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RESEARCH PAPER

Phosphorus acquisition by *Chlamydomonas acidophila* under autotrophic and osmo-mixotrophic growth conditions

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Abstract

Chlamydomonas acidophila **Negoro is a green algal species abundant in acidic waters where inorganic phosphorus (P_i) and carbon (CO_2) are considered the most important growth-limiting nutrients for the phytoplankton. This paper describes the P_i uptake and growth kinetics under varying carbon supply by cultivating the alga autotrophically, with and without CO_2 aeration, and osmo-mixotrophically with glucose under low P_i conditions at pH 2.7. The low minimum cellular phosphorus quota (Q_0; ranging from 0.6 to 1.1 mmol P mol^{-1} C) suggested P_i-limiting conditions under all different modes of carbon supply, and was lowest under CO_2-aerated conditions. The threshold P_i concentration for growth did not vary from zero, suggesting no detectable metabolic costs. Maximum P_i-uptake rates (V_{max}) were a better indication of P_i limitation when compared with the affinity constant for P_i uptake (K_m), as V_{max} was only high under P_i-limited conditions whereas K_m was low under both P_i-limited and P_i-replete conditions. Osmo-mixotrophic growth conditions did not result in decreased extracellular phosphatase activity, but often resulted in physiological characteristics comparable with CO_2-aerated cells, suggesting intracellular CO_2 production by glucose respiration. In addition, at low CO_2 and in autotrophic conditions, *C. acidophila* had a higher Q_0, lower dissolved organic carbon concentration, lower maximum P_i-uptake rates, and lower phosphatase activity, suggesting that growth was co-limited by CO_2 and P_i. Furthermore, cells may respond physiologically to both nutrient limitations simultaneously.**

Key words: Acidophilic algae, *Chlamydomonas acidophila*, CO_2, co-limitation, extremophile, glucose, growth, osmo-mixotrophy, phosphatase activity, P limitation.

Introduction

Very acidic lakes and rivers with pH values between 2.0 and 3.2 are found all over the world (Doi *et al*., 2001; Lopez-Archilla *et al*., 2001; Baffico *et al*., 2004; Kamjunke *et al*., 2004). In these waters, *Chlamydomonas acidophila* is often an abundant species that maintains an optimal growth rate when the external pH is acidic (Nishikawa and Tominaga, 2001; Gerloff-Elias *et al*., 2005; Spijkerman, 2005). Recent studies unequivocally showed that *C. acidophila* maintains a neutral intracellular pH (Messerli *et al*., 2005; Gerloff-Elias *et al*., 2006), which results in increased metabolic costs (Nishikawa *et al*., 2006). Increased metabolic costs can result in an increased cellular phosphorus (P) content, as suggested by Nishikawa *et al*. (2006) and Spijkerman *et al*. (2007), or increased ATP consumption rates (Messerli *et al*., 2005), for which proof is still under debate.

Under inorganic phosphorus (P_i)-deprivation, *Chlamydomonas reinhardtii* increased its maximum P_i-uptake rate (V_{max}) and P_i uptake affinity [reflected by a decrease in affinity constant for P_i uptake (K_m); Grossman, 2000] and in continuous cultures, these physiological characteristics generally increased with decreasing growth rate (Spijkerman and Coesel, 1996*b*). These physiological characteristics have not been described for *C. acidophila* thus far. In acidic lakes, both P_i and CO_2 are most likely the growth-limiting nutrients (Tittel *et al*., 2005; Spijkerman *et al*., 2007), which makes the interaction between these

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Abbreviations: DOC, dissolved organic carbon; K_m, affinity constant for P_i uptake; K_s, affinity constant for growth; μ, growth rate; μ_{max}, maximum growth rate; μ'_{max}, apparent maximal growth rate that would occur if Q_p became infinite; OD, optical density; P_i, inorganic phosphorus; $P_{i,t}$, threshold P_i concentration for growth; Q_0, minimum cellular P quota; Q_p, cellular P quota; SRP, soluble reactive P; SRP_t, threshold SRP value for growth; V, P_i-uptake rate; V_{max}, maximum P_i-uptake rate; WH medium, Woods Hole medium.

nutrients interesting to study. It appears likely that the uptake and growth kinetics for P_i and CO_2 interact as inorganic carbon acquisition varied with the P_i state of the single-celled chlorophyte *Chlorella* (Kozlowska-Szerenos *et al*., 2004; Beardall *et al*., 2005). The results from both studies on *Chlorella* were contrasting: in the study of Koslowska-Szerenos *et al*. (2004), the affinity constant for CO_2 uptake decreased with increasing P depletion, whereas the same parameter increased with increasing P depletion in the study of Beardall *et al*. (2005). In addition, the cellular P quota (Q_p) was higher at low CO_2 than at high CO_2 concentrations; for example, in the marine diatom *Skeletonema costatum* (Burkhardt and Riebesell, 1997). Under P_i-limited conditions, algal cells normally produce a surplus of organic carbon that is partly excreted (e.g. as glycolate). Therefore, P_i-limited cultures usually contain increased concentrations of dissolved organic carbon (DOC) and P_i-limited algae have an increased C:P ratio and are likely to be heavier.

In addition, P_i-limited algal cells synthesize an extracellular enzyme called alkaline or acid phosphatase (Spijkerman and Coesel, 1998; Beardall *et al*., 2001; Grossman and Takahashi, 2001). The synthesis of phosphatase enzymes under P_i-deplete, acidic conditions was reported for *C. acidophila* (Boavida and Heath, 1986; Spijkerman *et al*., 2007) and for *C. reinhardtii* (pH 4; Joseph *et al*., 1995). In phytoplankton from a eutrophic lake and an acid bog, the induction of this enzyme appeared especially important because P acquisition was faster from glucose-6-P than from P_i (Nedoma *et al*., 2003). The hydrolysis of glucose-6-P, results in glucose and P_i, both of which can be used by *C. acidophila* (Bissinger *et al*., 2000). Therefore, phosphatase enzyme activity could relate both to P_i depletion and carbon availability. As the production of P_i is known to inhibit phosphatase activity (O'Brien and Herschlag, 2001), it is possible that the presence of the other end-product, glucose, might also inhibit phosphatase activity.

