Effects of five heavy metals at sub-lethal concentrations on the growth and photosynthesis of *Chlorella vulgaris*

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The impact of sub-lethal concentrations (0.05, 0.5, 5 $\mu\text{mol L}^{-1}$) of the heavy metals copper (Cu), chromium (Cr), zinc (Zn), cadmium (Cd) and lead (Pb) on the growth and photosynthesis of *Chlorella vulgaris* was studied during 96 h exposure experiments. The results showed that the effects of these five metals on the growth of *Chlorella vulgaris* were dependent on both concentration and exposure time. It was found that 5 $\mu\text{mol L}^{-1}$ treatments of Cu, Cr, Zn, Cd and Pb significantly inhibited the growth of *Chlorella vulgaris*, and the effect became weaker with an increase in exposure duration. Different effects on chlorophyll fluorescence were found for different metals, with Cu and Cr having an inhibiting effect and Zn and Cd having a promoting effect. The effects of heavy metals upon the growth and photosynthesis of *Chlorella vulgaris* were independent of each other and not causally related.

heavy metal, sub-lethal, *Chlorella vulgaris*, growth, photosynthesis

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With the significant growth in both industry and urban development, the use of heavy metals has also risen, causing serious environmental problems in the water. Because algae play a role as primary producers in most aquatic ecosystems, estimating the effects of heavy metals on the growth and photosynthesis of algae can significantly impact the overall ecological risk assessment of heavy metals.

Heavy metals are highly abundant in nature. Among them, copper (Cu) and zinc (Zn) function as essential elements to plants while chromium (Cr), cadmium (Cd) and lead (Pb) show little biological usefulness. Cu is not only a key component of electron transport chains but also of co-enzyme factors that play important roles in plant photosynthesis and respiration [1,2]. Zn is a basic component of various enzymes involved in photosynthesis and metabolism, such as carbon anhydride, acidic phosphatase and alkaline phosphatase [3]. Plant growth is promoted once Cu and Zn are introduced into the plant's water supply. If lacking

either element, the plant's rate of growth and photosynthesis will be greatly reduced. However, both metals may become toxic at higher levels. Unlike Cu and Zn, the heavy metals Cr, Cd and Pb are not necessary for plant growth or respiration. However, with the rapid growth of industry, a great quantity of Cr, Cd and Pb is being deposited into the water, resulting in adverse effects on plants. The toxicity of Cr is form-dependent, with Cr(VI) being the most toxic one [4]. Cd, a potentially toxic metal that is very stable in aquatic environment, can directly affect the physiological processes of algae [5,6]. Pb is easily accumulated by algae and thus becomes a toxic influence [7].

The effects of heavy metal exposure upon algae manifest in suppressed cell division, inhibited growth rate, restrained enzyme activity and reduced photosynthesis [8–10]. Previous studies regarding the impacts of heavy metals on algae were mainly focused on finding the concentration that caused either 50% mortality (72 h- LC_{50} or 96 h- LC_{50} value) or 50% inhibition (72 h- EC_{50} or 96 h- EC_{50} value). Due to the development of chlorophyll fluorescence technology, it

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is now much easier to analyze the photosynthesis process, which has resulted in an abundance of new researches looking into factors that affect photosynthesis. Although the effects of heavy metals on algae have received significant attention and have been extensively studied, the exposure concentrations in those studies were necessarily high to calculate LC_{50} or EC_{50} values, and little information is available for evaluating the effects of these metals at sub-lethal concentrations.

In the present study, experiments were carried out on *Chlorella vulgaris*, a dominant type of green algae. Toxic effects of five heavy metals (Cu, Cr, Zn, Cd, Pb) at sub-lethal concentrations (0.05, 0.5 and $5 \mu\text{mol L}^{-1}$) on the growth and photosynthesis of *Chlorella vulgaris* were analyzed and compared, with the results intending to provide a more theoretical basis upon which to judge the toxic effects of heavy metals.

