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Article

Species-Specific Variations in the Nutritional Quality of Southern Ocean Phytoplankton in Response to Elevated pCO_2

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Abstract: Increased seawater pCO_2 has the potential to alter phytoplankton biochemistry, which in turn may negatively affect the nutritional quality of phytoplankton as food for grazers. Our aim was to identify how Antarctic phytoplankton, *Pyramimonas gelidicola*, *Phaeocystis antarctica*, and *Gymnodinium* sp., respond to increased pCO_2. Cultures were maintained in a continuous culture setup to ensure stable CO_2 concentrations. Cells were subjected to a range of pCO_2 from ambient to 993 µatm. We measured phytoplankton response in terms of cell size, cellular carbohydrate content, and elemental, pigment and fatty acid composition and content. We observed few changes in phytoplankton biochemistry with increasing CO_2 concentration which were species-specific and predominantly included differences in the fatty acid composition. The C:N ratio was unaffected by CO_2 concentration in the three species, while carbohydrate content decreased in *Pyramimonas gelidicola*, but increased in *Phaeocystis antarctica*. We found a significant reduction in the content of nutritionally important polyunsaturated fatty acids in

Pyramimonas gelidicola cultures under high CO_2 treatment, while cellular levels of the polyunsaturated fatty acid 20:5ω3, EPA, in *Gymnodinium* sp. increased. These changes in fatty acid profile could affect the nutritional quality of phytoplankton as food for grazers, however, further research is needed to identify the mechanisms for the observed species-specific changes and to improve our ability to extrapolate laboratory-based experiments on individual species to natural communities.

Keywords: Antarctic phytoplankton; biochemical changes; polyunsaturated fatty acids; nutritional quality; ocean acidification

1. Introduction

Human activities have led to an increase in atmospheric CO_2 concentration of which an estimated 30% have been absorbed by the oceans, causing global average surface seawater pH to drop by 0.1 units [1–3], a process termed ocean acidification [4,5]. High latitudes will be particularly vulnerable due to their capacity to store more CO_2, and upwelling and subsequent entrainment of CO_2-rich deep waters during winter [6–10], rendering its inhabitants among the first to be affected by ocean acidification. Yet, little is known presently about the susceptibility of polar organisms to increased pCO_2, and this is particularly true for Antarctic phytoplankton [11,12].

Ocean acidification has the potential to alter phytoplankton biochemistry. Elevated CO_2 concentration has been shown to influence the ratio of carbon to nutrient uptake rates in phytoplankton [13–16] and consequently an increase in C:N:P ratio [17–19]. A reduction in the percentage of polyunsaturated fatty acids (PUFA) in the diatom *Thalassiosira pseudonana* has also been reported [20].

Phytoplankton response to elevated CO_2 concentrations and lowered pH has been found to be species-specific and can lead to shifts in the species composition and bulk biochemical parameters of natural phytoplankton communities [8,21–23]. Predicting how phytoplankton will respond to ocean acidification has therefore been a difficult task. Contrary to the hypothesis that climate change will fertilize the oceans via increased availability of CO_2 and thereby stimulate primary productivity, on a global scale ocean primary productivity has declined since the early 1980's [24] and might continue to do so due to ocean warming [25].

Alterations to the composition of phytoplankton communities, as well as individual species' nutritional quality and availability, could have major ramifications for higher trophic levels [26–29]. The fatty acid profile of phytoplankton is of particular interest as an indicator of their nutritional quality for grazers. Fatty acids are divided into PUFA, monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). The composition of PUFA and the ratio of PUFA to MUFA and SFA are critically important for grazer development, reproduction and hatch rates, as has been well researched by the aquaculture and other industries [30–35].

To understand why and how phytoplankton communities change under the influence of ocean acidification, we need to understand the nature and implications of individual species' changes and what the common and species-specific changes are. Therefore, the aim of this research was to establish whether the biochemistry of Antarctic phytoplankton responds to elevated CO_2 concentrations and

if so, to identify similarities or differences in this response amongst species. Based on literature reports our hypothesis was to find an increase in C:N ratio, a decrease in PUFA and a decrease in cellular carbohydrate contents [36] with increasing pCO_2.

2. Materials and Methods

We conducted two experiments in which three Antarctic phytoplankton species were exposed to CO_2 concentrations ranging from ambient to 993 µatm following recommendations by Barry *et al.* [37]. The species chosen were the prasinophyte, *Pyramimonas gelidicola*, a single cell strain of the haptophyte *Phaeocystis antarctica*, and the dinoflagellate *Gymnodinium* sp. We chose these species to represent a mixture of phytoplankton types, commonly found in the waters off Davis Station, Antarctica, where these species were isolated from. We measured their pigment and fatty acid profiles, particulate carbohydrate content and carbon to nitrogen ratio immediately after acclimation to the experimental conditions. In agreement with recommendations in best practice guides [38], we used a continuous culture system with CO_2-enriched gas aeration to achieve stable carbonate chemistry that mimics the natural changes occurring due to ocean acidification. For a better understanding of how individual species will respond under natural conditions, we conducted our experiments at macronutrient concentrations reported for the area from which our strains were isolated (near shore coastal waters off Davis Station, Antarctica) and set the light intensity to a similarly high level, as light intensity can greatly alter the CO_2-induced response of phytoplankton [39–41]. Furthermore, we chose to expose all species to continuous light to minimize diurnal variations which can mask CO_2-induced changes [42].

