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Acute Toxicity of Bismuth to the Earthworm *Eisenia andrei*

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Abstract: Bismuth (Bi) is increasingly used in several industrial applications including the production of alloys, drugs, cosmetics and munitions formulations. However, little information is available on the environmental fate and ecotoxicological effects of Bi. The present study describes 14 days acute toxicity of Bi, added as Bi citrate to a natural sandy soil, to the adult earthworm *Eisenia andrei*. Total measured Bi concentrations were 298.0, 399.5, 431.0, and 469.5 mg Bi/kg dry soil. Data indicates that Bi was toxic to *Eisenia andrei*, as determined by LC_{50} and LOEC, i.e., 416.0 and 399.5 mg Bi/kg dry soil, respectively. At 14 days in the presence of *Eisenia andrei* the bioaccessible fraction of Bi in soil, as determined in KNO_3 aqueous soil extracts, increased by a factor ranging from 1.6 to 30.0 compared to those measured at the beginning of experiment. Moreover, this study shows that an increase in pH caused by the presence of earthworm in soil was accompanied by increase in Bi bioaccessibility and consequently toxicity. For example, when Bi bioaccessibility increased from 0.262 to 7.516 mg Bi/kg dry soil, the mortality rate increased from 0 to 79%. Assuming that there were at least two routes by which *Eisania andrei* enhanced Bi bioaccessibility; one route was guided by the mobility, the biochemical (mucus) and the biological (bacteria) interactions of *Eisenia andrei* with soil constituents, and the other route was marked by the death of earthworms and the release of the accumulated Bi from the carcass.

Keywords: Bismuth, Bioaccessibility, Soil, Acute Toxicity, *Eisenia andrei*

1. Introduction

Bismuth (Bi) is a nonessential element that occurs naturally in soil at low concentrations ranging between 0.048 and 0.2 µg/g [1]. It has two oxidation states (+3 and +5), but in environmental, biological and geochemical samples Bi is found mainly in the trivalent oxidation state [2]. Some of the industrial applications of Bi include manufacturing of drugs, cosmetics, hair dye formulations, and low melting solders. Bismuth has also been used as non-toxic replacement for lead (Pb) in the manufacturing of munitions formulations, hunting shots, fishing sinkers, plumbing fixtures [3, 4]. With new industrial applications for Bi taking place, global bismuth production increased from 5,880 tons to 13,600 tons between 2000 and 2014 [5, 6]. Hence, Bi concentrations in soils and aquatic environments could increase considerably and become a serious environmental problem. Indeed, samples of soil and biomass collected from Canadian military training sites showed the presence of Bi in high concentrations (e. g. up to 184.8 mg Bi/kg dry soil) and was attributed to the use of Bi-containing munitions at their training sites [7, 8]. Johnson, et al. [9] reported Bi concentrations ranging between 8 and 5140 mg/kg dry soil in soil samples taken from shooting ranges in Switzerland. Furthermore, Amneklev, et al. [10] reported an increase of 300% in Bi measured at water treatment plants in Stockholm in 2007 compared to 2006. Despite the fact that Bi content of soils increase quickly due to its increased to use in industrial, pharmaceutical and cosmetic production. Currently, little information is available on the ecotoxicological effects, transformation, and transport of Bi in terrestrial ecosystems.

Natural soil is a dynamic heterogeneous ecosystem characterized by having a complex environmental biogeochemical system that can play a critical role in determining the fate and ecological impact of pollutants on living organisms including earthworms. According to Hou, et al. [11] the mobility and availability of Bi in soil is governed by its binding and interaction mechanisms with soil chemical constituents such as organic matter, carbonates, pH and other metal oxides. A previous soil column study, with Bi deposited on the soil surface and exposed to water precipitation for 18 months, showed that the metal is retained mostly within the top 0-2 cm of the column [12].