In this study, the influence of varying carbon sources on P_i physiology and phosphatase activity in *C. acidophila* was investigated. Specimens were grown in semi-continuous, autotrophic P_i-limited culture conditions, with and without CO_2 aeration, and under osmo-mixotrophic P_i-limited conditions. Osmo-mixotrophic growth conditions were obtained by the addition of glucose to the growth medium. The minimum cellular P quota (Q_0), threshold P_i concentration for growth ($P_{i,t}$), P_i uptake kinetics, and phosphatase activity were obtained at pH 2.7.

Materials and methods

Cultures

Chlamydomonas acidophila Negoro, isolated from Lake 111 (SAG Göttingen, strain no. 2045), was grown in semi-continuous cultures at 19.5±1 °C in Woods Hole (WH) medium (Nichols, 1973), with no buffer, a P_i concentration of 1.6 μmol l^{-1}, and a pH adjusted to 2.7 with HCl. P_i-saturated batch cultures contained 50 μmol P_i l^{-1} and were harvested in the mid-exponential phase of growth, with a low cell density. Osmo-mixotrophic growth was established by the addition of 1 mmol glucose l^{-1} in the medium, and cultures were placed in light identical to that used for autotrophic cultures. Dilution rates were 0.1, 0.2, 0.3, 0.4, and 0.6 d^{-1} in non-aerated cultures and from 0.1, 0.2, 0.4, 0.6, and 0.8 d^{-1} in cultures aerated with 4.5% CO_2 in normal air (v/v) and in osmo-mixotrophic cultures (which were non-aerated). All treatments were performed in duplicate. Aeration did not result in significant mixing of the culture suspension and was ~15 ml h^{-1}. All cultures were mixed regularly and a total culture volume of 500 ml in a 1.0 l Erlenmeyer flask provided a large surface area for O_2 exchange with the air. Incident light supply was ~200 μmol photons m^{-2} s^{-1} with a light/dark period of 16/8 h. Daily dilution and harvesting were performed 4–5 h after the onset of light. Average CO_2 concentrations in the CO_2-aerated cultures were measured as dissolved inorganic carbon using a carbon analyser (HighTOC+N; Elementar) and were 0.33 (±0.05, n=20) mmol C l^{-1}. This rendered CO_2-limitation unlikely and this was confirmed by experiments where the CO_2 concentration in the medium was increased, but growth rates did not change. Non-aerated cultures had a CO_2 concentration below the detection limit of the carbon analyser (<0.04 mmol C l^{-1}) and were likely to be equivalent to equilibrium concentrations with the air (~0.02 mmol C l^{-1}). The pH of all cultures was 2.7, independent of aeration with CO_2. Average optical density (OD) ranged from 0.01 to 0.13. The OD of each culture was measured before and after dilution at 750 nm (UV1202, Shimadzu, Germany). After the cultures had reached a steady state (remaining at constant OD after an exchange of 3–5 times the culture volume), samples were taken for cell and bacterial counts, chemical analyses, P_i-uptake rates, and phosphatase activity. Cell numbers were determined using an automatic cell counter (CASY 1, Model TT, Schärfe, Reutlingen, Germany). Bacteria were enumerated under an epifluorescence microscope (Axioscop2, Zeiss) after staining with acridine orange on black 0.2 μm Nuclepore filters (Hobbie *et al*., 1977).

Phosphatase activity

Phosphatase activity was measured using difMUP (6,8-difluoro-4-methylumbelliferyl phosphate; Molecular Probes, Leiden, The Netherlands) on a fluorometer (Turner TD-700, ex: 365 nm, em: 410–610 nm; GAT Bremerhaven). The reaction solution contained 20 mmol Na-acetate l^{-1}, 20 mmol HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid] l^{-1}, and 2 mmol $MgCl_2$ l^{-1}. This solution was brought to pH 2.7, 6.0, and 9.0, to measure the activity at the culturing pH and at two previously determined optima (Spijkerman *et al*., 2007). Activity was measured in suspensions of P_i-limited and P_i-replete cultures, and in the supernatant of these cultures after centrifugation (2500 *g*, 5 min). The cellular activity was calculated by subtraction of these two fractions. Acid phosphatase from potato (EC 3.1.3.2; Sigma) and alkaline phosphatase from *Escherichia coli* (EC 3.1.3.1; Sigma) were similarly treated and served as standards. The increase in fluorescence was measured over a 3 min period after mixing 0.5 ml of 25 μmol difMUP l^{-1}, 0.5 ml of sample, or standard and 1.5 ml of buffer. Fluorescence changes over time and fluorescence intensity after 3 min were calibrated to the acid phosphatase at pH 2.7 and pH 6.0 and to alkaline phosphatase at pH 9.0. All measurements were performed in triplicate.

P_i-uptake kinetics

Cultures were centrifuged (1500 *g*, 5 min) and the pellet was resuspended in WH medium without P_i and iron-EDTA at a pH of 2.7. Final densities were between 1.1×10^5 and 4.4×10^5 cells ml^{-1}.

The culture was placed in the light (~90 µmol m^{-2} s^{-1} inside the flask) for about 15–30 min. Over a period of 1 min, ^{33}P uptake was measured by addition of $H_3^{33}PO_4$ (3000 Ci $mmol^{-1}$ specific activity; Amersham Biosciences, Freiburg) diluted in stock solutions of 50 or 500 µmol K_2HPO_4 l^{-1}. Uptake was terminated by filtration on 1.2 µm pore-size cellulose acetate filters and subsequent rinsing with 0.2 mol LiCl l^{-1}. The filters were embedded in Ultima Gold (Packard) and counted in a liquid scintillation analyser (2300 TR Packard). The P_i-uptake rate (V) was measured at eight concentrations ranging from 0 to 10 µmol P_i l^{-1} using six time points each. From these rates, maximum uptake rates (V_{max}) and the affinity for uptake (K_m) were estimated by fitting the data using SPSS software (version 11.5) to the Michaelis–Menten equation:

$$V = V_{max}[P_i/(P_i + K_m)] \quad (1)$$

Part of the culture was fixed with 0.2% Lugol's solution (final concentration) for cell enumeration. Cell numbers were determined as described above using a cell counter.