We used a continuous culture system with CO_2-enriched gas aeration to achieve a stable carbonate chemistry that mimics the natural changes occurring due to ocean acidification, following the recommendations by Andersson *et al.* [38]. The main advantages of a continuous culture system with automatic constant dilution are: (1) the reduced labour intensity compared to semi-continuous culturing, that require regular manual dilutions; and (2) the possibility to use low nutrient concentrations that are approximating natural conditions compared to artificially high nutrient concentrations, such as in the commonly used full-strength f/2 medium. We chose a base recipe of f/2 medium [43,44] as it lacks any buffer that could affect the carbonate chemistry and trace metal speciation in the growth medium, which would thereby affect medium pH and phytoplankton growth [45]. Iron concentrations were not adjusted to the realistically low concentrations of the Southern Ocean since iron limitation has been found to influence phytoplankton response to elevated CO_2 concentrations [46]; such changes would add a further level of complexity in interpreting our results. Nitrate and phosphate were adjusted to concentrations reported around O'Gorman Rocks, off Davis Station, Antarctica [47,48]. Silicate concentrations were lower than around O'Gorman Rocks but not limiting [49,50]. To achieve the target pCO_2, we bubbled the phytoplankton bag cultures with air containing predetermined CO_2 concentrations. Bubbling is a simple and effective way of altering the carbonate chemistry in agreement with natural changes [51]. The culture system is described in detail elsewhere [52] and will be summarized briefly below.

The system housed 24 × 2.3 L custom-made, transparent plastic bags (polyethylene, Entapack, Australia) thereby allowing two phytoplankton species to be studied simultaneously; each species was

examined with triplicate bags exposed to four CO_2 treatments (three CO_2 enriched treatments and an ambient control). For each of the four target CO_2 concentrations, two 28 L media reservoirs were bubbled with 0.2 µm filtered air. The reservoirs were used to supply fresh, sterile (0.2 µm filtered) medium by two 12-channel peristaltic pumps (Masterflex, John Morris Scientific Pty Ltd, Chatswood, NSW, Australia) to the culture bags corresponding to that pCO_2 treatment in order to maintain the cells in exponential growth (Figure 1). Each culture bag was inoculated with a clonal phytoplankton culture of the same parent population and randomly attributed to the treatments. Initial cell density for *Pyramimonas gelidicola* cultures was ~8000 cells/mL, ~9500 cells/mL for *Phaeocystis antarctica* cultures and only ~80 cells/mL for *Gymnodinium* sp. cultures. The peristaltic pumps flushed the culture bags with sterile nutrient medium at a rate equivalent to the species' growth rate once each species had reached exponential growth and sufficient cell density (Figure 2). *Gymnodinium* sp. was not constantly diluted before reaching the goal of 6–7 generations of acclimation at the end of the experiment, due to its slow growth rate.

Figure 1. Overview schematic diagram of the ocean acidification continuous culture system. For simplicity only one CO_2 treatment and culture bag is shown. (**a**) Eight 28 L media reservoirs supplied the cultures with media of the respective pCO_2. The reservoirs were continuously bubbled with CO_2-enriched air of the respective concentration; (**b**) Air bubbling through the reservoirs and all culture bags and overflow bottles was first dehumidified by passing through a silica-gel filled cylinder fitted with an activated charcoal stage at the end to remove any organic contaminants; (**c**) Peristaltic pumps delivered the media from the reservoirs to each culture bag at a rate equivalent to the growth rate of the culture; (**d**) Culture bags and overflow bottles were continuously bubbled to maintain stable carbonate chemistry. At the same rate as media was pumped in, culture overflowed into a sterile overflow bottle, which was sampled for subsequent biochemical and other analyses.

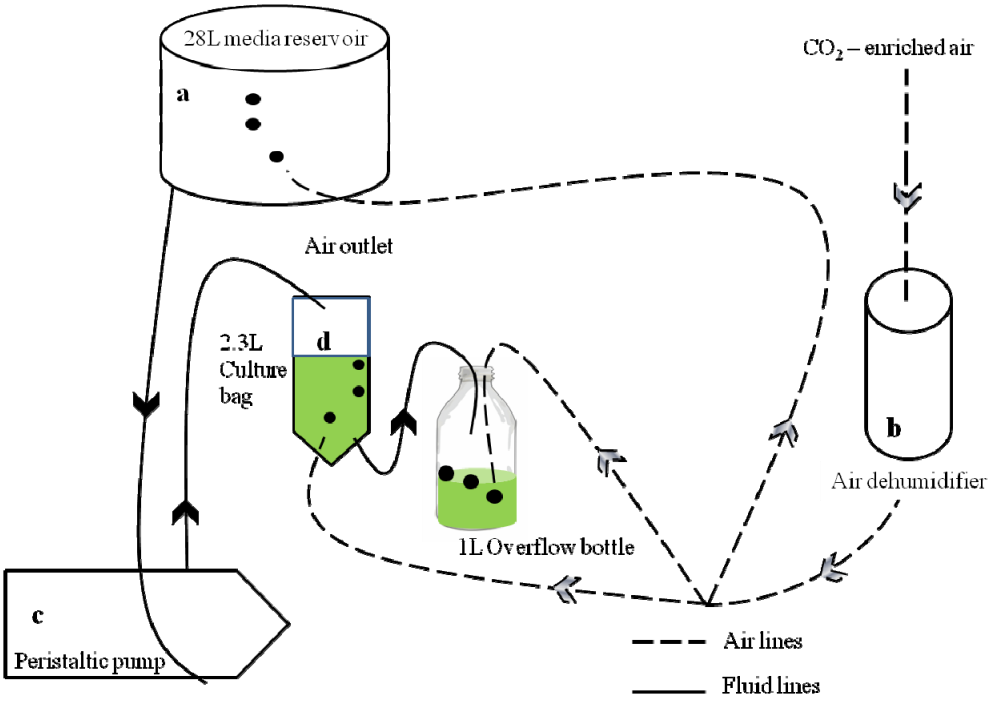
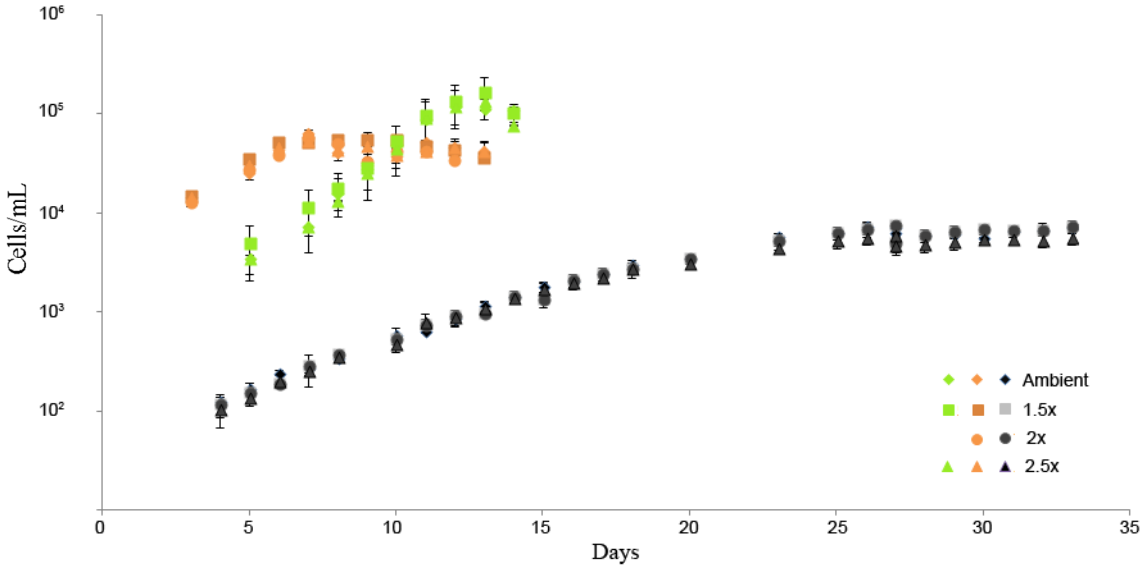


