A novel application of oriental beetle juvenile *Anomala corpulenta* to reflect Pb contamination in soil

Research Article

Xichao Xia*, Xinhua Zheng, Suxiang Lu, Zhiguo Chen, Xianguang Bai, Guina Liang, Shipeng Xue, Chuanxiu Hua, Guoying Song and Lianghong Guo

Objectives: Insects dwelled in soil play a key role in monitoring of metal contaminations. In order to explore the toxicity of lead (Pb) in soil, juvenile of oriental beetle *Anomala corpulenta*, were firstly applied to analyze effect of Pb.

Methods: In the current study, toxicity of different concentrations Pb on *A. corpulenta* in the laboratory was performed by measuring survival, growth and avoidance of animals. Meanwhile, activities of acetylcholinesterase (ChE), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in *A. corpulenta* were examined to quantify the extent of alterations caused by Pb treatments.

Result: Events of mortality were occurred in the Pb treated groups (1200 and 1600 mg Pb/kg soil). Administration of Pb treatments could result in a significant decrease of mean end weights in contrasted with that of control group. Treatment of Pb at 800 mg Pb/kg soil caused an obviously avoidance behavior. Expressions of ChE, SOD, CAT and GSH-Px of Pb treated groups were significant decreased compared with that of control group in the experiment observed.

Conclusion: Pb contamination of soil may cause multiple effects on juvenile *A. corpulenta* including occurrence of mortality, delay of growth and avoidance of contaminated environment.

Keywords: Pb contamination; *Anomala corpulenta*; Avoidance behavior; Acetylcholinesterase; Antioxidant enzymes.

Özet

Amaç: Topraka yaşayan böcekler metal kontaminasyonlarının izlenmesinde önemli bir rol oynamaktadır. Topraktaki kurşun (Pb) toksisitesini araştırmak için ilk önce oryantal bir böcek olan juvenil Anomala corpulenta kullanıldı.

Yöntemler: Bu çalışmada laboratuvar şartları altında, A. corpulenta’ya Pb’nin farklı konsantrasyonlarda toksisitesi, hayvanların hayatta kalma, büyüme ve kaçınma ölçümleri ile gerçekleştirilmiştir. Aynı zamanda, A. corpulentada asetilkolinesteraz (ChE), süperoksit dismutaz (SOD), katalaz (CAT) ve glutatyon peroksidaz (GSH-Px) aktiviteleri, Pb uygulamalarına bağlı olan değişikliklerin miktarını belirlemek için incelendi.
Bulgular: Pb ile muamele edilen gruplarda (1200 ve 1600 mg Pb/kg toprak) mortalite olayları meydana geldi. Kontrol grubu ile karşılaştırıldığında Pb verilen grupta mortalite oranları asıl olmakla birlikte, 800 mg Pb/kg toprakta uygulandığında belirgin bir kaçınılmaya neden olmuştur. Pb uygulanılan grupların ChE, SOD, CAT ve GSH-Px ifadeleri kontrol grubuna göre anlamlı olarak azalmıştır.

Sonuç: Topraktaki Pb kontaminasyonu, juvenil A. corpulenta üzerinde, mortalite, büyümenin geçikmesi ve kontamine çevreden kaçınma gibi bir çok etkiye neden olabilir.

Anahtar Kelimeler: Pb kontaminasyonu; Anomala corpulenta; Kaçınma davranışı; Asetilkolinesteraz; Antioksidan enzimler.

Introduction

Metal contamination, as an important part of environmental pollution, has been became a key challenge to soil ecosystem and water ecosystem with respect of intensive human activities, enormous industry productions and heavy traffic [1, 2]. Discharge of metals may cause a serious threat to soils in due to their characteristics of duration, enrichment, toxicity and concealment [3, 4]. For these reasons, soil is a good indicator of the level and extent of metal accumulation in the surface environment [5, 6]. Therefore, organisms dwelled in soil function as a key bioindicator to detect metal contaminations [7, 8]. Among of them, few invertebrates are highlighted to determine the potential biological effects of a given soil metal load [7, 9].

