Bacterial Diversity in Biological Filtration System for the Simultaneous Removal of Arsenic, Iron and Manganese from Groundwater

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ABSTRACT

Bacterial diversity of the microbial consortia in a biological filtration pilot plant for the simultaneous removal of arsenic (As), iron (Fe) and manganese (Mn) from groundwater was analyzed. PCR-based denaturing gradient gel electrophoresis (DGGE) of bacterial 16S ribosomal RNA (rRNA) genes represented at least 6 dominant signals and many weak signals. Phylogenetic analysis using the nucleotide sequences of the 16S rRNA gene clone library constructed from the pilot plant sample showed the presence of the bacteria closely related to *Gallionella* and *Leptothrix*, which are supposed to be involved in the production of Fe and Mn oxides utilized for adsorbents of As in this system. On the other hand, *aoxB* gene was not detected, suggesting that arsenite-oxidizing bacterium would not be involved in the As removal of the pilot plant. These results indicated that the simultaneous removal of As, Fe and Mn from groundwater was conducted by the physicochemical sorption of As by the biogenic Fe and Mn oxides produced by the bacteria closely related to *Gallionella* and *Leptothrix* included in the microbial consortia of the pilot plant.

Keywords: arsenic, biological filtration, *Leptothrix*

INTRODUCTION

Arsenic (As) contamination of the groundwater is a global problem affecting many countries. Because an increase of As concentration in the drinking water is associated with numerous health hazards, various treatment procedures are applied to reduce As level below the World Health Organization (WHO) guideline value of 10 µg/L (WHO, 1996). The conventional methods for the removal of As from groundwater, such as, precipitation-coagulation, ion-exchange, and reverse osmosis processes, generally require expensive devices or materials. Most of the As contaminations in groundwaters are permanent problems induced by natural mineral sources or mining activities, therefore, low-cost and easy-to-operate process for the removal of As should be required, especially for the developing economies. To solve this problem, alternative methods for As removal have been investigated using biological activities such as, biological incorporation, oxidation and volatilization (Čerňanský *et al.*, 2007; Ike *et al.*, 2008; Rahman and Hasegawa, 2011), or sorption of soluble As oxyanions onto biogenic

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materials (Katsoyiannis and Zouboulis, 2004, 2006; Sahabi *et al.*, 2009; Sari and Tuzen, 2009), which are less expensive than the conventional methods. Biological sorptive filtration for simultaneous removal of As, iron (Fe) and manganese (Mn) is one of the biological methods for As removal from groundwater, which utilizes biologically produced Fe and Mn oxides as the adsorbents for soluble As oxyanions. This method has an advantage over the other biological methods, since it is based on the biological filtration system for the removal of Fe and Mn from groundwater used in the drinking water treatment plants of many countries. In this system, a step of biological oxidation of Fe and Mn by the microorganisms in the reactor forms amorphous Fe and Mn oxide layers on the surface of filter media, and As species are subsequently removed from the groundwater by a combination of physicochemical adsorption and oxidation (Mohan and Pittman Jr., 2007; Hennebel *et al.*, 2009; Sahabi *et al.*, 2009). This method requires no chemical oxidant, and the sorbent materials are continuously produced *in situ*; therefore, the biological sorptive filtration is an economical, eco-friendly and easy-to-operate method for the treatment of groundwater containing As, Fe and Mn. It is known that high concentration of As in groundwater is often associated with high concentrations of Fe and Mn, thus the simultaneous removal of As, Fe and Mn by biological filtration is applicable. A number of studies using biological filtration for the simultaneous removal of As and Fe (Katsoyiannis and Zouboulis, 2004; Casiot *et al.*, 2006; Hassan *et al.*, 2009; Pokhrel and Viraraghavan, 2009), or the simultaneous removal of As, Fe and Mn (Katsoyiannis and Zouboulis, 2006; Sugahara *et al.*, 2008) exhibited effective removal of As from the water. However, composition and function of the microbial community in the biological filtration systems had not been studied in detail.

The biological filtration system has been considered to utilize the so-called “iron bacteria” for oxidation and deposition of Fe^{2+} and Mn^{2+} ions in groundwater (Mouchet, 1992; Pacini *et al.*, 2005). Iron bacteria is a common name of the bacteria found in Fe and Mn-containing groundwater and freshwater environments, which oxidize Fe (Fe-oxidizing bacteria; FeOB), or both Fe and Mn (Fe and Mn-oxidizing bacteria; MnOB). These bacteria have been well known not only for their particular habitats but also for their unique structures, such as twisted stalks (*Gallionella*), and filamentous tubes called “sheath” (*Leptothrix*, *Sphaerotilus*, and *Crenothrix*) (van Veen *et al.*, 1978; Ghiorse, 1984). These iron bacteria are assumed to deposit Fe and Mn oxides on their extracellular structures. However, because of the difficulty of their pure cultivation, their contribution on the removal of Fe and Mn from groundwater in the biological filtration system is poorly understood. Katsoyiannis and Zouboulis (2004) operated a biological filtration system for the simultaneous removal of As and Fe and showed the presence of *Gallionella* and *Leptothrix* in the biological filtration reactor (BFR) by microscopic analysis. This study used a raw groundwater containing Fe, which was spiked with As during the investigation. Battaglia-Brunet *et al.* (2006) investigated the simultaneous removal of As and Fe using the BFR inoculated with an enrichment culture of As-oxidizing microbial consortia and synthetic mine water, and isolated an As-oxidizing bacterium phylogenetically related to *Leptothrix* from the BFR. In addition, they studied the diversity of the bacterial consortia in the BFR using polymerase chain reaction (PCR)-based 16S ribosomal RNA (rRNA) gene sequence analysis, and showed the presence of 9 individual operational taxonomic units (OTUs) including the bacteria phylogenetically related to *Gallionella*. Casiot *et al.* (2006)