Figure 2. Daily cell densities with standard errors of *Pyramimonas gelidicola* (orange), *Phaeocystis antarctica* (green) and *Gymnodinium* sp. (black) cultures under the different CO_2 treatments.



The out-flowing culture was collected in sterile 1 L glass overflow bottles, providing culture material for subsequent analyses (Figure 1), as has been performed elsewhere [17]. Use of an overflow vessel to collect surplus culture allowed us to measure a suite of biochemical parameters for which large volumes of culture were required (up to 700 mL). By using the out-flowing culture rather than sample from within the culture bag, we avoided the possibility of contamination and also avoided any disturbances to the culture physiology by removing more than 10% of the culture volume [53]. Due to the slow growth rate of our three phytoplankton species and thus the slow dilution rate, surplus culture was left to accumulate for up to 2 days to provide the large volumes needed for the biochemical assays. These overflow bottles were located adjacent to the culture to ensure that the overflow bottles received the same light levels and temperatures as the cultures. Furthermore, the overflow bottles were bubbled with the same CO_2-air mix until sampling occurred. Thus the only property in the overflow bottles that potentially differed from conditions within the continuous system was the nutrient concentration. We cannot rule out the possibility that nutrient concentrations in the overflow bottles decreased over the 2 day period. However, it has been shown that diatoms have the ability to store nitrogen within the cells ([54] and references therein) and given the slow doubling time (~2 days) of our selected phytoplankton species, only those cells entering the overflow bottle at the start of the 2 day period would have undergone a maximum of one generation outside of the continuous culture system *versus* at least five generations of acclimation within the continuous culture system. Further, cell densities within the overflow bottles never exceeded the carrying capacity associated with our culture medium, as established prior to the experiments. Based on this, we have assumed that samples taken from the overflow bottles are representative of the culture within the continuous culture system. However, this uncertainty could be addressed in future experiments by monitoring nutrient concentrations in the overflow bottle as well as increasing culture bag volumes to shorten the timeframe required to accumulate enough culture volume for biochemical analyses.

2.1. Experimental Conditions

The cultures, overflow bottles and media reservoirs were kept in a temperature controlled refrigerator, maintained at an average 2.9 °C. The culture bags were positioned in front of fluorescent lights with an irradiance of 267 ± 6.9 µmol m^{-2} s^{-1}, approximating the light intensities at 5 m water depth around Davis Station, East Antarctica, from where most of these species were isolated [55]. Each culture, medium reservoir and overflow bottle was continuously bubbled with CO_2-regulated air, using mass flow controllers (Horiba STEC SEC-E-40), which mixed pure food grade CO_2 (BOC, Hobart, TAS, Australia) with ambient air to achieve specific CO_2 concentrations for the treatments. The resulting gas was passed through silica gel, activated charcoal, and a 0.2 µm filter to remove any contaminants before being used to adjust the CO_2 content of the culture bags, reservoirs and overflow bottles. Experimental conditions are listed in Table 1.

Table 1. Experimental conditions for all phytoplankton species. Values are averages with standard deviations, SD, in brackets. NO_x includes NO_3^- and NO_2^-.

Species	Acclimation in generations	Nutrient concentrations (µM)			CO_2 concentration (µatm)			
		NO_x	Si	P	Ambient	1.5×	2×	2.5×
Pyramimonas gelidicola	~5	17.5 (±4.5)	24.4 (±5.2)	5.7 (±1.3)	400 (±41)	612 (±54)	806 (±80)	977 (±106)
pH ± SD					8.04 (±0.04)	7.87 (±0.03)	7.75 (±0.04)	7.67 (±0.04)
Phaeocystis antarctica	~6	17.5 (±4.5)	24.4 (±5.2)	5.7 (±1.3)	413 (±31)	644 (±62)	-	993 (±83)
pH ± SD					8.02 (±0.03)	7.86 (±0.04)	-	7.67 (±0.03)
Gymnodinium sp.	~6	20.9 (±1.8)	17.6 (±0.5)	1.5 (±0.2)	458 (±48)	580 (±49)	797 (±79)	973 (±82)
pH ± SD					8.02 (±0.04)	7.89 (±0.03)	7.75 (±0.04)	7.70 (±0.03)

2.2. Carbonate Chemistry

Carbonate chemistry in the culture bags was monitored daily by measuring pH with a Mettler Toledo Multi Seven pH meter (Mettler Toledo, Melbourne, Australia). The pH meter was calibrated daily using freshly prepared tris- and aminopyridine buffers made in artificial seawater according to the SOP 6a in "Guide to best practices for ocean CO_2 measurements" [56]. Alkalinity samples (50 mL) were taken at regular intervals, poisoned with 25 µL saturated mercuric chloride solution and stored refrigerated in the dark until analysis in a closed cell using a Total Alkalinity Titrator ATT-05 (Kimoto, Osaka, Japan). A temperature probe logged the air temperature inside the refrigerator every 30 min (data not shown). CO_2 concentrations were calculated with the CO2SYS.BAS Excel programme [57] based on total alkalinity, pH, temperature, salinity and nutrient concentrations using the constants after Mehrbach *et al.* [58] as refitted after Dickson and Millero [59].

