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ABSTRACT

The aim of this study was to identify bacteria present in ripened manganese removal filters for drinking water production. The bacterial population was identified with 'next generation' DNA sequencing, and specific bacteria were quantified with quantitative polymerase chain reaction (qPCR) and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The 'next generation' DNA sequencing analysis showed a bacteria population shift from the iron oxidizing species *Gallionella* spp. in the Fe-filter to manganese and nitrite oxidizing species *Pseudomonas* spp. and *Nitrospira* spp., respectively, present in the manganese removal filter. qPCR analysis confirmed the presence of a low concentration of the well-known Mn^{2+}-oxidizing species *Ps. putida* in the manganese removal filter backwash water. Bacteria of the genus *Pseudomonas,* isolated from backwash water from a manganese removal filter were cultured and identified with MALDI-TOF MS analysis. Amongst others, *P. gessardii, P. grimontii, and P. koreensis* were identified. The presence of several manganese oxidizing bacteria species in ripened filter media supports the assumption that a microbial consortium is involved in the oxidation of manganese. Understanding the mechanisms by which manganese coating of filter media commences could endorse the creation of conditions favouring Birnessite formation, and possibly help in reducing typically long ripening periods of manganese removal filters with virgin filter media.

Key words | biological manganese oxidation, Birnessite, manganese removal ripening time, molecular DNA techniques, *Pseudomonas* spp.

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INTRODUCTION

An important drawback of aeration-rapid sand filtration, commonly applied in several West European countries to remove manganese from groundwater, is the long filter media ripening period.

Farnsworth *et al.* (2012) reported that manganese oxide formed on the filter media, responsible for manganese removal, is a Birnessite type of mineral. Due to its structure, Birnessite has outstanding properties to adsorb and subsequently oxidize Mn^{2+} (Post & Veblen 1990; Post 1999). Bruins *et al.* (2015a) showed that Birnessite present in the coating of a ripened manganese removing filter in operation for over 15 years, was of physicochemical origin. Chemical

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formation of Birnessite requires alkaline conditions (Feng *et al.* 2005). The redox potential – pH diagram for aqueous manganese (Stumm & Morgan 1996), suggests that besides a high pH, a high redox potential is required for chemical formation of MnO_x. Such water quality characteristics are not common for groundwater with, usually, low pH and low redox potential. Using electron paramagnetic resonance and Raman spectroscopy, Bruins *et al.* (2015b), showed that formation of Birnessite most likely starts through biological activity. In a number of other studies, it was also proposed that manganese removal is an obligatory biological process (Vandenabeele *et al.* 1992; Katsoyiannis & Zouboulis 2004; Burger *et al.* 2008a, 2008b; Tekerlekopoulou *et al.* 2008; Farnsworth *et al.* 2012). Several species of bacteria (*Pseudomonas* spp., *Leptothrix* spp. and *Bacillus* spores) able to oxidize or involved in oxidation of Mn^{2+} have been identified in (ground)water (Tebo *et al.* 2005; Kim *et al.* 2011). *Pseudomonas* spp., in particular *Pseudomonas putida*, were extensively studied in relation to manganese oxidation (DePalma 1993; Gounot 1994; Caspi *et al.* 1998; Brouwers *et al.* 1999; Villalobos *et al.* 2003, 2006; Tebo *et al.* 2004, 2005; Barger *et al.* 2009). Similar studies were performed for *Leptothrix* spp. (Adams & Ghiorse 1985; Boogerd & de Vrind 1987; Corstjens *et al.* 1997; Hope & Bott 2004; Tebo *et al.* 2004, 2005; Burger *et al.* 2008a, 2008b; Barger *et al.* 2009; El Gheriany *et al.* 2009) and *Bacillus* spp. spores (de Vrind *et al.* 1986; Mann *et al.* 1988; Brouwers *et al.* 2000; Tebo *et al.* 2004, 2005; Barger *et al.* 2005, 2009). Experiments with (spores of) *Bacillus* spp. were, however, often done with species of marine origin (Mann *et al.* 1988; Webb *et al.* 2005a, 2005b).