Some toxicity studies showed that Bi as one of the least harmful metals to human and wild animal life [13-16]. Others suggested that more experimental evidences are needed on the environmental behavior and toxicity of Bi [4, 17]. The lack of ecotoxicological studies has often been justified by the low concentration of Bi in environmental ecosystems as well the low solubility of Bi compounds [2]. However, the evidence on the release of Bi from munitions, shotshell, smelting activities and cosmetics to the environment as well the uptake of the metal by soil organisms were reported by some studies. For example, Berthelot, et al. [18] reported that exposing earthworm *Eisenia andrei* to soil samples contaminated with a mixture of metals and explosives, Bi was found to be among the most accumulated metal in the earthworm tissues (up to 22.8 mg Bi/kg dry tissue). A study on metals uptake from soil and accumulation in plants at an old antimony mine demonstrates high tolerance of plants to metals including Bi [19]. Furthermore, the application of sewage sludge of urban wastewater on arable land increase considerably the Bi content in the soil [10].

Earthworms are one of the most abundant organisms in soil. Through their movement in soil, earthworms are expected to introduce changes in the composition and properties of soil as a result of soil mixing and interactions with soil organic and inorganic constituents [20]. Earthworms can interact with the soil matrix and absorb metallic pollutants by at least two different routes; a dermal route where metal intake proceeds through the integument, and an oral route *via* which the metal enters the earthworm with the soil and pore water through its digestive tract. Whether the intake of metals by earthworms occurs internally or externally, either mechanism is bound to provide some useful insights into the accessibility and the toxic effects of the metals. The oligochaete *Eisenia andrei* is commonly used by ecotoxicologists to assess the acute and chronic toxicity of contaminants to soil. Also a database on the toxic effects of several metals on this species is already available. For example, Peijnenburg, et al. [21] reported that *E. andrei* can tolerate and accumulate high concentrations of metals (e.g., As, Cd, and Pb), causing a high risk of transferring contaminants to predators at higher trophic levels such as birds, reptiles, and mammals.

The aim of the present study is to investigate the acute toxicity effects of bismuth, added as bismuth citrate to a natural sandy soil, to the earthworm *E. andrei* and also to provide some insights into the role of earthworm presence in enhancing solubility and bioaccessibility of bismuth citrate in soil to eventually help understand the environmental fate and ecological impact of the metal.

2. Material and Methods

2.1. Chemicals and Reagents

Bismuth (III) citrate ($C_6H_5BiO_7$) and citric acid ($C_6H_8O_7$) were purchased from Sigma. The HNO_3 and HCl were of analytical grade. The standard reference soils TILL-2 and TILL-4 were obtained from CANMET Mining and Mineral Sciences Laboratories, Ottawa, ON, Canada. Information on preparation of soil, extraction of soil, and chemical characteristics of the two reference soil samples TILL-2 and TILL-4 are available on the Natural Resources Canada website (http://www.nrcan.gc.ca/mining-materials/certified-reference-materials/certificate-price-list/8137). ASTM Type II water was obtained using a Millipore Super-Q water purification system or Zenopure Mega-90. Glassware and polyethylene containers were washed with acetone, soaked in nitric acid solution (10%, v/v), and rinsed with deionized water.

2.2. Soil Characterization and Samples Preparation

The natural sandy soil used in this study was obtained from the Canadian Forces Base in Valcartier (Qc, CAN). The soil was collected from a site located in a non-contaminated area. This soil is representative of soil from Canadian range training area and similar soils were used in earlier toxicological studies to consider higher bioavailability of compounds [22-24]. After collection, soil was sieved (2 mm) to remove rocks, roots, and other large particles, then subjected to a battery of preliminary toxicity tests (i.e. plant germination, earthworm lethality test) to confirm that the soil was not toxic. Physical and chemical characteristics of this soil are shown in Table 1. Soil was spiked by adding bismuth citrate ($C_6H_5BiO_7$) in the powder form to soil samples separately to target the following nominal concentrations 450, 500, 550, and 600 mg Bi/kg dry soil. The nominal concentrations selected were based on the results of preliminary lethality tests (data not shown). A control (soil without Bi citrate) was prepared by adding Type II water only to the soil. Four replicates for each Bi concentration tested were prepared. Spiked soils were mixed for 20 ± 2 h in a rotary mixer to obtain a homogeneous distribution of Bi citrate. The soil was then hydrated to 75% of its water holding capacity (WHC). WHC was determined by saturating the soil with Type II water and by measuring the water content as described previously by Robidoux, et al. [25]. Water content was determined in separate studies, by measuring the loss of soil weight after drying for 18 h at 105°C in an oven. After hydration, the soil samples were then mixed overnight in a rotary mixer, and kept at room temperature for 2 weeks to stabilize. Soil aliquots were taken at the start (t= 0) and end of the exposure period (t=14 days),

to determine total and bioaccessible Bi, moisture content, and pH. The pH of the soil samples was measured using a 1:5 (v:v) soil/water suspension [26].

