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Long-term acclimation to elevated pCO_2 alters carbon metabolism and reduces growth in the Antarctic diatom *Nitzschia lecointei*

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Increasing atmospheric CO_2 levels are driving changes in the seawater carbonate system, resulting in higher pCO_2 and reduced pH (ocean acidification). Many studies on marine organisms have focused on short-term physiological responses to increased pCO_2, and few on slow-growing polar organisms with a relative low adaptation potential. In order to recognize the consequences of climate change in biological systems, acclimation and adaptation to new environments are crucial to address. In this study, physiological responses to long-term acclimation (194 days, approx. 60 asexual generations) of three pCO_2 levels (280, 390 and 960 µatm) were investigated in the psychrophilic sea ice diatom *Nitzschia lecointei*. After 147 days, a small reduction in growth was detected at 960 µatm pCO_2. Previous short-term experiments have failed to detect altered growth in *N. lecointei* at high pCO_2, which illustrates the importance of experimental duration in studies of climate change. In addition, carbon metabolism was significantly affected by the long-term treatments, resulting in higher cellular release of dissolved organic carbon (DOC). In turn, the release of labile organic carbon stimulated bacterial productivity in this system. We conclude that long-term acclimation to ocean acidification is important for *N. lecointei* and that carbon overconsumption and DOC exudation may increase in a high-CO_2 world.

1. Introduction

The projected increase of atmospheric pCO_2 from present day (approx. 400 µatm) to the end of the century (approx. 1000 µatm) will reduce the pH of the oceans by approximately 0.3 units [1]. High-latitude marine environments and ecosystems are particularly susceptible to ocean acidification as the solubility of CO_2 is higher in cold water [2,3]. The Southern Ocean has a naturally low carbonate saturation state and is believed to be one of the first oceans to become persistently undersaturated with respect to aragonite [3,4]. The ongoing ocean acidification is believed to affect organisms on all systematic levels [5], and especially in polar ecosystems [2].

Due to the low affinity of CO_2 to ribulose bisphosphate carboxylase/oxygenase (RuBisCO), ocean acidification has been suggested to stimulate primary productivity [6,7]. Antarctic sea ice algae are known to have widespread range of carbon concentrating mechanisms (CCMs) [8] that facilitate carbon fixation [9]. Furthermore, up to 40% of marine phytoplankton primary production is generally released as dissolved organic carbon (DOC) [10], although values over 70% have been observed [11]. Sea ice algae are known to excrete high amounts of extracellular polymeric substances (EPS) that constitute a substantial fraction of the DOC pool in sea ice [12,13]. EPS are produced as a normal cell function and used for motility, aggregation, desiccation, cryoprotection and protection

against ultraviolet radiation [13–15]. The release of DOC is an important nutrient source for heterotrophic bacteria and may therefore affect microbial ecology and biogeochemistry [16].

Sea ice is one of the most productive polar ecosystems, acting both by seeding pelagic phytoplankton blooms and as an important direct food source for higher trophic levels [17]. In addition, sea ice algae and bacteria are important in carbon biogeochemistry in polar areas. For instance, ice algae may contribute up to 25% of the annual primary production of ice-covered waters [18] and provide an important food source for grazers, such as krill [19]. In addition, approximately 20–30% of the primary production in sea ice is cycled through heterotrophic bacteria [20]. Diatoms are major primary producers in sea ice and play an important role in the sea ice microbial ecosystem [18], and could be sensitive to environmental change considering that they already grow in a stressful environment. Short-term responses in sea ice algae to increased $p\mathrm{CO_2}$ are highly variable, ranging from negative [21,22] to positive effects [9,22]. Results may also depend on temperature conditions [9] and manipulation technique [22]. Generally, effect sizes from ocean acidification experiments are quite small for sea ice algae. However, the potential of acclimation may be especially important in slow-growing organisms as short-term experiments may fail to address proper acclimation to high $\mathrm{CO_2}$.

Many recent studies that investigated physiological effects of ocean acidification on phytoplankton have been performed on a short-term basis, normally ranging from 7 to 15 days [9,21–23]. More specifically, short-term changes in $p\mathrm{CO_2}$ affect growth, fatty acid concentration and species competition of Antarctic microalgae [9,22,24,25]. Still, no long-term study (longer than one month) has previously been published on polar microalgae. In phytoplankton from other regions, long-term acclimation and adaptation have shown to affect community structure, growth, fatty acid and amino acid concentrations [26–28]. However, physiological responses to increased $p\mathrm{CO_2}$ are both strain- and species-specific [24,26,27,29,30]. In order to understand the impact of climate change, more long-term studies need to be performed to address the potential for acclimation and adaptation to ocean acidification. The aims of this study were to investigate growth and productivity in the Antarctic sea ice diatom *Nitzschia lecointei* van Heurck 1909 and its associated heterotrophic bacteria during long-term acclimation to different $p\mathrm{CO_2}$. Cells were maintained in semi-continuous cultures at three different $\mathrm{CO_2}$ levels (280, 390 and 960 μatm $p\mathrm{CO_2}$) over a period of 194 days, and sampled regularly throughout the experiment.

