**Biological manganese oxidation in biofilms from oxygen-supplemented biological activated carbon (BAC) filters**

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**Abstract**

Biological oxidation of manganese (Mn) under oligotrophic conditions results in the formation of biogenic Mn oxides (MnOx), which are known to be effective catalysts for water remediation. Manganese-oxidizing bacteria (MnOB) often develop in engineered systems for water treatment where such conditions apply. In this study, MnOB within biofilms sampled from a full-scale, oxygen-supplemented biological activated carbon (BAC) filter were investigated. Experimental evidence showed that the microbial community efficiently performed oxidation of Mn2+, growing into thick, aggregated biofilms at circumneutral pH, with manganese carbonate as the sole medium component. The amount of Mn oxidized was quantified using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Scanning electron microscopy (SEM) imaging and X-ray diffraction (XRD) analysis revealed that the MnOx formed was a birnessite-type or δ-MnO2. Comparison of the microbial community composition before and after the Mn enrichment by means of 16S rRNA gene-based profiling showed a drastic increase of members of the order *Rhizobiales*, together with genera *Burkholderiaceae, Rhodococcus, Ellin6067* and Pir4 lineage, never reported in Mn-oxidizing communities before. This work highlights the potential for harnessing the microbial community inside water filtration systems towards the oxidation of Mn2+ into MnOx, which can subsequently function as a catalyst boosting the water treatment performance.

# Introduction

Manganese (Mn) is the second most common trace metal on earth after iron and a critical micronutrient required for the growth and survival of many living organisms (Post, 1999; Sujith and Bharathi, 2011). Mn is found naturally in groundwater, surface water, freshwater, and seawater, mostly in the Mn2+ soluble form (Postawa et al., 2013). The concentration of soluble Mn2+ in the environment relates to redox conditions. In the presence of oxidizing agents (abiotic or biotic) and changes in pH, it is oxidized to insoluble Mn3+, Mn4+, or a higher oxidation state, depending on the oxidizing agents (Nealson, 2006). In aquatic environments, abiotic oxidation is not favored due to the high activation energy required for Mn2+ oxidation by O2 at a pH below 8 (Elzinga, 2011; Oldham et al., 2017). Fast abiotic oxidation in aquatic environments requires an oxidative agent, such as the presence of mineral surfaces (Junta and Hochella, 1994). In most naturally occurring environments with circumneutral pH, biogenic Mn2+ oxidation by bacteria, fungi, or algae is generally 4–5 orders of magnitude faster than abiotic ones (Zhou and Fu, 2020). This biotic mechanism is so ubiquitous that the majority of naturally occurring environmental Mn oxides (MnOx) are believed to be derived from biogenic Mn2+ oxidation or from subsequent alteration of biogenic oxides (Tebo et al., 2004). Biogenic MnOx are representative of the most highly reactive and important MnOx phases in the environment and are some of the strongest naturally occurring oxidizing agents (Spiro et al., 2010). Among manganese-oxidizing microorganisms, manganese-oxidizing bacteria (MnOB) are the most diverse, consisting of numerous species with a wide phylogenetic distribution (Nealson, 2006; Tebo et al., 2005). To date, type strains of MnOB have been isolated and characterized from 4 phyla (*Actinomycetota, Bacteroidota, Bacillota,* and *Pseudomonadota*) (Zhou and Fu, 2020). The reason why MnOB oxidize Mn2+ is poorly understood and unexplored. However, the properties of MnOx (adsorption capacity, cation exchange, and redox functionality) are hypothesized to provide bacteria protection from reactive oxygen species, heavy metal toxicity, and UV radiation, as well as support the biodegradation of recalcitrant organic matter (Tebo et al., 2005; Zhou and Fu, 2020). Some studies have highlighted that Mn oxidation can be coupled with adenosine triphosphate (ATP) synthesis, promoting autotrophic bacterial growth via chemolithotrophy in oligotrophic environments, with Mn2+ being the sole source of energy (Sujith and Bharathi, 2011; Yu and Leadbetter, 2020). A major fraction of biogenic MnOx is indeed produced under oligotrophic conditions (water pipes, hyporheic zones, and marine sediments) (Oldham et al., 2017; Tebo et al., 2005), thus communities rich in MnOB often develop in engineered systems for water treatment where such conditions prevail (Bernstein et al., 2022; X. Zhao et al., 2020). The presence of MnOx in water treatment systems in the past was often attributed to abiotic processes. Recently, many studies have shown the direct implication of bacteria growing in the form of biofilms on the filtration media (i.e., activated carbon, sand) in the removal of Mn2+ and the formation of black, insoluble MnOx particles (e.g., Breda et al., 2018; Sahabi et al., 2009). Insoluble MnOx particles can form and accumulate also within drinking water systems when Mn concentrations are in the range of 0.1 - 0.2 mg L-1 (World Health Organization, 2017). While this concentration is below levels that cause potential health effects (0.4 mg L-1), the resulting particles can cause water aesthetic and operation problems, coloring the delivered water and damaging household appliances (G. Li et al., 2019). For this reason, Mn is conventionally removed from drinking water, and the selection of the appropriate treatment system depends on the form of Mn (dissolved or particulate) present in the source water (World Health Organization, 2021). Treated water with Mn concentration below 0.02 mg L-1 is a common treatment goal for preventing chronic aesthetic and operational problems associated with Mn (Tobiason et al., 2016). Biofiltration, such as biological activated carbon (BAC) filters, can successfully remove Mn from groundwater and surface water, where MnOB in biofilms grow on BAC granules. The biofilms adsorb Mn2+ and the MnOB oxidize it, forming particulate oxides that are then removed by backwashing (Bernstein et al., 2022).

