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Combined effect of copper and cadmium on *Chlorella vulgaris* growth and photosynthesis-related gene transcription

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ABSTRACT

Chlorella vulgaris was tested to assess their toxicities in freshwater contaminated by the metal compounds of copper (Cu) and cadmium (Cd), both singly and combined. Exposure to 0.5 and 1.5 μM Cu or 1.0 and 2.0 μM Cd alone significantly decreased algal growth and chlorophyll content and increased reactive oxygen species (ROS) content. Two-way ANOVA analysis shows that the combination of these two metal compounds decreased cell growth, chlorophyll content and increased ROS content synergistically. The highest algal cell inhibition was 78.55%, the lowest levels of chl a, chl b and total-chl were 10.59%, 33.33% and 17.94% of the control, respectively. The highest increase in ROS was 9.15-fold greater than that of the control when exposed to Cu(1.5) + Cd(2.0). Real-time PCR shows that Cu and Cd reduced the transcript abundance of *psb*A and *rbc*L, but without a synergistic interaction, whereas Cu and Cd increased the transcript abundance of *psa*B synergistically. These results demonstrate that Cu and Cd independently inhibit PSII activity and CO_2 assimilation, but synergistically increase ROS content to disrupt chlorophyll synthesis and inhibit cell growth.

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1. Introduction

Aquatic ecosystems are particularly susceptible to accumulating contaminants. Due to their widespread industrial use, large quantities of metal compounds are discharged into freshwater ecosystems and the levels of these have increased substantially world-wide over the last century (Nriagu and Pacyna, 1988; Penuelas and Filella, 2002). Although metal compounds are originate from the bedrock, these chemicals largely enter the eco-environment through industrial and agricultural activities, and then transferred to the food chain (Schutzendubel and Polle, 2002), and can ultimately have significant toxic effects on organisms, causing ecological disturbances (Scoccianti et al., 2008). In China, industrial discharge and urban waste disposal into river and coastal regions are the major sources of aquatic pollution, the common components of which are metal compounds such as cadmium (Cd), copper (Cu), mercury, zinc, and lead (Wang et al., 2007). Unlike complex organic pollutants, metal compounds cannot be degraded by microorganisms; instead, they can be accumulated by organisms and also take part in the process of bioaccumulation throughout the food chain, thus threatening human health (Kong et al., 1995). Therefore, metal compounds are also one of the most persistent pollutants in aquatic environments.

Copper is an essential micronutrient for numerous physiological processes at low concentrations but a toxic metal at high concentrations (Gaetke and Chow, 2003). The progressive increase of Cu in aquatic ecosystems arises from various anthropogenic sources including copper mine drainage, copper-based pesticides, industrial and domestic wastes, and antifouling paints (Andrade et al., 2004; Ma et al., 2003). Cadmium is a non-essential element and extremely toxic to humans, animals and plants. In plants, Cd exposure interferes with plant uptake, transport and uses of different macro and micronutrients (Hart et al., 1998), and induces various symptoms of phytotoxicity, e.g., chlorosis, reduction of biomass, and finally death (Milone et al., 2003). Metals can directly or indirectly produce, by the production of reactive oxygen species, remarkable alterations on proteins, DNA and cellular lipids (Leonard et al., 2004; Valko et al., 2005) that can generate cell death. Cd and Cu were selected for this study because they play a relevant role in aquatic pollution and are potentially toxic to freshwater organisms.

In aquatic ecosystems, algae are primary producers, providing oxygen and organic substances to other life forms. In recent years, algae have been widely used in ecological risk assessment to evaluate the impacts of metal, herbicide and other xenobiotic contamination and bioavailability in aquatic systems, since they are sensitive to metal contaminants at environmentally relevant concentrations (Levy et al., 2007; Stauber and Davies, 2000; Qian et al., 2008a,b). Traditionally, most of the knowledge on the toxicity of metal compounds pollutants to freshwater algae is based upon

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the effects of a single metal compound tested in the laboratory by evaluating biochemical parameters such as EC_{50}, chlorophyll levels, biovolume, cell count, cell size, and so on (Wilde et al., 2006). However, in metal-polluted sites such as mines, two or more metal compounds are often found together in the environment. Since natural pools of water are normally polluted by a mixture of substances, these metals may exert their toxicity simultaneously (Shuhaimi-Othman and Pascoe, 2007). A few studies have been conducted to investigate the combined effect of metal compounds on plant species. The combined effects of Cu, Cd, and zinc on *Chlorella* sp. cell division rate, either as synergistic or antagonistic effects was observed in mixtures of different combinations of metals (Franklin et al., 2002). Sacan et al. (2007) proved that the combination of aluminum and lead synergistically caused cell membrane lysis in marine alga *Dunaliella tertiolecta*. Wong and Chang (1991) showed that various bimetallic combinations of copper, chromium and nickel interacted synergistically on growth, photosynthesis and chlorophyll a synthesis of *Chlorella pyrenoidosa*. Although there is an extensive literature on the biochemical and physiological influence of Cu and Cd on algae, to our knowledge, the interactive effects of Cu and Cd on plankton have rarely been assessed or reported at the gene transcription level.

In the present study, *Chlorella vulgaris* was chosen as a representative green microalga to evaluate the effect of Cu and Cd singly and in combination at the physiological (algal growth, chlorophyll and ROS content) and gene transcription (photosynthesis-related genes: *psa*B, *psb*A and *rbc*L) levels.

