J Appl Phycol (2010) 22:785–792
DOI 10.1007/s10811-010-9520-z

A fast algal bioassay for assessment of copper toxicity in water using *Euglena gracilis*

Hoda Ahmed · Donat-Peter Häder

Received: 15 December 2009 / Revised and accepted: 4 March 2010 / Published online: 13 April 2010

Abstract A rapid, sensitive algal bioassay was investigated to monitor copper toxicity using photosynthetic activity and motility parameters as end points in the photosynthetic flagellate *Euglena gracilis*. Effects on motility parameters were determined using the automatic bioassay ECOTOX. Photosynthetic efficiency was measured using chlorophyll fluorescence by means of a fast, noninvasive pulse amplitude modulated fluorometer. These parameters were assessed regarding their effectiveness as end points for short- (0–24 h) and long-term (1–5 days) toxicity tests. The model organism showed significant responses to the tested concentrations at both motility and photosynthesis examined levels. EC_{50} values for movement parameters immediately after the cells were mixed with copper are 19.09 (300.4 μM), 20.4 (312 μM) and 23.09 (363.35 μM)mg L^{-1} for motility, *r*-value and velocity, respectively. The current study proofs that *Euglena* is a convenient biotest organism where motility parameters show rapid sensitive responses to copper (direct exposure to copper). Also, the photosynthetic parameters appear to be appropriate for acute and chronic toxicity tests for higher and lower copper concentrations, respectively.

Keywords *Euglena gracilis* · Copper · PAM · Motility · Bioassay

Introduction

Copper is an essential microelement for all living organisms. It belongs to the group of heavy metals and exerts toxic effects at higher concentrations (Williams et al. 2000). Copper toxicity is known for its interference with essential physiological processes. In algae, excess copper suppresses cell growth, photosynthesis, chlorophyll synthesis and motility through a variety of toxicity mechanisms like replacement of essential metals, inhibition of enzymes and oxidative damage (Knauert and Knauer 2008). It contaminates the environment from different sources like mining, smelting and discarding of untreated industrial and agriculture wastes. Copper is recognised as a priority pollutant in aquatic environments with high potential threat to aquatic organisms and consequently to the food web (Macfarlane and Burchett 2001). An increase in copper concentrations in seawater of coastal environment of Northern Chile has resulted in loss of microalgae, macroalgae and invertebrates (Stauber et al. 2005). Heavy metal concentrations in seawater at the East London harbour range from 0.6 to 42.6 mg L^{-1} for Cu (Fatoki and Mathabatha 2001). Biomonitoring is of great interest providing better assessment of the potential toxicity to living organisms.

Algae are the primary producers in aquatic systems present in high concentrations. They show high sensitivity towards toxicants and can thus reveal any change in water quality (Turbak et al. 1986; Walsh and Merrill 1984). *Euglena gracilis* is a green unicellular alga inhabiting many freshwater ecosystems. Motility and photosynthesis in *Euglena* have been shown to be easily affected by environmental toxicity (Danilov and Ekelund 2001a; Häder et al. 1997). Danilov and Ekelund (2001a, b) have examined short- as well as long-term toxicity tests of wastewater using *E. gracilis*, concluding that 7-day tests could be more significant than short term (4-day test) with wastewater from the pulp and paper industry.

Euglena has been introduced previously as a biotest model which is significantly inhibited by heavy metals using the ECOTOX system (Tahedl and Häder 1999b,

H. Ahmed · D.-P. Häder (✉)
Department für Biologie, Friedrich-Alexander Universität,
Staudtstr. 5,
91058 Erlangen, Germany
e-mail: dphaeder@biologie.uni-erlangen.de

2001). This organism is a highly dedicated model in toxicology study since it shows plant (phototrophic) and animal (heterotrophic)-like characteristics and genetic stability due to its propagation by mitotic cell division (Watanabe et al. 2003). Einicker-Lamas et al. (2002) have proposed *E. gracilis* as a useful biological model for metal toxicity tests in eukaryotic cells.

