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Speciation Regulation and Biological Detoxification Mechanism of Copper Ions under the Synergistic Effect of Earthworms and Microorganisms

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Abstract: With the increasing prominence of heavy metal pollution in soil, the high reactivity and toxicity of copper ions pose a threat to ecosystems and biological health. Existing copper remediation strategies largely rely on physical adsorption or chemical fixation, making it difficult to achieve dynamic regulation of copper in the soil-biological system, and systematic research on the absorption and toxicity buffering mechanisms of copper in organisms is lacking. Therefore, this paper constructs an earthworm-microbe synergistic system, combining soil fractionation extraction and biological copper determination methods to explore the speciation, translocation, and biological detoxification mechanisms of copper ions. In the experiment, through earthworm feeding and microbial fixation, copper was transformed from exchangeable to organically bound and residual states. The results reveal that the synergistic effect between earthworms and microorganisms can achieve speciation regulation and toxicity buffering of copper at both the microscopic and ecological scales, providing a feasible technical approach for soil heavy metal risk management and ecological restoration.

1. Introduction

Heavy metal pollution in soil has become a significant issue in global ecological and environmental management. Copper, as a common essential micronutrient, participates in enzymatic reactions in plants and microorganisms at low concentrations, but high concentrations lead to decreased soil bioactivity, inhibited plant growth, and bioaccumulation in the food chain, posing significant ecological risks. The migration and transformation of copper in soil are influenced by chemical forms, soil physicochemical characteristics as well as biological processes.

Copper is easily migrated and is much bioavailable as exchangeable copper and the organically bound and residual copper is fairly stable and not as bio-toxic.

Based on this, this paper uses earthworm-microbe synergistic system as a model to conduct a systematic study with regard to the form transformation, migration and biological detoxification of copper in the soil-biological system. Specifically, the experiment first used a soils-graded chemical extraction process to sort copper into exchangeable, carbonate-bound, iron-manganese oxide-bound, organically bound and residual statuses and analyzed the responses of speciation of copper with different treatments. Subsequently, the paper discovered how copper was absorbed, transported and stored in earthworms through measuring the copper content in the earthworms and their excretions then multiplying it with the determination of the bioaccumulation factor (BAF) to understand the dynamics. Meanwhile, microbial action analysis, investigated the usefulness of the functions of the microorganisms in the fixation and slow release of copper through population adsorption, precipitation and metabolic transformation.

2. Related Work

The final nature of copper speciation in the mobility and biological effects to different levels including cells, environmental and catalytic systems has been discovered in the current research. Wu et al. discovered that, when it comes to the bioabsorption level, the accumulation in Caco-2 cells was significantly greater in copper ions compared with copper complexes, and the influence of several transport proteins and competing ions on both was significantly different in relation to the chemical specifying of copper that determines its expression of toxicity and its transmembrane transport route [1]. In the same breath, Li et al. in their research on marine diatoms reported that the nutritional status of organisms regulates the bioavailability of the organic copper complexes and inorganic copper. When there is copper shortage, the algae can consume the complexed copper through high-affinity transport systems and in high-copper environments, the algae relies mainly on diffusion of the free copper ions [2]. All the discoveries indicate that the biologic response of biological systems to copper is not a question of the overall quantity, but rather of the availability of speciation, and a dynamic transformation of the latter.

As to processes of environment behavior, in the study of the wastewater treatment by the method of high-resolution mass spectrometry, it was found that wastewater treatment removes some copper, but on the other hand, converts the binding form of copper that is attached to the organic ligands, which form new hydrophilic complexes, some of them containing nitrogen- and sulfur-containing organic compounds that have an influential chelating effect on copper [3]. This result indicates that copper is not only mined during environmental activities but continues to transform into other stable forms during the process of coordination exchange, and therefore is involved in the process of determining its movement and environmental risks. Nevertheless, in different coordination conditions as materials and catalysis researchers have since discovered, the copper species may be completely different regarding their reactivity and stability (Bao et al. and Iacobone et al.). As an example, one of the structures, such as $Cu(OH)]^+$ or $ZCu^{2+}(OH)^-$ can be the key active centers and can be changed in a reversible reaction to the change of water or temperature [4-5].