Table 1. *Summary of physical and chemical characteristics of natural soil collected at the non-contaminated area.*

Parameter	Measurement
pH	5.96
Humidity (%)	7.5
Total organic carbon (%)	2
Sand (%)	97.6
Silt (%)	1.6
Clay (%)	0.7
Silver (mg/kg)	< 2
Arsenic (mg/kg)	< 5
Barium (mg/kg)	11
Cadmium (mg/kg)	< 0.5
Cobalt (mg/kg)	2
Magnesium (mg/kg)	180
Mercury (mg/kg)	<0.02
Molybdenum (mg/kg)	<1
Nickel (mg/kg)	1
Lead (mg/kg)	<5
Zinc (mg/kg)	31
Bismuth (mg/kg)	<0.05

2.3. Lethality Test

Earthworms (*E. andrei*) used in this study were obtained from Carolina Biological Supply (Burlington, NC) and was initially used to establish laboratory culture. Earthworms were incubated in a bedding (Magic Products, Amherst Junction WI) supplemented with a dry grain-based food (Magic Worm Food, Magic Products) and maintained at 20 ± 1°C with 70% humidity, and a light-to-dark cycle of 16:8 h. For this toxicity test, only adult earthworms (ranging from 300 to 600 mg wet weight each) with well-developed clitellum were used. The earthworm lethality test was performed according to the OECD [27] method. In brief, after acclimation for 2 days in a non-contaminated soil, ten *E. andrei* were washed, individually weighed and placed in 1-L glass jar containing 350 g of spiked natural soil, with four replicates per concentration. The glass jars were closed using a geotextile and lids with 1.6-mm air holes. The number of surviving *E. andrei* was recorded after 7 and 14 d of exposure. In addition, monitoring of morphological changes and skin damage of surviving earthworms were recorded at the end of the test.

Control group, three in all, each containing soil amended with citric acid in the presence of *E. andrei,* were prepared to determine the effect of citrate on soil pH and on *E. andrei* survival. The three tested concentrations of citric acid were 200, 500, and 900 mg citric acid/kg dry soil. These citric acid concentrations matched the lowest, average and the highest citrate concentrations found in the tested concentrations of bismuth citrate including preliminary tests. The number of surviving *E. andrei* was recorded after 7 and 14 d of exposure. Soil pH was measured at the beginning and end of each experiment. Another control group included soil and bismuth citrate, in the absence of *E. andrei*, was prepared to determine the effect of earthworm presence on the soil pH and to help understand their role on Bi bioaccessibility.

2.4. Extraction and Analysis of Bismuth

First, solubility of Bi citrate in water was determined. Bi citrate was added to get 398, 39.8 and 3.9 mg/L of deionized water (containing 208, 20.8 and 2.08 mg Bi, respectively). The mixture was stirred overnight at room temperature then filtered through a 0.45 µm Millipore polytetrafluoroethylene membrane. The filtrate was acidified at 2% (v/v) with HNO_3 for subsequent analysis by Inductively Coupled Plasma/Optical Emission Spectrometry (ICP-OES, Agilent Technologies 5100). Total Bi was extracted using the nitric acid (HNO_3) digestion method as described by Sauvé, et al. [28]. Briefly, soil samples were dried at 105°C for 18 ±2 h, then about 0.5 g dry soil was digested in 10 mL of concentrated HNO_3 for 90 min at 85°C. The bioaccessible portion of Bi in soil was assessed using KNO_3 aqueous solution, as described previously [18, 28]. Briefly, 20 mL of 0.01 M KNO_3 solution was added to 10 g dry soil for subsequent shaking overnight (50 rpm). The resulting mixture in each extraction case was centrifuged (11000g) separately for 15 min. The supernatant was then filtered through a 0.45 µm Millipore membrane. Concentrations of total and bioaccessible Bi in soil extracts were determined using ICP-OES. Validation of the extraction and analysis method was performed using two standard reference soils TILL-2 and TILL-4.

