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Research

Chromium- and copper-induced inhibition of photosynthesis in *Euglena gracilis* analysed on the single-cell level by fluorescence kinetic microscopy

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Summary

• Here, we investigated effects of copper (Cu) and chromium (Cr) toxicity on two contrasting strains of *Euglena gracilis*, with and without chloroplasts, grown in culture media promoting either phototrophic or heterotrophic growth. This led to insights into Cr/Cu toxicity mechanisms and into the regulation of phototrophic vs heterotrophic metabolism.

• Our data strongly suggest that in Cu^{2+} and Cr^{6+} stressed *Euglena* photosynthesis is the primary target of damage. In the applied light conditions, this was mainly damage to the photosystem II reaction centre, as shown by single-cell measurements of photochemical fluorescence quenching. Respiration and photosynthetic dark reactions were less sensitive.

• The malfunctioning photosynthesis enhanced production of reactive oxygen species (mainly superoxide), leading to elevated amounts of carotenoid degradation products. At higher metal concentrations in chloroplast-containing cells, but not white cells, this oxidative stress resulted in increased respiratory oxygen uptake, likely by damage to mitochondria.

• During growth in nutrient solution promoting heterotrophic metabolism, the cells were able to repair the metal-induced damage to photosynthesis, moderating the inhibition of photochemistry. Growth in medium forcing the cells into photosynthesis increased the investment in photosynthetic pigments. Comparison of the two *Euglena* strains surprisingly showed that the previously metal-resistant strain lost this resistance during culture.

Abbreviations: Chl, chlorophyll; FKM, fluorescence kinetic microscope; F_0, minimal fluorescence yield of a dark-adapted sample, fluorescence in nonactinic measuring light; F_m, maximum fluorescence yield of a dark-adapted sample; $F_v/F_m = (F_m - F_0)/F_m$, maximal photochemical quantum yield of PSII in dark-adapted state; [hms]-Chl, heavy metal substituted Chl (i.e. Chl in which the central ion, Mg^{2+}, is replaced by a heavy metal ion); Mg-substitution, substitution of the natural central ion of Chl, Mg^{2+}, by heavy metals; NPQ, nonphotochemical quenching – in this paper, we measure nonphotochemical quenching as $q_{CN} = (F_m - F_m')/F_m$, 'complete non-photochemical quenching of Chl fluorescence', i.e. with normalisation to F_m; PSII, photosystem II; RC, photosynthetic reaction centre; Sm, Streptomycin; $\Phi_{PSII} = \Phi_e = (F_m' - F_t')/F_m'$, effective quantum yield of photochemical energy conversion in actinic light. Values of this parameter were also calculated for the relaxation period after the end of actinic light in order to follow the return of the system to its dark-acclimated state as measured by F_v/F_m.

Introduction

Metals such us copper (Cu), manganese (Mn), nickel (Ni) and Zinc (Zn) are essential trace elements for photosynthetic organisms. In animals, but so far not in photosynthetic organisms, even chromium (Cr) has been found to be essential (Hamilton & Wetterhahn, 1988). In higher concentrations, however, these metals have toxic effects. Copper is widely used as a pesticide in agriculture, and field runoff may easily reach concentrations of several μM (Gallagher *et al.*, 2001), which are lethal to many aquatic plants (review by Küpper & Kroneck, 2005). Also waste discharges without pretreatment play an important role in the pollution of aquatic systems. It is known that chromium can be found in several oxidation states, of which the most stable and common forms are trivalent Cr(III) and hexavalent Cr(VI), with different chemical properties (Bagchi *et al.*, 2002). The hexavalent form is the most toxic of this metal; it usually associates with oxygen to form chromate (CrO_4^{2-}). This molecule can easily go through cell membranes as an alternative substrate for the sulphate transport system (Riedel, 1985; Cieslak-Golonka, 1996).

Effects of heavy metals in algae have been the subject of extensive research for many years (Küpper *et al.*, 1998, 2002, 2003; Rai & Rai, 1998; Tam *et al.*, 2001; Pinto *et al.*, 2003; Rocchetta *et al.*, 2003, 2006a,b, 2007; Mendoza-Cozatl & Moreno-Sánchez, 2005). Effects of metal toxicity on metabolism depend on the species of organism, the nature and concentration of the metal, and the culture conditions (see reviews on photosynthetic organisms; Prasad & Hagemeyer, 1999; Küpper & Kroneck, 2005). The greatest damage caused by many heavy metals, including copper, in photosynthetic organisms results from the inhibition of photosynthesis, mainly of the light reactions (see reviews by Prasad & Strzałka, 1999; Küpper & Kroneck, 2005). One mechanism of inhibition of photosynthesis by heavy metals that was shown to occur, even at submicromolar concentrations, is the formation of heavy metal chlorophylls ([hms]-Chls), which are unsuitable for photosynthesis (Kowalewska & Hoffmann, 1989; Kowalewska *et al.*, 1992; Küpper *et al.*, 1996, 1998, 2002, 2003, 2006; Prasad *et al.*, 2001). This reaction strongly varies depending on the irradiance regime and the type of organism affected (reviewed by Küpper & Kroneck, 2005). It has also been postulated in various articles that reactive oxygen species (ROS) formation and, as a consequence, oxidative stress would be an important contribution to heavy metal stress (e.g. Pinto *et al.*, 2003). However, many experiments investigating oxidative stress in photosynthetic organisms have been carried out under conditions that are not environmentally relevant. In particular, the heavy metal concentrations used were far too high, values that almost never occur even in the most polluted environments. Oxidative stress can be caused directly by redox-active metals such as chromium and copper, but it can also be a secondary effect of the inhibition of photosynthesis and mitochondrial enzymes. It can cause changes in lipid composition, as well as severe damage to DNA (Watanabe & Suzuki, 2002; Pinto *et al.*, 2003; Watanabe *et al.*, 2003). As a result of metal-induced inhibition of metabolism, oxidative stress and genetic effects, alterations in cell morphology may occur (Kahoko *et al.*, 2003; Rocchetta *et al.*, 2007).

The unicellular protist *Euglena gracilis* is a useful model for studying cell damage caused by cytotoxic compounds, such as heavy metals (Einicker-Lamas *et al.*, 1996, 2002). Recently, it has been used to determine pollutant tolerance in urban aquatic ecosystems (Johnstone *et al.*, 2006). It grows under photoauxotrophic conditions like most euglenoids, but it is also able to develop under heterotrophic or photoheterotrophic conditions, depending on the culture medium and the light conditions (Barsanti *et al.*, 2000). *Euglena gracilis* cells grown under constant darkness can lose their chloroplast, but in a reversible manner (Schiff & Schwartzbach, 1982). Streptomycin (Sm) is an antibiotic that does not inhibit cell division or viability of *E. gracilis*, but 'bleaches' the cells by causing the permanent loss of plastids and plastid DNA in dividing photosynthetic cells and by blocking the development of chloroplasts in nondividing cells (Schwartzbach & Schiff, 1974). This effect may be linked to binding of Sm to the chloroplast ribosomes, resulting in a selective inhibition of plastid protein synthesis (Schwartzbach & Schiff, 1974).

Previous works revealed that toxic concentrations of Cr^{6+} in *E. gracilis* produce changes in cellular growth, proteins, lipids and carbohydrates (Rocchetta *et al.*, 2003, 2006a). Ultrastructural studies showed that the nucleus, mitochondria and chloroplasts were damaged by this metal, with the last organelles being the most affected (Rocchetta *et al.*, 2007). This was in concordance with changes in fatty acid contents. Polyunsaturated fatty acids (PUFAs) such as linolenic and linoleic acid, which are abundant in thylakoids and chloroplast membranes, were the most affected (Rocchetta *et al.*, 2006b).

In the present study, we analyse the inhibitory action of Cu^{2+} and Cr^{6+} on *E. gracilis* and the modulation of their toxicity by different culture media. We use two strains of *E. gracilis*: one isolated from a polluted river near Buenos Aires, Argentina (Ruiz *et al.*, 2004), and another obtained from a culture collection. The study was conducted under environmentally relevant light conditions and metal concentrations. The main method used for this study was the imaging microscopic measurement of photosynthesis biophysics via chlorophyll fluorescence kinetics under physiological conditions. This was performed with a new version of the fluorescence kinetic microscope (FKM; Küpper *et al.*, 2000a) described in Küpper *et al.* (2007a). Furthermore, photosynthesis and respiration were assessed via polarographic oxygen measurement. Changes in chlorophyll (including [hms]-Chl) and carotenoid composition, induced by metal treatments and growth media, were analysed in extracts of the algae via a novel spectroscopic method (Küpper *et al.*, 2007b). Finally, the involvement of

ROS in heavy metal toxicity was assessed in two ways. The formation of peroxides was traced in single cells in the FKM using a specific fluorescent dye, and superoxide anion released to the culture medium was measured using a luminescent dye.

