Removal of Mn(II) and Zn(II) Ions from Synthetic Mine Drainage Using a Laboratory-Scale Mn(II)-Oxidizing Bioreactor

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Abstract

Bioreactors with manganese (Mn)-oxidizing microorganisms are potent tools for removing Mn from pH-neutral mine drainages, based on their ability to form insoluble Mn oxides. We examined the effects of loadings of Mn and zinc (Zn) ions on the removal of these metals from synthetic mine drainages by a laboratory mini-size bioreactor, which was packed with limestones encrusted with Mn oxides and a Mn(II)-oxidizing bacterial community. The initial influent Mn(II) concentration and hydraulic retention time were set at 10 mg L⁻¹ and 24 h, respectively. The results showed that the Mn(II) removal rate was dependent on the amount of Mn oxide precipitates associated with the limestone and was not largely lowered even if the Mn(II) concentration was gradually increased to six times or soluble Zn(II) coexisted at 6 mg L⁻¹. The effluent Mn(II) concentrations met the effluent standard in Japan (< 10 mg L⁻¹) when Mn(II) was loaded up to 28 mg L⁻¹ bioreactor d⁻¹. The bioreactor also effectively removed dissolved Zn(II). The bacterial community structures of the Mn precipitates in the bioreactors consisted of diverse heterotrophs mainly belonging to Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Bacteroidetes. The bacterial communities also included species closely related to known Mn(II)-oxidizing heterotrophs. The results demonstrate that the bacterial communities with Mn(II)-oxidizing activity can be maintained under organic substrate-poor conditions, further supporting the usefulness of Mn(II)-oxidizing bioreactors in mine drainage remediation.

Key Words: biogenic manganese oxides, Mn(II)-oxidizing bacteria, mine drainage, packed bed bioreactor

INTRODUCTION

Abandoned mines often yield drainage waters containing toxic metal ions, which have adverse effects on human health and ecosystems. Their treatment is an important issue in the management of mine drainage-receiving environment¹,². Manganous ion [Mn(II)], a major metal contaminant in drainages, is highly soluble and is not readily hydrolyzed or air oxidized under neutral pH conditions³. The Mn(II)-containing mine drainage is commonly treated by increasing the pH to > 9 with caustic chemicals to

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facilitate Mn oxidation and precipitation\textsuperscript{3}. Hence, the alkaline effluent must be re-neutralized with an acidic reagent before being discharged. This alkaline treatment is a cost-intensive and energy-consuming process, making the maintenance of treatment facilities in abandoned mine sites difficult.

Based on the catalytic activity under near-neutral pH conditions, the Mn(II)-oxidizing microorganisms, including bacteria, fungi, and microalgae, can precipitate insoluble Mn(III, IV) oxides from Mn(II)-containing waters\textsuperscript{4-6}. Biological Mn(II) oxidation (along with inorganic surface catalytic reactions), in passive treatment systems such as constructed wetlands and aerobic limestone beds, contributes to metal removal from mine drainages containing various concentrations of Mn(II)\textsuperscript{7-10}. Owing to the relatively slow Mn oxidation, passive treatment systems are generally operated with hydraulic retention times (HRTs) of several days, although the Mn(II) removal efficiency is largely affected by treatment conditions (e.g., pH, temperature, and metal and organic substance concentrations)\textsuperscript{3}. Increased efficiencies of Mn removal may be achieved by using packed bed bioreactors inoculated with Mn(II)-oxidizing microbial communities\textsuperscript{12-15}, potentially leading to downsizing of drainage treatment facilities. In Japan, many abandoned mines are located in the mountains and lack sufficient space to construct vast passive treatment systems, such as constructed wetlands. Mn(II)-oxidizing bioreactors could be attractive alternatives, especially in such situations.