2. Material and methods

(a) Long-term acclimation set-up

The diatom *N. lecointei* was isolated from sea ice collected in the Amundsen Sea in January 2011 and was cultured at $-1.8°\mathrm{C}$ for 18 months before the experiment started. The diatoms were inoculated (3×10^6 cells $\mathrm{l^{-1}}$) under non-axenic conditions in f/2 medium [31], prepared from 0.2 μm filtered seawater with a salinity of 33. The culture was subsequently split into 15 identical, 1 l borosilicate flasks, and randomly placed ($n = 5$ per $\mathrm{CO_2}$ treatment) in a cooling water bath set at $-1.8°\mathrm{C}$. The $p\mathrm{CO_2}$ manipulation and agitation in each flask were performed by constant bubbling (10–15 ml $\mathrm{min^{-1}}$ flask^{-1}) of synthetic air (Air

Liquide, Malmö, Sweden) with different $p\mathrm{CO_2}$ (280, 390 and 960 μatm), using a system similar to that in Torstensson *et al.* [9]. The experiment was conducted under a 16 L : 8 D cycle at 30 μmol photons $\mathrm{m^{-2}\,s^{-1}}$ of photosynthetic active radiation (PAR 400–700 nm), provided by fluorescent tubes (Osram Lumilux Cool Daylight L36 W/865). Temperature was monitored throughout the experiment using HOBO Pendant data loggers (Onset Computer Corp., Pocasset, MA, USA). The long-term treatment lasted for 194 days and repeated sampling was conducted throughout the experiment. The cultures were maintained in active growth by semi-continuous dilution to a desired cell concentration (between 1 and 6×10^7 cells $\mathrm{l^{-1}}$, electronic supplementary material, figure S1) with pre-equilibrated f/2 medium from respective $\mathrm{CO_2}$-treatment. Dilutions were based on daily cell concentration in each flask, calculated and performed individually by carefully weighing (± 0.1 g) the removed culture and fresh medium. The positions of the flasks were rotated on a weekly basis to ensure equal radiation exposure. To reduce diatom and bacterial wall growth, the culture flasks were replaced with new, autoclaved and acid-washed flasks once a month (day 32, 62, 91, 125 and 159).

(b) Switched treatment assay

After the long-term acclimation, the treatments were switched during a short-term assay to investigate potential sustained long-term effects. Four flasks from the ambient (390 μatm) and four from the high $p\mathrm{CO_2}$ treatments (960 μatm) were chosen randomly and retained for the short-term assay. Cell suspensions from each flask were inoculated in two new flasks with f/2 medium. One of the two new flasks was then assayed under the same conditions as the long-term treatment (control), whereas the second flask was switched to the opposite condition (electronic supplementary material, figure S2), creating four assay treatments with four samples in each group. The short-term assay lasted for 13 days.

(c) Cell density and growth rate

Diatom cell density was counted in live samples using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) prior to dilution. For flow estimation, CountBright absolute counting beads (Invitrogen, Eugene, OR, USA) were added to each sample as an internal standard. Samples were analysed until a minimum of 300 cells were counted (minimum 1000 cells from day 32 and forward). The specific growth rate (μ, $\mathrm{d^{-1}}$) and number of asexual generations (n, number of times the cell population doubles during the time interval) generated was calculated using equations (2.1) and (2.2):

$$\mu = (\ln D_x - \ln D_y) \times (t_x - t_y)^{-1} \quad (2.1)$$

and

$$n = (\log D_x - \log D_y) \times (\log 2)^{-1}, \quad (2.2)$$

where D_x is the cell concentration at day x (before dilution), D_y is the cell concentration the previous day of measurement (after dilution), t_x is the time in days at day x (before dilution) and t_y is the time in days of the previous measurement (after dilution). Accumulation of generations for each day was subsequently used to estimate cumulative growth over time.

(d) Primary productivity

Primary productivity was measured using the radiocarbon technique. Ten millilitres of sample was incubated for 1 h with 3–4 μCi $^{14}\mathrm{C}$-sodium bicarbonate (PerkinElmer, Waltham, MA, USA) at experimental irradiance and temperature. A blank from each treatment was incubated in darkness to estimate dark uptake of $^{14}\mathrm{C}$. After incubation, formaldehyde (2%, final concentration) was added to all samples to stop carbon uptake.

At day 147 and 187, primary productivity was separated into primary productivity of dissolved organic carbon (PP_{DOC}) and particulate organic carbon (PP_{POC}) by gentle, low-pressure filtration on Whatman GF/F filters. Two drops of 1 M HCl were added to the liquid samples (PP_{DOC}) and the samples were bubbled with N_2 for 1 h to remove remaining dissolved inorganic carbon (DIC). Insta-Gel Plus scintillation cocktail (PerkinElmer) was added at a 1:1 ratio to the liquid samples and vials were shaken rigorously. The filters (PP_{POC}) were fumigated overnight after addition of 200 μl of 1 M HCl. Five millilitres of scintillation cocktail was added to the filters and samples were analysed in a liquid scintillation counter (Packard Tri-Carb 2900TR Liquid Scintillation Analyzer). Carbon uptake was corrected for dark uptake and calculated using equation (2.3):

$$^{12}C = \frac{^{14}C_s \times {}^{12}C_a \times 1.06}{^{14}C_a \times t},\qquad(2.3)$$

where ^{12}C is the carbon uptake rate (mg $C\,l^{-1}\,h^{-1}$), $^{14}C_s$ the radioactive activity (disintegrations per minute, DPM) in the sample, $^{12}C_a$ the available DIC (mg l^{-1}), 1.06 is the constant adjusting the fixation rate due to the discrimination of ^{14}C during carbon fixation, $^{14}C_a$ is the added activity of ^{14}C to the sample (DPM) and t is the incubation time (h).

