Analyses of the diversities of bacterial consortia involved in the biological removal of Arsenic, iron and manganese from water using molecular biological methods

（水中のヒ素、鉄およびマンガンの生物学的除去に関与する細菌群集構造の分子生物学的手法を用いた解析）

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ABSTRACT

Biological filtration plant is a simple, cost-effective and eco-friend system to remove soluble iron (Fe) and manganese (Mn) ions from groundwater. In this system, the combined biological activities along with chemical reactions are carried out by naturally occurring Fe- and Mn-oxidizing microorganisms. These microorganisms oxidize Fe and Mn to produce biogenic Fe and Mn oxides, which they are reactive to remove the soluble toxic metal ions like arsenic (As), cadmium (Cd), lead (Pb) from the groundwater. However, many biological filtration plants have been designed and developed for the drinking water purification in all over the world. In Japan, single stage rapid filtration plants have been operated to purify the drinking water by removing Fe and Mn from the raw groundwater. The biological filtration plant has been established to treat Fe and Mn at Joyo City, Kyoto Prefecture, Japan. But the biological removal method for toxic heavy metal for As is still under testing in the world. Similarly, the biological filtration pilot plant was operated at Muko City, Kyoto Prefecture to study the simultaneous biological removal of As, Fe and Mn from the raw groundwater. In these biological filtration plants, >70% of metal ions were removed without using any chemical reagents. The complete biological structures in these systems were not reported yet except few microscopic analyses. Therefore, the biological structures in these plants were studied via polymerase chain reaction (PCR) based culture-independent molecular biological techniques using 16S ribosomal RNA gene (16S rDNA).

Bacterial diversities in those plants were studied by denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), clone library analysis, real-time PCR. In Joyo plant, *Sideroxydans* and *Hyphomicrobium* related bacterial consortia were respectively detected as the dominant Fe-oxidizing bacteria (FeOB) and Mn-oxidizing bacteria (MnOB) via clone library analysis. These results showed that *Gallionella* and *Leptothrix* are not always the dominant bacteria in Fe and Mn removal biological plants to oxidize soluble Fe and Mn. However, *Gallionella* and *Leptothrix* related common iron bacteria were studied as the dominant FeOB and MnOB in As removal biological filtration plant by the same clone library analysis. The quantitative real-time PCR analysis estimated >10% of *Leptothrix* related 16S rRNA gene harboring PSP-6 sequences in both plants. The genes encoding ammonia monooxygenase subunit A (*amoA*) of ammonia

oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) were amplified that present in the DNA solution extracted from biological filter media (BFM) of Joyo plant, but only the AOB-amoA was amplified in extracted DNA solution from the microbial flocs of the pilot plant. But the *aoxB* gene encoding arsenite oxidase was not amplified in the DNA solution of the pilot plant. Therefore, it was considered in biological pilot plant that As-oxidizing bacteria (AsOB) were not prominent to remove As(III/V) ions, alternatively biogenic Fe and Mn oxides produced by FeOB and MnOB could remove soluble As(III/V).

To study the biological removal mechanism for these heavy metals, Mn-oxidizing microbial consortia were cultivated in modified PYM (mPYM) (Peptone: 1 g/l, Yeast-extract: 0.1 g/l and manganese: 30 mg/l of final Mn(II)) (Vandenabeele *et al.*, 1995) culture media to produce massive Mn oxides using the BFM of Joyo. In the cultured microbial consortia in mPYM, Mn oxides were produced and *Leptothrix* and *Hyphomicrobium* related MnOB were studied via PCR-DGGE analysis. Those reactive microbial consortia were applied for the simultaneous removal of Mn(II) and As(III/V). Ultimately, adequate As ions (>97% of As (III) and >75% of As(V) of initial concentration of 500 μg/l) were removed by those cultivated Mn-oxidizing microbial consortia (2-mL) in mPYM after three weeks incubation. MnOB such as *Leptothrix* and *Hyphomicrobium* related 16S rRNA gene copies were estimated in the cultured mPYM by real-time PCR. Naturally occurring AsOB in the BFM of Joyo plant became active after cultivation to remove As in mPYM. *Bosea*, *Agrobacterium*, *Sinorhizobium* related bacteria were isolated from the clone library analysis for BFM of Joyo plant and two weeks cultured microbial consortia. These Mn-oxidizing microbial consortia would be more applicable in new welcome plants for As removal, however its details is required to be studied.

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CHAPTER 1
(Introduction and outline)

1.1 Introduction

Groundwater is an important resource of drinking water in many small and medium-sized communities in the world. The natural groundwater is often contaminated by inorganic heavy metals such as iron (Fe), manganese (Mn), arsenic (As), and other species such as ammonia. The removal of Fe and Mn is an essential issue in the treatment of groundwater for drinking. Although relatively safe, the presence of these metal ions in water supplies even in low concentration causes aesthetic defects, organoleptic and operational problems. Conventional removal methods rely on physical-chemical reactions using intense aeration or chemical oxidation, coagulation and rapid sand filtration. However, these conventional processes have operating problems and they do not always meet the water quality objectives (Mouchet, 1992).

The conventional physical-chemical techniques for the removal of dissolved Fe and Mn ions from raw groundwater reflect some drawbacks, which persuaded the researchers and scientists to investigate an alternative, easy and simple water treatment method. Interestingly, biological method was accidently discovered during Fe^{2+} treatment in water treatment plant (Tanimoto, 1952; Kojima, 1972; Mouchet, 1992). Thereafter, a modern and useful biological filtration system has been developed for the removal of soluble Fe and Mn from natural groundwater to produce potable drinking water (Kojima, 1972; Mouchet, 1992; Tamura *et al.*, 1999). In the biological treatment method, naturally occurring microorganisms mediate the removal of dissolved Fe^{2+} and Mn^{2+} ions that present in raw groundwater. Soil and natural groundwater containing so-called iron bacteria; such as, *Gallionella*, *Crenothrix*, *Clonothrix*, *Leptothrix*, and these bacteria are assumed to oxidize soluble Fe and Mn to produce biogenic Fe and Mn oxides deposits (Ghiorse and Hirsch, 1982). These deposited metal oxides form very reactive complex, which enable the simultaneous removal of other toxic metal ions (Katsoyiannis *et al.*, 2004; Sahabi *et al.*, 2010). Eventually, these microorganisms are trapped onto filter media, and produce potable drinking water in the biological filtration system. On the filter bed of the system, dissolved Fe and Mn are catalytically oxidized by the combination of biological and chemical oxidation (Mouchet, 1992; Sahabi *et al.*, 2009a). The

sludge formation on the filter bed in the biological filtration system is frequently maintained by back washing process.

The biological water treatment method is carried out in single or two-stage water filtration system according to the composition and the concentrations of contaminants (Mouchet, 1992). Their treatment performance depends on chemical composition of groundwater, biological filter media and operational parameters. Long period filtration of Fe and Mn containing natural groundwater spontaneously coats the filter media by biofilms (Hopes and Bott, 2004; Mouchet, 1992; Sahabi *et al.*, 2009a). These naturally coated layers are composed of metal oxides-rich biomasses and microbial consortia including Fe and Mn oxidizing bacteria (Hopes and Bott, 2004; Sahabi *et al.*, 2009a). These filter media of the biological filtration plant is the called biological filter media (BFM) (Sahabi *et al.*, 2009a). In the BFM, the iron bacteria are trapped and immobilized to form the several layers of biofilms and biomass, which are responsible for subsequent removal of Fe^{2+} and Mn^{2+} via biological and autocatalytic chemical oxidation (Mouchet, 1992; Sahabi *et al.*, 2009a). However, despite biological and physicochemical oxidations have been reported, the complete biological structures in the biological filtration plants that reasonably mediate the removal of metal ions such as Fe, Mn, As have not been demonstrated. The common and dominant iron bacteria such as *Gallionella* and *Leptothrix* were analyzed by microscopic analysis (Czekella *et al.*, 1985; Mouchet, 1992; Pacini *et al.*, 2005), but it needs to be studied conceive microbial diversities to investigate the biological removal mechanism for Fe and Mn in biological filtration plant.

To study bacterial diversities that naturally occur in soil and groundwater environment, culture-independent molecular biological tools would be the most precise and reliable. The extracted microbial genomes from the environmental samples are analyzed using polymerase chain reaction (PCR), and subsequently applied for analyses via the molecular biological methods to investigate distinct microbial consortia. The molecular biological methods possibly grab both cultivable and uncultivable microorganisms present in environmental samples. The molecular-based bacterial composition study reflects the information about biological mechanism for the removal of Fe and Mn from the groundwater. It would explore the scant information on the biological mechanism not only for the removal of Fe and Mn from the natural groundwater but also the other toxic metal ions such as As, Cd and Pb in the

biological filtration plant.

For proper understanding the key mechanism on possible Fe and Mn removal by the biological filtration, it needs to be studied the structures of microbial consortia, complex microbial relationship and their potential for the removal of Fe and Mn. This profound study of bacterial communities in biological filtration plant for the removal of Fe, Mn, As and NH_3 from natural groundwater would assist to modify and develop the strategies on modern biological treatment system and methods. The extensive biological structural characteristics, existence conditions and their roles in oxidation and adsorption of dissolved metal ions as well as the removal of ammonia would make easier to find out the biological mechanism in drinking water production, using the biological filtration.

The main objective of this study is to investigate the structure of microbial community for the removal of Fe, Mn, As and NH_3 in drinking water production using the biological filtration. To fulfill this purpose, the bacterial diversities in biological filtration plants (Joyo biological filtration plant, operating for drinking water production by removing Fe and Mn from groundwater, and another Muko biological pilot-plant, used for simultaneous removal of Fe, Mn and As), operated at Kyoto prefecture in Japan were analyzed by PCR-based analyses using 16S ribosomal DNAs. These indispensable studies would render to understand bacterial composition and their role for the removal of Fe, Mn, As and NH_3 in the potable drinking water production using the biological filtration.

1.2 Groundwater quality and problems

Despite many conventional methods are exploited for the groundwater treatment by removing soluble metal ions such as Fe and Mn, many people still rely on untreated raw groundwater in many countries across the world. The guideline values for the dissolved Fe and Mn have been recommended in potable drinking water not to be more than 0.3 mg/L and 0.1 mg/L, respectively (World Health Organization: WHO-2006). The maximum recommended levels of Fe and Mn ions in drinking water are 0.3 mg/L and 0.05 mg/L, in Japan (Ministry of Health: MOH, Japan, 2003) and United States (United States Environmental Protection Agency: USEPA, 2010), and they have been recommended as 0.2 mg/L and 0.05 mg/L respectively in Europe (European Union Council Directive: EUCD, 1998). On the other hand,

the maximum recommended level of As ions in drinking water is 10 mg/L (WHO, 2006), because As contaminants in drinking water causes various health hazard problems.

The consumption of inorganic Mn and As through water over the long-term, causes aesthetic problems and hazardous diseases (WHO, 2006). It would extend the poverty in the societies and also affects in terms of environmental economics. Chronic exposure to high Mn (>0.4 mg/L) over the course of years has been associated with toxicity to the nervous system producing a syndrome that resembles Parkinsonism (Fact Sheet, 2001). In contrast, it has not demonstrated current health based guidelines for Fe concentration. In addition to these problems, excess level of Fe and Mn concentration confers the color of water, bad taste, deposition of metal oxides, staining of laundry, plumbing fixtures and increase in turbidity (Appelo & Postma, 1994). On the other hand, if toxic As ions are consumed, they are readily absorbed in the human gastrointestinal tract and cause cancer of the skin, lungs, urinary bladder, kidney and skin pigmentation (Abberantly & Ghanaian, 1993). It was reported in Thailand that chronic As exposure revealed the retardation of intelligence in children (Siripitayakunkit *et al.*, 1999).

1.3 Origin of macro components iron, manganese, arsenic and ammonium

1.3.1 Iron (Fe)

Fe is the fourth most abundant element in the earth's crust, which is a common constituent of anoxic groundwater. Iron exists in the environment dominantly in two valence states, the relatively water-soluble Fe^{2+} (ferrous iron) and the highly water-insoluble Fe^{3+} (ferric iron), with the latter being the stable form in oxygen-rich environments under neutral to alkaline pH conditions. In the normal pH range of groundwater (pH 6 to 8.5), dissolved iron is present as Fe^{2+}. The main sources of Fe^{2+} in groundwater are dissolution of Fe^{2+}-bearing minerals and reduction of iron-oxyhydroxides (FeOOH) present in the sediments. Important Fe^{2+}-bearing minerals commonly present in aquifer materials comprise minerals like magnetite, ilmenite, pyrite and siderite, and Fe^{2+}-bearing silicates like amphiboles, pyroxenes, olivine, biotite and glauconite, and clay minerals, such as smectites (Appelo and Postma, 1994). Reductive dissolution of FeOOH becomes important when an electron acceptor, such as dissolved organic matter, hydrogen sulphide, or methane, enters a sediment containing

FeOOH. The pool of FeOOH in the sediment covers a broad range of minerals with both variable stability and variable kinetic properties (Appelo and Postma, 1994). Sung and Morgan (1980) reported that the initial product of Fe^{2+} oxidation are lepidocrocite and amorphous FeOOH. In natural groundwater, inorganic Fe^{2+} concentration ranges from 0 to 40 mg/L with majority containing <5 mg/L (Water Quality Assessments: WQA, 1997), which simply react with oxygen to form insoluble Fe^{3+}. In the environment, Fe plays an important role as an electron donor during the oxidation of Fe^{2+} to Fe^{3+}, in its various mineral forms, as a precipitant or sorbent substrate (Takai, 1973; Sung & Morgan, 1980; Bendell-Young and Harvey, 1992). A long-term storage of contaminant metals and radionuclides on iron oxyhydroxide and oxide phases is, however, clearly pH and Eh dependant, as chemical and/or microbially-driven reduction reactions or an increase in acidity may lead to breakdown and solubilisation of iron phases and release of associated contaminants (Smedley and Kinniburgh, 2002).

1.3.2 Manganese (Mn)

Mn is the 10th most abundant element in the earth's crust and second only to iron as the most common heavy metal. On the average crustal rocks contain about 0.1% Mn (Davison, 1991). It can display various oxidation states (11 oxidation states) ranging from -3 to +7. In nature the oxidation states +2, +3 and +4 predominates, and are of greatest environmental importance. Dissolved Mn^{2+} state in aquatic environment is the most stable and can form a number of different metal complexes with ligands. These soluble Mn^{2+} ions are easily oxidized giving rise to more than 30 known Mn oxide/hydroxide minerals such as pyrolusite, birnessite, pyrochroite ([$Mn(OH)_2$], groutite (α-MnOOH), Feitknechite (β-MnOOH), Manganite (γ-MnOOH) (Post 1999). The thermodynamic stability of the different manganese (hydro) oxides is determined by the redox conditions, pH, temperature, oxygen partial pressure within the environment. For the most natural waters, the pH values range from 5.0 to 8.5, but Mn^{2+} is stable at pH below 9 (pH 6 to 9) because of its very low rate of oxidation. Their oxidation in such natural systems is thermodynamically favorable but often proceeds at very slow rates. The Mn^{2+} oxidation is autocatalytic with the Mn-(oxyhydr) oxide products adsorbing Mn^{2+} and catalyzing its oxidation (Stumm & Morgan, 1996). The dissolved Mn^{2+} ions can be oxidized directly to Mn^{4+} or to mixed Mn^{2+} and Mn^{3+} oxides and oxyhydroxides or to Mn_3O_4 (composed of $Mn_2O_3.MnO$) (Murray *et al.*, 1974). In the natural groundwater,

dissolved Mn^{2+} concentration ranges from 0 to 10 mg/L with the majority containing <2 mg/L.

The Mn^{3+} ion hydrolyses readily and is thermodynamically unstable in aqueous solutions, but could be stabilized at high pH in the form of MnOOH. Mn^{3+} does not occur in soluble form except in the presence of strong complexing agents such as humic acids or other organic acids (Kostka *et al.*, 1995). Mn^{3+} and Mn^{4+} primarily form insoluble oxides and oxyhydroxides. The solubility of Mn oxide (MnO_2) is very low within the pH range 3 to 10 (Stumn & Morgan, 1996). The concentration of dissolved Mn^{2+} in groundwater and surface waters is largely controlled by redox reactions between Mn^{2+} and $Mn^{3+, 4+}$ governed by pH. Recent studies suggest that microorganisms can increase the rate of Mn^{2+} oxidation by up to five orders of magnitude over abiotic oxidation and therefore are likely responsible for most of the natural Mn^{2+} oxidation (Tebo *et al.*, 1997). However at low Mn concentrations in the presence of bacteria the formation of amorphous Mn oxides has been reported (Tebo *et al.*, 2004). Laboratory experiments have shown that the sorption capacity of freshly precipitated Mn oxides is extremely high for a variety of metal cations (Murray, 1974; Jenne, 1968). Furthermore the adsorption of the heavy metals have been found to proceed with the release protons suggesting that the cations are bound into the Mn oxides atomic structure (Murray,1974).

1.3.3 Arsenic (As)

Arsenic (As) is the 20th most abundant element in the earth's crust and the 12th most abundant element in the human body (Borgono *et al.*, 1977; Abedin *et al.*, 2002); it has an average crustal concentration of 1.8 mg/Kg (Demayo, 1985). However through geogenic processing of crustal materials, arsenic can be concentrated in soils to a typical range of 2 to 20 mg/Kg. Concentrations as high as 70 mg/kg have been reported by Yan-chu, (1994). The toxicity of As(III) is 10 times higher than that of As(V) (Castro de Esparza, 2006). Arsenic is labile and readily changes oxidation or chemical form through chemical or biological reactions that are common in the environment. The mobility of arsenic is usually affected by redox conditions, pH, biological activity and adsorption / de-sorption reactions. In aqueous environment arsenic normally exist in the As(III) or As(V) states. Arsenic in groundwater most often originates from geogenic sources, although anthropogenic pollution sources do occur (Chakravarty *et al.*, 2002). Arsenic

occurs as a major constituent in more than 200 minerals, including elemental arsenic, arsenides sulphides, oxides, arsenates and arsenites etc. Arsenic can exist in the inorganic and organic forms. The concentration of arsenic in ground water ranges from less than 0.5 to 5,000 µg/L. In groundwater, the dominant As species is derived by pH and redox potential. Below pH 9, H_3AsO_3 is the dominant species, whereas $H_2AsO_3^-$ and $HAsO_3^{2-}$ dominate above pH 9 (Ferguson & Gavis, 1972; Sracek *et al.*, 2001). It is, however, noted that both the As(V) and As(III) forms occur in both oxic and reducing conditions due to the slow oxidation and reduction kinetics (Edwards, 1994).

In Terai region of Nepal, however the natural groundwater contains massive concentration of As (up to 75 µg/L), >90% people rely on raw groundwater for their drinking and irrigation purposes. The major contamination processes in this region are due to natural geogenic as well as insignificant anthropogenic activities. The geology of Nepal is complex system, centered on the great elevation change from the top of the Himalayas to the flatlands of the Terai (Halsey, 2000). Geologically, the Terai region is originally similar to Bengal Delta Plain, and the sedimentary layers consist of Holocene thick sand and gravel deposits interlocked with alluvium flood plains carried by rivers from Siwalik Hills (Pokhrel *et al.*, 2009; Bhattacharya *et al.*, 2002). This region is characterized as flat plains and farmland from west to east along the southern border with India. The multitude of minor rivers that originate in the mountains and foothills to the north, have massive deposition of naturally occurring As associated with their flow patterns. The high velocities achieved while the rivers flow down the steep mountains allow for massive transport elevations, suspended sediments in the water are deposited in huge quantities. These river systems, therefore, dictate a good deal of the geology contamination of As in the lower lands Terai region. The dissolution of arsenic-bearing rocks, sediments and minerals contribute As contamination to the groundwater via percolation (Bhattacharya *et al.*, 2003). Nepal is agricultural country and its 60% agricultural production is centered in Terai region. This region is densely populated with about half of total population (47%) of Nepal. Farmers in this region use various types of fertilizers and pesticides for their better agricultural production. Therefore, the reduced As ions are percolated via soil to the groundwater. In addition, the highly industrialized areas contain many industries, business and personal residences in the middle latitudes of Nepal as well as in Terai

region also receive extensive amounts of rainfall in monsoon season. Ultimately, the over flooding scatter the industrial complexes in this plain region, percolation of reduced As is becoming an insignificant contamination of As in groundwater (Nepal.com, 2000).

1.3.4 Ammonium (NH_4^+) and nitrate (NO_3^-)

Nitrogen (N) is found in groundwater as dissolved organic form such as NH_4^+, nitrite (NO_2^-) and NO_3^-. The most common N compound found in the groundwater is NO_3^-, but in strongly reducing environments NH_4^+ can be the dominant form. NH_4^+ is found in groundwater naturally as a result of anaerobic decomposition of organic material (Böhlke *et al.*, 2006). It is also commonly found in groundwater due to anthropogenic activities, primarily due to leaching from fertilizes, organic waste disposal or leaking sewage systems. NO_3^- is usually not found in concentrations over 2 mg/L in unpolluted groundwater, though this may be higher in arid environments (Kresic, 2007). Higher concentrations of NO_3^- in the groundwater are usually an effect of these anthropogenic activities. The WHO has set the recommended guide line values for NO_3^- in drinking water to 50 mg/L. The primary health concern regarding NH_4^+ and NO_2^- is so called methaemoglobinaemia (WHO, 2008). This state is also known as cyanosis or blue - baby syndrome, since infants are especially sensitive. The WHO has not issued any guidelines for NH_4^+ in drinking water since NH_4^+ has no known toxic effects in concentrations that can be expected to be found in drinking water. High concentrations of NH_4^+ in drinking water are however not desirable, since it can be converted to NO_3^- in the water distribution net, compromise disinfection quality, cause failure of filters for removal of Mn as well as taste and odour problems (WHO, 2008). If high levels of NH_4^+ are found in groundwater it is likely that the groundwater is being contaminated by anthropogenic sources and may as such contain pathogens, pesticides or other unwanted substances.

For NH_4^+, an obvious source is the degradation of nitrogenous (N - containing) organic matter in the confining clay and peat layer, in the river bottom, and to a lesser extent in the aquifer itself. The major pollutants in groundwater are nitrate (NO_3^-) and nitrite (NO_2^- : reduced NO_3^-). According to WHO and EUCD, the standards for drinking water quality are 50 mg-NO_3^-/L, whereas Japanese and United States federal standards are 10 mg-N/L. In usual level of ammonium ions (NH_4^+), other major component of

dissolved inorganic nitrogen does not pose a direct risk to human health. The biological method is more practicable process for removal of NH_4^+ from groundwater compared with physical-chemical methods.

1.4 Groundwater treatment methods

1.4.1 Conventional, physicochemical treatment methods

To avoid the problems caused by excess Fe, Mn and As ions in natural or polluted groundwater, different conventional methods such as, rapid sand filtration (occasionally supported by chemical oxidation and / or sedimentation), precipitation, and coagulation, are applied. The chemical oxidation (with O_3, $KMnO_4$ or ClO_2) method is predominantly referred to remove Fe, Mn and As from the groundwater in drinking water production. However, intense aeration and greensands are respectively used for the removal of Fe and Mn in the conventional method. In drinking water treatment process, the conventional methods attribute many drawbacks such as, operating problems during treatment, which causes poor quality in treated water and costing elevation (Gage *et al.*, 2001).

1.4.2 Biological treatment method

Naturally occurring microorganisms are trapped in the reactor and oxidize soluble Fe and Mn ions to insoluble Fe and Mn oxides. Those deposited oxides are removed by filtration to produce potable drinking water (Mouchet, 1992). For the past few decades, this method is being widespread to remove the dissolved Fe and Mn ions from drinking water in many countries in the world (Mouchet, 1992; Gage *et al.*, 2001; Pacini *et al.*, 2005). At neutral pH, Fe^{2+} ion is easily oxidized by dissolved oxygen, thus Fe oxidation is mainly achieved through aeration; in contrast, Mn^{2+} oxidation does not proceed at appreciable rates below pH 9 (Mouchet, 1992). Thus, the production of Mn oxides in the freshwater and marine environment is possibly due to biological activities (Tebo *et al.*, 2004).

1.4.3 Biological filtration system for the removal of Fe and Mn

1.4.3.1 Two stage biological filtration system

The first modern biological treatment systems using rapid sand filtration for treatment of Fe and Mn from groundwater was probably developed in France in the 1980s (Mouchet, 1992). For the past decades, Fe and Mn removal microbial activity was being operated in slow sand filtration without addition of chemical oxidants in several waterworks (Tanimoto, 1952;

Kojima, 1972; Mouchet, 1992; Czekalla *et al.*, 1985). Nowadays, most of the European countries such as France (more than 100), Germany, Belgium, Bulgaria, Finland, Netherland and Japan have developed and designed the biological treatment plants for the treatment of groundwater (Peitchev & Semov, 1988; Seppänen, 1991; Mouchet, 1992; Fujikawa *et al.*, 2008b). In France, biological Fe and Mn removal reactors consisted of aeration and rapid sand filtration have been developed and operated for the past few decades. In France, the most of the biological filtration plants employ two filtration steps to remove Fe and Mn from groundwater because of requirement of different optimum conditions for their biological oxidation. The steps include initial aeration followed by rapid filtration for Fe removal, and then secondary aeration, pH adjustment, and secondary rapid filtration for Mn removal (Mouchet, 1992). These systems require sophisticated devices to control dissolved oxygen concentration, pH, and redox potential of each filtration steps.

1.4.3.2 Single stage biological filtration system

Tanimoto (1952) has described Fe removal process by growing Fe and Mn oxidizing bacteria (MnOB) like *Leptothrix* sp. in slow sand filtration system at Tadotsu-cho, Kagawa, in Japan (established in 1924). This reactor plant was perhaps the first finding of biological filtration system (Tanimoto, 1952; Kojima, 1972). Consequently, inoculation of slow sand filters with iron bacteria, which is a common name of Fe-oxidizing bacteria (FeOB), and Fe- and Mn-oxidizing bacteria (MnOB), was practiced to improve the removal of these metals at several groundwater treatment systems (Kojima, 1972). Such observations were also reported in several European water works using slow and fast sand filtration (Czekalla *et al.*, 1985; Mouchet, 1992). In general, single filtration system requires porous filter media such as anthracite, zeolite for fixing various microorganisms in the reactor (Mouchet, 1992). This system is used for simultaneous removal of Fe and Mn, naturally found in Fe- and Mn-containing groundwater and freshwater environments.

1.4.3.3 Biological filtration plant in Joyo, Kyoto

The first single filtration model for biological Fe and Mn removal in Japan was constructed in 1993, in Joyo City of southern Kyoto Prefecture. It is the first mid-speed biological water filtration plant for removal of Fe and Mn in Japan. The system consisted of aeration by dropping, gravity filtration with dual-media filter (anthracite and sand), and final disinfection. This plant

supplies 11,100 m^3/day of drinking water, at where the filtration rate is 2.9 m/h (Tamura *et al.*, 1999). Dissolved oxygen was controlled at 5-7 mg/L by movable weir prior to the filtration. This system focuses on oxidation and sorption of dissolved Fe and Mn to remove these metal ions from groundwater. Backwashing of the filter is performed in every two weeks (14 days) to ensure optimal performance. This biological filtration plant was started with three wells in 1993, and the filter media was not replaced until now. After that, three extra new wells have been used since 2006, which successfully treat groundwater same as previously constructed wells.

1.4.3.4 Biological filtration plant in Yamato-Koriyama, Nara

Another biological Fe and Mn treatment system with rapid filtration rate is located in Yamato-Koriyama City, Nara Prefecture, Japan (established in 2001). The system is consisted of aeration by dropping and gravity filtration with single filter using spherical carriers of polyester fiber, which enable the filtration rate of up to 15 m/h (Fujikawa *et al.*, 2008b). This biological filtration system is used for the first treatment prior to the conventional rapid sand filtration.

1.4.3.5 Biological filtration pilot plant in Muko City, Kyoto

A modern test plant for biological filtration was established in 2005 for the simultaneous removal of Fe, Mn, As from natural groundwater at Muko City in Kyoto Prefecture, Japan (Fujikawa *et al.*, 2010). This test plant consists of aeration tank and filtration reactor (ϕ100 mm × 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). This pilot reactor removed >70% of As ions, which they present in influent raw groundwater (Fujikawa *et al.*, 2010).

1.5 Further applications of the biological Fe and Mn oxidation in advanced water treatment

The potential application of biological oxidation of Fe and Mn for removal of other toxic water contaminants is one of the most crucial research that led up to this research. The process of biological Fe and Mn oxidation invariably leads to the formation of Fe and Mn oxides that are associated with extracellular structures of microorganisms (Katsoyiannis and Zouboulis, 2004; Tebo *et al.*, 2004). The particular characteristics of these biogenic metal oxides, such as their large specific surface area and high reactivity

make them interested for various applications, for instance as adsorbents, catalysts, oxidants or reductants (Hennebel *et al.*, 2008).

In particular, the potential application of biological Fe and Mn oxidation for effective removal of toxic metal ions from contaminated water is an interesting area of biotechnology currently receiving enormous attention. Arsenic removal in conjunction with biological oxidation of Fe or Mn or both has been reported (Katsoyiannis *et al.*, 2002; Katsoyiannis and Zouboulis, 2006). The process may involved oxidation of As(III) to As(V) through the generation of oxidative Fe and Mn oxides (Katsoyiannis *et al.*, 2004; Sahabi *et al.*, 2010). In a similar vein, biogenic Fe and Mn oxides and their associated biomass are reported to have diverse adsorption capabilities for a variety of toxic heavy metal ions (Hennebel *et al.*, 2008; Miyata *et al.*, 2007; Sahabi *et al.*, 2010). Vandenabeele et al. (1995) has reported the cultivation of Mn-oxidizing microorganisms using the BFM of the rapid sand filtration plant, used for the removal of Mn^{2+} in Belgium (Vandenabeele *et al.*, 1995). It is thus, the biogenic Mn oxides were produced via microbial enrichment. It has been already known that the biogenic Mn oxides are more prominent for the removal of metal ions, such as Mn and As (Vandenabeele *et al.*, 1995; Katsoyiannis *et al.*, 2004; Tebo *et al.*, 2004; Sahabi *et al.*, 2010). The microbial flocs obtained from the cultivation of Mn-oxidizing microorganisms can be applied for the removal of Mn and As in natural and engineered environments.

1.6 Significant bacterial species in natural groundwater

1.6.1 Iron bacteria

Iron bacteria are common name of a kind of bacteria which generally found in the metal oxides-rich bio-mass or Fe and Mn-containing fresh water environment. These bacteria have long been well-known not only for their interesting habitats of Fe and Mn rich environment but also for their unique structures and shapes such as, twisted filaments (*Gallionella*), filamentous tubes called “sheath” (*Leptothrix*, *Sphaerotilus*, *Clonothrix*, and *Crenothrix*). But most of their characteristics on growth conditions and biological activities are still uncertain because of the difficulties of pure cultivation.

Gallionella ferruginea is an Fe oxidizing chemolithoautotroph which has unique spirally twisted stalks (Hanert *et al.*, 2006a), and able to utilize Fe^{2+} for its growth oxidation, whereas it does not oxidize Mn^{2+}. The genus

Leptothrix species is Fe and Mn oxidizing, sheathed heterotroph (van Veen *et al.*, 1978; Ghiorse, 1984; Spring, 2006). A number of *Leptothrix* species are well investigated in their characteristics by success of their pure cultures, however, pure culture of *Leptothrix ochracea*, which is the most common species found in biological treatment reactors, is not obtained yet. The genus *Sphaerotilus* is phylogenetically related to the genus *Leptothrix*, however, it does not oxidize Mn^{2+} (van Veen *et al.*, 1978; Spring, 2006). *Gallionella*, *Leptothrix*, and *Sphaerotilus* belong to beta subclass of *proteobacteria* according to phylogenetic analyses of 16S rRNA gene sequences. The other iron bacteria such as, *Clonothrix* (Hirsch *et al.*, 1989a) and *Crenothrix* (Hirsch *et al.*, 1989b), and the genus like *Toxothrix* (Krul *et al.*, 1970; Hirsch *et al.*, 2006) and *Siderocapsa* (Hanert *et al.*, 2006b), generally exist in Fe and Mn oxide deposits, although their metal oxidation activities and contribution for removal of Fe and Mn in biological treatment of groundwater are unclear. *Toxothrix*, a bacterium that produced twisted bundles of filaments containing oxidized Fe (Hirsch *et al.*, 2006), and *Siderocapsa*, which is considered for producing Fe and Mn oxides in aquatic environment (Hanert *et al.*, 2006b), are not yet isolated in pure culture. In contrast, *Metallogenium* is recently considered as Mn oxide particle produced by the other Mn oxidizing microorganisms rather than a bacterium (Maki *et al.*, 1987; Emerson *et al.*, 1989).