operated the biological filtration system using Fe and As-containing groundwater, and isolated an As-oxidizing bacterium from the BFR. In addition, they analyzed the diversity of the bacterial consortia in the biofilm from the BFR by PCR-based terminal-restriction fragment length polymorphism (T-RFLP) of the 16S rRNA genes, and showed the presence of 14 OTUs. On the other hand, bacterial diversity in the BFR for the simultaneous removal of As, Fe and Mn was not reported yet. Previously, we studied the bacterial diversity in the biological filtration system for the removal of Fe and Mn from groundwater used in the drinking water treatment plant located at Joyo City, Kyoto, Japan, using the PCR-based denaturing gradient gel electrophoresis (DGGE) and clone library analyses of the bacterial 16S rRNA genes (Thapa Chhetri *et al.*, 2013). The results showed the presence of FeOB such as *Gallionella* and *Sideroxydans*, and MnOB such as *Hyphomicrobium* in the BFR, which possibly contribute for the removal of Fe and Mn from groundwater. In this report, we studied bacterial diversity of the biological filtration plant for the simultaneous removal of As, Fe and Mn. A pilot plant for the biological treatment of groundwater containing As, Fe and Mn using a column reactor had been operated for 6 months (Sugahara *et al.*, 2008; Fujikawa *et al.*, 2010). During the operation, As, Fe and Mn in the raw groundwater were successfully removed. The aim of this study is to analyze the bacterial diversity in the pilot plant of biological filtration system for the simultaneous removal of As, Fe and Mn from groundwater, using PCR-based molecular biological techniques, such as, PCR-DGGE, clone library analysis, and real-time PCR. Furthermore, we compared the community structure of bacterial consortia on the pilot plant with that of the other biological filtration plant which we analyzed previously. This is the first study for the analysis of bacterial diversity in the biological filtration plant for the simultaneous removal of As, Fe and Mn, and it would be informative for researchers and engineers who are studying and developing the biological treatment system for the simultaneous removal of As, Fe and Mn.

MATERIALS AND METHODS

Sampling site

The microbial floc and raw groundwater samples were collected from the biological filtration pilot plant for the simultaneous removal of As, Fe and Mn, located at Muko City, Kyoto, Japan (Sugahara *et al.*, 2008; Fujikawa *et al.*, 2010). The system consists of aeration by dropping, and downflow filtration reactor ($\phi 100$ mm $\times$ 1,800 mm column) with dual-media filter of polypropylene tubes (5 – 8 mm in pore size, ca. 1,000 mm in height) and Mn-coated sands (ca. 200 mm in height). From May to November, 2006, this system was operated at the filtration rates of 150 to 600 m/day. The microbial floc sample in the filter bed of the reactor was collected from the backwashed water of the column reactor on September 21, 2006, and stored at -80°C . Raw groundwater sample was collected in polypropylene bottle, filtered through a $0.45\text{ }\mu\text{m}$ membrane filter, and then stored at room temperature until analysis. The concentrations of Fe and Mn in the groundwater were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES), while the concentration of total As was determined using hydride generation-atomic absorption spectrometry. The concentration of ammonium-nitrogen was determined by indophenol blue method (Solorzano, 1969). The biological filter media sample from Joyo biological filtration plant was collected as described previously (Thapa Chhetri *et al.*, 2013).

DNA extraction

Total DNA was extracted from 0.05 g (wet weight) of the microbial floc sample using FastDNA SPIN Kit for soil (MP Biochemicals, Solon, OH, USA) as described previously (Thapa Chhetri *et al.*, 2013). Total DNA from Joyo biological filter media sample was also prepared as described (Thapa Chhetri *et al.*, 2013).

Polymerase chain reaction (PCR)

Standard PCR procedure, and PCR procedure for the DGGE analysis with touchdown program, were carried out as described previously (Thapa Chhetri *et al.*, 2013).

Denaturing gradient gel electrophoresis

To analyze the molecular diversity of bacterial consortia in the samples, about 190 base pairs (bp) of DNA fragments containing the variable region 3 of bacterial 16S rRNA genes were amplified. Parallel DGGE analysis was carried out using mini-DGGE system NB-1490 (Nihon Eido, Tokyo, Japan) as described previously (Thapa Chhetri *et al.*, 2013). For the analysis, 12.5 μ L of PCR products were applied onto 8% (wt/vol) polyacrylamide gel with the denaturing gradients ranging from 30 to 60% denaturant, and electrophoresis was carried out in $1 \times$ TBE buffer at 50 V, 60°C for 4.5 h. The gel was visualized by SYBR Green I staining (Invitrogen, Carlsbad, CA, USA) using fluorescent image analyzer FLA-2000 (Fujifilm, Tokyo, Japan). A reference marker for DGGE analysis was prepared by mixing PCR-amplified 16S rRNA fragments of different Joyo clones obtained in the previous study (Thapa Chhetri *et al.*, 2013). The determination of nucleotide sequence of the separated DNA fragment in the gel was performed as described previously (Thapa Chhetri *et al.*, 2013).

