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Ecophysiology matters: linking inorganic carbon acquisition to ecological preference in four species of microalgae (Chlorophyceae)[1]

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Linking C_i acquisition to ecology

ABSTRACT

The effect of CO_2 supply is likely to play an important role in algal ecology. Since inorganic carbon (C_i) acquisition strategies are very diverse among microalgae and C_i availability varies greatly within and among habitats, we hypothesized that C_i acquisition depends on the pH of their preferred natural environment (adaptation) and that the efficiency of C_i uptake is affected by CO_2 availability (acclimation). To test this, four species of green algae originating from different habitats were studied. PH-drift and C_i uptake kinetic experiments were used to characterize C_i acquisition strategies and their ability to acclimate to *high* and *low* CO_2 conditions and *high* and low *pH* was evaluated. Results from pH drift experiments revealed that the acidophile and acidotolerant *Chlamydomonas* species were mainly restricted to CO_2, whereas the two neutrophiles were efficient bicarbonate users. CO_2 compensation points in *low* CO_2-acclimated cultures ranged between 0.6 and 1.4 µM CO_2 and acclimation to different culture pH and CO_2 conditions suggested that CO_2 concentrating mechanisms were present in most species. *High* CO_2 acclimated cultures adapted rapidly to low CO_2 condition during pH-drifts. C_i uptake kinetics at different pH values showed that the affinity for C_i was largely influenced by external pH, being highest under conditions where CO_2 dominated the C_i pool. In conclusion, C_i acquisition was highly variable among four species of green algae and linked to growth pH preference, suggesting that there is a connection between C_i acquisition and ecological distribution.

Key index words: acidophile; carbon acquisition; CCM; *Chlamydomonas; Chlorella*; CO_2 supply; extremophile; inorganic carbon uptake kinetics; pH-drift; *Scenedesmus*

Abbreviations: CA, carbonic anhydrase; CCM, carbon dioxide concentrating mechanism; C_i, dissolved inorganic carbon; CO_2, carbon dioxide; C_T, the concentration of C_i at the end of a pH-drift experiment; HCO_3^-, bicarbonate; EPPS, 4-(2-Hydroxyethyl)-piperazine-1-propane sulphonic acid; HEPES, N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid; MES, 2-

(N-Morpholino)-ethane sulphonic acid; TES, N-[Tris-(hydroxymethyl)-methyl]-2-aminoethane sulphonic acid

INTRODUCTION

Phytoplankton are the most important primary producers in oceans and many fresh waters (Häder et al. 1998). Freshwater habitats are very diverse in terms of size, bathymetry, hydrology, transparency and chemistry (Lampert and Sommer 1997, Wetzel 2001) yet phytoplankton are ubiquitous and present even in extreme habitats like volcanic lakes with very low pH (Pedrozo et al. 2001) or soda lakes with very high pH (Melack et al. 1982). This ubiquity results from rapid acclimation to environmental conditions and more importantly to adaptive features of different species, aided by a wide phylogenetic diversity (Falkowski et al. 2004, Maberly et al. 2010). Phytoplankton species have habitat preferences that depend on a wide range of factors (Reynolds 2012) including nutrients (e.g., P, N, Si) that determine ecological distribution (Spijkerman and Coesel 1998, Interlandi and Kilham 2001). Inorganic carbon is a major requirement and one that is highly variable. The geology of the catchment controls water alkalinity and background concentrations of bicarbonate (Raven and Maberly 2004) while biological processes in the catchment and water body can control the concentration of dissolved carbon dioxide (CO_2). The recent anthropogenic emissions of CO_2 to the atmosphere during the industrial period (Etheridge et al. 1996) as another factor of variability have been suggested to promote phytoplankton productivity in freshwaters (Schippers et al. 2004, Jansson et al. 2012) or increase of phytoplankton biomass (Low-Decarie et al. 2015) although if lake concentrations of CO_2 are determined by the catchment (Maberly et al. 2013) this response may be limited.

Over geological time, changing atmospheric CO_2 concentrations, such as the large decrease in the early Eocene (Pearson and Palmer 2000) is believed to have resulted in the

evolution of CO_2 concentrating mechanisms (CCMs; Raven et al. 2012). CCMs provide high CO_2 concentrations at the active side of RuBisCO to maximize carbon fixation and minimize photorespiration (Badger and Price 1992, Badger et al. 1998). They are very diverse among microalgae (Badger et al. 1998, Badger 2003, Giordano et al. 2005, Raven and Beardall 2014). Mechanisms include the active uptake of CO_2 and bicarbonate (HCO_3^-) (Maberly and Spence 1983, Sültemeyer et al. 1989, Kaplan and Reinhold 1999), the extracellular conversion of HCO_3^- to CO_2 by dehydration due to a carbonic anhydrase located in the periplasmic space (Moroney et al. 2001, van Hille et al. 2014) resulting in the uptake of CO_2 and the recapturing of CO_2 by barriers in the chloroplast (Yamano et al. 2010). The acclimation of algal cells to different pH and CO_2 levels cause variations in efficiency of CCMs (Trimborn et al. 2008, Raven and Beardall 2014) or even the occurrence of them which is for example shown for the detection of, predominantly periplasmic, carbonic anhydrases (Nimer et al. 1997, Tortell and Morel 2002).

In addition to acclimation to CO_2 availability caused by algal phenotypic plasticity, adaptive differences among species based on genotype has been described. For example, an earlier study showed that within a group of desmids, the pH of their original aquatic habitat roughly affects their C_i preference as determined in pH drift experiments (Spijkerman et al. 2005), while the chrysophytes as a group appear to lack a CCM (Maberly et al. 2009).

This study aimed to determine both adaptive and acclimatory responses of microalgae to pH and C_i availability. Adaptation was studied by comparing responses of four species of green microalgae from habitats of different pH. Acclimation was studied by growing these species under *high* and *low* CO_2 concentrations and *high* and low *pH*.