2. Materials and methods

2.1. Algal culture

All toxicity tests were conducted using the freshwater unicellular green alga, *C. vulgaris*, obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. The alga was cultured in 250 mL flasks containing 50 mL of sterilized shuisheng-4 medium (Zhou and Zhang, 1989), which is composed of the following chemical ingredients (mg/L): $(NH_4)_2SO_4$, 200; $Ca(H_2PO_4)_2{\cdot}H_2O$, 30; $MgSO_4{\cdot}7H_2O$, 80; $NaHCO_3$, 100; KCl, 25; $FeCl_3$, 1.5; K_2HPO_4, 10; and 3 mL/L soil extract. Algae were cultured at $25 \pm 0.5\,^{\circ}C$ on a 14:10-h light:dark cycle with a light intensity of approximately 46 $\mu mol\,m^{-2}\,s^{-1}$. Cells in the exponential growth phase were used for all experiments, and the initial cell density for each experiment was about 3.5×10^5 cells/mL.

2.2. Test chemicals

Cadmium chloride ($CdCl_2$, JingShanTing Chemical Co., China, reagent grade, 99.9% purity) and copper sulfate ($CuSO_4$, ZhengXin Chemical Co., China, reagent grade, 99.0% purity) were used. Metals were added as aqueous solutions. Stock solutions of metals were prepared in distilled water at a 100 μM concentration. Stock solutions were diluted with distilled water to obtain the desired concentrations for chemical tests.

2.3. Experimental design

The toxicities of copper sulfate and cadmium chloride were assessed independently in our preliminary experiments that generated the concentration–response curves for *C. vulgaris*. The concentration–response curves for copper sulfate and cadmium chloride were shown in Fig. 1. According to these curves, we selected two concentrations of $CuSO_4$ (0.5 and 1.5 μM) and $CdCl_2$ (1.0 and 2.0 μM) to research their combined toxicology. Inhibition of algal activity was expressed by the percentage inhibition (PI),

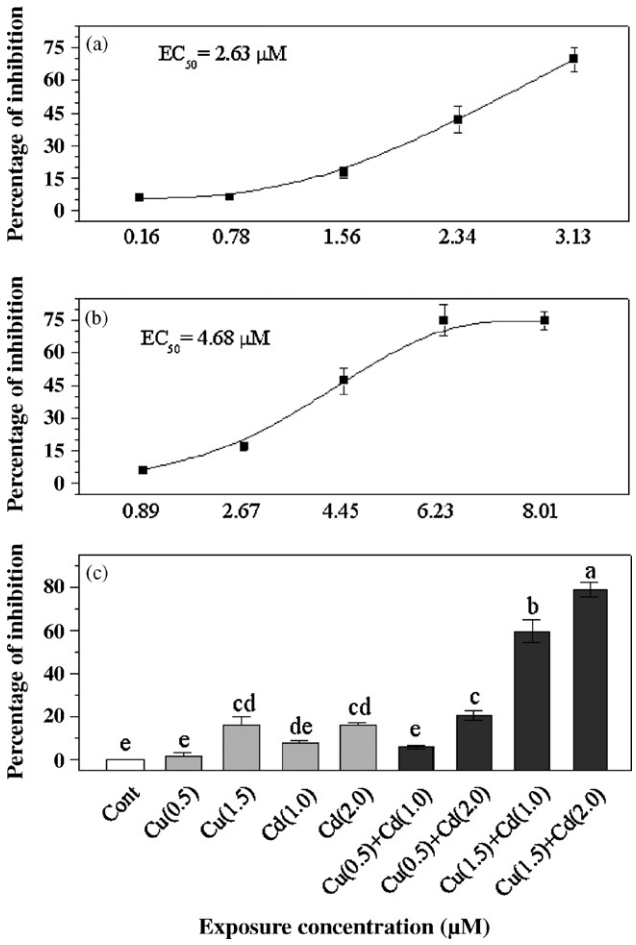

Fig. 1. Effects of copper and cadmium, singly and in combination, on the percentage inhibition of *Chlorella vulgaris* growth after 48 h. (a) Copper alone, (b) cadmium alone, and (c) copper and cadmium singly and in combination.

which was calculated as our previous report (Qian et al., 2009). Five replicates were made of each bioassay.

2.4. Pigment and ROS assays

To analyze chlorophyll a (chl a), chlorophyll b (chl b) and total chlorophyll (total-chl) content according to the method of Inskeep and Bloom (1985), 40 mL of each culture was collected. ROS were measured following the instructions supplied with the ROS kit (Beyotime Institute of Biotechnology, Haimen, China). In this kit, the non-fluorescent probe 2′, 7′-dichlorofluorescein diacetate (H2DCF-DA) passively diffuses into cells and is deacetylated by esterases to form non-fluorescent 2′, 7′-dichlorofluorescein (DCFH). DCFH reacts with ROS to form the fluorescent product DCF. The fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (Bio-TEK, USA). The intensity of fluorescence as compared to the control was viewed as the increase in intracellular ROS.

2.5. Gene transcription analysis

Total RNA was extracted from frozen, homogenized algae, which had been collected from 30 mL of algal culture using the RNAiso reagent (TaKaRa Biochemicals) according to the manufacturer's instructions. A given amount of total RNA (500 ng) was reverse transcribed using an M-MLV reverse transcriptase kit (TaKaRa Biochemicals). Real-time quantitative PCR was performed using a PCR

instrument (ABI 7300, USA) as described our previous report (Qian et al., 2008b).

