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Nutrient and pollutant metals within earthworm residues are immobilized in soil during decomposition

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ABSTRACT

Earthworms are known to bioaccumulate metals, making them a potential vector for metal transport in soils. However, the fate of metals within soil upon death of earthworms has not been characterized. We compared the fate of nutrient (Ca, Mg, Mn) and potentially toxic (Cu, Zn, Pb) metals during decomposition of *Amynthas agrestis* and *Lumbricus rubellus* in soil columns. Cumulative leachate pools, exchangeable pools (0.1 M KCl + 0.01 M acetic acid extracted), and stable pools (16 M HNO_3 + 12 M HCl extracted) were quantified in the soil columns after 7, 21, and 60 days of decomposition. Soil columns containing *A. agrestis* and *L. rubellus* had significantly higher cumulative leachate pools of Ca, Mn, Cu, and Pb than Control soil columns. Exchangeable and stable pools of Cu, Pb, and Zn were greater for *A. agrestis* and *L. rubellus* soil columns than Control soil columns. However, we estimated that >98% of metals from earthworm residues were immobilized in the soil in an exchangeable or stable form over the 60 days using a mass balance approach. Micro-XRF images of longitudinal thin sections of soil columns after 60 days containing *A. agrestis* confirm metals immobilization in earthworm residues. Our research demonstrates that nutrient and toxic metals are stabilized in soil within earthworm residues.

1. Introduction

Exotic and invasive earthworms are a driver of environmental change in forest soils throughout the northeastern United States. Earthworms enhance decomposition of vegetation detritus, the breakdown of soil organic matter and mix soil horizons (Edwards and Bohlen, 1996; Bohlen et al., 2004; Fahey et al., 2013). These actions affect elemental cycling in forests and yet their impacts on metal cycling in soils have been understudied (Ireland, 1983; Addison, 2009; Sizmur et al., 2011; Richardson et al., 2015a). Mobilization of macro- and micronutrients can decrease their storage in surface horizons, potentially decreasing their availability for shallow rooting plants of the forest understory (Christensen, 1988). Calcium, Mg, and Mn are important nutrients for plants that have been depleted from northeastern US forest soils as a result of acid deposition with concomitant reduction in base saturation and buffering capacity (Likens et al., 1996). The resultant deficiencies in Ca and Mn has diminished the establishment of sugar maple (*Acer saccharum*) saplings (St Clair et al., 2008), which is an economically important tree across the northeastern United States. In addition to nutrient metals, the mobilization of anthropogenic toxic metals currently stored in forest soils can impact terrestrial and aquatic ecosystems (Phillips, 1995; Fleeger et al., 2003; Ernst et al., 2008; Alloway, 2013). Soil concentrations of Cu, Zn and Pb have increased substantially across the northeastern United States due to pollution from smelting, automobile pollution, and other industrial processes (Johnson et al., 1982; Steinnes and Friedland, 2006; Richardson et al., 2015b). Forest soils act as a repository for these pollutant metals and retention is one of the ecosystem services soils provide. For example, a 10% mobilization of Pb from the organic horizons estimated by Richardson et al. (2014; 2015b) could potentially result in 12 kg of Pb mobilized to ground and surface waters in a 10 ha watershed. The effect could be even stronger in watersheds where earthworms inhabit soils close to point source-contaminated sites. Thus, understanding how exotic and invasive earthworms impact nutrient and toxic metal cycling is needed for identifying their effect on terrestrial and aquatic

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ecosystems of the region.

The effect of earthworms on soil metal pools has primarily focused on their ability to bioaccumulate metals in their tissue (see Sizmur and Hodson, 2009) to tissue concentrations of metals that are potentially hazardous to wildlife (Beyer and Cromartie, 1987; Neuhauser et al., 1995; Ernst et al., 2008; Richardson et al., 2015a). This pool may represent a substantial terrestrial amount of metals (Richardson et al., 2015a). However, the bioaccumulation of metals by living earthworms is only part of their life history; little is known regarding the mobilization of metals from their residues following their death. Many exotic species of earthworms found in the northeastern United States, such as *Aporrectodea longa* and *Amynthas agrestis,* exhibit >90% winter mortality (Christensen, 1988; Hodge et al., 2000; Görres et al., 2014). These previous studies have found that earthworm residues rapidly mineralize but the fate of their bioaccumulated metals is unclear (e.g. Hodge et al., 2000).

The overall goal of this study was to determine the extent to which metals in earthworms are mobilized or stabilized in soil during decomposition. *A. agrestis* is an Asian earthworm that has been observed recently to substantially impact soil processes in northern New England (Görres and Melnichuk, 2012; Görres et al., 2014; Richardson et al., 2015a). *Lumbricus rubellus* is a European earthworm that is widespread across Canada and North America (Bohlen et al., 2004; Addison, 2009). Both earthworm species are epi-endogeic earthworms, meaning that they consume both the organic and mineral soil horizons and thus mix materials from these two master horizons. Because of their proximity to the surface and preference not to burrow deep, populations of *A. agrestis* and *L. rubellus* are relatively sensitive to winter mortality. Furthermore, *A. agrestis*, is an annual species which overwinters only as cocoons (Görres et al., 2014). Metals from their residues may be mobilized from the soil via leaching, become weakly stabilized in soil as an exchangeable or bioavailable form, or be immobilized in a recalcitrant pool. Earthworm residues decompose rapidly. For example Christensen (1988) observed > 50% mass lost in 3 days at 21 °C under field and laboratory conditions. Thus, we hypothesized that nutrient metals (Ca, Mg, Mn) and potentially toxic metals (Cu, Zn and Pb) from earthworm residues would be mobilized from soil and primarily lost as leachate within 60 days in laboratory conditions. Increased leaching of nutrient and toxic metals could result in greater metal burdens to aquatic ecosystems and water bodies. The alternative hypothesis was that metals would be immobilized or stabilized in soil, furthering our understanding of earthworms as soil fertility regulators.

