Elsevier Editorial System(tm) for Science of the Total Environment

Manuscript Draft

Manuscript Number: STOTEN-D-17-09338R1

Title: Decreased phosphorus incorporation explains the negative effect of high iron concentrations in the green microalga Chlamydomonas acidophila

Article Type: Research Paper

Keywords: Chlamydomonas, ecotoxicology, extreme environment, iron toxicity, phosphate limitation, phytoplankton

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First Author: Elly Spijkerman

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Abstract: The green microalga Chlamydomonas acidophila is an important primary producer in very acidic lakes (pH 2.0-3.5), characterized by high concentrations of ferric iron (up to 1 g total Fe L-1) and low rates of primary production. It was previously suggested that these high iron concentrations result in high iron accumulation and inhibit photosynthesis in C. acidophila. To test this, the alga was grown in sterilized lake water and in medium with varying total iron concentrations under limiting and sufficient inorganic phosphorus (Pi) supply, because Pi is an important growth limiting nutrient in acidic waters. Photosynthesis and growth of C. acidophila as measured over 5 days were largely unaffected by high total iron concentrations and only decreased if free ionic Fe3+ concentrations exceeded 100 mg Fe3+ L-1. Although C. acidophila was relatively rich in iron (up to 5 mmol Fe: mol C), we found no evidence of iron toxicity. In contrast, a concentration of 260 mg total Fe L-1 (i.e. 15 mg free ionic Fe3+ L-1), which is common in many acidic lakes, reduced Pi-incorporation by 50% and will result in Pi-limited photosynthesis. The resulting Pi-limitation present at high iron and Pi concentrations was illustrated by elevated maximum Pi-uptake rates. No direct toxic effects of high iron were found, but unfavourable chemical Pi-speciation reduced growth of the acidophile alga.

Response to Reviewers: Ms. Ref. No.: STOTEN-D-17-09338
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Journal: Science of the Total Environment
Associate editor: Daniel Wunderlin

General remark:
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Specific replies to the reviewers:
We numbered (#) the comments and started the answer by A#:
Mention the page, paragraph, and line number of any revisions that are made.

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A13- Thank you!

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To the editor of Science of the Total Environment

Ms. Ref. No.: STOTEN-D-17-09338

Date: 18. January 2018

Dear editor,

This is a re-submission of our manuscript, now entitled: "Decreased phosphorus incorporation explains the negative effect of high iron concentrations in the green microalga *Chlamydomonas acidophila*", intended for publication as a regular research article in Science of the Total Environment.

We kindly thank the 4 reviewers for their constructive reviews and we are happy to follow all their queries and suggested changes / improvements.
We hope that our contribution is now acceptable for publication in Science of the Total Environment.

Please do not hesitate to contact us if there are any further questions. In anticipation of your kind consideration,

Yours sincerely,

Elly Spijkerman (corresponding author: spijker@uni-potsdam.de)
Hella Behrend (hella.behrend@googlemail.com),
Bettina Fach (bettina.fach@googlemail.com) &
Ursula Gaedke (gaedke@uni-potsdam.de)
All: University of Potsdam, Department of Ecology and Ecosystem Modelling, Am Neuen Palais 10, Potsdam, Germany.

Decreased phosphorus incorporation explains the negative effect of high iron concentrations in the green microalga *Chlamydomonas acidophila*

Elly Spijkerman*, Hella Behrend, Bettina Fach and Ursula Gaedke

Department of Ecology and Ecosystem Modelling, University of Potsdam, Am Neuen Palais 10, Potsdam, Germany

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Running head: High iron decreases phosphate-incorporation

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***Responses to Reviewers Comments**

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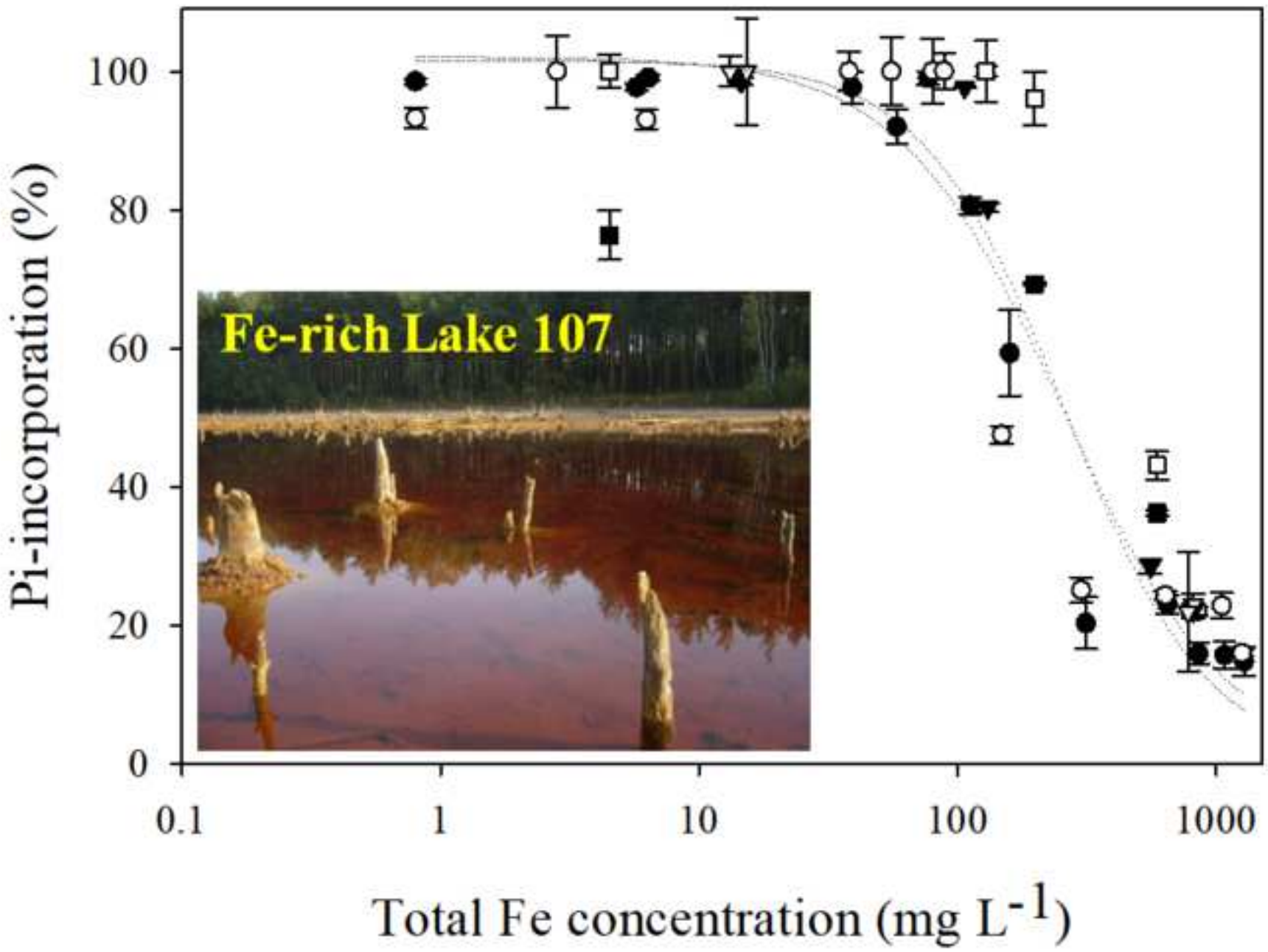
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

3-5 bulletpoints:

- Iron accumulated to up to 38 pg Fe $cell^{-1}$ in *Chlamydomonas acidophila*
- Accumulated iron did not result in oxidative stress response on photosynthesis
- Iron-phosphate complexes decrease bio-availability of phosphorus for uptake
- 260 mg iron per litre resulted in 50% inhibition of phosphate incorporation at pH 2.4
- Unfavourable chemical phosphorus speciation reduced algal growth

Decreased phosphorus incorporation explains the negative effect of high iron concentrations in ~~a~~ the green microalga *Chlamydomonas acidophila*

Elly Spijkerman*, Hella Behrend, Bettina Fach and Ursula Gaedke

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Abstract

The green microalga *Chlamydomonas acidophila* is an important primary producer in very acidic lakes (pH 2.0-3.5), characterized by high concentrations of ferric iron (up to 1 g total Fe L^{-1}) and low rates of primary production. It was previously suggested that these high iron concentrations result in high iron accumulation and inhibit photosynthesis in *C. acidophila.* To test this, the alga was grown in sterilized lake water and in medium with varying total iron concentrations under limiting and sufficient inorganic phosphorus (Pi) supply, because Pi is an important growth limiting nutrient in acidic waters. Photosynthesis and growth of *C. acidophila* as measured over 5 days were largely unaffected by high total iron concentrations and only decreased if free ionic Fe^{3+} concentrations exceeded 100 mg Fe^{3+} L^{-1}. Although *C. acidophila* was relatively rich in iron (up to 5 mmol Fe: mol C), we found no evidence of iron toxicity. In contrast, a concentration of 260 mg total Fe L^{-1} (i.e. 15 mg free ionic Fe^{3+} L^{-1}), which is common in many acidic lakes, reduced Pi-incorporation by 50% and will result in Pi-limited photosynthesis. The resulting Pi-limitation present at high iron and Pi concentrations was illustrated by elevated maximum Pi-uptake rates. No direct toxic effects of high iron were found, but unfavourable chemical Pi-speciation reduced growth of the acidophile alga.

Key words: Chlamydomonas, ecotoxicology, extreme environment, iron toxicity, phosphate limitation, phytoplankton

1. **Introduction**

Many very acid lakes and rivers are not only characterized by a low pH ranging between 2.0 and 3.5, but also by high concentrations of ferrous iron and sulphuric acid resulting from the weathering of rock such as pyrite and marcasite (iron-sulphur minerals) (Friese et al., 1998). Once oxidized, ferric iron hydroxide acts as a buffering substance and dominates the iron speciation constituent (Herzsprung et al., 1998). Understanding these iron-rich systems is of interest because they are found world-wide, and their chemical composition may threaten human health (e.g. the Kawah ljen crater lake in East Java; Lohr et al., 2006), whereas its extremophile inhabitants are used in a variety of biotechnological applications (Bell, 2012). In this contribution we unravel if high iron concentrations affect the ecophysiology of an important microalga inhabiting acid lakes: *Chlamydomonas acidophila*.

Iron is a redox-active heavy metal and therefore potentially toxic for organisms. It exists in different chemical forms and some of them, such as free ions of Fe^{2+} are highly toxic (Goshal et al., 2005). In the upper phototrophic layer of acidic lakes and rivers, concentrations of total iron can be high (up to 1000 mg Fe L^{-1}). However, the iron is mainly present as ferric iron (Herzsprung et al., 1998), from which a variable fraction is the potentially toxic, free ionic Fe^{3+}. It is known that most acidophilic algae are remarkably tolerant towards heavy metals (Whitton, 1970), possibly resulting from their positive charged membrane necessary to tolerate high proton concentrations. In the neutrophile *Chlamydomonas reinhardtii* high proton concentrations decreased metal uptake by non-competitive inhibition (Francois et al., 2007; Macfie et al., 1994), suggesting that iron accumulation might be restricted in *C. acidophila*.

Nonetheless, iron is considered to inhibit algal growth when concentrations are extremely high (Aguilera et al., 2006; Gross, 2000), for example in Rio Tinto, total iron concentrations can reach values over 2 g Fe L^{-1} (Lopez-Archilla et al., 2001). High iron

concentrations can cause bronzing symptoms in higher plants, which are mainly caused by oxidative stress or an antagonism between magnesium and iron in forming the chlorophyll prophyrin group (De Dorlodot et al., 2005). As a consequence, high iron concentrations affect the chlorophyll content and thereby primary productivity. Comparing the primary production of three acidic lakes confirmed that the two lakes with the higher iron concentrations had the lower primary production (Kamjunke et al., 2005). Moreover, *C. acidophila* cultured in water from Rio Tinto had a low Ribulose-1,5-bisphosphate carboxylase oxygenase content (Cid et al., 2010), and the algal natural abundance was negatively correlated with dissolved metal concentrations in the river (Aguilera et al., 2006). In laboratory experiments, ferric iron concentrations of 280 mg total Fe L^{-1} totally inhibited growth of a *Chlamydomonas* isolate from Rio Tinto (Rowe et al., 2007).

Metal tolerance is often connected with the storage of metals in polyphosphate bodies, and as a result, increased Pi availability often enhanced metal accumulation but mitigated its toxicity (Wang and Dei, 2006). In accordance, Pi-limiting conditions enhanced metal toxicity in micro-phytobenthos (Ivorra et al., 2002). Although most acidic environments contain relatively high concentrations of Pi, algal growth was often Pi-limited (Sabater et al., 2003; Spijkerman, 2008), where the mechanistic reasons remained unclear because acidic lakes ~~are rich in~~have high concentrations of ~~a great~~ many metal ions (Friese et al., 1998). In addition, high concentrations of metals may inhibit nutrient uptake and may have indirect effects on the biota by their precipitation with nutrients. It has been suggested that a coating of algae with Fe-hydroxides with an atomic Fe : P ratio higher than 100:1 will adsorb phosphorus efficiently and will promote a Pi-limitation (Kleeberg and Gruneberg, 2005).

We elucidate the effect of high iron concentrations on photosynthesis and growth of *C. acidophila* under variable iron and Pi concentrations. In contrast to expectations, we show that observed negative effects of high iron concentrations on primary production (Kamjunke

et al., 2005) are not directly related to toxicity but result from decreased Pi incorporation and consequently result from a Pi-limitation.

Experimental section

Culturing. To test for the effect of high iron concentrations, experiments with lake water and different media were performed. For the effect of naturally dissolved iron, lake water was sampled from the upper meter of the water column in four acidic lakes, situated in Brandenburg, Germany (Lakes 107, 111, 113 and 117; 51° 29' N, 13° 38' E) on October 19, 2006. Total iron concentrations were 590, 200, 130 and 5 mg Fe L^{-1} for Lakes 107, 113, 111 and 117, respectively, and consisted for over 90% of ferric iron (Spijkerman, 2008). The pH and Pi concentrations in the lake water were 2.3, 2.6, 2.7 and 3.0 and 26, 15, 12 and 8 µg P L^{-1} for Lakes 107, 113, 111 and 117, respectively (Spijkerman, 2008). Water was filter-sterilized through a 0.2 µm cellulose acetate filter (Sartorius, Göttingen, Germany) directly after sampling. Pi-replete lake water was obtained by addition of 310 µg Pi L^{-1} as phosphoric acid. The Pi-addition did not result in a visual precipitation or in a pH change.

