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Roles of different active metal-reducing bacteria in arsenic release from arsenic-contaminated paddy soil amended with biochar

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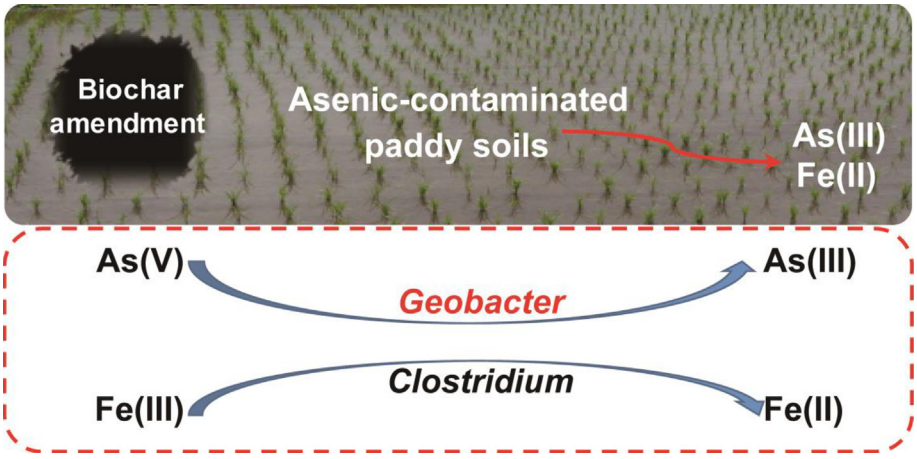
HIGHLIGHTS

- Biochar amendment simultaneously increased microbial reduction of As(V) and Fe(III).
- Biochar increased the abundance of *Geobacter*, *Anaeromyxobacter* and *Clostridium*.
- The abundance of *Geobacter* transcripts closely tracked with As(V) contents.
- The abundance of *Clostridium* transcripts closely tracked with Fe(III) contents.
- *Geobacter* has a role in As(V) reduction while *Clostridium* was in Fe(III) reduction.

GRAPHICAL ABSTRACT



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ABSTRACT

Although biochar has great potential for heavy metal removal from sediments or soils, its impact on arsenic biogeochemistry in contaminated paddy fields remains poorly characterized. In this study, anaerobic microcosms were established with arsenic-contaminated paddy soil to investigate arsenic transformation as well as the potentially active microbial community and their transcriptional activities in the presence of biochar. The results demonstrated that biochar can simultaneously stimulate microbial reduction of As(V) and Fe(III), releasing high levels of As(III) into the soil solution relative to the control. Total RNAs were extracted to profile the potentially active microbial communities, which suggested that biochar increased the abundance of arsenic- and iron-related bacteria, such as *Geobacter*, *Anaeromyxobacter* and *Clostridium* compared to the control. Reverse transcription, quantitative PCR (RT-qPCR) showed that the abundance of *Geobacter* transcripts were significantly stimulated by biochar throughout the incubation. Furthermore, significant positive correlations were observed between the abundance of *Geobacter* transcripts and As(V) concentrations, and between that of *Clostridium* transcripts and Fe(III) concentrations in biochar-amended microcosms. Our findings suggest that biochar can stimulate the activity of metal-reducing bacteria to promote arsenic mobility. The *Geobacter* may contribute to As(V) reduction in the presence of biochar, while *Clostridium* has a role in Fe(III) reduction.

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1. Introduction

Arsenic contamination of paddy fields is of increasing concern because of the threat it poses to food safety and human health worldwide [1,2]. Intermittent flooding and draining cycles can significantly affect the fate of arsenic in paddy fields. In particular, the flooding conditions in paddy fields can lead to the release of As(III) and its subsequent assimilation by rice plants [3,4]. It has been found that the dietary intake of rice has become a major source of arsenic in the human body [5,6]. Therefore, it is essential to understand the mechanism of arsenic release in anoxic paddy soils for the purpose of the remediation of arsenic-contaminated paddy fields.

Biochar, a carbon-rich solid product of the pyrolysis of organic material under low oxygen conditions, has attracted much attention recently due to its agronomic and environmental benefits [7–9]. The application of biochar to soil can enhance long-term carbon sequestration, modify soil quality properties, and increase crop yield [8,10]. Biochar also has a high pH, sorption capacity, surface area, cation exchange capacity and micropore volume [11,12], which enable it to be used for the removal of heavy metal contaminants from soils [13]. Furthermore, it has been demonstrated that biochar can facilitate electron transfer, leading to the facilitation of metal reactions [14–16]. In previous studies, biochar has had either an enhanced [17] or a negligible [18,19] influence on arsenic removal have been suggested using biochar. For instance, it has been reported that soluble arsenic concentrations did not decline after the addition of biochar to a contaminated soil for arsenic remediation in a column leaching experiment [18]. Conversely, a great increase in arsenic release was observed when biochar was applied to contaminated sediment or paddy soil [20,21]. It was implied that the enhanced arsenic release resulting from biochar amendment is mainly due to the shift in the microbial community and an increase in the abundance of Fe(III)-reducing bacteria [20,21].

Metal-reducing bacteria play a major role in controlling the mobility of metal contaminants through metal respiration [22]. In paddy fields, arsenic release is closely correlated with the redox reactivity of iron oxides [23,24]. Briefly, in drained paddy fields, the mobility of As(V) is low because Fe(III) (hydr) oxides can highly adsorb As(V) and constrain arsenic mobility. However, under flooding conditions, the microbially mediated Fe(III) reduction can result in the release of adsorbed As(V) into solution, and concomitantly, both adsorbed and dissolved As(V) can be reduced to As(III) by dissimilatory As(V)-reducing bacteria [25]. Some dissimilatory As(V)-respiring bacteria are also iron-reducers, such as *Geobacter* or *Shewanella*, which can simultaneously release Fe(II) and As(III) [23,26]. According to 16S rRNA-based microbial community profiling (the present communities), different arsenic- and iron-related members were identified in arsenic-contaminated soil or sediment. For example, *Clostridium*, *Bacillus* and *Caloramator* were predominant in arsenic-contaminated paddy soil amended with biochar [21], whereas biochar amendment increased the abundance of *Geobacter*, *Anaeromyxobacter*, *Desulfosporosinus* and *Pedobacter* in arsenic-contaminated sediment [20]. An increasing number of studies profile the microbial communities on the basis of RNA (the potentially active communities) and the transcriptomic activity, which is considered as a better indicator of microbial activity [27,28]. It is important to characterize the transcription activity of potentially active arsenic-related microbial community in paddy soil with biochar to better understand the effect of biochar on arsenic transformation. However, the potential influence of biochar amendment on transcriptional activity of microbial communities and their functions is still unknown in arsenic-contaminated paddy soils.