2.3. Physiological and Biochemical Analyses

After acclimation of the cultures of each phytoplankton species to the four CO_2 treatments, cultures in the overflow bottles were filtered to provide samples for fatty acid, pigment and C:N composition, and particulate carbohydrate content. Problems with the *Phaeocystis antarctica* cultures of the 2× ambient CO_2 treatment lead to their exclusion from the data set and the 1.5× and 2.5× CO_2 treatments were only comprised of two replicates each.

2.3.1. Cell Abundance

Daily cell abundance samples were taken to monitor exponential growth and relate measurements to a per cell basis. Samples were analysed using a BD FACSCalibur cytometer (Becton Dickson, San Diego, CA, USA) equipped with a 488 nm argon laser and cell concentrations were calculated by dividing event counts from bivariate scatter plots by the volume of culture analysed. PeakFlow Green 2.5 μm beads (Molecular Probes, Invitrogen, Mulgrave VIC, Australia) were added to each sample to monitor fluorescent signal strength.

2.3.2. Cell Dimensions

Cell dimensions were measured on Field Emission Scanning Electron Microscope images (JEOL JSM6701F, Frenchs Forest, NSW, Australia). For cells of *Gymnodinium* sp. and *Pyramimonas gelidicola* length and width were measured. Cell dimensions of *Phaeocystis antarctica* cells were not measured.

2.3.3. Pigments

Samples of each culture were vacuum filtered onto 13 mm GF/F filters (Whatman, GE Healthcare Life Sciences, Rydalmere, NSW, Australia), blotted dry and immediately frozen in liquid N_2 in cryovials. The samples were stored at −135 °C until analysis using a modified method [60]. Pigments were extracted with 300 μL dimethylformamide with 50 μL methanol containing 140 ng of apo-8'-carotenal (Fluka) internal standard and analysed by high pressure liquid chromatography (HPLC) [61] using a Waters 626 pump, Gilson 233 XL autoinjector, Waters Symmetry C8 column, a Waters 996 diode array detector and a Hitachi FT1000 fluorescence detector. Pigments were identified by comparison of their retention times and spectra with a sample of mixed standards from known cultures [62] injected at the start of each daily sample queue. Peaks were integrated using Waters Empower software, manually checked and corrected where necessary and quantified using an internal standard method [63]. Further details on the procedures are in [64].

2.3.4. Cellular Carbohydrates and C:N Ratio

Each culture was filtered onto a muffled quartz filter (Sartorius, Goettingen, Germany) and ¼ was used for C:N ratio analysis and the remaining ¾ for carbohydrate analysis. Cell distribution across the filters was even, but to minimize bias between the analyses through possible uneven filtration, the

filters were cut symmetrically into eight pieces and opposite ⅛ pieces of the filter were used to amount to the ¼ used for elemental analysis.

Phytoplankton particulate organic carbohydrates were first denatured into monosaccharides (adapted from [65]) and the carbohydrate content determined via a standard colorimetric analysis [66] on a GBC UV-Vis 916 spectrophotometer (GBC Scientific Equipment, Braeside, VIC, Australia).

Prior to C:N analysis, inorganic carbon was removed with 2 molar HCl. C:N composition was determined via a Thermo Finnigan EA 1112 Elemental Analyser (CEInstruments Ltd, Lancashire, UK).

2.3.5. Fatty Acid Analysis

Culture was filtered on pre-extracted (1:1 v/v chloroform:methanol) 25 mm GF/F (Whatman, GE Healthcare Life Sciences, Rydalmere, NSW, Australia) filters. Filters were trans-methylated to produce fatty acid methyl esters (FAME) by heating in methanol:chloroform:concentrated hydrochloric acid (3 mL, 10:1:1 v/v/v) at 80 °C for 2 h [67]. FAME were extracted into hexane:chloroform (4:1 v/v) and concentrated under a stream of nitrogen. An internal injection standard (19:0 FAME) was added and the fatty acid composition analysed by gas chromatography using an Agilent Technologies 7890A gas chromatography (Palo Alto, CA, USA) fitted with an Equity −1 fused silica capillary column (15 m × 0.1 mm internal diameter, 0.1 μm film thickness), a flame ionization detector, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was used as carrier gas. Operating conditions were as described in [30].

Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA) and individual components were identified using mass spectral data and comparison of retention time data with those obtained for authentic and laboratory standards. Gas chromatography-mass spectrometric analyses were performed on a Finnigan Thermoquest GCQ gas chromatography-mass spectrometer fitted with an on-column injector using Thermoquest Xcalibur software (Austin, TX, USA) and a capillary column of similar polarity to that described above.

2.3.6. Statistical Analysis

Biochemical results were analysed with a linear mixed effects model, including the interaction of culture bag and culture position in the three rows as nested random effects. Significant differences between treatment cultures and control cultures in all tests were accepted at $p \leq 0.05$. Calculations were performed with the R software environment 2.14.2 [68].

3. Results and Discussion

3.1. Cell Dimensions and Growth Rates

Pyramimonas gelidicola cells, grown at elevated CO_2 concentrations, were not significantly different from their respective control cultures. *Gymnodinium* sp. cell dimensions were not affected by CO_2 concentration (Table 2).

