Accumulation, Subcellular Distribution and Toxicity of Copper in Earthworm (*Eisenia fetida*) in the Presence of Ciprofloxacin

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Land application of wastes from concentrated animal feeding operations results in accumulation of copper (Cu) and antimicrobials in terrestrial systems. Interaction between Cu and antimicrobials may change Cu speciation in soil solution, and affect Cu bioavailability and toxicity. In this study, earthworms were exposed to guartz sand percolated with different concentrations of Cu and ciprofloxacin (CIP). Copper uptake by earthworms, its subcellular partition, and toxicity were studied. An increase in the applied CIP decreased the free Cu ion concentration in external solution and mortalities of earthworm, while Cu contents in earthworms increased. Copper and CIP in earthworms were fractionated into five fractions: a granular fraction (D), a fraction consisting of tissue fragments, cell membranes, and intact cells (E), a microsomal fraction (F), a denatured proteins fraction (G), and a heat-stable proteins fraction (H). Most of the CIP in earthworms was in fraction H. Copper was redistributed from the metal-sensitive fraction E to fractions D, F, G, and H with increasing CIP concentration. These results challenge the free ion activity model and suggested that Cu may be partly taken up as Cu-CIP complexes in earthworms, changing the bioavailability, subcellular distribution, and toxicity of Cu to earthworms.

Introduction

Land application of organic wastes produced by animal breeding not only brings the organic matter and nutrients to agricultural soils, but also releases some toxic substances into the environment. In particular, copper (Cu) is used abundantly as a feed additive for stimulating animal growth. Li et al. (1) found that Cu concentrations ranged 6.86–395.19 mg kg⁻¹ in swine feeds and 57.0–2016.7 mg kg⁻¹ in pig feces. Copper in swine sewage was one of the main contributors to the toxic effect on *Daphnia magna* (2). Long-term application of the wastes containing high levels of Cu can result in accumulation of Cu in the soil to a toxic level and pose a threat to groundwater quality (3).

The presence of antimicrobials in some animal wastes is another public and scientific concern because of their ecotoxicity and adverse effects on human health. Antimicrobials are widely used to treat diseases and protect the health of animals. They are also incorporated into animal feeds to improve growth rate and feed efficiency (4). Most of the antimicrobials orally administered may almost completely be excreted into feces or urine as their parent or metabolic products. The application of animal wastes as a fertilizer thereby results in a contamination of antimicrobials in the terrestrial environment. Hamscher et al. (5) reported that there were 4.0 and 0.1 mg kg $^{-1}$ of tetracycline and chlortetracyclines in liquid manure, with an average 86.2 $\mu \rm g$ kg $^{-1}$ in the top soil (0-10 cm). Concentrations of ciprofloxacin (CIP) and enrofloxacin in untreated raw sewage sludge were found to be 1.4 and 2.03 mg kg $^{-1}$ dry matter, respectively (6).

Copper is added to animal feeds as its sulfate salts or oxides, and presumably accumulated in ionic form in the litter. It can form stable soluble complexes with some antimicrobials. Quinolones, for example, have several potentially ionizable functional groups. The formation of complexes between divalent cations and quinolones have been demonstrated (7, 8). The roles of metal ions in the actions of quinolones were studied extensively, especially due to the interesting biological and chemical properties (9-11). For instance, Marshall and Piddock (11) found that in the presence of either magnesium or calcium chloride, the minimum inhibitory concentrations of 18 quinolones for Gram-positive and Gram-negative bacteria increased.

It is well accepted that bioavailability and toxicity of metals are strongly related to their speciation. Arnold et al. (12) found that an increase in the applications of the chelating agent EDTA resulted in the reduction of earthworm mortalities, consistent with the decrease of free copper ion (Cu²+) concentrations. Concentrations of free metal ions in the surface water are proved to be the best predictor for uptake of Cd, Cu, Ni, Pb, and Zn by two benthic species (Oligochaete Limnodrilus spp. and the midge Chironomus riparius) (13), and Cd uptake by the common carp Cyprinus carpio in the presence of humic acids (14). The presence of quinolones is assumed to change the speciation of metals, hence affecting their bioavailability and toxicity. However, limited information is available about the effect of quinolones on these aspects.

The aim of present study was to gain an insight into the effect of widely used quinolone CIP (7) on Cu bioaccumulation, subcellular distribution, and toxicity in earthworms. Earthworms were exposed to quartz sand. Quartz sand provides an inert matrix that does not bind any Cu and CIP from solution, which could help directly quantify Cu speciation in pore water and to evaluate the effect of Cu speciation on its uptake and toxicity to earthworm (15). Relevant mechanisms were investigated using time- and concentration-dependent uptakes of Cu with and without CIP.