2.5. Statistical Analyses

Data were analyzed using the ToxCalc program (Version 5.0; Tidepool Scientific Software, McKinleyville, CA). The toxicity endpoints such as point estimates (lethal concentration e.g., LC_{10}, LC_{25} and LC_{50}) were obtained by the maximum likelihood-probit regression method. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were estimated using the parametric hypothesis test (Bonferroni's Multiple Comparison Test). Normality of survival data was tested by the Shapiro-Wilk test and equality of variance using Bartlett's test. ToxCalc program include other statistical methods of analysis such as parametric hypothesis test (e.g., Dunnett's Multiple Comparison Test, Williams Multiple Comparison Test) and nonparametric hypothesis test (e.g. Steel's Many-One Rank Tests, Wilcoxon's, Two-Sample Test).

3. Results and Discussion

3.1. Lethality of Bi Citrate Spiked Sandy Soil to Eisenia andrei

The use of artificially contaminated soil allows investigators to control the exposure concentrations, and generate LC50, LC25, NOEC, and LOEC values. Table 2 and Figure 1 show that Bi was toxic to *E. andrei*. Data in Figure 1 indicate that from 399.5 to 469.5 mg Bi/kg dry soil measured total concentrations, the survival of *E. andrei* decreased after

7 and 14 d of exposure. The lethal effect was time dependent, the rate of *E. andrei* survival decreased from 77.0, 67.5, and 67.5 at 7 d to 57.5, 32.5, and 21 at 14 d for 399.5, 431, and 469.5 mg Bi/kg dry soil total concentration, respectively. The results from the control confirmed that the natural soil used in this study was not toxic to the earthworm. Furthermore, the results of control test with citric acid (concentrations range from 200 to 900 mg citric acid/kg dry soil) showed that citric acid did not cause any lethal effects on the earthworms (Table 3). Table 2 presents the acute toxicity parameters on *E. andrei* after 7 and 14 d of exposure in bismuth spiked natural soil. The 14 d LC_{50} was 416 mg Bi/kg, and no effect observed at 298 mg/kg.

Table 2. *Acute toxicity parameters on Eisenia andrei after 7 and 14 days (d) of exposure in bismuth spiked natural soil.*

Toxicity parameters	At 7 d (mg/kg)	At 14 d (mg/kg)
NOEC	298	298
LOEC	399.5	399.5
LC_{10}	364.88	340.56
LC_{25}	423.45	374.42
LC_{50}	499.61	416.00

NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; LC10: estimated lethal concentration for 10% of exposed earthworms. LC25: estimated lethal concentration for 25% of exposed earthworms; LC50: estimated lethal concentration for 50% of exposed earthworm.

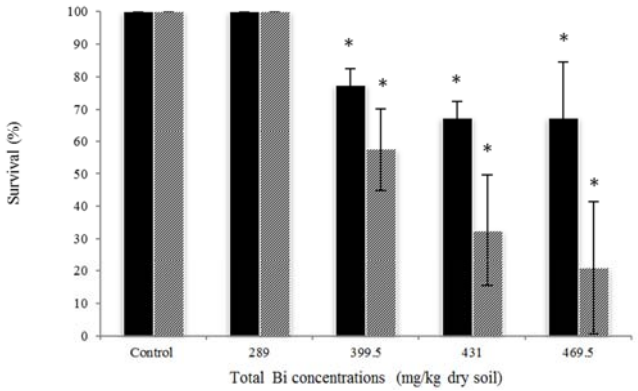

Figure 1. *Adult survival rate of Eisenia andrei after 7 and 14 days exposure in Bi citrate spiked natural sandy soil. Values are expressed as mean ± SD (Standard deviation), n = 4. *: Significantly different from the control (p <0.05).*

Table 3. *Acute effect of citric acid exposure in soil to the earthworm Eisenia andrei.*

Citric acid concentrations (mg/kg dry soil)	Survival (%) *	
	7 d	14 d
Control (0)	100(0)	100(0)
200	100 (0)	100 (0)
500	100 (0)	100 (0)
900	97.5 (5)	97.5 (5)

* Survival data are expressed as mean (SD), n=4.