1.6.2 Iron bacteria in the biological filtration reactor

The iron bacteria, which they are naturally trapped in microbial deposits on the surface of biological filtration reactors, have been reported by microscopic observations (Tanimoto, 1952; Czekalla *et al.*, 1985; Mouchet, 1992; Tamura *et al.*, 1999; Pacini *et al.*, 2005) because the unique extracellular structures of the iron bacteria are easily identified. However, viability of these bacterial cells was not reported yet. Probably, most of the cells in the deposits are just accumulated on the filter through raw groundwater but do not grow on the filter. As described above, Fe and Mn removal activities of the biofilters are recovered within one hour after backwashing (Mouchet, 1992; Tamura *et al.*, 1999; Fujikawa *et al.*, 2008b), suggesting that the microorganisms mainly contribute to Fe and Mn removal that exist in biofilms developed on the surface of the biofilter rather than the cells existing in the deposits.

For the biological treatment of As in the biological filtration plant, the

importance of iron bacteria has been reported (Katsoyiannis and Zouboulis, 2004; Katsoyiannis and Zouboulis, 2006). These naturally occurring Fe and Mn oxidizing microorganism oxidize the Fe^{2+} and Mn^{2+} ions to produce insoluble Fe and Mn oxides, which adsorb and oxidize As oxyanions as well as the other metal ions (Mouchet, 1992; Katsoyiannis and Zouboulis, 2004, Tebo *et al.*, 2004; Miyata *et al.*, 2007; Sahabi *et al.*, 2010). However, it needs to be studied the complete biological structures present in the biological filtration plant used for the simultaneous removal Fe, Mn and As from raw groundwater.

1.6.3 Effect of ammonium (NH_4^+) in groundwater for biological removal of Mn

It has been reported that biological Mn oxidation is initiated after completion of nitrification-denitrification, especially if the water contains relatively high concentration of ammonia (Frischherz *et al.*, 1985; Mouchet, 1992; Vandenabeele *et al.*, 1995; Gouzinis *et al.*, 1998; Stembal *et al.*, 2005). Gouzinis et al. (1998) investigated the biological oxidation of Fe, Mn and NH_4^+ when these ions simultaneously present in water. They found that high concentrations of Fe and ammonia (NH_3) affected Mn oxidation negatively, and Mn removal occurred after complete nitrification had taken place. An indirect link between nitrification-denitrification and Mn oxidation was reported by Vandenabeele et al. (1995) that presence of nitrate increased Mn-oxidizing activities in batch cultures of microbial consortia derived from biological filter media in Belgium biological filtration plant (Vandenabeele *et al.*, 1995). However, how nitrate influence the activity of MnOB in biological treatment system remains unknown.

1.6.3.1 Biological removal of NH_3 in water treatment system

In raw groundwater, NH_3 usually present in the form of NH_4^+, which is biologically removed via nitrification process by naturally occurring microorganisms. Two steps of nitrification process consist of initial oxidation of NH_3 to NO_2^- in presence of ammonium-oxidizing microorganisms, and thereafter another oxidation step of NO_2^- to NO_3^- is enhanced by nitrite-oxidizing microorganisms. Despite the biological removal of NH_3 is possible in the biological filtration plant to produce drinking water (Mouchet, 1992, Tamura *et al.*, 1999, Stembal *et al.*, 2005), however, the removal mechanism in biological filtration system is still unclear. Although many investigations on simultaneous biological removal of NH_4^+-N and Mn^{2+} have

been conducted (Stembal *et al.*, 2005 and Tekerlekopoulou and Vayenas, 2007), the specific effective microbes involved in the simultaneous removal are still unclear and unidentified.

1.6.3.2 Ammonium-oxidizing bacteria (AOB)

It has been known that NH_3 oxidation is carried out exclusively by AOB belonging to *Beta-* and *Gamma-proteobacteria*. The most frequently identified genus associated with the oxidation of NH_3 to NO_2^- is *Nitrosomonas*, although other genera like *Nitrosococcus* and *Nitrosospira* may be involved (Purkhold *et al.*, 2000). Similarly, some subgenera *Nitrosolobus* and *Nitrosovibrio* can also autotrophically oxidize NH_3. In chloraminated drinking water systems, the genus *Nitrosomonas* dominated over *Nitrosopira*, studied by molecular techniques (Regan *et al.*, 2002 and 2003). Aerobic AOB catalyze the first step of aerobic nitrification, the oxidation of NH_3 to NO_2^-. They are highly important for the turnover of inorganic N in many ecosystems and for biological wastewater treatment.

1.6.3.3 Ammonia oxidizing archaea (AOA)

Recently it was shown that in addition to AOB, archaea also are capable of NH_3 oxidation (Könneke *et al.*, 2005). AOA are widely distributed and frequently outnumber AOB in various environments (Francis *et al.*, 2005; Leininger *et al.*, 2006 and Wuchter *et al.*, 2006), however, only two reports describe the occurrence of AOA in drinking water treatment and distribution systems (de Vet *et al.*, 2009 and van der Wielen *et al.*, 2009). Different studies reported non-thermophilic AOA such as *Crenarchaeota* (Könneke *et al.*, 2005; Schleper *et al.*, 2005 and Treusch *et al.*, 2005). The contribution of AOA to NH_3 oxidation is a controversial issue (Prosser and Nicol, 2008 and You *et al.*, 2009). Some researchers argue that AOB could play a central role in NH_3 oxidation despite the predominance of AOA (Jia and Conrad, 2009 and Wells *et al.*, 2009). However, it is currently known that AOA are present in drinking water treatment processes and distribution systems (Paul *et al.*, 2009). It was demonstrated that AOA grow in a habitat with low NH_3 concentration (Martens-Habbena *et al.*, 2009). Wells et al. (2009) suggested that AOA were minor contributors to NH_3 oxidation in highly aerated activated sludge systems, but van der Wielen et al. (2009) suggested that AOA could be responsible for the removal of NH_3 in groundwater treatment plants. Other studies have demonstrated that AOA are indeed involved in nitrification (Herrmann *et al.*, 2008; Offre *et al.*, 2009 and Schauss *et al.*,

2009).

1.6.3.4 Nitrite-oxidizing bacteria (NOB)

Nitrifying bacteria can grow at pH range 7 to 10. *Nitrobacter* is the genus most frequently associated with this second step nitrification, although other genera, such as *Nitrospina*, *Nitrococcus*, and *Nitrospira*, can also autotrophically oxidize NO_2^- to NO_3^- (Watson *et al.*, 1981). NOB may also contribute to the growth of heterotrophs via the production of soluble organic products (Rittmann *et al.*, 1994). Having an active NOB community coupled with AOB within the drinking water distribution system may favor the biological oxidation of nitrite to nitrate (Regan et al., 2002 and 2003).

1.6.3.5 Denitrifying bacteria

Denitrification may be the process best suited to bioremediate NO_3^- contamination as it can utilize relatively large amounts of NO_3^- and it produces an innocuous end product. Denitrifying bacteria convert the NO_3^- to the atmospheric N. Some bacterial genera such as *Thiobacillus denitrificans*, *Paracoccus denitrificans*, and some species of *Serratia*, *Pseudomonas*, *Achromobacter* and *Burkholderia* implicated as denitrifiers (USEPA, 1999 and Takaya *et al.*, 2003).

1.7 Molecular biological tools for the detection of environmental bacteria

1.7.1 Culture-dependent analysis

To study the biological oxidation of Fe and Mn for specific bacterial species, culture-dependent experiment is generally performed using specific culture media via a pure cultivation of the isolated strain. However, it should be noted that most of the bacteria in environment are not yet cultured (viable but not culturable:VBNC-bacteria (*Gallionella*, *Toxothrix*, *Siderocapsa*). In the case of iron bacteria, though they had been known for more than 100 years by microscopic analyses, most of them were not isolated yet (Emerson *et al.*, 1997). Thus, culture-independent, molecular biological techniques would be the applicable procedures to study both cultivable and uncultivable microorganisms in environment.

1.7.2 Culture-independent molecular biological analyses

To study the microbial diversities, the culture-independent molecular biological tools are considered as convenient techniques to study environmental microorganisms, because cultivation method does not show

the real structure of microbial consortia. In contrast, culture-independent molecular method can directly analyze the structure of microbial consortia. The culture-independent methods are based on the molecular biological technique, such as polymerase chain reaction (PCR), DNA-DNA hybridization, and immunological assay. Among them, most suitable methods for the analysis of environmental microbial consortia are the PCR-based studies, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), clone-library analysis, real-time PCR, and next-generation sequencing.

1.7.2.1 DGGE

It is quick screening technique to reveal the microbial population composition in many natural and engineered systems. To analyze the genetic diversity of complex microbial populations, DGGE analysis was firstly performed by Gerard Muyzer (Muyzer *et al.*, 1993). PCR amplified double stranded DNA such as 16S ribosomal RNA genes (16S rDNAs) are separated by DGGE. According to length of various denatured DNA fragments, microbial diversities are studied by separation and isolation of DNA fragments. The separated amplified DNAs are excised to determine the nucleotide sequences. The nucleotide sequences are eventually compared to the sequences in online DNA databases to study the closely related bacterial species. This method is a useful process to determine the environmental bacterial consortia involved in natural groundwater treatment process in biological filtration system.

1.7.2.2 Clone-library analysis

Molecular cloning analysis is an essential molecular biological method to study environmental microbial diversities. DNA cloning techniques are developed by the laboratories of Boyer, Cohen, Berg, and their colleagues at Standford University and the University of California at San Francisco from 1972 to 1973. In this method, multiple DNA fragments of the target gene of the microbial consortia amplified by PCR are separated through cloning. The obtained clone library is applied for the determination of nucleotide sequences. Those determined nucleotide sequences are then compared to known DNA sequences in online DNA databases to search the closest relatives.

1.7.2.3 RFLP

It is a method of detecting the variability in the sequences of RNA or DNA within the genomes of the population of individuals (i.e. polymorphism) using the mutations at the sites of actions of restriction enzymes. Restriction enzymes are endonucleases that cut DNA at specific, very short base sequences, producing restriction fragments whose average size reflects the frequency with which the specific restriction sequence is found along the DNA. By separating the resulting restriction fragments through gel or capillary electrophoresis, on the basis of their lengths, the patterns of DNA bands can be compared in different individuals. This technique is limited by its ability to detect only mutation events occurring within enzyme restriction sites, which are sequences of only 4 to 6 nucleotides separated by hundreds or thousands of bases. A limited version of the RFLP method that used oligonucleotide probes was reported in 1985 (Saiki *et al.*, 1985). Despite this molecular biological technique is analyzed for microbial polymorphisms, it is not a very powerful method. This method has been modified to terminal restriction fragment length polymorphism (T-RFLP), at where the position of the restriction enzyme closest to a labeled end of an amplified gene is carried out for profiling of microorganisms.

1.7.2.4 FISH

To visualize the presence of the specific RNA or DNA in the environmental microbial consortia, fluorescent *in situ* hybridization analysis is carried out using specific fluorescent probe (used for hybridization with or "to be hybridized" RNA or DNA). Gall and Pardue (1969) started this study to detect the target RNA or DNA fragment in the microbial cell via hybridization. The hybridized probes are eventually observed under fluorescent microscope to study the specific microbial cell. This analysis is a useful method to study the specific microbial bacterium *in situ* condition without sequence analysis and cultivation.

1.7.2.5 Real-time PCR

The quantitative estimation of initial concentration of specific genes for microbial bacterial RNA or DNA is enabled using real-time PCR analysis. The short fragments of RNA or DNA (100 to 600 bp) are amplified with the presence of fluorescent dye or specific fluorescent-labeled probes. The PCR amplification is monitored by the fluorophores to estimate the initial amount of the target RNA or DNA fragments present in the various sample. This study reveals the relative quantity of microorganisms that present in

particular environmental samples. However, both FISH and real-time PCR are reliable for the RNA and DNA species of known sequences only.

1.8 Need of research

Interestingly, the biological filtration method for the removal of Fe and Mn was accidently discovered during Fe ion treatment in water treatment plant (Tanimoto, 1952; Kojima, 1972; Mouchet, 1992). Thereafter, for potable drinking water production, a modern, simple and useful biological filtration system has been developed for the treatment of Fe and Mn ions containing natural groundwater (Mouchet, 1992; Tamura *et al.*, 1999). However, the removal mechanism for Fe and Mn ions in the plant is still unknown in details. In biological filtration plants, the presence of several iron bacteria were shown by microscopic observations of their distinctive extracellular structures (Kojima, 1972; Czekalla *et al.*, 1985; Mouchet, 1992; Tamura *et al.*, 1999; Pacini *et al.*, 2005), but further studies on the diversity of these microbial consortia were poorly conducted. The compositions of microbial consortia in the biological filtration plant would be different to each other, possibly affected by the characteristics of the groundwater. However, there is no information about the commonality of the consortia, or the essential bacteria for the biological treatment of Fe and Mn containing groundwater.

For better understanding of the key mechanism and to develop the advanced strategies on biological treatment of the soluble Fe, Mn, As and NH_4^+ ions, it needs to be analyzed the structures of microbial communities and their complex relationship conditions of their habitats, and contribution on byproduct formation. Therefore, culture-independent molecular biological analysis to reveal the structure of microbial consortia in the biological filtration reactor would be needed.

1.9 Research objectives

This study particularly focuses on the structures of bacterial consortia involved in the biological filtration system for the treatment of Fe^{2+}, Mn^{2+}, As and NH_4^+ from natural groundwater to produce drinking water. In order to overview the structures of bacterial consortia in the biological filtration reactors, PCR-based culture-independent molecular biological analyses, such as DGGE, RFLP, clone-library analyses, and real-time PCR, were carried out, using 16S rDNAs and the other genes as the target. The filter media of two types of biological filtration reactors, Joyo biological filtration plant (Tamura

et al., 1999) and Muko biological filtration pilot plant (Fujikawa *et al.*, 2010) were selected to study the structures of bacterial consortia for the removal of Fe, Mn and As containing groundwater. These plants are situated at Joyo and Muko city, respectively in Kyoto, Japan. The spontaneously coated anthracites filter media that we call as BFM (Sahabi *et al.*, 2009a) of Joyo plant and bacterial flocs samples from Muko pilot plant (Fujikawa *et al.*, 2010) were collected for analyses.

<u>Chapter 2:</u> Study the bacterial diversity in the consortia on BFM of Joyo biological filtration plant was analyzed, using PCR-DGGE, clone library analysis and real-time PCR. The isolation of the known FeOB and MnOB are indicated, and their functions in the reactor are discussed.

<u>Chapter 3:</u> Study the bacterial diversity in the consortia in microbial flocs sample collected from the biological filtration pilot-plant in Muko City, Kyoto Prefecture, using PCR-DGGE, clone library analysis, real-time PCR. The dominant FeOB and MnOB in the flocs sample indicated, and their role on the removal of As from groundwater were indicated. The compared structures of bacterial consortia that involved in the well-established biological filtration plant and pilot-plant are discussed, which they are prominent for the removal of Fe, Mn and As to produce drinking water.

<u>Chapter 4</u>: Analyze different physico-chemical parameters for the removal of Mn^{2+} using the BFM of Joyo biological filtration plant. The optimal conditions for the biological removal of Mn^{2+} in batch experiment are performed, and the determined conditions for the biological removal of Mn^{2+} are discussed.

<u>Chapter 5:</u> Enrich the Mn-oxidizing microorganisms in batch experiment to produce the microbial flocs, using BFM of Joyo biological filtration plant. The carrier effect and the structure of MnOB are discussed.

<u>Chapter 6:</u> Study the simultaneous removal analysis for Mn and As by inoculating the enriched microbial flocs. The structures of bacterial composition are studied using PCR-DGGE, clone library analysis, real-time PCR. The removal of Mn and As ions in batch scale are discussed.

CHAPTER 2

Bacterial diversities in the biological filtration systems: I. Drinking water treatme plant for the removal of iron and manganese

Abstract

Bacterial diversity of the microbial consortia in a biological filtration plant for the elimination of iron (Fe) and manganese (Mn) from groundwater in Joyo City, Kyoto, Japan, was studied. PCR-based denaturing gradient gel electrophoresis (DGGE) analysis of bacterial 16S ribosomal RNA (rRNA) genes represented at least 15 signals, and nucleotide sequences of the dominant fragments showed similarities to *Gallionella* and *Nitrospira*. Phylogenetic analysis using the obtained nucleotide sequences of the 16S rRNA gene clone library showed the presence of the bacteria related to *Hyphomicrobium*, *Gallionella*, and *Sideroxydans*, which are supposed to be involved in Fe and Mn removal. In contrast, no 16S rRNA gene clone affiliated with the genus *Leptothrix*, which have been regarded as a major Fe- and Mn-oxidizer in biological filtration system, was observed. Though a large number of 16S rRNA gene clones closely related to *Nitrospira* was obtained, no clone showed high sequence similarity to known ammonium oxidizing bacteria (AOB). However, PCR-amplification of the ammonia monooxigenase gene (*amoA*) of AOB and ammonia oxidizing archaea (AOA) indicated the presence of both AOB and AOA in this biological filtration plant.

Keywords: biological filtration, *Gallionella*, *Hyphomicrobium*

2.1 Introduction

Groundwater, which is an important source of drinking water, often contains varying levels of iron (Fe) and manganese (Mn) as soluble divalent ions. Since contamination of these minerals in drinking water causes problems of bad tastes, color and deposition of metal oxides in the water distribution systems, Fe and Mn removal processes are required for such groundwaters. In Japan and the United States, the maximum recommended levels of Fe and Mn in drinking water are 0.3 and 0.05 mg/L, respectively. Conventional methods for the removal of Fe and Mn from groundwater are based on the physicochemical treatment processes such as aeration, chemical oxidation, coagulation and rapid sand filtration. An alternative method is based on a

biological process of Fe and Mn oxidation and deposition, which is mediated by microbial consortia in the biological filtration reactor (Mouchet, 1992). For many years, improved Fe and Mn removal was observed in several drinking water treatment plants when so-called "iron-bacteria" were grown in the traditional slow or rapid sand filters (Kojima, 1972; Czekalla *et al.*, 1985; Mouchet, 1992). Thereafter, modern biological water treatment systems had been developed in 1980s, using single filtration system with porous microbial carrier, such as anthracite or zeolite, for simultaneous removal of Fe and Mn (Mouchet, 1992; Tamura *et al.*, 1999), or two-stage filtration system to adjust the different optimum treatment conditions of the biological removal of Fe and Mn respectively (Mouchet, 1992). Advantages of these modern biological treatment systems over the conventional physicochemical methods include less use of chemicals, reduction of excess sludge, and lower operation and maintenance costs (Mouchet, 1992). However, though this method is called "biological filtration", the composition and function of the microorganisms involved in Fe and Mn removal are poorly understood.

The "iron bacteria", which have been considered to play important roles for the removal of Fe and Mn in the biological filtration process, are naturally found in Fe- and Mn-containing groundwater or freshwater environments, and Fe- or Mn-rich deposits in the water distribution pipelines. These bacteria have been well known not only for their particular habitats but also for their unique extracellular structures, such as twisted stalks (*Gallionella*), and filamentous tubes called "sheath" (*Leptothrix*, *Sphaerotilus*, *Clonothrix*, 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, their physiology and mechanisms for the deposition of Fe and Mn are still uncertain, except for chemolithotrophic, Fe-oxidizing bacteria (FeOB) *Gallionella,* and heterotrophic, Fe- and Mn-oxidizing bacteria (MnOB) *Leptothrix* (Ghiorse, 1984; Tebo, *et al.*, 2005; Emerson *et al.*, 2010; Hedrich *et al.*, 2011). In biological filtration plants, the presence of several iron bacteria were shown by microscopic observations of their distinctive extracellular structures (Kojima, 1972; Czekalla *et al.*, 1985; Mouchet, 1992; Tamura *et al.*, 1999; Pacini *et al.*, 2005), but further studies on the diversity of these microbial consortia were poorly conducted.

To analyze complex microbial systems, especially the unculturable microbial consortia in environmental samples, culture-independent molecular

biological methods such as, polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE), PCR-terminal restriction fragment length polymorphism (T-RFLP), cloning library analysis, and fluorescent in situ hybridization (FISH), are applicable. Though most of the iron bacteria are still difficult to culture, recent studies revealed the nucleotide sequences of 16S ribosomal RNA (rRNA) genes from several cultivable strains of *Sphaerotilus* and *Leptothrix*, and from enrichment cultures of *Gallionella ferruginea* (Hallbeck *et al.*, 1993), *Crenothrix polyspora* (Stoecker *et al.*, 2006), and *Clonothrix fusca* (Vigliotta *et al.*, 2007). These nucleotide sequence data enable us to confirm the presence of iron bacteria in microbial consortia of the biological filtration plant, which had been reported by microscopic observations. de Vet *et al.* (2009) analyzed the microbial diversities of the trickling sand filtration plant for the treatment of ammonia-containing groundwater in the Netherlands using PCR-DGGE, and determined nucleotide sequences of a number of dominant PCR-amplified fragments. Their results showed presence of the bacteria phylogenetically related to *Gallionella* and *Leptothrix*, since the raw groundwater of the plant contained Fe and Mn as well as ammonia. On the other hand, Burger *et al.* (2008a) analyzed the presence of *Leptothrix* in four biological filtration plants in Canada using real-time PCR, and indicated that *Leptothrix* was contained in only one plant. Their results suggested that *Leptothrix*, which had been regarded as major MnOB in the biological filtration system, is not necessarily the predominant organism. However, no other candidate bacteria had been suggested for oxidation of Mn in the biological filtration system yet.

The aim of this study is to reveal the bacterial diversity in the biological filtration plant for the removal of Fe and Mn from groundwater, using PCR-based DGGE and clone library analyses of bacterial 16S rRNA genes. Joyo biological filtration plant is the first mid-speed biological filtration plant for the removal of Fe and Mn from groundwater in Japan (Tamura *et al.*, 1999). The biological filtration system consists of aeration by dropping, gravity filtration with dual-media filter (anthracite and sand), and final disinfection. The filtration rate is 70 m/day and it supplies 11,100 m^3/day of drinking water. The average concentrations of Fe^{2+} and Mn^{2+} in the influent raw groundwater are 0.41 mg/L and 0.12 mg/L, respectively, while those in the treated water are <0.03 and <0.05, respectively. Dissolved oxygen was controlled at 5-7 mg/L by movable weir prior to the filtration. Backwashing

of the filter is performed every two weeks (14 days) to ensure optimal performance. The plant was established in 1993, and the filter media was not replaced until now. In our previous study, we showed the accumulation of Fe and Mn oxides on the surface of the biofilm-coated, "aged" anthracites which we call "biological filter media (BFM)" (Sahabi *et al.*, 2009a). It is supposed that these Fe and Mn oxides were produced by FeOB and MnOB existing in the microbial consortia of the biofilms. In this study, the molecular diversity of bacterial 16S rRNA genes in the microbial consortia from Joyo BFM sample was analyzed. Nucleotide sequence analysis of the obtained 16S rRNA gene clones indicated the presence or absence of the bacteria phylogenetically related to *Gallionella*, *Leptothrix*, and the other known FeOB and MnOB in Joyo biological filtration plant. These observations of bacterial compositions would be informative for scientists and engineers who are studying, developing and operating the biological treatment plants for the removal of Fe and Mn from groundwater.

2.2 Materials and methods

2.2.1 Sampling site

The BFM and raw groundwater samples were collected from Joyo biological filtration plant, which is located at the third drinking water treatment plant of Joyo City (N34° 51' 47", E135° 45' 50"), the southern part of Kyoto Prefecture, Japan (Tamura *et al.*, 1999; Sahabi *et al.*, 2009a). The BFM sample, approximately 5 mm in diameter of anthracites coated with Fe and Mn oxide-rich biofilms, was collected on September 19, 2005, and the collected sample was kept at –80°C. Raw groundwater sample was collected in 2 L polypropylene bottle and stored at 4°C until analysis. Characteristics of raw groundwater were provided by Joyo City Water Supply and Sewerage Department, except ammonium-nitrogen concentration which was determined by indophenol blue method (Solorzano, 1969).

2.2.2 DNA extraction

Total DNA was extracted from 0.5 g (wet weight) of Joyo BFM sample using FastDNA Spin Kit for Soil (MP Biochemicals, Solon, OH, USA). To prevent adhesion of DNA onto the metal oxides coated on the surface of the anthracites, 50 mg skim milk powder (biochemistry grade, Wako Pure Chemical, Osaka, Japan) was added prior to the cell lysis (Ikeda *et al.*, 2004). The sample was then subjected to DNA extraction according to the manufacturer's protocol, except with additional freeze-thaw cycles of –80°C

for 10 min and 80°C for 10 min, which were repeated for three times before the bead beating step. The extracted total DNA solution sample was subdivided into aliquots and stored at –20°C until use.

2.2.3 Polymerase chain reaction

Standard PCR procedure was carried out using *TaKaRa Ex Taq* polymerase (Takara Bio, Shiga, Japan). For each amplification, 50 µL of PCR mixture according to the manufacturer's protocol with 1 µL of ten times diluted total DNA solution as a template was prepared, and after 5 min of initial denaturation at 94°C, PCR was carried out with 0.5 min of denaturation at 94°C, 0.5 min of primer annealing at 55°C, and 0.5 min of primer extension at 72°C for 25 cycles, followed by 7 min of final primer extension. The resulted PCR product was applied for agarose gel electrophoresis, and then visualized using ethidium bromide staining and UV transilluminator. For DGGE analysis, 50 µL of PCR mixture was prepared as described above, and PCR was carried out with "touchdown" program. After 10 min of initial denaturation at 95°C, a "touchdown" PCR cycle protocol was used (Don *et al.*, 1991), and the annealing temperature was decreased by 1°C per cycle from 65°C to 56°C, then 20 additional cycles at 55°C were performed. Amplification was carried out with 0.5 min of denaturation at 95°C, 0.5 min of primer annealing, and 1 min of primer extension at 72°C, followed by 5 min of final primer extension.

2.2.4 Denaturing gradient gel electrophoresis

To analyze the molecular diversity of bacterial consortia in the sample, about 190 base pairs (bp) of DNA fragments containing the variable region 3 of bacterial 16S rRNA genes were amplified using universal bacterial primers GC341F (with 40 bp of GC-clamp) and 534R (Muyzer *et al.*, 1993) with touchdown program. Perpendicular DGGE analysis (Muyzer *et al.*, 1993) was carried out using mini-DGGE system NB-1490 (Nihon Eido, Tokyo, Japan). The DGGE gel plate was 10 mm × 10 mm in size and 1 mm thick. For the analysis, 200 µL of PCR products were applied onto 8% (wt/vol) polyacrylamide gels formed with acrylamide-N,N'-methylenebisacrylamide, 37.5:1 (Bio-Rad, Hercules, CA, USA) in 1×TBE buffer with the denaturing gradients ranging from 30 to 60% denaturant [100% denaturant contained 7 M urea and 25% (vol/vol) formamide]. Electrophoresis was carried out in 1×TBE buffer at 50 V, 60°C for 3 h, and then visualized by SYBR Green I staining (Invitrogen, Carlsbad, CA, USA) using fluorescent image analyzer

FLA-2000 (Fujifilm, Tokyo, Japan). For the determination of nucleotide sequence of the separated DNA fragment in the gel, the fragment was excised from the gel and eluted against 200 µL distilled-deionized water at 4°C overnight. The supernatant was applied for re-amplification of the eluted fragment using primers 341F (without GC-clamp) and 534R. The resulted PCR product was purified by Montage PCR filter unit (Millipore, Bedford, MA, USA), and then applied for the determination of nucleotide sequence using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 373 or 3100-Avant Genetic Analyzer (Applied Biosystems). The obtained nucleotide sequences were then compared to the sequences in online DNA databases using the Basic Local Alignment Search Tool (BLAST; http://blast.ddbj.nig.ac.jp/) (Altschul *et al.*, 1990).

2.2.5 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 8F and 1510R (Eden *et al.*, 1991). The resulted PCR product was introduced into pCR2.1 vector using TOPO-TA cloning kit (Invitrogen) according to the manufacturer's protocol. Plasmid DNA of each 16S rRNA gene clone was extracted using alkaline lysis miniprep method (Sambrook *et al.*, 1989) and then subjected to the restriction fragment length polymorphism (RFLP) analysis. At first, each inserted fragment in the plasmid was re-amplified using the primers 8F and 1510R, and then purified by Montage PCR filter unit. Each amplified DNA fragment was digested using *Hae*III and then separated by the agarose gel electrophoresis. The gel images were digitized by FLA-2000, and the resulted restriction fragment length patterns of the clones were analyzed. The additional restriction fragment length patterns using *Msp*I were obtained, and the clones were grouped by comparing their restriction fragment length patterns. Thereafter, the individual clone groups (operational taxonomic units; OTUs) were applied for sequence analysis as well as described above, using the universal primers 8F, 341F, 534R and 785R (Amann *et al.*, 1995). Then each obtained nucleotide sequence was examined for the presence of chimeric sequence using Bellerophon (Huber *et al.*, 2004). Each nucleotide sequence was then compared to known sequences in online DNA databases using BLAST. Phylogenetic trees were generated by CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/) (Thompson *et al.*, 1994) using the

neighbor-joining algorithm (Saitou and Nei, 1987).

2.2.6 Nucleotide sequence accession numbers

The nucleotide sequence data obtained in this study have been deposited in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under accession numbers AB591384 to AB591415.

2.2.7 Real-time PCR

Real-time PCR was carried out to analyze the relative amount of 16S rRNA genes of the bacteria related to *Leptothrix* to those of the total bacteria in the sample. Bacterial universal primers 341F and 534R were used to amplify 16S rRNA genes of the total bacteria in the sample. Primers PSP-6F (complementary sequence of the probe PSP-6, which was designed for the detection of *Leptothrix*; Siering and Ghiorse, 1997) and 341R (complementary sequence of the primer 341F) were used to amplify 16S rRNA genes of the bacteria related to *Leptothrix* in the sample. For the construction of a standard curve, 16S rRNA gene fragment in the plasmid clone (e.g., Jy04A06) was amplified by PCR using the primers 8F and 1510R, and then purified using Montage PCR filter unit. Copy number of 16S rRNA gene fragments in the solution was estimated by measurement of absorbance at 260 nm (Sambrook *et al.*, 1989). To generate the standard curve, real-time PCR was performed using SYBR *Premix Ex Taq* and Thermal Cycler Dice Real Time System TP800 (Takara Bio). For each reaction, 10 μL of PCR mixtures were prepared according to the manufacturer's protocol except the use of the ten-fold serial dilutions of the standard 16S rRNA gene fragment solution as templates. After 1 min of initial denaturation at 95°C, real-time PCR was carried out with 5 sec of denaturation at 95°C, 10 sec of primer annealing at 56°C, and 17 sec of primer extension at 72°C for 35 cycles, followed by dissociation cycle at 95°C for 15 sec and 65°C for 30sec. The threshold cycle value of each dilution was automatically identified and measured at the maximum acceleration of fluorescence using second derivative maximum method (Tichopad *et al.*, 2002) on the Thermal Cycler Dice Real Time System Software ver. 2.00B (Takara Bio), and then applied for the construction of standard curve. The copy numbers of the 16S rRNA genes in the total DNA solution were estimated by real-time PCR analyses as described above using the primer set 341F and 534R for the total bacteria, and the primer set PSP-6F and 341R for the bacteria related to *Leptothrix*.