Soil biota is continuously exposed to metal contaminants that can cause a negative effect on normal physiological activity of invertebrates [10]. Effects of metals on these animals are usually divided into strong direct and indirect impacts [11]. Direct physiological effects may result in alteration or inhibition of various enzymatic pathways, while indirect effects can be not only physiological dysfunction but may be also observed as pollution driven changes in growth or survival due to diminished energy budget [12]. Considering here, an important aspect in elucidating the side effect of metal contamination on soil is to identify and characterize direct and indirect effects from diverse organisms. Notably, applications of other invertebrate in this soil contamination are scarce in contrasted with that of earthworm.

The oriental beetle Anomala corpulenta Motsehulsiy (Coleoptera: Scarabaeidae) is widely distributed in China, from alpine tundra to seashores, deserts and tropical rainforests [13, 14]. The juvenile of A. corpulenta, like earthworm, lived in soil and contribute to leaf litter decomposition and organic matter recycling. Feeding directly on decaying materials and soil fungi, they provide an earlier indication of ecosystem disturbance than predaceous soil animals [14, 15]. In addition, juvenile of A. corpulenta are suite to use as a potential hallmark of soil pollution because they not only are easy to culture, but also have relative short life cycles [14, 16]. However, application of juvenile of A. corpulenta to determine the effects of metal contamination on soil remains unknown at our known.

Lead (Pb) is a typical heavy metal pollutant in agricultural land and toxic to soil microorganism, plants and soil fauna [17]. Moreover, excessive lead intake can inhibit the reproductive function and immunity of the human’s body, retard the intelligence development of children, resulting in various physical abnormalities [18]. Taken consideration of potential role of A. corpulenta in detecting soil pollution, toxicity of different concentrations Pb on juvenile A. corpulenta in laboratory experiment was fulfilled by measuring effects on survival, growth and avoidance of animals. In addition, activities of acetylcholinesterase (ChE) as well as some potential biomarkers of oxidative stress in the worms were also examined to quantify the extent of alterations caused by Pb.

Materials and methods

Experimental exposure

Juvenile of A. corpulenta specimens were obtained from farmland cultivated peanut and used for all exposures. They were of homogenous size and weight. The experiment was conducted in rectangular plastic boxes (40 cm × 25 cm; 10 cm height) containing 3000 g of dry soil derived from farmland above mentioned. Pb(NO\(_3\))\(_2\) were obtained from Beijing Yili Fine Chemicals Co. Appropriate amounts of Pb(NO\(_3\))\(_2\) dissolved in MilliQ water were spiked into premoistened soil to achieve the following nominal concentrations: 0, 200, 400, 800, 1200 and 1600 mg Pb/kg soil dry weight. Solvent was left to evaporate overnight and then deionized water was added to moisten the soil and reach to 40–60% of the water holding capacity. In this case, the control soil was also spiked with the same amount of water. Therefore, tests were run with a solvent control. The worms were exposed to different concentrations of Pb treatment and divided into different groups according to experiment arrangement. All exposures were conducted at 20°C under a 12/12h light/dark cycle.
Cow manure without chlortetracycline or other medications was selected as suitable food in order to avoid adverse affect on the worms during the test. Then, manure was dried at 100°C, finely ground, and sieved through a 2 mm sieve before use. Juvenile *A. corpulenta* were fed with 5 g of cow manure added to the soil surface at the beginning of the experiment and each week. Water lost was compensated with deionized water at every week. Three of *A. corpulenta* were randomly collected from each replicate container on the 0, 7, 14 and 21st day for enzymatic activities analysis.

### Mortality number of animals in each group was counted

**Survive, growth, avoidance**

Mortality number of animals in each group was counted once per 3 days in order to analysis animal survive. During the sequential exposures, every *A. corpulenta* in every group was weighed at the start of the experiment and the end of the exposure using an electronic laboratory balance. As there were no statistically significant differences between the mean start weights of the different groups, growth was expressed in terms of the end weight of the animals instead of weight change.

Avoidance behavior experiments were performed using rectangular plastic boxes (30 cm × 20 cm; 10 cm height) in which sampled soil were divided into two equal sections by a removable plastic wall. Before starting the test, 10 *A. corpulenta* were placed in a single box containing uncontaminated soil on both sides of the box and allowed to burrow into the substrate undisturbed for 48 h. This experiment had three replicates per group and the aim was to determine whether the worms dispersed randomly in the absence of a toxicant.