In conclusion, all the multi-scale analyses, such as cellular uptake, and ecological cycling, imply or suggest that it is not only its concentration that governs the behaviour of copper, but its coordination environment as well. However, all the existing studies are primarily being done on isolated systems and little concentration was given to the process of transformation and movement of copper in soil under the conditional influence of organisms and microorganisms, particularly without systematic study of the relation between the process of reducing copper activity and stabilizing it. Therefore, there is the need to explore changes in copper speciation and the ecological connotation in the perspective of ecological synergy within the concomitant action of a few biological factors.

3. Method

3.1 Regulatory Mechanism of Copper Ion Speciation Transformation

3.1.1 Transformation Process of Inorganic Bound Copper

The inner or outer coordination of copper that primarily achieves copper adsorption on clay mineral or surfaces of iron-manganese oxide surfaces $\equiv SO^- + Cu^{2+} \rightleftharpoons \equiv SOCu^+$. In case of the decrease of pH, the surface hydroxyl groups are protonated, leading to an enhanced desorption [6]:

$$\equiv SOCu^+ + H^+ \rightleftharpoons \equiv SOH + Cu^{2+} \tag{1}$$

Earthworms produce organic acids and make microorganisms breathe to produce CO_2 that minimizes the pH in the area thereby, enhancing copper movement.

One of the most significant reactions that are used in the control of stability is precipitation-dissolution reactions. When in an alkali or anion solution environment, the Cu^{2+} ion readily reacts to form salts that are sparingly soluble:

$$Cu^{2+} + 2OH^- \rightarrow Cu(OH)_2(s)* \tag{2}$$

$$Cu^{2+} + CO_3^{2-} \rightarrow CuCO_3(s) \tag{3}$$

$$Cu^{2+} + S^{2-} \rightarrow CuS(s) \tag{4}$$

CuS is highly insoluble but substantial stable form in the presence of reducing bacteria of sulfate but the precipitates can be redissolved in acidic or oxidizing conditions.

Redox reactions ($Cu^{2+} \rightleftharpoons Cu^+ \rightleftharpoons Cu^0$) can change the valence of copper and modify its toxicity and solubility.

Reducing microorganisms and organic matter can provide electrons to promote reduction, while oxygen can cause it to re-oxidize.

In addition, the hydroxyl groups on the surface of iron-manganese oxides can stabilize copper through inner-layer complexation ($\equiv FeOH + Cu^{2+} \rightleftharpoons \equiv FeOCu^+ + H^+$).

3.1.2 Formation mechanism of organically complexed copper

Under synergistic effects, dissolved or weakly bound Cu^{2+} easily forms stable complexes with various organic ligands, significantly altering its mobility and toxicity [7]. Carboxyl groups, phenolic hydroxyl groups, etc. in humic substances can form polydentate coordination:

$$2R\text{–}COO^- + Cu^{2+} \rightleftharpoons (R\text{–}COO)_2Cu \tag{5}$$

These complexes exhibit high stability and can reduce the concentration of free Cu^{2+}. Low-molecular-weight organic acids (such as oxalic acid and citric acid) are also important, as they can form precipitates or soluble complexes.

$$Cu^{2+} + C_2O_4^{2-} \rightarrow CuC_2O_4(s) \tag{6}$$

$$Cu^{2+} + Cit^{3-} \rightleftharpoons CuCit^- \tag{7}$$

Earthworm mucus and excrement contain proteins, polysaccharides, and amino acids, which can form more stable complexes through amino or thiol groups:

$$Cu^{2+} + 2R\text{–}NH_2 \rightleftharpoons [Cu(R\text{–}NH_2)_2]^{2+}* \tag{8}$$

$$Cu^{2+} + 2R\text{–}SH \rightarrow R\text{–}S\text{–}Cu\text{–}S\text{–}R + 2H^{+} \quad (9)$$

According to the Free Ion Activity Model (FIAM), metal toxicity is mainly determined by free Cu^{2+}. After the complexation, the amount of the free ions decreases, and large molecular complexes can hardly penetrate through the membrane, reducing biotoxicity [8].

3.1.3 Microbial-mediated Fixation and Mineralization

Microorganisms can help to fix precipitation and mineralization of copper by metabolites and cellular systems and to convert dissolved copper to an insoluble form that is stable. The sulfate reducing bacteria were grown under anaerobic conditions, and when this occurred, S^{2-} reacted with Cu^{2+} to form Cu_2S:

$$SO_4^{2-} + 8e^{-} + 8H^{+} \rightarrow S^{2-} + 4H_2O \quad (10)$$

$$Cu^{2+} + S^{2-} \rightarrow CuS(s) \quad (11)$$

CuS is highly insoluble and this inhibits movement to a large extent. The anoxic microzones created by the activity of earthworms favor this process.

