Treatment of groundwater containing Mn(II), Fe(II), As(III) and Sb(III) by bioaugmented quartz-sand filters

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ABSTRACT

High concentrations of iron (Fe(II)) and manganese (Mn(II)) often occur simultaneously in groundwater. Previously, we demonstrated that Fe(II) and Mn(II) could be oxidized to biogenic Fe-Mn oxides (BFMO) via aeration and microbial oxidation, and the formed BFMO could further oxidize and adsorb other pollutants (e.g., arsenic (As(III)) and antimony (Sb(III))). To apply this finding to groundwater remediation, we established four quartz-sand columns for treating groundwater containing Fe(II), Mn(II), As(III), and Sb(III). A Mn-oxidizing bacterium (*Pseudomonas* sp. QJX-1) was inoculated into two parallel bioaugmented columns. Long-term treatment (120 d) showed that bioaugmentation accelerated the formation of Fe-Mn oxides, resulting in an increase in As and Sb removal. The bioaugmented columns also exhibited higher overall treatment effect and anti-shock load capacity than that of the non-bioaugmented columns. To clarify the causal relationship between the microbial community and treatment effect, we compared the biomass of active bacteria (reverse-transcribed real-time PCR), bacterial community composition (Miseq 16S rRNA sequencing) and community function (metagenomic sequencing) between the bioaugmented and non-bioaugmented columns. Results indicated that the QJX1 strain grew steadily and attached onto the filter material surface in the bioaugmented columns. In general, the inoculated strain did not significantly alter the composition of the indigenous bacterial community, but did improve the relative abundances of xenobiotic metabolism genes and Mn oxidation gene. Thus, bioaugmentation intensified microbial degradation/utilization for the direct removal of pollutants and increased the formation of Fe-Mn oxides for the indirect removal of pollutants. Our study provides an alternative method for the treatment of groundwater containing high Fe(II), Mn(II) and As/Sb.

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1. Introduction

Arsenic (As) and antimony (Sb) are two toxic metalloids that can occur simultaneously in groundwater (Hiller et al., 2012; Luo et al., 2014; McCarty et al., 2004). Both enter the aquatic ecosystem from natural and anthropogenic sources and can threaten water quality (Guo et al., 2009; Smedley and Kinniburgh, 2002). Furthermore, given their positions on the periodic table, As and Sb have similar chemical and toxicological properties (Bentley and Chasteen, 2002), with their trivalent species exhibiting greater toxicological effects than that of their pentavalent species (Gebel, 1997). Due to

the high toxicities of As and Sb, the drinking water standards suggested by the World Health Organization (2011) are set at 10 µg/l (As) and 5 µg/l (Sb).

Different treatment techniques to remove As and/or Sb from groundwater have been studied intensively, and include coagulation/flocculation, iron exchange, adsorption, and chemical redox (Camacho et al., 2011; Pawlak et al., 2006; Ungureanu et al., 2015). These techniques are often adopted for pre- and deep-treatment in addition to traditional groundwater remediation (i.e., aeration-filter-disinfection). However, additional processing can lead to additional operation costs and increased complexity. Hence, improvement of the current treatment practices, rather than additional processing steps, would be an optimal way in which to treat As- and Sb-contaminated groundwater. Sand filtration is widely used in drinking water treatment, and greensand filters

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(Viraraghavan et al., 1999) and household-scale sand filters (Berg et al., 2006; Voegelin et al., 2014) have been shown to remove Fe, Mn, and As simultaneously. However its biological roles in pollutant removal still remain largely unknown. We previously demonstrated that abundant microbes exist on the surface of sand filters to form stable biofilms (Bai et al., 2013). As microbial community diversity and function in engineering settings are strongly related to treatment effects, optimizing the microbial community within a sand filter would be of benefit for the improvement of effluent quality.

In addition to As and Sb species, high concentrations of iron (Fe(II)) and manganese (Mn(II)) often occur together in groundwater (Huang et al., 2012; Postawa and Hayes, 2013). Therefore, the objective of this study was to explore the conditions under which Fe(II), Mn(II), As, and/or Sb co-exist, and to eliminate or decrease these four metal/metalloid ions in a quartz-sand filter as used in drinking water treatment. We proposed a bioaugmentation method to treat groundwater containing As(III) and/or Sb(III) in the presence of high concentrations of Fe(II) and Mn(II). Specifically, we added Mn-oxidizing bacteria to a quartz-sand filter to increase the formation of Fe-Mn oxides (BFMO, a mixture of biogenic Mn(IV) oxide and chemical FeOOH (Bai et al., 2016a)) on the surface of the filters. Our earlier studies demonstrated that BFMO has strong oxidation and adsorption capacity for As(III) (Bai et al., 2016a) and Sb(III) (Bai et al., 2016b). Thus, we hypothesized that an increase in BFMO yield and attachment on the filters would result in more efficient As and Sb removal.

