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Long-term acclimation of *Pseudokirchneriella subcapitata* (Korshikov) Hindak to different copper concentrations: changes in tolerance and physiology

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Abstract

The effect of long-term copper acclimation of the freshwater green algae *Pseudokirchneriella subcapitata* to copper was investigated using different physiological and toxicological endpoints. The algae were exposed to seven—five of which are ecologically relevant for European surface waters—copper concentration ranging from 0.5 to 100 μg Cu l^{-1} during a 3-month period. A standard medium was used as culture and test medium with an addition of 2 mg DOC l^{-1} (replacing EDTA). At certain intervals, experiments were performed to assess algal biomass, growth rate, chlorophyll and carotenoid content, pigment diversity, autotrophic index, intracellular and adsorbed copper, and the sensitivity of the algae to copper. Chronic copper tolerance (mean ± standard deviation) increased significantly from 88 ± 15 to 124 ± 25 μg Cu l^{-1} for *P. subcapitata* acclimated to 0.5 and 100 μg Cu l^{-1}, respectively. Based on the algal biomass, the growth rate, the pigment diversity and the autotrophic index, an optimal concentration range was observed between 1 and 35 μg Cu l^{-1}. Significant decreases in algal biomass, pigment diversity and autotrophic index were observed in algal cultures acclimated to 0.5 μg Cu l^{-1} and 100 μg Cu l^{-1}. Chlorophyll *a* content (mean ± standard deviation) increased from 8.4 ± 3.1 to 28.6 ± 7.5 × 10^{-14} g per cell and carotenoid content (mean ± standard deviation) increased from 3.7 ± 0.8 to 7.1 ± 1.2 × 10^{-14} g per cell for algae exposed to 1 and 100 μg Cu l^{-1}, respectively. Intracellular copper increased from 0.099 to 20.6 × 10^{-15} g Cu per cell and adsorbed copper increased from 0.026 to 1.8 × 10^{-15} g Cu per cell for algae acclimated for 12 weeks to 0.5 and 100 μg Cu l^{-1}, respectively. This research demonstrates that the use of standard culture media, some of which may be deficient in copper, can result in sub-optimal performance of the organisms, which in turn may affect toxicity test results. Additionally, this work also established an optimal concentration range for copper for this algal species. This phenomenon should be taken in consideration when performing environmental risk assessments of essential elements.

Keywords: *Pseudokirchneriella subcapitata*; Copper; Acclimation; Optimal concentration

1. Introduction

Copper is a trace element essential for all living organisms (Price and Morel, 1994). In plants, it participates in photosynthetic electron transport and also plays a role as a co-factor of several oxidizing en-

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zymes. Although copper can act at many different levels in the cell, one of the most important mechanisms of copper action in green plants is believed to be the inhibition of electron transfer in the chloroplasts (Shioi et al., 1978), the formation of reactive radicals (Flemming and Trevors, 1989; Fernandes and Henrique, 1991), the destruction of the chloroplast membrane (Sandmann and Böger, 1980), inhibition of the formation of photosynthetic pigments and decrease in intracellular K^+ and Na^+ concentrations (DeFilipis, 1979). A lack of copper, on the other hand, may interfere with a number of important functions, such as photosynthesis, respiration, protein synthesis, lignification, auxin regulation, disease resistance and reproduction (Shorocks and Alloway, 1985).

To cope with the dualism of copper's essential functions versus its potential toxicity, all living cells have developed strong capabilities to manage the cellular copper levels. Indeed, intracellular copper routes studies have demonstrated that the intracellular copper regulation is generally controlled by 2 key elements: P-type ATPases that can pump copper across biological membranes in either direction and copper chaperones, small intracellular copper binding proteins that allow safe intracellular transport of copper to copper requiring proteins. It is thereby interesting to know that these copper homeostasis mechanisms, only functional within certain concentration limits, have been found in yeast and bacteria to mammalian cells and are therefore considered as being highly conserved through evolution (Wunderli-Ye and Solioz, 1999; Harrison et al., 2000). Additionally, different organisms have developed species-specific copper management mechanisms resulting in different species-specific internal copper levels and copper susceptibilities. For algae, it was indeed demonstrated that copper regulating mechanisms involve the ability of algae to excrete metal-binding compounds to the surrounding medium inducing a reduction in the metal bioavailability and metal uptake (McKnight and Morel, 1979; Lumsden and Florence, 1983) and the production of intracellular phytochelatin, a metal-binding peptide, which can detoxify copper (Silverberg et al., 1976; Gekeler et al., 1988; Knauer et al., 1998). However, different algal species appear to differ in their ability to produce such compounds, which is reflected in the large variability in inter-species sensitivity of copper (Takamura et al., 1989). Additionally, intra-species variability in observed copper sensitivity can span several orders of magnitude (Janssen and Heijrick, 2003) and have been, at least partially, attributed to differences in test water chemistry: nutrient supply, pH, hardness, the concentration of organic complexing agents in the water (Knauer et al., 1997; Janssen and Heijrick, 2003).

The exposure history of the algal population community may however also play an important role in determining the response of the algae to a given copper concentration. The importance of exposure history in ecotoxicological studies with algae has been recognized since Kellner (1955) demonstrated that *Ankistrodesmus braunii* can develop a tolerance to elevated copper and rubidium concentrations. Since then, several studies have assessed the effect of metal-polluted environments on algal sensitivity (Foster, 1982; Whitton, 1984; Kuwabara and Leland, 1986; Hall et al., 1989; Muyssen and Janssen, 2001). Possible effects of—relatively low—natural (background) copper concentrations on the physiology and tolerance and the optimal concentration range of micro-algae to copper have, however, not been investigated.