In photosynthesis, both PSI and PSII are impaired upon heavy metal stress (Scordino et al. 2008). Copper has been reported to affect the electron transport in PSII especially (Baszynski et al. 1988). Baumann et al. (2008) showed significant effects on chlorophyll fluorescence yield of seven species of macroalgae as a result of Cu, Cr, Zn, Cd and Pb during a 14-day bioassay exposure test. Several end points have been used in the *Euglena* bioassay such as: cell growth (Aronsson and Ekelund 2005; Gajdosova and Reichrtova 1996; Stallwitz and Häder 1994), photosynthesis and respiration (De Filippis et al. 1981) and genetic expression (DNA, RNA, protein and gene expression; Deloménie et al. 2007; dos Santos Ferreira et al. 2007). The influences of two hepatotoxins, microcystin-LR and cylindrospermopsin, on growth, cell productivity, O_2 consumption and protein expression on *Euglena* were tested by Duval et al. (2005). Measured cell parameters showed toxin-specific effects depending on exposure time and concentration.

The main aim of this study was to test the hypothesis that motility and photosynthesis in *Euglena* can be used as a reliable bioassay end point for copper toxicity.

Materials and methods

Experiments were done using the unicellular green flagellate *E. gracilis* KLEBS strain Z obtained from the algal culture collection of the University of Göttingen (Schlösser 1994). Cells were grown in a mixture of complex and mineral media described elsewhere (Checcucci et al. 1976; Starr 1964) in static cultures at 22°C and in continuous white light of 20 Wm^{-2} in 100-mL Erlenmeyer flasks. Two-week-old axenic cultures were used for the current study.

Experimental procedure

Motion analysis

Movement and orientation parameters were determined using the automatic biosystem ECOTOX (Tahedl and Häder 1999a, 2001). The device consists of a miniaturised microscope with 6.3× object lens and infrared monitoring light source (λ=875 nm), a Firewire camera (DMK 21F04, Imaging Source, Bremen, Germany), a stainless steel frame cuvette with glass window and three stepper motors to transfer water, samples and cultures to the observation cuvette. The internal functions are controlled by a built-in microprocessor linked to an external host computer to ensure fully automatic performance. Many parameters are calculated automatically by the programme such as the *r*-value (the precision of orientation of the tested organism which has a value between 0 for un-oriented and 1 for perfectly oriented cells), motility (percentage of motile cells), upward-swimming cells (cells moving against the gravity field direction) and velocity of the cells. For more details, see Tahedl and Häder (1999a). All recorded data are saved in ASCII files and then imported into Excel for further processing.

Assay of photosynthesis efficiency

Exponentially growing cells were transferred into sterile 100-mL Erlenmeyer flasks and treated with different concentrations of copper for 5 days. Cu was used as $CuCl_2 2H_2O$ at a concentration of 0.5, 5, 10, 30 or 75 mg L^{-1} corresponding to 7.87, 78.7, 157.3, 472 or 1,180 μM, respectively, to test photosynthetic efficiency. Samples were taken for chlorophyll fluorescence analysis daily and up to 1-week exposure to Cu. The photosynthetic quantum yield was determined for light- and dark-adapted samples (20 min in darkness before measurement) at room temperature by means of a pulse amplitude modulated fluorometer (PAM 2000, Walz, Germany). Using the light curve function, electron transport rate (ETR) and photosynthetic yield were measured in a series of 13 increasing illumination steps after a 20-min dark adaptation and 5-day exposure to copper. Control samples were prepared with the same amount of distilled water instead of the toxin solute, keeping the same cell suspension concentration. For each treatment, three independent samples were measured and each experiment was repeated at least two times.

Statistical analysis

ECOTOX data were stored in files and then imported to Excel files. By means of SigmaPlot (2001), EC_{50} curves were calculated using per cent inhibition recorded after each treatment measurement. Data from PAM were averaged and analysed by one-way ANOVA to detect the significant differences between treatments and control.

Results

Exposure to copper resulted in a significant disturbance of the motility parameters in the tested alga *E. gracilis* even

after 3 min. Figure 1a–d presents the relation between short-term exposure to copper and the inhibition (%) of the orientation parameters (*r*-value and upward-swimming cell) as well as movement parameters (motility and velocity) of *E. gracilis*. The sensitivity of the motion parameters are in the following order: motility > *r*-value > velocity > upward swimming with EC_{50} values of 19.09, 20.4, 23.09 and 50 mg L^{-1} corresponding to 300.4, 3124, 363.35 and 787 μM, respectively. Incubation of *Euglena* with copper for 24 h produces a slight shift in the EC_{50} values in the case of motility and upward swimming (EC_{50} values are 23 and 56 mg L^{-1} equal to 361 and 881 μM, respectively; Fig. 2).