To verify this hypothesis, we established biological filters filled with quartz-sand and performed a long-term bioaugmentation study. The removal of the four metal ions was compared between bioaugmented (inoculated with Mn-oxidizing bacterium (*Pseudomonas* sp. QJX-1)) and non-bioaugmented columns. We determined whether the inoculated bacterial strain was retained in the filter after long-term operation, i.e., not washed out or outcompeted by other microbes, and whether it affected the indigenous microbial community (from groundwater community filtration) within the sand filter. We also attempted to clarify the causal relationships among the inoculated strain, indigenous microbial community, formation of BFMO, and treatment effect.

2. Materials and methods

2.1. Inoculated strain for bioaugmentation

A Mn-oxidizing bacterium, *Pseudomonas* sp. QJX-1 (GenBank accession number, KM242057) (Bai et al., 2016a), was used for this bioaugmentation study. QJX-1 is a gram-negative and rod-shaped bacterial strain, which is able to oxidize Mn(II) at an optimum temperature of 30 °C and pH of 7.5. The bacterial strain carries a *cumA* gene, which is proposed to be involved in Mn oxidation (Brouwers et al., 1999; de Vrind-de Jong et al., 2000) (Fig. S1). A culture of the strain was deposited in the China General Microorganism Culture Center (CGMCC; accession number, 6630).

Pseudomonas sp. QJX-1 was enriched in several 500-mL flasks containing 200 mL of PYG medium (Bai et al., 2016a), and then incubated at 30 °C and 170 rpm in a rotary shaker until the bacteria reached the logarithmic phase of growth (48 h). The bacterial cells were harvested by centrifugation at 3000g for 5 min, at room temperature. The cells were washed three times with 50 mL of sterile deionized water. The bacterial deposit was resuspended with 500 mL of sterile deionized water. The bacterial suspension was employed immediately as the inoculum in the following column experiment.

2.2. Column experiment startup and operation

Four parallel biofilter columns (height, 50 cm; diameter, 3 cm) were established (Fig. S2) to explore the effect of bioaugmentation on water treatment and microbial diversity and function inside the water treatment filters. The heights of the graded gravel layer and quartz-sand (2–5 mm particle size) filling layer were 3 cm and 17 cm, respectively.

All columns were seeded with raw groundwater (obtained from a drinking water treatment plant, with water quality described in Table S1) for microbial enrichment inside the filters. After 30 d, the reactor influent was replaced and prepared by adding certain amounts of Fe(II), Mn(II), As(III), and Sb(III) into the raw groundwater to simulate Fe(II)-Mn(II)-As(III)-Sb(III) co-contaminated groundwater. *Pseudomonas* sp. QJX-1 (approximately 0.63 g, dry weight at 105 °C) was inoculated into two replicate columns using a pump, thus representing the bioaugmented columns. Another two columns without inoculating bacteria were used as the negative controls, representing the non-bioaugmented columns. During the experiment, the influent containing Fe(II), Mn(II), As(III), and Sb(III) was aerated (dissolved oxygen was around 3.5 mg/L) using air pumps. The hydraulic retention time (HRT) was set at 0.17 h. Ambient temperature ranged from 18 °C to 33 °C during the experiment. Similar to actual quartz-sand filters in drinking water treatment, to prevent filter blockage by microbes and chemical substances in the effluent, we also performed backwashing every 48–72 h, with 8 L/(s m²) of backwash intensity and 3 min of backwash time.

We hypothesized that in the bioaugmented columns, the inoculated QJX-1 strain might oxidize Mn(II) in the groundwater to insoluble Mn(IV) oxides and then mix with chemical Fe(III) oxide originating from the chemical oxidation of Fe(II) by aeration (Bai et al., 2016a). The columns were operated for 120 d and influent and effluent water samples were then collected to compare the treatment effects between the bioaugmented and non-bioaugmented columns. After collection, the samples were filtered through a 0.45 µm membrane for Fe(II), Mn(II), total As, and total Sb determination. To compare the carbon (C) and nitrogen (N) utilization of the microbes between the bioaugmented and non-bioaugmented columns, dissolved organic carbon (DOC), NH₃-N, and NO₃⁻-N were analyzed in 116 d, 118 d, and 120 d influent and effluent samples when the column treatment was stable. In addition, at the end of the experiment (120 d), we sampled the sand filter material for surface structure analysis and also extracted DNA and RNA to compare the microbial community composition and function between the bioaugmented and non-bioaugmented columns.