2.2.8 PCR detection of ammonia monooxigenase genes

To examine the presence of the gene encoding ammonia monooxigenase subunit A (*amoA*) of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in the total DNA sample, primers amoA-1F and amoA-2R for AOB-*amoA* (Rotthauwe *et al.*, 1997), and primers Arch-amoAF and Arch-amoAR for AOA-*amoA* (Beman and Francis, 2006) were used for PCR amplification, respectively. PCR was performed with the standard PCR condition as described above.

2.3 Results and discussion

2.3.1 Analysis of bacterial diversity in Joyo biological filtration plant by PCR-DGGE

In Joyo biological filtration plant, raw groundwater contained 0.41 mg/L Fe^{2+}, 0.12 mg/L Mn^{2+}, 0.11 mg/L ammonia-nitrogen, 27.2 mg/L hardness as $CaCO_3$, 9.2 mg/L Na^+, 4.2 mg/L Cl^-, and <0.5 mg/L total organic carbon. The other metal ions, such as lead, zinc, and arsenic, were not detected. Temperature of

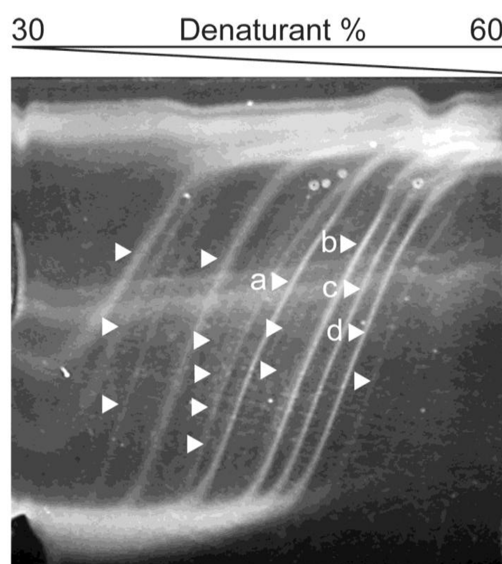

Fig. 2.1 - DGGE analysis of 16S rRNA gene fragments amplified from the total DNA extracted from Joyo BFM sample. Visible signals of the 16S rRNA gene fragments are indicated by arrows. The signals labeled a, b, c, and d, indicate the DNA fragments of which the sequences were determined.

the groundwater was 19.0°C, and pH was 6.78. This 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. To show the overview of bacterial diversity involved in the biological treatment of Fe- and Mn-containing groundwater in Joyo plant, the total DNA of the microbial consortia was extracted from the BFM sample of Joyo plant, and then applied for PCR-based DGGE analysis. Approximately 190 bp of PCR-amplified fragments containing the variable region 3 of bacterial 16S rRNA genes were separated by perpendicular DGGE, and at least fifteen signals of the amplified 16S rRNA gene fragments were observed (Fig. 2.1). These fragments were excised from the gel, re-amplified, and then applied for the determination of nucleotide sequences. Among them, nucleotide sequences of four dominant fragments (Fig. 2.1, signals a-d) were successfully determined. Nucleotide sequence of the fragment 'a' showed 90% similarity to *Gallionella capsiferriformans* ES-2 (Weiss *et al.*, 2007), while those of the fragments 'b', 'c', and 'd' showed 96-100% similarities to the nitrite-oxidizing bacteria (NOB) *Nitrospira*. Unfortunately, these dominant fragments did not show any similarity to the 16S rRNA gene sequence of known MnOB, such as *Leptothrix*. The remaining, recessive fragments showed ambiguous nucleotide sequences, because of the contamination of multiple fragments, which were probably due to the insufficient separation in the gel. Therefore, further investigation is required to obtain a more diverse 16S rRNA gene sequence information of the bacterial consortia in Joyo plant.

2.3.2 Analysis of 16S rRNA gene clone library constructed from bacterial consortia in Joyo plant

To study the bacterial diversity involved in the Fe and Mn removal processes of Joyo biological filtration plant in detail, 16S rRNA gene clone library was constructed. Approximately 1.5 kbp of PCR-amplified 16S rRNA gene fragments were applied for the construction of the clone library through the TOPO TA-cloning. Fifty seven clones were isolated, and then separated into 32 operational taxonomic units (OTUs) by RFLP analysis. Nucleotide sequence of the first half of each cloned 16S rRNA gene fragment (corresponds to *Escherichia coli* 16S rRNA positions 28 to 784) was determined, and then submitted to BLAST analysis to identify the closest known relative (Table 2.1). The dominant groups of the clones were affiliated with *Betaproteobacteria* (15 OTUs, 39% of total clones), and *Nitrospira* (5 OTUs, 30% of total clones). The other clones were affiliated to *Alphaproteobacteria* (5 OTUs, 14% of total clones), *Gammaproteobacteria* (4

OTUs, 9% of total clones), *Acidobacteria* (2 OTUs, 7% of total clones), and *Deltaproteobacteria* (1 OTU, 1% of total clones). It should be noted that four individual OTUs containing the identical sequences with the DGGE fragments 'a-d' were found. The OTU named Jy04A21 contained the nucleotide sequence of the DGGE fragment 'a', while OTUs named Jy04A34, Jy04A40, and Jy04A24 contained each nucleotide sequence of the DGGE fragments 'b', 'c', and 'd', respectively. The remaining OTUs were probably derived from the bacteria which were represented as weak or invisible signals of the 16S rRNA gene fragments in the DGGE gel.

BLAST searches indicated that a number of OTUs showed higher sequence similarities (>90%) to known FeOB and MnOB, such as *Gallionella* (Jy04A26, and Jy04A41), *Sideroxydans* (Jy04A14, Jy04A20, Jy04A21, Jy04A27, and Jy04A29), *Ferritrophicum radicicola* (Jy04A05), and *Hyphomicrobium* (Jy04A47) (Table 2.1). The OTU Jy04A31 showed 94.4% similarity to one of the sheathed iron bacteria *C. polyspora*, however, the Fe or Mn deposition activity of *C. polyspora* is still unknown (Emerson *et al.*, 2010). Surprisingly, no OTU closely related to *Leptothrix* was obtained, except three OTUs (Jy04A04, Jy04A06 and Jy04A46) which showed higher sequence similarities (>95%) to a *Sphaerotilus-Leptothrix* group of bacterium, *Methylibium petroleiphilum* PM1 (Nakatsu *et al.*, 2006). *M. petroleiphilum* PM1 is a methylotrophic, methyl *tert*-butyl ether-degrading bacterium, but its Mn^{2+} oxidation activity has not been reported.

Phylogenetic relationship among all OTUs and 16S rRNA gene sequences of the known relatives is shown in Fig. 2.2. This possible phylogenic tree indicates that the OTUs closely related to the family *Gallionellaceae*, and to the genus *Hyphomicrobium* are present in the bacterial consortia of Joyo BFM. *Sideroxydans* and *Ferritrophicum* 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 *et al.*, 1997; Weiss *et al.*, 2007; Emerson *et al.*, 2010). *Hyphomicrobium* spp. are methylotrophic, prosthecate bacteria which have buds on filamentous stalks, and some strains of *Hyphomicrobium* are known for the deposition of Fe and Mn oxides (Tyler, 1970; Moore, 1981). The presence of *Hyphomicrobium* in rapid sand filters for treatment of Fe- and Mn-containing groundwater in North Germany was described by Czekalla *et al.* (1985) using scanning electron microscopy. Thus, these OTUs were supposed to be involved in Fe and Mn

removal from groundwater in Joyo biological filtration plant, however, further studies would be required to confirm their Fe or Mn oxidation activities.

Table 2.1 - Similarities between 16S rRNA gene sequences of the clone library obtained from Joyo BFM sample and the most similar database sequences.

OTU name	Accession no.	No. of isolates[a]	Closest relative[b][Accessionno.]	Similarity (%)
Alphaproteobacteria				
Jy04A54	AB591414	1	*Bradyrhizobium yuanmingense* TTB5 [FJ540928]	99.7
Jy04A39	AB591406	3	*Brevundimonas vesicularis* Asd M7-3 [FM955876]	86.3
Jy04A47	AB591410	2	*Hyphomicrobium* sp. P-47 [AM411913]	97.6
Jy04A28	AB591397	1	*Sphingomonas* sp. IC081 [AB196250]	96.3
Jy04A16	AB591390	1	*Sphingomonas suberifaciens* IFO 15211 [D13737]	96.6
Betaproteobacteria				
Jy04A46	AB591409	1	*Methylibium petroleiphilum* PM1 [CP000555]	95.6
Jy04A10	AB591387	1	*Azoarcus evansii* KB740 [X77679]	92.8
Jy04A05	AB591385	1	*Ferritrophicum radicicola* CCJ [DQ386263]	90.4
Jy04A41	AB591408	1	*Gallionella capsiferriformans* ES-2 [CP002159]	95.6
Jy04A26	AB591395	2	*Gallionella ferruginea* [L07897]	93.1
Jy04A60	AB591415	1	*Gallionella ferruginea* [L07897]	87.7
Jy04A04	AB591384	1	*Methylibium petroleiphilum* PM1 [CP000555]	95.5
Jy04A06	AB591386	1	*Methylibium petroleiphilum* PM1 [CP000555]	97.2
Jy04A18	AB591391	1	*Methyloversatilis* sp. cd-1 [GU350457]	92.3
Jy04A20	AB591392	1	*Sideroxydans lithotrophicus* ES-1 [CP001965]	94.4
Jy04A21	AB591393	3	*Sideroxydans lithotrophicus* ES-1 [CP001965]	93.9
Jy04A27	AB591396	3	*Sideroxydans lithotrophicus* ES-1 [CP001965]	95.1
Jy04A14	AB591389	1	*Sideroxydans lithotrophicus* LD-1 [DQ386859]	93.6
Jy04A29	AB591398	3	*Sideroxydans lithotrophicus* LD-1 [DQ386859]	94.6
Jy04A11	AB591388	1	*Stenotrophomonas maltophilia* A1Y15 [AY512626]	91.2
Gammaproteobacteria				
Jy04A31	AB591400	1	*Crenothrix polyspora* clone 13[c] [DQ295887]	94.4
Jy04A33	AB591402	1	*Methylosarcina lacus* LW14 [AY007296]	92.8
Jy04A36	AB591404	2	*Methylovulum miyakonense* NBRC 106162 [AB501287]	91.7
Jy04A38	AB591405	1	*Nitrosococcus watsoni* C-113 [CP002086]	85.9
Deltaproteobacteria				

Jy04A52	AB591413	1	*Myxococcus xanthus* UCDaV5 [AY724798]	82.5
Nitrospira				
Jy04A40	AB591407	1	*Nitrospira moscoviensis* [X82558]	94.6
Jy04A34	AB591403	6	*Nitrospira* sp. [AF035813]	98.7
Jy04A24	AB591394	6	*Nitrospira* sp. GC86 [Y14644]	94.8
Jy04A32	AB591401	3	*Nitrospira* sp. GC86 [Y14644]	99.2
Jy04A51	AB591412	1	*Nitrospira* sp. GC86 [Y14644]	86.5
Acidobacteria				
Jy04A30	AB591399	3	Bacterium Ellin 6075[c] [AY234727]	94.3
Jy04A48	AB591411	1	Bacterium Ellin 6099[c] [AY234751]	84.7

a) Total 57 clones were isolated.
b) Based on BLAST analysis using the greengene database.
c) Based on BLAST analysis using the DDBJ database.

The OTUs Jy04A04, Jy04A06, and Jy04A46 were phylogenetically close to a cluster of the genus *Methylibium* but slightly distinct to a cluster including *Leptothrix cholodnii* SP-6*, Leptothrix mobilis* Feox-1 (Spring *et al*., 1996), *Leptothrix ochracea* (Fleming *et al*., 2011), *Leptothrix discophora* SS-1 and *Sphaerotilus natans* DSM 6575 (Siering and Ghiorse, 1996). Among these three OTUs, Jy04A06 and Jy04A46 contained the target sequence of a *Leptothrix*-specific probe PSP-6 (5'-CAGTA GTGGG GGATA GCC-3'; Siering and Ghiorse, 1997), which was used by Burger *et al.* (2008a) as a primer for the analysis of *Leptothrix*-related bacteria in the biological filtration plants using real-time PCR, while Jy04A04 contained the same target sequence except one base substitution at 5'-end C to T. Copy numbers of the 16S rRNA genes of the total bacteria and *Leptothrix*-related bacteria in the sample were estimated by real-time PCR. The amount of 16S rRNA genes of the total bacteria in the DNA solution extracted from Joyo BFM was 8.20×10^{8} copies per one gram BFM, whereas, that of the *Leptothrix*-related bacteria harboring PSP-6 sequence was 1.01×10^{8} copies per one gram BFM (12.5% of total 16S rRNA gene copies). This result raised a question of whether the other *Leptothrix*-related bacteria were present in Joyo bacterial consortia, therefore, the same DNA solution sample was applied for PCR amplification using primers PSP-6F and 1510R to construct another 16S rRNA gene clone library. BLAST search indicated that all of the obtained clones were affiliated with the *Sphaerotilus*-*Leptothrix* group of bacteria, however, these clones hardly showed high sequence similarity to known *Leptothrix* strains

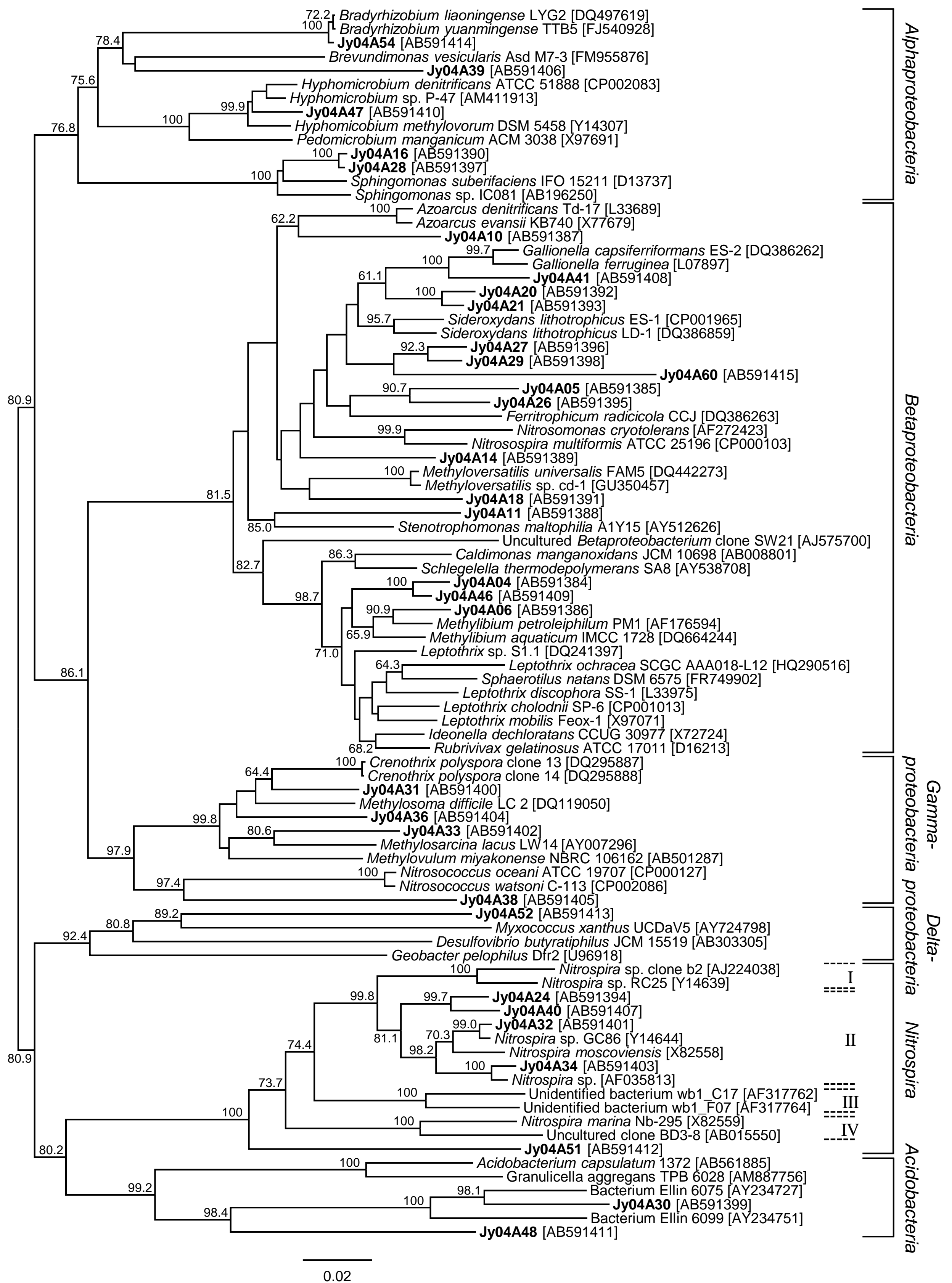

Fig. 2.2 - Phylogenetic relationships of bacterial 16S rRNA gene sequences obtained from the clone library of Joyo biological filtration plant and

relative bacteria. Bootstrap values of >60% (obtained with 1,000 resamplings) are shown above or near the relevant nodes. Clones from this study are indicated in boldface, and GenBank accession numbers are in parentheses.

(unpublished data). These results suggest that *Leptothrix*, which had been regarded to play a principal part in biological treatment for the removal of Fe and Mn from groundwater, was not the dominant MnOB in Joyo biological filtration plant. Similar observations were reported by Burger *et al.* (2008a) for three biological filtration plants in Canada which did not show PCR amplification of 16S rRNA gene fragment of *Leptothrix*-related bacteria. Whereas, de Vet *et al.* (2009) analyzed the microbial diversities of the trickling sand filters in the Netherlands using PCR-DGGE and obtained three DGGE fragments (LKF_DGGE_CTO27, CTO28, and CTO31) which that of the dominant bacterial strains, which made it difficult to detect by PCR-based DGGE and clone library analyses using universal 16S rRNA gene primers. This study also indicated that the primer PSP-6 was not suitable for PCR-detection of 16S rRNA gene of the genus *Leptothrix*. Therefore, development of newly designed primer for specific detection of the 16S rRNA gene of *Leptothrix* would be required to analyze the population of *Leptothrix* in Joyo bacterial consortia using real-time PCR. *Leptothrix* had been thought as a principal MnOB in the biological filtration system, and used as a model MnOB for laboratory-scale studies for biological removal of Fe and Mn from water (Hope and Bott, 2004; Katsoyiannis and Zouboulis, 2004; Burger *et al.*, 2008b). However, contribution of the other MnOB, such as *Hyphomicrobium*, for the Mn oxidation would be considered.

2.3.3 PCR detection of ammonia monooxygenase genes

It has been reported that complete nitrification is required to commence the biological removal of Mn (Mouchet 1992; Vandenabeele *et al.*, 1995; Štembal *et al.*, 2005), thus, contribution of the nitrifying microorganisms to the biological removal of Mn had been supposed. Though the ammonia concentration of Joyo groundwater was low (0.11 mg/L ammonia-nitrogen), PCR-DGGE and the clone library analyses indicated that a quite large number of bacteria affiliated with the genus *Nitrospira* were present in the microbial consortia of Joyo plant. In contrast, no clone closely related to known AOB was detected. To confirm the presence of ammonia-oxidizing

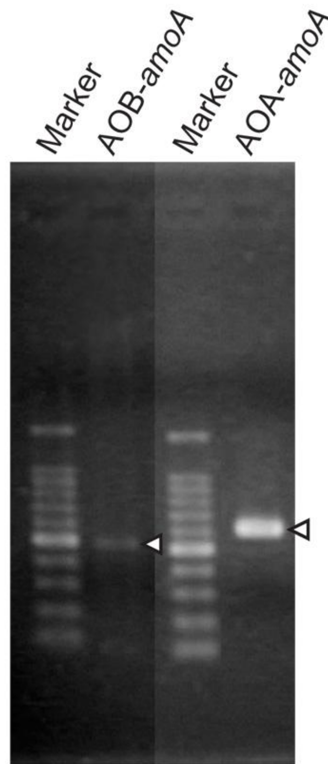

Fig. 2.3 - PCR amplification of archaeal and bacterial *amoA* genes, present in Joyo BFM sample. Each of the amplified *amoA* gene fragments are indicated by arrows. Marker: 100 bp ladder marker (TaKaRa Bio).

microorganisms in the microbial consortia of Joyo plant, genes encoding *amoA* of AOB and AOA were amplified by PCR. As shown in Fig. 2.3, PCR-amplified fragment of both AOB-*amoA* and AOA-*amoA* were detected. It was demonstrated that AOA grow in a habitat with low ammonia concentration (Martens-Habbena *et al.*, 2009). In addition, phylogenetic relationship (Fig. 2.2) indicated that all of the OTUs affiliated with *Nitrospira* except Jy04A51 were classified as *Nitrospira* sublineage II (Maixner *et al.*, 2006), which prefer the environment with lower concentration of nitrite. Accordingly, it is supposed that these AOA and *Nitrospira* would contribute to consume low levels of ammonia contained in Joyo groundwater. AOB was also present in the consortia, therefore further investigation would be needed for the contribution of AOA and AOB on ammonia removal of Joyo.

2.4 Conclusion

Molecular diversity of bacterial 16S rRNA genes in biological filtration plant for the removal of Fe and Mn from groundwater in Joyo City, Kyoto, Japan

was studied using PCR-based DGGE and clone library analyses. The results indicated the presence of the bacteria affiliated with *Gallionella, Sideroxydans*, *Ferritrophicum,* and *Hyphomicrobium*, which are supposed to be involved in Fe and Mn removal from groundwater in Joyo plant. In contrast, no clone closely related to *Leptothrix* was obtained, suggesting that *Leptothrix* is not a dominant MnOB in this plant. Though a large number of 16S rRNA gene clones affiliated with the genus *Nitrospira* were isolated, no clone closely related to known AOB was obtained. However, PCR-detection of bacterial and archaeal *amoA* gene showed that both AOB and AOA were present in Joyo plant. Although this study showed the microbial diversity of only one biological filtration plant, our finding would be informative for studying the biological Fe and Mn removal. However, further investigations of the structures of microbial consortia in other biological filtration plants should be required not only for understanding the mechanism of the biological elimination of Fe and Mn, but for optimizing the operation of the plant and developing advanced biological filtration system.

2.5 Acknowledgement

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CHAPTER 3

Bacterial diversities in the biological filtration systems: II. A pilot plant for the simultaneous removal of arsenic, iron and manganese

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 for 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: biological filtration, arsenic, *Leptothrix*

3.1 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 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 (Heinrich, 2006; Ike *et al.*, 2008; Rahman and Hasegawa, 2011), or sorption of soluble As oxyanions onto biogenic materials (Katsoyiannis and Zouboulis, 2004 and 2006; Sari and Tuzen, 2009; Sahabi *et al.*, 2009b), which are less expensive than the conventional methods. Biological sorptive filtration for simultaneous removal for 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, 2007; Hennebel *et al.*, 2009; Sahabi *et al.*, 2009b). 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 bacteria 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 bacteria 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 are possibly contribute for the removal of Fe and Mn from groundwater. In this report, bacterial diversity of the biological filtration plant for the simultaneous removal of As, Fe and Mn was studied. 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, the community structure of bacterial consortia on the pilot plant was compared 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.

3.2 Materials and methods

3.2.1 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 (□100 mm × 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 μm 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).

3.2.2 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 prepared as also described (Thapa Chhetri *et al.*, 2013).

3.2.3 Polymerase chain reaction (PCR)

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

3.2.4 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 µL 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×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). DGGE Marker 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).

3.2.5 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).

3.2.6 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.

3.2.7 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).

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

PCR detection of the genes encoding ammonia monooxygenase subunit A (*amoA*) of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in the total DNA sample were performed 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 (Quéméneur *et al.*, 2008) were used. PCR was carried out with the standard PCR condition as described above.

3.3 Results and discussion

3.3.1 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 were 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 µg/L, 2.11 mg/L and 0.48 mg/L, respectively, while those in the treated water are 5.5 µg/L, 0.01 mg/L and 0.13 mg/L, respectively. The concentration of As was successfully decreased under the WHO's guideline

Table 3.1 - 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

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. Fig. 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.

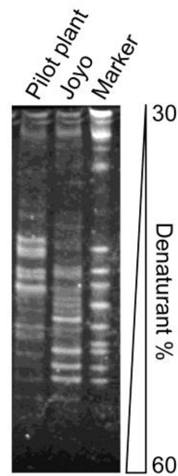


Fig. 3.1 - Comparison of the DNA fingerprints of PCR-amplified 16S rRNA genes from the pilot plant and Joyo biological filtration plant samples by parallel DGGE.

However, unfortunately, all of those DNA fragments showed ambiguous nucleotide sequences, because of the contamination of multiple fragments which were probably due to the insufficient separation in the gel.

3.3.2 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 *Escharichia 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).

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 ferricapsiformans* ES-2, which is a chemolithotrophic FeOB (Emerson and Moyer, 1997). The OTUs M07A007 and M07A046 showed >96% similarities to *Ideonella*. Fe and Mn oxidation 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 relationship 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 is 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. capsiferriformans* ES-2 and *Sideroxydans lithotrophicus* ES-1, and described that *Gallionella* ES-2 has more capacity to resist heavy metals than *Sideroxydans* 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 the all OTUs from Joyo and the pilot plants, these two OTUs and another four OTUs phylogenetically close to *Methylibium* (Jy04A04,

Table 3.2 - Similarities between 16S rRNA gene 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 (%)
Alphaproteobacteria				
M07A146	AB604837	1	*Beijerinckia fluminensis* LMG 2819 [EU401905]	94.3
M07A071	AB604819	1	*Bradyrhizobium* sp. II-47 [AB531406]	100.0
M07A072	AB604820	3	*Bradyrhizobium* sp. Wall28 [EF601950]	99.4
M07A022	AB604800	7	*Methylocystis* sp. B3 [DQ496232]	99.1
M07A144	AB604836	1	*Nordella oligomobilis* N21 [AF370880]	96.0
M07A009	AB604794	2	*Novosphingobium naphthalenivorans* TUT562 [AB177883]	96.1
M07A054	AB604814	7	*Novosphingobium naphthalenivorans* TUT562 [AB177883]	96.2
M07A017	AB604798	12	*Novosphingobium subterraneum* F-4 [FJ527720]	98.0
M07A121	AB604828	2	*Novosphingobium subterraneum* F-4 [FJ527720]	97.6
M07A034	AB604804	1	*Rhodobacter capsulatus* ATCC 11166 [DQ342320]	97.4
M07A128	AB604833	1	*Rhodobacter* sp. ZH15 [FJ872532]	94.4
Betaproteobacteria				
M07A125	AB604831	1	*Gallionella capsiferriformans* ES-2 [DQ386262]	95.2
M07A046	AB604808	1	*Ideonella* sp. 0-0013 [AB211233]	96.2
M07A007	AB604793	6	*Ideonella* sp. B513 [AB049107]	97.3
M07A053	AB604813	1	*Methylibium fulvum* Gsoil 328 [AB245357]	99.0
M07A023	AB604801	1	*Methylophilus* sp. ECd5 [AY436794]	95.4
M07A080	AB604821	1	*Methylophilus* sp. ECd5 [AY436794]	96.8
M07A047	AB604809	1	*Rhodoferax* sp. IMCC 1723 [DQ664242]	98.3
Gammaproteobacteria				
M07A030	AB604802	1	*Crenothrix polyspora* clone 6 [DQ295898][c]	94.8
M07A039	AB604806	2	*Crenothrix polyspora* clone 6 [DQ295898][c]	94.8
M07A102	AB604826	1	*Crenothrix polyspora* clone 6 [DQ295898][c]	93.4
M07A122	AB604829	1	*Legionella lytica* L2 [X97364]	95.3
M07A088	AB604823	1	*Legionella pneumophila* Paris [CR628336]	94.1
M07A052	AB604812	1	*Lysobacter brunescens* str. KCTC 12130 [AB161360]	99.5
M07A148	AB604838	1	*Lysobacter brunescens* str. KCTC 12130 [AB161360]	99.6
M07A069	AB604818	1	*Methylobacter tundripaludum* SV96 [AJ414655]	96.8
M07A090	AB604824	1	*Methylobacter tundripaludum* SV96 [AJ414655]	94.4
M07A015	AB604797	1	*Methylomonas* sp. LW16 [AF150796]	97.6
M07A096	AB604825	1	*Pseudomonas* sp. 24 [FJ867623][c]	89.2
Deltaproteobacteria				
M07A041	AB604807	1	*Anaeromyxobacter* sp. IN2 [FJ939131]	95.2
M07A060	AB604817	2	*Anaeromyxobacter* sp. K [CP001131]	92.6
M07A051	AB604811	2	*Bdellovibrio* sp. MPA [AY294215]	89.9
M07A049	AB604810	4	*Geobacter chapelleii* 172 [U41561]	93.3
M07A010	AB604795	2	*Geobacter argillaceus* G12 [DQ145534]	95.3
M07A082	AB604822	1	*Geobacter argillaceus* G12 [DQ145534]	94.9
M07A055	AB604815	1	*Geobacter psychrophilus* P11 [AY653551]	96.6
M07A031	AB604803	1	*Haliangium tepidum* SMP-10 [AB062751]	85.7
Firmicutes				
M07A124	AB604830	1	*Staphylococcus epidermidis* ATCC 12228 [AE015929]	99.9
Actinobacteria				
M07A059	AB604816	2	Actinobacterium YM22-133 [AB286031][c]	90.6
M07A126	AB604832	1	Actinobacterium 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	*Sporichthya polymorpha* DSM 46113 [X72377]	94.3
Acidobacteria				
M07A019	AB604799	1	*Acidobacteria* bacterium IGE-011 [GU187027][c]	89.8
M07A002	AB604792	3	*Geothrix fermentans* H5 [U41563]	94.7
M07A035	AB604805	1	*Geothrix fermentans* H5 [U41563]	96.5
M07A103	AB604827	2	*Geothrix fermentans* 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.

