Manganese removal in drinking water biofilters

from start-up to matured filters

Breda, Inês Lousinha Ribeiro

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FROM START-UP TO MATURED FILTERS

BY
INÊS L. BREDA

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AALBORG UNIVERSITY
DENMARK

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FROM START-UP TO MATURED FILTERS

PHD THESIS

INÊS L. BREDA

AALBORG UNIVERSITY
DENMARK

DEPARTMENT OF CHEMISTRY AND BIOSCIENCE
FACULTY OF ENGINEERING AND SCIENCE
AALBORG UNIVERSITY

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Dissertation submitted:	January 2019
Academic PhD supervisor:	Associate Professor Peter Roslev Department of Chemistry and Bioscience Aalborg University, Denmark
Academic PhD co-supervisors:	Associate Professor Loren Ramsay Research Group for Energy and Environment VIA University College, Denmark
	Associate Professor Ditte A. Søborg Research Group for Energy and Environment VIA University College, Denmark
Industrial PhD supervisor:	Head of Project & Development Group Christina Søndergaard, Skanderborg Forsyning A/S, Denmark
Industrial PhD co-supervisor:	Head of Operations Michael Pilc, Skanderborg Forsyning A/S, Denmark
PhD committee:	Associate Professor, Niels Iversen (chairman) Aalborg University
	Associate Professor, Catherine Jane Paul Lund University
	Water Quality Specialist, Laure Lopato HOFOR A/S, Copenhagen
PhD Series:	Faculty of Engineering and Science, Aalborg University
Department:	Department of Chemistry and Bioscience

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“I'm always horrified whenever I finish anything. Horrified and desolate. My instinct for perfection should inhibit me from ever finishing anything; it should in fact inhibit me from ever beginning. But I become distracted and do things.” – Fernando Pessoa, The Book of Disquiet.

- Let’s do it!

“Pasmo sempre quando acabo qualquer coisa. Pasmo e desolo-me. O meu instinto de perfeição deveria inibir-me de acabar; deveria inibir-me até de dar começo. Mas distraio-me e faço.” – Fernando Pessoa, Livro do Desassossego.

- Vamos equipa!

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Aos meus avós pelo exemplo de vida. À minha mãe pelo tempo de conversa e constante presença na minha vida, ao meu pai por me ensinar que sucesso vem com caminho diário, ao meu irmão pela proteção e partilha, e à minha irmã pela paciência em ouvir cada interminável explicação sobre este projecto. A todos aqueles com quem partilhei momentos de oração e entrega, que servem hoje de propósito e que me inspiram a contribuir para a construção do bem comum.

Til Loren Ramsay, Ditte A. Søborg og Torben L. Skovhus, min danske familie. I er altid der for mig. TAK.

To Lawan Subba. मेरो पतिलाई धन्यवाद। तपाईं मलाई हरेक दिन सबै भन्दा राम्रो दिन प्रेरणा दिनुहुन्छ।

Inês L. Breda - Porto, December 2018

PREFACE

This thesis is the result of an Industrial PhD study funded by *Skanderborg Forsyning A/S* and *Innovationsfonden* (grant nr.5016-00134B). The Industrial PhD study was carried out at *Skanderborg Forsyning A/S* and at the *Department of Chemistry and Bioscience Aalborg University* from February 2016 to February 2019.

The thesis is organised in two sections. The first section puts into context the main findings of the studies and is divided into four chapters: Chapter 1 describes the motivations and aims of this research; Chapter 2 describes the state-of-the-art, remarking the knowledge gap and practical challenges of the research topic; Chapter 3 provides an overview of the experimental work; and lastly, Chapter 4 summarizes the impact of the research.