Background copper concentrations can vary considerably with geographical area. According to Heijerick and Janssen (2000, update database 2003, personal communication) the 5th and 95th percentile of the (total) copper concentrations in unpolluted European waters is 0.4 and 16.0 $\mu g\ Cu\ l^{-1}$, respectively. The 5th and 95th percentile for dissolved copper is 0.6–10.9 $\mu g\ Cu\ l^{-1}$ (based on data only from The Netherlands, Germany and UK). Knauer et al. (1997) found a total dissolved copper concentration in lake Greifen (Switzerland) ranging between 0.2 and 1.8 $\mu g\ Cu\ l^{-1}$. In a survey of 11 pristine surface waters in five European countries, we found dissolved copper concentrations between 0.1 and 11 $\mu g\ Cu\ l^{-1}$ (Bossuyt et al., unpublished data; Heijerick et al., unpublished data).

As organisms are dependent on essential metals for optimal growth and development, it may be hypothesized that species occurring in ecosystems with different background concentrations are differentially acclimated or adapted. Consequently, as each organism has an optimal concentration range for copper which is dependent on the prevailing copper background, it is suggested that with increasing copper concentration the organism's copper tolerance will

increase. To test this hypothesis for algae, we exposed *Pseudokirchneriella subcapitata* to seven different copper concentrations during 3 months. At regular time intervals, experiments were performed to assess the effect of copper acclimation on the algal biomass, growth rate, pigment diversity, autotrophic index and their tolerance to copper.

2. Materials and methods

2.1. Acclimation of the algae

Acclimation experiments were performed with the green algae *P. subcapitata* (Korshikov) Hindak (CCAP 278/4, formerly known as *Selenastrum capricornutum* Printz and *Raphidocelis subcapitata* Korshikov) obtained from the Culture Collection of Algae and Protozoa (CCAP; CEH, Ambleside, UK). Preparation of the synthetic freshwater International Organisation for Standardisation (ISO) culture medium and the maintenance of algal cultures during the acclimation period of 3 months followed the procedures described in ISO protocol 8692 (ISO, 1987). In the medium used for culturing and toxicity testing, ethylenediaminetetraacetic acid disodium salt (EDTA, Sigma–Aldrich Chemie, Steinheim, Germany) was replaced by Aldrich humic acid (AHA, Sigma–Aldrich Chemie, Steinheim, Germany) at a concentration of 2 mg l^{-1} as dissolved organic carbon (DOC) (see Section 4); this medium will be referred to as modified ISO medium. The final pH of this medium was 7.8 ± 0.1. Seven different copper acclimation treatments were simultaneously started: <0.5 (nominal zero), 1, 5, 12, 35, 60 and 100 μg Cu l^{-1}. To minimise metal contamination, all materials in contact with the culture or test medium were soaked 24 h in 1% HNO_3 (p.a., VWR International, Leuven, Belgium) and rinsed with deionized water. All chemicals used for the preparation of the medium were reagent grade quality and were purchased from VWR International (Leuven, Belgium). Stock solutions of DOC were prepared by dissolving 5 g of AHA in 2 l of deionized water, equilibrating the solution for 24 h at 4 °C and filtering it through a 0.45 μm filter (Gelman Sciences, Ann Arbor, MI, USA). DOC was measured with a TOC-5000 analyser (Shimadzu, Duisburg, Germany).

During the acclimation period, the algae were cultured in 125 ml erlenmeyer flasks containing 50 ml of sterilized medium. The erlenmeyer flasks were autoclaved for 20 min at 121 °C and 1 kg cm^{-2} (pbi international S.p.A., Milano, Italy). After initiating the cultures (inoculum of 10^4 cells ml^{-1}), these flasks were closed with loose cotton stopper and shaken manually three times a day. The cultures were maintained at 22 ± 1 °C and illuminated continuously at 7250 cd m^{-2}. Each week, an inoculum (10^4 cells ml^{-1}) was taken from these cultures and transferred to fresh sterile medium to maintain exponential growth. Before inoculation, all cultures were visually inspected for contamination using a microscope (Kyowa, Tokyo, Japan) with 10×20 magnification. Cultures were performed in triplicate. Each week, cell densities were measured (Coulter, Model DN, Harpenden, Herts, England). Average weight of the algae was determined in triplicate for all tested concentrations and did not differ significantly between treatments (ANOVA, $P > 0.05$). Algal biomass was calculated using the particle counter data and average ($N = 21$) cell dry weight of $1.5 \pm 0.1 \times 10^{-11}$ g per cell.

2.2. Growth inhibition experiments

Prior to the acclimation experiments, a growth inhibition test was performed in standard ISO medium. During the acclimation experiment, the sensitivity of the acclimated algae to copper was also evaluated. Growth inhibition tests according to the ISO protocol 8692 (ISO, 1987) were performed every week up to week 4 and from then onwards every 2 weeks. Copper test concentrations were prepared by diluting a concentrated stock solution of $CuCl_2$ (0.1 g Cu l^{-1}) in modified ISO medium. Each test consisted of six copper concentrations ranging from <0.5 (nominal zero = modified ISO) to 320 μg Cu l^{-1} with three replicates per concentration. Test vessels were incubated as described for the acclimation cultures. Algal densities were measured after 24, 48 and 72 h as cell numbers (algal cells ml^{-1}) using a particle counter (Coulter Model DN, Harpenden, Herts, England). At the same time, the pH of the medium was measured and noted. Initial cell density in all experiments was 10^4 cells ml^{-1}. A test was considered as valid when pH variation at the end of the test was less than one

pH unit and when the final cell concentration in the control exceeded 1.6×10^5 cells ml^{-1}.

The growth rate μ (per day) was calculated as follows (ISO, 1987):

$$\mu = \frac{\ln N_t - \ln N_0}{t_n}$$

with N_t the final cell density (cells ml^{-1}), N_0 the initial cell density (cell ml^{-1}) and t_n the time (day) after the initiation of the test.

