**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 phylogeny showed a drastic increase of members of the order *Rhizobiales*, together with genera *Burkholderiaceae, Rhodococcus, Ellin6067* and Pir4 lineage, never reported among MnOB before. This work highlighted the potential of harnessing the microbial community growth in 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, and in the presence of oxidizing agents (abiotic or biotic) and changes in pH, it is oxidized to insoluble Mn, such as Mn3+, Mn4+, or a higher oxidation state, depending on the oxidizing agents (Nealson, 2006). In aquatic environments, abiotic oxidation is not favoured due to the high activation energy required for Mn2+ oxidation by O2 at a pH below 8 (Elzinga, 2011; Oldham et al., 2017). A 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 mediated by bacteria, fungi, or algae is generally faster than the abiotic one by 4–5 orders of magnitude (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 in the environment (Spiro et al., 2010). Among manganese-oxidizing microorganisms, manganese-oxidizing bacteria (MnOB) are the most diffused and ubiquitous (Nealson, 2006; Tebo et al., 2005). To date, type strains of MnOB have been isolated and characterized from 4 phyla (*Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria*) (Zhou and Fu, 2020). The reason why MnOB oxidize Mn2+ is yet not known or still 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 (freshwater, soil, and marine sediments) (Oldham et al., 2017), thus MnOB often develop in engineered systems for water treatment where such conditions apply (Bernstein et al., 2022; X. Zhao et al., 2020). The presence of MnOx in such systems in the past was often attributed to abiotic processes, but 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 (Breda et al., 2018; Sahabi et al., 2009). MnOx insoluble particles can form and accumulate also within drinking water supplies when Mn concentrations are in the range of 0.1 - 0.2 mg/L (World Health Organization, 2017). This concentration is below of that causing potential health effects (0.4 mg/L), but the resulting particles can cause water aesthetic and operation problems, colouring 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 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 growing on BAC granules adsorb and oxidize Mn2+, forming particulate oxides that are then removed by backwashing (Bernstein et al., 2022).

While several studies have investigated the whole microbial communities characterizing BAC filters biofilms (Lu et al., 2022), to the best of our knowledge, none of them have specifically targeted the MnOB populations. 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. 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 inactivating biofilms by autoclaving. 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 developing within the biofilms were characterized by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), X-ray diffraction (XRD), and visualized by Scanning electron microscope (SEM). The results showed that BAC biofilms were able to grow in oligotrophic conditions, producing a high amount of MnOx, thanks to the enhanced growth of several 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, Nieuw-Amsterdam, the Netherlands) 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 preserved at 4º C until further processing. Two different samplings were executed in September 2021 and January 2022, before and after a maintenance of the BAC filters (October 2021), where they were not in operation for 7 days. The samples are defined as “inoculum biofilm” (IB) along the text. The composition of the water containing the IB samples 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 (Duran, Germany) with inside walls coated with manganese carbonate (MnCO3, Alfa Aesar) slurry at a final concentration of 2 mmol, previously dried overnight, as described elsewhere (Yu and Leadbetter, 2020). To assess if the Mn oxidation was related to biological activity, control bottles were prepared using IB inactivated after sterilization by autoclaving at 121º C for 20 min. All experiments were run in triplicate per each condition (active and control biofilms) and each 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 analysed before and after the incubation, as described in the following sections. A water evaporation of 10% after the 42 days experiment was taken into account.

## 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 by SevenExcellence pH/Cond meterS470 (Mettler Toledo®).

### Inductively coupled plasma atomic emission spectroscopy

The Mn concentration in the IB and at the end of 42 days 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, USA) 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). As a method development, four Mn salts were tested based on their Mn oxidation state solubility in HNO3 (Table S2). The procedure of the Mn measurement was as follows: a series of 4.5 mL of Mn-contained sample was put inside a falcon centrifuge tube, and 0.5 mL of HNO3 69% (VWR) was added. The sample was then mixed in the dark overnight (~15 h) to reassure all the Mn2+ was soluble in the solution. 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) and then was subjected to microwave-assisted digestion (Ethos Easy, Milestone SRL) 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). The membrane was then washed thrice using the HEPES buffer solution 25 mM, pH 7.5 (Sigma Aldrich). Dehydration steps were performed using ethanol (VWR) by increasing its concentration from 30, 50, 70, 90, and 100% (v/v). The sample was in contact with ethanol for at least 15 min for each step. The final dehydration step was performed using critical point drying (Leica EM CPD3000), where ethanol was replaced by liquid CO2 at temperatures < 10 °C and 55 bar of pressure. After reaching the critical point, the pressure was released slowly at temperatures > 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 under optical microscopy on glass slides under a DM750 microscope (Leica, DE), and images were acquired using Leica LAS-X (version 4.12) software. Extracellular polymeric substance (EPS) within biofilms was further visualized by mixing 100 µl of the sample with 20 µl of crystal violet 0.1%, targeting protein and polysaccharides.

