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Subacute toxicity of copper and glyphosate and their interaction to earthworm (*Eisenia fetida*)

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ABSTRACT

Glyphosate (GPS) and copper (Cu) are common pollutants in soils, and commonly co-exist. Due to the chemical structure of GPS, it can form complexes of heavy metals and interface their bioavailability in soil environment. In order to explore the interactions between GPS and Cu, subacute toxicity tests of Cu and GPS on soil invertebrate earthworms (*Eisenia fetida*) were conducted. The relative weight loss and whole-worm metal burdens increased significantly with the increasing exposure concentration of Cu, while the toxicity of GPS was insignificant. The joint toxicity data showed that the relative weight loss and the uptake of Cu, as well as the superoxide dismutase, catalase and malondialdehyde activities, were significantly alleviated in the present of GPS, which indicated that GPS could reduce the toxicity and bioavailability of Cu in the soil because of its strong chelating effects.

1. Introduction

Pesticides have been consistently demonstrated their worth by increasing global agricultural production, reducing insect-borne diseases and protecting/restoring plantations, forests, harvested wood products, homes and fiber (Ecobichon, 2001). Copper-containing sprays have been used to control fungal diseases in pome and stone fruit orchards, vineyards and vegetable fields for over 100 years. In Australia over 7500 t per year of copper fungicides were used, accounting for 13% of the global total (Lepp et al., 1994). Copper has the potential to be gradually accumulated in surface horizons of agricultural soil with the continuing application of copper pesticides in agriculture (Wang et al., 2009b). Micronutrient Cu is toxic to organisms at high concentration. The yield and quality of crop could be affected by Cu contamination, which could further threaten the health and lives of animals and human beings as it entered food chains (Sparks, 2002).

Glyphosate (*N*-[phosphonomethyl]Glycine) is a broad-spectrum herbicide with high water solubility, which was relatively nonselective and very effective on deeply rooted perennial species of grasses, sedges, and broadleaf weeds (Blackburn and Boutin, 2003). According to the survey, the total consumption of glyphosate in agriculture was 74,045 tonnes technical acid in 1997, and the total glyphosate-applied area was approximated to 52 million hectares worldwide in 1994 (Carlisle and Trevors, 1988). The Environmental Protection Agency of U.S. estimated that about 82,000 to 84,000 tonnes of glyphosate has been used in 2007 (Grube et al., 2011).

If not considering soil adsorption of glyphosate, the top 13 cm soil concentration of glyphosate would be 0.45 mg kg^{-1} at the application rate of 1 kg ha^{-1}, which might be overestimated the real level of glyphosate in the top of the soil (Carlisle and Trevors, 1988). In a real contaminated soil, pollutants usually co-exist in the environment and potentially interact with each other, which complicate their reaction mechanisms (Renner, 2003; Vijver et al., 2010). Glyphosate can act as a chelating agent with divalent and trivalent metallic cations and form stable complexes. The chelation between of Cu and GPS could change the solubility and bioavailability of GPS and Cu, which might have some significant effects on soil organisms. Our recent studies on the acute toxicity of Cu and GPS showed that the presence of GPS significantly reduced the acute toxicity of Cu to earthworms. The mortality rate of Cu to earthworm decreased sharply and the uptake rate of Cu was nearly halted in the presence of GPS; and the (superoxide dismutase) SOD activity, glutathione (GSH) content, and acetylcholinesterase (AchE) activity almost declined to the control level (Zhou et al., 2012). However, these results were achieved in aqueous, which were different from the soil environment.

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In the present study, a laboratory experiment was conducted to investigate the interactions between GPS and Cu with respect to the subacute toxicity of earthworm (*Eisenia fetida*, *E. fetida*). Earthworms were exposed to the artificial soil spiked with different concentrations of Cu and GPS for 28d. The superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) activities of the earthworm *E. fetida* exposed to Cu and GPS were also investigated. The objective of this study was to investigate the single and joint subacute toxicity of Cu and GPS on earthworm, which will be useful to assess the toxicity of combined pollution and provide a scientific basis for rational pesticide application and ecological risk assessment of Cu and GPS in terrestrial ecosystem.

2. Materials and methods

2.1. Experiment conditions

The earthworm (*E. fetida*) used in the present study was purchased from a farm located at Dachang District of Nanjing, Jiangsu Province, China. Before usage, the earthworms were raised with decaying leaves (mainly *Zelkova schneideriana* from broad-leaved deciduous forest of Zhong Shan Hill Scenic Area) in a condition controlled chamber in dark. The temperature of the chamber was controlled at 25 °C and a relative humidity was between 60 and 70% (Zhou et al., 2012). All chemicals were in analytical reagent grade or higher purity. The containers would be cleaned with diluted HNO_3 (10%) and thoroughly rinsed with deionized water before usage.