Clone library analysis

To construct 16S rRNA gene clone library from the sample, PCR amplification was carried out by the standard PCR procedure as described above using universal primers 63F (Marchesi *et al.*, 1998) and 1392R (Lane *et al.*, 1985). The resulted PCR product was introduced into pCR4-TOPO vector using TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's protocol. Plasmid DNA harboring each 16S rRNA gene clone was prepared using E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA), and then subjected to sequence analysis. The determination and analysis of the nucleotide sequence of each plasmid clone was described previously (Thapa Chhetri *et al.*, 2013).

Nucleotide sequence accession numbers

The nucleotide sequence data obtained in this study are deposited in the DNA Data Bank of Japan (DDBJ) database under accession numbers AB604792 to AB604838.

Real-time PCR

Real-time PCR was carried out to analyze the relative amount of the 16S rRNA genes of *Leptothrix*-related bacteria to that of the total bacteria in the sample as described previously (Thapa Chhetri *et al.*, 2013).

PCR detection of the genes encoding bacterial ammonia monooxygenase and arsenic oxidase

The presence of the genes encoding ammonia monooxygenase subunit A (*amoA*) of

ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in the total DNA sample was examined using PCR as described previously (Thapa Chhetri *et al.*, 2013). For PCR detection of the gene encoding arsenite oxidase large subunit (*aoxB*) in the total DNA sample, primers *aoxBM1-2F* and *aoxBM3-2R* (Quéménéur *et al.*, 2008) were used. PCR was carried out with the standard PCR condition as described above.

RESULTS AND DISCUSSION

Analysis of bacterial diversity in the biological filtration pilot plant by PCR-DGGE

The biological filtration plant utilizes the microorganisms naturally present in raw groundwater. Therefore, the structure of microbial consortia in the plant would be affected by the characteristics of the influent groundwater. Basic characteristics of the raw groundwaters used in the pilot plant and Joyo biological filtration plant are listed in Table 1. The concentrations of Fe, Mn, As and ammonium ion (NH_4^+) in the raw groundwater of the pilot plant were higher than those of Joyo plant. To study the bacterial diversity in the biological filtration pilot plant for the simultaneous removal of As, Fe and Mn, microbial flocs accumulated in the BFR were collected. At that period, the concentrations of As, Fe and Mn in the influent raw groundwater were 22.5 $\mu\text{g/L}$, 2.11 mg/L and 0.48 mg/L, respectively, while those in the treated water were 5.5 $\mu\text{g/L}$, 0.01 mg/L and 0.13 mg/L, respectively. The concentration of As was successfully decreased under the WHO's guideline level. The concentration of NH_4^+ in the raw groundwater was 0.76 mg/L, which was not detected in the treated water. To depict the overview of bacterial diversity in the BFR of the pilot plant, the total DNA of the microbial consortia was extracted from the sample and applied for PCR-based DGGE analysis. Figure 1 shows that at least six strong signals and many weak signals of the amplified 16S rRNA gene fragments were observed. The patterns of PCR-amplified 16S rRNA genes separated by DGGE (DNA fingerprints) were compared between the samples from the pilot plant and Joyo biological filtration plant. However, no common signal was observed. In Joyo sample, one strong signal corresponded to the bacteria closely related to *Gallionella*, and the other three signals corresponded to the bacteria closely related to *Nitrospira* (Thapa Chhetri *et al.*, 2013). Casiot *et al.* (2006) analyzed the diversity of bacterial consortia in the BFR using T-RFLP and showed that the dominant OTU was arsenite-oxidizing bacterium strain B2. The 16S rRNA gene fragments observed in the pilot plant sample were excised from the gel and then applied for the determination of the nucleotide sequences. However, all these DNA fragments showed ambiguous nucleotide sequences, because of the contamination of multiple fragments which were probably due to the insufficient separation in the gel.

Table 1 - Basic characteristics of the pilot plant and Joyo biological filtration plant.

	Pilot plant ^a	Joyo ^b
Raw groundwater		
Temperature (°C)	18.9	19.0
pH	6.5	6.78
Fe (mg/L)	2.11	0.41
Mn (mg/L)	0.48	0.12
As (µg/L)	22.5	– ^d
NH ₄ ⁺ -N (mg/L)	0.76	0.11
TOC ^c (mg/L)	0.4	< 0.5
Hardness (mg/L as CaCO ₃)	54.0	27.2
Biological filtration reactor		
DO (mg/L)	3.5 ± 0.9 ^e	5 – 7
Filter media	Polypropylene tubes, Mn-coated sands	Anthracites, sands
Filter surface area (m ²)	7.85 × 10 ⁻³	189 (31.5 m ² × 6 filters)
Filtration rate (m/day)	150 – 600	70
Backwash frequency	1 – 2 days	14 days

a. On Oct. 5, 2006.

b. Thapa Chhetri *et al.*, 2013.

c. Total organic carbon.

d. Not detected.

e. Sugahara *et al.*, 2008.

Fig. 1 - Comparison of the DNA fingerprints of PCR-amplified 16S rRNA genes from the pilot plant and Joyo biological filtration plant samples by parallel DGGE.

Analysis of 16S rRNA gene clone library constructed from bacterial consortia in the pilot plant

To study the bacterial diversity of the pilot plant in detail, 16S rRNA gene clone library was constructed. PCR-amplified 16S rRNA gene fragments were applied for the construction of the clone library, and 92 clones were isolated. These clones were then subjected to the determination of nucleotide sequence, and separated into 47 OTUs according to their nucleotide sequences. Nucleotide sequence of the first half of each cloned 16S rRNA gene fragment (corresponds to *Escherichia coli* 16S rRNA positions 64 to 784) was submitted to BLAST analysis to identify the closest known relative (Table 2). The dominant groups of the clones were affiliated with four subclasses of the

Proteobacteria; *Alphaproteobacteria* (11 OTUs, 41% of total clones), *Betaproteobacteria* (7 OTUs, 13% of total clones), *Gammaproteobacteria* (11 OTUs, 13% of total clones), and *Deltaproteobacteria* (8 OTUs, 15% of total clones). The other clones were affiliated with *Actinobacteria* (5 OTUs, 9% of total clones), *Acidobacteria* (4 OTUs, 8% of total clones), and *Firmicutes* (1 OTU, 1% of total clones).