The presence of copper caused major changes in the photosynthetic parameters of the tested alga. The response of photosynthesis in *Euglena* to copper is quantified by changes in F_v/F_m and F_v'/F_m' (Fig. 3). Light-adapted cells showed a significant reduction in the photosynthetic yield even during the first minutes after exposure to 30 or 75 mg L^{-1} of copper equal to 472 or 1,180 μM, respectively (Fig. 3a). After 24 h of treatment, the photosynthetic yield exhibited an 88% decrease in cells treated with 75 mg L^{-1} (1,180 μM) and was completely inhibited with longer exposure times up to 3 days. Concentrations of 30 mg L^{-1} (472 μM) showed a significant inhibition in F_v'/F_m' during the whole experiment. Ten milligrams per litre (157.3 μM) showed a significant decrease in F_v'/F_m' after 1 day of incubation then recovered at days 2 and 3, and thereafter, there was a significant effect on the photosynthetic yield up to the end of the exposure times. Both 0.5 and 5 mg L^{-1} (equal to 7.87 and 78.7 μM) showed marginal disturbances of the light photosynthetic yield after 2 days followed by an adaptation in the longer incubation time. In dark-adapted samples, a significant inhibition was observed in cells exposed to 10, 30 or 75 mg L^{-1} corresponding to 157.3, 472 or 1,180 μM of copper during the entire experiments (Fig. 3b). In contrast to light-adapted samples, concentrations tested from 5 mg L^{-1} (78.6 μM) showed a significant reduction in the values of F_v/F_m after an incubation time of 3 days.

Results of the photosynthetic yield and ETR derived from light curve measurements of the control cells showed a typical form: The ETR increased with increasing irradiation until it reached a constant value at higher irradiation due to a reduction of PSII electron acceptor sites (Fig. 4a–d). This was also observed in treated samples with a decrease in both parameters in comparison to the control. This pronounced effect on photosynthetic efficiency increases with increasing copper concentrations and incubation time. Severe impacts were observed at copper concentrations of 30 and 75 mg L^{-1} corresponding to 472 or 1,180 μM after 20 min of incubation with copper. These

