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

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**Abstract**

Biological oxidation of manganese (Mn) by bacteria results in the formation of biogenic Mn oxides (MnOx), which are known to be strong oxidants and effective catalysts in several reactions. Manganese-oxidizing bacteria (MnOB) often develop in engineered systems for water treatment under oligotrophic conditions. In this study, we investigated the MnOB within biofilms sampled from a full-scale oxygen-supplemented biological activated carbon (BAC) filter performing the complete removal of Mn from wastewater. In enrichment cultures with manganese carbonate as the sole medium component and at circumneutral pH, the microbial community efficiently performed oxidation of Mn2+, growing into aggregated biofilms with numerous black nodules. The amount of Mn oxidized was quantified using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). X-ray diffraction (XRD) analysis and Scanning electron microscopy (SEM) revealed that the MnOx formed was a birnessite-type or δ-MnO2 with a nanoflower structure. Comparison of the microbial community composition before and after the Mn enrichment by means of 16S rRNA gene-based profiling showed a drastic increase in members of the order *Rhizobiales*, together with unclassified generaof *Burkholderiaceae* and *Pirellulaceae* Pir4 lineage*, Rhodococcus,* and *Ellin6067*, of which members have never been reported as Mn-oxidizing microorganisms. This work highlights the potential for harnessing the microbial community in water filtration systems towards the oxidation of Mn2+ into MnOx, which can subsequently function as a powerful catalyst boosting the removal of recalcitrant compounds from the treated stream.

# Introduction

Manganese (Mn) is the second most abundant transition elements on earth after iron and a critical micronutrient required for the growth and survival of many living organisms (Post, 1999; Sujith and Bharathi, 2011). It occurs 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 oxidants (abiotic or biotic) and changes in pH, it is oxidized to insoluble Mn3+, Mn4+, or a higher oxidation state, depending on the oxidant (Nealson, 2006). In aquatic environments, Mn2+ abiotic oxidation by O2 is not favored due to the high activation energy at pH below 8 (Elzinga, 2011), requiring an alternative oxidant such as mineral surfaces (Junta and Hochella, 1994). In contrast, biogenic Mn2+ oxidation by bacteria, fungi, or algae is generally 4–5 orders of magnitude faster than abiotic one (Zhou and Fu, 2020). This biotic mechanism is so ubiquitous that the majority of naturally occurring 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 natural oxidizing agents (Spiro et al., 2010). Among manganese-oxidizing microorganisms, manganese-oxidizing bacteria (MnOB) are the most diffused, consisting of numerous species with a wide phylogenetic distribution (Nealson, 2006; Tebo et al., 2005). Few type strains were characterized from 4 phyla, namely *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria* (Zhou and Fu, 2020). Two main hypotheses have been proposed as to why bacteria oxidize Mn2+: first, the properties of MnOx (adsorption, cation exchange, and redox functionality) may provide bacteria protection from oxidative stress, heavy metal toxicity, and UV radiation, and aid in the biodegradation of recalcitrant organics (Tebo et al., 2005; Zhou and Fu, 2020). Second, Mn oxidation can be coupled with adenosine triphosphate (ATP) synthesis, promoting autotrophic bacterial growth via chemolithotrophy in oligotrophic environments, with Mn2+ being the sole energy source (e.g., Sujith and Bharathi, 2011; Yu and Leadbetter, 2020). A major fraction of biogenic MnOx is indeed produced under oligotrophic conditions (Tebo et al., 2005), thus communities rich in MnOB often develop in water treatment processes where such conditions prevail (Bernstein et al., 2022; Hu 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 filtration media (i.e., activated carbon, sand) in the removal of Mn2+ and the formation of black, insoluble MnOx particles (Bernstein et al., 2022; Sahabi et al., 2009). MnOx particles can form also in drinking water systems when Mn concentrations are in the range of 0.1 - 0.2 mg L-1 (World Health Organization, 2017), causing water aesthetic and operational problems (Li et al., 2019). Thus, Mn2+ is conventionally removed from drinking water, aiming to reduce its concentration below 0.02 mg L-1 (Tobiason et al., 2016). In biofiltration systems, such as biological activated carbon (BAC), the Mn2+ is adsorbed in the biofilm matrix growing on the BAC surface and oxidized by MnOB to form particulate oxides that are then removed by backwashing (Bernstein et al., 2022). Recently, biofiltration research has focused on tailoring microbial communities development to enhance its performance (Kirisits et al., 2019). While several studies have investigated the microbial communities in BAC filter biofilms (e.g. Lu et al., 2022, 2020; Qi et al., 2019), 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 potential for biological oxidation of Mn2+ in a full-scale BAC filter, normally achieving complete removal of Mn from secondary wastewater treatment effluent (Bernadet et al., 2023). Biofilms backwashed from 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 volatile suspended solids (VSS) measurement and optical microscopy, while the microbial community was investigated with 16S rRNA gene amplicon sequencing. The degree of Mn oxidation was assessed by developing a dedicated method for Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), and the MnOx particles developed were characterized with Scanning Electron Microscopy (SEM) and X-ray diffraction (XRD). The results showed that biofilms were extensively developing in the applied 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 inoculum biofilm samples 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 is reported in Supplementary Information (Fig. S1). The BAC filters are operated with periodic pure-oxygen dosing to maintain full aerobic conditions (van der Maas et al., 2020), and always achieve a high Mn removal (Bernadet et al., 2023). The biofilms were harvested from the backwash water during the periodic backwashing of BAC filters with air and water. Backwash water was collected in acid-washed LDPE bottles from the top of the filters within the first 5 min after the backwashing started and stored at 4º C. The samples are referred to as “inoculum biofilm” (IB) hereafter. Two different samplings were executed in September 2021 and January 2022, before and after the BAC filters maintenance (October 2021) with 7 days of inoperability. The composition of the water containing the IB is reported in Table S1.

