

RESEARCH ARTICLE

Dissolved inorganic carbon driven dynamics of calcite shell formation in 12 strains of the freshwater algae *Phacotus lenticularis* (Chlorophyta)

Uta Gruenert^{1*}, Jan Benda¹, Oliver Bossdorf¹, Uta Raeder²

1 Eberhard Karls Universität Tübingen, Auf der Morgenstelle, Tübingen, Germany, **2** Limnological Research Station Iffeldorf, Technical University of Munich, Munich, Germany

* uta.gruenert@uni-tuebingen.de



OPEN ACCESS

Citation: Gruenert U, Benda J, Bossdorf O, Raeder U (2026) Dissolved inorganic carbon driven dynamics of calcite shell formation in 12 strains of the freshwater algae *Phacotus lenticularis* (Chlorophyta). PLoS One 21(6): e0349771. <https://doi.org/10.1371/journal.pone.0349771>

Editor: Susmita Lahiri (Ganguly), University of Kalyani, INDIA

Received: July 21, 2025

Accepted: May 5, 2026

Published: June 26, 2026

Copyright: © 2026 Gruenert et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data availability statement: The raw data on shell morphology, cell growth and the R-Script are deposited at Zenodo (<https://doi.org/10.5281/zenodo.17400610>).

Funding: The author(s) received no specific funding for this work.

Abstract

The present rise in temperature, pCO₂ and altered precipitation impact lake water alkalinity and dissolved inorganic carbon (DIC) dynamics. Such changes on carbonate chemistry have been shown to modify calcification of shell-forming phytoplankton in marine ecosystems. Similar responses in freshwater systems remain largely unexplored. In this study, we investigate the direct effects of DIC concentration changes on the calcification state of *Phacotus lenticularis*, a globally abundant unicellular freshwater phytoplankton. The flagellated green algae are major contributors to modern lake carbonate production during bloom formation. *P. lenticularis* shells have a high CaCO₃ content compared to other pelagic calcifiers and are likely more sensitive to changing lake water carbonate chemistry. We isolated 12 *P. lenticularis* strains and exposed them to an ecologically relevant range of DIC (0.2 to 12 mmol L⁻¹ total scale) in a culture experiment. By means of high resolution scanning electron microscopy (SEM) and automatic image analysis we measured functional responses and strain-specific variability in response to DIC changes. All *P. lenticularis* strains showed reduced shell thickness by up to 60% and dissolved calcite crystals structures at declining DIC < 4 mmol L⁻¹, while increasing DIC > 4 mmol L⁻¹ had no significant effect on shell morphology. We also found no dependence of growth rates up to a lethal DIC of > 10 mmol L⁻¹, pointing to an efficient photosynthetic rate of *P. lenticularis* in an under-saturated as well as saturated inorganic carbon environment. *Phacotus* strains showed a preadaptation to ambient DIC concentrations measured in their lake of origin. Strains from the more environmentally dynamic lake Gönningersee exhibited more variable growth rates and cell densities compared to strains from the more stable Großer Ostersee. We hypothesize, that reduced availability of dissolved inorganic carbon and a lowered saturation state with regard to calcite will drive a negative calcification response in *P. lenticularis*. However, intraspecific variations in

Competing interests: The authors have declared that no competing interests exist.

sensitivity to DIC changes were evident in our study and may represent a geographically available potential to adapt to new stressors.

Introduction

Biological calcification is an important climatic feedback and, in phytoplankton, is highly impacted by changes in water carbonate chemistry [1–6]. In alkaline lakes, which comprise more than half of all inland waters, dissolved inorganic carbon (DIC) is the dominant form of aquatic carbon [7]. DIC in waters is composed of bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}). These anions are associated with dissolved carbon dioxide (CO_2) via the carbonate equilibria. DIC levels in lake waters vary considerably on seasonal and daily scales with values between 0.1 to 100 mmol L^{-1} [8,9]. Variability depends primarily on carbonate inputs (riverine and groundwater loads, atmospheric CO_2 input) and carbonate outputs (water outflow, groundwater leakage, degassing of CO_2 to the atmosphere and calcite precipitation [10–13]. Importance of lake metabolism in driving DIC changes has been found to diminish with increasing alkalinity [7,14]. Various aspects of climate change, including altered precipitation patterns promoting higher weathering rates and ion transfer, falling lake and groundwater tables and increased soil respiration have been identified as relevant drivers of hard-water lake chemistry [15–18]. Future trends for these lakes remain uncertain and predict increasing ion concentrations and greater chemical variation [19,20]. While growth of freshwater phytoplankton is expected to be stimulated in future climate change scenarios it remains speculative how calcite production and competitive fitness of calcifying phytoplankton will be affected [21].

The most abundant modern species of freshwater calcifying phytoplankton is *Phacotus lenticularis*, a bloom-forming member of the order *Chlamydomonadales* which typically have two flagella for locomotion [22,23]. The unicellular alga fixes dissolved inorganic carbon by forming a lens-shaped calcite shell. Shell formation is thought to be genetically controlled. Ions are concentrated within intracellular vesicles to form small calcite crystals. The crystals exit the cell and fuse to form a thin continuous shell that covers the outer lorica surface [24]. A second phase of growth subsequently forms the highly organized ring-like calcite crystals structure of the mature shell. It is yet unknown if *P. lenticularis* uses specific ion channels and pumps in the daughter cell plasma membranes to increase Calcium concentrations and pH. Only two other unicellular marine calcifiers, coccolithophores and foraminifera, have been found to perform intracellular crystal formation [25,26].

P. lenticularis shells consist of 98% – 99% pure CaCO_3 and contribute significantly to the removal of epilimnetic carbon via the combined effects of calcification and the downward transport of organic carbon in sinking aggregates to the sediments [4,23,27,28]. At the same time, the precipitation of calcium carbonate during calcite shell formation releases CO_2 and this way reduces total alkalinity [29]. *P. lenticularis* influences carbonate flux in hard-water lakes, particularly during bloom periods [22,23]. Lake sediments containing significant amounts of *P. lenticularis* shells were reported from many continents [30–32].

A growing number of studies on marine pelagic coccolithophore species, strains and morphotypes suggests variable response patterns of calcification rates, shell morphology and growth to changes in carbonate chemistry in particular to elevated CO_2 partial pressures [1,33–35]. These findings have been linked to a high genetic variability in coccolithophores due to their global distribution [36,37]. Photosynthesis and calcification of *Emiliana huxleyi* were stimulated due to additional HCO_3^- uptake in a high DIC concentration culture experiment, whereas growth was unaffected [38]. The same experiment showed inhibited coccolithophorid growth and calcification at increased H^+ concentrations. Adaptation by increased tolerance to ocean acidification has been observed in experimental evolutionary studies on clonal replicates of coccolithophores [39]. Zhang et al. [40] additionally found higher optimal pCO_2 growth and higher tolerance to low pH in populations isolated from places with larger environmental variability. The work to date is already providing valuable information on marine coccolithophores sensitivity or resilience to climate change. Still so far, there has been no systematic examination of how projected changes in carbonate chemistry will affect the physiology and calcification of freshwater calcifying phytoplankton.

How DIC influences calcification of freshwater phytoplankton is a central component for understanding mechanisms of biological carbonate formation in lakes and their relationship with climate change. We fill this gap by performing trait based culture experiments on freshly isolated *P. lenticularis* wild-type strains over a wide DIC range. The spatial isolation of alkaline lakes can limit dispersal of freshwater phytoplankton, restrict gene flow between geographic populations, and result in their genetic differentiation [41–43]. Freshwater calcifying algae have rarely been studied because of difficulties to isolate and cultivate this algal group for controlled experiments. Furthermore, it is often argued that experiments using long-term laboratory cultures may document evolutionary adaptations to changes induced by the culture conditions [44]. We isolated 12 strains from two lakes in southern Germany differing in lake morphology and DIC content and immediately assessed their growth, cumulative inorganic carbon production and calcite shell morphology parameters as a function of DIC and carbon ion concentrations. Our study aims to investigate whether geographical variation in a lakes ambient carbonate chemistry exerts a selection pressure on *P. lenticularis* shell formation.

Methods

Sample collection

Plankton samples were collected by 100 μm mesh net tows taken from Lake Gönningersee (48°25'32.5"N 9°10'38.4"E) and Lake Großer Ostersee (47°47'30.6"N 11°18'03.5"E), South Germany between July and September during *Phacotus* bloom formation (Fig 1). Lake Gönningersee is a shallow (maximum depth: 4 m), mesotrophic lake receiving high but variable input of carbonate rich stream water. Lake Großer Ostersee is a deep (maximum depth: 29 m), oligotrophic lake with relatively little variation in pH and DIC and regular groundwater inflow of carbonates. Seasonal DIC concentrations in the lake surface water ranged from 4.8 to 5.7 mmol L^{-1} in Lake Gönningersee and from 4.2 to 4.7 mmol L^{-1} in Lake Großer Ostersee. Environmental parameters for the sampling locations were repeatedly measured directly before plankton sampling (temperature and pH) and *in vitro* (total alkalinity (T_A) and major nutrients). Permit for field sampling at Lake Großer Ostersee was granted by Regierung von Oberbayern, Bereich 5 – Umwelt, Gesundheit und Verbraucherschutz in cooperation with the Limnological Research Station of the Technical University Munich. No permission was needed for sampling at the publicly accessible Lake Gönningersee.

Phacotus lenticularis cells were isolated from plankton samples by serial dilution with specific microcapillaries (borosilicate; 1.5-mm outer diameter; GB150F-8P; Science Products, Hofheim, Germany) pulled on a P97 puller (Sutter Instruments, Novato, CA, USA) and broken to an appropriate diameter. N-HS-Ca culture medium was prepared as described by Schlegel 2000 [45]. Nitrate (NO_3^-), phosphate (PO_4^{3-}) and calcium (Ca^{2+}) were added in concentrations representing ambient lake nutrient concentrations of 160, 10 and 1500 $\mu\text{mol l}^{-1}$, respectively. *P. lenticularis* cells were placed in culture medium immediately after isolation. Strains from Lake Gönningersee likely belong to morphotype III while strains from Lake Ostersee belong to morphotype IV as distinguished by Schlegel [45].