Chemical analyses

Total P content of the cell suspension was determined on culture samples heated to 100 °C for 1 h with $K_2S_2O_8$ and 0.5 mol H_2SO_4 l^{-1}. For soluble reactive P (SRP), 12 ml culture samples were centrifuged at 2500 *g* for 5 min, and 10 ml of supernatant was taken for analysis. Measurements of total P and SRP were performed spectrophotometrically using molybdate and ascorbic acid (Murphy and Riley, 1962).

For carbon analyses, culture samples were filtered on pre-combusted, pre-weighed QF20 (Schleicher and Schuell) or GF/F filters. The filter was used for particulate and the filtrate for dissolved organic carbon (DOC) determination in the carbon analyser (HighTOC+N; Elementar). Before measuring particulate organic carbon, filters were dried for 1 week at 30 °C and the dry weight of the algae determined.

Calculation of P_i

Because SRP concentrations in the culture vessel were very low and their values would not equal P_i (Rigler, 1968), P_i was calculated by coupling the kinetics of steady-state nutrient uptake (V, equation 1) with the kinetics of growth (µ; Spijkerman and Coesel, 1996*b*). Growth rates were related to the cellular P quota (Q_p) by Droop's equation (Droop, 1973):

$$\mu = \mu'_{max}[1 - (Q_0/Q_p)] \quad (2)$$

in which Q_0 is the minimum cell quota of P (Q_p at µ=0) and μ'_{max} is the apparent maximal growth rate that would occur if Q_p became infinite. From a steady-state condition, it follows that the rate of cell quota increase due to uptake equals the dilution of cell quota due to growth (Turpin, 1988):

$$V = \mu Q_p \quad (3)$$

Because V is measured separately at various P_i concentrations (equation 1), the actual P_i concentration for a given steady-state condition can be calculated by combining equations 1 and 3. These P_i concentrations were used for the determination of maximal growth rate (μ_{max}), the affinity constant for growth (K_s), and the threshold P_i concentration for growth ($P_{i,t}$, i.e. P_i at µ=0) according to the Monod equation (modified by Tilman and Kilham, 1976):

$$\mu = \mu_{max}[(P_i - P_{i,t})/(K_s + P_i - P_{i,t})] \quad (4)$$

The non-linear regression module in SPSS software was used to fit the models. Statistical tests were also performed with SPSS (version 11.5) using Spearman correlation tests and the analysis of covariance (ANCOVA) as growth rates and carbon source are not totally independent factors.

Results

The growth rates of *C. acidophila* decreased with decreasing cellular quota and were significantly different under CO_2-aerated, non-aerated, and osmo-mixotrophic growth conditions (ANCOVA, P <0.005; Fig. 1A). Estimation of the minimum Q_p (Q_0) using equation 2 (Droop equation) showed that Q_0 is lowest in CO_2-aerated cells (+CO_2), intermediate for the osmo-mixotrophic cells (+glucose), and highest for the non-aerated cells (–CO_2). Estimated Q_0 values from the Droop equation are provided in Table 1.

Growth rates decreased with decreasing residual P_i concentrations in the medium, which allowed the Monod equation (equation 4; Fig. 1B) to be fitted. The estimated

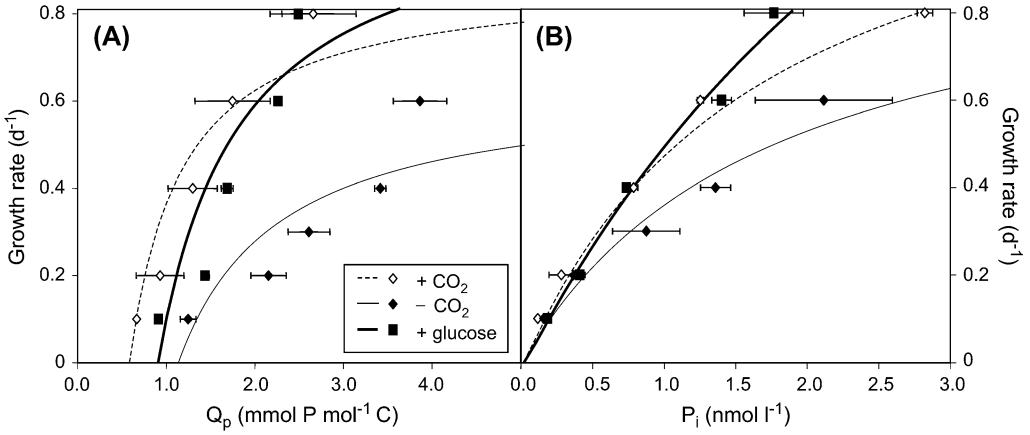

Fig. 1. Growth rates of *Chlamydomonas acidophila* (µ, d^{-1}) in relation to: (A) cellular phosphorus quota (Q_p, mmol P mol^{-1} C) and (B) residual P_i concentrations (in nmol l^{-1}) in CO_2-aerated (+CO_2), non-aerated (–CO_2), and osmo-mixotrophic (+glucose) cultures. Lines represent curves fitted to the Droop model (equation 2, A) or the Monod model (equation 4, B).

Table 1. *Results from non-linear fitting of calculated P_i concentrations to the Droop model (Q_0) and the Monod model (μ'_{max}, K_s and $P_{i,t}$)*

Values are mean ±standard error.

