Extremophiles (2007) 11:551–562
DOI 10.1007/s00792-007-0067-0

ORIGINAL PAPER

Stress responses and metal tolerance of *Chlamydomonas acidophila* in metal-enriched lake water and artificial medium

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Received: 18 July 2006 / Accepted: 15 February 2007 / Published online: 12 April 2007

Abstract *Chlamydomonas acidophila* faces high heavy-metal concentrations in acidic mining lakes, where it is a dominant phytoplankton species. To investigate the importance of metals to *C. acidophila* in these lakes, we examined the response of growth, photosynthesis, cell structure, heat-shock protein (Hsp) accumulation, and metal adsorption after incubation in metal-rich lake water and artificial growth medium enriched with metals (Fe, Zn). Incubation in both metal-rich lake water and medium caused large decreases in photosystem II function (though no differences among lakes), but no decrease in growth rate (except for medium + Fe). Concentrations of small Hsps were higher in algae incubated in metal-rich lake-water than in metal-enriched medium, whereas Hsp60 and Hsp70A were either less or equally expressed. Cellular Zn and Fe contents were lower, and metals adsorbed to the cell surface were higher, in lake-water-incubated algae than in medium-grown cells. The results indicate that high Zn or Fe levels are likely not the main or only contributor to the low primary production in mining lakes, and multiple adaptations of *C. acidophila* (e.g., high Hsp levels, decreased metal accumulation) increase its tolerance to metals and permit survival under such adverse environmental conditions. Supposedly, the main stress factor present in the lake water is an interaction between low P and high Fe concentrations.

Keywords *Chlamydomonas acidophila* · Heat-shock protein accumulation · Lake water incubation · Metal accumulation · Metal stress · Photosynthetic yield

Communicated by K. Horikoshi.

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Introduction

Chlamydomonas acidophila is a dominant phytoplankton species in very acidic mining lakes (Gyure et al. 1987; Lessmann et al. 2000), as well as in acidic volcanic lakes (Doi et al. 2001; Nishikawa and Tominaga 2001). Besides low pH, these lakes are characterised by high concentrations of metal ions, such as iron and zinc (Nixdorf et al. 1998; Lessmann et al. 1999; Nishikawa et al. 2003). Low pH, high metal concentrations, or other factors (e.g., low P, high S), could be the cause of the lower primary productivity in acidic lakes, compared to neutral ones (Nixdorf et al. 2003). Recent results suggest that low pH is not the main limitation to primary productivity of *C. acidophila* in acidic mining lakes (Gerloff-Elias et al. 2005a), whereas the importance of heavy metals has not been examined in this regard. Acidophilic algae have been described as extremely tolerant to high concentrations of heavy metals (Whitton 1970). This is thought to be a result of significantly reduced surface binding and uptake rates of heavy metals caused by the high proton concentration of acidic water (Olaveson and Stokes 1989; Nalewajko et al. 1997). A considerable metal tolerance of *C. acidophila* was

described in an isolate from an acidic volcanic lake in Japan grown in artificial medium (Nishikawa and Tominaga 2001; Nishikawa et al. 2003). In this study we investigated physiological responses to increased metal concentrations on *C. acidophila* under natural conditions.

Metal exposure is known to result in growth inhibition and toxicity symptoms, metal binding to the cell wall and extracellular exudates, or reduced uptake or efflux pumping of metals at the plasma membrane (Hall 2002). The relative metal concentration bound to the cell wall is considered a good indicator for measuring the toxic effects of a metal on algal growth, this toxic effect being higher when more metals are adsorbed to the cell wall (Ma et al. 2003). Exposure of plants and algae to excess concentrations of metals can damage cells directly (e.g., direct inhibition of enzymes), and indirectly, in the case of redox active metals such as Fe, by causing oxidative injury (de Vos et al. 1992; Hall 2002). Photosynthesis is among the processes that are very sensitive to metal stress, with Photosystem II (PSII) often being a target (Singh and Singh 1987; Krupa and Baszynski 1995; Poskuta et al. 1996; Ralph and Burchett 1998; Ivorra et al. 2002; Heckathorn et al. 2004; Ikegami et al. 2005).

One of the possible adaptive mechanisms to cope with increased metal concentrations is the induction of heat-shock proteins (Hsps) (e.g., Hall 2002; Heckathorn et al. 2004). The induction of these proteins is well-described under high temperatures, but other stress conditions can also induce Hsps such as low temperature, high light, heavy metals, high population density, bacterial and viral infection, and oxidative stress (Sørensen et al. 2003; Barua and Heckathorn 2006). In *C. acidophila*, extremely low (1.5) or high (6 and 7) pH can also induce Hsps (Gerloff-Elias et al. 2006). Hsps increase tolerance to heavy metal stress by preventing membrane damage (Neumann et al. 1994; Hall 2002) and protecting PSII (Schroda et al. 2001; Heckathorn et al. 2004), in addition to general roles in protecting and repairing proteins. Heavy metals can lower photosynthesis, in part, by increased photoinhibition from excess light, and Hsps can help protect photosynthesis under high light stress (Heckathorn et al. 2004 and references therein). For example, small (low-molecular-weight) Hsps, sHsps, can protect photosynthesis from photoinhibition in both plants (Downs et al. 1999) and algae (i.e., *Chlamydomonas reinhardtii*; Schuster et al. 1988). In maize mitochondria, sHsps were shown to improve mitochondrial electron transport, mainly by protection of the NADH:ubiquinone oxidoreductase activity (Hamilton and Heckathorn 2001). In *C. reinhardtii*, the increased expression of several nuclear Hsp70 genes, including *Hsp*70B, encoding a chloroplast-localized chaperone Hsp70, resulted in faster recovery from photoinhibition after exposure to high light conditions than control cells (Schroda et al. 1999). The authors concluded that Hsp70B may participate in vivo both in the molecular protection of the PSII during photoinhibition and in the process of PSII repair. Hsp60 is primarily found in mitochondria and chloroplasts and accumulation might consequently respond to redox-stress caused by metal toxicity (Lewis et al. 2001). In general, Hsps are ubiquitous in all extremophilic microorganisms and might therefore be a prerequisite to survival under stress conditions (Laksanalamai and Robb 2004).