(e) Bacterial abundance and productivity

In total, 1.5 ml of sample was fixed in 1% glutaraldehyde (final concentration) and stored at −80°C until analysis. Samples were stained with SYBR Green I Nucleic Acid Gel Stain (Invitrogen) for 10 min in darkness. Bacterial abundances were analysed using a FACSCalibur flow cytometer (BD Biosciences) using CountBright absolute counting beads (Invitrogen) as the internal standard in each sample.

Bacterial productivity was measured according to Fuhrman & Azam [32]. In total, 1.7 ml of sample was incubated in darkness at −1.8°C with ^{3}H-thymidine (100 nM final concentration, PerkinElmer) for 1 h. An additional sample from each treatment was used as a blank during the incubation, in which trichloroacetic acid (TCA, 5% final concentration) was added prior to the addition of ^{3}H-thymidine. To stop the incubations, TCA (5% final concentration) was added to all samples. Samples were centrifuged for 10 min at 21 000g and 4°C, and the supernatant was carefully aspirated. The pellets were rinsed with 5% TCA on ice and subsequently with 80% ethanol on ice. After rinsing, 0.5 ml of scintillation cocktail (Insta-Gel Plus, PerkinElmer) was added to the pellets. The ^{3}H-thymidine uptake was measured in a liquid scintillation counter (Packard Tri-Carb 2900TR Liquid Scintillation Analyzer), and converted into bacterial carbon productivity (BCP) by using 1.63×10^{18} cells mole^{-1} incorporated thymidine [33] and 20 fg C cell^{-1} [34]. The isotope concentrations were at satisfying levels according to saturation curves.

(f) Particulate organic carbon and nitrogen

A well-mixed sample (30–230 ml) was filtered onto pre-combusted (3 h at 450°C) Whatman GF/F filters. Samples were stored at −20°C until further analysis, subsequently dried at 60°C over a period of 48 h and the dry weight was measured. The filters were ground for 1 min using a Retsch MM301 ball mill (frequency 30 s^{-1}). Analysis of particulate organic carbon (POC) and nitrogen (PON) was performed with an EA 1108 CHNS-O elemental analyser (Fisons Instruments, Milano, Italy), using 2,5-bis(5-tert-butyl-bensoaxzol-2-yl)thiophen as the internal standard (provided by Säntis Analytical AG, Teufen, Switzerland).

(g) Dissolved inorganic nitrogen, phosphorus and silica

Dissolved inorganic nutrients ($NO_2^- + NO_3^-$, PO_4^{3-} and $Si(OH)_4$) were sampled to confirm that nutrients were not limiting growth.

Samples were filtered (0.2 μm) and stored at −20°C until colorimetric analyses [35] were performed at the Sven Lovén Centre for Marine Sciences, Kristineberg, Sweden.

(h) Dissolved inorganic carbon system

pH was measured spectrophotometrically on the total scale (pH_T), using m-cresol purple as an indicator [36]. After tempering at 25°C, the samples were analysed in a Shimadzu UV-1800 spectrophotometer (measured in the visible spectrum). The indicator was prepared from 0.2 μm-filtered seawater (salinity 33) and stored in an E.V.A. gastight container (Baxter, Mississauga, Canada) to avoid atmospheric perturbation of the indicator during the experiment.

Samples for total alkalinity (A_T) were stored in darkness overnight at 7°C. Prior to analysis, samples were tempered to 25°C and A_T and were measured potentiometrically in an open cell using an automatic titration system (Metrohm 888 Titrando, Metrohm Aquatrode Plus Pt1000). Each sample was titrated with 0.05 M HCl and the equivalence point was evaluated using the Gran function [37]. The accuracy for A_T data was checked and calibrated against analysed certified reference material (CRM), provided by Andrew G. Dickson, Scripps Institute of Oceanography, CA, USA. The CRM was titrated daily in triplicate with a variation of 0.5%.

Parameters of the carbonate system was calculated at *in situ* conditions (salinity, temperature and pressure) from measured values of pH_T, A_T, salinity and temperature using the program CO2SYS [38]. K_1 and K_2 constants used in calculations were determined by Mehrbach *et al.* [39] and refitted by Dickson & Millero [40]. The constant for SO_4^- was determined by Dickson [41].

