Bull. Environ. Contam. Toxicol. (2002) 69:546–553

DOI: 10.1007/s00128-002-0096-4

Bulletin of Environmental Contamination and Toxicology

Copper Tolerance and Accumulation Potential of *Chlamydomonas reinhardtii*

C. Boswell,[1] N. C. Sharma,[2] S. V. Sahi[1]

[1] Biotechnology Center, Department of Biology, Western Kentucky University, Bowling Green, KY 42101, USA
[2] S.B.S. P.G. Institute of Biomedical Sciences and Research, Dehradun 248161, India

Received: 30 September 2001/Accepted 27 April 2002

Toxic heavy metals enter to water bodies from various sources and aquatic plant species are threatened by their increasing concentrations (Prasad 1997). As unicellular chlorophytes are good indicators of water pollution, they are increasingly used in phytotoxicity tests for environmental monitoring (Fernandez-Leborans and Novillo 1996; Tadros et al. 1994; Wang and Freemark 1995). In this context, responses of several green algae to toxic metals have been investigated (Cain and Allen 1980; Prasad et al.1998; Visviki and Rachlin 1994; Weiss-Magasic et al. 1997) and some of these taxa have been found to display tolerance/resistance to toxic heavy metals (Bariaud et al; 1985; Foster 1977; Harding and Whitton 1976). The acquisition of heavy metal tolerance in algae from polluted areas, in line with acquisition of pesticide resistance in insects or herbicide resistance in weeds, is an example of evolution in action (Shaw 1990).

The mechanism of tolerance seems to be dependent on the algal species, the strain within a particular species and the metal involved. Thus in the present study *Chlamydomonas reinhardtii* strain CC125 was selected to determine toxic effects of higher doses of copper (Cu). *C. reinhardtii* constitutes a model system for genetic analysis and biochemical characterization among photosynthetic eukaryotes (Collard and Matagne 1990). Copper, though an essential micronutrient for photosynthetic eukaryotes, produces acute toxic effects to aquatic plants below the level of 1ppm (USEPA 1985). Therefore, the purpose of this study was to determine the algistatic/algicidal concentration of Cu for *C. reinhardtii* CC125 cells and explore the Cu accumulation potential of this strain, if any. This study illustrates the tolerance of *C. reinhardtii* cells at the toxic concentrations of 100 and 150 $\mu M CuSO_4$.

MATERIALS AND METHODS

Cultures of *Chlamydomonas reinhardtii* Dangeard strain CC125 (derived from Levine's 137c mt+ strain) were obtained from the *Chlamydomonas* Genetics Center at Duke University. Erlenmeyer flasks containing 100 ml of TAP medium (Harris 1989) supplemented with various levels of $CuSO_4.5H_2O$ (0, 50, 100, 150, 200 and 250 μM) were inoculated at 10^5 cells/ml (counted with a hemocytometer) as described by Harris (1989). In each experiment $CuSO_4.5H_2O$ was used as

Correspondence to: S. V. Sahi

source of copper in the media. Cultures were grown mixotrophically on a shaker (rpm 250) under constant light (20 $\mu E/m^2s$) and temperature (25^0C) for three weeks. Aliquots of 1 ml were taken every three days from each culture and the optical density was measured using a spectrophotometric procedure described by Lustigman et al. (1995) at 750 nm to determine cell growth.

In order to determine whether high concentrations of $CuSO_4$ were algistatic or algicidal, aliquots were taken every three days, centrifuged and washed several times to rid the cells of Cu contaminants. Cells were then diluted to 1:10, 1:100, and 1:1000 and plated on TAP-agar plates without $CuSO_4$. These plates were exposed to constant light (same as above) for 18 d at 25^0C. Colonies were then counted to determine the effects of increased concentrations of $CuSO_4$ on cell viability. All experiments were repeated twice with three replicates each (data presented in results refer to average values of the final experiment). The results were statistically evaluated by student's t-test at P=0.05. Cell morphology was observed for an entire growth period using light microscopy. Color, shape and size were observed to determine the health of the cells grown in TAP media containing $CuSO_4$ concentrations of 0, 50, 100, 150, 200 and 250 μM.

In order to be able to measure the effects of $CuSO_4$ on the total chlorophyll content even at higher levels (200 and 250 μM), cells from stationary phase cultures growing in the absence of $CuSO_4$ were centrifuged and re-suspended in an equal amount of TAP medium supplemented with 0, 50, 150 or 250 μM $CuSO_4$. An aliquot of 1.5 ml was removed from the cultures every three days, chlorophyll was extracted by the method described by Wang et al. (1975), and measured spectrophotometrically. Total chlorophyll was calculated by the method described by Arnon (1949).