To study the effect of different iron concentrations, culturing was done in filter-sterilized media. We used a chemically complex medium reflecting the chemical composition of the lake water and applied $Fe_2(SO_4)_3$ as the sole iron-source (Bissinger et al., 2000; Spijkerman, 2011). In addition we used a minimal medium (Gerloff-Elias et al., 2005) where we applied either $Fe_2(SO_4)_3$ to compare with the complex medium or $FeCl_3$ to vary free ionic Fe^{3+} concentrations (Appendix Table A.1). In both media the total iron concentrations varied between 1 and 1200 mg Fe L^{-1} (see Table 1 for a detailed composition of the main ions in both media). In the minimal medium the pH was kept constant at 2.36 which enabled total dissolving of all salts, whereas it naturally varied in the complex medium between 2.04 at the highest Fe concentration and 2.65 in media with $\leq$ 100 mg Fe L^{-1}, to enable a comparison with the lake water treatment (where neither pH adjustment nor additions of iron were made). Chemical speciation analysis of the complex medium in Visual MINTEQ software (2007)

112 suggested that inorganic Fe-P complexation increased with medium iron concentration
113 (Table. A.1 & A.2).
114
115 **Table 1.** Chemical composition (excluding the micronutrients) of the culture media prepared
116 with Fe concentrations ranging from 1 to 1200 mg L^{-1}. The complex medium is based on the
117 chemical composition of Lake 111 (Bissinger et al., 2000), whereas the minimal medium is a
118 slightly adapted Woods Hole medium (Gerloff-Elias et al., 2005). All concentrations in mg L^{-1},
119 unless stated otherwise.

Main salts	Complex medium	Minimal medium
Ca^{2+}	300	10.0
Mg^{2+}	28.1	3.65
Na^{+}	5.98	26.7
K^{+}	2.64	3.91
Total Fe	1; 10; 50; 100; 200; 400; 600; 800; 1000; 1200	10; 100; 800
N (as NO_3^{-})	0.28	14.0
N (as NH_4^{+})	2.30	0
P (as PO_4^{3-})	5; 310 µg L^{-1}	5; 310 µg L^{-1}
Cl^{-}	9.2	19.1
S (as SO_4^{2-})	417	4.80
pH	2.04 - 2.65	2.36

120
121 The unicellular green alga *Chlamydomonas acidophila* Negoro (SAG strain no. 2045),
122 isolated from Lake 111 (Gerloff-Elias et al., 2005) and maintained in a complex medium at
123 145 mg Fe L^{-1} for eight years, was used in all experiments. To adjust to Pi conditions, *C.*

acidophila was grown in Pi-limiting (i.e. 5 µg Pi L^{-1}) or Pi-replete (i.e. 310 µg Pi L^{-1}) medium containing a moderate Fe concentration (145 mg Fe L^{-1}) for eight days. This was followed by a pre-acclimation period of four days to experimental Pi and Fe concentrations or lake water. The subsequent growth experiments ran over a 5-day period, starting with 1-2 10^7 cells L^{-1}. By applying this short time-period and low starting cell density, we aimed for constant Pi-conditions during the experiment. Cultures were illuminated for 16 h per day (TLD 58W/930, Philips) at 150 µmol photons m^{-2} s^{-1} measured inside the culture flask (4π quantum sensor US-SQS, Walz, Effeltrich, Germany). Temperature was kept at 20° C ± 2. Before transfer into new medium or lake water, cells were washed once by centrifugation (1,500·*g*, 5 min) with the experimental water.

Growth rate and chemical measurements. Daily measurements of optical density (OD at 750 nm, UV-2401 PC; Shimadzu, Berlin, Germany) were done in a 5 cm cuvette over a 5-day period. A linear model was fitted to the natural logarithm of OD values over time to calculate the exponential growth rates. To standardize for differences in culturing conditions (Pi concentration and/or medium or lake water use), the growth rate in the low iron treatment (1 or 10 mg Fe L^{-1}) from every series of experiments was considered as a control and set to 100%. Growth rates at higher iron concentrations were related to this control. The low cell densities did not influence the medium iron concentrations over the 5-day growth period.

All chemical and activity measurements were done on algal suspensions from day 5, at the end of the growth experiment. We measured cellular phosphorus (P), carbon (C) and iron (Fe) content to obtain the elemental stoichiometry. For cell enumeration, samples were fixed with acidified Lugol's solution, and counted according to the method of Utermöhl using an inverted light microscope (Thalheim-Spezial-Optik TSO, Pulsnitz, Germany) and sedimentation chambers (Thalheim, Pulsnitz, Germany). At least 600 cells and 2 transects of the chamber were counted.

149 The cellular P content was determined in cells harvested on acid-drained nucleopore
150 filters (Track-Etch; Whatman, Göttingen, Germany), rinsed with de-ionized water. Particulate
151 P was macerated by autoclaving together with $K_2S_2O_8$ at low pH, reduced to form a
152 phosphate-molybdate complex and measured at 880 nm on a spectrophotometer (Murphy and
153 Riley, 1962). The residual Pi concentration in the medium was determined with the same
154 method, but without maceration, in the supernatant of centrifuged culture suspension (see
155 cellular Fe content). The Pi-incorporation was calculated as the ratio between the cellular P
156 and the sum of cellular and residual Pi concentration multiplied by 100%. If residual Pi
157 concentrations were below the detection limit we assumed that all Pi was taken up. The Pi-
158 incorporation was used to calculate the medium iron concentration at which 50 % of Pi was
159 incorporated (EC_{50}) by use of the model from Haanstra et al. (Haanstra et al., 1985) in
160 SigmaPlot (version 11.0):

$$Pi - incorporation\ (\%) = \frac{c}{1 + e^{(b \times (\log [\text{Fe}-\text{concentration}]-a)}}$$

161 in which c = Pi-incorporation at lowest Fe concentration (~ 100%), b =slope and a = log
162 EC_{50}.

163 To determine the cellular C content, cells were harvested on pre-combusted (4 h at
164 450 °C) glass fiber filters and measured after drying at 50°C in a HighTOC+N gas analyzer
165 (HighTOC+N; Elementar Analysensysteme, Hanau, Germany).

166 To measure the cellular Fe content, the culture suspension was centrifuged (2,500·g,
167 10 min) and the supernatant was used for the determination of medium iron and residual Pi
168 concentration. The pellet washed twice with de-ionized water and incubated with 0.5 ml
169 20mM K_2EDTA (pH 2.7) for 10 min (Bates et al., 1982). After centrifugation (12,000·g, 5
170 min, the supernatant (extra-cellular Fe) and the pellet (intra-cellular Fe) were separated and
171 lyophilized. Samples were extracted with 0.2 ml HNO_3 (69%, trace element Sigma,
172 München, Germany), dried in a thermo-block under a fume head at 80°C overnight and then

extracted with 0.2 ml of H_2O_2 (30%) and dried again at 80° C overnight. Finally, the pellet was dissolved in acidified water (1.5% HNO_3), and liquid samples of medium and lake water were acidified in a similar way. Iron concentrations were measured by atomic absorption spectroscopy (AAS1100B, Perkin Elmer).

The total Fe content (i.e. the sum of the intra- and extra-cellular fraction) was used in the calculation of cellular ratios. Concentrations of ferrous iron in the medium were determined using ferrozine reagent (Lovley and Phillips, 1987), and did not exceed 7% of total iron concentrations in lake water and medium. Iron and phosphorus complexation and free ionic Fe^{3+} concentrations in the different media were calculated with Visual MINTEQ software (including the full ionic composition and pH; the full ionic speciation of selected media is provided in Table A.2). Because the complete ionic composition of the lake water is unknown, free ionic Fe^{3+} concentrations in the lake waters were estimated based on the chemical composition of the complex medium and the measured pH. The chemical composition of the complex medium resembles that of Lake 111 but omits the aluminum concentration (Bissinger et al., 2000).

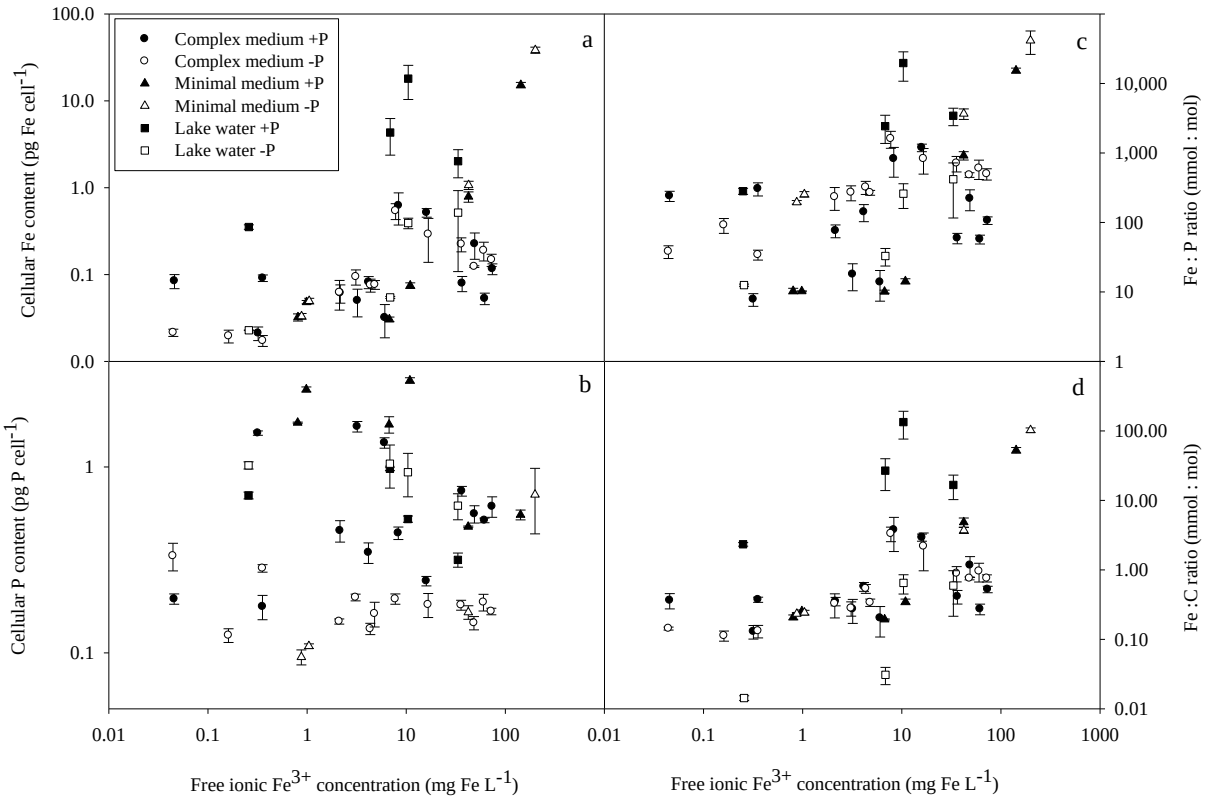
Chlorophyll a fluorescence transient. As a measure of maximum photosynthetic rate chlorophyll a fluorescence emission transients were determined in a double-modulation fluorometer (Dual-Modulation Kinetic Fluorometer FL-3000 with APD cuvette, PSI Photon Systems Instruments, Brno, Czech Republic). Dark adapted (for 30 min: minimal fluorescence, F_0) cells were exposed to saturating light (1 sec, 2100 µmol m^{-2} s^{-1}) and the maximum fluorescence (F_m) was measured. From these values, the maximum fluorescence yield of photosystem II (Φ_{II}, $(F_m-F_0)/F_m$, also known as F_v/F_m) was calculated.

Pi-uptake rates. For this measurement, *C. acidophila* was grown in semi-continuously diluted cultures (μ= 0.35 d^{-1}) ~~adapted~~ acclimated to four medium total iron concentrations (0.6, 11, 223 and 558 mg Fe L^{-1}) at Pi-replete (1.55 mg P L^{-1}) and Pi-limited

198 (31 µg P L^{-1}) conditions at pH 2.4. These Pi-concentrations resulted in Pi-replete and Pi-
199 limited conditions for this alga previously (Spijkerman et al., 2007b). Cells were centrifuged
200 (1,500·*g*, 5 min), the pellet washed once with medium lacking iron and Pi, and subsequently
201 resuspended in a Pi and Fe-free medium reaching a density of 2 10^8 cells L^{-1}. The culture was
202 placed in the light (approx. 90 µmol m^{-2} s^{-1} inside the flask) for about 15-30 minutes. Over a
203 period of 1 (Pi-limited) or 5 (Pi-replete) minutes, Pi uptake was measured by addition of
204 $H_3{}^{33}PO_4$ (565 TBq $mmol^{-1}$ specific activity, Perkin Elmer) diluted in a stock solution of 31
205 mg P L^{-1} adjusted to pH 2.4, resulting in a final concentration of 150 µg P L^{-1}. At this
206 concentration, Pi-uptake is maximal (Spijkerman, 2007). Uptake was terminated by filtration
207 on 1.2 µm pore-size cellulose acetate filter and subsequent rinsing with 0.2 M LiCl. The
208 filters were embedded in Ultima Gold (Packard) and counted in a liquid scintillation analyser
209 (Tri-Carb 2810 Tr, Perkin Elmer). Part of the diluted culture was fixed with 0.2% Lugol's
210 solution (f.c.) for cell enumeration. Cell numbers were determined as described above.
211 ***Statistics.*** All cultures were run in triplicate. Statistical analyses were performed in
212 SPSS 20.0. When providing statistical test results, the degrees of freedom, or sample size are
213 given as an index number.
214

Results

Figure 1. Cellular Fe content (a), cellular P content (b), Fe:P ratio (c) and Fe:C ratio (d) of *Chlamydomonas acidophila* grown in Pi-replete (310 µg P L^{-1}: +P) or low Pi (non-treated or 5 µg P L^{-1}: -P) medium or lake water over a range of calculated free ionic Fe^{3+} concentrations. Symbols show the mean ± SE of 3 independent replicates in complex medium (circles), in minimal medium (triangles) and lake water (squares). The free ionic Fe^{3+} concentrations in lake water were calculated using the chemical composition of complex medium and the measured pH. Please note the logarithmic scales on the x-axes and the y-axes.