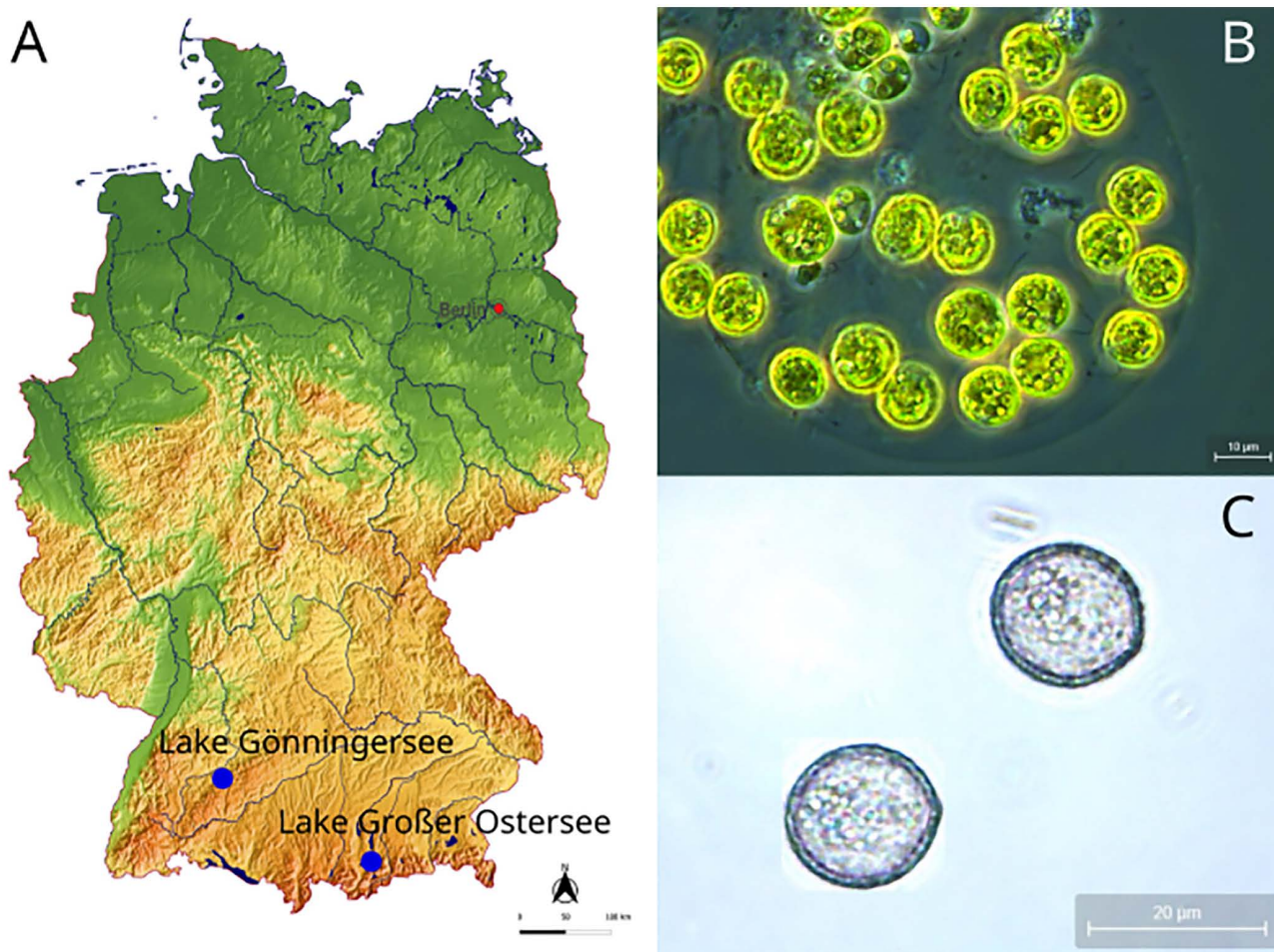


Fig 1. Locations of Lake Gönningersee in the federal state Baden-Württemberg and Lake Großer Ostersee in the federal state Bavaria (A), light microscope image of a *P. lenticularis* strain isolated from Lake Großer Ostersee (B, 100x magnification), light microscope image of two shell halves from a *P. lenticularis* strain isolated from Lake Gönningersee (C, 100x magnification).

<https://doi.org/10.1371/journal.pone.0349771.g001>

Experimental design

Phacotus lenticularis strains were inoculated in triplicate shake flasks and assigned to a range of DIC concentrations (7 treatments, DIC = 0.2, 1, 4, 6, 8, 10, 12 mmol L⁻¹) two to four months after isolation. The lower DIC levels corresponded to seasonal variations found in middle and hard water lakes (0.2–6 mmol L⁻¹, [46]). The higher DIC levels exceeded this range up to the point where no growth of *Phacotus* cells was monitored anymore. Treatment culture medium was prepared using particulate and dissolved inorganic carbon free N-HS-Ca culture medium. DIC and T_A were increased by addition of sodium bicarbonate and magnesium carbonate. Carbonate chemistry manipulation is more analogous to changes expected in lake surface waters in the next decades than gaseous CO₂ addition [13]. Cells were inoculated in treatment medium at an initial cell concentration of ~ 50 cell ml⁻¹ in 500 ml shake flasks and placed on a shaker. Cultures were grown at 20 °C (±0.15 °C) with an incident photon flux of 100 µmol photons m⁻² s⁻¹ PAR (ATUM LEDbar 18W, Klutronic GmbH, Klagenfurt) under a stable 12 hour light/dark cycle in the Plant Evolutionary Ecology Lab at University of Tuebingen. In the 4, 6 and 8 mmol L⁻¹ DIC treatments, where higher cell densities of approx. 40 000 cells ml⁻¹ were reached, 10 ml aliquot was sub-cultured into new medium after seven days to minimize changes in carbonate chemistry and nutrient availability.

and to avoid self-shading. Cultures were kept at experimental DIC concentrations for 14 days. T_A , DIC, pH and temperature were measured immediately after preparation of the medium, at the beginning of the experiment, after 7 days and after 14 days at the end of the experimental treatment to control for stable DIC and T_A concentrations. Calcium concentrations were kept constant at 1.5 mmol L^{-1} and pH was not manipulated.

Alkalinity was determined by acidimetric titration with 0.01 M HCl , in triplicates. DIC concentrations were calculated from measurements of acid neutralising capacity, pH, temperature, and specific conductivity according to Mackereth et. al [47] and reported as the average and standard deviations of triplicate measurements. The relative uncertainty for both DIC and T_A was $\pm 0.5\%$ of the final value. Temperature and pH were measured using a glass electrode (Xylem, pH535, Weilheim, Germany), which included a temperature sensor and was two-point calibrated with NBS buffers prior to every set of measurements. Average repeatability was found to be $\pm 0.03 \text{ pH units}$. Conductivity was measured using a Conductivity/TDS/Salinity Meter (Extech EC400). Ion exchange chromatography was used to obtain concentrations of inorganic ions in solution (Ca^{2+} , Mg^{2+} , K^+ , Na^+ , Cl^- , F^- , NO_3^- , PO_4^{3-} and SO_4^{2-}) (Dionex Dx-120, Thermo Fisher Scientific, USA, California). The carbonate system was calculated from direct measurement of pH, temperature, T_A and concentrations of primary ions using the software WinIAP (Sequentix, http://www.sequentix.de/software_winiap.php). Activity coefficients were estimated by means of the extended Debye–Hückel equation, valid for solutions of higher ionic strength and electrical conductivities [46].

Analysis

Plastic responses of functional morphological traits were measured as (i) shell diameter and thickness, (ii) shell ultra-structure, (iii) bulk calcification rates for each strain of *Phacotus* cells, as well as (iv) cell concentrations and (v) growth rates. Subsamples for measurements of cell concentrations were taken every second day and placed on a glass microscope slide (improved Neubauer haemocytometer). Four images per strain and treatment were taken using a light microscope with digital camera. By means of the ImageJ software package the images were converted to 8-bit images and cells were segmented from the image background. Cell counts as well as cell sizes were retrieved using the particle analyzer algorithm. Cell size was reported as the average and standard deviation of measurements retrieved from four images.

Cell densities were calculated by dividing the cell counts by the measurement volume of $0.1 \text{ }\mu\text{l}$. For cell cultures that have been diluted after 7 days, all cell densities after dilution were multiplied by $(1 - \Delta V/V)^{-1} = 4$, where $V = 40 \text{ ml}$ is the volume of the treatment medium and $\Delta V = 30 \text{ ml}$ is the removed volume, in order to correct for the dilution. Specific growth rates μ were obtained via a logistic function (1)

$$x(t) = x_{\max}/(1 + \exp(-\mu(t - t_0))) \quad (1)$$

that has been fitted to the cell densities x measured at times t ($=1, 3, 5 \dots$ days). $x_{\max}$ is the maximum cell density reached at saturation and t_0 is a shift parameter.