Table 2 - Similarities among 16S rDNA sequences of the clone library from the biological filtration pilot plant and the most similar database sequences.

OTU name	Accession no.	No. of isolates ^a	Closest relative ^b [Accession no.]	Similarity (%)
<i>Alphaproteobacteria</i>				
M07A146	AB604837	1	<i>Beijerinckia fluminensis</i> LMG 2819 [EU401905]	94.3
M07A071	AB604819	1	<i>Bradyrhizobium</i> sp. II-47 [AB531406]	100.0
M07A072	AB604820	3	<i>Bradyrhizobium</i> sp. Wall28 [EF601950]	99.4
M07A022	AB604800	7	<i>Methylocystis</i> sp. B3 [DQ496232]	99.1
M07A144	AB604836	1	<i>Nordella oligomobilis</i> N21 [AF370880]	96.0
M07A009	AB604794	2	<i>Novosphingobium naphthalenivorans</i> TUT562 [AB177883]	96.1
M07A054	AB604814	7	<i>Novosphingobium naphthalenivorans</i> TUT562 [AB177883]	96.2
M07A017	AB604798	12	<i>Novosphingobium subterraneum</i> F-4 [FJ527720]	98.0
M07A121	AB604828	2	<i>Novosphingobium subterraneum</i> F-4 [FJ527720]	97.6
M07A034	AB604804	1	<i>Rhodobacter capsulatus</i> ATCC 11166 [DQ342320]	97.4
M07A128	AB604833	1	<i>Rhodobacter</i> sp. ZH15 [FJ872532]	94.4
<i>Betaproteobacteria</i>				
M07A125	AB604831	1	<i>Gallionella capsiferiformans</i> ES-2 [DQ386262]	95.2
M07A046	AB604808	1	<i>Ideonella</i> sp. 0-0013 [AB211233]	96.2
M07A007	AB604793	6	<i>Ideonella</i> sp. B513 [AB049107]	97.3
M07A053	AB604813	1	<i>Methylibium fulvum</i> Gsoil 328 [AB245357]	99.0
M07A023	AB604801	1	<i>Methylophilus</i> sp. ECd5 [AY436794]	95.4
M07A080	AB604821	1	<i>Methylophilus</i> sp. ECd5 [AY436794]	96.8
M07A047	AB604809	1	<i>Rhodoferrax</i> sp. IMCC 1723 [DQ664242]	98.3
<i>Gammaproteobacteria</i>				
M07A030	AB604802	1	<i>Crenothrix polyspora</i> clone 6 [DQ295898] ^c	94.8
M07A039	AB604806	2	<i>Crenothrix polyspora</i> clone 6 [DQ295898] ^c	94.8
M07A102	AB604826	1	<i>Crenothrix polyspora</i> clone 6 [DQ295898] ^c	93.4
M07A122	AB604829	1	<i>Legionella lytica</i> L2 [X97364]	95.3
M07A088	AB604823	1	<i>Legionella pneumophila</i> Paris [CR628336]	94.1
M07A052	AB604812	1	<i>Lysobacter brunescens</i> str. KCTC 12130 [AB161360]	99.5
M07A148	AB604838	1	<i>Lysobacter brunescens</i> str. KCTC 12130 [AB161360]	99.6
M07A069	AB604818	1	<i>Methylobacter tundripaludum</i> SV96 [AJ414655]	96.8
M07A090	AB604824	1	<i>Methylobacter tundripaludum</i> SV96 [AJ414655]	94.4
M07A015	AB604797	1	<i>Methylomonas</i> sp. LW16 [AF150796]	97.6
M07A096	AB604825	1	<i>Pseudomonas</i> sp. 24 [FJ867623] ^c	89.2
<i>Deltaproteobacteria</i>				
M07A041	AB604807	1	<i>Anaeromyxobacter</i> sp. IN2 [FJ939131]	95.2
M07A060	AB604817	2	<i>Anaeromyxobacter</i> sp. K [CP001131]	92.6
M07A051	AB604811	2	<i>Bdellovibrio</i> sp. MPA [AY294215]	89.9
M07A049	AB604810	4	<i>Geobacter chapelleii</i> 172 [U41561]	93.3
M07A010	AB604795	2	<i>Geobacter argillaceus</i> G12 [DQ145534]	95.3
M07A082	AB604822	1	<i>Geobacter argillaceus</i> G12 [DQ145534]	94.9
M07A055	AB604815	1	<i>Geobacter psychrophilus</i> P11 [AY653551]	96.6
M07A031	AB604803	1	<i>Haliangium tepidum</i> SMP-10 [AB062751]	85.7
<i>Firmicutes</i>				
M07A124	AB604830	1	<i>Staphylococcus epidermidis</i> ATCC 12228 [AE015929]	99.9
<i>Actinobacteria</i>				
M07A059	AB604816	2	<i>Actinobacterium</i> YM22-133 [AB286031] ^c	90.6
M07A126	AB604832	1	<i>Actinobacterium</i> YM22-133 [AB286031] ^c	90.5
M07A140	AB604834	2	bacterium Ellin 5025 [AY234442] ^c	94.2
M07A142	AB604835	2	bacterium Ellin 5025 [AY234442] ^c	94.4
M07A014	AB604796	1	<i>Sporichthya polymorpha</i> DSM 46113 [X72377]	94.3
<i>Acidobacteria</i>				
M07A019	AB604799	1	<i>Acidobacteria</i> bacterium IGE-011 [GU187027] ^c	89.8
M07A002	AB604792	3	<i>Geothrix fermentans</i> H5 [U41563]	94.7
M07A035	AB604805	1	<i>Geothrix fermentans</i> H5 [U41563]	96.5
M07A103	AB604827	2	<i>Geothrix fermentans</i> H5 [U41563]	96.5