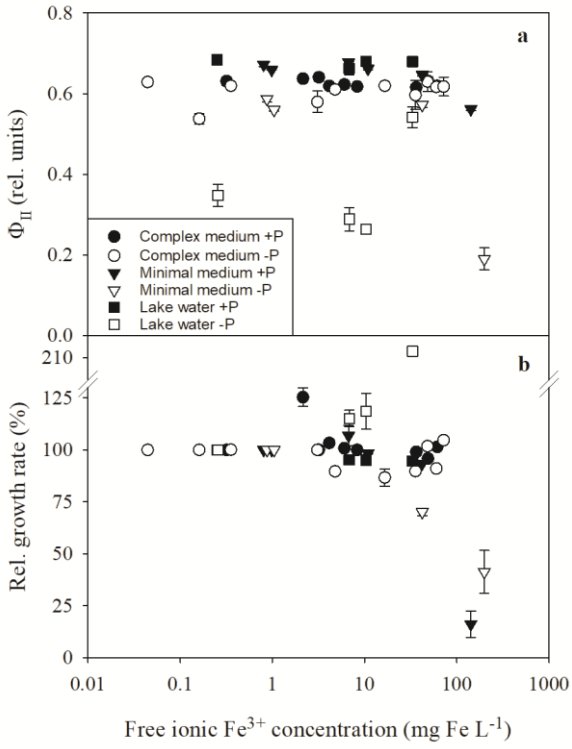
Although we expected a low iron accumulation into cells of *C. acidophila*, the cellular Fe content increased with increasing free ionic Fe^{3+} concentrations (Pearson, r_{36}=0.66, ~~P~~p<0.01; Fig. 1a), both in Pi-limited and in Pi-replete grown cells, but with a large scatter. The cellular Fe content predominantly varied between 0.02 and 1.07 pg Fe $cell^{-1}$, with higher values in three Pi-replete lake water incubations and in the two cultures with the highest free ionic Fe^{3+} concentrations (that resulted in 15 and 38 pg Fe $cell^{-1}$). In the latter 2 treatments the

total iron concentration was 800 mg Fe L^{-1}, and only the medium Pi concentration differed. The cellular P-analysis resolved that the most Fe-rich cells had an intermediate cellular P content (Fig. 1b), similar to cells grown in Pi-replete medium or in untreated lake water. Also the Pi-limited, high iron exposed cells grown in minimal medium had an intermediate cellular P content.

The cellular Fe:P and Fe:C ratios increased in Pi-limited *C. acidophila* with increasing free ionic Fe^{3+} concentration (Pearson, , $r_{25}>0.69$, ~~P~~p<0.01), but remained rather constant in Pi-replete cells (Pearson, $r_{19}<0.38$, ~~P~~p>0.10; Fig. 1c,d). Pi-limitation did not influence the mean accumulation of iron to P in the cells as the mean Fe:P ratio was similar in the Pi-limited (2750±1750, mean±se) and Pi-replete (1500±1000, mean±se) cultures (paired t-test based on 16 pairs of free ionic Fe^{3+} concentrations, ~~P~~p=0.30; Fig. 1c). The mmol Fe:mol P ratio from low to high free ionic Fe^{3+} concentrations ranged between 8 and 41,500 (Fig. 1c) and the mmol Fe:mol C ratio between 0.014 and 134 (Fig. 1d). When the cellular P content was set to 1, the lowest molar C:P ratio was 40:1 under Pi-replete conditions coinciding with the lowest molar Fe:P ratio (0.008:1). Under Pi-limiting conditions the molar C:P ratio was highest (1060:1) as was the Fe:P ratio (42:1).

The percentage of adsorbed extra-cellular iron related to the cellular iron content covered a wide range between 6 and 90%, without a clear pattern (Fig. A.1). In contrast to expectations (Kleeberg and Gruneberg, 2005), an external Fe:P ratio >100 did not result in an enhanced Fe adsorption on *C. acidophila*. In contrast, in lake water and under both Pi-conditions Fe adsorption was negatively related to external Fe:P ratio (Pearson, $r_{15}>0.62$ (n=15), ~~P~~p<0.05 in both cases).

Figure 2. Maximum fluorescence yield of photosystem II (Φ_{II}) measured at day 5(a) and the relative growth rate during 5 days (b) versus a range of calculated free ionic Fe^{3+} concentrations in Pi-replete (310 µg P L^{-1}: +P) or low Pi (non-treated or 5 µg P L^{-1}: -P) medium or lake water grown *Chlamydomonas acidophila* cells. Symbols as in fig. 1. The free ionic Fe^{3+} concentrations in lake water were calculated using the chemical composition of complex medium and the measured pH. Please note the logarithmic scale on the x-axis.



We expected that high external and internal iron concentrations would have a negative effect on the photosynthesis, which we measured by maximum fluorescence yield of photosystem II (Φ_{II}) and of growth of *C. acidophila*. Against expectations, no correlations were detected when considering the total iron concentration (Fig. A.2), and Φ_{II} was only weakly negatively correlated with calculated free ionic Fe^{3+} medium concentrations in Pi-replete cells (excluding Φ_{II} at >100 mg Fe^{3+} L^{-1} Pearson, r_{42} =-0.31, ~~P~~p<0.05; Fig. 2a). The Φ_{II} was 0.644±0.004 at lower Fe^{3+} concentrations (mean±se at < 10 mg Fe^{3+} L^{-1}) and 0.628±0.005 at higher Fe^{3+} concentrations (mean±se at > 30 mg Fe^{3+} L^{-1}). A contrasting trend was found if the cells were grown in Pi-limited medium (excluding 200 mg Fe^{3+} L^{-1}; Pearson,

r_{39}=+0.28, ~~P~~p=0.09; Fig. 2a). On average the Φ_{II} was 0.589±0.008 in the lowest Fe^{3+} concentrations (mean±se at < 10 mg Fe^{3+} L^{-1}) and 0.609±0.008 at the higher Fe^{3+} concentrations (mean±se at 20-80 mg Fe^{3+} L^{-1}). The contrasting trend might be related to the fact that all iron salts used to prepare media were contaminated with Pi, resulting in a positive correlation between total Fe and Pi concentrations. Pi concentrations in the Pi-limited media were a 20-fold higher in the media containing the highest total iron concentration compared to the lowest one. The minimal medium prepared with $FeCl_3$ as an iron source had higher free ionic Fe^{3+} concentrations (Table A.1) and only incubations in this medium and 800 mg total Fe L^{-1} resulted in a lower Φ_{II} and relative growth rate (triangles in Fig. 2).

When grown in lake water, the Φ_{II} of *C. acidophila* was lower than in the Pi-limited complex and minimal medium (excluding the value at 200 mg Fe^{3+} L^{-1}) suggesting the presence of additional stress factors in lake water (Table 2 and Fig. 2a). In Pi-enriched lake water the Φ_{II} was enhanced to values above those obtained in Pi-replete complex medium (T-test, t_{11}=-9.2, ~~P~~p<0.001; Fig. 2a), suggesting that Pi-addition compensated for the stress. Accordingly, but less pronounced than in lake water, the Φ_{II} of Pi-replete cells was higher than algae grown in Pi-limited medium (Mann-Whitney U, Z_{85}=-5.5, ~~P~~p<0.001).

At the highest calculated free ionic Fe^{3+} concentration (200 mg Fe^{3+} L^{-1}) the Φ_{II} in the Pi-limited cells was strongly reduced to 0.190±0.027 (mean±se), whereas in Pi-replete cells at 142 mg Fe^{3+} L^{-1} the Φ_{II} was still 0.562±0.004 (Fig. 2a). This contrasts with the effect on growth rates, which were strongly reduced at the two highest Fe^{3+} concentrations (Fig. 2b).

As expected, in absolute terms and covering all iron concentrations, exponential growth rates of Pi-limited cultures (mean ± se 0.48 ± 0.03 d^{-1}; n=57) were lower than Pi-replete cultures (0.77 ± 0.03 d^{-1}; n=45). This effect was even more pronounced in the lake water incubations (0.23 ± 0.03 d^{-1} and 0.80 ± 0.01 d^{-1} for Pi-limited and Pi-replete, respectively; Table 2). Within the non-treated lake water incubations, the Φ_{II} and growth rate

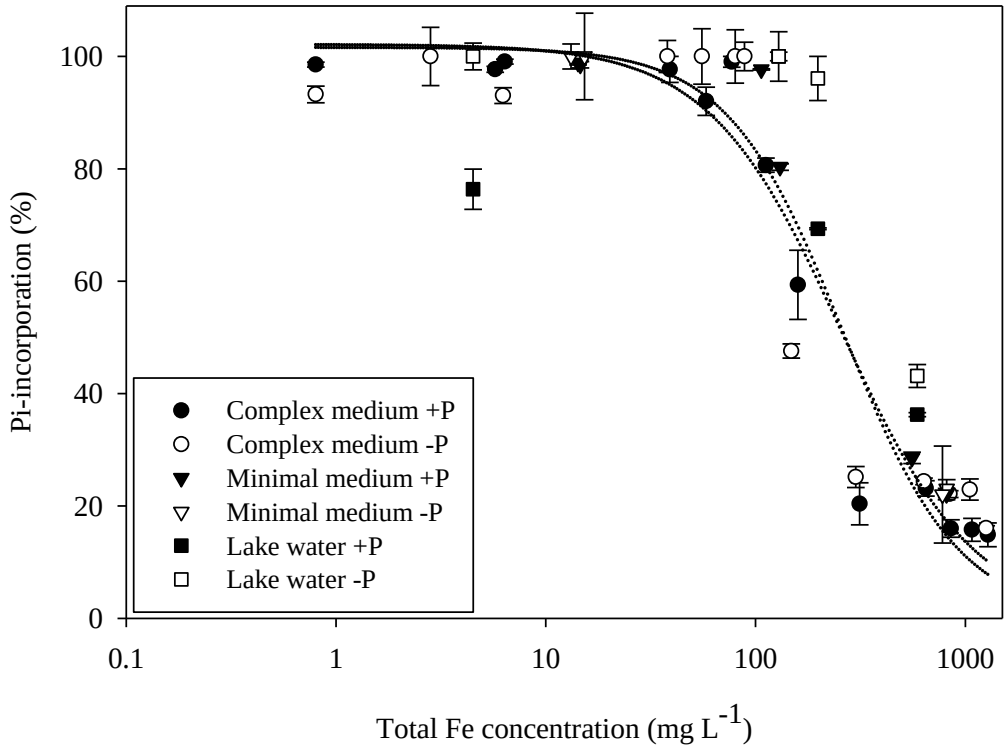
of *C. acidophila* increased in concert with the Pi-concentration and in contrast to the pH of the lake water (see methods section for details and Spijkerman, 2008).

Table 2. Calculated free ionic Fe^{3+} concentrations, measured maximum fluorescence yield of photosystem II (Φ_{II},), and relative and absolute growth rates in *Chlamydomonas acidophila* grown in water from 4 different acidic lakes. The water was either non-treated or enriched with Pi, resulting in Pi-limited and Pi-replete growth conditions, respectively. Mean ± SE of 3 replicate cultures. Calculated free ionic Fe^{3+} concentrations were based on the chemical composition known from Lake 111 (Bissinger et al., 2000) and the measured pH. The growth rates in water from Lake 117 (lowest Fe concentration) were set to 100% for calculation of the relative growth rate.

		non-treated lake water			P-enriched lake water (+ 310 µg P L^{-1})		
Lake	Fe^{3+} (mg L^{-1})	Φ_{II} (rel. units)	Relative growth (%)	Growth rate (d^{-1})	Φ_{II} (rel. units)	Relative growth (%)	Growth rate (d^{-1})
117	0.26	0.35 ± 0.03	100	0.16 ± 0.01	0.68 ± 0.01	100	0.83 ± 0.01
111	6.85	0.29 ± 0.03	115 ± 4	0.18 ± 0.01	0.66 ± 0.02	95 ± 0	0.79 ± 0.00
113	10.40	0.26 ± 0.00	119 ± 9	0.19 ± 0.01	0.68 ± 0.01	95 ± 1	0.79 ± 0.01
107	33.18	0.54 ± 0.03	213 ± 2	0.34 ± 0.00	0.68 ± 0.00	95 ± 1	0.78 ± 0.01

307 The Pi-incorporation decreased with total Fe concentrations in medium and lake water
308 (Fig. 3), suggesting that at higher iron concentrations, Pi-availability and consequently Pi-
309 uptake decreased. The EC_{50} of total Fe concentration in all media and lake water on Pi-
310 incorporation was 260 mg Fe L^{-1} for cells grown in either Pi concentration. The EC_{50} of free
311 ionic Fe^{3+} concentration in the medium on Pi-incorporation (calculated without the values
312 from the lake water incubations) was 15 mg Fe^{3+} L^{-1}. Calculations of phosphorus and iron
313 speciation in MINTEQ revealed that Pi-complexation to Fe increased strongly with increasing
314 total iron concentrations, but that the speciation was the same under Pi-replete and Pi-limited
315 conditions (Table A.1). While in low iron treatments $H_2PO_4^-$ made up more than 70% of total
316 phosphorus, 90% of Pi was in complex with iron in treatments with > 200 mg total Fe L^{-1}
317 (Table A.1). Consequently, the contamination of the Fe-salts that resulted in a 20-fold higher
318 Pi concentration in the Pi-limiting medium at the highest Fe-concentrations did only result in
319 a higher total Pi concentration which was only partly bio-available (~10%). The applied 5-day
320 growth period did not result in a pronounced decrease in cellular P content under Pi-replete
321 conditions (Fig. 1b) because the depletion of stored P requires more cell divisions and
322 consequently a prolonged exposure (another 3 to 7.5 days if stored P supports 6 cell
323 divisions).
324