Besides, phosphate released by microorganisms can be occurrences of soluble precipitates.

$$3Cu^{2+} + 2PO_4^{3-} \rightarrow Cu_3(PO_4)_2(s) \quad (12)$$

The other consideration is the biofilms (EPS) which are rich in carboxyl and phosphate groups and which have the capability of complex or ion exchange copper:

$$\equiv COO^{-} + Cu^{2+} \rightleftharpoons \equiv COOCu^{+} \quad (13)$$

As the membrane structure becomes thicker, more copper is added, and becomes a nucleation site of mineralization, and ultimately transforms to a stable solid form, which can only be reactivated in a state of high acidity or high oxidation.

3.2 Mechanisms of Bioaccumulation and Translocation Regulation

3.2.1 Copper Absorption and Distribution in Earthworms

Firstly, absorption on the body surface is mainly done through the use of a moist cuticle [9]. The earthworm epidermis is moistened with mucus and contains large concentrations of water to the extent that Cu^{2+} can take its path into the general epithelial cells by diffusion or ionic pathways:

$$Cu^{2+}(aq) \rightarrow Cu^{2+}(\text{epidermis}) \quad (14)$$

This is significantly enhanced by the increment in the copper level in the soil solution. The proteins and polysaccharides in the mucus can also initially bind Cu+ as a buffer layer and regulate the rate of ingestion [10-11].

Second, the greater is intestinal absorption. When copper is ingested by earthworms in copper-containing soil and when copper is absorbed in the digestive tract and absorbed, the copper is released and absorbed in the intestinal epithelium. The acid of the gastrointestinal juices and the activity of the enzymes transform solid copper into the dissolved form:

$$Cu(OH)_2(s) + 2H^{+} \rightarrow Cu^{2+} + 2H_2O \quad (15)$$

After that, the Cu^{2+} is delivered into the coelomic fluid by the effort of transmembrane transport proteins. The absorbed copper is again transported to the different tissues throughout the body via the coelomic fluid. Some of the copper is bound to metal-binding proteins and forms a complex $Cu^{2+} + MT \rightarrow Cu\text{–}MT$, where MT is metallothionein. This shape renders the free copper ions less hostile to the cells and simple to store and transport. The enrichment of copper is generally believed to be in the hepatoid tissue (chlorinated gland tissue) and in the intestinal wall cells.

3.2.2 Microbial Retention and Transport of Copper

Microbial communities play an important role as biofilter of copper in soil and regulate copper distribution through adsorption, uptake and excretion of copper.

To begin with, cell walls and extracellular polymeric substances (EPS) adsorption capacity is very high in copper. Functional groups in cell surfaces of bacteria that are negatively charged such as carboxyl, phosphate and amino functional groups have the ability to bind $Cu^{2+}$by ion exchange: $\equiv COO^- + Cu^{2+} \rightleftharpoons \equiv COOCu^+$. The EPS polysaccharide net can also multiplex copper ions and stabilize them outside the cell, in a complex form. Some of the copper can also be delivered to the cell by active transport, although to avoid toxicity, microorganisms tend to activate efflux systems to expel it. An illustration of a reaction process is $Cu^{2+}(in) \rightarrow Cu^{2+}(out)$.

3.2.3 Overall Migration Regulation of the Synergistic System

Earthworms and microorganisms form a complicated system of migrations in the biosphere, such that copper can be concentrated and diffusion-impeded. Another significant copper sink and reservoir is the biosphere. Copper in soil earthworms, in microbial cells and biofilms are included in a passage system: $Cu\ ^{2}(soil) \rightarrow Cu(biota)$. The process reduces the concentration of free copper ions of soil solution hence, transporting to either ground water or plants.

The assassination or detachment of organisms leads to re-introduction of copper into the environment. An example of this is that earthworm castings are characterized by a high level of bound copper and, once decomposed by microbes, they can participate in the reaction of speciation $Cu_{(biota)} \rightarrow Cu^{2+}{}_{(soil)}$. This emission is nearly always slow, and is succeeded by new adsorption or precipitation. This synergistic system creates accumulation and release balance because of dynamism. When the organisms die or change of environment leads to an increase in the release and a decrease in the absorption and fixation is increased at high biological activity. The cycle allows copper to remain long, not to migrate and diffuse rapidly in the local environment, which would allow the migration control and buffering of risks at the ecological level.