a. Total 92 clones were isolated.

b. Based on BLAST analysis using the greengenes database.

c. Based on BLAST analysis using the DDBJ database.

BLAST searches indicated that a number of OTUs showed higher sequence similarities (> 95%) to known FeOB and MnOB. The OTU M07A125 showed 95.2% similarity to *Gallionella capsiferriiformans* ES-2, which is a chemolithotrophic FeOB (Emerson and Moyer, 1997). The OTUs M07A007 and M07A046 showed > 96% similarities to *Ideonella*. The oxidation of Fe and Mn by *Ideonella* had not been reported, but it should be noted that these two OTUs also showed higher similarities to the Mn-depositing *Burkholderiales* bacterium JOSHI_001 isolated from the urban stream (Smith and Lewis, 2008), and arsenite-oxidizing bacterium *Leptothrix* sp. S1.1 isolated from the settling pond sediments of As and Fe-containing mine drainage water (Battaglia-Brunet *et al.*, 2006). The nucleotide sequence similarities of the OTUs M07A007 and M07A046 to JOSHI_001 were 96.7% and 96.2%, respectively, while those to *Leptothrix* sp. S1.1 were 96.9% and 95.1%, respectively. The OTUs M07A030, M07A039 and M07A102 showed > 93% similarities to one of the sheathed iron bacteria *Crenothrix polyspora* (Stoecker *et al.*, 2006), however, the Fe or Mn deposition activity of *C. polyspora* is still unknown (Emerson *et al.*, 2010).

Phylogenetic relationships among all OTUs from the pilot plant for the simultaneous removal of As, Fe and Mn, 32 OTUs from Joyo biological filtration plant (Thapa Chhetri *et al.*, 2013) and 16S rRNA gene sequences of the known relatives are shown in Fig. 2. Interestingly, the OTUs closely related to the genus *Gallionella* were present in the pilot plant and Joyo plant (M07A125 and Jy04A41), whereas the OTUs closely related to the other FeOB *Sideroxydans* were found in Joyo plant only. *Sideroxydans* spp. are microaerobic, chemolithotrophic bacteria which can utilize Fe^{2+} as an energy source the same as *Gallionella*, but they do not produce the twisted stalk (Emerson and Moyer, 1997; Emerson *et al.*, 2010). As well as the pilot plant, Battaglia-Brunet *et al.* (2006) showed the presence of the 16S rRNA gene clones phylogenetically close to *Gallionella* in the bacterial consortia from the BFR for the simultaneous removal of As and Fe. Recently, Emerson *et al.* (2013) compared the genomes of *G. capsiferriiformans* ES-2 and *Sideroxydans lithotrophicus* ES-1, and described that *G. capsiferriiformans* ES-2 has more capacity to resist heavy metals than *S. lithotrophicus* ES-1.

The OTUs phylogenetically close to *Leptothrix* were obtained from the pilot plant (M07A007 and M07A046), but not from Joyo biological filtration plant. Among all the OTUs from Joyo and the pilot plants, these two OTUs and another four OTUs phylogenetically close to *Methylibium* (Jy04A04, Jy04A06, Jy04A46, and M07A053) contained the target sequence of a *Leptothrix*-specific probe PSP-6 (Siering and Ghiorse, 1997). *Methylibium* spp. are methylotrophic bacteria (Nakatsu *et al.*, 2006), but their Mn^{2+} oxidation activity are unknown. The amounts of 16S rRNA genes of the total bacteria and *Leptothrix*-related bacteria harboring the PSP-6 sequence in the DNA solution extracted from the microbial floc sample of the pilot plant were estimated using real-time PCR. The copy number of the 16S rRNA genes of the *Leptothrix*-related harboring the PSP-6 sequence bacteria was 1.93×10^{10} copies per one gram of the flocs, while that of total bacteria was 1.89×10^{11} copies per one gram of the flocs; thus, the ratio was 10.2%. Our previous study of Joyo sample indicated that the relative amount of the 16S rRNA gene copies of *Leptothrix*-related bacteria to those of the total bacteria in Joyo bacterial consortia was 12.5% (Thapa Chhetri *et al.*, 2013); therefore, the ratios of the bacteria harboring PSP-6 sequence in the pilot plant and Joyo plant were quite similar. However, it is supposed that the proportion of the bacteria closely related to

Leptothrix in the pilot plant would be larger than that in Joyo plant, because 7 isolates (7.6% of total clones) were affiliated with the OTUs closely related to *Leptothrix* in the pilot plant clone library, whereas no isolate closely related to *Leptothrix* was obtained from Joyo clone library.