1 Materials and methods

1.1 Algal material and culture conditions

Chlorella vulgaris was chosen as algal material based on several considerations. Firstly, *C. vulgaris* is found all over the world and is a dominant type of green algae. Secondly, *C. vulgaris* is a suitable material for toxicity tests due to its simple growth conditions and strong tolerability. Finally, the results can be cross-referenced against a number of experiments where *C. vulgaris* has been used as the test algae.

C. vulgaris was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Sciences. It was cultured in BG11 medium at $(24 \pm 1)^\circ\text{C}$ with a cycle of light (14 h, 4000 Lux) and dark (10 h, 0 Lux) in a GXZ-280B illumination cultivation cabinet (China). *C. vulgaris* was cultivated statically, put through a wave cycle two to three times and had its position changed randomly.

1.2 Chemical materials

Copper as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (analytical grade), chromium as K_2CrO_4 (analytical grade), zinc as ZnCl_2 (analytical grade), cadmium as $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ (analytical grade) and lead as PbCl_2 (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd.

1.3 Exposure concentrations

An exposure concentration which is lower than LC_{50} is considered as sub-lethal concentration. In algal toxicity test, the LC_{50} value is affected by the initial cellular density, culture medium, temperature condition, etc., and even a small alteration in those conditions may give rise to a different LC_{50} value, even if the same algae and metal are used. LC_{50} values referenced from previous experiments showed

discrepancies, so exposure concentrations were determined by a preliminary experiment in this study.

The preliminary experiment showed that the 96 h- LC_{50} of Cu^{2+} for *C. vulgaris* was approximately $10 \mu\text{mol L}^{-1}$. Former studies reported that Cu was the most toxic one among the five heavy metals, so it can be inferred that the 96 h- LC_{50} of the other four metals should be above $10 \mu\text{mol L}^{-1}$. For the purpose of comparison, exposure concentrations were kept exactly the same for all metals, at 0.05, 0.5 and $5 \mu\text{mol L}^{-1}$. In addition, the results shown in section 2.1 manifested that the percentage of inhibition (PI) was below 50% in all conditions after 96 h under the setting concentrations. Hence, the exposure concentrations are indeed sub-lethal concentrations.

1.4 Algae pre-cultivation

Because Cu^{2+} and Zn^{2+} are both contained in BG11 medium, it is necessary to pre-cultivate *C. vulgaris* in a medium without Cu^{2+} or Zn^{2+} to control the experimental concentrations of the metals.

In a BG11 medium, the concentration of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ is 0.079 mg L^{-1} and 0.222 mg L^{-1} , respectively, which is approximately $0.31 \mu\text{mol L}^{-1}$ for Cu^{2+} and $0.77 \mu\text{mol L}^{-1}$ for Zn^{2+} . When diluted, the concentration of Cu^{2+} (Zn^{2+}) in the medium reduced to 0.01 (0.02) $\mu\text{mol L}^{-1}$, which represented 20% (40%) of a $0.05 \mu\text{mol L}^{-1}$ solution. Considering that both elements are required for the algae's growth, and taking into account the enrichment of the algae, the concentration will be further reduced over time. However, the concentrations of Cu^{2+} and Zn^{2+} remaining in the medium after dilution may still exert a slight effect on the treatments at the lowest exposure concentration. As a result, *C. vulgaris* was pre-cultivated in a medium without Cu^{2+} or Zn^{2+} . Our former study showed that the influence of phosphorus was eliminated by 3 days' pre-cultivation in a medium without phosphorus. Therefore, the time for pre-cultivation in this study was set at 3 d as well. Before the formal exposure experiment, *C. vulgaris* was diluted twice, including a 10-fold dilution before pre-cultivation followed by a 3–4 fold dilution. The effects of the dilution, combined with the consumption of Cu^{2+} and Zn^{2+} by *C. vulgaris* during growth, significantly reduced the concentration of the metals, even to the point of non-detection. From our point of view, pre-cultivation eliminated the effect of any remaining Cu^{2+} and Zn^{2+} from the $0.05 \mu\text{mol L}^{-1}$ treatment.