Materials and Methods

Organisms, culture conditions and metal toxicity assays

The strains of *Euglena gracilis* used were UTEX 753 from the Culture Collection of Algae of Texas University (USA) and MAT isolated from the Matanza River near Buenos Aires, Argentina (Ruiz *et al.*, 2004, generously provided by Dr Visitación Conforti, from the Protistology Laboratory of Buenos Aires University, Argentina). Both photosynthetic strains (MAT and UTEX) and their variants, bleached with streptomycin (100 μg ml^{-1} during 7 d), SmMAT and SmUTEX, were used for this study (Ruiz *et al.*, 2004). Master and experimental cultures were grown in two mineral media with pH 7; Cramer & Myers (C&M) as a medium without an organic carbon source and Buetow with sodium acetate as a carbon source, which *Euglena* can incorporate and assimilate (Buetow, 1982). To get the same pH in both media, C&M medium pH was adjusted with 2 mM MES-NaOH (2-morpholinoethanesulphonic acid). All chemicals were analytical grade and purchased from Merck (Darmstadt, Germany). The algae were grown in 300 ml glass tubes at 26 ± 1°C, with continuous aeration. Irradiance was provided with daylight-white fluorescent tubes (Osram Dulux L 55W/12-950: Osram, München, Germany) that were computer-controlled to provide a sinusoidal light cycle with a maximum of 300 μmol m^{-2} s^{-1} irradiance at noon and a total photoperiod of 14 h (i.e. 10 h dark). This irradiance was chosen because it resembles a usual intermediate intensity in the natural habitat of *Euglena gracilis* (e.g. the Matanza river from which the MAT strain was isolated), reaching between 200 and 500 μmol m^{-2} s^{-1} (Conforti *et al.*, 1995; Gomez, 1997; Lamagna *et al.*, 2008). A new culture was initiated 6 d before each experiment in order to obtain an inoculum in exponential growth.

Aliquots of master cultures in exponential growth containing 2×10^4 cells ml^{-1} were inoculated in each flask for the toxicity assays, following the culture conditions mentioned earlier. Altogether, nine experiments were carried out, each of which lasted 7 d. Samples for the different measurements were collected on the second, fourth and seventh days. The algae were treated with $K_2Cr_2O_7$ or $CuSO_4$ (p.a., Merck, Darmstadt, Germany), added from stock solutions at the beginning of each experiment. We tested several concentrations (0.5, 1, 2, 5, 10, 20, 50 μM) for both metals, in order to find one close to the minimum concentration necessary to obtain 50% growth ($IC_{50,96h}$, as Environmental Protection Agency protocols). For this study, we used two metal concentrations, one far from the $IC_{50,96h}$ and the other close, taking into account the cell survival during seven experiment days. For the chromium treatment, the final concentrations were 2 and 10 μM. For copper they were much higher, 10 and 50 μM, because our *Euglena* cultures turned out to be relatively resistant to this metal.

Determination of growth by cell counts

Cell counts were performed with an automatic cell counter (Coulter Counter TAII, Beckman Coulter GmbH, Krefeld, Germany) and checked with a Neubauer chamber, with a systematic error of less than 10%, $\alpha = 0.05$ (Venrick, 1978). However, the noise of the cell counts was quite high, and caused an elevated noise in all other data that had to be normalized to the cell counts (i.e. oxygen exchange by respiration and photosynthesis, formation of superoxide, pigment concentrations).

Imaging chlorophyll fluorescence kinetic measurements

Measurement of chlorophyll fluorescence kinetics is a powerful method for investigating the physiological status of photosynthetic organisms, in particular in cases like heavy metal-induced stress where photosynthesis is a primary target of inhibition (Joshi & Mohanty, 2004; Küpper & Kroneck, 2005; Küpper *et al.*, 2007a). Chlorophyll fluorescence kinetic measurement is a more direct way to assess photosynthetic activity than gas exchange measurements, which only measure the difference between photosynthetic oxygen release and oxygen consumption. Therefore, in the current study it was the most important method for analysing physiological performance of the algae. It was carried out by two-dimensional (imaging) microscopic measurements, so that photosynthetic performance could be assessed on a single-cell level (Küpper *et al.*, 2000a). The new version of the FKM, used in this study, was described in detail in Küpper *et al.* (2007a).

Measurements Blue (410–500 nm) excitation was used, which was provided by white LEDs with the excitation filter 2P-HQ 460/80 (AHF, Tübingen, Germany) and the dichroic mirror 505DCXR (AHF). Chl fluorescence was detected from 665 to 705 nm with the emitter filter D680/30 (AHF). Actinic light was *c.* 350 μmol m^{-2} s^{-1}, like the growth irradiance of the cells, supersaturating flashes were 3200 μmol m^{-2} s^{-1}, measuring light was less than 2.5 μmol m^{-2} s^{-1}. To perform a measurement, 3 ml of algae culture obtained from the second, fourth and seventh experimental days were centrifuged and the pellet was suspended in 80 μl of C&M medium modified by omitting micronutrients and vitamins. To 80 μl *Euglena* cell suspension, 20 μl of a H_2DCF-DA solution to detect intracellular ROS was added (see details later) and then allowed to stand for 30 min at room temperature in the darkness. Cell suspension (50 μl) was then placed on to the glass window of the measuring chamber, mixed with 1%

low-melting agarose in order to restrict cell movement, and covered with cellophane pre-soaked in the modified C&M medium to hold the sample in place (see details in Küpper *et al.*, 2000a, 2007a). A stream of the modified C&M medium saturated with air was pumped through the measuring chamber at 26°C and a flow rate of 20 ml min^{-1}. Further details of the construction and operation of the chamber, as well as all details of the measuring protocol, have been published before (Küpper *et al.*, 2000b, 2007a).

Analysis of fluorescence kinetics The data were analysed using the FluorCam 6.0 software from PSI, as described earlier (Küpper *et al.*, 2000a, 2007a).

Quantification of pigments in algae extracts

Euglena gracilis cells were harvested by centrifugation of 100 ml of the cultures on day 7 of each experiment. They were frozen in liquid nitrogen, then lyophilized, and subsequently extracted in 100% acetone. Spectra of pigment extracts were measured with the UV/VIS spectrophotometer Lambda 16 (Perkin-Elmer, Waltham, MA, USA) at a spectral bandwidth of 1 nm with 0.2 nm sampling interval from 350 to 750 nm. Pigments were quantified according to the 'Gauss peak spectra' method of Küpper *et al.* (2000b), in its new version, which allows for a detailed analysis of carotenoids in addition to Chls. Its performance was also verified for *Euglena* extracts during its development (Küpper *et al.*, 2007b).

Oxygen measurements

Culture samples of 5 ml were collected during days 2, 4 and 7 of each experiment. Net photosynthetic oxygen release and respiratory oxygen uptake were measured using a Clark-type electrode (Theta'99, Praha, Czech Republic) in a 2 ml temperature-controlled (26 ± 1°C) measuring chamber. The algae were kept in the dark to measure oxygen consumption by dark respiration and then exposed to the light (*c.* 300 μmol m^{-2} s^{-1}, i.e. growth irradiance) for measuring net photosynthetic oxygen release. Data were recorded using the OxyCorder measuring device with the software Oxywin 2.71 (Photon Systems Instruments, Brno, Czech Republic). This software was also used for analysis. The results were expressed as oxygen exchange per cell using the cell counts of the same samples (see earlier).

Reactive oxygen species (ROS) measurements

Superoxide anion determination by chemoluminescence Extracellular superoxide anion was detected by chemoluminescence using a LKB 1250 luminometer. Stock solutions of 10 mM 2-metil-6-(4metoxipentil)-3,7-dihydroimidazol 1,2-apirazin-3-1 hydrochlorhydrate (MCLA) were prepared with redistilled water under anoxic and dark conditions. Aliquots of these stocks were diluted in C&M medium (without micronutrients and vitamins to avoid any interference during the measurement) in anoxic conditions at a concentration of 1 μM. These aliquots were frozen with liquid nitrogen and stored at −80°C. Samples (2 ml) of the algal cultures were taken on the second, fourth and seventh experimental days and harvested by centrifugation. The pellet was resuspended in the modified C&M medium mentioned earlier, reaching a final volume of 2 ml before an MCLA aliquot was added (250 μl). We measured the initial MCLA luminescence to quantify the amount of superoxide anion released into the culture medium by the cells. The data were recorded by the analogue/digital converter input channel of the OxyCorder with the software Oxywin 2.71 (see earlier). The results were expressed as arbitrary units of chemoluminescence per cell (AUC/cell) using the cell counts of the same samples (see earlier). To asses the extent of interference from cell-independent chemical reactions of the medium, controls were measured using the culture medium without cells, with and without metals.

Detection of intracellular hydroperoxides with H2DCF-DA 5- (and 6-) -carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, purchased from Invitrogen) was used to detect intracellular peroxides in both strains of *E. gracilis*, MAT and UTEX, and their bleached cultures, SmMAT and SmUTEX, grown under the different conditions mentioned earlier. Aliquots of H_2DCFDA (100 μM) were dissolved under anoxic conditions in the modified C&M culture medium as for the MCLA measurements, frozen in liquid nitrogen and stored at −80°C. Culture samples (3 ml) obtained from the second, fourth and seventh experimental days, with and without treatment, were centrifuged and the pellet was suspended in the modified C&M medium (80 μl). To 80 μl *Euglena* cell suspension, 20 μl of H_2DCFDA was added and then allowed to stand for 30 min at room temperature. Incorporation of H_2DCFDA into *E. gracilis* was carried out in darkness in order to reduce decomposition of the probe. The peroxide-specific fluorescence was measured in the FKM using filter set F41-028 (HQ-YFP) from AHF (Tübingen, Germany) with an excitation 490–510 nm, 515 nm dichroic mirror and 520–550 emission filter. The measuring protocol consisted only of one flash of 1 s duration and 650 μmol m^{-2} s^{-1} intensity in order to minimize artefactual peroxide generation by the dye itself as well as photobleaching, which can cause serious problems in conventional fluorescence microscopes typically using many thousand μmol for a prolonged time of excitation (discussed in Küpper *et al.*, 2000a).