In the present study, anaerobic microcosms were established using arsenic-contaminated paddy soil with biochar. The aims of this study were: (i) to evaluate the potential impacts of biochar amendment on arsenic biogeochemistry in anoxic paddy soils by monitoring the dynamics of arsenic and iron speciation in biochar-amended and biochar-free microcosms throughout the incubation; (ii) to characterize the diversity and composition of the potentially active microbial community during arsenic transformation using high-throughput sequencing of the extracted total RNAs; and (iii) to quantify the number of transcripts from major arsenic-related bacteria and their potential roles using RT-qPCR and further analysis of the correlations between arsenic mobility and their transcripts.

2. Material and methods

2.1. Soil, biochar and microcosm preparation

The soil was collected from an arsenic-contaminated paddy field in the area downstream of the Lianhua Mountain tungsten mine, located in Shantou, Guangdong Province, China. The soil was randomly collected from 3 to 5 sampling points in the paddy field and mixed as a composite sample, then immediately transported to the laboratory under ice and stored at 4 °C for further analysis. The fundamental chemical properties of the soil are shown in Table S1. The biochar used in this study was made from oil palm fibers, sourced from Malaysia. After air-dried, the oil palm fibers were charred at 700 °C for 4 h in a muffle furnace using N_2 as the medium gas. The pyrolysis temperature was raised to the desired values at a ramp rate of 1.5 °C min^{-1}. Biochar was milled to pass a 2 mm sieve and stored in a drying oven for further analysis and incubation experiments. The elemental composition of the biochar is presented in Table S2. Fourier transform infrared (FT-IR) spectroscopy analysis was conducted to characterize the surface functional groups of biochar using methods described previously (Fig. S1) [29].

To simulate flooded conditions in the paddy field, the paddy soil was incubated with deionized water at 30 °C for approximately three weeks to activate the soil microbes and deplete the indigenous electron acceptors [30,31]. Anaerobic microcosm experiments were set up in 120 ml serum vials with 70 ml liquid volume (30 mM PIPES buffer, pH 7.3) and 7 g paddy soil (wet weight) at 30 °C under an atmosphere of N_2 in the dark without shaking. For each culture, 1 ml l^{-1} trace element solution, 1 ml l^{-1} vitamin solution [32] and 10 mM lactate were added. Three treatments were set up in triplicate: (i) paddy soil amended with 3% (w/w) biochar (biochar-amended), (ii) untreated paddy soil (control), and (iii) the sterile control (sterilized by 50 kGy of gamma-ray irradiation). Biochar was added at a ratio of 3% (w/w), which is reported to have greater potential to increase soil C/N ratio and plant productivity compared to a 1% application rate [33,34] and has been frequently chosen in previous studies [20,21,35]. At each sampling period (day 0, 1, 2, 5, 10 and 20), three microcosms were removed and subjected to destructive sampling for further arsenic and iron speciation, and total RNA extraction.

2.2. Arsenic and iron speciation

After centrifugation (9000 rpm, 15 min), the supernatant of each microcosm was passed through a sterilized 0.22 μm filter for analysis of aqueous arsenic speciation using hydride generation-atomic fluorescence spectrometry (HG-AFS, Jitian AFS-820, Beijing, China) by a previously described method [36]. The HPLC-HG-AFS system consists of an LC-15C liquid chromatograph system (Shimadzu Corporation, Japan) and an HG-AFS detection system (AFS-820, Jitian, Beijing). The separation of the arsenicals was performed on a Hamilton PRP-X 100 anion-exchange column (250 mm × 4.1 mm) at 30 °C with an isocratic elution by 15 mmol l^{-1} $(NH_4)_2HPO_4$ solution (adjusted to pH 6.0 with 10% HNO_3 solution, v/v) under a flow rate of 1.0 ml min^{-1}. The hydride (arsine) was generated by reduc-

tion with 7% HCl (v/v) solution and with freshly prepared 2.5% KBH_4 (w/v) stabilized in 0.5% KOH solution (w/v) at flow rates of 3.2 and 4.8 ml min^{-1}, respectively. After being separated by the gas-liquid separator, the analytes in the gas phase were carried into the atomizer and detected by the atomic fluorescence detector. The pellets were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The concentrations of Fe(II) and Fe(III) in the soil solutions were quantified based on the method described [37].

2.3. RNA extraction and reverse transcription

Total RNA from the biochar-amended and control microcosms was extracted at each time point (total 36 RNA extracts) using the MoBio PowerSoil Total RNA Isolation Kit (Mio Bio Laboratories, Inc., USA) according to the instructions of the manufacturer. The RNA was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Shiga, Japan) to eliminate the possibility of genomic DNA contamination following the manufacturer's instructions.

2.4. Illumina sequencing of the potentially active bacterial communities

To characterize the composition of the potentially active microbial communities in the biochar and control microcosms, cDNA samples from 3 replicates at each sampling time (total 36 cDNA) were subjected to high-throughput sequencing targeting the V4 regions 16S rRNA gene (amplified using primers 515F and 806R). The primer 806R included the Illumina sequencing adapter, a 12-bp barcode (unique to each sample), and a CC linker [38]. All of the amplifications were performed in 50 μl reactions containing 25 μl 2 × *Ex* Taq MasterMix (Takara, Shiga, Japan), 0.2 μM of each primer, 6 mM of bovine serum albumin (BSA) and *c.* 10 ng of total cDNA. The PCR products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek, Doraville, GA, USA). The concentrations were quantified using a Qubit 3.0 Fluorometer (Invitrogen, NY, USA). Paired-end sequencing was performed on an Illumina MiSeq 2500 sequencing platform (Illumina, San Diego, CA, USA) at Biomarker Technologies CO., LTD, Beijing, China (http://www.biomarker.com.cn/).