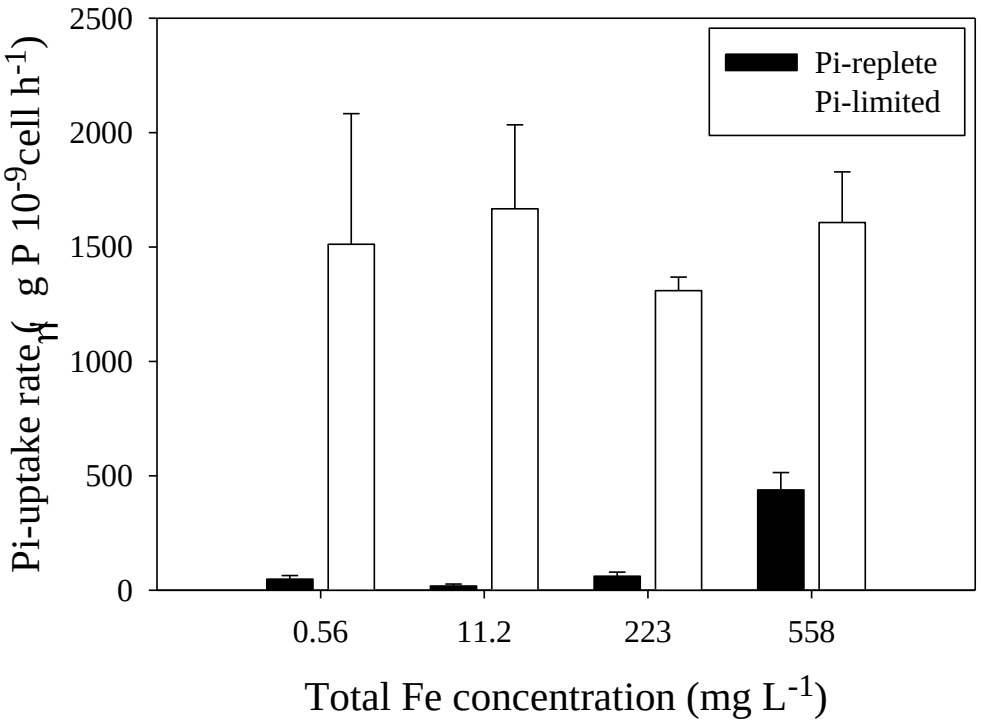
Figure 3. Phosphorus incorporation in *Chlamydomonas acidophila* grown in Pi-replete (310 µg P L^{-1}: +P) or Pi-limited (non-treated or 5 µg P L^{-1}: -P) medium or lake water in relation to the total Fe concentration in medium or lake water. Values are given in percent of the maximum Pi-incorporation measured at the lowest Fe concentration. Symbols as in Fig. 1. The lines illustrate the non-linear fit of equation 1 to the +P and -P data separately. Please note the logarithmic scale on x-axis.



Maximum ~~Pi~~ Pi-uptake as measured by ^{33}P incorporation in semi-continuous cultures was highest under Pi-limited growth conditions and independent of medium iron concentrations (Kruskall-Wallis, p=0.87, Fig. 4). The maximum Pi-uptake rates reflect the cellular Pi-demand, i.e. the rate increases under Pi-limiting conditions but may not be realised under *in situ* conditions. The maximum Pi-uptake rates under Pi-replete conditions were at least 3-fold lower than those of Pi-limited cultures (Kruskall-Wallis, p<0.001). Among Pi-replete cultures, maximum Pi-uptake rates were enhanced in the cells cultured in the medium containing most iron (Dunnett-T3 Post-hoc p<0.05; Fig. 4). This suggests that high total iron

concentrations inhibited active Pi-acquisition which underlies a decreased Pi-incorporation even when medium Pi-concentrations are sufficiently high (i.e. 1.55 mg P L^{-1}).

Figure 4. Maximum phosphorus uptake rates of *Chlamydomonas acidophila* grown in Pi-replete (1.55 mg P L^{-1}) or Pi-limited (31 µg P L^{-1}) semi-continuous cultures at 0.35 d^{-1} in relation to the total Fe concentration in medium. Rates were measured after an addition of a saturating Pi-concentration of 150 µg P L^{-1}. Values are mean ± SD of at least 3 cultures.



Discussion

The important primary producer in iron-rich acidic lakes, *Chlamydomonas acidophila*, accumulated significant cellular iron concentrations, but tolerated these in its photosynthesis and growth response. Only free ionic Fe^{3+} concentrations exceeding 100 mg Fe L^{-1}, that have not been found in extremely iron-rich waters, inhibited its growth. Our results strongly suggest that former effects showing inhibition of growth by ferric iron (Rowe et al., 2007; Spijkerman et al., 2007a) were a result of an indirect effect of the high iron concentration: a Pi-limitation caused by a decreased Pi-incorporation (see below). Although our study is rather specific towards species living in acidic mining lakes, Pi-starvation in the plant *Arabidopsis thaliana* resulted in a down-regulation of proteins potentially involved in iron homeostasis (Chevalier and Rossignol, 2011) and results might thus apply to other plants and systems.

We expected that the cellular iron content in *C. acidophila* would be enhanced as compared with other algae and phytoplankton. Indeed, Fe:P ratios in *C. acidophila* ranged between 8 and 42,000 (mmol Fe:mol P), whereas in some species of marine phytoplankton from the taxonomic green plastid lineage the Fe:P ratio only ranged between 16 and 55, with the highest value being 110 (Quigg et al., 2011). In another study on coastal phytoplankton a range in Fe:P ratio of 0.8 to 10 was described (Brand, 1991) and 7.5 was reported as an average in another study on oceanic phytoplankton (Quigg et al., 2003). In laboratory studies, the ratio in the marine diatom *Thalassiosira weissflogii* was 29 (Price, 2005), and in a freshwater *Chlorella* it ranged between 30 and 330 (Ji and Sherrell, 2008). This suggests that under the lowest total iron concentrations tested here, *C. acidophila* already had a moderate to high Fe:P ratio, and under high iron concentrations it accumulated iron to values much higher than its green algal relatives. Our values are also higher than those of cyanobacteria listed in the recent study of Quigg et al. (2011) that have Fe:P ratios up to 125, and which are considered high because of their evolutionary origin from anoxic oceans rich in bio-available iron, and their high iron demand necessary for N_2-fixation. Another example for ancient

adaptation to Fe-rich environments is provided in the extremophile archae *Ferroplasma acidiphilum* proteome that contains a uniquely high proportion of iron containing proteins (86% of total protein) that might contribute to the pH stability of enzymes at low pH (Ferrer et al., 2007). Unfortunately, no Fe:P ratios were reported for this organism.

More comparisons with other algae can be made if we consider the Fe:C ratio, that predominantly varied between 0.01 and 5 mmol Fe:mol C in this study (90% of the values). In a *C. acidophila* isolate from Rio Tinto, the intracellular concentration of iron was maximal 2.6 % of dry weight under high iron treatment (Garbayo et al., 2007). In contrast, in 'high iron-exposed' *C. reinhardtii* (that is, 1.7 mg Fe L^{-1}) the corresponding value of iron to dry weight was only 0.3-0.5 (Semin et al., 2003). Assuming 50% of dry weight consists of carbon (Spijkerman, 2007), these values are 11 mmol Fe:mol C for *C. acidophila* (Rio Tinto isolate; Garbayo et al., 2007) and 1-2 for *C. reinhardtii* (Semin et al., 2003). Both values fall into the range of values found for our *C. acidophila* isolate. Logically, the Fe:C ratio was lower in oceanic (Fe-limited) phytoplankton, where values varied between 0.01 and 0.14 mmol Fe:mol C (Tovar-Sanchez et al., 2003). Possibly, acidophilic green algae still contain ancient adaptations reflected in a relatively high Fe content. Five exceptionally high Fe:C ratios (17, 27, 53, 102 and 134 mmol Fe:mol C) from our study even approach values reported in the filamentous green alga *Mougeotia* sp. that accumulated iron to 25% of its dry weight in Australian acid waters (John, 2003), and the acidophilic euglenophyte, *Euglena* sp. that accumulated Fe up to 40-60% of its dry weight (Mann et al., 1987). Confirming our expectation, Fe:C ratios in *C. acidophila* were relatively high.

High concentrations of dissolved iron are a characteristic of many very acidic lakes (Herzsprung et al., 1998). Of the two chemical free ionic iron species, free ionic Fe^{2+} iron is considered the most toxic (De Dorlodot et al., 2005; Hanikenne et al., 2005), but little is known about the toxicity of free ionic Fe^{3+} iron which is the dominant free ionic iron species in the oxidized layers of very acidic waters (Herzsprung et al., 1998). A toxic effect of free

ionic iron has been revealed in many plants and algae: e.g. in rice, concentrations of 125 mg Fe^{2+} L^{-1} were toxic (De Dorlodot et al., 2005) and in *Chlamydomonas reinhardtii* concentrations above 14 mg Fe^{2+} L^{-1} reduced growth and resulted in signs of chlorosis (Hanikenne et al., 2005). Chlorosis, decreased chlorophyll content or decreased photosynthetic rates were often found in response to toxic metal exposure (e.g. Krupa and Baszynski, 1995). Lower photosynthetic rates in lakes with high iron concentrations compared to lakes with lower iron concentrations (Kamjunke et al., 2005) therefore suggested inhibiting effects of iron, but our results show that the photosynthetic yield in the main photoautotroph, *C. acidophila* was largely unaffected by high iron. Similarly, hyperaccumulating plants were often unaffected in their photosynthesis as they enhanced production of proteins involved in photosynthesis and had more efficient protein turnover (DalCorso et al., 2013). In addition, super oxide dismutase activity, as an indicator of oxidative stress (Janknegt et al., 2007), was the same in all treatments with $Fe_2(SO_4)_3$ as an iron salt (Table A.3~~S2~~), suggesting that high iron itself did not cause oxidative stress. The values of super oxide dismutase activity might be in general enhanced in acidophiles as genes involved in surviving harmful reactive oxygen substances were constitutively overexpressed in *Dunaliella acidophila* originating from the very acidic, metal-rich Rio Tinto (Puente-Sanchez et al., 2016).

Chemical iron speciation was largely similar in our complex and minimal media when $Fe_2(SO_4)_3$ was used as an iron salt (Table A.~~S~~1). In contrast, the free ionic Fe^{3+} concentrations were more than 3-fold higher when using $FeCl_3$ rather than $Fe_2(SO_4)_3$ as an iron salt in the minimal medium. In a chemical study, the two salts also largely differed in their iron speciation as 40% of total Fe consisted of free ionic Fe^{3+} in a solution of $FeCl_3$, whereas this was less than 1% in a $Fe_2(SO_4)_3$ solution at the same pH (2.3) and temperature (25 °C) (Welham et al., 2000). Only at high free ionic Fe^{3+} concentrations, we observed a strong inhibition in photosynthesis and growth in *C. acidophila* exposed to a medium with $FeCl_3$ as

429 an iron salt. In accordance, an inhibition of growth was previously observed by applying 840
430 mg total Fe L^{-1} (as $FeCl_3$) to a culture of *C. acidophila* (Spijkerman et al., 2007a). As
431 $Fe_2(SO_4)_3$ rather than $FeCl_3$ is likely to dominate in the acidic lakes (Bissinger et al., 2000), *in*
432 *situ* free ionic Fe^{3+} concentrations will be relatively low. High free ionic Fe^{3+} concentrations
433 thus only influenced the ecophysiology of *C. acidophila* when present at concentrations
434 higher than known for natural habitats.

435 *In situ* measurements in acidic lakes revealed that dissolved Fe:P ratios exceeding
436 100:1 resulted in an iron-coating on filamentous green algae (Kleeberg and Gruneberg, 2005),
437 which was speculated to result in Pi-limited growth of the plant. An enhanced Fe-adsorption
438 compared with its cellular Fe content was however not observed in *C. acidophila* and values
439 were on average 38 % with no clear trend over medium Fe:P ratios (Fig. A.1). Hence, our
440 observed Pi-limitation in *C. acidophila* was not caused by Fe-adsorption at high iron
441 concentrations. At total iron concentrations > 280 mg Fe L^{-1} Pi incorporation was inhibited by
442 > 50% which pattern coincided with a change in Pi speciation, although the total iron
443 concentration at which 50% of Pi was in complex with iron was only 12.6 mg Fe L^{-1} (Fig
444 A.3). Chemical speciation reveals that at low total iron concentrations $H_2PO_4^-$ is the dominant
445 Pi-species, whereas at high total iron $FeHPO_4^+$ predominates (Table A.1). At high total iron
446 concentrations Pi-incorporation was strongly reduced and maximum Pi-uptake capacity was
447 enhanced (Figs 3,4). This coincides with the observation that acidophile Pi-transporters used
448 $H_2PO_4^-$ (Hirsch et al., 1993) (which is available at low Fe), whereas those of neutrophile algae
449 use HPO_4^{2-} (Pedersen et al., 2013). Unfavourable chemical Pi-speciation therefore brings
450 about a Pi-limitation in acidophiles at high iron, and calculations in Visual MINTEQ software
451 further revealed that an acidification event will slightly decrease the iron-phosphate
452 complexation that could weaken this Pi-limitation. However, in nature Pi-limited conditions
453 might be mitigated as *C. acidophila* expresses phosphatase enzymes (Boavida and Heath,

1986; Lachmann et al., 2017; Spijkerman et al., 2007b) that enable the use of organic Pi-sources.

High iron concentrations effectively decreased Pi-incorporation, and 260 mg total Fe L^{-1} resulted in an inhibition by 50%. Consequently, an important result of this study is that high iron concentrations promote Pi-limitation. A Pi-limitation in the natural phytoplankton of all four lakes under study had already been revealed, although the Pi concentrations in lake water (ranging between 8 and 26 µg P L^{-1}) did not directly suggest the presence of a Pi-limitation (Spijkerman, 2008). Similarly, high phosphatase activities that were measured in the benthic algal community of Rio Tinto, despite high external Pi concentrations (ranging between 0.2 and 3.2 mg P L^{-1}!) also suggest a physiological acclimation to Pi-limitation (Sabater et al., 2003). In addition, observations that growth of a *Chlamydomonas* isolate from Rio Tinto was completely inhibited by a ferric iron concentration of 280 mg total Fe L^{-1} (Rowe et al., 2007) might also have resulted from halved Pi-incorporation. Low Pi availability due to Fe-P-complexation likely dominates the algal physiology in acidic lakes, and Pi-limitation resulting from high iron can explain the low primary production observed in Fe-rich, acidic lakes (Nixdorf et al., 2003).