MATERIAL AND METHODS

Algae and pH growth experiment. Four species of green algae were studied*:* *Chlamydomonas acidophila* Negoro (SAG 2045, from an acidic mining lake (pH 2.7), Lausitz, Germany)*, Chlamydomonas pitschmannii* Ettl (SAG 14.73, from a boggy spring near Brezová, Slovakia), *Chlamydomonas reinhardtii* Dangeard (SAG 11-32b, from soil from a potato field near Amherst, USA) and *Scenedesmus vacuolatus* Shihira et Krauss (SAG 211-8c, from tap water from Berlin, Germany, formerly designed as *Chlorella pyrenoidosa* (CCAP 211/8c), *Chlorella emersonii* (CCAP 211/8c) and *Chlorella fusca* (SAG 211-8c)*).* Algal habitats differ in their pH but they are all located in temperate climate zones, which means that they experience similar temperatures and light intensities. To measure the growth response to different external pH, algae were grown in modified Woods Hole medium (initially 150 µM $NaHCO_3$, without silicate; Gerloff-Elias et al. 2005) in Erlenmeyer flasks (300 mL) containing 150 mL of medium and closed with a foam stopper. The medium with MES was filter-sterilized, whereas others were autoclaved. The medium was regarded as P-replete (100 µM) and buffered following the desired pH range (2.0 – 3.5: 0.012 mM Fe Cl_3, 4.0 – 5.5: 2 mM MES, 6.0 – 7.0: 5 mM HEPES, 7.5 – 9.0: 5 mM TES). Cultures were inoculated with stationary-phase batch-cultured algae to obtain an optical density (OD) at 750 nm of 0.01 in a 5 cm pathlength cuvette. Cultures were placed in a temperature-controlled climate chamber at 20°C (± 1°) and received 130 – 150 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} of photosynthetically active radiation from fluorescent tubes (Philips TLD 58W/840, New Generation, Holland) as measured in air with a spherical light sensor (SQSA 0107, WALZ Mess-& Regeltechnik, Effeltrich, Germany) and which was provided in a 16 h day, 8 h night cycle. The OD was measured daily over 5 days in a spectrophotometer (UV-2401 PC, Shimadzu, Kyoto, Japan). In addition, the pH was measured daily (Knick Portamess®, SE 102N pH/Pt 1000 Sensor, Berlin, Germany) and adjusted when necessary by adding small volumes of NaOH or H_2SO_4. Exponential growth rates were calculated from a linear

regression through the natural logarithm of OD over time. All experiments were carried out at least in triplicate.

Growth of algae for pH drift experiments. For pH drift experiments, cultures were grown in a climate chamber at 20°C under continuous light at 155 – 180 µmol photon $\cdot m^{-2} \cdot s^{-1}$ (measured with the spherical light sensor Li-Cor, SPQA 1329 in air) from fluorescent lamps (Silvania luxline plus F58W/840 (184), coolwhite deluxe, Germany). All cultures were grown in P-replete medium (100 µM) and species-specific optimal pH conditions, which were determined by growth experiments at different pH conditions (Table 1). Growth was monitored by measuring the OD at a wavelength of 800 nm with a spectrophotometer (U-2800 Digital, Hitachi, Tokyo, Japan). Cultures were grown semi-continuously in 1 L flasks by daily exchange of 110 mL culture with fresh medium out of 600 mL total volume ($\mu = 0.2\ d^{-1}$) for at least 11 d prior to use in experiments.

Cells were grown under three further conditions with different pH and concentration of CO_2. *High pH* conditions with very low CO_2 concentrations (0.4 – 2.7 µM); ii) *low CO_2* (12 – 26 µM) conditions with optimal pH and iii) *high CO_2* conditions (~1000 µM) with optimal pH. *Low CO_2* was produced by not aerating the culture. *High CO_2* was produced in diluted cultures of 400 mL algal suspension in 1 L flasks which were bubbled with 2.1% CO_2 in air at a flow rate of about 60 mL $\cdot$ min^{-1} using gas mass-flow controllers (Bronkhorst (UK) Ltd, Cambridge). These *high CO_2* (2.1%) cultures were grown for 2 to 3 days without dilution at initial optimal pH conditions. The species were also grown at *high pH* (Table 1) which was selected to represent a pH above their optimum but within their tolerance. In this case cultures were grown in batch culture for 5 d in 1 L Erlenmeyer flasks containing 800 mL of medium. These cultures were inoculated at a high an OD as possible to maximize the demand for inorganic carbon and simulate very low CO_2 supply at the end of growth.

pH drift experiments. Experiments were conducted in an artificial test medium with an alkalinity of 1 mEq $\cdot$ L^{-1}, comprising 0.5 mM $NaHCO_3$ and 0.5 mM $KHCO_3$ plus 0.15 mM $CaCl_2$ and 0.25 mM $MgSO_4$. Algal cells were separated from the culture medium by centrifugation for 5 minutes at 1000*g* (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). The pellets were washed two times with the test medium and finally resuspended in the test medium to produce a maximal OD of 1.0 at 800 nm (5 cm pathlength cuvette). The resuspended cultures were briefly aerated with CO_2 to reach a starting pH-value of about 7.0 before being sealed from the atmosphere in 100 mL glass bottles containing a magnetic follower and a combination pH-electrode (CSIM11-PH-15L/K2, Campbell Scientific, Logan, USA). They were placed in a thermo-controlled glass water bath at 20°C with a 6-place stirring motor below (stirrer controller A-S 601, Electrothermal, Rochford, UK) that provided slow and continuous stirring. The electrodes had been pre-calibrated with buffers at pH 7 and 10 in the identical bottles, temperature and stirring regime. The suspensions received 310-340 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} continuous light from the side from Silvania Standard F15W/129 warm white and F18W/35 lamps (Mississauga, Canada) measured inside the water bath with a 4 π sensor (LI-COR-sensor, SPQA 1329, Lincoln, USA). The pH increase as a result of the uptake of CO_2 and HCO_3^- caused by the photosynthesis of the cells was recorded from each electrode every 10 seconds with a pH logger (CR10X with a AM 416/Relay Multiplexer, Campbell Scientific) connected to a computer. The end of the drift experiment was determined when a change in pH of < 0.01 units was observed within at least half an hour, which typically occurred between 12 and 20 h from the start of the experiment. The starting and final alkalinity was measured by Gran titration using 0.05 M HCl on a 20 mL sub-sample of the drifted culture.

Drift parameter and compensation points. The final pH was equivalent to the highest pH reached during the pH-drift experiment. The total C_i represents the dissolved inorganic carbon comprising free CO_2, HCO_3^- and CO_3^{2-} and was calculated from the alkalinity,

temperature, pH, and ionic strength of the medium (Stumm and Morgan 1970). The total C_i remaining at the end of the pH-drift was designated C_T. The quotient of C_T over final alkalinity (C_T/alkalinity) was calculated to express the ability of each species to deplete inorganic carbon reserves (Maberly and Spence 1983, Spijkerman et al. 2005). Total C_i uptake rate (C_i -uptake; in mmol C_i (g chl *a* · h)$^{-1}$) was calculated from the change of total C_i concentration over time standardized by chlorophyll *a* (chl *a*) concentration.