2.3. Analytical methods

The filtered water samples were adjusted to pH < 2 with 1 mol/L HCl, and Fe(II), Mn(II), total As, and total Sb were then measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES 710, Agilent, USA). We determined pH with a pH meter (PHS-3C, Shanghai INESA & Scientific Instrument Co., Ltd., China), and TOC was measured with a TOC analyzer (TOC-V_{CPH}, Shimadzu, Japan). The concentrations of NH₃-N and NO₃⁻-N were quantified using standard spectrometry (State Environmental Protection Administration of China, 1989) and ion chromatography (DIONEX ICS-2000, USA), respectively. The morphological and elemental distributions of the biofilm attached to the quartz-sand were captured by field emission scanning electron microscopy (FE-SEM) coupled with an energy-dispersive X-ray (EDX) analyzer (Hitachi

SU-8020, Japan) (Bai et al., 2016a).

2.4. DNA and RNA extraction

Triplicate sand filter samples (5 g, wet weight) from the upper, middle, and bottom layers of the four columns were collected after treatment. Each sample was transferred to a 15 ml tube. Total RNA and total DNA were extracted simultaneously with an RNA PowerSoil total RNA isolation kit and an RNA PowerSoil DNA elution accessory kit (Mobio, USA) according to the manufacturer's protocols. The extracted RNA was digested with RNase-Free DNase I (Tiangen, China) for DNA elimination and then immediately reverse transcribed using a Tiangen Quantiscript Reverse Transcription Kit for cDNA synthesis according to the manufacturer's instructions. Total DNA and synthesized cDNA were stored at -20°C in a freezer for reverse-transcribed (RT) quantitative real-time polymerase chain reaction (RT real-time PCR) assay, 16S rRNA sequencing, and metagenomic sequencing.

2.5. RT real-time PCR

We performed RT real-time PCR to evaluate the quantities of active bacteria in the four columns. Bacterial 16S rRNA and *cumA* gene expressions were quantified from total cDNA samples using an ABI 7300 fast RT real-time PCR system (USA) based on the SYBR Green I method. The primer pairs and thermal programs are listed in Table S2. Standard curve construction has been described in detail previously (Bai et al., 2012). The specificity of the PCR for the target genes was checked using melting curve analysis and gel electrophoresis.

2.6. MiSeq sequencing for the bacterial 16S rRNA gene

The cDNA samples from the middle layers of the four columns were used for 16S rRNA sequencing to explore the differences in active bacterial community composition between the bio-augmented and non-bioaugmented columns. The V4 region of the 16S rRNA gene was amplified using barcoded primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR products were then purified with an AxyPrep DNA Gel Extraction Kit (Axygen, USA) in accordance with the manufacturer's instructions. The DNA concentration of the purified PCR products was measured by a TBS-380 Fluorometer (Turner Biosystems, CA, USA). Prior to sequencing, the amplicons from triplicate samples were pooled in equal amounts based on concentration. Sequencing was performed using an Illumina MiSeq sequencing platform. The raw sequence reads were initially filtered to remove low quality reads and barcode primers. The remaining high quality paired-end reads were connected to tags based on overlaps. The connected tags were then analyzed using the QIIME standard pipeline (Caporaso et al., 2010). Briefly, the sequences were subjected to chimera removal by UCHIME algorithm (Edgar et al., 2011). Operational taxonomic units (OTUs) were clustered using the Uclust program at the 97% similarity level (Edgar, 2010). Taxonomic annotations were assigned to each OTU representative sequence against the GreenGenes 16S reference database. The 50 most abundant OTUs were selected and a circular approximately-maximum-likelihood phylogenetic tree was constructed based on the sequence representatives for each OTU using the FastTree program (Price et al., 2010). The tree was displayed using iTOL v3 software (Letunic and Bork, 2016). Further, Venn diagrams were constructed using Mothur 1.36.1 software (Schloss et al., 2009) to ascertain the shared OTUs in the four column

samples.

To calculate the proportions of the inoculated strain in the bacterial communities of the four columns, the V4 region of the 16S rDNA sequences of the inoculated strains were picked out from previously identified 16S rDNA sequences (GenBank No. KM242057), and BLASTed against the high-quality sequences of the four datasets. The four column sequences with 100% sequence similarity to the QJX1 16S rRNA sequence were selected to calculate the inoculated strain proportion.