Acknowledgements

Stefanie Branscheid is acknowledged for her contribution to this study as part of her bachelor degree. Dr. Bukowsky determined the total iron concentrations. Barbara Schmitz assisted during the practical work. ES acknowledges the DFG for financial support (SP695/2, SP695/4 and SP695/5). The authors have no competing interests to declare.

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Decreased phosphorus incorporation explains the negative effect of high iron concentrations in the green microalga *Chlamydomonas acidophila*

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Running head: High iron decreases phosphate-incorporation

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Abstract

The green microalga *Chlamydomonas acidophila* is an important primary producer in very acidic lakes (pH 2.0-3.5), characterized by high concentrations of ferric iron (up to 1 g total Fe L^{-1}) and low rates of primary production. It was previously suggested that these high iron concentrations result in high iron accumulation and inhibit photosynthesis in *C. acidophila.* To test this, the alga was grown in sterilized lake water and in medium with varying total iron concentrations under limiting and sufficient inorganic phosphorus (Pi) supply, because Pi is an important growth limiting nutrient in acidic waters. Photosynthesis and growth of *C. acidophila* as measured over 5 days were largely unaffected by high total iron concentrations and only decreased if free ionic Fe^{3+} concentrations exceeded 100 mg Fe^{3+} L^{-1}. Although *C. acidophila* was relatively rich in iron (up to 5 mmol Fe: mol C), we found no evidence of iron toxicity. In contrast, a concentration of 260 mg total Fe L^{-1} (i.e. 15 mg free ionic Fe^{3+} L^{-1}), which is common in many acidic lakes, reduced Pi-incorporation by 50% and will result in Pi-

limited photosynthesis. The resulting Pi-limitation present at high iron and Pi concentrations was illustrated by elevated maximum Pi-uptake rates. No direct toxic effects of high iron were found, but unfavourable chemical Pi-speciation reduced growth of the acidophile alga.

Key words: Chlamydomonas, ecotoxicology, extreme environment, iron toxicity, phosphate limitation, phytoplankton

1. **Introduction**

Many very acid lakes and rivers are not only characterized by a low pH ranging between 2.0 and 3.5, but also by high concentrations of ferrous iron and sulphuric acid resulting from the weathering of rock such as pyrite and marcasite (iron-sulphur minerals) (Friese et al., 1998). Once oxidized, ferric iron hydroxide acts as a buffering substance and dominates the iron speciation constituent (Herzsprung et al., 1998). Understanding these iron-rich systems is of interest because they are found world-wide, and their chemical composition may threaten human health (e.g. the Kawah ljen crater lake in East Java; Lohr et al., 2006), whereas its extremophile inhabitants are used in a variety of biotechnological applications (Bell, 2012). In this contribution we unravel if high iron concentrations affect the ecophysiology of an important microalga inhabiting acid lakes: *Chlamydomonas acidophila*.

Iron is a redox-active heavy metal and therefore potentially toxic for organisms. It exists in different chemical forms and some of them, such as free ions of Fe^{2+} are highly toxic (Goshal et al., 2005). In the upper phototrophic layer of acidic lakes and rivers, concentrations of total iron can be high (up to 1000 mg Fe L^{-1}). However, the iron is mainly present as ferric iron (Herzsprung et al., 1998), from which a variable fraction is the potentially toxic, free ionic Fe^{3+}. It is known that most acidophilic algae are remarkably tolerant towards heavy metals (Whitton, 1970), possibly resulting from their positive charged membrane necessary to tolerate high proton concentrations. In the neutrophile *Chlamydomonas reinhardtii* high

proton concentrations decreased metal uptake by non-competitive inhibition (Francois et al., 2007; Macfie et al., 1994), suggesting that iron accumulation might be restricted in *C. acidophila*.

Nonetheless, iron is considered to inhibit algal growth when concentrations are extremely high (Aguilera et al., 2006; Gross, 2000), for example in Rio Tinto, total iron concentrations can reach values over 2 g Fe L^{-1} (Lopez-Archilla et al., 2001). High iron concentrations can cause bronzing symptoms in higher plants, which are mainly caused by oxidative stress or an antagonism between magnesium and iron in forming the chlorophyll prophyrin group (De Dorlodot et al., 2005). As a consequence, high iron concentrations affect the chlorophyll content and thereby primary productivity. Comparing the primary production of three acidic lakes confirmed that the two lakes with the higher iron concentrations had the lower primary production (Kamjunke et al., 2005). Moreover, *C. acidophila* cultured in water from Rio Tinto had a low Ribulose-1,5-bisphosphate carboxylase oxygenase content (Cid et al., 2010), and the algal natural abundance was negatively correlated with dissolved metal concentrations in the river (Aguilera et al., 2006). In laboratory experiments, ferric iron concentrations of 280 mg total Fe L^{-1} totally inhibited growth of a *Chlamydomonas* isolate from Rio Tinto (Rowe et al., 2007).

Metal tolerance is often connected with the storage of metals in polyphosphate bodies, and as a result, increased Pi availability often enhanced metal accumulation but mitigated its toxicity (Wang and Dei, 2006). In accordance, Pi-limiting conditions enhanced metal toxicity in micro-phytobenthos (Ivorra et al., 2002). Although most acidic environments contain relatively high concentrations of Pi, algal growth was often Pi-limited (Sabater et al., 2003; Spijkerman, 2008), where the mechanistic reasons remained unclear because acidic lakes have high concentrations of many metal ions (Friese et al., 1998). In addition, high concentrations of metals may inhibit nutrient uptake and may have indirect effects on the biota by their precipitation with nutrients. It has been suggested that a coating of algae with Fe-hydroxides

79 with an atomic Fe : P ratio higher than 100:1 will adsorb phosphorus efficiently and will
80 promote a Pi-limitation (Kleeberg and Gruneberg, 2005).
81 We elucidate the effect of high iron concentrations on photosynthesis and growth of *C.*
82 *acidophila* under variable iron and Pi concentrations. In contrast to expectations, we show that
83 observed negative effects of high iron concentrations on primary production (Kamjunke et al.,
84 2005) are not directly related to toxicity but result from decreased Pi incorporation and
85 consequently result from a Pi-limitation.
86
87 **Experimental section**
88 ***Culturing.*** To test for the effect of high iron concentrations, experiments with lake water and
89 different media were performed. For the effect of naturally dissolved iron, lake water was
90 sampled from the upper meter of the water column in four acidic lakes, situated in
91 Brandenburg, Germany (Lakes 107, 111, 113 and 117; 51° 29' N, 13° 38' E) on October 19,
92 2006. Total iron concentrations were 590, 200, 130 and 5 mg Fe L^{-1} for Lakes 107, 113, 111
93 and 117, respectively, and consisted for over 90% of ferric iron (Spijkerman, 2008). The pH
94 and Pi concentrations in the lake water were 2.3, 2.6, 2.7 and 3.0 and 26, 15, 12 and 8 µg P L^{-1}
95 for Lakes 107, 113, 111 and 117, respectively (Spijkerman, 2008). Water was filter-sterilized
96 through a 0.2 µm cellulose acetate filter (Sartorius, Göttingen, Germany) directly after
97 sampling. Pi-replete lake water was obtained by addition of 310 µg Pi L^{-1} as phosphoric acid.
98 The Pi-addition did not result in a visual precipitation or in a pH change.
99 To study the effect of different iron concentrations, culturing was done in filter-
100 sterilized media. We used a chemically complex medium reflecting the chemical composition
101 of the lake water and applied $Fe_2(SO_4)_3$ as the sole iron-source (Bissinger et al., 2000;
102 Spijkerman, 2011). In addition we used a minimal medium (Gerloff-Elias et al., 2005) where
103 we applied either $Fe_2(SO_4)_3$ to compare with the complex medium or $FeCl_3$ to vary free ionic
104 Fe^{3+} concentrations (Appendix Table A.1). In both media the total iron concentrations varied

between 1 and 1200 mg Fe L^{-1} (see Table 1 for a detailed composition of the main ions in both media). In the minimal medium the pH was kept constant at 2.36 which enabled total dissolving of all salts, whereas it naturally varied in the complex medium between 2.04 at the highest Fe concentration and 2.65 in media with $\leq$ 100 mg Fe L^{-1}, to enable a comparison with the lake water treatment (where neither pH adjustment nor additions of iron were made). Chemical speciation analysis of the complex medium in Visual MINTEQ software (2007) suggested that inorganic Fe-P complexation increased with medium iron concentration (Table. A.1 & A.2).

The unicellular green alga *Chlamydomonas acidophila* Negoro (SAG strain no. 2045), isolated from Lake 111 (Gerloff-Elias et al., 2005) and maintained in a complex medium at 145 mg Fe L^{-1} for eight years, was used in all experiments. To adjust to Pi conditions, *C. acidophila* was grown in Pi-limiting (i.e. 5 μg Pi L^{-1}) or Pi-replete (i.e. 310 μg Pi L^{-1}) medium containing a moderate Fe concentration (145 mg Fe L^{-1}) for eight days. This was followed by a pre-acclimation period of four days to experimental Pi and Fe concentrations or lake water. The subsequent growth experiments ran over a 5-day period, starting with 1-2 10^7 cells L^{-1}. By applying this short time-period and low starting cell density, we aimed for constant Pi-conditions during the experiment. Cultures were illuminated for 16 h per day (TLD 58W/930, Philips) at 150 μmol photons m^{-2} s^{-1} measured inside the culture flask (4π quantum sensor US-SQS, Walz, Effeltrich, Germany). Temperature was kept at 20° C ± 2. Before transfer into new medium or lake water, cells were washed once by centrifugation (1,500·*g*, 5 min) with the experimental water.

Growth rate and chemical measurements. Daily measurements of optical density (OD at 750 nm, UV-2401 PC; Shimadzu, Berlin, Germany) were done in a 5 cm cuvette over a 5-day period. A linear model was fitted to the natural logarithm of OD values over time to calculate the exponential growth rates. To standardize for differences in culturing conditions

131 (Pi concentration and/or medium or lake water use), the growth rate in the low iron treatment
132 (1 or 10 mg Fe L^{-1}) from every series of experiments was considered as a control and set to
133 100%. Growth rates at higher iron concentrations were related to this control. The low cell
134 densities did not influence the medium iron concentrations over the 5-day growth period.
135 All chemical and activity measurements were done on algal suspensions from day 5, at
136 the end of the growth experiment. We measured cellular phosphorus (P), carbon (C) and iron
137 (Fe) content to obtain the elemental stoichiometry. For cell enumeration, samples were fixed
138 with acidified Lugol's solution, and counted according to the method of Utermöhl using an
139 inverted light microscope (Thalheim-Spezial-Optik TSO, Pulsnitz, Germany) and
140 sedimentation chambers (Thalheim, Pulsnitz, Germany). At least 600 cells and 2 transects of
141 the chamber were counted.
142 The cellular P content was determined in cells harvested on acid-drained nucleopore
143 filters (Track-Etch; Whatman, Göttingen, Germany), rinsed with de-ionized water. Particulate
144 P was macerated by autoclaving together with $K_2S_2O_8$ at low pH, reduced to form a
145 phosphate-molybdate complex and measured at 880 nm on a spectrophotometer (Murphy and
146 Riley, 1962). The residual Pi concentration in the medium was determined with the same
147 method, but without maceration, in the supernatant of centrifuged culture suspension (see
148 cellular Fe content). The Pi-incorporation was calculated as the ratio between the cellular P
149 and the sum of cellular and residual Pi concentration multiplied by 100%. If residual Pi
150 concentrations were below the detection limit we assumed that all Pi was taken up. The Pi-
151 incorporation was used to calculate the medium iron concentration at which 50 % of Pi was
152 incorporated (EC_{50}) by use of the model from Haanstra et al. (Haanstra et al., 1985) in
153 SigmaPlot (version 11.0):

$$Pi - incorporation\ (\%) = \frac{c}{1 + e^{(b \times (\log [\mathrm{Fe-concentration}] - a)}}$$

154 in which c = Pi-incorporation at lowest Fe concentration (~ 100%), b =slope and a = log EC_{50}.

To determine the cellular C content, cells were harvested on pre-combusted (4 h at 450 °C) glass fiber filters and measured after drying at 50°C in a HighTOC+N gas analyzer (HighTOC+N; Elementar Analysensysteme, Hanau, Germany).

To measure the cellular Fe content, the culture suspension was centrifuged (2,500·*g*, 10 min) and the supernatant was used for the determination of medium iron and residual Pi concentration. The pellet washed twice with de-ionized water and incubated with 0.5 ml 20mM K_2EDTA (pH 2.7) for 10 min (Bates et al., 1982). After centrifugation (12,000·*g*, 5 min, the supernatant (extra-cellular Fe) and the pellet (intra-cellular Fe) were separated and lyophilized. Samples were extracted with 0.2 ml HNO_3 (69%, trace element Sigma, München, Germany), dried in a thermo-block under a fume head at 80°C overnight and then extracted with 0.2 ml of H_2O_2 (30%) and dried again at 80° C overnight. Finally, the pellet was dissolved in acidified water (1.5% HNO_3), and liquid samples of medium and lake water were acidified in a similar way. Iron concentrations were measured by atomic absorption spectroscopy (AAS1100B, Perkin Elmer).

The total Fe content (i.e. the sum of the intra- and extra-cellular fraction) was used in the calculation of cellular ratios. Concentrations of ferrous iron in the medium were determined using ferrozine reagent (Lovley and Phillips, 1987), and did not exceed 7% of total iron concentrations in lake water and medium. Iron and phosphorus complexation and free ionic Fe^{3+} concentrations in the different media were calculated with Visual MINTEQ software (including the full ionic composition and pH; the full ionic speciation of selected media is provided in Table A.2). Because the complete ionic composition of the lake water is unknown, free ionic Fe^{3+} concentrations in the lake waters were estimated based on the chemical composition of the complex medium and the measured pH. The chemical composition of the complex medium resembles that of Lake 111 but omits the aluminum concentration (Bissinger et al., 2000).