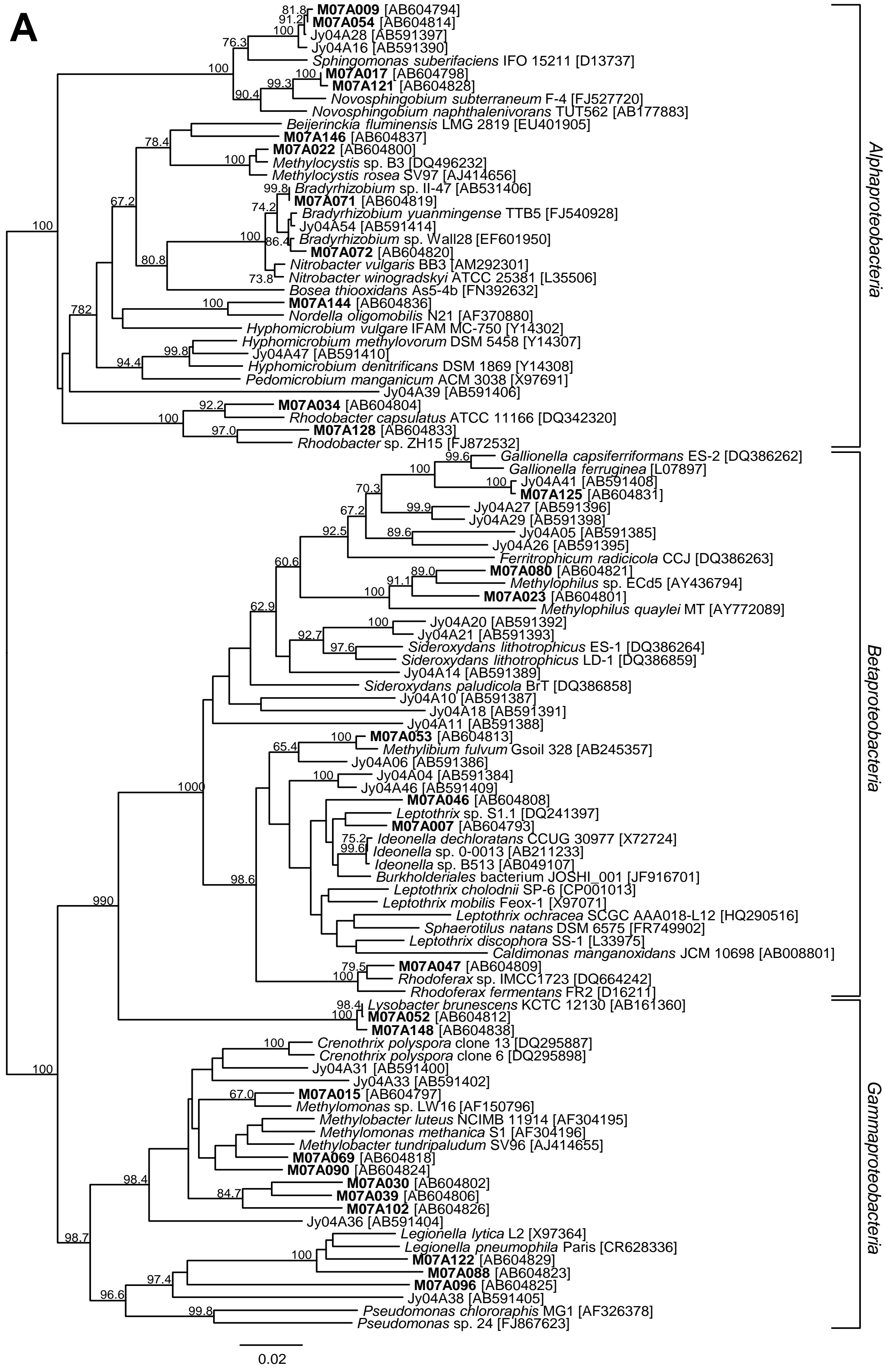
Jy04A46, and M07A053) contained the target sequence of a *Leptothrix*-specific probe PSP-6 (Siering and Ghiorse, 1997). *Methylibium* spp. are methylotrophic bacteria (Nakatsu *et al.*, 2006), but their Mn^{2+} oxidation activity are unknown. The amounts of 16S rRNA genes of the total bacteria and *Leptothrix*-related bacteria harboring the PSP-6 sequence in the DNA solution extracted from 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.

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, prosthecate 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.

3.3.3 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 the biological removal of Mn when the raw groundwater contains ammonia (Mouchet 1992; Štembal *et al.*, 2005). However, unfortunately, 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-Habbena *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. Quéméneur *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. PCR detection of *aoxB* gene in the DNA solution extracted from the microbial floc sample of the pilot plant was examined, 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


A
M07A009 [AB604794]
M07A054 [AB604814]
Jy04A28 [AB591397]
Jy04A16 [AB591390]
Sphingomonas suberifaciens IFO 15211 [D13737]
M07A017 [AB604798]
M07A121 [AB604828]
Novosphingobium subterraneum F-4 [FJ527720]
Novosphingobium naphthalenivorans TUT562 [AB177883]
Beijerinckia fluminensis LMG 2819 [EU401905]
M07A146 [AB604837]
M07A022 [AB604800]
Methylocystis sp. B3 [DQ496232]
Methylocystis rosea SV97 [AJ414656]
Bradyrhizobium sp. II-47 [AB531406]
M07A071 [AB604819]
Bradyrhizobium yuanmingense TTB5 [FJ540928]
Jy04A54 [AB591414]
Bradyrhizobium sp. Wall28 [EF601950]
M07A072 [AB604820]
Nitrobacter vulgaris BB3 [AM292301]
Nitrobacter winogradskyi ATCC 25381 [L35506]
Bosea thiooxidans As5-4b [FN392632]
M07A144 [AB604836]
Nordella oligomobilis N21 [AF370880]
Hyphomicrobium vulgare IFAM MC-750 [Y14302]
Hyphomicrobium methylovorum DSM 5458 [Y14307]
Jy04A47 [AB591410]
Hyphomicrobium denitrificans DSM 1869 [Y14308]
Pedomicrobium manganicum ACM 3038 [X97691]
Jy04A39 [AB591406]
M07A034 [AB604804]
Rhodobacter capsulatus ATCC 11166 [DQ342320]
M07A128 [AB604833]
Rhodobacter sp. ZH15 [FJ872532]
Alphaproteobacteria
Gallionella capsiferriformans ES-2 [DQ386262]
Gallionella ferruginea [L07897]
Jy04A41 [AB591408]
M07A125 [AB604831]
Jy04A27 [AB591396]
Jy04A29 [AB591398]
Jy04A05 [AB591385]
Jy04A26 [AB591395]
Ferritrophicum radicicola CCJ [DQ386263]
M07A080 [AB604821]
Methylophilus sp. ECd5 [AY436794]
M07A023 [AB604801]
Methylophilus quaylei MT [AY772089]
Jy04A20 [AB591392]
Jy04A21 [AB591393]
Sideroxydans lithotrophicus ES-1 [DQ386264]
Sideroxydans lithotrophicus LD-1 [DQ386859]
Jy04A14 [AB591389]
Sideroxydans paludicola BrT [DQ386858]
Jy04A10 [AB591387]
Jy04A18 [AB591391]
Jy04A11 [AB591388]
M07A053 [AB604813]
Methylibium fulvum Gsoil 328 [AB245357]
Jy04A06 [AB591386]
Jy04A04 [AB591384]
Jy04A46 [AB591409]
M07A046 [AB604808]
Leptothrix sp. S1.1 [DQ241397]
M07A007 [AB604793]
Ideonella dechloratans CCUG 30977 [X72724]
Ideonella sp. 0-0013 [AB211233]
Ideonella sp. B513 [AB049107]
Burkholderiales bacterium JOSHI_001 [JF916701]
Leptothrix cholodnii SP-6 [CP001013]
Leptothrix mobilis Feox-1 [X97071]
Leptothrix ochracea SCGC AAA018-L12 [HQ290516]
Sphaerotilus natans DSM 6575 [FR749902]
Leptothrix discophora SS-1 [L33975]
Caldimonas manganoxidans JCM 10698 [AB008801]
M07A047 [AB604809]
Rhodoferax sp. IMCC1723 [DQ664242]
Rhodoferax fermentans FR2 [D16211]
Betaproteobacteria
Lysobacter brunescens KCTC 12130 [AB161360]
M07A052 [AB604812]
M07A148 [AB604838]
Crenothrix polyspora clone 13 [DQ295887]
Crenothrix polyspora clone 6 [DQ295898]
Jy04A31 [AB591400]
Jy04A33 [AB591402]
M07A015 [AB604797]
Methylomonas sp. LW16 [AF150796]
Methylobacter luteus NCIMB 11914 [AF304195]
Methylomonas methanica S1 [AF304196]
Methylobacter tundripaludum SV96 [AJ414655]
M07A069 [AB604818]
M07A090 [AB604824]
M07A030 [AB604802]
M07A039 [AB604806]
M07A102 [AB604826]
Jy04A36 [AB591404]
Legionella lytica L2 [X97364]
Legionella pneumophila Paris [CR628336]
M07A122 [AB604829]
M07A088 [AB604823]
M07A096 [AB604825]
Jy04A38 [AB591405]
Pseudomonas chlororaphis MG1 [AF326378]
Pseudomonas sp. 24 [FJ867623]
Gammaproteobacteria
0.02

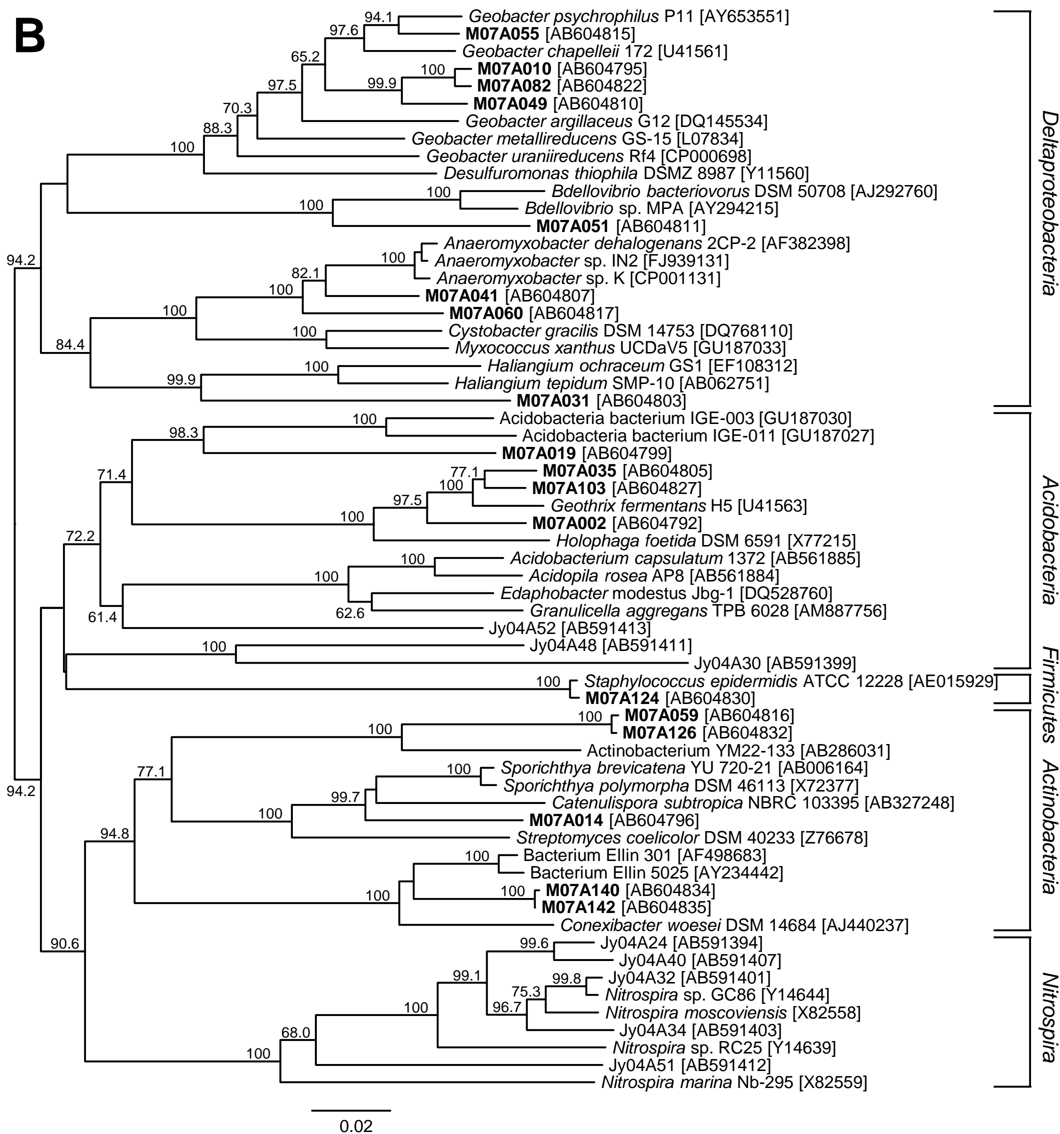


Fig. 3.2 – Phylogenetic relationships of bacterial 16S rRNA gene sequences obtained from the clone libraries of the biological filtration plants and relative bacteria. (A) *Alpha*, *Beta*, and *Gamma*-subclasses of *Proteobacteria*; (B) *Deltaproteobacteria*, *Acidobacteria*, *Firmicutes*, *Actinobacteria*, 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.

pilot plant sample although the microbial consortia in the pilot plant were

come from the As-containing groundwater. In contrast, the studies for the simultaneous removal of As and Fe reported by Battaglia-Brunet *et al.* (2002) 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.

3.4 Conclusion

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 as the other studies for the simultaneous removal of As and Fe using the microbial consortia containing arsenite-oxidizing bacteria. Those information of the compositions of microbial consortia in the biological filtration plants would be informative to improve the biological filtration systems for the removal of As from groundwater.

3.5 Acknowledgement

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CHAPTER 4

Biological removal of Mn^{2+} from water by aged biological filter media

Abstract

During the biological filtration process for the removal of iron (Fe) and manganese (Mn) from water, the filter media in the reactor were sequentially coated with biofilms and deposited Fe and Mn oxides, which show biological and physicochemical activities for the removal of Fe and Mn from water. These "aged" filter media are called "biological filter media (BFM)". In previous study, physicochemical activity for Mn^{2+} removal of the BFM collected from biological filtration plant in Joyo city, Kyoto, Japan, was characterized (Sahabi *et al.*, *J. Biosci. Bioeng.*, **107**, 151-157, 2009a). In this study, the biological activity of the BFM for the removal of Mn^{2+} from water was analyzed. Sodium azide, which inhibits the enzymatic oxidation of Mn^{2+}, was used to estimate the biological and physicochemical activities for Mn^{2+} removal of the BFM. The activity of Mn^{2+} removal from water by the BFM was considerably affected by pH. The effect of the temperature on Mn^{2+} removal rate was analyzed at 15-25°C. The physicochemical Mn^{2+} removal rate was higher at 15°C, but the biological Mn^{2+} removal rate did not show linear dependency on the temperature. The biological removal rates of Mn^{2+} from water per 1 g of BFM were ranged 1.58×10^{-2} to 1.89×10^{-2} mg/min at 15-20°C, which were adequate to remove Mn^{2+} ions contained in Joyo groundwater.

Keywords: biological filtration system, BFM, Mn removal, pH, temperature

4.1 Introduction

Biological removal of iron (Fe) and manganese (Mn) from water is a useful, cost-effective and eco-friendly drinking water treatment system (Mouchet, 1992; Pacini *et al.*, 2005). This system is called biological filtration, which utilizes microbial consortia containing Fe-oxidizing bacteria (FeOB) and Fe- and Mn-oxidizing bacteria (MnOB) by trapping them onto porous filter media packed in the reactor. In the biological filtration system, however, most of the Fe^{2+} in the water would be removed by the aeration, because soluble Fe^{2+} ion in the water is easily oxidized to $Fe(OH)_3$ and deposited in the neutral pH (Mouchet, 1992). In contrast, the oxidation of Mn^{2+} ion to insoluble Mn oxide by aeration alone is a slow process in the neutral and acidic pH (Aziz *et al.*, 1992; Berbenni *et al.*, 2000). When the start-up of the

biological filtration reactor (BFR), it had been reported that Fe removal occurs within a few days, while it requires a few weeks to one month for Mn removal to take place (Mouchet, 1992). Therefore, it is important to analyze the biological activity for Mn^{2+} removal in the BFR to understand the mechanism of the biological filtration process.

In the BFR, various microorganisms naturally present in the groundwater containing FeOB such as *Gallionella* and *Sideroxydans*, MnOB such as *Leptothrix* and *Hyphomicrobium*, and nitrifying bacteria, are trapped onto porous filter media (Thapa Chhetri *et al.*, 2013; see chapter 2 and 3). These microbial consortia would form biofilms on the surface of the filter media, and soluble Fe^{2+} and Mn^{2+} ions in the groundwater are adsorbed, oxidized and deposited onto the filter media (Sahabi *et al.* 2009a). Furthermore, the Fe and Mn oxides accumulated and coated on the surface of the filter media showed autocatalytic activity for Mn^{2+} oxidation (Sahabi *et al.*, 2009a), therefore Mn^{2+} removal of the biological filtration system is promoted by a combination of biological and physicochemical activities (Olańczuk-Neyman, and Bray, 2000; Sahabi *et al.*, 2009a). These "aged" filter media in the BFR coated with the catalytic Fe and Mn oxide layers and biofilms are called "biological filter media" (BFM). These catalytic oxide layers of the BFM showed sorptive activities not only for metal cations such as Mn, lead and cadmium but also for oxyanions such as arsenite and arsenate (Sahabi *et al.*, 2009a, 2009b and 2010).

Previously, Sahabi *et al* (2009a) investigated the physicochemical removal of Mn^{2+} by catalytic metal oxide layers coated on the surface of the BFM in detail. However, the mechanism of the biological removal of Mn^{2+} from water by the BFM is still unknown. In this study, the biological activity for Mn^{2+} removal from water of the BFM is analyzed by comparing total and physicochemical Mn^{2+} removal activities of the BFM, because Mn^{2+} removal activity of the BFM is a combination of the biological and physicochemical activities. Sodium azide was used as an inhibitor for the biological oxidation

of Mn^{2+} by the BFM, which enables to estimate biological activity for Mn^{2+} removal of the BFM. The influence of pH and temperature on the biological removal of Mn^{2+} by the BFM would be analyzed.

4.2 Materials and methods

4.2.1 Samples

The BFM (anthracites, 2.0-2.1 mm in diameter) coated with metal oxides-rich biofilms and raw groundwater samples were collected from Joyo biological filtration plant for the removal of Fe and Mn from groundwater, which is located at The Third Drinking Water Treatment Plant of Joyo City, Kyoto Prefecture, Japan (Tamura *et al.*, 1999; Sahabi *et al.*, 2009a). The characteristics of Joyo biological filtration plant is described in chapter 2. The BFM samples were collected in 250 ml polypropylene bottles and stored at 15°C and used within a month. Raw groundwater samples were collected in 2 L polypropylene bottles, filtered through 0.2 µm cellulose acetate membrane filter (Advantec, Tokyo, Japan), and stored at 4°C until analysis.

4.2.2 Manganese removal experiments

All experiments for the quantitative determination of Mn^{2+} removal from water were carried out with reaction mixture placed into 500-ml Erlenmeyer flasks. The flasks were incubated at various temperatures with gentle shaking (100 rpm) in a gyratory shaker (BR-300LF, Taitec, Saitama, Japan). Each test was run in duplicate.

Mn^{2+} removal assay was conducted with freshly collected BFM from Joyo biological filtration reactor (BFR). Approximately 0.5 g (wet weight) of fresh BFM was added to 100 ml of filter-sterilized groundwater spiked with $MnSO_4 \cdot 5H_2O$ solution to a final concentration of 15 mg/L Mn^{2+} at pH 7.4 ± 0.1 (adjusted by 15 mM HEPES-NaOH) or without HEPES buffer. At predetermined time intervals, 5 ml aliquots were withdrawn from the reaction mixtures and filtered through 0.2 µm syringe filter (Millex GP, Millipore, MA, USA). The filtrates were then analyzed for Mn^{2+} concentration using AA-6650F atomic absorption spectrophotometer (AAS)

(Shimadzu, Kyoto, Japan). To estimate the contribution of biological (enzymatic) activity in the Mn^{2+} removal from water by BFM, a set of the flasks were amended with 10 mM sodium azide (NaN_3) solution to inhibit the microbial oxidation of Mn (Rosson *et al.*, 1984). Blank controls containing the reaction mixture without the BFM sample were run with each test to correct for chemical precipitation of Mn on the course of the reaction.

To examine the effect of pH, all experimental procedures were applied the same as described above except different pH of HEPES-buffer (initially adjusted at pH4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively). Each pH was adjusted by NaOH solution. At predetermined time points (1 h and 24 h), 2 ml aliquots were withdrawn and filtered. Mn^{2+} concentration was analyzed using the AAS as described. To determine the effect of HEPES-NaOH buffer in biological removal of Mn^{2+} from water, again all experimental procedures were applied the same as described above except HEPES buffer concentrations. Final concentration of HEPES-buffer in each flask was 2, 5, 10, 15 and 20 mM, respectively. At predetermined time intervals, aliquots were withdrawn and filtered, and then Mn^{2+} concentration was analyzed using the AAS. To estimate the influence of temperature on Mn^{2+} removal of BFM, all experimental procedures were applied the same as described above except different incubation temperatures (15, 20, and 25°C). At predetermined time intervals, aliquots were withdrawn and filtered, and then Mn^{2+} concentrations were analyzed using the AAS. Biological activity for the removal of Mn^{2+} from water by BFM were estimated from the difference between the specific Mn^{2+} removal rates at the conditions with or without NaN_3.

4.3 Results and discussion

4.3.1 Biological removal of manganese by the biological filter media

To study the biological activity for Mn^{2+} removal from water of the BFM, experiments were performed using the BFM collected from Joyo biological filtration plant. To observe the biological activity for Mn^{2+} removal of the BFM, the experiments were carried out with or without NaN_3 as described in materials and methods. As shown in Figs. 4.1, the biological activities for Mn^{2+} removal of the BFM were observed as the difference between the

reaction conditions with and without NaN_3. These reactions were carried out under the buffered conditions with HEPES-NaOH to prevent increase of pH to basic values which causes chemical oxidation of Mn^{2+} (Fig. 4.1a), or the unbuffered conditions (Fig. 4.1b), respectively. When the reactions were carried out in HEPES-buffered conditions, Mn^{2+} was removed to 9.3 mg/L after 48 h incubation without NaN_3 inhibition, while that was decreased to 10.0 mg/L with the inhibition (Fig. 4.1a). The pH values did not change during the reactions. On the other hand, when the reactions were carried out without HEPES buffer, Mn^{2+} concentration was decreased to 5.6 mg/L after 48 h incubation without NaN_3 inhibition, while that was decreased to 8.3 mg/L with the inhibition (Fig. 4.1b). The pH values were 7.6 at initial 2 hours, and then raised to 7.7 after 4 hours incubation. It should be noted that both of the samples with and without NaN_3 inhibition showed nearly the same pH values at each sampling time, which suggests that the change of pH values during the incubation would be due to the physicochemical removal of Mn^{2+} by the BFM, but not to the microbial activity.

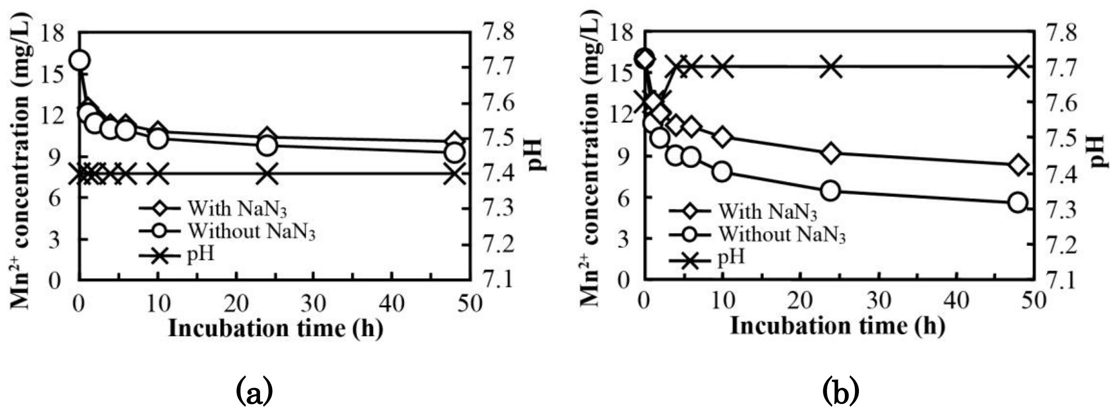

Fig. 4.1 - Mn^{2+} removal by Joyo BFM;
(a) HEPES-buffered conditions, (b) unbuffered conditions

Comparing to the experiments under the condition with HEPES buffer, those without the buffer showed much higher activity not only for the physicochemical removal of Mn^{2+} with NaN_3 but also for the biological removal of Mn^{2+} estimated form the difference between the conditions with

and without NaN_3 (Fig. 4.1a and b). These results suggested that both biological and physicochemical activities of Mn^{2+} removal were possibly affected by either by pH or presence of HEPES buffer.

4.3.2 Effect of pH and HEPES buffer on Mn^{2+} removal

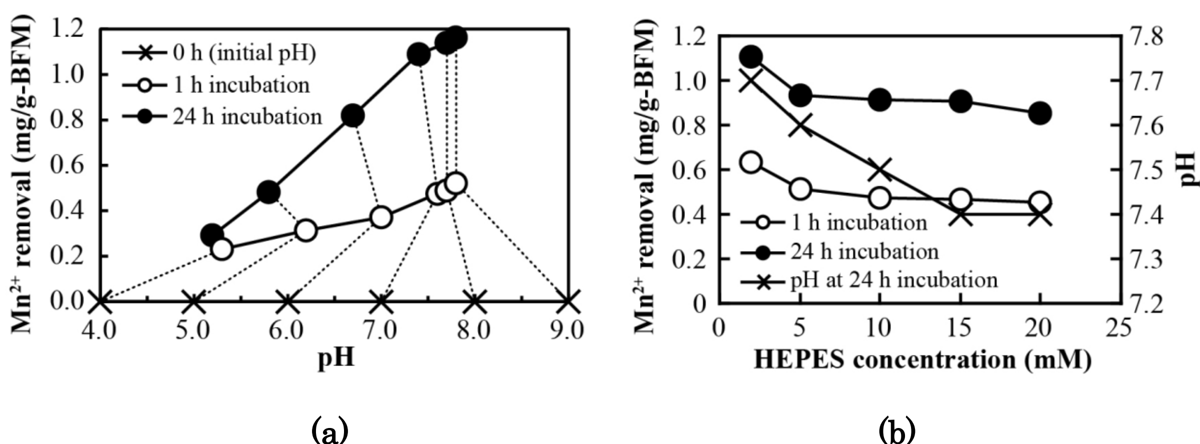

Fig. 4.2 - Effects of (a) pH and (b) concentration of HEPES on Mn^{2+} removal by Joyo BFM.

It has been reported that biological oxidation of Mn^{2+} is strongly affected by pH and oxidation-reduction potential (Mouchet, 1992; Pacini *et al.*, 2005; Stembal *et al.*, 2005). Effect of pH on Mn^{2+} removal by the BFM was examined as described in Materials and Methods. As shown in Fig. 4.2, Mn^{2+} removal by the BFM was dependent on pH of the solution, but not on the concentration of HEPES. Interestingly, pH values of the samples were changed during the incubation (Fig. 4.2a). The pH values of the samples initially adjusted below 7.0 were increased after 1 h incubation, and then decreased after 24 h incubation. On the other hand, the pH values of those initially adjusted over 8.0 were decreased after 1 h incubation, and then did not change after 24 h incubation. However, the mechanisms of those changes of pH values have not been cleared yet.

Unlike the effect of pH, no relationship was observed between Mn^{2+} removal by the BFM and the concentration of HEPES buffer (Fig. 4.2b). At the

concentrations of HEPES-NaOH buffer higher than 15 mM, pH values were kept at 7.4 after 24 h incubation. When the concentrations of HEPES-NaOH buffer were lower than 15 mM, pH values were raised after 24 h incubation, which slightly caused the increase of Mn^{2+} removal (Fig. 4.2b). These increases of the Mn^{2+} removal were probably due to the increases of the pH values, but not the decreases of HEPES buffer concentration. These results indicated that the experiments of Mn^{2+} removal by the BFM would be performed under the condition with 15 mM HEPES-NaOH buffer.

4.3.3 Mn^{2+} removal rate in different temperature conditions

Table 4.1 - Mn^{2+} removal rate of the BFM at different temperatures with or without NaN_3.

	Mn^{2+} removal rate [mg-Mn^{2+}/min/g-BFM]		
Temp. [°C]	Without NaN_3 *	With NaN_3 *	Biological activity
15	$7.93\times10^{-2} \pm 1.08\times10^{-3}$	$6.04\times10^{-2} \pm 1.59\times10^{-2}$	1.89×10^{-2}
20	$6.79\times10^{-2} \pm 1.77\times10^{-2}$	$5.21\times10^{-2} \pm 2.00\times10^{-4}$	1.58×10^{-2}
25	$7.70\times10^{-2} \pm 1.26\times10^{-2}$	$4.69\times10^{-2} \pm 5.91\times10^{-3}$	3.02×10^{-2}

* The standard errors were indicated.

In Joyo plant, temperature of the raw groundwater has been ranged from 16 to 18°C. The effect of temperature conditions on Mn^{2+} removal by the BFM was tested at 15-25°C with and without NaN_3 (Table 4.1). When the biological oxidation of Mn^{2+} was inhibited by NaN_3, the physicochemical removal rates of Mn^{2+} from water by the BFM were decreased at the higher temperatures. On the other hand, the biological removal rates of Mn^{2+} by the BFM did not show linear dependency on the temperature. It is supposed that Joyo BFM would contain several kinds of MnOB with different characteristics of Mn oxidizing enzymes. Therefore, further investigation would be required to study the characteristics of Mn oxidizing enzymes through the isolation of the MnOB from Joyo BFM.

The biological Mn^{2+} removal rates per 1 g of the BFM were ranged from

1.58×10^{-2} to 1.89×10^{-2} mg/min at 15-20°C. In Joyo, the BFR treatss 11,100 m^3 of drinking water in a day, and the concentration of Mn^{2+} in raw groundwater was 0.12 mg/L, thus 9.25×10^2 mg of Mn^{2+} have been removed in a minute by Joyo BFR. The biological filter of Joyo plant has a surface area of 189 m^2 (31.5 m^2 × 6 filters) and a height of 0.3 m. The density of the anthracite was 0.75%, and the total amount of the BFM in Joyo BFR is about 4.25×10^7 g. Therefore, 1 g of Joyo BFM removes 2.18×10^{-5} mg of Mn^{2+} per a minute in Joyo BFR. Our results indicated that the biological activity of Joyo BFM for Mn^{2+} removal is sufficient for the elimination of Mn^{2+} contained in the groundwater of Joyo plant.

4.4 Conclusion

Biological activity for the removal of Mn^{2+} from water using Joyo BFM was investigated. The biological and physicochemical activities for Mn^{2+} removal were estimated using NaN_3 as an inhibitor for biological Mn^{2+} oxidation. Mn^{2+} removal was strongly influenced by pH changes. The physicochemical Mn^{2+} removal rate was higher at 15°C, but the biological Mn^{2+} removal rate did not show linear dependency on the temperature. The biological removal rates of Mn^{2+} from water per 1 g of BFM were ranged 1.58×10^{-2} to 1.89×10^{-2} mg/min at 15-20°C, which were adequate to remove Mn^{2+} ions contained in Joyo groundwater.

4.5 Acknowledgement

We are grateful to T. Tamura of Joyo City Water Supply and Sewerage Department for providing us samples of BFM and giving us relevant information about the plant. We are thankful to M. Kondo of Instrumental Analysis Center, Yokohama National University.

CHAPTER 5

Cultivation of manganese oxidizing microbial consortia from biological filter media for the removal of dissolved manganese

Abstract

The natural MnOB present in soil and water environment influence to remove Mn in biological filtration plant by oxidation and deposition. To study the biological removal mechanism for Mn, the Mn-oxidizing microbial consortia were cultivated in mPYM (modified amount of 1.0 g/L-peptone, 0.1 g/L-yeast extract and $MnSO_4.5H_2O$ solution to a final Mn(II) concentration of 30 mg/L) (Vandenabeele *et al.*, 1995) culture media using BFM of Joyo biological filtration plant. The cultivated microbial consortia were applied to remove Mn(II) in presence of carriers in mPYM. In batch experiment, Mn(II) ions removal was detected higher (>3 mg/L) in carriers containing reaction mixtures, and that was almost similar (>2.5 mg/L) in the mPYM with no carriers. Interestingly, adequate Mn oxides formation occurred in carrier containing mPYM compared with no carrier containing culture media. The microbial consortia in the cultivated culture media were studied using amplified 16S rRNA gene in DGGE analysis. It was observed that *Leptothrix* and *Hyphomicrobium* were the dominant MnOB in carrier containing cultivated microbial consortia.