To explore whether the inoculated strain altered the indigenous bacterial community composition, we used "compare_categories.py" command in QIIME based on analysis of similarities (ANOSIM) to compare the bacterial OTUs between bioaugmented and non-bioaugmented columns, after excluding the OTUs belonging to the inoculated strain (QJX1).

All raw sequences generated from pyrosequencing were deposited in the NCBI Sequence Read Archive (SRA) database (accession no. SRX1738749, SRX1738754, SRX1738756, and SRX1738759).

2.7. Metagenomic sequencing

Total DNA samples from the middle layers of the four columns were used for metagenomic sequencing to explore the differences in community function between the bioaugmented and non-bioaugmented columns. We merged triplicate DNA samples (of equal amount) from each column into one individual metagenomic sample. The four metagenomic samples (of equal amount) were then used to construct two paired-end libraries (350 bp insert size) using an Illumina TruSeq DNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA) and cluster generation was performed on an Illumina cBot automated cluster generation system following the manufacturer's instructions. The prepared templates were loaded on the Illumina HiSeq 2000 sequencing system for sequencing (125 bp pair-end reads). Sequence reads were initially filtered to remove adapters, low quality reads, and reads that belonged to the host. The filtered reads (~12 Gbp in total for the four datasets) were uploaded to the MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology, v3.2.2) website (<http://metagenomics.anl.gov/>) for function annotation (Meyer et al., 2008). Function annotation in MG-RAST with set parameters (minimum alignment length, 30 bp; E-value cutoffs, $1e^{-5}$) were performed to assign reads to different functional groups (SEED) or metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG). In specific microbial degradation or transformation functions, we focused on the metabolism of xenobiotic compounds, inorganic N, and heavy metals (Mn, As, and Sb). For xenobiotic compounds, KEGG annotation in MG-RAST with set parameters (minimum alignment length, 30 bp; E-value cutoffs, $1e^{-5}$) was used. GenBank annotation was used as the reference database to search all major genes involved in nitrification and denitrification, and Mn, As, and Sb transformation. Metabolic genes present in the four columns were identified and the abundances of those gene sequences were calculated.

Statistical analysis of metagenomic profiles (STAMP) bioinformatics software (Parks and Beiko, 2010) was used to compare community function between the bioaugmented and non-bioaugmented samples. Statistical significance was calculated using two-sided Fisher's exact test, and the differences between proportions were analyzed using the Newcombe-Wilson method with a 95% confidence interval.

The four metagenomic datasets were deposited in the NCBI SRA database (accession no. SRX2184839, SRX2184841, SRX2184842,

and SRX2184843).

3. Results

3.1. Comparison of treatment effects between bioaugmented and non-bioaugmented columns

To compare treatment effects, we gradually increased Mn(II), As(III), and Sb(III) in the influent during the column study. At all times, the pH values did not significantly vary in the effluents of the four filter columns, and ranged from 7.0 to 7.2. The long-term experimental results (Fig. 1a) showed that in the initial phase, the removal of Mn increased from 80% to 100% within 20 d in the bioaugmented columns, but required 35 d to reach 100% in the non-bioaugmented columns. This revealed that the inoculated *Pseudomonas* sp. QJX-1 accelerated Mn(II) oxidation. As a result, the removal of Fe in bioaugmented columns was slightly higher than that in non-bioaugmented columns in the initial phase, which was due to a portion of Fe(II) being oxidized by the formed Mn oxides (Fig. 1b). After entering the stationary phase (35 d–72 d), both Fe and Mn were completely removed in the bioaugmented and non-bioaugmented columns. When the influent Mn(II) concentration increased from 2 mg/l to 4 mg/l (after 72 d), the removal of Mn decreased from 100% to 81–96% in the non-bioaugmented columns. However, Mn removal in the bioaugmented columns was still around 100%, which was likely due to the presence of the QJX1.