Chlorophyll a fluorescence transient. As a measure of maximum photosynthetic rate chlorophyll a fluorescence emission transients were determined in a double-modulation fluorometer (Dual-Modulation Kinetic Fluorometer FL-3000 with APD cuvette, PSI Photon Systems Instruments, Brno, Czech Republic). Dark adapted (for 30 min: minimal fluorescence, F_0) cells were exposed to saturating light (1 sec, 2100 µmol m^{-2} s^{-1}) and the maximum fluorescence (F_m) was measured. From these values, the maximum fluorescence yield of photosystem II (Φ_{II}, $(F_m-F_0)/F_m$, also known as F_v/F_m) was calculated.

Pi-uptake rates. For this measurement, *C. acidophila* was grown in semi-continuously diluted cultures (μ= 0.35 d^{-1}) acclimated to four medium total iron concentrations (0.6, 11, 223 and 558 mg Fe L^{-1}) at Pi-replete (1.55 mg P L^{-1}) and Pi-limited (31 µg P L^{-1}) conditions at pH 2.4. These Pi-concentrations resulted in Pi-replete and Pi-limited conditions for this alga previously (Spijkerman et al., 2007b). Cells were centrifuged (1,500·*g*, 5 min), the pellet washed once with medium lacking iron and Pi, and subsequently resuspended in a Pi and Fe-free medium reaching a density of 2 10^8 cells L^{-1}. The culture was placed in the light (approx. 90 µmol m^{-2} s^{-1} inside the flask) for about 15-30 minutes. Over a period of 1 (Pi-limited) or 5 (Pi-replete) minutes, Pi uptake was measured by addition of $H_3{}^{33}PO_4$ (565 TBq $mmol^{-1}$ specific activity, Perkin Elmer) diluted in a stock solution of 31 mg P L^{-1} adjusted to pH 2.4, resulting in a final concentration of 150 µg P L^{-1}. At this concentration, Pi-uptake is maximal (Spijkerman, 2007). Uptake was terminated by filtration on 1.2 µm pore-size cellulose acetate filter and subsequent rinsing with 0.2 M LiCl. The filters were embedded in Ultima Gold (Packard) and counted in a liquid scintillation analyser (Tri-Carb 2810 Tr, Perkin Elmer). Part of the diluted culture was fixed with 0.2% Lugol's solution (f.c.) for cell enumeration. Cell numbers were determined as described above.

Statistics. All cultures were run in triplicate. Statistical analyses were performed in SPSS 20.0. When providing statistical test results, the degrees of freedom, or sample size are given as an index number.

206

207 **Results**

208 Although we expected a low iron accumulation into cells of *C. acidophila*, the cellular
209 Fe content increased with increasing free ionic Fe^{3+} concentrations (Pearson, r_{36}=0.66,
210 p<0.01; Fig. 1a), both in Pi-limited and in Pi-replete grown cells, but with a large scatter. The
211 cellular Fe content predominantly varied between 0.02 and 1.07 pg Fe $cell^{-1}$, with higher
212 values in three Pi-replete lake water incubations and in the two cultures with the highest free
213 ionic Fe^{3+} concentrations (that resulted in 15 and 38 pg Fe $cell^{-1}$). In the latter 2 treatments the
214 total iron concentration was 800 mg Fe L^{-1}, and only the medium Pi concentration differed.
215 The cellular P-analysis resolved that the most Fe-rich cells had an intermediate cellular P
216 content (Fig. 1b), similar to cells grown in Pi-replete medium or in untreated lake water. Also
217 the Pi-limited, high iron exposed cells grown in minimal medium had an intermediate cellular
218 P content.

219 The cellular Fe:P and Fe:C ratios increased in Pi-limited *C. acidophila* with increasing
220 free ionic Fe^{3+} concentration (Pearson, , r_{25}>0.69, p<0.01), but remained rather constant in Pi-
221 replete cells (Pearson, r_{19}<0.38, p>0.10; Fig. 1c,d). Pi-limitation did not influence the mean
222 accumulation of iron to P in the cells as the mean Fe:P ratio was similar in the Pi-limited
223 (2750±1750, mean±se) and Pi-replete (1500±1000, mean±se) cultures (paired t-test based on
224 16 pairs of free ionic Fe^{3+} concentrations, p=0.30; Fig. 1c). The mmol Fe:mol P ratio from
225 low to high free ionic Fe^{3+} concentrations ranged between 8 and 41,500 (Fig. 1c) and the
226 mmol Fe:mol C ratio between 0.014 and 134 (Fig. 1d). When the cellular P content was set to
227 1, the lowest molar C:P ratio was 40:1 under Pi-replete conditions coinciding with the lowest
228 molar Fe:P ratio (0.008:1). Under Pi-limiting conditions the molar C:P ratio was highest
229 (1060:1) as was the Fe:P ratio (42:1).

230 The percentage of adsorbed extra-cellular iron related to the cellular iron content
231 covered a wide range between 6 and 90%, without a clear pattern (Fig. A.1). In contrast to

232 expectations (Kleeberg and Gruneberg, 2005), an external Fe:P ratio >100 did not result in an
233 enhanced Fe adsorption on *C. acidophila*. In contrast, in lake water and under both Pi-
234 conditions Fe adsorption was negatively related to external Fe:P ratio (Pearson, $r_{15}>0.62$
235 (n=15), $p<0.05$ in both cases).
236
237 We expected that high external and internal iron concentrations would have a negative
238 effect on the photosynthesis, which we measured by maximum fluorescence yield of
239 photosystem II (Φ_{II}) and of growth of *C. acidophila*. Against expectations, no correlations
240 were detected when considering the total iron concentration (Fig. A.2), and Φ_{II} was only
241 weakly negatively correlated with calculated free ionic Fe^{3+} medium concentrations in Pi-
242 replete cells (excluding Φ_{II} at >100 mg Fe^{3+} L^{-1} Pearson, r_{42} =-0.31, $p<0.05$; Fig. 2a). The Φ_{II}
243 was 0.644±0.004 at lower Fe^{3+} concentrations (mean±se at < 10 mg Fe^{3+} L^{-1}) and 0.628±0.005
244 at higher Fe^{3+} concentrations (mean±se at > 30 mg Fe^{3+} L^{-1}). A contrasting trend was found if
245 the cells were grown in Pi-limited medium (excluding 200 mg Fe^{3+} L^{-1}; Pearson, r_{39}=+0.28,
246 p=0.09; Fig. 2a). On average the Φ_{II} was 0.589±0.008 in the lowest Fe^{3+} concentrations
247 (mean±se at < 10 mg Fe^{3+} L^{-1}) and 0.609±0.008 at the higher Fe^{3+} concentrations (mean±se at
248 20-80 mg Fe^{3+} L^{-1}). The contrasting trend might be related to the fact that all iron salts used to
249 prepare media were contaminated with Pi, resulting in a positive correlation between total Fe
250 and Pi concentrations. Pi concentrations in the Pi-limited media were a 20-fold higher in the
251 media containing the highest total iron concentration compared to the lowest one. The
252 minimal medium prepared with $FeCl_3$ as an iron source had higher free ionic Fe^{3+}
253 concentrations (Table A.1) and only incubations in this medium and 800 mg total Fe L^{-1}
254 resulted in a lower Φ_{II} and relative growth rate (triangles in Fig. 2).
255 When grown in lake water, the Φ_{II} of *C. acidophila* was lower than in the Pi-limited
256 complex and minimal medium (excluding the value at 200 mg Fe^{3+} L^{-1}) suggesting the
257 presence of additional stress factors in lake water (Table 2 and Fig. 2a). In Pi-enriched lake

water the Φ_{II} was enhanced to values above those obtained in Pi-replete complex medium (T-test, t_{11}=-9.2, p<0.001; Fig. 2a), suggesting that Pi-addition compensated for the stress. Accordingly, but less pronounced than in lake water, the Φ_{II} of Pi-replete cells was higher than algae grown in Pi-limited medium (Mann-Whitney U, Z_{85}=-5.5, p<0.001).

At the highest calculated free ionic Fe^{3+} concentration (200 mg Fe^{3+} L^{-1}) the Φ_{II} in the Pi-limited cells was strongly reduced to 0.190±0.027 (mean±se), whereas in Pi-replete cells at 142 mg Fe^{3+} L^{-1} the Φ_{II} was still 0.562±0.004 (Fig. 2a). This contrasts with the effect on growth rates, which were strongly reduced at the two highest Fe^{3+} concentrations (Fig. 2b).

As expected, in absolute terms and covering all iron concentrations, exponential growth rates of Pi-limited cultures (mean ± se 0.48 ± 0.03 d^{-1}; n=57) were lower than Pi-replete cultures (0.77 ± 0.03 d^{-1}; n=45). This effect was even more pronounced in the lake water incubations (0.23 ± 0.03 d^{-1} and 0.80 ± 0.01 d^{-1} for Pi-limited and Pi-replete, respectively; Table 2). Within the non-treated lake water incubations, the Φ_{II} and growth rate of *C. acidophila* increased in concert with the Pi-concentration and in contrast to the pH of the lake water (see methods section for details and Spijkerman, 2008).

The Pi-incorporation decreased with total Fe concentrations in medium and lake water (Fig. 3), suggesting that at higher iron concentrations, Pi-availability and consequently Pi-uptake decreased. The EC_{50} of total Fe concentration in all media and lake water on Pi-incorporation was 260 mg Fe L^{-1} for cells grown in either Pi concentration. The EC_{50} of free ionic Fe^{3+} concentration in the medium on Pi-incorporation (calculated without the values from the lake water incubations) was 15 mg Fe^{3+} L^{-1}. Calculations of phosphorus and iron speciation in MINTEQ revealed that Pi-complexation to Fe increased strongly with increasing total iron concentrations, but that the speciation was the same under Pi-replete and Pi-limited conditions (Table A.1). While in low iron treatments $H_2PO_4^-$ made up more than 70% of total phosphorus, 90% of Pi was in complex with iron in treatments with > 200 mg total Fe L^{-1} (Table A.1). Consequently, the contamination of the Fe-salts that resulted in a 20-fold higher

Pi concentration in the Pi-limiting medium at the highest Fe-concentrations did only result in a higher total Pi concentration which was only partly bio-available (~10%). The applied 5-day growth period did not result in a pronounced decrease in cellular P content under Pi-replete conditions (Fig. 1b) because the depletion of stored P requires more cell divisions and consequently a prolonged exposure (another 3 to 7.5 days if stored P supports 6 cell divisions).

Maximum Pi-uptake as measured by ^{33}P incorporation in semi-continuous cultures was highest under Pi-limited growth conditions and independent of medium iron concentrations (Kruskall-Wallis, $p=0.87$, Fig. 4). The maximum Pi-uptake rates reflect the cellular Pi-demand, i.e. the rate increases under Pi-limiting conditions but may not be realised under *in situ* conditions. The maximum Pi-uptake rates under Pi-replete conditions were at least 3-fold lower than those of Pi-limited cultures (Kruskall-Wallis, $p<0.001$). Among Pi-replete cultures, maximum Pi-uptake rates were enhanced in the cells cultured in the medium containing most iron (Dunnett-T3 Post-hoc $p<0.05$; Fig. 4). This suggests that high total iron concentrations inhibited active Pi-acquisition which underlies a decreased Pi-incorporation even when medium Pi-concentrations are sufficiently high (i.e. 1.55 mg P L^{-1}).

Discussion

The important primary producer in iron-rich acidic lakes, *Chlamydomonas acidophila*, accumulated significant cellular iron concentrations, but tolerated these in its photosynthesis and growth response. Only free ionic Fe^{3+} concentrations exceeding 100 mg Fe L^{-1}, that have not been found in extremely iron-rich waters, inhibited its growth. Our results strongly suggest that former effects showing inhibition of growth by ferric iron (Rowe et al., 2007; Spijkerman et al., 2007a) were a result of an indirect effect of the high iron concentration: a Pi-limitation caused by a decreased Pi-incorporation (see below). Although our study is rather

specific towards species living in acidic mining lakes, Pi-starvation in the plant *Arabidopsis thaliana* resulted in a down-regulation of proteins potentially involved in iron homeostasis (Chevalier and Rossignol, 2011) and results might thus apply to other plants and systems.

We expected that the cellular iron content in *C. acidophila* would be enhanced as compared with other algae and phytoplankton. Indeed, Fe:P ratios in *C. acidophila* ranged between 8 and 42,000 (mmol Fe:mol P), whereas in some species of marine phytoplankton from the taxonomic green plastid lineage the Fe:P ratio only ranged between 16 and 55, with the highest value being 110 (Quigg et al., 2011). In another study on coastal phytoplankton a range in Fe:P ratio of 0.8 to 10 was described (Brand, 1991) and 7.5 was reported as an average in another study on oceanic phytoplankton (Quigg et al., 2003). In laboratory studies, the ratio in the marine diatom *Thalassiosira weissflogii* was 29 (Price, 2005), and in a freshwater *Chlorella* it ranged between 30 and 330 (Ji and Sherrell, 2008). This suggests that under the lowest total iron concentrations tested here, *C. acidophila* already had a moderate to high Fe:P ratio, and under high iron concentrations it accumulated iron to values much higher than its green algal relatives. Our values are also higher than those of cyanobacteria listed in the recent study of Quigg et al. (2011) that have Fe:P ratios up to 125, and which are considered high because of their evolutionary origin from anoxic oceans rich in bio-available iron, and their high iron demand necessary for N_2-fixation. Another example for ancient adaptation to Fe-rich environments is provided in the extremophile archae *Ferroplasma acidiphilum* proteome that contains a uniquely high proportion of iron containing proteins (86% of total protein) that might contribute to the pH stability of enzymes at low pH (Ferrer et al., 2007). Unfortunately, no Fe:P ratios were reported for this organism.