2.5. Quantification of transcripts with real-time PCR

Quantification of the transcripts of the bacterial 16S rRNA gene, *Geobacter*, *Shewanella*, *Clostridium* and *Anaeromyxobacter* was performed on an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) using the SYBR Green I detection method. Each 20 μl qPCR mixture contained 10 μl of 2 × IQ™ SYBR® Green Supermix (Bio-Rad, USA), 0.2 μM of each primer and 1 μl of cDNA (10 ng). For each reaction, the primers of 338F/518R (16S rRNA) [39,40], Geo494F/Geo825R (*Geobacter*) [41], Chis150f/ClostIr (*Clostridium*) [42], FAc12-66F/FAc12-432R (*Anaeromyxobacter*) [43] and She120F/She220R (*Shewanella*) [44], were used, respectively. Details describing the qPCR and primers used for each reaction are in Table S3. All of the qPCR assays were run in triplicates. The plasmid pGEM-T Easy Vector (Promega, Madison, WI, USA) was used in the cloning of specific fragments to establish standard curves [45]. The plasmid DNA was extracted using an EZNA Plasmid Mini Kit I (Omega Bio-Tek, Doraville, GA, USA) and the concentration was determined using a Qubit 3.0 Fluorometer.

2.6. High-throughput sequencing data processing and statistical analysis

The sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME 1.8.0) toolkit [46]. After removing low quality or ambiguous reads, high-quality paired-end reads were made using the FLASH (Fast Length Adjustment of Short reads) [47]. Operational taxonomic units (OTUs) were defined at the 97% sequence similarity level by UCLUST [48]. Representative sequences were chosen for each OTU [49], and the taxonomic assignment was performed using an RDP classifier [50]. The alpha diversity indices, including observed species, chao1, PD_whole tree, Shannon and Simpson were calculated using QIIME. Both unweighted and weighted UniFrac metrics were performed for the principle coordinates analysis (PCoA) [51]. The 16S rRNA gene sequence datasets were submitted to the NCBI Sequence Read Archive (SRA) (BioProject accession number PRJNA355907).

The data are presented as the mean ± standard deviation (SD). The statistical significance of the differences between the biochar and control microcosms was determined via one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests in SPSS 22.0, and $P < 0.05$ was considered to be statistically significant. The correlation analysis was conducted using the Pearson and Spearman correlation at the significance level of $P < 0.05$ (two-tailed) using SPSS 22.0.

3. Results and discussion

3.1. Arsenic and iron release

Fig. 1 illustrates the transformation of arsenic and iron in different samples over the course of the 20 days of incubation. After the incubation of the paddy soil with biochar under anoxic conditions, a rapid increase in dissolved Fe(III) was observed on day 1, and then gradually declined (Fig. 1a); and the concentrations of dissolved Fe(II) increased successively throughout the incubation, reaching 4.7 mg l^{-1} on day 20 (Fig. 1a), which is approximately a 2-fold increase relative to the control level of 2.3 mg l^{-1} (Fig. 1b). This suggests that the Fe(III) reduction in the anoxic paddy soil was stimulated by biochar. Similarly, dissolved As(V) was higher in the biochar-amended microcosms than in the control, indicating that biochar can enhance the desorption or release of As(V) from paddy soil (Fig. 1a-b). The dissolved As(III) in the biochar-amended microcosms increased up to approximately 3-fold higher over the control (biochar-amended: 3.2 mg l^{-1}; control: 0.88 mg l^{-1}) on day 20 (Fig. 1a-b). Furthermore, the concentrations of As(III) tracked closely with those of Fe(II) over the incubation period in the biochar-amended microcosms, implying that the reduction of Fe(III) and As(V) occurs simultaneously in the presence of biochar. In sterile microcosms (sterilized by gamma irradiation), no significant release of arsenic or iron was observed over the incubation period (Fig. 1c), suggesting that the reduction of arsenic and iron was microbially driven.

It has been reported that biochar amendment can increase the soil pH due to its alkaline effect, which may further increase arsenic release [52]. In this study, the pH of different microcosms did not significantly change throughout the incubation (pH 7.4-7.5), suggesting that the pH effect of biochar on arsenic transformation is negligible. The reductive dissolution of Fe(III) (hydr) oxide plays a unique role in arsenic release [25,53]. Paddy fields usually contain abundant levels of iron oxides, in which Fe(III) reduction is usually accompanied by an increase in As(V) release [21,25,53]. Due to its high content of aromatic carbon structures, biochar can function as electron shuttles like humic substances, thereby accelerating the reduction of Fe(III) oxides [15,54]. Accordingly, the stimulated release of arsenic from arsenic-contaminated sediments or paddy soils through biochar amendment was attributed to enhanced Fe(III) reduction [20,21]. In this study, the reduction of As(V) and Fe(III) increased stimultaneously over the incubation period after the addition of biochar (Fig. 1). These results suggested