Fig. 2 - Phylogenetic relationships of bacterial 16S rRNA gene sequences obtained from the clone libraries of the biological filtration plants and relative bacteria. (A) *Alpha*, *Beta*, and *Gamma*-subclasses of *Proteobacteria*; (B) *Deltaproteobacteria*, *Acidobacteria*, *Firmicutes*, *Actinobacteria*, and *Nitrospira*. Bootstrap values of > 60% (obtained with 1,000 resamplings) are shown above or near the relevant nodes. The OTUs from the pilot plant obtained in this study are indicated as M07Axxx in boldface, while the OTUs from Joyo biological filtration plant are indicated as Jy04Axx. GenBank accession numbers are in parentheses.

On the other hand, no OTU closely related to *Hyphomicrobium* was obtained from the clone library of the pilot plant, while the OTU Jy04A47 was obtained from Joyo clone library. *Hyphomicrobium* spp. are methylotrophic, prosthecae bacteria, and some strains of *Hyphomicrobium* are known for the deposition of Fe and Mn oxides (Moore, 1981). In conclusion, it is suggested that *Leptothrix*-related bacteria were the dominant MnOB in the pilot plant, while *Hyphomicrobium*-related bacteria were the dominant MnOB in Joyo plant. The dominant population of *Leptothrix*-related bacterium in the BFR was

also reported by Casiot *et al.* (2006). In contrast, Battaglia-Brunet *et al.* (2006) did not find the 16S rRNA gene clone of *Leptothrix* sp. S1.1 in the bacterial consortia of the BFR, although this strain was isolated from the sediments of the mine water they used. The relationship between the groundwater characteristics, especially As concentration and the compositions of MnOB are still unclear, so that further investigation is required.

PCR detection of the genes encoding ammonia monooxygenase and arsenite oxidase

It has been suggested that nitrifying bacteria would contribute to the biological removal of Mn, because complete nitrification is required to take place for the biological removal of Mn when the raw groundwater contains ammonia (Mouchet 1992; Štembal *et al.*, 2005). However, there was no OTU closely related to known AOB and nitrite oxidizing bacteria (NOB) in the 16S rRNA gene clone library from the pilot plant sample, although NH_4^+ in raw groundwater was completely removed after the filtration. The presence of the AOB was confirmed by PCR-based detection of AOA- and AOB-*amoA* genes, and PCR-amplified fragment of the AOB-*amoA* gene was detected (data not shown). Therefore, NH_4^+ oxidation in the biological filtration system of the pilot plant was promoted by AOB. In Joyo plant, AOA-*amoA* but not AOB-*amoA* was detected (Thapa Chhetri *et al.*, 2013). It has been known that AOA grow in a habitat with low NH_4^+ concentration (Martens-Habben *et al.*, 2009). Moreover, it was also shown that the growth rates of the AOB increased with increasing NH_4^+ concentrations, while the growth rates of the AOA decreased slightly (French *et al.*, 2012). The concentrations of NH_4^+ in the raw groundwater of the pilot plant and Joyo plant were 0.76 mg/L and 0.11 mg/L, respectively, and it is supposed that such a slight difference would affect the growth of AOA and AOB in the biological filtration reactor. The detection of NOB in the microbial consortia of the pilot plant has not succeeded yet, therefore further investigation would be needed.

Among the 16S rRNA gene clone library from the pilot plant sample, the OTU closely related to the arsenite-oxidizing bacterium *Leptothrix* sp. S1.1 was obtained as described above. Quéménéur *et al.* (2008) designed the oligonucleotide primer pair for PCR amplification of the gene encoding arsenite oxidase large subunit (*aoxB*), and cloned the *aoxB* gene from *Leptothrix* sp. S1.1. The detection of *aoxB* gene in the DNA solution extracted from the microbial floc sample of the pilot plant was examined using PCR. However, no obvious signal of the amplified *aoxB* gene fragment was observed (data not shown). It has been known that the distribution of *aoxB* gene is widely spread in prokaryotes probably due to the horizontal gene transfer, and does not match with the phylogenetic relationship of 16S rRNA genes (Heinrich-Salmeron *et al.*, 2011). The result suggested that the removal of As in the BFR of the pilot plant was conducted by the sorptive filtration of As with biogenic Fe and Mn oxides produced by the microbial consortia containing the bacteria closely related to *Gallionella* and *Leptothrix*. However, it is still unclear why *aoxB* was not detected in the pilot plant sample although the microbial consortia in the pilot plant came from the As-containing groundwater. In contrast, the studies for the simultaneous removal of As and Fe reported by Battaglia-Brunet *et al.* (2006) and Casiot *et al.* (2006) suggested that the removal of As was conducted by the combination of biological oxidation and physicochemical sorption. It should be noted that arsenite is oxidized to arsenate, and both of these soluble As oxyanions are adsorbed by the biogenic Fe and Mn oxides (Sahabi *et al.*,

2009). Thus, the principle of the biological filtration for the simultaneous removal of As, Fe and Mn is the production of Fe and Mn oxides by FeOB and MnOB. However, it is supposed that the presence (or inoculation) of arsenite-oxidizing bacteria in the biological filtration system would improve the efficiency of As removal. It is still difficult to reveal the mechanism of the simultaneous removal of As, Fe and Mn by the biological filtration system because of the difficulties of the pure cultivation of FeOB and MnOB in the microbial consortia. However, our analyses based on the nucleotide sequence information of the bacterial genes would help to understand the role of each FeOB, MnOB and arsenite-oxidizing bacteria in the BFR. The primers for specific detection of the genes of *Leptothrix*, *Gallionella*, and *Hyphomicrobium* would be useful to survey the presence of these bacteria in the groundwater for construction and improvement of the biological filtration system. However, as described in this study, the primers already reported for the detection of 16S rRNA genes of these bacteria showed unexpected amplification of the non-targeted clones in the clone libraries of the pilot plant and Joyo plant (unpublished data). Therefore, further investigation is required to develop the suitable primers for detection and quantification of FeOB and MnOB involved in the biological filtration system for the simultaneous removal of As, Fe and Mn from groundwater.