Keywords: biological filter media, Mn-oxidizing microbial consortia, Mn removal, 16S rRNA gene, DGGE

5.1 Introduction

A biological filtration system for the removal of Fe and Mn from groundwater has been operated in the drinking water treatment plant of many countries (Mouchet, 1992; Pacini *et al.,* 2005). In this system, soluble Fe^{2+} and Mn^{2+} ions are particularly removed via a combination of biologicaland physicochemical oxidation. Fe^{2+} ion in groundwater is easily oxidized and deposited by aeration at neutral pH, while Mn^{2+} ion requires catalytic materials or enzymes for oxidation at neutral pH; therefore, Mn oxidizing bacteria (MnOB) are essential for the biological filtration system to remove Mn-from groundwater.

The long period filtration of Fe and Mn containing raw groundwater

spontaneously coats the filter media with metal-oxides rich biofilms (Sahabi *et al.*, 2009a). These naturally coated filter media are the so-called biological filter media (BFM). In Joyo biological filtration plant, Fe and Mn are adequately removed from natural groundwater (Tamura *et al.*, 1999). In the biofilms on the BFM of Joyo plant, *Hyphomicrobium* has been reported as the dominant MnOB (Thapa Chhetri *et al.*, 2013). In addition, these BFM of Joyo plant were also reported as useful filter media not only for the removal of soluble Mn ions but also remove very carcinogenic heavy metal ions such as arsenic (As), lead (Pb) and cadmium (Cd) from engineered and natural water environments (Sahabi *et al.*, 2009b). Despite the metal ions removal kinetics and bacterial consortia onto these BFM were analyzed (Sahabi *et al.*, 2009a, 2009b and 2010; Thapa Chhetri *et al.*, 2013), the cultivation analyses for those environmental microorganisms have not been studied yet.

In Belgium, the cultivation of *Hyphomicrobium* was carried out in PYM culture media using the sand filter media of biological filtration plant (Vandenabeele *et al.*, 1995). However, some bacterial species of genera *Leptothrix* (Ghiorse, 1984), *Pedomicrobium* (Gebers, 1981), *Hyphomicrobium* (Tyler, 1970), *Bacillus*, *Pseudomonas* (Tebo *et al.*, 2004) have beene reported as MnOB. The Mn-oxidizing microbial consortia were cultivated using BFM of Joyo plant in already confirmed physicochemical incubation conditions (chapter 4). They can oxidize Mn(II) to insoluble Mn oxides to gain energy for their growth. Biogenic Mn-oxides have been reported as active catalyst to oxidize metal ions such as Mn, As, Co, Cu, Cd from the aquatic environment (Kastoyiannis *et al.*, 2004; Tebo *et al.*, 2004; Casiot *et al.*, 2006; Sahabi *et al.*, 2009b). Therefore, it is assumed in this study that the reactive biogenic Mn oxides would be prominent for the removal of soluble metal ions because the related analyses were already reported by various researchers (Tebo *et al.*, 2004; Sahabi *et al.*, 2009a & 2009b).

The characterization of MnOB that embedded onto the BFM of Joyo biological filtration plant have not been studied yet due to lack of well-known specific culture media and difficulties of isolation procedures. To study the biological removal mechanism of Mn in the Joyo biological filtration plant, it requires to be cultivated dominant MnOB and their application in metal ions removal. The aim of this study is to cultivate Mn-oxidizing microbial consortia using Joyo BFM to produce microbial flocs and to apply for the removal of dissolved Mn(II). In addition, this research also focus on effect of

carriers in the Mn-oxidizing microbial consortia cultivation and Mn(II) removal. Sterilized and uncoated sea sand and anthracites were used as carriers for favorable shaking and microbial enrichment. The bacterial diversities cultivated in mPYM were studied using culture-independent molecular biological analyses.

5.2 Material and methods

5.2.1 Samples

Approximately 5 mm in diameter of anthracites BFM (Sahabi *et al.*, 2009a) were collected from Joyo biological filtration plant. The location of this plant and concentrations of the contaminants in the raw groundwater and treated water were the same as mentioned in chapter 2 (Thapa Chhetri *et al.*, 2013). The raw (uncoated) anthracite used in the plant was also kindly provided by the plant personnel in Joyo city.

5.2.2 Cultivation of Mn oxidizing microbial consortia

Batch experiment was carried out in 500-mL and 100-mL Erlenmeyer flasks, and duplicate was used to each cultivation Each 500-ml and 100-mL flasks contained 100-ml and 30-ml of mPYM culture media (Vandenabeele *et al.*, 1995), respectively, with HEPES-NaOH buffer (pH 7.4 ± 0.1) of final concentration 15 mM. Additional 0.5 g (wet weight) of BFM of Joyo plant was inoculated in 100-mL of mPYM containing flasks. Experiments were performed at 25°C shaking with 100 rpm. Before starting batch experiments, except Mn(II) solution and BFM, the reaction mixtures of culture media along with uncoated anthracites and sea sands were sterilized by autoclaving. However, Mn(II) solution was filter sterilized before skipping into sterilized culture medium to avoid the chemical precipitation and complexation with organics in the culture medium. In this study, the reaction mixtures were prepared with and without sodium azide solution (15 mM final concentration) (Rosson *et al.*, 1984), and they were considered as abiotic and biotic culture media, respectively in primary and secondary cultivations.

5.2.3 Analyses of Mn oxidation and Mn^{2+} concentration

For the cultivation of Mn-oxidizing microbial consortia, 100-mL reaction mixture containing 500-mL Erlenmeyer flasks (as described above) were incubated at 25 °C with 100 rpm gentle shaking for two weeks. One set of experiment was designed and assumed as supported culture media

containing the same reaction mixture with additional 1.0 g of sterilized sea sand as carrier to obtain excess microbial growth in mPYM. At predetermined time interval, 5-mL aliquots were withdrawn from the reaction mixtures and filtered through 0.2 µm filter (Millex GP, Millipore, MA, USA). Filtrates were then analyzed for Mn(II) concentration using AA-6650F atomic absorption spectrophotometer (AAS) (Shimadzu, Kyoto, Japan). Two weeks cultivated microbial consortia (only the biotic culture media) were collected in 50-mL tubes (Sumilon, Sumitomo Bakelite Co., Ltd., Japan), and then centrifuged at 7000 rpm, 15°C for 3 min. 30-mL of cultured microbial pellets were collected separately after discarding the supernatants. At last, harvested microbial consortia were mixed well and used as working microbial consortia to study the effect of carrier in Mn removal, Mn-oxides formation and microscopic analyses. A small portion of these primary cultivated microbial consortia were preserved at -80°C to analyze molecular biological studies.

To evaluate the effects of carrier materials on Mn(II) removal in batch experiment, 100-ml Erlenmeyer flask with 30-ml buffered mPYM was used as mentioned above. One set of experiment was designed using the same culture media with additional sterilized 0.5 g (dried weight) of fresh (uncoated) anthracites and 1.0 g of sterilized sea sand as carriers. In addition, 2-mL of primary enriched microbial flocs (unsterilized) were inoculated into each flask and the incubation parameters were set the same as described previously except extended four weeks incubation period. In this study, all the batch cultures were run without sodium azide i.e. only for biotic cultures. 500-µL of aliquots were withdrawn at predetermined time and thereafter filtered through 0.2 µm syringe filter (Millex GP, Millipore, MA, USA) to analyze Mn(II) concentration. Mn(II) concentration was analyzed using AAS as described previously. Additional 2-mL aliquots were withdrawn from one and three week incubated culture media and preserved at -80°C to analyze further microbial analyses until complete the kinetic studies.

5.2.4 Microscopic analysis

The microscopic analyses were performed to study the cultivated microbial consortia. Cultivated microbial consortia in primary cultivation (two weeks cultured) and secondary incubation (three weeks incubated) were observed under phase-contrast microscopy (Axioskop, ZEISS, Germany). To observe the bacterial cells enriched in supported and unsupported primary

cultivation in mPYM, DAPI staining analysis was carried out. Thereafter, those stained cells were observed under fluorescence microscope (Axioskop, ZEISS, Germany).

5.2.5 DNA extraction

DNA was extracted using 0.5 g of BFM, 0.3 ml of primary cultivated (two weeks cultured) and secondary incubated (one and three weeks incubated) microbial consortia, respectively. To extract the total genome, FastDNA SPIN Kit for soil (MP Biochemicals, Solon, OH, USA) was used. DNA extraction procedures were followed the same as described in chapter 2 (Thapa Chhetri *et al.*, 2013). The extracted total DNA solution sample was subdivided into aliquots and stored at –20 °C until use.

5.2.6 Polymerase chain reaction

Standard PCR procedure was carried out using *TaKaRa Ex Taq* polymerase (Takara bio, Shiga, Japan). 50 μL of PCR mixture according to the manufacturer's protocol with 1 μL of ten-times diluted total DNA solution as a template was prepared. After 5 min of initial denaturation at 94°C, PCR was carried out with 0.5 min of denaturation at 94°C, 0.5 min of primer annealing at 55°C, and 0.5 min of primer extension at 72°C for 25 cycles, followed by 7 min of final primer extension. The resulted PCR product was applied for agarose gel electrophoresis, and then visualized using ethidium bromide staining and UV transilluminator. For DGGE analysis, 50 μL of PCR mixture was prepared as described above, and PCR was carried out with "touchdown" program. After 10 min of initial denaturation at 95°C, "touchdown" PCR cycle protocol was used (Don *et al.*, 1991), and the annealing temperature was decreased by 1°C per cycle from 65°C to 56°C, then 20 additional cycles at 55°C were performed. Amplification was carried out with 0.5 min of denaturation at 95°C, 0.5 min of primer annealing, and 1 min of primer extension at 72°C, followed by 5 min of final primer extension.

5.2.7 Denaturing gradient gel electrophoresis

To analyze molecular diversity of bacterial consortia that trapped on the Joyo BFM and cultivated in mPYM culture media, those extracted DNAs were used for DGGE analysis. Approximately 190 bp of variable region 3 of bacterial 16S rRNA gene were amplified using primers GC-341F (with 40 bp of GC-clamp) and 534R (Muyzer *et al.*, 1993). DGGE analysis was carried out with a mini-DGGE system NB-1490 (Nihon Eido, Tokyo, Japan). The DGGE

gel plate was 10 mm × 10 mm in size and 1 mm thick. Amplified PCR products were applied onto 8% (wt/vol) polyacrylamide gels formed with acrylamide-N,N'-methylenebisacrylamide, 37.5:1 (Bio-Rad, Hercules, CA, USA) in 1×TBE buffer [89 mM Tris-borate, 2 mM EDTA (Sambrook *et al.*, 1989)] with the denaturing gradients ranged from 30 to 60% denaturant [100% denaturant contained 7 M urea and 25% (vol/vol) formamide]. Electrophoresis was studied in 1×TBE buffer at 50 V and 60°C for 4.5 h, and then gel was stained in SYBR Green I (Invitrogen, Carlsbad, CA, USA). Stained gel was visualized by using UV transilluminator (Funakoshi, Japan) and DigiDoc-It Image System (UVP, Upland, CA, USA).

To determine the nucleotide sequences for amplified DNA fragments obtained from DGGE analysis, isolated fragments were excised and eluted in 40 µL of distilled-deionized water at 4°C for overnight by slightly shaking. Thereafter, supernatant was re-amplified, purified and then applied for nucleotide sequence analysis using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 373 or 3100-Avant Genetic Analyzer (Applied Biosystems). The obtained nucleotide sequences were then compared with DNA databases using BLAST (http://blast.ddbj.nig.ac.jp/) (Altschul *et al.*, 1990).

5.3 Resultis and discussion

5.3.1 Cultivation of Mn oxidizing microbial consortia and Mn^{2+} removal

The adequate removal of Fe and Mn from raw groundwater in well-established Joyo biological filtration reactor is carried out by spontaneously embedded BFM (Tamura *et al.*, 1999 and Sahabi *et al.*, 2009a). The naturally occurring Fe- and Mn-oxidizing microorganisms are trapped onto the BFM after long period filtration of Fe and Mn containing raw groundwater, which they are prominent for the removal of those soluble metal ions.

In Mn-oxidizing microbial consortia cultivation study, adequate Mn(II) ions were removed from the mPYM using BFM of Joyo plant. Interestingly, the microbial consortia both in supported (additionally inoculated sterilized and uncoated sea sand) and unsupported (without sterilized and uncoated sea sand) mPYM culture media were distinctly developed after two days incubation. However, heavier microbial growth was observed in supported culture media compared with unsupported cultivation. However, the removal

of Mn(II) from both supported and unsupported culture media was observed almost similar (data not shown). It was thus, the importance of carrier (sterilized sea sand) in primary cultivation was not seen to cultivate Mn-oxidizing microbial consortia and Mn(II) removal in mPYM. However, it was supposed to be studied the effect of carriers on Mn(II) removal in secondary cultivation by applying cultivated microbial consortia obtained from primary cultivation. In the batch experiment, the carriers were decided not to be used in Mn-oxidizing microbial consortia cultivation in primary cultivation.

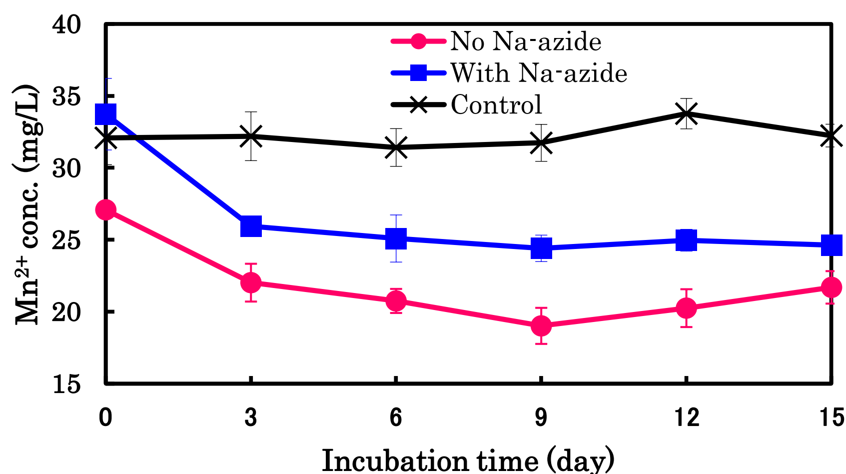

Fig. 5.1 – Mn(II) removal using BFM of Joyo plant in mPYM. The incubation conditions were set at 15°C, 100 rpm for two weeks (without carrier).

Interestingly in Mn(II) removal study using BFM of Joyo plant in mPYM, it was observed Mn(II) removal concentration was higher in biotic culture media compared with abiotic (Fig. 5.1). After two weeks cultivation, Mn(II) concentrations were reduced to 21.69 mg/L and 24.61 mg/L from initial concentration in biotic and abiotic mPYM, respectively (only unsupported cultivation data has been shown) (Fig. 5.1). The removal of Mn(II) ions were almost constant in control mPYM, however, the evaporation of water portion affected the final Mn(II) ions concentration for two weeks incubation (Fig. 5.1). In the abiotic culture media, the biological activities were inhibited by using sodium azide (Rosson *et al.*, 1984) so that only the physicochemical oxidation of Mn(II) was possible in abiotic culture media, whereas both biological and physicochemical activities were possible in biotic mPYM. The Fe- and Mn-oxidizing microorganisms including iron bacteria play the vital role for the oxidation of Fe(II) and Mn(II) to deposit insoluble and reactive Fe or Mn oxides (Mouchet, 1992; Katsoyiannis and Zouboulis, 2004; Tebo *et al.*,

2004). The deposited biogenic Mn oxides are prominent for the subsequent autocatalytic oxidation of Mn(II) ions and to remove various toxic metal ions from groundwater (Tebo *et al.*, 2004; Katsoyiannis *et al.*, 2004; Casiot *et al.*, 2006). It was considered in biotic mPYM, Mn-oxidizing microbial consortia and naturally coated Mn-oxides on the BFM of Joyo plant could remove Mn(II) by oxidation and adsorption, although only the autocatalytic oxidation might had been carried out by coated Mn-oxides on BFM of Joyo plant in abiotic mPYM. However, the complete mechanism is still unclear. It has been already reported that MnOB such as *Hyphomicrobium* and *Leptothrix* are trapped on the BFM surface of Joyo biological filtration plant (Thapa Chhetri *et al.*, 2013). Therefore, it was thus supposed that the MnOB such as *Hyphomicrobium* could be cultivated in mPYM and Mn(II) ions were possibly removed by microbial oxidation and thereafter autocatalytic oxidation by Mn oxides in biotic mPYM. Consequently, those enriched microbial consortia were supposed to apply in Mn(II) removal in our next batch experiment, which we called secondary cultivation.

5.3.2 Effect of carrier materials on Mn^{2+} removal using cultured microbial consortia

Mn(II) removal was analyzed using cultivated microbial consortia in the secondary cultivation with and without carriers. In four weeks incubated secondary cultivation, the cultivated microbial consortia (harvested from primary cultivation that cultured in unsupported mPYM) removed 2.4 mg/L of Mn(II) from the initial concentration of 29.8 mg/L in carrier containing mPYM. But in another set of experiment in carrier containing mPYM, >3.8 mg/L of Mn(II) ions were removed from the same initial concentration by the cultured microbial consortia (harvested from primary cultivation in supported mPYM) after four weeks incubation (Fig. 5.2). Surprisingly, in other sets of batch experiment in absence of carriers, 2 mg/L of Mn(II) ions were removed from the initial concentration of 29.8 mg/L by the cultivated microbial consortia (harvested from primary cultivation in unsupported mPYM) after four weeks incubation, while only 1.4 mg/L of Mn(II) was removed by the other inoculated microbial consortia (harvested from the primary cultivation in supported mPYM) from the same initial concentration in mPYM. These results distinctly revealed the effect of carrier in secondary incubation on Mn(II) removal in mPYM.

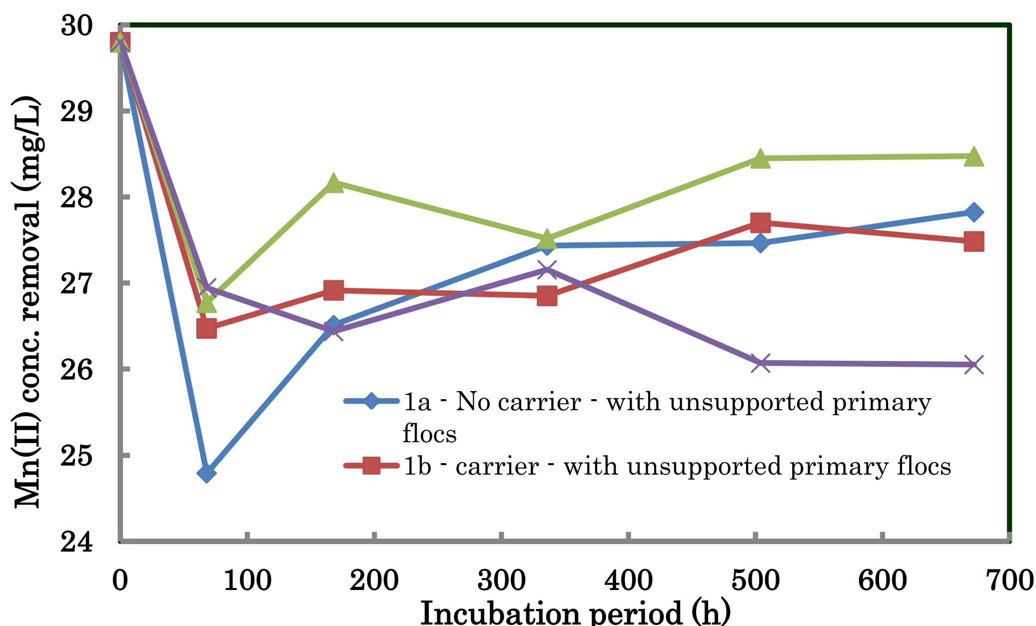

Fig. 5.2 – Mn(II) removal analysis using primary culured microbial consortia in mPYM. ‘a’ and ‘b’ indicate the culture media without carrier and with carrier (sterilized uncoated anthracites and sea sand), respectively. 1 and 2 indicate the incubated culture media after inoculation of primary cultivated microbial consortia (cultured from BFM of Joyo biological filtration plant) in unsupported and supported cultivation, respectively (sterilized sea sand was used as supporter).

In our other batch experiments for secondary cultivation, it was observed that the Mn(II) removal was observed higher in presence of carriers (sterilized uncoated anthracites and sea sand) in inoculation of cultivated microbial consortia (harvested from two weeks cultured in primary cultivation), whereas only modest Mn(II) removal was observed in absence of any carriers in mPYM (data not shown). It was thus at least one supporter or carrier (either sterilized uncoated anthracite or/and sea sand) was proposed to be used in Mn(II) removal when the cultivated microbial consortia is used in mPYM. These carriers such as uncoated fresh anthracites and sterilized sea sand do not have any chemical and biological effects themselves for Mn(II) ions oxidation and removal, however, it made feasible shaking in the culture media. However, the complete effect of carrier in Mn(II) removal mechanism is still unclear.

In addition to Mn(II) removal, the cultivated microbial consortia were analyzed using culture-independent molecular biological tools to investigate

the effect of carriers on Mn(II) removal. Microbial flocs were primarily cultivated in supported (sterilized uncoated sea sand) and unsupported mPYM using BFM of Joyo biological filtration plant. Despite the Mn(II) removal analysis was carried out in batch experiment, it was revealed almost similar removal concentration of dissolved Mn(II) ions both in supported and unsupported primary cultivation (data not shown). In the primary cultivation, microbial flocs were cultivated both in supported (used sterilized uncoated sea sand) and unsupported mPYM. Thereafter, those enriched flocs were applied in secondary cultivation to analyze the carrier effects in cultivation and Mn(II) removal. To make the feasible shaking for aerobic microorganisms, those carriers were used. In the culture media, these carriers do not have any biological and physicochemical effects on Mn(II) removal and microbial growth or microbial flocs formation.

5.3.3 Confirmation of Mn oxide production

In biotic mPYM, heavy growth of microbial consortia was observed along with dark brown particles. In those cultivated microbial consortia, Mn oxides formation was confirmed by qualitative colorimetric analysis method using leuco-crystal violet (LCV) staining solution (Dickinson *et al.*, 1996). Briefly, the harvested dark brown particles turned its color into violet with LCV reagent (data not shown). On the other hand, the Mn-oxidizing microbial consortia could not be cultured in abiotic mPYM culture media, but the modestly formed Mn oxides turned its color into violet. It resulted that Mn oxides were heavily formed in biotic mPYM compared with abiotic during cultivation of Mn-oxidizing microbial consortia in mPYM. Similarly in the secondary cultivation, Mn oxides were confirmed using LCV staining reagent. In all cultured mPYM culture media in presence and absence of carriers, biogenic Mn oxides were confirmed by turning their color into violet along with Leuco cryal violet reagent (data not shown).

5.3.4 Microscopic appearance of cultured Mn oxidizing microbial consortia

To analyze morphological characteristics of cultured microbial consortia, phase-contrast microscopy was carried out for primary cultivated microbial consortia cultured in mPYM using BFM of Joyo plant. It was observed the elongated, rod and oval-shaped bacteria both in supported and unsupported mPYM. In two weeks cultivated consortia, bacterial cells were loosely clustered around the brownish black Mn oxides (Fig. 5.3 (A)). Those Mn oxides revealed blackish around dark brownish with various sizes. In

addition in secondary cultivated mPYM, it was observed slightly more elongated bacterial consortia compared with primary cultivation, which they were also clustered around the blackish Mn oxides (data not shown).

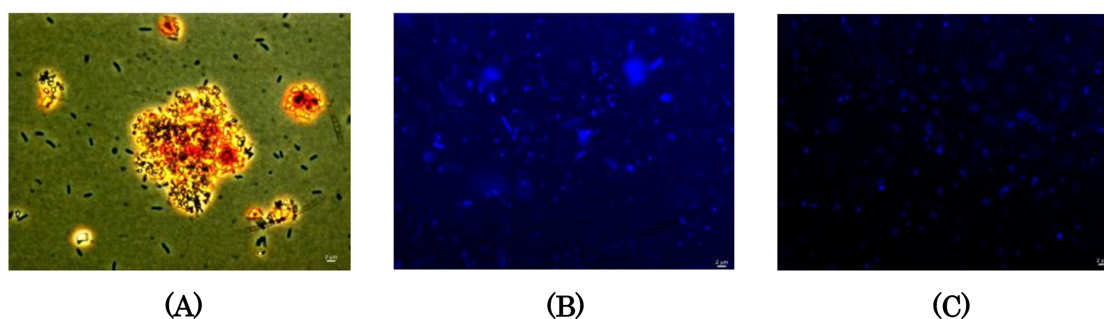

Fig. 5.3 – Microscopic analyses of cultivated microbial consortia cultured for two week in mPYM using BFM of Joyo plant. (A) depicts cultured microbial consortia observed under phase-contrast microscope. (B) and (C) represent the DAPI staining for cultivated microbial consortia revealed in supported and unsupported culture media, respectively.

On the other hand for DAPI staining, the elongated, rod and oval-shaped bacterial cells were observed in primary cultivation in supported and unsupported mPYM. However, the microbial cells in the cultivated culture media were not counted. In contrast, optical density (OD) were not also analyzed, although the higher growth of microbial cells were observed in supported mPYM compared with unsupported mPYM in primary cultivation (Fig. 5.3 (B) and (C)), although the more studies are required to know the complete biological structures.

5.3.5 Bacterial diversities analyzed by PCR-DGGE

The bacterial diversities in the cultivated microbial consortia were studied by DGGE using PCR amplified 16S rRNA gene fragments. The extracted DNAs for BFM of Joyo plant and secondary cultivated microbial consortia (cultured for one and three weeks in mPYM with and without carriers) were analyzed to determine the bacterial structure. The variable region three of 16S rRNA gene (about 190 bp) were amplified by PCR and applied for DGGE analysis as described previously (Chapter 2). It was observed in secondary cultivation that many amplified fragments were isolated in carrier containing mPYM compared with no carrier containing culture media (Fig. 5.4). However in the secondary cultivation, the isolated fragments were

observed almost similar in one and three weeks incubated mPYM, but the bacterial consortia were observed higher in carrier containing mPYM (Fig. 5.4). In this cultivation, the Mn-oxidizing microbial consortia harvested from unsupported primary cultivation were inoculated for Mn(II) removal in mPYM. This result showed that in carrier affect to increase the microbial consortia in secondary cultivation for Mn(II) removal. The isolated fragments were excised and re-amplified to determine the nucleotide sequences using sequence analysis. In order to study the closest relatives, those obtained nucleotide sequences were then compared with the sequences of DNA data base by using BLAST analysis (Altschul *et al.*, 1990).

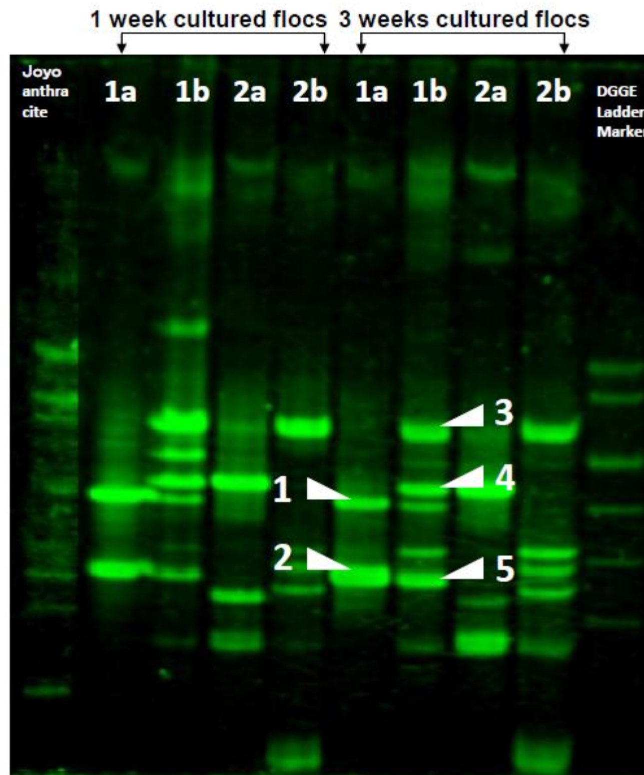

Fig. 5.4 – DGGE analysis using PCR-amplified 16S rRNA gene fragments. Indications: extracted DNA solution from, 1st lane - BFM of Joyo plant, lanes 2-9 indicate secondary cultivated microbial consortia using primary cultivated consortia. In the lanes, '1' represents incubated microbial consortia by inoculating unsupported primary cultivated consrotia and '2' for inoculation of supported primary cultivated microbial consortia. The lanes labeled 'a' indicate cultures without carriers, while 'b' indicate those with carriers. The band numbers with arrows indicate isolated 16S rRNA gene fragments used for sequencing analysis and closest relative study.

Table 5.1 – Similarities between 16S rRNA gene sequences for cultivated microbial consortia detected from DGGE analysis. The Mn-oxidizing microbial consortia were cultivated in mPYM culture media.

DGGE lane (3 weeks)	DNA band indication	Closest relative	Accession no.	Similarity (%)
1a	1	-	-	-
1a	2	*Mesorhizobium* sp.	[AY490108]	96
1b	3	*Leptothrix mobilis*	[X97071]	91
1b	4	*Hyphomicrobium* sp.	GQ131420]	91
1b	5	*Arthrobacter* sp.	[FM161611]	97

The phylogenetic study for three weeks incubated microbial flocs revealed the fragment '2' affiliated to *Mesorhizobium* with sequences similarities >95%. The isolated bands such as '3', '4' and '5' revealed their nucleotide sequences similar to *Leptothrix* (Ghiorse, 1984), *Hyphomicrobium* (Tyler, 1970), and *Arthrobacter*, respectively with >90% sequences similarities (Table 5.1). In Joyo biological filtration plant, microscopy showed the common iron bacteria like *Leptothrix* to oxidize the soluble Mn(II) into insoluble Mn oxides (Tamura *et al.*, 1999), but culture-independent 16S rRNA gene clone library analysis revealed *Hyphomicrobium* as the dominant MnOB (Thapa Chhetri *et al.*, 2013). In the mPYM, *Hyphomicrobium* related bacteria could be cultivated using the BFM of Joyo plant. Therefore, it was supposed that these *Hyphomicrobium* (Tyler, 1970) as well as *Leptothrix* (Ghiorse, 1984) could remove Mn(II) by oxidation in mPYM culture media. Similarly, it was also thought that those cultivated consortia further removed Mn(II) by combined biological and physicochemical oxidation and adsorption in mPYM. In contrast, other isolates showed ambiguous nucleotide sequences along with sequence analysis. It was perhaps due to cross contamination of isolated 16S rDNA fragments or no proper separation occurred in PCR-DGGE analysis.

In the embedded BFM of Joyo biological filtration plant, the dominant FeOB and MnOB such as *Sideroxydans* and *Hyphomicrobium* were detected via PCR-cloning analysis (Thapa Chhetri *et al.*, 2013). However, to estimate the common iron bacteria like *Leptothrix* related bacterial consortia in cultured mPYM, the quantitative real-time PCR analysis was carried out as described

previously. The relative quantity of total bacteria and *Leptothrix* related bacterial 16S rRNA gene copies were estimated in the extracted DNA solution for BFM of Joyo plant and cultivated microbial consortia. Surprisingly, the number of amplified copies of 16S rRNA gene fragments was estimated the highest (7.35×10^7 copies/μL) in three weeks incubated carrier containing secondary cultivated consortia. This cultivation contained two weeks cultured microbial consortia that harvested from supported primary cultivation. On the other hand, the amplified *Leptothrix* related 16S rRNA gene copies were detected the highest (7.50×10^4 copies/μL) in number in the extracted DNA for BFM of Joyo plant (Table 5.2).

Table 5.2 – The estimation of total and *Leptothrix*-related bacterial 16S rRNA gene copies in BFM and incubated microbial consortia by real-time PCR.