1.5 Experiment design

C. vulgaris was cultivated in 250 mL flask and exposed to Cu, Cr, Zn, Cd and Pb at given concentrations of 0.05, 0.5 and $5 \mu\text{mol L}^{-1}$, with a blank included for each of the experimental groups. Three parallels were set to ensure the repeatability of each sample concentration. The alga tested

was in exponential phase with a density of 2×10^6 cells mL^{-1} . The *C. vulgaris* exposed to Cu and Zn was pre-cultivated for 3 d. The treatments were not aerated because shaking 2–3 times every day introduced the possibility of keeping the algae in vigor. Chlorophyll fluorescence and biomass were measured every 24 h over the duration of 96 h.

1.6 Algal growth analysis

Growth was measured by a microplate reader (Model-680, Bio-Rad) at 450 nm (optical density) and then converted into unit of biomass (cells mL^{-1}). The inhibition, P , is calculated using the following formula:

$$P = \frac{\lg N_{t \text{ control}} - \lg N_t}{\lg N_{t \text{ control}} - \lg N_{0 \text{ control}}},$$

where $N_{0 \text{ control}}$ is the biomass of control at time 0, and $N_{t \text{ control}}$ and N_t are the biomass of control and treatment at time t .

1.7 Chlorophyll fluorescence analysis

The maximal quantum yield of PSII (F_v/F_m) was chosen to indicate the photosynthetic capacity of *C. vulgaris* and

measured by MAXI-Imaging-PAM (WALZ, Germany) after 25 min dark adaptation. F_v/F_m was calculated by F_0 (the minimal level of fluorescence) and F_m (the maximal level of fluorescence), which were directly measured and where $F_v/F_m = (F_m - F_0)/F_m$.

1.8 Data analysis

Data were analyzed by the program SPSS 16.0 and Excel 2010 for Windows.

2 Results and discussions

2.1 Effects of five metals on the growth

The effects of five heavy metals on the growth of *C. vulgaris* are shown in Figure 1. There was no significant effect under the $0.05 \mu\text{mol L}^{-1}$ treatment when Cd or Zn was added to the medium ($P > 0.05$), though an inhibiting effect was found when the same concentration of Cu, Cr or Pb was added ($P < 0.05$). However, biomass were lower than the blank in all cases when exposure concentration increased to $5 \mu\text{mol L}^{-1}$, indicating that specific concentration had an inhibiting impact on algal growth. The inhibition effect

Figure 1 Effects of Cu, Cr, Zn, Cd and Pb treatments ($0, 0.05, 0.5$ and $5 \mu\text{mol L}^{-1}$) on the growth of *Chlorella vulgaris*.

became weaker with the increase of exposure time. For example, the percentages of inhibition (PI) under 5 $\mu\text{mol L}^{-1}$ Cu were 85.5%, 67.8%, 55.05% and 38.3% after exposure times of 24, 48, 72 and 96 h, showing a downward trend.

As shown in Table 1, the toxic effects of heavy metals were dependent on both concentration and exposure time. The growth of *C. vulgaris* was significantly affected by metal concentration and exposure time together with their interaction in the tests of Cu, Cr, Zn and Pb ($P<0.05$). As for Cd, metal concentration and exposure time also influenced algal growth rate, but their interaction showed no effect ($P=0.055$).

Qian et al. [5] found that the EC_{50} value of Cu was lower than that of Cd with respect to the growth of *C. vulgaris*, suggesting that Cu was more toxic than Cd. A similar result was found by Ismail et al. [9] when *Isochrysi galbana*, *Tetraselmis tetrahele* and *Tetraselmis sp.* were used as test algae. Franklin et al. [11] recorded the toxic sequence as $\text{Cu}>\text{Cd}>\text{Zn}$ for *Chlorella sp.* while Liang et al. [12] reported the sequence as $\text{Cd}>\text{Cu}>\text{Zn}$ for *Phaeodactylum tricornutum*. Obviously, the toxic rank for different metals is not static and depends on the species of algae. Our results revealed that Cu was the most toxic of the five metals, but the rank varied as the exposure time changed. The toxic sequence after 24 h was $\text{Cu}>\text{Cd}>\text{Pb}>\text{Cr}>\text{Zn}$, but after 96 h, it changed to $\text{Cu}>\text{Cr}>\text{Cd}>\text{Zn}>\text{Pb}$ (5 $\mu\text{mol L}^{-1}$ treatments were used to compare). Considering that Cu and Zn are indispensable elements for algae growth, the difference threshold might be the reason why Cu was more poisonous than Zn. As to other three metals, the difference in ranking order may arise from differential tolerances when under stress or from the sequence during detoxification.