Following this preliminary dispersal experiment, control soil (1000 g wet weight) was placed on one side of the test vessel and the same amount of test soil was placed on the opposite side. Same experimental design was followed but the worms were offered a choice between uncontaminated soil and soil contaminated with Pb at a specific concentration. The removable plastic wall was then removed carefully and ten worms placed on the soil surface along the center line and allowed to burrow. At the end of the test, the movable wall was again placed in the center and each side of the box was independently searched for animals. Following this the soil in the exposure box was divided into two equal parts by pulling a metal spatula through the midline of the box and inserting the plastic divider. Worms in the middle of the box were picked from the soil and these *A. corpulenta* were judged not to be on either side of the line and were not counted. The two halves of soil were removed separately from the box and the number of *A. corpulenta* in the soil on each side of the divider counted.

### Enzymatic assays

After the temporal exposure with different Pb concentrations, five worms per replicate were sacrificed by freezing, weighed and transferred to pre-chilled mortar kept on ice. Cold phosphate buffered saline used as homogenizing buffer was added in a ratio of 1:4 (w/v) and the animals were homogenized using a Polytron Kinematika tissue homogenizer. Homogenate was centrifuged at 10,000×g for 15 min at 4°C and supernatants were stored at −80°C until determination of the enzymatic activities and protein content. Protein content of samples was determined according to Bradford method [19]. A standard curve was constructed using bovine serum albumin Fraction V and absorbance was determined at 595 nm using a Multiskan micro plate reader after incubating the sample and reagents for 10 min at room temperature.

ChE activity was determined according to combination of Askar method and Haigh method [20, 21]. The assay was conducted at a temperature of 20°C and a phosphate buffer with pH 8.0 was used. Three replicates of every sample were measured and blanks added to each row in the microtiter plate. Controls with all substrates except the tissue homogenate were also used to correct for non-enzymatic hydrolysis of substrate. Enzyme activity was determined spectrophotometrically at 412 nm and expressed as micromoles of product – thiocholine – formed per minute per microgram of protein per sample. ChE activity was expressed as units (U) per permilligram of protein (U/mg protein).

Superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit photochemical reduction of pyrogallol as described [22]. The rate of pyrogallol reduction was recorded at 325 nm. One unit of SOD activity was defined as the amount of enzyme required for inhibiting pyrogallol autoxidation by 50% per min at 25°C, and SOD activity was expressed as units (U) per permilligram of protein (U/mg protein).

Catalase (CAT) activity was measured by determining the decrease of absorbance at 240 nm in respect of the transformation of hydrogen peroxide (55 mM H₂O₂ in 50 mM KPB pH 7.0) into H₂O [23]. The reaction was treated for 2 min, and the final activity was expressed as units per milligram of protein: one unit of CAT activity was defined as the enzyme quantity required consuming half of H₂O₂ in 120 s at 25°C.
The glutathione peroxidase (GSH-Px) activity was determined using a spectrophotometric method. First, the 0.1 mL of the homogenate and 1.7 mL of 100 mM potassium phosphate buffer (pH 7.4, containing 1 mM NaN₃, 1 mM EDTA, 0.2 mM NADPH, 1 unit/mL glutathione reductase, and 1 mM glutathione) were mixed and preincubated at ambient temperature for 10 min. Second, 0.2 mL of 2.5 mM H₂O₂ solution was added and mixed with the original solution for enzymatic reaction. Third, the resulting solution was incubated at ambient temperature, and the absorbance calculated at 340 nm was determined in a period of 5 min. GSH-Px activity was expressed as units of nmol/min/mg protein (nM of NADPH/min/mg protein).

Statistical analysis

Data obtained in the avoidance behavior experiment was tested using a $\chi^2$ test to compare the observed and expected number of animals in the two soils and to determine whether an avoidance response was present. For the endpoints of the sequential exposures (growth and end weight), means and standard deviations were determined using the MS Excel software program and analyzed for statistical differences using Statistica Version 7. In enzyme assays, the data were expressed as the mean ± SD. The results of the study were analyzed by one-way analysis of variance (ANOVA) with the aid of SPSS statistics software. $p < 0.05$ were considered statistically significant.

