Independent Colimitation for Carbon Dioxide and Inorganic Phosphorus

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Abstract
Simultaneous limitation of plant growth by two or more nutrients is increasingly acknowledged as a common phenomenon in nature, but its cellular mechanisms are far from understood. We investigated the uptake kinetics of CO₂ and phosphorus of the algae Chlamydomonas acidophila in response to growth at limiting conditions of CO₂ and phosphorus. In addition, we fitted the data to four different Monod-type models: one assuming Liebig’s Law of the minimum, one assuming that the affinity for the uptake of one nutrient is not influenced by the supply of the other (independent colimitation) and two where the uptake affinity for one nutrient depends on the supply of the other (dependent colimitation). In addition we asked whether the physiological response under colimitation differs from that under single nutrient limitation. We found no negative correlation between the affinities for uptake of the two nutrients, thereby rejecting a dependent colimitation. Kinetic data were supported by a better model fit assuming independent uptake of colimiting nutrients than when assuming Liebig’s Law of the minimum or a dependent colimitation. Results show that cell nutrient homeostasis regulated nutrient acquisition which resulted in a trade-off in the maximum uptake rates of CO₂ and phosphorus, possibly driven by space limitation on the cell membrane for porters for the different nutrients. Hence, the response to colimitation deviated from that to a single nutrient limitation. In conclusion, responses to single nutrient limitation cannot be extrapolated to situations where multiple nutrients are limiting, which calls for colimitation experiments and models to properly predict growth responses to a changing natural environment. These deviations from single nutrient limitation response under colimiting conditions and independent colimitation may also hold for other nutrients in algae and in higher plants.

Introduction
Plant biomass forms the basis of food webs and its primary production promotes global economic and ecosystem services such as crop harvest, fish yield and carbon sequestration. Because plant photosynthesis and growth is often nutrient limited, knowledge on how nutrients limit plant growth is both, ecologically and economically important. On the other hand, massive plant growth such as large scale algal blooms, arising from an excess of nutrients, negatively affect biodiversity and are a nuisance to human activity. This excessive plant growth often results from plants acclimated to scavenge the limiting nutrient but which are suddenly faced with saturating conditions often due to anthropogenic impacts. Thus, knowledge on plant nutrient uptake kinetics and response to changes of the limiting nutrient provides important insights to predict plant growth response.

Most previous studies have focused on the effect of a single limiting nutrient, such as inorganic phosphate (P) which often limits algal growth in freshwater (e.g. [1]). In many cases, however, this approach was unsatisfactory, which has recently been explained by the occurrence of colimitation by two or more nutrients under natural conditions [2,3]. For example, a colimitation by nitrogen, P and iron was shown in the phytoplankton communities of Lake Kasumigaura [4] and Lake Erie [2]. As illustrated in these two studies, nutrient supplementation alleviates each incremental limitation and produces a synergistic effect when all limiting nutrients are added together (see [5] for a nice illustration). In the case of entire plankton communities a stepwise increase in growth and biomass after addition of all limiting nutrients, possibly results from the independent response of different species. In addition, a single species can also show the effects of colimitation [6,7].

In general, algal cells respond to nutrient limiting conditions by increasing their ability for nutrient uptake. This can be achieved in two ways which are not mutually exclusive (Fig. 1); either increasing the maximum uptake rate (V_max) and/or increasing the affinity for uptake. The latter is typically reflected in a decrease of the half saturation constant (K_m), and an increase of the initial slope of the curve (affinity characterized by V_max*K_m). V_max is positively related to the number of porters in the cytoplasmic membrane [8] whereas changes in K_m reflect different types of porters. The affinity thus reflects the physiological combination of the two strategies of acclimation.

In response to a P-limitation most algae increased V_max as found in the green alga Scenedesmus sp. [9], the diatom Thalassiosira pseudonana [10] and the cyanobacterium Anabaena flos-aquae [11], while the K_m often remained relatively constant. In contrast, in response to low CO₂ many algae decrease their K_m [12–14]. Both responses often result in an enhanced affinity for nutrient uptake; likely driven by the need to maintain a balanced cell nutrient
Colimitation by Essential Nutrients

Figure 1. Illustration of P uptake kinetics: Cell-based rates related to the initial P-concentration in the medium (μM P). A) Data from [61] on the high (∆m P) and low (∆m P) affinity P transporter in Escherichia coli that have similar Vmax P. B) Data from [31] on P-limited (high Vmax P) and P-saturated (low Vmax P) Chlamydomonas acidophila with the same Km P (0.1–0.5 μM P). doi:10.1371/journal.pone.0028219.g001

content. Cell homeostasis is of great importance for a proper functioning of enzymes and proteins, and consequently nutrient uptake and excretion rates are regulated to balance nutrient ratios within a certain range [15,16].

Even if colimitation is a rather common phenomenon in phytoplankton and plant growth in general [3,5], the cellular response mechanisms in a single algal species are far from understood. They may involve interactions between two or more nutrients in short supply which questions the common practice to infer the consequences of nutrient limitation by considering only a single nutrient at a time. Consequently, we ask whether a colimitation by two macronutrients alters their respective uptake kinetics, compared to situations where a single nutrient is limiting, to better understand the mechanism(s) of algal growth acclimation and cellular response to colimiting conditions.

Here, we study the uptake and growth of the single-celled flagellated green alga Chlamydomonas acidophila to limiting conditions of CO2 and P, two important macro-nutrients for phytoplankton which are prone to change in the future and often limit phytoplankton blooms in very acidic and neutral fresh waters, as well as in marine systems [17–20]. Inorganic carbon occurs in three forms in aquatic systems: CO2, HCO3− and CO32−, but here we consider only the dissolved gas, CO2, which is the only inorganic C source present in the low pH environment (pH 2.7) of our model organism [21]. In C. acidophila, CO2 uptake is considered an active process supported by a high affinity uptake mechanism at least under low CO2 conditions [12], thus allowing us to consider CO2 as a ‘normal’ macro-nutrient.