Pseudomonas spp. and *Leptothrix* spp. are heterotrophic bacteria. *Leptothrix* spp. are able to oxidize iron as well as manganese, whereas *Pseudomonas* spp. are known to oxidize manganese only (Daum *et al.* 1998; Fleming *et al.* 2011). However, recently a novel autotrophic iron oxidizing *Pseudomonas* species was found (Su *et al.* 2015). Despite extensive research, it is still not clear how heterotrophic bacteria benefit from manganese oxidation (Tebo *et al.* 2005; De Schamphelaire *et al.* 2007; Geszvain *et al.* 2013). However, from literature it is known that Mn^{2+} oxidation to MnO_2, by *Pseudomonas putida,* could be beneficial for the co-metabolic degradation of micropollutants, resulting in the formation of easily accessible organic compounds for their metabolism (Sabirova *et al.* 2008; Forrez *et al.* 2010; Meerburg *et al.* 2012). Similarly, it is proposed that complex organic molecules (*e.g.*, natural organic matter (NOM), such as humic acids in groundwater) undergo degradation by the same process, performed by *e.g., Pseudomonas putida* (Verstraete 2013). Based on the proposed model of Mn^{2+} oxidation by *e.g., Pseudomonas putida* and co-metabolic degradation of organic micro pollutants from Meerburg *et al.* (2012), a simplified degradation scheme for NOM is adopted (Figure 1). The process of co-metabolic degradation is known as 'bio cracking'.

From Figure 1 it can be seen that MnO_x, formed during the process of bio cracking, is reduced again to Mn^{2+}, in a self-supporting metabolic cycle. Literature suggested that not one bacterium is responsible for manganese oxidation, but a microbial consortia (Vandenabeele *et al.* 1992; Vandenabeele 1993; Verstraete 2013). Once biological Birnessite (MnO_x) is formed on filter media, it has extremely high adsorptive capacity for metal ions, such as Mn^{2+} (Webb *et al.* 2005a, 2005b; Jiang *et al.* 2010). In this way biologically produced Birnessite promotes and accelerates manganese removal through physicochemical autocatalytic adsorption and subsequent oxidation of adsorbed manganese according to the oxidation kinetics of dissolved Mn^{2+} by oxygen in aqueous solution (Stumm & Morgan 1996).

The goal of this study was to provide additional insight into the role of microbes in manganese removal. A specific objective of the study was to identify (with molecular (DNA) techniques) species of bacteria present in iron removal filters, and freshly ripened manganese removal filters. Furthermore the capability of selected bacterial species found in manganese

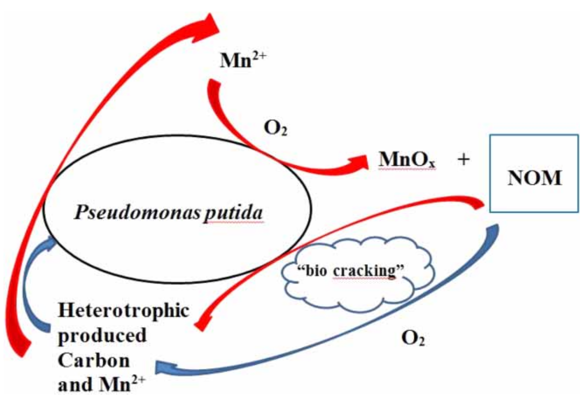

Figure 1 | Simplified co-metabolic degradation scheme of NOM by biological MnO_x oxidation (adopted from Meerburg *et al.* (2012) and Verstraete (2013)).

removal filters to oxidize Mn^{2+} was investigated in the laboratory. This study will enhance knowledge of the role of biological activity in the ripening of manganese filters in practice and show how to create conditions favorable for the biological manganese oxidation process.

MATERIALS AND METHODS

The experiments presented in this paper were carried out on a pilot plant located at a full scale groundwater treatment plant (GWTP) 'Grobbendonk', operated by the water supply company Pidpa, Belgium.