2. Methods

2.1. Earthworms and soils

Earthworms were collected from a complex of Inceptisols in Norwich, Vermont, U.S. The soils are a coarse-silty, mixed, active, mesic Typic Dystrudepts (Soil Survey Staff, 2010) and classified as a complex of Hartland-Hitchcock soil series according to the Web Soil Survey (Soil Survey Staff, 2013). The soils were formed from silty lacustrine material and support an uneven-aged forest comprised primarily of northern hardwoods (e.g., *Acer saccharum*, *Betula papyrifera*, *Betula alleghaniensis*, and *Fagus grandifolia*) interspersed with coniferous vegetation (e.g., *Picea rubrus*, *Pinus strobus*, and *Tsuga canadensis*). Only adult, clitellated earthworms were collected using the hand-sort method from the organic horizon and top 20 cm of the soil profile (Görres et al., 2015). They were stored alive in their horizon material and identified live in the laboratory using a dichotomous key (Great Lakes Worm Watch, University of Minnesota, 2011). *Amynthas agrestis* and *Lumbricus rubellus* were chosen for study because of their high rate of winter mortality, their active movement between the organic horizon and mineral soil as epi-endogeic earthworms, and presence throughout the northeastern United States (Görres and Melnichuk, 2012; Görres et al., 2014). In addition, the two earthworms have different life histories. *A. agrestis* is an annual species that does not tolerate temperatures below 5 °C (Richardson et al., 2009). Although both species are regarded as frost intolerant, *L. rubellus* may be cold-hardy, avoiding frost damage by desiccation (Holmstrup et al., 1999) and migration beyond freezing depth. *L. rubellus* may have lifespans greater than 2 years under those circumstances (Klok et al., 1997). Earthworm physiological data is presented in Table 1.

2.2. Earthworm incubation

Earthworms were incubated in large bins containing the silt-loam A horizon material they inhabited at the time of collection, which had been amended with inorganic chloride salts of Cu, Zn and Pb to produce soil concentrations of Cu (50 μg g^{-1} soil), Zn (100 μg g^{-1} soil), and Pb (100 μg g^{-1} soil). The incubation period lasted for 3 weeks to increase the metal concentrations in their tissue. Following incubation, the adult earthworms were rinsed in deionized water and transferred to plastic petri dishes with moist Whatman No. 1 filter paper disks (3 earthworms per dish) to allow for evacuation of their digestive tracts. The Petri dishes were kept in the dark at 22 °C for 3 days with daily filter paper changes to prevent coprophagy. Fifteen earthworms from each species were analyzed to determine the tissue concentration and the total amount of metal taken up by each earthworm. These earthworms were individually weighed for wet mass, preserved using cryodesiccation and weighed for dry weight (dw) biomass after drying to a constant weight.

2.3. Decomposition experiment

Silt-Loam Bw horizon material was collected from the same soil as the earthworms, the Hartland- Hitchcock complex. Material from the Bw horizon was chosen because of its lower concentrations of organic matter and metals and also because epi-endogeic earthworms burrow from the A horizon to the Bw horizon as an attempt to avoid colder air temperatures in early winter. The Bw horizon soil was air-dried, sieved to <2 mm, rocks and roots were removed, and the remaining material was thoroughly homogenized. Soil columns were constructed from 6 cm diameter, 24 cm long poly-vinyl chloride tubes. Eighty-one soil columns were partially filled with 0.50 kg of Bw horizon material and held in place with a synthetic mesh cloth with 50 μm pores. A funnel was sealed to the bottom of the column to collect leachate. The synthetic mesh and funnel were washed in a weak-acid solution (0.001 M HCl) prior to use.

We compared two earthworm treatments (*A. agrestis* and *L. rubellus*) to a set of Control soil columns with no earthworms added. All soil columns were hydrated with deionized water to 40% water-holding capacity (WHC) and incubated for 10 days prior to introduction of the earthworms. Twenty-seven soil columns were stocked with live *A. agrestis*, another twenty-seven soil columns were stocked with live *L. rubellus*, and 27 soil Control columns did not receive any earthworms. The stocking rates of earthworms were at biomass densities similar to those of A horizons determined from previous studies conducted in this region (Richardson et al., 2015a); Live *A. agrestis* was added at equivalent dw of 3.0 ± 0.1 g dw per soil column and live *L. rubellus* were stocked at 2.0 ± 0.1 g dw per soil column. Approximately 1 h after introduction, soil columns containing earthworms were placed into freezers at −18 °C for 21 days to mimic winter mortality. Because freezing of

Table 1
Earthworm ecological and physiological properties.

Species	Native range	Ecophysiological group	Average length live	Average wet weight	Average dry weight	Average worm mass added to soil columns
			cm	g per individual	g per individual	g dw per soil column
Amynthas agrestis	Eastern Asia	Epi-endogeic	7 ± 2	0.31 ± 0.07	0.14 ± 0.03	3.0 ± 0.1
Lumbricus rubellus	Europe	Epi-endogeic	6 ± 1	0.38 ± 0.05	0.16 ± 0.05	2.0 ± 0.1

the earthworm tissues occurred gradually as opposed to instantaneously, this likely promoted the growth of large crystals in the earthworm cellular tissues. Soil columns were then thawed for 3 days prior to the beginning of the experiment.

Day 0 for the decomposition experiment occurred after thawing the columns for 3 days and started with the soil columns reaching room temperature. Subsequently, the soil columns were maintained at 22 °C, between 40 and 60% WHC, and saturated with deionized water every 7 days to 120% WHC to generate soil leachate. Leachate was collected in acid-washed polypropylene bottles cumulatively from Day 0. Of the 81 soil columns and leachate containers in total, 9 of each were collected after 7, 21, and 60 days of decomposition. Day 7 cumulative leachate was from the first induced leaching. The Day 21 leachate was from three cumulative events (7, 14, and 21 days). Day 60 leachates represented cumulative volumes from eight percolation events. The leachate was filtered with a Whatman 2 filter to remove any colloids >8 μm. Because leachate mass varied among columns and collection periods, all cumulative leachates were weighed and diluted with 5% acid (9:1, HNO_3: HCl) to 100 g. Soil columns were oven-dried at 40 °C for 2 days to stop decomposition. Soil pH was determined with a soil suspension in 0.01 M $CaCl_2$ using a soil-to-solution ratio of 2:5 by mass. The % soil organic matter (SOM) was determined using loss on ignition, in which 4 g of soil was held at 475 °C for 8 h.