Recent theoretical studies suggest two types of colimitation for macro-nutrients: 1) independent and 2) dependent colimitation [6,7,22]. An independent colimitation arises when the concentration of more than one nutrient is below the optimal concentration for uptake (a multi-nutrient colimitation sensu [6]). Under these conditions, the cell will increase Vmax and/or decrease the Km for the uptake of both limiting nutrients, i.e. exhibit a multiplicative response to the two concurrent limitations. The response to an independent colimitation might be restricted according to theoretical considerations showing that Vmax is positively related to the number of nutrient transporter proteins [8] and as space on the cell surface for these proteins may be limited [8,23], the total number of nutrient transporters is thus restricted. Hence, theory predicts a trade-off between the Vmax of both limiting nutrients on a cellular level depending on the limiting nutrient in highest demand [24]. However, as far as we know, this hypothesis has never been tested with empirical data.

Alternatively, a dependent colimitation for nutrients exists if the uptake of one nutrient is enhanced by the availability of another one (a biochemical colimitation sensu [6]). In our situation, a P-limitation may inhibit the high affinity uptake of inorganic carbon (i.e. prevent a low affinity constant for CO2 uptake (Km,C)) and of a CO2 concentrating mechanism (CCM, [25–27]) which are both active processes [26,28] that depend, directly or indirectly, on ATP. Under P-saturated and low CO2 conditions, C. acidophila had a low Km,C and a CCM, both of which were absent under high CO2 conditions [12,29] indicating some costs which promote their down regulation at sufficient C-supply. If CO2 uptake depends on P-supply, the Km for CO2 uptake should increase with increasing P-limitation (as shown in the green alga Chlorella emersonii [25]). The dependent colimitation hypothesis implies that a minimum P concentration is required to acclimate to low CO2. If that minimum is not satisfied it results in a high Km,C when both P and CO2 are low. The alternative, that P uptake ability depends on CO2-concentration, is also possible. Recent studies on C. acidophila [30] support this option: at high CO2 concentrations, cells could deplete the P concentration in the medium more strongly than at low CO2 concentrations. Also, the minimum cellular P quota was lower in high than in low CO2 cells [31], suggesting that cellular P requirements for growth are lower at high CO2. Following this hypothesis, the P uptake ability (enhanced maximum P uptake rate (Vmax,P) and/or decreased affinity constant for P uptake (Km,P)) should increase with increasing CO2-concentration.

We analyzed the response of C. acidophila to a combination of CO2 and P limitations. Our objectives are first, to test the hypothesis that responses to two limiting factors cannot be inferred from single-nutrient studies; second to decide which of the above-described mechanisms of colimitation is more plausible. For this, four different models, two of independent colimitation and two of dependent colimitation (CO2 limiting P uptake and vice versa), were fitted to the data by maximum likelihood. We also provide empirical evidence for the theoretically expected trade-off between the Vmax for both nutrients.
Methods

Cultures and analyses

Triplicate semi-continuous cultures of C. acidophila Negoro (CCAP 11/137) were grown at 20±1 °C in Woods Hole medium [32] with 1.6 μM P and a pH adjusted to 2.7 with HCl. Daily diluted cultures at growth rates of 0.1, 0.2, 0.3, 0.4 and 0.6 d⁻¹ in low CO₂ and 0.1, 0.2, 0.4, 0.6 and 0.8 d⁻¹ in high CO₂ treatments resulted in a decrease of P-limitation with increasing growth rate. Cell densities ranged between 1.1×10⁵ cells ml⁻¹ in the highest growth rates to 1.5×10⁵ cells ml⁻¹ in the lowest growth rates. Cell densities were on average 1.6-fold higher in the high CO₂ cultures than in the low CO₂. Incident light was approximately 200 μmol photons m⁻² s⁻¹ in all cultures with a light/dark period of 16/8 h. Daily dilution and harvesting were done 4–5 hours after the onset of light. High CO₂ cultures were mildly aerated with a mixture of 4.5% CO₂ in air, resulting in an average CO₂ concentration in the medium of 0.33 (±0.05, n=20) mM C, whereas low CO₂ cultures were non-aerated to realise CO₂ limiting conditions and contained approximately 0.02 mM CO₂ (HighToc, Elementar, Hanau, Germany). These concentrations were measured in the medium, but do not reflect concentrations nearby the cell (see discussion for further details).

Inorganic iron buffered the pH of the medium, thus resulting in a constant pH independent of CO₂ concentration. At balanced growth (remaining at constant cell density 4–5 hours after the onset of light after an exchange of three to five times the culture volume), samples were taken for measurements of algal density, chemical analyses and CO₂- and P-uptake kinetics.

Cellular phosphorus quota (Qₚ), cellular C and P content were determined by measuring the particulate P and C in the cultures. The particulate P concentration was determined on filtered culture suspension (0.2 μm Whatman nucleopore) extracted at 100°C for 1 h with K₂S₂O₇ and 0.5 M H₂SO₄ and measured spectrophotometrically using molybdate and ascorbic acid [33]. Particulate C was analysed on culture suspension filtered on pre-combusted GF/F filters (Whatman), dried for one week at 50°C, and combusted in a carbon analyser (HighTOC+N, Elementar or EuroVector CHNS-O Elementaranalysator, Wegberg, Germany). Cell numbers were determined using an automatic cell counter (CASY 1, Model TT, Scharfe, Reutlingen, Germany).

Uptake kinetics

The CO₂-uptake kinetics was obtained in a temperature regulated light dispersion system (Topgalant LLC, Salt Lake City, Utah, USA) providing 300 μmol photons m⁻² s⁻¹ and measuring oxygen evolution in a Clark type electrode (Microelectrode Inc., Bedford, Ohio, USA) as described for P-replete C. acidophila in [12,29]. Cells were centrifuged (1500g, 5 min) and resuspended in C-free medium to an optical density of 0.2 at 750 nm in a 1 cm cuvette. After O₂ evolution ceased, one of six different concentrations of HCO₃⁻ was added and the response recorded on a computer. Each concentration was measured in three-fold, resulting in 18 data points for establishing one kinetic curve. At pH 2.65, 95% of the added HCO₃⁻ was assumed to be dehydrated to CO₂ within 60 s and no delay in response in O₂ evolution was observed. There was no significant effect of the addition of HCO₃⁻ on the pH: on average pH decreased by 0.001 units over the total run of 6 additions. Part of the algal suspension was fixed with iodine and cell densities were determined as described above. Oxygen evolution rates were related to cell densities and fitted to the Michaelis Menten model using the non-linear regression module in SPSS software (using the Levenberg-Marquardt estimation, version 12.01) to obtain the Kₘₐₓₚ and the maximum CO₂ uptake rate by photosynthesis (Vₘₐₓₚ,CO₂).