Materials and Methods

Reagents. A 1.000 g Cu $\rm L^{-1}$ standard solution (Cu(NO₃)₂ in 0.5 M HNO₃ (Fisher, USA)) was used to prepare a serial of working solutions of Cu. Standard solution of ciprofloxacin (CIP) (Bayer, Wuppertal and Leverkusen, Germany) was prepared by dissolving 100 mg of CIP in 100 mL of methanol containing 0.02 M sodium hydroxide (1 mg/mL), and then stored in the dark at 0–4 °C. Working solutions of Cu and CIP were prepared weekly or daily depending on their concentrations.

All organic solvents such as acetonitrile, methanol, and formic acid (Sigma-Aldrich, St. Louis, MO) were of residue analysis grade. Purified Milli-Q water (Millipore Corporation,

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Bedford, MA) was used throughout. $HClO_4$ and HNO_3 were of guaranteed reagent grade, and all other reagents were of analytical reagent grade (purity >99%). All glassware and plastic containers were soaked in 5% (v/v) HNO_3 for at least 12 h and thoroughly rinsed initially with tap water and subsequently with deionized water before use.

Earthworms. Mature (clitellate) earthworms (*Eisenia fetida*) were purchased from the Agricultural University of China (Beijing, China), and maintained in a laboratory culture box (54 cm \times 40 cm \times 30 cm) filled with sterilized loam and peat moss (70%:30%, fresh weight) (Changbaishan Ltd., China) at 20 \pm 2 °C for at least two weeks before use. Wet weight of individual earthworms was on average 345 \pm 10 mg (n = 30). Earthworms were allowed to depurate for 24 h on moist filter paper before commencing the experiment. Earthworms were kept in the dark during all tests.

Accumulation of Cu in the Presence of CIP. Earthworms were exposed to a 500 mL glass beaker containing 400 g of rinsed quartz sands that had been percolated with 100 mL of aqueous solution containing various Cu and CIP concentrations for 1 day. The pH of the test solution was adjusted to 6.0 ± 0.1 using NaOH or HNO₃. Earthworms exposed in the quartz sand with Milli-Q water (pH 6.0) was used as blank. Earthworm exposed in the quartz sand with solution containing Cu but no CIP was used as control. No significant adsorption of Cu or CIP on quartz sands was found. The earthworms were transferred to a new glass beaker containing the quartz sands that had been percolated with test solution every day to maintain a consistent solution concentration and to prevent a build-up of complexing exudates. Each container held 7 worms. After exposure 4 earthworms were collected randomly, placed on moist filter paper for 24 h to void their gut, and frozen at -18 °C.

Time-Dependent Kinetics of Cu Uptake by Earthworms under the Influence of CIP. Earthworms were transferred to a control solution containing $20~\mu M$ Cu without CIP, while in the other three uptake experiments varying amounts of CIP were added separately to the control solution to make the CIP to Cu molar concentration ratios of 0.5, 1, and 5. At increasing exposure time intervals (1, 2, 3, 5, and 7 d), earthworms were sampled. The concentrations of Cu in earthworms were determined. All experiments were performed in quadruplicate.

Concentration-Dependent Uptake of Cu by Earthworms under the Influence of CIP. Serial experiments were performed simultaneously to test Cu uptake at different Cu concentrations by earthworms. Earthworms were transferred to solution containing various concentrations of Cu (0, 20, 50, 100 μ M). Of these experiments, three different amounts of CIP were added, separately, to make the CIP to Cu molar concentration ratios of 0.5, 1, and 5. After 7 d exposure, the earthworms were sampled. The concentrations of Cu in earthworms were determined. All experiments were performed in quadruplicate.

Determination of Cu and CIP Influx Rate. Two earthworms (about 0.7 g fresh wet weight) were incubated with 5 mL of fresh solutions (pH 6.0 \pm 0.1) containing various concentrations of Cu (0, 2, 4, 6, 8, 10, and 20 μ M). Ciprofloxacin was added to make the CIP to Cu concentration ratio of 1. After incubating for 20 min, earthworms were sampled and washed with ice-cold distilled water for 30 min to remove the surface-adsorbed Cu and CIP. Copper and CIP concentrations in the solutions and the washing water were determined immediately using inductively coupled plasma mass spectrometry (ICP MS) and high-performance liquid chromatography (HPLC), respectively. All experiments were performed in septuplicate.

Effects of Cu and CIP on the Mortality of Earthworm. Four hundred and fifty five earthworms were incubated in 65 pots. Earthworms were exposed to quartz sand percolated with solution containing different Cu concentrations (0, 20,

50, and $100\,\mu\mathrm{M}$) at the different CIP to Cu ratios (0.5, 1, and 5). Earthworms were checked daily and the dead ones were picked out and discarded to avoid any influence on other earthworms. After 7 days of exposure, surviving individuals were removed and counted. A pilot experiment (data not shown) indicated that the presence of $500\,\mu\mathrm{M}$ CIP in solution (contained no Cu) did not induce any earthworm death. Six replicates were carried out for each experimental point.