As shown in Fig. 1c, As removal in the bioaugmented columns was slightly higher than that in the non-bioaugmented columns in the initial phase, but reached almost 100% in both columns at 13 d. However, when influent As concentration increased from 0.1 mg/l to 0.2 mg/l, As removal continuously decreased in both the bioaugmented and non-bioaugmented columns. Remarkably, along with the increase in influent Mn(II) concentration (Fig. 1a), As removal in the bioaugmented columns gradually increased, but As removal in the non-bioaugmented columns decreased to 16%. The further increase in influent As concentration from 0.2 mg/l to 0.4 mg/l led to a greater difference in As removal between the bioaugmented and non-bioaugmented columns. These data indicate that the bioaugmented columns had a stronger ability on anti-shock load capacity than that of the non-bioaugmented columns due to the greater production of Mn oxides. Compared to As removal, the removal of Sb in the four columns (Fig. 1d) was comparatively low. In general, Sb removal in the bioaugmented columns was slightly higher than that in the non-bioaugmented columns when the influent Sb concentration was 0.1 mg/l. However, after the influent Sb concentration increased to 0.2 mg/l, the removal of Sb in the bioaugmented columns was significantly higher than that in the non-bioaugmented columns ($p < 0.01$, paired t -test) due to the oxidation and adsorption of Mn oxides (Bai et al., 2016b). The column experiments thus demonstrated that the bioaugmented quartz-sand filter had more efficient As and Sb removal and anti-shock load capacity than that of the non-

Fig. 1. Treatment efficiencies of total Mn (a), Fe (b), As (c), and Sb (d) in the bioaugmented and non-bioaugmented quartz-sand filters. Removal data from two parallel bioaugmented or non-bioaugmented columns were averaged.

bioaugmented filters.

We also compared the treatment of DOC, $\text{NH}_3\text{-N}$, and $\text{NO}_3^- \text{-N}$ between the bioaugmented and non-bioaugmented columns at 116, 118, and 120 d when the column treatments were stable. Results (Fig. S3) revealed that the bioaugmented columns had significantly higher treatment effect on DOC and $\text{NO}_3^- \text{-N}$ than that of the non-bioaugmented columns ($p < 0.05$, Student's t -test).

3.2. Comparisons of biomass, surface morphology, and elemental distribution

The RT real-time PCR results (Fig. 2) showed that the biomass of total active bacteria (reflected in 16S rRNA) and putative active inoculated strain (reflected in the *cumA* gene) in the bioaugmented and non-bioaugmented columns both displayed the layer pattern of upper > middle > bottom, though the biomass in the bioaugmented columns was several magnitudes higher than that in the non-bioaugmented columns. The abundances of the *cumA* gene in the different layers of the bioaugmented columns (0.48–0.69) were obviously higher than those of the non-bioaugmented column layers (0.04–0.10). These data demonstrate that the inoculated strain grew steadily on the surface of the sand filter material over the course of the long-term experiment.

SEM observations (Data not shown) showed that in the non-bioaugmented columns, the biofilm on the filter material had a granular structure and the microbes attached on the surface were of short-rod, long-rod, and spherical shapes. In the bioaugmented columns, however, the biofilm contained a large amount of flocs

and most biofilm microbes were of short-rod shape. The EDX analysis (Fig. S4) revealed that elements Fe, Mn, As, and Sb all existed in the sand filters, indicating the occurrence of BFMO formation and As and Sb adsorption.

3.3. Comparison of bacterial community composition

A total of 110,004 16S rRNA sequences from PCR of the four cDNA samples were obtained after pre-filtration of ambiguous sequences, with 25,768–28,632 16S rRNA sequences for each sample. Diversity indices showed that bacterial estimated richness (3244, average of Chao1 in two replicates) and phylogenetic diversity (121.58, average of PD whole tree values) in the non-bioaugmented columns were significantly higher than those in the bioaugmented columns (2642 for richness and 97.36 for diversity), suggesting that the inoculated strain outcompeted some indigenous bacteria. The Venn diagram (Fig. S5) showed that 94.5% of OTUs in the bioaugmented columns were identical to those in the non-bioaugmented columns. Of the identified OTUs, 32 bacterial phyla were present across the four samples. *Proteobacteria* was the most abundant phylum in all columns, but its abundance was higher in the bioaugmented (around 70%) than non-bioaugmented columns (around 40%) due to the presence of the inoculated strain (Fig. S6). The dominant bacterial phyla (>1%) between the non- and bioaugmented columns were not obviously different, with only two phyla not identical. We subsequently compared the taxonomic affiliations of the 50 most abundant OTUs among the four samples (Fig. 3). These OTUs belonged to 11 phyla, with most found in *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes*. We calculated the abundances of the inoculated strain (Fig. 3). The sequences identical to *Pseudomonas* sp. QJX-1 accounted for 43.8% (average) in the bioaugmented columns, which was significantly higher than that in the non-bioaugmented columns (6.9%). After removing the OTU representing the inoculant strain, we tested whether the community composition between the columns was significantly different based on the OTU abundances using ANOSIM. Results showed that the two groups were not significantly different ($p = 0.317$), suggesting that the bioaugmented column maintained the predominant indigenous bacterial structure.