To investigate the relative importance of high levels of heavy metals to growth and primary productivity of algae in acidic mining lakes, we quantified growth, quantum yield of PSII, Hsp levels and metal adsorption and content of *C. acidophila* after incubation in metal-rich lake water and in artificial medium enriched with Zn or Fe. We show that high concentrations of Zn had minor effects on the physiology of *C. acidophila*, whereas increased concentrations of Fe affected photosynthesis and morphology. Incubation in natural lake water induced a stress response in *C. acidophila*, but this response was largely different from that observed in the metal incubations. Therefore, the main stress factor present in the lake water is not likely the presence of the metals Zn and Fe, but might be an interaction between low P and high Fe concentrations.

Materials and methods

Chlamydomonas acidophila Negoro (strain nr. SAG 2045, Gerloff-Elias et al. 2005a) isolated from an acidic mining lake in eastern Germany (Lake 111), was grown in modified Woods Hole (WH) medium (Gerloff-Elias et al. 2005a) or in Medium 111 (Bissinger et al. 2000) at a pH of 2.7. The modifications to the WH medium consisted of the exclusion of silicate and an organic buffer, and adjustment of pH to 2.7 by HCl. The chemical composition of Medium 111 reflects that of the acidic mining lake 111, except the phosphorus concentration which is higher (1.6 mg P l^{-1}). All culturing was performed in triplicate.

Lake water experiment

Lake water from mining lake 107 (pH 2.3), 111 (pH 2.7) and 117 (pH 3.0), situated in eastern part of Germany (51°29′N; 13°38′E; described in Packroff 2000), was collected on 10 January 2003 by drilling a hole through approximately 15 cm ice. As the solubility of metals is highly pH dependent, the lake with the lowest pH (Lake 107) contains the highest concentrations of metals and total phosphorus (TP, pH 2.3, 3.3 mg Zn l^{-1}, 400 mg Fe l^{-1}, TP 13 μg P l^{-1}), Lake 111 (pH 2.7) has lower concentrations (0.75 mg Zn l^{-1}, 140 mg Fe l^{-1}, TP 8 μg P l^{-1}), and Lake 117 (pH 3.0) has the lowest metal concentrations

(0.022 mg Zn l^{-1}; 49 mg Fe l^{-1}, TP 6 μg P l^{-1}) (Friese et al. 1998; Nixdorf et al. 1998; Packroff 2000). Lake water was collected from 1 m depth using a 3.5 l Limnos universal water sampler. Lake water was filter-sterilized through a 0.2 μm filter in the laboratory before use in experiments. *C. acidophila*, grown in Medium 111, was inoculated in the lake water at 0.5–1.5 × 10^8 cells l^{-1}. Cultures were aerated with normal air and kept at 20 ± 1°C in a light:dark cycle of 16:8 h; light irradiance was approximately 150 μmol m^{-2} s^{-1} PAR, as measured with a spherical quantum sensor (US-SQS, Walz, Heidelberg, Germany). After 1, 3, and 14 days, algae were collected by centrifugation (1,500×*g*, 5 min) for protein detection and fluorescence analysis. For protein extractions, algal pellets were immediately frozen in liquid nitrogen and stored at –80°C until utilisation. Fluorescence of PSII was measured on concentrated cultures with an optical density at 750 nm of 0.2. A pulse-amplitude-modulated chlorophyll fluorometer (PAM101/103, Walz, Germany) provided saturating light pulses (600 m, approximately 2,500 μmol photons m^{-2} s^{-1}) in order to measure maximal (F_m) and minimal (F_0) fluorescence in dark-acclimated samples, or maximal fluorescence in actinic light-acclimated cells (F_m' measured at 150 μmol m^{-2} s^{-1}). From this, the dark fluorescence yield of PS II $\left(\Phi_{II} = \frac{F_m - F_0}{F_m}\right)$ was determined after 15 min of dark acclimation. Quenching of maximum fluorescence in the light (at 150 μmol m^{-2} s^{-1}) was calculated as: $\frac{F_m - F_m'}{F_m'}$ (Spijkerman et al. 2004).

Hsp detection

For the preparation of a standard sample, *Chlamydomonas reinhardtii* Dangeard (strain nr. SAG 11-32b) was grown in WH medium buffered with 0.12 g N-[Tris(hydroxymethyl)methyl]–2-aminoethanesulfonic acid (TES) l^{-1} to a pH of 7.2 (Nichols 1973). *C. reinhardtii* was heat shocked at 41°C for 2 h in an illuminated water bath, conforming to optimal conditions for the induction of several Hsps (Hsp70, Hsp60, and Hsp22; Tanaka et al. 2000). The sample originating from this 41°C, 2 h treatment will hereafter be referred to as a standard heat-shock sample, and a subsample of this standard was run on every gel to calibrate the Hsp response of other samples to 100%.

Proteins were extracted, separated by 1D SDS-PAGE, and electroblotted to a polyvinylidine difluoride (PVDF) membrane as described previously (Gerloff-Elias et al. 2006). PVDF membranes were probed with protein-specific antibodies. Antibodies to the chloroplast small HSPs were polyclonal raised against oligopeptides of conserved sequences (Heckathorn et al. 2002). The Hsp 70A antibody (a kind gift of Dr. Pederson, Yale University, USA) was raised by Yale Immunization Services (New Haven, CT) against a His6 fusion protein containing the C-terminal 293 amino acids from the *C. reinhardtii* HSP 70A gene (Edward Savino and Joel Rosenbaum, unpublished), and this antibody detects multiple members of the Hsp70 family of proteins in the cytosol (constitutive and induced). The Hsp 60 antibody (a kind gift from R. A. Hallberg, Hallberg et al. 1993) detects mitochondrial and chloroplastic members of the Hsp60 family. The secondary antibody was conjugated to alkaline phosphatase (Goat Anti-Rabbit IgG-AP, Bio-Rad, Germany), enabling colorimetric detection with nitroblue tetrazolium/5-bromo–4-chloro–3-indolyl phosphate. Following development, blots were scanned and analysed using digital imaging software, Scion Image Beta 4.02 Win software (Scion Corp., USA). From every independent sample, a minimum of six densitometric analyses per antibody were performed to determine expression levels as a percent of the heat-shock sample.