Statistical analysis

The data shown in the results section are averages from three independent experiments for each strain and growth medium,

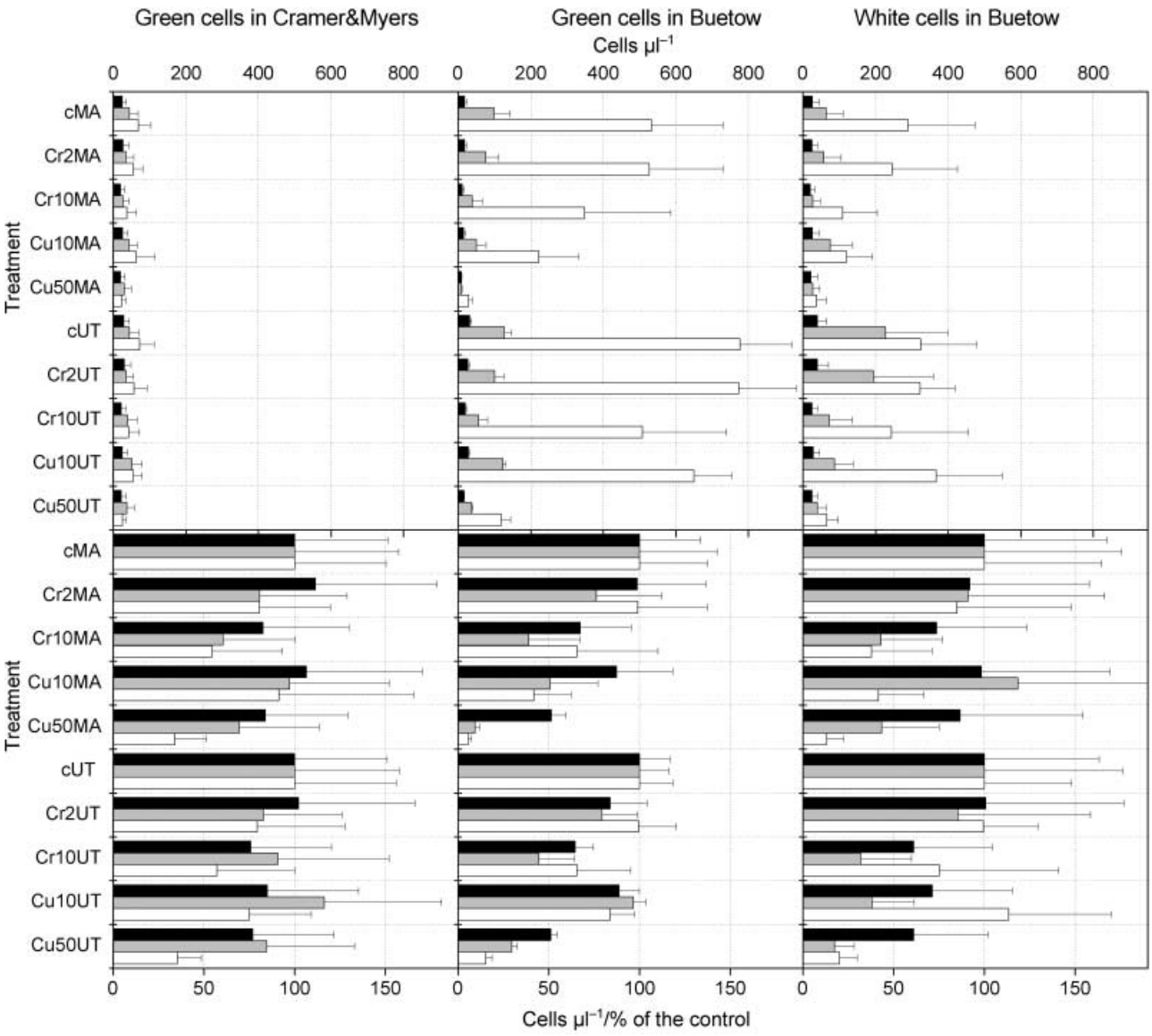

Fig. 1 Effects of culture media and metal treatments on growth of green and white cells of the UTEX and MAT *Euglena gracilis* strains. The absolute cell counts (top panels) show the effects of the media and the increasing cell density during the experiment, while the relative cell counts (bottom panel) emphasize the effect of the metal treatments. Time after start of metal treatment: black bars, day 2; grey bars, day 4; white bars, day 7. The data shown are averages and standard errors from three experiments.

each experiment consisting of the control and the metal treatments. Repeated-measures ANOVAs were made for the cellular growth, the oxygen exchange and the superoxide anion release determinations, in order to compare metal treatment and time, for each culture medium and strain. Comparisons were performed using Tuckey's test from STATISTICA 8.0 and the significance level was at least $P < 0.05$. Absolute values were used for analyses. For the pigment content, two-way ANOVAs were made in order to evaluate the differences between metal treatments and the culture media, for each strain. Further comparisons were carried out using Duncan's test from STATISTICA 8.0 ($P < 0.05$).

Results

Growth characteristics

Growth was analysed in terms of cell numbers in order to find out about the overall vitality of the cells under the different treatment conditions.

First, it should be noted that growth of the green cells of both strains was generally much faster in Buetow medium, which allows for a mixed autotrophic/heterotrophic metabolism, than in C&M medium, which forces the cells into photoauxotrophic growth (Fig. 1).

Cellular growth was affected in both MAT and UTEX strains, depending on the treatment assayed and the culture conditions (Fig. 1). Mostly, cell numbers as a percentage of the control decreased depending on the metal concentration and the exposure time. The strongest growth inhibition, *c.* 30–50% on the fourth and 80% on the seventh day, was observed at 50 μM Cu^{2+}. With 10 μM Cr^{6+} in C&M medium, growth of both strains was inhibited by 10% compared with the controls on the second treatment day, reaching 30–50% on the fourth day. Green cells cultured in Buetow medium in the presence of metal showed the same tendency as those grown in C&M. On the second day, a growth inhibition of 40% was observed for both strains at 10 μM Cr^{6+} and 50 μM Cu^{2+}, and 60–70% inhibition was reached on the fourth treatment day. Growth inhibition of 10% with 10 μM Cu^{2+} was observed on this day for both strains. On the last (seventh) day, a total recovery was observed for cells treated with 10 μM Cr^{6+}. By contrast, for cells treated with 50 μM Cu^{2+}, growth inhibition increased significantly ($P < 0.05$) to 90%. White cells of UTEX and MAT strains showed an inhibition of 20–40%, with the higher concentrations of both metals on the second day of treatment. On the fourth day, the inhibition reached 60–70% for 10 μM Cr^{6+} and 50 μM Cu^{2+}, and 20–30% for 10 μM Cu^{2+}. A recovery was observed on the last exposure day for cultures treated with the highest chromium concentration (reaching 40% of inhibition), while the opposite was found for 50 μM Cu^{2+}, increasing the cellular growth inhibition to 80%.

Oxygen exchange

Oxygen exchange was analysed in order to find out how the different treatments affected the balance between photosynthetic oxygen release and respiratory oxygen uptake.

Oxygen exchange was altered by both metal treatments, in both strains cultured under all conditions (Fig. 2). Photosynthetic oxygen release and respiratory oxygen uptake were evaluated during days 2, 4 and 7 of treatment. No changes in respiration were observed in green cells of both strains when exposed to 2 μM Cr^{6+}. In white cells, by contrast, this treatment seems to increase respiration in MAT cells on day 7 and in UTEX cells on days 2 and 4, while decreasing it in UTEX cells on day 7 of treatment. Furthermore, 10 μM Cr^{6+} and both copper concentrations increased respiration in white MAT cells throughout the experiment, while in white UTEX cells this enhancement of respiration was observed only on the second day. A very strong decrease of respiration was observed for 10 μM Cr^{6+} and both copper concentrations on the seventh treatment day. In green cells, similar trends of enhancement of respiration were observed, but strong inhibition in the UTEX strain on the seventh treatment day did not occur at any of the tested metal concentrations.

At most treatment time-points and at all metal concentrations, an inhibition of photosynthesis was observed (Fig. 2). However, since the respiration data were already noisy and gross photosynthesis had to be calculated by subtracting respiration from the net oxygen release data, the data of photosynthetic oxygen release became even more noisy, so that trends were less consistent than those observed in the respiration data. Nevertheless, three important observations could be made. First, even the 2 μM Cr^{6+} treatment, which did not affect respiration in the green cells, decreased photosynthetic oxygen release in the MAT strain. This was observed in both growth media and occurred mainly at the beginning of the stress. In UTEX cells, this treatment only decreased photosynthesis on day 2 in Buetow medium. Second, metal-induced inhibition of photosynthetic oxygen release was generally stronger in Buetow than in C&M medium for both strains and under almost all treatment regimes. The strongest inhibition was found for both MAT and UTEX cells upon treatment with 50 μM Cu^{2+}. Third, most metal treatments in Buetow medium resulted in a significant decrease of photosynthetic oxygen production on day 2, and a recovery on days 4 and 7, except treatment with 50 μM Cu^{2+}. The latter treatment produced a decrease during these days in cells of both strains, being significant ($P < 0.05$) only for day 7 (Fig. 2).