For P-uptake kinetics, cells were centrifuged (1500g, 5 min) and the pellet resuspended in medium without P and iron-EDTA at a pH of 2.7. Final densities were adjusted to an optical density of 0.02 at 750 nm in a 1 cm cuvette. After an acclimation of 15 to 30 min at 90 μmol photons m⁻² s⁻¹, P-uptake was determined over a period of one minute after the addition of different concentrations of H₃PO₄ (111 TBq mmol⁻¹ specific activity, Amersham biosciences, Freiburg, Germany) diluted in stock solutions of 50 or 500 μM K₂HPO₄ at pH 2.7 as described in [31]. Uptake was terminated by filtration on 1.2 μm cellulose acetate filters and subsequently rinsed with 0.2 M LiCl. P-uptake kinetics from two out of three replicate P-limited cultures and data of P-replete cells were published before [31]. Similar as for CO₂-uptake kinetics, cell specific data were fitted to the Michaelis-Menten model to estimate the Kₘₐₓₚ and Vₘₐₓₚ.

Modelling and statistics

We fitted four models describing colimitation to our data, following terminology and equations from [22] and [7]. This was done by calculating the external P and CO₂ (C) concentrations from individual uptake kinetics and cellular quota for each balanced growth rate following principle characteristics of the (semi-) continuous culture as explained by several authors [31,34–37]. Direct measurements of the limiting nutrient concentrations in the medium were not possible as they were too low to be measured directly, but the calculation of external concentration is a reasonable procedure despite the inevitable uncertainty involved [36]. Prior to this, we tested single nutrient models with the standard Monod function for P and C. In both cases the fit was poorer than in two-nutrient (colimitation) models, so we do not show detailed results here (Fig. S1). We used the following models describing colimitation:

1a. Independent co-limitation, multiplicative form:

\[ \mu = \mu_{\text{max}} \left( \frac{P}{P + K_P} \right) \left( \frac{C}{C + K_C} \right) \]

1b. Independent co-limitation, minimum form (Liebig’s Law):

\[ \mu = \mu_{\text{max}} \text{min} \left( \frac{P}{P + K_P}, \frac{C}{C + K_C} \right) \]

2a. Dependent co-limitation with C acquisition depending on P-limitation:

\[ \mu = \frac{\mu_{\text{max}} C}{(P + K_P) \mu_{\text{max}} C_{\text{max}}} + C \]

where the first term of the denominator comes from:

\[ K_C = \frac{\mu_{\text{max}}}{\alpha_C} \]

and since: \[ \alpha_C = \frac{P}{\mu_{\text{max}} K_P + P} \] it follows that:

\[ K_C = \frac{(P + K_P) \mu_{\text{max}}}{P \alpha_C} \]

2b. Dependent co-limitation with P acquisition depending on C-limitation:

\[ \mu = \frac{\mu_{\text{max}} P}{(C + K_C) \mu_{\text{max}} C_{\text{max}}} + P \]

The symbols used in the equations are \( \mu \), growth rate (in h⁻¹); \( \mu_{\text{max}} \), maximum growth rate (in h⁻¹); \( \alpha_C \), \( \alpha_P \), affinity for growth at
C or P-limiting conditions; $a_{C_{\text{max}}}$, $a_{P_{\text{max}}}$ maximum affinity for growth at C or P-limiting conditions; $K_{C}$, half saturation constant for growth in relation to external CO$_2$ concentration and $K_{P}$, half saturation constant for growth in relation to external P concentration (for details see [7,22]). We used maximum likelihood to fit the models to the data, assuming a normal distribution for the stochastic component of the models.

In addition to the Monod curves we established contour plots to distinguish between the effects of the cellular C and P contents on the uptake kinetics using Matlab 7.8 and interpolation based on Sandwell [38].

Statistical tests were performed with SPSS (version 12.01). Homogeneity of variances was checked with a Levene test.

Results

We tested the nutrient uptake response in the green alga *Chlamydomonas acidophila* to different CO$_2$ and P colimiting conditions. By using semi-continuous cultures the extent of limitation decreases with increasing dilution rate and, thus, with increasing balanced growth rate.

CO$_2$ and P uptake kinetics differed in the high and low CO$_2$ acclimated algae (Fig. 2), e.g. $V_{\text{max,C}}$ was higher in the low CO$_2$ acclimated cultures than in the high CO$_2$ ones, when the effect of growth rate was accounted for (Fig. 2A; ANCOVA, $F_{1,27} = 27.0$, $p<0.001$). Possibly, this kinetic difference resulted from the lower cellular C content (Fig. S2A) in the low CO$_2$ than in the high CO$_2$ cells at a given steady state growth rate. The higher $V_{\text{max,C}}$ in cells with low C content supports the nutrient kinetic response theory (Fig. 1B) that at a cellular level, a C-deficiency results in a higher CO$_2$ demand and thus a higher $V_{\text{max,C}}$. The potential growth rate (growth capacity) calculated from $V_{\text{max,C}}$ and the cellular carbon content supports the idea that conditions were limiting for CO$_2$, as the growth capacity was between 1.7 and 4.4-fold higher than balanced growth rates in the low but not in the high CO$_2$ cultures (Table S1). The ratio between growth capacity and balanced growth rate increased with decreasing growth rate, i.e. with increasing CO$_2$ limitation (see also [39]). In high CO$_2$ cells the growth capacity equaled the balanced growth rate, but enrichment experiments showed that these cells were nevertheless colimited for P and CO$_2$ [39] meaning that results from C-uptake kinetics alone were not conclusive.

