Comparison of mercury and methylmercury bioaccumulation in earthworms (*Bimastus parvus*) native to landfill-leachate-contaminated forest soil

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(Received February 22, 2018; Accepted May 7, 2018)

**ABSTRACT** — Total mercury (THg) and methylmercury (MeHg) bioaccumulation was explored in the *Bimastus parvus* species of earthworm (*B. parvus*) native to the leachate-contaminated forest soils around a Hg-polluted traditional landfill in Japan. General soil properties and concentrations of THg and MeHg in forest soils and in *B. parvus* were determined. The results indicated that the average THg concentrations in *B. parvus* and in forest soils in the leachate-contaminated sites were 10.21 and 14.90 times higher than those in the reference sites, respectively, whereas similar average MeHg concentrations were observed in forest soils (< 0.01 mg kg⁻¹) and in *B. parvus* (0.100-0.114 mg kg⁻¹) across all sampled sites. The average bioaccumulation factors of THg in *B. parvus* (BAF₉Hg) in forest soil were similar between the leachate-contaminated sites and the reference sites. Cluster and regression analyzes demonstrated that the *B. parvus* Hg (THg and MeHg) and soil THg were positively correlated with each other and with soil organic matter (SOM) and clays, but were negatively correlated with sand and hardly correlated with silts and pH in leachate-contaminated forest soils. From these results, it was proposed that Hg exposure to food chains is possible through *B. parvus*, because *B. parvus* showed a high ability to accumulate THg and MeHg in both leachate-contaminated and reference forest soils. Together, these findings indicated that the role of *B. parvus* in MeHg production is not clear, and it is possible that the MeHg in *B. parvus* was firstly formed within forest soils and then accumulated in their tissues.

**Key words:** Bioaccumulation, Mercury, Forest soil, Earthworms, Landfill

**INTRODUCTION**

Mercury can be accumulated and amplified in predatory animals and humans through the food chain, causing many hazardous health effects, such as neurotoxicity, mortality, and reproductive toxicity (Scheuhammer *et al*., 2007). It is also considered one of the most toxic nonessential contaminants to human health and the environment (Buch *et al*., 2017). Hence, the level of Hg in the environment has been controlled by many countries and international organizations, such as the World Health Organization (WHO), United Nations Environment Programme (UNEP), and Food and Agriculture Organization (FAO) (Ding *et al*., 2007).

Earthworms are considered useful bio-indicators for assessing soil health and quality in numerous studies of contaminated sites and in laboratory experiments (Nannoni *et al*., 2011; Teršič and Gosar, 2012). They spend most of their life in soil and play an important role in soil structure maintenance; approximately 80% of soil fauna biomass is constituted by earthworms in terrestrial ecosystems (Buch *et al*., 2017). Earthworms can be sampled easily, have proven to be a good biological model, and can accumulate trace metals from soil through direct dermal contact and/or diet uptake by gut ingestion from soils. Also, Hg accumulation in earthworms has been
demonstrated in many studies (Burton et al., 2006). It has been generally accepted that inorganic forms of Hg can be methylated by soil bacterial activity (mostly under anaerobic conditions); methylation enhances bioavailability and bio-toxicity and increases potential threats to soil ecosystem functions (Shao et al., 2012). However, recent studies have found that inorganic Hg is methylated within the guts of earthworms and not by soil bacterial action, based on the fact that sulfate-reducing bacteria were detected in the gut tract of earthworms (Kaschak et al., 2014; Rieder et al., 2013).

Globally, up to 70% of municipal solid waste (MSW) is disposed of in landfills, and Hg is inevitably and unavoidably deposited in traditional landfills via a variety of Hg-containing MSW, such as household batteries, devices, lamps, mirror coatings, and thermometers (Zhu et al., 2013; Li et al., 2010). About 1,010-4,070 tons of Hg are discharged into the environment every year from natural sources, such as volcano eruptions, forest fires, and re-emission from surface soils and oceans, and from anthropogenic sources, such as ore mining, fossil fuel combustion, and MSW (United Nations Environment Programme, 2013). In 2005, it was estimated that approximately 8% of total anthropogenic Hg emissions have been contributed by disposal procedures in landfills and waste utilization (Gworek et al., 2015; Zhu et al., 2013). Hg-containing components in MSW can enter and impact surrounding groundwater, soil, and air through Hg-contaminated leachates and gas emissions after traditional burial (Cheng and Hu, 2012). Large amounts of Hg have been found in inorganic forms, such as Hg, HgCl₂, HgS, and HgO, in leachates and gases from MSW landfills. Furthermore, inorganic Hg can be methylated by soil bacterial action, which causes high toxicity to surrounding ecosystems under different conditions (Cheng and Hu, 2012; Earle et al., 1999; Lee et al., 2016). Similar to landfills in other countries over the last century, about 70% of MSW landfills in Japan were built into mountainsides using the natural space between ridges to limit land usage (Tanaka et al., 2005). It has been reported that between 1976 and 1979, only 40% of traditional landfills were constructed with a liner system for preventing leachate leaks; this increased to 100% for landfills constructed in 1990 (Tanaka et al., 2005). Meanwhile, the double-liner system for preventing leachate leaks became mandatory for new Japanese landfills after 1997 (Tanaka et al., 2005). Therefore, the potential leachate pollution caused by those traditional landfills without anti-leaching systems have been a societal concern (Moody and Townsend, 2017).