Uptake kinetics. Algal species were grown in Wood Hole medium adjusted to low and high pH and in continuous light (70 - 130 µmol photons · m^{-2} · s^{-1}) and 20°C. The definition of low and high pH depended on the optimal range of pH for growth of species analyzed in previous experiments, but were below pH 6.3 for low pH and above for high pH conditions (Table 1). Similar to pH drift cultures, low pH cultures consisted of a dilute suspension of 400 mL medium in 1 L Erlenmeyer flasks, whereas high pH cultures were more densely inoculated and the flasks contained 800 mL. Cultures were checked for pH, OD and concentration of C_i before harvest after 5 or 6 days of growth in the middle of exponential growth phase. Harvested cells were centrifuged (1500*g*, 5 min) and resuspended in medium low in HCO_3^- at both, low and high pH, with buffers mentioned above (Table 1), but with a stronger buffering capacity consisting of 10 mM (except for media at pH 2.5, which were unchanged). Resuspended algae were brought to an OD 0.3 (low pH) and OD 0.4 (high pH) both at 800 nm and a 5 cm pathlength and injected into the cuvette of a light dispensing system (Illuminova, Uppsala, Sweden). After depleting C_i in the cuvette, different amount of a stock solution of $NaHCO_3$ was added repeatedly and O_2 evolution recorded at saturating light intensity (~500 µmol photons · m^{-2} · s^{-1}) with a Clark type electrode (MI-730 oxygen electrode, Microelectrodes, New Hampshire, USA). O_2 evolution rates after every HCO_3^- addition were related to chl *a* content and modelled to C_i concentration using the Michaelis-Menten equation. Linearization following Hofstee (Hofstee 1952) provided the maximal uptake rate (V_{max}) and the half-saturation-constant ($K_{0.5}(C_i)$), which represents the substrate

concentration at which the uptake rate is half of the maximum (V_{max}). Additionally, we calculated the $K_{0.5}(CO_2)$ by multiplication of $K_{0.5}(C_i)$ with the proportion of CO_2 in the C_i pool. The affinity for C_i, as the initial slope, was calculated from $V_{max}/K_{0.5}(C_i)$. Experiments were performed in triplicates.

Measurement of C_i and calculation of CO_2. We measured the C_i concentration remaining in the media after cultivation by centrifugation (2500 g, 5 min) and careful filtration though 0.22 µm filters called Minisart High Flow (Satorius Stedim Biotech GmbH, Göttingen, Germany). These samples were injected in the elemantar highTOC (Elementar Analysensysteme GmbH, Hanau, Germany) and concentrations of C_i were determined by comparing values with a standard curve. CO_2 concentrations were calculated by multiplying the proportion of CO_2 given by pH with the concentration of C_i.

Cell density, volume and chlorophyll a content. Cells were fixed with Lugol's iodine (1%) and counted on an automatic cell counter (CASY®1 TT, Schärfe System, Reutlingen, Germany). The diameter of the cells were also measured with the CASY®1 TT and converted into volume by assuming that cells were spherical. Chl *a* was extracted by collecting the cells on glass microfiber filters (GF/F, diameter: 25 mm, Whatman, Buckinghamshire, UK), heating them with 90% ethanol at 60°C for 15 min and incubating them at room temperature overnight in the dark. The chl *a* was measured the next day on a fluorometer (T.D 700, Turner Designs, Gamma Analysen Technik GmbH) as described by Welschmeyer (1994). The calibration of the fluorometer originated from a dilution range of commercially obtained chl *a* (Sigma). The chl *a* content per cell volume was calculated from the chl *a* concentration, cell density and the volume of the cells.

Statistical analyses. Data were analysed statistically with Sigma plot (Version 13.0). After testing for normal distribution and homogeneity of variance three-way, two-way- or one-way-ANOVAs were used to detect differences between treatments and algal species. The

Holm-Sidak test was used as a post-hoc test to identify differences within groups of treatments or species. Data that were not normally distributed were analysed with Kruskal-Wallis One Way Analysis of Variance on Ranks followed by a Tukey test (post-hoc).

RESULTS

pH-dependent growth rates. The exponential growth rates of the four algal species depended on the external pH and the physiology of the species (Fig. 1). The optimal pH conditions and the range of this optimum for growth differed among algal species (Table 2). The results for *C. acidophila* confirmed the acidophilic nature of this species (Gerloff-Elias et al. 2005) as growth ceased at pH 7 and was optimal between pH 2.5 and 5.3. Relative to the other species the pH tolerance was narrow in *C. acidophila* (Table 2). In contrast, *C. pitschmannii* and *C.reinhardtii* both had a wide range in pH optimum, with a mean optimum in slightly acidic pH (5.6 and 6.3, respectively). We detected a broad tolerance, but narrow pH optimum range for *S. vacuolatus* which grew best in neutral, up to slightly alkaline, pH conditions (6.7 – 9.5) but could tolerate pH between 3.5 and higher than 10. In this species we observed a sudden increase in growth rate above pH 6.0, which was not related to a change in the buffer used as the increase happened within the range of one buffer.

Cell volume and chlorophyll a content. The CO_2 concentration influenced the volume of algal cells (Table 3; 2-Way-ANOVA, $F_{2,26} = 20.39$, $P < 0.001$), where in all species, except *C. reinhardtii*, cells were larger when grown at *high* CO_2 conditions than when grown at *low* CO_2 (Table 3). In all species, except *C. acidophila* (Holm-Sidak, $P < 0.001$), the cell volume was similar in cells grown at *high pH* and *low* CO_2. In contrast, the chl *a* content per cell (pg · $cell^{-1}$) or per cell volume (fg · μm^{-3}) did not differ significantly among any treatments. Cell volumes were different for each species (2-Way-ANOVA, $F_{3,26} = 169$, $P < 0.001$); with *C. pitschmannii* having the smallest and *C. reinhardtii* the largest. The cells of *S. vacuolatus*

were intermediate in volume between *C. pitschmannii* and *C. acidophila* (Table 3). The chl *a* content per cell was highest in *C. reinhardtii* (2-Way-ANOVA, $F_{3,26} = 41.2$, $P < 0.001$; Holm-Sidak, $P < 0.001$) independent of treatment. The chl *a* content per cell volume also differed among species (2-Way-ANOVA, $F_{3,26} = 10.0$, $P < 0.001$). It was higher in *C. reinhardtii* and C. *pitschmannii* than in the other two species (Holm-Sidak, $P < 0.01$).