The full scale plant consists of a pre-aeration step (cascade), 1st filtration stage, post aeration (cascade) with pH correction, and post filtration. GWTP Grobbendonk treats groundwater containing iron, manganese and ammonia. Very effective removal of iron (>98%) is achieved in the 1st filtration step. Iron is removed predominantly through adsorptive and biological mechanisms, with support of the bacterium *Gallionella* sp. Filter media ripening of the post filters in this plant, concerning manganese removal, is a very fast process (typically complete manganese removal is achieved within approximately 16 days). It is believed that the filter media ripening process at this location is initially biological. Similar to post filters in the full scale plant, the pilot filter column was fed with re-aerated filtrate from the first filter stage of the full scale GWTP, after pH correction (pH =7.6). The pilot filter column is named in the rest of the paper as 'column A1'. Details of GWTP Grobbendonk and the pilot filter column are given in Bruins *et al.* (2015b).

To determine the presence and composition of the bacteria population, during the filter media ripening process samples were taken from backwash water from the 1st filtration step in the full scale plant Grobbendonk ('BW 1st RSF'), and backwash water from the pilot filter column A1 ('BW A1'). Backwash water was used to obtain a higher bacteria yield. 'BW A1' was sampled when filter media ripening was almost completed (*i.e.,* manganese removal efficiency in the filter was >90%).

Measurements and subsequent identification of bacteria present in 'BW 1st RSF' and 'BW A1' were carried out with 'next generation DNA sequencing', quantitative polymerase chain reaction (qPCR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Finally, growth tests were performed in a fermenter with selected bacteria from a sample of 'BW A1', to examine if MnO_x could be produced biologically. In addition a pure culture of *Pseudomonas putida* (ATCC 23483, LMG 2321), grown on a selective growth medium, was used as a reference.

Next generation DNA sequencing

Samples were taken from both, 'BW 1st RSF' and 'BW A1'. The DNA in the samples was extracted using the power biofilm DNA isolation kit including a beat-beating step for enhanced cell disruption (MoBio Laboratories, Carlsbad, USA). A part of the 16S rRNA gene (approximately 900 bp) was amplified from these samples, using a eubacterial forward primer GM3 (5′-AGA GTT TGA TCM TGG C-3′), and the universal reverse primer 926r (5′-CCG TCA ATT CMT TTG AGT TT-3′) with identifiable sample bar codes. The pyrosequencing analysis of the amplified 16S rRNA genes was performed using LGC genomics (Berlin, Germany) with a 454 Life Sciences GS FLX series genome sequencer upgraded to long read length (Roche, The Netherlands). The returned 16S rRNA gene sequences were analysed, trimmed, aligned, and identified using the metagenomics tool in the software package Bionumercs (Schloss *et al.* 2011) based on the 454 SOP (Schloss *et al.* 2011) and the Mothur pipeline software tool (Schloss *et al.* 2009).

In short, sequences were analysed and sequencing errors were reduced using flowgrams. To reduce computing time the maximum number of flows was set to 650–900 depending on the number of available sequences (50,000–200,000). Subsequently, sequences were trimmed (arbitrary choices: only sequences with minimum lengths of 200 bp and with both primer sequences were selected, tdiffs: 3, and max homop: 8). Sequences were identified against the Silva reference file release 111 (www.arb-silva.de). To display sequence abundance a taxonomic tree was calculated with a minimal percentage of all observations of >1%.

qPCR

For the qPCR measurements employed to determine *Leptothrix* spp., a Light cycler 480 II from Roche was used.

Samples were taken from both 'BW 1st RSF' and 'BW A1'. A sample of 100 mL water was filtered through a 0.45 μm membrane filter. The filter was used in the 'powerbiofilm DNA extraction kit' from MoBio (article number: 24000-50). DNA extraction is based on mechanical and chemical lysis. DNA binds to a silica membrane, followed by wash steps. After that, DNA is eluted in 100 μl elution buffer from the MoBio kit. Primers and probe for *Leptothrix* spp. were heterogeneous for its 16S rRNA. Two upstream primers for *Leptothrix* spp. are required to get all relevant species.

Forward primer1 PS-1: 5′ ACGGTAGAGGAGCAATC 3′ (Burger *et al.* 2008a).