Table 1 2-way-ANOVA on the biomass of *Chlorella vulgaris* exposed to different metal concentrations for different time

Metal	Factor	F	P-level
Cu	Concentration ($\mu\text{mol L}^{-1}$)	180.848	$P<0.001$
	Exposure time (h)	796.695	$P<0.001$
	Concentration $\times$ time	26.094	$P<0.001$
Cr	Concentration ($\mu\text{mol L}^{-1}$)	111.172	$P<0.001$
	Exposure time (h)	765.912	$P<0.001$
	Concentration $\times$ time	14.372	$P<0.001$
Zn	Concentration ($\mu\text{mol L}^{-1}$)	9.218	$P<0.001$
	Exposure time (h)	547.360	$P<0.001$
	Concentration $\times$ time	2.010	$P=0.049$
Cd	Concentration ($\mu\text{mol L}^{-1}$)	9.330	$P<0.001$
	Exposure time (h)	364.299	$P<0.001$
	Concentration $\times$ time	1.965	$P=0.055$
Pb	Concentration ($\mu\text{mol L}^{-1}$)	15.888	$P<0.001$
	Exposure time (h)	979.575	$P<0.001$
	Concentration $\times$ time	5.499	$P<0.001$

2.2 Effects of five metals on the photosynthesis

F_v/F_m was used to indicate the influences of different metals on the photosynthesis of *C. vulgaris*. The variations of F_v/F_m for *C. vulgaris* are shown in Figure 2. Photosynthesis yield declined at first and then rose sharply with the increase of exposure time. The decline can be attributed to both the change of cultivation as well as the state of the algae. Because *C. vulgaris* was diluted to the same initial density for each treatment, a recovery process was required, thus resulting in a lower F_v/F_m over the initial 24 h. With the increase of biomass, the chlorophyll fluorescence absorbed and shaded by the medium also increased, leading to a more reduction of maximum fluorescence (F_m) compared to minimum fluorescence (F_0), which resulted in a higher $F_v/F_m(1-F_m/F_0)$. Even though, the influence of heavy metals on the quantum yield of PSII can still be distinguished. Chlorophyll fluorescence images were taken of all treatments at the end of their exposure period to display the change of F_v/F_m (Figure 3). F_v/F_m of *C. vulgaris* in 5 $\mu\text{mol L}^{-1}$ Cu and Cr treatments were lower than their corresponding controls, indicating that a suppression of photosynthesis was caused by Cu and Cr. Figure 3 also shows that F_v/F_m values of the control samples in Cu and Zn treatments were relatively lower than those in the other control samples, which was attributed to the lack of necessary Cu or Zn in those controls.

No common conclusion could be drawn about the effects of the five metals on the photosynthesis of *C. vulgaris*. Except for Cd, no significant effect was found for the lowest exposure concentration (0.05 $\mu\text{mol L}^{-1}$). Treatment concentration of 5 $\mu\text{mol L}^{-1}$ showed that Cu and Cr reduced the quantum yield of PS II, Zn and Cd increased the yield and Pb displayed no impact. After 96 h of exposure, the proportion of the maximum quantum yield of PS II promoted by Zn and Cd was approximately 10%.

Recent studies regarding photosynthesis have increased due to the development of chlorophyll fluorescence technology and included researches into factors affecting photosynthesis and to what degree these factors affect the photosynthetic process. Few comparisons between studies have been carried out, however. During a study of seven species of marine macroalgae, Bauman et al. [10] found that the chlorophyll fluorescence was reduced by Cu, Zn, Cd and Cr but was not affected by Pb under the concentration of 10 $\mu\text{mol L}^{-1}$. Our study found that under a concentration of 5 $\mu\text{mol L}^{-1}$, the quantum yield of PS II was reduced by Cu and Cr, promoted by Zn and Cd and not affected by Pb. The promotion effect was not observed in the previous studies and could be a result of stimulatory effect at low concentrations.