(i) Statistical analyses

Individual and combined effects of CO_2 treatment and sampling day were analysed with linear mixed effects (LME) models in R v. 3.0.1 [42], using the package nlme [43]. Models were fitted using restricted maximum likelihood using both time and CO_2 concentration as fixed factors in a full model with interaction. The individual replicate flask was used as random factor in all analyses to account for pseudoreplication over time. Due to unsuccessful transformation of heterogeneous variances across residuals for cumulative growth data, modelling of the within-error group (per day) was performed to control for heteroscedasticity using the varIdent argument [43,44]. LME models are useful tools that can provide a better fit than generalized linear models (e.g. repeated measure ANOVA) on longitudinal data, especially when dealing with missing data and unequal spacing over time [45]. Multiple comparisons of means were performed on significant effects using generalized linear hypothesis test (glht) and Tukey's (HSD) test in the multcomp package [46]. Interaction in growth data was explored by contrasts defined to compare means between CO_2 levels for each day, and Bonferroni correction was applied to account for multiple testing. Statistical significance was determined using a probability level of $\alpha < 0.05$.

3. Results

(a) Growth rate

During the 194 days of experiment, 55–62 asexual generations of *N. lecointei* were accumulated, and the accumulation of generations was used to estimate cumulative growth over time. There was a significant interaction between pCO₂ treatments and sampling day on accumulated generations ($p < 0.0001$, LME). Accumulation of generations decreased in the 960 μatm pCO₂ treatment relative to the 280 and 390 μatm pCO₂ treatments (figure 1). The deviation in accumulated
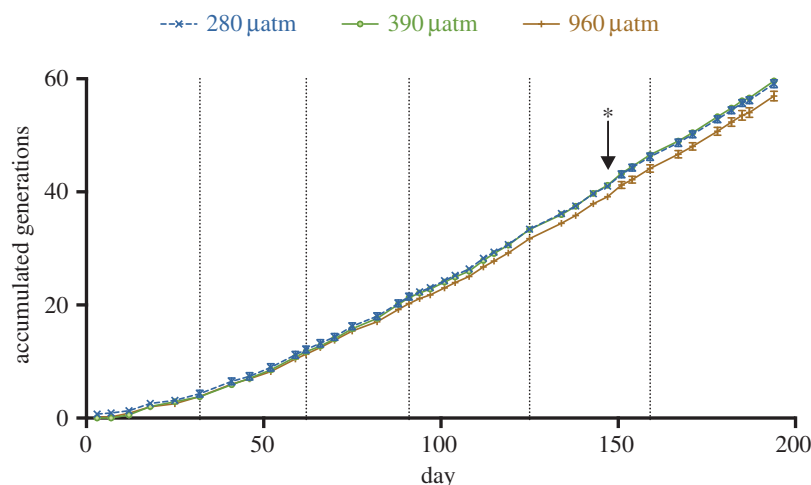
Figure 1. Accumulation of cell generations (cumulative growth) of *N. lecointei* in three pCO_2 treatments over a period of 194 days. Accumulation of generations at 960 μatm was significantly lower compared with 280 and 390 μatm pCO_2 at day 147*. Dotted vertical lines show the days where the culture flasks were acid-washed and autoclaved. Error bars represent standard error ($n = 5$). (Online version in colour.)

generations from the 280 and 390 μatm treatments started at day 147 (after 40 accumulated generations), and persisted throughout the experiment ($p < 0.05$, Tukey's test). However, analysis of the specific growth rate did not detect a significant interaction between sampling day and pCO_2 (electronic supplementary material, figure S3a, $p = 0.084$, LME), although the main effects of pCO_2 and sampling day were significant ($p = 0.038$ and $p < 0.0001$, respectively, LME). The specific growth rate was lower at 960 μatm compared with 280 and 390 μatm pCO_2 ($p = 0.017$ and $p = 0.022$, respectively, Tukey's test).

(b) Primary productivity

Total primary productivity (PP_{TOT}) was significantly affected by CO_2 treatment and sampling day, respectively (figure 2a, $p = 0.004$ and $p < 0.0001$, respectively, LME). A significant difference in PP_{TOT} between the 280 μatm and the 960 μatm treatments was detected ($p < 0.01$, Tukey's test). In addition, the 280 μatm treatment was significantly lower than the 390 μatm level ($p = 0.03$, Tukey's test). Specific PP_{TOT} was highest at day 97 and decreased significantly at the following two sampling days ($p < 0.01$, Tukey's test).

When separating PP_{TOT} into PP_{DOC} and PP_{POC} at day 147 and 187, various effects of treatments and sampling day were found. Significant effects of pCO_2 treatment and sampling day were detected on PP_{DOC} (figure 2b, $p = 0.006$ and $p = 0.01$, respectively, LME). PP_{DOC} in the 960 μatm pCO_2 treatment was significantly higher than the other two treatments ($p < 0.02$, Tukey's test). In addition, a significant increase in PP_{DOC} was detected from the first to the second sampling day ($p < 0.02$, Tukey's test).

There was a significant interaction of pCO_2 treatment and sampling day on PP_{POC} (figure 2c, $p = 0.003$, LME). At day 147, PP_{POC} was significantly lower at 280 μatm compared with 390 and 960 μatm pCO_2 ($p = 0.001$ and $p < 0.001$, respectively, Tukey's test). However, at day 187, PP_{POC} was reduced at 960 μatm compared to 390 μatm pCO_2 ($p = 0.004$, Tukey's test).