3.4. Comparison of microbial community function

To determine how the inoculated strain affected the pollutant degradation or transformation function of the sand filter, we performed metagenomic analysis of the sand filter samples from the four columns. Around 10^7 protein-encoding sequences in each sample were identified with known function (Table S3). The specific functions (SEED based annotation) differentially expressed between the bioaugmented and non-bioaugmented columns were reflected in four categories: Cell Wall and Capsule, Iron Acquisition and Metabolism, Potassium Metabolism, and Virulence, Disease, and Defense (Fig. S7). Using KEGG mapper (Supplemental Fig. S8), we found that the two datasets (bioaugmented and non-bioaugmented) shared most metabolic pathways, though the metabolic pathways in the bioaugmented samples were comparatively diverse compared with those in the non-bioaugmented samples.

The genes related to xenobiotic degradation, nitrification and denitrification, and metal (Mn, As, Sb) transformation were investigated due to their important roles in water purification. The community functional genes involved in xenobiotic metabolism are shown in Table 1. Fourteen xenobiotic compound degradation genes were identified, with 11 from the bioaugmented and 11 from the non-bioaugmented columns. Generally, the abundances of overlapped degradation genes ($n = 7$) in the bioaugmented

Fig. 2. Comparison of active bacteria (16S rRNA) and inoculated strain (*cumA*) in the bioaugmented and non-bioaugmented columns. Data were obtained from RT real-time PCR assay. Data points represent the mean value (combined three replicate target gene copy numbers in two parallel bioaugmented or non-bioaugmented columns, $n = 6$) $\pm$ standard deviation.

Fig. 3. Abundances of dominant bacterial OTUs and inoculated strain in bioaugmented and non-bioaugmented columns. Upper figure depicts a circular approximately-maximum-likelihood phylogenetic tree based on the 50 most abundant bacterial OTUs detected in the bioaugmented and non-bioaugmented columns. Bars in the outer band represent OTU abundances. Lower figure depicts the abundances of the *Pseudomonas* genus and inoculated *Pseudomonas* QJX1 strain. NB: non-bioaugmented, BA: bioaugmented.

columns were much higher than those in the non-bioaugmented columns ($p < 0.05$, paired sample sign test). Specifically, the genes associated with benzoate biodegradation were the most abundant in both bioaugmented and non-bioaugmented columns as it is a key intermediary in the microbial metabolism of various aromatic compounds (Silva et al., 2012); however, the abundances in the bioaugmented columns were two times higher than those in the non-bioaugmented columns. These results suggest that xenobiotic compound degradation might be more effective in the bioaugmented columns than that in the non-bioaugmented columns. Nitrification encoded ammonia monooxygenase (*amo*) and denitrification gene encoded nitrite reductase (*nir*) were identified and compared. The abundances of *amo* and *nir* in the bioaugmented and non-bioaugmented columns were not significantly different. We observed As transformation genes, including arsenite oxidation genes and arsenate reduction genes, but no Sb transformation genes, which might be due to the limited study of Sb transformation and transformation gene information in the GenBank database. For Mn oxidation genes, we determined (i) the Mn

oxidation genes in the non-bioaugmented columns and (ii) how much *cumA* (inoculated strain) was present in the bioaugmented columns. As shown in Fig. 4, two putative Mn(II) oxidation genes, i.e., *moxA* and *cumA*, were present in the four columns, though *cumA* gene abundance in the bioaugmented columns was much higher than that in the non-bioaugmented columns due to the introduction of the QJX1 strain. Results also suggest that other Mn-oxidizing *Pseudomonas* may exist in the columns as the *cumA* gene is frequently found in different *Pseudomonas* strains (Brouwers et al., 1999; de Vrind-de Jong et al., 2000). The *mox* genes in columns were similar to those found in genera *Aurantimonas* and *Methylobacterium* (Dick et al., 2008; Vuilleumier et al., 2009).

4. Discussion

Bioaugmentation has been studied and applied intensively in wastewater (Guo et al., 2010; Zhu et al., 2015), soil (Kuppusamy et al., 2016; Tribedi and Sil, 2013), and sediment (Ahn et al., 2008; Wang et al., 2015) treatment to solve practical problems

Table 1

Relative abundances of xenobiotic metabolism-related, nitrification- and denitrification-related, and arsenic transformation-related gene sequences retrieved from the four metagenomic datasets. Values were normalized by randomly sampling 10,000,000 sequences per sample. BA: bioaugmented, NB: non-bioaugmented.