Fractionation of Cu and CIP among Earthworms. Earthworms were exposed to quartz sand percolated with solution containing 100 μ M Cu at different CIP to Cu ratios (0.5, 1, and 5). Three replicates were carried out for each experimental point. Three earthworms (1000–1100 mg fresh wet weight) randomly picked were thawed, weighed, and homogenized (Omni International, Warranton, VA) in 5.0 mL of ice-cold 0.01 M Tris-HCl buffer (pH 7.5, Fisher Scientific, Houston, TX). Homogenates were then centrifuged at 10,000g for 30 min at 5 °C. Fractionation was performed following the protocol described by Vijver et al. (16). Briefly, Cu and CIP in earthworms were fractionated into five fractions: a granular fraction (D), a fraction consisting of tissue fragments, cell membranes, and intact cells (E), a microsomal fraction (F), a denatured proteins fraction (G), and a heat-stable proteins fraction (H). All fractions were dried under a gentle nitrogen stream. Copper and CIP concentrations in the earthworms and the earthworm fractions were determined.

Determination of Cu^{2+} Concentration and Speciation of Cu in Pore Water with and without CIP. Copper ion-selective electrodes (Cu-ISE) in combination with a voltmeter of 0.1 mV resolution (model 9629, Orion Research, Cambridge, MA) were used to measure Cu^{2+} concentrations. The Cu-ISE was activated in 1 N $\text{Cu}(\text{NO}_3)_2$ for 2 h and rinsed with Milli-Q water before use. The limit of detection was 0.7 μ M Cu^{2+} . The activity of Cu^{2+} at each calibration point and the chemical speciation of Cu in the solutions with and without CIP were calculated using the speciation program ChemEQL version 3.01 based on the total Cu concentration together with other parameters including pH, alkalinity, and major ion concentrations constituting inputs to the model. The stability constants were taken from Wallis et al. (8).

Determination of Cu in Earthworms and Earthworm Fractions. The total Cu contents in earthworms and earthworm fractions were analyzed after sample digestion with 3 mL of concentrated HNO₃–HClO₄ (2:1, v:v) under high pressure condition (17). Copper concentrations were determined by ICP-MS (Optima 2000 DV, Perkin-Elmer Co.). During digestion, reference sample of NCS ZC 78005 (a mussel) obtained from the Chinese National Analysis Centre for Iron and Steel was applied and an average recovery of 98.6 \pm 2.51% (n=6) was obtained for Cu.

Determination of CIP in Earthworms and Earthworm Fractions. CIP was extracted from earthworms according to the method of Ramos et al. (*18*) (Supporting Information) with some modification. Briefly, the earthworms were thawed. The CIP in earthworms was extracted with 0.05 M phosphate buffer (pH 7.4), and cleaned with Oasis HLB cartridge (6 mL/300 mg, Waters, MA).

All measurements were performed using a 1200 LC instrument equipped with gradient pump and fluorescence detector (Agilent Technologies, Wilmington, DE). HPLC separations were conducted using injection volumes of 20 μ L and linear gradient elution as follows: from 90% A (0.1% formic acid, pH 2.8) and 10% B (acetonitrile) to 85% A and 15% B in 8 min, returning to initial conditions in 3 min. The excitation and emission wavelengths for detection were 280 and 450 nm, respectively. The chromatographic separation was carried out in an Atlantis-dC18 ODS HPLC column (4.6

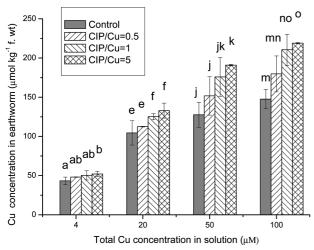


FIGURE 1. Cu concentration in earthworms E. fetida after 7 days of exposure to quartz sand percolated with Cu and CIP solution. The molar ratios of CIP to Cu were 0, 0.5, 1, and 5. Mean values are shown with standard deviations (n = 4).

i.d. \times 150 mm, 5 μ m) (Waters, MA). Under these HPLC conditions CIP showed a single peak at the retention time of 8.5 min.

Data Analysis. All statistical analyses were conducted with the software SPSS 11.5 for Windows (SPSS Inc., Chicago, IL). One-way ANOVA was used to assess the significance of the difference between groups, and linear regression analyses were conducted by the least-squares method. Statements of significant differences are based on P < 0.05.

Results

Effect of Cu to CIP ratio on Cu^{2^+} Concentration in Pore Water. The concentration of Cu^{2^+} in solutions measured by Cu-ISE and the speciation of Cu as computed with the ChemEQL program in the absence and presence of CIP are shown in Table S1. Good agreement was obtained between the data measured and those calculated. More than 95% of total Cu in control solution is Cu^{2^+} . The presence of CIP decreased Cu^{2^+} concentration significantly (p < 0.05). When the CIP to Cu ratios were 0.5 and 1, the percentages of Cu^{2^+} were 51.3–60.0% and 16.6–33.0% of the total Cu concentrations in pore water, respectively. The Cu^{2^+} concentration in pore water was lower than the limit of detection when the CIP to Cu ratio was 5.