2.6. Data analysis

Experimental data were checked for normality and homogeneity of variance using the Kolmogorov–Smirnov one-sample test and Levene's test, respectively. When necessary, data were transformed for normalization to reduce heterogeneity of variance. Intergroup differences were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. In order to assess the individual effects of Cu and Cd, a factorial experiment design consisting of three concentrations of Cu and Cd, was performed, and followed by two-way ANOVA. All statistical analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL, USA) and Origin 7.0 (OriginLab, Northampton, MA, USA). The critical value for statistical significance was $p < 0.05$.

3. Results

3.1. Effect of Cu and Cd compounds on algal growth

Panels a and b of Fig. 1 show the cell growth percentage of growth inhibition *C. vulgaris* exposed to either Cu or Cd alone. The influence of metal compounds on algal cell growth was expressed as a PI of the algal growth compared to the control treatment. The algal growth was adversely affected upon Cu and Cd exposure. The severity of the response increased with increasing concentrations of Cu and Cd. From Fig. 1a and b, we calculated that the EC_{50} values of Cu and Cd were 2.63 and 4.68 μM, respectively, such that 0.5 and 1.5 μM concentrations of Cu and 1.0 and 2.0 μM concentrations of Cd were selected to analyze the toxicological interaction between Cd and Cu. In our preliminary study, we had selected higher concentrations of Cu and Cd to assess their interaction, and we found that the PI of alga was too high to perform additional assessments (PI >90%).

Fig. 1c shows the inhibitory ratio of Cu and Cd, both singly and combined. The percentage of inhibitions (PI) was 1.59% and 7.90% after exposure to Cu(0.5) and Cd(1.0), respectively, and growth in Cu(1.5) and Cd(2.0) induced a similar PI (∼16%). The combined treatments were Cu(0.5) + Cd(1.0), Cu(0.5) + Cd(2.0), Cu(1.5) + Cd(1.0), and Cu(1.5) + Cd(2.0), where their respective PIs were 6.01%, 20.80%, 59.64% and 78.55%. By one-way ANOVA, the inhibitory effects of Cu(1.5), Cd(2.0), and Cu(0.5–1.5) + Cd(2.0) treatments were significantly increased as compared with the control. Furthermore, PIs resulting from Cu(0.5–1.5) + Cd(2.0) combinations were much greater than those of any other treatment. Two-way ANOVA indicated that either Cu or Cd alone had a significant inhibitory effect on algal growth ($p < 0.001$, Table 1), and that they also exhibited a strong synergistic interaction on algal growth inhibition ($p < 0.001$, Table 1).

3.2. Effect of Cu and Cd compounds on chlorophyll content

The inhibitory effects of Cu and Cd, singly and in combination, on chl a, chl b and total-chl in *C. vulgaris* cells after 48 h of exposure are shown in Fig. 2. The 0.5 and 1.5 μM concentrations of Cu significantly inhibited the content of chl a to 81.78%, 72.46% of the control, respectively, while the 1.0 and 2.0 μM concentrations of Cd significantly inhibited the content of chl a to about 84% of the control, respectively. The combined treatments of two metal compounds also had an inhibitory effect on chl a content, being only 23.24% and 10.59% of that of the control after exposure to Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0), respectively, levels far lower than those of other treatments.

The influence of these two metal compounds on chl b and total chlorophyll content was similar to that of chl a. Each metal

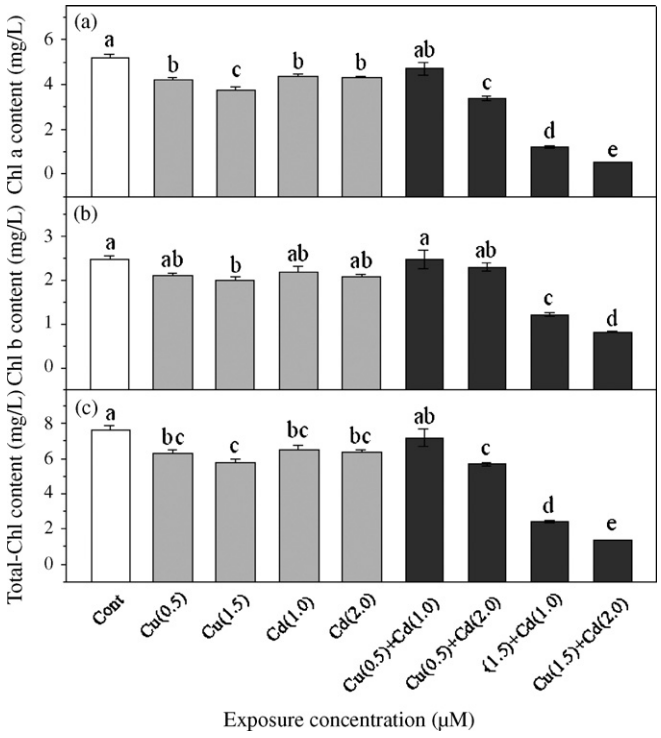

Fig. 2. Effects of copper and cadmium, singly and in combination, on the inhibition of *Chlorella vulgaris* chlorophyll content after 48 h. (a) Chlorophyll a, (b) chlorophyll b, and (c) total chlorophyll. Different letters represent statistically significant differences at $p < 0.05$ from one-way ANOVA.

compound was found to significantly decrease chl b and total-chl by two-way ANOVA. The combination of Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0) caused a decrease in chl b and total-chl content, to levels much lower than those seen after other treatments. Chl b content was 49.51% and 33.33% of the control after exposure to Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0), respectively, and the total-chl content was 31.75% and 17.94% of the control, respectively. Two-way ANOVA indicated that either Cu or Cd alone had a significant inhibitory effect on the content of chl b and total-chl ($p < 0.001$, Table 1), and that they exhibited a strong synergistic interaction on chlorophyll content ($p < 0.001$, Table 1).