The 72 h EC_{50} values were calculated based on the percentage inhibition of the growth rate (72 h E_rC_{50}) compared to the control (OECD, 1996). The inhibition percentages were plotted against the measured copper concentrations and a statistically significant ($P < 0.05$) sigmoidal concentration-effect relationship was fitted through the data points using the software package SigmaPlot® 2000 (SPSS, Chicago, IL, USA). From this relationship, the 72 h E_rC_{50} and the 95% confidence intervals were derived.

2.3. Absorbed and adsorbed metal concentration

The method used to measure the absorbed and adsorbed metal concentration is based on Franklin et al. (2000). These measurements were performed every week up to week 4 and from then onwards every 2 weeks. At the moment an inoculum of the acclimated algae was transferred to a sterile medium, 40 ml of the algal culture was transferred to 50 ml acid-washed Teflon centrifuge tubes. Samples were centrifuged (Centra-8, IEC, USA) at $500 \times g$ for 20 min and 20 °C. Ten milliliters of the supernatant solution was then pipetted into acid washed polypropylene vials (AAS tubes; Laborimpex, Brussels, Belgium). These samples were analyzed for dissolved copper. The remaining supernatant was removed and the algal pellet was resuspended in 20 ml of 5×10^{-3} M EDTA (Sigma–Aldrich Chemie, Steinheim, Germany) and shaken for approximately 30 s to remove the copper adsorbed to the cells (Florence and Stauber, 1986). Franklin et al. (2000) have shown that no cell lysis occurred due to this EDTA treatment. The samples were then centrifuged again at $500 \times g$ for 20 min and the supernatant removed for copper analysis. These samples are referred to as the adsorbed copper fraction. The remaining algal pellets were left to dry in a laminar flow hood for 2 days and subsequently acid digested with 2 ml of 14N HNO_3 for 30 min. These samples were heated in a microwave oven (Samsung MF245, Korea) at a power setting of 150 W for 5 min. After cooling to room temperature, the copper concentration of these samples—referred to as the absorbed copper fraction—was determined.

2.4. Copper measurements

All copper concentrations were analyzed using a flame-atomic absorption spectrophotometer (AAS, for Cu >20 μg Cu l^{-1}, SpectrAA100, Varian, Mulgrave, Australia) or a graphite furnace atomic absorption spectrophotometer (for Cu <20 μg Cu l^{-1}, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Calibration standards (Sigma–Aldrich Chemie, Steinheim, Germany) and reagent blank were analyzed with every ten samples. The quantification limit of the flame AAS and the graphite furnace AAS is 17 and 1.5 μg Cu l^{-1}, respectively (as determined bi-monthly by the method described in APHA et al., 1998). Ten mL of the water samples was acidified (pH < 2) with 14 N HNO_3 (p.a., VWR International, Leuven, Belgium) and measured. All reported copper concentrations of the prepared media are dissolved copper concentrations (0.45 μm filtered). Absorbed and adsorbed metal concentrations were reported as g Cu per cell. Analysis of the nominal zero concentrations could only assure that copper concentrations were <0.5 μg Cu l^{-1} (detection limit of the graphite furnace AAS). Results of acclimation to nominal zero concentration will therefore be referred to as 0.5 μg Cu l^{-1}.

2.5. Pigment determination

Extraction and spectrophotometric determination of algal pigments were based on APHA (1998). These measurements were performed every week up to week 4 and from then onwards every 2 weeks. At the moment an inoculum of the acclimated algae was transferred to a sterile medium, 20 ml of algae was taken from each algal culture and filtered over 0.7 μm Whatman® GF/C filter (Whatman International, Maidstone, UK). The filter was placed in a tissue grinder, covered with 3 ml of 90% aqueous acetone solution (90 ml acetone with 10 ml of 10 g $MgCO_3$ l^{-1}) and

macerated for 1 min with a grinder. The sample was transferred to a glass centrifuge tube and the volume was adjusted to 10 ml with 90% aqueous acetone solution. Samples were kept in the dark at 4 °C for 24 h after which the solution was centrifuged (Centra-8, IEC, USA) at 500 × *g* for 20 min. The resulting supernatant was used for spectrophotometric pigment determination (Spectronic 601, Milton Roy, USA). The following formulae were used to calculate chlorophyll and carotenoid concentrations (Rowan, 1989):

$$\text{chlorophyll } a\ (\text{mg l}^{-1}) = \frac{2.67(664_b - 665_a)V_1}{V_2 L}$$

$$\text{chlorophyll } b\ (\text{mg l}^{-1}) = \frac{[21.03(\text{OD647}) - 5.43(\text{OD664}) - 2.66(\text{OD630})]V_1}{V_2}$$

$$\text{carotenoid}\ (\text{mg l}^{-1}) = \frac{[4.1(\text{OD480}) - 0.043C_a - 0.367C_b]V_1}{V_2}$$

in which V_1 is the extract volume (l); V_2 the volume of the sample (l); L the length of the cuvette used (m); 664_a and 665_b the optical density of 90% acetone extract before and after acidification at 664 and 665 nm; OD480, OD647, OD664 and OD630 the optical density at 480, 647, 664 and 630 nm; C_a and C_b concentration of chlorophylls *a* and *b*, respectively (mg l^{-1}). Pigment concentrations were reported as g per cell. Autotrophic index (AI) was calculated as the ratio of the biomass (dry weight) to the chlorophyll *a* concentration. Pigment diversity (PD) was calculated as the ratio of carotenoid concentration to the chlorophyll *a* concentration (Rai et al., 1990).

2.6. Statistical analysis

The effect of the various acclimation concentrations on the algal response were compared using one-way analysis of variance (ANOVA) and Duncan's multiple range test (STATISTICA® software, Tulsa, OK, USA). The ANOVA assumptions on homogeneity of variance and normality were tested using Bartlett's and Kolmogorov-Smirnov's test, respectively. In case these assumptions were not met, log 10 transformation of the data was sufficient in order to proceed with the ANOVA. Statements of significant differences are based on accepting $P < 0.05$.