Forest soil is essential in biological cycling of forest ecosystems. Previous studies have shown that forests can deposit large amounts of trace metals, including Hg, through canopies from atmospheric aerosols; Hg can reach the forest soil through litterfall or rain (Driscoll et al., 2007; Gong et al., 2014). Atmospheric Hg accumulation in litter and soil in tropical forests has been reported in recent research (Luo et al., 2015; Siudek et al., 2016). Generally, Hg is relatively immobile because it binds with organic matter and accumulates in the top layers of soil (Ravichandran, 2004). To our knowledge, limited studies have focused on the assessment of risks from total mercury (THg) bioaccumulation by earthworms in forest soil environments, and only a few reports determined the bioaccumulation of methylmercury (MeHg) (Ernst et al., 2008; Rieder et al., 2011). There have been no studies on Hg accumulation in earthworms from Hg-containing leachate-contaminated soils around traditional municipal landfills, particularly forest soils. Therefore, exploring the Hg (THg and MeHg) accumulation in native earthworms in forest soils will improve our understanding of the risks of secondary Hg poisoning to predators, such as birds and mammals, from earthworms in forest ecosystems (Dang et al., 2015), especially in Hg-containing leachate-contaminated terrestrial ecosystems.

This study investigated the current status of Hg (THg and MeHg) accumulation in native earthworms and forest soils around a traditional Hg-polluted MSW landfill compared to that of non-contaminated reference sites. The focus was on the characteristic relationships and impacts of Hg (THg and MeHg) levels in Bimastus parvus earthworms and general soil properties of Hg-containing leachate-contaminated forest soils compared to Hg background levels in uncontaminated forest soils.

**MATERIALS AND METHODS**

**Sampling sites and sample preparation**

A traditional MSW landfill was built on forested mountainsides of Mt. Sanpou (32.876°N, 129.743°E) in suburban Nagasaki (north-west of Kyushu, Japan) in 1975. Large amounts of traditional MSW that might have contained Hg, such as incinerated ash, industrial wastes, and municipal sludge, were buried in this landfill between 1975 and the early 1990s (Earle et al., 1999; Jung et al., 2004). A large flood in Nagasaki in 1982 enhanced the Hg pollution in the area by bringing in mixed Hg-contaminated municipal garbage, such as damaged fluorescent lights, switch devices, consumer electronics, and household products. A leachate-contaminated forest zone gradually formed about 500 m below the landfill. Previous investigations indicated that THg concentrations in the leachate-contaminated forest soil ranged from
0.81 mg kg\(^{-1}\) to 8.48 mg kg\(^{-1}\), which was about 13-140 times higher than natural background levels of Hg (0.06 mg kg\(^{-1}\)) in 1998 (Arisawa et al., 2000, Commission of Water Environment Protection and SanPouZan, 2017). All sampling sites for earthworms and soils were located in the surface of the landfill-leachate-contaminated soil along the ditches and in small, scattered puddles formed by leachate and rainwater, which were in relatively soft and wet areas (Fig. 1). Earthworms and 2 kg soil samples (S1-S13) were collected from each site. The earthworms were found after digging in a 0.25 m\(^2\) area at a depth of 0-15 cm. Another mountainside with similar natural environmental properties, which was about 1.6 km from the contaminated study site, was selected as the reference site (R1-R3) for natural background Hg levels. The contaminated and reference sites were densely covered by a canopy of local deciduous forest.

Earthworms were maintained in the dark at 22 ± 2°C without feeding for 3 days, until they emptied their gut contents. Mature earthworms with obvious developed clitella were classified by site and species, and the identification was combined with morphologic characteristics (e.g., clitellum shapes, tubercula pubertatis, genital, ventral view, tail, setal pairing arrangements, color, size, etc.) and ecological distribution information in accordance with earthworm monographs (Minamiya, 2014; Sims and Gerard, 1985). Thereafter, the earthworms, with lengths ranging between 4.5 cm and 5.5 cm, were lyophilized and crushed into a fine powder using an agate pestle, and then stored at -20°C until analysis. The soil samples were air-dried to constant dry weight (dw) at laboratory conditions of 25 ± 3°C and approximately 40% humidity. Earthworms and soil samples were gently ground into a fine powder with an agate mortar and pestle, and soil powder was sieved through a 2 mm nylon mesh.