3.3 Biological Detoxification and Tolerance Mechanisms

3.3.1 Physiological Mechanisms of Earthworm Detoxification

Earthworms can produce metallothionein (MT), a high-cysteine protein, whose thiol group (-SH) can form a stable coordination of Cu^+ or Cu^{2+}.

$$Cu^{2+} + 2R\text{–}SH \rightarrow R\text{–}S\text{–}Cu\text{–}S\text{–}R + 2H^+ \tag{16}$$

Further formation of multi-coordination complex:

$$nCu^+ + MT \rightarrow Cu_n\text{–}MT \tag{17}$$

The significant result of this binding is the decrease of the concentration of the free copper ions, which otherwise were destructively reacting with valuable enzymes and membrane structures. Secondly, Copper ions can also catalyze the synthesis of reactive oxygen species (ROS) in reactions of a Fenton-like character and lead to oxidative damage.

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + \cdot OH + OH^- \tag{18}$$

In response to oxidative stress, earthworms recruit an antioxidant defense system of superoxide dismutase (SOD), catalase (CAT) and glutathione system. Reactive oxygen species may be converted to non-toxic products under such enzymatic reactions, such as:

$$2O_2^{-}\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \tag{19}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{20}$$

3.3.2 Microbial Anti-Copper Strategies

Microorganisms release Cu^+ or Cu^{2+} through cell transmembrane proteins:

$$Cu^+\mathit{(in)} \rightarrow \mathit{Cu}^+\mathrm{(out)} \tag{21}$$

It is mostly an energy-consuming process and regulated by copper-tolerant genes. The more copper the gene is expressed in the environment, and this will lead to an increase in rate of excretion. Microorganisms can be assisted to get rid of copper toxicity through biological remediation. Reductively, Cu^{2+} can in reducing environment be reduced to the less soluble Cu^+ or the metallic copper.

$$Cu^{2+} + e^- \rightarrow Cu^+ \tag{22}$$

$$Cu^+ + e^- \rightarrow Cu^0(s) \tag{23}$$

Sulfides or phosphates may also be formed by other others, which make copper insoluble in form of precipitates (e.g. CuS), and therefore reduces bioavailability. Besides, at the community level, microorganisms receive protection through the establishment of biofilms. Extracellular polymeric substances (EPS) are able to adsorb large amounts of copper ions:

$$\equiv COO^- + Cu^{2+} \rightleftharpoons \equiv COOCu^+ \tag{24}$$

3.3.3 Systemic Effects of Synergistic Detoxification

This is a buffering process of copper toxicity that is manifested in the multi-level fixation and transformation process superposition: fixation and secondary storage in the earthworms reduces the free copper, adsorption and precipitation by microorganisms reduces the concentration in the environment, and thereby maintains a low concentration of Cu^{2+} in the solution.

$$Cu^{2+}\mathit{(free)} \rightarrow \mathit{Cu}\mathrm{(bound/solid)} \tag{25}$$

Copper also flows through localized hotspots of fixation in organic-rich areas such as feces by earthworms with the fixation and excretion process to induce copper transfer between the high and low-activity zones, as well as through micro organisms. The outcome of this synergist effect is a path to overall reduction in ecotoxicity.

Dissolved copper → Bioabsorption → Binding/precipitation → Isolated storage → Stable retention

Copper is in a low-activity form as the cycle is long-lived and could be maintained by comparatively constant environmental conditions. A re-release will be immediately re-adsorbed or converted thus preserving the tolerance of the system.

4. Results and Discussion

4.1 Experimental Materials

Topsoil (0-20 cm) was sampled and was untaminated, the plant material and stone removed and the soil dried in the air, ground and sieved (2 mm sieve). Simple physicochemical properties of soil, including, pH, organic matter and cation exchange capacity were determined. The soil was artificially contaminated with copper solution through copper sulfate ($CuSO_4 \cdot 5H_2O$) solution. The solution was thoroughly blended and pre-cultured at unvarying humidity to allow copper to settle down to a fairly consistent position in the soil.

As experimental organisms, adult earthworms were selected; healthy, equally-sized adult worms. They had been initially incarcerated on dampened filter papers 24 hours prior to the experiment, to empty their intestinal contents, and those injured or abnormally active had been eliminated. The source of microbes was the in situ soil microbial community; in some treatments the microbial activity was inhibited by sterilization of the soil or by adding antimicrobial substances to the soil.