Protein identification in selected bands from 1D SDS-PAGE gels was performed at the Campus Chemical Instrument Center of Ohio State University, Columbus OH, USA. Protein spots were excised and digested by trypsin. The masses of the extracted peptides were determined by capillary-liquid-chromatography nano-electrospray-ionization tandem mass-spectrometry (nano-LC/MS/MS). Sequence information from the MS/MS data was processed using Turbo SEQUEST algorithm in BioWorks 3.1 Software (Thermo Electron Corporation, Waltham), and the Mascot search engine (http://www.matrixscience.com) was used to search protein MS/MS match-up from the NCBInr 20041122 database. Probability scores were used as the criterion for correct identification.

In situ lake incubations and metal extraction

In situ incubations were performed in June 2003 in Lake 111. Schott flasks (500 ml), either filled with WH medium (pH of 2.7) or freshly filter-sterilized (over 0.2 μm cellulose-acetate filter) Lake 111 water, were inoculated with *C. acidophila* (grown in WH medium) and incubated in the lake (see also Gerloff-Elias et al. 2005b). In situ incubations lasted 4 days, after which samples for cell counts and metal determination were collected within 24 h after harvest. Metal determinations were performed on the supernatant after centrifugation (2,500×*g*, 5 min). The pellet was washed once with distilled water, incubated with 0.5 ml of 20 mM K_2EDTA for 2 min, and subsequently centrifuged (12,000×*g*, 1 min). The supernatant was removed (EDTA-extractable fraction, Ma et al. 2003). Both fractions were lyophilised, after which they were extracted with 0.2 ml HNO_3 (69%, trace select Sigma) and 0.2 ml of H_2O_2 (30%), with intermediate drying overnight at 80°C. Finally, acidified double-distilled water (1.5% HNO_3) was added, and all samples were measured for concentrations of total Fe and Zn by atomic absorption (AAS vario 6). Both flame

and electrothermal atomisations were utilized. Throughout the measurements, deuterium correction was applied. Appropriate blanks were analysed in parallel with samples. Although metals dissolved in acidic water will likely be protonated, we only determined total Fe (and Zn) and therefore only reference to total concentrations of metal have been made throughout the paper.

Metal-rich medium incubations

In laboratory experiments, WH medium (pH of 2.7) was enriched with either 840 mg Fe l^{-1} or 3.3 mg Zn l^{-1}, equalling maximum metal concentrations of Lake 107. In the case of Fe, the pH dropped to 2.3 and could not be raised without Fe precipitation. Medium was inoculated with *C. acidophila* (grown in WH medium) and kept under comparable conditions as the lake water incubation experiment described above. After 1, 3, and 14 days, Φ_{II} and F_m quenching were determined on a PAM101/103 as described above.

Growth rate determination

Samples from the lake water and metal incubation experiment were collected every day over the first 5 days after inoculation. Samples were fixed with lugol (Woelfl and Whitton 2000) and cell densities determined by using an automatic particle counter (CASY 1, Schärfe, Reutlingen, Germany). The cell diameter provided by the Casy was used to calculate cell volume assuming spherical cell morphology. Cell densities were used to calculate growth rates assuming exponential growth.

Pigment determination

Chlorophyll a (Chl *a*) content was determined spectrophotometrically after extraction in 90% acetone (Jeffrey and Humphrey 1975). Pigments were measured as in Gilmore and Yamamoto (1991) on a HPLC with a Vertex ODS-1 column (5 µm particle size, 250 × 4.6 mm I.D., Knauer, Berlin) on a variable UV/VIS detector (PHD601, GAT, Bremerhaven, Germany).

Results

The ecological response to metal-rich lake water and medium was studied by determining exponential growth rates and cellular chlorophyll content. Exponential growth rates were similar in water of Lake 107, 111, and 117 (Table 1, ANOVA, $F = 0.45$, $P = 0.66$). Although Lake 107 contained the highest concentration of dissolved metals and had the lowest pH, growth rates equalled those measured in WH medium without additions (control), as well as those in Zn-enriched medium (Table 1). This result indicates that growth of *C. acidophila* is not hampered by the Zn-enriched environment, at least not when inoculated cells were P-saturated, as here. Furthermore, the difference in pH did not play a role in the growth of the algal cells as water from Lake 107 had a pH of 2.3, instead of 2.7 of the medium or 3.0 of water from Lake 117 (Table 1). In Fe-enriched WH medium, no growth was detected and this partly resulted from the formation of a large mucous layer enclosing cells (not shown). Also in WH medium containing 400 mg Fe l^{-1} no growth was observed (results not shown). The high Fe concentration in the Lake 107 water did not have such an effect on the morphology of the cells.