**Analyses**

**General soil properties**

General soil properties that might affect Hg accumulation in *B. parvus* were analyzed. Soil organic matter (SOM) was analyzed by determining the loss on ignition (LOI) at 550°C for 2 hr (ISUZU AT-S13) (Heiri et al., 2001). Soil pH was measured in 0.01 M CaCl\(_2\) with an extractant ratio of 1:2 v/v (soil-liquid) using a pH meter (Eutech instruments Cyberscan, pH 1100) (Xu et al., 2014). The soil particle size distributions (PSD) were classified as sands (0.050 mm < % < 2 mm), silts (0.002 mm < % < 0.050 mm), and clays (% < 0.002 mm) (Kettler et al., 2001). All analyses were tested in triplicate at minimum.

**Extraction of mercury and methylmercury**

THg and MeHg bioaccumulation of earthworms in landfill-leachate-contaminated soil

THg and MeHg extractions from earthworms were performed based on the reported protocol for biological tissue (Miyamoto et al., 2010; Yoshimoto et al., 2016). For THg extraction, an aliquot of approximately 0.1 g (dw) of each earthworm sample was weighed in 15 mL polypropylene (PP) tubes with screw caps, in triplicate. First, the samples were hydrolyzed by adding 0.1% L-Cys solution (1.0 mL) and 5 M NaOH (1.0 mL). Samples were placed in a water bath (80°C) for 1 hr with vortexing every 10 min until incubating hydrolysis was completed. Samples were then cooled in the hydrolyzed tubes to room temperature (22 ± 2°C) in tap water. Second, the hydrolyzed sample solution was adjusted to 5 mL with ultrapure water and degreased for 10 min with 6 mL methyl isobutyl ketone (MIBK) and a shaking machine (220 rpm). The upper MIBK suspension was removed after 10 min of centrifugation (3,000 rpm); the residual MIBK was eliminated by adding and removing 5 mL of hexane after shaking (5 min, 220 rpm) and centrifugation (5 min, 3,000 rpm), and the final liquid was used for THg determination in earthworms.

For MeHg extraction, a 2 mL solution of the THg extraction was added to 2 mL HBr (5 M), 0.5 mL CuCl\(_2\), and 6 mL toluene, followed by shaking (10 min, 220 rpm) and centrifuging (10 min, 3,000 rpm). Then, 5 mL of the upper toluene supernatant was collected into a new 15 mL PP tube; 1 mL of the mixed solution was added (0.2% L-Cys and 2% NaOAc) to the toluene extract and then shaken (5 min, 220 rpm) and centrifuged (5 min, 3,000 rpm). After removing the upper toluene, the final liquid was used for MeHg determination in earthworms.

**Mercury determination, quality control (QC), and quality assurance (QA)**

A direct thermal decomposition Hg analyzer (MA-3000, Nippon Instruments Corporation, Tokyo, Japan) was used to analyze soil samples for THg concentration and earthworm extracts for THg and MeHg concentrations. The instrument uses thermal decomposition, gold amalgam collection, and cold vapor-atomic absorption spectroscopy. The concentration of total Hg in the sample was determined by direct preparation and placement into sample determining boats. Soil MeHg was detected using the electron capture detector gas chromatography (GC-ECD) method used by Japan Food Research Laboratories.

All reagents used were of analytical grade or higher. All containers used in the analytical procedures were soaked in 10% HNO\(_3\) for 24 hr, rinsed three times in deionized water, and dried before use. Hg concentrations in all reagents and blanks were always below the detec-
tion limits for each relevant analytical procedure.

All recovery tests for the extraction procedures for THg and MeHg were conducted using certified reference materials (CRM) from swordfish 7403-a (CRM165) and codfish 7402-a (CRM250). Digestion of CRM using this extraction procedure on replicates (n = 6) showed a MeHg recovery of 98.2 ± 3.7% (i.e., 4.910 ± 0.190 mg kg⁻¹) for CRM165 (i.e., 5.000 ± 0.220 mg kg⁻¹) with a coefficient of variation (CV) of 3.79%, and 96.9 ± 2.2% (i.e., 0.550 ± 0.010 mg kg⁻¹) recovery for CRM250 (i.e., 0.580 ± 0.020 mg kg⁻¹) with a CV of 2.32%. CRM165 (5.340 ± 0.140 mg kg⁻¹) showed a THg recovery of 97.6 ± 4.3%
THg and MeHg bioaccumulation of earthworms in landfill-leachate-contaminated soil

Table 1. Sampling locations, earthworm species, and general soil properties in the study.