pH drift. The pH at the end of a drift differed significantly among species when grown in *low* CO_2 conditions (ANOVA, $F_{3,12} = 397.9$, $P > 0.001$, Fig. 2). We found a high final pH for *C. reinhardtii* and *S. vacuolatus* and relatively low values for *C. pitschmannii* and *C. acidophila* (Holm-Sidak, $P < 0.001$). The shape of the pH-drift curves over time (Fig. 2) indicated a differential reliance on the available inorganic carbon sources among species which was confirmed when analysed as rate of photosynthesis versus concentration of CO_2 (Fig. 3). At low external pH a rapid increase and thus a rapid uptake of CO_2 is shown in all species. The acidophile *C. acidophila* and the acidotolerant C. *pitschmannii* drifted only until a pH between 9 and 10, suggesting that they mainly relied on CO_2 for their external source of C_i. The observation in all pH-drifts that the pH remained constant at the end of the drifts of *C. acidophila* although this final pH lies well above its tolerated pH for growth (see Fig. 1), suggests that the exposure to alkaline pH for several hours was not lethal. The other two algae continued to raise pH to much higher values, indicating that either they acclimated during the drift to acquire bicarbonate as the concentration of CO_2 is extremely low at these high pH values or that they had this capacity already. The decrease of the C_i-uptake rate in that part of the curve shown by a slower increase of pH is linked to a physiological change from CO_2 to HCO_3^- uptake (Fig. 2).

C_i-uptake rate during pH drift. We analysed the C_i-uptake characteristics of the different species of algae grown at *high* and *low* CO_2 conditions when plotted against CO_2-concentration (in µM); which declined during the pH-drift (Fig. 3). When the curves consisted

of just one linear section, the algae used mainly CO_2, whereas the presence of a second section of uptake rates at extremely low CO_2 concentrations implied the additional use of bicarbonate (Allen and Spence 1981, Maberly and Spence 1983). The C_i-uptake rates at very low CO_2 concentrations of the neutrophilic algae have to be interpreted with caution as they are based on very small changes in pH at the end of the drift. Nevertheless, the results in Fig. 3 showed that in all algal species, uptake rates varied with carbon concentration - expressed here as CO_2 concentration. The slope of the C_i-uptake rate of the algae grown in *high CO_2* conditions often converged to the slope of the *low CO_2* curve, which implied an acclimation to low CO_2 conditions during the drift. The reduction of the C_i-uptake rate between CO_2-dependent and bicarbonate-dependent sections indicated the operation or induction of a CCM. In contrast to algae growing at *low CO_2* conditions, algae growing in *high CO_2* conditions decreased their C_i-uptake rate faster with declining CO_2-concentrations which resulted in a lower C_i-uptake rate at 1 µM CO_2 (Fig.4). As a consequence, a clearer separation between CO_2 and HCO_3^- use in the pH-drift experiments of the neutrophilic algae acclimated to *high CO_2* conditions was observed.

C_T/alkalinity quotient. We hypothesized that the C_T/alkalinity quotient would be higher in species originating from habitats with low pH where CO_2 is the dominant form of inorganic carbon and lower in species originating from habitats with higher pH where bicarbonate is present and algae are often able to use it as a C_i source. The C_T/alkalinity quotient (the remaining total C_i at the end of the drift, C_T related to the alkalinity) describes the effectiveness to deplete C_i. A low value indicates that a large proportion of the inorganic carbon pool is available for acquisition.

The C_T/alkalinity quotient differed among species (2-Way ANOVA, $F_{3,25} = 382.6$, $P < 0.001$) and an interaction between treatment and species was found (2-Way ANOVA $F_{2,25} = 10.93$, $P < 0.001$), meaning that there were species-specific responses to the treatments. As

expected from their pH preference for growth, the quotients of *C. acidophila* and *C. pitschmannii* were higher than those of the two other species within all treatments (Holm-Sidak, $P < 0.001$) as their ability to use bicarbonate was limited (Fig. 3). In addition, the C_T/alkalinity quotient was influenced by at least one treatment in species originated from acidic environments. In the following we analysed the influence of the treatments within a single species more closely.

There were significant differences in the C_T/alkalinity quotient of *C. acidophila* among treatments (Fig. 5a, One-Way ANOVA, $F_{2,7} = 27.3$, $P < 0.001$). Compared to the *low* CO_2 treatment, the quotient was significantly higher under *high* CO_2 and *high pH* conditions (Holm-Sidak, $P < 0.001$ for both). Similar values were measured in the C_T/alkalinity quotient of *C. acidophila* grown at *high pH* and *high* CO_2 conditions (Holm-Sidak, $P = 0.624$). Thus, the algae were able to take up C_i more efficiently when grown in *low* CO_2 conditions.

Significant differences in the C_T/alkalinity quotient were found between growth conditions of *C. pitschmannii* (Fig. 5b; One-Way-ANOVA, $F_{2,8} = 19.3$, $P < 0.01$). Like for *C. acidophila*, the C_T/alkalinity quotient was lowest in cells grown at *low* CO_2 (Holm-Sidak, $P < 0.05$) and highest in cells grown at *high* CO_2 conditions (Holm-Sidak, $P < 0.05$). The C_T/alkalinity quotient of cells grown under *high pH* conditions was intermediate and significantly different from cells in the two other treatments (Holm-Sidak, $P < 0.05$). Similar to the results in *C. acidophila,* a *high*er concentration of CO_2 and *higher pH* conditions lowered the ability to deplete total inorganic carbon during a pH drift.

Against expectations, the C_T/alkalinity quotient did not vary significantly with growth conditions for *C. reinhardtii* (ANOVA, $F_{2,8} = 3.99$, $P = 0.079$), suggesting that growth conditions did not influence the ability to deplete C_i at the end of pH-drift which is consistent with an acclimation to low CO_2 conditions during the drift. Acclimation seemed to enable the algae to use bicarbonate effectively at the end of the drift (see also Fig. 3) for example by

activation of CCMs during drift, thereby masking the acclimation to *high CO_2* conditions during growth.

Similar to the results described for *C. reinhardtii*, there were no significant differences in the C_T/alkalinity quotient among treatments for *S. vacuolatus* (Kruskal-Wallis One Way ANOVA on ranks, P = 0.05).