The earthworms exposed to Bi were getting shorter in size, with deformed and cut skins. The severity of the effect on the decrease of the body length was concentration-dependent. Skin deterioration and deformation were observed mainly in the posterior part of earthworm (Figure 2). This can be explained by the fact that the chloragogenous tissue surrounding the posterior alimentary canal is the main area of metal accumulation in earthworms. He, et al. [29] observed similar effects, such as smaller head size and shorter body length in zebrafish embryos exposed to bismuth−asparagine coordination polymer due to changes in expression of genes involved in cell migration and growth. Other researchers showed that high doses of Bi (form colloidal bismuth subcitrate) might induce epithelial cells death in rats by destabilizing the cell membrane [30].

(a)

(b)

(c)

Figure 2. *Deformation and disconnection observed on the skin of the posterior part of Eisenia andrei exposed to bismuth citrate spiked soil, a (Control), b (431 mg Bi/kg dry soil), c (469.5 mg Bi/kg dry soil).*

In addition, the results of the present study showed that Bi was more toxic to *E. andrei* than Pb. Indeed, the LC_{50} of Pb reported by Berthelot (2008) [8] was 579 mg Pb/kg dry soil in a sandy soil after 28 d exposure, which is higher than that obtained for Bi after 14 d in the present study (416 mg Bi/kg dry soil). Khangarot and Das [31] have also showed similar comparisons for freshwater ostracod *Cypris subglobosa*, the 48 h LC_{50} were 37.08 and 40.19 mg/L for Bi (form Bi nitrate) and Pb, respectively. Furthermore, Bi is found to be more toxic to *Tubifex tubifex* species with a 96 h LC_{50} = 0.662 mg de Bi/L [32]. However, it should be mentioned that despite its wide utilization in the ammunition, pharmaceutical and cosmetic industry, Bi remains the least studied metal in the environment under the pretext of the low solubility of the metal and its salts [9, 33, 34]. Because of this, many considered the element to be unavailable for uptake by living organisms, and thus less harmful to the environment [2, 35]. In contrast, the present study has shown that bioaccessibility and bioavailability of Bi in soil was, to some extent, linked to the presence of earthworm *E. andrei* and its interaction with the metal, rather than to its water solubility. It is known that the bioavailability of metals to earthworms is complex because of the existence of multiple routes of exposure. Literature reports are still debating whether the dermal or the ingestion route is the dominant mechanism for the uptake/absorption of metals by earthworms [36, 37]. Reputedly, the route of metal uptake depends on the form of metal ions and how the metal partitions itself in soil. Pore water exchangeable metal that is extracted by a weak aqueous salt solution (e.g. KNO_3, $NaNO_3$) passes to the receptor through dermal uptake, but sequestered metal rather mobilized by the ingestion and digestion mechanism [38, 39]. One of the key mechanisms involved in the dermal intake route presumably involves the complexation of Bi with the chemical constituents in the skin including polysaccharides, lipids, and proteins through their HS-, NH-, or OH-containing functionalities. Also the –OH in bismuth citrate can potentially interact with earthworm biological tissues through H-bonding. Such interactions would lead to morphological and physiological changes in the skin thus causing toxic effects and eventually death of *E. andrei.* As for the second intake mechanism Bi in soil sequestered, or irreversibly sorbed to soil might become potentially bioavailable for inner cellular intake through the digestive duct. The presence of Bi inside the earthworm might also lead to a disruption in the inter and intra cellular functions and the enzymatic processes responsible for earthworm survival.