| Site | Longitude (°E) | Latitude (°N) | Earthworm species (Count) | pH | SOM(%) | Soil PSD |
|------|----------------|---------------|---------------------------|----|--------|----------|
|      |                |               |                           |    |        | Sand (%) | Silt (%) | Clay (%) |
| Leachate contaminated sites | | | | | | | | |
| S1   | 129.7435       | 32.8743       | B. parvus (3)             | 5.57 | 4.82  | 52.64    | 33.60    | 13.76    |
| S2   | 129.7437       | 32.8743       | B. parvus (13)            | 4.58 | 8.82  | 49.18    | 18.16    | 32.66    |
| S3   | 129.7434       | 32.8739       | B. parvus (43)            | 4.55 | 17.59 | 28.27    | 23.14    | 48.59    |
| S4   | 129.7438       | 32.8738       | B. parvus (60), D. octaedra (1) | 4.82 | 9.15  | 32.62    | 21.93    | 45.45    |
| S5   | 129.7435       | 32.8738       | B. parvus (35), D. octaedra (2) | 4.91 | 9.39  | 49.94    | 18.37    | 31.69    |
| S6   | 129.7440       | 32.8737       | B. parvus (38)            | 4.94 | 8.98  | 53.92    | 24.32    | 21.76    |
| S7   | 129.7441       | 32.8738       | B. parvus (20)            | 4.84 | 6.99  | 60.73    | 15.19    | 24.08    |
| S8   | 129.7435       | 32.8734       | B. parvus (21)            | 5.15 | 10.92 | 46.19    | 12.90    | 40.91    |
| S9   | 129.7433       | 32.8736       | B. parvus (22)            | 4.82 | 13.52 | 38.30    | 20.74    | 40.96    |
| S10  | 129.7433       | 32.8733       | B. parvus (24)            | 4.63 | 15.29 | 37.17    | 15.86    | 46.97    |
| S11  | 129.7434       | 32.8731       | B. parvus (18)            | 4.18 | 13.42 | 30.95    | 22.35    | 46.70    |
| S12  | 129.7436       | 32.8730       | B. parvus (17)            | 4.08 | 6.20  | 55.99    | 23.84    | 20.17    |
| S13  | 129.7437       | 32.8736       | B. parvus (25)            | 4.96 | 5.41  | 59.15    | 19.43    | 21.42    |
| Ave. |                |               |                           | 4.77 | 10.04 | 45.77    | 20.76    | 33.47    |
| Reference sites | | | | | | | | |
| R1   | 129.7533       | 32.8857       | B. parvus (27), D. octaedra (6) | 4.58 | 9.78  | 45.37    | 25.32    |
| R2   | 129.7545       | 32.8840       | B. parvus (17)            | 5.21 | 8.76  | 5.21     | 38.19    | 29.46    |
| R3   | 129.7551       | 32.8839       | B. parvus (19), D. octaedra (9) | 4.76 | 10.55 | 37.93    | 28.31    |
| Ave. |                |               |                           | 4.85 | 9.70  | 40.50    | 27.70    | 31.81    |

Note: 1. SOM: soil organic materials. 2. Soil PSD: soil particle size distribution (Sands: 0.050 < % < 2 mm, Silts: 0.002 < % < 0.050 mm, Clays: % < 0.002 mm). 3. Ave.: average.

Data statistical analysis

Bioaccumulation factors (BAFs) were calculated as Hg (THg and MeHg) concentrations in earthworm tissues divided by the corresponding Hg (THg and MeHg) concentrations in forest soils (BAF_{THg} and BAF_{MeHg}). All data were presented as mean ± standard deviation (SD), except for the general forest soil properties, which were only presented as means. Data were analyzed using Statistical Product and Service Solutions (SPSS) version 21.0, and Excel 2016 was used for data statistical treatment. Statistical analyses included one-way ANOVA tests, or the non-parametric Kruskal-Wallis test if the data had a non-normal distribution. Hierarchical cluster, correlation, and linear regression analyses were used for evaluating the relationships between general soil properties and Hg (THg and MeHg) concentrations in earthworm tissues and forest soils. Less than 0.05 was considered the level of statistical significance (p value) in all statistical treatments of the data. Figures were generated using GraphPad Prism version 6.04 and SPSS 21.0.