2.4. Soil physical and chemical parameters

Material from the Bw horizon of the Hartland-Hitchcock soil was analyzed for physical and chemical properties. The WHC of the soil was measured by saturating a 100 g of soil, and measuring the water mass after gravimetric drainage ceased. The pH was measured from a filtered 2:5, soil: water suspension in 0.01 M $CaCl_2$. Soil C concentration in the soil matrix and leachate was measured by a Carlo-Erba elemental analyzer. Every 20 samples included one blank, one Atropine standard reference material (SRM), and triplicate analysis of one sample. Total C concentration in Atropine SRMs was within 4% of the certified value and <5% relative percent difference among triplicates.

2.5. Metal extraction, quantification and quality control

We utilized a weak acid-salt and strong acid extraction to quantify exchangeable and stable metal concentrations and pools by destructively sampling and homogenizing the soil columns following 7, 21, and 60 days of decomposition. Exchangeable metal pools were operationally-defined as readily exchangeable from cation exchange sites with a 0.1 M KCl, 0.01 M acetic acid to extract, which is similar to the USEPA method 1311 Toxicity Characteristic Leaching Procedure (TCLP) (USEPA, 1990). In our procedure, 2 g of soil were shaken in 10 mL of the weak acid-salt extraction solution for 24 h. The slurry was centrifuged at 6000 rpm for 20 min and the supernatant was decanted. This extraction process was repeated, but with 10 mL of deionized water, and the resulting supernatant was combined with the previous supernatant. Stable metal pools were operationally-defined as strongly-bound metals complexed by SOM or strongly-sorbed to mineral surfaces and were not desorbed into solution with a weak acid solution, requiring strong acid digestion ($HCl + HNO_3$) to dissolve (Sizmur and Hodson, 2009). However, the strong acid digestion is unable to dissolve primary minerals, and thus metals within primary mineral matrix were not considered part of the stable pool in this study (Chen and Ma, 2001). For the strong acid digestion, 5 mL of strong acid (9:1, HNO_3: HCl, trace metal grade) was added to the slurry from the weak-acid-salt extractions. After 12 h of degassing, the samples were microwave digested at 90 °C for 45 min. Earthworm tissue concentrations were measured using strong acid digestion following EPA method 3051A (USEPA, 1995). Entire earthworms were lyophilized to a constant weight (ranging between between 100 and 400 mg dw) and digested overnight in 5 ml of strong acid (9:1, HNO_3: HCl, trace metal grade).

Soil weak acid-salt extractions, soil strong acid digestions, acidified leachates, and earthworm digestions were further diluted with deionized water and analyzed with an Agilent 7700x ICP-MS (Agilent Technologies, Santa Clara, CA) for Ca, Cu, Mg, Mn, Zn, and Pb. Every 25 samples included a digestion blank, a randomly spiked sample, a duplicate and 1 standard reference material (SRM). Digestion blanks were below detection limits. A random sample was spiked with 50 μL of multi-element standard 71A from Inorganic Ventures (Inorganic Ventures, Christiansburg, Virginia) and recoveries of the added elements ranged between 86 and 103% with an average of 93% for all spiked samples. Matching sample matrices from the National Institute of Standards and Technology were used: San Joaquin 2709 for soil digests and Mussel Tissue 2976 for earthworm tissue analysis (National Institute of Standards and Technology, Gaithersburg, MD). All metal concentrations for SRMs were within 12% of their certified values.

2.6. Micro X-ray fluorescence earthworm decomposition experiment

The goal of the synchrotron-based Micro X-ray Fluorescence experiments (μ-XRF) was to elucidate if metals were retained in the earthworm residues from *A. agrestis* or entered the soil matrix during decomposition by characterizing the distribution of metals at different time points. The samples analyzed by μ-XRF were similar to the previously described soil columns except that the column diameter was 3 cm and accommodated 100 g of soil. To create the thin-section samples, average adult *A. agrestis* from the metal incubation were rinsed, depurated, and frozen at − 20 °C for 3 days. The earthworms were lyophilized to remove all moisture and temporarily fixed along the center axis of smaller cylindrical columns (2 cm diameter, 10 cm in length) using nylon threads. The earthworms were then encased with the Bw horizon soil, the nylon thread was removed, and the soil was wetted to 40–60% WHC and allowed to decompose for 7 or 60 days. Every 7 days, the columns were moistened to 120% WHC to allow for leaching, which was collected. Following the 7 and 60 day decomposition period, the soil columns were frozen at − 20 °C for 3 days and lyophilized to remove water. The soil and decomposing earthworm were cemented in place using, a room-temperature-curing epoxy-resin (EPO-TEK 301-2FL). The hardened soil columns were cut into 30 μm thin-sections along the vertical axis, along the alimentary canal of

the earthworms.

The earthworm-soil boundary of the Day 7 and 60 soil columns were analyzed by μ-XRF imaging at the Stanford Synchrotron Radiation Lightsource (SSRL) with the Stanford Positron Electron Accelerating Ring (SPEAR) storage ring, containing 150–200 mA at 3.0 GeV. The μXRF imaging and spectroscopy was collected using beam line 2–3. The incident x-ray energy was set to 10 keV using a Si (111) double crystal monochromator. The microfocused beam of 2 × 2 μm was provided by a Pt-coated Kirkpatrick-Baez mirror pair (Xradia Inc.). The fluorescence lines of Ca, Cu, Fe, K, Mn, S, Si, Ti, and Zn, as well as the intensity of the total scattered X-rays, were monitored using a silicon drift Vortex detector (SII NanoTechnology USA Inc.). The fluorescence lines for Mg and Pb could not be measured due to interferences from other elements. In addition to the fluorescence lines for the metals of interest, the entire fluorescence spectrum was also collected at each data point. Samples were mounted at 45° to the incident x-ray beam and were spatially rastered in the microbeam using a Newport VP-25XA-XYZ stage. Beam exposure was 25–50 ms per pixel. Images were created and analyzed using SMAK MicroAnalysis Toolkit v 1.10 (Webb, 2011). Data from all pixels in the image and selected earthworm-residue pixels in the μXRF images were explored for linear least squares regressions among metals using SMAK.