Copper Uptake by Earthworms in the Presence of CIP. Time-Dependent Kinetics of Cu Uptake. The uptake of Cu did not reach saturation on day 7 (Figure S1). The Cu uptake patterns were similar to that of the control. A linear relationship was found between exposure time and earthworm uptake (p < 0.01). Overall, the uptake rate of Cu was significantly enhanced by CIP. The uptake rates of Cu were 22.1 ± 0.5 , 25.7 ± 0.8 , and $28.7 \pm 0.4 \ \mu mol \ kg^{-1} \ d^{-1}$ in the presence of CIP at CIP to Cu ratio of 0.5, 1, and 5, respectively. These values were higher than uptake rate of Cu in the absence of CIP (17.6 \pm 0.4 μ mol kg⁻¹ d⁻¹). With increasing ratios of CIP to Cu, the Cu uptake rates increased, which suggested that earthworms might take up both Cu²⁺ and Cu-CIP complexes simultaneously. The relative contribution of both Cu species was estimated by fitting a multiple linear regression model to the data:

Cu uptake rate =
$$a[Cu^{2+}] + b[Cu-CIP]$$
 (1)

where $[Cu^{2+}]$ and [Cu-CIP] are the concentrations of Cu^{2+} and Cu-CIP complexes (the sum of $(Cu-CIP)^+$ and $Cu(CIP)_2$) in the pore water (μ mol L^{-1}) (Table S1). The a and b obtained by multiple regression analysis are the conditional uptake

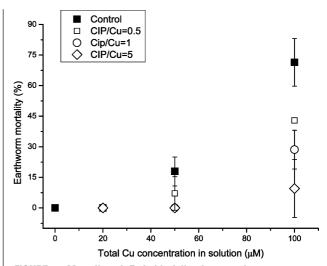


FIGURE 2. Mortality of *E. fetida* following a 7 day exposure to quartz sand. Mean values are shown with standard deviations(n = 6).

rate constants for the uptake of Cu^{2+} and the Cu-CIP complexes, respectively (L kg $^{-1}$ d $^{-1}$). The unit of Cu uptake rate is μ mol kg $^{-1}$ d $^{-1}$. The results show that the uptake rate constant of Cu-CIP complex (1.421 \pm 0.014 L kg $^{-1}$ d $^{-1}$) is about 1.64 times higher than the uptake rate constant of Cu^{2+} (0.863 \pm 0.016 L kg $^{-1}$ d $^{-1}$). Thus, the uptake of Cu-CIP complex is faster than that of Cu^{2+} .

Concentration-Dependent Uptake of Cu and CIP. Copper uptake by earthworms increased with increasing total concentration of Cu in solution (Figure 1). The presence of CIP enhanced Cu accumulation in earthworms; the larger the CIP to Cu ratio, the more significant the increased accumulation effect. The Cu concentrations in earthworms were 1.09–1.22, 1.20–1.43, and 1.28–1.50 times higher than that in the control when the CIP to Cu ratios were 0.5, 1, and 5, respectively.

Influx of Cu and CIP. Data from the relatively short uptake period (<20 min) were used to investigate the uptake while minimizing the possible efflux of Cu and CIP across the plasma membrane back into the external solution. Figure S2 shows that the CIP increased Cu uptake over the entire range of applied Cu in the uptake solutions. The uptake kinetics of Cu in the presence and absence of CIP and the uptake kinetics of CIP was described with a Michaelis—Menten equation:

$$V = V_{\text{max}} C / (K_{\text{m}} + C) \tag{2}$$

where V is the uptake rate (μ mol kg $^{-1}$ min $^{-1}$), C is the total Cu or CIP concentration in the solution (μ mol L $^{-1}$), and $V_{\rm max}$ (μ mol kg $^{-1}$ min $^{-1}$) and $K_{\rm m}$ (μ mol L $^{-1}$) are the maximum uptake rate and Michaelis—Menten rate constant, respectively. The experimental data and model calculation for CIP and Cu with and without CIP fit well with $R^2 > 0.99$ (Figure S2, Table S2). Data derived from the Michaelis—Menten equation showed that CIP increased $V_{\rm max}$ for Cu influx rate by 1.4 times compared to the control, while had no significant effect on $K_{\rm m}$. When earthworms were exposed to the solution containing both CIP and Cu (the ratio is 1), the $V_{\rm max}$ of CIP was equivalent to that of Cu.

Effect of CIP on the Cu Toxicity to Earthworms. The mortality of earthworms after exposure to Cu and CIP for 7 days is given in Figure 2. The results showed that all earthworms survived when worms were exposured to $20\,\mu\mathrm{M}$ Cu in the presence and absence of CIP. When the Cu concentration increased to $100\,\mu\mathrm{m}$, 71.4% of the total earthworms were dead in the absence of CIP. However, in

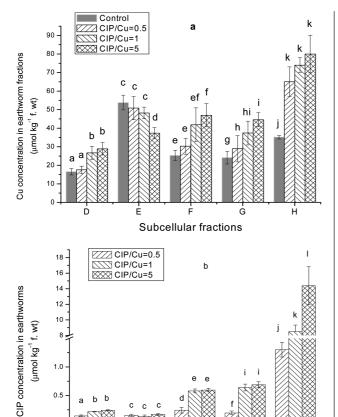


FIGURE 3. Subcellular partition of Cu (a) and CIP (b) in E. fetida following a 7-day exposure to quartz sand when the total solution Cu concentration was 100 μ M. Mean values are shown with standard deviations (n = 4).