RESULTS

Characteristics of forest soils and native earthworms

The physicochemical characteristics of forest soil for all samples are summarized in Table 1. The measured pH ranged from 4.08 to 5.57 (mean: 4.77) in leachate-contaminated soils, which were similarly acidic to the reference soils, whose pH ranged from 4.58 to 5.21 (mean: 4.85). SOM was slightly higher in the leachate-contaminated soils, which varied between 4.09% and 17.59% (i.e., 5.210 ± 0.170 mg kg⁻¹) with a CV of 4.4%, and CRM250 (i.e., 0.610 ± 0.020 mg kg⁻¹) showed a recovery of 98.01 ± 2.9% (i.e., 0.580 ± 0.020 mg kg⁻¹) with a CV of 2.95%. Therefore, we used 97.5% (the mean CRM MeHg recovery for swordfish and codfish) as the correction factor for calculating the concentration of MeHg extracted from earthworm samples (Yoshimoto et al., 2016; Miyamoto et al., 2010). The recovery tests for soil THg were measured using CRM lake sediments 7303-a (CRM E-0190) and marine sediments 7302-a (CRM A-0210). The mean (n = 6) THg recovery was 96.8 ± 5.3% (i.e., 0.065 ± 0.004 mg kg⁻¹) for CRM E-0190 (i.e., 0.067 ± 0.006 mg kg⁻¹) with a CV of 5.51%, and 96.7 ± 3.1% (i.e., 0.503 ± 0.160 mg kg⁻¹) for CRM A-0210 (i.e., 0.520 ± 0.030 mg kg⁻¹) with a CV of 3.19%. All CRM for this study were purchased from the National Metrology Institute of Japan (NMIJ).
The highest THg concentrations (2.750 ± 0.264 mg kg\(^{-1}\) dw, respectively) in leachate-contaminated forest soils, respectively. Three sites (S3, S8, and S10) showed the highest earthworm BAF\(_{\text{THg}}\) values of 11.65, 10.56, and 5.83 in reference soils. Sites S2, S5, and S7 had the top three highest earthworm tissue THg concentrations were found in the soils with the corresponding maximum THg concentrations (6.775 ± 0.171 mg kg\(^{-1}\) dw for S3, 6.168 ± 0.065 mg kg\(^{-1}\) dw for S8, and 6.551 ± 0.268 mg kg\(^{-1}\) dw for S10) in the leachate-contaminated forest sites (Fig. 2a). The dendrogram branches in the cluster analysis indicated that the THg and MeHg concentrations in \(B.\ parvus\) tissues were correlated with each other and with soil THg, SOM, and clays, and were not correlated with the soil pH, sands, and silts in leachate-contaminated forest soil (Fig. 3). Further linear regression results also indicated that there were close relationships between earthworm THg and MeHg concentrations and forest soil THg from the leachate-contaminated sites (Figs. 4a, 4b, and 4c).

Mercury accumulation in earthworm tissues
The measured \(B.\ parvus\) BAF for forest soil THg contained a wide range of values, varying between 2.04 and 11.65 in leachate-contaminated soils and between 4.17 and 5.83 in reference soils. Sites S2, S5, and S7 had the highest earthworm BAF\(_{\text{THg}}\) values of 11.65, 10.56, and 11.55, respectively, in the leachate-contaminated forest soils compared to the values of other study sites (Fig. 5a). The mean BAF\(_{\text{MeHg}}\) of \(B.\ parvus\) in the leachate-contaminated forest sites (5.42) was slightly higher than that of the reference sites (mean: 4.91) (Fig. 5a). The ratio of MeHg to THg (MeHg/THg %) in \(B.\ parvus\) ranged between 2.04% and 3.33% (mean: 2.59%) in leachate-contaminated soils, which was significantly lower than the range (MeHg/THg %) in reference soils, which
THg and MeHg bioaccumulation of earthworms in landfill-leachate-contaminated soil

**DISCUSSION**

Mercury concentrations in forest soils and native earthworms

Most studies on soil THg have been conducted in polluted sites, and have found a wide range in concentration of THg, from a few milligrams to several thousand milligrams per kilogram of soil (Fernández-Martínez et al., 2015; Reis et al., 2009). According to a survey of environmental Hg levels conducted in 18 prefectures of Japan, the residual soil THg concentrations varied between 0.002 mg kg⁻¹ and 78.6 mg kg⁻¹ (Nakagawa, 2008). However, it is difficult to find studies analyzing THg and MeHg concentrations in earthworms from MSW leachate-contaminated forest soils in the literature; the general consensus of previous studies is that atmospheric Hg deposition is the major source of Hg in forest soils (Rieder et al., 2011, Buch et al., 2017).

Our results indicated that THg concentrations in the reference forest soil surface varied between 0.074 mg kg⁻¹ and 0.103 mg kg⁻¹ dw, with a mean value of 0.087 mg kg⁻¹ dw. These were similar to measurements from forest soils in Switzerland, which varied between 0.07 mg kg⁻¹ dw and 0.55 mg kg⁻¹ dw (Rieder et al., 2011, Buch et al., 2017).
et al., 2011), and soils in Brazil, which varied between 0.02 mg kg\(^{-1}\) and 0.15 mg kg\(^{-1}\) (Buch et al., 2017, 2016). The concentrations were lower than those from soils in French Guiana, which were between 0.25 mg kg\(^{-1}\) and 0.55 mg kg\(^{-1}\) (Da Silva et al., 2016). THg concentrations in landfill-leachate-contaminated forest soils varied between 0.227 mg kg\(^{-1}\) dw and 2.919 mg kg\(^{-1}\) dw, and were significantly higher (approximately 15 times higher) than the background values of THg in uncontaminated forest soils. Higher THg concentrations were correlated with higher SOM content in leachate-contaminated forest soils, which was consistent with other reports (Buch et al., 2017; Rieder et al., 2011). This observation may be explained by SOM having a strong affinity for binding with Hg, especially for SOM containing thiol groups (Skyllberg and Drott, 2010). In addition, soil clays were correlated with THg concentrations and SOM, and these relationships have also been found in previous reports (Buch et al., 2017; Soares et al., 2015). In contrast, the pH value had no significant correlation with soil THg contents in leachate-contaminated forest sites, potentially because of the relatively narrow and proximate pH range (4.08-5.57); previous reports showed that SOM had better affinity to Hg when the pH values ranged between 4 and 5 in tropical forest soils (Buch et al., 2017). This acidity might cause most Hg to accumulate in organic matter and