Reagents used in the experiment were copper sulfate, hydrochloric acid, nitric acid, deionized water, and buffer solutions, and were of analytical grade.

4.2 Experimental Apparatus and Cultivation Conditions

The treated soil was placed in polyethylene cultivation containers. An equal amount of soil was experimentally placed in each of the containers and the content of moisture in each container was adjusted to 60-70 per cent of its total water retention capacity. There were even holes in the containers to allow them to exchange oxygen. The test was conducted in an incubator of constant temperature maintained at 20-25 degC in the absence of light interference.

Different treatments were developed regarding the biological make up and part of these treatments included; earthworm treatment, microorganism treatment, earthworm and microorganism synergistic treatment, and a control treatment which did not contain an organism. Treatments were paralleled. Every so often we added deionized water so that we had that much moisture.

4.3 Experimental Procedure

$CuSO_4$ solution was added to the soil in the necessary concentration and stirred. The suspension was left standing after some time to increase the adsorption and redistribution of copper in the soil. In the corresponding treatment groups Earthworms were then introduced and the soil kept wet and well-aerated.

The earthworms were observed regularly during the cultivation period and dead earthworms were eliminated and the soil was turned loosely, without compacting it. After cultivation, samples of soil, body and excrement of earthworms were collected to be analyzed in the future.

4.4 Sample Processing and Measurement Methods

In this study, the researcher will adopt qualitative and quantitative techniques to analyze the data obtained. Air drying and sieving of soil samples were performed. Copper was extracted into the different forms of the solution to be tested by the chemical fractionation method and obtained by centrifugation and filtration. Earthworm samples were washed with deionized water to remove the surface deposits, dried and ground. The samples were digested using an acid to convert the copper in them to a solution.

The distribution of copper in soil and organisms was determined using instrumental analysis to measure the copper content in the extract and in the digestion solution. Meanwhile, the parameters such as the pH and moisture of the culture soil were measured to assist in the analysis of copper migration properties.

4.5 Instruments and Equipment

The major equipment used in the experiment was a constant temperature incubator, electronic balance, pH meter, centrifuge, shaker, drying oven and an acid digestion instrument. The amount of copper in it was determined by using the atomic absorption spectrometry or inductively coupled plasma optical emission spectrometry (ICP-OES). Measurement results were calibrated prior to use to ensure the accuracy and repeatability of the results.

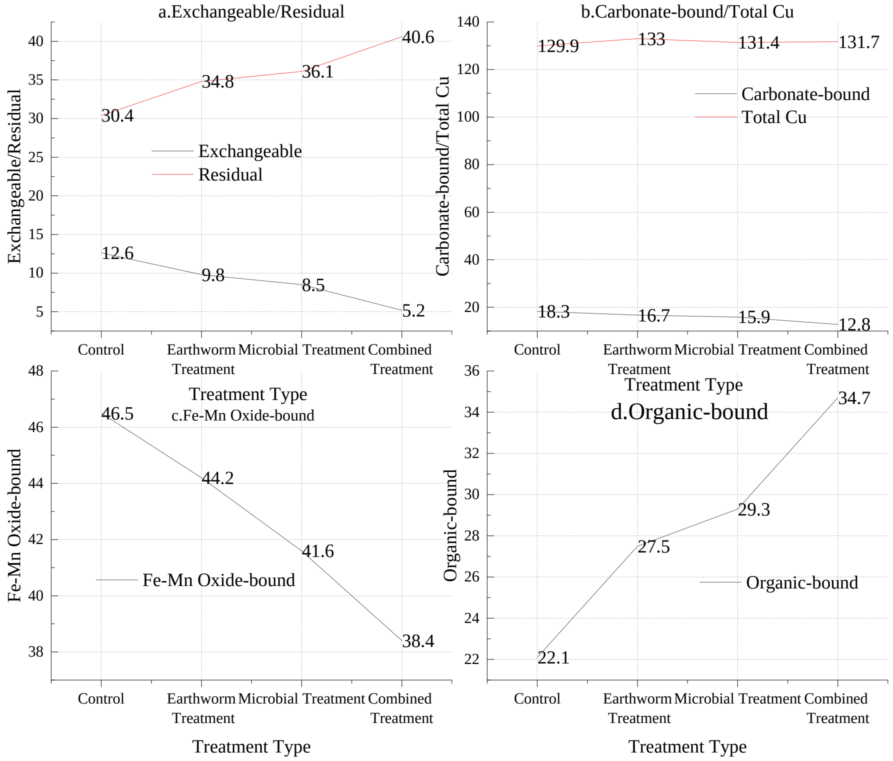

Figure 1. Distribution of copper speciation in soil under different treatments (mg·kg⁻¹)

Note: A decrease in exchangeable copper and an increase in stable copper (organically bound and residual forms) generally indicate a decline in copper activity.