Compensation point of CO_2. By plotting the total C_i -uptake against total C_i a two-phased graph can often be observed. The part with a positive slope at high concentration of C_i shows the uptake of CO_2, and the intercept of this linear regression with the x-axis (total C_i) represents the compensation point of CO_2 (Maberly and Spence 1983). At that point the net photosynthetic rate reaches zero, if CO_2 is the only used C_i source. No differences were found between the CO_2 compensation points of the algal species (Fig. 5b; 2-Way-ANOVA, $F_{3,29}$ = 0.78, P = 0.51), although there was an influence of the growth conditions on the CO_2 compensation point (2-Way-ANOVA, $F_{2,29}$ = 11.0, P < 0.001). Two algal species (*C. pitschmannii* and *S. vacuolatus*) grown under *high CO_2* conditions had significantly higher CO_2 compensation points than when grown at *low CO_2* (Holm-Sidak, P < 0.05). The CO_2 compensation points of *C. acidophila* and *C. pitschmannii* grown in *high pH* were significantly higher than the compensation points of these species grown in *low CO_2* (Holm-Sidak, P < 0.05 for both), suggesting that pH in itself (as proton concentration) had an influence on C_i-acquisition.

C_i uptake kinetics. It was not possible to separate fully the influence of pH from the influence of CO_2, as algae growing in low pH conditions had a higher concentration of CO_2 (19 ± 7 µM) than algae growing at high pH (1.2 ± 1.1 µM) although the C_i concentration did not differ significantly (21 ± 13 µM). The influence of pH/CO_2 in both growth condition and measuring condition was studied in order to separate the influence of pH/CO_2 on physiological properties during growth from the influence of the available CO_2 during C_i

uptake. Most parameters for uptake kinetics are only calculated for C_i as the uptake of CO_2 and HCO_3^- cannot be readily separated in these experiments. However, in order to compare C_i uptake kinetics with values from literature, we calculated $K_{0.5}(CO_2)$ from pH and the $K_{0.5}(C_i)$.

Uptake kinetics for C_i exemplified for *C. acidophila* varied with pH treatment as shown by different maximal rates of oxygen evolution (V_{max}) and affinity constants ($K_{0.5}(C_i)$) (Fig. 6). In this species, the measurement pH largely influenced $K_{0.5}(C_i)$ (2-Way-ANOVA, $F_{1,11}$ = 145.1, $P < 0.001$) and the pH of the culture during growth determined V_{max} (2-Way-ANOVA, $F_{1,11} = 43.7$, $P < 0.001$). For *C. acidophila*, cells cultured at pH 7 and measured at pH 7 were unable to use low concentrations of C_i leading to the observation that there was a threshold in C_i for the uptake rate under these conditions. Also in *C. reinhardtii* growth conditions affected V_{max} (2-Way-ANOVA, $F_{1,11} = 17.4$, $P < 0.01$) and we saw higher values in low pH conditions. *C. reinhardtii* had the highest V_{max} values in all treatments.

Neither V_{max} in the other two species nor $K_{0.5}(C_i)$ in the other three species varied significantly among different pH conditions (Table S1) which means that within a species C_i is taken up at a similar rate independent of treatment. Therefore we restrict our results to $K_{0.5}(CO_2)$ and the affinity for C_i uptake.

The half-saturation constant $K_{0.5}$ for CO_2 varied among species (3-way-ANOVA, $F_{3,35}$ = 99,3, $P < 0.001$) and was also affected by pH during measurement (3-way-ANOVA, $F_{1,35}$ = 146.5, $P < 0.001$), but pH conditions during growth had no influence (3-way-ANOVA, $F_{1,35}$ = 0.0217, $P = 0.884$; Fig. 7). *Chlamydomonas pitschmannii*, *C. reinhardtii* and *S. vacuolatus* had a higher $K_{0.5}(CO_2)$ when the external pH was low (i.e. when the proportion of CO_2 to C_i is higher). In contrast, *C. acidophila* had a lower $K_{0.5}(CO_2)$ at low pH. Because the absolute value of $K_{0.5}(CO_2)$ depends on that of V_{max}, we analysed the affinity for C_i uptake more closely.

The affinity ($V_{max}/K_{0.5}$) for C_i uptake differed among species (3-way-ANOVA, $F_{3,35}$=17.1, $P < 0.001$), growth conditions (3-way-ANOVA, $F_{1,35} = 22.9$, $P < 0.001$) and pH during the measurement (3-way-ANOVA, $F_{1,35} = 401.2$, $P < 0.001$). Because these differences were not always the same within species, interactions between these factors were statistically significant, therefore requiring analysis of individual species (Fig. 8).

For *C. acidophila*, *C. reinhardtii* and *S. vacuolatus*, the highest affinity for total inorganic carbon (C_i) was found for algae cells grown and measured at low pH (LL, Holm-Sidak, $P < 0.01$). Also, a high affinity although significantly lower than mentioned above, was present in algae grown at high pH but measured at low pH (HL, Holm-Sidak, $P < 0.01$). These species measured at high pH had a significantly lower affinity for C_i uptake than algae measured at low pH (Holm-Sidak, $P < 0.05$), which was independent of growth pH (Holm-Sidak, $P \geq 0.125$).

Chlamydomonas pitschmannii measured at high pH had a higher affinity for C_i (Holm-Sidak, $P < 0.05$) compared to measurements at low pH, but no influence of growth conditions to the affinity was observed (Holm-Sidak, $P \geq 0.882$).

In summary, the greatest differences in C_i uptake were established in cultures differing in pH during the measurement. Therefore the C_i uptake was influenced more by changes in carbon speciation caused by pH than by possible physiological disadvantages during growth in higher pH conditions.

C_T/alkalinity vs. pH growth optimum. There was a negative correlation between the mean of the pH optimum range for growth and the ability to take up C_i (Fig. 9). Consequently, for these algae there was a greater ability to take up C_i (lower C_T/alkalinity quotient), as the preferred growth pH increased.

DISCUSSION

The diversity of phytoplankton phylogeny (Falkowski et al. 2004), environmental variability (Lampert and Sommer 1997, Wetzel 2001, Kim et al. 2006), and also ecophysiological variation found within one species (Moore and Chisholm 1999, West and Scanlan 1999, Spijkerman 2005), results in adaption and acclimation being important processes determining distribution and local adaptation. Among the different resource acquisition processes, the energy requiring uptake of C_i is one mechanism underlying habitat preference, and one which may be regulated depending on environmental changes or circumstances like pH -or CO_2 concentrations. In this study we generally confirmed our hypothesis that for four species of green algae there is a relationship between the pH of the environment at which each species is typically found, the pH optimum for growth and their C_i uptake strategy.