3. Results

A decrease in algal biomass and growth rate was noted with increasing acclimation concentration after the first week of acclimation (Table 1). In contrast, an increase was observed in all pigment concentrations. Algae acclimated to 100 μg Cu l^{-1} had a significantly lower algal biomass (mean ± standard deviation: 4.7 ± 0.7 mg DW l^{-1}; $N = 3$) and growth rate (mean ± standard deviation: 0.489 ± 0.021 per day; $N = 3$) and high pigment concentrations (84.7, 14.8 and 20.9 × 10^{-14} g per cell for chlorophylls *a*, *b* and carotenoid concentration) compared to the algae acclimated to 0.5 μg Cu l^{-1}. Algae ac-

Table 1
Biomass (mean ± standard deviation (S.D.), $N = 3$), growth rate (mean ± standard deviation (S.D.), $N = 3$), chlorophyll *a* (Chl *a*) and *b* (Chl *b*) and carotenoid (car) concentration of algae acclimated to seven copper concentration after the first week of acclimation

Concentration (μg Cu l^{-1})	Biomass ± S.D. (mg DW l^{-1})	Growth rate ± S.D. (per day)	Chl *a* (10^{-14} g per cell)	Chl *b* (10^{-14} g per cell)	Car (10^{-14} g per cell)
0.5	111.6 ± 2.4 a	0.944 ± 0.001 a	3.8	0.5	2.2
1	95.0 ± 3.1 b	0.921 ± 0.004 b	4.4	0.6	2.9
5	93.1 ± 1.8 b	0.919 ± 0.003 b	6.7	1.1	3.5
12	93.4 ± 3.6 b	0.919 ± 0.005 b	9.8	1.5	4.1
35	93.2 ± 4.1 b	0.919 ± 0.006 b	10.3	1.1	4.7
60	30.5 ± 2.4 c	0.759 ± 0.011 c	39.3	7.7	9.0
100	4.7 ± 0.7 d	0.489 ± 0.021 d	84.7	20.9	14.8

Values with same letter are not significantly different (analysis of variance, $P < 0.05$).

climated to 60 μg Cu l^{-1} showed similar results. The algal biomass and growth rate of algae acclimated to 0.5 μg Cu l^{-1} was significantly higher than those of algae acclimated to higher copper concentrations.

Except for the results obtained during the first (shock) week of acclimation, all parameters did not change significantly during the 3 months acclimation period. Hence, the mean values ($N = 8$) of the algal biomass, the growth rate, the pigment concentrations, the pigment diversity and the autotrophic index of the different *P. subcapitata* acclimation cultures, are presented in Table 2. In weeks 2–12 pooled, the highest biomass is observed for algal populations exposed to 1, 5 and 12 μg Cu l^{-1}. Severe reduction of algal biomass is observed in the cultures acclimated to 100 μg Cu l^{-1}. The growth rate (mean ± standard deviation) of the latter was 0.777 ± 0.038 per day compared to 0.917 ± 0.015 per day for algae acclimated to 0.5 μg Cu l^{-1}. A Student's *t*-test for independent samples ($P < 0.05$) for algal biomass and growth rate revealed a no observed effect concentration (NOEC) at 35 μg Cu l^{-1} for acclimated algal populations. The autrophic index and pigment diversity gave a NOEC at 60 μg Cu l^{-1}.

A significant increase in chlorophyll *a* and carotenoid concentrations is observed in algae acclimated to 60 and 100 μg Cu l^{-1}. Chlorophyll *a* and *b* concentrations increased with a factor of 3.4 and 2.7, respectively, with increasing acclimation concentration. Carotenoid concentrations increased only with a factor of 2. Chlorophyll *a* concentrations were 5–6-fold higher than chlorophyll *b* concentrations and 2–4-fold higher than carotenoid concentrations. The pigment diversity of algae acclimated to 1–35 μg Cu l^{-1} was significantly higher than that of algae acclimated to lower and higher copper concentrations. Similar results were noted for the autotrophic index. A positive linear correlation ($R^2 = 0.76$; $N = 60$) was observed between pigment diversity and autotrophic index (data not shown). A negative logarithmic correlation was observed between chlorophyll *a* ($R^2 = 0.95$; $N = 60$), chlorophyll *b* ($R^2 = 0.87$; $N = 60$) or carotenoid ($R^2 = 0.89$; $N = 60$) concentrations and the algal biomass (Fig. 1).

Experiments with *P. subcapitata* performed in standard ISO-medium (ISO, 1987) resulted in a 72 h E_rC_{50} of 37 ± 3 μg Cu l^{-1} (mean ± standard deviation, $N = 3$). The chronic copper sensitivity ($N = 8$), performed in modified ISO medium, of *P. subcapitata* acclimated to the seven different copper concentrations is presented in Fig. 2. An increase in copper toxicity values is observed with increasing copper acclimation concentrations. The 72 h E_rC_{50} (mean ± standard deviation) only increased significantly from 88 ± 12 μg Cu l^{-1} to 124 ± 21 μg Cu l^{-1} for algae acclimated to 0.5 and 100 μg Cu l^{-1}, respectively. The data fitted a positive linear regression equation: $a = 0.33b + 90$ ($R^2 = 0.94$; $P < 0.05$) with *a* the 72 h E_rC_{50} and *b* the acclimation concentration (μg Cu l^{-1}). For each toxicity test, the pH drift was less than 0.5 pH units over 72 h. pH drift in acclimation cultures was sometimes as much as 1 pH unit as a result of the high final algal cell densities (>10^6 cells ml^{-1}).