Metabolism	BA1	BA2	NB1	NB2
Xenobiotic degradation or transformation				
Bisphenol	0	0	6	9
Fluorobenzoate	0	0	20	0
Dioxin	0	1	5	5
Xylene	0	0	7	8
Toluene	397	334	48	33
Chloroalkane and chloroalkene	262	196	0	0
Aminobenzoate	1149	814	86	50
Nitrotoluene	1086	1030	678	707
Styrene	266	224	104	104
Atrazine	544	429	289	330
Caprolactam	811	649	0	0
Drug	138	167	0	0
Chlorocyclohexane and chlorobenzene	785	581	302	660
Benzoate	3958	3182	1530	1755
Nitrogen transformation				
Nitrification (<i>amo</i>)	194	245	217	101
Denitrification (<i>nir</i>)	2843	3254	2426	4910
Arsonic transformation				
Arsenite oxidation	114	64	111	105
Arsenate reduction	282	78	312	420

(e.g., improve catabolism of refractory organics) and enhance removal efficiency (Herrero and Stuckey, 2015). It is seldom used in drinking water treatment, however, due to lower nutrient influent, backwash, and undefined microbial roles in the treatment system. Benner et al. (2013) once proposed in a review that bioaugmentation is “a potential targeted, cost-effective, and sustainable alternative to existing treatment processes”. Christian et al. (2015) and Haig et al. (2016) used bioaugmentation in sand filters to successfully improve the removal of 2,6-dichlorobenzamide and estrogens, respectively. In this study we explored the feasibility of using bioaugmentation in the sand filters for groundwater treatment. The bioaugmentation used here increased the formation of Fe-Mn oxides and indirectly improved the removal of target pollutants.

4.1. Role of bioaugmentation on As and Sb removal

In the bioaugmented columns, the added *Pseudomonas* sp. QJX-1

could not oxidize or adsorb As and Sb according to our previous studies (Bai et al., 2016a, 2016b) and therefore did not have direct role in As and Sb removal. Bioaugmentation increased the formation of biogenic Mn oxides (reflecting in lower Mn concentrations in the effluents). The formed biogenic Mn oxides could oxidize Fe(II) to Fe oxides and thus accelerated the formation of Fe-Mn oxides. We previously demonstrated in batch experiments (Fe(II), Mn(II), As(III) and/or Sb(III), and QJX1 co-existed) that the As(III) and Sb(III) could be first oxidized to As(V) and Sb(V) by Mn oxides part of BFMO, and then adsorbed on the surface of BFMO (Bai et al., 2016b). Hence the enhancement of As and Sb removal in the bioaugmented columns was mainly attributed to the increasing generation of BFMO.

In addition, when As and Sb coexist in water, Fe-Mn oxides adsorb As prior to Sb (Bai et al., 2016b). This was also reflected in our long-term experiment, with much greater As than Sb removal.

4.2. Impact of bioaugmentation on microbial community in the sand filter

The decrease in richness and diversity in the bioaugmented columns indicated that the inoculated strain outcompeted some indigenous microbes. Inconsistent with this, previous studies have reported that bioaugmentation used in wastewater treatment may recover or increase richness and diversity (Schauer-Gimenez et al., 2010; Shi et al., 2015; Zhu et al., 2015). The reason for these earlier results might be that the influent wastewater used contained one or more compounds lethal to some microbes (e.g., some toxic xenobiotics), and thus bioaugmentation might recover the growth of bacteria sensitive to the toxic compound via its degradation or transformation. Hence, the effect of bioaugmentation on community diversity might be determined by both influent water quality and the inoculated strain.

Most bioaugmentation studies on wastewater have revealed that the inoculated strain(s) may significantly alter indigenous microbial community composition (Bai et al., 2011; Shi et al., 2015; Wang et al., 2009; Zhu et al., 2015). In our study, however, the indigenous microbial community maintained their predominant structure after the introduction of strain QJX1. The possible reason is that the microbes in the oligotrophic environment (groundwater) were all tolerant to starvation stress and thus could survive even in that a portion of C and N source were deprived by inoculated strain. This is of great importance in regards to bioaugmentation as the biodegradation of a broad range of pollutants relies on indigenous

Fig. 4. Phylogenetic analysis illustrating the potential Mn(II)-oxidation genes present in the bioaugmented and non-bioaugmented columns. Metagenomic sequences were BLASTed against the GenBank protein database and the potential Mn(II)-oxidizing sequences were identified. As the metagenomic sequences were too short to be aligned, the sequences of their closest relatives from the GenBank database were selected for phylogenetic tree construction. The *cumA* gene in QJX1 was translated into a protein sequence and then included in the tree. The numbers on the branch nodes demonstrate the percentage of bootstrap support for the clades based on 1000 bootstrap resampling. The abundances of Mn(II) oxidation enzymes are list on the right and the numbers represent the abundances of CumA or MoxA sequences per 10,000,000 raw metagenomic sequences. Symbols: ■ bioaugmented 1, □ bioaugmented 2, ● non-bioaugmented 1, ○ non-bioaugmented 2.

bacteria.