Large quantities of *Chlamydomonas* cells were grown in TAP media containing 0, 50, 100 and 150 μM $CuSO_4$ for 18 days. Cells were then centrifuged, washed several times and the pellets were collected. The cellular Cu concentrations in these pellets were measured by Inductively Coupled Plasma Spectrophotometry.

RESULTS AND DISCUSSION

Growth rates of *C. reinhardtii* were recorded in TAP media containing various elevated doses of $CuSO_4$ (Figure 1). Up to the treatment of 100 μM $CuSO_4$, cultures grew quickly attaining stationary phase after 6d and the growth curves generated are identical to the control. Cells grown in media containing 150 μM $CuSO_4$ had a different pattern since they took longer to reach exponential growth phase (9d), although during stationary phase the cell concentration was not significantly lower than the control (P>0.05) (Figure 1). However, there was very little growth observed when cells were grown on media containing 200 and 250 μM $CuSO_4$.

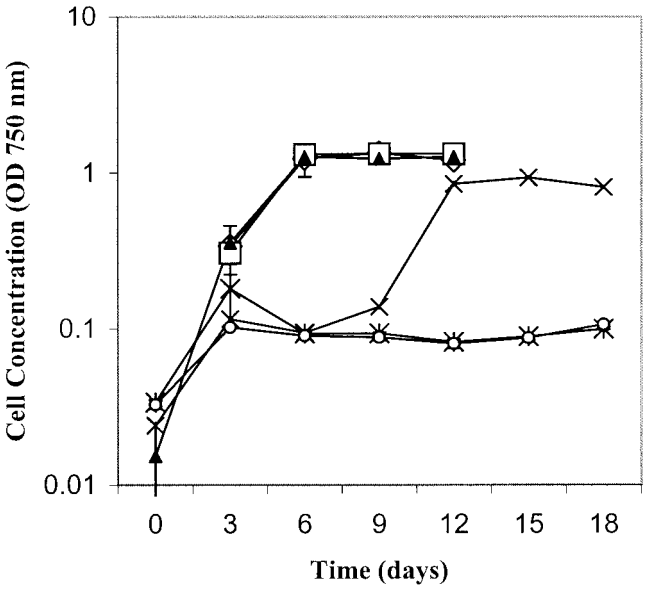

Figure 1. Growth of *Chlamydomonas reinhardtii* in TAP medium containing $CuS0_4$ at the concentrations of 0 –250 μM ($\diamond$ = 0 μM, $\square$ = 50 μM, $\blacktriangle$ = 100 μM, X = 150 μM, $\ast$ = 200 μM, $\circ$ = 250 μM). Each number represents the average ($\pm$ standard error of the mean) of three replicates.

These results clearly demonstrate that $CuSO_4$ up to a level of 100 μM is not toxic to *C. reinhardtii* cells (strain CC125). These results are contrary to the findings of Prasad et al. (1998) who observed substantial reduction of growth at 50 and 100 μM $CuSO_4$ in case of *C. reinhardtii* strain WT2137. Data presented here are also not compatible with the work on *C. bullosa* and *Dunnaliella salina* where $CuSO_4$ inhibited growth, and EC (50)s were determined to be 0.78 μM and 5.94 μM, respectively (Visviki and Rachlin 1994). Thus these findings reflect development of tolerance by this strain against elevated levels of $CuSO_4$. This is further demonstrated by the behavior of cultures with 150 μM $CuSO_4$ wherein growth picked up after an extended period of incubation (9d instead of 3d) and the plateau was reached after 12d instead of 6d.

In order to determine the number of cells that survived the higher concentrations of $CuSO_4$, aliquots were plated on TAP agar media (without $CuSO_4$) and their ability to form colonies was tested. Plating was done every three days until stationary phase was reached. Figure 2 shows that most cells were inactivated at the higher concentrations of 150 and 200 μM $CuSO_4$. Those that managed to survive adapted well and were thus able to grow normally. In the 200 μM $CuSO_4$ cultures growth was inhibited although a small amount of cells managed to