Despite their potential application in mine drainages, research on Mn(II)-oxidizing bioreactors, to reveal the effects of operational conditions on Mn removal and their capacities against influent Mn loadings, is limited. In addition, the microorganisms that contribute to Mn(II) oxidation in bioreactors have not been fully identified. In a packed bed bioreactor composed of dolomite, bentonite, and MnO\textsubscript{2} powder, the Mn removal of > 95\% was attained at an HRT of 8 h for continuous flow of mine water containing ~20 mg L\textsuperscript{-1} Mn\textsuperscript{13}. The participation of microbial activity in Mn removal was suggested by adding a disinfectant\textsuperscript{13}. In the batch treatment of Mn(II)-containing stream water\textsuperscript{14}, a bioreactor packed with Mn oxide-coated pebbles reduced Mn(II) from 10 to < 0.25 mg L\textsuperscript{-1} when operated in a fill-and-draw mode every two days. The workers isolated two species of ascomycete fungi and an alphaproteobacterium, Bosea sp., and demonstrated that the fungal isolates were the dominant eukaryotes in the microbial community via rRNA-targeted molecular analysis (i.e., T-RFLP analysis)\textsuperscript{14}. A recent continuous-flow bioreactor study showed > 99\% removal of Mn at pH 6.5 and an HRT of 45 h during treatment of a partly processed synthetic mine water\textsuperscript{15}. The rRNA-based molecular techniques identified two Mn(II)-oxidizing microorganisms, a Leptothrix discophora-related bacterium and an ascomycete fungus. Mn(II)-oxidizing fungi have often been isolated from passive treatment facilities\textsuperscript{10,16} and laboratory bioreactors\textsuperscript{14,15}. Although research suggests the significance of fungal Mn(II) oxidizers in mine drainage treatment\textsuperscript{10,16}, bacterial Mn(II) oxidizers appear to be less identified and their role in the mine drainage treatment has not been clarified.

Furthermore, the metal ions often coexisting with Mn may affect the Mn removal in bioreactors. The solid Mn minerals occurring in limestone beds of coal mine drainages were shown to be poorly crystalline birnessite and todorokite; these incorporate trace metals such as Zn, Ni, and Co ions in their mineral structures\textsuperscript{11}. Such Mn minerals appear to be ubiquitous in mine drainage treatment\textsuperscript{3}. Given that Mn oxide minerals have a high sorption capacity for metal cations\textsuperscript{5}, Mn oxide precipitation could also serve in the removal of these metal cations coexisting in the drainages. Previous research has demonstrated the simultaneous removal of Mn(II) and Zn(II) from mine drainage in bioreactor experiment\textsuperscript{15}. However, this may not be universal because microbial Mn(II) oxidation is known to be inhibited by a low concentration (1 mg L\textsuperscript{-1} or less ) of Zn(II)\textsuperscript{17,18}.

In this study, the removal of Mn(II) from a synthetic mine drainage was examined using laboratory limestone-packed bioreactor. Bioreactor characteristics, with respect to the Mn removal efficiencies at different
concentrations of Mn(II) and in the presence of Zn(II), were elucidated. Analysis of 16S rRNA gene amplicon sequencing revealed the bacterial communities associated with the limestone beds exhibiting Mn(II) oxidation activity. This study supports the potential application of Mn(II)-oxidizing bioreactors in mine drainage remediation.

MATERIALS AND METHODS

Bioreactor experiments A non-sterile, synthetic mine drainage used in this study contained (per liter of tap water) MnSO₄, [up to 60 mg L⁻¹ as Mn(II)], 25 mg of KNO₃, 5 mg of KH₂PO₄, 220 mg of MgSO₄, 155 mg of CaCl₂, 100 mg of NaHCO₃, 200 mg of Na₂SO₄, 10 mg of K₂SO₄, and 10 mL of trace metals solution. In some experiments, MnSO₄ was dissolved at a concentration of 6 mg L⁻¹ Zn. The pH was adjusted to 7.0–7.2 using a dilute H₂SO₄ or NaOH solution.

Limestones (2–4 cm diameter; Yoshizawa Lime Industry, Tochigi, Japan) encrusted with a Mn oxide phase, including the microbial community, were prepared in batches as described by K. Sunouchi and N. Miyata; unpublished results). An 8-L plastic container was packed with limestones, filled with synthetic mine drainage containing 20 mg L⁻¹ Mn(II) and 6 mg L⁻¹ Zn(II) ions, and then inoculated with Mn oxide-containing sediment (dry weight, 21 g) collected from drainage ditch in a mine tunnel, under permission of the Department of Commerce, Industry and Labor, Aomori Prefecture. The continuous-flow bioreactor operation was conducted with aeration at 18–20 °C and an HRT of 72 h, under dark conditions to prevent growth of phototrophs. During the operation of several weeks, black Mn oxide coatings developed on the limestones.