3.2. Total and Bioaccessible Bi in Soil

From the solubility test Bi citrate was found poorly soluble in water, the amount of Bi measured was < than 4 mg Bi/L. Table 4 shows total and bioaccessible Bi as measured by ICP-OES in extracts of soil obtained by digestion with HNO_3 and by extraction with KNO_3, respectively. Potassium nitrate is a neutral salt that is known to displace other metal ions in soil [40], which in the present study K^+ presumably displaced Bi^{3+} ions. Average percent recovery of total Bi from contaminated soil, calculated as the ratio of measured total concentrations and calculated nominal concentrations, varied between 66.05 ± 3.05% and 78.84 ± 2.20% (Table 4). Whereas, recoveries of Bi in the two reference soils TILL-4 and TILL-2 were ranging between 84.98 ± 6.08% and 91.66 ± 23.62%, respectively (data not shown). Compared to the sandy soil, the higher Bi recoveries obtained using the standard reference soil was probably caused by intrinsic variations in the physical and chemical characteristics of the soils. For example, the standard reference soil had finer texture (≤ 0.074 mm) compared to the sandy soils used in the present study (≤ 2 mm). In the case of the standard reference soil, HNO_3 completely dissolved the fine soil particles, thus enhancing recovery of Bi. This was not observed using the sandy soil, where nitric acid failed to dissolve the sand particles. The link between extractability of heavy metals from soil and the size of soil particles was reported earlier [11, 41, 42].

Table 4. *Total and bioaccessible bismuth measured in soil samples at the beginning (t=0) and the end of experiment (t = 14d) with and without the presence of Eisenia andrei.*

Nominal Bi (mg/kg)	Total Bi [a,] * (mg/kg)	Total recovery* (%)	Bioaccessible Bi [b,] * (mg/Kg) At t=0	Bioaccessible Bi [b,] * (mg/kg) at t=14 d	
				Without *E. andrei*	With *E. andrei*
0 (Control)	< DL	ND	< DL	< DL	< DL
450	298 (13.58)	66.05 (3.05)	0.165 (0.009)	0.111 (0.001)	0.262 (0.031)
500	399.5 (9.19)	79.9 (2.20)	0.194 (0.008)	0.171 (0.028)	0.974 (0.076)
550	431 (11.31)	76.67 (3.26)	0.212 (0.024)	0.185 (0.001)	3.96 (1.845)
600	469.5 (11.96)	77.52 (2.14)	0.250 (0.014)	0.167 (0.001)	7.516 (1.411)

* Values are expressed as mean (SD), N=3. When Bi was not detected, results are presented as < 0.05 detection limit (DL); a; extracted by HNO_3; b: extracted by aqueous KNO_3.

However, it is widely acknowledged that the total content of metals in soil is not a relevant parameter to determine potential risks from soil contamination. In general, the potential toxicity of a metal in soil depends rather on its concentration in soil aqueous phase, the nature of its association with other soluble species, and on the ability of soil to release the metal from the solid phase [43]. Table 4 shows that for similar total contents of Bi, the bioaccessible fraction of the metal in soil varied with time and with the presence of earthworm *E. andrei*. After 14 d in the absence of earthworms, the bioaccessible Bi fraction decreased, probably due to soil aging accompanied with some sort of complexation of the metal with other soil chemical constituents. In contrast, in the presence of earthworms, Bi bioaccessibility increased considerably after 14 d of incubation (Table 4). The amount of Bi bioaccessible in soil at t=0 d was ≤ 0.252 mg/kg dry soil, which is too low to cause lethal effects