## Preparation of Mn enrichment cultures

To prepare enrichment cultures of MnOB, 100 mL of the IB collected 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 as source of soluble Mn2+, with final concentration of ~20 mM, and dried overnight in the dark (Yu and Leadbetter, 2020). To assess if the Mn oxidation was related to biological activity, control bottles were prepared with IB samples inactivated by autoclaving (121 ºC, 103 kPa, 20 min). All experiments were run in triplicate per condition (active and non-active biofilms) and per sampling events (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. Samples 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 (VSS) and pH measurement

The biomass growth in the biofilm cultures was monitored by measuring VSS before and after the experiment, using the standard protocol EPA 160.4 (EPA, 1975). The pH during the experiment was measured using a 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 biofilms was measured on unfiltered samples using an Optima 5300 DV ICP-OES (Perkin Elmer, US) with argon as carrier gas, after addition of nitric acid (HNO3) (2% final concentration). Since biological Mn oxidation converts soluble Mn2+ into insoluble MnOx (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 (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, Supplementary Information). The procedure for measuring Mn fractions in both reference and experimental samples was as follows: 4.5 mL of sample and 0.5 mL of HNO3 69% (VWR, FR) were put inside a centrifuge tube. The tube was mixed in the dark overnight (~15 h) to reassure all the Mn2+ was in soluble state. A 1 mL of the sample was measured using ICP-OES and is called “acid-soluble fraction”. A 2 mL of the sample was further mixed with 4.8 mL of HNO3 69% and 4 mL H2O2 30% (VWR, UK) and subjected to microwave-assisted digestion (Ethos Easy, Milestone SRL, IT) at 180 °C for 30 min in ramp mode. The sample was then diluted until it contained 2% of HNO3 and measured by ICP-OES as the “total Mn”. The subtraction between “total Mn” and “acid-soluble fraction” results in the “acid-insoluble fraction”.

### X-ray diffraction

Phase identification of MnOx particles was carried out on freeze dried samples via powder X-ray diffraction (XRD), 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 samples.

### Scanning electron microscopy

Samples preparation followed the protocol described by Bernadet et al. (2023) and modified according to Yu and Leadbetter (2020), with fixation in 2.5 % glutaraldehyde, washing with HEPES buffer 25 mM at pH 7.5, dehydration with ethanol, and a final dehydration step with critical point drying (EM CPD3000, Leica, DE), where ethanol was replaced by liquid CO2 at <10 °C and 55 bar. Scanning electron microscope (SEM) imaging was performed on the dried samples using a JSM-6480LV (JEOL, JP) at an operating voltage of 6 kV.