In low CO$_2$, $V_{\text{max,C}}$ was the same in P-limited cells (data from the three highest growth rates in Fig. 2) and P-replete (data from [12]), whereas in high CO$_2$ it was lower in P-limited than in P-replete cells (Table 1). These results illustrate that the kinetic response to a colimitation differs from that to a single nutrient limitation. In contrast, the $K_{\text{m,C}}$ was the same in P-limited and P-replete cultures (Table 1), but depended on the CO$_2$ concentration: $K_{\text{m,C}}$ was higher in the high CO$_2$ than in the low CO$_2$ cultures (ANCOVA, $F_{1,27} = 10.5$, $p<0.01$). Contrary to expectations, $K_{\text{m,C}}$ did not increase with decreasing growth rate in the low CO$_2$ cultures (Fig. 2B; Pearson $r_{15} = 0.40$, $p = 0.14$), which should happen if CO$_2$ uptake depended on P-limitation (model 2a). Moreover, $K_{\text{m,C}}$ decreased with decreasing growth rate in the high CO$_2$ cultures (Fig. 2B; Pearson $r_{15} = 0.55$, $p<0.05$), thus suggesting that the high CO$_2$ cells became more severely CO$_2$-limited with lower growth rates (see discussion for a possible mechanism). Although at such low growth rates high CO$_2$ cells were severely P-limited, a high affinity CO$_2$ uptake kinetics was established also suggesting that a P-limitation did not influence CO$_2$-acquisition.

Under low CO$_2$ conditions $V_{\text{max,P}}$ did not vary over growth rate (Fig. 2C), suggesting that all cultures were severely P-limited. This $V_{\text{max,P}}$ was 20-fold higher in P-limited than in P-replete cells (data from [31]; Table 1). In the colimited cultures $V_{\text{max,P}}$ was on average higher in high CO$_2$ than in the low CO$_2$ cultures (ANCOVA, $F_{1,21} = 12.4$, $p<0.01$) and coincides with a lower cellular P content in the high CO$_2$ cells (Fig. S2B). Thus, high CO$_2$ cells could exploit the external P concentration more and were possibly more severely P-limited than low CO$_2$ cells resulting in a higher calculated growth capacity (Table S1). Growth P capacities (i.e. the hypothetical growth rate at $V_{\text{max}}$) greatly exceeded the

![Figure 2. CO$_2$ and P uptake kinetics of *Chlamydomonas acidophila* in relation to balanced growth rates at high CO$_2$ (+CO$_2$) and low CO$_2$ (−CO$_2$) P-limited conditions.](https://doi.org/10.1371/journal.pone.0028219.g002)
balanced growth rate in all cultures. The higher \( V_{\text{max},P} \) in the high CO\(_2\) cultures suggests that P-uptake depended on CO\(_2\) during growth (possibly supporting model 2b).

\( K_{m,P} \) did not vary over growth rate in either high or low CO\(_2\) treatments, but was higher in the high CO\(_2\) than in the low CO\(_2\) cultures (Fig. 2D; T-test, \( t_{27} = 3.1, p < 0.005 \)), suggesting that low CO\(_2\) cells had a higher affinity P-uptake system. Possibly, \( V_{\text{max},P} \) influenced the estimation of this parameter as \( C. \) acidophila had a high affinity P-uptake system under all conditions, including P-replete conditions (Table 1). Because the cellular C and P content of the high and low CO\(_2\)-acclimated cells differed at a given balanced growth rate (Fig. S2) and we expected cell homeostasis to play a role, we will now relate the uptake kinetics to the cellular P to C quota which is independent of cell size.

Independent of CO\(_2\) conditions the \( Q_p \) determined \( V_{\text{max}} \) of both nutrients. \( V_{\text{max},C} \) increased (Pearson \( r_{30} = 0.83, p < 0.001 \)) and \( V_{\text{max},P} \) decreased with increasing \( Q_p \) (Pearson \( r_{22} = -0.69, p < 0.001 \); Fig. 3A, C). Consequently, there was a clear trade-off in the \( V_{\text{max}} \) for both nutrients (Pearson \( r_{22} = -0.59, p < 0.005 \); Fig. 4). The variation in \( V_{\text{max},C} \) was much larger than that in \( V_{\text{max},P} \) hence, a small increase of \( V_{\text{max},P} \) can only be obtained by a substantial lowering of \( V_{\text{max},C} \), implying high costs involved in this adaptation (P-starvation). Cells relatively rich in P (high Q\(_{p,P} \)) had a lower \( V_{\text{max},P} \), and cells relatively rich in C (low Q\(_{p,C} \)) had a much lower \( V_{\text{max},C} \). Contour plots which display the cellular C and P content on 2 separate axes, reveal that a low cellular C content resulted in the highest \( V_{\text{max},C} \) and a low cellular P content in the highest \( V_{\text{max},P} \) (Fig. 5A, B). In addition, there is a tendency for an even higher \( V_{\text{max},C} \) at higher cellular P and maximum values of \( V_{\text{max},P} \) at higher cellular C contents.

In both high and low CO\(_2\) conditions, \( K_{m,C} \) increased with increasing \( Q_p \) when data from high and low CO\(_2\) cultures were analyzed separately (Fig. 3B; Pearson \( r_{15} = 0.53, p < 0.05 \) for high CO\(_2\) and Pearson \( r_{15} = 0.67, p < 0.01 \) for low CO\(_2\)). In addition, \( K_{m,C} \) was higher in high than in low CO\(_2\) cells (\( Q_p \) as a co-variate; ANCOVA, \( F_{1, 29} = 25.3, p < 0.001 \)). In contrast, no changes in \( K_{m,P} \) were observed over \( Q_p \) (Fig. 3D). The contour plots that separate the cellular C and P content on 2 axes, reveal rather complex patterns of \( K_m \) in relation to the cellular C and P content (Fig. 5C, D). Against theory (Fig. 1A), cells with a low nutrient content often had a high \( K_m \) for that nutrient. Observed changes in \( K_m \) were however small compared to the changes in \( V_{\text{max}} \), suggesting that overall \( V_{\text{max}} \) dominated responses in uptake kinetics. As a result, the affinity for C or P uptake was highest at