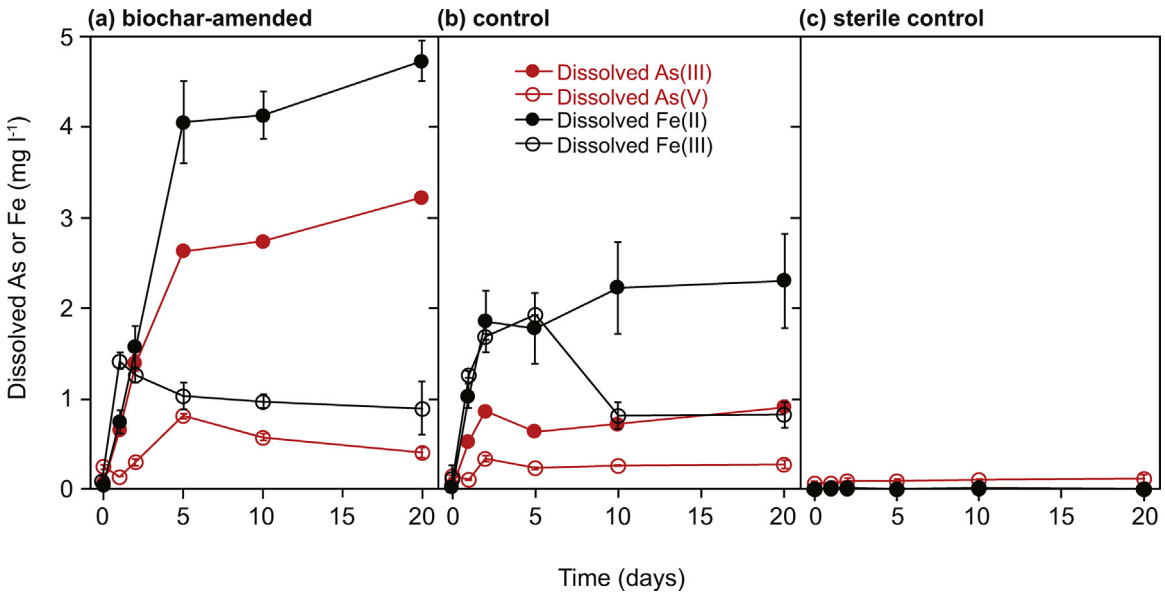

Fig. 1. The concentrations of dissolved As(III)/As(V), and dissolved Fe(II)/Fe(III) from the paddy soils in different treatments over the course of the incubation period in the (a) biochar-amended (the biochar-amended microcosms), (b) control (the biochar-free microcosms), and (c) sterile control. Bars represent standard errors (n = 3).

that microbial Fe(III) reduction was coupled with the arsenic reduction and release in the presence of biochar, both of which showed positive responses to the addition of biochar. Additionally, it can be speculated that biochar amendment also enhances the microbial reduction of As(V), likely due to the electron transfer capacity of biochar (electron shuttle), releasing high levels of As(III) and Fe(II) to soil solutions.

3.2. Composition of the potentially active bacterial community

After quality filtering, a total of 1,285,803 bacterial 16S rRNA sequences were obtained, with an average of 34,751 sequences for each sample, generating 5134 OTUs at the 97% similarity level (Table S4). Rarefaction analysis showed that the accumulation of observed species did not reach a clear saturation, indicating that even at the sequencing depth of 12,500, the diversity of soil bacteria had partially covered (Fig. S2). There were no significant differences in the alpha diversity indices (observed species, chao1, PD whole tree, Shannon and Simpson) between the biochar-amended and control microcosms at the same sequencing depth (12,500 sequences per sample) (Table S4), which suggests that biochar amendment had a negligible influence on the bacterial community diversity in the tested paddy soil.

Within the domain *Bacteria*, 16 distinct active phyla and classes were detected (with a relative abundance > 1% in at least one sample). The abundant bacterial communities were *β-Proteobacteria* (biochar-amended: 25–39%; control: 19–43%), *δ-Proteobacteria* (21–39%; 13–27%), *γ-Proteobacteria* (2–11%; 2–7%), *Actinobacteria* (2–3%; 2–6%) and *Acidobacteria* (3–7%; 2–4%) in both the biochar and control microcosms over the incubation period (Fig. 2). The abundance of *Firmicutes* increased significantly from 1% (biochar-amended) and 14% (control) on day 0–11-30% (biochar-amended) and 28–41% (control) within the first two days, respectively, and then rapidly declined to 3–4% over the rest of the incubation period. The difference in the abundance of *Firmicutes* between biochar-amended and control microcosms on day 0 can be reasonably attributed to changes in transcriptional-level response induced by biochar. This finding demonstrates that the transcriptomic analysis is a powerful approach to reflect the physiological activity of microbial population.

At the genus level, 14 active genera were detected with relative abundance > 1% in at least one sample. *Azoarcus* (4–27%), *Geobacter* (2–17%) and *Anaeromyxobacter* (4–9%) represented the dominant active bacteria in both the biochar-amended and control microcosms throughout the incubation period (Fig. 3). A high abundance of *Azoarcus* was observed in both treatments (biochar-amended: 4–27%; control: 5–27%), indicating that *Azoarcus* was the indigenous soil bacteria and that it may be associated with arsenic and iron transformation. Members of *Azoarcus* are known to be capable of performing nitrogen-fixation or denitrification, without any Fe(III) or As(V) respiration previously recorded [55]. However, results of whole-genome sequencing have demonstrated that many *Azoarcus* species contained the *ars* operon in their genomes [56–59]. In fact, previous studies have reported that *Azoarcus* can frequently be identified by targeting the 16S rRNA gene when trying to isolate arsenic-resistant bacteria with high arsenic concentration [60]. It remains to be clarified whether there is a direct association between *Azoarcus* members and arsenic/iron reduction. Considering the uncertainty surrounding the effects of the *Azoarcus* on As(V) reduction and the difficulty in universal primer design, we did not quantify the number of transcripts from *Azoarcus* in this study.

The second most abundant genus is *Geobacter*. Compared to the control (2–11%), the abundance of *Geobacter* (8–17%) was significantly increased by biochar throughout the incubation period (Fig. 3). Members of the *Geobacter* genus are the dominant Fe(III)-reducing bacteria in a variety of anoxic subsurface environments and have been shown to be involved in the bioremediation of both organic and metal contaminants including arsenic [22,23]. The presence of some *Geobacter* species such as *Geobacter uraniireducens* Rf4, *Geobacter lovleyi* SZ and *Geobacter* sp. OR-1, has been frequently reported to be found in arsenic-rich sediments or soils [23,61–63]. These results demonstrated that *Geobacter* could play an important role in arsenic transformation in flooded paddy soils.