Subcellular fractions

1.0

0.5

the presence of CIP the moralities of earthworms were significantly lower than that of the control (p < 0.05) even when the CIP to Cu ratio was 0.5. Such alleviation effects were more pronounced when the CIP to Cu ratio was increased. For example, the earthworm mortalities were 42.9, 28.6, and 9.5% for the CIP to Cu ratios of 0.5, 1, and 5, respectively, when Cu concentration was set at 100 μ M.

Effect of CIP on the Subcellular Distribution of Cu in Earthworms. To evaluate the validity of the fractionation and determination performance, earthworms were subjected to the fractionation and subsequent determination of Cu for six replications. The relative standard deviation of Cu concentrations in fractions D, E, F, G, and H was in the range of 3.8-14.8%. The sum of Cu in the five fractions was comparable with the total obtained by acid digestion of earthworms, thereby showing the validity of the fractionation and determination performance (data not shown).

The subcellular partitioning of Cu among the fractions D, E, F, G, and H following 7-day exposure to quartz sand percolated with Cu and CIP is shown in Figure 3a. In the absence of CIP, Cu associated with fractions D, E, F, G, and H was found to be 16.5, 53.8, 25.3, 24.0, and 35.2 μ mol kg⁻¹, respectively. In the presence of CIP, the contents of Cu associated with fractions D, F, G, and H increased, while those associated with fraction E decreased. When the CIP to Cu ratios were 0.5, 1, and 5, the average Cu contents in fraction E were 50.9, 48.2, and 37.4 μ mol kg⁻¹, respectively.

More than 60% of total CIP in earthworms was associated with fraction H (Figure 3b). When the CIP to Cu molar ratio increased from 0.5 to 5, the Cu concentration in fractions F, G, and H increased obviously. Significantly positive correlation was found between Cu and CIP in fraction H (P < 0.01).

Discussion

Effect of CIP on the Cu²⁺ Concentration. Ouinolones are amphoteric compounds because of the presence of both Lewis base and Lewis acid functional groups, and may exist as a cation, zwitterions, or a net negatively charged ion at environmentally relevant pH range. Between quinolone and some divalent cations, a 1:1 complex was reported to be formed by ion-dipole interaction (7). Zhang et al. (19) found that in the presence of CIP, Cu²⁺ concentration decreased significantly. They used a mercury electrode to study the voltammetric behavior of CIP-Cu complexes and suggested that the composition of the electroactive complex of CIP-Cu(II) was 2:1, and the stability constant of the complex. β , was 4.79 \times 10¹¹. Results of Wallis et al. (8) showed that the addition of CIP to Cu formed Cu-CIP and Cu-(CIP)2 complexes in a stepwise manner and their formation constants were $\log K_1$ 6.2 and $\log K_2$ 11.1. In this study, the decrease of Cu²⁺ concentration (Table S1) with increasing CIP concentration demonstrated the variation of Cu speciation as a result of CIP addition.

Cu Uptake by Earthworms in the Presence of CIP. Several models have been developed to predict metal bioavailability, focusing on identifying which speciation is present in the external environment and their concentrations, and investigating their interactions with the biological sites. According to the free ion activity model (FIAM), the free metal ion activity reflects the chemical reactivity of the metal, the extent to which the metal reacts with the binding sites on the cell membrane surface, and hence its bioavailability (20). The uptake of metal ions was assumed to have occurred over specific carrier ligands in the membrane. The presence of ligands in bulk solution will tend to reduce the free metal ion concentration, and thus attenuate the bioavailability of metals. Results obtained by the present experiment showed that in the presence of CIP the Cu²⁺ concentration in the external solution was significantly decreased, but Cu uptake by earthworms was slightly increased. This result was in a clear contradiction with FIAM, and was also in disagreement with the studies using other types of hydrophilic organic ligands (12, 21, 22).

Increases of metal uptake by addition of small hydrophilic organic ligands have been reported occasionally (23-26). One of the explanations was the rate-limited diffusion of the free metal ion to the cell surface (26, 28). The presence of small hydrophilic organic ligands could effectively thin out the unstirred layer of metal adjacent to the organism, leading to a more rapid diffusion toward the organism (23).