For the treatment of Mn(II)-containing drainages, the limestones (weight, 1.13 kg) were collected from the 8-L bioreactor operated for 6 months and placed in a rectangular section of acrylic container (each size, 5.5 × 5 × 30 cm; volume, 795 mL), and the effective volume of each section was 460 mL (Fig. 1). To examine the effect of the amount of Mn oxide precipitates on Mn removal, the Mn-coated limestones were mixed with unused limestones at 33% or 66% of the total weight of the limestones. For the control experiment, only unused limestones were placed in a section (i.e., 0% Mn-coated). To treat drainages containing both Mn(II) and Zn(II) ions, only the first half of each reactor section was packed with limestones encrusted with a solid Mn phase. All reactor sections were filled with synthetic mine drainage and supplied with non-sterilized air through a small diffuser placed at the near-center position. The upper surface of the reactor was covered with light shading plate. The continuous flow bioreactor was operated at 15 °C and an HRT of 24 h.

Chemical analyses To determine dissolved Mn(II) and Zn(II), the influent and effluent water samples were centrifuged at 12,000 × g for 10 min at 10 °C and the supernatants were stored at 4 °C after addition of 1 M HNO₃. To determine the amount of solid Mn associated with limestones, each limestone was immersed overnight in 0.1 M NH₂OH·HCl solution to solubilize oxidized Mn. The supernatant solutions were obtained by centrifugation and stored as described above. The concentrations of dissolved Mn and Zn in the supernatants were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; iCAP 6000, Thermo Fisher Scientific, San Jose, USA). The total organic carbon (TOC) concentrations of tap water samples used for the synthetic mine drainage preparation were determined using a TOC–L analyzer (Shimadzu, Kyoto, Japan).
DNA extraction and 16S rRNA gene amplicon sequencing The Mn precipitates associated with limestones were collected by scraping with a sterilized spatula at the end of the bioreactor experiments and stored at -80 °C until use. These were resuspended in 0.1 M ascorbic acid and mixed gently until the Mn oxides were dissolved. Thereafter, the suspensions were subjected to total DNA extraction using ISOIL for Beads Beating Kit (Nippon Gene, Tokyo, Japan). For 16S rRNA gene sequencing, amplicon libraries were constructed by the two-step PCR\textsuperscript{21).}

The first PCR was conducted with the 515F~806R primer set targeting the 16S rRNA gene\textsuperscript{22).} The PCR products were purified with AMPure XP (Beckman Coulter, Brea, USA) and subjected to a second PCR, which was conducted by a tailed-PCR procedure that allowed for the addition of the index and adapter sequences using a MiSeq sequencer (Illumina, San Diego, USA). All the second-PCR products were treated with an EB buffer to adjust to iso-density and then mixed to prepare one sequence sample. Subsequently, the mixed sample was further purified using BluePippin (Sage Science, Beverly, USA), and amplicon sequencing was performed. The sequences obtained were phylogenetically classified into unique operational taxonomic units (OTUs) based on 97% sequence identity using Claident v0.2 (https://www.claident.org). The relative abundances of the targeted bacteria were obtained as ratios of the OTU reads to the total eubacteria reads.

RESULTS AND DISCUSSION

Effect of Mn loadings on Mn removal In the treatment of synthetic drainage, the Mn removal efficiency was dependent on the amount of active limestones, which were encrusted with a solid Mn phase, including the bacterial community (Fig. 2). The amount of solid-state Mn was \(1.36 ± 0.42 \text{ mg g}^{-1} \text{limestone (mean ± SD; } n = 9).\) Introducing unused limestones resulted in a decrease in the Mn removal rate (Fig. 2). During the bioreactor operation, small amounts of black Mn coatings occurred on the unused limestones that were placed along with Mn-coated limestones. This suggested a gradual increase in the Mn removal ability during the operation. However, most Mn(II) in the influents passed through the section packed with only unused limestones, which did not yield visible Mn coatings. In the bioreactors containing Mn-coated limestones at 100%, 66%, and 33%, the amounts of solid Mn were estimated to be 1.9, 1.3, and 0.66 g L\(^{-1}\) bioreactor, respectively. These results indicate that the active limestones retaining Mn oxides and Mn-oxidizing microbial communities contribute directly to Mn removal in the bioreactors.