Regarding *Anaeromyxobacter*, another dominant active genus, the biochar amendment slightly increased its abundance (5–9%) relative to the control (3–7%), with no significant differences observed between these two treatments (Fig. 3). The *Anaeromyxobacter* genus can utilize metals, including Fe(III) and uranium, as electron acceptors for respiration [44,64]. A dissimilatory As(V)-reducing bacterium, *Anaeromyxobacter* sp. strain PSR-1, was isolated from the contaminated soil [65], which demonstrated

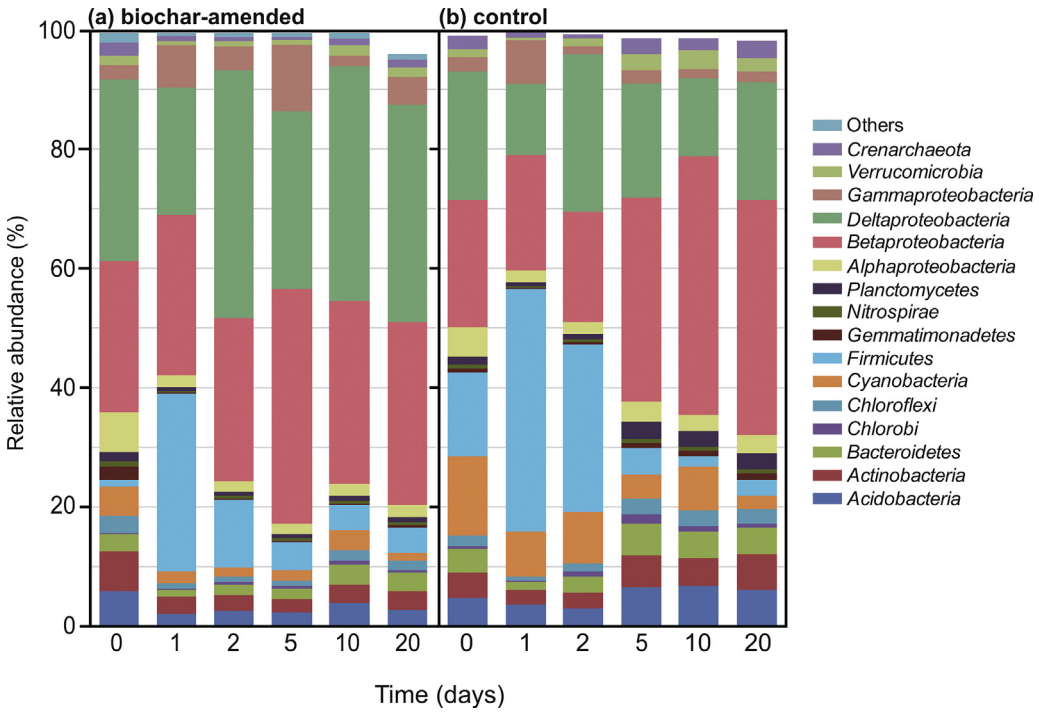

Fig. 2. The relative abundance of the potentially active bacterial community at the phylum and class levels detected in the (a) biochar-amended and (b) control microcosms using 16S rRNA high-throughput sequencing at each sampling time (RNA-based). Only the phyla with relative abundance higher than 1% in at least one sample were selected for further analysis. The abundance is presented in terms of an average percentage of the three replicates, classified by the RDP Classifier at a confidence threshold of 97%. "Other" refers to the sum of the unclassified sequences in each sample.

that the *Anaeromyxobacter* could also involve in As(V) reduction. Additionally, a large increase in the abundance of *Clostridium* was also observed in the biochar-amended and control microcosms on day 1 (28–39%) and day 2 (9–15%), followed by a rapid decreased to 1–2% over the remaining incubation period (Fig. 3). Members of *Clostridium* are known to be fermentative bacteria and are also capable of reducing a range of metals, including arsenic and iron [66]; this suggests that *Clostridium* could also affect arsenic and iron transformation in flooded paddy soils. Thus, the addition of biochar to the flooded paddy soils can increase the abundance of active arsenic and iron-related bacteria such as *Geobacter* and *Anaeromyxobacter*, releasing As(III) into the soil solution. In flooded paddy soils, *Clostridium* was the dominant indigenous active bacteria that could be involved in As(V) and Fe(III) reduction. As for the *Azoarcus*, its influence on As(V) reduction and release has remained unclear, although it was also represented as an abundant indigenous active bacteria.

In this study, biochar increased the abundance of arsenic- and iron-related bacteria, such as *Geobacter*, *Anaeromyxobacter* and *Clostridium*. One of the possible reasons for this is that biochar has porous structure and large specific surface area, which can enhance the probability of contact between bacteria and organic nutrients by providing a good habitat or surface [67,68]. In addition, another key aspect is the feature of biochar's electron transfer capacity, which can also stimulate the activity of As(V)- and Fe(III)-reducing bacteria [20,69]. Due to its condensed aromatic structures and quinine/hydroquinone moieties, biochar can facilitate electron transfer from microbes to terminal electron acceptor [15,70]. The biochar used in this study had a high C/H ratio (> 55), which suggests that the biochar may have a high C aromaticity [71] and a high electron accepting capacity [72]. The FTIR spectrum of the biochar suggested the presence of aryl carbonyl carbon group (C═O) at 1653 cm^{-1} and methyl group (C—H) at 1385 cm^{-1} [73,74] (Fig. S1), again demonstrating that it has the potential in facilitating electron transfer.

3.3. Transcriptions of the potentially active metal-reducing bacteria

In this study, the four genera of *Geobacter*, *Clostridium*, *Anaeromyxobacter*, and *Shewanella* were selected for transcriptomic analysis using RT-qPCR. *Geobacter*, *Clostridium* and *Anaeromyxobacter* were selected for their (i) high relative abundance in both biochar-amended and control microcosms, and (ii) capacity for As(V) and Fe(III) reduction under anoxic conditions [23,44,66]. Although the *Shewanella* genus was not detected in this study, it is a well-known Fe(III)-reducing genus and some members could also respire As(V) [22,75]; therefore, this genus was also selected.

