

Multiple organic substrates support Mn(II) removal with enrichment of Mn(II)-oxidizing bacteria

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Abstract

Three different organic substrates, K-medium, sterilized activated sludge (SAS), and methanol, were examined for utility as substrates for enriching manganese-oxidizing bacteria (MnOB) in an open bioreactor. The differences in Mn(II) oxidation performance between the substrates were investigated using three down-flow hanging sponge (DHS) reactors continuously treating artificial Mn(II)-containing water over 131 days. The results revealed that all three substrates were useful for enriching MnOB. Surprisingly, we observed only slight differences in Mn(II) removal between the substrates. The highest Mn(II) removal rate for the SAS-supplied reactor was $0.41 \text{ kg Mn} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, which was greater than that of K-medium, although the SAS performance was unstable. In contrast, the methanol-supplied reactor had more stable performance and the highest Mn(II) removal rate. We conclude that multiple genera of *Comamonas*, *Pseudomonas*, *Mycobacterium*, *Nocardia* and *Hyphomicrobium* play a role in Mn(II) oxidation and that their relative predominance was dependent on the substrate. Moreover, the initial inclusion of abiotic-MnO₂ in the reactors promoted early MnOB enrichment.

Keywords: biological manganese oxidation, down-flow hanging sponge reactor, K-medium, manganese-oxidizing bacteria, methanol, sterilized activated sludge

1. Introduction

A wide variety of organisms, including bacteria, fungi, algae, and plants, have been used in bioremediation processes to remove heavy metals and hazardous organics from contaminated waters and wastewaters (Bahar et al., 2016; Jaiswal et al., 2018; Zeraatkar et al., 2016; Yin et al., 2017). Of all microbes used, manganese-oxidizing bacteria (MnOB) are gaining attention for their unique characteristics that promote the removal of heavy metals. Heterotrophic MnOB, capable of oxidizing Mn(II) to Mn(III/IV), yield manganese oxides, which also are referred to as biogenic manganese oxides (Bio-MnO_x) (Francis and Tebo, 2002). Bio-MnO_x can adsorb cationic metals and oxidize inorganic contaminants because of their poorly crystalline-layered materials that have many vacant sites (Meng et al., 2009; Droz et al., 2015; Tang et al., 2014; Bai et al., 2016a). MnOB are ubiquitous and widespread in nature; they have been detected in oligotrophic environments including caves (Cloutier et al., 2017), deep oceanic sediments (Blothe et al., 2015), and river estuaries (Anderson et al., 2011). They play a key role not only in the biogeochemical cycle of manganese (Tebo et al., 2005) but also in carbon (Jones et al., 2018), nitrogen (Lin and Tallefert, 2014) and sulfur (Geszvain et al., 2012) cycles in nature.

Exploiting these characteristics of Bio-MnO_x in MnOB-enriched bioreactors is promising for water and wastewater treatments. Therefore, many studies on the applications of MnOB for removal of heavy metals have been reported. In most previous studies, pure MnOB cultures were employed for batch-scale and column experiments (Meng et al., 2009; Bai et al., 2016b). Pure cultures present problems for large-scale use, because it is difficult to prevent contamination by other bacteria in open systems. Even if bacterial contamination occurs, it is essential to maintain the presence and prevalence of MnOB. In a previous study, we successfully enriched MnOB in an opened down-flow hanging sponge (DHS) reactor to continuously remove the minor metals,

nickel (Ni)(II) and cobalt (Co)(II), from synthetic wastewater by coupling nitrification (Cao et al., 2015). The growth of MnOB was promoted by providing a natural substrate of soluble microbial products (SMPs) generated by nitrifying bacteria. However, a long time was required to confirm significant Mn(II) oxidation. MnO₂ inhibited bacterial activity due to its toxicity (Matsushita et al., 2018). By slowly but steadily increasing the accumulation of produced Bio-MnO_x over time, the activity of MnOB might be gradually enhanced by the MnO_x inhibition of growth of other bacteria. We hypothesized that starting with a large amount of MnO₂ would provide early MnOB enrichment and high Mn(II) oxidation performance in a shorter time.

There is little information on organic substrates that promote the enrichment of MnOB. K-medium, consisting of peptone and yeast extract, has been widely used for MnOB growth in pure cultures (Yang et al., 2013). Methanol is a promising substrate because Matsushita et al. (2018) successfully enriched methanol-utilizing MnOB in a methane oxidation reactor, where methanol was generated as a utilization-associated product (UAP) of methanotrophs. Methanol is abundant in nature and is produced in soils from the decomposition of dead plants (Kolb S., 2009). Matsushita et al. (2018) also found that Mn(II) oxidation occurred under starvation conditions without exogenous methane and concluded that MnOB could utilize biomass-associated products (BAPs) derived from cell lysis in biofilms. The bulk liquid from dead microbes contains high concentrations of proteins, carbohydrates and lipids (Ramstedt et al., 2011). Combined, these reports suggest that activated sludge is a good candidate substrate for MnOB enrichment.