(c) Bacterial abundance and productivity

Bacterial cell concentrations ranged between 0.3 and 8.1 × 10^6 cells ml^{-1} during the semi-continuous culturing of

N. lecointei (electronic supplementary material, figure S3b). Cell-specific BCP was significantly affected by pCO_2 treatment ($p < 0.001$, LME). Post hoc test indicated that all treatments differed and that bacterial productivity was stimulated by increased pCO_2 (figure 2d, $p < 0.02$, Tukey's test). Heterotrophic bacteria consumed on average 5.6% of the daily DOC produced by *N. lecointei* in all treatments (figure 3).

(d) Particulate organic carbon and nitrogen

No difference in cellular POC between pCO_2 treatments was detected ($p > 0.05$, LME), however, there was a difference over time. Cellular POC at day 18 was significantly higher compared with all other sampling occasions (figure 4a, $p < 0.01$, Tukey's test). In addition, there was no difference in cellular dry weight (250 ± 107 pg cell^{-1}, average ± s.d. throughout the experiment) between the CO_2 treatments ($p > 0.05$, LME), indicating that pCO_2 treatment did not affect the cell size of *N. lecointei*.

Cellular PON varied significantly throughout the long-term exposure (figure 4b, $p < 0.0001$, LME). PON values at day 147 and 171 were significantly lower than at day 18 and 97 ($p < 0.002$, Tukey's test). However, no difference between pCO_2 treatments was detected ($p > 0.05$, LME). The PON of three samples were below the detection limit and thereby omitted from the analysis. CN ratios varied between 5.5 and 10.2 in all samples, with the highest levels at day 18. No difference in CN ratio between pCO_2 treatments was detected ($p > 0.05$, LME).

(e) Sustained effect of dissolved organic carbon excretion after switched treatment assay

To determine the possibility of sustained effects from long-term exposure of elevated pCO_2, long-term acclimated cells from the 390 and 960 μatm pCO_2 treatments were assayed under switched pCO_2 conditions in a 13-day short-term assay (electronic supplementary material, figure S2). Although PP_{DOC} were higher compared with the long-term study, there was a significant interaction in the PP_{DOC} fraction between long-term pCO_2 treatments and assay treatments (figure 5, $p = 0.048$, LME). For diatoms acclimated to 960 μatm, the
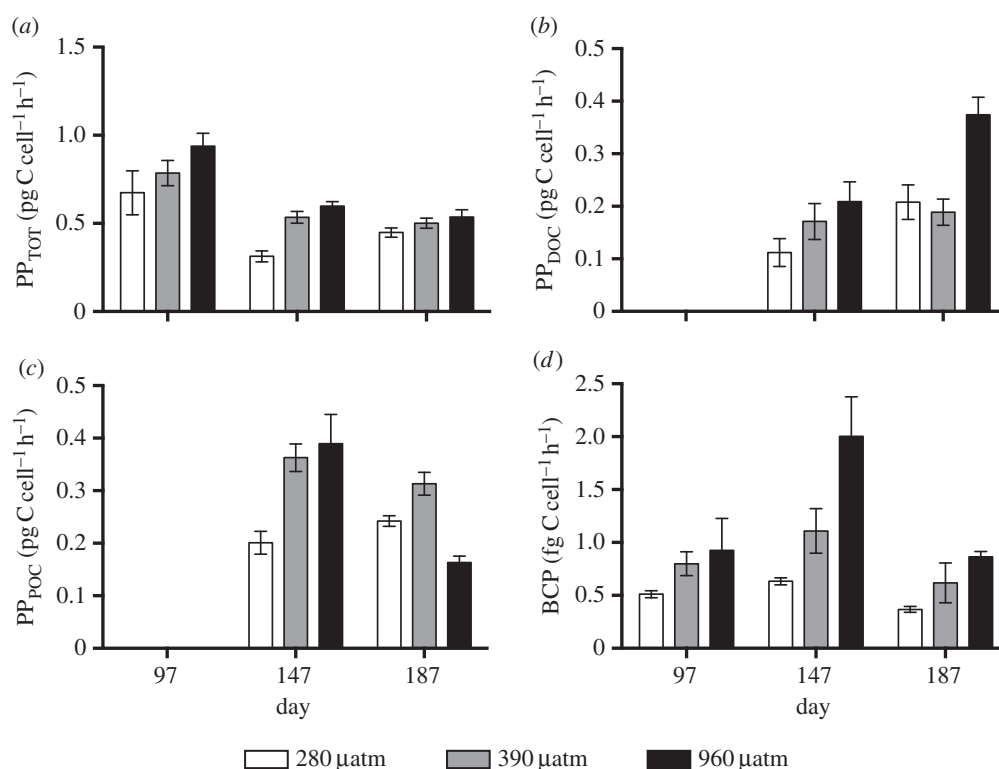
Figure 2. Carbon production rates of *N. lecointei* cultures during long-term exposure of three $p\mathrm{CO_2}$ levels (280, 390 and 960 µatm). (*a*) Total primary productivity (PP$_{TOT}$). (*b*) Dissolved organic carbon primary productivity (PP$_{DOC}$). (*c*) Particulate organic carbon primary productivity (PP$_{POC}$) and (*d*) bacterial carbon productivity (BCP). All values are normalized to cell count and error bars represent standard error ($n = 5$).
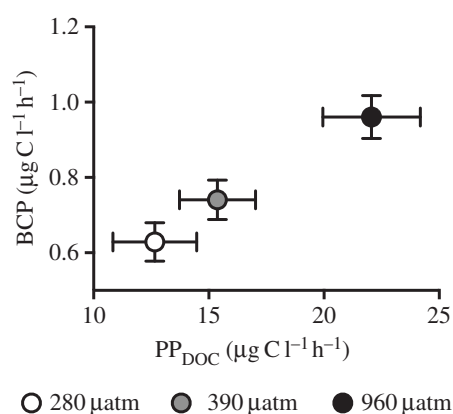