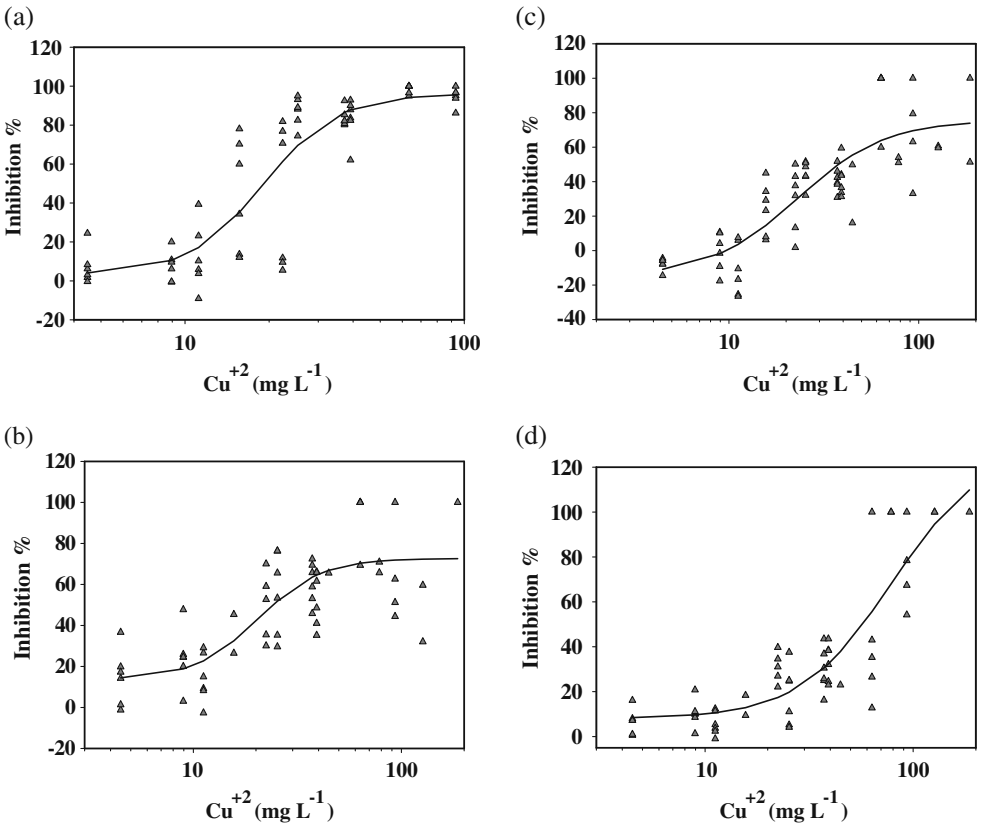

Fig. 1 Inhibition by copper of the **a** motility (EC_{50} 19.09 mg L^{-1}= 300.4 μM), **b** *r*-value (20.4 mg L^{-1}=3,124 μM), **c** velocity (EC_{50}=23.09 mg L^{-1}= 363.35 μM) and **d** upward swimming (EC_{50} 50 mg L^{-1}= 787 μM) in *E. gracilis* after short-term exposure

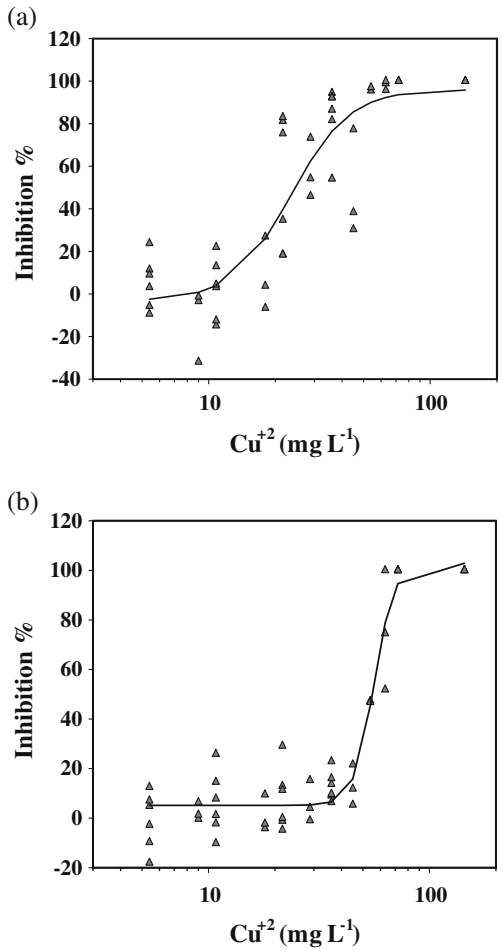

Fig. 2 Inhibition by copper of **a** motility (EC_{50} 23.4 mg L^{-1}= 368.2 μM) and **b** upward swimming (EC_{50} 56 mg L^{-1}=881 μM) of *E. gracilis* after 24 h incubation time

concentrations showed maximum effects after 5 days of exposure where photosynthetic yield and ETR in 75 mg L^{-1} (1,180 μM) treated samples were zero, whereas at 10 and 30 mg L^{-1} equal to 157.3 and 472 μM, both parameters were very significantly decreased in comparison to the controls and low copper concentrations.

Discussion

With increasing pollution problems at all levels (water, soil, air etc.), the development of bioassays is of special interest. Two factors are essential for choosing new bioassays: organism and end points. The organism should have a short life cycle, easy culture condition and handling, as well as short response time and sensitivity. End points should be clear, easy to measure and reliable. *E. gracilis* fulfils this requirement as a biotest organism.