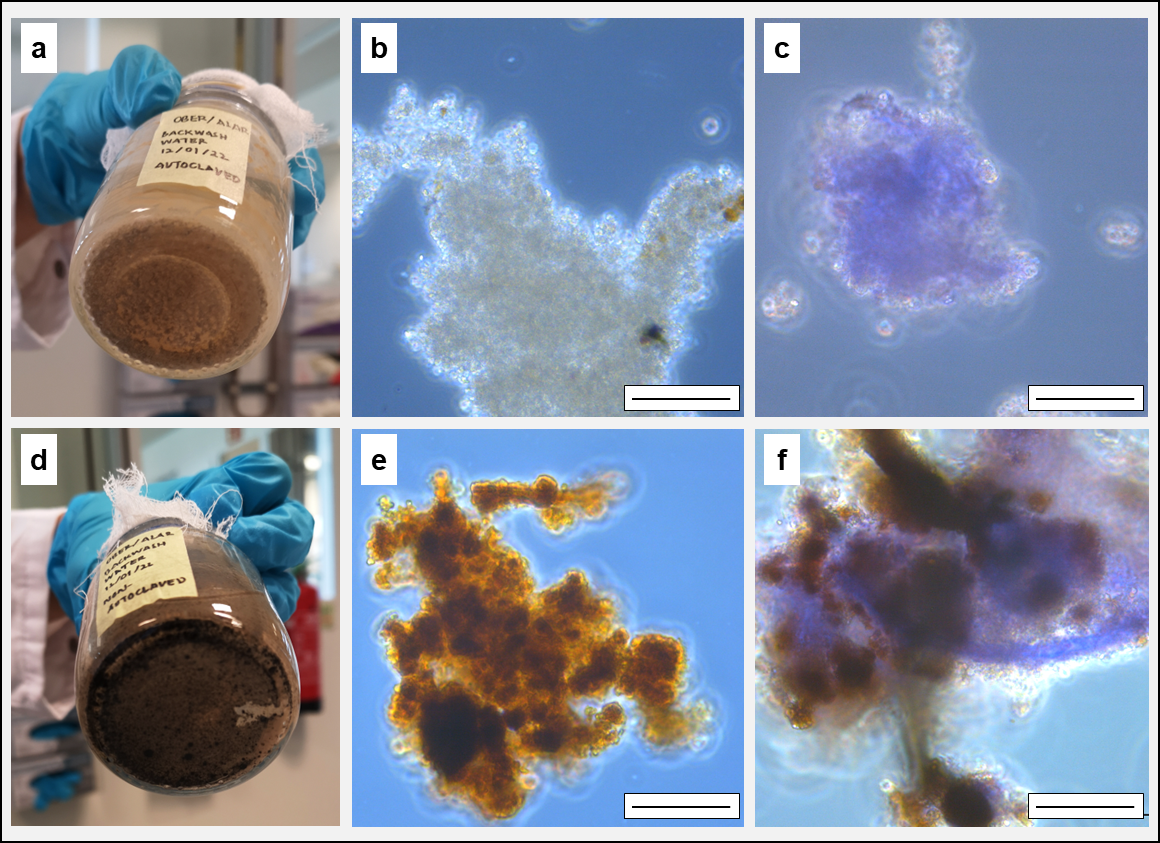
## Microbial community analysis

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 experiment. A volume of 10 mL of the mixture of biofilm and liquid from two of the three replicate experimental bottles was centrifuged at 4750 xg, at 4 °C, for 5 mins. 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, USA) on a MiSeq (Illumina, USA) with 2x300 bp (V3) paired-end sequencing. Quality check 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). Sequencing data were deposited in the European Nucleotide Archive (ENA) under the project number …..

