growth and survival, and must obtain them from their food.

4.1. Impact of temperature and salinity on carbon content and assemblage composition

A temperature increase of 4 °C resulted in a significant increase in total carbon biomass in the microcosms in mid-experiment (days 2–4), but in contrast with previous findings (Hernando et al., 2015), there was no significant effect of decreased salinity. The higher biomass at elevated temperatures coincided with a significant increase in the relative abundance of large centric diatoms (i.e. *Porosira glacialis* and *Thalassiosira antarctica*), whereas relative abundance of small prasinophytes decreased under higher temperature throughout the incubation period. Temperature has an essential role in enzymatic activity and metabolic processes by which reaction rates increase with increasing temperature as seen in the laboratory (e.g. Eppley, 1972; Berges et al., 2002) and in the field (e.g. Montagnes and Franklin, 2001). In our experiments, higher temperature enhanced the carbon accumulation, implying a faster growth rate. Although large diatom species (with a higher carbon content) were indeed favored in both high temperature and low salinity treatments, it was only under high temperature that carbon accumulation was enhanced. Lionard et al. (2012) showed that the cumulative biomass of large centric diatoms were favored when natural marine phytoplankton assemblages were exposed to high temperature in temperate environments. In contrast, naturally dominant large diatoms in the Bering Sea were replaced by smaller nanophytoplankton during incubations at high temperature (Hare et al., 2007). We found small (10–20 μm) diatoms to increase at the end of the incubation only under S0T+ but not in the S-T+ treatment. This probably indicated that these cells were affected by low salinity, and that this effect was not compensated by temperature-induced metabolic changes. Therefore, responses to salinity fluctuations may be species-specific (Aizdaicher and Markina, 2010; Piquet et al., 2011). Hernando et al. (2015) described a negative osmotic effect of decreased salinity on phytoplankton assemblages in the early experimental stages (2 days of a 6-day incubation), which were dominated by big centric diatoms like *Odontella weissflogii*, *Chaetoceros tortissimus,* and chains of *Chaetoceros socialis*. However, after 6 days of exposure at 30 psu, assemblages of small diatoms (such as *Fragilariopsis cylindrus/nana*) became more abundant, reflecting their better capacity to cope with osmotic stress. Other studies have shown that the photophysiological and phenotypic plasticity (i.e. light energy allocation and macromolecular composition) of Antarctic diatoms during changes in salinity and temperature is highly species-specific (Petrou et al., 2011; Sackett et al., 2013). The response of phytoplankton assemblage to low salinity and temperature is therefore dependent on the initial assemblage' composition at the time of perturbation, and probably also on the species-specific capacity for acclimation (Villafañe et al., 1995; Huertas et al., 2011).

4.2. Impact of temperature and salinity on FA profiles

In polar oceans, PUFAs represent 20–60% of the total FA content in individual algae and in natural assemblages (Teoh et al., 2004), which is consistent with our average of 50–60% of ambient PUFA levels. High levels of PUFAs in cellular membranes preserve membrane fluidity at low temperatures (Lodish et al., 2000), and therefore ensure the functioning of integral membrane proteins. The increase in total FA content throughout the experiment was in the same range as that reported for culture diatoms. This increase was probably related to the storage of cellular lipids, which occurs when phytoplankton's cell division is blocked due to nutritional deficiency while the cell is still functional (Siron et al., 1989).

It has been previously shown that temperature affects the lipid content of Antarctic phytoplankton (Smith and Morris, 1980), and low salinity regulates the production of PUFAs in diatoms (Guan-Qun et al., 2008). In this study, a significant increase in the proportion of PUFA/SFA was measured at 48 h, when phytoplankton were exposed to either low salinity or high temperature, which resulted in measurable oxidative damage (see Section 4.3). In contrast, the combined treatment of low salinity and high temperature resulted in no change in the PUFA/SFA proportions, and consequently no oxidative damage was detected under the synergistic effect of both stressors. The lack of variation in the FA index, could be due to the presence of genes participating in the synthesis of molecules (e.g. osmoregulation proteins) involved in survival mechanisms.

