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Effects of Arsenic on Gut Microbiota and Its Biotransformation Genes in Earthworm *Metaphire sieboldi*

Hong-Tao Wang,[†,‡] Dong Zhu,[†,‡] Gang Li,[†] Fei Zheng,[†,‡] Jing Ding,[§] Patrick J O'Connor,[∥] Yong-Guan Zhu,[†,‡,§] and Xi-Mei Xue*[,†]

[†]Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China

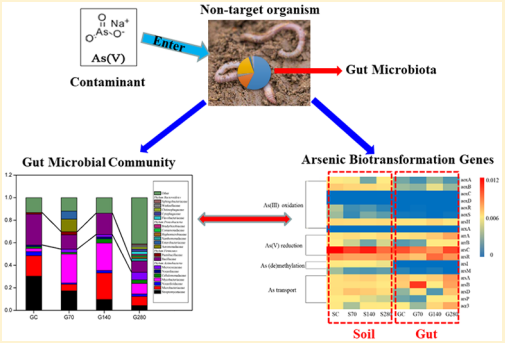
[‡]University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China

[§]State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[∥]Centre for Global Food and Resources, University of Adelaide, Adelaide 5005, Australia

Supporting Information

ABSTRACT: Arsenic biotransformation mediated by gut microbiota can affect arsenic bioavailability and microbial community. Arsenic species, arsenic biotransformation genes (ABGs), and the composition of gut microbial community were characterized after the earthworm *Metaphire sieboldi* was cultured in soils spiked with different arsenic concentrations. Arsenite (As(III)) was the major component in the earthworm gut, whereas arsenate (As(V)) was predominant in the soil. A total of 16 ABGs were quantified by high-throughput quantitative polymerase chain reaction (HT-qPCR). Genes involved in arsenic redox and efflux were predominant in all samples, and the abundance of ABGs involved in arsenic methylation and demethylation in the gut was very low. These results reveal that the earthworm gut can be a reservoir of microbes with the capability of reducing As(V) and extruding As(III) but with little methylation of arsenic. Moreover, gut microbial communities were dominated by Actinobacteria, Firmicutes, and Proteobacteria at the phylum level and were considerably different from those in the surrounding soil. Our work demonstrates that exposure to As(V) disturbs the gut microbiota of earthworms and provides some insights into arsenic biotransformation in the earthworm gut.



INTRODUCTION

Arsenic is widely distributed in the soil and can be bioaccumulated through the food web in soil, plants, and animals.[1−3] Arsenic bioaccumulation can result in toxicity for soil biota and may ultimately affect human health. Most previous studies concentrated on the bioconcentration and toxicity of environmental arsenic in the earthworm. Using a laboratory vermicomposting system, Fischer and Koszorus[4] studied the sublethal effects, lethal concentrations, accumulation, and elimination of arsenic, selenium, and mercury in *Eisenia fetida*. The effect of edaphic factors (pH, depth in soil profile, and soil organic matter content) on the toxicity and accumulation of arsenate (As(V)) were investigated in the earthworm *Lumbricus terrestris*.[5] Furthermore, a few laboratory and field surveys investigated diverse arsenic species in earthworm body tissues.[6,7] The only organoarsenical found in *L. rubellus* and *Dendrodrilus rubidus* collected from an arsenic mine and an uncontaminated soil was arsenobetaine.[8] In addition, earthworm activity may influence the species, mobility, and partitioning of metals and metalloids in soil and pore waters by changing soil pH, soluble organic carbon, or microbial communities.[9] However, these studies mostly focused on arsenic toxicity on earthworm and arsenic species in earthworm body tissues or gut, with little attention to the mechanism of arsenic detoxification or biotransformation in the earthworm gut.

Many of the ecosystem processes attributed to soil fauna may in fact be mediated by the microbiome of those fauna, which is an important part of the soil microorganism process and will exert influence on the host metabolism and health.[10−12] For example, earthworm gut microbiota have been shown to be involved in organic matter decomposition, denitrification, nutrient stabilization, and other bio-geo-chemical cycles.[13−15] The earthworm gut is considered to be a transient habitat for the microbes of aerated soils.[14] During passage through the gut, the unique microenvironment of the gut of different species of earthworms from different environments appears to selectively stimulate a specific subset of ingested soil microbes.[14,15] This selection of earthworm gut

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microbiota has been shown to be influenced in the presence of environment contaminants such as triclosan[16] and arsenic.[17] In addition, the use of the earthworm as a bioindicator of arsenic contamination was assessed by analyzing the effects of arsenic on earthworm toxicity.[18] Only a recent study revealed that soil arsenic contamination could alter the microbiome of the earthworm.[17] However, comprehensive studies of the interactions between arsenic species and the gut bacterial flora of earthworms have not been undertaken.

Microbe-mediated arsenic metabolism plays an important role in the arsenic bio-geochemical cycle; different types of genes involved in arsenic metabolism encode proteins that regulate arsenic species and solubility in the environment.[19−21] For example, arsenic redox genes encoding cytoplasmic As(V) reductase (ArsC), respiratory As(V) reductase (ArrAB), and As(III) oxidase (AioAB) impact the species transformation between As(V) and As(III)[21] and then change arsenic toxicity and bioavailability. Arsenic methylation or demethylation is catalyzed by an As(III) *S*-adenosylmethionine methyltransferase (ArsM) or a non-heme iron-dependent dioxygenase with C·As lyase activity (ArsI).[22] Organisms can also mobilize arsenic via phosphate transporters, aquaglyceroporins, or As(III) efflux systems.[23] Using qPCR amplifications with five primer pairs, Zhang et al.[24] showed that ABGs involved in arsenic redox reactions and methylation were widely distributed in paddy soils. Metagenomic analysis was applied to reveal the coexistence of a different arsenic resistance system of microbes in low-arsenic soil habitats.[25] Arsenic species changes in earthworm body tissues are associated with the related genes of arsenic detoxification and biotransformation in the earthworm gut, which is an anaerobic environment, like that of flooded paddy soil.[15] However, there is still relatively little known about the distribution, diversity, and abundance of arsenic biotransformation genes (ABGs) in soil fauna, not to mention the relationship between arsenic species and ABGs in the gut of soil fauna.