While several studies have investigated the microbial communities in BAC filter biofilms (Du et al., 2020; Lu et al., 2022; Zhang et al., 2018), to the best of our knowledge, none of them have specifically emphasized MnOB subpopulations in descriptions of the biofilm community profiles. This study focused on further characterization of the biological oxidation of Mn in a full-scale BAC filter achieving complete removal of Mn from secondary wastewater effluents (Bernadet et al., 2023). Biofilms detached from aged BAC granules were used to set-up enrichment cultures fed with manganese carbonate (MnCO3) as the sole medium component, and control cultures were prepared by autoclaving biofilms for inactivation. The biomass and biofilm growth were assessed via solids measurement and optical microscopy, while the microbial community was investigated with 16S rRNA gene amplicon sequencing. The MnOx particles developed within the biofilms were characterized by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), X-ray diffraction (XRD), and visualized by Scanning Electron Microscopy (SEM). The results showed that BAC biofilms were able to grow in oligotrophic conditions, producing a high amount of MnOx, due to the selective growth of bacteria belonging to the orders *Betaproteobacteriales* and *Rhizobiales*. The aim of this research was to shed light on the importance and predominance of biological Mn removal in biofiltration systems, with the perspective to apply these natural, Mn-removing microbial communities, for different biotechnological applications.

# Materials and Methods

## **Inoculum source**

The biofilm samples used as inoculum to prepare the enrichment cultures were obtained from the BAC filters of a plant producing ultrapure water (UPW factory, Emmen, NL) from secondary wastewater treatment effluent. A description of the UPW factory treatment line is reported in Supplementary Information (Fig. S1). The BAC filters are operated with periodical pure-oxygen dosing, to maintain full aerobic conditions (van der Maas et al., 2020) and always achieve full removal of Mn after the water treatment (Bernadet et al., 2023). Detached biofilms from the BAC granules’ surface were harvested from backwash water samples collected during the periodical backwashing of BAC filters with air and water. Samples were collected in acid-washed LDPE bottles from the top of the filters within the first 5 min after the backwashing started, and then stored at 4º C until further processing. Two different samplings were executed in September 2021 and January 2022, before and after maintenance of the BAC filters (October 2021) where they were not in operation for 7 days. The samples are referred to as “inoculum biofilm” (IB) hereafter. The composition of the water containing the IB is reported in Table S1.

## **Preparation of Mn enrichment cultures**

To prepare enrichment cultures of manganese oxidizing bacteria (MnOB), 100 mL of the IB collected from BAC filters in September 2021 and January 2022 were placed into 100 mL borosilicate glass bottles with inside walls coated with manganese carbonate (MnCO3 (Alfa Aesar, US)) slurry at a final concentration of approximately 20 mM, and dried overnight in the dark as described elsewhere (Yu and Leadbetter, 2020). To assess if the Mn oxidation was related to biological activity, control bottles were prepared using inactivated IB, sterilized by autoclaving at 121 ºC at 103 kPa for 20 min. All experiments were run in triplicate per condition (active and control biofilms) and per sampling point (September and January). The bottles were closed with gauze cloth to ensure air exchange, and then incubated at room temperature in the dark for 42 days. The Mn and biomass growth were analyzed before and after the incubation, as described in the following sections. Water evaporation of 10% after the 42-day experiment was considered.

## **Characterization of Mn oxides and biofilms**

### Volatile suspended solid and pH measurement

The biomass growth in the active and control biofilm cultures was monitored by measuring volatile suspended solids (VSS) in the sample before and after the experiment, following the EPA standard protocol (EPA 160.4) (Environmental Protection Agency (EPA), 1975). The pH of the samples at the end of the experiment was measured using SevenExcellence pH meterS470 (Mettler Toledo®, CH).

### Inductively coupled plasma atomic emission spectroscopy

The Mn concentration in the IB and at the end of the 42-day experiment for the active and control biofilm was measured on unfiltered samples using an Optima 5300 DV Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Perkin Elmer, US) with argon as the carrier gas, after addition of nitric acid (HNO3) (2% final concentration in solution). Since biogenic Mn oxidation converts soluble Mn2+ into insoluble MnOx (i.e., Mn3+ and Mn4+), the Mn concentration was classified, on the basis of Mn solubility in HNO3, as “acid-soluble fraction” and ‘acid-insoluble” fractions, as described previously by Yu and Leadbetter (2020). For the method development and assessment, four Mn salts were used as reference based on their Mn oxidation state and the solubility of the Mn species in HNO3 (see Table S2 and Supplementary Information section). The procedure to measure Mn fractions in both reference and experimental samples was as follows: 4.5 mL of sample was put inside a falcon centrifuge tube, and 0.5 mL of HNO3 69% (VWR, FR) was added. The sample was then mixed in the dark overnight (~15 h) to reassure all the Mn2+ was in soluble state. The mixed sample with acid was measured using ICP-OES and is called “acid-soluble fraction”. A 2 mL of the sample was mixed with 4.8 mL of HNO3 69% and 4 mL H2O2 30% (VWR, UK) and was then subjected to microwave-assisted digestion (Ethos Easy, Milestone SRL, IT) at a temperature of 180 °C for 30 min in ramp mode. Once finished, the sample was diluted until it contained 2% of HNO3 and measured as the total Mn. The subtraction between total Mn and “acid-soluble fraction” results in “acid-insoluble fraction”.