Growth rates were not affected by CO_2 concentrations in any of the three species (Figure 2). Based on the exponential growth phase prior to culture dilutions, growth rates of our cultures were:

Pyramimonas gelidicola 0.38 d^{-1}, SE ± 0.03

Phaeocystis antarctica 0.53 d^{-1}, SE ± 0.03

Gymnodinium sp. 0.24 d^{-1}, SE ± 0.01

Table 2. *Pyramimonas gelidicola* and *Gymnodinium* sp. cell dimensions of the respective CO_2 treatments. SD in brackets, n = number of samples.

CO_2 treatment	**Ambient**	**1.5×**	**2×**	**2.5×**
		Pyramimonas gelidicola		
Length (µm)	6.72 (±1.01)	7.04 (±1.23)	6.82 (±1.03)	7.01 (±1.37)
Width (µm)	5.58 (±0.59)	5.80 (±0.64)	5.73 (±0.55)	5.70 (±0.72)
	n = 60	n = 60	n = 60	n = 60
		Gymnodinium sp.		
Length (µm)	16.62 (±2.60)	17.21 (±2.65)	17.24 (±2.44)	16.54 (±2.42)
Width (µm)	8.16 (±1.08)	8.59 (±1.49)	8.97 (±1.63)	8.07 (±1.37)
	n = 45	n = 45	n = 58	n = 57

3.2. Pigments

There were no significant changes in pigment contents and ratios with increasing CO_2 levels in the three phytoplankton species (Table 3).

Table 3. Phytoplankton pigment ratios and contents in pg $cell^{-1}$ of the respective CO_2 treatments. Chl = Chlorophyll, ant = antheraxanthin, violax = violaxanthin, zea = zeaxanthin, Ddx = diadinoxanthin, dtx = diatoxanthin, SD in brackets, n = number of samples.

CO_2 treatment	**Ambient**	**1.5×**	**2×**	**2.5×**
		Pyramimonas gelidicola		
Chl *a*	0.62 (±0.18)	0.47 (±0.11)	0.53 (±0.14)	0.70 (±0.06)
Chl *a* + *b*	0.75 (±0.22)	0.58 (±0.14)	0.66 (±0.18)	0.86 (±0.07)
(ant + violax + zea)/Chl *a*	0.32 (±0.02)	0.32 (±0.03)	0.36 (±0.01)	0.32 (±0.01)
γ-carotene/Chl *a*	0.04 (±0.004)	0.04 (±0.001)	0.04 (±0.002)	0.04 (±0.001)
Lutein/Chl *a*	0.12 (±0.01)	0.14 (±0.03)	0.16 (±0.01)	0.12 (±0.001)
	n = 3	n = 3	n = 3	n = 3
		Phaeocystis antarctica		
Chl *a*	0.15 (±0.06)	0.10 (±0.02)	-	0.08 (±0.02)
Chl $a + c_2 + c_3$	0.17 (±0.08)	0.12 (±0.03)	-	0.09 (±0.03)
(Ddx + dtx)/Chl *a*	0.49 (±0.18)	0.68 (±0.18)	-	0.86 (±0.01)
	n = 3	n = 2	-	n = 2
		Gymnodinium sp.		
Chl *a*	1.71 (±0.84)	2.32 (±0.06)	2.07 (±0.22)	1.95 (±0.71)
Chl $a + c_2$	1.80 (±0.89)	2.43 (±0.06)	2.19 (±0.23)	2.04 (±0.74)
(Ddx + dtx)/Chl *a*	0.82 (±0.12)	0.77 (±0.07)	0.77 (±0.11)	0.84 (±0.09)
β-carotene/Chl *a*	0.07 (±0.01)	0.06 (±0.02)	0.04 (±0.02)	0.04 (±0.04)
	n = 3	n = 3	n = 3	n = 3

3.3. *Elemental Composition*

There was no significant change in elemental composition of the three phytoplankton species cells under the different CO_2 treatments (Table 4), although all cultures had lower %C and C:N ratios compared to control cultures at the highest CO_2 concentration.

Table 4. Phytoplankton elemental composition of the respective CO_2 treatments. Percentages of dry mass, SD in brackets, n = number of samples.

CO_2 treatment	**Ambient**	**1.5×**	**2×**	**2.5×**
		Pyramimonas gelidicola		
%C	10.7 (±2.2)	6.0 (±2.6)	8.1 (±9.8)	7.7 (±3.3)
%N	1.1 (±0.1)	0.7 (±0.0)	0.9 (±0.6)	0.9 (±0.1)
C:N	9.9 (±2.4)	8.6 (±4.1)	6.6 (±5.6)	8.5 (±3.9)
	n = 3	n = 3	n = 3	n = 3
		Phaeocystis antarctica		
%C	11.8 (±5.4)	13.0 (±5.4)	-	9.4 (±5.3)
%N	1.2 (±0.03)	1.1 (±0.1)	-	1.4 (±0.6)
C:N	9.4 (±4.1)	11.6 (±4.1)	-	6.7 (±1.0)
	n = 3	n = 2	-	n = 2
		Gymnodinium sp.		
%C	14.1 (±2.6)	14.2 (±2.8)	15.6 (±4.5)	10.5 (±2.5)
%N	2.0 (±0.2)	2.2 (±0.6)	2.0 (±0.3)	1.8 (±0.4)
C:N	6.9 (±0.9)	6.8 (±1.5)	7.6 (±1.1)	5.7 (±0.3)
	n = 3	n = 3	n = 3	n = 3

3.4. Particulate Carbohydrates

Particulate organic carbohydrate (CHO) content per cell in *Pyramimonas gelidicola* cultures were significantly lower at 2× and 2.5× pCO_2 than in control cells ($p < 0.05$) (Figure 3 and Table 5). In *Phaeocystis antarctica* cultures, CHO content were significantly higher in the highest CO_2 treatments than in control cultures ($p < 0.05$). CHO content per cell in *Gymnodinium* sp. cultures were not affected by elevated CO_2 concentrations.