2.7. Descriptive and statistical analyses

Descriptive statistics for soil properties, earthworm, soil extracts and soil digest concentrations were calculated in MATLAB R2011b (MathWorks Inc, Natick, MA). For the figures and in-text data, mean values are given ±1 standard error of the mean. Differences among earthworm treatments were compared using Student's t-test after evaluating the normality of their distribution using the Lilliefors test (Lilliefors, 1967). One-way ANOVAs among earthworm treatments were performed on cumulative leachate concentrations, exchangeable pools, and stable pools for each metal (MATLAB R2011b). Subsequently, when significantly differences were observed, we utilized Repeated Measures One-way ANOVAs to determine if there was a significant different among treatments for the duration of the experiment (MATLAB 2015a). A mass balance approach was utilized to estimate the distribution of metals in soil columns with *A. agrestis*, and *L. rubellus*, using Control columns without earthworms as a blank. The total pool of each metal was calculated as the summation of the cumulative leachate, exchangeable, and stable metal pools for each treatment at each collection time (see Table 2).

3. Results and discussion

3.1. Earthworm incubation metal concentrations

Following incubation in the metal enriched soil, the earthworms attained Ca, Mg, Mn, Cu, Zn, and Pb concentrations in tissues (Table 2) that were comparable to concentrations observed in earthworms of the same species from forest soils in a regional sampling of northern New England (Richardson et al., 2015a). However, Cu, Zn, and Pb concentrations were lower than concentrations observed at uncontaminated and contaminated sites (e.g. *L. rubellus* in Wales, UK, Morgan and Morgan, 1999). Tissue concentrations of Ca, Mg, Mn, and Cu in *A. agrestis* were significantly greater than in *L. rubellus* ($p < 0.05$; Table 2). Only Zn tissue concentrations were lower in *A. agrestis* than in *L. rubellus* ($p < 0.05$). Lead tissue concentrations were similar for both species. Preferences in soil consumption and ecophysiology (e.g. earthworms of Lumbricidae have a calciferous gland) may have caused the differences in observed tissue concentrations (Morgan and Morgan, 1990). Since *A. agrestis* were stocked in the soil columns in greater dw biomass per individual (Table 1) and had higher metal concentrations in their tissues (Table 2), we estimated that *A. agrestis* would add significantly more Ca, Mg, Mn, Cu, and Pb to the soil columns than *L. rubellus* ($p < 0.05$; Table 2).

3.2. Metals in cumulative leachate

Soil column leachate pH was 6.6 ± 0.2 and C concentration was 11 ± 5 mg C L^{-1} and did not vary among treatments or through the experiment. Soil columns containing *A. agrestis* and *L. rubellus* had significantly greater pools of Ca and Mn in their cumulative leachate than Control soil columns throughout the decomposition experiment ($p < 0.05$, Fig. 1). Soil columns containing *A. agrestis* and *L. rubellus* had greater Cu in their cumulative leachate than the Control columns for Day 7 ($p < 0.05$, Fig. 1). Over 60 days, The *A. agrestis* and *L. rubellus* soil columns leached significantly more Pb than the Control soil columns ($p < 0.05$, Fig. 1). Using a post-hoc repeated measures one-way ANOVA, we determined that Ca, Mn, and Pb cumulative leachates were different during the duration of the experiment ($p < 0.05$). These results demonstrate that a fraction of the metals introduced to the soil column in earthworm residues were lost as leachate. Since all materials and methods were the same for all treatments except for inclusion of earthworm residues, the metals in the leachate are either the result of direct leaching from the earthworm residues or leaching from the soil matrix due to interaction with earthworm residues. We were unable to

Table 2
Earthworm tissue metal concentrations were measured using strong acid digestion. Estimation of metals added to the soil columns from earthworm biomass (tissue concentration × earthworm dw mass) and mass balance (exchangeable pool + stable pool + leachate) differences between earthworm treatments and control soil columns. (*) indicates a significant difference between earthworm treatments. Error bars are ±1 standard error.

Treatment	n[a]	Ca	Mg	Mn	Cu	Zn	Pb
Earthworm tissue concentrations		mg g^{-1}	mg g^{-1}	mg g^{-1}	μg g^{-1}	μg g^{-1}	μg g^{-1}
A. agrestis	15	9.8 ± 1.9*	4.6 ± 0.9*	1.5 ± 0.3*	17 ± 3*	65 ± 13	43 ± 3
L. rubellus	15	3.8 ± 0.4	0.9 ± 0.1	0.3 ± 0.1	9 ± 1	148 ± 21*	46 ± 2
Estimated from earthworm biomass		mg	mg	mg	mg	mg	mg
A. agrestis	15	29 ± 7	14 ± 3	4.43 ± 0.12	0.05 ± 0.01	0.19 ± 0.05	0.13 ± 0.03
L. rubellus	15	8 ± 3	1.8 ± 0.4	0.65 ± 0.09	0.02 ± 0.00	0.30 ± 0.04	0.09 ± 0.02
Calculated from soil column mass balance		mg	mg	mg	mg	mg	mg
A. agrestis	27	315 ± 189	65 ± 28	1.53 ± 0.71	0.17 ± 0.07	0.92 ± 0.27	0.70 ± 0.23
L. rubellus	27	215 ± 151	36 ± 19	0.82 ± 0.33	0.15 ± 0.05	0.38 ± 0.14	0.34 ± 0.13

[a] n are the number of earthworms analyzed for the earthworm biomass and the number of soil columns used to estimate metal pools added to the soil columns.

Table 3
Distribution of added metal pools from earthworms to soil columns for the Day 60 soil columns based upon the leachate, exchangeable and stable pools mass balance.