To minimize the variances in background bacterial abundances, and extraction and analytical efficiencies, the absolute transcript copy number of *Geobacter*, *Clostridium*, *Anaeromyxobacter*, and *Shewanella* were normalized to those of ambient 16S rRNA genes in the individual microcosm (Fig. S3). The relative abundance of transcripts for *Geobacter* were significantly higher in biochar microcosms ($0.6\text{-}3.6 \times 10^{-2}$) than in control microcosms ($0.3\text{-}3.1 \times 10^{-3}$) over the incubation period (Fig. 4a). The relative transcript abundance of *Clostridium* rapidly reached its maximum on day 1 (4.3×10^{5}), and then gradually declined for the rest of the incubation, with higher transcripts observed in biochar-amended microcosms than in control late in the incubation (10-20 days) (Fig. 4b). The relative abundance of *Anaeromyxobacter* transcripts tended to be higher in biochar-amended microcosms than in the control, except for on day 5 and day 20 (Fig. 4c). Additionally, biochar significantly increased the transcript copies of *Shewanella* over the first two days of the incubation (5.5×10^{-4}) relative to the control (1.5×10^{-4}) (Fig. 4d). Overall, the addition of biochar to arsenic-contaminated paddy soil significantly stimulated the transcriptional activities of *Geobacter*. Furthermore, biochar also has a positive impact on the transcriptions of *Anaeromyxobacter* and *Shewanella* in the early stages of incubation, while *Clostridium* exhibits the reverse behavior.

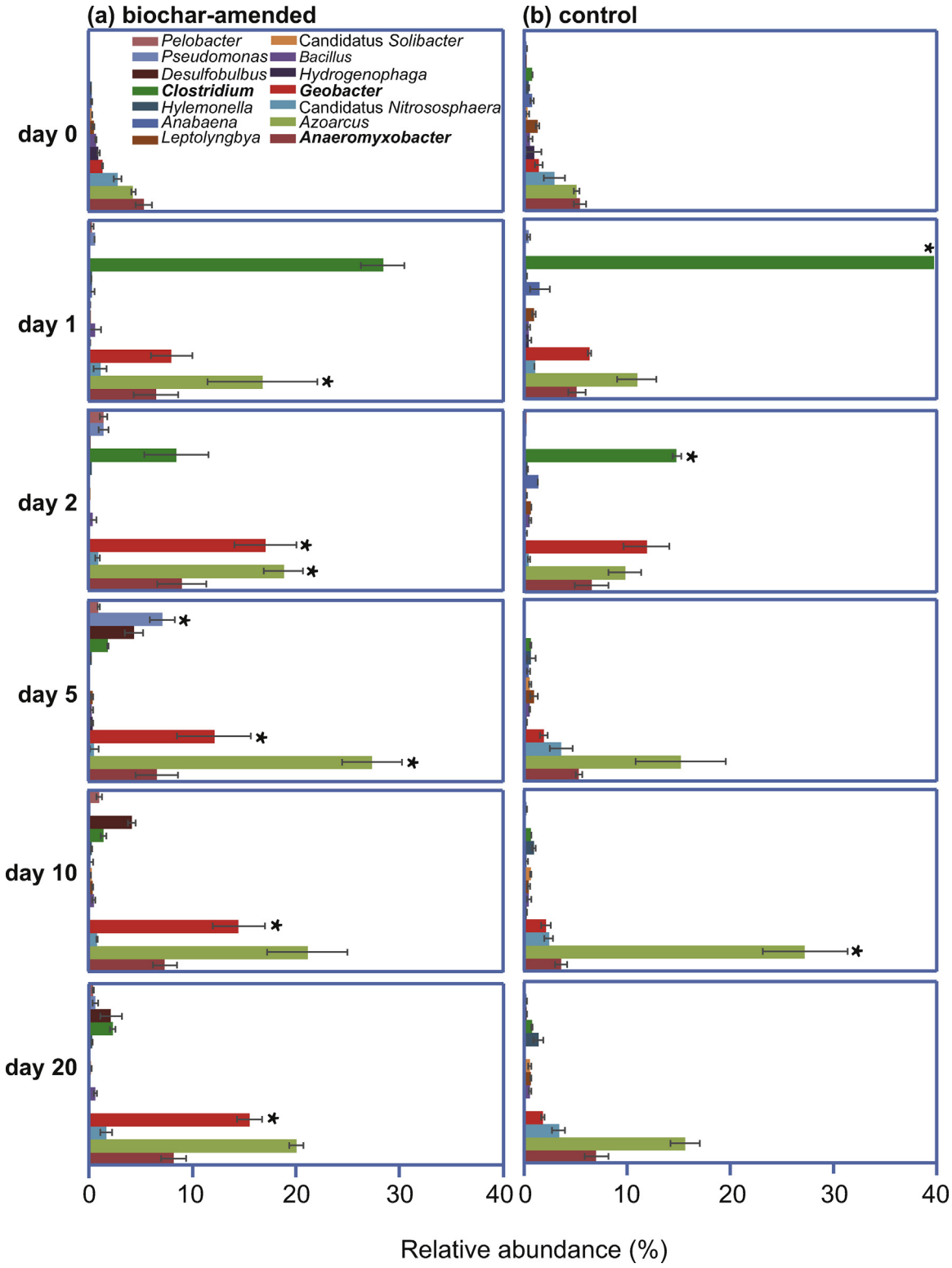

Fig. 3. The relative abundance of the potentially active bacterial community at the genus level detected in the (a) biochar-amended and (b) control microcosms throughout the incubation (with a relative abundance over 1% in at least one sample). Asterisks indicate significant differences ($P < 0.05$).