Therefore, expected climatic changes in coastal Antarctica may favor a FA composition in phytoplankton that can protect the cells from oxidative damage. According to the hypothesis of homeoviscous adaptation, changes in FA composition with increasing temperature favor the proper functioning of membranes (Hazel, 1995). Temperature changes may then induce the replacement of an enzyme by an isoenzyme with better heat- or cold-tolerance (Steele and Fry, 2000). Several authors have shown an inverse relationship between temperature and FA unsaturation in microalgae (Wada et al., 1990; Zhu et al., 1997). Jiang and Gao (2004) explained that elevated levels of unsaturated FAs in the diatom *P. tricornutum* after 12 h of exposure to low temperatures were due to an increased activity of a desaturase, a temperature-sensitive enzyme, which inserts the double bonds into the hydrocarbon chains of FAs (Sato and Murata, 1982). Other studies have shown similar changes in the composition of FAs in diatoms within a similar period of time (12–48 h) (Tonon et al., 2002; Siron et al., 1989; Liang et al., 2006). Mayzaud et al. (2013) demonstrated that high temperatures decreased the amount of ω-3 PUFAs (such as EPA and 18:4ω3) in natural blooming phytoplankton in an Arctic fjord. However, because in our study the total concentration of unsaturated FAs increased in S0T+ and in S-T0 as a function of time, it is likely that desaturase enzymes (not measured in this study) were active due to temperature or salinity changes. It has been suggested that FA desaturation activity and the availability of potential FA precursors could also explain the variations in FA content (Suutari et al., 1996). Desaturase genes can receive signals from a specific sensor in the cytoplasmic membrane and increase the level of PUFAs in low temperature conditions (Vigh et al., 1993; Guschina and Harwood, 2006). Studies of the microalgal genome have revealed the presence of genes participating in the synthesis of molecules involved in microalgal survival mechanisms (e.g. osmoregulation enzymes) and in FA synthesis (Azachi et al., 2002; Arisz and Munnik, 2011; Li et al., 2012). Gene expression for FA synthesis has been studied in several microalgal species, demonstrating that up- and/or down-regulation of genes occurs as a result of changes in external conditions such as salinity (Msanne et al., 2012). Allakhverdiev et al. (2001) used targeted mutagenesis to alter genes responsible for the production of FA desaturases in *Synechocystis* sp. and produced strains with decreased levels of unsaturated FAs in their membrane lipids (Tasaka et al., 1996) as well as decreased tolerance to salt (Allakhverdiev et al., 1999). The adaptation of *Porosira glacialis* and *Thalassiosira antarctica*, which accounted for the highest carbon biomass in all treatments and highest relative abundance in S-T+, could be related to the regulation of such genes.

4.3. Oxidative metabolism

Photosynthetic organisms are exposed to ROS because they simultaneously produce and consume oxygen during exposure to light (Asada, 1999). The production of highly oxidants ROS has to be tightly controlled by antioxidants and radical-scavenging biochemical