There was a significant trend of cellular Chl *a* being highest in cells grown in water from Lake 107 and lowest in those from Lake 117 (Pearson, $n = 9$, $r = -0.75$, $P < 0.05$, Table 1). Notably, the cellular Chl *a* content in the medium-grown cells was greater than in lake-water-grown cells, except for cells grown in medium + Fe. In medium-grown cells, Chl *a* content decreased with Zn, and sharply with Fe additions (ANOVA, $df = 3$, $P < 0.05$). Chl *a*:*b* ratios were barely affected by the treatments, excluding

Table 1 Total iron, zinc and phosphorus concentrations (mg l^{-1}), growth rates (μ, day^{-1}), pH, cellular Chl *a* content (pg $cell^{-1}$), and Chl *a*:*b* ratio (g:g) of *C. acidophila* incubated for 3 days in water from Lake 107, 111 and 117, WH medium without additions (control) or WH medium enriched with metals (results are mean ± STD, *n*=3)

Lake	[Fe][a]	[Zn][a]	[P][a]	μ	pH	Cellular Chl *a*	Chl *a*:*b*
107	400–800	3.3	0.013–0.046	0.59 ± 0.06	2.3	1.30 ± 0.18	3.0 ± 0.04
111	120–190	0.75	0.008–0.016	0.55 ± 0.03	2.7	1.02 ± 0.09	2.9 ± 0.03
117	17–25	0.022	0.006–0.018	0.52 ± 0.02	3.0	0.82 ± 0.30	2.8 ± 0.04
Control	0.64	0.009	1.55	0.55 ± 0.03	2.7	2.21 ± 0.20	3.3 ± 0.7
M + zinc	0.64	3.25	1.55	0.58 ± 0.02	2.7	1.61 ± 0.21	2.5 ± 0.4
M + iron	401–890	0.009	1.55	–0.21 ± 0.08	2.3	0.63 ± 0.18	0.4 ± 0.0

[a] Concentrations in Lake 107, 111 and 117 taken from Friese et al. (1998), Nixdorf et al. (1998) and Packroff (2000)

the Fe-enriched medium where this ratio was very low, indicating relatively great damage to reaction centre cores versus light-harvesting complexes.

Photosynthesis is among the processes that are very sensitive to metal stress and therefore photosynthetic quantum yield and F_m quenching were determined. In both lake water and metal-enriched WH medium, a time-dependent pattern in Φ_{II} and F_m quenching was observed (Fig. 1). Φ_{II} was lower on the first day after inoculation than after 3 days, possibly related to inoculation stress (Fig. 1a, b). Three days after inoculation, the cells were in mid-exponential growth, coinciding with higher quantum yield. After 14 days of incubation, the cells were in a stationary phase coinciding with a lower Φ_{II}. A similar pattern in quantum yield was observed in the medium-grown cells, but smaller decreases occurred from day 3 to 14 that were only significant in the control and medium + Zn treatment (*t*-test, $P < 0.05$ or $P = 0.09$ for medium + Fe, Fig. 1b). The Φ_{II} in the medium + Fe cultures was lower than those in both control and medium + Zn cultures on all measuring days (ANOVA, $P < 0.05$).

A negative F_m quenching was measured after 1 and 3 days of incubation in lake water (Fig. 1c), as well as after 1 day of incubation in all of the medium treatments and in the medium + Fe on days 3 and 14 (Fig. 1d). This was a result of the lower F_m measured in the dark in comparison with F_m measured after light acclimation. After 14 days of lake water incubation, F_m quenching rose to approximately +0.4 in all different lake waters (Fig. 1c), indicating protective down-regulation of photosynthesis from excess light.

One general response to stress is the induction of heat shock proteins. We studied the accumulation of Hsp70A, Hsp60 and sHsps and compared our western blotting results with changes over time in the total protein extract. Cellular levels of Hsp70A, Hsp60 and sHsps differed in cells incubated in lake water compared to cells in medium, and depending on the Hsp, changed or remained constant over the incubation period (Fig. 2). For example, Hsp70A content of *C. acidophila* grown in lake water was first low, increased to values similar to that of algae grown in medium either with or without Zn or Fe, and decreased again after 14 days (Kruskal Wallis, $P < 0.05$); meanwhile, Hsp70A levels were relatively constant over time in the medium-grown cells (Fig. 2a, b). Quantification of Hsp70B, a chloroplastic Hsp70 (Drzymalla et al. 1996) in lake water incubations, resulted in a similar pattern (results not shown). In contrast to Hsp70A, the content of Hsp60 remained fairly constant over time in lake-water-grown cells, increasing only slightly at the end of the experiment (Kruskal Wallis, $P = 0.21$, 0.06 and 0.02 for Lake 107, 111 and 117, respectively, Fig. 2c). Compared to lake-water-grown cells, cells grown in medium generally had higher levels of Hsp60, excluding cells in control and medium + Zn on day 14 (Fig. 2d). Hsp60 levels were slightly lower in metal-treated cells versus controls, and decreased with time for controls and medium + Zn; in contrast, the Hsp60 content in Fe-enriched medium remained high throughout the experiment (Fig. 2d). Compared to the cells grown in medium, the accumulation of sHsps in lake water was pronounced, being approximately 1.5-fold greater. Small Hsp accumulation was similar in all 3 types of lake water, but over time the concentration in algae grown in water from Lake 111 and 117 decreased (ANOVA, $P < 0.05$ in both cases, Fig. 2e). The sHsp content was similar between treatments in the medium experiment, and also decreased over time (ANOVA, $P < 0.01$ in all cases, Fig. 2f). Notably, relative to the heat-shocked standard (*C. reinhardtii*), Hsp levels were often quite high (e.g., for Hsp