Adsorbed and intracellular (absorbed) copper concentrations for the different acclimation cultures are shown in Fig. 3. An increase in surface-bound and intracellular copper with increasing copper acclimation concentrations is observed. Algae acclimated to the highest copper acclimation concentrations (60 and 100 μg Cu l^{-1}) exhibited a strong increase in both adsorbed and intracellular copper after the first week of acclimation. Intracellular copper concentration for algae acclimated to 0.5 and 100 μg Cu l^{-1} increased from 0.001 to 59.3×10^{-15} g Cu per cell, while adsorbed copper concentration ranged from 0.033 to 35.1×10^{-15} g Cu per cell. From week 3 onwards, a strong decrease of both parameters is observed. Compared to the first week measurements, intracellular copper concentrations of algae acclimated to 100 μg Cu l^{-1} for 12 weeks decreased from 59.3×10^{-15} to 20.6×10^{-15} g Cu per cell and adsorbed copper concentrations from 35.1×10^{-15} to 1.8×10^{-15} g Cu per cell. Except for the lowest copper acclimation concentration, more than 80% (50% for 0.5 μg Cu l^{-1}) of the total cellular copper (adsorbed + absorbed) was located intracellularly.

Finally, the adsorbed and absorbed copper concentrations were plotted against the algal biomass inhibition of the acclimation cultures (Fig. 4). This figure gives an indication whether it are mechanisms on the cell wall or in the algal cell which regulate the copper homeostasis at a certain copper exposure concentration. Based on a breakpoint–regression analysis with the log 10 transformed values of adsorbed and

Table 2
Mean ($N = 8$) biomass, growth rate, chlorophyll *a* (Chl *a*) and *b* (Chl *b*) concentration, carotenoid concentration (Car), pigment diversity (PD) and autotrophic index (AI) with standard deviation (S.D.) of algae acclimated to seven copper concentrations (pooled data of weeks 2–12)

Concentration (μg Cu L^{-1})	Biomass ± S.D. (mg DW l^{-1})	Growth rate ± S.D. (per day)	Chl *a* ± S.D. (10^{-14} g per cell)	Chl *b* ± S.D. (10^{-14} g per cell)	Car ± S.D. (10^{-14} g per cell)	PD ± S.D. (–)	AI ± S.D. (–)
0.5	88.1 ± 8.9 b	0.917 ± 0.015 a	8.4 ± 3.1 a,c	1.7 ± 0.5 b	3.7 ± 0.8 c	0.390 ± 0.065 b	137 ± 26 b,c
1	95.6 ± 6.0 a	0.920 ± 0.016 a	9.4 ± 2.0 a,c	1.8 ± 0.6 b	3.8 ± 0.2 c	0.422 ± 0.081 a,b	178 ± 34 a
5	96.4 ± 7.8 a	0.919 ± 0.016 a	9.9 ± 3.6 a,c	1.9 ± 1.0 b	4.1 ± 0.6 b,c	0.465 ± 0.059 a	183 ± 39 a
12	95.6 ± 4.8 a	0.920 ± 0.016 a	10.6 ± 2.8 b,c	1.9 ± 0.7 b	4.2 ± 1.1 b,c	0.401 ± 0.058 a,b	158 ± 44 a,b
35	88.9 ± 6.6 b	0.906 ± 0.019 b	10.3 ± 2.0 b,c	2.0 ± 0.6 b	4.4 ± 0.8 b,c	0.425 ± 0.045 a,b	150 ± 29 a,b,c
60	75.2 ± 4.4 c	0.888 ± 0.008 c	13.5 ± 3.2 b	2.3 ± 0.8 b	4.9 ± 1.1 b	0.370 ± 0.051 b	118 ± 33 c
100	35.7 ± 9.8 d	0.777 ± 0.038 d	28.6 ± 7.5 a	4.6 ± 1.6 a	7.1 ± 1.2 a	0.251 ± 0.024 c	56 ± 14 d

Values with the same letter are not significantly different at $P < 0.05$ (analysis of variance).

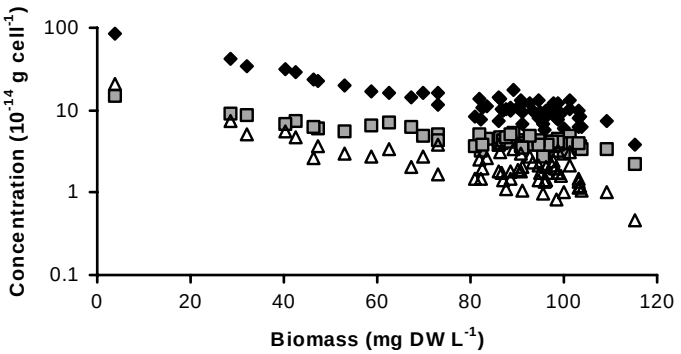

Fig. 1. Relation between algal biomass and chlorophyll *a* (◆), chlorophyll *b* (△) and carotenoid (■) concentration of the acclimated *P. subcapitata*.

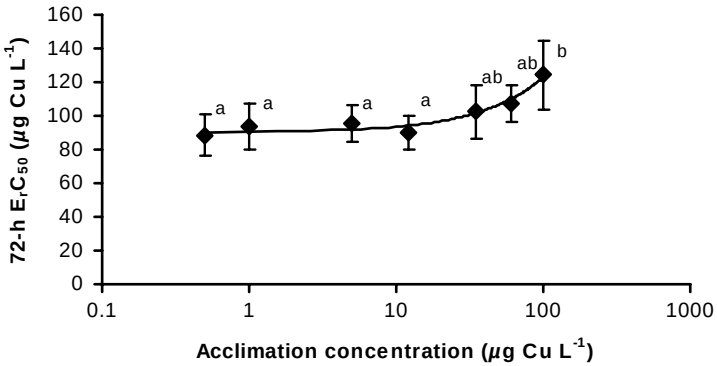

Fig. 2. Chronic copper tolerance (72 h E_rC_{50}) of *P. subcapitata* acclimated to different copper concentrations. Error bars represent standard deviations. Mean ($N = 8$) values with the same letter are not significantly different at $P < 0.05$.

absorbed copper concentrations, a critical accumulation concentration (with 95% confidence limits) of copper on the algal cell wall ($0.12(0.09–0.17) \times 10^{-15}$ g Cu per cell; $R^2 = 0.9$, $N = 48$, F-test: $P < 0.001$) and in the algal cell ($2.9(2.4–3.6) \times 10^{-15}$ g Cu per cell; $R^2 = 0.9$, $N = 51$, F-test: $P < 0.001$) could be determined, resulting in no algal biomass inhibition. When inhibition of growth was considered, similar results were obtained. Adsorbed and absorbed copper concentrations of 2.6×10^{-15} and 13.7×10^{-15} g Cu per cell were noted at an algal population biomass inhibition of 50%. A poor positive correlation was observed between internal copper concentrations of the acclimated algae used in 72 h toxicity assays and their chronic copper sensitivity ($R^2 = 0.10$; $N = 42$, $P > 0.05$).