3.5. Fatty Acids

Phaeocystis antarctica cultures showed no changes in the total amount of fatty acids per cell, the sum of monounsaturated fatty acids (MUFA), saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), nor the sum of ω3 and ω6 PUFA, particularly DHA and EPA (Table 6). *Pyramimonas gelidicola* cultures showed decreased PUFA, ω3 and ω6 PUFA, DHA and total fatty acid content per cell at 2.5× pCO_2 (Table 6), but did not differ from control cultures in SFA and EPA content.

The cellular PUFA, MUFA, SFA, ω3 and ω6 PUFA and total fatty acid content of *Gymnodinium* sp. cultures were unaffected by CO_2. EPA contents per cell were significantly elevated at all high CO_2 treatments compared to control cultures (Figure 4 and Table 6).

Figure 3. Cellular carbohydrate (CHO) content (pg/cell) with standard deviation of *Pyramimonas gelidicola* cultures under the different CO_2 concentrations.

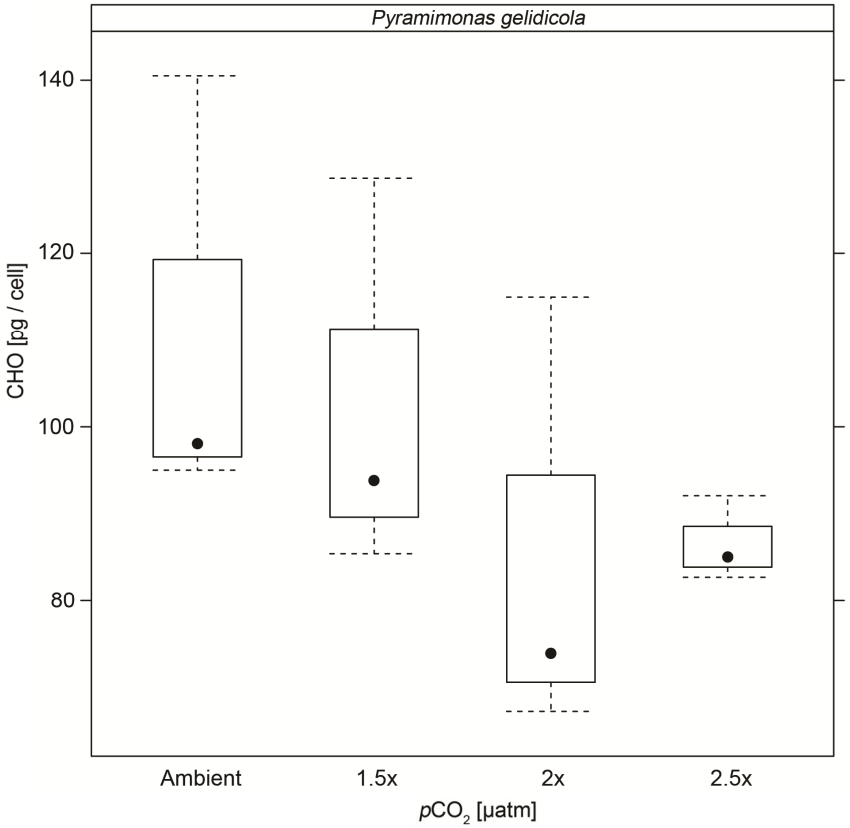


Table 5. Phytoplankton particulate organic carbohydrate (CHO) content in pg cell^{-1} of the respective CO_2 treatments. SD in brackets, n = number of samples. * $p < 0.05$.

CO_2 treatment	**Ambient**	**1.5×**	**2×**	**2.5×**
		Pyramimonas gelidicola		
CHO	111.2 (±25.5)	102.7 (±22.9)	85.4 (±25.8) *	86.7 (±4.9) *
	n = 3	n = 3	n = 3	n = 3
		Phaeocystis antarctica		
CHO	7.6 (±0.5)	8.3 (±0.7)	-	9.9 (±0.5) *
	n = 3	n = 2	-	n = 2
		Gymnodinium sp.		
CHO	93.4 (±30.3)	92.1 (±12.3)	110.4 (±26.5)	91.9 (±16.5)
	n = 3	n = 3	n = 3	n = 3

Based on literature reports, our hypothesis was that an increase in C:N ratio, a decrease in PUFA and a decrease in cellular carbohydrate contents may occur with increasing pCO_2. Between the three Antarctic phytoplankton species examined here, we found subtle but highly variable responses under elevated CO_2 concentrations, with opposite changes observed in cellular carbohydrate contents as well as in fatty acid profiles.

Contrary to our hypothesis we found no changes in C:N ratio in the three phytoplankton species examined. This is consistent with some literature results [14,42], which showed only minor responses of C:N ratios in a range of species under CO_2 concentrations above present day levels, but it is

in contrast to other studies that have shown increased C:N ratios under elevated pCO_2 [17–19]. A species-specific and even life stage-specific response of C:N ratio to elevated pCO_2 has been reported elsewhere [69].