### X-ray diffraction

To characterize MnOx particles, powder X-ray diffraction (XRD) patterns of IB and the Mn-enriched biofilms were obtained using a diffractometer (Bruker D8 advance, Bruker GR) with Cu Kα radiation (30 kV, 30 mA, λ= 1.034 Å). A low background silicon sample holder was used during the analysis. The obtained XRD patterns were compared with the Crystallography Open Database (COD, <http://www.crystallography.net/cod/>) using Mn as the mandatory element to determine the mineral phases of the samples. Prior to XRD analysis, all the IB and Mn-enriched biofilms in both active and non-active samples were freeze-dried.

### Scanning electron microscopy

A 1 mL sample from each experimental bottle was fixed in 2.5% glutaraldehyde at 4 °C overnight (~15 h). The sample was then attached to a 0.2 µm hydrophilic polycarbonate membrane (IsoporeTM, US). The membrane was then washed thrice using a HEPES buffer solution 25 mM, pH 7.5 (Sigma Aldrich, US). Dehydration steps were performed using ethanol (VWR) for 15 min by increasing each step its concentration from 30, 50, 70, 90, to 100% (v/v). A final dehydration step was performed using critical point drying (Leica EM CPD3000), where ethanol was replaced by liquid CO2 at <10 °C and 55 bar. After reaching the critical point, the pressure was released slowly at >35 °C until atmospheric conditions. The fixation and drying method was modified from (Yu and Leadbetter, 2020). Scanning electron microscope (SEM) imaging was performed on the dried samples using JEOL JSM-6480LV at an operating voltage of 6 and 15 kV, respectively.

### Optical microscopy and biofilm staining

Biofilm aggregates from experimental bottles were analyzed on glass slides using optical microscopy (DM750, Leica, DE), and images were acquired using Leica LAS-X (version 4.12) software. Extracellular polymeric substances (EPS) of biofilms were further visualized by mixing 100 µl of the sample with 20 µl of 0.1% crystal violet, targeting proteins and polysaccharides (O’Toole et al., 1999).