Chlorophyll fluorescence kinetics

General features of *Euglena* photosynthesis and growth In order to understand the differences in effects of the metal treatments that were caused by the different growth media and *Euglena* strains, the influence of these factors was first analysed in the control cells. Growth in C&M medium led to more investment in the photosynthetic apparatus. This was already clear from the pigment contents, which were reflected in this study in high total fluorescence (F_0 and F_m) in cells grown in C&M medium. A decreasing tendency was observed in these parameters during the fourth and seventh culture day in C&M medium (Fig. 3).

Photosynthetic quantum efficiency of PSII in the dark-adapted state (measured by F_v/F_m) and in the light-adapted state (measured by the Φ_{PSII} parameter; Genty *et al.*, 1989) was not higher in C&M compared with Buetow medium. An interesting side-effect was the change of activity of the cells during the 7 d of culture. On day 2, cells grown in C&M medium were photosynthetically rather inactive, with a very low value of F_v/F_m (0.05). In the subsequent days, photosynthetic activity increased, but remained generally low, with a maximum of 0.2, while corresponding values for healthy higher plants are 0.83 (Fig. 4).

Often, negative NPQ values were observed. These were most pronounced on day 4 of growing UTEX cells in either Buetow or C&M medium without metal stress, but sometimes also occurred under metal stress and in the MAT strain (Fig. 5). Whenever such negative NPQ values were observed, they were usually correlated with higher Φ_{PSII} in

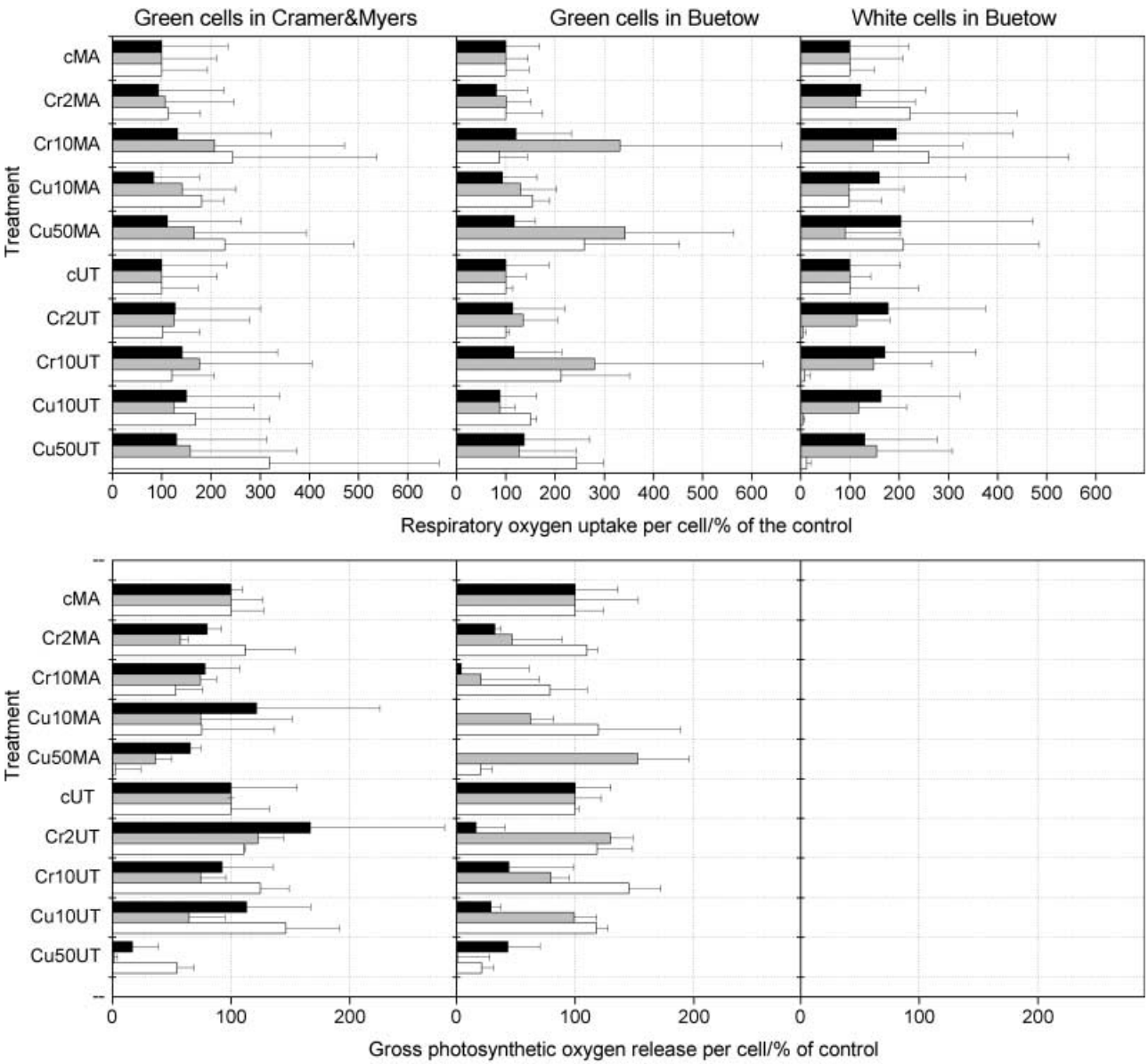

Fig. 2 Changes in oxygen exchange in *Euglena gracilis* cells during stress and acclimation in response to chromium and copper treatment. Time after start of metal treatment: black bars, day 2; grey bars, day 4; white bars, day 7. The data shown are averages and standard errors from three experiments.

actinic light (SIPs 1–4) compared with the dark-adapted F_v/F_m (= SIP0; cf. Figs 4 and 5).

Modulation of metal effects on *E. gracilis* by different growth media Changes in the absolute fluorescence parameters (F_0 and F_m) were observed in *E. gracilis* cells of both strains, but only in C&M medium and only in those cells cultured in C&M treated with 50 μM Cu^{2+} after 4 and 7 d of treatment, where a significant increase occurred (Fig. 3).

The metal-induced inhibition of photochemistry was generally stronger in C&M than in Buetow medium. This difference was best visible in the MAT strain, on day 4 of treatment with 10 μM Cr^{6+} and on day 2 of treatment with 10 μM Cu^{2+} (Fig. 4). Metal-induced changes in photochemistry as judged by F_v/F_m and Φ_{PSII} parameters were significantly stronger than the changes in F_0 and F_m. The strongest inhibition of photochemistry was found in cells treated with 50 μM Cu^{2+} and mostly occurred on the days 4 and 7. No changes were observed with 2 μM Cr^{6+}, while for 10 μM of either Cr^{2+} or Cu^{2+}, a significant inhibition was observed on days 2 and 4. For both treatments in both the MAT and the UTEX strain, in Buetow medium a recovery of photochemistry occurred during the last treatment day, while in C&M medium the inhibition was identical on days 4 and 7 (Fig. 4).