to *E. andrei*. According to the 14 d toxicity data shown in (Table 2), the no observed effect concentration (NOEC) (298 mg Bi/kg dry soil) corresponding to 0.262 mg Bi/kg dry soil bioaccessible concentration. Whereas, a significant lethal effect was observed at 399.5 mg Bi/kg soil with a bioaccessible fraction reaching 0.974 mg Bi/kg soil (Table 4). The lethal effect of Bi on *E. andrei* is caused by the increase in Bi bioaccessible fraction in soil, and not according to the total content of metal in soil (Table 4 and Fig. 1). In line with the results of this study, Berthelot [8] has also found that bioaccessibility of Bi in soil doesn't correlate with measured total concentrations, but rather is influenced by changes in the physical, chemical and biological characteristics of soil. At day 0, the bioaccessible Bi fraction in soil increased slightly with increasing total concentrations of bismuth citrate. Average bioaccessible fraction of Bi increased from 0.165 to 0.250 mg/kg dry soil when the average measured total Bi increased from 298 to 469.5 mg/kg dry, respectively (Table 4). While after 14 d, bioaccessible fraction of Bi measured in soil with earthworm *E. andrei* was much higher than those measured in soil in the absence of earthworms. The results showed also that measured bioaccessible Bi concentrations at 14 d were higher than those obtained at t=0 before the addition of earthworms (Table 4). For example, after 14 d exposure, bioaccessible concentrations of Bi in soil with earthworms were 1.6, 5.0, 18.6, and 30.0 times higher than the values measured at t=0 d using the following Bi total concentrations 298.0, 399.5, 431.0, and 469.5 mg Bi/kg dry soil, respectively. Whereas the bioaccessible Bi fraction in soil with earthworm increased by a factor of 2.4, 5.7, 21.4, and 45.0 after 14 d, compared to those measured after 14 d in soil without earthworms. Experimental evidence gathered thus far confirmed that the lethal effect observed in earthworms was caused by the high concentrations of Bi made bioaccessible in the presence of *E. andrei* in the soil. In the absence of earthworms, the fraction of bioaccessible Bi decreased in soil, possibly caused by adsorption of the metal onto soil. Increased Bi bioaccessibility in the presence of earthworms could be attributed to the digestive mechanism connected to the earthworm gut. Ma, et al. [39] reported that the increase in bioaccessibility of arsenic, copper, and zinc in soil in the presence of *E. andrei* is caused by metal sequestration carried out by enzymatic digestion. Furthermore, a novel test, namely, simulated earthworm gut, recently developed based on the enzymatic composition of the gastrointestinal fluid of *E. andrei* appeared to be most promising for predicting availability of metals in soil than chemical extraction methods [44]. The concentration of bioaccessible Bi recorded in the present study due to *E. andrei* activity was greater than those reported by Wen, et al. [45] for Cr, Co, Ni, Zn, Cu, Cd, and Pb using *E. fetida*. This was possibly caused by a stronger affinity of Bi to form chelating complexes with metallophores and other biomolecule produced by earthworm. On the other hand, some studies suggested that the earthworms could stimulate the growth of soil bacterial populations that enzymatically degrade soil organic matter causing the release of the organically bound metal [45-47]. Bi bioaccessibility increased significantly following the death of the earthworms. *E. andrei* can accumulate high concentrations of Bi within its body. After decomposition, high metal burdens might return to the soil, thus causing the observed increase in bioaccessible Bi concentrations in soil with the death rate of earthworms. Similar results have been reported by Ireland [48] for the high quantities of lead and zinc extracted from decayed earthworm *Dendrobaena rubida*. As mentioned above, evidence gathered thus far suggest that earthworms increase the availability and mobility of Bi in soil, but the mechanism responsible for this increase is not clear yet. One may speculate that Bi (III) ion coming out of the citrate salt might form some sort of complexes with sulfhydryl, hydroxyl, or amine-containing ligands in the chemical fabric of the earthworm.