### 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 v4.12 software. Extracellular polymeric substances (EPS) of biofilms were 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 IB samples and the enrichment cultures after 42 days. 10 mL of culture from two replicate experimental bottles was centrifuged at 4750 x*g* at 4 °C for 5 min. The liquid was decanted, and the pellet was washed with PBS 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). Taxonomic classification of ASVs was based on SILVA v.138 database (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 were observed in the active cultures

After 42 days, the difference between non-active and active biofilms was clearly visible, with control bottles retaining the light-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).

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**Fig. 1** – Representative photos and micrographs showing the difference between control and active biofilms after 42 days incubation with MnCO3. Control bottles with non-active biofilms preserved the light-brown color of MnCO3 slurry (a), and microscopy observation showed persistence of MnCO3 as salt (b) and the original EPS matrix (c). Bottles inoculated with active biofilms were dark brown to black (d) and the biofilm grown accumulating black nodules (e) and EPS (f). Scale bars are 20 μm.

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 unconverted MnCO3 was still visible (Fig. 1 B and C). Similar black MnOx nodules from biological oxidation were previously observed microscopically under laboratory conditions (Furuta et al., 2023; Yu and Leadbetter, 2020).

Further visual analysis by SEM showed that the raw MnCO3 slurry particles (Fig. 2 A) covered the control, non-active biofilms (Fig. 2 B), confirming the lack of conversion into nodules. On the other hand, the aggregates in active biofilms (Fig. 2 C and E) mostly contained Mn in the form of crystals with nanoflower structures, clearly distinguishable by shape in comparison to the raw MnCO3 particles (Fig. 2 D and F). Most MnOx formed via biological oxidation in the environment were found to have a 3D flower-like layered structure, such as buserite and birnessite (δ-MnO2) (Li et al., 2021).

It is worth noting that the active biofilms originated from IB in September showed a higher presence of nanoflower structures (Fig. 2 C) than the bottles inoculated with the 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 preceding the January sampling, which left the BAC granules without nutrients and minerals for 7 days, potentially changing the microbial community composition. Based on VSS measurements, active biofilms developed over time compared to the start-up IB, while control, non-active biofilms remained stable (Table 1). Assuming that the growth is only related to either heterotrophic utilization of total organics (indicated as total chemical oxygen demand (tCOD), Table S1) or autotrophic consumption of (bi)carbonate ions from MnCO3 (Nealson, 2006), VSS values for both experiments were relatively high compared to BAC biofilms growing under similar oligotrophic experimental conditions (Lin and Ho, 2022; Piai et al., 2022).

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**Figure 2** – Scanning Electron Microscopy (SEM) images of samples taken before and after the 42-day experiment. The MnCO3 slurry alone was composed of small, tightly aggregated round particles (a). In (b), the control biofilm (non-active) at the end of the experiment, covered with MnCO3 particles. After 42 days, in active biofilms from September (c and d) and January (e and f) bottles MnCO3 particles (white arrows) were converted into Mn oxide crystals with a nanoflower structure (black arrows).

Thus, we can hypothesize that Mn2+ was actively used for growth, as it can be the sole energy source for some bacteria when oxidation is coupled with ATP synthesis via chemolithoautotrophy, as discovered in early studies on single strains from oligotrophic environments (Arcuri and Ehrlich, 1980; Ehrlich, 1978; Ehrlich and Salerno, 1990).