		Unsupported primary enriched flocs		Supported primary enriched flocs	
	Joyo BFM	Secondary culture with no carrier	Secondary culture with carrier	Secondary culture with no carrier	Secondary culture with carrier
Total 16S rDNA(copies/μL)	1.00×10^5	2.25×10^7	4.64×10^7	4.54×10^7	7.35×10^7
Leptothrix related bacteria (copies/μL)	7.50×10^4	3.56×10^6	2.85×10^6	1.57×10^3	1.95×10^3

Interestingly, although mPYM culture media was demonstrated for the cultivation of *Hyphomicrobium* (Vandenabeele *et al.*, 1995), *Leptothrix* related bacteria were also cultivated simultaneously. But these *Leptothrix* related bacteria were not adequately cultivated in mPYM, however, about 10% of *Leptothrix* related 16S rRNA gene copies were estimated in the BFM of Joyo biological filtration plant in our previous analyses (Thapa Chhetri *et al.*, 2013). In this study, it was observed that the carrier did not affect *Leptothrix* related bacterial cultivation, despite the other MnOB cultivation might had been affected in mPYM. This study spurred some information on Mn-oxidizing microbial consortia cultivation and Mn(II) removal in carrier containing mPYM, although it requires to be studied the detail to investigate the complete mechanism for cultivation and Mn(II) removal in mPYM.

5.4 Conclusion

In this batch study, Mn(II) removal and the cultivation of Mn-oxidizing

microbial consortia was successfully carried out using the BFM of Joyo plant in mPYM. MnOB related *Letothrix* and *Hyphomicrobium* were isolated in the cultivated microbial consortia via PCR-DGGE analysis. In addition, cultured microbial consortia (2-mL; two weeks cultured in primary cultivation) removed >1.4 mg/L of Mn(II) ions from the mPYM culture media for four weeks incubation. It was observed that Mn(II) ions removal and bacterial consortia were slightly higher in carrier (sterilized uncoated anthracites and sea sand) containing mPYM, whereas adequate Mn(II) removal was not observed in absence of any carriers. However, the effect of carrier on cultivation of Mn-oxidizing microbial consortia was observed via DGGE analysis (Fig. 5.4), but its effect was not detected for the cultivation of *Leptothrix* by real-time PCR analysis (Table 5.2).

5.5 Acknowledgement

We are grateful to T. Tamura of Joyo City Water Supply and Sewerage Department for providing us samples of biofilter media and giving us relevant information about the plant. We are thankful to M. Kondo of Instrumental Analysis Center, Yokohama National University.

CHAPTER 6

Simultaneous removal of Mn and As by the cultured microbial consortia, and analysis of their bacterial diversities

Abstract

The soil and groundwater containing Mn oxidizing bacteria (MnOB) that embedded on the biological filter media (BFM) in biological water filtration plant were cultivated in mPYM [modified amount of peptone-0.25 g/L, yeast extract-0.025 g/L and manganese (Mn(II))-30 mg/L] culture media. In the cultivated microbial flocs, active biogenic Mn oxides were developed, which they were further applied for the simultaneous removal of Mn and arsenic (As(III/V)). Those flocs removed >97% and >75% of dissolved As(III) and As(V) ions respectively from 500 μg/L of initial concentration indifferently after three weeks incubation. However, the modest concentration of Mn(II) was removed in simultaneous removal analysis in mPYM. The biological structures in the cultivated microbial flocs were revealed via PCR-DGGE analysis by using PCR amplified 16S rDNAs. MnOB and As-oxidizing bacteria (AsOB) related bacterial consortia were not detected in the isolated fragments. In contrast, quantitative real-time PCR analysis showed heavy growth of MnOB like *Leptothrix* and *Hyphomicrobium* in enriched microbial flocs. The *aoxB* gene that encodes As-oxidase revealed the clones related to *Bosea, Agrobacterium*, *Sinorhizobium* via clone library analysis.

Keywords: biological filter media, Mn-oxidizing bacteria, As removal, 16S rRNA gene

6.1 Introduction

Biological removal of reduced inorganic metal ions such as Fe and Mn from contaminated raw groundwater is executed in biological filtration plant (Mouchet, 1992). In addition to the Fe and Mn, soluble toxic heavy metal such as arsenic (As) oxyanions occurs in raw groundwater, which is being an effective problem in many countries in the world (Sogaard *et al.*, 2001). However, briefly, different physicochemical conventional methods such as coagulation and filtration, ion exchange, membrane and adsorption technologies are used to treat dissolved metal ions. These conventional treatment methods are more costly, create problems in sludge generation and disposal, render maintenance problems during treatment and provide

not completely treated water after treatment. Although the biological filtration method is a useful, cost-effective and eco-friend system to remove Fe and Mn from the groundwater (Mouchet, 1992), the biological method for As removal from the raw groundwater is under study in the world. To remove the soluble Fe and Mn, the biological oxidation and precipitation technique is almost 1000 time faster than physicochemical methods to remove from water environment (Sogaard *et al.*, 2001). In the biological method, long period filtration of natural groundwater coats the filter media in the filter bed with biofilms containing metal-oxide rich biomass (Mouchet, 1992; Casiot *et al.*, 2006; Sahabi *et al.*, 2009a). These naturally coated filter media are the so-called biological filter media (BFM), reactive for simultaneous removal of Mn and Fe (Mouchet, 1992; Tebo *et al.*, 2004; Sahabi *et al.*, 2009a). But Sahabi et al. (2009a and b) have reported the characteristics of BFM and their removal kinetics and efficiencies for the dissolved Mn(II) and As(III/V)) in water environment (Sahabi *et al.*, 2009a and 2009b).

It is considered in the biofilms on the BFM that the various bacterial diversities embedded and participated for the removal of Fe and Mn from the raw groundwater. In order to oxidize Fe and Mn, many FeOB and MnOB has been demonstrated via microscopy (Czekella *et al.*, 1985; Mouchet, 1992; Pacini *et al.*, 2005). But some bacteria such as *Leptothrix* (Siering & Ghirose, 1996), *Pedomicrobium* (Gebers, 1981), *Hyphomicrobium* (Tyler, 1970), *Pseudomonas* (Jung *et al.*, 1979), *Bacillus* (Tebo *et al.*, 2004) were demonstrated as MnOB. These bacteria are responsible for the enzymatic oxidation of Mn(II) so that very reactive biogenic Mn oxides can be deposited. In biological removal process, the biogenic Mn oxides have been also reported to remove the dissolved As(III/V) (Katsoyiannis and Zouboulish, 2004 and 2006). In addition, these biogenic oxides are also considered as prominent oxides for autocatalytic oxidation of dissolved Mn(II) (Tebo *et al.*, 2004; Sahabi *et al.*, 2009a). However, the simultaneous removal study for dissolved As, Fe and Mn ions was carried out to produce potable drinking water (Katsoyiannis *et al.*, 2004; Casiot *et al.*, 2006; Fujikawa *et al.*, 2010), the complete removal mechanisms for these metal ions is still unclear.

However, the characteristics of BFM of Joyo biological filtration plant and the bacterial diversities involved in the plants were already studied (Sahabi *et al.*, 2009a; Thapa Chhetri *et al.*, 2013), the cultivation of Mn-oxidizing bacterial consortia and their application on the removal of Mn and As has

not been studied yet. However, *Hyphomicrobium* was reported as dominant Mn-oxidizing bacteria (MnOB) via 16S rRNA clone library analysis (Thapa Chhetri *et al.*, 2013), their culturing procedures have been studied using the sand filter media of Fe and Mn removal bioreactor (Vandenabeele *et al.*, 1995). Therefore, the MnOB such as *Hyphomicrobium* were considered to be cultured to harvest adequate microbial consortia and Mn oxides using the BFM of Joyo plant. These cultivated microbial consortia were thought to apply for the simultaneous removal of As(III/V) and Mn(II) in batch experiments. The dissolved metal ions such as Mn(II) and As(III/V) removal using these cultivated microbial consortia were studied along with its biological structure study using PCR amplified 16S rRNA gene.. To investigate the biological removal mechanism for As, this study would spur the scant information on the biological removal of As. In addition, it would be more applicable for natural environmental as well as human impacted ecological bioremediation particularly for the removal of As.

6.2 Materials and methods

6.2.1 Samples

The sampling site and freshly collected BFM anthracites details were the same as mentioned in chapter 2 except sampling date (Thapa Chhetri *et al.*, 2013). This biological filtration plant is well-established system for drinking water production in Joyo City, Kyoto Prefecture, Japan (Sahabi *et al.*, 2009a; Thapa Chhetri *et al.*, 2013). Briefly, the unit is a medium speed filtration system with a dual filter media (anthracite on top of sand). The raw (uncoated) anthracite used in the plant was also kindly provided by the plant personnel in Joyo city.

6.2.2 Cultivation of Mn oxidizing microbial consortia

All batch experiments were carried out into 500-mL Erlenmeyer flasks to cultivate Mn-oxidizing microorganisms to harvest the microbial consortia having massive biogenic Mn oxides. Those microbial flocs were considered for the removal study of Mn & As ions. Each 500-mL-flask contained 100-mL of modified PYM (mPYM) culture media (peptone: 0.25 g/L, yeast extract: 0.025 g/L, final Mn^{2+}: 30 mg/L using $MnSO_4.5H_2O$ solution) (Ehrlich *et al.*, 1985) with HEPES-NaOH buffer (pH 7.4 ± 0.1) of final concentration of 15 mM. Additional 0.5 g (wet weight) of freshly collected BFM was skipped into the same flasks. Each test was run in duplicate. Before starting batch experiments, reaction mixtures of culture media except Mn(II) solution and

BFM were sterilized by autoclaving. However, Mn(II) solution was filter sterilized before skipping into sterilized culture medium to avoid the chemical precipitation and complexation with organics in the culture medium. In this study, the reaction mixtures were prepared with and without sodium azide solution (final concentration of 15 mM) (Rosson *et al.*, 1984), and they were considered as abiotic and biotic culture media, respectively, in all cultivation batch studies.

Each 500-mL flasks contained 100-mL of mPYM culture media as described above. The reaction mixtures were incubated at 25°C with gentle shaking of 100 rpm for two weeks. At predetermined time interval, 5-mL aliquots were withdrawn from the reaction mixture and centrifuged at 7,000 rpm, 25°C for 3 min to separate supernatant and microbial flocs. Thereafter, supernatant was filtered through 0.2 μm syringe filter (Millex GP, Millipore, MA, USA) to avoid microbial cells and biomass that present in the culture media. Filtrates were then analyzed for Mn(II) concentration using AA-6650F atomic absorption spectrophotometer (AAS) (Shimadzu, Kyoto, Japan).

Two weeks cultivated microbial consortia (only in biotic culture media) were collected in 50-mL tubes (Sumilon, Sumitomo Bakelite Co., Ltd., Japan) and then centrifuged at 7,000 rpm, 25 °C for 3 min. After centrifugation, about 30-mL of cultured microbial pellets were collected after discarding the supernatants indifferently. Ultimately, those harvested microbial consortia were mixed well and supposed as working microbial consortia for the simultaneous removal of Mn(II) and As(III/V) in the mPYM. In addition, the biological structures in those cultivated consortia were studied by microscopic analyses. On the other hand, a small portion of those primary cultured microbial consortia were preserved at -80°C to study the biological structure using molecular biological analyses.

To study the removal performance of those cultured microbial consortia for the Mn(II) and As(III/V), the reaction mixtures were prepared as mentioned above. In each flask, alternative 0.5 g (dried weight) of sterilized uncoated anthracites was added instead of Joyo's freshly collected BFM. Those uncoated anthracites were supposed to be excellent carrier, which would make feasible shaking and aeration for microorganisms in the reaction mixture during cultivation and metal ions removal. Before starting secondary enrichments, all reaction mixtures except Mn(II), As (As(III) and

As(V)) solutions and working microbial consortia were also sterilized by autoclaving. All the tests in batch experiments were set in duplicates for abiotic and biotic culture media as mentioned above. In each flasks, additional 2-mL of working microbial consortia (unsterilized) were inoculated. The incubation conditions were set the same as previously described in primary cultivation except extended three weeks incubation period.

Dissolved Mn(II) removal performance was studied in mPYM for working microbial consortia. Incubation conditions were maintained as mentioned before. 5-mL of aliquots was withdrawn at predetermined time and centrifuged at 7000 rpm, 25°C for 3 min before filtering through 0.2 μm syringe filter (Millex GP, Millipore, MA, USA). The filtrates were analyzed for Mn(II) concentration as described above. Additional 2-mL aliquots from each one and three weeks incubated culture media were withdrawn and then preserved at -80°C to analyze microbial analyses.

For the simultaneous removal of Mn(II) and As (As(III) and As(V)) using cultured microbial consortia, two sets of experiments were designed using the same reaction mixtures as described in Mn(II) removal analysis. Additionally, 500 μg/L of final concentration of filter sterilized As(III) and As(V) solutions were skipped in each of them separately. Except As (As(III) and As(V)) solutions, all the composition and incubation conditions were exactly the same as described above in Mn(II) removal analysis. At predetermined time interval, 5-mL aliquots were withdrawn from the cultured media and centrifuged as mentioned above. Thereafter, supernatant was filtered through 0.2 μm syringe filter (Millex GP, Millipore, MA, USA) and divided into two halves to analyze Mn(II) and As (As(III) and As(V)) concentrations, respectively. Additional 2-mL aliquots were withdrawn from each cultured consortia for one and three weeks and then preserved at -80°C to analyze microbial analyses. In this analysis, Mn(II) concentration was analyzed using AAS (Shimadzu, Kyoto, Japan). On the other hand, As (As(III) and As(V)) concentration was analyzed by the molybdenum blue spectrophotometric method of Johnson and Pilson (Johnson & Pilson, 1972), as optimized by Dhar et al (Dhar *et al.*, 2004). The method is based on the formation of a blue complex between As(V) and molybdenum under acidic conditions, in the presence of antimony as catalyst. Phosphate when present in the solution also reacts with the same

stoichiometry, thus, a reducing reagent was used to lower the oxidation state of arsenic from As(V) to As(III). Since As(III) does not react with molybdenum, the difference between the "untreated" and the "reduced" aliquots gives the absorbance due to As(V) at 880 nm (Hitachi U-100 spectrophotometer, Japan). The concentration of As(III) was determined by treating a third aliquot with an oxidizing agent (KIO_3), which oxidizes As(III) to As(V) and the concentration of As(III) was calculated by difference.

6.2.3 Confirmation of Mn oxides production

In order to confirm Mn oxides formation in the cultured culture media, qualitative colorimetric analysis was carried out using leuco-crystal violet (LCV) staining reagents (Dickinson *et al.*, 1996). The reaction between Mn(IV) and LCV staining reagent reveal the violet color at end product.

6.2.4 Microscopic analyses

To study the biological structures that enriched in the cultivated microbial flocs, microscopic analyses were carried out using each microbial flocs samples of primary cultivated (two week enriched) and secondary cultured (three week enriched). Cultured microbial consortia were analyzed under phase-contrast microscopy (Axioskop, ZEISS, Germany) and scanning electron microscopy (SEM) (JSM-6700-NT, Nihon Densi, Japan). This analysis was performed to study morphological characteristics of microbial flocs and microbial structures.

6.2.5 DNA extraction

DNA was extracted using 0.5 g of BFM, and 0.25 g of enriched flocs (primary cultivation - two week cultured and secondary cultivation - one and three weeks incubated) samples using FastDNA SPIN Kit for soil (MP Biochemicals, Solon, OH, USA). The DNA extraction and preservation were performed as described in chapter 2 (Thapa chhetri *et al.*, 2013).

6.2.6 Polymerase chain reaction

In order to study the molecular biological structures using those extracted DNA solutions for all samples, standard and touchdown PCR were carried out. The PCR procedures were followed the same as mentioned in chapter 2 (Thapa Chhetri *et al.*, 2013). Arsenic oxidizing enzyme encoding *aoxB* gene that present in the extracted DNA solutions for BFM and cultivated microbial flocs samples were amplified by PCR. The details of the

experimental procedures were as mentioned in chapter 3.

6.2.7 Denaturing gradient gel electrophoresis

To analyze the comparative biological diversities and their closest relatives in cultivated microbial flocs samples, parallel DGGE analysis (Muyzer *et al.*, 1993) was performed using PCR-amplified 16S rDNAs. In this analysis, the procedures were obeyed as mentioned in chapter 5.

6.2.8 Real-time PCR

The estimation of the relative quantity of 16S rRNA gene closely related to MnOB such as *Leptothrix* (Ghiorse, 1984) in BFM and cultured samples was studied as described in chapter 2 (Thapa Chhetri *et al.*, 2013). In addition, the relative estimation of *Hyphomicrobium* (Tyler, 1970) related 16S rRNA gene copies in total bacterial 16S rDNAs were also analyzed by the real-time PCR. To estimate *Hyphomicrobium* related 16S rRNA genes, the DNA plasmid of clone Jy04A47 was detected, which harbored 16S DNA related to *Hyphomicrobium* (Thapa Chhetri *et al.*, 2013). The quantification procedures were followed the same as mentioned above.

6.2.9 Clone library analysis

In order to construct the clone library using PCR-amplified *aoxB* gene, the extracted total genomes for BFM and cultivated microbial flocs (obtained from primary enrichment) samples were amplified by PCR using the primer set aoxBM1-2F and aoxBM3-2R (Quéméneur *et al.*, 2008), and TaKaRa *Ex Taq* polymerase (Takara, Bio, Japan) as described previously. Those amplified fragments were introduced into the pCR4-TOPO vector using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The phylogenetic analysis of constructed clones were studied as same as mentioned before in chapter 2 (Thapa Chhetri *et al.*, 2013).

6.3 Results and discussion

6.3.1 Simultaneous removal of Mn and As by cultured microbial consortia

6.3.1.1 Primary culture of Mn oxidizing microbial consortia and Mn^{2+} removal

In Joyo biological filtration plant (Tamura *et al.*, 1999), the Fe and Mn oxides-rich biomass spontaneously embed the filter media and produce the layers of biofilms (Sahabi *et al.*, 2009a). In the biofilms, different bacterial

diversities including FeOB and MnOB related bacteria were entrapped on the BFM of Joyo plant. *Hyphomicrobium* related bacteria have been reported as the dominant MnOB in the BFM of Joyo plant (Thapa Chhetri *et al.*, 2013). The naturally occurring bacteria oxidize raw groundwater containing dissolved Fe(II) and Mn(II) to produce insoluble Fe and Mn oxides (Mouchet, 1992; Tebo *et al.*, 2004; Pacini *et al.*, 2005). Vandenabeele et al. (1995) reported the culturing of MnOB in batch experiment using the sand filter media of the biological filtration reactor. In this study, Mn-oxidizing microbial consortia were cultured using the BFM of Joyo plant. The biogenic metal oxides particularly Mn oxides facilitate the oxidation and removal of dissolved Mn and As ions in aquatic environment (Katsoyiannis *et al.*, 2004; Tebo *et al.*, 2004; Casiot *et al.*, 2006; Sahabi *et al.*, 2009a and 2009b). It was hypothesized that these cultivated microbial consortia would be more prominent for simultaneous removal of Mn(II) and As(III/V) ions.

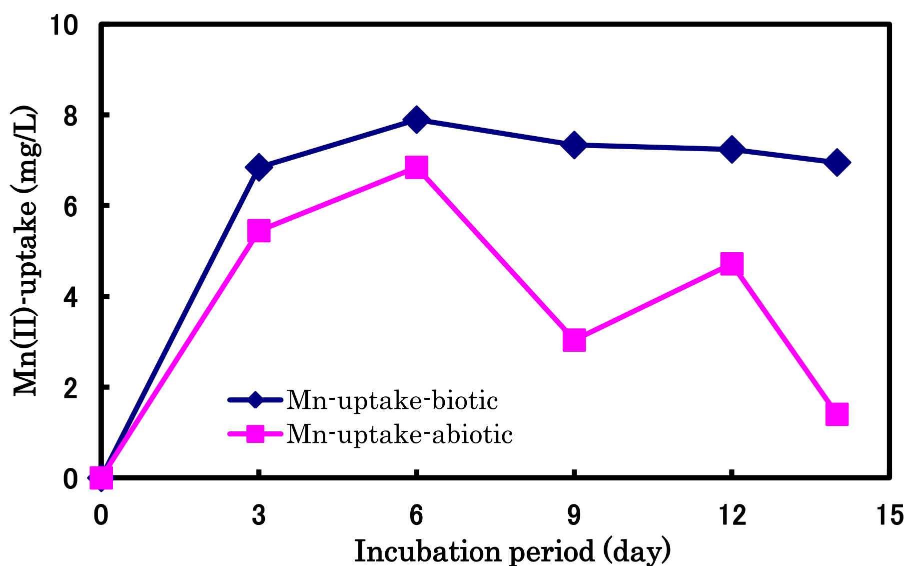

Fig. 6.1 – Mn (II) uptake during Mn-oxidizing microbial consortia cultivation using BFM of Joyo plant in mPYM, the cultivation conditions were set at 25°C, 100 rpm for two weeks.

In the primary culturing (two weeks cultivation), heavy growth of microbial cells and Mn oxides formation were taken place only in biotic mPYM culture media. But in abiotic culture media, microbial growth was not observed and Mn oxides formation was insignificantly shown. It was considered the Mn oxides spontaneously embedded onto the BFM of Joyo plant could autocatalytically oxidize Mn(II) (Sahabi *et al.*, 2009a). In Mn mPYM, 6.9 and

1.4 mg/L of Mn(II) ions were removed respectively from biotic and abiotic mPYM using 0.5 g of BFM for two weeks incubation (Fig. 6.1). Microbial enzymatic as well as autocatalytic oxidation remove dissolved Mn (II) (Nealson *et al.*, 1988; Sahabi *et al.*, 2009a), and additional adsorption is also another access of removal (Tebo *et al.*, 2004). The possible combined oxidation and adsorption activities were taken place for the Mn removal in Mn oxidizing microbial consortia cultivation in biotic mPYM using BFM of Joyo plant. In our study, rapid depletion of dissolved Mn(II) ions was detected at initial incubation period up to 6 days in both biotic and abiotic reaction mixtures. It was supposed that there might had been occurred expeditious adsorption of Mn(II) on aged BFM, however, the complete mechanism is not well-known. Those Mn(II) removals were subsequently decreased along with incubation period. Ultimately, the removal was observed higher in biotic culture media compared with abiotic mPYM. It was because both biological and physicochemical activities were possible to oxidize soluble Mn(II) in biotic culture, whereas only the physicochemical removal was possible in abiotic reaction mixture due to microbial inhibition using sodium azide (Rosson *et al.*, 1984). But, it needs to be studied the details to investigate the complete mechanism.

6.3.1.2 Secondary cultures for the simultaneous removal of Mn^{2+} and As(III) or As(V)

To study the Mn(II) removal using the cultured microbial consortia, harvested microbial consortia obtained from primary cultivation was used to remove Mn(II) ions of 30 mg/L of initial concentration. 2-mL of working microbial consortia (primarily cultured) removed <1.0 mg/L i.e. 0.49 mg/L (1.63%) from initial Mn(II) in biotic culture, whereas 0.09 mg/L (0.3%) was removed in abiotic mPYM after three weeks incubation (Fig. 6.2). However, our previous study (chapter 5) showed 1.4 to 3.8 mg/L of Mn(II) removal in the mPYM culture media in presence and/or absence of carrier. But in this study, the Mn(II) removal concentration was observed very low, although uncoated sterilized anthracites were used to make feasible shaking and aeration in the mPYM. But the Mn(II) removal differences between this and previous study could not be known clearly.

The microbial enzymatic and autocatalytic oxidation of Mn(II) by Mn oxides have been already reported (Tebo *et al.*, 2004; Miyata *et al.*, 2007; Sahabi *et al.*, 2009a). It was supposed in our study that the autocatalytic oxidation

removed very few Mn(II) ions in abiotic mPYM, whereas both enzymatic and autocatalytic oxidation of Mn(II) were possible in biotic culture media to remove more Mn(II) ions (Fig. 6.2). It is because, both microbial activities were not inhibited in biotic mPYM, but the microbial activities were inhibited using sodium azide (15 mM) in abiotic culture media in Mn(II) removal study (without using any As ions). The strong biological activities removed the higher Mn(II) ions concentration in biotic mPYM compared with abiotic culture media (Fig. 6.2). In addition to the oxidation, Mn(II) ions are removed by adsorption on microbial cell-surface and biogenic Mn-oxides (Mn(IV)) (Stone & Morgan, 1984; Nelson *et al.*, 1988; Tebo *et al.*, 2004). The adsorption might had been occurred in mPYM during Mn(II) removal, however, the complete removal mechanism is still unclear.

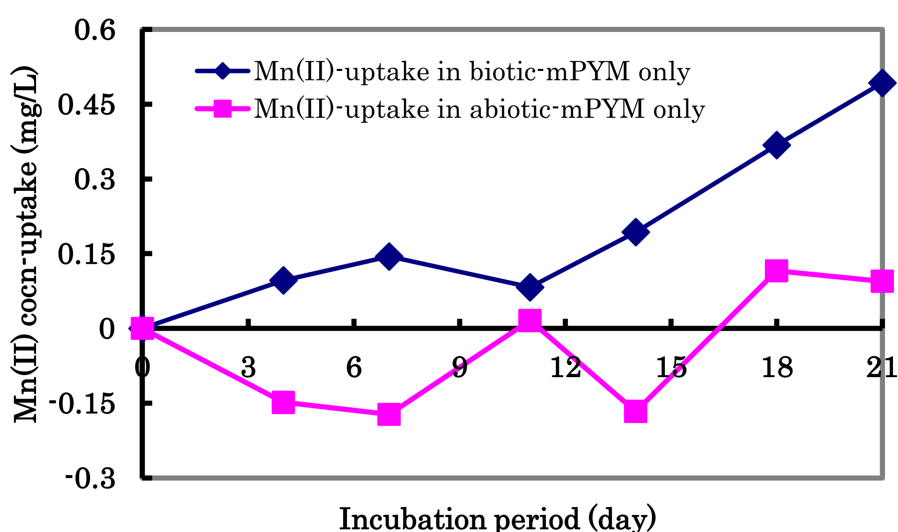

Fig. 6.2 – Mn(II)-uptake using the cultivated Mn-oxidizing microbial consortia in mPYM culture media in absence of As(III/V), the cultivation was carried out at 25°C, 100 rpm for three weeks.

But on the other hand in simultaneous removal study for Mn(II) and As(III), the dissolved As(III) ions were removed both in biotic and abiotic mPYM using primarily cultured microbial consortia. These carcinogenic As(III) ions were removed relatively higher in biotic condition compared with abiotic culture media (Fig. 6.3 (A)). About 97% of dissolved As(III) ions were removed in biotic mPYM from initial concentration of 570 μg/L, whereas only 45% of As(III) ions removal was observed in abiotic culture media after three weeks incubation (Fig. 6.3(A)). It was considered that the combined activities of cultured Mn-oxidizing microorganisms and active biogenic Mn oxides or

biomass (developed in primary cultivation) increased the As(III) removal in biotic mPYM because the microbial inhibition in abiotic culture media revealed comparatively lower As(IIII) ions removal.

In the same simultaneous removal study of Mn(II) and As(III), it was observed that >50% of As(III) ions were oxidized into As(V) in biotic mPYM by the cultured Mn oxidizing microbial consortia (Fig. 6.3 (C)). It has been reported that the As(V) ions are more stable and easier to remove via adsorption onto the metal oxides (Katsoyiannis and Zouboulis, 2004). In our study, although >50% of dissolved As(III) ions were removed by the oxidation, the remaining As(III) ions should be removed via adsorption. But in abiotic mPYM, <50% of As(III) removal were observed without any As(III) oxidation (Fig. 6.3 (C). It means, only the biogenic metal oxides obtained from primary cultivation were possibly prominent for the removal of As(III) via adsorption. Interestingly, however, the rapid removal of As(III) by one week incubation was alternatively decreased into stable removal from one to three weeks incubation in abiotic culture media, subsequent removal of As(III) was observed in biotic mPYM by three weeks incubation (Fig. 6.3 (A)). The As(III) oxidation is carried out by microbial enzymatic activity and Mn oxides to less toxic As(V), which they are ultimately removed by adsorption (Scott & Morgan, 1995; Oscarson *et al.*, 1981; Tebo *et al.*, 2004; Katsoyiannis and Zouboulis, 2006; Sahabi *et al.*, 2009a). In our biotic culture media, the highest As(V) formation (341.1 μg/L) was detected in eighteen days incubation (Fig. 6.3 (C)), while in abiotic mPYM the As(V) (124.9 μg/L) formation was detected the highest in one week incubation (Fig. 6.3 (C)).

Alternatively, the Mn(II) ions were released in the simultaneous removal analysis for Mn(II) and As(III). Scott and Morgan (1995) have reported that Mn(II) ions are released during As(III) oxidation by Mn oxides. Interestingly, few Mn(II) ions (0.26 mg/L) were ultimately removed in biotic mPYM, but those ions were released in spite of removal in abiotic mPYM after three weeks incubation (Fig. 6.3(B)). It was considered that microbial activities influenced to remove modest concentration of released Mn(II) in biotic culture media, while that was not detected in abiotic mPYM because the microbial activities were inhibited using sodium azide (15 mM). In this simultaneous study, the imbalanced initial concentration of Mn(II) (30 mg/L) and As(III) (500 μg/L) were ultimately removed, although more Mn(II) ions were expected to be removed by cultured microbial consortia in mPYM

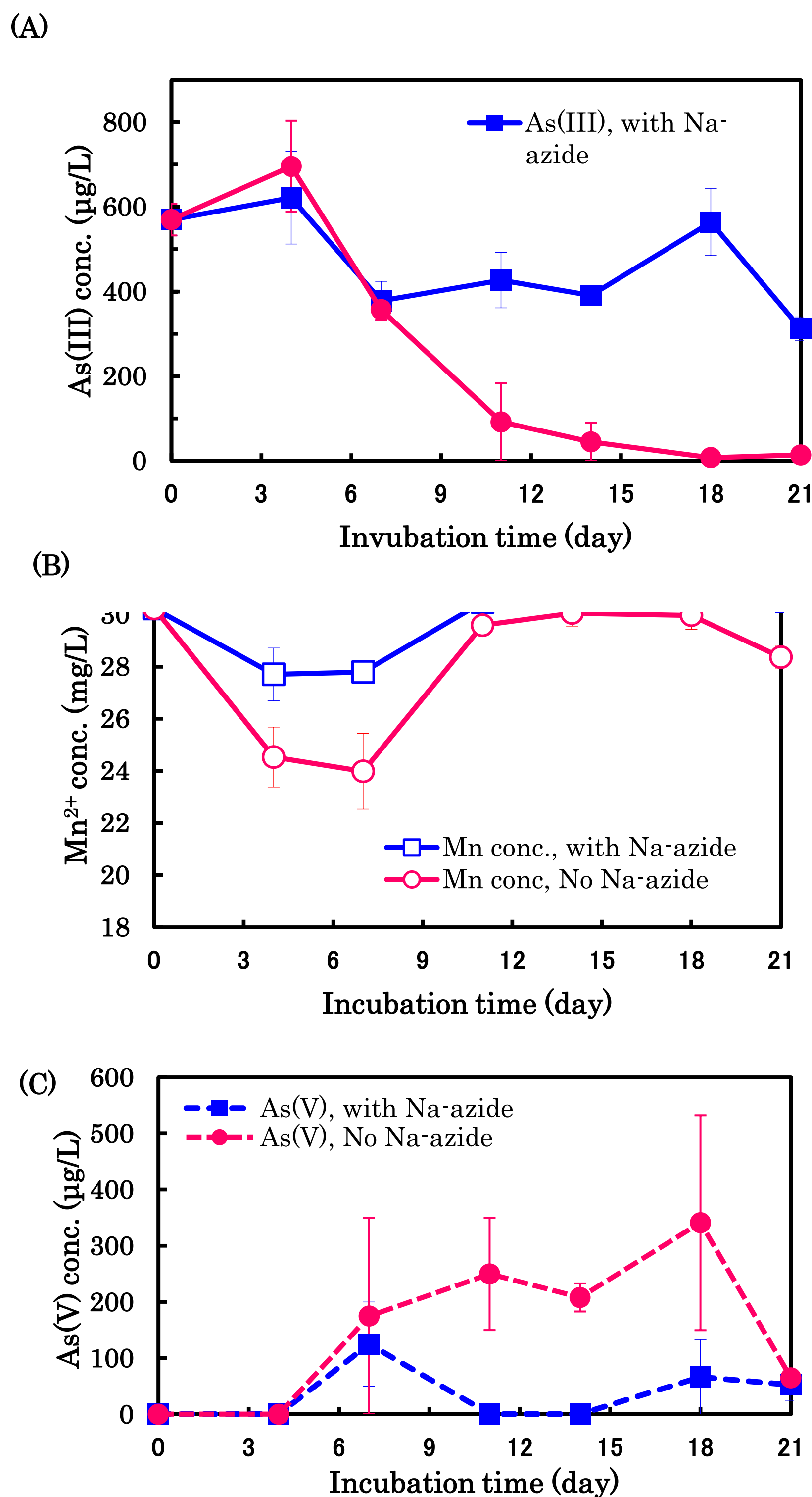

Fig. 6.3 – Simultaneous removal of Mn(II) and As(III) in mPYM using cultured Mn-oxidizing consortia. (A) As(III) removal, (B) Mn(II) removal and (C) deposition of oxidized As(V) in batch study, physical parameters for incubation were set at at 25°C, 100 rpm for three weeks.

culture media. Some entities might had interfered in excess Mn(II) ions removal compared with As(III) ions in both biotic and abiotic mPYM culture media, however, further study is required to be analyzed to know in details.