Samples for scanning electron microscope (SEM) were taken during exponential growth from the optimum as well as the lower and upper boundaries of the growth response functions (DIC 1 (low), 4 (optimum) and 8 mmol L^{-1} (upper)) and placed onto wafers, air-dried at room temperature for 24 hours and stored in a desiccator. Wafers were sputter coated with gold-palladium using an EmiTech K500 Sputter Coater. Imaging was carried out with a EVO LS10 SEM (ZEISS, Oberkochen, Germany) at an acceleration voltage of 20 kV . Calcite shell morphological traits were measured as (i) shell diameter, (ii) shell thickness, (iii) pore size, and (iv) calcite crystal length for each *Phacotus* strain (total number of images $N = 300$). Shell thickness was measured as rim width from images showing shell halves. Calcite crystal length was estimated from shell surface images as follows. First, images showing the lens-shaped shell from the top were cropped to a square focusing on the shell's center (Fig 2A, 2C), transformed to gray-scale, and slightly smoothed using a Gaussian

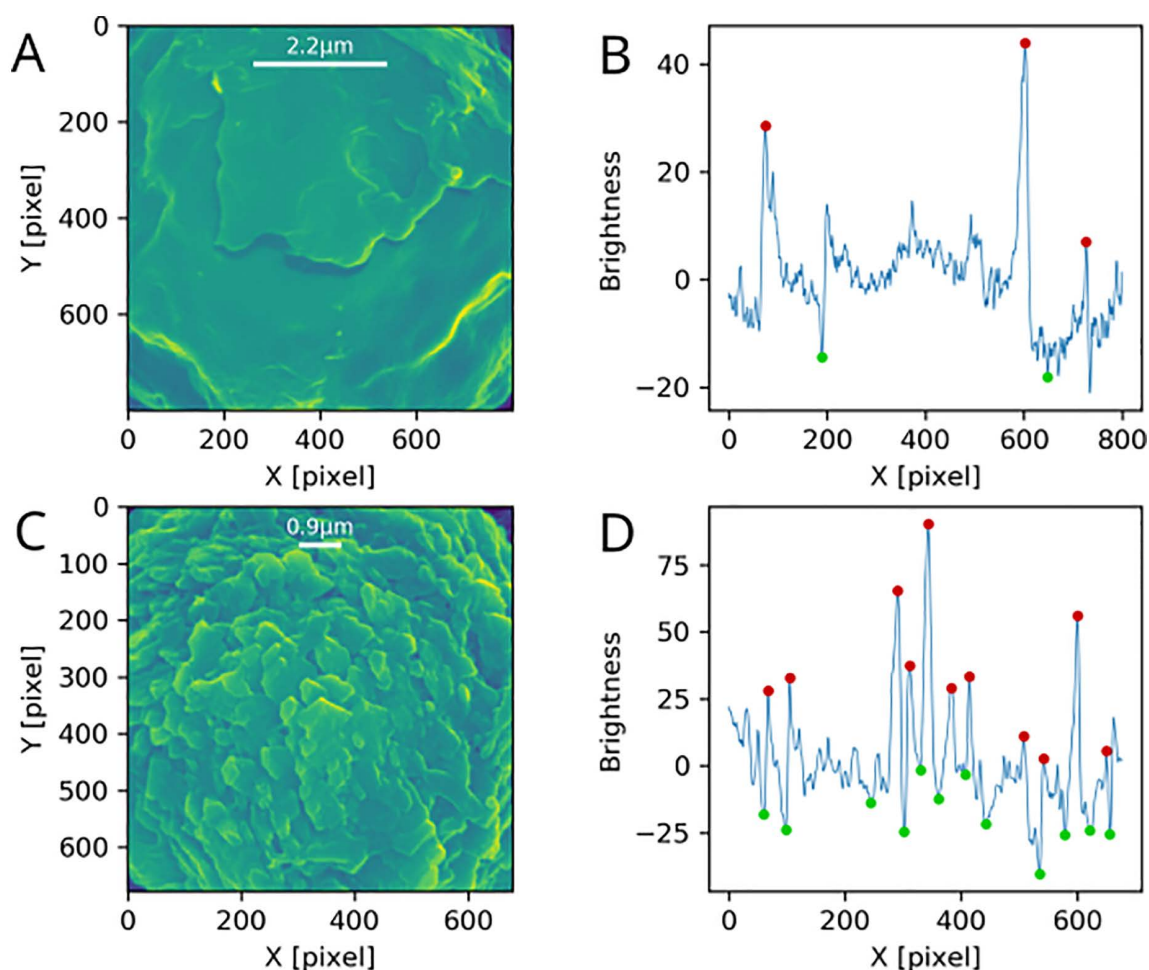


Fig 2. Smoothed gray-scale shell surface images showing bright or dark edges of individual crystals A,C; Peaks and troughs of brightness were detected along a section through the image B,D.

<https://doi.org/10.1371/journal.pone.0349771.g002>

filter with a sigma of one pixel. The lens-shaped shell surface caused a gradual change of the mean brightness towards the rim. We corrected for this by fitting and then subtracting a 2-dimensional parabola from the image.

The crystals are characterized by particularly bright or dark edges resulting in prominent peaks and troughs in brightness along a section through the image. In each row and column of pixels we detected these peaks and troughs (Fig 2B, 2D), computed a typical crystal length by dividing the image width by the averaged number of peaks and troughs, and calculated the median. All shell measurements are reported as means with standard deviations per DIC treatment and lake.

Statistical analysis

We used least square regression and Pearson's correlation coefficient for linear correlations and nonlinear least squares functions for nonlinear models to explore relationships between environmental parameters (T_A , pH, HCO_3^- , CO_3^{2-}).

An exponential function with a rate constant set to the average of the calculated growth rates λ_i was then fitted to the cell counts in order to calculate exponential growth [4]. From final cell densities at the end of the treatment position, height, and width of the optimum peak were estimated by least-square fitting of a parameterized Gaussian function to the

data across all DIC concentrations. The functions were selected based on fit quality measures (correlation between fit and data, root-mean-square difference, Gaussian distribution of residuals). Differences of response curves between the different *P. lenticularis* lineages and DIC concentrations were assessed based on confidence intervals of the fitted curves obtained by bootstrapping. Differences in the parameters obtained from the fits, like peak position and width of the optima, were tested by a one-way ANOVA with the strains, lake origin, and DIC concentration as factors. Tests were performed on shell characteristics (shell diameter, shell thickness, calcite crystal size and pore size) to evaluate differences in all strains. All calculations were made using R Version 3.4.3.

Results

The DIC culturing system (DIC gradient between 0.2 to 12 mmol L⁻¹) proved effective at maintaining a steady carbonate system in treatment replicates over the course of the experiment. DIC and total alkalinity (T_A) depletion by the physiological activity of *Phacotus* cells was higher in low DIC treatments but never exceeded 6% of the initial concentrations in the culture medium. The calcite saturation state (Ω_{Calcite}) was 0.5 at DIC 1 mmol L⁻¹, 9.3 at DIC 4 and 51 mmol L⁻¹ at DIC 8 mmol L⁻¹. As expected, alkalinity was proportional to DIC with a proportionality factor of 0.97 meq/mol (Fig 3A). Values of pH increased quickly from 6.2 to approx. 7.3 for DIC values < 1 mmol L⁻¹. For larger DIC values pH further increased

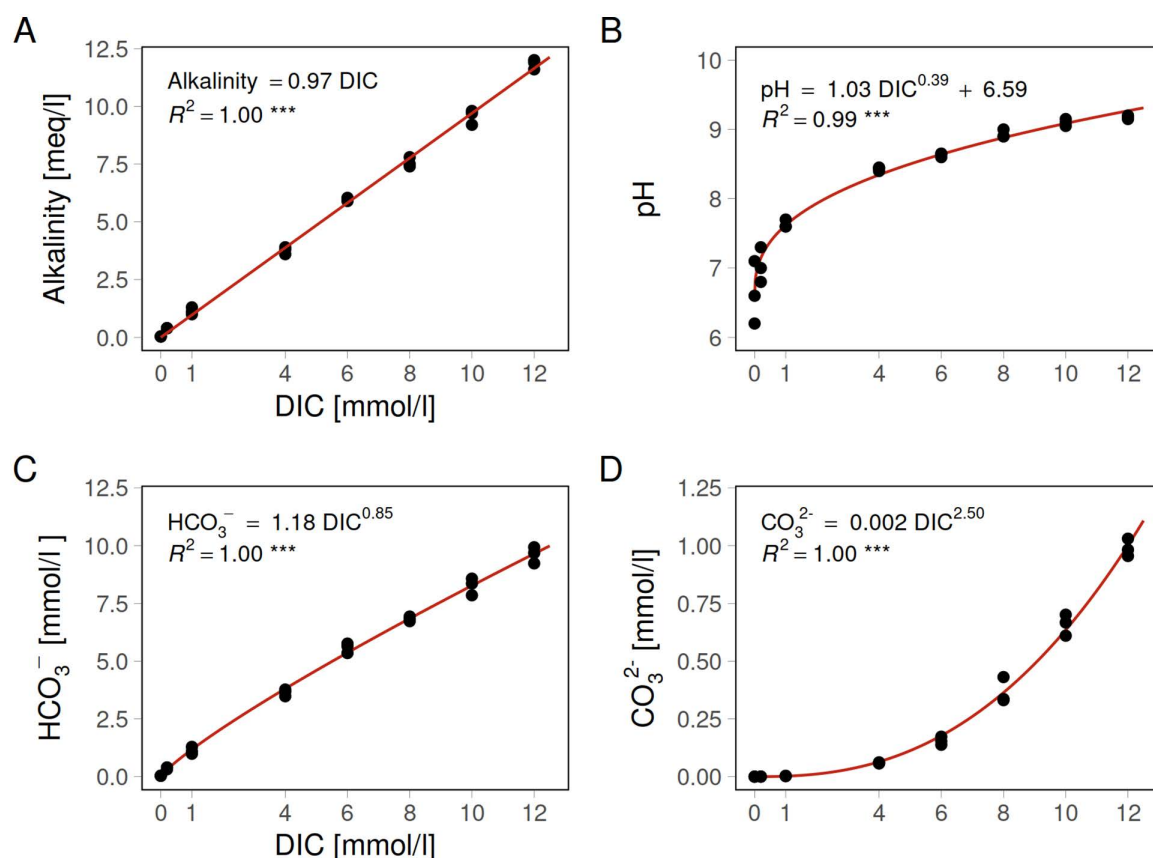


Fig 3. Non-linear and linear relationships between Alkalinity (A), pH (B), HCO_3^- (C), CO_3^{2-} (D) and DIC concentrations in the modified culture medium N-HS-Ca. Coefficient of determination R^2 as a measure of the goodness of fit and significance levels are presented for each regression. Curves were fitted based on nonlinear least squares functions for nonlinear models and least square regression and Pearson's correlation coefficient for linear correlations.