To understand the influence of arsenic contamination in soils on earthworm gut microflora and the relationships between arsenic species and ABGs in the gut, this study was designed to (1) determine arsenic species in the earthworm gut and body tissues after exposure to arsenic contaminated soils; (2) characterize the diversity and abundance of ABGs in the earthworm gut; (3) profile the microbial communities in the soil and the earthworm gut; and (4) examine the difference in the diversity of gut microbial communities after earthworms were exposed to different arsenic concentrations.

MATERIALS AND METHODS

Soil and Earthworm Preparation. Soil samples, which were not contaminated with arsenic, were collected in 2017 from disused farmland in Xiamen City, southeast China. Detailed physical−chemical properties of the soil are described in Table S1 of the Supporting Information (SI). The soil was ground in an agate mortar and sieved through 2 mm nylon sieves after being separated from gravel particles and litter and then air-dried at room temperature. The stock solution of As(V) (448 mg L^{-1}), prepared by dissolving $Na_3AsO_4{\cdot}12H_2O$ (chemically pure, Chemical Reagent Purchasing and Supply Station, Shanghai, China) in Milli-Q water (18.2 MΩ, Millipore, UK), was mixed thoroughly with 900 g of the homogenized soil in polyethylene plastic containers (25 × 15 × 12 cm) (Runpeng Plastic Co., Ltd., Jieyang, Guangdong, China) to yield a final concentration of control (7), 70, 140, and 280 mg of As(V) kg^{-1} dry soil. The spiked soils had a water content of 30% and were activated for 14 days under laboratory conditions (20 °C, 12 h light/12 h dark cycle).

Adult earthworms (*M. sieboldi*) of the same age, with well-developed clitellum, purchased from an earthworm company in Nanjing City, were acclimated for 14 days in the untreated soil under laboratory conditions prior to the experiment. Oatmeal (Nanguo Food Industry Co., Ltd., Haikou, Hainan, China) as a food source was added to the soil. The earthworm species was confirmed by sequencing the cytochrome oxidase I (COI) barcode gene (primers: LCO-1490 (5′-GGTCAACAAA-TCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAAC-TTCAGGGTGACCAAAAAATCA-3′)).[17,26,27] The sequences obtained were submitted to the National Center for Biotechnology Information (NCBI) via the Basic Local Alignment Search Tool (BLAST) to identify the species of earthworm. *M. sieboldi* was rinsed with Milli-Q water to remove adhering soils. A total of 10 earthworms with similar magnitude and wet mass were transferred to the spiked soils. Arsenic-free oatmeal was mixed into the soil as a food source at the initial stage of the experiment. The culture containers were covered with a lid, in which holes were punched to maintain ventilation, and containers were maintained in an artificial incubator (Saifu Laboratory Instruments Co., Ltd., Ningbo, Zhejiang, China) (20 °C, 70% relative humidity) with a 12:12 h light/dark cycle. The experiment consisted of a control (no added As(V)) and 3 treatments with 3 replicates of each treatment, giving a total of 12 containers. During the culture, dead earthworms were observed and removed. The soil water content was maintained at 30% through providing Milli-Q water at regular intervals.

DNA Extraction, High-Throughput Sequencing, and Bioinformatic Analysis. The earthworms, exposed to As(V) for 28 days, according to the US Environmental Protection Agency (USEPA)[28] and the Organization for Economic Co-operation and Development (OECD) (No. 222) protocols,[29] were collected and immediately killed with absolute ethyl alcohol (analytically pure, Sinopharm Chemical Reagent Co., Ltd., Ningbo, Zhejiang, China). Worms were gently shaken and rinsed in sterilized water five times to remove surface microbiota. The earthworm gut was obtained using sterile forceps under sterile conditions.

Earthworm gut and 0.5 g of soil from each treatment (a total of 24 samples including 12 gut and 12 soil, i.e., control soil and gut (SC/GC); 70 mg of As(V) kg^{-1} soil and gut (S70/G70); 140 mg of As(V) kg^{-1} of soil and gut (S140/G140); 280 mg of As(V) kg^{-1} of soil and gut (S280/G280)) were used to extract DNA using a FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, California, USA) according to the manufacturer's instructions. In the end, 100 and 70 μL of DNA elution solution were used to dissolve the soil and earthworm gut DNA, respectively. The extracted DNA was stored at −20 °C after concentration, and the quality of DNA was measured by Nanodrop ND-1000 (Thermo Fisher, USA) and agarose gel electrophoresis.

The V4−V5 region of 16S rRNA was amplified and sequenced on the Illumina platform (Majorbio, China) as previously described.[30] Briefly, a 392 bp fragment of 16S rRNA was PCR amplified using the forward primer F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer R907 (5′-CCGTCAATCMTTTRAGTTT-3′), with R907 modified to contain a unique 8 nt barcode at the 5′ terminus

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to distinguish different source samples in the same pool. The amplicons were purified and combined into one pool.