More comparisons with other algae can be made if we consider the Fe:C ratio, that predominantly varied between 0.01 and 5 mmol Fe:mol C in this study (90% of the values). In a *C. acidophila* isolate from Rio Tinto, the intracellular concentration of iron was maximal 2.6 % of dry weight under high iron treatment (Garbayo et al., 2007). In contrast, in ‘high iron-

336 exposed' *C. reinhardtii* (that is, 1.7 mg Fe L^{-1}) the corresponding value of iron to dry weight
337 was only 0.3-0.5 (Semin et al., 2003). Assuming 50% of dry weight consists of carbon
338 (Spijkerman, 2007), these values are 11 mmol Fe:mol C for *C. acidophila* (Rio Tinto isolate;
339 Garbayo et al., 2007) and 1-2 for *C. reinhardtii* (Semin et al., 2003). Both values fall into the
340 range of values found for our *C. acidophila* isolate. Logically, the Fe:C ratio was lower in
341 oceanic (Fe-limited) phytoplankton, where values varied between 0.01 and 0.14 mmol Fe:mol
342 C (Tovar-Sanchez et al., 2003). Possibly, acidophilic green algae still contain ancient
343 adaptations reflected in a relatively high Fe content. Five exceptionally high Fe:C ratios (17,
344 27, 53, 102 and 134 mmol Fe:mol C) from our study even approach values reported in the
345 filamentous green alga *Mougeotia* sp. that accumulated iron to 25% of its dry weight in
346 Australian acid waters (John, 2003), and the acidophilic euglenophyte, *Euglena* sp. that
347 accumulated Fe up to 40-60% of its dry weight (Mann et al., 1987). Confirming our
348 expectation, Fe:C ratios in *C. acidophila* were relatively high.
349 High concentrations of dissolved iron are a characteristic of many very acidic lakes
350 (Herzsprung et al., 1998). Of the two chemical free ionic iron species, free ionic Fe^{2+} iron is
351 considered the most toxic (De Dorlodot et al., 2005; Hanikenne et al., 2005), but little is
352 known about the toxicity of free ionic Fe^{3+} iron which is the dominant free ionic iron species
353 in the oxidized layers of very acidic waters (Herzsprung et al., 1998). A toxic effect of free
354 ionic iron has been revealed in many plants and algae: e.g. in rice, concentrations of 125 mg
355 Fe^{2+} L^{-1} were toxic (De Dorlodot et al., 2005) and in *Chlamydomonas reinhardtii*
356 concentrations above 14 mg Fe^{2+} L^{-1} reduced growth and resulted in signs of chlorosis
357 (Hanikenne et al., 2005). Chlorosis, decreased chlorophyll content or decreased
358 photosynthetic rates were often found in response to toxic metal exposure (e.g. Krupa and
359 Baszynski, 1995). Lower photosynthetic rates in lakes with high iron concentrations
360 compared to lakes with lower iron concentrations (Kamjunke et al., 2005) therefore suggested
361 inhibiting effects of iron, but our results show that the photosynthetic yield in the main

362 photoautotroph, *C. acidophila* was largely unaffected by high iron. Similarly,
363 hyperaccumulating plants were often unaffected in their photosynthesis as they enhanced
364 production of proteins involved in photosynthesis and had more efficient protein turnover
365 (DalCorso et al., 2013). In addition, super oxide dismutase activity, as an indicator of
366 oxidative stress (Janknegt et al., 2007), was the same in all treatments with $Fe_2(SO_4)_3$ as an
367 iron salt (Table A.3), suggesting that high iron itself did not cause oxidative stress. The values
368 of super oxide dismutase activity might be in general enhanced in acidophiles as genes
369 involved in surviving harmful reactive oxygen substances were constitutively overexpressed
370 in *Dunaliella acidophila* originating from the very acidic, metal-rich Rio Tinto (Puente-
371 Sanchez et al., 2016).

372 Chemical iron speciation was largely similar in our complex and minimal media when
373 $Fe_2(SO_4)_3$ was used as an iron salt (Table A.1). In contrast, the free ionic Fe^{3+} concentrations
374 were more than 3-fold higher when using $FeCl_3$ rather than $Fe_2(SO_4)_3$ as an iron salt in the
375 minimal medium. In a chemical study, the two salts also largely differed in their iron
376 speciation as 40% of total Fe consisted of free ionic Fe^{3+} in a solution of $FeCl_3$, whereas this
377 was less than 1% in a $Fe_2(SO_4)_3$ solution at the same pH (2.3) and temperature (25 °C)
378 (Welham et al., 2000). Only at high free ionic Fe^{3+} concentrations, we observed a strong
379 inhibition in photosynthesis and growth in *C. acidophila* exposed to a medium with $FeCl_3$ as
380 an iron salt. In accordance, an inhibition of growth was previously observed by applying 840
381 mg total Fe L^{-1} (as $FeCl_3$) to a culture of *C. acidophila* (Spijkerman et al., 2007a). As
382 $Fe_2(SO_4)_3$ rather than $FeCl_3$ is likely to dominate in the acidic lakes (Bissinger et al., 2000), *in*
383 *situ* free ionic Fe^{3+} concentrations will be relatively low. High free ionic Fe^{3+} concentrations
384 thus only influenced the ecophysiology of *C. acidophila* when present at concentrations
385 higher than known for natural habitats.

386 *In situ* measurements in acidic lakes revealed that dissolved Fe:P ratios exceeding
387 100:1 resulted in an iron-coating on filamentous green algae (Kleeberg and Gruneberg, 2005),

388 which was speculated to result in Pi-limited growth of the plant. An enhanced Fe-adsorption
389 compared with its cellular Fe content was however not observed in *C. acidophila* and values
390 were on average 38 % with no clear trend over medium Fe:P ratios (Fig. A.1). Hence, our
391 observed Pi-limitation in *C. acidophila* was not caused by Fe-adsorption at high iron
392 concentrations. At total iron concentrations > 280 mg Fe L^{-1} Pi incorporation was inhibited by
393 > 50% which pattern coincided with a change in Pi speciation, although the total iron
394 concentration at which 50% of Pi was in complex with iron was only 12.6 mg Fe L^{-1} (Fig
395 A.3). Chemical speciation reveals that at low total iron concentrations $H_2PO_4^-$ is the dominant
396 Pi-species, whereas at high total iron $FeHPO_4^+$ predominates (Table A.1). At high total iron
397 concentrations Pi-incorporation was strongly reduced and maximum Pi-uptake capacity was
398 enhanced (Figs 3,4). This coincides with the observation that acidophile Pi-transporters used
399 $H_2PO_4^-$ (Hirsch et al., 1993) (which is available at low Fe), whereas those of neutrophile algae
400 use HPO_4^{2-} (Pedersen et al., 2013). Unfavourable chemical Pi-speciation therefore brings
401 about a Pi-limitation in acidophiles at high iron, and calculations in Visual MINTEQ software
402 further revealed that an acidification event will slightly decrease the iron-phosphate
403 complexation that could weaken this Pi-limitation. However, in nature Pi-limited conditions
404 might be mitigated as *C. acidophila* expresses phosphatase enzymes (Boavida and Heath,
405 1986; Lachmann et al., 2017; Spijkerman et al., 2007b) that enable the use of organic Pi-
406 sources.

407 High iron concentrations effectively decreased Pi-incorporation, and 260 mg total Fe
408 L^{-1} resulted in an inhibition by 50%. Consequently, an important result of this study is that
409 high iron concentrations promote Pi-limitation. A Pi-limitation in the natural phytoplankton of
410 all four lakes under study had already been revealed, although the Pi concentrations in lake
411 water (ranging between 8 and 26 µg P L^{-1}) did not directly suggest the presence of a Pi-
412 limitation (Spijkerman, 2008). Similarly, high phosphatase activities that were measured in
413 the benthic algal community of Rio Tinto, despite high external Pi concentrations (ranging

between 0.2 and 3.2 mg P L^{-1}!) also suggest a physiological acclimation to Pi-limitation (Sabater et al., 2003). In addition, observations that growth of a *Chlamydomonas* isolate from Rio Tinto was completely inhibited by a ferric iron concentration of 280 mg total Fe L^{-1} (Rowe et al., 2007) might also have resulted from halved Pi-incorporation. Low Pi availability due to Fe-P-complexation likely dominates the algal physiology in acidic lakes, and Pi-limitation resulting from high iron can explain the low primary production observed in Fe-rich, acidic lakes (Nixdorf et al., 2003).

Acknowledgements

Stefanie Branscheid is acknowledged for her contribution to this study as part of her bachelor degree. Dr. Bukowsky determined the total iron concentrations. Barbara Schmitz assisted during the practical work. ES acknowledges the DFG for financial support (SP695/2, SP695/4 and SP695/5). The authors have no competing interests to declare.

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591 **Figure legends**

592 **Figure 1.** Cellular Fe content (a), cellular P content (b), Fe:P ratio (c) and Fe:C ratio (d) of
593 *Chlamydomonas acidophila* grown in Pi-replete (310 µg P L^{-1}: +P) or low Pi (non-treated or
594 5 µg P L^{-1}: -P) medium or lake water over a range of calculated free ionic Fe^{3+}
595 concentrations. Symbols show the mean ± SE of 3 independent replicates in complex medium
596 (circles), in minimal medium (triangles) and lake water (squares). The free ionic Fe^{3+}
597 concentrations in lake water were calculated using the chemical composition of complex
598 medium and the measured pH. Please note the logarithmic scales on the x-axes and the y-
599 axes.
600

601 **Figure 2.** Maximum fluorescence yield of photosystem II (Φ_{II}) measured at day 5(a) and the
602 relative growth rate during 5 days (b) versus a range of calculated free ionic Fe^{3+}
603 concentrations in Pi-replete (310 µg P L^{-1}: +P) or low Pi (non-treated or 5 µg P L^{-1}: -P)
604 medium or lake water grown *Chlamydomonas acidophila* cells. Symbols as in fig. 1. The free
605 ionic Fe^{3+} concentrations in lake water were calculated using the chemical composition of
606 complex medium and the measured pH. Please note the logarithmic scale on the x-axis.
607

608 **Figure 3.** Phosphorus incorporation in *Chlamydomonas acidophila* grown in Pi-replete (310
609 µg P L^{-1}: +P) or Pi-limited (non-treated or 5 µg P L^{-1}: -P) medium or lake water in relation to
610 the total Fe concentration in medium or lake water. Values are given in percent of the
611 maximum Pi-incorporation measured at the lowest Fe concentration. Symbols as in Fig. 1.

612 The lines illustrate the non-linear fit of equation 1 to the +P and -P data separately. Please
613 note the logarithmic scale on x-axis.
614
615 **Figure 4.** Maximum phosphorus uptake rates of *Chlamydomonas acidophila* grown in Pi-
616 replete (1.55 mg P L^{-1}) or Pi-limited (31 µg P L^{-1}) semi-continuous cultures at 0.35 d^{-1} in
617 relation to the total Fe concentration in medium. Rates were measured after an addition of a
618 saturating Pi-concentration of 150 µg P L^{-1}. Values are mean ± SD of at least 3 cultures.

Table 1. Chemical composition (excluding the micronutrients) of the culture media prepared with Fe concentrations ranging from 1 to 1200 mg L^{-1}. The complex medium is based on the chemical composition of Lake 111 (Bissinger et al., 2000), whereas the minimal medium is a slightly adapted Woods Hole medium (Gerloff-Elias et al., 2005). All concentrations in mg L^{-1}, unless stated otherwise.

Main salts	Complex medium	Minimal medium
Ca^{2+}	300	10.0
Mg^{2+}	28.1	3.65
Na^{+}	5.98	26.7
K^{+}	2.64	3.91
Total Fe	1; 10; 50; 100; 200; 400; 600; 800; 1000; 1200	10; 100; 800
N (as NO_3^{-})	0.28	14.0
N (as NH_4^{+})	2.30	0
P (as PO_4^{3-})	5; 310 µg L^{-1}	5; 310 µg L^{-1}
Cl^{-}	9.2	19.1
S (as SO_4^{2-})	417	4.80
pH	2.04 - 2.65	2.36

Table 2. Calculated free ionic Fe^{3+} concentrations, measured maximum fluorescence yield of photosystem II (Φ_{II},), and relative and absolute growth rates in *Chlamydomonas acidophila* grown in water from 4 different acidic lakes. The water was either non-treated or enriched with Pi, resulting in Pi-limited and Pi-replete growth conditions, respectively. Mean ± SE of 3 replicate cultures. Calculated free ionic Fe^{3+} concentrations were based on the chemical composition known from Lake 111 (Bissinger et al., 2000) and the measured pH. The growth rates in water from Lake 117 (lowest Fe concentration) were set to 100% for calculation of the relative growth rate.

		non-treated lake water			P-enriched lake water (+ 310 µg P L^{-1})		
Lake	Fe^{3+} (mg L^{-1})	Φ_{II} (rel. units)	Relative growth (%)	Growth rate (d^{-1})	Φ_{II} (rel. units)	Relative growth (%)	Growth rate (d^{-1})
117	0.26	0.35 ± 0.03	100	0.16 ± 0.01	0.68 ± 0.01	100	0.83 ± 0.01
111	6.85	0.29 ± 0.03	115 ± 4	0.18 ± 0.01	0.66 ± 0.02	95 ± 0	0.79 ± 0.00
113	10.40	0.26 ± 0.00	119 ± 9	0.19 ± 0.01	0.68 ± 0.01	95 ± 1	0.79 ± 0.01
107	33.18	0.54 ± 0.03	213 ± 2	0.34 ± 0.00	0.68 ± 0.00	95 ± 1	0.78 ± 0.01