3.3. Effect of Cu and Cd compounds on ROS content

Lower concentrations of single metal compounds caused a small increase in ROS (Fig. 3); ROS content in the Cu(0.5), Cu(1.5), and Cd(1.0) single treatments are 104%, 124%, and 120% of that of the control, respectively, and are not significantly different by one-way ANOVA. The Cd(2.0) treatment increased ROS content significantly. The combined Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0) treatment stimulated ROS content to levels 3.58- and 9.15-fold greater of that of the control, and were higher than levels observed after other treatments. Two-way ANOVA indicated that both Cu and Cd alone had a significant positive effect on ROS formation ($p < 0.001$, Table 1). Furthermore, we also observed a strong synergistic interaction between Cd and Cu on algal growth inhibition ($p < 0.001$, Table 1) and on ROS content by two-way ANOVA ($p < 0.001$, Table 1).

3.4. Effect of Cu and Cd compounds on gene transcription

Fig. 4 shows the change in three photosynthesis-related gene transcripts (*psb*A, *psa*B and *rbc*L) after *C. vulgaris* exposure to Cu and Cd. *psb*A gene transcription is slightly inhibited by Cu(1.5) and Cd(2.0) treatments; mRNA levels are 82.74% and 92.53% of the con-

Table 1
Results of 3 × 3 factorial analysis of variance (ANOVA) between copper and cadmium on growth inhibition, ROS content, chlorophyll and *psb*A, *psa*B and *rbc*L mRNA expression in *Chlorella vulgaris*.

Factors	*F*(*p*)							
	Percent inhibition	Chl a	Chl b	Total-Chl	ROS	*psb*A	*psa*B	*rbc*L
Cu	638.50 (<0.001)	286.99 (<0.001)	73.42 (<0.001)	209.46 (<0.001)	384.52 (<0.001)	38.61 (<0.001)	14.12 (<0.001)	19.59 (<0.001)
Cd	248.20 (<0.001)	86.85 (<0.001)	13.29 (<0.001)	56.47 (<0.001)	199.65 (<0.001)	14.31 (<0.001)	49.09 (<0.001)	5.63 (<0.05)
Cu + Cd	106.41 (<0.001)	33.11 (<0.001)	12.59 (<0.001)	26.08 (<0.001)	172.67 (<0.001)	1.06 (0.390)	2.94 (0.035)	0.60 (0.668)

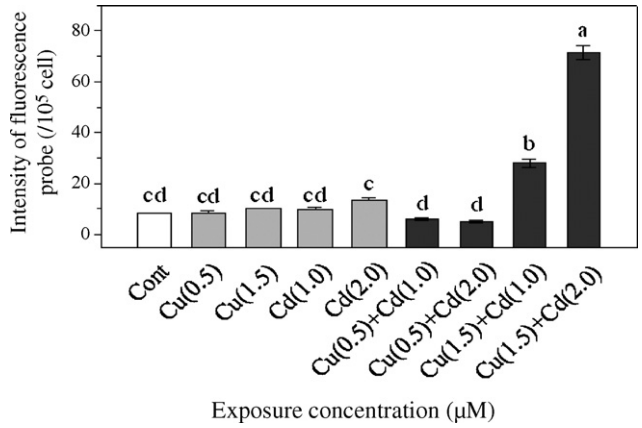

Fig. 3. Effects of copper and cadmium, singly and in combination, on the inhibition of *Chlorella vulgaris* ROS content after 48 h. Different letters represent statistically significant differences at $p < 0.05$ from one-way ANOVA.

trol, respectively (Fig. 4a). Lower concentrations of Cu and Cd had no influence on *psb*A gene transcription. The Cu(0.5) + Cd(1.0–2.0) combinations also had no influence on *psb*A gene transcription, however, the Cu(1.5) + Cd(1.0–2.0) combinations significantly

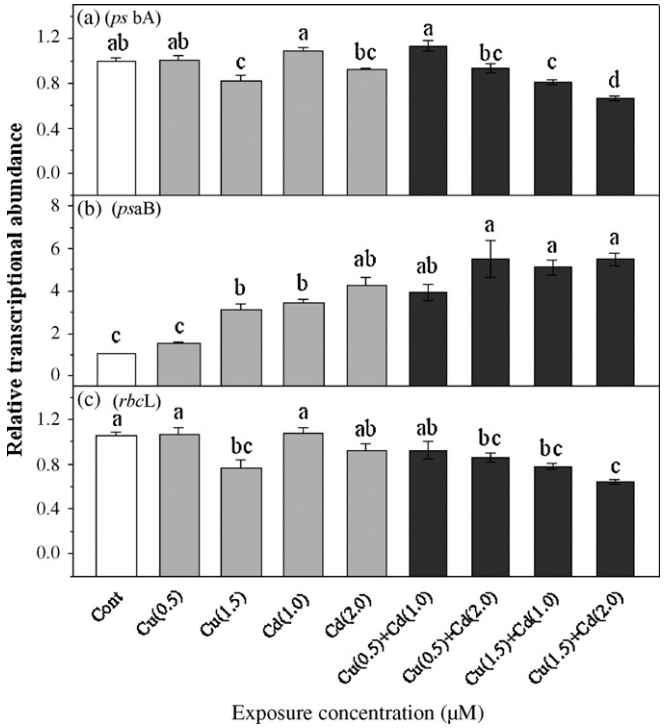

Fig. 4. Effects of copper and cadmium, singly and in combination, on the *Chlorella vulgaris* mRNA expression after 48 h. (a) *psb*A, (b) *psa*B, and (c) *rbc*L. Values were normalized against 18S rRNA, and represent relative the mean mRNA expression value ± SEM of three replicate cultures. Different letters represent statistically significant differences at $p < 0.05$ from one-way ANOVA.