The second section of this thesis consists of four scientific papers, listed below, which are a direct result of the three-year period of the studies:

PAPER I **Breda, I. L.**; Ramsay, L.; Roslev, P. (2017) Manganese oxidation and bacterial diversity on different filter media coatings during the start-up of drinking water biofilters. *Journal of Water Supply: Research and Technology. AQUA*, Vol. 66, No. 8, p. 641-650. https://doi.org/10.2166/aqua.2017.084

PAPER II **Breda, I. L.**; Søborg, D.A.; Ramsay, L.; Roslev, P. (2018) Manganese removal processes during start-up of inoculated and non-inoculated drinking water biofilters. *Water Quality Research Journal.* https://doi.org/10.2166/wqrj.2018.016

PAPER III **Breda, I. L.**; Ramsay, L.; Søborg, D.A.; Dimitrova, R.; Roslev, P. Manganese removal processes in 10 groundwater-fed full-scale drinking water biofilters in Denmark. (*submitted to Water Quality Research Journal)*

PAPER IV Søborg, D.A.; **Breda, I. L.**; Roslev, P. Microbial diversity and distribution in 10 groundwater fed full-scale drinking water biofilters in Denmark. (*in preparation)*

The following papers, not included in this thesis, were also published during the PhD period:

- Ramsay, L.; **Breda, I. L.**; Søborg, D.A. (2018). Comprehensive analysis of the start-up period of a full-scale drinking water biofilter provides guidance for optimisation. *Drinking Water Engineering and Science*, 11, 87-100. https://doi.org/10.5194/dwes-11-87-2018

- **Breda, I. L.**; Søborg, D.A.; Ramsay, L. (2016). The role of backwash in start-up of full-scale drinking water biofilters. *Journal of Water Supply: Research and Technology. AQUA*, Vol. 65, No. 3, p. 234-243. https://doi.org/10.2166/aqua.2016.093

In addition to the scientific papers, the results obtained in this study were disseminated in conferences and seminars. A list of activities is available below, categorised as international dissemination activities, national dissemination activities, and accepted abstracts for future dissemination activities:

International dissemination activities:

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2018). Effect of filter media and inoculation on manganese oxidation and microbial diversity in drinking water biofilters. Platform presentation at *World Water Congress & Exhibition 2018*, Tokyo, Japan.

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2018). Manganese oxidation and microbial diversity during start-up of non-inoculated and inoculated drinking water biofilters. Poster presentation at *Nordic Drinking Water Conference*, Oslo, Norway.

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2018). Biostimulation strategies to enhance manganese removal in drinking water biofilters. Poster presentation at *International Symposium on Biological Treatment*, Austin, United States.

Breda, I. L.; Ramsay, L.; Roslev, P. (2016). Prokaryotic communities in drinking water biofilters using alternative filter medium. Poster presentation at *World Water Congress & Exhibition 2016*, Brisbane, Australia.

National dissemination activities:

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2019). Manganese removal processes at 10 waterworks. Presentation at *Danish Water Forum*, Copenhagen, Denmark.

Breda, I. L.; Ramsay, L.; Roslev, P. (2019). Effect of different filter media on manganese oxidation and microbial diversity during the start-up period. Presentation at *Aalborg Forsyning seminar*, Aalborg, Denmark.

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2018). Effect of filter media and inoculation on manganese oxidation and microbial diversity in drinking water biofilters. Presentation at 4th *Young Water Professionals Denmark Conference*, Copenhagen, Denmark.

Breda, I. L.; Ramsay, L.; Roslev, P. (2017). Removal of manganese by alternative filter media. Presentation at 3rd *Young Water Professionals Denmark Conference*, Odense, Denmark.

Breda, I. L.; Roslev, P.; Ramsay, L. (2017). Removing manganese from groundwater for drinking water consumption using alternative filter materials. Presentation at *2nd FoU Conference – VIA Byggeri, Energi & Miljø*, Horsens, Denmark.

Breda, I. L.; Ramsay, L.; Roslev, P. (2016). Industrial PhD: Biostimulation of prokaryotic communities in drinking water biofilters. Presentation at 2nd *Young Water Professionals Denmark Conference*, Aarhus, Denmark.

Breda, I. L.; Roslev, P.; Ramsay, L. (2016). Presentation of an Industrial PhD: Biostimulation of prokaryotic communities in drinking water biofilters. Presentation at *1st FoU Conference – VIA Byggeri, Energi & Miljø*, Horsens, Denmark.

Accepted abstracts for future dissemination activities:

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2019). Manganese removal in drinking water biofilters. Poster presentation at IWA *Young Water Professionals Conference*, Toronto, Canada.