	Q_0 (mmol P mol C^{-1})	μ'_{max} (d^{-1})	K_s (nmol l^{-1})	$P_{i,t}$ (nmol l^{-1})
+ CO_2	0.59±0.06	1.30±0.17	1.70±0.51	0.028±0.06
No aeration	1.14±0.14	0.99±0.67	1.73±0.27	0.013±0.10
+ Glucose	0.91±0.07	2.55±2.15	4.06±4.97	0.030±0.12

threshold P_i concentration for growth ($P_{i,t}$) of *C. acidophila* was not significantly different from zero and not significantly different under the various culture conditions (Table 1). $P_{i,t}$ concentrations were on average 0.023 nmol P_i l^{-1}. The largest difference among the various culture conditions was detected in the estimated maximum growth rate. The μ_{max} was about 1.5-fold greater in the CO_2-aerated and 2.5-fold greater in the osmo-mixotrophic cultures than in the non-aerated cultures. There was no significant difference in K_s between cultures due to the high standard error, although the estimated value of the autotrophic cultures appeared lower than that of the osmo-mixotrophic cultures (Table 1). The conductance to P_i (μ_{max}/K_s) was highest in the CO_2-aerated cells, intermediate in the osmo-mixotrophs, and lowest in the non-aerated cells. This implies that at the lower P_i concentrations, CO_2-aerated cells can achieve the highest growth rates.

SRP concentrations at the lowest growth rates (≤0.4 d^{-1}) were averaged and considered to be the threshold SRP concentration for growth (SRP_t). The SRP_t concentrations were calculated to be 0.12±0.03, 0.12±0.02, and 0.10±0.02 µmol P l^{-1} (mean ±standard error) with and without CO_2 aeration and with glucose, respectively. These values were not significantly different and were >1000-fold higher than the $P_{i,t}$ concentrations.

The phosphatase activity of *C. acidophila* was highest at pH 6, whereas both at pH 2.7 and pH 9.0 very low activities were determined (ANCOVA, P <0.001; Fig. 2). Phosphatase activities increased with decreasing growth rates once the effect of the C-source was accounted for (ANCOVA, P <0.05). In the cultures at low growth rates (i.e. μ=0.1 and 0.2 d^{-1}), phosphatase activities were lower in the non-aerated than in CO_2-aerated and osmo-mixotrophic cultures (Friedman test paired over the measurements at all three pH values, n=6, df=2, P <0.05).

Maximum P_i-uptake rates (V_{max}) were independent of growth rate in all different P_i-limited cultures (Pearson, P >0.05) and were only significantly higher in the high CO_2 than in the low CO_2 cultures (ANOVA, P <0.05; Fig. 3). In the P-saturated cultures, V_{max} was 1.3 µmol P 10^{-9} cells h^{-1}, this being ~60-fold lower than in P_i-limited cultures. The affinity constant for uptake (K_m) was also independent of growth rate (Pearson, P >0.05) and did not differ significantly between the application of different

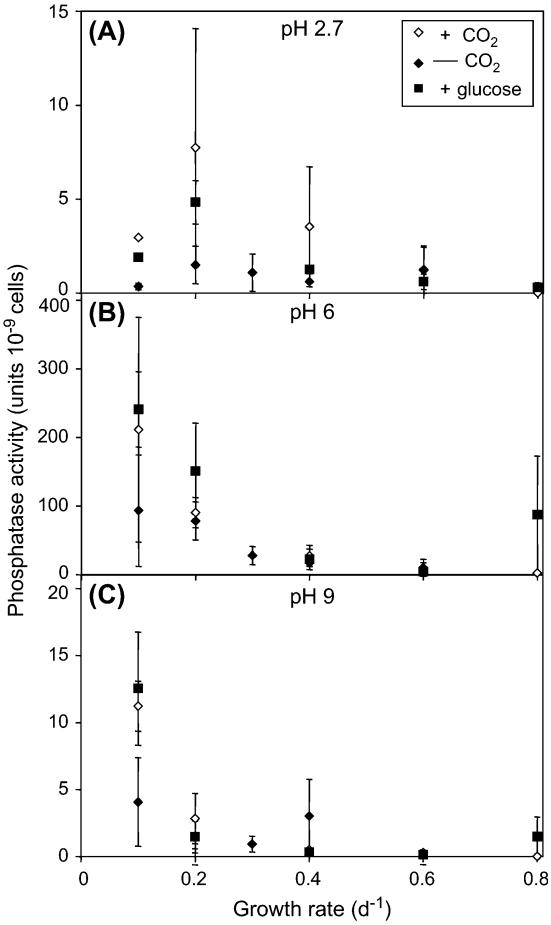

Fig. 2. Phosphatase activity of *Chlamydomonas acidophila* grown over a range of P_i-limited conditions at pH 2.7, measured at pH 2.7 (A), pH 6.0 (B), and pH 9.0 (C). For further details, see Fig. 1 legend. Note the different scales on the *y*-axis.

C-sources (ANOVA, P >0.05). Remarkably, in P-saturated cultures, K_m was significantly lower than in the P_i-limited cultures independent of the application of different C-sources (ANOVA, P <0.05).

The DOC concentrations in CO_2-aerated cultures were negatively correlated to the growth rate (Pearson, P <0.005), whereas a correlation was not found in the non-aerated cultures (Table 2; the presence of glucose prevented the possibility of determining DOC in the osmo-mixotrophic cultures). In addition, DOC concentrations were significantly higher in CO_2-aerated than in non-aerated cultures once the effect of growth rate was accounted for (ANCOVA, P <0.05; Table 2). The relative carbon content of *C. acidophila* (calculated as mg carbon per mg dry weight) did not correlate with growth rate nor differ with C-source (ANCOVA, all P >0.05; Table 2). In CO_2-aerated and osmo-mixotrophic cultures, cell volume had a negative correlation with growth rate (Pearson, P <0.05), whereas in non-aerated cultures, this correlation was only found at growth rates between 0.1 d^{-1} and 0.5 d^{-1} (Table 2). The cell volume was largest in

CO_2-aerated, intermediate in size in osmo-mixotrophic, and smallest in non-aerated cells once the effect of growth rate was accounted for (ANCOVA, P <0.05; Table 2). Consequently, the cellular carbon content and cellular dry weight were highest in CO_2-aerated, intermediate in osmo-mixotrophic, and smallest in non-aerated cells (results not shown). Bacterial densities were independent of growth rate, DOC, and CO_2 aeration (not shown), and were considered too low (max. 1×10^9 bacteria l^{-1}) to influence algal physiology.