Several studies have proven significant disturbance of gravitaxis and motility parameters in response to abiotic environmental stresses like temperature, pH, oxygen concentration, high light and salinity (Richter et al. 2002a, b), UV radiation (Häder and Liu 1990), herbicides (Pettersson and Ekelund 2006) and heavy metals (Gajdosova and Reichrtova 1996; Stallwitz and Häder 1993). These stress factors cause changing negative gravitaxis to positive or no gravitactic orientation or sometimes immobility.

In the current bioassay, motility, velocity and orientation (*r*-value) have a clear direct sensitivity toward copper showing 50% inhibition at concentrations of about 19–23 mg L^{-1} equal to 300 and 363 μM. During long-term exposure, the parameters showed quite similar sensitivity. This is in agreement with Tahedl (2000) who noticed a significant effect of copper on *E. gracilis* motility parameters, recording EC_{50} values of 10.2 mg L^{-1} (*r*-value), 43.6 mg L^{-1} (motility) and 53.1 mg L^{-1} (velocity) corresponding to 160.5, 686.1 and 835 μM, respectively. Using motility parameters, Willemann (2002) has determined an EC_{50} value of 0.54 mM equal to 34.0 mg L^{-1} in *E. gracilis*. In addition, young *E. gracilis* cell culture showed positive gravitaxis which switched to negative upon growing from 4 to 11 days in media containing copper (Stallwitz and Häder 1994). Cell speed and motility were inhibited with an EC_{50} value of 50 μM≈3.18 mg L^{-1}. Although EC_{50} values by these authors are lower than the current values, one important factor should be taken into consideration, which is cell age. Young cells seem to be more susceptible to heavy metals, but at this age, they could not show any stable orientation behaviour since cell strategy at this time is mainly directed to cell division. The current results point out that the motility of the organism was the most sensitive parameter in response to copper. This is in good agreement with Eisner and Fomin (2002) who reported that motility in *E. gracilis* is an effective parameter to assess the varying toxic potential of incinerator stack gas condensate. In addition, cell velocity showed an instant response to copper exposure, proving its sensitivity to toxins. This was reported previously by Pettersson and Ekelund (2006) who tested the effects of two herbicides (Avans and Roundup) on movement and gravitaxis parameters of *Euglena.* After short-term exposure, cell velocity showed obvious sensitivity to both tested herbicides. The EC_{50} values obtained in the current study compare well with those from the MICROTOX bioassay, which is based on a decrease of luminescence in *Vibrio phosphoreum* in response to copper with an EC_{50} of 34.4 mg L^{-1} (541.3 μM; Info 04 1997). The current results are in agreement with Einicker-Lamas et al. (2002) who reported alterations in cell motility and shape of *E. gracilis* cells grown for 24 h in the presence of $CuCl_2$. The cells displayed an apparent LC_{50} for Cu^{2+} at 0.22 mM (13.96 mg L^{-1}). Ultrastructural analysis of copper-treated *Euglena* cells showed chloroplast disorganisation. The