inhibited *psb*A mRNA expression. ANOVA indicated that both Cu and Cd alone had a significant inhibitory effect on *psb*A gene transcription ($p < 0.001$, Table 1). However, we observe no significant interaction between Cd and Cu on *psb*A gene transcription by two-way ANOVA ($p > 0.05$, Table 1).

Fig. 4b shows *psa*B gene transcription after exposure to Cu and Cd. The addition administration of Cu (both 0.5 and1.5 μM doses) or Cd (both 1.0 and 2.0 μM doses) stimulated *psa*B mRNA levels to be 1.43-, 2.98-, 3.30- and 4.08-fold greater than the control. Cu and Cd combined also stimulated *psa*B mRNA expression, and the level of stimulation is significantly higher than that of the single treatments. *psa*B mRNA levels were 3.80-, 5.32-, 4.94- and 5.31-fold greater than the control when after treatment with Cu(0.5) + Cd(1.0), Cu(0.5) + Cd(2.0), Cu(1.5) + Cd(1.0), or Cu(1.5) + Cd(2.0). ANOVA indicated that both Cu and Cd alone had a significant stimulated effect on *psa*B gene transcription ($p < 0.05$, Table 1). We also observed a significant interaction between Cd and Cu on *psa*B gene transcription by two-way ANOVA ($p < 0.05$, Table 1), and we surmise that this interaction should be synergistic by the increase trend.

*rbc*L exhibited similar responses to Cu and Cd treatment as *psb*A (Fig. 4c). At both of the higher Cu and Cd concentrations used in this study, abundance of *rbc*L transcripts decreased. Two-way ANOVA indicated that both Cu and Cd alone had a significant inhibitory effect on *rbc*L gene transcription ($p < 0.05$, Table 1). The combined treatment of Cu and Cd also decreased *rbc*L mRNA expression, and the maximum decrease in the *rbc*L transcript abundance is observed after exposure to Cu(1.5) + Cd(2.0). However, we did not observe a significant interaction of Cd and Cu combined on *rbc*L gene transcription by two-way ANOVA ($p > 0.05$, Table 1).

4. Discussion

Photosynthetic organisms are highly sensitive to metal compounds. The effect of metal ions on higher plants includes the disruption of many physiological functions such as water uptake, respiration, mineral nutrient uptake and photosynthesis (Burzynski and Zurek, 2007). Copper is an essential micronutrient for algal growth and also plays a vital role as an enzymatic cofactor and electron carrier in the photosynthetic and respiratory processes (Andrade et al., 2004). Cadmium is a non-essential metal with high toxicity, it can substitute for other metal ions (mainly Zn^{2+}, Cu^{2+} and Ca^{2+}) in metalloenzymes and shows a very strong affinity to biological structures containing −SH groups.

Several studies have focused on the inhibition of photosynthesis; however, the mechanisms of the toxic effects that metal compounds (including Cu and Cd) have on photosynthetic processes have remained for the most part, elusive. It is known that Cu and Cd can decrease the activity of photosystem II (PSII). Baron et al. (1995) reviewed that Cu has a direct impact on photosynthesis by inhibiting photosynthetic electron transport, especially in PSII. Geiken et al. (1998) demonstrated that Cd could alter the activity of the oxygen-evolving complex in pea and broad bean, ultimately causing the disassembly of their PSII, and Herbette et al. (2006) demonstrated that Cd down-regulates PSII proteins in

Arabidopsis thaliana. Zhou et al. (2006) showed that the inhibitory site of Cd in *M. aeruginosa* is not located at the PSII or PSI level, but is probably situated on the ferredoxin/$NADP^+$-oxidoreductase enzyme at the terminal of the whole electron transport chain. In this study, we have proved that concentrations of Cu (0.5 and 1.5 μM) and Cd (1.0 and 2.0 μM) inhibit the abundance of *psb*A in *C. vulgaris*, a gene that codes for an integral membrane protein D1 of photosystem II (PSII). The inhibition of *psb*A mRNA transcripts may decrease the activity of photosystem II (PSII) and electron transfer rates in *C. vulgaris*, which was observed by a decrease in chlorophyll content.