# Results and Discussion

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

After 42 days of experiment, the difference between non-active and active biofilms was already visible via naked eye observation, with control bottles preserving the brownish colour of MnCO3 slurry (Fig. 1 A), while all the cultures fed with active biofilms were turning dark to black colour (Fig 1 D). A closer look via microscopy on the active biofilms showed the growth of a robust EPS matrix extensively loaded with black nodules (Fig. 1 D and E), opposite to the non-active biofilms, where Mn was still visible as non-converted crystals (Fig. 1 B and C).



**Fig. 1** – Images showing the difference between active and control cultures after 42 days in presence of MnCO3. Control bottles with non-active biofilms did not turn black, preserving the brownish colour of MnCO3 slurry (a), and microscopy observation showed persistence of MnCO3 as salt crystals (b), while the original EPS matrix was preserved (c). Experimental bottles inoculated with active biofilms, were dark black (c) and the biofilm inside accumulated black nodules (e) and were rich in EPS (f).

Further visual analysis of the samples by SEM showed that raw MnCO3 slurry particles (Fig. 2 A) were covering the non-active biofilms in control bottles (Fig. 2 B), suggesting that in these cultures Mn was not converted into its mineral form. On the other hand, in the bottles inoculated with active biofilms, the aggregates (Fig. 2 C and E) contained mostly the Mn crystal form, clearly distinguishable in comparison to the MnCO3 particles shape (Fig. 2 D and F, indicated by arrows). It is worth noticing that Mn-enriched biofilms taken from the bottles containing the IB from September showed a higher number of crystals (Fig. 2 C) in comparison to the January bottles, where numerous, unconverted MnCO3 particles were visible (Fig. 2 E). This could likely be a consequence of the maintenance of the BAC filters preceding the January sampling, which left the BAC granules without any nutrients and minerals for 7 days (see section 2.1).

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 experiment. In a, appearance of the MnCO3 slurry before being fed to the experimental bottles, with small, round particles tightly aggregated. In b, the control biofilm (non-active) 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 (black arrows).

VSS measurements indicated that active biofilms were growing in time in comparison to the start-up IB in the bottle, while the VSS values observed in control, non-active biofilms, remained comparable with the 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 (Table S1), 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).

**Table 1** - Volatile suspended solids (VSS) and pH values measured 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.



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.

## Characterization of the conversion of soluble into insoluble Mn

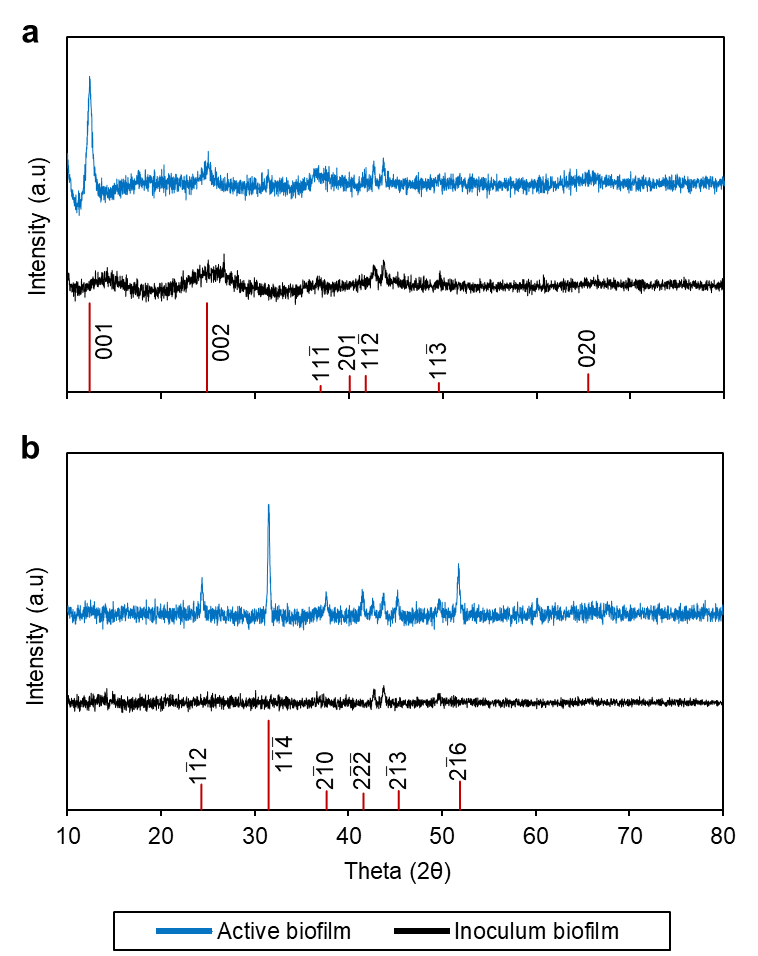
To determine the fraction of soluble Mn2+, fed as MnCO3 into the experimental bottles, converted into insoluble Mn (i.e., Mn3+ and Mn4+), a dedicated method was developed applying ICP-OES, determining Mn concentrations as “acid-soluble fraction” and ‘acid-insoluble” fractions (see section 2.3.2). At the end of the 42 days experiment, the bottles with control and active biofilms contained the same amount of total Mn. Still, the amount of acid-insoluble Mn, corresponding to the oxidized Mn, was higher for the active biofilms (Table 2), further supporting the hypothesis that biological Mn oxidation occurred. 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 form, where the fraction in active biofilm within September bottles (average of 84.8%) was higher than in the January ones (average of 52.1%) (Table 2).