2.2. Soil preparation

Artificial soil was prepared according to the OECD protocol (OECD, 2004), which was composed by dry weight: finely ground peat, 10%; kaolin clay (kaolinite content 100%), 20%; washed non-sharp industrial sand (50% of the particles between 50 and 200 microns), 70%. The pH of the artificial soil was not modified over the experiments ranging from 5.80 to 6.20. Deionized water was added to maintain soil moisture at 35%.

2.3. Test procedures and experimental setup

Toxicity tests on earthworms were referred to OECD Guidelines (OECD, 2004), including a single exposure of earthworm to copper or glyphosate, respectively, and a joint exposure of earthworm to both copper and glyphosate. Three indicators were analyzed, which were explained in details below.

2.3.1. Toxicity of Cu or GPS to earthworm

Stock solutions of Cu ($Cu(NO_3)_2 \cdot 3H_2O$) and GPS (in 95% Technical, Sigma) (pH was adjusted to 6.0 ± 0.1 with NaOH) were spiked into 300 g soil to make soil concentrations of Cu and GPS at 0, 25, 50, 100 and 200 mg kg^{-1} dry weight soil. After mixed well, the amended soils were placed in plastic container for equilibration in 2 d. Then, 10 mature earthworms were weighed and introduced into each container, which was covered with perforated lid to keep worms inside and maintain moisture, and kept in a controlled incubator for 28 days. Soil moisture content was adjusted gravimetrically every two days. After one week of exposure, each earthworm was fed with about 0.5 g of cow dung. The earthworms were removed from the substrates at the end of the exposure, and starved on moist filter for 24 h to void their gut. The paper was changed twice during the starvation period (at 6 and 18 h). After starvation, the worms were washed with distilled water, dried with paper towels, weighed and stored at −70 °C till further analysis.

2.3.2. Joint toxicity of Cu and GPS to earthworms

To examine the toxicity interaction between Cu and GPS, earthworms were exposed to soils spiked with Cu concentrations at 50 or 200 mg kg^{-1} and GPS concentrations at 0, 25, 50, 100, or 200 mg kg^{-1} respectively. Control experiment was conducted in deionized water. The absorption kinetics of copper in earthworm were also investigated, where earthworms were exposed to soil with 50 or 200 mg kg^{-1} of Cu and 0 or 200 mg kg^{-1} of GPS, and the exposure periods were 3, 7, 14, 21 and 28 days. The amended soils and worms were prepared as described in experiment 1 (single pollutant exposure). Each treatment (of 10 worms each) was in four replicates.

2.3.3. Weight loss and cocoon production

The weights of the earthworms in each concentration group after exposure were then used to calculate the relative weight loss rate, as follows:

$$WL_n = (W_0 - W_t)/W_0 \times 100\%$$

where WL_n (%) is the relative weight loss rate for the *n*th concentration group, W_0 (g) is the weight at day 0 and W_t (g) is the weight after *t* days of exposure. The number of cocoons was measured at the end of the exposure by wet sieving the substrates (through a 2.0- and 1.0-mm sieve system) (Owojori et al., 2009).

2.3.4. Uptake of Cu in earthworm

The frozen earthworms were lyophilized and digested in 5.0 ml concentrated HNO_3 for 2 h at 120 °C. Then, 1.0 ml H_2O_2 was added in three times to oxidize the organism, and the temperature was then increased to 150 °C and maintained for 1 h. The solution was transferred to a 10 ml flask, and the Cu concentration was measured by a Hitachi Z2000 flame atomic absorption spectrophotometer (Zhou et al., 2012).