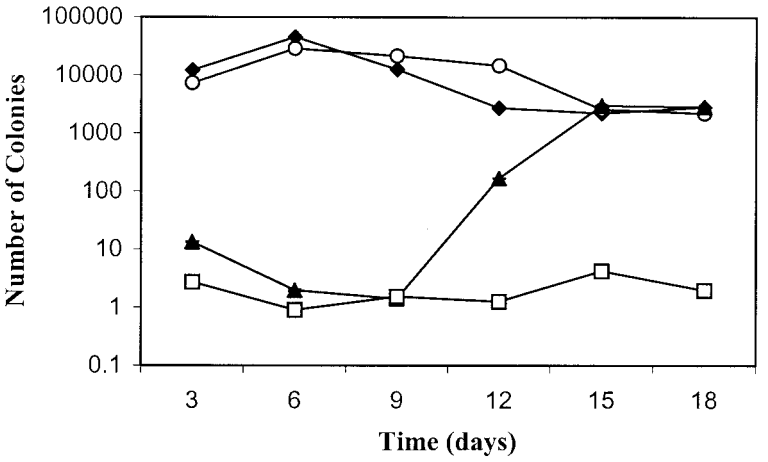

Figure 2. Viability of *Chlamydomonas* cells previously grown in different concentrations of $CuSO_4$ (◆ = 0 μM, ○ = 50 μM, ▲ = 150 μM, □ = 200 μM). Each number represents the average (± standard error of the mean) of three replicates.

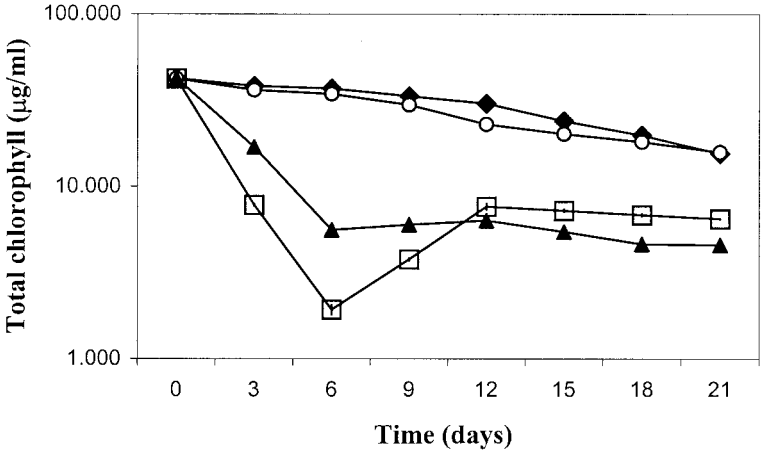

Figure 3. The chlorophyll degradation pattern of *Chlamydomonas* cells grown in different concentrations of $CuSO_4$ (◆ = 0 μM, ○= 50 μM, ▲= 150 μM, □ = 200 μM). Each number represents the average(± standard error of the mean) of total chlorophyll contents of three replicates.

survive the treatment. The effect of high $CuSO_4$ doses on cell morphology was also studied and it was found that $CuSO_4$ up to 100 μM had no adverse effect on cell shape, size or color (data not presented). At 150 μM $CuSO_4$, cells exhibited some degree of toxicity as reflected by their change of color (yellow) and shape. Changes in cell morphology as a result of heavy metal–stress have been reported (in *C. reinhardtii*) by other workers (Weiss-Magasic et al.1997).

Since cell color was affected, the chlorophyll content in cultures was also determined. The results as depicted in figure 3 show a decline in total chlorophyll content in cultures at and above 150 μM $CuSO_4$. It is interesting to note that the steep decline leveled off or even reversed after 6d for both the 150 and 200 μM $CuSO_4$ treatments. Reduction in chlorophyll content in *C. reinhardtii* at 50 and 100 μM of $CuSO_4$ has been reported by Prasad et al. (1998).

The last question to be answered was how much $CuSO_4$ do these cells take up and in which way does the accumulated $CuSO_4$ affect the profile of other elements within the cell. Figure 4 indicates that Cu accumulation within the cell increases with increasing $CuSO_4$ in medium. The accumulation of copper by cells in 100 and 150 μM $CuSO_4$ is about six fold of the control value. Thus *C. reinhardtii* strain CC125 stands as a potential candidate for remediation of Cu- contaminated sites. A trend whereby intracellular P, Ca and Mg declined with increasing Cu accumulation was also recorded (Figure 5). Uptake of Cu was negatively correlated with P ($r = -0.896$), Ca ($r = -0.649$) and Mg ($r = -0.829$) content. The relationship between Cu and both P and Mg was significant based on Bonferroni-adjusted probabilities ($p = 0.0002$ and $p = 0.0016$, respectively).