The 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), 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.

|  |  |  |  |
| --- | --- | --- | --- |
| **Experiment** | **Sample** | **VSS (mg/L)** | **pH** |
| September | Inoculum biofilm | 51 ± 1 | 7.9 ± 0.0 |
| Control biofilm | 55 ± 23.5 | 8.2 ± 0.1 |
| Active biofilm | 255 ± 7.1 | 7.4 ± 0.1 |
| January | Inoculum biofilm | 23 ± 1 | 7.3 ± 0.0 |
| Control biofilm | 58.3 ± 29.2 | 7.8 ± 0.1 |
| Active biofilm | 410 ± 96.4 | 7.4 ± 0.0 |

## Characterization of the conversion of soluble into insoluble Mn

To determine the fraction of soluble Mn2+ added as MnCO3 into the experimental bottles that was converted into insoluble Mn3+ and/or Mn4+, a dedicated method was developed applying ICP-OES to determine Mn concentrations as “acid-soluble” and ‘acid-insoluble” fractions (section 2.3.2). At the end of the 42-day experiment, the bottles with control and active biofilms contained the same amount of total Mn, while the amount of acid-insoluble Mn (Mn3+ and/or Mn4+) was 6- to 10-fold higher for the active biofilms (Table 2), further supporting the biological Mn oxidation. The fractions of acid-soluble (the unconverted Mn2+) and insoluble Mn present in the control bottles were similar to those in the IB, indicating that the abiotic oxidation did not occur even though the water pH values were higher (7.8 – 8.2) than in the active biofilm bottles (7.4) (Table 1). Active biofilms converted most of the soluble Mn2+ to acid-insoluble forms, and the converted fraction in the active biofilm of September bottles was higher (average of 84.8%) than in the January ones (average of 52.1%) (Table 2). Differences in the oxidation of Mn between the September and January samples were also observed by XRD analysis (Fig. 3). These XRD patterns from the September and January samples were relatively low in peak intensity and showed peak broadening, likely indicating either small crystal size, low crystallinity, amorphous structure or the presence of impurities (Holder and Schaak, 2019). The XRD patterns of the active biofilms in the September experiment identified the produced MnOx as birnessite-type (also called δ-MnO2) (Fig. 3 A), based on the COD database (COD 9013652, (Lopano et al., 2009), referring to the two distinctive peaks from the 00*l* reflections at 12.01° 2θ (λ = 7.36 Å) and 24.16° 2θ (3.68 Å). Another two peaks appearing at 36.9° 2θ (2.43 Å) and 66.2° 2θ (1.4 Å) are indexed to birnessite, which are broad and have low signal-to-noise ratios, likely corresponding to small sized crystals (Holder and Schaak, 2019). 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 the MnOx formed in the September experiment were similar to abiotically synthesized H+-birnessite or H+ inserted between layers (Saratovsky et al., 2006). Their morphology (Fig. 2 D) was also similar to δ-MnO2 chemically synthesized using potassium permanganate (KMnO4) and hydrochloric acid (HCl), where the oxide structure is built-up of nanosheets, forming nanoflower structures (Liang et al., 2017).

**Table 2** – Mn2+oxidation trends analyzed via ICP-OES in the IB compared to control and active biofilms after 42-day experiment with MnCO3 (n=3). The amount of soluble Mn2+ and insoluble MnOx (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.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **Mn concentration (mmol/L)** | | | **Mn fraction (%)** | |
| **Experiment** | **Sample** | **Total** | **Acid-soluble** | **Acid-insoluble** | **Acid-soluble** | **Acid-insoluble** |
| September | Inoculum biofilm | 0.11 ± 0.0 | 0.10 ± 0.0 | 0.01 ± 0.0 | 93.7% ± 4.5% | 6.3% ± 4.6% |
| Control biofilm | 22.4 ± 0.8 | 20.6 ± 1.2 | 1.8 ± 1.4 | 91.9% ± 6.2% | 8.1% ± 6.3% |
| Active biofilm | 22.4 ± 0.3 | 3.4 ± 0.2 | 19 ± 0.4 | 15.2% ± 0.8% | 84.8% ± 2.1% |
| January | Inoculum biofilm | 0.036 ± 0.0 | 0.034 ± 0.0 | 0.003 ± 0.0 | 92.9% ± 8.9% | 7.1% ± 9.2% |
| Control biofilm | 20.6 ± 0.7 | 18.9 ± 0.5 | 1.6 ± 0.9 | 92.3% ± 4.1% | 7.7% ± 4.3% |
| Active biofilm | 20.3 ± 0.5 | 9.7 ± 0.1 | 10.6 ± 0.5 | 47.9% ± 1.3% | 52.1% ± 2.7% |

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**Figure 3** – XRD patterns of the IB (black) and the active biofilms (blue) after the 42-day incubation for September (a) and January (b) experiments. The red lines indicate peaks position and Miller indices (hkl) defining reflection, adapted from Crystallography Open Database (COD) for birnessite (in a) (COD 9013652, Lopano et al., 2009) and rhodochrosite (in b) (COD 9007691, Maslen et al., 1995).