This study aims to clarify whether it is possible to use methanol and sterilized activated sludge (SAS) as substrates for MnOB enrichment. We also set out to evaluate the performance of Mn(II) oxidation relative to that of K-medium through a continuous Mn(II) removal experiment. A unique DHS reactor, with abiotic MnO₂ initially installed, was employed with the goal of

promoting early MnOB growth. Microbial community analyses of each retained biomass were performed to identify the predominant MnOB related to the Mn(II) oxidation performance.

2. Materials and methods

2.1. Reactor configurations, inoculation, and operational conditions

Enrichments of MnOB were conducted in three DHS reactors of 75 cm height and 5 cm diameter, in which a set of 20 polyurethane sponge cubes (each $2 \times 2 \times 2 \text{ cm}^3$, total volume of 160 cm^3) were hung diagonally in series on a nylon string (Fig. S1). The sponge carriers were inoculated with activated sludge from the aeration tank of a municipal sewage treatment plant in Higashihiroshima, by squeezing and soaking in its suspension liquid mixed with abiotic-MnO₂ $100 \text{ g} \cdot \text{L}^{-1}$ (Kishida Chemical Co. Ltd., Japan). The activated sludge, consisting of a large variety of microorganisms, was expected to contain MnOB (Abu Hasan et al., 2012). Prior to using abiotic-MnO₂ for inoculation, it was pretreated by Mn(II) adsorption to equilibrium, allowing us to judge whether the Mn(II) removal performance of the reactors was caused by the oxidation or the adsorption. The reactors were placed in a dark room at 26°C .

The reactors, R-1, R-2, and R-3, were supplied with different organic substrates: K-medium, sterilized activated sludge (SAS) and methanol, respectively. K-medium is composed of peptone and yeast extract (4:1, w/w). SAS was made by heating activated sludge at 100°C for 24 hours in a drying oven (DVS 602 Yamato Scientific Co., Ltd., Japan). The concentrations of organic substrates in the influent are provided in Table 1, except for SAS, because the concentration of SAS in the influent was adjusted based on the COD concentration of SAS stock solution. The substrates contained Mn(II) ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), minerals ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ $0.05 \text{ mg} \cdot \text{L}^{-1}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ $0.2 \text{ mg} \cdot \text{L}^{-1}$, $\text{Fe}_2\text{SO}_4 \cdot 5\text{H}_2\text{O}$ $0.1 \text{ mg} \cdot \text{L}^{-1}$, KH_2PO_4 $1.156 \text{ mg} \cdot \text{L}^{-1}$, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ $49.08 \text{ mg} \cdot \text{L}^{-1}$), and

trace elements ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ $0.025 \text{ mg} \cdot \text{L}^{-1}$, NaSeO_4 $0.005 \text{ mg} \cdot \text{L}^{-1}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ $0.019 \text{ mg} \cdot \text{L}^{-1}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ $0.024 \text{ mg} \cdot \text{L}^{-1}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ $0.022 \text{ mg} \cdot \text{L}^{-1}$, H_3BO_3 $0.001 \text{ mg} \cdot \text{L}^{-1}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ $0.043 \text{ mg} \cdot \text{L}^{-1}$), [all final concentration](#). Before the substrates were supplied to the respective reactors, the substrate tanks were purged with nitrogen gas. Air was provided to the reactors at a flow rate of $15.6 \text{ L} \cdot \text{h}^{-1}$ to create aerobic conditions. The organic and Mn(II) loading rates were set by controlling the hydraulic retention time (HRT) based on the sponge volumes and concentrations (Table 1). The reactor operation was conducted for 131 days and the condition was divided into three phases. The effluent waters were recirculated at a ratio of 1:10 (Q influent:Q recirculation).

Table 1. Operational conditions for the DHS reactors.

2.2. Analytical methods

Water samples were filtered through a $0.45\text{-}\mu\text{m}$ membrane filter (Advantec, Tokyo, Japan). Mn(II) and chemical oxygen demand (COD) concentrations were determined by the colorimetric method using a Hach DR2800 spectrophotometer (Hach Co., Loveland, CO, USA).

2.3. Microbial community analysis

Biomass samples were collected by squeezing sponge carriers taken from the upper, middle and lower portions of each reactor at the end of Phase 2 (day 60) and Phase 3 (day 131). DNA was extracted using a Fast DNA spin kit for soil (MP Biomedicals, Irvine, CA, USA). Polymerase

chain reaction (PCR) amplification of the 16S rRNA gene was performed using the primers 341F (5'-CCTACGGGGNGGCWGCAG) and 805R (5'-GGACTACCAGGGTATCTAATCC). The PCR conditions were as follows: 3 min initial denaturation at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and final extension at 72 °C for 5 min. PCR products were purified using an Agencourt AMPure XP kit (Beckman Coulter, CA, USA) and subsequently sent to Hokkaido System Science Co. Ltd (Sapporo, Japan) for sequencing on the Illumina MiSeq platform with a Miseq Reagent Kit v3 (Illumina Inc., San Diego, CA, USA). Sequence data were analyzed using QIIME software version 1.8.0 (Caporaso et al., 2010). Sequences showing more than 97% identity of DNA were grouped into the same operational taxonomic units (OTUs) using the UCLUST method (Edgar, 2010) and the OTUs were classified using the MiDAS v1.20 database (McIlroy et al., 2015).