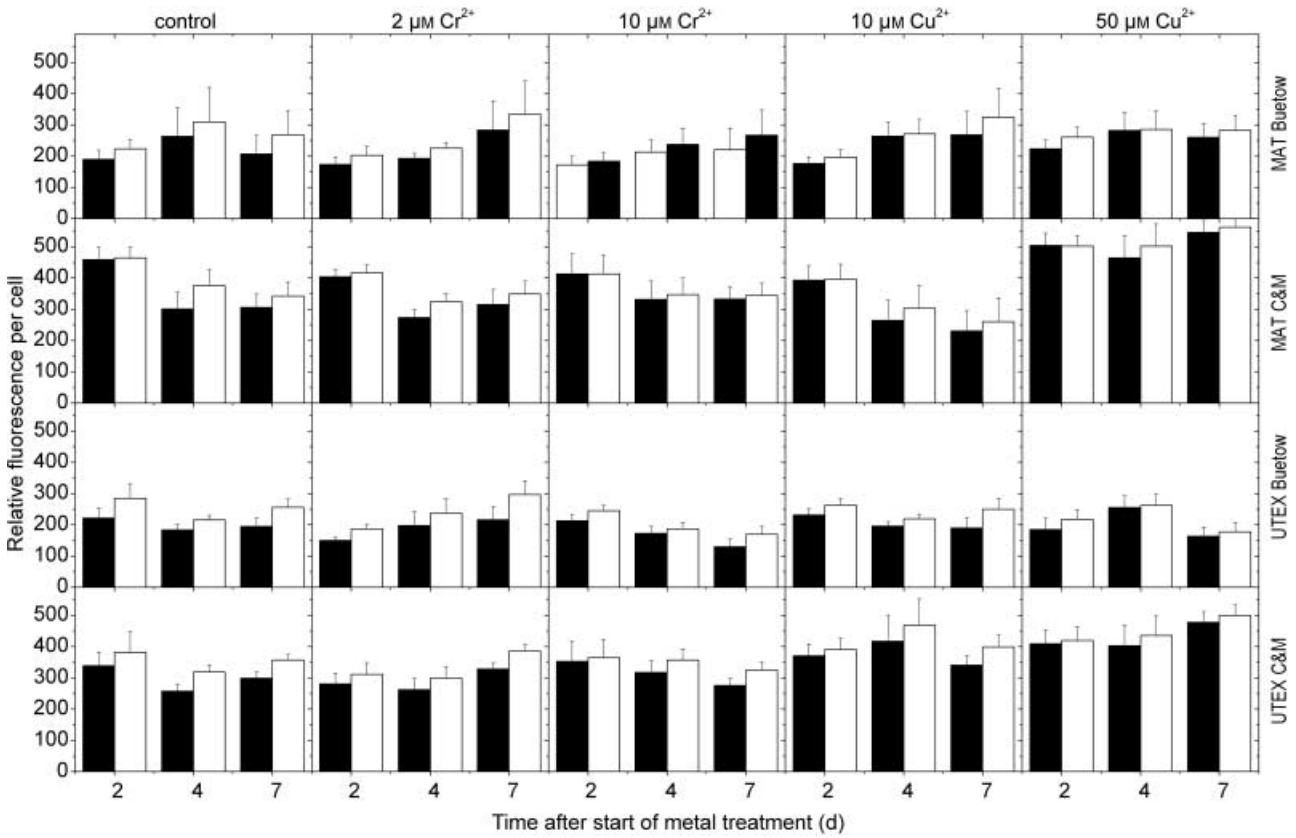

Fig. 3 Changes of absolute fluorescence parameters F_0 (black bars) and F_m (white bars) in *Euglena gracilis* cells during stress and acclimatization in response to chromium and copper treatment. The data shown are averages and standard errors from three experiments and were measured with the fluorescence kinetic microscope (FKM) at an actinic irradiance of 350 μmol photons m^{-2} s^{-1}.

Heavy metal treatment led to an increase of NPQ. This was already obvious in the 2 μM Cr^{6+} treatment of the MAT strain in Buetow medium, and became evident in both strains and both media at 10 μM Cr^{6+}, mainly on the second day of exposure, and at 50 μM Cu^{2+} on all days (Fig. 5).

Differences in the inhibitory action between copper and chromium Treatment with copper led to clearer effects on photochemistry than with chromium. Although treatment with 50 μM Cu^{2+} led to similar inhibition of cell growth as treatment with 10 μM Cr^{6+} for a period of 96 h (following the EPA protocols as $IC_{50,96\,h}$), the inhibition of photochemistry was much stronger in the former than in the latter (cf. Figs 1 and 4). The increase in NPQ was also strongest in the latter treatment (Fig. 5), but the effect of Cr^{6+} and Cu^{2+} was generally more similar than for the photochemical fluorescence quenching parameters.

Differences between the two *Euglena* strains (MAT vs UTEX) Differences between the strains were not very pronounced. Unexpectedly, however, MAT was somewhat more sensitive to both copper and chromium toxicity. This was most evident in the stronger inhibition of photochemical activity in both the 10 and 50 μM copper treatments, but it was also visible in the stronger inhibition of photochemistry (Fig. 4) and stronger increase of NPQ (Fig. 5) on day 2 of 10 μM Cr^{6+} treatment. The increase in NPQ in response to copper stress, by contrast, was generally not stronger in MAT than in UTEX.

Pigment composition

Control cells grown in C&M contained a higher amount of total pigments than cells grown in Buetow medium (Fig. 6). MAT cultures, in both culture media, showed basal amounts of oxidative degradation products of carotenoids (e.g. diadinochrome = diadinoxanthin-5,8-epoxide and aurochrome = β-carotene-5,8,5′,8′-diepoxide). The most abundant pigment in *E. gracilis* was Chl*a*, followed by diadinoxanthin, Chl*b* and neoxanthin. Keto-carotenoinds and β-carotene-like carotenoids were also found in very low concentrations (Fig. 6). The ratio Chl : carotenoids decreased significantly ($P < 0.05$) in both strains grown in Buetow medium with the 50 μM Cu^{2+} treatments compared with the controls. The MAT strain showed a decreasing Chl : carotenoid ratio with both copper concentrations and with 10 μM chromium.

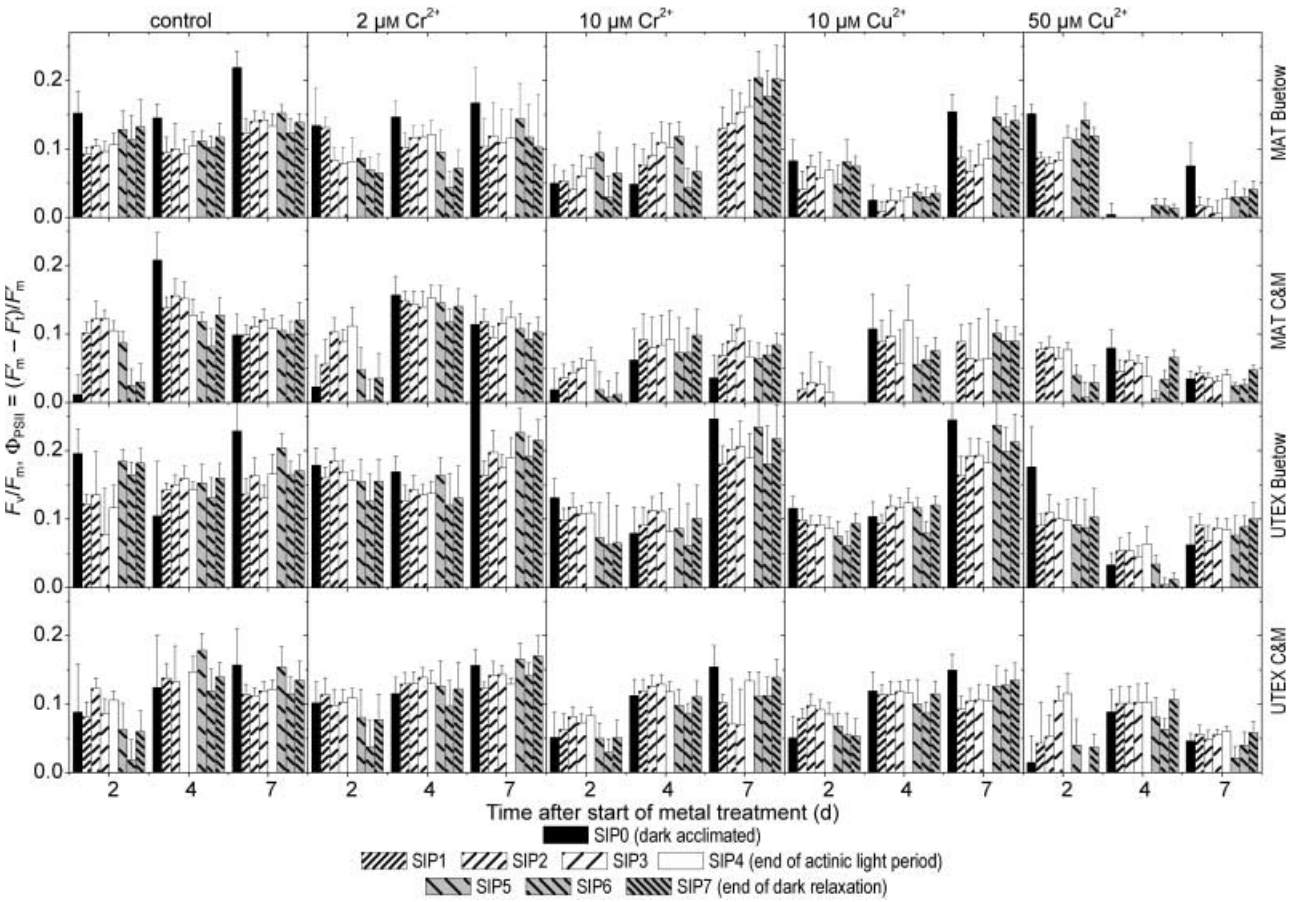

Fig. 4 Changes of fluorescence kinetic parameters related to photochemistry, F_v/F_m and Φ_{PSII} during stress and acclimatization in response to Cr^{6+} and Cu^{2+} treatment. The data are averages and standard errors from three experiments and were measured with the fluorescence kinetic microscope (FKM) at an actinic irradiance of 350 μmol photons m^{-2} s^{-1}. SIP, 'saturating irradiation pulse', that is, the flashes used for measuring photochemical vs nonphotochemical quenching. SIPs 1–4 were in the actinic light period of the measuring protocol; SIPs 4–6 were in the dark relaxation afterwards.

It was possible to detect small amounts of [Cu]-Chl*a* in cells of both strains in both growth media when the cells were treated with 50 μM Cu^{2+}. [Cu]-Chl*a* was also detected in the 10 μM Cu^{2+} treatments, but only for cells grown in Buetow medium. As expected, controls did not contain any detectable amounts of Cu-Chl. The possibility of formation of [Cr]-Chl was tested in methanolic solution of pure Chl*a* with subsequent purification by HPLC, but it was not found in extracts of the Cr-stressed cells, meaning that either the amounts were below the detection limit or there was no *in vivo* Cr-Chl formation. In the Buetow medium, Cr and Cu stress led to reduced Mg-Chl*a* amounts per cell, with the lowest values found in cells grown with 50 μM copper, being significant ($P < 0.05$) only for UTEX strain. Chl*b* was less affected, except for the 50 μM Cu^{2+} treatment, where a significant difference was found for the UTEX strain. Cells grown in C&M presented no significant differences in the Chl content, after treatment with either metal.