2.3 Comparison of the effects on the growth and photosynthesis

In previous studies, the effects of heavy metals on the

Figure 2 Effects of Cu, Cr, Zn, Cd and Pb treatments ($0, 0.05, 0.5$ and $5 \mu\text{mol L}^{-1}$) on the photosynthesis of *Chlorella vulgaris*.

Figure 3 Chlorophyll fluorescence images of *Chlorella vulgaris* exposed to Cu, Cr, Zn, Cd and Pb after 96 h.

growth and photosynthesis have been discussed separately. In our study, a comparison was conducted by analyzing the effects of the five metals on the growth and chlorophyll fluorescence of *C. vulgaris*. Figure 4 shows the impacts of the five metals on the growth at different time, which is represented by PI. When the PI was positive, the effect was regarded as inhibition, with a higher PI indicating a higher toxic level of the metal. Figure 5 shows the influence of five metals on the photosynthesis over time, which is represented by relative F_v/F_m value compared to the corresponding control. When the ratio is above 1, the effect is considered as promotion, with result less than 1 considered as inhibition.

As shown in Figures 4 and 5, Cu and Cr both reduced biomass and chlorophyll fluorescence at the same time. The inhibition upon growth reached a climax within 24 h in the treatments of $5 \mu\text{mol L}^{-1}$ Cu and Cr, and then weakened with the increased exposure time. The inhibition on the photosynthesis for Cr was observed after 24 h and reached the peak at 48 h. But for Cu, the inhibition was observed after 48 h and reached the peak at 72 h. As a result, Cu was found to be more toxic than Cr when evaluated by the growth, but Cr was found to be the more toxic when evaluated by the photosynthesis. It also indicates that the effects upon chlorophyll fluorescence showed a lagged response comparing to the growth rate, although the effects of

Figure 4 PIs of different treatments on the growth of *Chlorella vulgaris* in different time (PI means the percentage of inhibition).

Figure 5 Effects of different treatments on the F_v/F_m of *Chlorella vulgaris* at different times.

inhibition on the growth and photosynthesis both decreased over time due to the adaptive mechanism of *C. vulgaris*.

Zn and Cd reduced biomass but enhanced the quantum yield of photochemistry. Similar to Cu, the effect of Cd on algae growth culminated quickly and then declined. At $5 \mu\text{mol L}^{-1}$, the suppression effect of Zn reached a peak at 48 h. Cd was more toxic than Zn within 48 h, but became less toxic than Zn after exposure for a longer time. Meanwhile, a significant promoting effect on the photosynthesis by Zn and Cd was observed after 72 h. Overall, it can be

concluded that the mechanisms of these two metals upon growth and photosynthesis were totally different because a suppression of growth and a promotion of photosynthesis were found simultaneously.

Pb reduced biomass, with the metal presenting a high inhibition effect at first but rapidly declining thereafter. Chlorophyll fluorescence was not affected by Pb. Hence, sub-lethal concentrations of Pb did not produce a significant toxic effect for *C. vulgaris* within 96 h.

Perales-Vela et al. [13] reported that the growth of

Table 2 Comparison of effects on the growth and photosynthesis of *Chlorella vulgaris* by different metals under different concentrations

Metal	Factor	Exposure concentration($\mu\text{mol L}^{-1}$)		
		0.05	0.5	5
Cu	Growth	– ^{a)} ($P < 0.001$)	– ($P = 0.002$)	– ($P < 0.001$)
	Photosynthesis	0 ^{b)} ($P = 0.877$)	0 ($P = 0.657$)	– ($P < 0.001$)
Cr	Growth	– ($P = 0.013$)	0 ($P = 0.826$)	– ($P < 0.001$)
	Photosynthesis	0 ($P = 0.421$)	0 ($P = 0.165$)	– ($P < 0.001$)
Zn	Growth	0 ($P = 0.090$)	0 ($P = 0.255$)	– ($P < 0.001$)
	Photosynthesis	0 ($P = 0.910$)	+ ^{c)} ($P = 0.020$)	+ ($P < 0.001$)
Cd	Growth	0 ($P = 0.485$)	0 ($P = 0.281$)	– ($P < 0.001$)
	Photosynthesis	+ ($P < 0.001$)	+ ($P < 0.001$)	+ ($P < 0.001$)
Pb	Growth	– ($P < 0.001$)	0 ($P = 0.058$)	– ($P < 0.001$)
	Photosynthesis	0 ($P = 0.857$)	0 ($P = 0.548$)	0 ($P = 0.084$)