Breda, I. L.; Amengor, J. (2019). From Research to Practice: Changing the Narrative. Workshop at IWA *Young Water Professionals Conference*, Toronto, Canada.

Breda, I. L. (2019). Manganese removal processes at 10 waterworks. Presentation at *Nordic Drinking Water Biofilm Symposium*, Horsens, Denmark.

Breda, I. L.; Ramsay, L.; Søborg, D.A.; Roslev, P. (2019). Manganese removal in drinking water biofilters. Presentation at *5th Young Water Professionals Denmark Conference*, Skanderborg, Denmark.

ABSTRACT

The reoccurring theme of this thesis is manganese removal in drinking water biofilters using groundwater sources. Manganese removal in biofilters is based on physicochemical and biological processes. Practical concerns of manganese removal in drinking water treatment plants include an extended period until the onset of removal during the start-up period of new filters and deterioration of the removal process during operations of matured filters.

The primary ambition of this thesis was to provide further insight into manganese removal in drinking water biofilters by characterising the removal process in biofilters at different stages of maturation and different depths of the filter. More specifically, this study investigated possible solutions to shorten the start-up period by accelerating the onset of manganese removal in new filters and characterized manganese removal processes and the microbial community distribution in early matured and fully matured biofilters across different depths.

The results obtained during this research were based on batch, column, pilot and full-scale assays, including a selection of five different filter materials and collection of over one hundred filter material samples throughout the studies.

Before this research, it was considered that after initial sorption, manganese removal by a non-coated virgin material was initiated biologically, evolving to a predominantly physicochemical removal process over time. This thesis confirms that manganese removal processes are indeed dynamic over time, by showing that manganese removal is dominated by biological processes during the early stages of filter maturation, moving to a physicochemical dominance after the filters' maturation.

It was shown that solutions to shorten the start-up by inherent inoculation (using alternative filter materials) or proactive inoculation (using matured quartz) could accelerate the onset of manganese removal of virgin filters. Furthermore, the use of alternative filter material was shown to influence the microbial community developed during the initial stage of maturation of the filter, whereas proactive inoculation by addition of matured quartz had limited effect on the microbial community of the filters.

It was also found that initial selection of filter material becomes of less importance over time with regards to manganese removal capacity. This observation is of value to water utilities when considering investing in alternative filter materials.

Regarding the microbial diversity in drinking water biofilters, results suggest that *Hydrogenophaga*, *Pseudomonas*, *Hyphomicrobium*, and *Pedomicrobium* genus might be of importance during the onset of manganese removal. Further, preliminary results on the spatial distribution of microbial communities across the depth of matured filters suggest that genera known to include manganese oxidising bacteria have limited correlation with the manganese removal profiles registered along the depth of matured filters.

Finally, the results showed that concentration profiles of main treatment substances can change over time. This observation suggests that the design of drinking water biofilters should be adaptable over time, not only to accommodate fluctuations in production and groundwater quality, but also to follow the maturation stage of the filter.

The active collaboration between the academic and industrial partners in this applied research study was favourable and indispensable to the practical conclusions obtained throughout the studies. Based on the knowledge presented in this thesis, water supply companies can take measures to change the start of operations of virgin filters, rethink filter design, and revise operational conditions at drinking water treatment plants.

RESUMÉ

Denne ph.d.-afhandlings gennemgående tema er manganfjernelse i drikkevand biofiltre, ved brug af grundvandskilder. Manganfjernelse i biofiltre baseres på fysisk-kemiske såvel som biologiske processer. Praktiske udfordringer vedrørende manganfjernelse i drikkevandsrensefaciliteter indebærer: En længerevarende tidsperiode indtil manganfjernelsen påbegyndes i opstartsperioden af nyetablerede filtre, såvel som forringelse af fjernelsesprocessen under drift af fuldt modnede filtre.

Denne afhandling har til formål, at give yderligere indsigt i vandværkers manganfjernelse i drikkevand biofiltre, ved karakterisering af fjernelsesprocessen i biofiltre ved forskellige modningsstadier og filterdybder. Dette studie undersøger hermed muligheder for at forkorte opstartsperioden, ved at accelerere igangsættelsen af manganfjernelse i nyetablerede filtre. Medvidere karakteriseres fjernelsesprocessen for mangan samt fordelingen af mikrobielle samfund i nyligt og fuldt modnede biofiltre ved forskellige filterdybder.