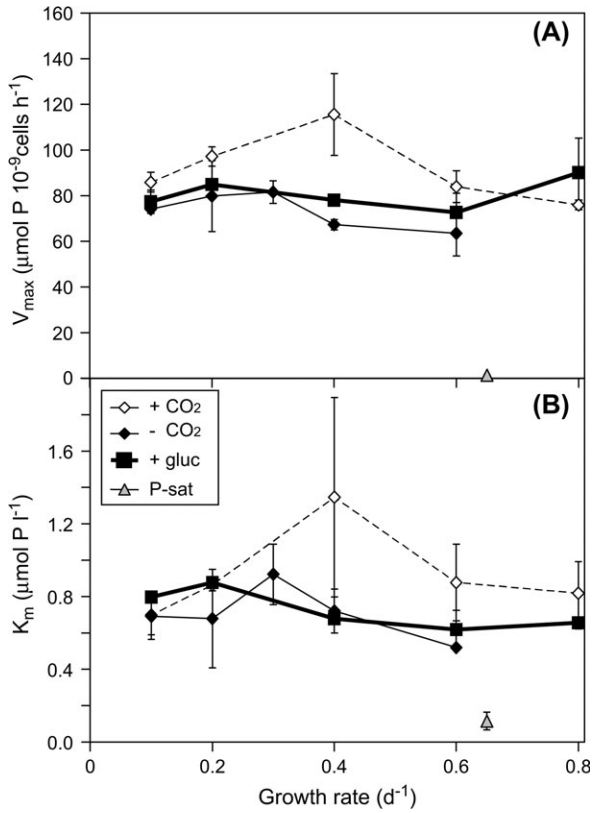

Fig. 3. Maximum P_i-uptake rates (A; in µmol P 10^{-9} cells h^{-1}) and affinity constant for P_i uptake (B; K_m, in µmol P l^{-1}) of *Chlamydomonas acidophila* grown over a range of P_i-limited conditions and under P_i-saturated (P-sat) growth at pH 2.7. For further details, see Fig. 1 legend.

Discussion

Chlamydomonas acidophila had unconventional adaptations to P_i-limiting conditions, as K_m was low under both P_i-limited and P_i-replete conditions. The presence of different C-sources had a clear influence on the P_i physiology of *C. acidophila*, and both P_i and CO_2 most likely limited growth under autotrophic, non-aerated P_i-limited conditions.

Growth and minimal P_i requirements

In accordance with previous studies, maximum growth rate of *C. acidophila* was increased by both CO_2 aeration and glucose (Tittel *et al.*, 2005). The extent of the stimulation of μ_{max} by CO_2 was comparable under the P_i-replete (Tittel *et al.*, 2005) and P_i-limiting conditions (this study) and indeed comparable CO_2 concentrations were applied in both studies. The μ_{max} in the osmo-mixotrophic (i.e. in the presence of glucose) cultures in this study was higher than that reported for P_i-replete cultures (Tittel *et al.*, 2005), and was likely to be the result of the higher glucose concentrations used in this study (1 mmol l^{-1} in this study compared with 0.4 mmol l^{-1} in Tittel *et al.*, 2005).

The calculated Monod relationshops revealed that there was no detectable threshold concentration of P_i for growth ($P_{i,t}$) in *C. acidophila*. The $P_{i,t}$ was similar in all cultures and was estimated to be 0.023 nmol P_i l^{-1} on average, but this was not significantly different from zero. A $P_{i,t}$ was described in the green algae *Cosmarium abbreviatum*, *Staurastrum pingue*, and *Staurastrum chaetoceras* (Spijkerman and Coesel, 1996*a*) but the $P_{i,t}$ of these desmids was 1000-fold higher (ranging from 17 to 43 nmol P_i l^{-1}) than the $P_{i,t}$ described here, underlining the non-significance of the $P_{i,t}$ concentration in this study. The absence of a $P_{i,t}$ in *C. acidophila* suggests that all P_i can be directly converted into biomass and therefore indicates no measurable increased metabolic costs. This does not exclude the metabolic costs suggested for an isolate of *C. acidophila* from the Rio Tinto because these costs consisted of only slightly increased ATP

Table 2. *Dissolved organic carbon in the medium (DOC), relative carbon content, and cell volume in relation to the growth rate (µ) in non-aerated (–CO_2), CO_2-aerated (+CO_2), and osmo-mixotrophic (+Gluc) cultures of* C. acidophila

Values are averages of two replicates. The standard deviation was lower than 20% of average. n.d.=not determined.

µ (d^{-1})	DOC (µmol Cl^{-1})		Rel active C content (% of DW)			Cell volume ($µm^3$)		
	–CO_2	+CO_2	–CO_2	+CO_2	+Gluc	–CO_2	+CO_2	+Gluc
0.1	256	350	52	54	53	71	114	94
0.2	144	305	51	48	51	61	111	87
0.3	117	n.d.	51	n.d.	n.d.	57	n.d.	n.d.
0.4	147	102	46	50	50	52	94	80
0.6	161	184	60	49	51	59	73	78
0.8		69		50	54		79	75

consumption rates that cannot be measured with the methods used in this study (Messerli *et al*., 2005).

In an earlier study of P_i limitation in *C. acidophila* using a complex medium containing high concentrations of iron, aluminium, and zinc (the composition of this medium is described in Spijkerman *et al*., 2007), threshold SRP values for growth (SRP_t) were determined from the SRP concentrations at the lowest growth rates ($\leqslant 0.4\ d^{-1}$; Spijkerman *et al*., 2007). In the metal-rich medium, SRP_t was estimated as 0.19 and 0.22 µmol P l^{-1} with and without CO_2 aeration, respectively. In the present study, using WH medium that contains low concentrations of iron and zinc and no aluminium, SRP_t values ranged from 0.10 to 0.12 µmol P l^{-1}. These ~2-fold lower concentrations can be explained by increased Fe–P complexation in the metal-rich medium that is obviously unavailable for growth of *C. acidophila*, but is detected in the SRP fraction. Most of the acidic lakes are rich in Fe, suggesting therefore that the SRP measured is not 100% available for algal growth.