As shown in Figure 1, the distribution of copper speciation in the soil differed significantly under different treatments. In the control group, the exchangeable copper content was 12.6 mg·kg^{-1}, accounting for approximately 9.7% of the total copper, which is a highly active form, indicating that copper has high bioavailability in the soil (Figure 1a). After earthworm treatment, the exchangeable copper content decreased to 9.8 mg·kg^{-1}, while the organically bound and residual copper contents increased to 27.5 mg·kg^{-1} and 34.8 mg·kg^{-1}, respectively, indicating that earthworm feeding and soil disturbance promoted the conversion of copper from a highly active to a stable state (Figures 1a and 1b). Microbial treatment further reduced the exchangeable copper content to 8.5 mg·kg^{-1}, while the iron-manganese oxide bound and organically bound copper contents slightly increased, indicating that microorganisms play a role in copper fixation through adsorption, precipitation, and metabolic transformation (Figures 1c and 1d). The most significant change was observed in the synergistic treatment group, where exchangeable copper decreased to 5.2 mg·kg^{-1}, while organically bound and residual copper reached 34.7 mg·kg^{-1} and 40.6 mg·kg^{-1}, respectively. The total copper content remained relatively stable (131.7 mg·kg^{-1}), indicating that the synergistic effect of earthworms and microorganisms effectively reduced the proportion of active copper while maintaining the stability of total copper, thus forming a stable storage state.

Table 1. Characteristics of copper enrichment and migration in organisms under different treatments

Treatment Type	Copper in Earthworms (mg·kg^{-1})	Soil Solution Cu Concentration (mg·L^{-1})	Cast Cu Content (mg·kg^{-1})	Bioaccumulation Factor (BAF)
Control	—	1.42	—	—
Earthworm Treatment	68.3	0.96	82.5	0.52
Microbial Treatment	—	0.88	—	—
Combined Treatment	74.6	0.61	95.7	0.57

Note: BAF = Copper content in organisms / Total copper content in soil; "—" indicates that the corresponding organism was not present in this treatment.

As shown in Table 1, The most significant effect was observed in the earthworm-microbe synergistic treatment group, where the copper content in earthworms increased to 74.6 mg·kg^{-1}, the copper content in feces increased to 95.7 mg·kg^{-1}, while the copper concentration in soil solution decreased to 0.61 mg·L^{-1}, and the BAF increased to 0.57. This indicates that, through synergy, earthworms and microorganisms can more efficiently regulate the distribution of copper in the soil-biosystem, achieving the bioabsorption and stabilization of copper, while reducing the ecological risks of soluble copper in the soil.

5. Conclusion

The current research was a systemic investigation of the speciation, migration and biode-toxicity of copper in a soil-biosystem during the synergist activity of earthworms and microorganisms. The results of the experiment showed that by feeding, tilling and excretion, earthworms increase the conversion of the existing copper in high reactive form in the soil to the organically bonded form and residual form. Microorganisms, on the other hand, also may further dilute the level of copper in the soil solution through adsorption, precipitation, and fixation of copper through the metabolism processes, and can thus stabilize copper. The study also revealed the key roles of induction of earthworm metal-binding protein, antioxidant defense and isolation storage, and microbial efflux pumps and precipitation fixation pathways in overall copper regulation, providing mechanistic support to copper pollution ecological risk management. The experiment was done under artificially controlled conditions and did not exhaustively take into consideration the effects of temperature, humidity, and the type of soil and the diversity of the microbial communities on the transformation of copper in the natural environment. The future study will undertake long-term follow-up experiments in the real polluted soil environment to integrate high-throughput microbial sequencing with molecular biology to further understand the regulatory mechanisms of important microorganisms and earthworm physiological reactions on copper cycling within the synergistic system and to seek the possible strategies that can be used to optimize soil copper stabilization and ecological restoration by using different combinations of biological factors, and provide scalable technical solutions to the remediation of heavy metal pollution.

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