### Table 1. CO\(_2\) and P uptake kinetics of \( C. \) acidophila in P-replete batch cultures (data from [12,31]) and in P-limited cultures (data from the three highest growth rates in Fig. 2 in this contribution).

|                  | P-replete | P-limited | Statistical result          |
|------------------|-----------|-----------|-----------------------------|
| Low CO\(_2\) \( V_{\text{max},C} \) | 178 ± 20  | 202 ± 14  | ANOVA, \( F = 0.86, df = 1,12, p = 0.37 \) |
| High CO\(_2\) \( V_{\text{max},C} \) | 311 ± 16  | 161 ± 13  | ANOVA, \( F = 47.1, df = 1,17, p < 0.001 \) |
| Low CO\(_2\) \( K_{m,C} \)          | 2.4 ± 0.3 | 3.1 ± 0.4 | ANOVA, \( F = 1.5, df = 1,12, p = 0.24 \) |
| High CO\(_2\) \( K_{m,C} \)          | 5.7 ± 0.5 | 5.7 ± 0.6 | ANOVA, \( F = 23.9, df = 1,9, p < 0.001 \) |
| Low CO\(_2\) \( V_{\text{max},P} \) | 3 ± 1     | 69 ± 9    | ANOVA, \( F = 5.6, df = 1,9, p < 0.05 \) |
| Low CO\(_2\) \( K_{m,P} \)          | 0.23 ± 0.10 | 0.64 ± 0.07 | ANOVA, \( F = 6.5, df = 1,9, p < 0.05 \) |

Values of \( V_{\text{max}} \) given in mmol O\(_2\) 10\(^{-12}\) cells h\(^{-1}\), \( K_{m,C} \) in \( \mu \text{M} \) O\(_2\), \( V_{\text{max},P} \) in mmol P 10\(^{-12}\) cells h\(^{-1}\) and \( K_{m,P} \) in \( \mu \text{M} \) P. Mean ± SE of at least 3 measurements.

doi:10.1371/journal.pone.0028219.t001

Figure 3. CO\(_2\) and P uptake kinetics of \( Chlamydomonas \) acidophila in relation to the cellular P quota (\( Q_p \), mmol P mol C\(^{-1}\)) grown in high CO\(_2\) (+CO\(_2\)) and low CO\(_2\) (−CO\(_2\)) P-limited conditions. A) Maximum CO\(_2\) uptake rate (\( V_{\text{max},C} \), mmol O\(_2\) 10\(^{-12}\) cells h\(^{-1}\)), B) affinity constant for CO\(_2\) uptake by photosynthesis (\( K_{m,C} \), \( \mu \text{M} \) CO\(_2\)), C) maximum P uptake rate (\( V_{\text{max},P} \), mmol P 10\(^{-12}\) cells h\(^{-1}\)) and, D) affinity constant for P uptake (\( K_{m,P} \), \( \mu \text{M} \) P).

doi:10.1371/journal.pone.0028219.g003
the lowest cellular C and P content, respectively (Fig. 5E, F), although the pattern is less clear than with V_max (Fig. 5A, B). That is, the highest affinity for C uptake was realized at the lowest cellular C content with a tendency that the maximum affinity was present in cells with a higher P content (Fig. 5E). The highest affinity for P uptake was realized at the lowest cellular P content but values varied little over cellular C and P content (Fig. 5F).

To test for an independent or dependent colimitation, the external P and CO2 concentrations were calculated from the kinetic data (concentrations were too low to be measured), thus combining the kinetic characteristics of uptake with the cellular C and P content into a external nutrient concentration present in the medium. Growth rates in relation to these external nutrient concentrations were first fitted to single-nutrient Monod models and revealed that external P concentration could satisfactorily explain growth rate, whereas CO2 concentration could not (Fig. S1). Then, we fitted the data to 4 models, reflecting 4 types of colimitation. The best fit of the data was obtained when assuming the multiplicative form of independent colimitation (model 1a; Fig. 6, Table 2) suggesting no interaction between the uptake kinetics of the two limiting nutrients. The fit was better than the single Monod model (Fig. S1), supporting the presence of a colimitation. Figure 6A reveals a strong effect of the external P concentrations on the growth rate, while the effect of the CO2 concentration is much weaker. This agrees with the response in the enrichment experiments, where growth was enhanced by P-addition, and CO2 addition only stimulated growth when provided in concert with P [39]. Fitting model 1b to the growth rates, which also assumes independent nutrient uptake kinetics but that only the most limiting nutrient (either C or P) is affecting the growth rate (ultimately, Liebig’s law) resulted in a more angular shape given the sudden changes in nutrient limitation (Fig. 6B; Table 2). The fit of this model to the data was also good, but significantly less than that of model 1A, according to the difference of -5 in the Akaike Information Criterion (AICc) between both fits [40].

The models 2a, and 2b (Fig. 6C,D; Table 2) assume a dependent colimitation. In the model 2a, CO2 uptake depends on the P-limitation, resulting in an angular shape of the surface that shows an even stronger effect of P concentration on the growth rate (lowest Kp, Table 2) and a weaker effect of CO2 concentration. Especially the data points at high CO2 concentration were badly fitted by the model. Given that the fitting was highly sensitive to starting values and that we found a difference in AICc compared to the fit of model 1a of -50, we conclude that model 2a does not reflect the underlying mechanisms of colimitation. In contrast, if we assume that P uptake depends on CO2-limitation (model 2b) we get a fit to the data similar to that obtained with the model 1b (Fig. 6D). There are no strong differences between the goodness of fit of model 1b and 2b as the AICc is similar (Table 2). The independent, multiplicative colimitation of model 1a resulted in the best fit, but the difference in AICc between model 1a and 2b is only -4.5. The residuals (observed-predicted) for the four models shown in Fig. 6 are visualised in Fig. S3.