The Illumina sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0) following the instructions at Getting Help with QIIME.[31] The default method was used to pick the operational taxonomic units (OTUs), which were defined at the 97% similarity level by UCLUST clustering[32] after the raw reads were filtered and representative sequences of the OUTs obtained. A phylogenetic tree was constructed using the FastTree algorithm[33] based on the multiple sequence alignment generated by a PyNAST prior to downstream analysis.[34] Phylogenetic distance (PD) whole tree, Chao1 estimator, rarefaction curves, and Shannon entropy were used to describe alpha diversity for each sample. Principal coordinate analysis (PCoA) based on Bray−Curtis distance was performed to evaluate the profiles of microbial communities in different treatments. The sequence has been deposited in the NCBI Sequence Read Archive under the accession number PRJNA516551.

HT-qPCR of ABGs. The abundance of ABGs in the gut and soil was estimated using the Wafergen SmartChip Real-Time PCR System (WaferGen Biosystems, Fremont, CA) as described in ref 35 with minor modifications. A total of 80 primer pairs (Table S2) was designed to target 19 ABGs and a 16S rRNA gene. The 100 nL HT-qPCR reaction contained LightCycler 480 SYBR Green I Master, primers, nuclease-free PCR-grade water, bovine serum albumin, and DNA template. The thermal conditions were 95 °C denaturation for 10 min, 40 cycles of 95 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and a final 30 s extension step at 72 °C.[47] The gene copy number of ABGs or 16S rRNA was calculated using eq 1, and a threshold cycle (C_T) of 31 was utilized as the detection limit. When three replicates for each DNA sample were all above the detection limit, ABGs were considered to be detected. Moreover, in order to minimize bias from background bacterial abundances and DNA extraction efficiencies, the ABG copy number was normalized to the number of ABG copies per bacterial cell using eq 2. The bacterial cell numbers in one sample were estimated by dividing 16S rRNA gene quantities by 4.1 based on the Ribosomal RNA Operon Copy Number Database.[36]

$$\text{relative gene copy number} = 10^{(31-C_T)/(10/3)} \quad (1)$$

$$\text{normalized ABG abundance} = 4.1 \times (\text{relative ABG copy number} / \text{relative 16S rRNA gene copy number}) \quad (2)$$

Determination of Total Arsenic Concentration and Arsenic Species. The freeze-dried earthworm body tissues and gut contents were ground in an agate mortar with liquid nitrogen to a fine powder prior to digestion and arsenic extraction. The soil (200 mg, weighed to a precision of 0.1 mg), earthworm body tissues (30 mg, weighed to a precision of 0.1 mg), or gut contents (30 mg, weighed to a precision of 0.1 mg) for the analysis of total arsenic concentration was precisely weighed into 50 mL polypropylene digestion tubes. A HNO_3 (Merck Millipore, 65%, Darmstadt, Germany)/HF (Thermo Fisher Scientific, 49%, USA) mixture (5 + 1 v/v, 6 mL) for soil or a HNO_3/H_2O_2 (Merck Millipore, 30%, Darmstadt, Germany) mixture (2 + 1 v/v, 6 mL) for earthworm body tissues or gut contents was added to the samples and allowed to stand at room temperature for 2 h before the tubes were covered with caps and transferred to the microwave-accelerated system (CEM Microwave Technology Ltd., Buckingham, UK). The microwave-assisted digestion for earthworm body tissues or gut contents in closed tubes was carried out as described previously.[37] The temperature ramping program in the microwave digested for soil samples was shown as the following: 105 °C for 20 min, 180 °C for 10 min, 180 °C for 30 min. Upon reaching room temperature, samples were diluted to 50 mL with Milli-Q water and filtered through 0.45 μm syringe filters (PVDF, Millipore, USA). Arsenic concentrations of soil, earthworm body tissues, and gut contents were determined by ICP-MS (Agilent 7500 ce, Agilent Technologies, USA) in a collision cell mode to avoid interference from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As). The total arsenic measurement was validated against the certified reference material, GBW07403, GBW07406, and GBW10050 bought from the National Institute of Metrology of China with certified values for arsenic of 4.4 ± 0.6 mg kg^{-1}, 220 ± 14 mg kg^{-1}, and 2.5 mg kg^{-1} (reference value); we obtained 4.3 ± 0.4 mg kg^{-1}, 216 ± 15 mg kg^{-1}, and 2.4 ± 0.4 mg kg^{-1} (n = 4), respectively. The recovery rates of the CRMs ranged from 90.0 to 108.2%.