Another possible explanation for the observed extra availability of Cu in the presence of CIP is the formation of ternary complexes (R-Cu-CIP). One of the FIAM assumptions is that if a metal complex reacts at the cell surface, the reaction proceeds by ligand exchange. If the whole complex interacts with the plasma membrane, then FIAM will no longer apply. The possible uptake of small organic ligandmetal complex has been discussed and demonstrated. Errecalde et al. (25) observed that the uptake of Cd by an alga increased in the presence of citrate. They found that the uptake rate of citrate was 4 times greater than that of Cd, and assumed that this increase was attributed to the accidental transport of the Cd-citrate complex over a citrate transporter. Zheng et al. (26) observed that snails fed Fe-EDTA pellets could accumulate 10-100 times more Fe in tissues than did snails fed FePO₄ pellets. They used hydrophilic interaction liquid chromatography (HILIC) coupled with Fourier transform electrospray ionization mass spectrometry (FT-ESIMS) to analyze Fe-EDTA complex and found that the Fe-EDTA complex was present at very high concentrations in the tissues. Results obtained by Chuang and Wang (27) indicated that free metal ion cannot fully explain metal uptake since

metal complex species were also available to the mussels to some extent, apparently through a cotransport process.

Nowadays little information is available about the effect of CIP on the uptake of metals. However, varying and even controversial results have been reported concerning the effect of metals on CIP accumulation and toxicity. Some pharmacokinetic studies revealed a decreased bioavailability of fluoroquinolones after simultaneous administration of metal ions (11, 29). Whereas Imran et al. (30) reported that when CIP complexed with Cu(II), Ni(II), Co(II), and Zn(II), the complexes are more antibacterial compared to the uncomplexed ligands. Once complexes were formed the polarity of a metal ion was reduced to a greater extent due to the overlap of ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Further formation of complexes increases the delocalization of π -electrons over the whole chelate ring and enhances the lipophilicity of complexes. This increased lipophilicity enhances the penetration of complexes into the lipid membranes. In this study, the Cu uptake rate was higher in the presence of CIP than that of the control. The finding of the existence of CIP in earthworms and the similar maximum uptake rate between Cu and CIP suggests that earthworms are able to take up the Cu-CIP complexes.

Effect of CIP on the Subcellular Cu Partition and **Toxicity.** Toxicity is defined as accumulation of a metal at the biotic ligand at or above a critical threshold concentration (31). The diverse biological effects are highly determined by the concentration of the metal in the target tissue (32). The presence of hydrophilic organic ligands, such as nitrilotriacetic acid (NTA) or EDTA, has been reported to decrease the Cu toxicity to the earthworms and fish, which is attributed to the decreases of Cu accumulation (21, 22, 33). However, in this study, the presence of CIP was found to increase Cu accumulation while decreasing morality of earthworms, indicating the differences of the internal Cu speciation between the treatments with and without CIP. A similar phenomenon of bioavailability independent toxicity was also found by Su et al. (34). They reported that when the ratio of atrazine and Cd in solution was 1:1, Cd uptake by rice increased, but the Cd and atrazine toxicity decreased, presuming the formation of Cd-atrazine complex in both nutrient solution and within plant.

It is suggested that toxicity cannot be predicted only from total metal concentration in the organism because biological significance of metal concentration depends on specific tissue in which the metal is deposited (16, 35). The subcellular partitioning that takes into account the cellular fates of metals has been proposed to predict metal toxicity in organisms. Several metal forms in organism are not biologically reactive, including binding to proteins and other ligands, and storage in the inorganic matrix of granules. Wallace and Lopez (36) developed an original method to quantify different metal sequestration forms on the basis of several centrifugation steps. In this procedure, an organism is separated into granular, tissue and cell membrane, and cytosolic fractions. Then the cytosolic fraction was further isolated into a microsomal fraction, a heat-stable proteins (HSP; e.g., metallothioneins) fraction, and a heat denatured proteins (HDP; e.g., enzymes) fraction (37). Wallace et al. (37) suggested that D and H were biologically detoxified fractions, while F and G were metal sensitive fractions. However, Vijver et al. (16) proposed that for most metals in earthworms, granular (D) and cytosolic (the sum of F, G and H) fractions were responsible for metal immobilization, and the metals in tissue fragments, cell membrane, and intact cell fractions (E) were mostly incorporated in the metabolically required pools. It should be pointed out that all fraction ascriptions in the above-mentioned reports were based on the uptake and elimination kinetics rather than toxicological test. In view of the decreases of fraction E and decrease of earthworm morality in the presence of CIP, one can assume that the fraction E was the metal-sensitive fraction, which is consistent with the results of Vijver et al. (16). However, to investigate which fraction can be associated with toxicity requires more research.

More than 60% of the CIP in earthworms is present in fraction H due to its hydrophilic nature. Good relationship between the CIP in fraction H and increased Cu concentrations in this fraction as compared with the control implied that the existence of Cu–CIP complex in earthworms is one of the important factors affecting the Cu subcellular distribution.

The results from this study showed that the presence of CIP could decrease the Cu²⁺ concentration in solutions, while enhancing the Cu uptake by earthworms. The subcellular distribution of Cu in earthworms changed, which was responsible for the decreased Cu toxicity. The above evidence indicated that earthworms could take up, at least partly, the Cu–CIP complexes. This finding challenges the general applicability of the FIAM for metals in general. However, this effect remains to be clarified with a more decisive analytical technique, and further effort should be taken to detail the process of metal biouptake, physiological response, internal redistribution, and their relation with external condition and toxicological response.