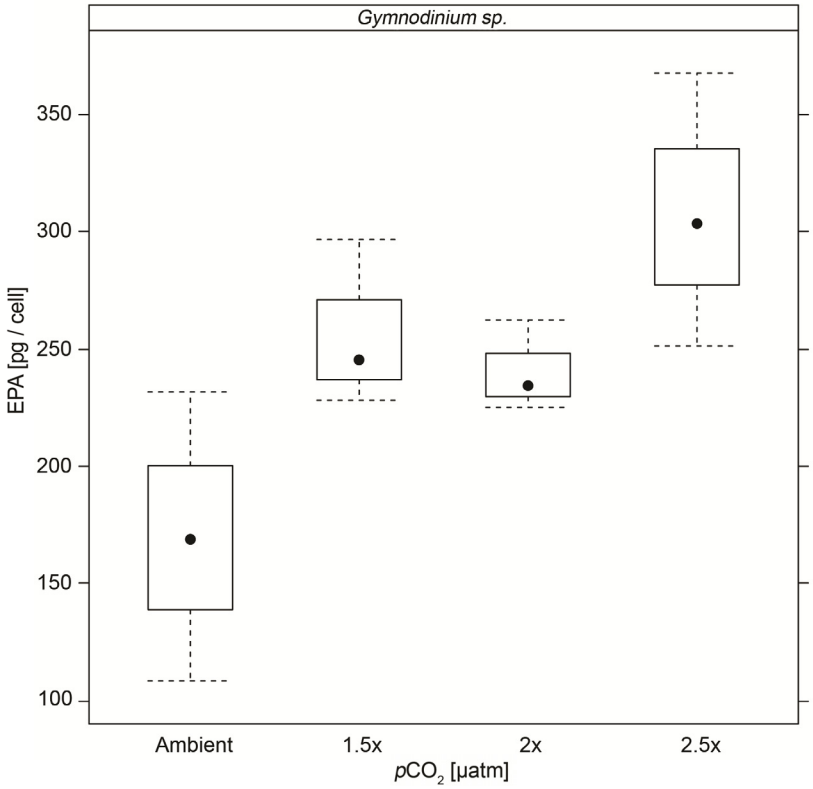
Table 6. Fatty acid content in pg/cell for all three phytoplankton species at the respective CO_2 treatments. SD in brackets, n = number of samples. * $p < 0.05$; ** $p < 0.01$.

CO_2 treatment	Ambient	1.5×	2×	2.5×
		Pyramimonas gelidicola		
SFA	0.3 (±0.08)	0.4 (±0.12)	0.4 (±0.12)	0.3 (±0.21)
MUFA	3.0 (±0.36)	4.6 (±0.92) *	3.8 (±0.72)	2.2 (±0.85)
PUFA	9.9 (±1.10)	9.4 (±0.90)	9.5 (±1.38)	6.5 (±2.72) *
ω3	4.9 (±0.67)	4.3 (±0.36)	4.7 (±0.63)	3.4 (±1.29) *
ω6	2.5 (±0.31)	2.7 (±0.34)	2.6 (±0.47)	1.5 (±1.00) *
DHA	1.2 (±0.19)	1.1 (±0.10)	1.2 (±0.15)	0.8 (±0.36) *
EPA	0.1 (±0.01)	0.1 (±0.02)	0.1 (±0.01)	0.1 (±0.03)
Total FA	14.9 (±1.5)	16.3 (±2.3)	15.4 (±2.2)	10.2 (±3.9) *
	n = 3	n = 3	n = 3	n = 3
		Phaeocystis antarctica		
SFA	0.2 (±0.15)	0.2 (±0.03)	-	0.3 (±0.06)
MUFA	0.7 (±0.58)	0.5 (±0.17)	-	0.7 (±0.11)
PUFA	1.9 (±1.42)	1.4 (±0.04)	-	1.6 (±0.25)
ω3	1.1 (±0.81)	0.8 (±0.00)	-	0.9 (±0.13)
ω6	0.8 (±0.61)	0.6 (±0.04)	-	0.7 (±0.11)
DHA	0.5 (±0.37)	0.4 (±0.02)	-	0.5 (±0.08)
EPA	0.1 (±0.09)	0.1 (±0.00)	-	0.1 (±0.01)
Total FA	3.1 (±2.37)	2.3 (±0.28)	-	2.9 (±0.48)
	n = 3	n = 2	-	n = 2
		Gymnodinium sp.		
SFA	28.0 (±16.47)	26.4 (±3.93)	35.4 (±30.07)	20.0 (±9.40)
MUFA	27.0 (±8.65)	27.0 (±2.86)	37.0 (±7.98) *	31.2 (±5.31)
PUFA	80.6 (±38.52)	96.8 (±7.58)	117.1 (±52.75)	94.1 (±27.36)
ω3	33.7 (±12.41)	39.7 (±2.55)	44.7 (±14.12)	38.2 (±10.79)
ω6	47.0 (±26.42)	57.2 (±5.14)	72.6 (±38.80)	55.7 (±16.60)
DHA	18.0 (±7.75)	22.0 (±1.92)	25.3 (±9.77)	22.6 (±5.13)
EPA	0.17 (±0.061)	0.26 (±0.036) **	0.24 (±0.020) **	0.31 (±0.058) **
Total FA	153.6 (±72.45)	170.5 (±11.91)	216.2 (±104.09)	164.9 (±44.92)
	n = 3	n = 3	n = 3	n = 3

Cellular carbohydrate content of the prasinophyte, *Pyramimonas gelidicola*, and the haptophyte, *Phaeocystis antarctica*, cultures showed significant changes under elevated CO_2 concentrations. In agreement with the findings of Thornton [36], who studied the marine diatom *Chaetoceros muelleri*, cellular carbohydrate contents of *Pyramimonas gelidicola*, cultures decreased about 22% under 2× (pH = 7.75) and 2.5× (pH = 7.67) ambient CO_2 concentrations, compared to a ~20% decrease at pH 7.9 reported by Thornton [36]. Extracellular carbohydrate content was not measured in our experiment, thus we cannot exclude the possibility of excess carbon being accumulated and excreted as

transparent exopolymers or low molecular weight carbohydrates in *Pyramimonas gelidicola* cultures, as suggested by Engel [70] and Thornton [36]. Cellular carbohydrate content increased by ~30% in *Phaeocystis antarctica* cultures at 2.5× ambient pCO_2 (pH = 7.67). This is a larger increase than the 23% increase in cellular glucan content, a storage carbohydrate, reported in the marine diatom *Skeletonema costatum* grown at pH 7.5 [71]. Taraldsvik and Myklestad [71] manipulated the pH of the growth medium via addition of acid/base in contrast to the use of CO_2 as occurred here, which might explain the difference in the magnitude between our result and theirs. This opposing trend in cellular carbohydrate contents between our two species highlights the importance of analysing the changes in both particulate and dissolved carbohydrates in future studies to identify the fate of C in the cellular and extracellular C pool. It also highlights the possibility that different phytoplankton types respond differently to increased CO_2 concentrations. However, as the results by Thornton [36] and Taraldsvik and Myklestad [71] show, even within the same phytoplankton types, in this case diatoms, opposing response to CO_2 has been found.