2.3.5. Biochemical assays

Prior to biochemical analyses, the earthworm was cut into pieces and mixed with 0.86% NaCl in ice bath. The mixture was homogenized with a high speed blender (XHF-D, Zhejiang, China), and then centrifuged at 4000 rev min^{-1} for 10 min at 4 °C. The resulting supernatant was used to test superoxide dismutase (SOD) activity, catalase (CAT) activities, and malondialdehyde (MDA) content examinations. The activity of SOD was assayed using the xanthine exanthine oxidase and nitroblue tetrazolium (NBT) system. One unit of SOD was defined as the amount of protein that inhibited the rate of NBT reduction by 50% (Sun et al., 1988). The CAT activity was assayed immediately after centrifugation of the homogenates according to the method of Saint-Denis et al. (1998). One unit of the CAT activity was defined as the enzyme quantity required to consume half of H_2O_2 in 120 s at 25 °C. The specific activity was defined as the unit of the enzyme activity per mg of protein. The content of MDA was estimated by the formation of thiobarbituric acid reactive substances according to the method described by Livingstone et al. (1990). Protein content was determined by the Bradford dye-binding assay using bovine serum albumin as standard (Bradford, 1976).

2.3.6. Statistical analysis

All statistical analyses were conducted with the software SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). One-way ANOVA was applied to assess the significant difference ($p < 0.05$) between groups; and linear regression analyses were conducted by the S−N−K method using software Origin 8.0 (OriginLab).

3. Results

3.1. Effects on earthworm growth

The earthworms in all treatments survived after 28 days of exposure. The relative weight loss rates of earthworms in Cu treatment changed significantly compared to the control. In the control, the relative weight loss rates were 3.7% after 28 days. And, the relative weight loss rates were significantly increased to 5.8%, 11.2%, 21.2%, and 25.4% compared to the control after 28 d of exposure at 25, 50, 100 and 200 mg kg^{-1} of Cu, respectively (Fig. 1A). The ANOVA analysis revealed that Cu concentration had significant effects on weight loss rates ($p < 0.01$). However, there were no significant difference of relative weight loss rates between treatments and control after 28 days of exposure with GPS ($p > 0.05$) (Fig. 1A).

The joint toxicity test showed that no sufficient decrease of the relative weight loss rates was detected at 50 mg kg^{-1} Cu concentration, in the presence of GPS. While increased Cu concentration to 200 mg kg^{-1}, the relative weight loss rate was significantly decreased ($p < 0.05$) in the presence of GPS, especially, when GPS was above 50 mg kg^{-1} (Fig. 1B).

3.2. Effects on cocoon production

The number of cocoons collected from the control group was higher than those collected from the Cu treated groups (Table 1). A significant reduction in cocoon production during the course of the exposure was observed when compared with the control ($p < 0.05$). The worms exposed to 200 mg kg^{-1} Cu had the lowest cocoon production after 28 d exposure, which only accounted for 50.5% of that from the control. While the average numbers of cocoons produced in the other four concentration of Cu group are 108%, 77%, and 68% of the control, respectively. Nevertheless, no significant difference was observed in total number of cocoons exposed to GPS spiked soil (Table 1). In the combined test at