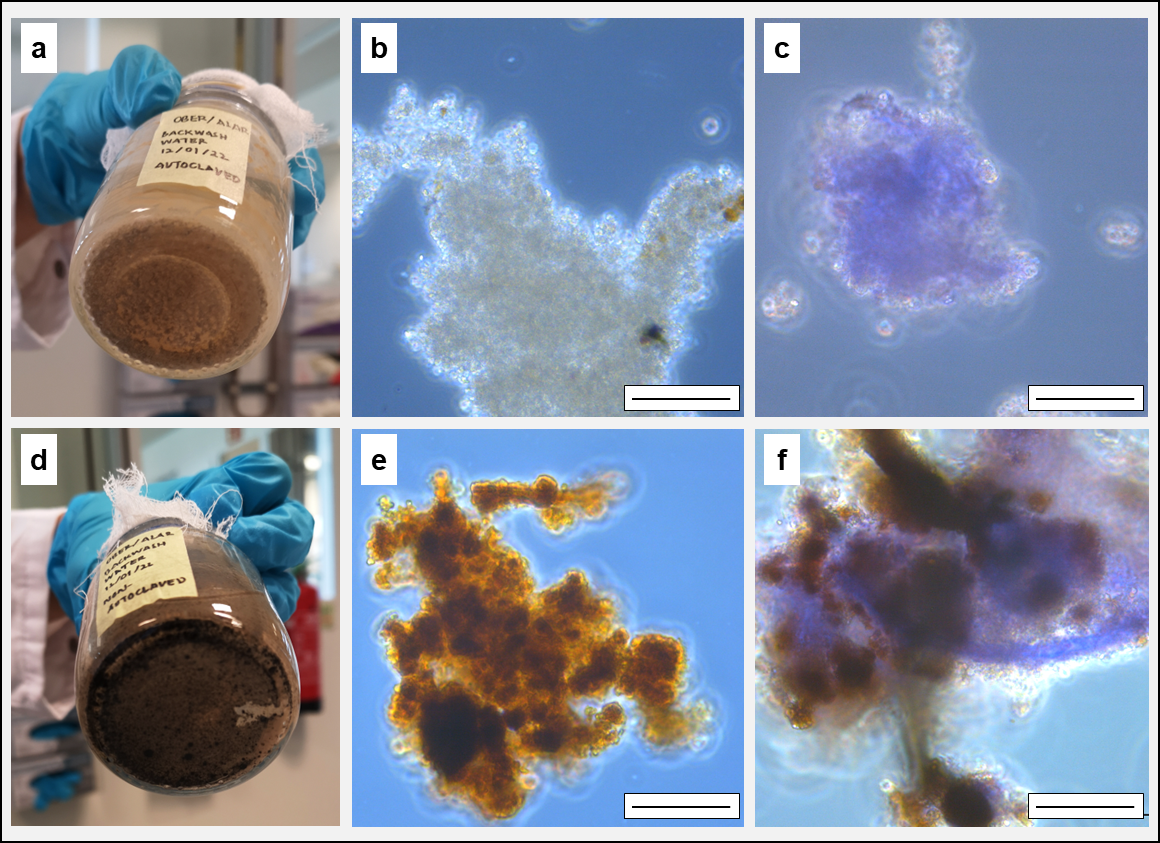
## Microbial community analysis

Microbial community analysis based on 16S rRNA gene amplicon sequencing (NGS) analysis was performed on the initial IB samples and the enrichment cultures after 42 days. 10 mL of the mixture of biofilm and liquid from two of the three replicate experimental bottles was centrifuged at 4750 x*g* at 4 °C for 5 min. The liquid was then decanted, and the pellet was washed with PBS buffer and used for DNA extraction with the FastDNA™ Spin kit for soil (MP Biomedicals, US), following the manufacturer’s instructions. The extracted DNA was quantified using fluorescence spectroscopy (QuantiFluor dsDNA system and Quantus™ Fluorometer (Promega, US)). The V4 - V5 region of the 16S rRNA gene of bacteria and archaea was amplified using PCR primers 515F (Parada et al., 2016) and 926R (Quince et al., 2011). Amplicon sequencing was performed at MrDNAlab (Shallowater, US) on a MiSeq (Illumina, US) with 2x300 bp (V3) paired-end sequencing. Quality control of sequences was performed in QIIME2 (v. 2019.10) (Bolyen et al., 2019), where DADA2 (Callahan et al., 2016) was used for error-correction and inference of exact amplicon sequence variants (ASVs). For the taxonomic classification of ASVs, reference sequences of SILVA v.138 database were used (Quast et al., 2013). QIIME2 scripts and an Rmarkdown document are accessible from Github (<https://github.com/pietervanveelen/Larasati_Mn_oxidation_16SrRNA>). Sequencing data were deposited in the European Nucleotide Archive (ENA) under the project number PRJEB64232.

# Results and Discussion

## Biomass growth and Mn nodules observed in the enriched cultures

After 42 days, the difference between non-active and active biofilms was clearly visible, with control bottles that preserved the sandy brown color of MnCO3 slurry (Fig. 1 A), while all the cultures fed with active biofilms had turned dark brown to black (Fig 1 D). A closer look at the active biofilms using microscopy showed the presence of a robust EPS matrix extensively loaded with black nodules (Fig. 1 E and F), opposite to the non-active biofilms, where Mn was still visible as non-converted form (Fig. 1 B and C). Similar black MnOx nodules resulting from biological oxidation were observed previously via microscopy in lab-scale conditions (Cavazos and Glass, 2020; Furuta et al., 2023; Yu and Leadbetter, 2020).



**Fig. 1** – Representative photos and micrographs showing the difference between control and active cultures after 42 days in the presence of MnCO3. Control bottles with non-active biofilms did not turn black, preserving the sandy color of MnCO3 slurry (a), and microscopy observation showed persistence of MnCO3 as salt (b), while the original EPS matrix was preserved (c). Experimental bottles inoculated with active biofilms were dark brown to black (d) and the biofilm accumulated black nodules (e) and was rich in EPS (f). Scale bars are 20 μm.

Further visual analysis of the samples by SEM showed that raw MnCO3 slurry particles (Fig. 2 A) covered the non-active biofilms in the control bottles (Fig. 2 B), confirming the lack of conversion into nodules. On the other hand, in the bottles inoculated with active biofilms, the aggregates (Fig. 2 C and E) mostly contained the Mn crystals form as nanoflower structures, clearly distinguishable by shape in comparison to the raw MnCO3 particles (Fig. 2 D and F, indicated by arrows).

A picture containing reef, screenshot, coral

Description automatically generated

**Figure 2** – Scanning Electron Microscopy (SEM) images of samples taken before and after the 42 days. Before coating the experimental bottles, the appearance of the MnCO3 slurry (a) was with small, tightly aggregated round particles. In (b), the control biofilm (non-active) was covered with MnCO3 particles at the end of the experiment. At the same time point, active biofilms from September (c and d) and January (e and f) showed the conversion of the MnCO3 particles (white arrows) into Mn oxide crystals with a nanoflower structure (black arrows).

Most biological MnOx under actual environmental conditions, formed via biological catalytic oxidation, were found to have a 3D flower-like layered structure, such as buserite, birnessite and δ-MnO2 (Li et al., 2021). It is worth noting that Mn-enriched biofilms from the bottles that originated from IB in September showed a higher number of nanoflower structures (Fig. 2 C) than the bottles inoculated with January IB, in which more unconverted MnCO3 particles were visible (Fig. 2 E). This could be a consequence of maintenance procedures to the BAC filters that preceded the January sampling, which left the BAC granules without nutrients and minerals for 7 days (see section 2.1).

VSS measurements indicated that active biofilms increasingly developed over time from the start-up moment of IB in the bottle, while the VSS values measured in control, non-active biofilms, remained stable from the start-up IB (Table 1), highlighting that MnCO3 was likely actively utilized as an energy source. Assuming the complete degradation of the total chemical oxygen demand (tCOD) present in the biofilm inoculum solution at the start of the experiment (Table S1) (which was the only organics source present in the cultures), the observed biomass growth in terms of VSS for the September and January experiments was relatively high in comparison to BAC biofilms growing in similar oligotrophic, experimental conditions (Lin and Ho, 2022; Piai et al., 2022). In some bacteria, Mn oxidation can be coupled with ATP synthesis via chemolithoautotrophy, with Mn2+ being the sole source of energy, as discovered already in early studies on single strains from oligotrophic environments (Arcuri and Ehrlich, 1980; Ehrilch and Salerno, 1990; Ehrlich, 1980, 1978).

pH values in all bottles at the end of the experiments were around neutral, with a slightly higher pH in the control cultures (Table 1). In the experimental conditions applied (atmospheric pressure and 20% O2 from air), and at a pH below 8, the abiotic Mn2+ oxidation is not favored since a high activation energy is required for oxidation by O2 (Elzinga, 2011; Oldham et al., 2017), as also depicted in the adapted Pourbaix diagram in Fig. S2.