Acknowledgments

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Supporting Information Available

Concentrations of free Cu ions measured by ion-selective electrodes and Cu speciation calculated by program ChemEQL in 4 series of exposure solutions (Table S1), kinetic parameters of Cu and Ciprofloxacin uptake in earthworm (*Eisenia fetida*) (Table S2), uptake kinetics of Cu in earthworm *E. fetida* following a 7 day exposure (Figure S1), overall kinetics curves for Cu (a) and CIP (b) uptake influx (Figure S2). This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

- (1) Li, Y.; Li, W.; Wu, J.; Xu, L.; Su, Q.; Xiong, X. Contribution of additives Cu to its accumulation in pig feces: study in Beijing and Fuxin of China. *J. Environ. Sci.* **2007**, *19*, 610–615.
- (2) De la Torre, A. I.; Jiménez, J. A.; Carballo, M.; Fernandez, C.; Roset, J.; Munoz, M. J. Ecotoxicological evaluation of pig slurry. *Chemosphere* 2000, 41, 1629–1635.
 (3) Hao, X. Z.; Zhou, D. M.; Chen, H. M.; Dong, Y. H. Leaching of
- (3) Hao, X. Z.; Zhou, D. M.; Chen, H. M.; Dong, Y. H. Leaching of copper and zinc in a garden soil receiving poultry and livestock manures from intensive farming. *Pedosphere* 2008, 18, 69–76.
- (4) Sarmah, A. K.; Meyer, M. T.; Boxall, A. B. A. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* **2006**, *65*, 725–759.
- (5) Hamscher, G.; Sczesny, S.; Hoper, H.; Nau, H. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal. Chem.* 2002. 74, 1509–1518.
- (6) Picó, Y.; Andreu, V. Fluoroquinolones in soil—risks and challenges. Anal. Bioanal. Chem. 2007, 387, 1287–1299.
- (7) Park, H. R.; Kim, T. H.; Bark, K. M. Physicochemical properties of quinolone antibiotics in various environments. *Eur. J. Med. Chem.* 2002, 37, 443–460.
- (8) Wallis, S. C.; Gahan, L. R.; Charles, B. G.; Hambley, T. W.; Duckworth, P. A. Copper(II) complexes of the fluoroquinolone antimicrobial ciprofloxacin. Synthesis, X-ray structural characterization, and potentiometric study. *J. Inorg. Biochem.* 1996, 62, 1–16.
- (9) Drevenšek, P.; Zupančič, T.; Pihlar, B.; Jerala, R.; Kolitsch, U.; Plaper, A.; Turel, I. Mixed-valence Cu(II)/Cu(I) complex of

- quinolone ciprofloxacin isolated by a hydrothermal reaction in the presence of l-histidine: comparison of biological activities of various copper-ciprofloxacin compounds. *J. Inorg. Biochem.* **2005**, 99, 432–442.
- (10) Turel, I. The interactions of metal ions with quinolone antibacterial agents. Coord. Chem. Rev. 2002, 232, 27–47.
- (11) Marshall, A. J. H.; Piddock, L. J. V. Interaction of divalent cations, quinolones and bacteria. *J. Antimicrob. Chemother.* 1994, 34, 465–483.
- (12) Arnold, R. E.; Hodson, M. E.; Comber, S. Effect of organic complexation on the toxicity of Cu to the earthworm *Eisenia fetida*. *Appl. Geochem.* **2007**, *22*, 2397–2405.
- (13) Vink, J. P. M. The origin of speciation: Trace metal kinetics over natural water/sediment interfaces and the consequences for bioaccumulation. *Environ. Pollut.* **2009**, *157*, 519–527.
- (14) Van Ginneken, L.; Bervoets, L.; Blust, R. Bioavailability of Cd to the common carp, *Cyprinus carpio*, in the presence of humic acid. *Aquat. Toxicol.* 2001, 52, 13–27.
- (15) Vijver, M. G.; Koster, M.; Peijnenburg, W. J. G. M. Impact of pH on Cu accumulation kinetics in Earthworm cytosol. *Environ. Sci. Technol.* 2007, 41, 2255–2260.
- (16) Vijver, M. G.; van Gestel, C. A. M.; van Straalen, N. M.; Lanno, R. P.; Peijnenburg, W. J. G. M. Biological significance of metals partitioned to subcellular fractions within earthworms (*Apor*rectodea caliginosa). Environ. Toxicol. Chem. 2006, 25, 807– 814.
- (17) Zhang, S.; Shan, X. The determination of rare earth elements in soil by inductively coupled plasma mass spectrometry. At. Spectrosc. 1997, 18, 140–144.
- (18) Ramos, M.; Aranda, A.; Garcia, E.; Reuvers, T.; Hooghuis, H. Simple and sensitive determination of five quinolones in food by liquid chromatography with fluorescence detection. *J. Chro*matogr., B 2003, 789, 373–381.
- (19) Zhang, N.; Zhang, X.; Zhao, Y. Voltammetric study of the interaction of ciprofloxacin-copper with nucleic acids and the determination of nucleic acids. *Talanta* 2004, 62, 1041–1045.
- (20) Morel, F. M. M. Principles of Aquatic Chemistry, John Wiley and Sons: New York, 1983.
- (21) Steenbergen, N. T. T. M.; Iaccino, F.; Dewinkel, M.; Reijnders, L.; Peijnenburg, W. J. G. M. Development of a biotic ligand model and a regression model predicting acute copper toxicity to the earthworm *Aporrectodea caliginosa*. *Environ. Sci. Technol.* 2005, 39, 5694–5702.
- (22) Tao, S.; Long, A.; Pan, B.; Xu, F.; Dawson, R. Uptake of copper complexed to EDTA, diaminothane, oxalic acid, or tartaric acid by Neon Tetras (*Paracheirodon innesi*). *Ecotoxicol. Environ. Saf.* 2002, 53, 317–322.
- (23) Wang, H.; Shan, X.; Liu, T.; Xie, Y.; Wen, B.; Zhang, S.; Han, F.; van Genuchten, M. Th. Organic acids enhance the uptake of lead by wheat roots. *Planta* **2007**, *25*, 1483–1494.
- (24) Degryse, F.; Smolders, E.; Parker, D. Metal complexes increase uptake of Zn and Cu by plants: implications for uptake and