Results

Effect of Pb on survive of *A. corpulenta*

No mortality was observed in control as well as animals exposed to 200, 400 and 800 mg Pb/kg soil at a 14 day treatment interval, but mortality did occur in groups exposed to 1200 and 1600 mg Pb/kg soil. Mortality of one, two and one of juvenile *A. corpulenta* were respectively observed in the three 1200 mg /kg soil replicates by the end of day 14 exposure period. For the 1600 mg Pb/kg soil group, mortality of two, three and three worms were respectively detected. In addition, dead body of juvenile *A. corpulenta* characterized by white-stiff status was widely fund in 1200 and 1600 mg Pb/kg soil treated group (Figure 1). After day 14, no mortality of juvenile *A. corpulenta* was occurred in all groups.

Effect of Pb on weight of *A. corpulenta*

Mean start weight of juvenile *A. corpulenta* was not significantly different among every group (0.904 ± 0.093 g) before Pb treatment. At the end of experiment, except of 200 mg Pb/kg soil group, a significant decrease of mean end weight was observed in Pb treated groups (400, 800, 1200 and 1600 mg Pb/kg soil) compared with that of control group ($p < 0.05$) (Figure 2). Meanwhile, mean end weights of the survived juvenile *A. corpulenta* were significantly decreased in Pb treated groups (1200 and 1600 mg Pb/kg soil) compared with that of 200 mg Pb/kg soil group ($p < 0.05$) (Figure 2).

Figure 1: Partial corpses of juvenile *A. corpulenta* occurred in the 1200 mg Pb/kg soil group and 1600 mg Pb/kg soil group at a 14-day interval exposure.

Figure 2: Effect of different Pb concentrations on the growth of juvenile *A. corpulenta*.

*p < 0.05, *p < 0.01 vs. the control group, *p < 0.05, *p < 0.01 vs. the 200 mg Pb/kg soil group.
Effect of Pb on avoidance of *A. corpulenta*

Statistical analysis derived from results of preliminary dispersal experiment indicated that juvenile *A. corpulenta* distributed randomly in the uncontaminated soil. When allowing the worms a choice between uncontaminated soil and soil contaminated with the different concentrations of Pb, ratios of avoidance were respectively 52.00%, 64.00%, 72.72%, 85.71%, 60.96% and 63.53% in the 0, 200, 400, 800, 1200 and 1600 mg Pb/kg soil groups (Figure 3).

Effect of Pb on ChE expression of *A. corpulenta*

ChE activity in control group as well as each Pb-treated group showed a fluctuation change from day 0 to 21. When the animals were exposed with different concentrations of Pb, significant decrease of enzyme activities were observed among the treated groups in contrasted with that of control group (Figure 4). In the whole experiment test, ChE activity in Pb-treated groups derived from survived animals was inhibited by more than 30.18% compared with that of the control. Beetles exposed to the 1600 mg at the day 21, expression of ChE was the most severely affected with a reduce of more than 84.28% (p < 0.01) enzyme activity with respect with that of control group (Figure 4). Nonetheless, enzyme activity of ChE derived from 800, 1200 and 1600 mg Pb/kg soil treatment respectively showed a significant decrease in contrasted with that of 200 mg Pb/kg soil group (p < 0.05).

Effect of Pb on SOD expression of *A. corpulenta*

Administration of different concentrations of Pb could result in a down-regulation of SOD activity. Juvenile *A. corpulenta* with a challenge of 200 mg Pb/kg soil, a significant decrease of SOD activities was detected at day 7 in contrasted with that of control group (Figure 5). When those exposed to 400, 800, 1200 and 1600 mg Pb/kg soil, SOD activities reduced more than 30.61% (p < 0.05), even to 60.76% (p < 0.01) in the whole test (Figure 5). Notably, a dose-dependent of decrease of SOD activity among Pb treated groups was observed from day 7 to 21 (Figure 5).

Effect of Pb on CAT expression of *A. corpulenta*

When the animals treated with the different concentrations of Pb, CAT activities showed a fluctuation during experiment. Juvenile *A. corpulenta* challenged with 200 mg Pb/kg soil in where CAT expressions showed an increase trend compared with that of control group, although there were not statistically differences (Figure 6). However, CAT activities in groups administrated with 400, 800, 1200 and 1600 mg Pb/kg soil showed a significant decrease with respect of control group (p < 0.05) (Figure 6). Compared with that of day 7, up-regulations of CAT expression were fund in Pb treated groups (400, 800, 1200 and 1600 mg Pb/kg soil), and reached to 36.12%, 62.83%, 138.95% and 36.79% at day 21, respectively (Figure 6).
Effect of Pb on GSH-Px expression of *A. corpulenta*

During experiment observed, levels of GSH-Px of beetles treated with different concentrations of Pb showed a dose-dependant decrease. Among of them, a significant decrease was observed in 800, 1200 and 1600 mg Pb/kg soil groups (Figure 7). In these three groups, activities of GSH-Px decreased more than 53.81% ($p < 0.05$), 38.06% ($p < 0.05$) and 32.51% ($p < 0.05$) at day 7, day 14 and day 21 in contrasted with that of control group, respectively (Figure 7). Like SOD and CAT, levels of GSH-Px in Pb-treated groups also showed an increase trend after day 7.