The following extractants for arsenic species were varied for each sample type. Soil (200 mg, weighed to a precision of 0.1 mg) was extracted with 5 mL of 0.05 M aqueous ammonium sulfate,[38] and freeze-dried earthworm body tissues or gut (30 mg, weighed to a precision of 0.1 mg) was weighed into a polyethylene vessel with 5 mL of a MeOH (HPLC grade, Thermo Fisher Scientific, USA)/H_2O mixture (1:1 v/v).[7] Arsenic species were extracted on a rotary wheel at 150 rpm overnight. The mixture was centrifuged (4754 g, 15 min) at 4 °C to separate the supernatant from the pellet. Soil extract was filtered through a 0.22 μm filter and stored at −80 °C prior to analysis. The extract containing methanol was evaporated to dryness under a nitrogen stream at room temperature and redissolved in Milli-Q water. HPLC (Agilent 1200, Agilent Technologies, USA)−ICP-MS (Agilent 7700, Agilent Technologies, USA) was used to analyze arsenic species. The species analysis was carried out on a PRP-X100 anion column (250 × 4.1 mm length, 10 μm particle size) and a precolumn (11.2 mm length, 12−20 μm particle size) from Hamilton (Reno, NV, USA) with a mobile phase consisting of 10 mM diammonium hydrogen phosphate and 10 mM ammonium nitrate (pH = 9.25, adjusted with aqueous ammonia).[39] In addition, four normal arsenic species including As(III), monomethylarsonic acid (MAs(V)), dimethylarsinic acid (DMAs(V)), and As(V), four arsenosugars (glycerol arsenosugar (sugar 1), phosphate arsenosugar (sugar 2), sulfonate arsenosugar (sugar 3), and sulfate arsenosugar (sugar 4)) purified from *Fucus serratus*, and arsenobetaine from shrimp were analyzed in the column. The injection volume was 20 μL, and the flow rate was 1.0 mL min^{-1}. ICP-MS signals were recorded at m/z 75 (^{75}As and $^{40}Ar^{35}Cl$) at a dwell time of 300 ms, at m/z 77 (^{77}Se and $^{40}Ar^{37}Cl$) for possible chloride interferences, and at m/z 74 (^{74}Ge) for an internal standard at a dwell time of 100 ms. Quantification was based on peak areas against external calibration with standards (0, 0.1, 0.5, 1, 5, 10, 50, and 100 μg L^{-1}) containing four arsenic species (As(III), As(V), MAs(V), and DMAs(V)). The calibration standard (10 ppb) and blank were analyzed every 10 samples to ensure instrumental stability based on our previous study.[39]

Statistical Analysis. The data analysis including basic statistics (mean value, variable coefficient, standard deviation

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Table 1. Characterization of Lethality and Bioconcentration in *M. sieboldi* in the Arsenic-Spiked Soil after 28 Days[a]

	control	As70	As140	As280
no. (28 days)	8.3 ± 0.6^{c}	5.7 ± 1.2^{b}	2.4 ± 1.2^{a}	1.3 ± 0.6^{a}
mass(28 days/FW) (mg)	227 ± 11^{c}	203 ± 21^{bc}	168 ± 8^{ab}	140 ± 13^{a}
As in soil (mg kg^{-1})	6.5 ± 0.1^{a}	71.7 ± 1.6^{b}	141.6 ± 4^{c}	284 ± 4.8^{d}
As in worm (DW) (mg kg^{-1})	6.4 ± 1.9^{a}	97.8 ± 9.1^{b}	200.3 ± 9^{c}	343.1 ± 23^{d}
As in gut (DW) (mg kg^{-1})	6.0 ± 1.8^{a}	73.1 ± 8.6^{b}	155.2 ± 14^{c}	-
mortality (%)	16.7 ± 0.1^{a}	43.3 ± 0.1^{b}	73.3 ± 0.1^{c}	86.7 ± 0.1^{c}
BAF	1.0 ± 0.3^{a}	1.4 ± 0.2^{a}	1.4 ± 0.1^{a}	1.2 ± 0.1^{a}

[a]Mean ± SD, n = 3. Note: "-" indicates no data, FW indicates fresh weight of earthworm, DW indicates dry weight of earthworm, BAF indicates bioaccumulation factor of arsenic in earthworm. Different letters show significant differences between different treatments at the 0.05 level (ANOVA).

(SD), standard error (SE), the percentage of arsenic species, and ABGs value) and alpha diversity (PD whole tree, Chao1, rarefaction curves, and Shannon index) were performed in Origin 8.5 (OriginLab, USA). The PCoA and Adonis test were carried out using R version 3.4.3. Analysis of variance (ANOVA) and the Pearson correlation test were performed using the statistical software SPSS V18.0 (IBM, USA). Microsoft Excel 2010 (Microsoft, USA) was used to generate other tabulations and graphics.

RESULTS

Body Weight, Mortality, and Arsenic Bioaccumulation. The body weight of *M. sieboldi* was remarkably lower in treatments with additions of 140 and 280 mg of As(V) kg^{-1} than in the control, corresponding to a reduction of 35.5 and 41.6%, respectively (ANOVA, $P < 0.01$) after 28 days (Table 1). Earthworm mortality was significantly different between all treatments (ANOVA, $P < 0.001$), and exhibited a clear dose−response relationship (Table 1). Earthworm mortality increased sharply with exposure to arsenic at 280 mg kg^{-1} (86.7%) compared to the control (16.7%) (ANOVA, $P < 0.001$). With the increase in arsenic concentration in soil, total arsenic concentrations of *M. sieboldi* body tissues or gut contents increased significantly (ANOVA, $P < 0.05$). The bioaccumulation factor (BAF) of earthworms was estimated to be 1.0−1.4.

Arsenic Species and Abundance of ABGs in Soil and Gut. Figure S1 shows that cationic species including arsenobetaine and sugar 1, which usually coelute at the solvent front with As(III), can be separated from As(III) by the anion-exchange column under this condition. The results indicated that inorganic arsenic (As(III) and As(V)) alone was detectable in soil, earthworm body tissues, and gut contents (Table S3). The average extraction rate was 12.6, 52.6, and 79.5% for soil, body tissues, and gut contents, respectively (Table S3). The concentration of extracted arsenic species was up to 119.3 mg kg^{-1} in G140 consisting of 90.2 mg kg^{-1} As(III) and 29.1 mg kg^{-1}As(V) (Table S3). As(V) (>71.7%) was the dominant species found in soils, whereas the major form of arsenic in earthworm body tissues was As(III) (>77.9%) (Figure 1). Moreover, As(III) was the predominant arsenic species (>75.6%) detected in the gut contents (Figure 1).