2.4. Data analysis

Alpha-diversity indexes including Simpson's index of diversity (1-D), Shannon (H), Evenness and Chao-1 were calculated using PAST (PAleontological STatistics) 3.20 software (Hammer et al., 2001). One-way analysis of variance (ANOVA) was performed using Microsoft Excel to measure statistical significance between the Mn(II) oxidation performance of the three reactors.

3. Results

3.1. Reactor performances

The performance data are shown in Fig. 1. Mn(II) removal was immediately observed for all reactors, although the Mn(II) loading rate was very low for each. Since the influent Mn(II) concentration of 5 mg Mn(II) L⁻¹ was reduced to almost zero in the effluent, we gradually increased the Mn(II) loading rate by reducing HRT and increasing the influent Mn(II)

concentration to find the maximum Mn(II) oxidation potentials in Phase 1, with the COD loading rate set at approximately $0.3 \text{ kg COD m}^{-3} \text{ d}^{-1}$. The Mn(II) removal rates steadily increased to result in effluent concentrations of less than $0.5 \text{ mg Mn(II) L}^{-1}$. At the end of Phase 1, R-1 (day 30), R-2 (day 38) and R-3 (day 36) achieved Mn(II) removal rates of 0.26, 0.41 and $0.49 \text{ kg Mn} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, respectively, at a HRT of 0.4 h, with effluent Mn(II) concentrations exceeding $0.5 \text{ mg Mn(II)} \cdot \text{L}^{-1}$. Thus, as expected, Mn(II) removal with oxidation was successful with K-medium, SAS, or methanol used as substrate, although Mn(II) oxidation potential was different for each substrate at almost the same COD removal rate. In addition, a slight decrease in the effluent pH was observed in three reactors because of Mn(II) oxidation.

Fig. 1 The performance of R-1, R-2, and R-3. The arrows indicate biofilm sampling on days-60 and 131.

In Phase 2, the operational conditions were kept the same as the last conditions in Phase 1 to investigate performance stability. With elapsed time, the effluent Mn(II) concentration tended to increase for all three reactors. It was difficult to maintain stable reactor performance at high Mn(II) loading, resulting in a deterioration of the Mn(II) removal rate to 0.23, 0.36, and $0.34 \text{ kg Mn} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ on average for R-1, R-2 and R-3, respectively (Figs. 1 and 2).

Mn(II) removal performance of the reactor should be related to the population size of microbes with manganese oxidation ability. We expected that the Mn(II) removal rate would be improved by increasing COD loading rate. In Phase 3, we therefore doubled the COD loading rate to $0.6 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ and increased the Mn(II) concentration from 5 to 10 mg L^{-1} in the influent without

changing the Mn(II) loading rate of the reactors. Despite the change, the Mn(II) removal rates did not improve, although the COD removal rates were slightly higher. In R-1 and R-3, although the Mn(II) removal rates fluctuated greatly, their averages were almost the same as those achieved at the end of Phase 2 (Figs. 1 and 2). In R-2, in contrast, Mn(II) removal gradually deteriorated over time (Fig. 1). Therefore, to restore the Mn(II) removal performance in R-2, we decreased the influent Mn(II) concentration for the last 5 days because we hypothesized that high concentrations of Mn(II) were inhibiting removal. However, the deterioration of Mn(II) removal performance did not stop and therefore, this attempt failed. The results in Phase 3 suggested that the COD loading rate was less important in MnOB enrichment.

Until the low Mn(II) loading rate of approximately $0.25 \text{ kg Mn} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$, three reactors could completely remove Mn(II), as shown in the plots on the line of slope 1 in Fig. 3. Higher Mn(II) loading rates resulted in incomplete Mn(II) removal and unstable performance. Surprisingly, the SAS substrate of R-2 yielded higher Mn(II) removal than K-medium, which is usually used for MnOB cultivation (Fig. 3). Thus, we conclude that SAS is a useful substrate for enriching MnOB. As expected, the methanol in R-3 was a preferred substrate for Mn(II) removal and yielded high and stable performance. During the operation, a black particulate precipitate was observed at the bottoms of all reactors, consistent with the removal of Mn(II) by oxidation, yielding Bio-MnO_x.

Fig. 2 Mn(II) removal rates of each reactor during Phase 2 and 3.

Fig. 3 Relationship between Mn(II) loading and removal rates.

3.2. Microbial community-

In a microbial community analysis of the six biomass samples, more than 90,000 reads were obtained. Each sample yielded more than 1,600 OTUs, based on 97% similarity threshold of the 16S rRNA gene sequences (Table S1). Microbial diversity was assessed by determining the alpha-diversity indices of Simpson (1-D), Shannon (H), Evenness, and Chao-1 estimator (Table S1). No significant difference was found between the diversity of the three reactors, suggesting organic substrate had little effect on diversity.