The minimal cell quota (Q_0) represents the amount of phosphate associated with the structural and metabolic components that are essential for cellular integrity and viability (Droop, 1974). In this study, using WH medium that contains NO_3^- as the sole N-source, Q_0 was higher in non-aerated than in CO_2-aerated cultures (1.14 ± 0.14 and 0.59 ± 0.06 mmol P mol^{-1} C, respectively). In a previous study, using a metal-rich medium that contained NH_4^+ as the primary N-source, a non-significant trend towards a similar difference was found (0.81 ± 0.04 and 0.77 ± 0.04 mmol P mol^{-1} C in non-aerated and CO_2-aerated cultures, respectively; Spijkerman *et al*., 2007). The Q_0 in CO_2-aerated cells was not significantly different in WH medium or metal-rich medium (ANCOVA; df=24, 2; $F=11$; $P > 0.05$). The cellular carbon content and cell volume determinations support the similar Q_0 in CO_2-aerated cells cultured in both media as these cellular characteristics are the same in the cells grown in WH medium as those in metal-rich medium (compare 4.1 pmol C $cell^{-1}$ and 111 μm^3 in WH medium and 3.7 pmol C $cell^{-1}$ and 106 μm^3 in metal-rich medium, both grown at 0.2 d^{-1}). By contrast, the Q_0 in non-aerated cells cultured in WH medium was significantly higher than cells cultured in the metal-rich medium (ANCOVA; df=18, 2; $F=25$; $P < 0.05$), which might be a consequence of increased metabolic costs resulting from the reduction of NO_3^- to NH_4^+ before the incorporation of N into amino acids. The reduction of NO_3^- to NH_4^+ requires electrons delivered from photosynthesis (Toepel *et al*., 2004) thereby increasing the ATP and NADPH demand compared with a situation where NH_4^+ is the acquired N-species. As a consequence μ_{max} was 1.5-fold higher in *Dunaliella saline* when NH_4^+, rather than NO_3^-, was the N-source used (Giordano, 2001). In addition, the affinity for CO_2 uptake was higher in cells of *Dunaliella salina* and *D. parva* grown in NH_4^+-N than in NO_3^--N medium (reviewed in Giordano *et al*., 2005). To summarize, algae grown in medium containing NH_4^+-N might have a lower cellular P and a higher cellular C content than cells in NO_3^--N medium.

Phosphatase activity

In this study, very little extracellular phosphatase activity was found in measurements performed at pH 2.7 and pH 9.0. The latter is in accordance with a previous study on the induction of phosphatase enzymes in *C. acidophila* (Boavida and Heath, 1986). The present results at pH 9.0, however, contrast with a previous study (Spijkerman *et al*., 2007) where inducible alkaline phosphatase activity was found in *C. acidophila*. This alkaline phosphatase activity was detected in natural phytoplankton as well as in cultures grown in metal-rich medium reflecting the chemical composition of the lake water. Some components of the mining lake water, such as high concentrations of sulphur, iron, or other metal ions, might possibly stimulate the induction of alkaline phosphatase enzymes (Sabater *et al*., 2003). However, the alkaline phosphatase activity was only detected when the algae were grown in P_i-deplete, ion-rich medium, and consequently high concentrations of ions alone do not induce these enzymes. At a growth rate of 0.1 d^{-1}, phosphatase activity of *C. acidophila* measured at pH 6.0 was about 2-fold lower in this study using WH medium, than when cultured in metal-rich medium (Spijkerman *et al*., 2007). Therefore, the activity at pH 6.0 might also have been increased by the high concentrations of ions in the metal-rich medium. The negative correlation between phosphatase activity and dilution rates is in accordance with findings from studies of other algae, for example, *C. reinhardtii* (Olsen *et al*., 1983).

The phosphatase activity was lowest in the non-aerated cultures, which suggests that growth of *C. acidophila* in the non-aerated cultures was not P_i-limited to the same extent as CO_2-aerated or osmo-mixotrophic cultures. Presumably, growth of *C. acidophila* was co-limited by P_i and CO_2 in the non-aerated cultures (also see below).

Phosphatase activities in osmo-mixotrophic cultures were similar to those in CO_2-aerated cultures, although they were also non-aerated. Therefore, the presence of glucose did not inhibit enzyme activity. Possibly, CO_2 was produced intracellularly by the respiration of the glucose (Chen and Gibbs, 1991; Villarejo *et al*., 1995), resulting in high CO_2 conditions.

P_i uptake kinetics

By contrast to expectations based on other studies (Fu *et al*., 2006), the maximum uptake rate of P_i was independent of the growth rate, although the cellular P

quota increased at least 2-fold with increasing growth rate. Although maximum P_i-uptake rates (V_{max}) are inhibited by an increased Q_p (Nieuwenhuis and Borst-Pauwels, 1984), only under P_i-replete conditions, when Q_p was 10-fold higher than under P_i-limited conditions (54±32 mmol P mol^{-1} C), was V_{max} 30-fold lower. Similarly, V_{max} and K_m in the green alga *Cosmarium abbreviatium* remained stable over a 2-fold change in Q_p and only changed when cells were nearly flushed out of the continuous cultures by high dilution rates (Spijkerman and Coesel, 1996*b*).

Chlamydomonas acidophila had a high affinity uptake system for P_i, independent of aeration with CO_2, availability of glucose, or P_i saturation (Fig. 3). With a K_m value varying between 0.1 and 1.2 µmol P_i l^{-1}, this high affinity uptake system is comparable with that described in a broad range of other green algal species (Rhee, 1973; Gotham and Rhee, 1981; Healey and Hendzel, 1988; Jansson, 1993; Spijkerman and Coesel, 1996*b*; Grossman, 2000).