Synthetic birnessite-type, δ-MnO2 with nanoflower morphology exhibits excellent ability to remove various 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. The XRD patterns of the active biofilms from the January experiments (Fig. 3 B) showed that Mn was not fully oxidized and was still present as MnCO3, as compared to XRD peaks of rhodochrosite (a pure MnCO3 mineral) from the COD database (COD 9007691, (Maslen et al., 1995). This finding aligns with the ICP results, accounting for just half of the Mn2+ oxidized to insoluble forms (Table 2), and the SEM images showing the presence of residual MnCO3 (Fig. 2 E and F). In contrast to the high VSS values measured at the end of both experiments (Table 2), the lower yield of Mn oxidation suggests that the MnOB developed from the IB samples from January samples were less active than the ones from September. Nevertheless, similar nanoflower structures were observed in January samples as in September ones (Fig. 2 E and F). However, the exact type of MnOx forming these nanoflowers could not be distinguished using XRD, likely due to the high concentration of MnCO3 mixed with amorphous and/or crystalline 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 increase of 55% in the relative abundance of *Pseudomonadota* (formerly called *Proteobacteria*) when feeding Mn2+, with a concurrent 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 (Fig. S3). The majority of *Bacteroidota* in the IB,which relative abundance decreased after 42 days, 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; Ouyang et al., 2019), similarly to the BAC filters from which the IB were sampled (Bernadet et al., 2023).

A core microbiome of eight bacterial groups increased significantly in both September and January experiments compared to their IBs (Fig. 4). This core dominated the community in all the replicates analyzed, and comprised genera (of) *Pseudomonas, Hyphomicrobium*, *Rhodococcus*, *Nitrosomonadaceae* *-* Ellin6067 and *Pirellulaceae*- Pir4 lineage, the families *Burkholderiaceae* and *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 for which pathways and enzymes were partially characterized (Cömert and Tepe, 2020; Geszvain et al., 2013; Okazaki et al., 1997). *Hyphomicrobium* isone of the first Mn oxidizers studied, responsible for the accumulation of MnOx in water pipelines (Tyler and Marshall, 1967) and biofilters (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 (Marcus et al., 2017; Sjöberg et al., 2020). A well-known Mn-oxidizing *Rhizobiales* detected in the experimental bottles was *Pedomicrobium* (Larsen et al., 1999)(Fig. 4),in whichdeposition of MnOx occurs in close association with EPS 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 in the Mn-enriched communities, potentially correspond to undescribed Mn-oxidizing species. The other groups within the core microbiome have rarely been associated with Mn oxidation. *Burkholderiaceae* was a dominant family in all the samples analyzed, with average relative abundances of 9.1% and 7.9% in the September and January experiments, respectively.

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**Fig. 4** - Relative abundances (between 0% and 12%) of dominant bacterial groups (>1% relative abundance in at least one of the samples reported) comparing the inoculum biofilms (IB) and active biofilms sampled at the end of the September and January experiments in duplicate bottles (B1 and B2). Microbial groups were identified using 16S rRNA gene amplicon sequencing, and their taxonomy classification is reported at the identified level. Arrows indicate the trend in comparison to the inoculum: green (increasing), yellow (stable), and red (decreasing).

This genus was associated once with Mn-oxidation communities found in caves (Carmichael and Bräuer, 2015), and reported as putative Mn-oxidizers (Li et al., 2013) and heavy metal resistant bacteria (Drewniak et al., 2016).