**Discussion**

When facing nutrient limiting conditions plants respond by increasing their ability for nutrient uptake. This can be established by increasing their maximum uptake rate (V_max) and/or increasing their affinity for uptake (lower affinity constant, K_m). V_max is positively related to the number of porters on the cytoplasmic membrane [8] whereas a change in K_m reflects the presence of a different porter type [23]. Because the response to single-nutrient or colimitation may differ, we analyzed the uptake kinetics of the green alga *Chlamydomonas acidophila* to different CO2 and P colimiting conditions and fitted the data to 4 different models describing colimitation.

![Figure 4. Maximum CO2 uptake rate (Vmax,C in mmol O2 10^-12 cells h^-1) in relation to the maximum P uptake rate (Vmax,P in mmol P 10^-12 cells h^-1) of the same colimited culture of Chlamydomonas acidophila.](https://example.com/fig4)

**Figure 4.** Maximum CO2 uptake rate (V_max,C in mmol O2 10^{-12} cells h^{-1}) in relation to the maximum P uptake rate (V_max,P in mmol P 10^{-12} cells h^{-1}) of the same colimited culture of *Chlamydomonas acidophila*. doi:10.1371/journal.pone.0028219.g004
We used semi-continuous cultures which implies that the strength of limitation decreases with increasing balanced growth rate. Moreover, uptake kinetics and cellular quota were directly converted into external nutrient concentrations that could then be related to balanced growth rate [41] and used to test different colimitation models. Because growth rates were used to calculate the external nutrient concentrations, the modeling results were considered carefully and conclusions were based in concert with the contour plots that show only measured kinetic characteristics.

Enrichment experiments had revealed that growth in all cultures was colimited by CO₂ and P, since the growth rates of both high and low CO₂ acclimated cells were enhanced by increased P concentration (3.8-fold) but even more when both CO₂ and P were supplemented (4.8-fold; [39]).

**Single vs. multiple nutrient limitation**

Cell homeostasis of balanced nutrient content is of the greatest importance for a proper functioning of enzymes and proteins, and
Table 2. Estimated parameter values and their 95% confidence intervals as well as the maximum log-likelihoods (L) and corrected Akaike Information Criterion (AICc) for each co-limitation model as presented in Fig. 6.

| Parameter | Model 1a | Model 1b | Model 2a | Model 2b |
|-----------|----------|----------|----------|----------|
| Kc (µM)   | 1.09 [0.77, 1.49] | 1.46 [1.06, 1.85] | 0.70 | N/A (0.88) |
| Km (µM)   | 0.38 [0.21, 0.57] | 1.43 [1.00, 2.16] | N/A (1.51) | 0.77 [0.36–1.57] |
| Vmax,C (2a) | N/A | N/A | 0.039 [-0.987, 0.193] | 0.075 [0.056, 0.109] |
| Vmax,P (2b) | 0.073 [0.063, 0.083] | 0.076 [0.065, 0.092] | 0.059 [0.0432, 0.0588] | 0.066 [0.057, 0.078] |
| L         | 128      | 126      | 102      | 126      |
| AlCc      | -250     | -245     | -198     | -246     |

Model 1a: Independent co-limitation, multiplicative form; model 1b: Independent co-limitation, minimum form (Liebig’s Law); model 2a: Dependent co-limitation (CO₂ uptake depends on P-limitation), and; model 2b: Dependent co-limitation (P uptake depends on CO₂-limitation). N/A = not applicable (K is calculated from \( \frac{V_{\text{max},P}}{V_{\text{max},C}} \)).

doi:10.1371/journal.pone.0028219.t002

Consequently nutrient uptake and excretion rates are regulated to balance nutrient ratios within a certain range [15,16]. Thus, survival and growth critically depend on an increase of the uptake capacity of all nutrients available in sub-optimal concentrations. This was reflected in the uptake response of colimited C. acidophila that had a high \( V_{\text{max},C} \) when the cellular C content was low, and a high \( V_{\text{max},P} \) when the cellular P content was low (Fig. 5). \( V_{\text{max}} \) was strongly related to \( Q_m \) (Fig. 3). Cell homeostasis therefore determined the limitation status of the cell, and consequently its nutrient uptake kinetics. The relationships of \( V_{\text{max},P} \) and \( V_{\text{max},C} \) versus \( Q_m \) for both high and low CO₂ cells support previous observations that all cells were colimited for CO₂ and P [39] and that acclimation to nutrient limiting conditions resulted mainly in changes in \( V_{\text{max}} \) (as exemplified in Fig. 1B; [31]). High CO₂ cells had a lower cellular P content and were more severely P-limited whereas low CO₂ cells had the lower cellular C content and were thus more severely CO₂-limited. In addition, high CO₂ cells possibly contained more C as a result of luxury accumulation caused by high CO₂ concentrations, or their C content was increased by cellular accumulation of photosynthetic products resulting from P-limitation (mainly lipids; [42]). The fact that \( V_{\text{max},P} \) increases with increasing P-limitation has been amply demonstrated by a large number of observations in single nutrient, P-limited algal species [9–11]; however, our colimitation results contrast with many single-nutrient studies in which \( V_{\text{max},C} \) decreased with increasing CO₂-limitation (e.g. [12,43]). Thus, under colimiting conditions, CO₂ and P uptake interact and single nutrient limitation studies cannot predict the cell response adequately. A similar conclusion was recently obtained in a cyanobacterium grown under N, Fe and N/Fe colimited conditions [44].

In P-replete cells of C. acidophila \( V_{\text{max},C} \) was higher in high CO₂ than in low CO₂ conditions [12]. The same pattern was observed in many other P-replete algal species, which is explained by enhanced growth rates at high CO₂ [14,43]. If growth is not limited by either nutrient supply or light, high CO₂ concentrations stimulate photosynthesis and consequently growth. In our experiments uptake and growth response to changing CO₂ conditions was uncoupled in P-limited cells, revealed by the lower \( V_{\text{max},C} \) in high CO₂ P-limited cells that still have a higher maximum growth rate [31] than the low CO₂ P-limited cells.