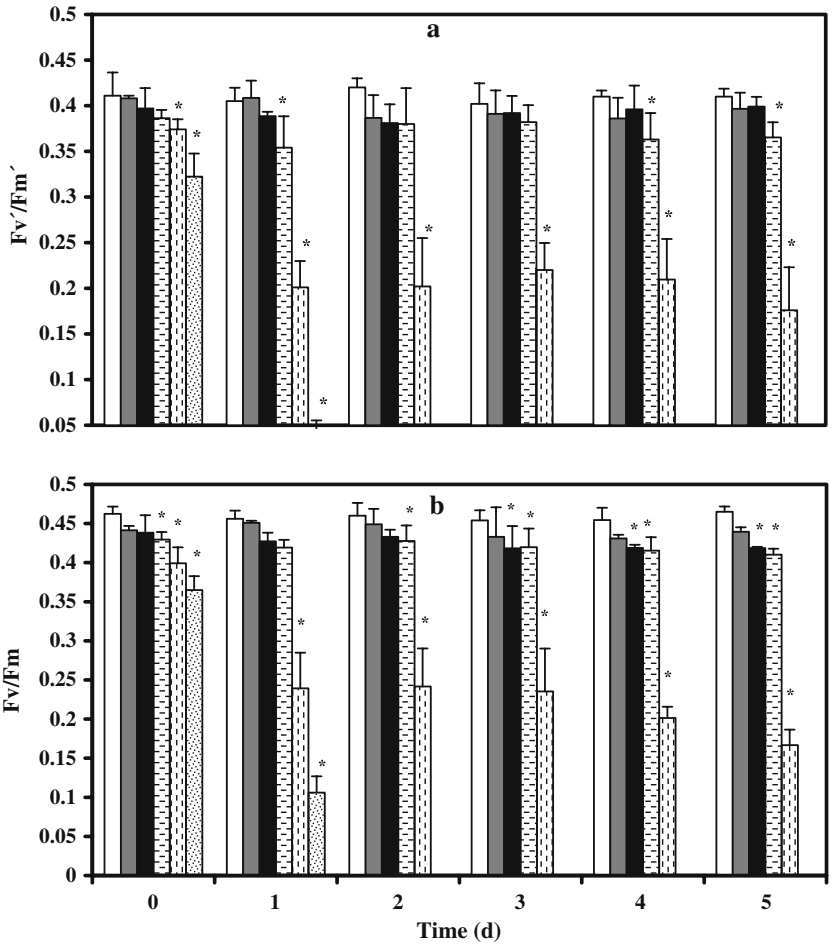

Fig. 3 Photosynthetic quantum yield for light- and dark-adapted samples treated with copper at different incubation times. *Bars* indicate standard deviations of three independent data points. *Asterisks* mean significance at 0.05 levels (ANOVA). □ control, ▩ 0.5 mg L^{-1} (7.87 μM), ■ 5 mg L^{-1} (78.7 μM), ⊟ 10 mg L^{-1} (157.3 μM), ⊡ 30 mg L^{-1} (472 μM), ⊠ 75 mg L^{-1} (1,180 μM)

authors found an accumulation of copper in the vacuoles, suggesting a detoxification role played by the vacuoles. They also indicate that copper had an overall toxic effect on *E. gracilis*, chiefly on the structure of chloroplast membranes.

Euglena gracilis motility was dedicated as a suitable bioassay to investigate mining lake samples of extreme acidic character (Fomina et al. 2000). Moreover, Millán de Kuhn et al. (2006) have tested motility inhibition of nine marine flagellates upon direct copper exposure. The lowest sensitive species to copper was *Dunaliella* species with EC_{50} values from 176 to 220 mg L^{-1}. In contrast, *Porocentrum* species were the most sensitive algae, with EC_{50} values from 7.5 to 13.5 mg L^{-1}; *Heterocapsa triquetra* has an EC_{50} value of 0.11 mM (7.0 mg L^{-1}). In this regard, EC_{50} value obtained by *Euglena* compare well with the most sensitive alga (*Porocentrum* sp.) determined in this study.

Many advantages of the ECOTOX system can be noticed, including short time measurement, automatic measurement of data reducing personal error, as well as determination of several parameters in parallel. In comparison to other used organisms like *Daphnia*, fish, invertebrates, etc., *Euglena* is easy to grow and handle without any special growth condition. An additional important characteristic of *Euglena* is its photo- and heterotrophic manner, which makes *E. gracilis* a suitable bioindicator in polluted areas (Einicker-Lamas et al. 1996). In this context, motility as well as orientation parameters in *E. gracilis* are especially suitable as end points for short-term copper toxicity tests.

Although copper is an essential micronutrient for autotrophic organisms like plants and algae, it has toxic potential at higher concentrations mainly to the photosynthetic process since it is mainly accumulated in the chloroplast (Braune et al. 1994). In this context, the plant uptakes actively copper which may exceed metabolic requirements, causing toxic effects (Ralph and Burchett 1998). This may explain the tolerance of organism to lower copper concentrations and toxicity at relatively higher concentrations. Measurements of chlorophyll fluorescence using PAM have become a common method to study the effects of environmental factors on the photosynthesis of plants, macro- as well as microalgae (Nielsen and Nielsen 2008).