<https://doi.org/10.1371/journal.pone.0349771.g003>

approximately linearly up to 9.2 (Fig 3B). HCO_3^- was almost linearly related to DIC (Fig 3C). CO_3^{2+} concentrations were about an order of magnitude smaller than HCO_3^- and increased according to a power law with exponent 2.5 with DIC (Fig 3D). The concentration changes of both CO_3^{2+} and HCO_3^- follow the expected dependency on pH.

Effect of DIC concentrations on shell characteristics

We measured shell morphology parameters of 300 complete *P. lenticularis* calcite shells and 170 shell halves based on SEM observations at three DIC concentrations (1, 4 and 8 mmol L⁻¹, at least 10 replicates per strain). The images provided high resolution information about shell diameter, thickness, shape and arrangement of crystals, as well as pore size (Fig 5). Measured average diameter of *Phacotus* strains was $12.0 \pm 1.9 \mu\text{m}$ in OS strains across DIC concentrations of 1, 4 and 8 mmol L⁻¹ and was significantly smaller than average diameter of GS strains ($13.6 \pm 1.8 \mu\text{m}$) (Wilcoxon rank test, $W=8092$, $p<0.0001$). This corresponds well with SEM image measurements of *Phacotus* shell diameters from both lakes (Fig 4 and Lenz et al. 2018). Shell diameters were largest at ambient DIC values of 4 mmol L⁻¹ and decreased by 1.5

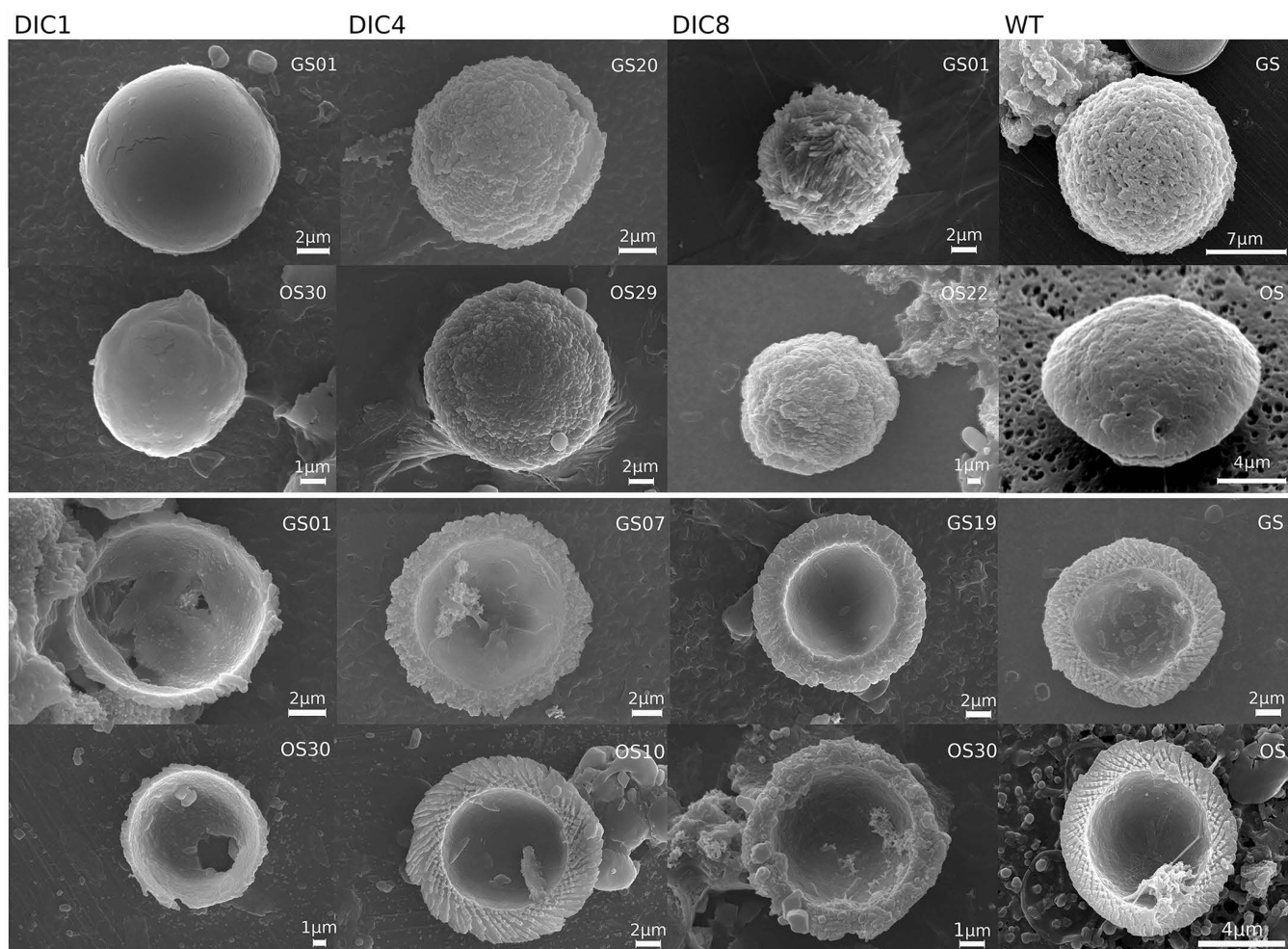


Fig 4. Scanning electron micrographs showing complete shells (upper panel) and open shell halves (lower panel) of *P. lenticularis* strains across three DIC concentrations; calcite shells are composed of two shell halves attached to one another at the rim. The column WT on the right side displays original wildtype *Phacotus* shells from Lake Gönningersee (GS) and Lake Großer Ostersee (OS). Strain IDs are shown for each image.

<https://doi.org/10.1371/journal.pone.0349771.g004>

and 1.2 μm when DIC was changed to 1 and 8 mmol L^{-1} , respectively (Fig 5A). A large decrease in shell thickness was found when DIC concentrations were lowered from 4 mmol L^{-1} to 1 mmol L^{-1} in all strains (Wilcoxon rank test, $W=590$, $p<0.0001$). In strains isolated from lake OS shell thickness decreased from $1.7\pm0.3\text{ }\mu\text{m}$ to $0.6\pm0.2\text{ }\mu\text{m}$, while GS strains showed a smaller reduction from $1.8\pm0.3\text{ }\mu\text{m}$ to $1.2\pm0.2\text{ }\mu\text{m}$. In contrast, shell-thickness of *P. lenticularis* strains from both lakes did not change noticeably when DIC concentration was doubled to 8 mmol L^{-1} (Fig 5B).

All shells exposed to a low DIC concentration of 1 mmol L^{-1} lost their distinct crystalline structure with the calcite surface becoming smoother and more fragile (Fig 4). Adjacent crystals merged to a larger calcite field accompanied by a loss of crystal thickness. This is reflected by a significant increase in average crystal length from $1.0\pm0.4\text{ }\mu\text{m}$ to $2.8\pm1.9\text{ }\mu\text{m}$ in OS strains and from $1.4\pm0.7\text{ }\mu\text{m}$ to $3.0\pm2.0\text{ }\mu\text{m}$ in GS strains (Fig 5C, Wilcoxon rank test, $W=2235$, $p<0.0001$). An increase in DIC from 4 to 8 mmol L^{-1} , however, had no significant effect on average crystal length. Ambient crystal length was preserved despite a high tendency for CaCO_3 precipitation when DIC was doubled. Our SEM image analysis, however, demonstrated lake-specific differences in crystal shape when DIC increased from 4 to 8 mmol L^{-1} . GS strains largely belong to morphotype III defined by a rhombohedral crystalline shape with a high density of nanoscale pores at ambient DIC. OS strains belong to morphotype IV defined by a smooth shell surface and lower pore density. While almost all shells lost their crystalline structure at DIC 1, the crystals of GS strain shells elongated to spines that extended outward at a DIC

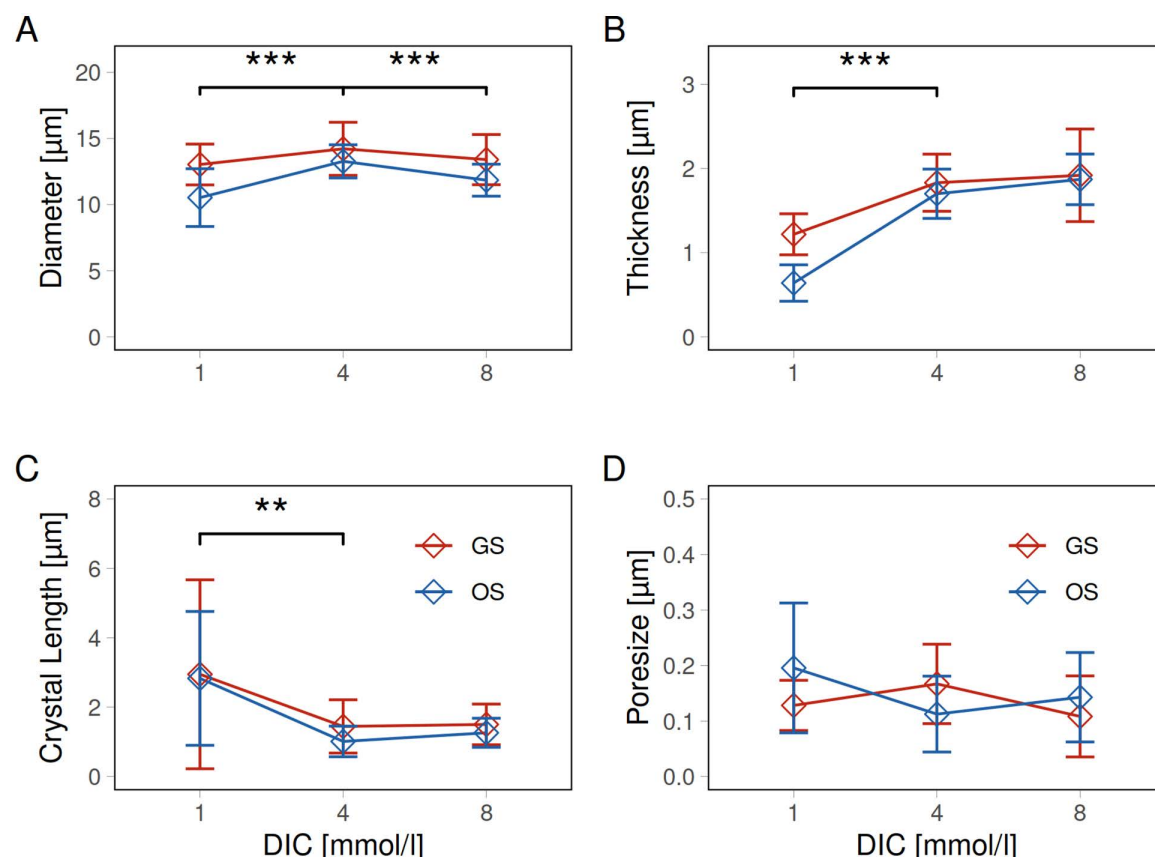


Fig 5. Calcite shell parameters versus DIC for *P. lenticularis* strains per lake; diameter(A), thickness (B), crystal length (C) and pore size (D) were measured with the aid of a scanning electron microscope, vertical error bars represent standard deviations of between 20 and 40 measurements for each strain isolated from Lake Gönningersee (GS) and Grosser Ostersee (OS).