4. Discussion

The toxicity of copper to *P. subcapitata* has been reported by a number of workers (Bartlett et al., 1974; Christensen et al., 1979; Blaylock et al., 1985; Haley et al., 1986; Franklin et al., 2001, 2002). EC50 values range from 8 to 400 µg Cu l^{-1}. It has been suggested that the main reason for this high variability is attributable to the differences in culture and test media composition affecting both the algal performance and the metal's bioavailability (Janssen and Heijrick, 2003). The EC50s (>70 µg Cu l^{-1}) obtained in the present study are considerably higher than those derived from tests (37 µg Cu l^{-1}) performed in standard ISO medium (ISO, 1987). This is due to differences in copper complexation capacities of the two media:

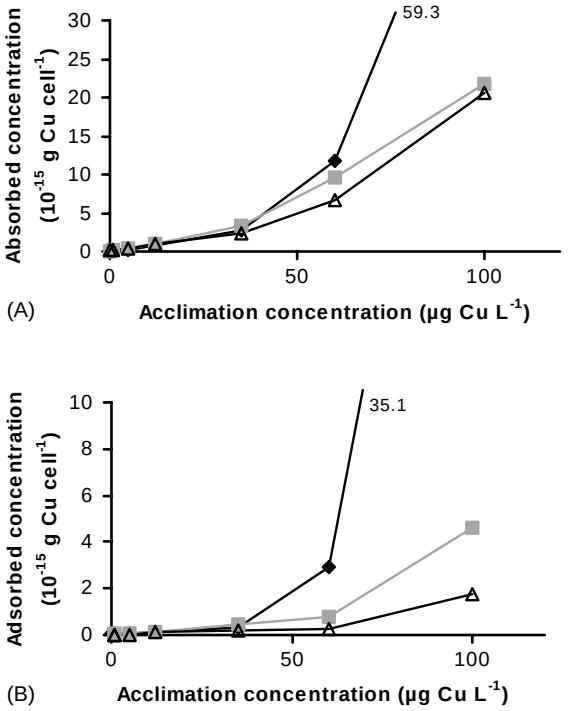

Fig. 3. Absorbed (A) and adsorbed (B) copper concentration of *P. subcapitata* acclimated to different copper concentrations. ((◆) week 1; (■) week 3 and (△) week 12).

different DOC sources (AHA versus EDTA) and different concentrations (2 mg C l^{-1} in our test media compared to 0.32 mg C l^{-1} in standard media). Indeed, several authors have demonstrated that copper toxicity for algae decreases with increasing DOC (Winner and Owen, 1991; Garvey et al., 1991; Janssen and Heijrick, 2003).

It is well recognized that concentrations of a chelator are necessary to prevent iron (Fe) precipitation by forming Fe-complexes in algal culture and test media. Therefore, omitting chelators will lead to nutrient limited algal growth (Lewis, 1995). Consequently, although organic complexes will reduce the copper toxicity, some complexing capacity is needed in culture media if some degree of natural realism is to be attained. Although AHA—used in our study—is not a naturally produced component of aquatic ecosystems, but derived from terrestrial humic acid and brought in the aquatic compartment through run-off, it will better reflect the buffering capacity of natural organic matter in surface waters compared to EDTA. The selection of the DOC concentration used in the test and culture media was based on an analysis of the Surface Water Database (SWAD; Heijerick and Janssen, 2000, update database 2003, personal communication), a database containing the physico-chemistry of approximately 200,000 European surface water monitoring stations. The 10th and 50th percentile of the DOC concentration was around 2 and 5 mg l^{-1}, respectively (Bossuyt and Janssen, 2003).

The influence of essential trace elements on the sensitivity of micro-algae to environmental contaminants has—up to now—not been examined in-depth. This factor may be especially important in toxicity assessments of metals and metal compounds, as it has been reported that pre-exposure to metals during culturing can affect the sensitivity of algae in

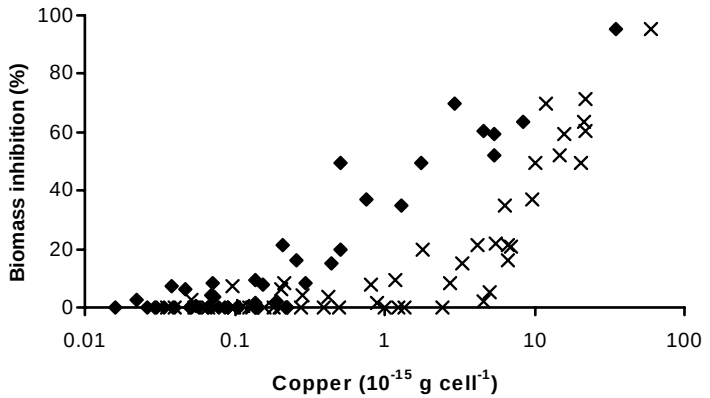

Fig. 4. Relationship between the algal biomass inhibition and the adsorbed (◆) and absorbed copper (×) concentration in *P. subcapitata* acclimated to different copper concentrations.