The ABGs were divided into four types based on their function, namely, As(III) oxidation, As(V) reduction, arsenic (de)methylation, and arsenic transport. A total of 16 ABGs were detected in the soil and gut samples (Figure S2), in which some genes involved in As(III) oxidation (*aoxC*, *aoxD*, and *arxA*) were absent (Figure 2A). More ABG species in soil were found than those in gut contents (Figure S2); for example, *aoxR*, *aoxS*, and *arsI* were found only in soil and were not detected in earthworm gut (Figure 2A). The normalized copy numbers of ABGs ranged from 0.017 to 0.032 copies per cell, with S280 and G280 harboring the lowest and highest gene copies of ABGs, respectively (Figure 2B). The *arsI* and *arsM* were rarely detected in gut and soil. The predominant ABGs in gut and soil were involved in As(V) reduction and arsenic transport (Figure 2B).

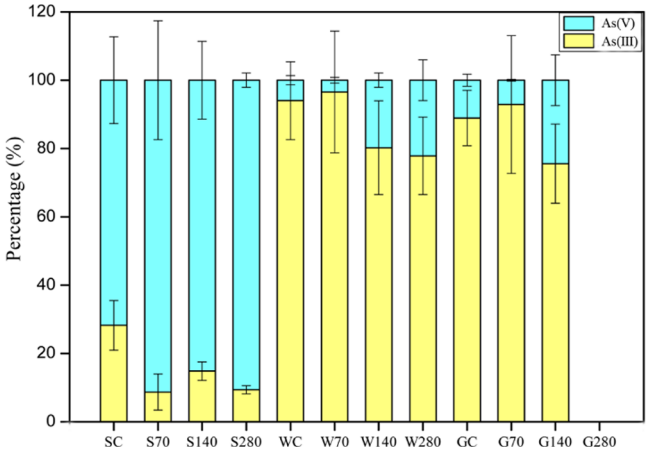

Figure 1. Proportion of arsenic species in the soil−earthworm−gut system. C, control; S, soil; W, earthworm tissues; G, gut; 70, 140, 280, treatment with 70, 140, and 280 mg of As(V) kg^{-1} of dry soil, respectively. The data for G280 is missing, because gut contents were of insufficient volume for arsenic species analysis.

Differences in Microbial Communities between Earthworm Gut Contents and the Surrounding Soil. Across all samples, 2 635 484 nonsingleton reads were calculated, and counts of sequences per sample ranged from 41 999 to 173 030. A total of 49 993 OTUs in gut samples and 67 807 OTUs in soil samples were obtained, respectively. The Venn diagram shows that a total of 28 381 OTUs are shared by the two sources, accounting for 56.8 and 41.9% of the total reads from the gut and soil sources, respectively (Figure S3A). Actinobacteria (accounting for 57.5% of the total reads), Firmicutes (20.7%), and Proteobacteria (13.5%) were the major phyla in the gut, whereas the predominant phyla in soil were Bacteroidetes (26.1%), Proteobacteria (27.0%), and Actinobacteria (24.6%) (Figure S4). Actinobacteria, Bacteroidetes, and Firmicutes varied greatly in earthworm guts and inhabited soil (t-test, $P < 0.05$) (Figure S4). The relative abundance of Rhizobiales in the soil (7.8%) was significantly higher than that in the gut (1.8%) (t-test, $P < 0.01$). The relative abundance of the shared bacterial families shows a

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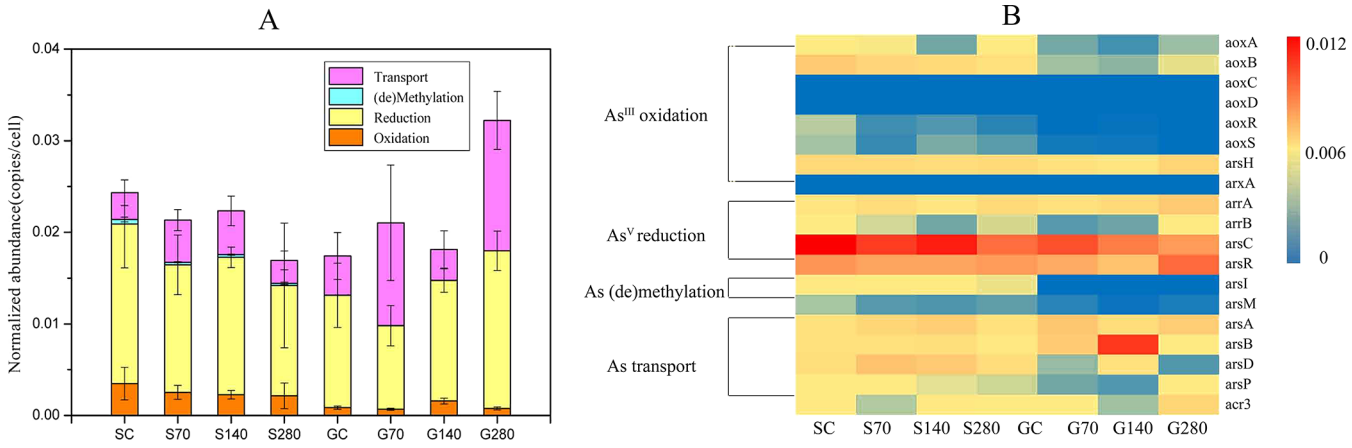

Figure 2. (A) Heat maps of ABGs in soil and gut samples. (B) Normalized abundance of ABGs per bacterial cell. The data are presented as the mean value ± standard error (SE) (n = 3).