The results demonstrated that the Mn removal rate depended on the Mn loading rate (Figs. 2B), indicating that such a range

![Fig. 2](image-url)
of Mn loadings does not suppress the Mn removal ability of the bioreactors. In the bioreactor where only active limestones were placed, the removal efficiency remained at 80.0% even if the influent Mn reached 62 mg L\(^{-1}\) (Fig. 3; for the Zn-coexisting influents, see below). Thus, the effluent Mn(II) concentrations met the effluent standard in Japan (< 10 mg L\(^{-1}\)) when the influent Mn(II) was loaded up to 50 mg L\(^{-1}\), which corresponded to a volumetric Mn loading rate of 28 mg L\(^{-1}\) bioreactor d\(^{-1}\).

**Effect of coexisting Zn on Mn removal and the Mn removal capacity** The addition of 6 mg L\(^{-1}\) Zn(II) to the drainage did not have a negative effect on the ability of the bioreactor because, during the course of operation for 340 h, the Mn removal efficiency and volumetric Mn removal rate were not lowered by the coexistence of Zn(II) (Table 1 and Fig. 3). In addition to Mn(II), Zn(II) was also effectively removed to a level below the effluent standard (2 mg L\(^{-1}\)) despite the different Mn(II) concentrations (Table 1). Biogenic Mn oxides serve as fine adsorbents for metal cations\(^{30}\), and this likely contributes to the Zn(II) removal in the Mn(II)-oxidizing bioreactor. A fungal Mn oxide was reported to incorporate Zn(II) in a Zn/Mn molar ratio of up to 0.23\(^{25}\), this ratio corresponds to 6 mg Zn/22 mg Mn. The lower Mn(II) concentrations (Table 1; 1 to 10 mg L\(^{-1}\)) appeared to be insufficient to yield Mn oxides that fully incorporated Zn(II) ions added at 6 mg L\(^{-1}\). In addition to sorption by Mn oxides, the formation of insoluble Zn species such as hydrate and carbonate could be involved in the limestone-packed system\(^{24}\).

In the bioreactors, Mn(II) removal was not affected by coexisting Zn at 6 mg L\(^{-1}\) under the conditions examined (Table 1 and Fig. 3). Observations with pure bacterial cultures\(^{17}\) and enrichment cultures\(^{18}\) indicated that Mn(II) oxidation was largely inhibited by Zn(II) concentrations of 1 mg L\(^{-1}\) or less. In this study, while microbial communities in the bioreactors seemed more resistant to Zn(II) than those reported in the cultures used in previous studies, the Mn oxides associated with the limestone surfaces may be responsible for resistance to Zn(II). The Mn(II) oxidation by an ascomycete fungus was inhibited completely by addition of Zn(II) at ~2 mg L\(^{-1}\)\(^{25}\). In the cultures retaining Mn oxides, however, the fungal Mn(II) oxidation was no longer affected by Zn(II) at ~30 mg L\(^{-1}\)\(^{25}\). The existence of Mn oxides on limestones is likely involved in the Zn-resistance of the bioreactors used in this study.

**Bacterial community structures in solid Mn phases on limestones** Analysis of bacterial community structures revealed that diverse heterotrophic bacteria lived in the solid Mn phases on limestones loaded with synthetic drainage waters containing Mn(II) and Zn(II) (Table 2). Interestingly, the microbial Mn(II)-oxidizing ability was maintained in organic carbon-poor drainage waters. The tap water used for the drainage preparation contained 1.2–1.4 mg L\(^{-1}\) TOC (a range of

![Fig. 3 Relationship between Mn(II) removal rate and volumetric Mn(II) loading rate. Data points were collected in the treatment of synthetic mine drainages containing Mn(II) alone (10–60 mg L\(^{-1}\); closed circle) and Mn(II) and Zn(II) (1–40 mg L\(^{-1}\) and 6 mg L\(^{-1}\); respectively; open triangle).](image)