Forward primer2 PSP-6: 5′ CAGTAGTGGGGGATAGCC 3′ (Burger *et al.* 2008a).

Reverse primer DSP-6: 5′ GCTTTTGTCAGGGAAGAAATC 3′ (Burger *et al.* 2008a).

Lepto-pr6 forward: 5′ CACGCGGCATGGCT 3′ *Cy5 (developed by WLN).

The PCR program was as follows: 10 minutes room temperature (uracil glycosylase), 5 minutes 95 °C (denaturation), 50 × 30 seconds at 95 °C; 1 minute at 55 °C and 10 seconds at 72 °C.

To quantify the number of bacteria, a WLN-III plasmid was developed for target genes. The start concentration of this plasmid is referenced to the mip gene of the standardized Minerva plasmid, and is about 160,000 cDNA/L for *Leptothrix* spp.

To determine the gene copy numbers of *Pseudomonas* spp. and *Pseudomonas putida*, a qPCR protocol, using newly developed primers was used. For the specific detection of *Pseudomonas* spp. primers were developed targeting the 16S rRNA gene, for detection of *P. putida,* the more variable gyrB gene was selected. The primers and probe used for detection of *Pseudomonas* spp. were Pspp16Sf1 (5′-GAG CCT AGG TCG GAT TAG-3′), Psppr3 (5′-CGC TAC ACA GGA AAT TCC AC-3′), and probe PspP1 (5′-CGC GTG TGT GAA GAA GGT CTT CG-3′). For quantification of *P. putida* the primers PpgyrBf3 (5′-GAC ATC CTG GCC AAG CGT-3′) and PpgyrBr3 (5′-CTT CCT GYT CGA TGT AGC-3′) and probe PpgyrBp1 (5′-CTG CAR TGG AAY GAC AGC TTC AAC G-3′) were selected. Primer specificity and selectivity were analysed and PCR conditions were optimized.

PCR was conducted in 50 μl reaction volumes containing 25 μl of 2x IQ Supermix (Bio-Rad Laboratories BV, The Netherlands), 10 pmol of the forward primer, reverse primer and probe, 20 mg of bovine serum albumin, and 10 μl of the DNA template. The amplification program consisted of 2 min at 95 °C; 43 cycles of 20 s at 95 °C and 30 sec at 60 °C. Amplification, detection, and data analysis were performed in an iCycler IQ real-time detection system (Bio-Rad Laboratories BV, The Netherlands). The PCR cycle after which the fluorescence signal of the amplified DNA and the probe was detected (threshold cycle [*Cq*]) was used to quantify the gene copy concentration. Quantification was based on comparison of the sample *Cq* value with the *Cq* values of a calibration curve based on known copy numbers of the plasmin containing the 16S rRNA gene of *P. putida* (U70977.1) and the gyrB gene of *P. putida* (HF545867.1).

MALDI-TOF MS

With this technique microorganisms can be identified directly after culturing on selective agar media. Spectra were generated with the MALDI-TOF MS biotyper from Brŭker Daltonik GmbH, and compared with approximately 4,000 spectra in the Brŭker Daltonik GmbH database. In a log score from 1 to 3, the MALDI-TOF biotyper defined the similarity of the known and unknown spectra.

When the log score is between 2 and 2.3, the genus identification is secure and probably the species is also identified. With a log score >2.3, it is highly probable that the species is identified.

MALDI-TOF MS is based on the chemotaxonomy of microorganisms. This 'fingerprint' is based on identified proteins of the microorganism. These proteins are always present in a living cell and make it possible to characterize microorganisms. In this project we expected *Pseudomonas* to grow in the filter column where Mn^{2+} was oxidized ('BW A1'). Samples were filtered through a 0.45 μm membrane and incubated on *Pseudomonas* specific agar, a media containing cetrimide and sodium nalidixate to inhibit gram positive bacteria and some gram negatives other than *Pseudomonas*.