Figure

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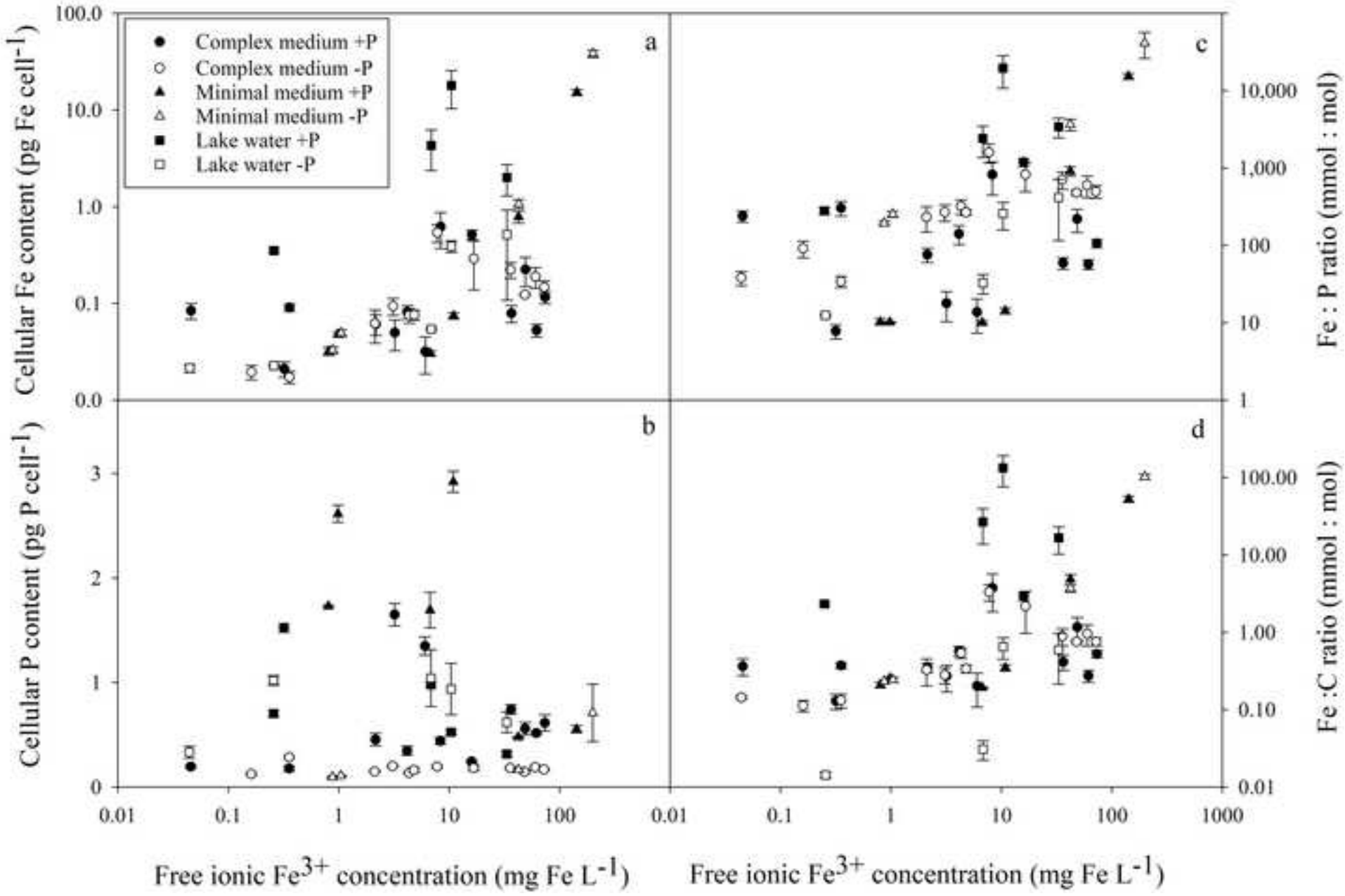

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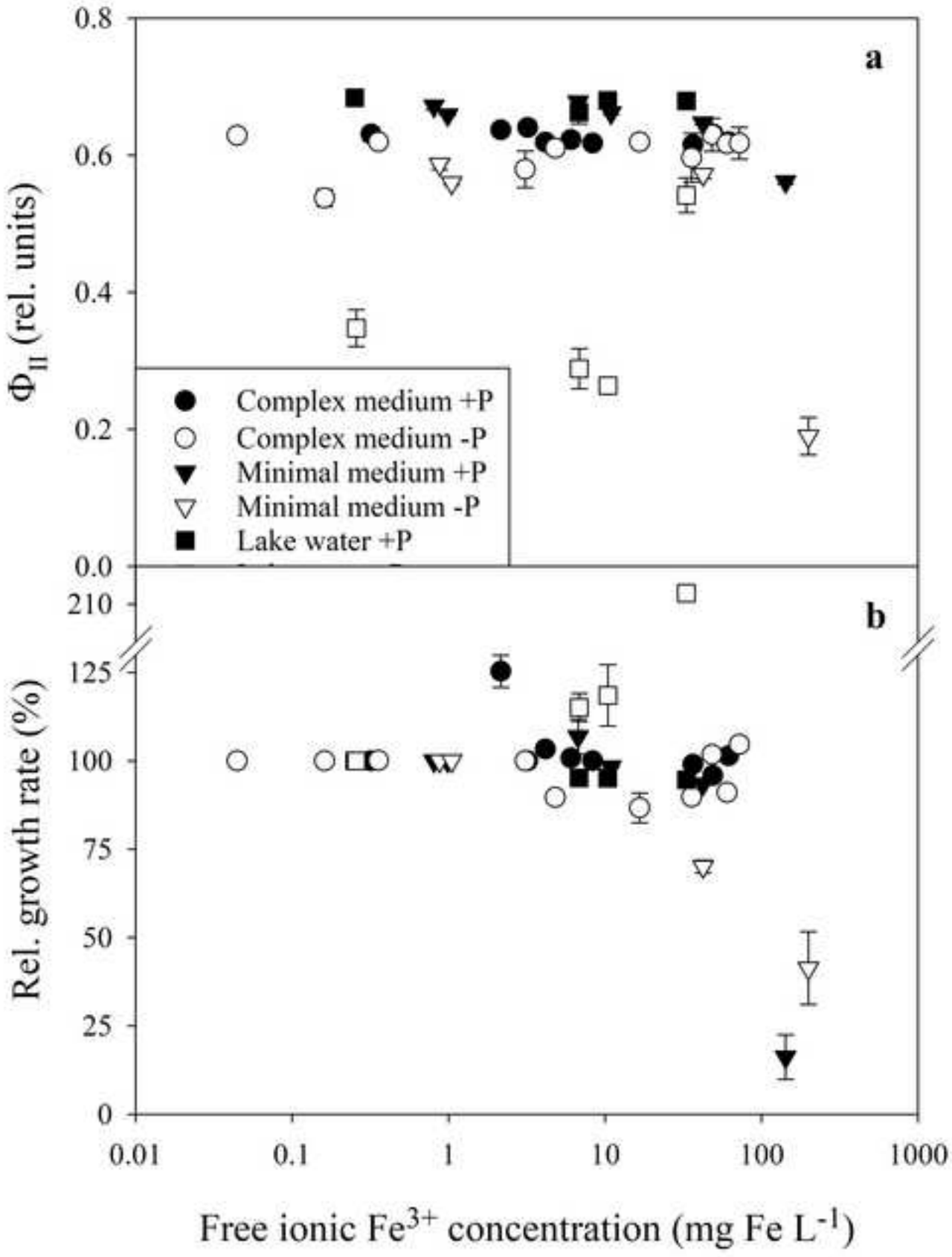

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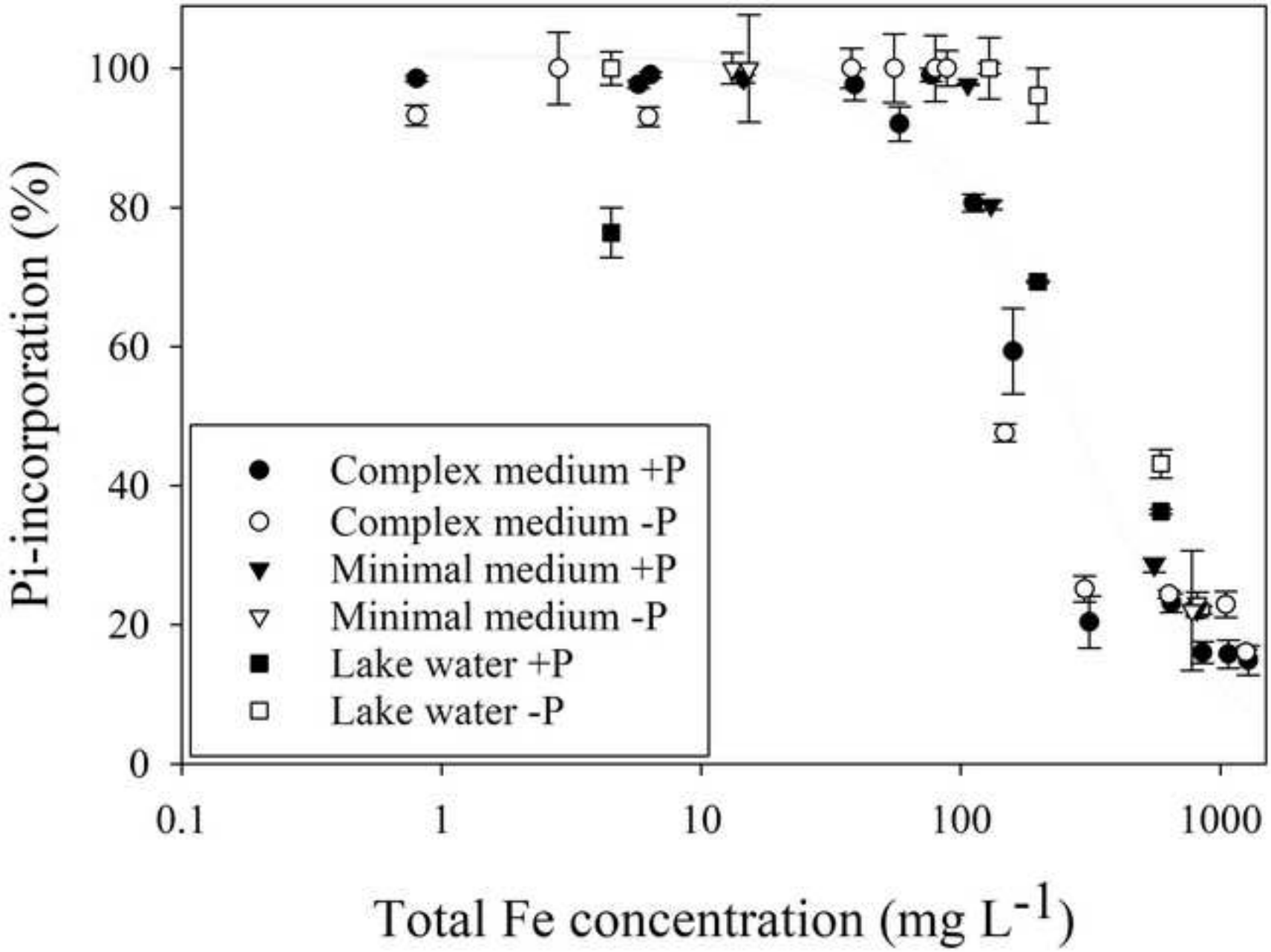

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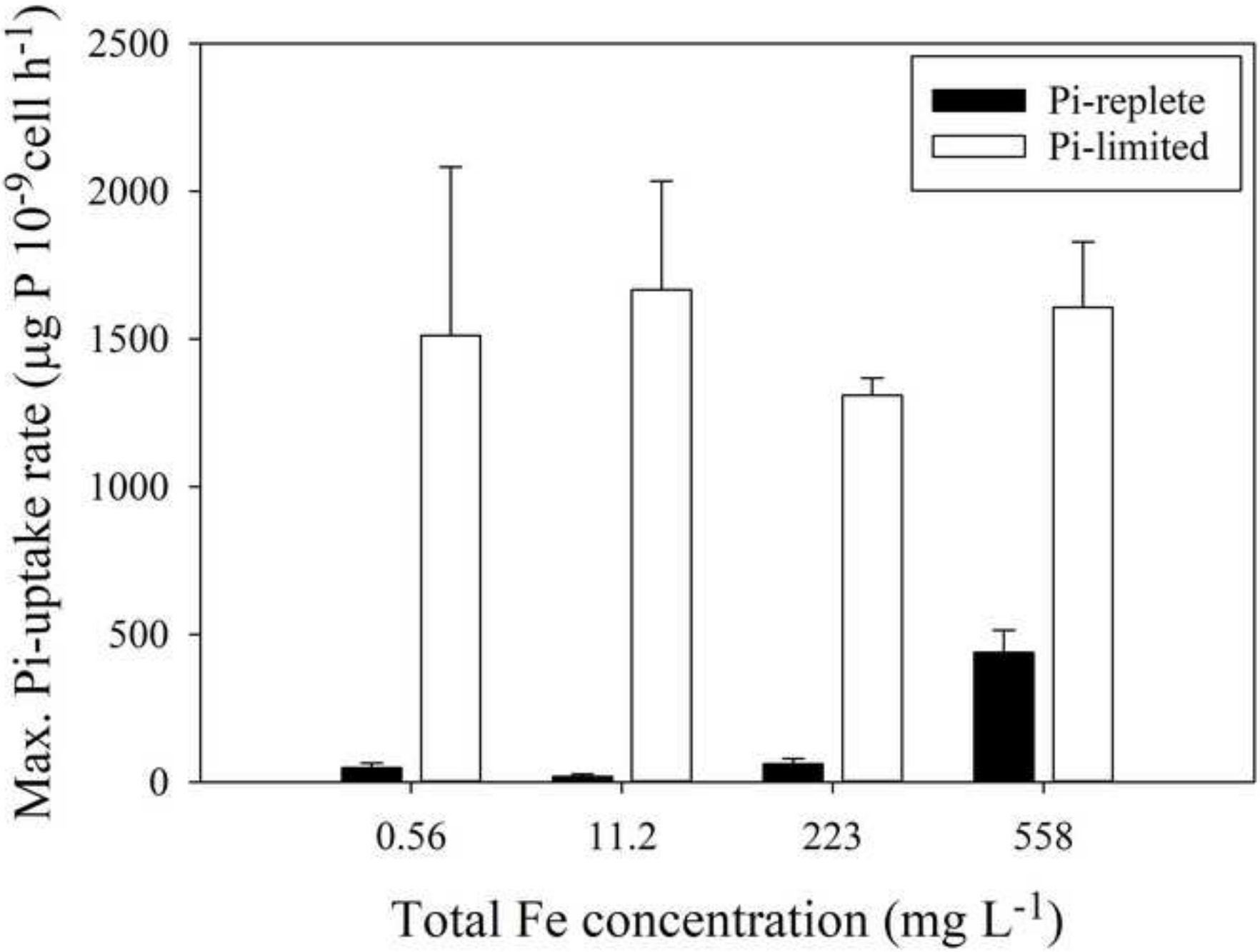

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