The V_{max} of P_i uptake by *C. acidophila* under non-aerated conditions was lower than that under CO_2-aerated growth conditions, which together with the higher Q_0, lower DOC, and lower phosphatase activity indicated that the non-aerated cells were less stringently P_i-limited than the CO_2-aerated or osmo-mixotrophic cells. Presumably, non-aerated cells were co-limited by CO_2 and P_i under low CO_2 and low P_i conditions, and invested metabolic energy in physiological responses related to both nutrient limitations. Co-limiting conditions for P_i and NO_3^- in phytoplankton growth have often been described (Elser *et al.*, 1990; Davies *et al.*, 2003), but not many studies describe the regulatory influence of both resource limitations on the physiological acclimation. From the few studies on the physiological acclimation to low CO_2 and low P_i concentrations, a down-regulation of the inorganic carbon uptake was shown under P_i-limited conditions in *Chlorella emersonii* (Beardall *et al.*, 2005). Inorganic carbon acquisition under low CO_2 conditions is an active process in most micro-algae, requiring ATP, and it might therefore be expected that P_i limitation could have a direct regulatory influence on carbon acquisition. At present, it is not clear if active CO_2 uptake is an important process compared with CO_2 diffusion in *C. acidophila*, and this requires further investigation. A recent paper on CO_2 acquisition in an acid-tolerant *Chlamydomonas* suggests solely diffusive uptake (Balkos and Colman, 2007), whereas an energy-demanding carbon-concentrating mechanism was found in *C. acidophila* under low CO_2 conditions (Spijkerman, 2005). Future work will report on the effect of different C-sources on CO_2-acquisition during P_i-limitation in *C. acidophila*. This study, however, provides the first indications of a co-limitation for CO_2 and P_i in *C. acidophila* and the possible regulation of CO_2 limitation on P_i acquisition.

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References

Baffico GD, Diaz MM, Wenzel MT, Koschorreck M, Schimmele M, Neu TR, Pedrozo F. 2004. Community structure and photosynthetic activity of epilithon from a highly acidic (pH <= 2) mountain stream in Patagonia, Argentina. *Extremophiles* **8,** 463–473.

Balkos KD, Colman B. 2007. Mechanism of CO_2 acquisition in an acid-tolerant *Chlamydomonas*. *Plant, Cell and Environment* **30,** 745–752.

Beardall J, Roberts S, Raven JA. 2005. Regulation of inorganic carbon acquisition by phosphorus limitation in the green alga *Chlorella emersonii*. *Canadian Journal of Botany* **83,** 859–864.

Beardall J, Young E, Roberts S. 2001. Approaches for determining phytoplankton nutrient limitation. *Aquatic Sciences* **63,** 44–69.

Bissinger V, Jander J, Tittel J. 2000. A new medium free of organic carbon to cultivate organisms from extremely acidic mining lakes (pH 2.7). *Acta Hydrochimica et Hydrobiologica* **28,** 310–312.

Boavida MJ, Heath RT. 1986. Phosphatase acitivity of *Chlamydomonas acidophila* Negoro (Volvocales, Chlorophyceae). *Phycologia* **25,** 400–404.

Burkhardt S, Riebesell U. 1997. CO_2 availability affects elemental composition (C:N:P) of the marine diatom *Skeletonema costatum*. *Marine Ecology-Progress Series* **155,** 67–76.

Chen CG, Gibbs M. 1991. Glucose respiration in the intact chloroplast of *Chlamydomonas reinhardtii*. *Plant Physiology* **95,** 82–87.

Davies JM, Hesslein RH, Kelly CA, Hecky RE. 2003. PCO_2 method for measuring photosynthesis and respiration in freshwater lakes. *Journal of Plankton Research* **25,** 385–395.

Doi H, Kikuchi E, Shikano S. 2001. Carbon and nitrogen stable isotope ratios analysis of food sources for *Chironomus acerbiphilus* larvae (Diptera, Chironomidae) in strongly acidic lake Katanuma. *Radioisotopes* **50,** 601–611.

Droop MR. 1973. Some thoughts on nutrient limitation in algae. *Journal of Phycology* **9,** 264–272.

Droop MR. 1974. The nutrient status of algal cells in continuous culture. *Journal of the Marine Biological Association of the United Kingdom* **54,** 825–855.

Elser JJ, Marzolf ER, Goldman CR. 1990. Phosphorus and nitrogen limitation of phytoplankton growth in the freshwaters of North America: a review and critique of experimental enrichments. *Canadian Journal of Fisheries and Aquatic Sciences* **47,** 1468–1477.

Fu FX, Zhang YH, Feng YY, Hutchins DA. 2006. Phosphate and ATP uptake and growth kinetics in axenic cultures of the cyanobacterium *Synechococcus* CCMP 1334. *European Journal of Phycology* **41,** 15–28.

Gerloff-Elias A, Barua D, Molich A, Spijkerman E. 2006. Temperature- and pH-dependent accumulation of heat-shock proteins in the acidophilic green alga *Chlamydomonas acidophila*. *FEMS Microbiology Ecology* **56,** 345–354.

Gerloff-Elias A, Spijkerman E, Proschold T. 2005. Effect of external pH on the growth, photosynthesis and photosynthetic electron transport of *Chlamydomonas acidophila* Negoro, isolated from an extremely acidic lake (pH 2.6). *Plant, Cell and Environment* **28,** 1218–1229.

Giordano M. 2001. Interactions between C and N metabolism in *Dunaliella salina* cells cultured at elevated CO_2 and high N concentrations. *Journal of Plant Physiology* **158,** 577–581.

Giordano M, Beardall J, Raven JA. 2005. CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annual Review of Plant Biology* **56,** 99–131.

Gotham IJ, Rhee G-Y. 1981. Comparative kinetic studies of phosphate-limited growth and phosphate uptake in phytoplankton in continuous culture. *Journal of Phycology* **17,** 257–265.

Grossman A. 2000. Acclimation of *Chlamydomonas reinhardtii* to its nutrient environment. *Protist* **151,** 201–224.

Grossman A, Takahashi H. 2001. Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 163–210.