Some reports have shown that Cu and Cd can cause a decrease in the assimilation of CO_2 by influencing the activities of photosynthetic carbon reduction cycle enzymes. Sheoran et al. (1990) observed the inhibitory effect of Cd on 3-phosphoglyceric acid kinase (PGK, one of photosynthetic carbon reduction cycle enzymes) in pigeon pea leaves. Burzynski and Zurek (2007) showed that Cu and Cd not only decreased the activities of PGK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, another photosynthetic carbon reduction cycle enzyme), but also the synthesis of these proteins in cucumber cotyledons; both metals affected PGK in the experiments of Stiborová et al. (1986) on maize leaves. Rubisco is the carboxylase that controls the rate-limiting step of carbon assimilation as it enables the continuation of growth and development of plants. In this study, we have performed real-time PCR to evaluate the abundance of *rbc*L, which codes for the large subunit of Rubisco, and we have shown that Cu and Cd decrease *rbc*L gene transcription. Therefore we speculate that Cu and Cd may decrease of the assimilation of CO_2 not only by influencing the activities of enzymes, but also by inhibiting the mRNA expression of genes coding for related enzymes, at least for *rbc*L.

A few reports have shown a relationship between Cu or Cd and the activity of photosystem I (PSI), and some of which have been controversial. Neelam and Rai (2003) reported that Cd inhibited PSI activity in *Microcystis* sp. However, Zhou et al. (2006) reported that Cd increased the activity of PSI in *M. aeruginosa*. Our results show that exposure to Cu and Cd results in a higher transcript abundance of *psa*B, which is part of the *psa*A/B operon of the chloroplast genome and encodes P700 chlorophyll A2 apoproteins. The increase in *psa*B transcript abundance should improve the activity of PSI. Zhou et al. (2006) suggested that the increase in PSI activity results from the increase of cyclic electron transport around PSI, which increases of ATP synthesis but decreases the generation of NADPH, which is regarded as reducing equivalents to fix CO_2. Therefore, we speculate that NADPH content should decrease. The decreases in NADPH and *rbc*L gene transcription disrupt carbon assimilation, and retard the algal growth or cause cell death. At the same time, this cyclic electron transport around PSI is suggested to play an important role in the synthesis of more ATP, which could be provided as energy to synthesize more anti-stress molecules. Several studies have also confirmed the increase of cyclic electron flow in cyanobacterial cells under salinity stress (Howitt et al., 2001; Jeanjean et al., 1998; Tanaka et al., 1997). Thus, the increase in PSI activity resulting from the increase of cyclic electron transport around PSI could be one of the adaptive mechanisms to stress (Howitt et al., 2001; Zhou et al., 2006). Furthermore, our previous research also found that the abundance of *psa*B in *C. vulgaris* increased after short exposure to low concentrations of glufosinate (Qian et al., 2008a).

In this study, we found that ROS content increases not very significantly after exposure to Cu and Cd in single, except for the treatment of Cd(2.0). It showed that low concentration of metal compound was difficult to promote ROS content in short-time exposure. The combined Cu(1.5) + Cd(1.0–2.0) treatment stimulated ROS significantly, which meant these two combinations were the strongest oxidative stress among all treatments. ROS included free radicals (hydroxyl radical $OH^•$, phenoxy radicals $RO^•$, peroxy radicals $ROO^•$) and other ROS (superoxide radical anion $O_2^{•-}$, singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2). As it is known, chloroplasts are the main source of ROS, which can cause cell damage in various ways when photosynthesis-related genes are inhibited and electron transport is blocked. These surplus electrons are transported to molecular oxygen, generating ROS (Kumar et al., 2008). Consequences of ROS formation include the gradual peroxidation of lipid structures (Baryla et al., 2000), oxidative DNA damage (Kasprzak, 2002) and photosynthetic apparatus damage (Dewez et al., 2005; Vajpayee et al., 2005). In our study, we have found that the content of chl a, chl b and total chlorophyll decrease, with lower chlorophyll content indicating a decrease in the antenna size of the photosynthetic reaction center complexes (Björkman, 1981). This result is consistent with the report of Devos et al. (1991) showing that Cu disturbed the integrity of thylakoid membranes and with the report of Yruela (2005) showing that ROS interfered with the biosynthesis of photosynthetic machinery and decreased the photosynthetic rate.

Compared with metal compound stress of single metals, few studies have looked at the interactions between metal compounds. An et al. (2004) observed three types of interactions: strictly additive, synergistic and antagonistic in *Cucumis sativus*. It is particularly complex to understand the mechanisms involved in the interaction between metal compounds, including Cd and Cu (Gallego et al., 2007). Our present study shows that Cu and Cd have no significant synergistic interaction on the expression of *psb*A and *rbc*L at the transcriptional level; however, they show a significant synergistic interaction on the expression of *psa*B. We speculate that Cu and Cd have different interaction sites with which they inhibit *psb*A and *rbc*L gene expression, and that these interaction sites have no relationship. Cu and Cd then indirectly interact to cause synergistic effects on *psa*B gene expression because of the anti-stress mechanism, the cyclic electron transport around PSI.

Our results also show that Cu and Cd synergistically interact to affect ROS formation and chlorophyll content. Some reports have shown that copper ions are prone to participate in ROS formation via the so-called Fenton reaction (Mehta et al., 2006). Furthermore, Cd can replace Cu in various cytoplasmic and membrane proteins, thus increasing the amount of unbound free Cu ions participating in oxidative stress via the Fenton reaction (Valko et al., 2005). This Cd-induced displacement may explain the synergistic toxicity that we have detected in ROS formation, which has also been found in *Tetrahymena thermophila* in high metal concentrations of Cu and Cd (Gallego et al., 2007). ROS either disrupted photosynthetic pigments or damaged the photosynthetic apparatus (Bernal et al., 2006), causing a decrease in chlorophyll, while the more-than-additive increase in ROS content by the combination of Cu and Cd decreased chlorophyll content synergistically, resulting in the synergistic inhibition of algal cell growth.