Table 1 illustrates the potential correlation between the relative abundance of individual transcripts and dissolved As(V) or Fe(III) concentrations throughout the incubation. There was a significantly strong positive correlation between the number of *Geobacter* transcripts and dissolved As(V) concentrations in biochar-amended microcosms (Pearson's correlation r = 0.985, $P < 0.001$) and Spearman's correlation R = 1 ($P < 0.001$), while no significant correlation was found in the control (Table 1 and Fig. S4). Additionally, transcript abundances of *Clostridium*, *Anaeromyxobacter* and *Shewanella* did not correspond well with dissolved As(V) concentrations throughout the incubation in biochar-amended or control microcosms. There were no significant correlations

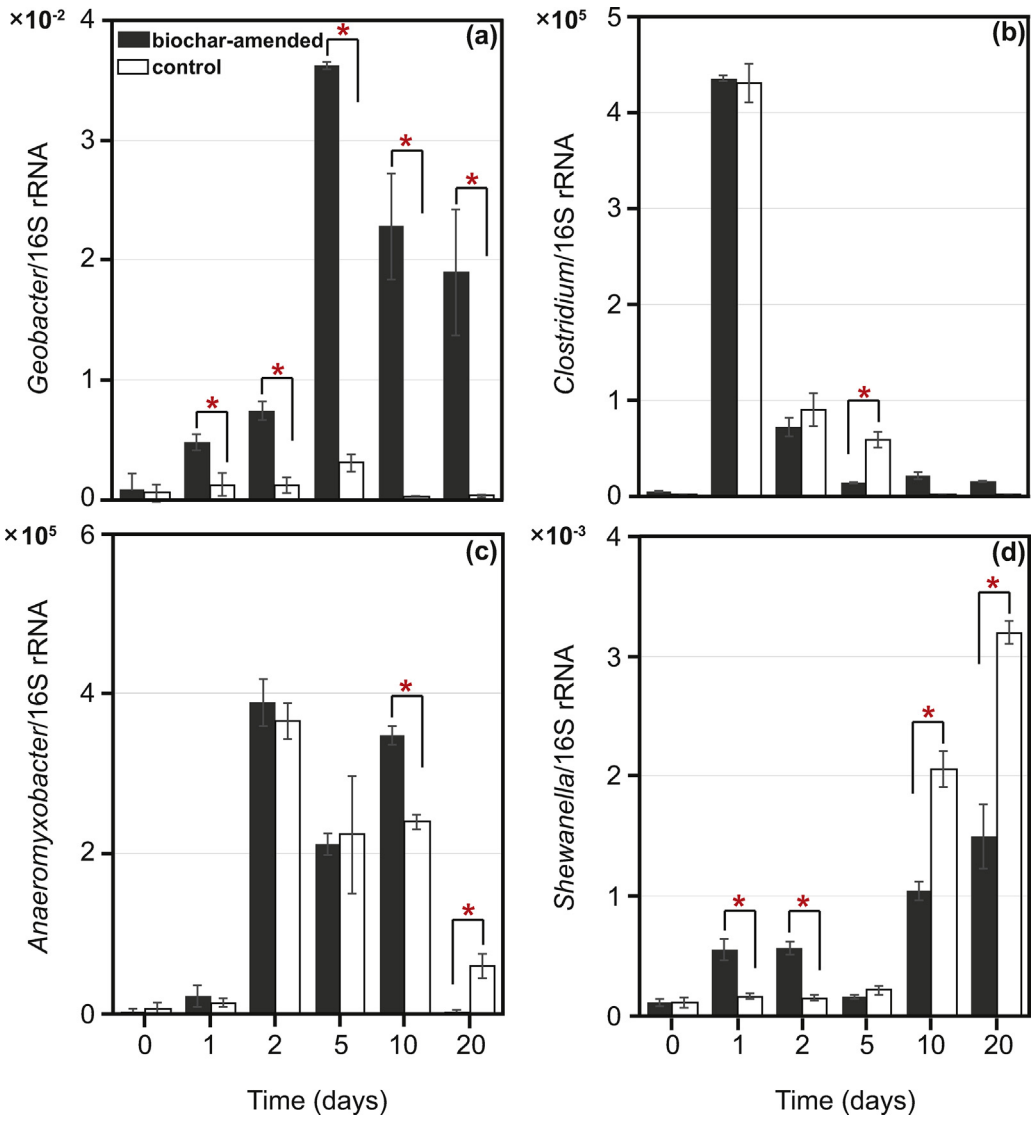

Fig. 4. The relative transcript abundance of (a) *Geobacter*, (b) *Clostridium*, (c) *Anaeromyxobacter* and (d) *Shewanella* in the biochar-amended and control microcosms throughout the incubation. Asterisks indicate significant differences ($P < 0.05$). Bars represent standard errors (n = 3).

Table 1
Pearson and Spearman correlation matrix for transcripts levels of *Geobacter*, *Clostridium*, *Anaeromyxobacter* and *Shewanella* normalized to 16S rRNA gene, dissolved As(V) and dissolved Fe(III) in biochar-amended and control microcosms.

Treatments	Dissolved As(V)		Dissolved Fe(III)	
	biochar-amended	control	biochar-amended	control
Pearson correlation				
Geobacter/16S rRNA	**0.985**(*P* <0.001)**	0.343 (*P* = 0.505)	0.201 (*P* = 0.702)	−0.257 (*P* = 0.623)
Clostridium/16S rRNA	−0.351 (*P* = 0.495)	−0.350 (*P* = 0.496)	0.593 (*P* = 0.215)	−0.143 (*P* = 0.787)
Anaeromyxobacter/16S rRNA	0.427 (*P* = 0.399)	0.575 (*P* = 0.232)	0.382 (*P* = 0.455)	0.543 (*P* = 0.266)
Shewanella/16S rRNA	0.046 (*P* = 0.930)	0.246 (*P* = 0.638)	0.246 (*P* = 0.638)	−0.029 (*P* = 0.957)
Spearman's correlation				
Geobacter/16S rRNA	**1.000** (*P* <0.001)**	−0.257 (P = 0.623)	0.086 (*P* = 0.872)	0.771 (*P* = 0.072)
Clostridium/16S rRNA	−0.086 (*P* = 0.872)	−0.143 (*P* = 0.787)	**0.829* (P = 0.042)**	0.600 (*P* = 0.208)
Anaeromyxobacter/16S rRNA	0.486 (*P* = 0.329)	0.543 (*P* = 0.266)	0.600 (*P* = 0.208)	0.371 (*P* = 0.468)
Shewanella/16S rRNA	0.371 (*P* = 0.468)	−0.029 (*P* = 0.957)	0.029 (*P* = 0.957)	−0.600 (*P* = 0.208)