Discussion

Metal contamination of soil has been regarded as a serious environmental problem in many developing countries [1, 2]. In order to prevent and control metal contamination, few non-targets insects lived in soil were selected to guard side effect of metal [7, 9]. Among of them, beetle has been recommended as an important biomarker [8]. In current study, the beetle juvenile *A. corpulenta* was firstly used to shed light of Pb contamination in soil through determination of avoiding behavior, growth, survive and activities of ChE, SOD, CAT, and GSH-Px.

The avoidance effect was characterized by percentage of affected animals. In accordance with the previously mentioned Guideline for the Earthworm Avoidance Test, the habitat environment of soils is considered to be limited if on average $>80\%$ of worms are detected in the control soil [24]. With this regard, it was only fund that administration of 800 mg Pb/kg soil could result in a statistical change of avoidance behavior among treated groups. Notably, if the 800 Pb/kg soil was considered as threshold of concentration of avoidance event, significant effect of avoidance behavior should also be occurred in the Pb treated groups (1200 and 1600 mg Pb/kg soil) while it was other. As to this phenomenon, one interpretation can be reasonably accepted is that physiological response of animals involved into treating different concentrations of Pb challenge are different. Animals administrated 200 and 400 mg Pb/kg soil challenge with 48 h exposure, almost of them were able to adapt or overcome environmental stress through enhancing antioxidant ability, and stay in the original dwelling place. Once Pb concentration...
reached to 800 mg Pb/kg soil, most worms could not be tolerated to the environmental stress and choice avoidance event. When Pb concentration persistently evaluated to 1200 or 1600 mg Pb/kg soil, most of worms should lose escaping ability and also stayed in the Pb contaminated soils because ROS derived from Pb treatment have caused a serious impact on normal physiological function. It has been established that avoidance behavior is considered as a well-studied endpoint in various earthworm species following exposure to a number of contaminants [25, 26]. Evidence from avoidance response to Pb, it appears that such a response is inconsistent with others of influenced concentration of the metal [27, 28].

For the parameter end weight, no difference was observed between Pb treated group (200 mg Pb/kg soil) and control group because these animals were likely able to recover homeostasis at a low Pb concentration with a longer interval. The impaired growth observed in other treatment groups can possibly be explained by what the combination of a higher exposure concentration and a successive exposure interval can result in a negative effect on feeding behavior and metabolism [29]. Treatment of 400, 800, 1200 and 1600 mg Pb/kg soil could cause an imbalance of body homeostasis that in turn impairs feeding efficiency of animals at same food supply. Poor obtainment of food results in a reduction in available energy to maintain metabolic and physiological functions, as well as leaving less available energy to cope with the physiological demands of being exposed to a toxicant [30]. Once animals under this condition, they lose basic body functions because energy is likely to be diverted away from growth and reproduction, and shunted towards coping with the effect of a chemical stress [25].

During the present study, a down-regulation of ChE activity was observed in all the Pb treated groups. Inter-individual variation of ChE activity in control group as well as treated groups was also observed, which is consistent with the results of previous studies. Notably, all animals exposed to Pb had a significant decrease of enzyme activity with respect with that of control group during whole experiment and this down-regulation of ChE exhibited a dose and time-dependent matter in Pb treated groups. This illustrates that combination a relatively high exposure concentration with a relatively longer exposure intervals plays a key role in inhibition of ChE activity. The most significant inhibition of ChE expression was occurred in 1600 mg Pb/kg soil group at a 21 day. However, the scenario of mortality was respectively found in Pb treated groups (1200 and 1600 mg Pb/kg soil) in the present study. It was suggested juvenile *A. corpulenta* can survive at a high level of ChE inhibition, but significant inhibition of ChE is prone to result in a stance of mortality. As we all known, metal contamination may inhibit ChE activity and disturb the normal transfer of nerve impulses that can cause dysfunction of the nervous system and result in a side effect on feeding behavior, energy supply and mobility of organisms [31]. In this environment, metal-induced mortality in invertebrate and vertebrate species including nematodes, earthworm, fish and small mammals has been extensively reported that is close with the down-regulation of ChE expression [31].