**Table 1 Bioreactor treatments of synthetic mine drainages containing Mn(II) and Zn(II) ions**

| Influent Mn(II) and Zn(II) conditions (mg L\(^{-1}\)) | Effluent Mn(II) (mg L\(^{-1}\)) | Effluent Zn(II) (mg L\(^{-1}\)) |
|--------------------------------------------------|-------------------------------|-------------------------------|
| Mn : Zn = 1 : 6                                  | 0.02 ± 0.01                   | 0.09 ± 0.03                   |
| 5 : 6                                            | 0.17 ± 0.09                   | 0.19 ± 0.07                   |
| 10 : 6                                           | 0.10 ± 0.05                   | 0.17 ± 0.06                   |
| 20 : 6                                           | 1.05 ± 0.43                   | 0.54 ± 0.18                   |
| 40 : 6                                           | 7.24 ± 3.29                   | 0.81 ± 0.19                   |

\(^{a}\) Mean ± SD for the data during the experimental period of 120–335 h (9 data points for each run).
three measurements). The kinetics of microbial growth under such conditions were not analyzed in this study; further studies are needed to reveal these. The abundant species found within all the solid phases belonged to the phyla *Proteobacteria* (Alpha-proteobacteria, 19%–33% of total sequences; Betaproteobacteria, 37%–56%; Deltaproteobacteria, 0.5%–7.3%), *Bacteroidetes* (3.2%–27%), *Actinobacteria* (1.5%–3.4%), and *Planctomycetes* (0.3%–1.6%) (Fig. 4). Among these, the relative abundances of *Deltaproteobacteria* and *Bacteroidetes* were low in the drainage with 40 mg L\(^{-1}\) Mn(II), suggesting that these included the bacterial species sensitive to such a high concentration of Mn(II).

*Hyphomicrobium* and *Rhodobacter* species included in the class *Alphaproteobacteria* were detected at > 5% of the total sequences in at least one specimen (Table 2). *Hyphomicrobium* and *Rhodobacter* species included in the class *Alphaproteobacteria* were detected at > 5% of the total sequences in at least one specimen (Table 2).

![Bacterial community structures on limestones, encrusted with a solid Mn phase, loaded with synthetic drainages containing 1–40 mg L\(^{-1}\) Mn(II) and 6 mg L\(^{-1}\) Zn(II).](image)

**Table 2** Bacterial species present in solid Mn phases on limestones loaded with synthetic drainages containing Mn(II) and Zn(II).

| OTU_ID\(^a\) | Identified taxon | Phylum or class; order | Functions\(^b\) | % total sequences\(^c\) |
|-------------|-----------------|------------------------|-----------------|--------------------------|
| OTU0304     | *Hyphomicrobium* sp.| Alphaproteobacteria; Rhizobiales | MT, OF, MnOx   | 12.17  4.67  6.71  4.25  4.75  4.11 |
| OTU3728     | *Rhodobacter* sp. | Alphaproteobacteria; Rhodobacteriales | CF, OF, AP      | 1.16  2.13  2.97  3.41  2.12  5.89 |
| OTU2470     | *Methylloversatilis* sp. | Betaproteobacteria; Nitrosomonadales | MT, OF  | 33.21  22.02  12.05  21.50  31.34  15.40 |
| OTU4172     | Unclassified *Methylphilaeeae* sp. | Betaproteobacteria; Nitrosomonadales | MT | 0.72  1.52  3.27  5.40  5.52  14.57 |
| OTU2382     | *Methylibium* sp. | Betaproteobacteria; Burkholderiales | MT, OF, AF       | 6.34  10.49  5.88  6.60  3.91  16.94 |
| OTU1606     | *Aquabacterium* sp. | Betaproteobacteria; Burkholderiales | OF | 2.80  4.79  4.52  6.10  3.52  0.67 |
| OTU3433     | *Curvibacter* sp. | Betaproteobacteria; Burkholderiales | CF   | 2.00  1.44  3.34  3.92  2.22  8.08 |
| OTU3062     | *Hydrogenophaga* sp. | Betaproteobacteria; Burkholderiales | CF, OF, AF, HO | 0.82  4.26  6.35  4.00  2.66  1.73 |
| OTU0512     | *Pajaroellobacter abortivobis* | Betaproteobacteria; Myxococcales | IP | 6.62  1.45  2.69  1.35  1.80  0.11 |
| OTU2531     | *Terrimonas* sp. | Bacteroidetes; Chitinophagales | CF | 4.73  7.68  11.64  6.75  5.85  0.79 |
| OTU0955     | Unclassified *Saprospirales* sp. | Bacteroidetes; Saprospirales | — | 0.89  10.33  9.88  6.35  7.47  0.44 |