The effects of Cu and Cd singly and in combination on *C. vulgaris* growth, ROS content and the transcription of photosynthesis-related genes have been assessed in this study. The results show that Cu and Cd independently inhibit *psb*A and *rbc*L gene expression, and that they can synergistically stimulate *psa*B gene expression. Furthermore, Cu and Cd can increase ROS formation and decrease chlorophyll content synergistically. To our knowledge, this report is among the first to study the effects of Cu and Cd, singly and in combination, on physiological parameters and gene expression levels in *C. vulgaris*.

Acknowledgments

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References

An, Y.J., Kim, Y.M., Kwon, T.I., Jeong, S.W., 2004. Combined effect of copper, cadmium, and lead upon *Cucumis sativus* growth and bioaccumulation. Sci. Total Environ. 326, 85–93.

Andrade, L.R., Farina, M., Amado, G.M., 2004. Effects of copper on *Enteromorpha flexuosa* (Chlorophyta) in vitro. Ecotoxicol. Environ. Safe. 58, 117–125.

Baron, M., Arellano, J.B., Gorge, J.L., 1995. Copper and photosystem-II—a controversial relationship. Physiol. Plant. 94, 174–180.

Baryla, A., Laborde, C., Montillet, J.L., Triantaphylides, C., Chagvardieff, P., 2000. Evaluation of lipid peroxidation as a toxicity bioassay for plants exposed to copper. Environ. Pollut. 109, 131–135.

Bernal, M., Sanchez-Testillano, P., Risueiio, M.C., Yruela, I., 2006. Excess copper induces structural changes in cultured photosynthetic soybean cells. Funct. Plant Biol. 33, 1001–1012.

Björkman, O., 1981. Responses to different quantum flux densities. In: Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (Eds.), Physiological Plant Ecology. I. Encyclopedia of Plant Physiology. Springer-Verlag, Berlin.

Burzynski, M., Zurek, A., 2007. Effects of copper and cadmium on photosynthesis in cucumber cotyledons. Photosynthetica 45, 239–244.

Devos, C.H.R., Schat, H., Dewaal, M.A.M., Vooijs, R., Ernst, W.H.O., 1991. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant silene-cucubalus. Physiol. Plant. 82, 523–528.

Dewez, D., Geoffroy, L., Vernet, G., Popovic, R., 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga *Scenedesmus obliquus*. Aquat. Toxicol. 74, 150–159.

Franklin, N.M., Stauber, J.L., Lim, R.P., Petocz, P., 2002. Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environ. Toxicol. Chem. 21, 2412–2422.

Gaetke, L.M., Chow, C.K., 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189, 147–163.

Gallego, A., Martin-Gonzalez, A., Ortega, R., Gutierrez, J.C., 2007. Flow cytometry assessment of cytotoxicity and reactive oxygen species generation by single and binary mixtures of cadmium, zinc and copper on populations of the ciliated protozoan *Tetrahymena thermophila*. Chemosphere 68, 647–661.

Geiken, B., Masojidek, J., Rizzuto, M., Pompili, M.L., Giardi, M.T., 1998. Incorporation of [S-35]methionine in higher plants reveals that stimulation of the D1 reaction centre. II. Protein turnover accompanies tolerance to heavy metal stress. Plant Cell Environ. 21, 1265–1273.

Hart, J.J., Welch, R.M., Norvell, W.A., Sullivan, L.A., Kochian, L.V., 1998. Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. Plant Physiol. 116, 1413–1420.

Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L.M., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J.P., Vavasseur, A., Leonhardt, N., 2006. Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. Biochimie 88, 1751–1765.

Howitt, C.A., Cooley, J.W., Wiskich, J.T., Vermaas, W.F., 2001. A strain of *Synechocystis* sp. PCC 6803 without photosynthetic oxygen evolution and respiratory oxygen consumption: implications for the study of cyclic photosynthetic electron transport. Planta 214, 46–56.

Inskeep, W.P., Bloom, P.R., 1985. Extinction coefficients of chlorophyll a and b in N,N-dimethylformamide and 80% acetone. Plant Physiol. 77, 483–485.

Jeanjean, R., Bedu, S., Havaux, M., Matthijs, H.C.P., Joset, F., 1998. Salt-induced photosystem I cyclic electron transfer restores growth on low inorganic carbon in a type I NAD(P)H dehydrogenase deficient mutant of *Synechocystis* PCC6803. FEMS Microbiol. Lett. 167, 131–137.

Kasprzak, K.S., 2002. Oxidative DNA and protein damage in metal-induced toxicity and carcinogenesis. Free Radic. Biol. Med. 32, 958–967.

Kong, I.C., Bitton, G., Koopman, B., Jung, K.H., 1995. Heavy metal toxicity testing in environmental samples. Rev. Environ. Contam. T. 142, 119–149.

Kumar, S., Habib, K., Fatma, T., 2008. Endosulfan induced biochemical changes in nitrogen-fixing cyanobacteria. Sci. Total Environ. 403, 130–138.

Leonard, S.S., Harris, G.K., Shi, X., 2004. Metal-induced oxidative stress and signal transduction. Free Radic. Biol. Med. 37, 1921–1942.

Levy, J.L., Stauber, J.L., Jolley, D.F., 2007. Sensitivity of marine microalgae to copper: the effect of biotic factors on copper adsorption and toxicity. Sci. Total Environ. 387, 141–154.