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) reveal that Mn was oxidized into birnessite-type oxide or δ-MnO2, as compared with 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 intensities and had peak broadening, indicating low crystallinity or amorphous structure of the MnOx formed and/or the presence of impurities.

**Table 2** – Observed yields of Mn2+oxidation, analyzed via ICP-OES, in the inoculum biofilm and the experimental bottles (control and active biofilms) after 42 days 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 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).



**Figure 3** – XRD patterns of the inoculum biofilm and the experimental bottles with active biofilms after 42 days 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 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 hydrochloride acid (HCl), where the oxide structure is built up of nanosheets, forming nanoflower structure (Cheng et al., 2022; Cremonezzi et al., 2020). A 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 Mn 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 rhodochrosite or MnCO3, as compared with XRD peaks of rhodochrosite from the database (COD 9007690) (Maslen et al., 1995). This finding aligns with the fraction of Mn2+ oxidized of only ~52% in January samples (Table 2) and the still presence of MnCO3 indicated by the SEM images (Fig. 2 E and F). Opposite 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 sampled in January was less active than the one sampled in September, which could be related 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 MnCO3 was oxidized and transformed from acid-soluble Mn into acid-insoluble Mn (Table 2), and similar nanoflowers structures were observed (Fig. 2 E and F). The exact form of the oxidized Mn in these samples was challenging to distinguish using XRD due to the still presence of MnCO3 in the sample.

## Microbial community shift to specialized MnOB populations

Different (putative) MnOB have been found in BAC filters (Bernstein et al., 2022), while the known, isolated species are affiliated to the phyla *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* (Zhou and Fu, 2020). At the phylum level, in both September and January experiments, there was an evident increase of 55% in relative abundance for *Proteobacteria* when feeding Mn, but a net decrease in members of the phylum *Bacteroidetes*, dominating the IB in both cases (Fig. S2). Within the phylum *Proteobacteria*, members of the orders *Rhizobiales* and *Betaproteobacteriales* had the highest increase (between 70 and 80% in comparison to the start-up IB) after 42 days incubation with MnCO3 (Fig. S2). The majority of *Bacteroidetes* identified in the IB,which did not grow in the Mn enrichment cultures, were affiliated with the order *Chitinophagales* (Fig. S2)*.* 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 prevalent 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 or Chapter 3).