Our metagenomic analysis indicated that the inoculated strain promoted the biodegradation of some xenobiotics, reflected in the increase in degradation gene abundances (Table 1). One possibility is that the inoculated strain might carry some specific degradation function genes, or that horizontal transfer of the xenobiotic degradation genes (e.g., conjugation of plasmids) may more frequently occur in the bioaugmented column as its biofilm (“an environmental hotspot for gene transfer”) was more active (Fig. 2) than that in the non-bioaugmented column.

Taken together, the higher biomass, stable indigenous community structure, augmentation of xenobiotic biodegradation/transformation, and increase in BFMO formation in the bioaugmented columns resulted in more efficient treatment than that in the non-bioaugmented columns.

4.3. Bioaugmentation treatment of groundwater containing high Mn(II) and Fe(II)

Our study indicated that bioaugmentation could be applied in a sand filter for the treatment of groundwater containing high Fe(II) and Mn(II). Further, two issues should be addressed in practice.

(i) Inoculation method

All bacterial inoculants are sensitive to temperature. In low temperature, the growth rate will decrease and the occurrence of Mn(II) oxidation will be delayed. In winter, for example, bioaugmentation will have a significant reduction in BFMO formation due to low bacterial activity. In such cases, the reactor may need a greater biomass concentration in the winter period than that in the summer period (Herrero and Stuckey, 2015). In addition, although the inoculant in our experiment survived in the column over the entire experimental period, previous studies also have shown a significant increase in bioreactor performance soon after bioaugmentation, with the positive effects only maintained for a short time after inoculation (Boon et al., 2000; Christian et al., 2015; Herrero and Stuckey, 2015). Hence, adding inoculants at regular intervals might be needed in actual treatment.

(ii) Optimization of environmental conditions

Whether inoculants can survive and grow in a sand filter biofilm is related to ambient physicochemical parameters. Prolonging the contact time to oxygen (or aeration) is more useful for Mn(II) and Fe(II) oxidation. An appropriate hydraulic retention time (HRT) is also essential. On the one hand, a longer HRT may increase reaction time, and thus improve the treatment effect; on the other hand, a shorter HRT may increase substrate support and diminish the diffusive boundary layer surrounding each filter particle leading to more efficient degradation (Benner et al., 2013). Therefore, optimizing the HRT for pollutant removal should be done prior to the operation of a sand filter. In addition, to overcome washout, bioaugmentation could use filter material with a crude surface to improve microorganism attachment within the biosystem.

5. Conclusions

For the treatment of groundwater containing Fe(II), Mn(II), As(III), and Sb(III), we established four quartz-sand filter columns, i.e., two bioaugmented columns (inoculated with Mn-oxidizing strain *Pseudomonas* sp. QJX-1) and two non-bioaugmented controls. We experimentally verified the hypothesis that the inoculated strain accelerated the formation of Fe-Mn oxides, thus resulting in an improvement in As(III) and Sb(III) removal in the sand filter. This

bioaugmentation method exhibited several advantages, i.e. shortened start-up time, anti-shock load capacity, and improved TOC and NO_3^- -N removal. Combining RT real-time PCR, 16S rRNA sequencing, and metagenomic sequencing, we found that the inoculated strain could survive and continuously grow in the sand filters within the experimental time, with a relative abundance of 43.8% (estimated by 16S rRNA sequencing) in the bacterial community after 120 d. Although the inoculant outcompeted a small portion of indigenous microbes and led to a decrease in community richness and diversity, the predominant structure of the indigenous bacterial community was not significantly changed. In the bioaugmented columns, the biodegradation gene abundances of some xenobiotics were significantly increased, suggesting bioaugmentation intensified the water treatment effect. Overall, our study indicated that bioaugmentation could be used in sand filters for the treatment of groundwater containing high Fe(II), Mn(II), and As/Sb.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2016.09.040>.

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