**Table 1** - Volatile suspended solids (VSS) and pH values measured in duplicate on the start-up inoculum biofilm, in comparison to the control and active enrichment cultures after 42 days. For each experiment, values and standard deviations were calculated on triplicate bottles.



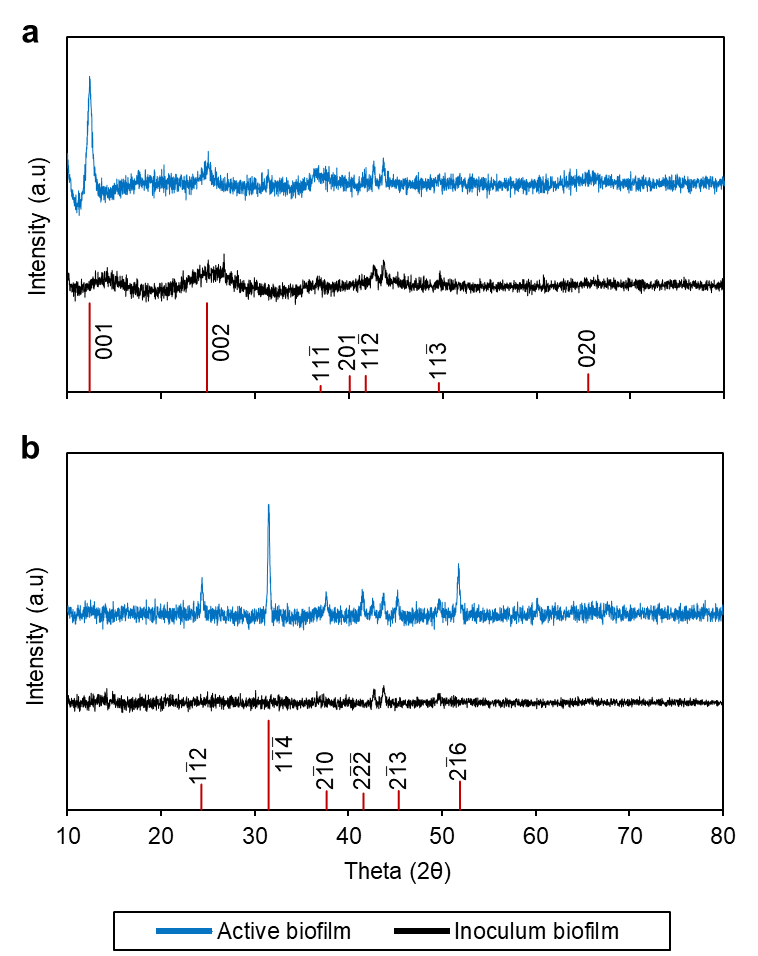
## Characterization of the conversion of soluble into insoluble Mn

To determine the fraction of soluble Mn2+, fed as MnCO3 into the experimental bottles, that was converted into insoluble Mn (i.e., Mn3+ and Mn4+), a dedicated method was developed applying ICP-OES to determine Mn concentrations as “acid-soluble” and ‘acid-insoluble” fractions (see section 2.3.2). At the end of 42-day experiment, the bottles with control and active biofilms contained the same amount of total Mn. However, the amount of acid-insoluble Mn, corresponding to the oxidized ones (Mn3+ andMn4+), was 6- to 10-fold higher for the active biofilms (Table 2), further supporting the hypothesized biological Mn oxidation. The fraction (%) of acid-soluble (corresponding to the unconverted Mn2+) and insoluble Mn present in the control bottles was similar to the values calculated for the IB, suggesting the abiotic oxidation did not occur in the control bottles, even though the water pH values in the control biofilm bottles were higher (7.8 – 8.2) than in the active biofilm bottles (7.4) (Table 1). Active biofilms converted most of the soluble Mn2+ into acid-insoluble forms, where the fraction in the active biofilm of September bottles was higher (average of 84.8%) than in the January bottles (average of 52.1%) (Table 2).

**Table 2** – Mn2+oxidation yields analyzed via ICP-OES in the inoculum biofilm and the experimental bottles (control and active biofilms) after 42 days of incubation with MnCO3 as the sole medium component added. For each sample type, average values and standard deviations were calculated on triplicate bottles. The amount of soluble Mn2+ and insoluble MnOx (i.e., Mn3+ and Mn4+) are called “acid-soluble” and “acid-insoluble” fraction, respectively. The “acid-insoluble” fraction was calculated as difference between total Mn and “acid-soluble” fraction.