There was little difference in microbial composition among the six samples, even in phylum level (Fig. S2). Of the 17 phyla identified, which were the major microbial communities with a relative abundance greater than 1% of the total sequence reads, Proteobacteria, Chloroflexi and Bacteroidetes were found to be dominant in all samples. However, differences in microbial composition were clearly observed at the genus level. The genera with relative abundances of more than 3% are shown in Fig. 4. Dominant genera were *Comamonas*, *unclassified Caldilineaceae* and *Methylobacter* in the R-1 reactor. In R-2, although *unclassified Caldilineaceae* dominated, *Comamonas* was barely present. In R-3, *Hyphomicrobium* and *Methylobacterium* were the dominant genera. Thus, the microbial community was strongly influenced by organic substrate even though high Mn(II) oxidation was observed at similar levels. An effect of the organic loading rate on the microbial community was also observed, but it was insignificant relative to the effect of the substrates (samples on days 60 and 131 in Fig. 4).

Fig. 4 Most dominant genera in the reactors with relative abundance >3% of the total sequence reads in each sample.

During Mn(II) removal, certain MnOB should have played a role in Mn(II) oxidation. Unfortunately, we were unable to identify enrichment for known MnOB in any of the reactors because of a limitation of DNA sequence data obtained in this study and the probable presence of unknown MnOB. However several genera that contain strains previously identified as MnOB were detected, so we tentatively refer to them as “putative MnOB” responsible for Mn(II) oxidation, with the caveat that not all putative MnOB are able to oxidize Mn(II) (Francis and Tebo, 2001; Francis and Tebo, 2002). Many putative MnOB were detected (Fig. 5); comprising 8–25% of the total bacteria. Most putative MnOB in R-1 belonged to either *Comamonas* or *Pseudomonas*. In R-2, multiple genera including *Mycobacterium*, *Nocardia*, and *Hyphomicrobium* were identified as putative MnOB. *Hyphomicrobium* were the dominant putative MnOB genus in R-3. These results indicated that even though three kinds of substrate used in this study were completely different, high Mn(II) oxidation rates were possible because different MnOB were highly enriched depending on the substrate.

Fig. 5 Relative abundance of putative manganese-oxidizing bacteria (MnOB).

4. Discussion

Our microbial community analysis showed that the three reactors were dominated by different putative MnOB genera (Fig. 5). These genera have been reported to inhabit different environments. *Comamonas testosteroni*, belonging to Proteobacteria phylum, have been found to contribute to the formation of Bio-MnO_x on the leaf surface of submerged aquatic plants in a lake (Tsuji et al., 2017). *Pseudomonas* and *Mycobacterium* have been reported to play a major

role in manganese removal in drinking water systems (Cerrato et al., 2010; Marcus et al., 2017). *Nocardia* and *Hyphomicrobium* have been detected in ferromanganese deposits in a cave (Carmichael and Brauer, 2015; Lozano and Rossi, 2012), and *Hyphomicrobium* have been found to oxidize Mn(II) in a methane-fed reactor (Matsushita et al., 2018). We presumed that different environments provide different available nutrients leading to differences in the microbial community. This study revealed that the distinct putative MnOB community among the three reactors was due to the adaptation of microbial communities to the different substrates.

We found that all three substrates used in this study supported MnOB communities (Fig. 5). It is unsurprising that *Comamonas* and *Pseudomonas* dominated in R-1, as they perform ammonification and nitrogen removal, because K-medium containing peptone and yeast extract has been commonly used to culture MnOB (Yang et al., 2013). In addition, MnOB have been successfully enriched even during the nitrification process (Cao et al., 2015). Both the *Mycobacterium* and the *Nocardia* belonging to Actinobacteria were suggested to be the predominant putative MnOB in the R-2 fed with SAS. The genus *Mycobacterium* can use a wide range of organic compounds as carbon sources, including recalcitrant compounds such as polycyclic aromatic hydrocarbons (PAHs) (Hartmans, et al., 2006; Zeng et al., 2010); it was also detected in similar abundance in all reactors. The DHS reactor employed in this study has a longer sludge retention time and allows a higher degree of sludge autolysis because of a biofilm system that results in reduced sludge generation (Tandukar et al., 2007). Even though different substrates were provided, *Mycobacterium* probably utilized the generated cell lysate in biofilm as a nutrient source. A previous study reported that in a methane providing reactor, *Hyphomicrobium* played a role in M(II) oxidation by growing on methanol associated with the metabolism of methanotrophic bacteria (Matsushita et al., 2018). As we expected, putative

MnOB belonging to *Hyphomicrobium* were enriched with the methanol substrate of R-3. Our results demonstrate that a wide variety of organic substrates can be used for Mn(II) removal and MnO₂ production in engineered ecosystems, since MnOB are diverse and ubiquitous in natural environments.