Ma, M., Zhu, W., Wang, Z., Witkamp, G.J., 2003. Accumulation, assimilation and growth inhibition of copper on freshwater alga (*Scenedesmus subspicatus* 86.81 SAG) in the presence of EDTA and fulvic acid. Aquat. Toxicol. 63, 221–228.

Mehta, R., Templeton, D.M., O'Brien, P.J., 2006. Mitochondrial involvement in genetically determined transition metal toxicity. II. Copper toxicity. Chem. Biol. Interact. 163, 77–85.

Milone, M.T., Sgherri, C., Clijsters, H., Navari-Izzo, F., 2003. Antioxidative responses of wheat treated with realistic concentration of cadmium. Environ. Exp. Bot. 50, 265–276.

Neelam, A., Rai, L.C., 2003. Differential responses of three cyanobacteria to UV-B and Cd. J. Microbiol. Biotechnol. 13, 544–551.

Nriagu, J.O., Pacyna, J.M., 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333, 134–139.

Penuelas, J., Filella, I., 2002. Metal pollution in Spanish terrestrial ecosystems during the twentieth century. Chemosphere 46, 501–505.

Qian, H.F., Chen, W., Sheng, G.D., Xu, X.Y., Liu, W.P., Fu, Z.W., 2008a. Effects of glufosinate on antioxidant enzymes, subcellular structure, and gene expression in the unicellular green alga *Chlorella vulgaris*. Aquat. Toxicol. 88, 301–307.

Qian, H.F., Sheng, G.D., Liu, W.P., Lu, Y.C., Liu, Z.G., Fu, Z.W., 2008b. Inhibitory effects of atrazine on *Chlorella vulgaris* as assessed by real-time polymerase chain reaction. Environ. Toxicol. Chem. 27, 182–187.

Qian, H.F., Xu, X.Y., Chen, W., Jiang, H., Jin, Y.X., Liu, W.P., Fu, Z.W., 2009. Allelochemical stress causes oxidative damage and inhibition of photosynthesis in *Chlorella vulgaris*. Chemosphere 75, 368–375.

Sacan, M.T., Oztay, F., Bolkent, S., 2007. Exposure of *Dunaliella tertiolecta* to lead and aluminum: toxicity and effects on ultrastructure. Biol. Trace Elem. Res. 120, 264–272.

Schutzendubel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351–1365.

Scoccianti, V., Iacobucci, M., Paoletti, M.F., Fraternale, A., Speranza, A., 2008. Species-dependent chromium accumulation, lipid peroxidation, and glutathione levels in germinating kiwifruit pollen under Cr(III) and Cr(VI) stress. Chemosphere 73, 1042–1048.

Sheoran, I.S., Singal, H.R., Singh, R., 1990. Effect of cadmium and nickel on photosynthesis and the enzymes of the photosynthetic carbon-reduction cycle in Pigeonpea (*Cajanus Cajan* L). Photosynth. Res. 23, 345–351.

Shuhaimi-Othman, M., Pascoe, D., 2007. Bioconcentration and depuration of copper, cadmium, and zinc mixtures by the freshwater amphipod *Hyalella azteca*. Ecotoxicol. Environ. Safe. 66, 29–35.

Stauber, J.L., Davies, C.M., 2000. Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. Environ. Rev. 8, 255–301.

Stiborová, M., Doubravová, M., Leblová, S., 1986. A comparative study of the effect of heavy metal ions on ribulose-1,5-bisphosphate carboxylase and phosphoenolpyruvate carboxylase. Biochem. Physiol. Pflanz. 181, 373–379.

Tanaka, Y., Katada, S., Ishikawa, H., Ogawa, T., Takabe, T., 1997. Electron flow front NAD(P)H dehydrogenase to photosystem I is required for adaptation to salt shock in the Cyanobacterium *Synechocystis* sp. PCC 6803. Plant Cell Physiol. 38, 1311–1318.

Vajpayee, P., Rai, U.N., Ali, M.B., Tripathi, R.D., Kumar, A., Singh, S.N., 2005. Possible involvement of oxidative stress in copper induced inhibition of nitrate reductase activity in *Vallisneria spiralis* L. Bull. Environ. Contam. Toxicol. 74, 745–754.

Valko, M., Morris, H., Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161–1208.

Wang, Z.S., Kong, H.N., Wu, D.Y., 2007. Acute and chronic copper toxicity to a saltwater cladoceran *Moina monogolica daday*. Arch. Environ. Contam. Toxicol. 53, 50–56.

Wilde, K.L., Stauber, J.L., Markich, S.J., Franklin, N.M., Brown, P.L., 2006. The effect of pH on the uptake and toxicity of copper and zinc in a tropical freshwater alga (*Chlorella* sp.). Arch. Environ. Contam. Toxicol. 51, 174–185.

Wong, P.K., Chang, L., 1991. Effects of copper, chromium and nickel on growth, photosynthesis and chlorophyll a synthesis of *Chlorella pyrenoidosa* 251. Environ. Pollut. 72, 127–139.

Yruela, I., 2005. Copper in plants. Braz. J. Plant Physiol. 17, 145–156.

Zhou, W., Juneau, P., Qiu, B., 2006. Growth and photosynthetic responses of the bloom-forming cyanobacterium *Microcystis aeruginosa* to elevated levels of cadmium. Chemosphere 65, 1738–1746.

Zhou, Y., Zhang, Z., 1989. The Toxicity Methods in Aquatic Living. Agriculture Press, Beijing.