Pseudomonas secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown). Coloured colonies on a *Pseudomonas* agar are suspected to be *Pseudomonas,* and they were identified using the MALDI-TOF MS biotyper from Brüker. A single colony of a target organism is put directly on a 96 target plate. After deposition, the spots were overlaid with 1 μl matrix solution (2.5 mg α-Cyano-4-hydroxycinnamic dissolved in 50% acetonitrile, 2.5% trifluoro acetic acid, 47.5% ultra-pure water). The matrix opens the cell wall. A laser irradiates the matrix sample, to divide it into little portions of proteins. The matrix evaporates and positively charged proteins become free. In the strong electric field, the positively charged proteins are lined up. So these proteins have the same starting point, before they accelerate in the flight tube to get to their specific time-of-flight corresponding with their specific mass.

Fermenter growth test with selected bacteria to produce biological MnOx

A Bioflow III fermenter from New Brunswick Scientific was inoculated with pure cultures of *Pseudomonas putida* (ATCC 23483, LMG 2321), as well as with *P. grimontii* and *P. koreensis*, which were isolated from 'BW A1'. The growth medium used in the fermenter is described by Jiang *et al.* (2010). Growth and subsequent manganese oxidation in the fermenter took place over 4 days. Growth of the *Pseudomonas* spp. was achieved by use of the standard *Pseudomonas* agar growth medium (48.4 g agar and 10 ml glycerol per liter, sterilized for 15 min at 121 °C). The incubation time for growth was 24–48 hr. at 30 °C. Colonies were identified by MALDI-TOF MS and stored in pentone-glycerol at −80 °C. Formation of MnO_x was identified as black deposits and was verified by inductively coupled plasma mass spectrometry (ICP-MS) and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX).

RESULTS AND DISCUSSION

Next generation DNA sequencing

Table 1 provides an overview of the bacterial population found in backwash water samples from the first stage filter ('BW 1st RSF') and the pilot filter column ('BW A1'). The sequencing results of sample 'BW 1st RSF' are based on 188,241 sequences, whereas the results of sample 'BW A1' are based on 55,298 sequences. The taxonomic trees are shown in Appendices A and B (Supplementary Information, available with the online version of this paper).

Table 1 | Bacterial population in samples of 'BW 1st RSF' and 'BW A1'

Sample	Identification	% of population
BW 1st RSF	*Gallionella* spp.	97.0
	Other	3.0
BW A1	*Nitrospira* spp.	25.7
	Pseudomonas spp.	14.3
	Gallionella spp.	12.4
	Other	47.6

Table 1 shows a clear difference in bacterial population present in sample 'BW 1st RSF' (iron removal filter of the full scale plant) in comparison to bacteria present in 'BW A1' of the freshly ripened (manganese removal) pilot filter column.

The majority (97%) of bacteria found in 'BW 1st RSF' were *Gallionella* spp. The abundance of *Gallionella* spp. is understandable as iron removal takes place by a biological removal mechanism. Very similar bacterial composition is present in the feed water to the 2nd stage full scale and thus also in the feed water of the pilot filter column. Identification of bacteria present in 'BW A1', showed that only 12.4% of the bacterial population was *Gallionella* spp., *Pseudomonas* spp. and *Nitrospira* spp. represented 14.3% and 25.7% of the total population, respectively. Almost half of the population found in 'BW A1' belongs to smaller populations or could not be identified. The presence of the bacterium *Nitrospira* spp. (25.7%) was expected, because this species is involved in the oxidation of ammonia and specifically conversion of nitrite to nitrate, which takes place in this filter. *Pseudomonas* spp. might be involved in the biological manganese removal process. The literature suggests that besides oxidation of manganese, *Pseudomonas* spp. are able to oxidize ammonia (Daum *et al.* 1998; Nemergut & Schmidt 2002). This finding also explains the strong relation between manganese and ammonia oxidation observed in practice (Bruins *et al.* 2014). The observation that 14.3% of the bacterial population found in 'BW A1' consists of *Pseudomonas* spp., a potential manganese oxidizing bacterium, supports the assumption that manganese removal starts biologically (Bruins *et al.* 2015b).

qPCR

Leptothrix spp. and *Pseudomonas* spp., are both able to oxidize dissolved manganese. Their concentration was quantified with qPCR as the number of DNA copies (n [cDNA/L]) present in 'BW A1'. Furthermore the concentration of the species *Pseudomons putida* was quantified with the same technique. Table 2 gives an overview of the number of quantified species, expressed as DNA copies/L.