Figure 3. Relationship between volumetric bacterial production and primary productivity of dissolved organic carbon. Average primary productivity of DOC (PP$_{DOC}$) and bacterial carbon productivity (BCP) are calculated from all data points during the experiment. Error bars represent standard error ($n = 10$).

short-term assayed cells at 960 µatm had a higher proportion of PP$_{DOC}$ compared with cells assayed at 390 µatm $p\mathrm{CO_2}$ (figure 5). However, there was no difference between the assays for cells that were acclimated to 390 µatm $p\mathrm{CO_2}$. No differences between treatments were detected in PP$_{TOT}$, PP$_{POC}$, POC, PON or BCP ($p > 0.05$, LME).

(f) Dissolved inorganic carbonate system

The pH$_T$ and $p\mathrm{CO_2}$ were significantly differentiated in the three $p\mathrm{CO_2}$ treatments ($p < 0.001$, LME), indicating that the carbonate system was successfully controlled by the $p\mathrm{CO_2}$ manipulations (electronic supplementary material, figure S4a,b). Values of the high $p\mathrm{CO_2}$ treatment were in the ranges of predicted atmospheric $p\mathrm{CO_2}$ levels by year 2100 [1]. However, high primary productivity resulted in slightly lower $p\mathrm{CO_2}$ than the anticipated levels (i.e. 280, 390 and 960 µatm $p\mathrm{CO_2}$, respectively). Inorganic nutrient concentrations did not indicate nutrient (NO$_2^-$ + NO$_3^-$, PO$_4^{3-}$, Si(OH)$_4$) depletion during the study (electronic supplementary material, tables S1 and S2).

4. Discussion

In this study, we investigated long-term acclimation (194 days, approx. 60 generations) of the sea ice diatom *N. lecointei* to changes in $p\mathrm{CO_2}$. Acclimation and adaptation are crucial in understanding the consequences of ocean acidification, but no published studies have previously addressed long-term responses to increased $p\mathrm{CO_2}$ in slow-growing psychrophiles. We observed that long-term exposure to elevated $p\mathrm{CO_2}$ affected growth rate and carbon metabolism in *N. lecointei*.

In comparison with cultures exposed to pre-industrial (280 µatm) and ambient (390 µatm) $p\mathrm{CO_2}$, the accumulation of generations was slightly reduced at high $p\mathrm{CO_2}$ (960 µatm) after long-term acclimation. These results are complementary to a recent short-term study where no change in specific growth rate was detected in *N. lecointei* after 14 days exposure to 960 µatm $p\mathrm{CO_2}$ at -1.8°C, although increased $p\mathrm{CO_2}$ had a positive effect on growth at higher temperatures [9]. Another recent short-term study also concluded that natural sea ice algal communities could be very tolerant to changes in $p\mathrm{CO_2}$ and pH [22]. We would like to emphasize that reduction in growth was first observed after 147 days in this study. Hence, it is unlikely that small changes in growth, like the ones illustrated in this study, are detected during short experimental periods. Proper acclimation to different conditions may also influence interpretations of short-term experiments. Therefore, long-term studies are crucial for estimating potential consequences of climate change
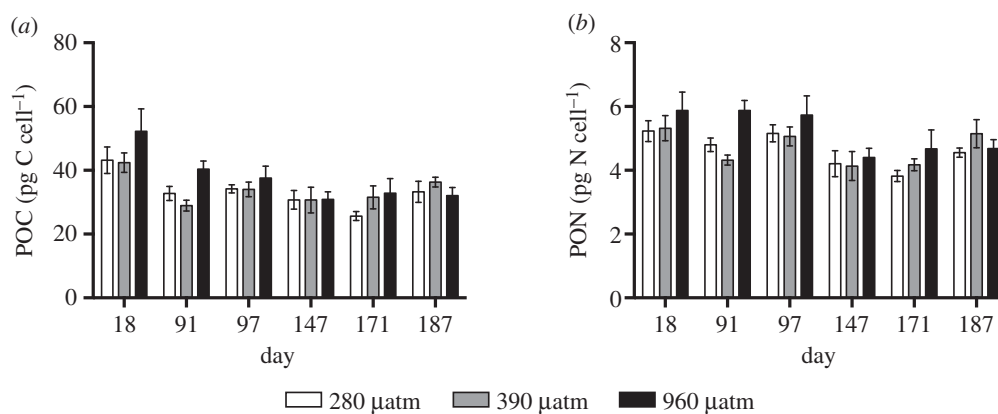
Figure 4. Particulate organic carbon (POC) and nitrogen (PON) of *N. lecointei* under the long-term acclimation to three pCO_2 levels (280, 390 and 960 µatm). (*a*) Cellular POC values. (*b*) Cellular PON values. Error bars represent standard error ($n = 5$).
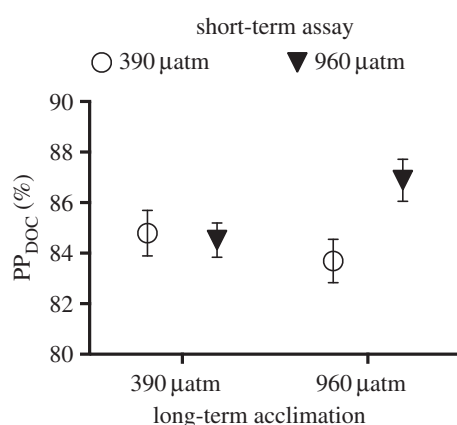