The presence of MnO₂ has been reported to shorten the acclimation period of MnOB in biofilter and inhibit the activity of non-MnOB by its toxicity (Zhang et al., 2015; Matsushita et al., 2018). In this study, we employed the sponge carriers combined with abiotic-MnO₂ in the hope of enhancing MnOB enrichment by inhibiting the growth other bacteria. Our results showed that the maximum Mn(II) removal rate was achieved within 42 days in all three reactors (Fig. 1a, g and m). In contrast, studies using sponge carriers without abiotic-MnO₂ took a longer time to achieve similar results, even for Mn(II) removal (Cao et al., 2015; Matsushita et al., 2018). Not only higher initial Mn(II) removal performance but also much higher Mn(II) oxidation rates were obtained with sponge carriers with abiotic-MnO₂ relative to those without abiotic-MnO₂. The highest Mn(II) oxidation rates of 0.26 and 0.41 kg Mn·m⁻³·d⁻¹ for R-1 and R-2, respectively (Fig. 1b and 1h), were almost 10-fold higher than those of 0.048 and 0.011 kg Mn·m⁻³·d⁻¹, which were achieved by other groups in Mn(II) oxidation reactors combined with nitrification (Cao et al., 2015) and methane oxidation under marine conditions (Kato et al., 2017), respectively. Interestingly, however, the highest Mn(II) oxidation rate of 0.49 kg Mn·m⁻³·d⁻¹ in R-3 was almost the same as the highest rate in a DHS reactor coupled with methane oxidation in a freshwater environment, even though it took a long time to reach that rate (Matsushita et al. 2018). These similar Mn(II) oxidation rates might be caused by having the same predominant MnOB, *Hyphomicrobium* in both reactors. Once significant Bio-MnO_x is produced, Mn(II) oxidation might reach a similar rate regardless of the initial installation of abiotic-MnO₂.

Although R-2 had a significantly lower proportion of putative MnOB relative to the other reactors (Fig. 5), their respective Mn(II) removal rates were almost the same (Fig. 2). Since many uncultured bacteria were detected, unknown MnOB might be included in them and play a major role in Mn(II) removal in R-2. Two factors, the amount of MnOB retained in the reactor and their Mn(II) oxidation potential, should directly affect the Mn(II) removal performance of the reactor. Even if the proportion of MnOB is low, a high Mn(II) removal performance will be obtained when MnOB possess high Mn(II) oxidation potentials. In addition, interactions between two non-MnOB in co-culture could induce Mn(II)-oxidizing activity (Liang et al., 2016). If such interactions occurred in R-2, the high Mn(II) removal would be expected even if unknown MnOB did not exist. It is hard to explain why so little difference in Mn(II) removal performance was observed among the three reactors despite of the feasible reasons mentioned above.

An unstable Mn(II) oxidation rate was observed at higher Mn(II) loading rates of greater than approximately $0.25 \text{ kg Mn} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ in Phase 3 (Fig. 3). This instability could be explained by decreased Mn(II) transfer into biomass with increased production of Bio-MnO_x covering MnOB by mass transfer resistance, resulting in deterioration of Mn(II) oxidation, as reported elsewhere (Jiang et al., 2010). These investigators also reported that the Mn(II) concentration would respond to the instability. Microorganisms have different tolerances for Mn(II) (Zhou et al., 2016). The residual Mn(II) concentration should depend on an Mn(II) loading rate due to incomplete oxidation. As shown in Fig. 1, Mn(II) oxidation rate tended to decrease with increased effluent Mn(II) concentration in Phase 3 of R-2. Thus, high Mn(II) loading rates would affect the stability of Mn(II) oxidation performance.

Our proposed bioreactor having high Mn(II) oxidative capacity could be applied for remediation of water contaminated by high concentrations of Mn(II), such as acid-mine drainage (Tutu et al.,

2008). However, because MnOB are heterotrophs, organic substrates have to be provided to MnOB enriched bioreactors to remove heavy metals or to recover minor metals from wastewaters containing low or no organics. This study demonstrated that multiple substrates enriched MnOB and produced Bio-MnO_x at a high rate. Methanol would be preferred over K-medium for its low cost, and somewhat higher Mn(II) oxidation performance. In addition, if combined with the treatment of methanol-containing wastewaters generated from methanol production plants (G. Cao et al., 2015) or pulp and paper mills (Meyer and Edwards, 2014), MnOB enrichment of using waste methanol is preferable. The employment of SAS may also be very attractive. SAS is the most cost-effective and eco-friendly solution for the disposal of large amounts of sewage sludge. Nevertheless, our trial of SAS had the minor disadvantage of unstable Mn(II) oxidation performance. However, if we could identify the cause of instability and overcome the disadvantage, SAS becomes the most promising substrate for MnOB enrichment. SAS is the bulk of dead cells, which consist of cell walls and cytoplasm. It is unclear whether the cell wall or cytoplasm is preferable for MnOB enrichment. If we could determine the preferable substrate for MnOB enrichment, Mn(II) oxidation performance could be enhanced. Future experiments on SAS as a substrate for MnOB are therefore promising.

5. Conclusions

Three different organic substrates, K-medium, SAS and methanol, successfully enriched MnOB with high Mn(II) oxidation rates during continuous operation of a DHS reactor employing sponge carriers initially combined with abiotic-MnO₂. Microbial analysis revealed that the major MnOB players were significantly different at the genus level among the substrates. We found that organic substances other than K-medium could enrich MnOB. Methanol was most effective in achieving the highest Mn(II) removal with stability. Our data suggest that SAS is a promising

substrate for MnOB enrichment, despite less-stable performance. To better understand the instability, a study on the effect of Mn(II) concentration on MnOB activity is necessary.