The difference in the oxidation of Mn between the September and January experiments was also observed in the results obtained from XRD analysis (Fig. 3). The XRD patterns of MnOx produced by active biofilms in the September experiment (Fig. 3 A) revealed that Mn was oxidized into birnessite-type oxide or δ-MnO2, based on XRD peaks of birnessite obtained from the database (COD 9013652) (Lopano et al., 2009). The XRD patterns from the September experiment (Fig. 3 A) were relatively low in peak intensity and showed peak broadening, indicating low crystallinity or amorphous structure of the MnOx formed and/or the presence of impurities. The two peaks at 7.36 Å (12.01° 2θ) and 3.68 Å (24.16° 2θ) could be indexed to δ-MnO2 (Lopano et al., 2009). Peaks appearing around 2.43 Å (36.9° 2θ) and 1.4 Å (66.2° 2θ) are hk0 bands, which are mostly found to be broad and have low signal-to-noise ratios and could correspond to small sizes of the crystals formed (Holder and Schaak, 2019). The basic building block of synthetic and natural MnOx is the MnO6 octahedron, which can be joined together in two structures: (1) chain and tunnel structures (tectomanganates) and (2) layer structures (phyllomanganates) (Webb et al., 2005b). Biogenic MnOx were reported to be primarily composed of birnessite and/or bursite types of phyllomanganates (Tebo et al., 2004), consisting of stacked hexagonal sheets of MnO6 octahedra, being extremely small with numerous structural defects (Spiro et al., 2010). A δ-MnO2 is defined as a layered polymorph with repeating single layers of MnO2, separated by cations and/or water molecules (Robinson et al., 2013). The XRD patterns of MnOx formed in the September experiment were similar to abiotically synthesized H+-birnessite or H+ inserted between layers in the δ-MnO2 structure (Saratovsky et al., 2006). The morphology of δ-MnO2 formed by the active biofilm samples (Fig. 2 D) was also similar to δ-MnO2 that was chemically synthesized using potassium permanganate (KMnO4) and hydrochloric acid (HCl), where the oxide structure is built up of nanosheets, forming nanoflower structure (Cheng et al., 2022; Cremonezzi et al., 2020). Synthetic birnessite-type, δ-MnO2 with nanoflower morphology exhibits excellent abilities for removal of different chemical species (Qin et al., 2016; Wang et al., 2015), showing higher oxidation activity than other morphologies, such as nanosheet or nanowire (Hou et al., 2017). δ-MnO2 is known as a promising and effective catalyst for removing ammonia (Cheng et al., 2022), organic water contaminants (Remucal and Ginder-Vogel, 2014), and oxidize metals (Li et al., 2022); therefore, its presence in BAC filters could be beneficial to perform a robust water treatment process to remove these types of contaminants.



**Figure 3** – XRD patterns of the inoculum biofilm and the experimental bottles with active biofilms after 42-day incubation with MnCO3 as the sole medium component, for September (a) and January (b) experiments. The red line corresponds to Miller indices (hkl) adapted from Crystallography Open Database (COD): (a) birnessite COD 9013652 (Lopano et al., 2009) and (b) rhodochrosite COD 9007690 (Maslen et al., 1995).

The XRD patterns of the samples after 42 days of incubation by active biofilms in January (Fig. 3 B) show that Mn was not fully oxidized and still present as crystalline MnCO3, as compared with XRD peaks of rhodochrosite (a pure MnCO3 mineral) from the database (COD 9007690) (Maslen et al., 1995). This finding aligns with only a fraction of ~52% of Mn2+ oxidized in January samples (Table 2) and the presence of some remaining MnCO3, as indicated by the SEM images (Fig. 2 E and F). In contrast to the VSS values measured at the end of the experiments (Table 2), the lower yield of Mn oxidation suggests that the MnOB present in the IB samples from January were less active than the ones from September; an observation that can be linked to the maintenance procedure of the BAC filters in between the two sampling dates (see section 2.1). Nevertheless, in the January experiment, half of the MnCO3 was oxidized and transformed from acid-soluble Mn into acid-insoluble Mn (Table 2), and similar nanoflower structures were observed as in September (Fig. 2 E and F). The exact form of the oxidized Mn in these samples was challenging to distinguish using XRD due to the residual MnCO3 and the presence of amorphous MnOx.

## Microbial community enrichment into specialized MnOB populations

Different (putative) MnOB have been found in BAC filters (Bernstein et al., 2022), and better-described isolated species are affiliated with the phyla *Actinomycetota, Bacteroidota, Bacillota,* and *Pseudomonadota* (Zhou and Fu, 2020). At the phylum level, in both September and January experiments, there was an evident relative increase of 55% in the abundance of *Pseudomonadota* (formerly called *Proteobacteria*) when feeding Mn, with a concurrent relative decrease in members of the phylum *Bacteroidota*, that dominated the IB of both samplings (Fig. S3). Within the phylum *Pseudomonadota*, members of the orders *Rhizobiales* and *Betaproteobacteriales* had the highest increase (between 70 and 80% in comparison to the start-up IB) after 42-day incubation with MnCO3 (Fig. S3). The majority of *Bacteroidota* identified in the IB,which relative abundance in the community decreased in the Mn enrichment cultures, were affiliated with the order *Chitinophagales* (Fig. S3)*.* Members of the two families of the order, *Chitinophagaceae,* and *Saprospiraceae*,were identified in biological filtration systems where nitrification, protein degradation, and removal of micropollutants were the prevailing metabolisms (Bartelme et al., 2017; Gomez-Alvarez et al., 2013; Jeon et al., 2020; Ouyang et al., 2019). Such metabolisms were indeed prevalent in the BAC filters from which the IB were sampled (Bernadet et al., 2023).