Fig. 3. Hierarchical cluster analysis between the determined soil THg, Hg (THg and MeHg) concentrations of B. parvus earthworms and soil properties (Soil organic materials (SOM), Clay, Silt, Sand, pH) in the leachate-contaminated forest soils.

Fig. 4. Bivariate relationships of regression analyzing among Hg concentration in soil and tissue of native earthworm, (a) Between soil THg and B. parvus earthworm THg, (b) Between soil THg and B. parvus earthworm MeHg, (c) Between THg and MeHg in B. parvus earthworms.
clays of the surface forest soils, which also represents the main food source for most soil dwelling fauna, including earthworms (Meili et al., 2003).

However, there has been limited understanding of the influence of Hg (THg and MeHg) from leachate-contaminated soils on soil dwelling organisms. Hg limits of 0.13 mg kg\(^{-1}\) in soils and 3.3 mg kg\(^{-1}\) in organic matter have been recommended as guidelines; it is assumed that Hg poses no harm to soil organisms below these critical levels (Tipping et al., 2010). Based on this critical limit, THg concentrations of all leachate-contaminated forest soils (13) exceeded this guideline, while THg concentrations at three reference sites were below this reference guideline.

THg, especially MeHg, in earthworms has the potential for bio-magnification in natural food chains of earthworm predators, such as birds, reptiles, and other invertebrates (Han et al., 2012). The results in this study indicated that the THg concentrations in B. parvus tissues in leachate-contaminated forest soils (1.242-6.775 mg kg\(^{-1}\) dw) were significantly higher than those in reference forest soils (0.350-0.490 mg kg\(^{-1}\) dw), and mean concentrations of MeHg in B. parvus tissues were similar to those in both the leachate-contaminated and uncontaminated sites (0.114 mg kg\(^{-1}\) dw and 0.104 mg kg\(^{-1}\) dw, respectively). Standards have not been published for Hg in earthworm tissues. Hg transfer coefficients in the food chain are 0.88 and 2.35 in herbivorous/omnivorous and carnivorous invertebrates, respectively, as proposed by Canadian soil quality guidelines (Buch et al., 2017). Accordingly, the acceptable earthworm Hg limit would be below approximately 0.2 mg kg\(^{-1}\), referencing the fish Hg limit of 0.5 mg kg\(^{-1}\) (WHO, 2008). Therefore, it cannot be ignored that Hg will have potential effects on the food chain via earthworms native to leachate-contaminated forest soil ecosystems.

**Mercury accumulation in native earthworms**

The current results demonstrated that B. parvus can accumulate Hg in their tissues from leachate-contaminated or uncontaminated forest soil, as the average BAF values for soil THg were similar in all sites, which were 4.91 (reference forest soils) and 5.42 (leachate-contaminated soils). The BAF\(_{\text{THg}}\) varied between 2.04 and 11.65 in leachate-contaminated forest soils, which was close to the BAF\(_{\text{THg}}\) found for four endogeic species of earthworm (between 3 and 15) reported in Swiss forest soils (Rieder et al., 2011). However, it is generally accepted that the assimilated accumulation of soil inorganic Hg is much lower in earthworms and varies across species in many studies. The BAF\(_{\text{THg}}\) of *Diplocardia* earthworm species varied between 0.47 and 1.75 in floodplain soil (Han et al., 2012); the BAF\(_{\text{THg}}\) of three earthworm species (*D. alpina*, *A. hirudinacea*, and *L. hoffmeisteri*) varied between 0.040 and 0.539 in soils polluted by chloralkali and smelting industries (Zhang et al., 2009); and *Eisenia fetida* in dredge spoil soils had a BAF value of 0.4 (Edwards et al., 1998). These previously measured BAF\(_{\text{THg}}\) values were different from our results, most likely due to different geochemical characteristics and earthworm species in forest soils. For example, Hg accumulation in earthworms is influenced by SOM contents, the soil components for binding Hg, and earthworm exposure time or life expectancy (Buch et al., 2017; Dang et al., 2015; Han et al., 2012).