between the abundance of transcripts for *Geobacter* and dissolved Fe(III) in biochar-amended microcosms (Table 1). In contrast, the number of transcripts from *Clostridium* had a significant positive correction with Fe(III) in biochar-amended microcosms (Spearman's correlation R = 0.829 ($P < 0.05$)) (Table 1 and Fig. S5). Similar to dissolved As(V), the abundance of transcripts for *Anaeromyxobacter* and *Shewanella* did not correspond well with dissolved Fe(III) concentrations in the biochar and control microcosms (Table 1). Thus, in the presence of biochar in anaerobic paddy soil, members of *Geobacter* and *Clostridium* can rapidly respond to As(V) and Fe(III) concentrations by improving their transcripts.

In fact, *Geobacter* species have frequently been detected based on microbial community analysis of the sequences of 16S rRNA or dissimilatory As(V)-reducing gene in arsenic-rich sediments from South and Southeast Asia [20,21,61–63,76,77]. Genome sequencing of *Geobacter uraniireducens* Rf4, *Geobacter lovleyi* SZ and *Geobacter* sp. OR-1 demonstrated that they all possess the *arr* operon for As(V) respiration [78–80]. Additionally, direct evidence for As(V) respiration by *Geobacter* sp. OR-1 isolated from paddy soil has been verified [23]. Thus, our results indicate that *Geobacter* should primarily participate in the reduction of As(V) in the presence of biochar. While some species of the Clostridium genus can utilize As(V) as an electron acceptor for respiration, such as *Clostridium* sp. OhiLAs [81], many isolates from this genus have been identified as dissimilatory Fe(III)-reducing bacteria, such as *Clostridium beijerinckii* [82], *Clostridium butyricum* [83] and *Clostridium saccarobutylicum* BS2 [84]. In this study, the abundance of *Clostridium* transcripts closely tracked only with dissolved Fe(III) concentrations in the presence of biochar, indicating that *Clostridium* is mainly involved in Fe(III) reduction.

4. Conclusions and implications

Our study demonstrated that the addition of biochar to arsenic-contaminated paddy soils can simultaneously enhance the microbial reduction of As(V) and Fe(III), releasing high levels of As(III) into the soil solution under anoxic conditions. Biochar amendment increased the relative abundance of the potentially active arsenic- and iron-related bacteria, such as *Geobacter*, *Anaeromyxobacter* and *Clostridium*. Furthermore, biochar stimulated the transcriptional activities of the potentially active metal-reducing bacteria, particularly the *Geobacter*. The *Geobacter* plays a major role in the reduction of As(V) and the *Clostridium* predominantly participates in Fe(III) reduction.

Based on previous studies, the effect of biochar amendment on arsenic mobility is complicated. As(III) was effectively immobilized using sludge-derived biochar via adsorption [85], while biochar did not influence arsenic removal from water [17,18]. Our results and a recent study demonstrated that the application of biochar to arsenic-contaminated paddy soils can lead to an increase in arsenic reduction and release [21]. In contrast to the previous study, we used a transcriptomic approach to evaluate the effect of biochar on the arsenic-related microbial community and their transcriptional activities during arsenic transformation. The results obtained in this study demonstrated the roles of different potentially active metal-reducing bacteria in arsenic release from an arsenic-contaminated paddy soil amended with biochar. Because As(III) commonly exists as uncharged molecules ($H_3AsO_3^0$) at a pH of most natural environments and is much less adsorptive than negatively charged As(V) ($H_2AsO_4^-$, $HAsO_4^{2-}$) [86,87], the microbial reduction of As(V) contained in soils can lead to the mobilization and release of As(III) into the aqueous phase [88,89]. Thus, it appears possible that to use microbial reduction of As(V) to mobile As(III) as a technique for promoting the removal of As from arsenic-contaminated soils [90]. In fact, using quinone compounds such as anthraquione-2,6-disulfonate (AQDS) to improve the removal efficiency of arsenic by a dissimilatory arsenate-reducing bacterium have showed great potential for application in arsenic bioremediation of industrially contaminated soils [91]. Furthermore, it is verified that the natural occurring electron shutting mediator, biogenic riboflavin that could also stimulate soil microbial community for arsenic removal can be used as a remediation strategy for arsenic-contaminated soils [92]. In our study, biochar addition to arsenic-contaminated paddy soils significantly stimulated microbial arsenic reduction, releasing amounts of mobilized As(III) into soil solution (Fig. 1). Although dissolved As(III) in the biochar-amended microcosms was about 32 mg kg^{-1}, which only accounted for 10% of the initial soil-solid arsenic (300 mg kg^{-1}) (Table S1), it may still have the potential for arsenic removal after optimizing the application ratio of biochar, production temperature and biomass material in future. In addition, considering the fact that rice is particularly efficient in accumulating As(III) with high toxicity and mobility, high concentrations of dissolved As(III) resulting from biochar amendment under anoxic conditions may increase arsenic bioavailability to rice plants. According to a previous pot trial with rice and biochar, biochar amendments significantly increased pore water concentrations of arsenic, and rice shoot concentrations of arsenic increased by up to 327% due to biochar amendment [93]. Hence, further evaluation of the effects of biochar amendment on arsenic biogeochemistry and bioaccumulation should be conducted, considering the functional microbial community and arsenic-related genes and their correlations with biochar amendment. At present, the application of biochar to arsenic-contaminated paddy soil should be carefully considered, particularly for flooded paddy soil with rice.

Conflict of interest

We have no conflict of interest to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhazmat.2017.11.025.

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