Our fluorescence results clearly indicate that the effects on the photosynthetic parameters are dependent on the

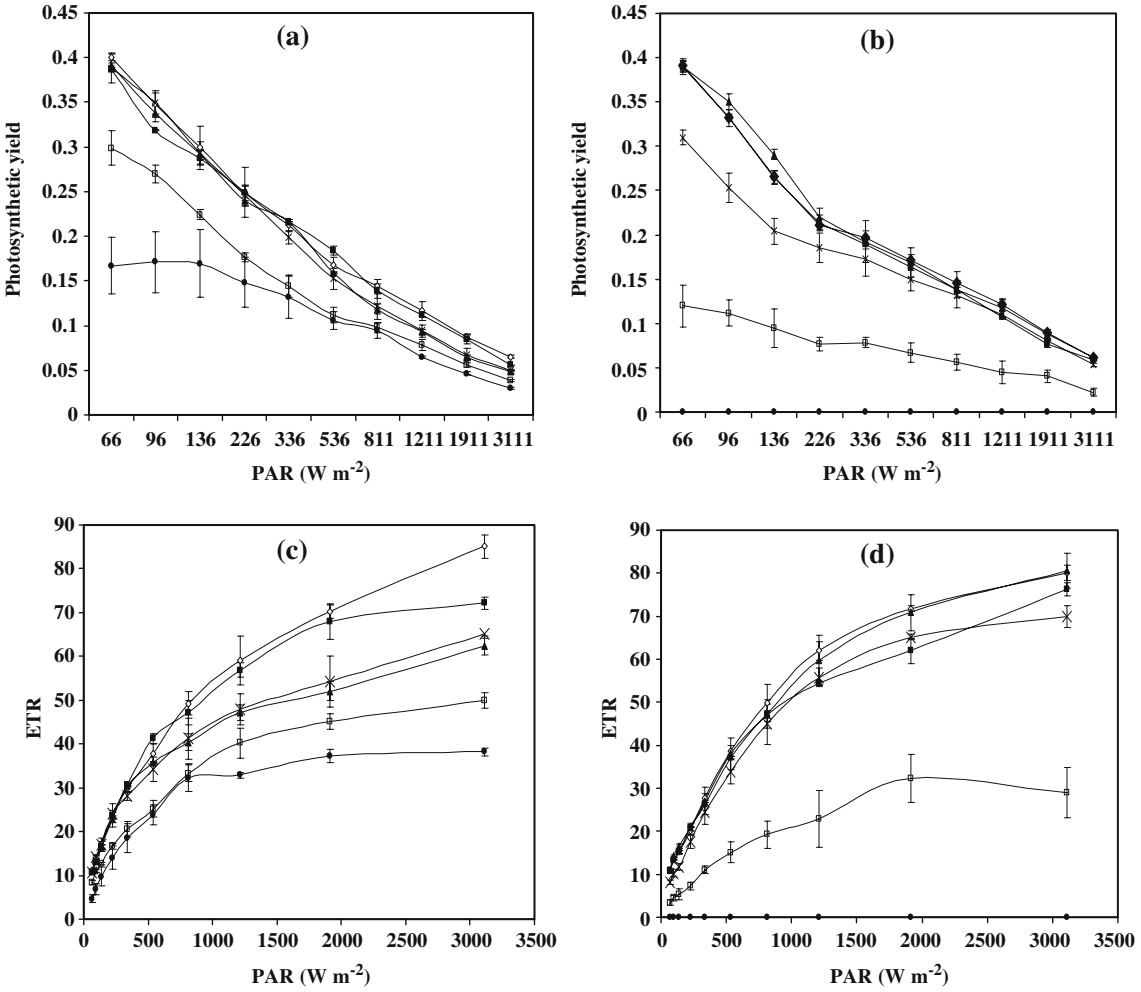

Fig. 4 Effective quantum yield of PS II (**a**, **b**) and electron transport rate (*ETR*) (**c**, **d**) of *E. gracilis* in response to different copper concentrations after short-term (*left diagrams*) and 5 days of exposure time (*right diagrams*). *Bars* standard deviations (n=3). ◊ control, ■ 0.5 mg L^{-1} (7.87 μM), ▲ 5 mg L^{-1} (78.7 μM), X 10 mg L^{-1} (157.3 μM), □ 30 mg L^{-1} (472 μM), ● 75 mg L^{-1} (1,180 μM)