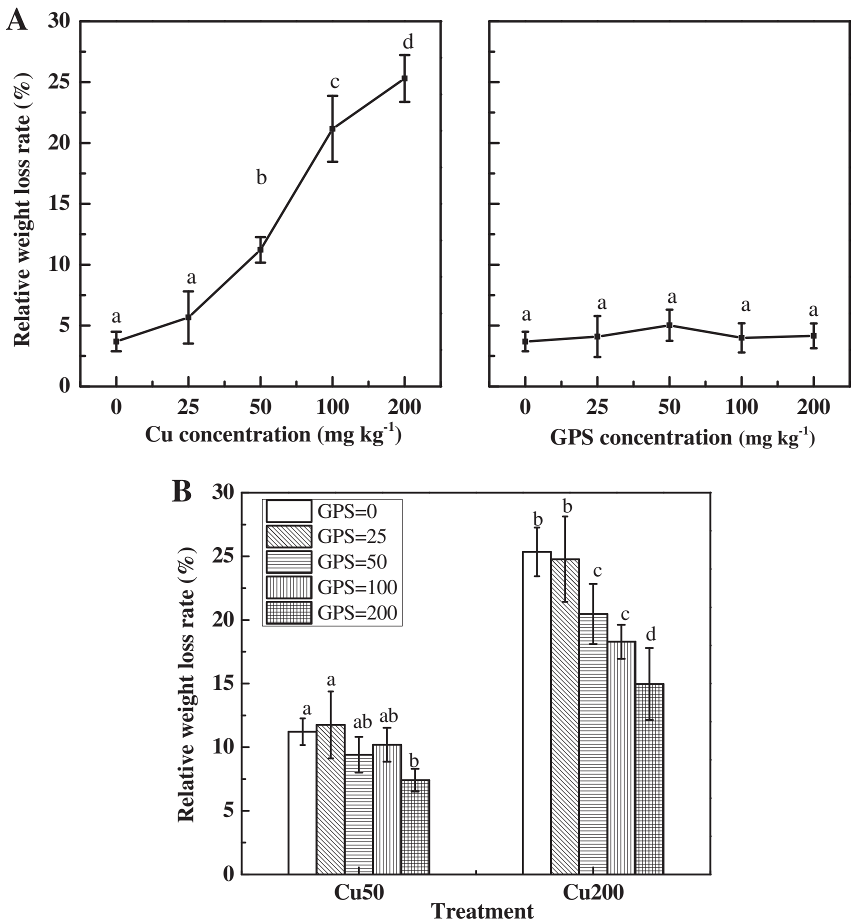

Fig. 1. Relative growth rates of *E. fetida* as exposed to different concentrations of toxicant (A, Cu/GPS. B, their joint). All data are presented as the means of four replications with standard deviations; means with different lowercase letters are significantly different ($p < 0.05$).

200 mg kg^{-1} Cu, the number of cocoons increased from 16.3 to 17.5, 15.5, 20.8, and 22.5 in the presence of GPS at 25, 50,100, and 200 mg kg^{-1}, respectively.

3.3. Effects on Cu uptake in the presence of GPS

Fig. 2 illustrated Cu uptake by earthworms after 28 d exposure to Cu without GPS, which increased linearly with the total concentration of Cu in artificial soil. A sharp increase of the Cu burden in earthworm was noted compared with the control (13.4 mg kg^{-1}). The accumulation of Cu was positively correlated ($r^2 = 0.97$, $p < 0.01$) with the soils concentration of Cu (Fig. 3). There was significant difference ($p < 0.01$) between the control and the treated groups.

Table 1
Production of cocoons of *Eisenia fetida* exposed to Cu and GPS spiked soil after 28 d of exposure.[a]

Treatment (mg kg-1)	Number of cocoons				
	0	25	50	100	200
Cu	32.3 ± 3.8	35.0 ± 4.9	24.8 ± 4.3*	22.0 ± 2.9**	16.3 ± 3.3**
GPS	32.3 ± 3.8	32.8 ± 5.8	29.5 ± 5.4	30.3 ± 2.8	29.5 ± 6.2
Cu50 + GPS	24.8 ± 4.3	22.3 ± 1.5	23.0 ± 4.2	23.5 ± 3.7	25.5 ± 3.9
Cu200 + GPS	16.3 ± 3.3	17.5 ± 2.1	15.5 ± 6.2	20.8 ± 3.3	22.5 ± 4.5*

*$p < 0.05$, **$p < 0.01$.
[a] Results are expressed as means standard deviations ($n = 4$). Statistical significance versus control group.

The copper uptake by earthworms did not change significantly with time in the control. The uptake of copper increased with both the increase of Cu concentration in spike soils and exposure times, while the presence of GPS could partially retard the absorption process of Cu by earthworms (Fig. 4). For example, the uptake of Cu

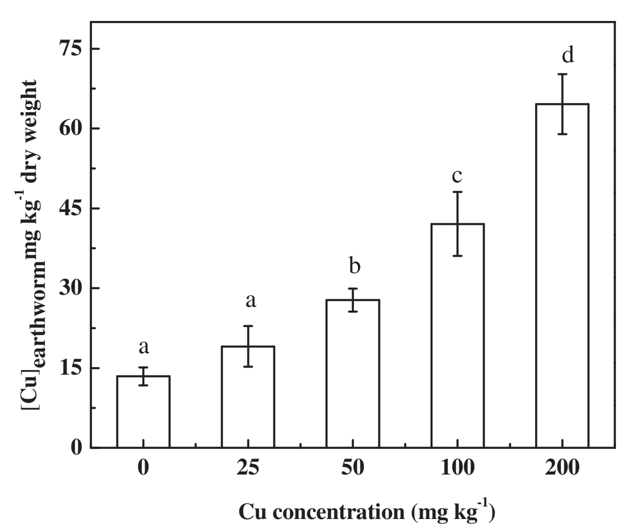

Fig. 2. Correlation between internal body concentrations of Cu and the relative weight loss ratio of earthworm exposed to Cu via an artificial substrate for 28 d.