Figure 4. EPA content (pg/cell) with standard deviation of *Gymnodinium* sp. cultures under the different CO_2 concentrations.



Equally, no universal response in fatty acid content to increased pCO_2 was found in the three phytoplankton species investigated here. However, the changes in fatty acid profiles found in *Pyramimonas gelidicola* are of particular interest with regards to the availability of essential fatty acids for grazers. A decrease in PUFA content could constitute deterioration in the nutritional quality of phytoplankton for grazers, while an increase in these essential fatty acids such as EPA in *Gymnodinium* sp. could improve their quality as food. The effects of ocean acidification on the nutritional quality of phytoplankton are still mostly unknown with only a few studies conducted to

date. While Leu *et al.* [72] could not find any deterioration in a natural plankton community in the Arctic, Rossoll *et al.* [20] reported that *Thalassiosira pseudonana* grown at high pCO_2 negatively affected growth and reproduction of the copepod *Acartia tonsa*. Similarly Wynn-Edwards *et al.* [73] found negative effects of *Pseudonitzschia subcurvata* grown at high pCO_2 on Antarctic krill, *Euphausia superba*, larval mortality rates. The PUFA content in *Pyramimonas gelidicola* cultures under elevated CO_2 was about 34% lower than under ambient conditions and this agrees well with the 36% decrease in PUFA content in the diatom *Thalassiosira pseudonana* under 761 µatm pCO_2 reported by Rossoll *et al.* [20]. Detailed studies of the mechanisms and pathways of lipid and fatty acid production in other organisms suggest that external and internal pH influence lipid and fatty acid production. A decrease in external pH can translate into a decrease in internal pH [74]. Decreased internal pH in turn was reported to suppress phospholipid metabolic genes in yeasts [75] and a lower degree of unsaturation of fatty acids in CO_2-enriched cultures of *Chlorella kessleri* compared to ambient CO_2 concentrations was at least partially attributed to suppressed fatty acid synthesis and thus the promotion of desaturation of pre-existing fatty acids [76]. A higher degree of membrane lipid fatty acid saturation could be a mechanism to maintain internal pH, since a higher degree of fatty acid saturation leads to lowered fluidity and lower CO_2-permeability of cell membranes [20].

Ocean acidification experiments generally aim to allow the prediction of the test organisms' future response to elevated pCO_2 A recent study by Tortensson *et al.* [77] highlighted the need of conducting ocean acidification experiments under as close to natural conditions as possible, *i.e.*, nutrient concentrations, temperature, light intensities, *etc*. In the latter study the authors exposed the Antarctic diatom *Nitzschia lecointei* to 390 and 960 µatm CO_2 for 14 days under −1.8 and 2.5 °C temperature. They only found a significant difference in PUFA levels between the two CO_2 treatments at −1.8 °C. Increased temperature affected the PUFA levels more drastically than CO_2 concentrations. If this temperature dependency of the CO_2 response is universal for Antarctic phytoplankton, then this could explain why we saw very little significant differences in PUFA levels across our three species which were grown at approximately 2.9 °C. Furthermore, if the CO_2-induced response of phytoplankton biochemistry is greatly reduced under warmer temperatures, it is possible that a CO_2-signal was not detected under the noise of the data. Since ocean acidification experiments are often resource intensive, use of a large number of replicates is not always practical, which can make the detection of subtle differences difficult. While we performed every treatment in triplicate in our experiments, a larger number of replicates would improve our statistical power and ability to detect subtle differences between treatments. To facilitate increased replication the number of CO_2 treatments can be reduced in favor of more replicates per treatment. However, this reduces the resolution of a possible CO_2 dose-response-curve and the ability to detect any potential tipping points.

Except for EPA contents in *Gymnodinium* sp. and carbohydrate content in *Pyramimonas gelidicola*, we could not detect a CO_2 signal across all CO_2 concentrations, and often only found significantly different biochemical contents in the highest pCO_2 cultures compared to control cultures. As noted above, this could be due to noise in the data due to low replication, which only allows strong differences to be statistically significant. However, the chance of false positive results also increases with the number of parameters that are tested for. Increased replication and possibly reduced experimental temperatures are therefore recommended for future research.

4. Conclusions

In this study we have shown that an elevated CO_2 concentration has, at most, only modest effects on the biochemistry of three Antarctic phytoplankton species, although the responses were species-specific. It is unlikely that any phytoplankton species will be completely unaffected by changes in CO_2 concentration; however, the degree to which different species will be capable of tolerating ocean acidification, while simultaneously exposed to other climate-induced stressors, will determine which species will be the "winners and losers" in the future [39,78–80]. While some of the species studied here showed responses in line with current literature, some of the results were contradictory. Laboratory experiments are not able to include all phytoplankton species of the oceans. Thus, to improve our ability to predict future changes of mixed phytoplankton communities, we need to increase our understanding of the underlying mechanisms by which pH and CO_2 availability affect phytoplankton physiology. An enhanced understanding will help explain the differences in species-specific responses and thereby improve our ability to extrapolate laboratory based results of individual species to natural communities. Our study suggests that increases in pCO_2 have the potential to alter the nutritional quality of individual phytoplankton species available for grazers via species-specific changes in their biochemistry, particularly the fatty acid profiles as emphasized by examination of the essential long-chain PUFA, and this adds to the importance of understanding how phytoplankton will change in a high-CO_2 ocean.

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Author Contributions

The first author was responsible for all experimental work and sample analysis. The first and second authors were responsible for the concept and design of the experimental facilities. The third, fourth and fifth authors provided guidance during the experimental and analytical work and together with the seventh and eighth authors helped with the writing and presentation of the manuscript. The sixth author provided guidance with statistical analysis of the data.

Conflicts of Interest

The authors declare no conflict of interest.

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