<https://doi.org/10.1371/journal.pone.0349771.g005>

of 8 mmol L⁻¹ (Fig 6). Calcite crystals of OS strain shells developed a rhombohedral shape at DIC of 8 mmol L⁻¹, similar to the shells of GS strains at ambient DIC concentrations of 4 mmol L⁻¹.

Pore size of *Phacotus* shells did not change significantly between treatments (Fig 5D). Surprisingly, many shells lacked pores entirely at all three DIC concentrations with no visible effect on cell growth (see below). A small space between the rims where the two shell halves attach to one another may allow for a selective uptake of molecules from the surrounding medium when pores are absent.

Growth rate and cell density

The 12 *P. lenticularis* strains did not alter their growth rate in response to DIC manipulations across a broad range between 0.2 and 10 mmol L⁻¹ (Fig 7A, 7B). Estimated growth rates did not differ between OS strains (0.49 ± 0.15 d⁻¹) and GS strains (0.55 ± 0.14 d⁻¹, *t*-test, *t* = 0.53, *p* = 0.60). At a concentration of 12 mmol L⁻¹ all strains except GS20 died within a few days into the experiment.

In contrast to growth rates, maximum cell densities of *P. lenticularis* strains strongly depended on DIC concentrations (Fig 7). Cell densities measured at day 14 followed a Gaussian response profile. At low and high DIC concentrations cell cultures reached their stationary phase earlier in the experiment, resulting in a strongly reduced cell density compared to the one at ambient DIC concentrations. However, based on maximum cell counts, all 12 strains showed a wide tolerance to DIC concentrations between 1 and 8 mmol L⁻¹. Peak height (*F*-test, *F* = 3.5, *p* = 0.09) and width (*F*-test, *F* = 0.002, *p* = 0.97) of the Gaussian functions fitted to the cell densities did not differ between lakes. GS strains, however, preferred slightly higher DIC concentrations than OS strains (*F*-test, *F* = 6.8, *p* = 0.03).

Optimum growth of GS strains occurred at DIC concentrations of 5.2 ± 0.3 mmol L⁻¹ and of OS strains at 4.7 ± 0.2 mmol L⁻¹, matching ambient lake concentrations in their lake of origin (Fig 8C, 8D). Variability in peak height of the Gaussian preference function was larger in GS strains than in OS strains (*F*-test, *F* = 7.4, *p* = 0.046, Fig 7C and 7D).

To look in more detail into local adaptations of the OS and GS strains we compared the maximum cell density reached at saturation for DIC concentrations of 4 and 6 mmol L⁻¹ (Fig 8). Indeed, the strains originating from OS reached higher

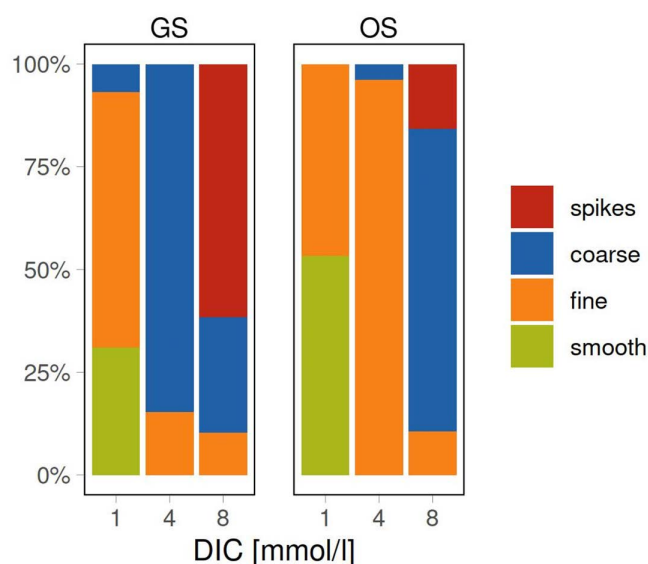


Fig 6. Percent stacked bar plots representing the proportions of four categories of calcite crystal shape at the surface of *P. lenticularis* shells across three DIC concentrations comparing Lakes Gönningersee (GS) and Großer Ostersee (OS). Crystal shape estimates were obtained from SEM pictures of *P. lenticularis* shells.

<https://doi.org/10.1371/journal.pone.0349771.g006>

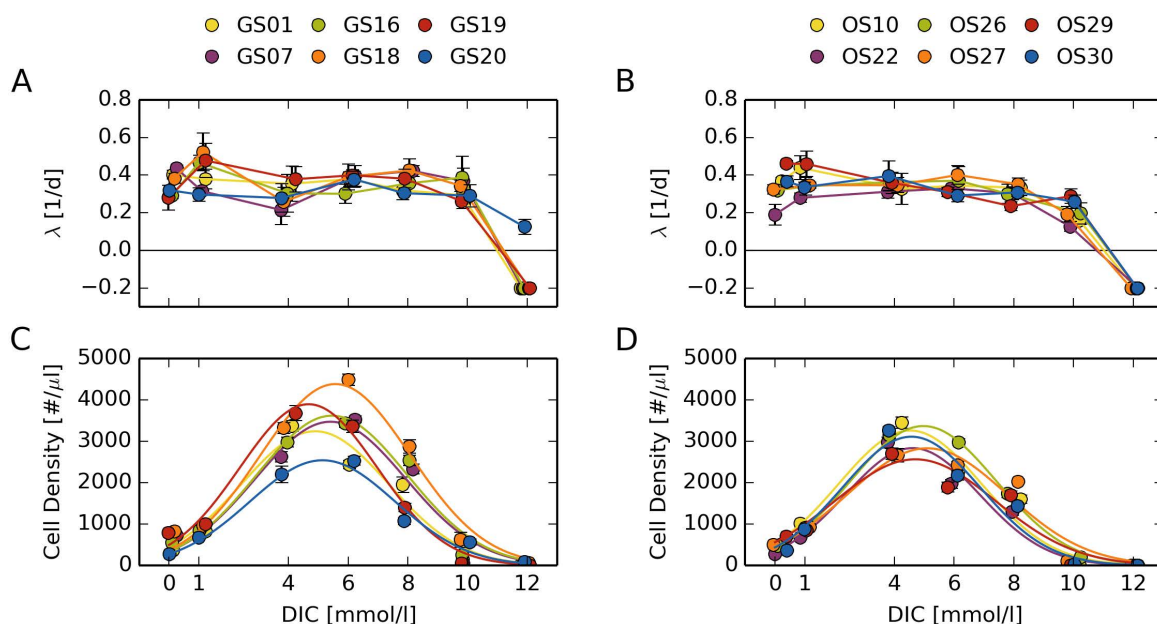


Fig 7. Optimum curve responses of measured growth (λ) and cell density at the final day of the experiment (14 days) across a DIC gradient, individual *P. lenticularis* strains from Lake Gönningersee (left) and Lake Großer Ostersee (right) are plotted.

<https://doi.org/10.1371/journal.pone.0349771.g007>

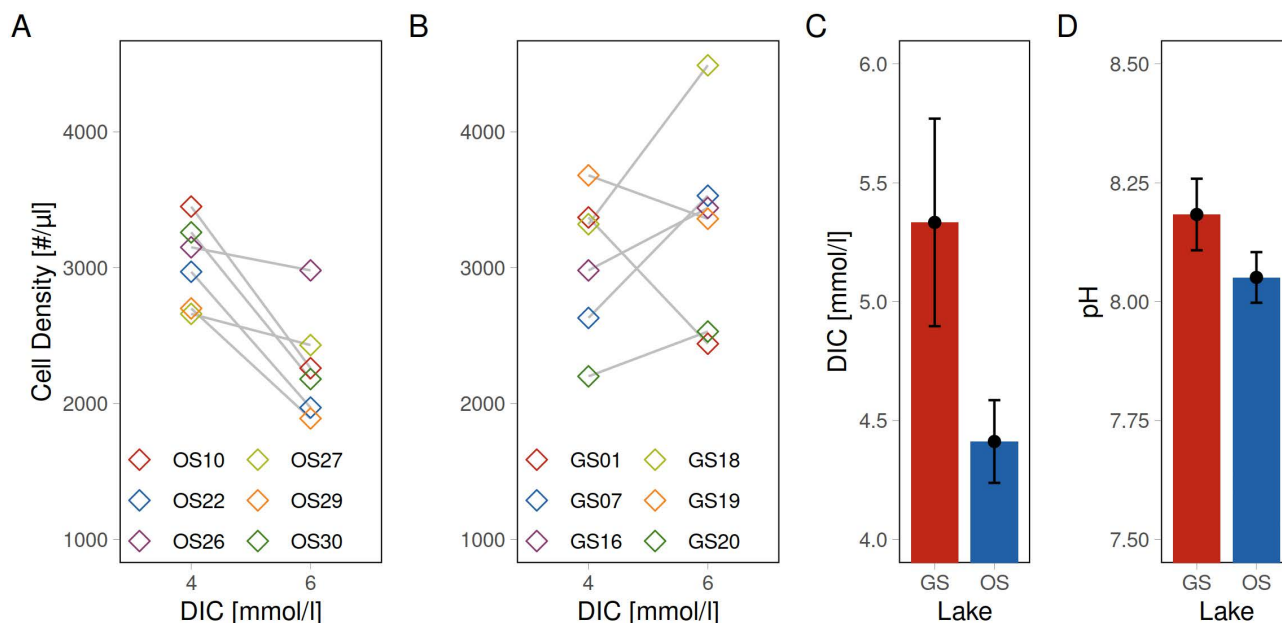


Fig 8. Results from reciprocal crosses show response of cell density at the final day of the experiment for individual *P. lenticularis* strains at two DIC concentration representing ambient conditions in the two lakes of strain origin (A, B); average DIC concentration with standard deviation (C), average pH and standard deviation during the vegetation period (C) in Lake Gönningersee (GS) and Lake Großer Ostersee (OS).