Total

| % total sequences\(^c\) |
|--------------------------|
| 71.16  70.78  69.30  69.63  71.16  63.73 |

\(^a\) Selected for abundant OTUs accounting for > 5% of the total sequences of at least one solid Mn phase sample. \(^b\) Deduced based on identified taxa. Abbreviations: MT, methylotrophic; CF, carbohydrate fermentative; OF, organic acid fermentative; AF, amino acid fermentative; HO, hydrogen oxidation; AP, anoxygenic phototrophic; IP, intracellular plastics; MnOx, Mn(II) oxidation. \(^c\) Total read numbers were 10,592, 20,423, 12,560, 14,586, 18,144, and 22,256 for the samples of initial solids, 1:6, 5:6, 10:6, 20:6, and 40:6, respectively. \(^d\) Potential Mn(II) oxidizing bacterium (see Table 3).
microbium species have been demonstrated to be Mn(II) oxidizers. The relative abundance of *Hyphomicrobium* did not vary largely among the bioreactors operated under different Mn(II) conditions. Major species within the *Betaproteobacteria* were affiliated with the genera *Methylloversatilis*, *Methylibium*, *Aquabacterium*, *Curvibacter*, and *Hydrogenophaga* and unclassified *Methylophilaceae*. Members of the genera *Pajaroellobacter* (*Deltaproteobacteria*), *Terrimonas*, and unclassified *Saprospirales* bacterium (*Bacteroidetes*) were detected as the other abundant species. The bacterial group sensitive to the high concentration of Mn(II) (40 mg L\(^{-1}\); Fig. 4) included the species of *Aquabacterium*, *Pajaroellobacter*, *Terrimonas*, and unclassified *Saprospirales* (Table 2). The other species appeared to be less sensitive because these showed considerably high ratios at 40 mg L\(^{-1}\) Mn.

The 10 taxa found in the bacterial communities were shown to be closely related to known Mn(II)-oxidizing bacteria (Table 3), including the genera *Hyphomicrobium*, *Bosea*, *Mesorhizobium*, *Terricaulis*, *Erythrobacter*, *Pseudomonas*, *Leptothrix*, *Acinetobacter*, and *Rhodococcus*. As described above, *Hyphomicrobium* species were found to be abundant in all samples (Tables 2 and 3). A recently reported autotrophic Mn(II)-oxidizing bacterium, *Candidatus Manganitrophus noduliformans*, was not detected in all samples (< 0.01%). Although the heterotrophic species detected are considered potential Mn(II) oxidizers, further studies are needed to identify, isolate, and characterize the bacteria responsible for the Mn(II) oxidation.

The bacterial communities associated with the Mn precipitates in bioreactors could be characterized by a high abundance of *methylophilaceae* bacteria responsible for the Mn(II) oxidation. A previous study demonstrated that Mn(II) oxidation in a bioreactor was stimulated by the supply of methane gas, which promoted the growth of *C.1*-utilizing bacteria and their biomass and organic exudates could serve as substrates for Mn(II)-oxidizing bacteria. Based on the

### Table 3: Potential Mn(II)-oxidizing bacteria present in solid Mn phases on limestones loaded with synthetic drainages containing Mn(II) and Zn(II).