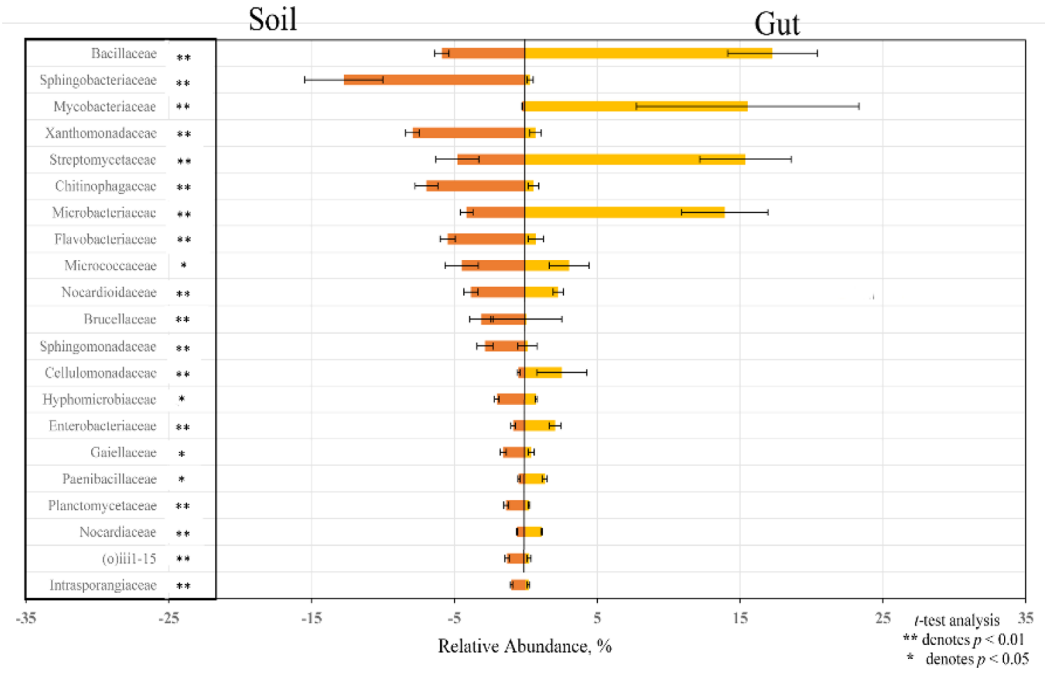

Figure 3. Relative abundance (%) of bacterial families with significant differences between soil and *M. sieboldi* gut. The order is labeled (o) if family level annotation was impossible. Only families with >1% reads are displayed.

statistically significant difference between the gut and soil sources (t-test, $P < 0.05$) (Figure 3). Bacillaceae (17.3%), Mycobacteriaceae (15.5%), Streptomycetaceae (15.4%), and Microbacteriaceae (13.9%) were the four most abundant families in the gut, whereas their abundance was only 3.1, 7.8, 3.2, and 3.0% in soil, respectively. Sphingobacteriaceae (12.7%) and Xanthomonadaceae (7.9%) were the most abundance families detected in soil (Figure 3). In addition, the dominant genera in soil and earthworm gut microbiome were different (Figure S7). The relative abundance of *Bacillus* in the gut (12.2%) was significantly higher than that in the soil (4.4%) (t-test, $P < 0.01$).

The Chao1 index shows that the diversity of the soil microbial community is significantly higher than that in gut (t-test, $P < 0.01$) (Figure S5). The result was further confirmed by the PD whole tree and Shannon index measures (Figure S5). PCoA analysis (Figure 4) demonstrates a significant shift (Adonis test, $P < 0.01$) between gut contents and soil along with PC1 (which explained 30.8% of the total variance).

Effect of Arsenic on the *M. sieboldi* Gut Microbial Community. The maximum OTUs (26 452) and minimum OTUs (12 071) were found in G280 and G70, respectively. Only 2202 OTUs were shared (Figure S3B) between gut contents from different treatments, accounting for 4.4% of the total OTUs in gut samples. The unique OTUs in the GC, G70, G140, and G280 account for 11.7, 8.6, 11.0, and 27.6% of the total OTUs in gut contents, respectively. At the phylum level, the abundance of Bacteroidetes increased significantly with increasing arsenic concentration (t-test, $P < 0.05$) in the earthworm gut (Figure S6). Compared to the control (GC), Acidobacteria and Gemmatimonadetes in G280 were both notably increased (t-test, $P < 0.05$). At the family level, the abundance of Flavobacteriaceae, Cytophagaceae, Chitinophagaceae, Weeksellaceae, and Sphingobacteriaceae in G280 dramatically exceeded that in gut samples from other arsenic