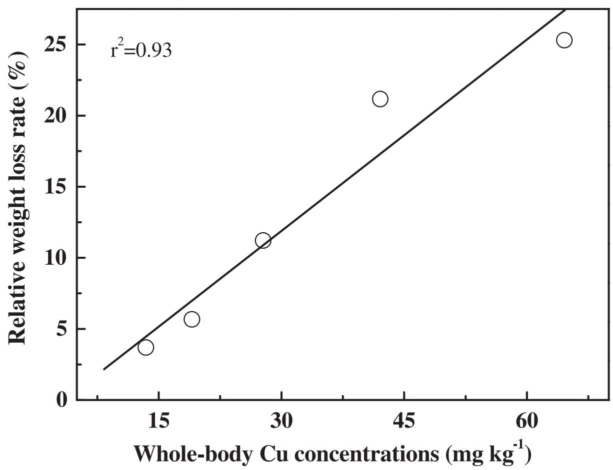

Fig. 3. Concentration of Cu in earthworm as exposed to different concentrations of Cu. All data are presented as the means of four replications with standard deviations; means with different lowercase letters are significantly different ($p < 0.05$).

by earthworms increased rapidly to 31.3 mg kg^{-1} after 3 d exposure to soil containing 50 mg kg^{-1} of Cu, whereas 200 mg kg^{-1} of GPS could reduce the Cu uptake to 23.2 mg kg^{-1}. Furthermore, when exposed to 200 mg kg^{-1} Cu spiked soil, the earthworms took up 46.5 and 28.5 mg kg^{-1} of Cu in the absence or presence of 200 mg kg^{-1} of GPS, respectively. GPS can significantly reduce the absorption of Cu in the first three days, but the inhibition slowed down as exposure time increased. Similarly, after 28 days' exposure the Cu burden in earthworm still decreased with the increasing of GPS (Fig. 5).

3.4. *Effects on anti-oxidant enzyme activities and MDA content*

Table 2 shows the effects of Cu on the SOD and CAT activities of earthworms. After 28 days' exposure, SOD activity in all treatment groups except the 25 mg kg^{-1} group decreased significantly ($p < 0.05$) when compared to the control. The CAT activity was markedly lower at 200 mg kg^{-1} Cu ($p < 0.01$) (Table 2). Lipid peroxidation was determined by evaluating earthworm's MDA content. The results (Table 2) showed MDA content was increased depending on Cu concentration, ($p < 0.01$).

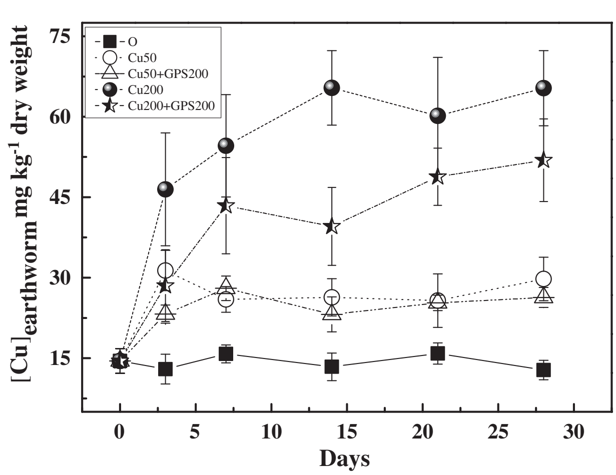

Fig. 4. Effect of glyphosate on earthworm absorption of Cu in different time exposure. O means no toxicant, Cu 50 means Cu at 50 mg kg^{-1}, Cu 50 + GPS 200 means Cu at 50 mg kg^{-1} and GPS at 200 mg kg^{-1}, Cu 200 means Cu at 200 mg kg^{-1}, Cu 200 + GPS200 means Cu at 200 mg kg^{-1} and GPS 200 mg kg^{-1}. All data are presented as the means of four replications with standard deviations; means with different lowercase letters are significantly different ($p < 0.05$).

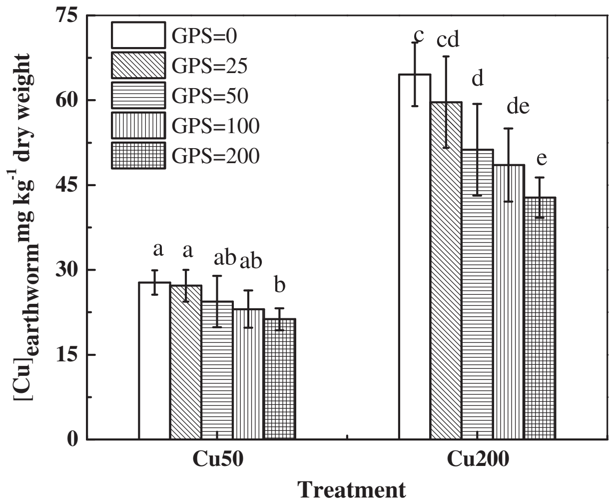

Fig. 5. Effect of glyphosate on earthworm absorption of Cu. All data are presented as the means of four replications with standard deviations; means with different lowercase letters are significantly different ($p < 0.05$).