Table 3
Essential metal (copper: Cu; zinc: Zn) and EDTA concentrations in culture and test media commonly used in experiments with freshwater Chlorophyceae

Medium	Cu ($\mu g\,l^{-1}$)	Zn ($\mu g\,l^{-1}$)	EDTA ($\mu g\,l^{-1}$)	Reference
Chu no 10	0	0	0	Chu, 1942
DSW	0	0	0	Tubbing et al., 1994
JM/5	0	0	3600	Thompson et al., 1988
Volvox	0	7.2	1845	Starr, 1969
OECD/ISO	0.0037	1.4	82	OECD, 1996; ISO, 1987
ASTM/USEPA	0.004	1.57	246	ASTM, 1998; USEPA, 1994
SAAM	0.0041	15.7	246	Christensen et al., 1979
Fraquil	0.065	0.3	1525	Morel et al., 1975
SANM	0.127	1.56	1100	Kuwabara and Leland, 1986
BBM	400	2000	50000	Nichols and Bold, 1965

metal toxicity tests. The copper concentration in standard algal media can differ up to a factor of 10,000 (Table 3). Reports of acquired resistance to copper by different algal species growing in copper contaminated freshwater systems are numerous (Stokes et al., 1973; Stokes, 1975; Butler et al., 1980; Foster, 1982; Takamura et al., 1989; Lombardi and Vieira, 1998). Stokes and Dreier (1981) noted that *Scenedesmus* cells cultured in medium with copper addition (500 $\mu g\ Cu\,l^{-1}$) were—after only three generations—more tolerant than those cultured in copper deficient medium. Sandmann and Böger (1980) reported acclimation to copper by *S. acutus* in the first 24 h of exposure. The present study demonstrated that *P. subcapitata* can also acquire increased copper tolerance, but no significant changes were observed at lower—more environmentally realistic—copper concentrations (Fig. 2). A population NOEC value of 35 $\mu g\,Cu\,l^{-1}$ was observed based on the algal biomass and growth rate of the acclimated algae. As the test set-up allowed for a gradual acclimation and induction of the homeostasis processes, which is probably naturally occurring in surface waters, this NOEC may be of very high ecological relevance and hence directly comparable to a field situation. A maximum resistance factor (RF = 72 h E_rC_{50} of acclimated organisms divided by the 72 h E_rC_{50} of non-acclimated organisms) of 1.7 was noted. Muyssen and Janssen (2001) found a maximum RF of 3.2 for *P. subcapitata* and *Chlorella vulgaris* when cultured in medium containing 65 $\mu g\,Zn\,l^{-1}$. This zinc concentration was, however, higher than background concentrations in European lowland freshwaters (mean: 18.5 $\mu g\,Zn\,l^{-1}$; range 8.0–42.7 $\mu g\,Zn\,l^{-1}$; Zuurdeeg et al., 1992).

From the observed changes in biomass, growth rate, pigment diversity and autotrophic index (Table 2) as a function of the copper acclimation concentrations, the optimal concentration range (OCEE curve) or Window of Essentiality can be derived (Van Assche et al., 1997; Hopkin, 1989; respectively). In Table 2, significant optimal concentrations were observed in biomass (1–12 $\mu g\ Cu\,l^{-1}$), growth rate (up to 12 $\mu g\ Cu\,l^{-1}$), pigment diversity (1–35 $\mu g\ Cu\,l^{-1}$) and autotrophic index (1–35 $\mu g\ Cu\,l^{-1}$). Toxicity and deficiency effects (reduced values) were noted at higher and lower concentrations, respectively. Peterson et al. (1984) and Reuter et al. (1987) found an optimal growth for *S. quadricauda* at a Cu^{2+} concentration lower than 10^{-11} M. They did not observe limitation of growth at the lowest copper concentrations tested ($10^{-12.5}$ M Cu^{2+}). Knauer et al. (1997) determined the optimal copper range for three different species of unicellular green algae: 10^{-15} to 10^{-7} M Cu^{2+} for *S. subspicatus*, 10^{-11} M Cu^{2+} for *Chlamydomonas reinhardtii* and 10^{-13} to 10^{-10} M Cu^{2+} for *Chlorella fusca*. Expressed as Cu^{2+}, the optimal concentration range of *P. subcapitata* obtained in our study was 10^{-14} to 10^{-10} M Cu^{2+} (see further), which is in agreement with these reported in literature. In contrast to the algal growth rate (biomass), pigment concentrations were significantly higher in the highest acclimation concentration. Cid et al. (1995) also observed an increase in chlorophyll *a* content in *Phaeodactylum tricornutum* when exposed to copper up to 100 $\mu g\ Cu\,l^{-1}$ (i.e. concentration that induced

about 50% growth reduction), followed by a decrease at higher concentrations. At copper concentrations lower than the optimal concentration range, low values of biomass, pigment diversity and autotrophic index were observed and this may be due to possible copper deficiency. However, no significant lower values were demonstrated using the chronic toxicity values and chlorophyll and carotenoid endpoints.

It is suggested that continuous culture over several years in such deficient media may affect the health of the organisms. Standard ISO medium (<0.1 μg Cu l^{-1}) and other international media vary considerably in essential metal concentrations (Table 3) and may be copper deficient for culturing algae, resulting in significantly affected toxicity test results (depending on the parameter investigated).