One intriguing result of our study is the colimitation for CO₂ and P in cells growing at high CO₂. The CO₂ concentration measured in the CO₂ aerated, algae-containing vessels was 330 µM independent of whether cultures contained 7.8 × 10⁵ or 1.5 × 10⁶ cells ml⁻¹, nonetheless, calculations of external CO₂ concentrations in the medium were a 100-fold lower (i.e 3 µM). A possible explanation is the presence of a diffusion barrier around the cells [45] despite the fact that C. acidophila is an active swimming flagellate with a strong chemotactic response to CO₂ (pers. obs. and see [46] for Chlamydomonas moewussii).

The presence of such diffusion barrier can be inferred more clearly by looking closer at the difference between \( V_{\text{max},C} \) and \( V_{\text{max},P} \) and also at the definition of \( V_{\text{max}} \). \( V_{\text{max}} \) is a function of two parameters [8]: The number of porters divided by the handling time for nutrient uptake. High CO₂ acclimated cells had the highest \( V_{\text{max},P} \) showing they were under stringent P-limitation. Under P-limitation, cells excrete superfluously produced sugars from photosynthesis and cells producing a polysaccharide mucous layer will extend such layer under severe P-depletion [47], thus surrounding the cell with a diffusion barrier for nutrient uptake that increases handling time. Indeed, high CO₂, P-limited cultures of C. acidophila had higher concentrations of dissolved organic substances than low CO₂ cultures suggesting more [poly]saccharide excretion [31]. Presumably, this affects P uptake much less than C-uptake as \( V_{\text{max},P} \) did not decline in the presence of a thick mucous layer in two other green algal species (mucilage twice cell radius; [48]), whereas CO₂-uptake is considered seriously hampered by such diffusion barrier [49,50]. This implies that high CO₂, P-limited cells had to cope with increased handling time for CO₂ uptake, which dampened the increase in \( V_{\text{max},C} \) and the measured values of \( V_{\text{max},C} \) underestimate the number of CO₂-uptake porters actually present. Because low CO₂ cells were also P-limited, a (lesser) diffusion barrier for CO₂ uptake may likewise be present in these cells.

The low \( K_{\text{m},C} \) in the high CO₂, P-limited cells at low growth rate (Fig. 2b) also indicates that the CO₂ concentration in direct vicinity of the cell was limiting and supports the presence of a diffusion barrier around the cells. This layer presumably increased in size with decreasing growth rate. The impact of a mucilage layer possibly explains the difference in response between a single nutrient limitation and colimitation in CO₂-uptake kinetics, because P-replete cells will have a small or no diffusion barrier around the cells.

Independent colimitation

The fact that model 1a delivered the best fit suggests that an independent, multiplicative colimitation for CO₂ and P in C. acidophila is the best explanation. Liebig’s Law of the minimum (model 1b), another form of independent colimitation, showed a
worse fit, as did the two dependent colimitation models. But, beyond differences in goodness of fit, there are other arguments that support an independent colimitation. For instance, a basic assumption of Liebig’s Law (model 1b) does not really apply, because the instantaneous maximum growth in a nutrient enrichment experiment was obtained when both CO2 and P were added [39]. The model 1b nevertheless fits reasonably well because one limitation (P) is considerably stronger than the other (CO2), which was confirmed by single-nutrient models (Fig. S1).

Regarding the dependent colimitation models (2a,b), their basic assumption does not agree with the results. The models assume changes in Km over the range of nutrient limitation that did not occur in the experiments (see below for further discussion). The data show that, irrespective of the other nutrient limitation, the cells responded to a low nutrient content by increasing their Vmax for that nutrient. On a cellular level this presumably resulted in a trade-off in space for porters of either nutrient on the cytoplasm membrane (see below). The contour plots support the conclusions from the model fittings as they reveal a trade-off in Vmax for both limiting nutrients (Fig. 5A,B).

Dependent colimitation

We expected to find that CO2-acquisition depends on P-limitation [25,31,51], since the realization of a low Km,C and a CCM [25] are both active processes hampered by insufficient ATP during P-limitation [28]. Under P-replete, low CO2 conditions, C. acidophila had both a low Km,C and a CCM [12,29]. However, the Km,C was also unexpectedly low in stringent P-limited cells (Figs. 2B, 3B). Thus, our results support those of Kozlowska et al. [51], who showed a lower Km,C in P-limited than in P-replete cells of Chlorella vulgaris. Possibly, Chlorella vulgaris was also colimited by P and CO2 in their study as cell densities were high, whereas in the study of Beareall et al. (Km,C higher in P-limited than P-replete low CO2 cells) the cell density of Chlorella emersonii was low [25]. Of course other explanations such as the presence of entirely different adaptation mechanisms in the different species of Chlorella are also possible. Nonetheless, the poor fit of our data to model 2a (which reflects these assumptions) clearly results in a rejection of a dependent colimitation in which CO2 acquisition depends on P-limitation in C. acidophila.

Model 2b (P-uptake depended on CO2 limitation) provided a reasonable fit to the data and it supports our earlier finding that high CO2, P-depleted cells of C. acidophila had a higher P uptake ability (i.e. realized a lower external P concentration) than low CO2 cultures [30]. However, we think that the model fits for the wrong reason, as the presence of a dependent colimitation would imply that the Km,P is higher in the low CO2 cells (i.e. more stringent CO2-limited), whereas we find the direct opposite (related to growth rate) or no difference (related to Qp).

The relative good fit of model 2b must therefore be a result of the enhanced growth capacity for P in the high CO2 cells (Table S1) compared to low CO2 cells. Again, the contour plots support the conclusions from the model fittings, as changes in Km,P and affinity for both limiting nutrients did not follow the expected changes based on dependent colimitation models (Fig. 5).

Space limitation

Our data show a trade-off between Vmax,P and Vmax,C (Fig. 4): when P uptake ability (Vmax,P) was high, that for CO2 (Vmax,C) was low and vice versa. Nutrient uptake modeling revealed that Vmax is directly and positively related to the number of porters, although increased handling time can dampen this relation [8]. Also experimentally, in higher plant cell cultures (guard cells of Solanum tuberosum, Nicotiana tabacum and Vicia faba) a positive relation was found between the K+ uptake porter density and K+ transport capacity [32]. Therefore, Vmax can be used as an indicator for the number of active porters. Accordingly, we conclude that the number of porters was related to the cellular content of the limiting nutrient, but also to its external concentration. This relation not only holds for micro-organisms or plant cell cultures but also for higher plants as, for example, the density of stomata (porter for gas) declined linearly with air CO2 concentration [53], although there is much debate at this point [54].