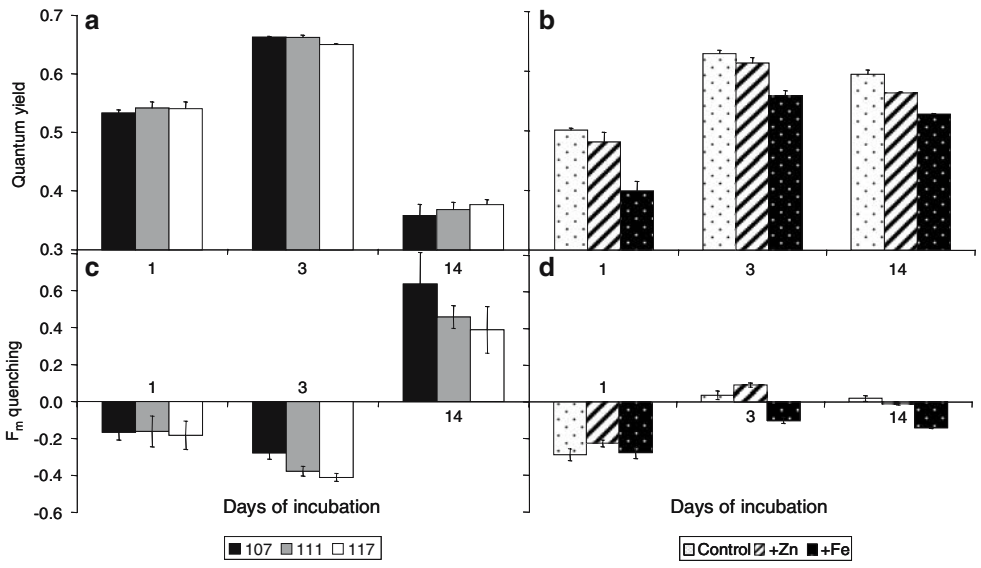

Fig. 1 Quantum yield of Photosystem II (Φ_{II}, **a**, **b**) and F_m quenching (light vs. dark) (**c**, **d**) of *C. acidophila* after 1, 3, or 14 days incubation in water from Lake 107, 111, and 117 (**a**, **c**) or after 1, 3, or 14 days incubation in medium enriched with different metals in a concentration similar to those present in Lake 107 (**b**, **d**). Results are mean ± SE of 3 incubations

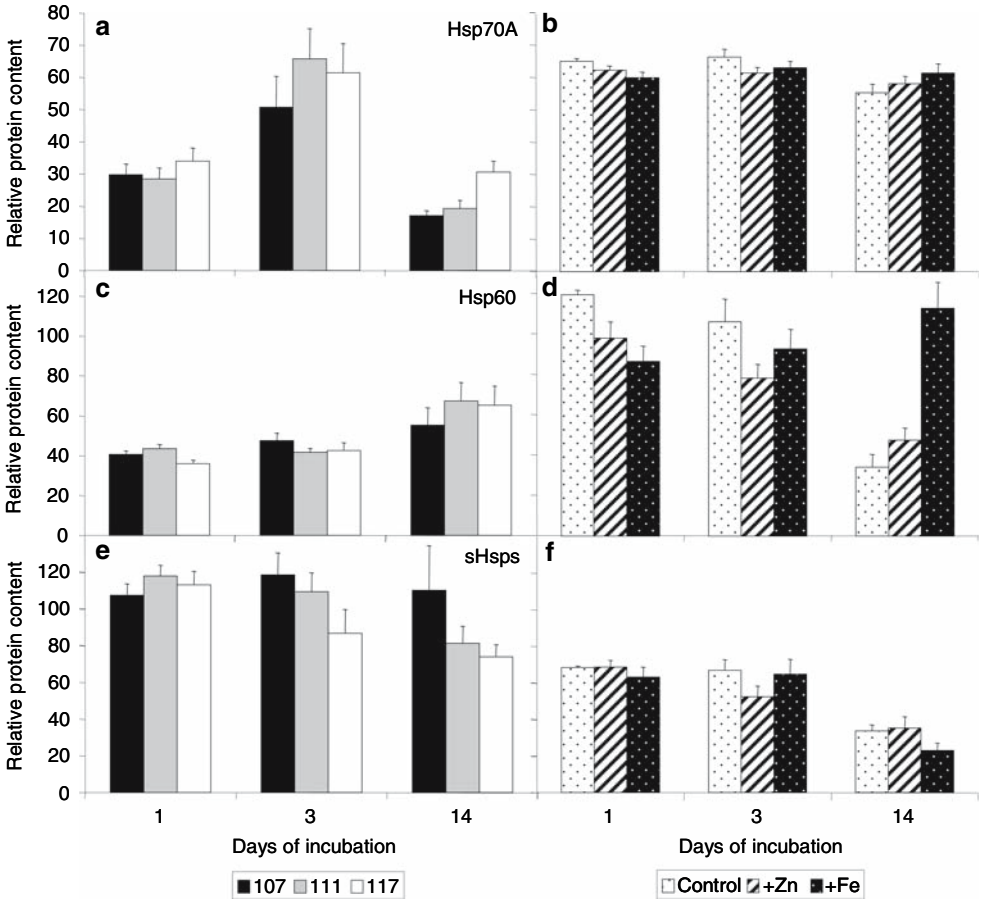

Fig. 2 Levels of Hsp70A (**a**, **b**), Hsp60 (**c**, **d**) and sHsps (**e**, **f**) after 1, 3, and 14 days incubation in water from Lake 107, 111, and 117 (**a**, **c** and **e**) or medium enriched with Zn or Fe (**b**, **d** and **f**). Results are means of at least 6 separate measurements ±SE. The protein content is expressed in percent of levels found in *C. reinhardtii* after a heat-shock treatment

70A in medium and in lake water on day 3, for Hsp60 in medium, and for sHsps in lake water).

The separation of total cell proteins of algae incubated in lake water on a large 1-dimension gel showed the pronounced increase with time in the density of 2 protein bands with molecular weights of approximately 70–75 and 90 kDa (Fig. 3). The density of these protein bands was highest in the algae grown in water from Lake 107 and lowest in those from Lake 117. Although the method does not allow quantitative measurements, mass spectrometry analysis of these bands suggested that the lower band was rich in Hsp70 on day 1, but that Hsp70 was less abundant on day 14 (Table 2), consistent with the immunoblotting results (Fig. 2a). The lower band also contained abundant starch synthase, the higher abundance of which, on day 14 compared to day 1, could explain the increased protein density. Analysis of the upper band yielded inconclusive results. The 2 week incubation in medium + Fe also resulted in pronounced protein bands with similar molecular weights, whereas these bands were much less distinct in 'normal' WH medium or medium + Zn (Fig. 3).