No significant differences in the SOD activity of earthworms was observed when exposed to GPS, nor did the CAT activity ($p > 0.05$) (Table 3). The MDA content of earthworm increased but not significantly ($p > 0.05$) (Table 3). Changes of the SOD, CAT activities and MDA content in earthworms exposed to Cu and GPS were shown in Table 3. Compared with the treatment of Cu at 200 mg kg^{-1} only, the SOD activity in earthworm increased with GPS concentration increasing ($p < 0.01$). Such detoxifying effects were not significant in the treatment with 50 mg kg^{-1} of Cu. However, the presence of Cu and GPS together had no effect on the CAT activity for all exposure periods ($P > 0.05$). But the co-presence of Cu and GPS significantly influenced the MDA content when the concentration of Cu was 200 mg kg^{-1}. MDA level decreased markedly in the presence of 100 or 200 mg kg^{-1} GPS ($P < 0.05$).

4. Discussion

4.1. *The toxicity of Cu to earthworms*

Cu induced dose-dependent weight loss of earthworms when it was added to the substrate (Fig. 1A). The weights of the earthworms in all treatments including the control were inhibited, probably due to the lack of suitable food in the OECD standard soil medium used, which was in agreement with the finding of Žaltauskaitė and Sodienė (2010). The results of the present work indicated that earthworms exposed to higher Cu concentrations in soil tended to lose more weight than those in control, in accord with other studies (Van Gestel et al., 1991; Helling et al., 2000; Spurgeon et al., 1994). Furthermore, the tissues of earthworms in the contaminated soil had times greater concentrations of Cu than those in the uncontaminated soil, which indicated that elevated heavy metal concentrations in environment resulted in more uptake of the heavy metal into the bodies of earthworms. These findings are consistent with similar studies, which clearly demonstrated that the internal concentrations of heavy metal in earthworms increased with total heavy metal contents in the environment (Fisker et al., 2011; Steenbergen et al., 2005). Our results also demonstrated that the weight loss ratio increased gradually with the increase of Cu burden in the earthworms, and there was a good linear correlation between them (Fig. 3).

Cocoon production is a sensitive parameter for pesticide and heavy metal toxicity. It was reported that pesticides reduced worm

Table 2
Biochemical responses of earthworms exposed to Cu spiked soil after 28 d of exposure.[a]

Biochemical measurements	CK	Cu concentrations (mg kg^{-1})				GPS concentrations (mg kg^{-1})			
	0	25	50	100	200	25	50	100	200
SOD activity (U mg^{-1} protein)	2.67 ± 0.36	2.92 ± 0.34	1.97 ± 0.14*	2.18 ± 0.11*	1.56 ± 0.17**	2.24 ± 0.30	2.16 ± 0.26	2.17 ± 0.28	2.53 ± 0.28
CAT activity (U mg^{-1} protein)	56.88 ± 4.45	54.71 ± 6.56	48.97 ± 4.93	49.39 ± 4.95	40.38 ± 3.66**	48.79 ± 5.47	50.57 ± 4.33	47.43 ± 5.97	48.07 ± 8.98
MDA content (μM mg^{-1} protein)	0.98 ± 0.05	1.53 ± 0.21*	1.86 ± 0.34**	2.14 ± 0.48**	2.45 ± 0.31**	1.33 ± 0.30	1.08 ± 0.39	1.17 ± 0.52	1.45 ± 0.09

[a] Results are expressed as means and standard deviations ($n = 4$). Statistical significance versus control group (CK); *$p < 0.05$, **$p < 0.01$.

cocoon production in a dose-dependent manner, with greater impact at higher chemical concentration (Reinecke et al., 2001; Spurgeon et al., 1994). In the present study, significant difference in the total cocoon production was also observed between the control groups and the groups of earthworm exposed to Cu contaminated soil after 28 days. This finding was also supported by previous studies (Helling et al., 2000; Ma, 1984), in which indicated that heavy metals such as Cu might affect cocoon viability. We concluded that food availability could have played a major role and could explain the different observations. The availability of sufficient food is of the prime importance for maintaining a high cocoon production rate (Žaltauskaitė and Sodienė, 2010).