A core microbiome of eight bacterial groups was significantly increased in bottle experiments of both September and January in comparison to their IB (Fig. 4). This core dominated the community in all the replicates analyzed, and comprised genera (of) *Burkholderiaceae, Pseudomonas, Hyphomicrobium*, *Rhodococcus*, *Nitrosomonadaceae* *-* Ellin6067 and *Pirellulaceae*- Pir4 lineage, the family *Rhizobiales* A0839 and unidentified *Rhizobiales*. Most of these have been associated with Mn oxidation before. For example, *Pseudomonas spp.* are model microorganisms for Mn oxidation identified in many different settings (Zhou and Fu, 2020), for which pathways and enzymes involved were partially characterized (Geszvain et al., 2013; Okazaki et al., 1997; Villalobos et al., 2003). *Hyphomicrobium* isone of the first Mn oxidizers studied since it is responsible for the accumulation of MnOx into water pipelines (Tyler and Marshall, 1967), and it was identified in several other water environments (Albers et al., 2015; X. Zhao et al., 2020). Mn-oxidizing *Rhizobiales* were previously identified in various natural habitats (Tebo et al., 2005), and recent studies have discovered new species within this orderhaving Mn-oxidizing ability (Anderson et al., 2009; Marcus et al., 2017; Sjöberg et al., 2020). Another known Mn-oxidizing *Rhizobiales* growing in the experimental bottles was *Pedomicrobium* (Larsen et al., 1999)(Fig. 4),in whichdeposition of MnOx occurs in close association with an extracellular polymeric matrix excretion (Sly et al., 1990), as also observed in our cultures (Fig. 1 F). Conversely, the family *Rhizobiales* A0839 has not been associated with Mn-oxidizing ability before. All the *Rhizobiales* not identified at the genus level, accounting for an average 7% relative abundance of these enrichment bottles, potentially correspond to undescribed Mn-oxidizing species. The other groups within the core microbiome have rarely or never been associated with Mn oxidation. *Burkholderiaceae* was the dominant genus in all the samples analyzed, with average relative abundances of 9.1% and 7.9% in the September and January experiments, respectively. This genus was associated once with Mn-oxidation communities found in caves (Carmichael and Bräuer, 2015). However, *Burkholderiaceae* were reported among metal-oxidizing communities in several natural environments (Chakraborty et al., 2020; Li et al., 2013; Vander Roost et al., 2018), and in microbial mats showing heavy metal resistance abilities (Drewniak et al., 2016). In the core microbiome, *Rhodococcus* was also included*,* a genus of soil actinomycetes that utilize a wide variety of organic compounds as growth substrates, also used in biotechnology to remove pollutants or produce drugs (Yam et al., 2011). One species, *Rhodococcus opacus,* was recently found to bioadsorb Mn2+ (Pimentel et al., 2022). The putative ammonia-oxidizing *Ellin6067* is a genus of *Nitrosomonadaceae* identified at high relative abundance in all the experiments (Fig. 4), commonly found in soil (Ye et al., 2016), which growth was stimulated in lab-scale conditions where a stress factor was present, such as high pharmaceuticals concentrations (Vega et al., 2022), light irradiation (L. Wang et al., 2021), or cadmium stress (Qu et al., 2022; G. Wang et al., 2021). *Nitrosomonadaceae,* in general, were found in nitrifying communities associated with MnOB, supporting their growth (Cao et al., 2015; Van Le et al., 2022), while *Ellin6067* is hypothesized to perform autotrophic denitrification under low C/N or carbon source-free conditions (Chen et al., 2020; Qiu et al., 2020). Thus, their strong increase in relative abundance from IB by 90% and 74% increase in the September and January experiments, respectively, could be associated with the oxidation of ammonia originating from biomass decay during 42 days in batch conditions.



**Fig. 4** - Relative abundances and trends of dominant bacterial groups (>1% relative abundance in at least one of the samples reported) comparing the inoculum biofilms (IB) and active biofilms sampled after 42 days from the September and January experiments in duplicate bottles (B1 and B2). Microbial groups were identified via microbial community analysis using 16S rRNA gene amplicon sequencing, and their taxonomy classification is reported at the identified level. Values are reported as percentages (in the range 0% to 12%), while arrows indicate the trend in comparison to the inoculum: green (increasing), yellow (stable), and red (decreasing).

Members of the family *Pirellulaceae* are commonly found in oligotrophic and extreme environments (Schlesner et al., 2004). The Pir4 lineage, included in our core microbiome, consists of a large number of uncharacterized isolates, with one novel genus isolated from metalliferous deposits of hydrothermal vent fields (Storesund et al., 2018). *Terrimonas*, one of the dominant bacteria in both IBs from the September and January experiments (Fig. 4), decreased in relative abundance in both experiments, but remained present at averages of 2.6% and 6.6% in the September and January experiments, respectively. Members of this genus are soil bacteria, often associated with the degradation of recalcitrant organics (Madeira et al., 2019), and in some cases, were reported to be able to oxidize Mn in natural environments such as rock varnish (Carmichael and Bräuer, 2015) and birnessite-type manganese deposits (Sjöberg et al., 2020).