Regarding MeHg accumulation, previous studies have found that BAF\(_{\text{MeHg}}\) values for earthworms were higher than BAF\(_{\text{THg}}\) values (Rieder et al., 2011). Unfortunate-
lly, we were not able to accurately calculate the value of BAF_{MeHg} in B. parvus because all soil MeHg concentrations were below the detection limit (< 0.01 mg kg\(^{-1}\) dw). Therefore, MeHg accumulation in B. parvus in this study cannot be quantitatively compared with other reports. MeHg concentrations in soils were low, and the relative contents ranged from 0.5% to 1.5% of soil THg in previous studies (Cristol et al., 2008; Rieder et al., 2011). Referencing this ratio of MeHg, we extrapolated from soil THg to estimate MeHg (0.001 mg kg\(^{-1}\) to 0.044 mg kg\(^{-1}\)) and BAF_{MeHg} (between 3 and 62) of B. parvus in this study. These estimated results were consistent with the BAF_{MeHg} values measured in other earthworm species (between 10 and 249) for soil MeHg (Burton et al., 2006; Rieder et al., 2011; Zhang et al., 2009), and were about 20 to 150 times higher than the BAF_{MeHg} in earthworms (Zhang et al., 2009). This difference may be due to MeHg being highly lipophilic, which makes it easier than inorganic Hg to be absorbed into earthworm bodies (Rieder et al., 2011). MeHg also has a strong adsorption tendency with organic matter, maintains high solubility for accumulation by invertebrates, and can be magnified along food chains (Ernst et al., 2008). In addition, the long lifespan of earthworms, usually from a few months to eight years, provides conditions for biological enrichment of soil Hg in their bodies (Han et al., 2012). The role of B. parvus as a bioindicator in Hg-contaminated environmental surveillance was proposed in this study. This study also showed that B. parvus has a high ability to accumulate MeHg (or THg) while reducing soil MeHg (or THg) contents in both leachate-contaminated and reference forest soils. Considering earthworms can be as an important protein source for predators in the ecosystem (e.g., birds, fish, reptiles, insects, and many mammals) (Dang et al., 2015). Thus, it was proposed that B. parvus can be a significant source of MeHg (or THg) exposure for food chains, which cannot be ignored.

**Mercury methylation characteristics**

It is normally accepted that inorganic forms of Hg can be methylated by soil bacterial activities, which enhances the bioavailability and bio-toxicity of Hg, and increases the potential threat of Hg to terrestrial organisms in soil ecosystems (Dang et al., 2015). However, recent research has indicated that this may not be universally true. Earthworms (Eisenia fetida) were found to potentially methylate Hg in their digestive tract (Hinton and Veiga, 2002). In addition, earthworms (Lumbricus terrestris) showed MeHg synthesis in vivo due to the sulfate-reducing bacteria found in earthworms but not in soils (Rieder et al., 2013). In our study, MeHg concentrations in the forest soil and native B. parvus earthworms were expected to increase due to the higher soil THg concentrations in the leachate-contaminated forest sites compared to the reference sites. Unexpectedly, no significant differences in MeHg concentrations were found between the leachate-contaminated soils and the reference soils (< 0.01 mg kg\(^{-1}\) dw in all sampled forest soil), or between the average MeHg concentrations in B. parvus tissues (0.114-0.104 mg kg\(^{-1}\)).

In addition, the ratio of MeHg to THg in B. parvus varied between 2.04% and 3.33% in leachate-contaminated forest soils, which was significantly lower than that of B. parvus in the uncontaminated forest soils, which ranged between 20.70% and 33.71%, because of the different THg concentrations in the forest soils. The ratio of MeHg to THg in B. parvus from leachate-contaminated forest soils was similar to the reported value of 3.01% in *D. fi* earthworms (Zhang et al., 2009), but was below the range of 5.7% to 10.1% found in seven different forest earthworm species (Rieder et al., 2011) and below the ratio of 12.02% found in *A. oblongopora* earthworms (Zhang et al., 2009). The similar MeHg levels found among B. parvus tissues in the contaminated forest soils and reference forest soils might have been due to the similar MeHg levels in the forest soil of them (< 0.01 mg kg\(^{-1}\) dw). The results support the proposal that MeHg was firstly formed by inorganic Hg being methylated in the forest soil, which was then ingested and accumulated in B. parvus via the lipophilic action, rather than that the MeHg was formed in the digestive tracts of B. parvus containing MeHg synthesizing bacteria (e.g., sulfate-reducing bacteria) (Rieder et al., 2013; Shao et al., 2012; Lee et al., 2016). The later will lead to more MeHg synthesized and accumulated in B. parvus as the average THg concentrations in forest soils and earthworms in leachate-contaminated soils were significantly higher than those in uncontaminated forest soils (about 14.9 times and 10.2 times higher, respectively). Another possibility was that the B. parvus had similar bioaccumulation factors for soil MeHg between the contaminated and reference sites. The similar BAF_{MeHg} of B. parvus between the contaminated forest sites and reference forest sites supported this possibility (Fig. 5a), even if we could not calculate the BAF_{MeHg} of B. parvus accurately. It was believed that B. parvus accumulated similar MeHg levels from both leachate-contaminated and reference forest soils in our study. Therefore, we reasoned that the *in vivo* MeHg biosynthesis of B. parvus was not obvious in our current investigation results.