The relative metal concentration bound to the cell wall is considered a good indicator for measuring the toxic effects of a metal on algal growth. The concentrations of Fe and Zn

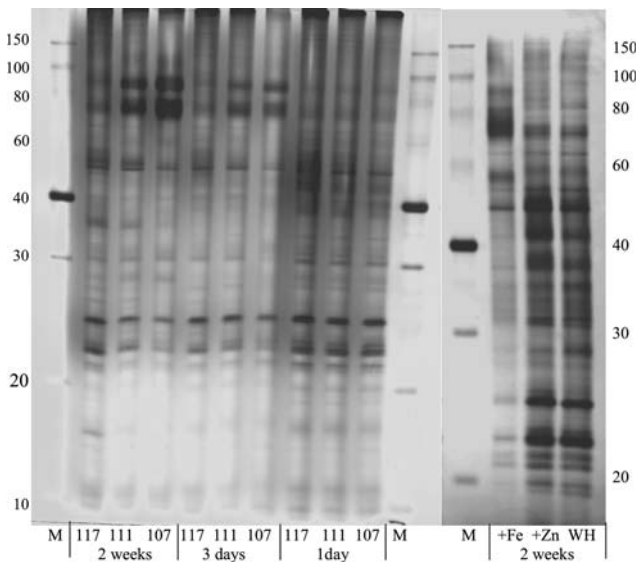

Fig. 3 One-dimensional fractionation of algal total cell protein by SDS-PAGE, followed by silver staining (equal total protein per *lane*, 5 μg). *M* molecular mass marker lane

in the WH medium from the incubation experiment in Lake 111 (Table 3) confirmed that no metal contamination between the lake water and the incubation bottles took place.

Table 2 Results from mass spectrometry analysis of the prominent lower protein band of ca. 70 kDa observed on 1D SDS-PAGE (cf. Fig. 3)

Day[a]	Identified Protein (rank)[b]	Database matches[c]	Mowse scores[d]	Peptide matches[e]
1	Hsp70 (1–9)	9 (including *C. reinhardtii*)	Ranging from 421 to 166	6,5,3,3,4,3,3,3,3
	Starch synthase I (10)	1 (*C. reinhardtii*)	86	1
	Un-named protein[f] (11)	1	61	1
14	Starch synthase I (1)	1 (*C. reinhardtii*)	145	2
	Un-named protein[f] (2)	1	66	1
	Hsp70 (3)	2	91–59	1,1

[a] Only results from days 1 and 14 were analyzed. Results shown for Lake 111 only, as similar results were obtained for Lakes 107 and 117

[b] Ranked by Mowse scores. All positive matches indicated

[c] Number of different Hsp70 proteins (either from different species or from different Hsp70s within a species) from database that match with *C. acidophila*

[d] Scores greater than 56 indicate identity or extensive homology at $P<0.05$

[e] Number of peptides from sample that matched with database peptides for each protein

[f] Database entry GI4722005 (from *Tetraodon nigroviridis*)

Cellular concentrations of Fe and Zn were higher in the medium-incubated than in the lake water incubated algae (Mann Whitney U, $P < 0.05$ in both cases), whereas the amount of metals adsorbed to the cell wall (EDTA removable) was higher in the lake-water-incubated algae (Mann Whitney U, $P < 0.05$ in both cases, Table 3). The total Zn concentration of the cells incubated in lake water and control (cellular + EDTA extractable fraction) were not significantly different (Mann Whitney U, $P = 0.058$, data not shown), suggesting that Zn uptake was decreased in the lake water by increased adsorption. After the incubation of *C. acidophila* in metal-enriched medium, the cellular metal concentration was about equal to the external metal concentration (accumulation close to one). In contrast, metal accumulation was lower in lake water, suggesting metal homeostasis regulation in lake water (Table 3).

Discussion

The results presented in this paper show that the extreme chemical conditions of the acidic mining lakes, wherein *Chlamydomonas acidophila* is common, induce cellular stress responses. These responses were evident in decreased quantum yield, increased small Hsp levels, changes in total cell protein profiles, and changes in cell metal content-but not in growth rate. Our results suggest that the stress responses associated with lake water were not primarily related to the high levels of the metals Zn or Fe in lake water, and hence, are not the main limit to productivity in these lakes. Our results partly coincide with those described for acid mining drainage in the USA Appalachian coal region, where soluble reactive phosphate concentrations explained most of the variance in primary

Table 3 Concentrations of iron and zinc in WH medium (M) and water from Lake 111, as well as cellular concentrations (fraction bound to the cell wall excluded, fg cell^{-1}) and the percentage of metal bound to the cells removable with EDTA (%) in *C. acidophila* incubated in WH medium or water from Lake 111

	4 days incubation		14 days incubation		
	Control	Lake 111	Control	M + Fe	M + Zn
Fe-soluble (mg l^{-1})	0.82 ± 0.05	136 ± 5	0.66 ± 0.04	892 ± 2	0.65 ± 0.04
Zn-soluble (μg l^{-1})	11 ± 1	737 ± 27	9 ± 1	–	3,252 ± 1
Fe-cellular (fg cell^{-1})	182 ± 26	58 ± 12	19 ± 2	15,584 ± 20	52 ± 28
Fe-EDTA (%)	15 ± 1	76 ± 5	39 ± 7	4 ± 0	23 ± 2
Fe-accumulation	19	0.07	4.2	0.94	15
Zn-cellular (fg cell^{-1})	5.8 ± 1.0	1.5 ± 0.2	4.4 ± 0.2	–	17 ± 3
Zn-EDTA (%)	55 ± 6	69 ± 3	24 ± 4	–	39 ± 3
Zn-accumulation	65	0.33	69	–	0.73

Metal accumulation was calculated by dividing the cellular (using the cell volume) by the external concentration. Results are mean ± SD of at least 6 measurements

– Depicts no measurements

productivity, and metals and pH were considered relatively unimportant (Simmons et al. 2004).