| OTU_ID       | Identified taxon                  | Phylum or class; order | % total sequences | Mn:Zn | Mn:Zn | Mn:Zn | Mn:Zn | Mn:Zn | Mn:Zn | Ref. |
|--------------|----------------------------------|------------------------|-------------------|-------|-------|-------|-------|-------|-------|------|
| OTU0304, OTU1231, OTU1673, OTU2574, OTU3622 | *Hyphomicrobium* spp. | Alphaproteobacteria; Rhizobiales | 13.20  | 5.22 | 7.34 | 4.70 | 5.19 | 5.17 | 26 |
| OTU3864      | *Bosea* sp.                      | Alphaproteobacteria; Rhizobiales | 0.06  | 0.03 | 0.00 | 0.01 | 0.01 | 0.02 | 14 |
| OTU0360, OTU3830 | *Mesorhizobium* spp. (2 OTUs) | Alphaproteobacteria; Rhizobiales | 0.05  | 0.03 | 0.03 | 0.03 | 0.02 | 0.11 | 27 |
| OTU3229      | *Terricaulis* sp.               | Alphaproteobacteria; Caulobacteriales | 0.12  | 0.05 | 0.05 | 0.09 | 0.17 | 0.14 | 28, 29 |
| OTU0436      | *Erythrobacter* sp.             | Alphaproteobacteria; Sphingomonadales | 0.01  | 0.01 | 0.02 | 0.03 | 0.03 | 0.03 | 30 |
| OTU3192      | *Leptothrix* sp.                | Betaproteobacteria; Burkholderiales | 0.00  | 0.01 | 0.02 | 0.02 | 0.01 | 0.01 | 26 |
| OTU2958      | *Acinetobacter* sp.             | Gammaproteobacteria; Pseudomonadales | 0.16  | 0.03 | 0.04 | 0.06 | 0.05 | 0.22 | 31 |
| OTU0689, OTU2425 | *Pseudomonas* spp. (2 OTUs) | Gammaproteobacteria; Pseudomonadales | 0.05  | 0.13 | 0.34 | 0.70 | 0.17 | 0.66 | 26 |
| OTU0459      | *Flavobacterium* sp.            | Gammaproteobacteria; Pseudomonadales | 0.03  | 0.00 | 0.20 | 0.30 | 0.02 | 0.02 | 31 |
| OTU0568, OTU2494 | *Rhodococcus* spp. (2 OTUs) | Gammaproteobacteria; Pseudomonadales | 0.40  | 0.29 | 0.35 | 0.25 | 0.26 | 0.49 | 32 |
results of this study, it can be suggested that the high abundance of methylotrophs was maintained in the bioreactor; however, the supply of C1 substrates was absent. These bacterial groups can utilize multiple carbon compounds so that the supply of C1 substrates is not essential for their growth. Although the grown methylotrophs may yield organic substrates for Mn(II)-oxidizing bacteria, as shown previously, further studies are needed to determine if the methylotrophs contribute directly or indirectly to the Mn(II) oxidation in the bioreactor.

**Implications of this study** Based on the heterotrophy in Mn(II)-oxidizing microorganisms, a supply of organic substrates as the carbon and energy sources has been focused on for its application to mine drainage remediation. The addition of organic substrates or the use of substrate-containing culture medium has been examined for the role of Mn(II)-oxidizing microorganisms in mine drainages and is a strategy for enhancing the biological treatment process. The limitation of Mn(II) oxidation rates in the incubation experiments was suggested to be due to the lower microbial biomass in organic carbon-limited mine drainages. This study used a tap water-based synthetic medium, containing approximately 1 mg L\(^{-1}\) TOC, without the supply of certain organic substrates and demonstrated that microbial Mn(II) oxidation can be maintained under such organic carbon-poor conditions. A similar observation was reported for the batch culture experiment with Mn-oxide rich sediments collected from mine drainage treatment systems (limestone beds). In this study, several potential Mn(II)-oxidizing bacteria, along with other heterotrophs, were found in the bacterial communities in the bioreactors. Most Mn(II)-oxidizing bioreactors applied to mine drainage have been developed as processes that presuppose the supply of organic substrates. However, this study suggests that the bioreactor can maintain heterotrophic bacterial communities with Mn(II) oxidation activity under organic carbon-poor conditions and serve for mine drainage remediation. The fact that the bioreactor experiments were conducted under the dark or light shading conditions could eliminate the contribution of phototrophs in the organic substrates supply; however, unknown intrinsic mechanism for the carbon supply may be involved in the maintenance of Mn(II) oxidation in bioreactors.

**CONCLUSIONS**

The mini-size bioreactor packed with limestones retaining Mn(II)-oxidizing bacterial communities and operated at an HRT of 24 h effectively removed dissolved Mn(II) from synthetic mine drainages. The Mn(II) removal efficiency was not largely lowered even if the Mn(II) concentration was increased up to 60 mg L\(^{-1}\) or soluble Zn(II) coexisted at 6 mg L\(^{-1}\). The microbial communities associated with limestones included diverse heterotrophic bacteria mainly belonging to the Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Bacteroidetes, several species of which were found to be closely related to known Mn(II)-oxidizing bacteria. The Mn(II)-oxidizing bioreactor functioned without the supply of certain organic substrates, further supporting the contention that such bioprocesses serve as a useful tool for mine drainage remediation.

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