Adaption: Effect of pH preference on C_i acquisition. The pH of the medium is the 'master variable' that determines the ratio of CO_2 to HCO_3^-; at a pH higher than about 6.4 the concentration of HCO_3^- exceeds that of CO_2 (Stumm and Morgan 1970). Therefore we hypothesised that pH preference for growth would correlate with the species' ability to acquire CO_2 and / or HCO_3^-. The acidophile *C. acidophila* grew optimally under acid conditions as reported before (Nishikawa and Tominaga 2001, Gerloff-Elias et al. 2005, Spijkerman 2005, Cuaresma et al. 2006). The acidotolerant species *C. pitschmannii* had a broad tolerance to pH. With the exception of high growth rates at alkaline pH, this pH tolerance fitted to the expected low pH tolerance as reported by Pollio and colleagues (Pollio et al. 2005). Neither of the neutrophiles, *S. vacuolatus* and *C. reinhardtii*, could grow at highly acidic pH conditions as has been reported before for *C. reinhardtii* (Erlbaum Cassin 1974, Spijkerman 2005).

As expected, the main C_i source used for photosynthesis, as determined from the pH drift experiments, was related to the ability to grow in low pH conditions: *C. acidophila* and

C. pitschmannii mainly relied on CO_2 (also suggested for *C. acidophila* because of low $K_{0.5}(C_i)$; Cuaresma et al. 2006) whereas the two other algae could use both CO_2 and HCO_3^- as previously described for *C. reinhardtii* (Sültemeyer et al. 1989, Amoroso et al. 1998). Beardall and Raven (1981) have shown an active uptake of bicarbonate via uniports in *S. vacuolatus* (then named *Chlorella emersonii*).

A positive correlation was detected between the pH optimum for growth and the ability to deplete C_i during the pH drift experiment. That means that algae preferentially growing in neutral waters and using both CO_2 and HCO_3^- were more efficient in C_i depletion, than species growing under acidic conditions and restricted to CO_2. This restriction seems to result in a lower exponential growth rate in *C. acidophila* compared to the other species (Fig. 1). In contrast, the acidotolerant alga *C. pitschmannii* reached growth rates as high as in the neutrophilic algae and a high exponential growth rate was even found at high pH (pH > 7 up to 9). Interestingly, *C. acidophila* could not grow at pH values above pH 7.5 (Fig. 1) although pH rose during the drift to pH 9 (Fig. 2). This might be caused by the rate of change in pH in these two types of experiment, but might also result from high pH restricting the acquisition of other resources such as phosphate, the speciation of which is also affected by pH. Additional research is needed to unravel further the reason why the growth of *C. acidophila* is restricted to low pH.

The detection of high affinity C_i-uptake as well as a low affinity constant for CO_2 under low CO_2 conditions in conjunction with the presence of a pyrenoid (visible under the microscope) in all four species of green algae suggests that they have a CCM (Meyer and Griffiths 2013, Moroney and Chen 1998), although Maberly et al. (2009) found pyrenoids in chrysophytes that appeared to lack a CCM.

Similar to the presence of a pyrenoid, all species had a high affinity C_i-uptake. To compare the data from the kinetic measurements with values of other studies, we calculated $K_{0.5}(CO_2)$. The values for *C. acidophila* are within the same range as Spijkerman (2005)

found in her study (2 and 7 µM CO_2 in pH 2.65 and pH 6 respectively). Also the affinity constants of *C. reinhardtii* are similar to values from literature (0.8 – 8.3 µM CO_2 depending on CO_2 supply, Amoroso et al. 1998). Our values for *S. vacuolatus* differ slightly from values of Beardall and Raven (1981, a species then called *Chlorella emersonii*), although they lie well within their reported range (5.7 µM CO_2 at a pH about 6.5).

Acclimation: Effects of CO_2 supply on C_i acquisition. There is a large literature documenting the down-regulation of CCMs when microalgae, including *C. reinhardtii,* are grown at high concentrations of CO_2 (Giordano et al. 2005, Sültemeyer et al. 1988, Beardall and Giordano 2002, Raven and Beardall 2014). The CO_2 compensation point represents one measure of CO_2 uptake efficiency. The range of the estimated compensation points reported here in *low CO_2* conditions is close to the ones Maberly and Spence (1983) found for *C. reinhardtii* (0.5 µM CO_2) and also close the range of the ones Diaz and Maberly (2009) have shown for some acidophilic algae (2 – 12 µM CO_2). The CO_2 compensation point was generally lowest in cells grown at *low CO_2* and statistically so in *C. pitschmannii* and *S. vacuolatus*. Growth at *high pH*, despite growth concentrations of CO_2 being low, generally resulted in a high CO_2 compensation point and this was statistically significant in *C. acidophila* and *C. pitschmannii*.

Because pH-regulation is an energy requiring process (Messerli et al. 2005) this is consistent with *high pH* during growth in these species causing physiological stress to the algae. This stress might result in, for example, higher respiration rates and lower net photosynthetic rates as shown for *C. acidophila* at pH 7 (Gerloff-Elias et al. 2005), lowering the adaptability to low CO_2 conditions. The difference in cell volumes of *C. acidophila* between *high pH* and *low CO_2* (low pH) treatments (Table 3) support such a physiological influence. In contrast, we found no change of cell volume between low (=*low CO_2* treatment) and *high pH* conditions in the neutrophiles. Although the acidotolerant species *C.*

pitschmannii could grow rapidly at high pH conditions (Fig. 1) the physiological stress might occur for example by a reduced final biomass.

During a pH-drift, there is a reduction in rate of photosynthesis as the concentration of CO_2 declines towards the CO_2 compensation point and then, in some species, an increase as carbon becomes depleted further and HCO_3^- becomes the sole carbon source available (see Allen and Spence 1981, Maberly and Spence 1983). This bi-phasic pattern was evident in the two neutrophile species indicating that they were able to use HCO_3^-. At 1 µM CO_2, around this transition point, the rate of C_i uptake was much greater in the neutrophile than the acidophile species when grown at *low CO_2*, but lower and similar across species when grown at *high CO_2* indicating an acclimation by neutrophile species to *low CO_2* but a much less-pronounced acclimation in the acidophile/acidotolerant species (Fig. 4). Possibly, neutrophiles growing on *low CO_2* already started their transition to HCO_3^- uptake at CO_2 concentrations higher than 1 µM.