Acknowledgments

This research was supported by the Japan Society for the Promotion of Science as a Grant-in-Aid for Scientific Research (A) Grant Number JP23241029, and the Environment Research and Technology Development Fund (2-3K133004) of the Ministry of the Environment, Japan. We thank the Ministry of Research, Technology and Higher Education of the Republic of Indonesia through Riset-Pro for PhD scholarship (World Bank Loan No. 8245-ID) to AS.

Declaration of interests

None.

Supplementary material

Tables captions

Table S1. Sequence reads, total number of OTU and diversity of each reactor

Figure captions

Figure S1. Reactor configuration.

Figure S2. Microbial community at the phyla level. [Phyla with relative abundance <1% were grouped as “Others”.](#)

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Table captions

Table 1. Operational conditions for the DHS reactors.

Figure captions

Fig. 1 The performance of R-1, R-2, and R-3. The arrows indicate biofilm sampling on days-60 and 131.

Fig. 2 Mn(II) removal rates of each reactor during Phase 2 and 3.

Fig. 3 Relationship between Mn(II) loading and removal rates.

Fig. 4 Most dominant genera in the reactors with relative abundance >3% of the total sequence reads in each sample.

Fig. 5 Relative abundance of putative manganese-oxidizing bacteria (MnOB).

Table 1. Operational conditions for the DHS reactors

	R-1			R-2			R-3		
	Phase 1 (0–30d)	Phase 2 (31–59d)	Phase 3 (60–131d)	Phase 1 (0–42d)	Phase 2 (43–59d)	Phase 3 (60–131d)	Phase 1 (0–36d)	Phase 2 (37–59d)	Phase 3 (60–131d)
Organic substrate:									
Yeast extract (mg·L ⁻¹)	1.6–10.0	1.0	2.0	-	-	-	-	-	-
Peptone (mg·L ⁻¹)	6.4–40.0	4.0	8.0	-	-	-	-	-	-
Methanol (μL·L ⁻¹)	-	-	-	-	-	-	6.7–42.1	4.2	8.4
Equivalent COD (mg·L ⁻¹)	8–50	5	10	8–50	5	10	8–50	5	10
Mn(II) (mg·L ⁻¹)	5	5	5	5–7.5	7.5	7.5	5–7.5	7.5	7.5
HRT (h)	4.2–0.6	0.4	0.4	4.2–0.6	0.4	0.4	4.2–0.6	0.4	0.4