<https://doi.org/10.1371/journal.pone.0349771.g008>

cell densities at a DIC of 4 mmol L⁻¹, matching the ambient condition in Lake Großer Ostersee. Vice versa, the majority of GS strains reached higher densities at a DIC of 6 mmol L⁻¹ close to the ambient condition in Lake Gönningersee.

Discussion

Climate and land use changes are globally accelerating terrestrial and aquatic carbon cycling rates and thereby modify chemical processes in aquatic systems [48]. CO₂-induced acidification in oceans is predicted to be particularly detrimental for marine shell-forming organism and a diversity of negative and variable responses to calcification between species and strains in marine phytoplankton has been reported [49–51]. Responses of freshwater calcifying phytoplankton have received far less attention and are more difficult to predict as effects on bicarbonate and carbonate ion concentrations in lakes operate via complex geochemical pathways [52,53].

Using experimental manipulation of the carbonate system, we have shown that dissolved inorganic carbon concentration appears to be an important driver controlling *P. lenticularis* calcification and shell morphology parameters. The 12 *P. lenticularis* strains responded with a significant reduction in calcification, shell thickness and formation of calcite crystals under declined DIC concentration, while these parameters remained constant at elevated DIC after 14 days of incubation. A decreased calcification and reduction in shell thickness of *P. lenticularis* shells at low DIC concentrations ≤ 1 mmol L⁻¹ is likely explained by the algae's inability to extract sufficient dissolved inorganic carbon from the culture medium and efficiently convert it into calcite, the major constituent of their shells. Declining DIC and pH result in decreased concentration of carbonate ions (CO₃²⁻) and consequently in a decreased calcite saturation state, on which aquatic calcifiers depend. CaCO₃ supersaturation occurs when the ionic activity product of Ca²⁺ and CO₃²⁻ exceeds the solubility product, creating a high potential for CaCO₃ precipitation. A high DIC and elevated carbonate ion levels directly contribute to CaCO₃ supersaturation. The resulting formation of biogenic calcite by phytoplankton causes a removal of DIC and alkalinity from the water lowering the CaCO₃ saturation state.

The final external maturation stage in *P. lenticularis* shell formation may render little control and therefore limits the capacity for adaptation to decreased carbonate concentrations. The mineralization pathways in *P. lenticularis* are just being investigated [24]. Through endocytosis calcium and dissolved inorganic carbon ions are concentrated in vesicles within the algal cell, allowing for the nucleation and growth of small crystals. In contrast to coccolith formation, crystals are not fully formed before exiting the cell of *P. lenticularis*. These small crystals are located on the outer surface of the daughter cell lorica, and more crystals form around them until the gaps between the crystals on the outer surface of the lorica are closed. Observations of cells with complete shells show that there likely are additional stages of shell maturation after the reproduction cycle is complete [54]. Fully formed shells can have different shell thicknesses and various levels of organization according to their environment [23].

The capability of maintaining elevated pH at the sites of calcification in artificially acidified waters was recently demonstrated across a range of phyla [29]. Studies of single-celled organisms in marine environments demonstrate that control of cytoplasmic pH in coccolithophores involved voltage-gated H⁺ channels in the plasma membrane pushing H⁺ out of the cell in order to maintain saturation conditions for calcite formation [55,56]. H⁺ channels showed greatly reduced activity in cells acclimated to low pH which impaired the ability to remove H⁺ generated by the internal calcification process [35]. The energization of plasma membrane transport in freshwater algae is less clear. *P. lenticularis* belongs to the *Chlamydomonadales* family of freshwater green algae. An ability to generate electrical voltage differences across their membrane has been found in *Chlamydomonas* and *Characean* algae [57,58]. There is also evidence suggesting that several freshwater algal taxa utilize a combination of H⁺- and Na⁺-energized transport at the plasma membrane, which may enable them to adapt to changes in the pH and ionic composition of their environments [59]. The capacity of pH regulation through voltage-gated proton channels may be central to counter the effects of declining DIC and pH, but needs further investigation in freshwater algae.

In the last decades, studies investigating responses to ocean acidification of *E. huxleyi* observed that high H⁺ concentration in seawater likely correspond with high H⁺ concentration inside phytoplankton cells and lead to reduced CO₂

fixation efficiency in the chloroplasts and decreased algal growth [60]. We showed, that *P. lenticularis* growth rates remained stable over a wide range of pH and DIC levels in the culture experiments. *P. lenticularis* seems to be capable of keeping their cell division rate constant over a pH range between approx. 6–9 and exponentially increasing carbonate ion concentration up to a DIC of 8 mmol/l. A potential ability to activate and deactivate a CO₂ concentrating mechanism (CCM) in response to changing CO₂ and HCO₃⁻ conditions could explain the acclimation of *P. lenticularis* to variable DIC concentrations [38]. In *Chlamydomonas reinhardtii*, a well studied member of the Chlamydomonadales family, at least 12 genes that encode carbonic anhydrase isoforms, have been detected [61]. Carbonic anhydrases (CAs) are zinc-containing metalloenzymes and important components of the CCM. CAs catalyze the reversible interconversion of CO₂ and HCO₃⁻. A sudden and entire reduction in growth of most *P. lenticularis* strains at ≥ 10 mmol L⁻¹ DIC may be explained by the cells disability to regulate the intracellular pH at high extracellular pH > 9. At the cellular level, a reaction to elevated carbonate concentrations involves regulating of membrane fluidity, metabolism, and photosynthesis. These costly regulatory mechanisms may slow down cell division, while enhancing survival at higher DIC.

The two lakes chosen for isolation of *P. lenticularis* differ substantially in morphology, trophical status and carbonate inputs offering a comparison between strains that were exposed to higher variation in one lake compared to the other. The *P. lenticularis* strains used in this study were isolated a few month prior to the experiment. Adaptation in monoclonal cultures is assumed to be slow due to asexual reproduction and genetic changes are based mainly on mutations. The phenotypic differences observed in the experiments are likely based on genetic or plastic differences the strains established prior to cultivation. Exploring intra-population variability in physiological experiments offers a wider perspective for evaluating species traits and is essential to predict future responses to environmental changes.

Our results demonstrate that *P. lenticularis* strains are adapted to the specific environmental conditions in their lake of origin. Local adaptation occurs when resident individuals have a higher fitness in their local environment than those from other populations of the same species due to genetic change [62]. In our experiments OS strains developed a clear optimum for growth at DIC 4 mmol L⁻¹, the ambient average DIC level in their lake of origin. Growth optima of GS strains were more variable and tended to shift to higher DIC levels between 5 and 6 mmol L⁻¹, the ambient average DIC level Lake Gönningersee. Consistent growth optima for specific DIC concentrations by strains from the same lake suggest genetic differentiation of populations into distinct phenotypes and local adaptation.

In comparison to OS strains, GS strains developed higher growth rates and significantly larger as well as heavier calcite shells across a wider DIC gradient. Furthermore, intra-population variability in final cell density was higher in GS strains compared to OS strains. Local adaptation in phytoplankton occurs by lineage selection on new arrivals, or by natural selection on standing genetic variation leading to selection of individuals best adapted to the local environment [63]. A cell's physiological reaction to the current environment is determined by complex interactions between the individuals history of environmental exposure and mechanisms governing its plasticity [64]. Our results demonstrate the importance of using multiple strains in order to make conclusions regarding species traits. DIC concentrations and pH measured during the time of strain isolation were more dynamic in Lake Gönningersee compared to measurements from Lake Großer Ostersee (by 2 units). Lake Gönningersee is smaller than Lake Großer Ostersee and receives direct input from a tributary that frequently carries high loads of carbonate ions from limestone bedrock in the surrounding watershed. The difference in environmental dynamics in Gönningersee may have caused an increased tolerance to higher variation in DIC in GS strains resulting in their higher tolerance to the experimental DIC manipulations. Schaum et al. [65] have shown that phytoplankton populations from more variable environments are more tolerant to a changing environment. The adaptation to higher variation in carbonate concentrations and generally heavier calcite shells compared to OS strains may stabilize the quantitative contribution of *Phacotus* shells to long term carbonate sequestration in Lake Gönningersee under future climate scenarios. Lenz et al. [32] found that *Phacotus* shells contributed at least 10% to the autochthonous carbonate precipitation in Bavarian hard-water lakes during mass development. Shell diameters in the top sediment layers remained equal to those of living individuals found in the water column indicating that *Phacotus* shells contribute to persistent

CaCO₃ deposition in the lake sediment. We can confirm strain and lake specific adaptation to higher variation in carbonate concentrations with our experimental results. The exact mechanisms underlying adaptation and genetic composition in freshwater calcifiers remain an important topic of study.

Freshwater habitats are often isolated water bodies with large variation in environmental variables that provide stronger dispersal barriers and potential for differentiation than marine habitats. The functional diversity of freshwater calcifying algae may be considerably larger than is presently assumed based upon only a limited number of studies and strains. The ability of *P. lenticularis* to continue its contribution to autochthonous calcite precipitated in the surface layer of lakes and ponds will depend on their ability to survive shifts in the composition and function forecasted for freshwater lakes over the next centuries [66]. Individual variability will maintain high functional diversity in *P. lenticularis* populations and increases success to persist in a changing environment.