Figure 5. Percentage of primary productivity released as dissolved organic carbon (PP_{DOC}) after the switched treatment assay. Cultures have been acclimated to 390 and 960 µatm pCO_2 for 194 days, and subsequently short-term assayed for 13 days with the opposite treatment to study sustained effects of pCO_2 acclimation. Error bars represent standard error ($n = 4$).

on algal physiology. Increasing CO_2 levels (from approx. 400 to approx. 1000 µatm) seem to have rather limited effects on growth of sea ice associated diatoms, ranging from 0 to 5% ([9,21], this study), but can have more significant effects at higher pCO_2 levels (more than 1700 µatm) [22]. Cumulative growth has the advantage of including small changes that do not occur simultaneously in all replicates, which is not unlikely over long experimental durations and could explain why there was no interaction in specific growth rate. Although cumulation is more sensitive towards systematic errors at specific time points, analysis of the specific growth rate suggests that there were no treatment-specific biases at specific sampling days (no interaction). In terms of climate change within the next century, other factors than increased CO_2, such as increased temperature [9,47] and sea ice thinning [48], will most likely play a more important ecological role in polar ecosystems. Synergisms between combined environmental stressors may also alleviate effects [9] and are important to consider. However, our study still illustrates the importance of experimental duration in ocean acidification studies, as acclimation may delay certain responses. In addition, changes in algal carbon metabolism (i.e. increased DOC release) may have greater ecological and biogeochemical consequences than a 3–4% reduction of growth.

Earlier long-term studies on marine and freshwater phytoplankton have shown varying growth responses to high pCO_2. Schaum & Collins [30] cultured the chlorophyte *Ostreococcus* for 400 generations and observed high growth rates, although growth rate at high pCO_2 was not suppressed until 100 cell cycles. In contrast, Low-Décarie *et al.* [49] reported increasing growth rates after less than 340 generations (184 days) of high pCO_2 conditioning in various freshwater species of diatoms, chlorophytes and cyanobacteria. In addition, no difference in growth was reported after 1000 generations in the chlorophyte *Chlamydomonas reinhardtii* grown at ambient and high pCO_2 [50]. Furthermore, high pCO_2 did not promote growth after long-term conditioning of six diatom genera [51], nor various dinoflagellates [27]. In contrast, to other long-term studies, there were considerably fewer cell cycles in our experiment (approx. 60 generations) due to the natural slow growth rate of ice algae. Hence, the potential for biological adaptation is less plausible in our experiment. In contrast, slow-growing organisms will also accumulate less cell cycles within the next century, and acclimation to climate change will be an important factor in determining polar species' responses to climate change.

Species [51] and strain-specific [26,29] responses to elevated pCO_2 are surprisingly not occurring and this is probably related to the alga's carbon metabolism and CCM efficiency. In our study, carbon metabolism was altered both during the long-term exposure, and also during the switched treatment assay. For instance, PP_{DOC} was promoted by elevated pCO_2 during the long-term acclimation. This may be due to a response referred to as carbon overconsumption, i.e. an increase in carbon relative to nitrogen and phosphorus assimilation as compared to the Redfield ratio of 106C : 16N : 1P [52]. This imbalance generally occurs when nutrients are depleted and often results in increased DOC leakage from the cells. Carbon overconsumption may also occur as a response to increased pCO_2 [7,53]. Since nutrient levels were kept replete in this study, the increased DOC excretion is most likely a direct effect of elevated DIC levels. Hence, an imbalance in the C : N : P ratio can have significant effects on the carbon metabolism in *N. lecointei*. However, it is also important to consider that nutrient levels are much higher in this study than expected in natural samples. Thus, care should be taken when interpreting results from laboratory studies.

In this study, release of DOC was generally high compared with phytoplankton communities. For instance, Engel et al. [53] reported on average 21–23% PP_{DOC} of PP_{TOT} in mesocosms with a natural Arctic phytoplankton community exposed to different pCO_2 treatments. Generally, DOC release in natural marine phytoplankton communities does not exceed 40% of the total primary production [10]. Nevertheless, sea ice algae are known to exude high amounts of EPS [12], which is believed to play an important role in cryoprotection in microorganisms [14]. DOC release is also related to the state and age of the culture, and can exceed values measured in the field [54]. Hence, high levels of DOC release can be anticipated in cultured sea ice algae, growing in temperatures close to the freezing point of seawater [13]. As a result, the absolute values of DOC release are probably overestimated compared with natural environments. However, it is unlikely that the relative differences between the treatments are artefacts from culturing conditions.