From Table 2 it is clear that from the potential manganese oxidizers the presence of *Pseudomonas* spp. was much more pronounced than the presence of *Leptothrix* spp. This supports the fact that *Leptothrix* spp. were not found with the next generation DNA sequencing. Also *Pseudomonas putida* was present in relatively low concentrations, compared to the genus *Pseudomonas*. In literature *Pseudomonas putida* is often associated with biological manganese removal. However, the species *Pseudomonas putida* was not ubiquitous and thus not likely responsible for the fast ripening of manganese removal filters in the pilot testing performed in this study. At the same time it is plausible that other closely related *Pseudomonas* species contributed to this process.

Table 2 | Manganese oxidizing bacteria in sample 'BW A1', quantified by qPCR

Bacterium	n (cDNA/L)	%
Pseudomonas spp.	2.3×10^{11}	>99.99%
Pseudomonas putida	1.5×10^{7}	<0.01%
Leptothrix spp.	3.8×10^{6}	<0.01%

Table 3 | Pseudomonas species identified by MALDI-TOF MS (including Log score)

Identified species	Log score
Pseudomonas gessardii	2.40
Pseudomonas libanensis	2.22
Pseudomonas synxantha	2.21
Pseudomonas veronii	2.17
Pseudomonas grimontii (Figure 2)	2.15
Pseudomonas koreensis (Figure 2)	2.14
Pseudomonas extremorientalis	2.07
Pseudomonas marginalis	2.04
Pseudomonas tolaasii	2.03
Pseudomonas azotoformans	2.03
Pseudomonas rhodesiae	2.00

MALDI-TOF MS

Several colonies isolated from sample 'BW A1', were cultured with a *Pseudomonas* agar and were identified by using the MALDI-TOF MS biotyper. Table 3 shows an overview of all identified *Pseudomonas* species present in the backwash water of a freshly ripened manganese removal filter.

The genus *Pseudomonas* consists of many very closely related species. No *Pseudomonas putida* was identified in any of the samples with MALDI-TOF MS. This was expected based on the low contribution of the strain *P. putida* to the total bacterial population determined with qPCR (<0.01%). The list of *Pseudomonas* species (Table 3) is incomplete, as only a limited number of colonies are identified. SEM images (Figure 2) show two *Pseudomonas* species, namely *Pseudomonas grimontii and Pseudomonas koreensis* in the backwash water of a freshly ripened manganese removal filter.

Fermenter growth test with selected bacteria for the biological production of MnOx

Pseudomonas grimontii (log: 2.15) and *Pseudomonas koreensis* (log: 2.14), obtained from 'BW A1', were used as inoculum in a fermenter to investigate their growth and MnO_x production under controlled laboratory conditions. As a reference, a similar growth test was conducted with the laboratory species *Pseudomonas putida* (ATCC 23483, LMG 2321). Although to a very limited extent, *Pseudomonas putida* was able to produce MnO_x. Results obtained from the fermenter growth test showed that *Pseudomonas grimontii* and *Pseudomonas koreensis* were not able to oxidize Mn^{2+} producing MnO_x, under the laboratory conditions.

Summarizing, results from this study show that the population of bacteria present in the backwash water of the 1st stage (iron removal) filter and the freshly ripened 2nd stage (manganese removal) pilot filter column dramatically changed. Furthermore the presence of *Pseudomonas putida* was very limited (<0.01% of the potential manganese oxidizing bacteria present). This indicates that the role of *Pseudomonas putida*, concerning manganese removal at the Grobbendonk treatment plant, is limited. However, related *Pseudomonas* species, may play an important role in the process of