reactions within the cell to avoid cell damage. Although all classes of macromolecules are susceptible to oxygen radical attack, PUFAs are extremely labile to oxidation due to their conjugated double-bond structures. Therefore, a high percentage of lipid unsaturation could exacerbate membrane susceptibility to radical attack (González et al., 2015). In this study we measured an increase in lipid damage and the concentration of the unsaturated FAs, which is expected since ROS preferentially attack the double-bond. Using TBARS content as an indicator of lipid damage, we observed a significant increase of it (and therefore higher damage) at the beginning of the high temperature and low salinity conditions, with the largest oxidative effect under low salinity during the first 48 h. These results were observed in the presence of a significant increase in unsaturated FAs. The physiological responses to lipid damage by the antioxidant compounds (see in the following paragraphs of this section) could explain the lack of a positive correlation when we try to statistically relate TBARS with a higher unsaturated FAs concentration as was expected. The change in ambient salinity mainly affects the internal homeostasis of the cells (Erdmann and Hagemann, 2001). Osmotic stress caused by a flux of water across the semipermeable cell membrane leads to a change in the cellular water potential. In particular, hypoosmotic conditions cause water influx into the cell resulting in increased turgor pressure and osmotic stress (Bisson and Kirst, 1995). When photosynthetic organisms are exposed to salt stress, the membrane FAs are desaturated (Singh et al., 2002) and the degree of osmotic stress that can be tolerated by phytoplankton cells ultimately depends on the ability of the cytoplasmic membrane to alter its FA composition (Elkahoui et al., 2004) in response to an external change in salt content. Interestingly, contrary to the responses observed under low salinity or high temperature, the combined low salinity and high temperature effect (S-T+) resulted in a lack of change in TBARS, with concentrations significantly lower than the control in mid-experiment (days 2 and 4). This decreased lipid damage for the low salinity and high temperature condition could be explained in two ways: (1) a lower relative abundance of unsaturated FAs and (2) a significant increase in α-T production. The oxidative damage to cellular components is limited under normal growing conditions due to efficient processing of ROS through a well-coordinated and responsive antioxidant system consisting of several enzymes and redox metabolites (González et al., 2015; Hernando et al., 2015). Lipophilic molecules, such as α-T, are able to deactivate singlet oxygen ($^1O_2$), reduce oxygen and terminate lipid radical chain reactions (Häubner et al., 2014). Hernando et al. (2011, 2015) showed that Antarctic diatoms are able to produce α-T rapidly (within hours) in response to stress, such as UV radiation or low salinity. In our experiments, α-T was lower on day 1 compared to a higher concentration in the control (data not shown here). On day 2, there was a significantly higher α-T production in S-T0 and in S0T+, which decreased days later because of consumption (data not shown). These results suggest protection from lipid damage due to the presence of enzymatic or non-enzymatic antioxidants or, as we previously suggested, regulation of genes involved in osmoregulation proteins and acclimation response, as seen in large diatoms. In all our treatments, α-T increased over the course of the experiment (data not shown), in agreement with a decrease in TBARS content. Hernando et al. (2015) reported the same effects in phytoplankton assemblages from the same location. The differential responses in lipid damage to combined or separate temperature and salinity effects could demonstrate that specific protection mechanisms are in effect when cells are exposed to both stressors simultaneously.

5. Conclusion

This study is the first to report oxidative stress resulting from changes in FA in diatom-dominated Antarctic phytoplankton assemblages exposed to temperature and salinity variations. Temperature seems to have a larger impact than salinity on the eco-physiological responses, both in terms of carbon accumulation and FA content. However, caution should be exercised when extrapolating results from microcosms to natural systems, since enclosed experiments exclude the effects of water mixing that exposes phytoplankton to daily changing conditions throughout the water column. We also found differential responses of phytoplankton size to increased temperature resulting in a significant change in the relative abundance of the main groups of primary producers towards the end of the experiment, with large diatoms better acclimating to the higher temperature. Furthermore, the relative abundance of small diatoms (10–20 μm) increased only under high temperature (S0T+), and small prasinophytes decreased under the combined condition (S-T+) at the end of the incubation.

Interestingly, the FA content changed rapidly, with an increased proportion of unsaturated FAs and lipid damage at 48 h. After 48 h, no further variations in the relative abundance of FAs was detected, indicating that antioxidant responses may have avoided further cellular damage (Hernando et al., 2015). The combination of both stressors affected the relative abundance of the main phytoplankton groups but did not increase lipid damage, probably due to a lower relative abundance of unsaturated FAs. A reduction in phytoplankton PUFAs under high temperature and low salinity may result in reduced food quality for grazers (Rossoll et al., 2012), with potentially serious ecological consequences for energy flow in Antarctic coastal systems. For example, low EPA content in microalgae was shown to be related to low zooplankton growth and egg production (Danielsdottir et al., 2007), which implies decreased energy transfer efficiency between trophic levels. Such differences in lipid content in phytoplankton could therefore have ecological implications at even higher trophic levels with regard to the availability of essential FAs.

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