The mortality time of animals of 1200 and 1600 mg Pb/kg soil groups was only observed in a period from day 0 to 14, not other time. Interestingly, a significant inhibition of ChE activity was occurred in whole experiment observed. This phenomenon is likely related with recovery time for survived worm in this process. Recovery of animal is largely dependent on the synthesis of new enzyme molecules due to the fact that metal form a stable irreversible bond with the target enzyme [32]. As to ChE, it has indicated that the length of the exposure period has important consequences in terms of mortality of earthworm [28, 29]. Nearly complete cholinesterase inhibition followed by a rapid recovery in the absence of a toxicant, whereas a slower recovery in the persistent of a toxicant [28, 29, 31].

In the whole test, the characterizations of SOD, CAT and GSH-Px expression in experiment observed were heterogeneous in which every anti-oxidant enzyme derived from different concentrations of Pb treatment had own features. Such heterogeneous pattern indicated that process of what different anti-oxidant enzymes coped with ROS in spatio-temporal condition is a complex event. In healthy worm, total ROS level should be tightly controlled at a relatively low level and excess ROS will be rapidly eliminated by an array of anti-oxidant enzymes to maintain a balance between ROS level and anti-oxidant enzyme activity [33]. Therefore, orchestra of anti-oxidant enzymes is a key hallmark of organism homeostasis. Administration of a little oxidative stress in a shorter time may function as an important player to induce different anti-oxidant enzyme expressions to deal with ROS [33, 34]. Once level of ROS exceeds a certain level in a longer time, anti-oxidant enzyme activities in animal body should be adjusted to adapt to tremendous oxidative stress [33]. So, characterizations of anti-oxidant enzyme activities should have a close relation with corresponding ROS level to some extent. Study the expression levels of anti-oxidant enzymes in *A. corpulenta* might help us to deep understand how the anti-oxidant defense system works to protect animals from excess oxidative damage.
Within a 7 days exposure, different anti-oxidant enzymes showed different sensitivity to different Pb concentrations. When animals exposed at higher concentrations of Pb treatment (800, 1200 and 1600 mg Pb/kg soil), the significant decreases of SOD, CAT and GSH-Px activity were detected. As a result, the balance between anti-oxidant enzymes scavenging and ROS production is breakdown base on above mentioned [33]. Meanwhile, it is suggested that 800 mg Pb/kg soil likely works as a threshold of concentration for animal survival. The phenomenon is benefit to further elucidate the avoidance behavior. On the other hand, administration of 1200 and 1600 mg Pb/kg soil could results in death of animals that is associated with a significant decrease of SOS, CAT and GSH-Px activities across the experiment. Animals suffered severe ROS stress which eventually causes substantial and irreversible damage to the cell membrane system along increasingly evaluated exposure magnitude.

At last exposure time, SOD, CAT and GSH-Px activities in 200 mg Pb/kg soil group (a low Pb stress condition) declined to normal level. Compared with that of control group, no significant up-regulation of CAT activity was observed in this group from day 7 to 21. Notably, other enzymes did not show this profile. The phenomenon assuming that a delay of process of different anti-oxidant enzymes from significantly inhibited status to normal status and even to activated status is not uniform. In the present study, it seems that the restoration and induction SOD and GSH-Px needed a longer delay than CAT. Similar phenomenon is also found in earthworm [33, 34].

In conclusion, the effects of various exposure regimes on growth, survive, avoiding behavior and activities of ChE, SOD, CAT, and GSH-Px in beetle juvenile *Anomala corpulenta* indicated that administration of Pb with longer intervals and higher concentration are likely to be more detrimental to non-target organisms than Pb with shorter intervals and lower concentration. In order to prevent or mitigate extensive negative impacts on non-target soil organisms in agricultural areas and conserve soil organism biodiversity, it would thus be of critical importance for government to be aware of the effects of sequential metal exposure and control diffusion of Pb.

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