a) – means inhibition; b) 0 means no significant effect; c) + means promotion.

Scenedesmus incrassatulus was much more sensitive than its photosynthetic electron transport when the alga was exposed to Cu^{2+} at sub-lethal concentrations of 0–3.145 $\mu\text{mol L}^{-1}$. A similar result was found in our experiments when *C. vulgaris* was exposed to Cu and Cr. Because it is very important to balance fundamental functions to be survival, algae tend to sacrifice its growth to maintain essential photosynthesis processes under stress [14]. Therefore, the exposure of heavy metals might not affect photosynthesis as quickly as it does growth, as was shown in exposure experiments of Cu and Cr. Cu^{2+} can oxidize membrane lipids, resulting in an increased quantity of active oxygen which can break the thylakoid layer structure, thus affecting photosynthesis of the plant [15,16]. The results showed that photosynthesis rates remained at normal levels longer than growth rates did under the stress of Cu^{2+} . So, it may be necessary for heavy metals to get into the cell before they can affect the photosynthesis of algae. Thus, it can be speculated that a phenomenon may be occurring whereby growth is partially restrained but photosynthesis continues normally. Andrade et al. [17] observed a self-protection mechanism against Cd and Pb toxicity in which the cell walls in brown algae overproduced polysaccharides. Thus, heavy metals may increase the production of polysaccharides, which ask for an increase of photochemistry efficiency since photosynthesis is the key process for transferring solar energy into chemical energy, with the result being a stimulation effect upon the algae.

Comparison of the effects of Cu, Cr, Zn, Cd and Pb on the growth and photosynthesis of *C. vulgaris* is listed in Table 2. It can be inferred that the different factors effecting growth and photosynthesis are not causally related. Nielsen H D and Nielsen S L [18] found the effect of Cu^{2+} on the photosynthesis of *Fucus serratus* was unrelated to its effect on growth inhibition. They recognized Cu^{2+} could inhibit the growth rate of algae by affecting the growth process directly, and the rate of growth inhibition caused by Cu^{2+} was not related to any effects that Cu^{2+} might have

upon photosynthesis. As a result, it has been shown that growth and photosynthesis are independent processes unrelated to each other. Thus, it is necessary to take both the growth process and the photosynthesis process into account when estimating the ecological risk of a toxicant, especially under sub-lethal concentrations.

3 Conclusions

All the five metals inhibited the growth of *C. vulgaris*, and the effects were both dose-dependent and time-dependent. In treatments using PIs of 5 $\mu\text{mol L}^{-1}$, the toxic order was $\text{Cu} > \text{Cd} > \text{Pb} > \text{Cr} > \text{Zn}$ after 24 h but changed to $\text{Cu} > \text{Cr} > \text{Cd} > \text{Zn} > \text{Pb}$ after 96 h. Unlike the effects on the growth, the impacts on the photosynthesis were more complicated. Cu and Cr reduced the quantum yield of PSII while Zn and Cd increased the yield, with Pb showed a negligible impact. Thus, the effects of heavy metals on the growth and photosynthesis of *C. vulgaris* are independent to each other and are not causally related.

In general, Cu and Cr were the most harmful metals among the five heavy metals investigated, even at sub-lethal concentrations. Although heavy metals had differential influences on the growth and photosynthesis, heavy metals at sub-lethal concentrations can reduce the algal growth rate, which indicates that the presence of all five metals should be paid attention to, even at sub-lethal concentrations.

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