An algal cell requires many different nutrients for growth, and all need transportation through the cytoplasm membrane by (often) nutrient-specific porters. Calculations on the number of nutrient porters in a hypothetical algal cell revealed that 5.5% of the cell surface may be covered by just one type of porter [8,25]. Within the constraint of an overall fixed number of porters, a cell can only increase the density of porters for a specific limiting nutrient at the expense of others [23]. Under colimiting conditions, it seems plausible that a space limitation for porters on the membrane results in a trade-off in the investment for porters for those limiting nutrients [24], for which we provide the first empirical evidence.

At the cellular level, the trade-off in Vmax can be interpreted as a kind of dependent colimitation: The space freed by a decrease in number of porters for one nutrient is used for an increase of porters of another one. In contrast, current dependent colimitation models assume that the concentration of one limiting nutrient has an effect on the Km for the acquisition of another one and not on Vmax. This requires a different set of models where adaptation in Vmax is considered (possibly starting from [55]).

Increasing evidence reveals that phytoplankton in marine and freshwater ecosystems and plants in general are colimited in their growth, and our study enhances the understanding of phytoplankton growth response and physiological adaptation under a colimitation for CO2 and P. In conclusion, cell nutrient homeostasis regulated nutrient acquisition in C. acidophila, and the most plausible mechanism was a multiplicative, independent colimitation. Given the space constraints on the cytoplasm membrane a trade-off in the number of porters for the uptake of different nutrients seems plausible under colimiting conditions. Responses to colimitation cannot be predicted from those to single nutrient limitation and therefore experiments on colimited plants are required to properly predict growth responses to a complex and changing natural environment. Our conclusions may also apply for other nutrients such as K and P [30], Si and P [56], N and CO2 [27,57–59] in algae and in higher plants [52,53,60].

Supporting Information

Figure S1 Balanced growth rates (h−1) fitted to external CO2 concentration (A: Sc, in μM) and P concentration (B: Sp, in nM) using a single nutrient Monod model. Prior to testing colimitation models, we fitted single-nutrient models to check if one nutrient alone can satisfactorily explain the growth response of C. acidophila. For this, we used a standard Monod function with the external concentration of either carbon or phosphorous as predictors, as:

\[
\mu = \mu_{\text{max}} \frac{S}{S + K}
\]

where S represents either carbon or phosphorous concentrations in the medium. The ability of carbon concentration to explain growth response was quite low, compared with other models, which is coherent with the high dispersion evident in the data (Fig. S1A). Additionally, the maximum growth rate predicted by this
model was much lower than all the others. On the other hand, phosphorus had a much better predictive ability (Fig. S1b), which is also consistent with the stronger effect of phosphorus detected in the colimitation models. Still, the model with phosphorus alone had a worse fit than most of the colimitation models, suggesting again that growth is better described based on a multiple-nutrient colimitation. The modelling of $\mu$ to CO$_2$ resulted in an estimation and 95% confidence interval of $\mu_{\text{est}}$ of 0.036 [0.029 0.043], $K_{\text{C}}$ of 0.97 [0.42 1.74], Log-likelihood of 75.5, and corrected Akaike Information Criterion ($\text{AIC}_C$) of $\text{AIC}_C$ of $-148.3$. The modelling of $\mu$ to P resulted in an estimation and 95% confidence interval of $\mu_{\text{est}}$ of 0.059 [0.034 0.065], $K_{\text{P}}$ of 1.08 [0.9 1.38], Log-likelihood of 105.8, and $\text{AIC}_C$ of $-208.9$.

**Figure S2** Cellular carbon ($A$, in pmol C cell$^{-1}$) and cellular phosphorus ($B$, in fmol P cell$^{-1}$) content of *Chlamydomonas acidophila* in relation to balanced growth rate ($d^{-1}$) of high CO$_2$ (+CO$_2$) and low CO$_2$ (−CO$_2$) P-limited cultures. Mean ± SE of 3 measurements. CO$_2$ concentration had a significant effect on the cellular C content (ANOVA, df = 1, 27, $F = 5.9$, $p = 0.05$) and cellular P content (ANOVA, df = 1, 27, $F = 12.5$, $p < 0.01$) when the effect of growth rate is accounted for.

**Figure S3** Residuals (observed-predicted) for the four models shown in Fig. 6 in the main text. A) model 1a; B) model 1b; C) model 2a; and D) model 2b.

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**Table S1** Calculated growth capacity, i.e. $V_{\text{max}}$/cellular nutrient content ($d^{-1}$), of *Chlamydomonas acidophila* in relation to balanced growth rate ($d^{-1}$) of high CO$_2$ (+CO$_2$) and low CO$_2$ (−CO$_2$) P-limited cultures at pH 2.7. The following assumptions were made: 1) The maximum uptake rate is for 100% converted into growth during the 16 h light period per day, and 2) 1 mol O$_2$ is released when 1 mol CO$_2$ is fixed (required to calculate $V_{\text{max}}$). Calculated growth capacity based on maximum CO$_2$ uptake rates revealed a higher capacity in the low CO$_2$ cells than needed to maintain balanced growth rate, whereas capacity equalled balanced growth rate in high CO$_2$ cells. Calculated growth capacity based on maximum P uptake rate were >100-fold higher than balanced growth rates. Such overcapacity has been found more often in P-limited algal cultures [10,62]. In addition, growth capacity was higher in the high CO$_2$ than in the low CO$_2$ cells.

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**Acknowledgments**

ES acknowledges several colleagues for stimulating discussions.

**Author Contributions**

Conceived and designed the experiments: ES. Performed the experiments: ES. Analyzed the data: ES FIUCU. Wrote the paper: ES FIUCU.
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