In another set of experimental for the simultaneous removal of Mn(II) and As(V), >75% of dissolved As(V) ions were removed using primary cultivated Mn-oxidizing microbial consortia in mPYM (Fig. 6.4 (A)). In the biological removal analysis for As(V), the sorption and precipitation procedures have been reported to remove from water environment (Katsoyiannis *et al.*, 2004; Casiot *et al.*, 2006). In the biotic mPYM, it was thought that dissolved As(V) ions were adsorbed onto the biomass as well as microbial cell surfaces. It was because the biological removal of As(V) have been already reported via adsorptions (Katsoyiannis and Zouboulis, 2006; Sahabi *et al.*, 2009a).

The As(V) removal efficiencies of cultivated Mn-oxidizing microbial consortia were observed quite similar in both biotic and abiotic mPYM culture media (Fig. 6.4 (A)). The sorption of As(V) on microbial cell-surface (live and/or dead) (Tebo *et al.*, 2004) and/or biogenic Mn oxides (Katsoyiannis *et al.*, 2004; Sahabi *et al.*, 2009a) have been demonstrated as the prevalent biological removal methods. The phenomenon of retention of cations in aquatic environment by microbial cells has been termed as biosorption (Santini *et al.*, 2004).

Interestingly, Mn(II) ions were readily removed in initial incubation period (up to one week) in this simultaneous removal for the Mn(II) and As(V), however, the ultimate removal of Mn(II) ions (1.06 mg/L in biotic and 1.54 mg/L in abiotic) were observed very low both in biotic and abiotic mPYM. The rapid removal of Mn(II) was perhaps due to sorption by cultivated microbial consortia (two week cultured primary cultivation), however, it requires to be studied more detail to know the complete mechanism. The complexion of Mn and As solid can be removed by deposition and precipitation process in various environmental conditions. In our biotic study, the Mn(II) ions were supposed subsequently released in to mPYM culture media, which were facilitated to be oxidized (by a combination of enzymatic activities and biogenic Mn oxides) and/or again adsorbed in biotic culture media. However in abiotic mPYM, the released Mn(II) ions were increased because of microbial activities inhibition by sodium azide (Rosson *et al.*, 1984).

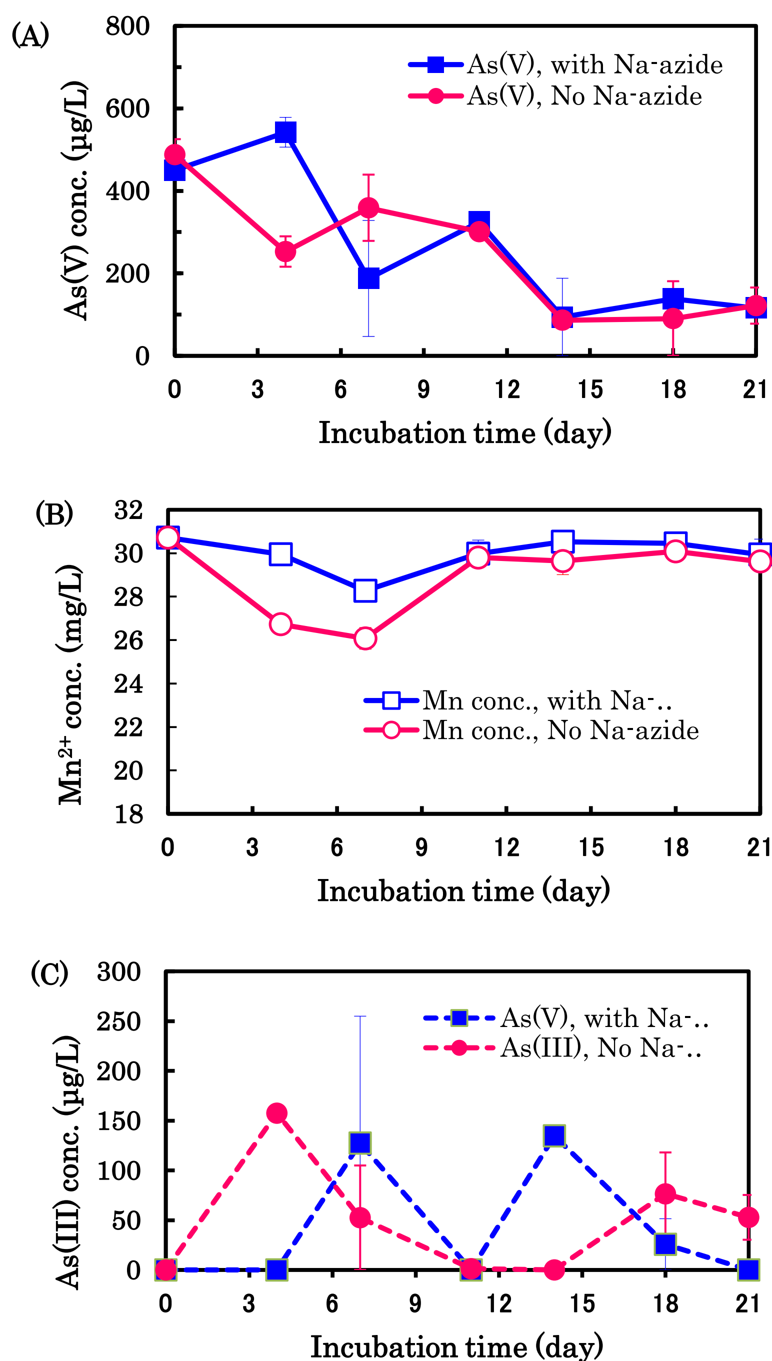

Fig. 6.4 – Simultaneous removal of Mn(II) and As(V) in mPYM using cultured Mn-oxidizing consortia. (A) As(V) removal, (B) Mn(II) removal and (C) releasing of As(III) in batch study, physical parameters for incubation were set at at 25°C, 100 rpm for three weeks.

Therefore, only the autocatalytic oxidation (inoculated primary cultivated

consortia containing Mn oxides) and adsorption (on to the dead microbial flocs) were possible to remove Mn(II) ions removal in abiotic mPYM. However, Mn(II) ions removal was not observed very high in the simultaneous removal analysis for Mn(II) and As(V) ions, the adequate As(V) ions (>110μg/L) were removed by cultivated microbial consortia in mPYM culture media.

Although the initial concentration of Mn(II) ions (30 mg/L) were comparatively higher than As (III/V) (500 μg/L) in simultaneous removal analysis for Mn(II) and As(III/V) in mPYM, Mn(II) removal concentration was observed very low. But the primary cultured Mn-oxidizing microbial consortia could remove adequate Mn(II) ions (>3 mg/L) from mPYM in our previous batch study (chapter 5). However, Mn oxidizing microbial consortia removed adequate As(III/V) ions (>97% of As(III) and >75% of As(V) from 500 μg/L of initial concentration) from mPYM for three weeks incubation. It is also considered that these reactive cultured microbial flocs has enough efficiency to remove dissolved As(III/V) ions from raw groundwater in natural environment as well as engineered biological filtration plant, but the various studies are required to be analyzed in details.

6.3.2 Confirmation of Mn oxide production

In biotic mPYM, heavy growth of microbial consortia was observed with dark brown particles during culturing the Mn-oxidizing microbial consortia using BFM of Joyo plant. The microbial cell growth and formation of particles were revealed after two days incubation in biotic reaction media, whereas no microbial growth was occurred in abiotic mPYM during that period. Those dark brown particles were tested, whether they were Mn oxides or not using leuco-crystal violet (LCV) staining reagent (Dickinson *et al.*, 1996). Two weeks cultivated microbial consortia with blackish particles turned into dark violet color with LCV staining reagent for biotic culture media, while slightly turned violet color was observed for the incubated sample of abiotic culture media. This results confirmed the formation of Mn oxides (Mn(IV)) in mPYM culture media comparatively higher in biotic rather than abiotic mPYM (data not shown).

After inoculation of primary cultivated microbial consortia (working microbial consortia sample) in the simultaneous removal batch experiments

for Mn(II) and As(III/V), Mn oxides formation were observed by the same staining analysis as described above (data not shown). It is considered that during removal of Mn(II), the Mn-oxidizing microbial consortia could oxidize Mn(II) into insoluble blackish particles i.e. Mn(IV) in the in mPYM by microbial enzymatic and /or autocatalytic activity of biogenic Mn oxides.

6.3.3 Microscopic appearance of cultured microbial consortia

To study the microbial morphology and characteristics of metal oxides in cultivated microbial consortia using BFM of Joyo in mPYM (particularly for biotic mPYM), the primary cultivated microbial consortia (two weeks cultured) and the secondary cultured microbial consortia (one and three weeks cultured during the simultaneous removal of Mn(II) and As(III/V)) were used for microscopy. In the mPYM, the Mn-oxidizing microorganisms could oxidize Mn(II) for their energy and insoluble dark brown particles were produced as the by-product. Phase-contrast microscopy revealed the maximum dark brownish biogenic Mn oxides in the biotic mPYM, which were depicted in Fig. 6.5 (A), (C), (E). These oxides formation were excessively observed in primary cultivation of Mn-oxidizing microbial consortia, whereas few Mn oxides were observed in As(III/V) containing secondary cultured mPYM. Besides these biogenic Mn oxides, heavy growth of elongated, rod and oval-shaped microbial cells were observed in biotic mPYM via phase-contrast microscopy. But in the secondary cultures used for the simultaneous removal study for Mn(II) and As(III/V) revealed more longer rod-shaped microbial cells separately compared with the microbial cells those were cultivated in primary cultivated microbial consortia (Fig. 6.5).

In order to analyze the complete microbial cell morphology, cultured Mn-oxidizing microbial cells were observed under SEM. This analysis showed the cultured microbial cells having elongated, rod and oval shaped with irregular size and concave shape (Fig. 6.5 (B), (D) & (F)). Interestingly in the secondary cultured mPYM used for simultaneous removal of Mn(II) and As(III/V), it was observed that some microbial cells surfaces embedded with precipitates. Those precipitates were considered as the complexes substances formed after the reaction between Mn oxides and As(III/V), which were consequently adsorbed on the microbial cell surfaces (Fig. 6.5 (D) and (F)). But it is still unclear and requires to be studied in details. On the other hand, Katsoyiannis and Zouboulis (2004) have reported the As(V) removal by

the sorption on biogenic Mn oxides. In our batch analysis, As(III) ions were oxidized into As(V) and biogenic Mn oxides were formed during simultaneous removal of Mn(II) and As(III/V) as described previously. It was supposed those As(V) were subsequently removed via sorption on the Mn oxides and/or microbial cell surfaces (Fig. 6.5 (D) & (F)). The precipitates were observed higher on the microbial cell surfaces that cultured in mPYM with As(III) compared with As(V) containing culture media ions (Fig. 6.5 (D) and (F)), however, the detail is still unclear. Despite the lack of complete removal mechanism for Mn(II) and As(III/V) in simultaneous removal of these metal ions in mPYM the complex reaction should be occurred between dissolved As(III) and/or As(V) with the biogenic Mn oxides via different chemical or ionic reactions.

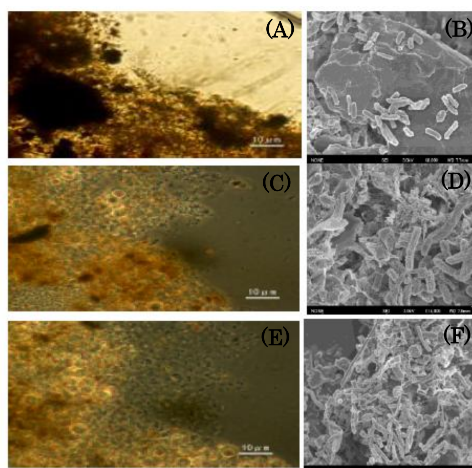

Fig. 6.5 – Microscopic analyses of cultured microbial consortia in mPYM. (A), (C) & (E) represent Phase-contrast microscopy and (B), (D) & (F) depict scanning electron microscopy (SEM) analyses. The indications, (A) & (B) were microbial flocs formed in primary cultivation using BFM of Joyo plant and cultured for two weeks, (C) & (D), and (E) & (F) were the cultured microbial consortia in secondary cultivation in mPYM with As(III) and As(V), respectively. (C) and (E) depict phase-contrast microscopy for one week cultivated secondary consortia and (D) and (F) represent SEM analysis for three weeks cultured consortia.

6.3.4 Bacterial diversities analyzed by PCR-DGGE

The biological structures in the cultured microbial consortia were studied using culture-independent molecular biological DGGE (Muyzer *et al.*, 1993). The amplified variable region 3 (about 190 base pairs) of 16S rRNA gene was

applied in DGGE analysis as described previously previously. The DNA solutions extracted from BFM of Joyo biological filtration plant, cultured microbial consortia obtained from primary and secondary cultivations were carried out to detect discrete biological structures. The parallel DGGE analysis revealed the isolated 16S rRNA gene fragments of various microbial bacterial diversities (Fig. 6.6).

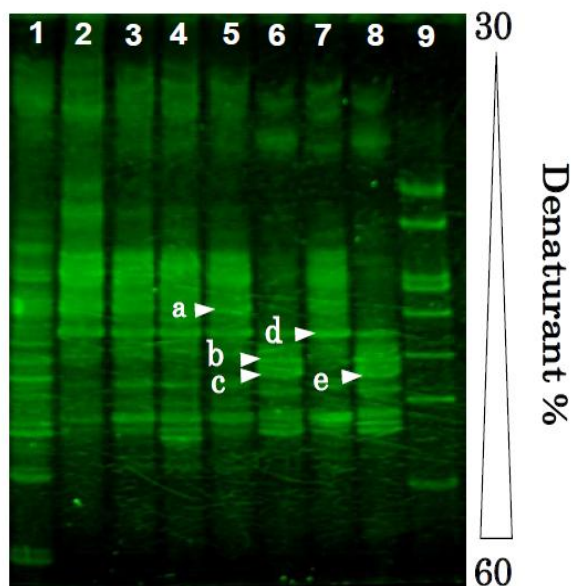

Fig. 6.6 – DGGE analysis of amplified 16S rRNA gene fragments for different samples. The lanes 2–8 indicate amplified 16S rRNA genes for microbial consortia cultured in mPYM. The individual lanes represent the amplified 16S rRNA gene for, 1 – BFM of Joyo biological filtration plant, 2 – primary cultivated microbial consorita (two weeks enriched) using BFM of Joyo plant, 3, 5, 7 – one week incubated secondary cultured microbial consortia at where primary cultivated consortia were inoculated in absence of As(III/V), with As(III) and with As(V), respectively, 4, 6, 8 – three weeks incubated secondary cultured microbial consortia that mentioned the same in lanes 3, 5 and 7, respectively, 9 – DGGE ladder marker (10 bands). The signals labeled a, b, c, d, and e, indicate the DNA fragments of which the sequences were determined.

In this study, the bacterial composition of Mn-oxidizing microbial consortia that cultured in mPYM for two weeks in primary cultivation was shown almost similar to those bacterial diversities for BFM of Joyo biological filtration plant. However, the amplified DNA fragments were observed lower

in number rather than that of original bacterial consortia of BFM of Joyo biological filtration plant. In secondary cultured microbial consortia that contained primary cultivated consortia in absence of As(III/V), similar bacterial diversities were detected in one and three weeks cultivations (Fig. 6.6). In addition, in the secondary cultured microbial consortia in presence of As(III) and As(V), indifferently, the isolated 16S rRNA gene fragments were revealed the similar to each other for one week cultivation, which were corresponding to those isolated fragments of secondary cultivation without As containing mPYM as described above (Fig. 6.6). Briefly, the cultured bacterial diversities in secondary cultivation for one week were almost similar to each other in mPYM whether there absence and/or presence of As(III/V) in the culture media. Interestingly, the amplified fragments isolated from secondary cultivated for three weeks incubation in mPYM in presence of As(III/V) revealed the lower in number compared with those previously described bacterial consortia, but quite similar to each other. This result indicates that dissolved As (III/V) definitely affects the existence and growth of microbial consortia in accordance to incubation period.

However, the closest relatives of isolated and excised DGGE fragments were studied by BLAST analysis after determination of nucleotide sequences. In one week cultured secondary cultivated consortia, the natural groundwater and soil containing common bacteria related consortia were isolated in As(III) containing mPYM. Phylogenetic analysis for isolated 16S rRNA gene fragment 'a' revealed the bacteria closed to *Bacillus* with high sequence similarities (100%) (Table 6.1). It has been reported that *Bacillus* sp. strain SG-1 is prominent for Mn(II) oxidation (van Waasbergen *et al.*, 1996). Two isolated fragments 'b' and 'c' obtained from three weeks cultured secondary cultivation revealed the bacteria closed to nitrogen fixing soil bacteria like *Mesorhizobium* and nitrite oxidizing bacteria (NOB) like *Nitrobacter*, respectively, with >97% sequences similarities. These bacteria related consortia were cultivated in presence of As(III) in mPYM. The amplified 16S rRNA gene fragment such as 'd' for one week cultured in secondary cultivation with As(V) exhibited the bacteria related to *Rhodoferax* with 99% of sequence similarities. It has been reported that *Rhodoferax ferrireducens* related bacteria possesses iron-reducing characteristics (Finneran *et al.*, 2003). In contrast, the isolated fragment like 'e' for the cultivated microbial consortia cultured for three weeks in secondary cultivation in presence of As(V) in mPYM, showed the sequence similarities of 95% with

Aquamicrobium related bacteria (Table 6.1). However, the prevalent MnOB such as *Leptothrix*, *Hyphomicrobium* related nucleotide sequences were not detected in excised fragments, but further analyses need to be studied for all other remaining isolated fragments to analyze the Mn-oxidizing bacterial diversities. In all cultivated culture media, MnOB related consortia might had been cultivated, although they were not detected in isolated and excised DNA fragments obtained from DGGE due to ambiguous nucleotide sequences. The ambiguous nucleotide sequences were probably due to multiple fragments contamination or insufficient separation in this analysis.

Table 6.1 – Similarities between 16S rRNA gene sequences for amplified fragments obtained from DGGE analysis. The bacterial diversities were cultured in mPYM culture media.

DNA fragment	Closest relative	Accession no.	Similarity (%)	Affiliation
a	*Bacillus* sp.	[FJ596447]	100	*Fermicutes*
b	*Mesorhizobium lurifarium*	[Y14161]	98	*Alphaprotoebacteria*
c	*Nitrobacter* sp.	[L35511]	97	*Alphaproteobacteria*
d	*Rhodoferax* sp.	[DQ664242]	99	*Betaproteobacteria*
e	*Aquamicrobium* sp.	[GQ254286]	95	*Alphaproteobacteria*

Interestingly, cultivated Mn-oxidizing microbial consortia were particularly involved to remove excess level of dissolved As(III/V) during simultaneous removal batch study for Mn and As in the mPYM culture media. It was supposed that there might had been simultaneously cultivated microbial consortia that relevant to As(III) oxidation along with Mn-oxidizing microorganisms cultivation. To analyze As-oxidizing bacteria (AsOB), *aoxB* gene that encodes arsentie-oxidase was amplified by PCR using the specific primers aoxBM1-2F and aoxBM3-2R (Quéméneur *et al.*, 2008). The amplified *aoxB* gene was detected in the extracted DNA for BFM of Joyo biological filtration plant and all cultivated microbial consortia for primary and secondary cultivations (data not shown). It was resulted that the naturally occurring AsOB could exist with inactive activity on BFM, and thereafter simultaneously cultivated during Mn-oxidizing microbial consortia cultivation in mPYM. This study further persuaded to be studied cloning analysis particularly to detect AsOB in those cultured microbial consortia in the mPYM.

6.3.5 Molecular diversity of the aoxB genes in BFM of Joyo plant and cultured microbial consortia

Table 6.2 – Similarities between *aoxB* gene sequences of the clone library obtained from BFM of Joyo plant and the most similar database sequences.

OUT name	No. of isolates	Closest relative [Accession no.]	Affiliation	Similarity (%)
aoxB11J11	1	Putative arsenite oxidase (aoxB) gene [EU311947]	*Agrobacterium* sp. LY4	89
aoxB11J20	1	Putative arsenite oxidase (aoxB) gene [EU311947]	*Agrobacterium* sp. LY4	89
aoxB11J24	2	Arsenite oxidase alpha subunit (aoxB) gene [GU731253]	*Methylobacterium* sp. S47	88
aoxB11J48	1	arsenite oxidase (aroA) gene [EF015458]	*Sinorhizobium* sp. DAO10	80
aoxB11J59	1	Arsenite oxidase large subunit [FP929003]	*Candidatus Nitrospira defluvii chromosome*	80
aoxB11J69	1	Arsenite oxidase (aroA) gene, [EF015463]	*Bosea* sp.WAO	89
aoxB11J79	1	Arsenite oxidase alpha subunit (aoxB) gene [GU731253]	*Methylobacterium* sp. S47	87
aoxB11J92	2	Arsenite oxidase alpha subunit (aoxB) gene [GU731253]	*Methylobacterium* sp. S47	88

In the Fe and Mn removal Joyo biological water filtration plant, 16S rRNA gene clone library analysis revealed *Siderooxydans* and *Hyphomicrobium* as the dominant FeOB and MnOB, respectively, but no AsOB related bacteria were isolated (Thapa Chhetri *et al.*, 2013). The BFM of Joyo biological filtration plant has been reported as very reactive to remove As(III/V) from aquatic environment (Sahabi *et al.*, 2009b). But the *aoxB* gene encoding arsenite oxidase could be amplified in the extracted DNA solution for BFM of Joyo plant as well as primary and secondary cultured microbial consortia in mPYM (data not shown). Therefore, to analyze the AsOB, the clone library was constructed using amplified *aoxB* gene. The determined nucleotide sequences of the isolates were submitted to BLAST to study the closest relatives.

Phylogenetic analysis of the isolates for Joyo BFM revealed the AsOB related bacteria such as *Agrobacterium*, *Methylobacterium*, *Sinorhizobium*, *Bosea* with the sequences similarities >80% (Table 6.2). These bacterial diversities harbor *aoxB* gene encoding the arsenic-oxidizing enzyme to oxidize As(III)

(Quéméneur *et al.*, 2008).

Table 6.3 – Similarities between *aoxB* gene sequences of the clone library obtained from cultivated microbial consortia of primary cultivation (two weeks cultured) in mPYM culture media using BFM of Joyo plant.

OUT name	No. of isolates	Closest relative[Accession no.]	Affiliation	Similarity (%)
aoxB11P22	2	Arsenite oxidase (aroA) gene [EF015463]	*Bosea* sp.WAO	84
aoxB11P54	1	Arsenite oxidase (aroA) gene [EF015463]	*Bosea* sp.WAO	84
aoxB11P69	1	Arsenite oxidase large subunit (aoxB) gene [EU304278]	*Aminobacter* sp. 86	88
aoxB11P76	1	Arsenite oxidase (aroA) gene [EF637043]	*Bosea* sp. L7506	85
aoxB11P85	1	Arsenite oxidase large subunit [CP002568]	*Polymorphum gilvum* SL003B-26A1	81

On the other hand, *aoxB* gene clone library constructed for two weeks cultivated microbial consortia obtained from primary cultivation revealed the isolates related to *Bosea*, *Aminobacter*, *Polymorphum*. Their sequences similarities to the closest relatives were ranged from 84 to 88% (Table 6.3). In batch scale primary cultivation, those As-oxidizing bacterial diversities were also cultured simultaneously along with MnOB cultivation in mPYM culture media. Although the bacterial genera harbor the gene encoding arsenite oxidase have been demonstrated for unstable As(III) oxidation into stable As(V) (Kashyap *et al.*, 2006; Santini *et al.*, 2000; Silver & Phung, 2005; Rhine *et al.*, 2007), their complete roles for simultaneous removal of Mn and As(III/V) from aquatic environments has not been well known yet.

The phylogenetic study clustered the closest relatives of isolates obtained from *aoxB* gene clone library for the BFM of Joyo plant and two weeks cultivated microbial consortia obtained from primary cultivation (Fig. 6.7). In the spontaneously coated BFM and cultured microbial consortia samples, the isolates exhibited the various bacterial diversities related to the genera such as *Agrobacterium*, *Methylobacterium*, *Sinorhizobium*, *Bosea*, *Aminobacter*, *Polymorphum gilvum*, although their involvement and complex mechanism in As(III/V) is still uncertain. The neighbor-joining tree showed the isolates clustered into different diversities. This result indicated that in the Joyo BFM naturally occurring different AsOB were embedded spontaneously and they could be cultured in mPYM for the further study for

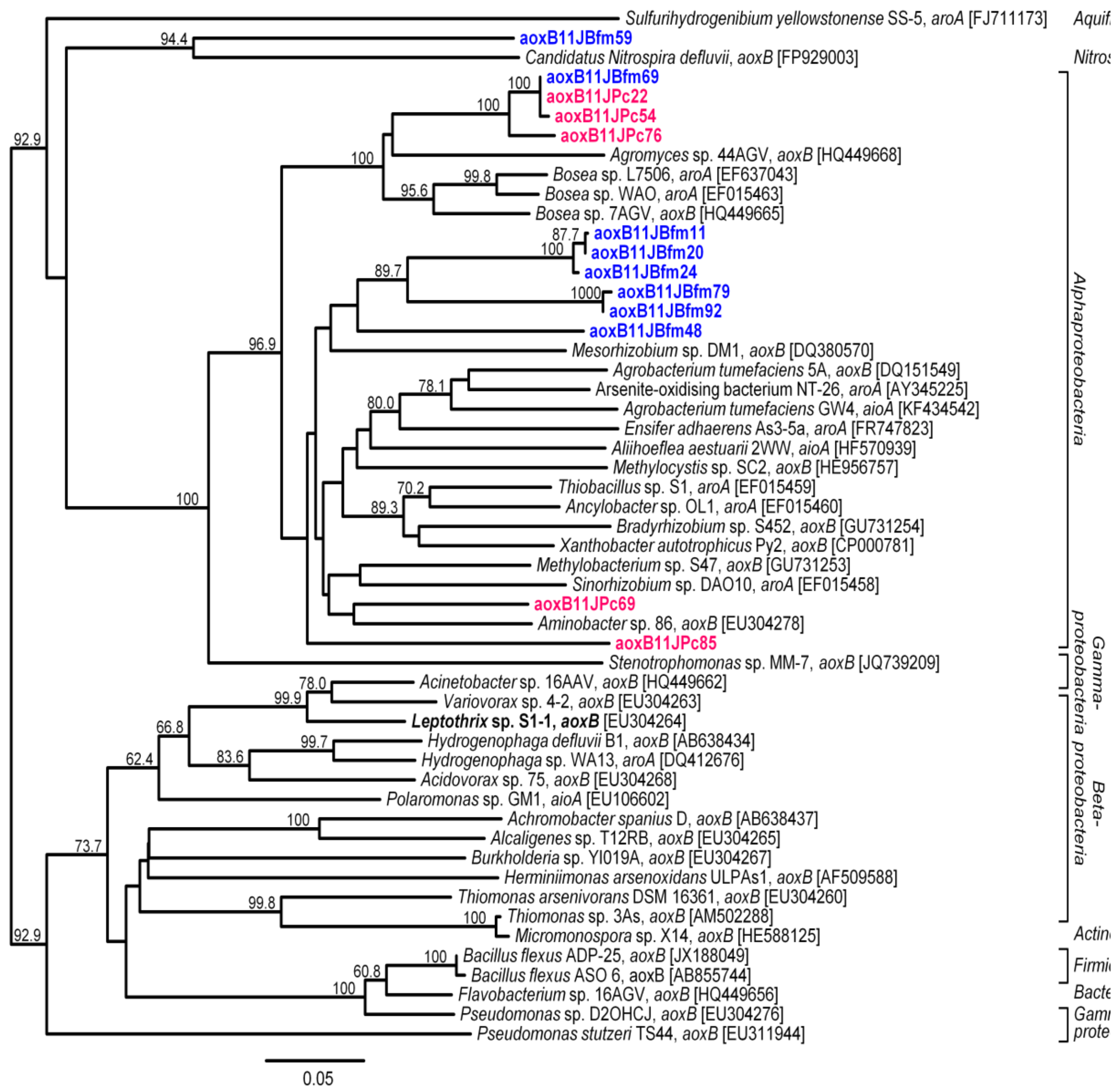

Fig. 6.7 – Phylogenetic relationship of bacterial aoxB gene clones obtained from clone library of Joyo BFM and primary cultured microbial consortia, and known relatives. Bootstrap values are shown for nodes that had >60% support in a bootstrap analysis of 1,000 replicates. Clones studied herein are represented in blue colored indicate the clones constructed for BFM of Joyo water treatment plant and in red colored indicate for primary cultured microbial consortia in mPYM culture media. Detected clones were clustered with the AsOB having *aoxB* gene and are listed on the same tree branch.

simultaneous removal of Mn and As (Fig. 6.7). However, their inactive existence in biological water filtration plant and cultivated AsOB and their

active role for simultaneous removal of Mn and As(III/V) in batch experiments is still unclear. It was also supposed that the cultivated As-oxidizing bacterial consortia might had conferred insignificant role for the simultaneous removal of As(III) along with Mn(II). But the prominent role of biogenic Mn oxides for the removal of dissolved As(III/V) has been already demonstrated (Katsoyiannis and Zouboulis, 2004 and 2006; Tebo *et al.*, 2004), and the importance of As-oxidizing bacterial activities in As(III/V) removal is required to be studied in details.