Treatment	Pool	n[a]	Ca	Mg	Mn	Cu	Zn	Pb
			g g^{-1}	g g^{-1}	g g^{-1}	mg mg^{-1}	mg mg^{-1}	mg mg^{-1}
A. agrestis	Leachate	9	1.1%	1.1%	0.7%	0.4%	0.9%	0.2%
A. agrestis	Exchangeable	9	79.7%	18.2%	86.1%	20.9%	16.8%	0.2%
A. agrestis	Stable	9	19.3%	80.7%	13.2%	78.7%	82.4%	99.6%
L. rubellus	Leachate	9	0.8%	0.4%	0.5%	0.4%	0.5%	0.2%
L. rubellus	Exchangeable	9	89.4%	26.4%	93.6%	54.0%	56.3%	1.4%
L. rubellus	Stable	9	9.8%	73.1%	5.9%	45.7%	43.1%	98.5%

[a] n are the number of replicate soil columns.

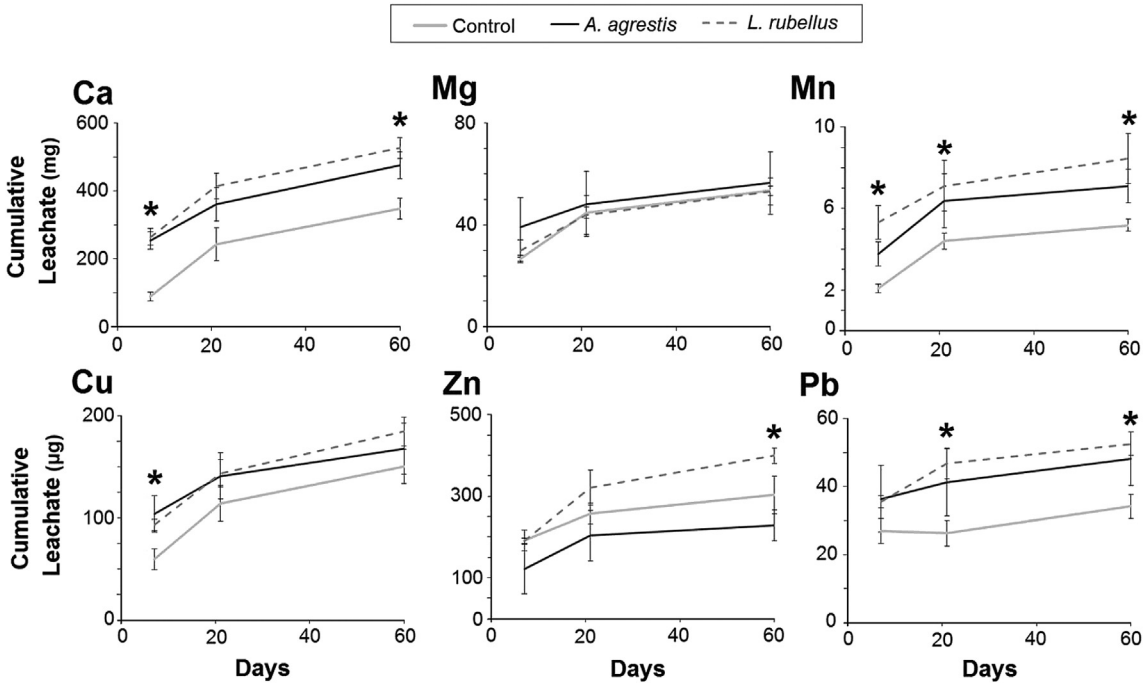

Fig. 1. Mean metal pools in cumulative leachate collected after 7, 21, and 60 days of earthworm residue decomposition and weekly flushes with deionized water. (*) indicates a significant difference among treatments for one of the cumulative leachate collections. Error bars are ±1 standard error.

distinctly link the mechanism of metal transport with DOC in the leachate. Thus, DOC compounds in the leachate, inorganic colloids smaller than the filter pore size (<8 μm), and dissolved metal species are possible mechanisms for metal leaching from the soil column.

3.3. Soil column exchangeable and stable pools

The soil column pH was 6.4 ± 0.2 and soil C concentration was 56 ± 6 mg C g^{-1} soil and did not vary significantly over time among the various leaching experiments (Data not shown). Exchangeable pools of Ca were not significantly different among treatments (Fig. 2). Differences in exchangeable Ca pools among earthworm and Control soil columns were not observed, likely due to the large Ca pools in the soil matrix relative to within earthworm residues. Generally, exchangeable pools of Mg and Mn were greater for soil columns containing *A. agrestis* and *L. rubellus* than Control columns throughout the decomposition experiment (Fig. 2, $p < 0.05$). In contrast, exchangeable pools of Cu, Zn, and Pb were significantly greater than the control columns for Day 7 and Day 21, but not significantly different for Day 60 (Fig. 2, $p < 0.05$). Using a post-hoc repeated measures one-way ANOVA, we determined that earthworm treatments significantly affected the exchangeable pools of Mg, Mn, Cu, and Pb for the duration of the experiment.

Stable pools of Ca, Mg, and Mn were similar among treatments for the duration of the experiment (Fig. 3), likely due to their large pools in the soil matrix relative to the metal masses within the earthworm residues. Stable pools of Cu and Zn were significantly greater for soil columns containing *A. agrestis* than the Control soil columns (Fig. 3, $p < 0.05$). Generally, stable pools of Cu and Zn were not significantly different for soil columns containing *L. rubellus* than Control soil columns. Stable pools of Pb were significantly greater for soil columns containing *A. agrestis* and *L. rubellus* than Control soil columns throughout the decomposition experiment (Fig. 3). We determined that earthworm treatments significantly affected the stable pools of Cu, Zn and Pb for the duration of the experiment using repeated measures one-way ANOVA.