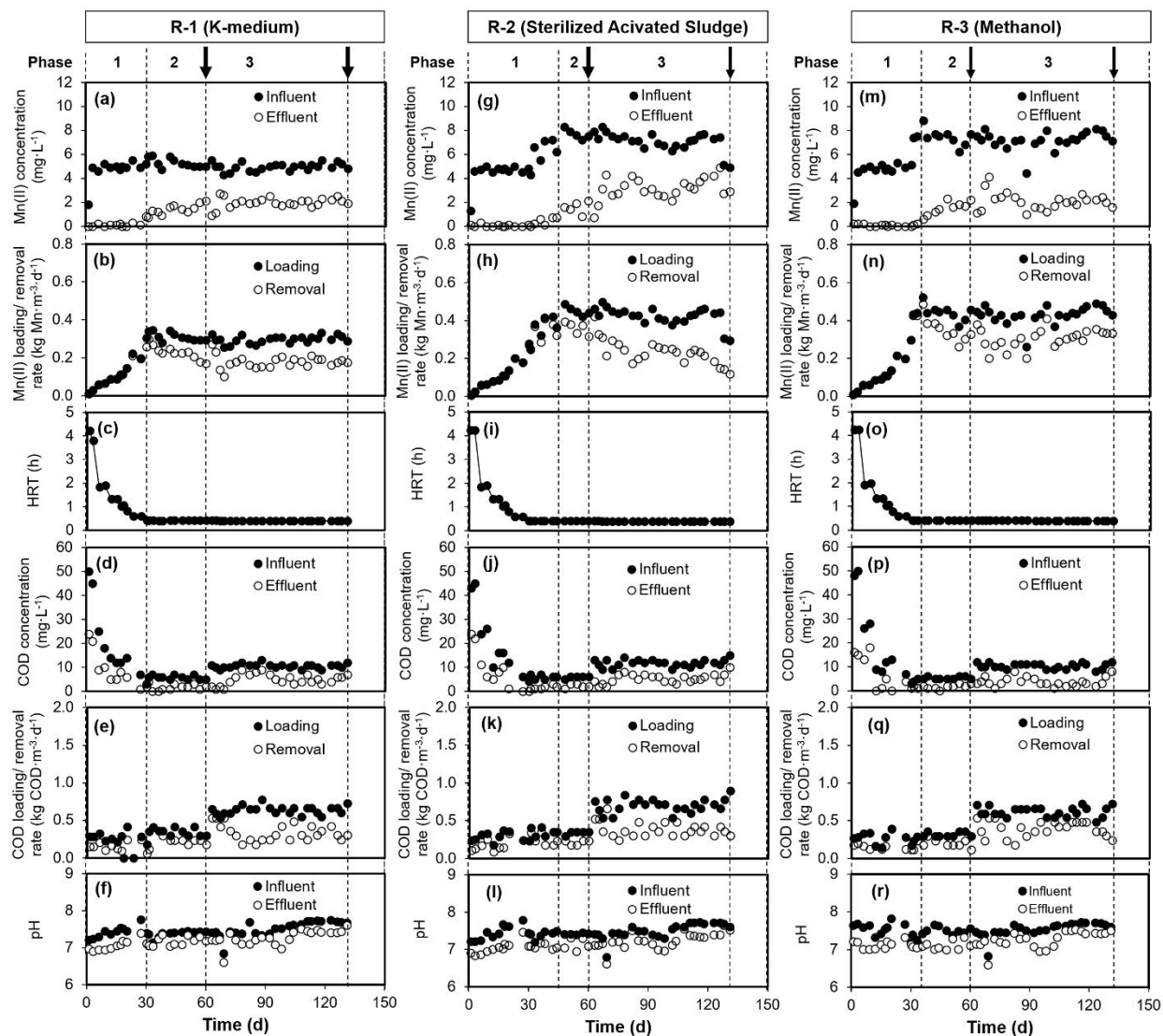


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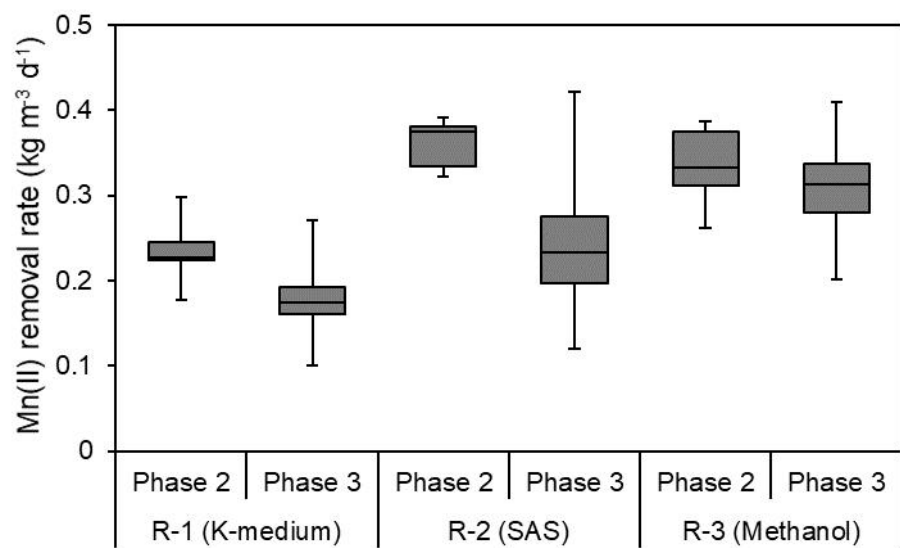