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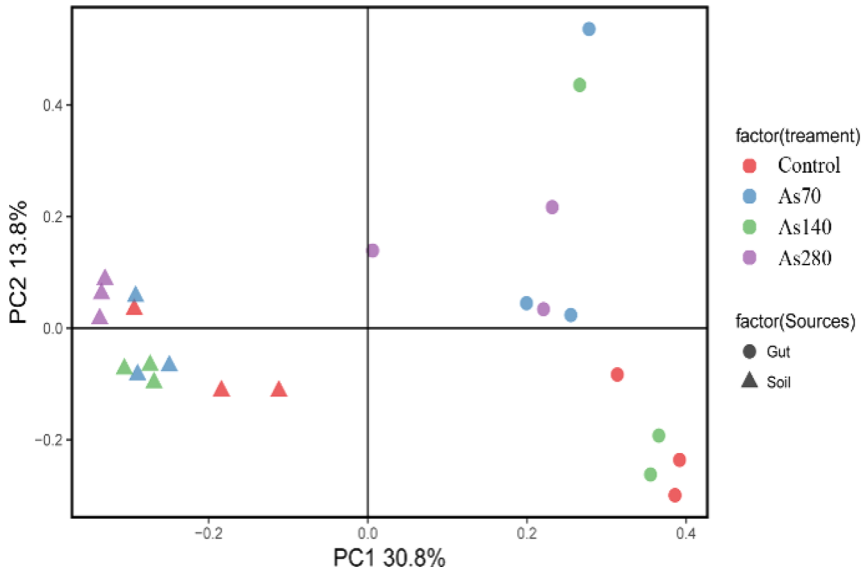

Figure 4. Principal coordinates analysis (PCoA) plots based on unweighted unifrac distances.

concentration treatments (*t*-test, $P < 0.05$). The relative abundance of Streptomycetaceae significantly declined with increasing arsenic exposure (ANOVA, $p < 0.05$) (Figure 5).

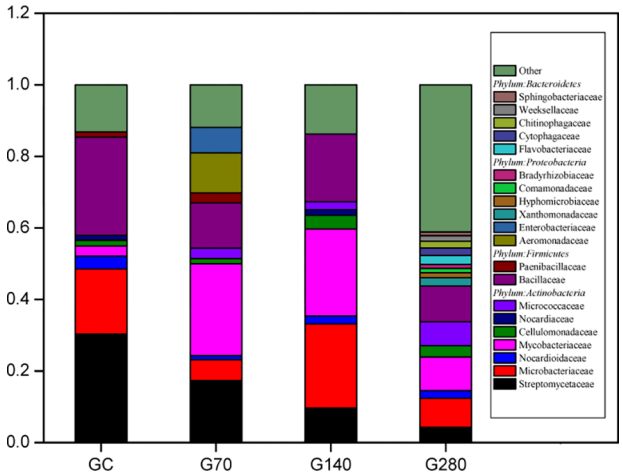

Figure 5. Family level diversity (mean, $n = 3$) of the gut microbiota affected by arsenic. Family level groups with <1% of the total number of reads are categorized into "Other".

The gut and soil from different arsenic spiking levels were clearly separated from each other (Adonis test, $P < 0.05$) using PCoA analysis along with PC2 (explained 13.8% of the total variance) (Figure 4). The PD whole tree indicates that the diversity of the gut microbiome first decreased and then increased with increasing arsenic concentration in soil, and the Chao1 and Shannon index show the same trend (Figure S5).

DISCUSSION

This study demonstrated that As(V) was toxic to *M. sieboldi*, resulting in significant decline in earthworm body weight and extremely high mortality. It is known that arsenic can cause pathological damage, likely because of arsenic uptake and accumulation by earthworms through gut and epidermal contact with the polluted soil.[4,40,41] Moreover, the mortality of earthworms was highly dependent on arsenic species and arsenic concentration in earthworm body tissues.[6,18] Meharg et al.[5] found that dead earthworms had a higher arsenic concentration than living worms. Dominant As(III) and higher arsenic concentration in the earthworm gut have been observed in arsenic exposure treatments, and this can explain the higher mortality of earthworms.

In this study, we found that As(V) was the most prevalent arsenic species in soil, with only traces of As(III) present probably due to high Eh and the low bioavailability of arsenic. This was further confirmed by our study with the lower extraction efficiency of arsenic in soil than in body tissues or the gut contents. The earthworm gut is anoxic,[14] which alters arsenic adsorption on soil particles, arsenic species, and arsenic bioavailability in the gut where As(V) can be reduced to As(III) by microbes. Furthermore, our results showed that ABGs encoding As(V) reductases and arsenic efflux transporters in the gut were much more abundant than other genes; similar levels were detected in estuarine wetland sediments.[42] No organoarsenicals and predominant As(V) were detected in any earthworm body tissues or gut contents, confirming previous reports of this for *E. fetida*,[43] and this supports our observation. Additionally, genes involved in arsenic oxidation and methylation were much less abundant in the earthworm gut than in the soil, suggesting that As(V) from soil was first reduced to As(III) in microbe cells, and then, As(III) was pumped out via arsenic efflux transporters without leaving detectable methylated arsenic in the gut. Thus, the more toxic As(III) generated by gut microbes was present in the gut and accumulated in earthworm body tissues. In order to alleviate the effects of arsenic toxicity, earthworms may chelate As(III) with the sulfur-rich protein metallothioneins (MTs) in chloragogen tissue by forming As(III)-thiol.[6] Previous work has indicated that genes encoding As(V) reductases (e.g., *arsC*) were identified in both aerobic and anaerobic microbes, implying that arsenic reduction and efflux could occur in the soil microbiota under aerobic conditions.[44] Despite the fact that many organoarsenics have been identified in *L. terrestris*, *L. rubellus*, and *D. rubidus* from contaminated soils,[5,6,8,45] no organoarsenics were observed in *M. sieboldi*. Organoarsenics in earthworms have three possible sources: transformation of inorganic arsenic by the earthworm, arsenic biotransformation

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by the gut microbes of the earthworm, and arsenic accumulation from the soil.[7] Button et al.[7] proposed that organoarsenics in field-collected *L. terrestris* were from symbiotic processes and ingestion of leaf litter in the natural soil. Moreover, it has been confirmed that ArsM was necessary for arsenic methylation and glycosylation.[46] Thus, the lack of *arsM* genes in either the gut or the soil may be an important explanation for this.