copper concentration and the exposure time. Copper has been shown to partially inhibit reaction centres in PSII. The remaining ones keep functioning effectively; this may explain the observed response in light-adapted samples at some concentrations in comparison to dark-adapted samples. A bioassay study does not only reflect the potential toxicity of a certain pollutant but also provides important information on the optimal conditions for the organism. Sensitivity of photosynthesis to copper is also confirmed by studies of the ETR which is decreased upon incubation with copper. As a result, photosynthesis is a sensitive parameter in chronic toxicity tests. It is also proven by Rocchetta and Küpper (2009) that in photosynthesis in *Euglena*, mainly the photosystem II reaction centre is the primary target of damage pon copper stress. This is in agreement with Fernandes and Henriques (1991), Mallick and Mohn (2003) and Perales-Vela et al. (2007) who found that the photosynthetic apparatus in algae is the main target of copper toxicity, reducing both photosynthetic efficiency and electron transfer rate of PSII. In parallel Shioi et al. (1978) have proven the inhibition of electron transport in PSII of green algae broken cells *Ankistrodesmus falcatus* by copper. Several studies have been done to identify the mechanism by which copper inhibits electron transport in photosynthesis, of which are alterations in the amino acid or lipid structure close to the acceptor side or donor side of electron transport, substitution of magnesium atom in the chlorophyll by heavy metals or direct interaction of heavy metals to the photosynthetic apparatus reducing electron flow (Knauert and Knauer 2008; Küpper et al. 1996; Yruela et al. 1996a, b). This is in addition to the formation of reactive oxygen species induced by copper stress which showed an indirect effect on photosynthesis (Knauert and Knauer 2008).

Toxicity of copper is very much dependent on the organism and growth condition. For example, marine algae are believed to be more sensitive than freshwater algae. Even so, among marine algae, the toxicity varies considerably. EC_{50} values for a variety of marine algae using cell division rate as end point have been reported to range from 0.45 to 58 mg L^{-1} for *Thoracosphaera heimii* and *Monallantus salina*, respectively (Brand et al. 1986; Gavis et al. 1981). An important factor playing a role in toxicity tests is the medium pH since it affects the concentration of free metals. Schuler et al. (2008) have reported that the amount of free Cu^{2+} in laboratory water declines rapidly from a pH of 6 (90%) to 8 (5%). Adverse sublethal effects, including altered free cysteine metabolism, were recorded in *E. gracilis* by Coppellotti (1989) after a 5-day exposure to 10 mg L^{-1} copper. The freshwater protozoan ciliate *Tetrahymena pyriformis* has an EC_{50} value of growth inhibition of 8 mg L^{-1} copper after 48 h. In addition, Wang and Chang (1991) found that addition of 0.1 mg L^{-1} chromium or nickel very much increases the toxicity of the same copper concentration to the growth of the freshwater green algae *Chlorella pyrenoidosa* from 7% to 100%.

Although many organisms as well as end points are used to test copper toxicity, the need for a standardised method is required. When we talk about copper water toxicity, then algae are a good example to be used since they inhabit aquatic ecosystems. Unicellular algae are better compared to multicellular, so our choice for *E. gracilis* is ideally suitable to test copper water toxicity. Growth inhibition, effects on cell numbers, fresh or dry weight, protein and nucleic acid content, morphology and photosynthesis are mainly used as end points in algal bioassays (Rai et al. 1994). Motility parameters as end points provide an early warning biomonitoring test for water toxicity. In addition, the use of fluorescence parameters has facilitated the use of photosynthesis parameters if a chronic toxicity test was performed.

In conclusion, rapid, sensitive and automatic measurements of several parameters are the main advantages of the studied bioassay. An important advantage is short time measurement which ranges from 8 to 10 min for one complete measurement using ECOTOX and PAM, respectively. *E. gracilis* is easy to cultivate and to handle. *E. gracilis* showed definite sensitivity towards copper using both motility and photosynthesis as measured parameters. Movement parameters proved to be suitable for short-term toxicity assessments. Photosynthesis in *Euglena* can be used in long-term bioassays (5 days) for the determination of copper toxicity.

Acknowledgements We thank the Deutsche Akademischer Austauschdienst (DAAD) for granting a scholarship to Hoda Ahmed.

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