Although the percentage of DOC release in the switched treatment assay was relatively high, it indicated that increased PP_{DOC} was a sustained alteration of physiology caused by long-term exposure to high pCO_2. On the other hand, this sustained effect after the assay treatment was only reflected in PP_{DOC}, and not in growth. Although DOC excretion is a response of algal growth, DOC leakage does not promote growth. Increased cellular DOC excretion has also been correlated to combinations of environmental stressors [55] and carbon overconsumption [52,53]. Acclimation to high pCO_2 may have affected the gene expression in N. lecointei, and needs to be further investigated. Sustained alteration of physiology as a result of long-term exposure of high pCO_2 has been observed in other studies. Consistent with the results from this study, Lohbeck et al. [26] reported decreased growth rates in the haptophyte Emiliania huxleyi after less than 430 generations of high pCO_2 conditions. It could be debated whether this should be considered a positive effect, as the overall growth rate decreased in comparison to E. huxleyi treated at ambient pCO_2. However, high pCO_2 treated multi-clone samples had higher growth rates compared to the controls when assayed at high pCO_2 after 500 generations. In our study, we confirm sustained effects after considerably less cell cycles, suggesting that the response in N. lecointei is rather acclimation, and probably not adaptation. The number of generations is indeed important for adaption to a changing environment, and may be of special concern in slow-growing organisms such as psychrophilic algae.

The mechanisms behind potential stress of ocean acidification on microalgae are not yet fully understood. It is possible that ocean acidification affects the membrane potential in algae by altering intracellular pH [56] and can affect enzymatic processes and energy partitioning in the cells [57,58]. A 5% reduction in growth rate at high pCO_2 has previously been reported in the Arctic sea ice diatom Navicula directa [21]. Increased release of DOC in N. lecointei could be interpreted as an indication of physiological stress. One plausible explanation for reduced growth rate and increased DOC exudation could be due to changes in enzyme activities. For instance, elevated CO_2 levels have recently been described to affect the activity of carbonic anhydrase and nitrate reductase in the rhodophyte Corallina officinalis [58]. If enzymes related to the assimilation of inorganic nutrients are suppressed in N. lecointei, e.g. nitrate reductase [58], carbon overconsumption and reduced growth rate are

expected to occur simultaneously. Although enzymes are highly influenced by pH, there are few published data on enzymatic processes in algae exposed to CO_2 levels projected within this century. However, it has been shown that several extracellular hydrolytic enzymes in bacterial communities, including leucine aminopeptidase and lipase, are negatively affected by ocean acidification [59]. The effect of ocean acidification on different enzyme activities and pH homeostasis in algae may be important in algal physiology [56,58] and should be further investigated.

Sea ice algal communities live associated with bacteria in aggregations of EPS predominantly produced by diatoms [12]. Bacterial abundance and BCP in this study were comparable to natural environments [14,47]. In addition, bacterial activity was closely related to PP_{DOC}, suggesting that bacterial growth was limited by labile organic carbon in the cultures. Hence, we assume that carbon overconsumption in N. lecointei nourished heterotrophic bacteria at high pCO_2 and therefore had an indirect effect on bacterial productivity. Similar observations have been seen in natural Arctic spring bloom assemblages [53]. DOC release also plays an important role in carbon biogeochemistry. It promotes the microbial loop [16] and aggregation of cells [13], the latter possibly resulting in an enhanced biological carbon pump due to increased sinking speeds. Hence, the coupling between primary producers and heterotrophic bacteria may cause indirect effects of ocean acidification, and may be important to consider when interpreting experimental data. Other factors influencing these observations may be pH sensitive enzymes and bacterial community evolution. It has been shown that extracellular hydrolytic enzymes are negatively affected by ocean acidification [59], which could directly influence bacterial productivity. Still, this does not fully explain an increase in BCP at high pCO_2 as we identified a positive effect of increased pCO_2. There is also a potential for uncontrolled bacterial evolution and changes in bacterial community composition in our system as it also allows for bacterial growth. Although this is a very important aspect, this needs a different approach, as we do not fully know the origin and history of the bacteria in this study. Therefore, we choose not to discuss bacterial evolution in detail. Carbon dioxide induced changes in the bacterial community composition may, however, influence the feedback mechanisms of carbon metabolism in this study. On the other hand, effects of increased CO_2 (up to approx. 1 050 μatm) in Arctic bacterioplankton communities have previously been shown to be rather small [60]. Therefore, we believe that increased BCP was most likely an indirect response of increased DOC release from N. lecointei.

Polar areas are believed to be particularly susceptible to climate change [2], and sea ice algae are a vital part of primary production in polar oceans [17]. This is the first published study that addresses long-term acclimation to elevated pCO_2 in psychrophilic organisms and illustrates that long-term acclimation is important in determining mechanistic responses of diatoms to ocean acidification. We conclude that certain, small changes in growth can only be detected over longer periods of time (147 days in this study). Long-term acclimation to high pCO_2 may also affect the carbon metabolism of N. lecointei, with increased rates of DOC release as an outcome. In turn, this promotes bacterial growth and productivity, important for carbon biogeochemistry and microbial ecology.

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