Our results suggest that exchangeable and stable Cu, Zn, and Pb pools in the soil columns were significantly influenced by the presence of earthworm residues (Figs. 2 and 3). Differences in Ca, Mg, and Mn for both exchangeable and stable pools among treatments may have been difficult to detect due to comparatively larger pools within the soil matrix compared with the added earthworm residues. The exchangeable and stable pools of Cu, Zn, and Pb in the

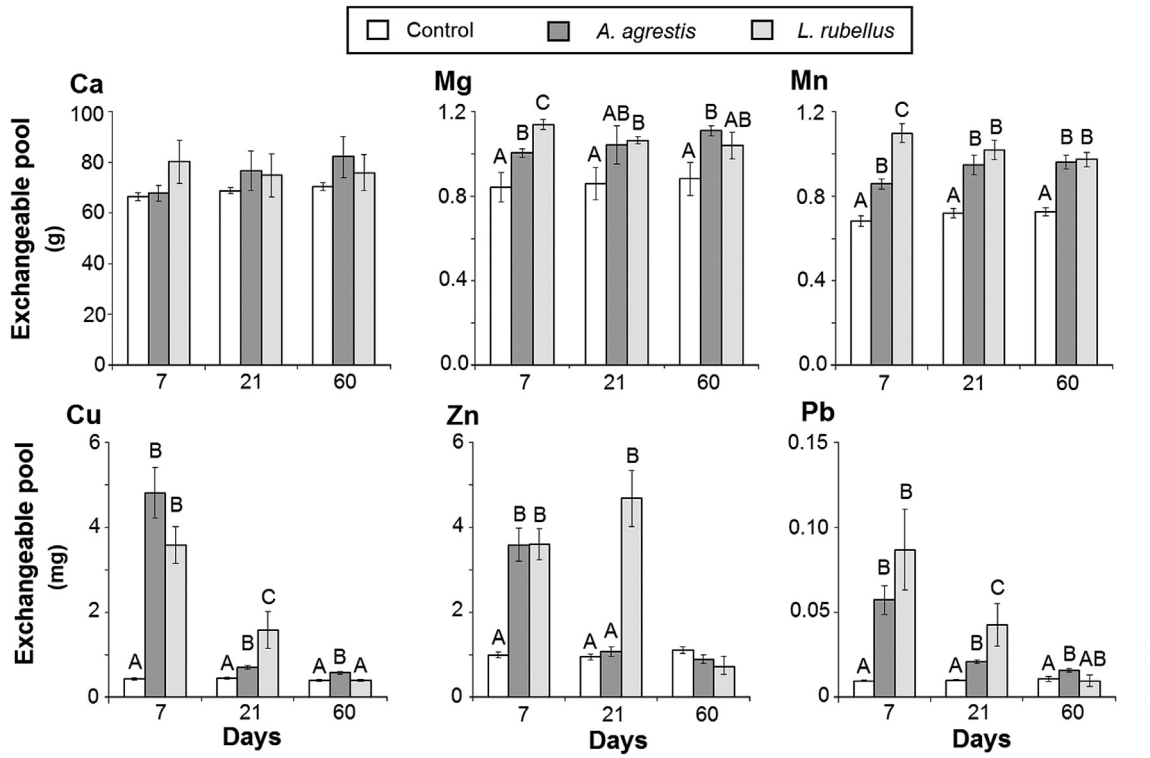

Fig. 2. Exchangeable pools of metals in each 0.5 kg soil column after 7, 21, and 60 days of earthworm residue decomposition. Letters (A, B, C) represent grouping by significant differences at the $p < 0.05$ level. Error bars are ±1 standard error.

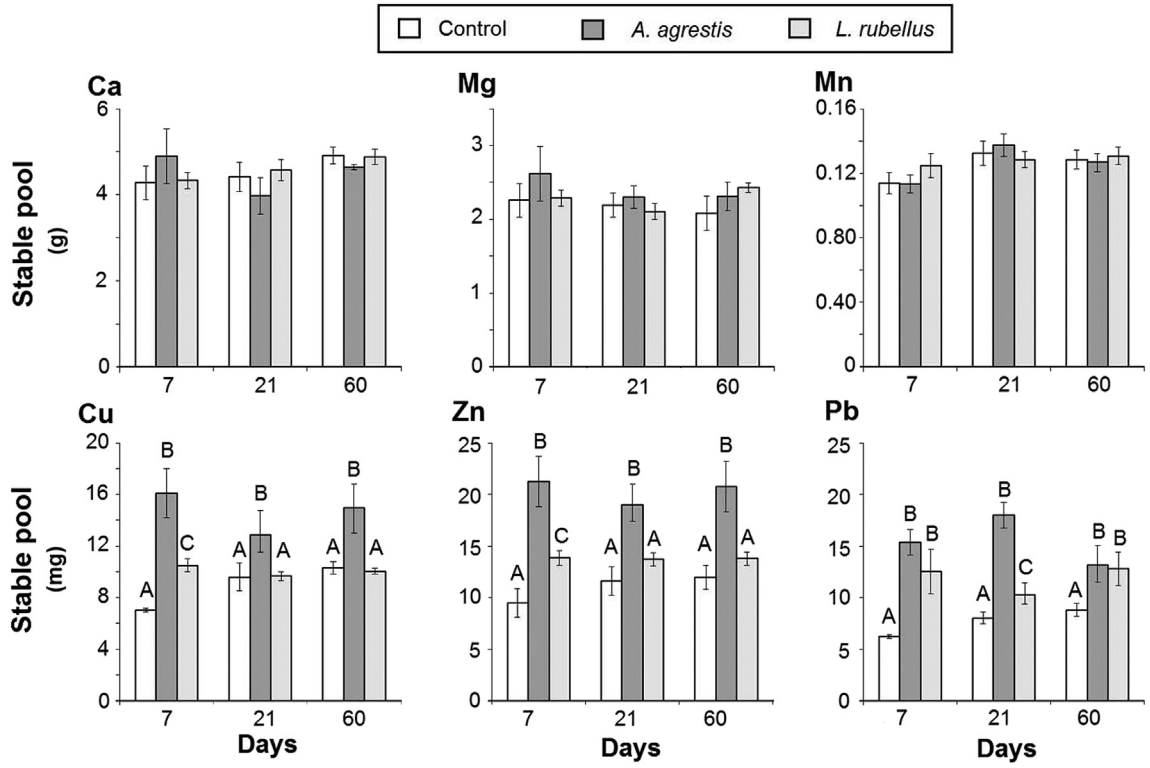

Fig. 3. Stable pools of metals in each 0.5 kg soil columns after 7, 21, and 60 days of earthworm residue decomposition. Letters (A, B, C) represent grouping by significant differences at the $p < 0.05$ level. Error bars are ±1 standard error.

soil matrix and earthworm residues were relatively closer in magnitude than Ca, Mg, and Mn. Thus, changes in Cu, Zn, and Pb

pools resulting from earthworm residues were easier to detect, such as the observed temporal trend in Cu, Zn and Pb. The decreasing trend in exchangeable pools of Cu, Zn, and Pb suggests that the metals within the earthworm residues were undergoing a biological or chemical change. It is unclear if the decreasing trend of exchangeable pools of Cu, Zn, and Pb was caused by stabilization in stronger sorption sites or potentially taken up by colonizing microorganisms, such as fungi and bacteria.