Conclusion

The impact of carbonate chemistry on marine coccolithophore shell formation is well studied. This study provides additional evidence of the important role of dissolved inorganic carbon changes to calcite shell functionality and growth in an abundant freshwater calcifying phytoplankton. Calcification and shell morphology of *P. lenticularis* was sensitive to decreasing but not to increasing DIC levels up to a biological threshold. Our studies revealed decreased shell thickness, loss of a distinct crystalline structure and decrease in cell diameter at decreased DIC from 4 to 1 mmol L⁻¹ in all cultured strains. Calcification rates scale positively with bicarbonate and carbonate ion concentrations in lake water. Our results suggest that in a future scenario of more variable carbon and alkalinity cycles *P. lenticularis* will continue to calcify, but their shells will be structurally weakened during prolonged periods of reduced CaCO₃ saturation state.

The isolation of individual strains from two lakes differing in DIC dynamics allowed us to investigate intraspecific variability in morphological and physiological traits. *P. lenticularis* strains have demonstrated a high potential for acclimation and plasticity to a wide range of DIC changes in the culture experiments. Strains originating from Lake Gönningersee developed higher and significantly larger as well as heavier calcite shells across a wider DIC gradient compared to strains from Lake Großer Ostersee. While all strains maintained high CO₂ fixation efficiency and unaltered algal growth at a wide range of DIC, GS strains responded with a higher variability in growth and final cell density compared to OS strains. This indicates that physiological functioning might be phenotypically buffered in the Gönningersee *Phacotus* populations increasing their competitiveness. We hypothesize, that *Phacotus* populations with a lower clone variability could be at greater risk to expected future carbonate chemistry alterations, endangering the ecosystem services they provide. Our work provides a foundation to further identify ecological strategies of freshwater calcifiers in response to anthropogenic change.

Acknowledgments

We thank Maren Lentz and Sebastian Lenz for laboratory assistance during the experiments. The help and advice from Lothar Krienitz concerning the strain isolation and experimental set-up of the DIC experiment was very appreciated. We gratefully acknowledge the Tübingen Structural Microscopy Core Facility (funded by the Excellence Strategy of the German Federal and State Governments) for their support and assistance in this work. We thank two anonymous reviewers whose comments greatly improved this paper.

Author contributions

Conceptualization: Uta Gruenert, Uta Raeder.

Data curation: Uta Gruenert.

Formal analysis: Jan Benda.

Methodology: Uta Gruenert.

Project administration: Uta Gruenert.

Resources: Uta Gruenert.

Software: Jan Benda.

Supervision: Oliver Bossdorf, Uta Raeder.

Validation: Uta Gruenert.

Visualization: Uta Gruenert, Jan Benda.

Writing – original draft: Uta Gruenert, Jan Benda.

Writing – review & editing: Uta Raeder.

References

1. Langer G, Nehrke G, Probert I, Ly J, Ziveri P. Strain-specific responses of *Emiliana huxleyi* to changing seawater carbonate chemistry. *Biogeosciences*. 2009;6:2637–46. <https://doi.org/10.5194/bg-6-2637-2009>
2. Müller MN, Schulz KG, Riebesell U. Effects of long-term high CO₂ exposure on two species of coccolithophores. *Biogeosciences*. 2010;7:1109–16.
3. Rokitta SD, Rost B. Effects of CO₂ and their modulation by light in the life-cycle stages of the coccolithophore *Emiliana huxleyi*. *Limnology & Oceanography*. 2012;57(2):607–18. <https://doi.org/10.4319/lo.2012.57.2.0607>
4. Gruenert U, Raeder U. Growth responses of the calcite-loricated freshwater phytoflagellate *Phacotus lenticularis* (Chlorophyta) to the CaCO₃ saturation state and meteorological changes. *Journal of Plankton Research*. 2014;36(3):630–40. <https://doi.org/10.1093/plankt/fbu006>
5. Raven JA, Beardall J. Influence of global environmental Change on plankton. *Journal of Plankton Research*. 2021;43(6):779–800. <https://doi.org/10.1093/plankt/fbab075>
6. Page HN, Bahr KD, Cyronak T, Jewett EB, Johnson MD, McCoy SJ. Responses of benthic calcifying algae to ocean acidification differ between laboratory and field settings. *ICES Journal of Marine Science*. 2022;79(1):1–11. <https://doi.org/10.1093/icesjms/fsab232>
7. Khan H, Laas A, Marce' R, Obrador B. Major effects of alkalinity on the relationship between metabolism and dissolved inorganic carbon dynamics in lakes. *Ecosystems*. 2020;23:1566–80. <https://doi.org/10.1007/s10021-020-00488-6>
8. Cole JJ. The carbon cycle. In: Weathers KC, Strayer DL, Likens GE. *Fundamentals of ecosystem science*. New York: Elsevier. 2013. 109–35.
9. Song K, Wen Z, Xu Y, Yang H, Lyu L, Zhao Y, et al. Dissolved carbon in a large variety of lakes across five limnetic regions in China. *Journal of Hydrology*. 2018;563:43–154.
10. Finlay K, Leavitt PR, Wissel B, Prairie YT. Regulation of spatial and temporal variability of carbon flux in six hard-water lakes of the northern Great Plains. *Limnology & Oceanography*. 2009;54(6part2):2553–64. https://doi.org/10.4319/lo.2009.54.6_part_2.2553
11. Müller B, Meyer JS, Gächter R. Alkalinity regulation in calcium carbonate-buffered lakes. *Limnology and Oceanography*. 2016;61:341–52. <https://doi.org/10.1002/lno.10213>
12. Hammer KJ, Kragh T, Sand-Jensen K. Inorganic carbon promotes photosynthesis, growth, and maximum biomass of phytoplankton in eutrophic water bodies. *Freshwater Biology*. 2019;64:1956–70. <https://doi.org/10.1111/fwb.13385>
13. Engel F, Farrell KJ, McCullough IM, Scordo F, Denfeld BA, Dugan HA, et al. A lake classification concept for a more accurate global estimate of the dissolved inorganic carbon export from terrestrial ecosystems to inland waters. *Naturwissenschaften*. 2018;105(3–4):25. <https://doi.org/10.1007/s00114-018-1547-z> PMID: 29582138
14. Marcé R, Obrador B, Morguí J-A, Lluís Riera J, López P, Armengol J. Carbonate weathering as a driver of CO₂ supersaturation in lakes. *Nature Geosci*. 2015;8(2):107–11. <https://doi.org/10.1038/ngeo2341>
15. Battin TJ, Luyssaert S, Kaplan LA, Aufdenkampe AK, Richter A, Tranvik LJ. The boundless carbon cycle. *Nature Geoscience*. 2009;2:598–600.
16. Tranvik LJ, Downing JA, Cotner JB, Loiselle SA, Striegl RG, Ballatore TJ. Lakes and reservoirs as regulators of carbon cycling and climate. *Limnology and Oceanography*. 2009;54(6):2283–97. <https://doi.org/10.4319/lo.2009.54.6>
17. Li Y, Wang N, Li Z, Zhou X, Zhang C, Wang Y. Carbonate formation and water level changes in a paleo-lake and its implication for carbon cycle and climate change, arid China. *Frontiers of Earth Science*. 2013;7:487–500. <https://doi.org/10.1007/s11707-013-0392-9>
18. Rogora M, Somaschini L, Marchetto A, Mosello R, Tartari GA, Paro L. Decadal trends in water chemistry of Alpine lakes in calcareous catchments driven by climate change. *Sci Total Environ*. 2020;708:135180. <https://doi.org/10.1016/j.scitotenv.2019.135180> PMID: 31812417
19. Rinta P, Bastviken D, van Hardenbroek M, Kankaala P, Leuenberger M, Schilder J, et al. An inter-regional assessment of concentrations and $\delta^{13}\text{C}$ values of methane and dissolved inorganic carbon in small European lakes. *Aquatic Science*. 2015;77:667–80. <https://doi.org/10.1007/s00027-015-0410-y>