6.3.6 Quatitative analyses of 16S rRNA genes related to Leptothrix and Hyphomicrobium in the cultured microbial consortia

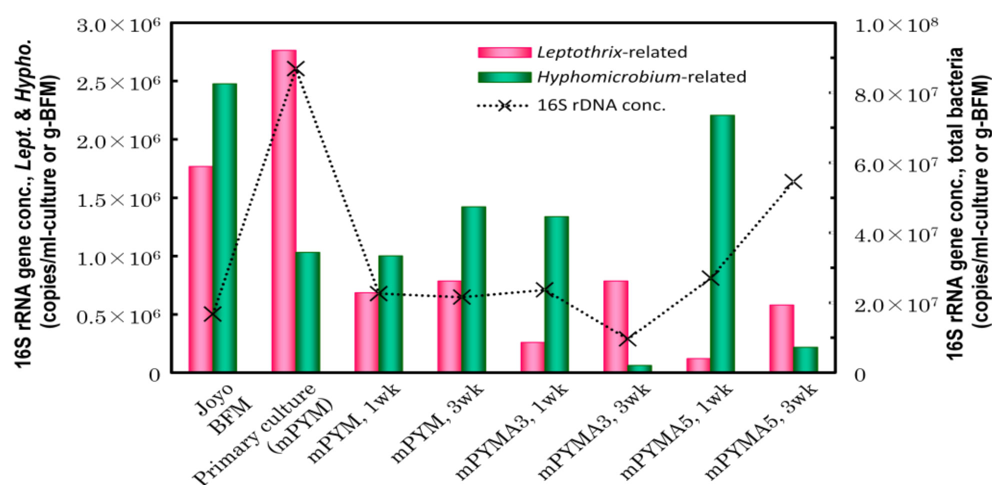

Fig. 6.8 – Real-time PCR analysis for bacterial consortia using 16S rRNA gene. The pink and green colored indicate *Leptothrix* and *Hyphomicrobium* related 16S rRNA gene copies, respectively, and cross marks depict 16S rRNA gene copies for total bacteria. Indications: extracted DNA solutions for; Joyo BFM, primary culture (mPYM) - primary cultivation of microbial consortia for two weeks, mPYM, 1wk - secondary cultivation for one week without As, mPYM, 3wk - secondary cultivation for three weeks without As, mPYMA3, 1wk - secondary cultivation for one week with As(III), mPYMA3, 3wk - secondary cultivation for three weeks with As(III), mPYMA5, 1wk - secondary cultivation for one week with As(V) and mPYMA5, 3wk - secondary cultivation for three weeks with As(V), respectively. Primary cultivated microbial consortia were inoculated in secondary cultivations.

The biogenic Mn oxides formation in Mn-oxidizing microbial consortia using

BFM of Joyo plant revealed the cultivation of MnOB in mPYM. *Leptothrix* (Ghiorse, 1984) and *Hyphomicrobium* (Tyler, 1970) have been reported as MnOB; and *Hyphomicrobium* related bacteria were cultured in PYM media using sand filter media collected from Fe and Mn removal bioreactor (Vandenabeele *et al.*, 1995). It was thought that *Hyphomicrobium* related bacteria could be cultured in mPYM using BFM of Joyo plant. It was because *Hyphomicrobium* related bacteria were detected as dominant MnOB in Joyo biological filtration plant via 16S rRNA gene clone library analysis (Thapa Chhetri *et al.*, 2013). Therefore, the quantitative real-time PCR was proposed to estimate the MnOB like *Leptothrix* and *Hyphomicrobium* related 16S rRNA gene copies present in the cultivated microbial consortia as described above. The bacterial 16S rRNA gene copies present in extracted total DNA solution for Joyo BFM and cultured microbial consortia were analyzed by real-time PCR. It was estimated the highest (8.68×10^5 copies/μL) total 16S rRNA gene copies in two weeks cultivated microbial consortia obtained from primary cultivation, whereas *Leptothrix* and *Hyphomicrobium* related 16S rRNA gene copies were detected the highest in number in the extracted DNA solution of the microbial consortia obtained from primary cultivation (2.76×10^4 copies/μL) and BFM of Joyo biological filtration plant (2.48×10^4 copies/μL), respectively (Fig. 6.8).

6.4 Conclusion

The BFM of Joyo biological filtration plant is very reactive for the removal of dissolved Mn and As(III/V) (Sahabi *et al.*, 2009a and 2009b). *Hyphomicrobium* related bacterial consortia were studied as the dominant MnOB in biofilms of Joyo BFM via 16S rRNA clone library analysis (Thapa Chhetri *et al.*, 2013). The cultivation of the Mn-oxidizing bacterial consortia was carried out in mPYM using the BFM of Joyo plant. The cultivated microbial consortia removed excess level of As(III) (>97%) and As(V) (>75%) ions in the mPYM culture media. The adequate biogenic Mn oxides were formed and implicated for the removal of Mn(II) and As(III/V), the common MnOB were not detected in the excised isolates obtained from DGGE analysis. Interestingly, *Bosea*, *Agrobacerium*, *Sinorhizobium* related bacterial diversities were also simultaneously cultivated along with Mn-oxidizing microbial consortia in mPYM, although their complete role in simultaneous Mn(II) and As(III/V) removal is still unclear. It is supposed that the cultivated microbial consortia would be more active and applicable for removal of dissolved Mn and As from drinking water, although the

complete analyses are required to be studied and confirmed.

6.5 Acknowledgement

We wish to thank T. Tsunai and T. Tamura of Joyo City Water Supply and Sewerage Department, Kyoto, Japan, who helped for sample collection and for providing the operating data of the biological filtration plant.

CHAPTER 7

(Discussion, conclusion and recommendation)

7.1 Discussion

7.1.1 Bacterial diversities in the biological filtration system for the removal of Fe and Mn from groundwater

In the Joyo biological filtration plant, raw groundwater containing Fe and Mn are adequately removed without addition of any chemical reagents. The BFM of the plant was spontaneously embedded by biofilms, which contained metal oxides-rich biomass along with various microorganisms (Sahabi *et al.*, 2009a). However, the complete biological mechanism for the removal of Fe and Mn from raw groundwater is still unclear. In this biological filtration plant, the *Leptothrix* related bacterial cells were reported as the iron bacteria via optical microscopy (Tamura *et al.*, 1999), and *Leptothrix* sp. OUMS1, which is closely related to *Leptothrix cholodnii*, were successfully isolated from the ocherous of the Joyo plant (Sawayama *et al.*, 2011), but the apparent biological structure in this plant has not been studied yet. In most of the Fe and Mn removal biological filtration plant, the iron bacteria such as *Gallionella* and *Leptothrix* were reported as the common Fe- and Mn oxidizing bacteria, which oxidize soluble Fe and Mn ions (Czekella *et al.*, 1985; Mouchet, 1992; Tamura *et al.*, 1999; Pacini *et al.*, 2005). These bacterial oxidation of Fe(II) and Mn(II) produce insoluble Fe and Mn oxides, which they are eventually removed by filtration. To study the biological removal mechanisms for Fe and Mn, it required to be analyzed the biological structures that present in raw groundwater in the biological filtration reactor. Therefore, the bacterial diversities were studied for the Joyo biological filtration plant to determine the biological structures by culture-independent molecular biological methods using PCR amplified 16S rDNA. This is because the culture-dependent analysis is not quite enough to study the biological mechanism for the removal of Fe and Mn from groundwater due to lack of adequate culture medium. Therefore, PCR-based DGGE, RFLP, clone library analysis, real-time PCR were carried out to analyze the biological structures in the BFM of Joyo plant (Sahabi *et al.*, 2009a).

The combined biological (microbial enzymatic oxidation) and physicochemical (auto-catalytic oxidation and adsorption) activities occur on

the filter bed in the biological filtration plant to remove the soluble Fe and Mn from groundwater. Although PCR-DGGE analysis revealed the amplified 16S rRNA gene closely related to *Gallionella capsiferrifomans* ES-2 (FeOB) and *Nitrospira* (NOB), but no any distinct MnOB were isolated. Interestingly, clone library analysis showed *Sideoxydans* (Weiss *et al.*, 2007) and *Hyphomicrobium* (Tyler, 1970) as the dominant FeOB and MnOB respectively in the Joyo biological filter plant. These results exhibited *Gallionella* and *Leptothrix* were not the dominant iron bacteria in the Fe and Mn removal Joyo biological filtration plant. Another study on Mn removal biological filtration reactors in Canada also showed no *Leptothrix* bacteria were the dominant in three reactors among four by real-time PCR analysis (Burger *et al.*, 2008). Similarly in Netherland, although *Leptothrix* related fragments (fragments: LKF_DGGE_CTO27, CTO28, and CTO31) were reported via PCR-DGGE analysis (de Vet *et al.*, 2009), those band were not distinctly closed to *Leptothrix* sp. when we repeatedly submitted in BLAST search ourselves. These two results strongly supported our study to demonstrate the common *Leptothrix* was not the dominant Fe- and MnOB particularly in Joyo biological filtration plant, but *Siderooxydans* and *Hyphomicrobium* were the dominant bacteria as well. The predominance of Fe- and Mn-oxidizing bacteria in the bioreactor depends on the water composition, plant site and other environmental conditions.

In the biological water filtration system, the Fe- and Mn-oxidizing bacteria such as *Gallionella*, *Siderooxidans*, *Siderocapsa*, *Crenothrix*, *Leptothrix*, *Hyphomicrobium*, *Pedomicrobium* oxidize inorganic Fe and Mn ions indifferently to produce insoluble Fe and Mn oxides. These biogenic metal oxides are very reactive to remove additional heavy and toxic metal ions such as As, Cd, Pb from the groundwater (Katsoyiannis and Zouboulis, 2004 and 2006; Sahabi *et al.*, 2009b and 2010) as well as Mn (Tebo *et al.*, 2004). This essential biological method subsequently removes Fe and Mn from the raw groundwater during drinking water purification so that other heavy and toxic metal ions would be removed simultaneously (Katsoyiannis and Zouboulis, 2004 and 2006). However, the complete biological removal method for Fe and Mn including other toxic metal ions is required to be studied in details.

In addition to Fe and Mn, despite influent NH_4^+-N (27.2 mg/L) was removed from raw groundwater in the Joyo biological filtration plant, although AOB

related bacteria were not detected from PCR-DGGE and clone library analyses. Surprisingly, the gene encoding ammonia oxidizing monooxygenase was amplified in the DNA solution extracted from the BFM. PCR amplification could amplify the *amoA* gene with intense fragment for AOA compared with AOB. It has been also reported that archaea exist in low level of NH_4^+-N containing aquatic environment (Martens-Habbena *et al.*, 2009). Therefore, in low level of NH_4^+-N containing Joyo raw groundwater, archaea could exist, and adequate removal of ammonia was ultimately occurred due to its presence in the bioreactor during drinking water treatment. In the spontaneously embedded BFM of Joyo plant, the bacterial consortia related to NOB were also detected via PCR-DGGE as well as clone library analysis. It is supposed that nitrification occurred in the plant due to presence of NOB and AOA, therefore, insignificant Mn oxidation could be possible in the filter bed of the plant. This is because the completion of nitrification commences Mn oxidation in the biological filtration plant (Vandenabeele *et al.*, 1995). However, the contribution of the various environmental bacterial consortia could not be understood, although it is required to be studied in detail to investigate the biological removal mechanism for Fe and Mn along with heavy metal and ammonia in the biological filtration plant.

Despite many conventional physico-chemical processes are applied for the removal of soluble As oxyanion, readily simple biological method is under testing in the world. Katsoyiannis and Zouboulis (2004) have demonstrated the simultaneous biological removal method for As, Fe and Mn in the biological filtration reactor. On the similar vein, a biological pilot plant for simultaneous removal of As, Fe and Mn from the raw groundwater was operated to purify the drinking water at Muko City, Kyoto Prefecture, Japan (Sugahara *et al.*, 2008). It was considered that the biological removal mechanisms for dissolved As(III/V) along with Fe and Mn needs to be studied in the biological pilot plant. To study the simultaneous biological removal mechanism for these metal ions, it requires to be analyzed the biological structure that involved in biological filter bed during treatment of the raw groundwater. Therefore, the biological structure in the pilot plant was studied using the culture independent molecular biological methods using PCR amplified 16S rRNA gene. The aim of our study is to reveal the biological structures to investigate the biological removal mechanism of As in aquatic environment.

The biological structure in the pilot plant was studied by PCR-based DGGE, clone library analysis and real-time PCR using bacterial flocs sample. In the DGGE analysis, the amplified DNA fragments were isolated, and revealed ambiguous nucleotide sequences. These indefinite fragments of 16S rRNA gene could not show distinct bacterial diversities that present in flocs sample in the pilot plant. This is the first case study for the As removal biological filtration plant to detect the bacterial diversities that involved in the simultaneous removal of As, Fe and Mn from raw groundwater.

On the other hand, the clone library analysis revealed some isolated clones closely related to the FeOB such as *Gallionella* (Ghiorse, 1984) and *Crenothrix polyspora* (Stoecker *et al.*, 2006), although Fe- and Mn-oxidizing activity of *C. polyspora* is still unclear (Emerson *et al.*, 2010). In addition to FeOB, some isolates in the clone library were closely related to *Ideonella* with >96% sequences similarities (chapter 3). Fe and Mn oxidation by *Ideonella* had not been reported, but their higher sequences similarities were closed to the Mn-depositing *Burkholderiales* bacterium JOSHI_001 isolated (Smith and Lewis, 2008), and arsenite-oxidizing bacterium *Leptothrix* sp. S1.1 isolated from As and Fe-containing mine drainage water Battaglia-Brunet *et al.*, 2006) (chapter 3). To remove the soluble As(III/V) ions from the groundwater, Fe- and Mn-oxidizing bacteria have essential role to produce the biogenic Fe and Mn in the bioreactor (Katsoyiannis and Zouboulis, 2004 and 2006; Casiot *et al.*, 2006). Therefore, the dissolved As(III/V) is subsequently removed by biogenic Fe and Mn oxides by adsorption. The raw groundwater containing As(III/V) ions are simultaneously removed via subsequent oxidation and/or adsorption on the insoluble Fe and Mn oxides (Katsoyiannis and Zouboulis, 2004 and 2006; Sahabi *et al.*, 2009b and 2010). In the biological filtration system during the simultaneous removal of As along with Fe and Mn from the raw groundwater, the sludge formation occurs on the filter bed, which effectively disturb the filtration of contaminated water until obviate. Therefore, it requires frequent back washing to remove that metal oxides complex formation from the filter bed. To study the biological removal mechanism As from the raw groundwater without using any chemical reagents, it needs to be known more details on biological structure in the biological filtration system. Thus, our study supports to demonstrate the biological removal of As(III/V) ions simultaneously with Fe and Mn, however, to find out the complete biological

removal mechanism for As ions, it requires to be studied further analyses in details.

In the bacterial consortia study, PCR-DGGE and 16S rRNA gene clone library analysis could not reveal the bacterial diversities closely related with AsOB in the bacterial flocs sample of the pilot plant. Interestingly, even the gene encoding arsenite oxidase catalytic subunit B (*aoxB*) were not amplified in the extracted DNA solution for bacterial flocs samples of the pilot plant by PCR (data not shown) (chapter 3). It resulted that the AOB in the biological pilot plant was not the dominant consortia to remove As oxyanion from the raw groundwater, however, Fe- and Mn-oxidizing bacterial diversities revealed the essential role for As(III/V) removal. On the other hand, AOB and AOA including NOB related consortia were not isolated via PCR-DGG and 16S rRNA gene clone library analysis, but ammonia monooxygenase A (*amoA*) encoding gene (Juretschko *et al.*, 1998) was amplified by PCR only for AOB (chapter 3). Therefore, it was considered that dissolved ammonia (0.31 mg/l) was adequately removed (0.02 mg/l) from the raw groundwater in the pilot plant by these AOB which they exist in the high level of NH_4^+-N compared with AOA (Martens-Habbena *et al.*, 2009). The concentration of NH_4^+-N was higher in the raw groundwater of the pilot plan compared with the raw groundwater of Joyo biological filtration plant (chapter 3). It has been demonstrated that the completion of nitrification commences Mn oxidation (Vandenabeele *et al.*, 1995). However, the essential contribution of nitrification for the oxidation of Mn to produce insoluble Mn oxides is still unclear, the role of Fe- and Mn-oxidizing bacteria such as *Gallionella* and *Leptothrix* were supposed as the dominant consortia for the simultaneous removal of As, Fe and Mn in the pilot plant (Katsoyiannis and Zouboulis, 2004). But it requires to be studied role of various bacterial consortia in details to demonstrate the simultaneous biological removal mechanism for As, Fe and Mn in the biological filtration reactor.

In the drinking water treatment biological filtration plants (chapter 2 and 3), located at Kyoto Prefecture, Japan; revealed various bacterial consortia using culture-independent molecular biological analyses. Although the isolated bacterial diversities showed *Siderooxydans* and *Hyphomicrobium* as dominant Fe and Mn oxidizing bacteria in Joyo biological filtration plant (chapter 2), *Gallionella* and *Leptothrix* were studied as predominance iron bacteria in the biological pilot plant (chapter 3). The existence of microbial

consortia depends on characteristics of the raw groundwater and treatment location, filter media and operational conditions. Our main objective is to find out the simultaneous biological removal mechanism for As, Fe and Mn in biological filtration plant. To analyze scant information on simultaneous removal mechanism for As, Fe and Mn, batch experiment was thought to be studied as pre-experiments. Therefore, the BFM of well-established Joyo biological filtration plant was taken to cultivate MnOB and to produce the bacterial flocs same as biological pilot plant. Thereafter, those cultivated microbial flocs containing biogenic Mn oxides were furthermore considered to be applied in simultaneous removal of As and Mn ions. This batch study would support to investigate the simultaneous removal mechanism for As, Fe and Mn. In addition, it would also spur additional notification on biological removal of As ions particularly in biological filtration system.

7.1.2 Biological removal of Mn and As using microbial consortia cultured from BFM

Before initiating the enrichment of FeOB and MnOB to produce Fe and Mn oxides, it was considered to be confirmed the biological removal activity of BFM to remove Mn(II). It was because the Mn(II) removal kinetics of BFM of Joyo plant and the bacterial consortia present on the filer media have been demonstrated (Sahabi *et al.*, 2009a), although their biological activity for Mn(II) removal had not been reported. Therefore, these BFM were thought to be used to culture microbial consortia so that that could be furthermore applied for the removal of heavy metal ions such as As and Mn same as in the biological pilot plant used for the simultaneous removal of As, Fe and Mn. Before initiating the culturing of Mn oxidizing microbial consortia, Mn(II) removal biological activity of BFM of Joyo plant was analyzed in the water environment. Its biological activities were analyzed at different pH and temperature in presence and absence of HEPES-buffer (chapter 4). It was observed that the biological removal activity of BFM for Mn(II) was higher in the reaction mixture in absence of HEPES-buffer, but in buffered (with HEPES-buffer; 15 mM, pH 7.4) reaction mixture, Mn(II) removal was detected higher and the pH remained stable at 7.4 in the reaction mixtures (chapter 4). The physico-chemical removal for dissolved Mn(II) using Joyo's BFM was observed highest at 15 °C, however, the total biological activities occurred the highest at 25 °C (chapter 4). These results ultimately showed that the BFM of Joyo biological filtration plant could be used for the cultivation of MnOB at 25 °C and pH 7.4. The simultaneous production of Fe

and Mn oxides by culturing of FeOB and MnOB using BFM of Joyo plant was very difficult to illustrate the dissolved metal ions removal mechanism for each Fe and Mn oxides in batch experiment. It was thus, the study on FeOB cultivation was not studied until complete the study on cultivation of Mn oxidizing microbial consortia and their application to analyze the simultaneous removal of Mn(II) and As(III/V) ions.

In the Joyo biological filtration plant, *Hyphomicrobium* related bacteria were isolated as dominant MnOB by clone library analysis that was embedded onto the BFM of that plant (chapter 2). On the other hand, to cultivate the MnOB such as *Hyphomicrobium*, PYM culture media has been reported (Vandenabeele *et al.*, 1995). It was thought that the dominant MnOB could be cultured in PYM culture media by using the BFM (chapter 2 and 4). The culturing of MnOB was carried out in buffered (HEPES-buffer; 15 mM, pH 7.4) mPYM at 25 °C via gentle shaking of 100 rpm. The massive growth of microbial consortia and consecutive Mn(II) removal was analyzed in mPYM that based on those previous studies. Those cultured microbial consortia were furthermore applied for the Mn(II) removal in the same mPYM culture media. However, modest Mn(II) ions were removed by those cultured microbial consortia, many fluctuated data was studied in the determined incubation period for Mn(II) removal in batch experiment (data not shown). In order to remove Mn(II) ions properly using cultured microbial consortia, it was supposed that there required to be used additional carriers or supporters, which could deplete proper concentration of dissolved Mn(II) in the mPYM. Surprisingly, Mn(II) removal concentration was observed slightly higher in mPYM after skipping the carrier (sterilized sea sand) compared with that previous analysis without using carriers (data not shown). It was considered that the feasible shaking generated aeration when the carrier was used in the culture media. In addition, that slightly formed aerated environment assisted aerobic microorganism for culturing and Mn(II) removal in the mPYM, even though the mechanism is still unclear.

However, the effect of carriers (using sterilized uncoated sea sand) was studied in MnOB culturing mPYM in batch experiment, the Mn(II) removal concentration was not distinctly affected. It was concluded that the effect of carrier such as sterilized uncoated sand in the primary culture was not detected, whereas, the combined effect of the carriers such as sterilized uncoated anthracite and sea sand was revealed in secondary incubation on

Mn(II) removal (negligible) and biological structures. It means their effects were observed only in the secondary incubated mPYM culture media, at where primarily cultured microbial consortia were inoculated for Mn(II) removal (chapter 5). Therefore, it was decided that not to use additional carrier in primary culture, but the carrier was supposed to be used in secondary culture when the primarily cultured microbial consortia are used in Mn(II) removal analysis. In our next set batch experiment, two carriers such as sterilized uncoated sea sand and anthracites were selected to find out their dual and individual effects on Mn(II) removal and microbial consortia culture in secondary incubation using primarily cultured microbial consortia in mPYM. It was observed that sterilized and uncoated sea sand did not show any dual and single effect on the secondary incubation, although only the sterilized and uncoated anthracite was quite enough to create favorable environment for microbial flocs formation and Mn(II) removal (data not shown). Ultimately, it was decided not to be used of any extra carrier (sterilized and uncoated sea sand) in primary enrichment, and only one carrier (sterilized uncoated anthracites) was confirmed to be used in the secondary cultivation during Mn(II) removal by cultured microbial consortia instead of BFM in mPYM (chapter 5).

In addition to the Mn(II) removal, the biological structures were studied in the cultured microbial consortia obtained from primary culture and secondary cultivation by PCR-DGGE analysis. The amplified 16S rRNA gene extracted from the bacterial flocs samples (collected from the carrier containing secondary cultivation) revealed the dominant MnOB such as *Hyphomicrobium* and *Leptothrix* related bacteria with >90% of sequences similarities. It was considered that these dominant MnOB such as *Hyphomicrobium* (chapter 2) embedded on the BFM of Joyo plant was cultured in mPYM along with common iron bacteria like *Leptothrix* studied via microscopy in the Joyo biological filtration plant (Tamura *et al.*, 1999). In addition to these bacteria, the enriched bacterial diversities related to genera *Mesorhizobium* and *Arthrobacter* were also isolated with >95% of sequences similarities (chapter 5). In our study, the real-time PCR analysis revealed no pertinent effect of carrier in the cultivation of *Leptothrix* related bacteria in the biotic mPYM. The aim of our ultimate study was to produce biogenic Mn oxides and microbial consortia, and their utilization for simultaneous removal of Mn(II) and As(III/V).

In order to study the simultaneous removal of Mn and As, the Mn-oxidizing microbial consortia were harvested by cultivation in mPYM using the BFM of Joyo plant for two weeks (chapter 5 and 6). Those cultured microbial consortia were further used to remove Mn and As in mPYM in presence of carrier like sterilized uncoated new anthracites. In this batch analysis, it was studied that very few Mn(II) ions were removed from initial concentration of 30 mg/L in mPYM, but soluble As(III/V) ions were adequately (>97% removal from the initial concentration of 500 μg/L) removed after three weeks incubation. On the other hand, the biological structure via PCR-DGGE analysis could not reveal any commonly known MnOB related bacteria such as *Leptothrix* and *Hyphomicrobium*. But in the extracted DNA solution for cultured microbial consortia, the DNA copies related to these MnOB (*Leptothrix* and *Hyphomicrobium*) were studied by real-time PCR analysis (chapter 6). Before culturing the MnOB, the DNA copies related to *Leptothrix* bacterial DNA were analyzed near about 10% of total 16S rRNA gene copies (chapter 2). However in cultured media, it was considered that these MnOB related bacterial consortia were heavily cultivated during heavy metal ions removal. Interestingly, the *aoxB* gene fragments were amplified by PCR that present in the DNA solution extracted from the BFM of Joyo plant and the cultivated microbial consortia (primary cultivation for two weeks cultured in mPYM using BFM of Joyo plant) (chapter 6). These results motivated us to construct the clone library using PCR amplified *aoxB* gene fragments. TOPO TA cloning was carried out to study the biological structures in the embedded biofilms on BFM of Joyo plant and primary cultivated microbial consortia (two weeks cultured in mPYM). It was isolated that the AsOB related bacteria such as *Bosea*, *Agrobacteria* were present on BFM of Joyo and primary culture via clone library analysis with >80% of sequences similarities (chapter 6).

The Mn-oxidizing microorganisms that cultivated in mPYM using BFM of Joyo plant produced very reactive microbial consortia. In those cultured microbial consortia, the biogenic Mn oxides were produced, which could remove Mn(II) and As(III/V) ions simultaneously from the mPYM. It has been also reported that biological removal of As(III/V) is carried out by biogenic Fe and Mn oxides (Katsoyiannis and Zouboulis, 2004 and 2006; Fujikawa *et al.*, 2010) (chapter 3). Therefore in our batch study, biogenic Mn oxides were produced during cultivation of Mn oxidizing microorganism, and these very reactive Mn oxides were considered as quite essential resources

for simultaneous removal of Mn and As by sorption in the mPYM (chapter 6). It was thought that the microbial Mn-oxidizing microbial consortia can be cultivated in mPYM using Joyo BFM, and would be more applicable for the removal of As(III/V) ions. The combined biological and chemical activities has been reported for the removal of heavy metals such as Mn and As (Oscarsion *et al.*, 1981; Katsoyiannis & Zouboulis, 2004; Tebo *et al.*, 2004; Sahabi *et al.*, 2009a and 2009b), however, it requires to be analyzed the details to investigate As(III/V) removal mechanism.

7.2 Conclusion

To study the biological removal mechanism particularly for As, Fe and Mn in groundwater, the biological structures in the biological filtration plants that used for the drinking water treatment were studied. PCR-based culture-independent molecular biological methods were carried out to analyze the biological structures. However, clone library analysis revealed *Siderooxydans* (Emerson *et al.*, 1997; Weiss *et al.*, 2007) and *Hyphomicrobium* (Moore, 1981) respectively as the dominant FeOB and MnOB in the BFM of Joyo biological filtration plant, whereas the common iron bacteria such as *Gallionella* (Ghiorse, 1984) and *Leptothrix* (Siering and Ghirose, 1996) were isolated as predominance to produce biogenic Fe and Mn oxides for simultaneous removal of As, Fe and Mn by sorption in biological pilot plant in Muko City, Kyoto Prefecture. In the Joyo plant, *amoA* gene encodes ammonium monooxygenase for AOA was amplified by PCR in the DNA solutions extracted from the samples of biological filtration plant (chapter 2).

In addition to the biological structure study, the particular MnOB such as *Hyphomicrobium* (Tyler, 1970, Moore, 1981) was cultured in mPYM (Vandenabeele *et al.*, 1995). The Mn oxidizing microbial consortia cultured using the BFM of Joyo plant in mPYM was applied for the simultaneous removal of As(III/V) and Mn(II) in presence of carrier in batch experiments (chapter 5 and 6). The MnOB such as *Hyphomicrobium* and *Leptothrix* could be cultured to produce biogenic Mn oxides (chapter 5). Therefore, those cultured microbial consortia adequately removed As (>97% of As(III) and >75% of As(V)) from mPYM culture media, at where moderate Mn(II) ions were simultaneously removed. Besides the MnOB, the AsOB related bacteria such as *Bosea* and *Agrobacterium* were isolated from clone library analysis constructed using *aoxB* gene fragment for the BFM of Joyo plant and

cultured microbial consortia (two weeks cultured microbial consortia using those BFM in mPYM). This leading conclusion from this systematic study revealed the simultaneous removal of Mn and As by cultured microbial consortia using BFM of Joyo plant in mPYM. It can be suggested that the cultured microbial consortia is quite essential to remove dissolved As(III/V) by sorption in the aquatic environment instead of BFM, however, it needs to be studied in the details to find out the biological removal mechanism for As.

7.3 Recommendation for future research

Despite the reactive BFM of Joyo biological filtration plant has been demonstrated as prominent filter media to treat drinking water by removing Fe and Mn from raw groundwater (Sahabi *et al.*, 2009a), the cultivation of Mn-oxidizing microbial consortia had not been studied. But those microbial consortia were cultured using the BFM of Joyo plant in mPYM because *Hyphomicrobium* was detected as the dominant MnOB in Joyo BFM (Thapa Chhetri *et al.*, 2013) and its cultivation was reported using the sand filter media of bioreactor (Vandenabeele *et al.*, 1995). In our batch study, cultured microbial consortia removed adequate Mn(II) and As(III/V) ions in mPYM, but their removal efficiencies for the soluble metal ions like Mn(II) and As(III/V) in engineered and natural water environments need to be studied in details. However, it is considered that these cultured microbial consortia would be more applicable to remove Mn and As in the drinking water treatment plants. In addition, it is also proposed for the simultaneous removal of various toxic metal ions such as Cd, Cr, Pb from the raw groundwater although the complete study is required to be investigated these metal ions removal during drinking water treatment in the biological filtration plant. Similarly, the study on the culturing of Fe-oxidizing microbial consortia using the BFM of Joyo plant would innovate the new theory on biological removal method for soluble As(III/V) ions in the field of biological drinking water purification.

Appendix 1: Relevant publications and conferences

Publications

1) Thapa Chhetri R., Suzuki I., Takezaki J., Tabusa H., Takeda M. and Koizumi J. (2013) Bacterial diversity in biological filtration plant for removal of iron and manganese from groundwater, *JWET*., **11**(1), 33-47.

2) Thapa Chhetri R., Suzuki I., Fujita T., Takeda M., Fujikawa Y., Minami A., Hamazaki T., Sugahara M. and Koizumi J. (2013) Bacterial diversity in

biological filtration system for simultaneous removal of arsenic, iron and manganese from groundwater, *JWET.*, (*accepted on 19th Octob. 2013*).

3) Thapa Chhetri R., Sahabi D. M., Oda T., Suzuki I., Takeda M. and Koizumi J. (2013) Biological removal of Mn^{2+} from water by aged biological filter media, (under preparation).

4) Thapa Chhetri R., Sahabi D. M., Suzuki I., Takeda M. and Koizumi J. (2013) Cultivation of manganese oxidizing microbial consortia from biological filter media for the removal of dissolved manganese (under preparation).

5) Thapa Chhetri R., Suzuki I., Takeda M. and Koizumi J. (2013) Simultaneous removal of Mn and As by the cultured microbial consortia, and analysis of their bacterial diversities (under preparation).

Conference presentations

1) Thapa Chhetri R., Fujita T., Suzuki I. and Koizumi J. Bacterial consortium in groundwater treatment plant for simultaneous removal of As, Fe and Mn using biological filtration. Presented at 24th annual meeting of Japanese Society of Microbial Ecology, held on 25th–28th November 2008 at Hokkaido University.
2) Thapa Chhetri R., Sahabi D. M., Suzuki I., Takeda M. and Koizumi J. Manganese removal by microbial consortia from biological filter media. Presented at 25th annual meeting of Japanese Society of Microbial Ecology held, on 21-23rd November, 2009, at University of Hiroshima.
3) Thapa Chhetri R., Fujita T., Iguchi Y., Suzuki I. and Koizumi J. Bacterial consortium in groundwater treatment plant for simultaneous removal of As, Fe and Mn using biological filtration. Presented at international conference for Asia Pacific Biochemical Engineering held on 24-28th November, 2009, at Kobe, Japan.
4) Thapa Chhetri R., Sahabi D. M., Oda T., Suzuki I., Takeda M. and Koizumi J. Biological removal of Mn^{2+} from water by mature biofilter media. Presented at international conference for water and environment Technology conference held on 25–26th June, 2010, at Yokohama National University, Japan.
5) Thapa Chhetri R., Sahabi D. M., Suzuki I., Takeda M. and Koizumi J. Effect of culture conditions on manganese oxidation of microbial consortia from biological filter media. Presented in 13th International Symposium on Microbial Ecology held on 22-27th August, 2010, at Seattle, WA, USA.

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