But an inescapable problem is that the Hg methylation process within B. parvus could not be completely ruled out, and further studies are needed to address the poten-
tial sources of MeHg accumulation in B. parvus. Also, it is worth to investigate the underlying reasons of some of the uncertainties. First, there were uncertainties about the amount of mobile Hg (Hg⁺) both in the leachate-contaminated soils and the reference forest soils. Ideally, Hg exists in soils in different chemical forms with different mobility and biotoxicity to organisms (Issaro et al., 2009). Previous reports indicated that THg concentration in soil cannot completely represent the biological behavior in ecosystems (Issaro et al., 2009; Pinedo-Hernández et al., 2015), and Hg biotoxicity effects are mainly determined by the actual amount of soluble Hg (e.g., HgCl₂) (Zagury et al., 2006), because the soluble soil-Hg fractions were high mobility and potential bioavailability compared with other stable or low soluble soil-Hg fractions (Bloom et al., 2003). That is why soluble Hg (e.g., HgCl₂) is often used for testing Hg methylation in earthworm digestive tracts in laboratory studies (Shao et al., 2012; Rieder et al., 2013). Second, comparing with other studies, B. parvus is a different earthworm species sampled from different forest soils in contaminated and reference sites, and some uncertainties arose from potential differences in anaerobic bacterial communities which associated with the Hg methylation in earthworm digestive tracts or in soils of sampling sites from the previous results (Kaschak et al., 2014; Rieder et al., 2013; Lee et al., 2016). The long-term effects of landfill leachate can greatly influence Hg speciation through changing the associated microbial communities under anaerobic conditions (Lee et al., 2016). It is difficult to determine the distribution of bacterial Hg methylation in the forest soils and in B. parvus, especially considering that the landfill leachate might differently affect the activity of bacterial Hg methylation in different sampling areas of leachate-contaminated forest soils. Third, the results from our cluster and linear regression analyses indicated that the THg and MeHg concentrations in B. parvus tissues were correlated with soil THg, SOM, and clays (Fig. 4). Based on previous studies, this suggested that the Hg forms were affected by multiple aspects of soil geochemical properties in forest soil because Hg specific compounds can combine with the soil matrix (e.g., SOM) to codetermine the Hg mobility and bioavailability in the natural environment (Bloom et al., 2003). The Hg combination formed by Hg compounds and the soil matrix was likely variable under the complex environment of the leachate-contaminated forest zone. The leachate Hg speciation, biogeochemical transformations, and mobilization were affected by changes in the redox potential and microbial communities of anaerobic conditions associated with Hg methylation during the long-term changes in landfill-leachate-contaminated soil (Lee et al., 2016).

In conclusions, most Hg accumulation studies have focused on aquatic ecosystems; however, the role of Hg (THg or MeHg) accumulated and transferred into food chains of terrestrial ecosystems has not attracted enough attention, and little academic knowledge is available on this topic. The present work showed that Hg deposited in leachate-contaminated forest soils around traditional Hg-polluted landfills and background levels of Hg in uncontaminated soils deposited via natural mechanisms (e.g., atmospheric deposition) can be accumulated by B. parvus over time. B. parvus showed similar BAF₃₁₃ in both leachate-contaminated (mean 5.42) soils and uncontaminated (mean 4.91) forest soils. Although THg concentrations in the leachate-contaminated forest soils (mean 2.919 mg kg⁻¹) were significantly higher than those in uncontaminated forest soils (mean 0.087 mg kg⁻¹), the average MeHg concentrations were similar in all sampled forest soils and in B. parvus. Based on the current results, the role of B. parvus in MeHg production is not clear; MeHg in B. parvus could have first formed in forest soils, and then accumulated into their tissues. Nonetheless, potential Hg exposure of food chains through B. parvus in leachate-contaminated forest soils cannot be ignored because B. parvus shows a high ability to accumulate THg and MeHg from forest soils. To better understand the ecological risks of secondary Hg poisoning to organisms in terrestrial ecosystems, further studies of forest ecosystems around Hg-contaminated traditional landfills are necessary to confirm the possibility of an associated bacterial community for Hg methylation in B. parvus and soil.

ACKNOWLEDGMENTS

This work was supported by funding from the International Postgraduate Scholarship for Research on Mercury from Kumamoto Prefecture, Japan. We thank Prof. J. Morrow for his help in checking language and grammar. We are grateful to Hiroaki Jubashi, Atsushi Sakai, and Shinji Toyota for their sampling support.

Conflict of interest— The authors declare that there is no conflict of interest.

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