- deficiency studies in chelator-buffered solutions. *Plant Soil* **2006**, 289, 171–185.
- (25) Errecalde, O.; Seidl, M.; Campbell, P. G. C. Influence of a low molecular weight metabolite (citrate) on the toxicity of cadmium and zinc to the unicellular green alga *Selenastrum carpricor-nutum*: An exception to the free-ion model. *Water Res.* 1998, 32, 419–429.
- (26) Zheng, L.; Watson, D. G.; Tettey, J. N. A.; Clements, C. A. The determination of iron as its EDTA complex in *Helix aspera* by hydrophilicinteraction liquid chromatography coupled to Fourier transform electrospray ionisation mass spectrometry. *Talanta* 2008, 76, 1165–1169.
- (27) Chuang, C. Y.; Wang, W. X. Co-transport of metal complexes by the green mussel *Perna viridis*. Environ. Sci. Technol. 2006, 40, 4523–4527.
- (28) Jansen, S.; Blust, R.; Van Leeuwen, H. P. Metal speciation dynamics and bioavailability: Zn(II) and Cd(II) uptake by mussel (*Mytilus edulis*) and carp (*Cyprinus carpio*). Environ. Sci. Technol. 2002, 36, 2164–2170.
- (29) Kozjek, F.; Palka, E.; Krizman, I.; Vodopivec, P. Pharmacokinetics of ciprofloxacin metal complexes. *Acta Pharm.* 1996, 46, 109– 114.
- (30) Imran, M.; Iqbal, J.; Iqbal, S.; Ijaz, N. In vitro antibacterial studies of ciprofloxacin-imines and their complexes with Cu(II), Ni(II), Co(II), and Zn(II). *Turk. J. Biol.* 2007, 31, 67–72.
- (31) Santore, R. C.; Di Toro, D. M.; Paquin, P. R.; Allen, H. E.; Meyer, J. S. Biotic ligand model of the acute toxicity of metals. 2. Application to acute coppertoxicity in freshwater fish and Daphnia. *Environ. Toxicol. Chem.* 2001, 20, 2397–2402.
- (32) Hickie, B. E.; McCarty, L. S.; Dixon, D. G. A residue-based toxicokinetic model for pulse-exposure toxicity in aquatic systems. *Environ. Toxicol. Chem.* 1995, 14, 2187–2197.
- (33) DiToro, D. M.; Allen, H. E.; Bergman, H. L.; Meyer, J. S.; Paquin, P. R.; Santore, R. C. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environ. Toxicol. Chem.* 2001, 20, 2383–2396.
- (34) Su, Y.; Zhu, Y.; Lin, A.; Zhang, X. Interaction between cadmium and atrazine during uptake by rice seedlings (*Oryza sativa L.*). *Chemosphere* **2005**, *60*, 802–809.
- (35) Vijver, M. G.; Lanno, R. P.; van Straalen, N. M.; van Gestel, C. A. M.; Peijnenburg, W. J. G. M. Internal metal sequestration and its ecotoxicological relevance: A review. *Environ. Sci. Technol.* 2004, 38, 4705–4712.
- (36) Wallace, W. G.; Lopez, G. R. Relathionship between subcellular cadmium distribution in prey and cadmium trophic transfer to a predator. *Estuaries* 1996, 19, 923–930.
- (37) Wallace, W. G.; Lee, B. G.; Luoma, S. N. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol.: Prog. Ser.* 2003, 249, 183–197.

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