Photosynthesis

In both Φ_{II} and F_m quenching, a stress response was measured on the first day after inoculation that might relate to a lag phase in growth just after inoculation. In both lake-water-incubated cultures and in algae grown in medium, Φ_{II} in the dark was highest after 3 days. There was no difference in Φ_{II} in algae incubated in water from lakes having different levels of Fe and Zn, whereas an increased Zn concentration in medium led to a minor, and increased Fe concentrations to a major effect on Φ_{II}. The concentrations of Zn and Fe in Lake 107 are therefore potentially toxic, given the similar Fe and Zn levels in the lakes and medium. The metal toxicity is probably lowered by the presence of natural dissolved organic carbon (DOC) present in the lake water or the toxic effect is overruled by the effect of low P_i concentrations. In the case of the lake water incubation, Φ_{II} was strongly decreased after 14 days of incubation, which suggests that *C. acidophila* was very stressed by this time. This effect was absent in the medium-grown algae and correlates with low TP concentrations measured in these lakes (6–46 μg P l^{-1}, Packroff 2000) compared to the medium (1.6 mg P l^{-1}, Nichols 1973). Although the P_i concentrations in the water of Lake 107 are approximately tenfold and the Fe concentrations 100-fold higher than those in Lake 117, the effect on Φ_{II} after 2 weeks of incubation in lake water was similar for all three lakes. We hypothesize that after 14 days of growth, low P_i concentrations dominated the stress response in lake water incubated algae, and this hypothesis is supported by the high abundance of lipid bodies (not shown) and suggested increase in starch synthase with time in lake-water-incubated algae (Table 2).

F_m quenching analysis revealed a lower F_m in the dark than after light acclimation (150 μmol m^{-2} s^{-1}). This relatively lower F_m measured in the dark can be a result of the phosphorylation of part of the antenna complexes connected to PSII that then move to photosystem I (PSI, Kromkamp et al. 2001) in a process called state 2 transition. F_m quenching in the dark can also result from the utilization of organic carbon in a process called chlororespiration (Endo and Asada 1996). In our results, the quenching of F_m in the dark was likely a result of state 2 transition because Φ_{II} was not higher in the light than in the dark (which would be the case during chlororespiration, data not shown). In the medium-cultivated algae, F_m quenching remained negative over the 14 days incubation period in the Fe-contaminated treatment. Thus, state 2 transition is stimulated in lake water, possibly related to the high Fe concentrations. The increased F_m quenching after 14 days cultivation in lake water to values of 0.4–0.6 is consistent with P_i limitation as quenching increases under nutrient-limited conditions (Parkhill et al. 2001).

Growth and pigment content

In *C. acidophila*, deleterious effects of high metal concentrations on growth were only detected in P-rich medium enriched with Fe. Zinc exposures and metal-rich lake water incubations did not result in decreased growth rates. Our results with Zn are consistent to other studies on Zn tolerance in acidophilic algae. Nishikawa and Tominaga (2001) reported an EC_{50} concentration of 75 mg Zn l^{-1} for growth of *C. acidophila*, this concentration being 20-fold higher than used here. The acid-tolerant filamentous green alga *Mougeotia* had comparable growth rates under high levels of Zn and under control conditions (Graham et al. 1996). *Mougeotia* has been found growing in waters polluted by mine waste with Zn concentrations up to 34 mg l^{-1}. Also, in the metal-contaminated Lake Cospuden (pH 2.65, approximately 2 mg Zn l^{-1}), increased biomass of a *Chlamydomonas* species (likely being *C. acidophila*) was observed (Woelfl et al. 2000).

The presence of natural DOC can decrease the effect of toxic metals by chelating them (Ivorra et al. 1995). Vigneault and Campbell (2005) postulated that DOC does not appear to influence metal bioavailability other than by reducing the free metal ion concentration in solution. If we assume that Fe is predominantly found in a 1:1 complex with a chelating agent (Shaked et al. 2005) and assume a conservative 12-C organic carbon molecule, the maximal 3.1 mg C l^{-1} DOC (Packroff 2000) in the lake water has a maximum chelating capacity of 1.5 mg Fe l^{-1}. This is far below the 49–400 mg Fe l^{-1} present in the lake water. Hence, it remains unclear what component in the lake water decreased Fe toxicity.

In algae grown in artificial medium for 3 days, Chl *a* concentration and the ratio of chlorophyll *a:b* decreased slightly with high Zn, and decreased dramatically with high Fe. In *Chlorella kessleri*, a similar decrease in the cellular Chl *a* and Chl *a:b* ratio was described after Zn accumulation (Ikegami et al. 2005). Decreases in chlorophyll concentration, in the absence of decreases in Φ_{II}, as observed here for medium + Zn during the first 3–5 days of incubation, indicate a decrease in the concentration of PSII complexes with no decrease in the function of remaining PSII. For algae in medium + Fe, decreases in Φ_{II} and chlorophyll *a* were accompanied by decreases in chlorophyll *a:b*, indicating Fe-related damage to PSII and photosynthesis. However, in the lake water incubations, the highest cellular Chl *a* content and Chl *a:b* ratio were observed in the metal-richest Lake 107-incubated cultures. Therefore, the pigment content of *C. acidophila* in lake

water, over the time-period investigated, was not decreased by metal contamination.

Hsp accumulation

Heavy metal exposure is known to increase Hsp levels, as for example found in *Synechococcus* sp. (Borbely et al. 1990). Small Hsps (e.g., Hsp17) increased in cell cultures of *Silene vulgaris* and *Lycopersicon peruvianum* in response to a range of heavy metal treatments (Wollgiehn and Neumann 1999). Centipedes (*Lithobius*) collected near a smelter had higher Hsp70 levels than those collected from unpolluted areas (Pyza et al. 1997).