3.4. Mass balance

A mass balance was utilized to estimate the fate and mobility of metals added to the soil columns within earthworm residues. Control metal pools were subtracted from *A. agrestis* and *L. rubellus* soil column metal pools to determine the metal mass added from earthworm residues. This was calculated for the Day 60 cumulative leachate, Day 60 exchangeable and Day 60 stable metal pools. The differences between earthworm and Control pools were summed as the total metal added to the columns from earthworm residues as a mass balance (Table 3). The % relative distribution of the added metals from the earthworm residues were calculated for leachate, exchangeable, and stable pools (Table 3).

Our mass balance results agree with findings from studies of N in earthworm residues. Christensen (1988) and Whalen et al. (1999) observed that N from earthworm residues was primarily incorporated into the soil as exchangeable and non-extractable forms as opposed to leachable forms. The experiment conducted in this study show that >98% of metals from within earthworm residues are immobilized in soil during decomposition. Calcium and Mn from *A. agrestis* and *L. rubellus* were primarily added to the exchangeable fraction (≥80%) and only a small portion was lost as leachate (<2%)(Table 3). Magnesium and Pb from *A. agrestis* and *L. rubellus* were primarily added to the stable pools (>70%)(Table 3). Zinc and Cu differed between *A. agrestis* and *L. rubellus*; Zn and Cu were predominantly in the stable pools for *A. agrestis* but a greater fraction of Zn and Cu were in the exchangeable pools for *L. rubellus* (Table 3). Although dw biomass and metal concentrations were different, the mass balance estimated that *A. agrestis* did not add significantly more mass of metals to the soil columns than *L. rubellus*. We compared the metal mass estimated by the mass balance to expected metal mass calculated from only earthworm biomass and tissue concentrations. These results are given in Table 3 and show that the mass balance approach estimated 2–11 times greater metal masses than the earthworm biomass approach. It must be noted that the mass balance based upon exchangeable, stable, and leachate pools accumulated larger error due to the use of more variables than the earthworm biomass-based calculation and thus had greater standard deviations and standard errors (Table 3). In addition, the mass balance values were affected by the variation in the soil matrix chemistry. Homogenizing the soil column material can decrease the ability to detect differences due to earthworms (Görres et al., 1997). Despite these limitations, the mass balance provides an estimate of the distribution of metals and only a small fraction of the metals from the earthworm-residues were lost from the soil column in leachate over the 60 day experiment.

3.5. Micro X-ray fluorescence analyses

Earthworm-residues from *A. agrestis* within the thin-sections for μ-XRF were distinguishable from the soil matrix. The μ-XRF images in Fig. 4 are of typical boundaries of the interface between earthworm residue and the soil matrix. A void space was visible along most of the earthworm-soil matrix interface. The lyophilization processes or introduction of the epoxy resin caused the earthworm-tissue to contract and separate from the soil matrix. In addition, silt and clay sized particles have become entrapped on the surface and incorporated into the earthworm residues during decomposition. The use of C in the epoxy resin means earthworm residue cannot be distinguished using ionization energies for C. However, Ca is a common structural cation in organic molecules and served as an identifier of SOM derived from earthworm residue (e.g. Kaste et al., 2006).

The μ-XRF work of soil thin sections of soil columns containing *A. agrestis* revealed that earthworm residue contains metals either widely dispersed throughout or concentrated in discrete areas. Calcium, Cu, and Zn are distributed throughout earthworm residue in the Day 7 and Day 60 thin-sections images in Fig. 4. Manganese appears in a few isolated areas in the earthworm residue. The 60 day μ-XRF images show Ca, Cu, and Zn are present in the earthworm residue on Day 7 and Day 60 (Fig. 4). Linear regressions were applied to the μXRF images for each pixel to determine if metals were spatially correlated with each other in the entire image and only in the identified earthworm residue. Calcium, Cu, Mn, Fe, Si, and Zn were poorly correlated among each other using linear regressions with $r^2 < 0.25$ for all combinations for both the 7 Da y and 60 Da y μXRF images (Data not shown). Linear regressions were also weak when only focusing on earthworm residues rather than the bulk image (Data not shown). On the basis of their separate occurrences in the images, we suggest that Ca, Cu, Mn, and Zn in the earthworm tissues and mineral soil matrix do not appear to be driven by interferences with other metals, particularly Fe or Si.

3.6. Potential biological and inorganic processes

It is unclear if the metals from the earthworm residues were stabilized in the soil due to biological or inorganic processes. Metals can be stabilized in soil by bacteria and fungi, through uptake into living tissue (e.g. Baldrian, 2003), precipitation of insoluble compounds (e.g. Sayer and Gadd, 1997), or through adsorption to their derivative organic compounds (e.g. Toner et al., 2005). Although bacteria and fungi were not quantified, colonization of the earthworm residues by fungal hyphae was visible upon destruction of the soil columns from all collection periods. In addition, the upper left portion of the 60 Da y μXRF image may be a microorganism colonizing the earthworm residue. In addition to biological reactions, inorganic reactions may have potentially stabilized metals in the soil matrix. The pH of the soil and leachate were close to neutral, discouraging the dissolution and solubility of the metals. Although a significant difference in C concentration between Control and earthworm soil columns was not detectable, we observed in the μXRF images that many of the metals remain in the organic matter derived from the earthworm residues. Soil organic matter from the earthworm residues may also have increased the adsorption capacity of the soil. In the 7 and 60 day Cu and Zn μXRF images in Fig. 4, Cu and Zn are dispersed throughout the earthworm residue, indicating that they were not readily leached during the decomposition experiment. On the basis of the metal pools and μXRF images, we conclude metals were stabilized in the soil columns within earthworm residues.