We expected the C_T/alkalinity quotient also to vary with the CO_2 treatment, especially for the neutrophile species, but in fact this was not the case. Probably as a results of the duration of the drift, the ability of the well-studied alga, *C. reinhardtii* and *S. vacuolatus* to take up C_i was not significantly affected by variation of CO_2 supply during growth. The observed rapid acclimation of the cells to low CO_2 conditions during a drift (Fig. 3) probably masked the acclimation to *high CO_2* conditions in the culture as the ability to take up C_i was based on measurements at the end of the drift (i.e. after 12 to 20 h). This is consistent with the literature showing in *C. reinhardtii* an induction of a CCM on transfer from low to high CO_2 of only a few hours (Wang et al. 2015).

Among the major constituents of a CCM is the induction of periplasmic carbonic anhydrase. In *C. reinhardtii* and *C. acidophila* this enzyme has been reported to be present under low CO_2 conditions (Wang et al. 2014, Spijkerman et al. 2014), whereas in *C.*

pitschmannii and *S. vacuolatus* its presence is still unclear. The presence of external carbonic anhydrase invalidates the approach of Miller and Colman (1980) that compares rates of CO_2-uptake to physico-chemical rates of CO_2 production from the carbonate equilibria, preventing this approach from being used.

The fully-factorial experiment on C_i-uptake kinetics allowed the separation of pH/CO_2 as a stress parameter (growth conditions) from the effect of pH on the CO_2 proportion (to C_i) during photosynthesis measurements. The affinity for C_i was mainly influenced by pH/CO_2 conditions during the measurement and less by conditions during growth. Differences in the affinity for C_i therefore depended more on the dominant C_i species in the media during the measurement than on any physiological disadvantages by growing in certain pH/CO_2 conditions suggesting that green algal species generally prefer CO_2 over bicarbonate. Thus, the C_i-acquisition characteristic we determined were likely intrinsic, species-specific adaptations, mainly revealing that all species had a low affinity constant for CO_2 uptake and a high affinity for C_i. However, for algae with a limited tolerance range for pH (*C. acidophila* and *S. vacuolatus*) the affinity for C_i when growing in high pH/very low CO_2 conditions was lower indicating that these conditions resulted in a physiological disadvantage. In contrast, *C. pitschmannii* was unaffected in C_i uptake kinetic by high medium pH during growth. Contrary to the results from pH-drift experiments, there was no evidence for upregulation of CCMs in C_i-uptake kinetics resulting from growth at low CO_2 conditions. Probably the addition of high bicarbonate concentrations partly converted to CO_2 hid the acclimation to low CO_2 conditions in that method. Additionally, a fast inactivation of a high affinity CO_2 concentration mechanism was possibly realized. Already for 18 years fast posttranslational acclimation to low CO_2 was acknowledged in *Synechococcus* sp. by Sültemeyer et al. (1998), which might well occur also *vice versa*.

Ecological relevance. Tortell and Morel (2002) have shown a high variability in mechanisms of HCO_3^- usage among marine phytoplankton populations which were dominated by different phylogenetic groups, but we demonstrate here that this range of C_i acquisition ability can also be found within one taxonomic group, the Chlorophyceae. This is concordant with pH drift results found for a variety of desmid strains (Spijkerman et al. 2005), for the CCM variability among different isolates of the coccolithophore *Emiliania huxleyi* (Stojkovic et al. 2013) and the large variability in RuBisCO kinetics in diatoms (Young et al. 2016).

The pH drifts from the two algae from acidic environments showed an influence of CO_2 concentration during growth on the ability to take up C_i and for *C. pitschmannii* also on the uptake efficiency. In contrast to the results for neutrophile algae, this indicates that these algae were less able to acclimate to the low CO_2 condition during the drift. This might result from environmental factors such as the general oversaturation with CO_2 in acidic waters, where *C. acidophila* blooms (Doi et al. 2001, Doi et al. 2003, Spijkerman et al. 2007, Clegg et al. 2012), the lower variability of daily CO_2 conditions in an acidic environment caused by strong pH buffering by iron (Herzsprung et al. 1998) and low photosynthetic demand because of phytoplankton densities (Nixdorf et al. 2003). Furthermore, the apparent absence of an ability to use HCO_3^- will restrict the variability in Ci-uptake capability. The rapid acclimation of the neutrophile algae indicates an ecological relevant plasticity which allows algae to handle fluctuations of C_i supply within hours. Cells of the green alga *C. reinhardtii* increase their intracellular CA activity up to ten-fold within 4 hours of being transferred to low CO_2 conditions (Sültemeyer et al. 1995). Fluctuations are also possible within a day as a result of C_i depletion during the day and in particular by entrainment of CO_2-rich water from depth (Maberly 1996, Reis and Barbosa 2014). Consequently, the ability to acclimate to changing CO_2 conditions, for example by using HCO_3^- more effectively, is ecologically relevant.

The differences in carbon acquisition efficiency of the studied green algae may play an important role in determining community composition. Species competitive exclusion or co-existence depends on (varying) carbon and nutrient concentrations (Jansson et al. 2012, Low-Decarie et al. 2015), as well as on intrinsic ecophysiological characteristics. Species which are able to use HCO_3^- in addition to CO_2 will be at an advantage at high pH by being less dependent on CO_2 availability. Although our results show more rapid acclimation to low CO_2 conditions during pH drift in the neutrophile algae, the reverse situation seems less straightforward as long-term cultivation at high CO_2 has resulted in a different physiological response in *C. reinhardtii* (Low-Decarie et al. 2013) than short term acclimation. During competition for C_i, a high plasticity as present in neutrophile algal species might be advantageous which could be the reason for a missing evolutionary response to high CO_2 conditions instead of an acclimation.

Conclusion. Although acclimation occurred in all species, a rapid acclimation from *high* to low CO_2 conditions during drift experiments was especially pronounced in the neutrophiles and these two species exhibited greater plasticity than the two acidophile/acidotolerant species. However, the magnitude of acclamatory responses was smaller than the adaptation differences among species which were strongly linked to the pH preference for growth and therefore probably to the availability of C_i in their natural environment.

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Table 1: Buffers used in modified Woods Hole medium to reach different pH conditions for every species.