The same tendencies as for Chl*a* were observed for neoxanthin in cells grown in Buetow medium. In C&M medium, only 50 μM Cu^{2+} in the MAT strain caused a significant ($P < 0.05$) decrease in neoxanthin concentrations. Diadinoxanthin concentrations varied most in response to the copper treatments, especially in cells grown in Buetow medium. In response to metal stress, an increase in the amounts of β-carotene-like carotenoids was observed in cells of both strains cultured in C&M medium. An increase in diatoxanthin was observed only in the UTEX strain treated with 50 μM Cu^{2+}; this was most pronounced in C&M medium. Keto-carotenoids did not change remarkably under metal stress and no consistent trend was observed. An increase in oxidative degradation products of carotenoids (measured as aurochrome and diadinochrome) was found in most of the treated cells. This was most pronounced in C&M medium under both Cr and Cu stress (Fig. 6).

ROS measurements

Comparing the control cultures, release of superoxide into the medium was stronger in cells grown in Buetow compared

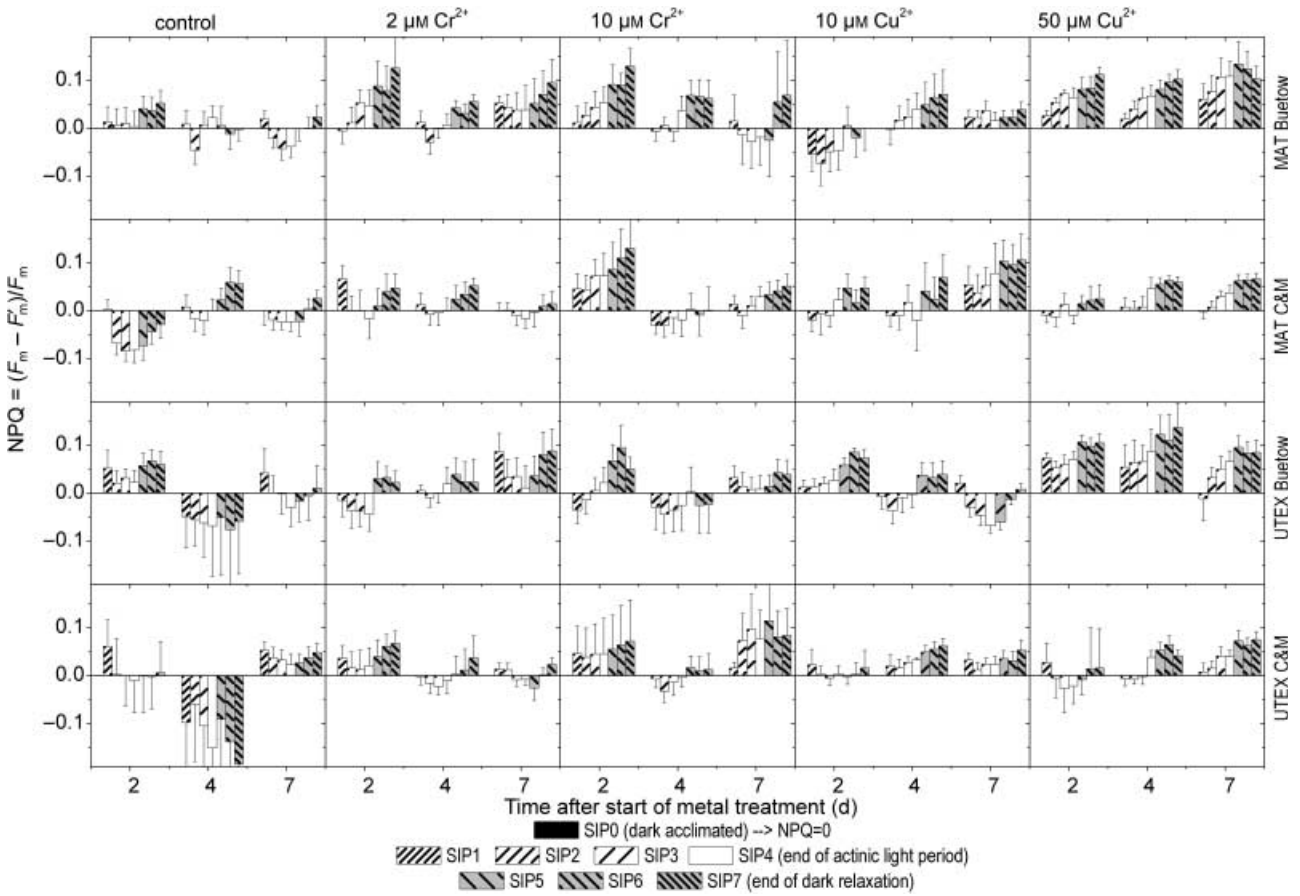

Fig. 5 Changes of fluorescence kinetic parameters related to nonphotochemical energy dissipation, NPQ, in *Euglena gracilis* cells during stress and acclimatization in response to Cr^{6+} and Cu^{2+} treatment. The data are averages and standard errors from three experiments and were measured with the fluorescence kinetic microscope (FKM) at an actinic irradiance of 350 μmol photons m^{-2} s^{-1}. SIP, 'saturating irradiation pulse', that is, the flashes used for measuring photochemical vs nonphotochemical quenching. SIPs 1–4 were in the actinic light period of the measuring protocol; SIPs 4–6 were in the dark relaxation afterwards.

with C&M medium for the MAT strain (Fig. 7). In Buetow medium, the green MAT cells released more superoxide than the white MAT cells. The superoxide release from green MAT and UTEX cells in Buetow medium decreased with the culturing time, while in C&M medium it was highest on day 4.

Furthermore, independent of metal treatment effects, cells of the MAT strain generally released more superoxide into both culture media compared with the UTEX strain.

In response to metal toxicity, MAT cells cultured in C&M medium showed an increase in superoxide release. This was observed in all metal treatments with the highest O_2^- release usually on day 4 of treatment (also in the controls), except for 50 μM Cu^{2+} where it was highest on day 7. In UTEX cells grown in C&M medium, the effect was evident only in the higher metal concentrations. On day 2, no changes of O_2^- release compared with the control were observed with the treatments of green cells, except for an increased O_2^- release in the 50 μM Cu^{2+} treatment in the MAT strain grown on C&M. In white MAT and UTEX cells, by contrast, the increases in superoxide release were already fully present on day 2. They did not change much during the following days in the white MAT cells, but decreased in the white UTEX cells (Fig. 7).

Attempts were made to correlate the photosynthetic response in one cell with the ROS generated by the oxidative damage. Unfortunately, we could not detect enough cells with hydroperoxide vacuoles to be statistically significant. Only very rarely, in far less than 1% of the cells, were we able to detect the presence of hydroperoxide in vacuoles of Cu- or Cr-treated cells (Fig. 8). Surprisingly, these cells with peroxide staining, appeared only in the lower of the two metal concentrations applied.

Discussion

In the current study, we have investigated the effects of toxic concentrations of Cu and Cr on two contrasting strains of *E. gracilis*, with and without chloroplasts, grown in culture media promoting either phototrophic or heterotrophic growth. This has led to insights into the mode of action of these