The mechanism of heavy metal tolerance in green algae and in *C. reinhardtii* particularly is still under investigation. A variety of strategies are employed by organisms to reduce heavy metal toxicity depending on the nature of the heavy metal and the organisms under stress. Copper tolerant strains of *Chlorella* secrete organic material that induces a decrease in the concentration of free copper ions in the medium (Prasad et al. 1998). Button and Hostetter (1977) have shown that cells of *C. reinhardtii* when exposed to exogenous Cu, accumulate this metal, much of which remains bound to the cell wall. Prasad et al. (1998) also supported this contention while working on Cu and Cd toxicity in *C. reinhardtii*. However, one case of cadmium resistance in *C. reinhardtii* was found to be due to a nuclear mutation (Collard and Matagne 1990). Accumulation of Cd in the chloroplast and mitochondria of *C. reinhardtii* has also been reported (Nagel et al. 1996). Recently, thioredoxin gene expression in *C. reinhardtii* has been implicated in the defense mechanism against heavy metals (Lemaire et al. 1999). In the present study, Cu tolerance in *C. reinhardtii* might be the result of a mutation that preexisted in the population of strain CC125. Tolerance of *C. reinhardtii*, without any scar on its growth profile, to a level of 100 μM may be a strong indicator of some preexisting genetic adaptation. The growth kinetics of cells grown at 150 μM $CuSO_4$ point to some additional adaptive mechanism induced as a result of metal stress. However, this toxic concentration of $CuSO_4$ not only suppresses

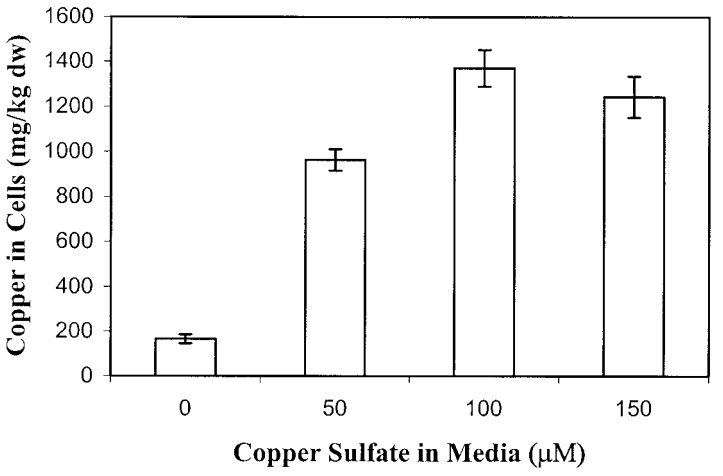

Figure 4. Accumulation of copper by *Chlamydomonas* cells grown in different concentrations of $CuSO_4$ (0-150 μ*M*). Each value represents the mean (± SE) of three replicates.

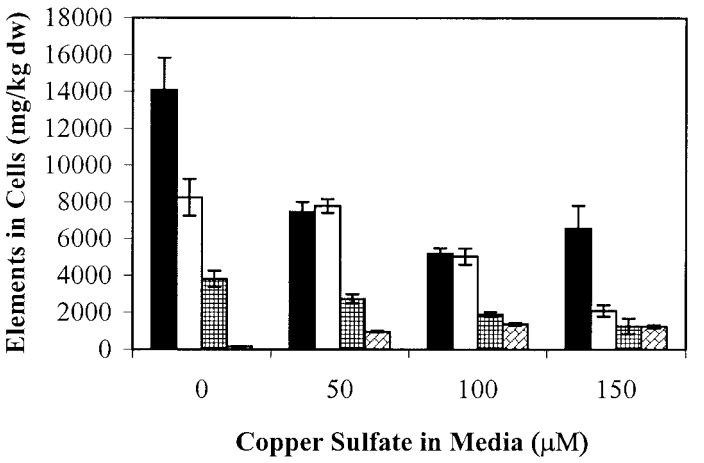

Figure 5. Concentrations of P, Ca, Mg, and Cu in *Chlamydomonas* cells grown at different levels of $CuSO_4$ (0-150 μ*M*). At each Cu concentration, bars represent (from left) P, Ca, Mg and Cu content in cells, respectively. Each value represents the mean (± SE) of three replicates.

growth but also kills many cells as revealed by the viability test (Figure 2). But once the cells that remain alive adapt to the stress conditions they grow as well as the control cells. Higher levels of $CuSO_4$ (200 and 250 μM) could not be counteracted and thus prove to be algicidal.

Acknowledgments. This research was supported partially by the Western Kentucky University in form of Junior Faculty Award to S. Sahi. We thank Drs. S. Jacobshagen and D. McElroy, Biology Department, WKU, for providing *Chlamydomonas* strain and statistical analyses, respectively. The graphical assistance from Mr. S. Cheepala is duly acknowledged.

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