## Importance of investigating Mn oxides from mixed microbial cultures

Biogenic MnOx are frequently detected in water treatment systems such as biofilters, where black nodules form within the biofilm matrix growing on top of the filtration medium (Bernstein et al., 2022; Duckworth et al., 2017; Hu et al., 2020). These MnOx are of technological importance since they can be utilized to improve water treatment performances and environmental remediation. Biogenic MnOx indeed can adsorb and oxidize toxic metals (e.g., lead (Pb), chromium (Cr), arsenic (As), and cadmium (Cd)), and organic matter (Zhou and Fu, 2020), boosting the degradation of a wide array of compounds, including recalcitrant pollutants, through various mechanisms (Tebo et al., 2004). Moreover, accumulation of labile Mn3+ intermediates during the bacterial Mn oxidation has been reported, which is a strong oxidant that can be utilized by the microorganisms to metabolize recalcitrant organics or to protect against reactive oxygen species (Spiro et al., 2010; Toner et al., 2005; Webb et al., 2005a). A better understanding of the properties of MnOx formed by biofilm mixed microbial cultures in such filtration systems is thus of interest, particularly to determine if it improves the water treatment performances. In addition, the manganese-oxidizing microbial communities within water filtration systems could be applied as a cost-effective method to efficiently produce Mn3+, a high-quality catalyst. In this study, we showed that mixed microbial communities in biofilms sampled from an oxygen-augmented BAC filtration system efficiently convert Mn2+ into MnOx in oligotrophic conditions, growing a consistent biofilm EPS matrix (Fig. 1, Table 1) that encompasses black nodules of birnessite-type minerals (Fig. 1 and 3), organized in nanoflowers structures with nano-layered crystals (Fig. 1 and 2). The EPS matrix of biofilms plays a fundamental role in Mn adsorption and oxidation (as assessed in pure culture bacteria (Li et al., 2016; Toner et al., 2005)), as Mn-oxidizing organisms utilize secreted organic molecules as templates for mineral growth (Emerson et al., 1989). There is a strong connection between biomacromolecules and MnOx deposition in natural environments (Huangfu et al., 2019); this connects with the role of MnOx not only in converting, but likely also preserving organic carbon in oligotrophic conditions (Estes et al., 2017). The presence of microbial enzymatic oxidation activity contributes to the continuous regeneration of biogenic MnOx nodules within the biofilm matrix, making their catalytic properties potentially unlimited as long as Mn2+ is present in the medium (Martínez-Ruiz et al., 2020; Tran et al., 2018).

The reactivity of MnOx with metal cations depends on the fine structure of the oxides (Spiro et al., 2010). Therefore, detailed knowledge of MnOx structures formed by microorganisms living in a variety of environmental conditions can provide insight into how they can ultimately be applied as catalysts. Most of the knowledge on the catalytic potential of biogenic MnOx comes from the isolated model microorganisms *Bacillus* sp. strain SG-1, *Pseudomonas putida* strain MnB1, and *Leptothrix discophora* strain SS-1 (Spiro et al., 2010; Toner et al., 2005; Webb et al., 2005b). These bacteria form a mixed-valent, layered MnOx similar to hexagonal birnessite, with a poorly crystalline structure. Extended X-ray absorption fine structure (EXAFS) spectroscopy showed that biogenic MnOx from these type strains, has a higher number of negative charges than the chemically synthesized one, which can allocate numerous hydrated metal cations (Saratovsky et al., 2006; Webb et al., 2005b), and the embedding organic material of the biofilm do not compete with this adsorption (Spiro et al., 2010). In contrast to the above-mentioned investigations of Mn oxidation by pure cultures in rich culture media, in this work, we used mixed microbial biofilms that were not provided with any macronutrient besides the ones already present in the inoculum water (Table S1). Under these oligotrophic conditions, XRD profiles showed the formation of a δ-MnO2 (Fig. 3 A) with nanoflower morphology (Fig. 2) similar to the chemically synthesized δ-MnO2 (K. Li et al., 2019), already applied as catalysts for the decomposition of recalcitrant compounds or oxidation of toxic metals (Li et al., 2020; H. Zhao et al., 2020). The 3D nanoflower morphology ensures a high surface to volume ratio, enhancing surface adsorption and charge transfer, accelerating the kinetics of reactions (Shende et al., 2018). The redox and adsorption properties of biogenic MnOx may provide increased access to both biodegradable and recalcitrant materials for microbial degradation, thus being beneficial for water treatment in general.

By means of 16S rRNA gene sequencing, we identified several dominant bacteria, of which most have never been detected within Mn oxidizing communities before (Fig. 4). To date, the characterized biochemical mechanisms associated with Mn oxidation are limited to a few isolated strains, thus the list of Mn oxidation enzymes is still very limited (Tebo et al., 2005). Overall, future work should focus on further structural characterization of MnOx formed by mixed microbial culture and link to the catalytic potential, as well as on unraveling the enzymology behind this almost ubiquitous yet poorly understood microbial function.

# Conclusions

Biofiltration, such as a BAC filter, can remove and retain Mn, thanks to the activity of MnOB in biofilms growing on the surface of BAC granules. Our results indicate that the detached biofilms from aged BAC granules harbor a diverse microbial community that has the capability to grow via Mn oxidation. We specifically enriched MnOB groups using MnCO3 as the sole medium component in the experiment, resulting in the formation of biogenic MnOx characterized as a birnessite-type MnO2 or δ-MnO2 via SEM visualization and XRD analysis. Within a wider context, the results are relevant to understanding the characteristics of MnOx formed biologically by mixed microbial cultures in biotechnological contexts where MnOB likely coming from soil and groundwater, finding a suitable habitat to adhere and grow. The presence of biogenic MnOx in biofilters may be advantageous in terms of removing recalcitrant compounds from water.

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