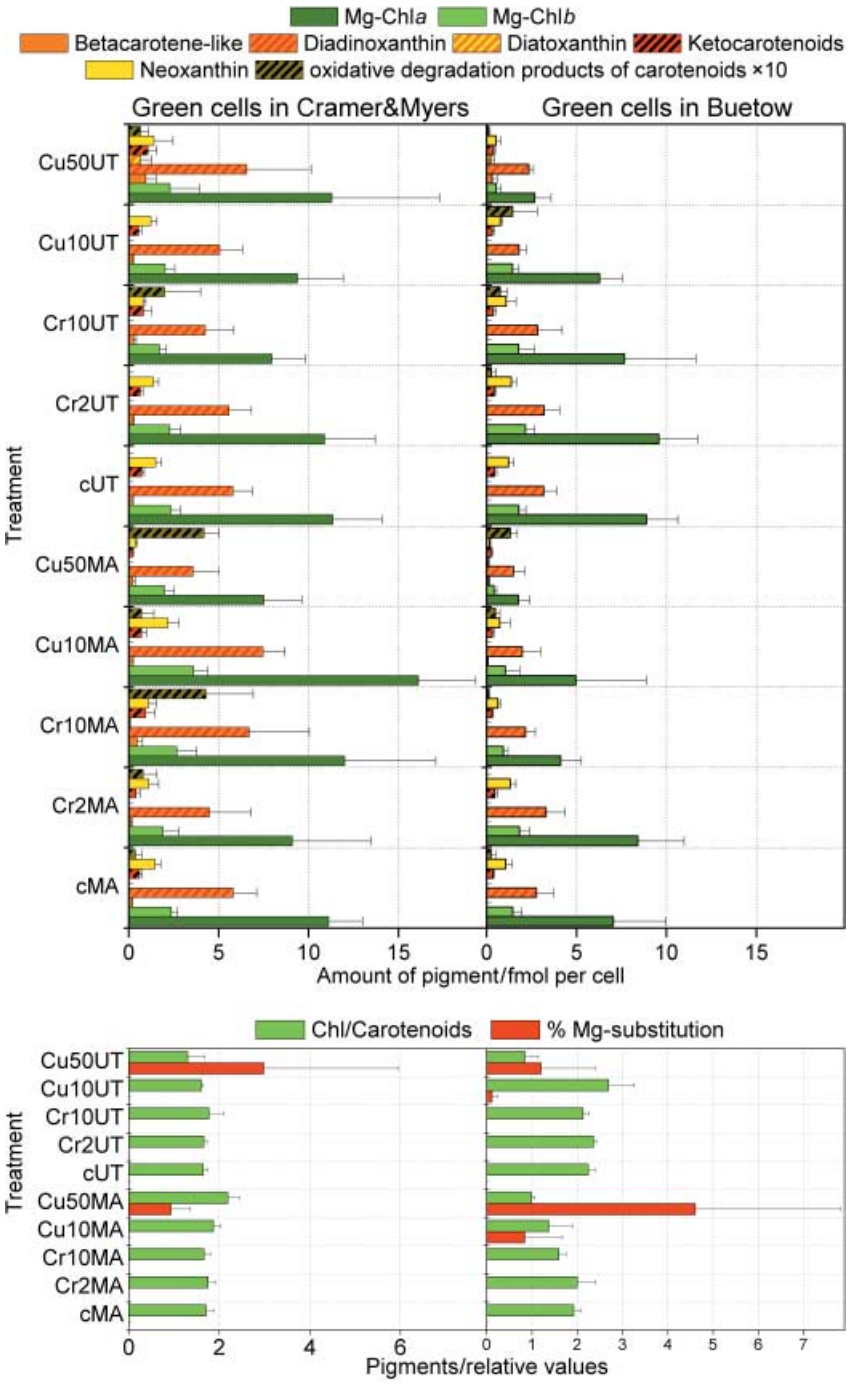

Fig. 6 Changes of chlorophyll and carotenoid content in *Euglena gracilis* cells during stress and acclimatization in response to chromium and copper treatment. The data shown are averages from three experiments.

metals, as well as into the regulation of phototrophic ('plant-like') vs heterotrophic ('animal-like') metabolic pathways in *Euglena*.

Mode of action of copper and chromium toxicity in *Euglena*

Our data have shown that *Euglena*, in contrast to many higher Chlorophyta (Küpper *et al.*, 1996), is rather resistant to copper toxicity; this applied to both strains examined. This is probably related to the fact that Euglenophyte*s* are phylogenetically rather distant to Chlorophyta, and are probably more related to animals, which are usually less copper-sensitive than plants (Melkonian, 2001; Nudelman *et al.*, 2003).

Sequence of events (inhibitions) during copper and chromium stress In the current study, when both metals reached the threshold of toxicity, they inhibited growth and photosynthesis,

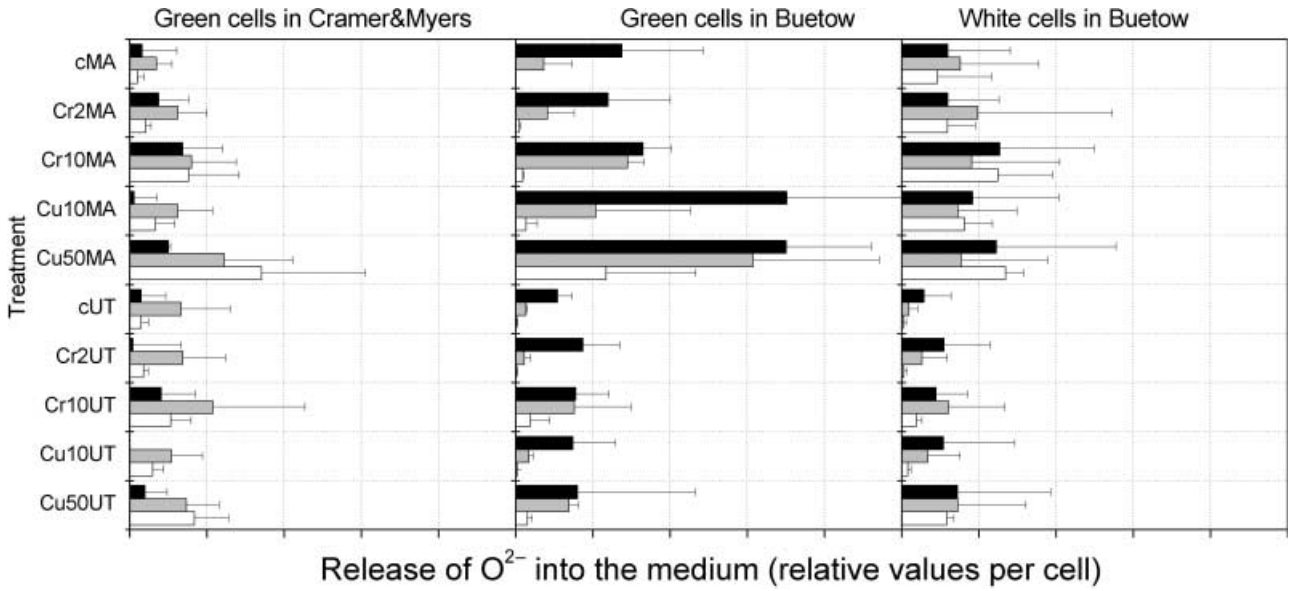

Fig. 7 Changes in superoxide release by *Euglena gracilis* cells during stress and acclimatization in response to chromium and copper treatment. Time after start of metal treatment: black bars, day 2; grey bars, day 4; white bars, day 7. The data shown are averages and standard errors from three experiments.

decreased the amounts of pigments needed for photosynthesis, changed respiration and increased the amounts of ROS. While all these facts, viewed alone, are not new, the combination of treatments and measuring methods applied in the current study revealed some insights into causal relationships between these changes.

As a starting point, it should be noted that, obviously, the presence of a photosynthetic apparatus drastically changes the effects of metals on the respiratory system. While a strong inhibition of respiration occurred in white UTEX cells on day 7 of all Cr and Cu stresses applied, this did not occur at all in the green cells, indicating that these metals have a higher affinity to binding targets in the photosynthetic apparatus than in the respiratory system. This conclusion is further supported by the observation that small Cr^{6+} concentrations (2 μM) did inhibit photosynthetic oxygen release, but not respiration; even after 2 d in the MAT strain, and also in most other cases the inhibition of photosynthetic oxygen release was stronger and/or faster than any alteration in respiration.

This comparison may already help to explain the next observed phenomena: the increases of respiration and ROS (mainly superoxide) during metal toxicity stress in the green cells. A malfunctioning photosynthetic apparatus will inevitably divert the absorbed light energy towards processes other than the desired photochemical electron transport chain and subsequent dark reactions. If the excited states of Chls cannot be used, they will either be emitted as fluorescence, as observed here in increased F_0 and F_m values, or released as heat (nonphotochemical quenching), or they will be accidentally transferred to oxygen, resulting in the production of ROS. In addition, metals such us Cr^{6+} have been shown to generate ·OH radicals from H_2O_2 via a Fenton-type mechanism (Shi & Dalal, 1990), although the *in vivo* environmental relevance of the Fenton reaction in heavy metal-stressed plants or algae has never been shown. We could measure ROS directly (mainly as superoxide via MCLA) and indirectly (as carotenoid oxidation products) in our study. The latter way of 'indirect detection' is actually another hint that the ROS originated from photosynthesis. Furthermore, in our experiments, enhanced superoxide release into the medium was mainly observed in green cells, not in white cells grown at the same metal concentration in the same growth medium. This is a strong hint that the ROS were not generated by the Fenton reaction, which would have been the same in both white and green cells, but rather by malfunctioning photosynthesis, which was present only in the green cells.

Regardless of how the ROS are generated, they attack thylakoid lipids, mainly unsaturated fatty acids. This initiates peroxyl-radical chain reactions, destroying membranes and damaging indirectly structural pigment–protein complexes located in chloroplast membranes. This was observed in *Chlamydomonas* treated with Cr^{6+} (Rodríguez *et al.*, 2007). Some authors described important mitochondria alterations, chloroplast disorganization and changes in the protein and lipid composition caused by Cd, Cu and Zn in *E. gracilis* (Einicker-Lamas *et al.*, 2002, Mendoza-Cozatl & Moreno-Sánchez, 2005). Observations by TEM in chromium-treated *Euglena* cells showed chloroplasts with disorganized thylakoids, and mitochondrial nets with hypertrophic development (Rocchetta *et al.*, 2007). Such hypertrophic development