Hsps can protect the cell against heavy metal stress. A short heat stress, resulting in the accumulation of Hsp70, increased subsequent metal tolerance in tomato cell cultures by preventing membrane damage (Neumann et al. 1994). Circumstantial evidence indicates that two small Hsps may be associated with membranes during metal exposure, resulting in the protective effects (Lin et al. 1985; Panaretou and Piper 1992). Compared to *C. reinhardtii* grown under optimal control conditions, *C. acidophila* has relatively high Hsps levels (Gerloff-Elias et al. 2006), suggesting that its adaptation to its natural environment resulted in relatively high basal levels of Hsps. Interestingly, cellular levels of small Hsps upon metal-rich lake-water incubation were even higher than those found in algae cultured in the metal-enriched artificial medium. The increased levels of sHsps in natural lake water indicate a stress response that is different from metal stress alone. In contrast, the levels of Hsp70A and Hsp60 were either decreased or similar in lake water, compared to medium (+metals) incubations. The difference in accumulation of Hsp70A and Hsp60 in lake water or medium after 1 day of incubation might partly have been a result of a difference in Hsp accumulation in the inoculum cultures as these had grown in different media. The accumulation of Hsp70A in *C. acidophila* after 14 days in lake water was lower than that in all medium cultures including the control, suggesting that the accumulation of this protein was not related to metal stress. This is in contrast to the accumulation of Hsp60 that remained high in the algae grown in Fe-enriched medium, whereas the levels in the other medium-cultures dropped to levels found in the lake-water-incubated algae.

Hsp induction related to acid stress described in *Escherichia coli* (Heyde and Portalier 1990), can likely be ruled out in *C. acidophila* as within the range of pH of the lake water (pH 2.3–3.0), no major effect of pH on Hsp concentration was found (Gerloff-Elias et al. 2006).

The lower protein band from the 1D SDS-gel yielded two clear positive hits: Hsp70 and starch synthase 1, with Hsp70 being the primary signal in day 1 samples, and starch synthase 1 in day 14. The detection of starch synthase 1 coincides with microscopic observations after 14 days of growth in lake water when cells of *C. acidophila* showed signs of a P_i-limitation by the abundance of lipid bodies inside the cell. Preliminary results for the upper band indicated high levels of Hsp90 and AA-type ATPases. The lower protein band (and to lesser extend the upper band) was also prominent in algae incubated in Fe-enriched medium for 2 weeks, suggesting that part of the protein response in natural lake water resulted from the high Fe concentrations.

Metal content in *C. acidophila*

Several algal species accumulate metals on the cell surface (reviewed in Gaur and Rai 2001) but the positive zeta potential of an acidophilic organism decreases metal binding at an external pH < 3 (Gimmler 2001). The metal-tolerant *Silene vulgaris* spp. *humilis* accumulated a range of metals in the epidermal cell walls, either bound to a protein or as silicates (Bringezu et al. 1999). The adsorption of Fe and Zn to *C. acidophila* was higher in lake water than in control or medium enriched with metals. The 30% of Zn adsorbed to cells of *Chlamydomonas variabilis* (at pH 7.0 Harrison et al. 1986) was similar to that of *C. acidophila* in Zn-enriched medium at pH 2.7 after 14 days of growth (39%). Zinc adsorbed to semi-continuously grown *Chlamydomonas variabilis* after 10 min of Zn uptake were higher (54–82%, Bates et al. 1982) and more in the range of values found for *C. acidophila* after 4 days of growth (69%). The relatively larger fraction of Zn adsorbed to *C. acidophila* after 4 days, compared to those collected after 14 days, coincides with the decreased binding of Zn to *C. variabilis* with increasing culture age (Harrison et al. 1986).

In lake water, the cellular Fe and Zn content of *C. acidophila* was lower than that in control medium, whereas the reverse was obtained in the metal enriched medium. Therefore, Fe and Zn uptake was enhanced in the medium, and decreased in lake water. This contrasts with the metal adsorption data and suggests that increased binding decreased uptake. Supposedly these differences between metal uptake in medium and lake water were related to DOC. Although the DOC concentration in lake water is too low to decrease toxicity by complexation with the metal-ions (see above), in Lake 111 DOC largely consists of fulvic acids (Poerschmann et al. 2004). Fulvic acids can either change binding to the cell wall (Ma et al. 2003) or the permeability of the membrane (Vigneault et al. 2000) and thereby compensate for the toxic effects of high metal concentrations. The uptake of Zn by semi-continuously grown *C. variabilis* resulted in cellular zinc concentrations of 2.4–4.8 fg $cell^{-1}$ after 10 min (Bates et al. 1982) being

in the range of those found for *C. acidophila* (2–17 fg cell^{-1}). Within the range of uncertainty given by the different incubation times, the cellular Zn concentration in *C. acidophila* was similar to those found in a neutral *Chlamydomonas* species. The lower cellular metal concentration of *C. acidophila* in natural lake water can explain the lesser effect of metals in natural lake water on growth and cellular Chl *a* concentration than in medium and contrasts the view that the toxic effect of a metal is higher when more metals are adsorbed to the cell wall (Ma et al. 2003).

In contrast to the acidophilic species *Euglena mutabilis*, *C. acidophila* did not accumulate Fe from the natural lake water (Mann et al. 1987; Mann and Fyfe 1989). Besides that, the cellular Fe content was higher in the Zn-enriched cultures suggesting that an increased Zn content resulted in a higher Fe accumulation. This can be explained by the presence of the IRT1 transporter on the plasma membrane that links the uptake of Zn with Fe as found in *Arabidopsis* (Varotto et al. 2002).

Conclusion

It is unlikely that the low primary productivity in acidic mining lakes in the Lusatia region of Germany results exclusively from the high concentrations of Fe and Zn in the lake water. Although incubation in natural lake water induced a stress response in *C. acidophila*, this response was largely different from that observed in the incubations in metal-enriched medium and may be (co-)related to a phosphorus limitation in lake water. Although *C. acidophila* faces potential metal stress in its natural environment, it has multiple adaptations to increase tolerance to metals (e.g., high accumulation of small Hsps, decreased accumulation of Zn and Fe resulting from increased adsorption) that permit its survival. The effect of high Fe concentrations and its interaction with DOC and P_i on the photosynthesis of *C. acidophila* is currently under investigation.

Acknowledgements We thank Klaus Hausmann for the TEM work that supports our results. This work has been supported by the German research foundation (DFG) to AG-E and ES, a European Union Marie Curie Development Host Fellowship received by ES, a scholarship from the FAZIT-foundation (Frankfurt/Main, Germany) received by AG-E and the German Academic Exchange Service (DAAD) as part of the International Quality Network (IQN) to DB and SH.

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