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Integration of chemical and toxicological tools to assess the bioavailability of copper derived from different copper-based fungicides in soil

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ABSTRACT

Because the extensive use of Cu-based fungicides, the accumulation of Cu in agricultural soil has been widely reported. However, little information is known about the bioavailability of Cu deriving from different fungicides in soil. This paper investigated both the distribution behaviors of Cu from two commonly used fungicides (Bordeaux mixture and copper oxychloride) during the aging process and the toxicological effects of Cu on earthworms. Copper nitrate was selected as a comparison during the aging process. The distribution process of exogenous Cu into different soil fractions involved an initial rapid retention (the first 8 weeks) and a following slow continuous retention. Moreover, Cu mainly moved from exchangeable and carbonate fractions to Fe-Mn oxides-combined fraction during the aging process. The Elovich model fit well with the available Cu aging process, and the transformation rate was in the order of $Cu(NO_3)_2$ > Bordeaux mixture > copper oxychloride. On the other hand, the biological responses of earthworms showed that catalase activities and malondialdehyde contents of the copper oxychloride treated earthworms were significantly higher than those of Bordeaux mixture treated earthworms. Also, body Cu loads of earthworms from different Cu compounds spiked soils were in the following order: copper oxychloride > Bordeaux mixture. Thus, the bioavailability of Cu from copper oxychloride in soil was significantly higher than that of Bordeaux mixture, and different Cu compounds should be taken into consideration when studying the bioavailability of Cu-based fungicides in the soil.

1. Introduction

Copper-based fungicides have been widely applied to agriculture, especially in the vineyards, to prevent and treat plant downy mildew (Arias et al., 2004; Proffit et al., 2015). The extensive usage of Cu-based fungicides can lead to Cu accumulations in agricultural soils. By reviewing the studies about Cu retention in vineyards worldwide, Komárek et al. (2010) found that the majority of vineyards had soil Cu concentration values exceeding the warning or critical legislative limits of local governments. For example, soil Cu concentrations with 1000 $mg\,kg^{-1}$ and 3000 $mg\,kg^{-1}$ were found in France and Brazilian vineyards, respectively (Flores-Vélez et al., 1996; Mirlean et al., 2007). Nowadays, since the use of Cu-based fungicides is still allowed in organic farming for most countries, a decrease in Cu accumulation of agricultural soil, especially for the fruit producing area, is not expected (Pérez-Rodríguez et al., 2016).

After being input into soil, heavy metal can gradually combine with soil constitutes, and this distribution process is regarded as “aging” (Ma et al., 2006a; Sayen et al., 2009). To understand the influences of aging process on metal bioavailability, studies usually use the indirect chemical measures, i.e., the fractionation of heavy metals in soils (Jalali and Khanlari, 2008). Water soluble and exchangeable fractions are considered readily mobile and bioavailable, while other metal fractions are considered immobile and tightly bound which are less available and toxic to organism (Tang et al., 2006). Changes of metal bioavailability during the aging processes have been widely studied, e.g. As, Cd, Cu and Pb, by measuring their chemical fractions after various periods from several weeks up to years (Safari-Sinegani and Mirahamdi-Araki, 2010; Guo et al., 2011; Wang et al., 2015). Moreover, the phenomena that aged soils and freshly spiked soils have different metal bioavailability have been reported (Alexander, 2000; Tom-Petersen et al., 2004). When fungicides-derived Cu enters into soil, as a result of the direct application, drift, or wash-off from leaves, its speciation rapidly changes and Cu is sorbed and (co)precipitated in the soil (Paradelo et al., 2008). This redistribution of Cu into different chemical fractions of soils may impact its bioavailability. Although the accumulation or distribution characteristics of fungicides-derived Cu in soil have been widely studied (Jalali and Khanlari, 2008; Guo et al., 2011), few studies

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have investigated the aging process of fungicides-derived Cu in soil (Sayen et al., 2009). Present studies usually use $CuCl_2$, $Cu(NO_3)_2$ or $CuSO_4$ as research materials which do not represent the actual chemical forms of the fungicides applied (Sayen et al., 2009). It has been observed that the physicochemical behaviors of fungicides-derived Cu in soil differ from each other (Pose et al., 2009). Based on the results from Bordeaux mixture, Cu oxychloride and $Cu(NO_3)_2$, Komárek et al. (2009) and Wang et al. (2016) have found that Cu retention behaviors and retention amounts in soils were directly controlled by the compositions of Cu compounds, and Bordeaux mixture can result in more Cu retention than Cu oxychloride. Additionally, the results from $Cu(NO_3)_2$-based experiments could not stand for the actual adsorption behaviors of Cu-based fungicides in soils. Since aging reactions can modify Cu bioavailability and toxicity over time, it is important to take the aging process into consideration when evaluating the bioavailability of fungicides-derived Cu in soil.

Copper-based fungicides can not only control fungal diseases, but also can affect non-target soil organism. Helling et al. (2000) have found that even at a concentration of 16 mg kg^{-1}, Cu oxychloride can negatively impact the populations of earthworm (*Eisenia fetida*). Maboeta et al. (2002) showed that earthworm started to accumulate Cu after the eighth spraying applications of Cu oxychloride, while the neutral red retention time values of earthworm coelomocytes decreased significantly after two spraying applications. Snyman et al. (2009) also observed toxicological responses of snails after being exposed to Cu oxychloride treated soils. Moreover, despite chemical approaches are usually used in assessing metal bioavailability, bioavailability is basically biological in nature (Lanno et al., 2004). To better explain metal bioavailability in soil, biological endpoints should also be taken into consideration. Currently, when it comes to the bioavailability of different Cu-based fungicides on soil organism, studies are mostly carried out for one type of Cu-based fungicide and do not consider the different compounds of Cu-based fungicides. Although El-Gendy et al. (2009) have compared the oxidative stress of snail (*Thebapisana*) caused by different Cu-based fungicides, their study was carried out based on the solution assays.