Species	***Buffer***	***Concentration (mM)***	***pH***
C. acidophila (optimal pH)	$FeCl_3$	0.012	2.5
C. acidophila (high pH)	HEPES	5	7
C. pitschmannii (optimal pH)	MES	2	5
C. pitschmannii (high pH)	EPPS	5	8
C. reinhardtii (optimal pH)	HEPES	5	7
C. reinhardtii (high pH)	EPPS	5	8
S. vacuolatus (optimal pH)	HEPES	5	7
S. vacuolatus (high pH)	EPPS	5	8

Table 2: Effect of external pH on the exponential growth rate of *Chlamydomonas acidophila*, *C. pitschmannii*, *C. reinhardtii* and *Scenedesmus vacuolatus*. Maximum growth rate presented as mean and standard deviation (in parentheses) of three replicates. The tolerance range represents the pH where growth is positive. The optimum pH represents the interpolated pH where the growth rate is above 80% of the maximum rate; the mid pH is the center of the optimum range.

Species	Max growth rate (d^{-1})	Tolerance range (pH)	Optimum range (pH)	Width of optimal range (pH)	Mid pH
C. acidophila	1.10 (0.03)	< 7.5	2.5 – 5.3	2.8	3.9
C. pitschmannii	1.58 (0.10)	> 2.0 and < 9.0	2.8 – 8.4	5.7	5.6
C. reinhardtii	1.54 (0.05)	> 3.5	3.9 – 8.7	4.8	6.3
S. vacuolatus	1.25 (0.03)	> 3.0	6.7 – 9.5	2.8	8.1

Table 3: Effect of growth treatment (*high CO_2*: 2-3 d batch culture, *high pH*: 4 d batch culture, *low CO_2*: 9 – 20 days semi-continuous cultivation) and species on cell volume, chlorophyll *a* per cell and chlorophyll *a* per cell volume. Values are the mean and standard deviation of three replicates given in parentheses. Species or treatments (within species) that are significantly different are indicated by different letters.

Characteristic	Treatment	*C. acidophila*	*C. pitschmannii*	*C. reinhardtii*	*S. vacuolatus*
Cell volume (μm^3)	High CO_2	79 (5)^a	40 (6)^a	86 (6)^a	68 (16)^a
	Low CO_2	58 (2)^b	19 (1)^b	100 (6)^a	32 (2)^b
	High pH	71 (5)^c	24 (2)^b	94 (5)^a	32 (1)^b
	Species difference	c	a	d	b
Chl a per cell ($pg \cdot cell^{-1}$)	High CO_2	1.22 (0.22)^a	1.26 (0.24)^a	2.71 (1.42)^a	1.17 (0.13)^a
	Low CO_2	0.90 (0.08)^a	0.95 (0.59)^a	3.36 (1.04)^a	0.52 (0.04)^a
	High pH	1.08 (0.12)^a	0.70 (0.10)^a	3.57 (0.38)^a	0.53 (0.24)^a
	Species difference	a	a	b	a
Chl a per cell volume ($fg \cdot \mu m^{-3}$)	High CO_2	15.2 (1.8)^a	31.8 (0.4)^a	30.8 (13.6)^a	17.7 (2.8)^a
	Low CO_2	15.7 (1.0)^a	49.6 (32.2)^a	34.8 (13.0)^a	16.2 (1.4)^a
	High pH	15.3 (2.4)^a	29.5 (4.9)^a	38.1 (5.9)^a	15.3 (2.4)^a
	Species difference	a	b	b	a

Figure 1: Effect of external pH on the exponential growth rate of *Chlamydomonas acidophila*, *C. pitschmannii*, *C. reinhardtii* and *Scenedesmus vacuolatus*. The mean and standard deviation of three replicates is presented.

Figure 2: Typical examples of pH-drift over time (one replicate) for *Chlamydomonas acidophila*, *C. pitschmannii*, *C. reinhardtii* and *Scendesmus vacuolatus* grown at air-levels of CO_2 and optimal pH and tested at an initial alkalinity of 1 mequiv $\cdot L^{-1}$.

Figure 2: Rate of photosynthesis during pH drift as a function of CO_2 concentration (log scale) for *Chlamydomonas acidophila*, *C. pitschmannii*, *C. reinhardtii* and *Scenedesmus vacuolatus* grown at air-levels of CO_2 (*low* CO_2) or 2.1% CO_2 in air (*high* CO_2). Smoothed curves by 10-data-point moving average of all three replicates are shown.

Figure 4: C_i uptake rates (mmol $C_i \cdot$ (g chl $a \cdot$ h)$^{-1}$) at a CO_2 concentration about 1 µM for *low* and *high* CO_2 treatments. Values represent mean and standard deviation of rates immediately above and below the CO_2 concentration of 1 µM for three replicates.

Figure 5: Response of the C_T/Alk quotient (A) and CO_2 compensation point (B) for *Chlamydomonas acidophila*, *C. pitschmannii*, *C. reinhardtii* and *Scenedesmus vacuolatus* grown at *low* CO_2 (grey), *high* CO_2 (black) and *high pH* (white). Mean plus or minus one standard deviation of three replicates are shown. Different letters show significant differences among treatments within a species (One way-ANOVA, Holm-Sidak, $P < 0.05$).

Figure 6: Modelled values of typical C_i uptake kinetics as measured by oxygen evolution versus total inorganic carbon (C_i) for the alga *Chlamydomonas acidophila*. The first value in the legend gives the external pH at which the alga was grown for 5 d and the second value gives the pH at which the measurement was performed.

Figure 7: Half-saturation constant $K_{0.5}(CO_2)$ for four species grown and tested under different conditions. The different letters show significant differences among treatments within species

(2-Way-ANOVA, Holm-Sidak, $P < 0.05$). The first letter in the legends represents the pH in the growth media and the second letter represents the pH during the measurement; L = low/optimal, H = high (Table 1).

Figure 8: Affinity for total inorganic carbon ((mmol $O_2 \cdot$ (g Chl *a* $\cdot$ h)$^{-1}$) $\cdot$ (µM C_i)$^{-1}$)) for four species grown and tested under different conditions. The different letters show significant differences among treatments within species (One-way-ANOVA, Holm-Sidak, $P < 0.001$). The first letter in the legends represents the pH in the growth media and the second letter represents the pH during the measurement; L = low/optimal, H = high (Table 1).

Figure 9: Relationship between the ability to deplete C_i represented by the parameter 'C_T/alkalinity' and the mean of the optimal pH conditions for growth for four species of algae grown at three different treatments (*low CO_2, high CO_2, high pH*). The line represents the correlation (Spearman, $P < 0.001$).