A core microbiome of eight bacterial groups was significantly increasing in both September and January experiments in comparison to the IB (Fig. 4) and dominating the community in all the replicates analysed, which includes the genera *Burkholderiaceae, Pseudomonas, Hyphomicrobium*, *Rhodococcus*, Ellin6067 (*Nitrosomonadaceae*) and Pir4 lineage (*Pirellulaceae*), the family *Rhizobiales* A0839 and unidentified *Rhizobiales*. Among these, most were already associated in the past with Mn oxidation. *Pseudomonas spp.* are model microorganisms for Mn oxidation identified in many different settings (Zhou and Fu, 2020), which pathways and the 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 different environments (Tebo et al., 2005), and recent studies have discovered new species under this order having this ability (Anderson et al., 2009; Marcus et al., 2017; Sjöberg et al., 2020), while family *Rhizobiales* A0839 was never associated to it. All the *Rhizobiales* not identified at the genus level, accounting for an average 7% relative abundance out of the total community, could correspond to new species growing in these enrichment cultures. Another known Mn-oxidizing *Rhizobiales* growing in the experimental bottles was *Pedomicrobium* (Larsen et al., 1999)(Fig. 4),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). The other groups within the core microbiome were rarely or never 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, associated just once with Mn-oxidizing communities in caves (Carmichael and Bräuer, 2015). *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). *Rhodococcus* is 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 affiliated with *Rhodococcus, R.opacus,* was recently found to adsorb Mn2+ (Pimentel et al., 2022). *Ellin6067* is a genus of *Nitrosomonadaceae*, putative ammonia-oxidizing bacteria 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 concentration (Vega et al., 2022), light irradiation (L. Wang et al., 2021), 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 high increase in relative abundance in comparison to the IB (90% and 74% increase in the September and January experiments, respectively) could be associated with the oxidation of ammonia originating from biomass decay during the 42 days experiments. Members of the family *Pirellulaceae* are commonly found in oligotrophic and extreme environments (Schlesner et al., 2004); Pir4 lineage consists of a large number of uncharacterized isolates, with one novel genus recently isolated from metalliferous deposits of hydrothermal vent fields (Storesund et al., 2018). *Terrimonas*, one of the dominant bacterial groups in both IBs in September and January experiments (Fig. 4), did decrease its relative abundance in both experiments, but it remained relevant with an average 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).



**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).

## 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; 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 (i.e., lead or Pb, chromium or Cr, arsenic or As, and cadmium or Cd) and organic matter (Zhou and Fu, 2020), boosting the degradation of a wide array of compounds, including recalcitrant pollutants, through multiple mechanisms (Tebo et al., 2004). Moreover, it has been shown the accumulation of labile Mn3+ intermediates in the bacterial Mn oxidation, which is a strong oxidant that can be utilized by the microorganisms to metabolize recalcitrant organics or to protect against toxic oxygen species (Spiro et al., 2010; Toner et al., 2005; Webb et al., 2005a). It is thus of particular interest to better understand the properties of MnOx formed by biofilm mixed microbial cultures in such filtration systems, to determine if they can improve the water treatment performances. In addition, these mixed microbial cultures clustering together within the water filtration systems could be applied as a relatively cheap method to efficiently produce such a high-quality catalyst. In this study, we showed that mixed microbial communities in biofilms sampled from an oxygen-augmented BAC filtration system could efficiently convert Mn2+ into MnOx in oligotrophic conditions, growing a consistent biofilm EPS matrix (Fig. 1, Table 1), including 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 biofilm plays a fundamental role in Mn adsorption and oxidation, as shown in studies investigating single-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 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 soon 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 their fine structure (Spiro et al., 2010), thus 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. An extended X-ray absorption fine structure (EXAFS) spectroscopy allowed to show 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 biofilm matrix do not outcompete with the adsorption (Toner et al., 2005). Differently from the above-mentioned studies, which investigated Mn oxidation by pure cultures in rich culture media, in this work, we used mixed microbial biofilms to which we did not provide any macronutrient besides the ones already present in the inoculum water (Table S1). XRD profiles showed the formation of a δ-MnO2 (Fig. 3 A), which nanoflower morphology (Fig. 2) is similar to the chemically synthesized one (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 redox and adsorption properties of such MnOx may provide increased access to a biodegradable material for microbial metabolism, thus being beneficial for water treatment in general. By means of 16S rRNA gene sequencing, we identified several dominant bacteria, of which most were never associated with Mn oxidation (Fig. 4). To date, the biochemical mechanisms associated with Mn oxidation are limited to the isolated strains, thus the list of enzymes corresponding with this pathway is still very limited (Tebo et al., 2005). Overall, future works should focus on further structural characterization of MnOx formed by mixed microbial culture to the catalytic potential, as well as on unravelling 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 harbour 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, allowing to 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 find 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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