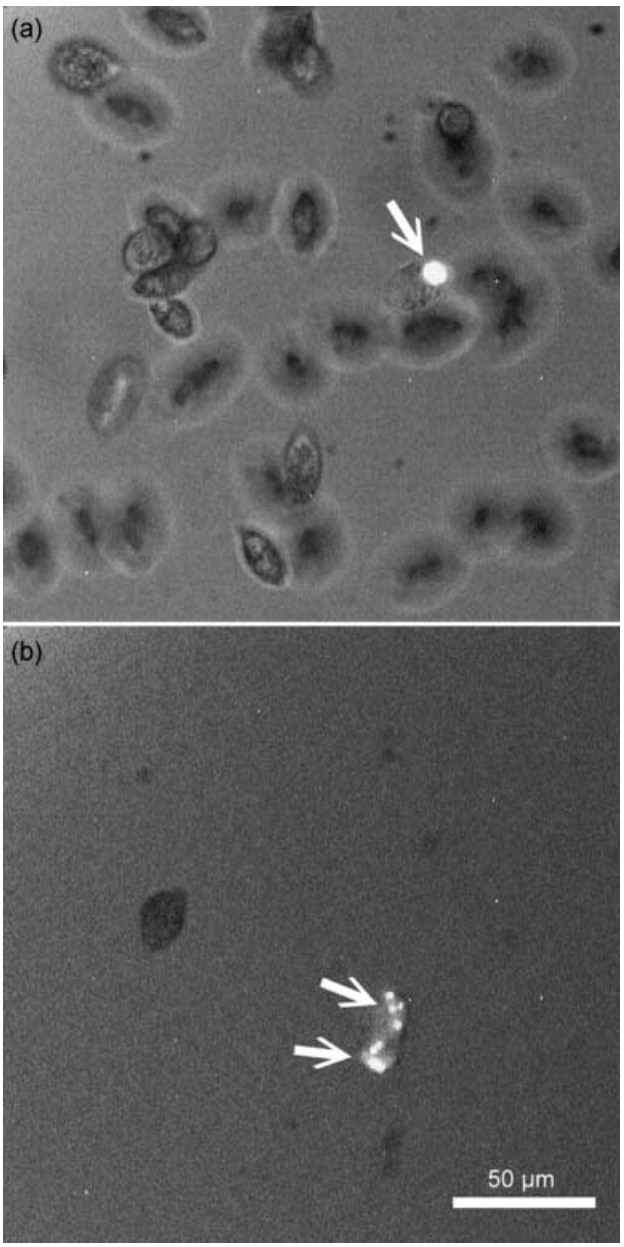

Fig. 8 *Euglena gracilis* cells treated with the lower chromium and copper concentrations. The pictures show the presence of hydroperoxide in vacuoles of Cu-treated (a) or Cr-treated (b) cells (arrows).

could add to the increase in the respiratory oxygen uptake observed in the current study, mainly at the highest metal concentrations, which could otherwise be related to uncoupling. Previous works investigating lipid composition in *E. gracilis* showed that the fatty acids damaged most by chromium exposure were polyunsaturated fatty acids (PUFAs) related to photosynthetic activity, besides the increase of the malondialdehyde (MDA) content measured as a lipid peroxidation product (Rocchetta *et al.*, 2006b).

Mechanisms of Cr^{6+}- and Cu^{2+}-induced photosynthesis inhibition As photosynthesis was obviously a prime target of Cr^{6+} and Cu^{2+} toxicity, a closer look at the mechanisms of this inhibition may be appropriate at this point. The treatment with copper led to clearer effects on photosynthesis than the chromium treatments. Whilst 50 μM Cu^{2+} treatment led to similar inhibition of cell growth as the 10 μM Cr^{6+} treatment for a period of 96 h (following the EPA protocols as $IC_{50,96\,h}$), the inhibition of photochemistry was much stronger in the former. Furthermore, while the 2 μM Cr^{6+} treatment did not cause a reduction in growth, it did decrease photosynthesis in the MAT cells grown on Buetow medium. It seems that the cells were still able to compensate for the decreased photosynthesis by enhancing heterotrophic pathways, which are obviously less metal-sensitive.

Overall, the Cr- and Cu- induced decrease of F_v/F_m showed that the reaction centres were affected more than the antenna, that is, the 'sun reaction' type of damage took place with an insertion of the heavy metals into the PSII reaction centre (Küpper *et al.*, 1996, 1998, 2002), at least for copper in the pheophytin a of the PS II RC (Küpper *et al.*, 2002). Furthermore, neither Cr nor Cu stress caused a decrease of total fluorescence per cell; rather, an increase occurred at 50 μM copper, consistent with the 'sun reaction' type of damage (Küpper *et al.*, 1998, 2002). Finally, pigment analysis indicated that only a low percentage of Mg substitution by copper had occurred after treatment with 50 μM Cu^{2+}, again typical for the 'sun reaction'. However, the improved sensitivity of the pigment analysis (Küpper *et al.*, 2007b) allowed even these small amounts of [Cu]-Chl in extracts of the stressed cells to be determined. Despite having some [Cu]-Chl in the 10 μM Cu treated cells grown in Buetow medium, the cultures did not show inhibition according to the oxygen measurements conducted on the seventh treatment day. However, a large decrease of oxygen production and photochemical fluorescence quenching occurred during the first days of treatment, followed by a total recovery during the last days. Therefore, in combination with the appearance of the [Cu]-Chl, we can make several conclusions. First, the [Cu]-Chl formation, decrease of oxygen release and photochemical quenching show that photosynthesis was again sensitive to inhibition. Second, the cells were obviously able to overcome the inhibition, probably again by enhancing their alternative heterotrophic lifestyle until the copper concentration in the medium had decreased enough that newly synthesized photosystems would no longer be in danger of Mg substitution. The [Cu]-Chl in the harvested recovered cells most likely originated from the inhibition in the previous days. In contrast to Mg-Chl, [Cu]-Chl is stable towards light, oxygen and acids, so that it should stay in the cells even if it is no longer bound in a pigment–protein complex. No [Cu]-Chl was detectable in the 10 μM Cu^{2+}-treated cells in C&M medium, which is in line with the oxygen measurements and growth data that show no photosynthesis inhibition, and also broadly agrees with the cell counts.

In our experiments, NPQ increased during heavy metal stress. This might be, at first glance, interpreted as an inhibition of the Calvin cycle. However, if the Calvin cycle really were inhibited more than the light reactions, this would lead to a strong decline in Φ_{PSII} (photochemical quenching in actinic light), because the $NADP^+$ reduction to $NADPH/H^+$ as well as the proton gradient over the thylakoid membrane would saturate since ATP and NADPH would no longer be consumed. This would lead to a feedback-downregulation of photosynthesis, so that Φ_{PSII} would be more affected by heavy metal stress than F_v/F_m. In our *Euglena* measurements, the opposite was actually the case; F_v/F_m decreased slightly more than Φ_{PSII}, showing that the photosynthetic light reactions were more affected (by PS II RC inhibition, see earlier) than the dark reactions.

Regulation of phototrophic vs heterotrophic pathways in *Euglena*

Regulation of photosynthesis in the control cultures Cells cultured in C&M medium, in comparison with cells grown in Buetow medium, invested more in the photosynthetic apparatus, which was reflected in higher pigment amounts (chlorophylls and carotenoids) leading to higher fluorescence (F_0 and F_m). This difference is related to the fact that cells cultured in a mineral medium such as C&M use photosynthetic activity as their main energy source , as opposed to the Buetow culture medium which has an alternative carbon source available to be incorporated by the cells. Although the cells grown in C&M had higher amounts of photosynthetic units, they were not more efficient per unit. The ratio F_v/F_m indicated that the culture was generally stressed by high light, though it adapted to the irradiation during the course of the experiment (from 0.05 to 0.2). However, even these values are low compared with healthy higher plants, where F_v/F_m is usually above 0.8. Negative NPQ indicated that *E. gracilis* fluorescence yield is strongly influenced by state transitions, that is, reversible coupling/moving of antenna chlorophyll complexes between the photosynthetic reaction centres, which can lead to an increase in fluorescence yield and thus overcompensate the nonphotochemical fluorescence quenching, leading to negative NPQ values. The state transitions were also indicated by the higher photochemical quenching in actinic light (SIPs 1-4) compared with the dark-adapted F_v/F_m (= SIP0). Such mechanisms are commonly applied by photosynthetic organisms to balance the energy delivery between PSI and PSII.

Modulation of metal toxicity Metal toxicity-induced changes in the absolute fluorescence parameters (F_0 and F_m) were observed in cells of both strains, but only in the C&M medium that forces *Euglena* to live mainly on photosynthesis. The metal-induced inhibition of photochemistry was also generally stronger in C&M than in Buetow medium. Moreover, while a recovery of photochemical yield of PSII was observed on day 7 of the 10 μM Cr^{6+} and Cu^{2+} treatment in Buetow medium, no recovery occurred in C&M medium. All this indicates that *Euglena* cells grown in a nutrient-rich medium such as Buetow use their heterotrophic pathways to replace damaged components of the photosynthetic apparatus. The energy invested in such repairs, however, may be one reason why, in the end, growth of the cells on Buetow medium was more inhibited than cells cultured in the C&M medium.

In Buetow medium, however, heavy metal treatment led to a stronger increase of NPQ compared with C&M medium over the course of the experiment, especially in cells exposed to the highest copper concentration. The reason for this is difficult to delineate, because the measurable NPQ was clearly an overlay of real NPQ and the strong effects of state transitions that often overcompensated NPQ (see earlier). Thus, an inhibition of state transitions could also cause the observed increase in NPQ in addition to various factors that can affect NPQ itself.

Differences between the analysed *Euglena* strains

Only a few differences were observed between the strains, and these were not in the expected direction. In previous works, the MAT strain, which had been isolated from a highly polluted river, had a much greater IC_{50} (50% inhibitory concentration with respect to cellular growth; 96 h bioassay, EPA protocols) than the UTEX strain (Rocchetta *et al.*, 2003). Molecular studies showed that the MAT strain could overexpress genes related to the antioxidant system (especially SOD) upon chromium treatment compared with UTEX (dos Santos Ferreira *et al.*, 2007). Our current results, however, seem to indicate that during the years of cultivation on Cr-free medium in the laboratory, the MAT strain lost its tolerance, so that it is now actually more Cr- and Cu-sensitive than the UTEX strain. This reversal of resistance and the mechanisms underlying it would be an interesting topic for future studies.

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