Copper could be not only accumulated in earthworms, but also induce adverse physiological effects. The main toxic effect of Cu was to produce oxidative stress, linked with lipid peroxidation of cell membranes and underlying the neurotoxicity (Stegeman et al., 1992). In this study, significant changes in SOD, CAT activities, and MDA contents were found in earthworms after exposing to Cu (Table 2).

Enzymatic activities have been considered as biomarkers of the environmental pollution, among which some can protect cells against the adverse effects of reactive oxygen species (ROS), such as SOD and CAT. Normally, oxygen would be reduced to water in cell by these enzymes to protect itself from ROS damage. Superoxide dismutase is an important enzyme in the anti-oxidant system of an organism. In the present study, SOD and CAT activity decreased with 28 d exposure (Table 2). However, the enzyme change in our study was different from other studies (Nagalakshmi and Prasad, 1998; Zhou et al., 2012). The differences may be due to different species used, exposure medium and especially exposure duration. Because under normal physiological conditions, SOD maintains a dynamic equilibrium, which is need for the organism to eliminate $O_2^{\bullet-}$. SOD activity were induced at the early period of incubation, which can prevent lipid peroxidation by catalyzing the disproportionation of the lipid peroxidation initiator and the transformation of superoxide radicals to H_2O_2 and $O_2^{\bullet-}$. The reason might be that the natural anti-oxidant defenses were saturated. The generated superfluous ROS exceeds the capacity of scavenging the ROS of SOD and CAT. As a result, the activities of SOD and CAT gradually decreased (Cui et al., 1999).

Malondialdehyde is the product of the reaction between free radicals and unsaturated fatty acids in cellular membranes. They may react with the free amino ends of proteins to form inter and intra-molecular protein crosslink, which can cause cellular injury. Our study indicated that MDA levels in earthworms in all treatment groups were significantly higher than those observed in control groups after 28 d of Cu exposure, which indicated the damage of the lipid membranes.

Table 3
Biochemical responses of earthworms exposed to Cu and GPS spiked soil after 28 d of exposure.[a]

Cu (mg kg^{-1})	GPS (mg kg^{-1})	SOD activity (U mg^{-1} protein)	CAT activity (U mg^{-1} protein)	MDA content (μM mg^{-1} protein)
50	0	1.97 ± 0.14	48.97 ± 4.93	1.86 ± 0.34
	25	2.06 ± 0.50	48.39 ± 1.82	1.40 ± 0.23
	50	2.72 ± 0.10*	43.51 ± 4.96	1.98 ± 0.39
	100	2.07 ± 0.27	50.35 ± 6.97	1.75 ± 0.17
	200	2.27 ± 0.36	47.43 ± 11.06	1.25 ± 0.25
200	0	1.56 ± 0.17	40.38 ± 3.66	2.45 ± 0.31
	25	1.89 ± 0.22	40.63 ± 3.78	2.51 ± 0.13
	50	2.16 ± 0.34	42.53 ± 3.83	2.22 ± 0.31
	100	2.05 ± 0.16*	44.49 ± 7.70	1.80 ± 0.39*
	200	2.57 ± 0.26**	46.85 ± 5.19	1.73 ± 0.24*

[a] The soils were spiked with different Cu (50, 200 mg kg^{-1}) and interaction with different GPS (0, 25, 50, 100, 200 mg kg^{-1}). Results are expressed as means standard deviations ($n = 4$). Statistical significance versus control group (Cu50 or Cu200) *$p < 0.05$, **$p < 0.01$.

4.2. The toxicity of GPS to earthworms

During the 28 d exposures, there was no significant difference in weight or the cocoon production between the control group and the GPS group. Our results showed that GPS had very low toxicity to the earthworms, which is consistent with other studies. For instance, the results of Howe et al. showed that GPS in technical-grade had no acute or chronic effects on developing tadpoles (Howe et al., 2004). Uchida et al. (2012) also found that no significant gene expression changes in the medaka liver after exposure to glyphosate. No acute toxic effect on earthworm was observed in our previous study when earthworm exposed to GPS solution (Zhou et al., 2012). Also, chronic feeding studies did not indicate carcinogenic activity of this herbicide in rats and dogs. No adverse effects on reproduction or fetal development have been observed in three-generation studies on rats and in a developmental toxicity study on rabbits (Bolognesi et al., 1997).