Fig. 2 Mn(II) removal rates of each reactor during Phase 2 and 3

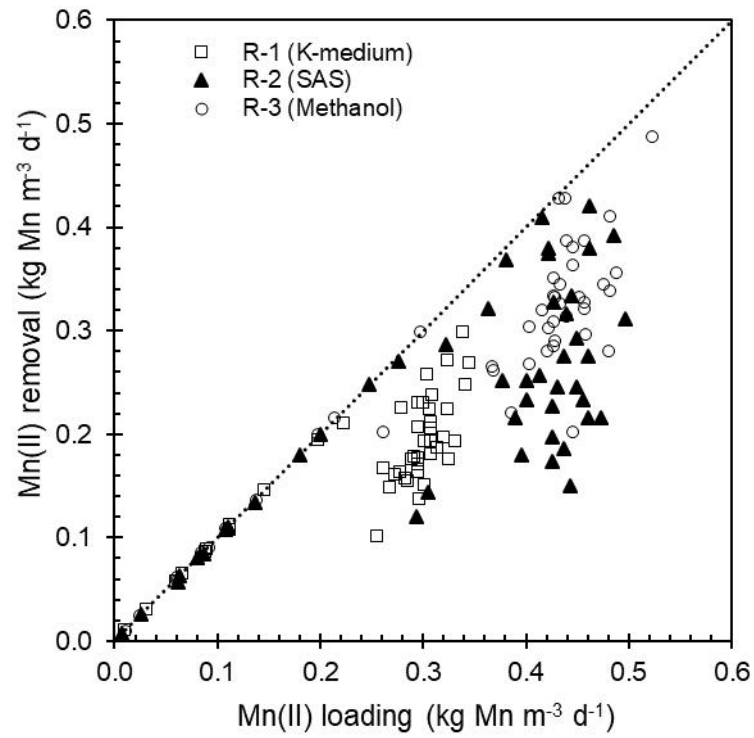


Fig. 3 Relationship between Mn(II) loading and removal rates

Phylum	Genus	R-1 (K-medium)		R-2 (SAS)		R-3 (Methanol)	
		R1-d60	R1-d131	R2-d60	R2-d131	R3-d60	R3-d131
Actinobacteria	<i>Mycobacterium</i>	3.8%	1.2%	2.8%	2.0%	1.4%	2.3%
	<i>Nocardia</i>	0.3%	0.4%	0.9%	3.2%	0.4%	2.5%
Bacteroidetes	unclassified <i>Chitinophagaceae</i>	1.5%	1.7%	0.9%	4.4%	1.4%	2.5%
	unclassified <i>Cytophagaceae</i>	2.8%	3.2%	1.3%	1.5%	1.9%	1.0%
	unclassified <i>Saprospiraceae</i>	2.7%	0.0%	0.5%	0.0%	5.5%	2.1%
Chloroflexi	unclassified <i>Caldilineaceae</i>	11.8%	4.5%	6.3%	9.2%	4.0%	0.6%
	<i>Candidatus Defluviifilum</i>	0.4%	0.3%	4.0%	1.2%	3.9%	1.1%
	<i>Kouleothrix</i>	0.5%	1.3%	1.5%	1.4%	3.6%	4.4%
Proteobacteria	<i>Comamonas</i>	15.9%	1.9%	0.7%	0.2%	0.4%	0.3%
	<i>Hyphomicrobium</i>	0.6%	0.7%	1.5%	1.2%	6.9%	11.1%
	<i>Methylobacter</i>	1.8%	10.3%	0.4%	2.7%	2.4%	0.0%
	<i>Methylobacterium</i>	0.0%	0.0%	0.0%	0.0%	0.1%	8.4%
	<i>Methylocaldum</i>	0.6%	3.5%	0.2%	0.9%	0.9%	0.0%
	<i>Methylophilus</i>	0.1%	0.7%	0.1%	0.3%	5.8%	1.7%
	<i>Pseudomonas</i>	2.8%	4.7%	0.4%	0.4%	0.1%	1.6%
	<i>Zymomonas</i>	1.3%	0.6%	3.0%	1.5%	1.8%	2.4%
Verrucomicrobia	<i>Prostheco bacter</i>	2.8%	0.9%	9.5%	1.5%	4.3%	1.3%

Fig. 4 Most dominant genera in the reactors with relative abundance >3% of the total sequence reads in each sample.

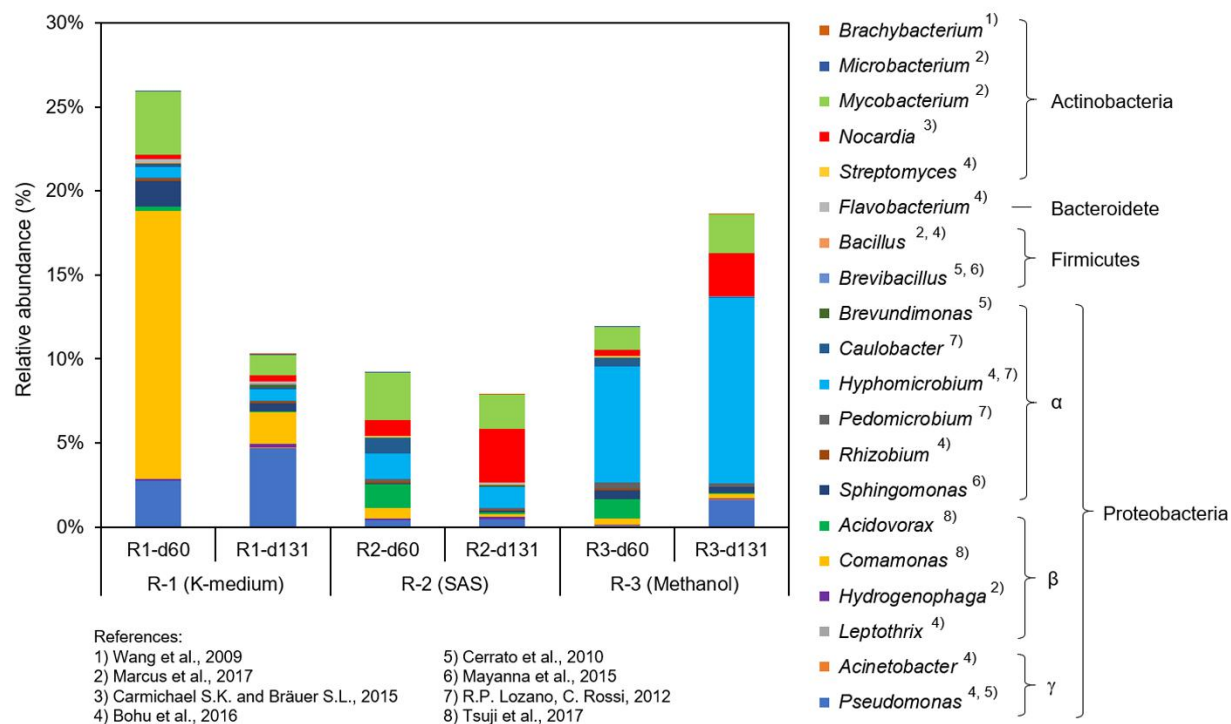


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