The objective of this work was to achieve a bioavailability assessment of Cu originating from different Cu-based fungicides in soils by adopting an integrated approach which combines both chemical and biological tools. To achieve the above aims, changes in soil Cu fractionation with time were measured using sequential extraction. Furthermore, earthworms, which are more susceptible to metal pollution than many other soil invertebrates, are used as biological tools in this study. Additionally, it is well recognized that different earthworm toxicity endpoints show different sensitivity to contaminants, and early biochemical reactions of earthworms are fairly important for assessing the potential adverse effects of pollutants on the environment (Wu et al., 2011). Catalase (CAT) and malondialdehyde (MDA) of earthworm, which have been widely regarded as early warning indices of pollution, were studied to determine the bioavailability of Cu-based fungicides on earthworms (Song et al., 2009). Through the findings of this study, we can get a more comprehensive understanding of the bioavailability of fungicides-derived Cu in soil and gain more information about the potential ecological risks of Cu-based fungicides on soil ecosystem.

2. Materials and methods

2.1. Soil sampling and tested animals

Soil was sampled from a one-year-old apple orchard at a depth of 0–20 cm (after removing the litter layer). Soil sample was combined by twelve subsamples which were collected from different plots of the orchard following a “W” scheme. No Cu-based fungicide was used for the orchard, and soil sample can be regarded as unpolluted soil. Soil type of the sampling site is Mollisols. Soil sample was air-dried and sieved through a 2-mm nylon-fiber sieve. Soil pH and organic carbon values were 5.95 and 18.60 g kg^{-1}, respectively.

Earthworms (*Eisenia fetida*) were collected from an earthworm breeding company in Jiangsu Province, China. Before the experiments, earthworms were fed with clean cow dung in the laboratory.

2.2. Soil incubation

About 1.0 kg soil samples were spiked with two commonly used fungicides-Bordeaux mixture ($CuSO_4$ + $Ca(OH)_2$; $CuSO_4/Ca(OH)_2$ = 1/1 by weight) and Cu oxychloride ($3Cu(OH)_2 \cdot CuCl_2$, AR ≥ 99%), respectively. Copper nitrate ($Cu(NO_3)_2 \cdot 4H_2O$), which has been widely used as a studying material to investigate the distribution behaviors of fungicide-derived Cu in soils, was used as a comparison in this study. Copper concentrations of the soils were set at 200 mg kg^{-1} (the maximum Cu permissible value for the soil environmental quality standard of the orchard in China is 100 mg kg^{-1} ($pH < 6.5$)). All incubation experiments were performed in triplicate. The moisture contents of soil samples were maintained at 60% of the maximum water holding capacity throughout the experiments. Then, soil samples were incubated at the laboratory without controlling the incubation temperature. During the aging process, soil samples were mixed with a laboratory spoon once per week. Soil samples were taken at 7d, 14d, 28d, 42d, 63d and 77d. The room temperature during the day time ranged from 26 to 30 °C, and varied from 13 to 20 °C during the night.

2.3. Earthworm exposure

Earthworm exposure experiments were carried out at sublethal Cu concentrations of 100 and 200 mg kg^{-1} based on the preliminary experiments of soil acute toxicity tests. Bordeaux mixture and Cu oxychloride were used in the earthworm exposure experiments, and the incubation experiments were performed in triplicate. Soil moisture content was kept at 70% of the water holding capacity during the experiments. About 1.0 kg of Cu-spiked soil was put into the test box, and then soil samples were incubated for 3 days before the experiments. The test box had a transparent lid with small holes for ventilation. Ten earthworms were transferred to each container after voiding their gut. The containers were maintained in an artificial climate chamber, which had a culture condition of 20 °C, 80% ambient humidity and a 12 h light/12 h dark cycle. Control groups were also prepared in the same way without adding Cu. About 0.50 g of clean cow dung was added onto the soil surface per container every week. After 28 days exposure, survival earthworms were determined. Then, earthworms were frozen by liquid nitrogen after being depurated to void their gut. Then, earthworms were stored either at − 20 °C for whole body metal measurements or at − 80 °C for biomarker analyses.

2.4. Soil fractionation analysis

The Tessier Method was applied for Cu fractionation analysis (Tessier et al., 1979). The exchangeable fraction (F1) was extracted after 1 h agitation of 1.00 g soil with 8 mL of 1.00 mol L^{-1} $MgCl_2$ (pH 7.0). The carbonate fraction (F2) was extracted from the residual soil sample after 5 h agitation with 8 mL of 1.00 mol L^{-1} CH_3COONa (pH 5.0). The soil Fe-Mn oxides combined fraction (F3) was extracted after 5 h agitation of the sample with 20 mL of 0.04 mol L^{-1} $NH_2OH \cdot HCl$ in 25% (v/v) CH_3COOH at 96 °C. The organic matter bound fraction (F4) was obtained by the following steps:1) heating the soil residual in 3 mL of 0.02 mol L^{-1} HNO_3 and 5 mL of 30% H_2O_2 (adjusted to pH 2 with HNO_3) for 2 h at 85 °C with occasional agitation; 2) extracting with 3 mL of 30% H_2O_2 (pH 2 with HNO_3) at 85 °C for 3 h with intermittent agitation; 3) 5 mL of 3.20 mol L^{-1} CH_3COONH_4 in 20% (v/v) HNO_3 was added after cooling, and the sample was diluted to 20 mL and agitated continuously for 30 min. The residual fraction (F5) was obtained after digesting the residual samples with a HF-$HClO_4$ mixture. The Cu