Because GPS is a competitive inhibitor of the enolpyruvylshikimate-phosphate synthase (ESP-synthase) to the synthesis of aromatic amino acids in plants, it had low toxicity to the non-target animal, which had no ESP-synthase. These aromatic amino acids interfered in protein production and with biomolecules that require these amino acids as precursors, such as auxins and polyphenols. Some studies have shown that the toxicity of commercial product of GPS is much stronger than the GPS acid, because the former contained toxic surfactants (Glusczak et al., 2006; Howe et al., 2004; Tsui and Chu, 2003). Nevertheless, adverse effects of glyphosate on non-target organisms were also found, such as a lower toxicity to *Lumbriculus variegates* reported by Contardo-Jara et al. (2009), and a higher toxicity to tadpole of *Litoria moorei* reported by Mann and Bidwell (1999). Our study suggests that these differences could be due to varied exposure pH. Because glyphosate is a Lewis acid, adding this acid may decrease the test media pH. Thus, buffered medium or pH-adjustment should be included in the toxicity tests for acidic toxicants to eliminate pH-induced toxicity since the natural soil and water has higher buffering capacity than the test media (Tsui and Chu, 2003).

4.3. The combine toxicity of Cu and GPS to earthworms

Physicochemical properties of soil pore-water, such as pH, ionic strength, and the speciation and concentrations of complexing ligands are important for the actual speciation of heavy metals. The total metal concentrations in soils are poor predictors of toxicity to terrestrial organisms, because soil characteristics and complexing ligands greatly influence the metal bioavailability. Glyphosate is widely used in the environments, including farmland and orchard, where heavy metals are very often found in elevated levels (Peterson et al., 1994). Therefore, interaction between these two chemicals was expected to occur in the environments. In our study, the subacute toxicity of Cu to earthworms was lowered by GPS, which was similar to our previous results that the acute toxicity of Cu to earthworms was reduced in the presence of GPS (Zhou et al., 2012), and was in agreement with the findings of Tsui et al. (2005) who found that GPS addition could significantly reduce the acute toxicity of Cu and other heavy metals to *Ceriodaphnia dubia.* The growth and cocoon production inhibitions were alleviated as the total Cu absorption by earthworms decreased. In addition, the SOD, CAT activities, and MDA contents were restored to a certain degree in presence of GPS. The marked decline in toxicity and accumulation of Cu by glyphosate may be related to the glyphosate ability to form immobile stable complexes with Cu (Zhou et al., 2012). Similar as GPS with functional groups of amine, carboxylate and phosphonate (Peterson et al., 1994) could be effective chelating agents for heavy metals (Subramaniam and Hoggard, 1988; Wang et al., 2006) and organic cations (Abate et al., 1999; Glass, 1984). Generally, the three functional groups of GPS can vigorously bind cation, especially the hard transition metals (Steenbergen et al., 2005). It was predicted that a formation of 1:1 of CuHG:CuG^{-} and the concentration of free Cu^{2+} ion increased with total Cu concentration by a computer model (Morillo et al., 1997).

The free ion activity model implies that only free ions were expected to be toxic or bioavailable. Because of the high complexation capacity with metals, GPS could potentially affect the bioavailability, toxicity, and bioaccumulation of metals when applied directly into the ecosystems receiving both chemical groups (Tsui et al., 2005). Several studies were carried out to examine the interactions of metal and glyphosate and their influence to organism. Fe^{2+}, Fe^{3+} and Al^{3+} significantly reduced the activity of glyphosate (Hensley et al., 1978), and the presence of Cu^{2+} could decrease the inhibition of glyphosate to wheat germination and sprout length and decrease the toxicity of glyphosate to root elongation when glyphosate was at high concentrations (Wang et al., 2009a). Similarly Fe and Mn in spray solutions were known to inhibit glyphosate herbicidal activity by limiting absorption and translocation of glyphosate in treated leaves. However, glyphosate residues or drift may result in severe impairments in Fe and Mn nutrition of non-target plants, possibly due to the formation of poorly soluble glyphosate-metal complexes in plant tissues and/or rhizosphere interactions (Eker et al., 2006).

5. Conclusion

The present study showed that Cu exhibited subacute toxicities on earthworms as Cu concentration increased. GPS was nearly nontoxic to earthworms in the presence of GPS, which could lessen the toxicity and bioavailability of heavy metals to earthworm, similar to other synthetic ligands such as EDTA and NTA. In other words, glyphosate could potentially affect the bioavailability, toxicity and bioaccumulation of heavy metals for a longer term when applied directly into the soil ecosystems.

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