Healey FP, Hendzel LL. 1988. Competition for phosphorus between desmids. *Journal of Phycology* **24,** 287–292.

Hobbie JE, Daley RJ, Jasper S. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology* **33,** 1225–1228.

Jansson M. 1993. Uptake, exchange, and excretion of orthophosphate in phosphate-starved *Scenedesmus quadricauda* and *Pseudomonas* K 7. *Limnology and Oceanography* **38,** 1162–1178.

Joseph EM, Morel FMM, Price NM. 1995. Effects of aluminum and fluoride on phosphorus acquisition by *Chlamydomonas reinhardtii*. *Canadian Journal of Fisheries and Aquatic Sciences* **52,** 353–357.

Kamjunke N, Gaedke U, Tittel J, Weithoff G, Bell EM. 2004. Strong vertical differences in the plankton composition of an extremely acidic lake. *Archiv für Hydrobiologie* **161,** 289–306.

Kozlowska-Szerenos B, Bialuk I, Maleszewski S. 2004. Enhancement of photosynthetic O_2 evolution in *Chlorella vulgaris* under high light and increased CO_2 concentration as a sign of acclimation to phosphate deficiency. *Plant Physiology and Biochemistry* **42,** 403–409.

Lopez-Archilla AI, Marin I, Amils R. 2001. Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. *Microbial Ecology* **41,** 20–35.

Messerli MA, Amaral-Zettler LA, Zettler E, Jung SK, Smith PJS, Sogin ML. 2005. Life at acidic pH imposes an increased energetic cost for a eukaryotic acidophile. *Journal of Experimental Biology* **208,** 2569–2579.

Murphy J, Riley JP. 1962. A modified single solution method for determination of phosphate in natural waters. *Analytica Chimica Acta* **26,** 31–36.

Nedoma J, Padisak J, Koschel R. 2003. Utilisation of ^{32}P-labelled nucleotide- and non-nucleotide dissolved organic phosphorus by freshwater plankton. *Archiv für Hydrobiologie* **58,** 87–99.

Nichols HW. 1973. Growth media – freshwater. In: Stein JR, ed. *Handbook of phycological methods: culture methods and growth measurements*. Cambridge: Cambridge University Press, 7–24.

Nieuwenhuis BJW, Borst-Pauwels GWFH. 1984. Derepression of the high-affinity phosphate uptake in the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **770,** 40–46.

Nishikawa K, Machida H, Yamakoshi Y, Ohtomo R, Saito K, Saito M, Tominaga N. 2006. Polyphosphate metabolism in an acidophilic alga *Chlamydomonas acidophila* KT-1 (Chlorophyta) under phosphate stress. *Plant Science* **170,** 307–313.

Nishikawa K, Tominaga N. 2001. Isolation, growth, ultrastructure, and metal tolerance of the green alga. *Chlamydomonas acidophila* (Chlorophyta). *Bioscience Biotechnology and Biochemistry* **65,** 2650–2656.

O'Brien PJ, Herschlag D. 2001. Functional interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of *Escherichia coli* alkaline phosphatase. *Biochemistry* **40,** 5691–5699.

Olsen Y, Knutsen G, Lien T. 1983. Characteristics of phosphorus limitation in *Chlamydomonas reinhardtii* (Chlorophyceae) and its palmelloids. *Journal of Phycology* **19,** 313–319.

Rhee G-Y. 1973. A continous culture study of phosphate uptake, growth rate and polyphosphate in *Scenedesmus* sp. *Journal of Phycology* **9,** 495–506.

Rigler FH. 1968. Further observations inconsistent with the hypothesis that the molybdenum blue method measures orthophosphate in lake water. *Limnology and Oceanography* **13,** 7–13.

Sabater S, Buchaca T, Cambra J, Catalan J, Guasch H, Ivorra N, Munoz I, Navarro E, Real M, Romani A. 2003. Structure and function of benthic algal communities in an extremely acid river. *Journal of Phycology* **39,** 481–489.

Spijkerman E. 2005. Inorganic carbon acquisition by *Chlamydomonas acidophila* across a pH range. *Canadian Journal of Botany* **83,** 872–878.

Spijkerman E, Bissinger V, Meister A, Gaedke U. 2007. Low potassium and inorganic carbon concentrations influence a possible phosphorus limitation in *Chlamydomonas acidophila* (Chlorophyceae). *European Journal of Phycology* **42,** 327–339.

Spijkerman E, Coesel PFM. 1996*a*. Competition for phosphorus among planktonic desmid species in continuous-flow culture. *Journal of Phycology* **32,** 939–948.

Spijkerman E, Coesel PFM. 1996*b*. Phosphorus uptake and growth kinetics of two planktonic desmid species. *European Journal of Phycology* **31,** 53–60.

Spijkerman E, Coesel PFM. 1998. Alkaline phosphatase activity in two planktonic desmid species and the possible role of an extracellular envelope. *Freshwater Biology* **39,** 503–513.

Tilman D, Kilham SS. 1976. Phosphate and silicate growth and uptake kinetics of the diatoms *Asterionella formosa* and *Cyclotella meneghiniana* in batch and semicontinuous culture. *Journal of Phycology* **12,** 375–383.

Tittel J, Bissinger V, Gaedke U, Kamjunke N. 2005. Inorganic carbon limitation and mixotrophic growth in *Chlamydomonas* from an acidic mining lake. *Protist* **156,** 63–75.

Toepel J, Gilbert M, Wilhelm C. 2004. Can chlorophyll-*a in-vivo* fluorescence be used for quantification of carbon-based primary production in absolute terms? *Archiv für Hydrobiologie* **160,** 515–526.

Turpin DH. 1988. Physiological mechanisms in phytoplankton resource competition. In: Sandgren CD, ed. *Growth and reproductive strategies of freshwater phytoplankton*. Cambridge: Cambridge University Press, 316–368.

Villarejo A, Orus MI, Martinez F. 1995. Coordination of photosynthetic and respiratory metabolism in *Chlorella vulgaris* UAM-101 in the light. *Physiologia Plantarum* **94,** 680–686.