The use of soil columns to determine the fate and transport of metals from earthworm residues was useful for quantifying leachates but had drawbacks in measuring changes in soil pools from earthworms and applying results to field conditions. Although our experiment utilized Bw horizons from a field site, the pH may be higher than many of the soils commonly found in forested areas of the northeastern United States. The soil pH of the soil matrix (pH 6.4 ± 0.2) was higher than most forest soils, which are mildly to strongly acidic (pH 4.0–5.5) depending on the soil horizon, parent material, and overlying forest vegetation (Pritchett and Fisher,

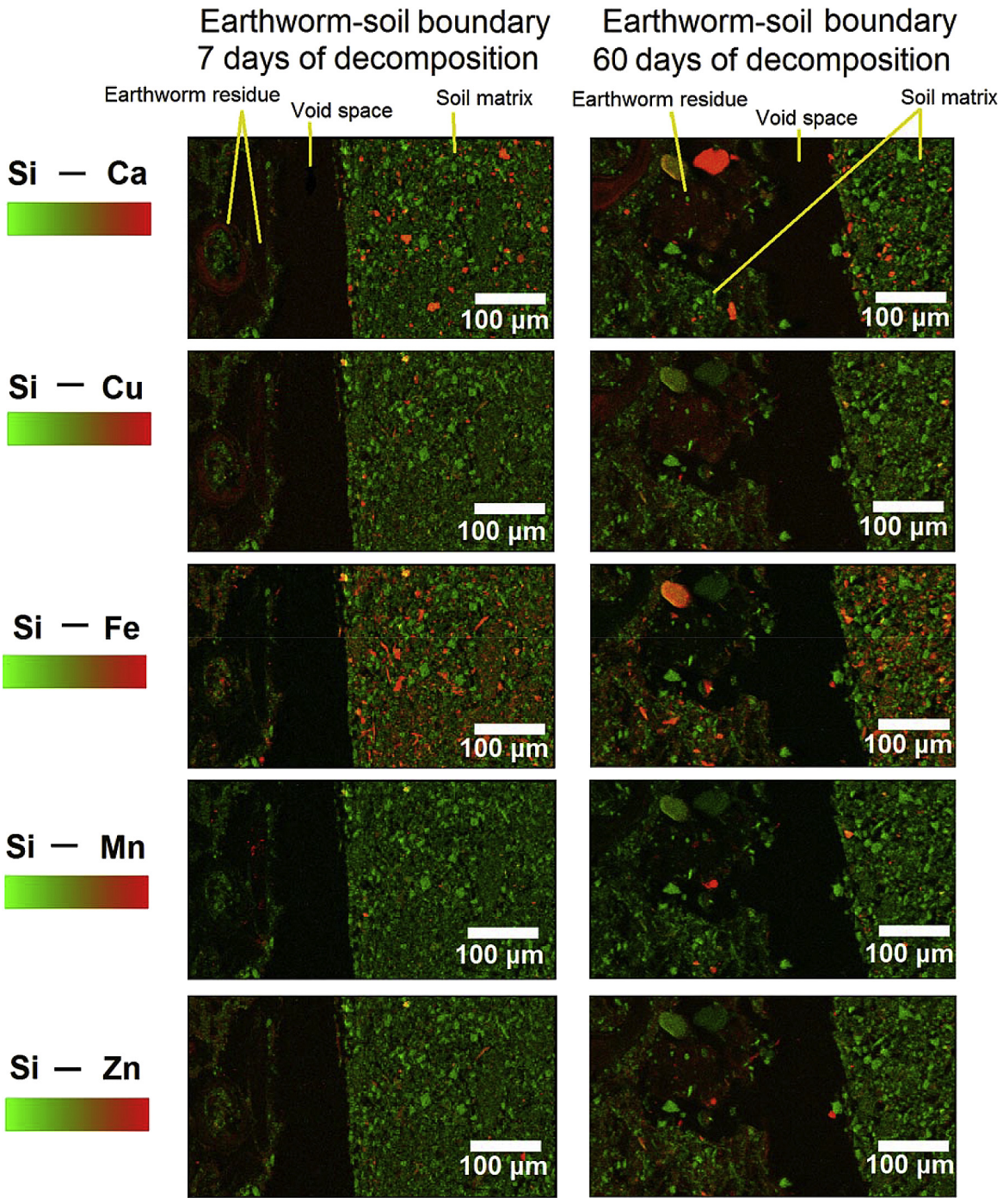

Fig. 4. Micro-XRF images of Ca, Cu, Fe, Mn, and Zn (in red) with Si (in green) in the earthworm-soil matrix interface after 7 and 60 days of earthworm residue decomposition.

1987). Lastly, the controlled conditions of the laboratory prevented the effect of soil dwelling organisms, particularly soil macro fauna and plant roots, from influencing the decomposition of the earthworm residues. Macrofauna and plant roots may play a substantial role in decomposition if the earthworm were to expire in the organic horizons or A horizons. Ion exchange for plant roots could increase the mobility of metals while the higher cation exchange capacity of A horizons may increase retention of metals. Future experiments conducted *in situ* in forested ecosystems may find greater exchangeability or leaching fraction due to lower pH and macrofauna and potentially flora can play a substantial role. In spite of these higher soil pH and laboratory conditions, our study provides the first experimental results showing metal immobilization in soil following earthworm mortality.

3.7. Conclusions

The overall goal of this study was to determine if metals in earthworm residues are mobilized or stabilized in soil. Although we observed significant increases in Ca, Mn, and Pb leaching, we reject our hypothesis that metals within earthworm residues are mobilized from the soil profile. Despite previous studies showing rapid

decomposition of earthworm tissues, we were able to identify their residues in the soil matrix. Moreover, we were able to identify Ca, Cu and Zn distributed in the earthworm residues. Lastly, our mass balance estimated that >98% of added metals were immobilized in soil as exchangeable and stable pools. We conclude that over the 60 day experiment, nutrient and pollutant metals from earthworms were stabilized within the soil matrix. The added Ca and Mn were primarily weakly stabilized in the soil and may be readily available for plants or leached from the soil profile. These results have implications for soil fertility and availability of metals in earthworm inhabited soils, but further experiments are required. Investigating the influence of soil microbial communities on metals in earthworm residues is warranted, both under laboratory and field conditions.

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