3.3. Effect of pH on Bi Bioaccessibility in Soil

Table 5 summarizes pH measurements obtained in the soil toxicity assays. At the beginning of the experiment (t=0), soil pH decreased slightly from 5.85 ± 0.06 to 5.24 ± 0.03 for control (soil without Bi citrate) and the highest bismuth citrate concentration tested (469.5 mg/kg dry soil), respectively. After 14 days, in the absence of *E. andrei* in contaminated soil pH did not change drastically. However, in the presence of *E. andrei* soil pH increased significantly compared to pH values obtained at t=0, as Bi citrate concentration increased. For example, in the assay with the control pH increased from 5,85 ± 0,058 to 6.01 ± 0.162 whereas in the assay with 499.5 mg Bi/kg dry soil pH increased from 5,24 ± 0,03 to 7.30 ± 0.18 (Table 5). The observed increase in soil pH in the presence of earthworms is consistent with the findings of several other studies on metals bioaccessibility including As, Cu, Pb, Zn and Cd in soil [18, 49, 50]. In this study, the values of contaminated soil pH in the presence of earthworm increased from 0.16 to 2.06 units. Similarly Wen, et al. [47] have found that soil pH increased from 0.2 to 1.1 due to earthworm (*Eisenia fetida*) activity. This noticeable increase in soil pH by earthworm has been attributed to the granules of calcium carbonate produced by the calciferous glands located into the earthworm esophagus [51]. Indeed, Garcı´a-Montero, et al. [52] showed that the increase of calcium carbonate content in earthworm casts cannot be explained by the original carbonate level in soil but rather by the synthesis of calcite granules by earthworms. On the other hand, Schrader [53] reported the role of cutaneous mucus secreted by three earthworm species to increase and neutralize the pH of their environment. Another study by Salmon [54] showed that an increase in nitrogen associated with earthworm alkaline urine lead to an increase in soil pH. Thus, the increase of soil pH caused by earthworm activities could influence mobility and availability of metal as Bi in soil. Several literature reports consider pH as a primary factor in determining bioaccessibility and availability of metals in soil, and thus their toxic effects to the living organism [55, 56]. The decline in heavy metals availability in soil with increasing soil pH has been reported earlier [57, 58]. In contrast, the results of this study showed that the fraction of Bi bioaccessible in soil, and consequently the fraction of Bi bioavailable to uptake by soils organisms increased despite the

pH rise induced by earthworm activity. The rise in soil pH might increase the mobility of metals including Bi, due to an increase in the number of pH-dependent cation-exchange sites on the soil surface as suggested by Hou, et al. [11]. Indeed, the present Bi study showed an increase in earthworm lethality with the increase of soil pH (Fig. 2 and Table 5). However, it is worth mentioning here that besides soil pH, other physico-chemical properties of soil and biological parameters in the earthworm could play significant roles in determining Bi bioaccessiblity and thus toxicity. Among other heavy-metal-chelating metallophores produced by earthworms might be responsible for the increase in Bi bioaccessibility in soil, as proposed by Wen, et al. [45] for Zn, Cu and Cr.

Table 5. *Variation of pH in soil samples in the presence and absence of earthworms.*

Measured total Bi concentrations (mg/kg dry soil)	pH*		
	0 d	14 d without *E. andrei*	14 d with *E. andrei*
0 (Control)	5.85 (0.058)	5.95 (0.072)	6.01 (0.162)
298	5.44 (0.020)	5.74 (0.036)	6.40 (0.072)
399.5	5.32 (0.011)	5.35 (0.035)	7.03 (0.105)
431	5.25 (0.015)	5.37 (0.025)	7.31 (0.251)
469.5	5.24 (0.030)	5.36 (0.023)	7.30 (0.179)

* Values of pH (H_20) measured in suspension of soil in water are expressed as mean ± SD, n=4

4. Conclusions

The present study demonstrated that bismuth is toxic to *E. andrei* and the extent of its lethality was tied to its bioaccessibility. In general the results showed that Bi bioaccessiblity and pH increased drastically in the presence of earthworms. Mobility and other physiological and biochemical activities associated with *E. andrei* might have been partially responsible for the noticeable increase in Bi bioaccessibility in soil. Enhanced bioaccessibility of Bi could have been caused by intrinsic microbial and other enzymatic activities associated with the earthworms and by the release of the metal after ingestion and digestion of soil containing Bi. To say the least, the production of chelating agents such as metallophores by *E. andrei* might enhance Bi solubility and thus its bioaccessibility. Finally, the fact that Bi was found to be more toxic than Pb to *E. andrei*, it would be highly recommended to follow cautious procedures while handling and using Bi and its salts. Some may argue that Bi-containing chemicals should not be considered harmful based on their very poor water solubility (< 4mg/L for Bi citrate). However, because of the potential exposure of bismuth to various interaction mechanisms in the environment, e.g., interactions with chemical constituents in soil and with nearby biological receptors, then bismuth should be considered as a potential labile health hazard.

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