Our results also demonstrated that the microbiota of the earthworm gut is different from that in the surrounding soil. The difference is probably due to the unique conditions in the gut (anoxia, neutral pH, and intestinal mucus enzyme) and because the surrounding soil is characterized by aerobic conditions and a complex mineral composition.[15,17] For example, the increased abundance of Enterobacteriaceae in the earthworm gut is believed to be due to its capability of anaerobiosis.[47] In addition, the microbial diversity was lower in the gut than in the surrounding soil, giving support to the findings of a previous study.[17] The likely factors driving the difference are as follows. *M. sieboldi* living in the topsoil environment fed on the decaying organic matter from the soil, and the transient soil consisting of potential pathogenic microorganisms and organic residues was ingested and selectively assimilated through the intestinal digestive tract by the specialized antibacterial immune system.[14] Accordingly, large numbers of OTUs were shared by the soil and the *M. sieboldi* gut, implying that the soil microbial community plays an initial role in shaping the bacterial composition of the earthworm gut. Specifically, a reduction in abundance of the genera *Chitinophaga*, *Ochrobactrum*, and *Sphingobacterium* was observed when soil passed through the gut, which is likely a result of gut filtration processes. Conversely, there was a sharp enhancement of abundance of the genera *Mycobacterium*, *Streptomyces*, and *Bacillus* in gut contents in comparison with the soil. This is likely due to the stimulation effect of the nutrient utilization.[15,17,47] For instance, *Mycobacterium*[48] and *Streptomyces*[15] are known to use organic remains of plants (humic and fluvic acids) and participate in the metabolism of hemicellulose, respectively. Increased *Bacillus* species in the gut are considered to accelerate phosphate mineralization and the reduction of nitrides.[49] Furthermore, the microbiota play a critical role in arsenic biotransformation.[19,21] Rhizobiales has been confirmed to make contributions to the oxidation of As(III) in soils,[24] and As(V) reductase encoded by *arsC* in *Bacillus*[50] may be involved in reducing As(V) to As(III) in the gut. Higher abundances of Rhizobiales (7.8%) in the soil and *Bacillus* (12.2%) in the earthworm gut observed in this study indicated an active role of bacterial community in driving the transformations of arsenic species in earthworm gut and soil.

The addition of arsenic to soil could significantly alter the community structure of the earthworm gut microbiota. This is supported by a large number of unique unshared OTUs that were observed in the arsenic-treated earthworm gut. The soil environment and unique gut habitat contribute to shaping the earthworm gut microbiota. The surrounding soil is the primary source of earthworm gut contents.[14] The structure of soil microbial communities changed significantly under the stress of arsenic toxicity, that is, arsenic exposure caused changes in the earthworm gut microflora. In addition, arsenic can dramatically inhibit the growth of earthworms and influence gut metabolism, which may also lead to changes in the gut microbial community. For example, reductions in the abundance of Streptomycetaceae genus (including *Streptomyces*) in the earthworm gut after exposure to arsenic implies an inhibition of biological activity, and this probably slowed down the cellulose degradation capacity by inhibiting enzymatic activity or causing complete loss in enzyme activity.[15,17]

In summary, two main reasons why As(III) was the major arsenic species in the gut and body tissues after the earthworm *M. sieboldi* was exposed to As(V) for 28 days are the unique microhabitats and predominant genes involved in reducing As(V) and extruding As(III) in the earthworm gut. As(V) reduction was a key process of the arsenic metabolic pathway in the earthworm gut. As(III) accumulation in earthworm body tissues exerts a critical influence on its growth and mortality, and high-concentration As(III) in the gut disturbs its microbial communities, resulting in a mass change in the unique OTUs. Moreover, the significant shifts in microbial communities from the surrounding soil to earthworm gut are probably due to the selection and stimulation in the unique earthworm gut microhabitat. These findings offer us a new perspective for establishing an association between arsenic biotransformation and ABGs or gut microbial communities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b06695.

Basic physicochemical information on soil (Table S1); information on 80 genes detected in gene chip (Table S2); extracted arsenic species and extraction efficiency in soil, earthworm body tissues, and gut (Table S3). Anion-exchange HPLC−ICP-MS chromatograms of arsenic species (Figure S1); numbers of ABGs detected in the different arsenic exposures (Figure S2); Venn diagrams displaying the number of microbial OTUs' shared groups (Figure S3); average percentages of the bacterial OTUs at the phylum level in the soil and gut (Figure S4); abundance and diversity of the microbial community in soil and earthworm gut (Figure S5); the diversity of soil and gut microbiota at the phylum level (Figure S6); the microbiota composition in soil and earthworm gut at the genus level (Figure S7) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86(0)592 6190559; fax: +86(0)592 6190977; e-mail: xmxue@iue.ac.cn. (X.-M.X.)

ORCID

Dong Zhu: 0000-0002-0826-6423

Yong-Guan Zhu: 0000-0003-3861-8482

Notes

The authors declare no competing financial interest.

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