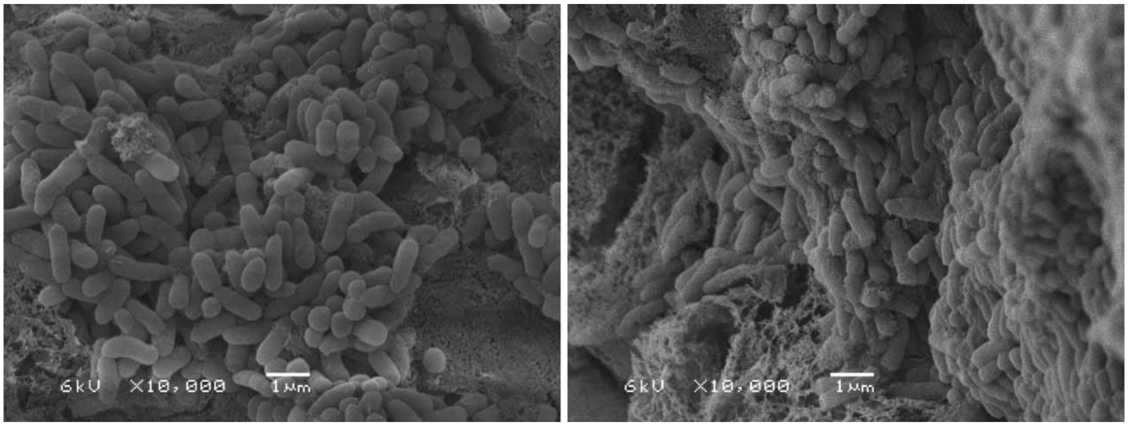

Figure 2 | SEM images (10,000×) of *Pseudomonas grimontii (L)* and *Pseudomonas koreensis (R)*, isolated from sample 'BW A1'.

manganese removal (Table 3), taking into account that the Birnessite (MnO_x) produced in the pilot filter column during the ripening period was of biological origin (Bruins *et al.* 2015b). It remains, however, unclear if *Pseudomonas* spp. are the only manganese oxidizing bacteria involved in the initial Mn^{2+} oxidation, or if other species form a microbial consortium, together responsible for the oxidation of Mn^{2+}. The knowledge that manganese removal in aeration-rapid sand filtration treatment is initiated biologically, together with an insight into the manganese oxidizing bacteria species involved, may enable typically long ripening times to be reduced by creating conditions favorable for the growth of these manganese oxidizing species. Therefore, the focus of the follow-up research will be on the inoculation of a consortium of bacteria, identified in manganese removing filters, to enhance filter media ripening. Also, conditions supporting the fast growth of Mn^{2+} oxidizing bacteria should be investigated in follow-up research.

CONCLUSIONS

From this study the following can be concluded:

- Based on 'next generation DNA sequencing' analyses, the population of bacteria present in backwash water from an iron removal filter (first step filter in a full scale plant), and the freshly ripened pilot manganese removal filter showed a clear population shift from the iron oxidizing *Gallionella* spp. (97%) to manganese and nitrite oxidizing species (*Pseudomonas* spp. (14%) and *Nitrospira* spp. (26%), respectively). However, it should be noted that 47.6% of the bacterial population in the manganese removal filter belongs to smaller populations or could not be identified.
- qPCR analysis showed that less than 0.01% of the genus *Pseudomonas* present in the freshly ripened manganese removal pilot filter column was the *Pseudomonas putida* species.
- *Pseudomonas* is most likely one of the manganese oxidizing bacteria genera that play an important role in the initial stage of the ripening of the manganese removal filters at the full scale GWTP Grobbendonk. However, it is still unclear whether this genus of bacteria is acting alone or as part of a microbial consortium.
- Amongst others, *P. gessardii, P. grimontii and P. koreensis*, closely related *Pseudomonas* species, were detected by the MALDI-TOF MS analysis, and are probably involved in the manganese removal process, possibly as part of a bacterial consortium.
- Selected *Pseudomonas* species from the ripened filter media column, namely *Pseudomonas grimontii* and *Pseudomonas koreensis*, were not able to produce MnO_x under controlled laboratory conditions, whereas the reference species *Pseudomonas putida* was able to do so.

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