CONCLUSIONS

Bacterial diversity of the microbial consortia in the biological filtration pilot plant for the simultaneous removal of As, Fe and Mn was analyzed. PCR-DGGE analysis of bacterial 16S rRNA genes showed at least six dominant signals and many weak signals in the sample. Nucleotide sequence analysis of the 16S rRNA gene clone library showed 47 OTUs affiliated with four subclasses of the *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Firmicutes*. The phylogenetic analysis of the clone sequences indicated the presence of the bacteria closely related to *Gallionella* and *Leptothrix*, which are supposed to be involved in Fe and Mn oxidation in the pilot plant. In contrast, PCR-based survey of *aoxB* gene suggested that arsenite-oxidizing bacterium was not involved in the removal of As in the pilot plant. These results indicated that the biological filtration system of the pilot plant was conducted by the physicochemical sorption of As by the biogenic Fe and Mn oxides produced by the bacteria closely related to *Gallionella* and *Leptothrix* included in the microbial consortia of the reactor, unlike other studies for the simultaneous removal of As and Fe using the microbial consortia containing arsenite-oxidizing bacteria. Information of the compositions of microbial consortia in the biological filtration plants would be significant to improve the biological filtration systems for the removal of As from groundwater.

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REFERENCES

- Battaglia-Brunet F., Itard Y., Garrido F., Delorme F., Crouzet C., Greffié C. and Joulian C. (2006) A simple biogeochemical process removing arsenic from a mine drainage water. *Geomicrobiol. J.*, **23**(3-4), 201-211.
- Casiot C., Pedron V., Bruneel O., Duran R., Personné J. C., Grapin G., Drakidès C. and Elbaz-Poulichet F. (2006) A new bacterial strain mediating As oxidation in the Fe-rich biofilm naturally growing in a groundwater Fe treatment pilot unit. *Chemosphere*, **64**(3), 492-496.
- Čerňanský S., Urík M., Ševc J. and Khun M. (2007) Biosorption and biovolatilization of arsenic by heat-resistant fungi. *Environ. Sci. Pollut. Res. Int.*, **14**, Suppl. 1, 31-35.
- Emerson D., Field E. K., Chertkov O., Davenport K. W., Goodwin L., Munk C., Nolan M. and Woyke T. (2013) Comparative genomics of freshwater Fe-oxidizing bacteria: Implications for physiology, ecology, and systematics. *Front. Microbiol.*, **4**, 254. (doi: 10.3389/fmicb.2013.00254)
- Emerson D., Fleming E. J. and McBeth J. M. (2010) Iron-oxidizing bacteria: An environmental and genomic perspective. *Annu. Rev. Microbiol.*, **64**, 561-583.
- Emerson D. and Moyer C. (1997) Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl. Environ. Microbiol.*, **63**(12), 4784-4792.
- French E., Kozłowski J. A., Mukherjee M., Bullerjahn G. and Bollmann A. (2012) Ecophysiological characterization of ammonia-oxidizing archaea and bacteria from freshwater. *Appl. Environ. Microbiol.*, **78**(16), 5773-5780.
- Fujikawa Y., Sugahara M., Hamasaki T., Yoneda D., Minami A., Sugimoto Y. and Iwasaki H. (2010) Biological filtration using iron bacteria for simultaneous removal of arsenic, iron, manganese and ammonia-Application to waterworks facilities in Japan and developing countries. *Osaka Sangyo Univ. J. Hum. Environ. Stud.*, **9**, 261-276. (<http://journal.osaka-sandai.ac.jp/pdf/n9261-276.pdf>, in Japanese) (accessed on March 6, 2014)
- Ghiorse W. C. (1984) Biology of iron-and manganese-depositing bacteria. *Annu. Rev. Microbiol.*, **38**, 515-550.
- Hassan K. M., Fukuhara T., Hai F. I., Bari Q. H. and Islam K. M. S. (2009) Development of a bio-physicochemical technique for arsenic removal from groundwater. *Desalin.*, **249**(1), 224-229.
- Heinrich-Salmeron A., Cordi A., Brochier-Armanet C., Halter D., Pagnout C., Abbaszadeh-fard E., Montaut D., Seby F., Bertin P. N., Bauda P. and Arsène-Ploetze F. (2011) Unsuspected diversity of arsenite-oxidizing bacteria as revealed by widespread distribution of the *aoxB* gene in prokaryotes. *Appl. Environ. Microbiol.*, **77**(13), 4685-4692.
- Hennebel T., De Gusseme B., Boon N. and Verstraete W. (2009) Biogenic metals in advanced water treatment. *Trends Biotechnol.*, **27**(2), 90-98.
- Ike M., Miyazaki T., Yamamoto N., Sei K. and Soda S. (2008) Removal of arsenic from groundwater by arsenite-oxidizing bacteria. *Water Sci. Technol.*, **58**(5), 1095-1100.
- Katsoyiannis I. A. and Zouboulis A. I. (2004) Application of biological processes for the removal of arsenic from groundwaters. *Water Res.*, **38**(1), 17-26.
- Katsoyiannis I. A. and Zouboulis A. I. (2006) Use of iron- and manganese-oxidizing bacteria for the combined removal of iron, manganese and arsenic from contaminated groundwater. *Water Qual. Res. J. Can.*, **41**(2), 117-129.
- Lane D. J., Pace B., Olsen G. J., Stahl D. A., Sogin M. L. and Pace N. R. (1985) Rapid

- determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. U.S.A.*, **82**(20), 6955-6959.
- Marchesi J. R., Sato T., Weightman A. J., Martin T. A., Fry J. C., Hiom S. J. and Wade W. G. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.*, **64**(2), 795-799.
- Martens-Habbena W., Berube P. M., Urakawa H., de la Torre J. R. and Stahl D. A. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. *Nature*, **461**(7266), 976-979.
- Mohan D. and Pittman Jr. C. U. (2007) Arsenic removal from water/wastewater using adsorbents - A critical review. *J. Hazard. Mater.*, **142**(1-2), 1-53.
- Moore R. L. (1981) The biology of *Hyphomicrobium* and other prosthecae, budding bacteria. *Annu. Rev. Microbiol.*, **35**, 567-594.
- Mouchet P. (1992) From conventional to biological removal of iron and manganese in France. *J. Am. Water Works Assn.*, **84**(4), 158-167.
- Nakatsu C. H., Hristova K., Hanada S., Meng X. Y., Hanson J. R., Scow K. M. and Kamagata Y. (2006) *Methylibium petroleiphilum* gen. nov., sp. nov., a novel methyl *tert*-butyl ether-degrading methylotroph of the *Betaproteobacteria*. *Int. J. Syst. Evol. Microbiol.*, **56**(5), 983-989.
- Pacini V. A., Ingallinella A. M. and Sanguinetti G. (2005) Removal of iron and manganese using biological roughing up flow filtration technology. *Water Res.*, **39**(18), 4463-4475.
- Pokhrel D. and Viraraghavan T. (2009) Biological filtration for removal of arsenic from drinking water. *J. Environ. Manage.*, **90**(5), 1956-1961.
- Quéménéur M., Heinrich-Salmeron A., Muller D., Lièvreumont D., Jauzein M., Bertin P. N., Garrido F. and Joulain C. (2008) Diversity surveys and evolutionary relationships of *aoxB* genes in aerobic arsenite-oxidizing bacteria. *Appl. Environ. Microbiol.*, **74**(14), 4567-4573.
- Rahman M. A. and Hasegawa H. (2011) Aquatic arsenic: Phytoremediation using floating macrophytes. *Chemosphere*, **83**(5), 633-646.
- Sahabi D. M., Takeda M., Suzuki I. and Koizumi J. (2009) Adsorption and abiotic oxidation of arsenic by aged biofilter media: Equilibrium and kinetics. *J. Hazard. Mater.*, **168**(2-3), 1310-1318.
- Sari A. and Tuzen M. (2009) Biosorption of As(III) and As(V) from aqueous solution by macrofungus (*Inonotus hispidus*) biomass: Equilibrium and kinetic studies. *J. Hazard. Mater.*, **164**(2-3), 1372-1378.
- Siering P. L. and Ghiorse W. C. (1997) Development and application of 16S rRNA-targeted probes for detection of iron- and manganese-oxidizing sheathed bacteria in environmental samples. *Appl. Environ. Microbiol.*, **63**(2), 644-651.
- Smith J. P. and Lewis G. D. (2008) Unusual bacterially mediated manganese-based structures within biofilms from urban streams. The 12 th International Symposium on Microbial Ecology (ISME12), Aug. 17-22, Cairns, Australia, 0183.
- Solorzano L. (1969) Determination of ammonia in natural waters by the phenol hypochlorite method. *Limnol. Oceanogr.*, **14**(5) 799-801.
- Štembal T., Markić M., Ribčić N., Briški F. and Sipos L. (2005) Removal of ammonia, iron and manganese from groundwaters of northern Croatia—pilot plant studies. *Process Biochem.*, **40**(1), 327-335.
- Stoecker K., Bendinger B., Schöning B., Nielsen P. H., Nielsen J. L., Baranyi C.,

- Toenshoff E. R., Daims H. and Wagner M. (2006) Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane mono-oxygenase. *Proc. Natl. Acad. Sci. U.S.A.*, **103**(7), 2363-2367.
- Sugahara M., Fujikawa Y., Hamasaki T., Minami A., Yoneda D., Tonokai K., Tani S., and Nishida K. (2008) Arsenic removal from groundwater using indigenous iron and manganese oxidizing bacteria. *Annu. Report FY 2007, The Core University Program between Japan Society for the Promotion of Science (JSPS) and Vietnamese Academy of Science and Technology (VAST)*, 403-407. (http://ir.library.osaka-u.ac.jp/dspace/bitstream/11094/13060/1/arfyjsps2007_403.pdf) (accessed on March 6, 2014)
- Thapa Chhetri R., Suzuki I., Takezaki J., Tabusa H., Takeda M. and Koizumi J. (2013) Bacterial diversity in biological filtration plant for the removal of iron and manganese from groundwater. *J. Water Environ. Technol.*, **11**(1), 33-47.
- van Veen W. L., Mulder E. G. and Deinema M. H. (1978) The *Sphaerotilus-Leptothrix* group of bacteria. *Microbiol. Rev.*, **42**(2), 329-356.
- WHO (1996) *Guidelines for drinking-water quality. Health criteria and other supporting information, 2nd ed.* World Health Organization, Geneva, Switzerland, pp.940-949.