Members of the *Rhodococcus* genus are soil actinomycetes applied in biotechnology to remove pollutants or produce drugs (Yam et al., 2011), and the species *R. 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), detected at lab-scale conditions in presence of a stress factor, such as high pharmaceuticals concentrations (Vega et al., 2022), light irradiation (Wang et al., 2021), or cadmium (Qu et al., 2022). *Nitrosomonadaceae* in general were found in nitrifying communities associated with MnOB, supporting their growth (Cao et al., 2015), while *Ellin6067* is hypothesized to perform autotrophic denitrification under carbon limited conditions (Chen et al., 2020). Thus, their strong increase in relative abundance from IB by 90% and 74% 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. Members of the family *Pirellulaceae* are commonly found in oligotrophic and extreme environments (Schlesner et al., 2004), and one novel genus of Pir4 lineage was isolated from hydrothermal metalliferous deposits (Storesund et al., 2018). *Terrimonas*, one of the dominant genera in both IBs (Fig. 4), decreased in both experiments but persisted at averages of 2.6% and 6.6% relative abundances in the September and January experiments, respectively. They 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).

The observed dissimilarities in Mn oxidation behavior between the September and January experiments (Table 2, Fig. 3) could be related to the different inoculum composition. Considering the Mn-enriched core microbiome only, a higher relative abundance of *Burkholderiaceae, Hyphomicrobium, Hydrogenophaga, Rhodococcus* and *Rhizobiales* was seen in the September IB compared to the January one (Fig. 4). However, after the 42-day experiment, a relevant difference in the relative abundances of key microbial groups was observed only for *Hydrogenophaga* and *Pseudomonas* (Fig. 4)*. Hydrogenophaga* species, barely detected in the January bottles compared to the September ones, are associated with Mn oxidation in drinking water systems (Marcus et al., 2017) and other natural environments (e.g. Sjöberg et al., 2020). Interestingly, *Pseudomonas*, a dominant genus in the January bottles, had a 76% higher relative abundance in these samples than in the September ones, and this could relate to the presence of amorphous MnOx, as hypothesized after XRD analysis (Fig. 3) and previously characterized from *Pseudomonas* pure cultures (Cömert and Tepe, 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 media (Bernstein et al., 2022; Hu et al., 2020). These MnOx are of technological importance because they can be used to improve water treatment performances and environmental remediation. Biogenic MnOx indeed can adsorb and oxidize toxic metals (e.g., lead, chromium, arsenic, and cadmium), 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, a strong oxidant which can boost the biodegradation of recalcitrant organics or protect against oxidative stress (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, MnOB 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 have shown that mixed microbial communities in biofilms sampled from an oxygen-augmented BAC filtration system efficiently convert Mn2+ into MnOx under 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 nanoflower structures with nano-layered crystals (Fig. 2).

The EPS matrix of biofilms plays a fundamental role in Mn adsorption and oxidation (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 microbial enzymatic oxidation activity contributes to the continuous regeneration of MnOx nodules within the biofilm, making their catalytic properties potentially unlimited as long as Mn2+ is present in the medium (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 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 layered birnessite with a poorly crystalline structure (Spiro et al., 2010) in a rich culture media. In contrast, in this work we used mixed microbial communities 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 birnessite (or δ-MnO2) (Fig. 3 A) with nanoflower morphology (Fig. 2), similar to the chemically synthesized δ-MnO2 (Li et al., 2021; Liang et al., 2017), applied as catalyst for the decomposition of recalcitrant compounds (Li 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 biodegradation, 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 cultures and link to the catalytic potential, as well as on unraveling the enzymology behind this almost ubiquitous yet poorly understood microbial function.

# Conclusions

* The detached biofilms from aged BAC granules harbor a diverse microbial community that has the capability to grow via Mn2+ oxidation.
* Opposite to pure cultures of model microorganisms, enriched oligotrophic communities with a variety of MnOB groups result in the formation of nanoflower crystals of δ-MnO2.
* Biotechnological contexts where MnOB, likely coming from soil and groundwater, find a suitable habitat to adhere and grow in mixed microbial cultures, are potential sources of biological MnOx catalysts.
* The understanding of biogenic MnOx formation and role in biofilters may be advantageous to optimize performances towards the removal of recalcitrant organics and metals from water.

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