20. Weyhenmeyer GA, Chukwuka AV, Anneville O, Brookes J, Carvalho CR, Cotner JB. Global lake health in the Anthropocene: societal implications and treatment strategies. *Earth's Future*. 2023;12:e2023EF004387. <https://doi.org/10.1029/2023EF004387>
21. Shi X, Li S, Wei L, Qin B, Brookes JD. CO₂ alters community composition of freshwater phytoplankton: A microcosm experiment. *Sci Total Environ*. 2017;607–608:69–77. <https://doi.org/10.1016/j.scitotenv.2017.06.224> PMID: 28688257
22. Gruenert U, Benda J, Müller C, Raeder U. Vertical segregation of two cell-cycle phases of the calcifying freshwater phytoflagellate *Phacotus lenticularis* (Chlorophyta). *Journal of Plankton Research*. 2016;38(1):94–105.
23. Lenz S, Gruenert U, Geist J, Stiefel M, Lentz M, Raeder U. Calcite production by the calcifying green alga *Phacotus lenticularis*. *Journal of Limnology*. 2018;77(2):209–19.
24. Shaked N, Addadi S, Goliand I, Fox S, Barinova S, Addadi L, et al. Intra- to extracellular crystallization of calcite in the freshwater green algae *Phacotus lenticularis*. *Acta Biomater*. 2023;167:583–92. <https://doi.org/10.1016/j.actbio.2023.06.019> PMID: 37348777
25. de Nooijer LJ, Toyofuku T, Kitazato H. Foraminifera promote calcification by elevating their intracellular pH. *Proc Natl Acad Sci U S A*. 2009;106(36):15374–8. <https://doi.org/10.1073/pnas.0904306106> PMID: 19706891
26. Mackinder L, Wheeler G, Schroeder D, Riebesell U, Brownlee C. Molecular mechanisms underlying calcification in coccolithophores. *Geomicrobiology Journal*. 2010;27:585–95.
27. Krienitz L, Koschel R, Giering B, Casper SJ, Hepperle D. Phenomenology of organismic calcite precipitation by *Phacotus* in hardwater lakes and ponds of northeastern Germany. *Verhandlungen des Internationalen Verein Limnologie*. 1993;25:170–4.
28. Klaas C, Archer DE. Association of sinking organic matter with various types of mineral ballast in the deep sea: Implications for the rain ratio. *Global Biogeochemical Cycles*. 2002;16(4). <https://doi.org/10.1029/2001gb001765>
29. Gilbert PUPA, Bergmann KD, Boekelheide N, Tambutté S, Mass T, Marin F, et al. Biomineralization: Integrating mechanism and evolutionary history. *Sci Adv*. 2022;8(10):eabl9653. <https://doi.org/10.1126/sciadv.abl9653> PMID: 35263127
30. Burns CW, Mitchell SF. Seasonal succession and vertical distribution of phytoplankton in Lake Hayes and Lake Johnson, South Island, New Zealand. *N Z Journal of Marine and Freshwater Research*. 1974;8(1):167–209.
31. Haberzettl T, Corbella H, Fey M, Janssen S, Lücke A, Mayr C, et al. Lateglacial and Holocene wet-dry cycles in southern Patagonia: chronology, sedimentology and geochemistry of a lacustrine record from Laguna Potrok Aike, Argentina. *Holocene*. 2007;17(3):297–310.
32. Lenz S, Dubois N, Geist J, Raeder U. *Phacotus lenticularis* content in carbonate sediments and epilimnion in four German hard water lakes. *Journal of Limnology*. 2020;79(2):187–97.
33. Müller M, Trull T, Hallegraeff G. Differing responses of three Southern Ocean *Emiliana huxleyi* ecotypes to changing seawater carbonate chemistry. *Mar Ecol Prog Ser*. 2015;531:81–90.
34. Feng Y, Roleda MY, Armstrong E, Boyd PW, Hurd CL. Environmental controls on the growth, photosynthetic and calcification rates of a Southern Hemisphere strain of the coccolithophore *Emiliana huxleyi*. *Limnology and Oceanography*. 2017;62:519–40.
35. Kottmeier DM, Chrachri A, Langer G, Helliwell KE, Wheeler GL, Brownlee C. Reduced H⁺ channel activity disrupts pH homeostasis and calcification in coccolithophores at low ocean pH. *Proc Natl Acad Sci U S A*. 2022;119(19):e2118009119. <https://doi.org/10.1073/pnas.2118009119> PMID: 35522711
36. Medlin LK, Barker GLA, Campbell L, Green JC. Genetic characterisation of *Emiliana huxleyi* (Haptophyta). *Journal of Marine Systems*. 1996;9:13–31.
37. Iglesias-Rodríguez MD, Probert I, Batley J. Microsatellite cross-amplification in coccolithophores: application in population diversity studies. *Heredity*. 2006;143(2006):99–102. <https://doi.org/10.1111/j.2006.0018-0661.01933.x> PMID: 17362341
38. Kottmeier DM, Rokitta SD, Rost B. H₁-driven increase in CO₂ uptake and decrease in HCO₃⁻ uptake explain coccolithophores' acclimation responses to ocean acidification. *Limnology and Oceanography*. 2016;61:2045–57.
39. Chan WY, Oakeshott JG, Buerger P, Edwards OR, van Oppen MJH. Adaptive responses of free-living and symbiotic microalgae to simulated future ocean conditions. *Glob Chang Biol*. 2021;27(9):1737–54. <https://doi.org/10.1111/gcb.15546> PMID: 33547698
40. Zhang Y, Bach LT, Lohbeck KT, Schulz KG, Listmann L, Klapper R, et al. Population-specific responses in physiological rates of *Emiliana huxleyi* to a broad CO₂ range. *Biogeosciences*. 2018;15(12):3691–701. <https://doi.org/10.5194/bg-15-3691-2018>
41. Van den Wyngaert S, Möst M, Freimann R, Ibelings BW, Spaak P. Hidden diversity in the freshwater planktonic diatom *Asterionella formosa*. *Mol Ecol*. 2015;24(12):2955–72. <https://doi.org/10.1111/mec.13218> PMID: 25919789
42. Regenfors K, Kremp A, Reusch TBH, Wood M. Genetic diversity and evolution in eukaryotic phytoplankton: revelations from population genetic studies. *Journal of Plankton Research*. 2017;39(2):165–79.
43. Ryderheim F, Kjørboe T. Intraspecific genetic diversity and coexistence in phytoplankton populations. *Limnology and Oceanography*. 2024;69:1450–63.
44. Zhang B, Wu J, Meng F. Adaptive Laboratory Evolution of Microalgae: A Review of the Regulation of Growth, Stress Resistance, Metabolic Processes, and Biodegradation of Pollutants. *Front Microbiol*. 2021;12:737248. <https://doi.org/10.3389/fmicb.2021.737248> PMID: 34484172
45. Schlegel I, Krienitz L, Hepperle D. Variability of calcification of *Phacotus lenticularis* (Chlorophyta, Chlamydomonadales) in nature and culture. *Phycologia*. 2000;39:318–22.
46. Stumm W, Morgan JJ. Aquatic chemistry: Chemical equilibria and rates in natural waters. New York: John Wiley & Sons, Inc. 2012.

47. Mackereth FJH, Heron J, Talling JF. Water analysis: Some revised methods for limnologists. Freshwater Biological Association. 1978.
48. de Wit HA, Garmo ØA, Jackson-Blake LA, Clayer F, Vogt RD, Austnes K, et al. Changing water chemistry in one thousand Norwegian lakes during three decades of cleaner air and climate change. *Global Biogeochemical Cycles*. 2023;37. <https://doi.org/10.1029/2022GB007509>
49. Comeau S, Carpenter RC, Edmunds PJ. Effects of pCO₂ on photosynthesis and respiration of tropical scleractinian corals and calcified algae. *ICES Journal of Marine Science*. 2017;74(4):1092–102. <https://doi.org/10.1093/icesjms/fsv267>
50. Page HN, Bahr KD, Cyronak T, Jewett EB, Johnson MD, McCoy SJ. Responses of benthic calcifying algae to ocean acidification differ between laboratory and field settings. *ICES Journal of Marine Science*. 2021;79(1):1–11. <https://doi.org/10.1093/icesjms/fsab232>
51. Ziveri P, Langer G, Chaabane S, de Vries J, Gray WR, Keul N, et al. Calcifying plankton: From biomineralization to global change. *Science*. 2025;390(6771):eadq8520. <https://doi.org/10.1126/science.adq8520> PMID: 41129633
52. Hasler CT, Butman D, Jeffrey JD, Suski CD. Freshwater biota and rising pCO₂? *Ecology Letters*. 2016;19:98–108.
53. Ninokawa AT, Ries J. Responses of freshwater calcifiers to carbon-dioxide-induced acidification. *Journal of Marine Science and Engineering*. 2022;10(8):1098.
54. Hepperle D, Krienitz L. The extracellular calcification of zoospores of *Phacotus lenticularis* (Chlorophyta, Chlamydomonadales). *European Journal of Phycology*. 1996;31:11–21. <https://doi.org/10.1080/09670269600651141>
55. Taylor AR, Brownlee C, Wheeler GL. Coccolithophore cell biology: Chalking up progress. *Annual Review of Marine Science*. 2017;9:283–310.
56. von Dassow P. Voltage-gated proton channels explain coccolithophore sensitivity to ocean acidification. *Proc Natl Acad Sci U S A*. 2022;119(25):e2206426119. <https://doi.org/10.1073/pnas.2206426119> PMID: 35687664
57. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, et al. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science*. 2007;318(5848):245–50. <https://doi.org/10.1126/science.1143609> PMID: 17932292
58. Beilby MJ, Bisson MA, Schneider SC. How characean algae take up needed and excrete unwanted ions – an overview explaining how insights from electrophysiology are useful to understand the ecology of aquatic macrophytes. *Aquatic Botany*. 2022;181:1–9.
59. Taylor AR, Brownlee C, Wheeler GL. Proton channels in algae: reasons to be excited. *Trends Plant Sci*. 2012;17(11):675–84. <https://doi.org/10.1016/j.tplants.2012.06.009> PMID: 22819465
60. Bach LT, Riebesell U, Schulz KG. Distinguishing between the effects of ocean acidification and ocean carbonation in the coccolithophore *Emiliania huxleyi*. *Limnology & Oceanography*. 2011;56(6):2040–50. <https://doi.org/10.4319/lo.2011.56.6.2040>
61. Moroney JV, Ma Y, Frey WD, Fusilier KA, Pham TT, Simms TA, et al. The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles. *Photosynth Res*. 2011;109(1–3):133–49. <https://doi.org/10.1007/s11120-011-9635-3> PMID: 21365258
62. Pigliucci M. Characters and environments. In: Wagner GP. *The Character and Concept in Evolutionary Biology*. San Diego, CA, USA: Academic Press. 2001.
63. Orsini L, Vanoverbeke J, Swillen I, Mergeay J, De Meester L. Drivers of population genetic differentiation in the wild: isolation by dispersal limitation, isolation by adaptation and isolation by colonization. *Mol Ecol*. 2013;22(24):5983–99. <https://doi.org/10.1111/mec.12561> PMID: 24128305
64. Kremer CT, Fey SB, Arellano AA, Vasseur DA. Gradual plasticity alters population dynamics in variable environments: thermal acclimation in the green alga *Chlamydomonas reinhardtii*. *Proc Biol Sci*. 2018;285(1870):20171942. <https://doi.org/10.1098/rspb.2017.1942> PMID: 29321297
65. Schaum E, Rost B, Millar AJ, Collins MS. Variation in plastic responses of a globally distributed picoplankton species to ocean acidification. *Nature Climate Change*. 2013;3:298–302. <https://doi.org/10.1038/NCLIMATE1774>
66. Capon SJ, Steward-Koster B, Bunn SE. Future of freshwater ecosystems in a 1.5°C warmer world. *Frontiers in Environmental Science / Freshwater Science*. 2021;9. <https://doi.org/10.3389/fenvs.2021.784642>