Measurements of intracellular and adsorbed copper of *P. subcapitata* indicated that 80% of the total cellular copper was located in the algal cells. Knauer et al. (1997) made a similar observation for *S. subspicatus* exposed to copper. Franklin et al. (2000) found that 60% of the copper content was internally bound in *Chlorella* sp. exposed to 10 μg Cu l^{-1}. Remarkable was that—for copper concentrations of 5–100 μg Cu l^{-1}—algae took up 50% of the copper added to the medium within 1 week. Franklin et al. (2002) noted similar results for *Chlorella* sp. and *P. subcapitata*. Knauer et al. (1997) observed that the maximal intracellular copper content for *S. subspicatus* exposed for 5 days to 6.4 mg Cu l^{-1} was 2.5×10^{-14} g Cu per cell. They found that the total cellular copper concentration increased with increasing copper concentration. Hall et al. (1989) also observed an increase in cellular copper content in *C. vulgaris* and *Chlamydomonas geitleri* (isolated from a Cu and Zn contaminated lake) when they were transferred from 2.5 to 2860 μg Cu l^{-1}. They found an increase in cellular copper content from 10^{-15} up to 10^{-12} g Cu per cell for both *Chlorella* and *Chlamydomonas*, respectively. This is an increase with a factor of 1000, which is similar to that observed in our study (factor 100–10,000). According to our results, optimal growth occurs at intracellular copper concentrations of 10^{-17} to 10^{-15} g Cu per cell. This rather broad range suggests that cells regulate the intracellular copper concentration, presumably through immobilization of excess copper, as demonstrated by Silverberg et al. (1976) and Knauer et al. (1998).

The internal metal concentrations of culture algae used in the growth inhibition experiments are poorly correlated with the observed copper toxicity in *P. subcapitata*. Franklin et al. (2002) observed a relationship between both extracellular and intracellular copper concentrations and 72 h growth inhibition test results for *Chlorella* sp. and *P. subcapitata*. They found that 50% growth inhibition occurred at 3×10^{-14} g Cu per cell of intracellular copper and 8×10^{-15} g Cu per cell of extracellular copper. In our study, the latter copper concentration only occurred in algae acclimated to 100 μg Cu l^{-1}, which did exhibit a strong decrease in biomass production (>60%). It has to be noted that, in our study, algae with this high internal copper concentration had an increased copper tolerance. The observed decrease of surface-bound and intracellular copper with longer acclimation duration (Fig. 3) can be the result of a defence mechanism of the algae such as cell wall exclusion of copper (Knauer et al., 1997) and changes in the permeability of the algal plasma membrane (DeFilipis, 1979). Further research will be needed to determine the exact detoxification mechanism.

From our results, it seems that *P. subcapitata* has different mechanisms to compete with copper stress. From Fig. 4, it was observed that starting from an adsorbed copper concentration of 0.12×10^{-15} g Cu per cell (i.e. critical accumulation concentration) to the algal cell wall, inhibition of the algal biomass (and growth rate) occurred. This adsorbed copper concentration was noted in algae cultures acclimated to 5 and 12 μg Cu l^{-1}. Ma et al. (2003) stated that copper toxicity to unicellular algae could be interpreted by its accumulation at a discrete site or biotic ligand at the algal cell wall. At a copper concentration equal to the 72 h EC50 of *S. subspicatus*, they observed a critical accumulation of adsorbed copper of 6.4×10^{-13} μg Cu per cell. The lower value in our study (2.6×10^{-15} g Cu per cell) can be explained by the use of a different algal species and because our critical value is determined on an algal population of 1 week old (high algal density). *Scenedesmus* sp. is already described as more tolerant to copper than *P. subcapitata* (Fargašová et al., 1999). It may be hypothesized that at copper concentrations lower than the critical accumulation concentration (i.e. acclimation concentrations <12 μg Cu l^{-1}), algae probably have an active copper accumulation in order to fulfil their metabolic require-

ments (1 and 5 μg Cu l^{-1} are optimal concentrations; Table 2). Hence, it may be suggested that—in this acclimation interval of copper—copper uptake in the algae is regulated by the algal cell membrane. Copper uptake is regulated at the cell wall level and will be taken up intracellularly by the cell through specific canals (Harrison et al., 2000). Internal algal copper concentrations (absorbed copper) up to 2.9×10^{-15} g Cu per cell, which occurs in algae acclimated to 12 and 35 μg Cu l^{-1}, resulted in a non-significant inhibition. At higher concentration, significant inhibition of the algal biomass and growth rate occurs (population NOEC = 35 μg Cu l^{-1}). Hence, for copper acclimation concentrations between 12 and 35 μg Cu l^{-1}, copper regulation may be a combination of regulation on the algal cell wall and intracellular copper regulation.

No literature was found on the influence of the cupric ion at relevant background concentrations on the acclimation potential of micro-algae. Free copper activities of the acclimation concentrations used in this study were calculated with the geochemical speciation program Windermere Humic Aqueous Model VI (Tipping, 1994; WHAM 6.0.1, Centre for Ecology and Hydrology, Windermere, UK) and ranged from 10^{-18} to 10^{-8} M Cu^{2+} (1.3×10^{-18}; 2.1×10^{-14}; 1.3×10^{-12}; 1.3×10^{-11}; 3.1×10^{-10}; 4.1×10^{-9}; 1.9×10^{-8} M Cu^{2+}). Based on the results described above, it can be concluded that concentrations ranging from 2.1×10^{-14} to 6.1×10^{-10} M Cu^{2+} are optimal for growth of *P. subcapitata*. Concentrations $>10^{-9}$ M Cu^{2+} resulted in toxic effects in long-term acclimation experiments, while concentrations $= 10^{-14}$ M Cu^{2+} resulted in deficiency effects. Calculated 10–90 percentile Cu^{2+} activity intervals for Europe were 2.1×10^{-14} to 4.6×10^{-8} M Cu^{2+} (Bossuyt and Janssen, unpublished data). This confirms that the concentration range tested in this study is relevant for Europe and that the optimal range for copper is situated within the copper activity range found across Europe. When the total copper concentrations mentioned in Table 3, are expressed as copper activities, all standard media have values lower than 10^{-14} M Cu^{2+}. According to the results of this study, these media are or may become copper deficient for algae and are only relevant for extreme environments, like mountain lakes (Knauer et al., 1997). Results of experiments performed with algae cultured or tested in these media may not be representative for most aquatic ecosystems.

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