Resultaterne blev baseret på batch-, kolonne-, pilot- og fuldskalaforsøg som inkluderede et udvalg af 5 forskellige filtermaterialer og indsamling af 100 filtermaterialeprøver i løbet af studiet.

Den foregående betragtning for studiet var, at efter indledende sorption, initieres manganfjernelse af ikke-belagt nyt materiale biologisk, hvorefter manganfjernelsen over tid udvikles til en overvejende fysisk-kemisk proces. Denne afhandling bekræfter, at manganfjernelsesprocesser er dynamiske over tid, og viser, at manganfjernelse primært faciliteres af biologiske processer i de tidlige stadier af filtermodning, hvorefter fysisk-kemiske processer dominerer manganfjernelsen efter filtermodning.

Studiet viser, at iboende podning (ved brug af alternative filtermaterialer) eller proaktiv podning (ved brug af modnet kvarts) kan accelerere påbegyndelsen af manganfjernelse i nye filtre og hermed forkorte opstartstiden. Medvidere konstateres at valget a filtermateriale med tid bliver mindre afgørende i forhold til kapaciteten for manganfjernelse. Dette er værdifuldt i forbindelse med vandforsyningers overvejelser om investeringer i alternative filtermaterialer.

Vedrørende mikrobiel diversitet in drikkevand biofiltre indikerer resultaterne, at *Hydrogenophaga*, *Pseudomonas*, *Hyphomicrobium* og *Pedomicrobium* slægter er af særlig betydning i begyndelsen af manganfjernelse. Medvidere, foreløbige resultater vedrørende rummelig fordeling af mikrobielle samfund i modnede filtre indikerer, at slægter som indeholder MnOB i begrænset omfang korrelerer med mangankoncentrationsprofiler over dybden af modnede filtrer.

Endeligt indikerer resultaterne, at koncentrationsprofiler for primære behandlingsstoffer ændres over tid. Denne observation foreslår, at drikkevand biofilterdesign burde være udskifteligt, for at i imødekomme fluktuationer i produktionsbehov og grundvandskvalitet, såvel som at have muligheden for at følge filterets modningsstadie.

Det stærke samarbejde mellem akademiske og industrielle partnere, var fordelagtigt og uundværligt, for at opnå de praktiske konklusioner i dette anvendt forskningsstudie. Baseret på det vidensgrundlag som denne afhandling præsenterer, kan vandforsyninger træffe foranstaltninger for at ændre driftsstart af nye filtrer, gentænke filterdesign og genoverveje driftsforhold ved vandværker.

ABBREVIATIONS

ADHD	*attention deficit hyperactivity disorder*
As	*arsenic*
CH_4	*methane*
Cu	*copper*
DWTP	*drinking water treatment plant*
Fe	*iron*
GEUS	*Geological Survey of Denmark and Greenland*
H_2S	*hydrogen sulphide*
k	*first order constant*
Mn	*manganese*
MnOB	*manganese oxidising bacteria*
MnO_x	*manganese oxide*
NaN_3	*sodium azide*
NH_4	*ammonium*
NO_2	*nitrite*
NO_3	*nitrate*
O_2	*oxygen*
P	*phosphorous*

TABLE OF CONTENTS

1. INTRODUCTION

1.1 MOTIVATION

When using anaerobic groundwater for production of drinking water, the primary water treatment concerns are reduced compounds such as dissolved iron (Fe), ammonium (NH_4) and manganese (Mn). Biofilters have been in use for decades in some European countries and are currently gaining interest in North America for production of drinking water from anaerobic groundwater [1]. However, it is still possible to find references within the last ten years using the term "black box" to describe biofilters [2,3]. Further understanding of the removal processes occurring in drinking water biofilters is of interest to both academia and water utilities.

Mn removal in drinking water biofilters is facilitated by biological and physicochemical processes. Recent studies suggest that after initial sorption, Mn removal by a non-coated virgin material is initiated biologically, evolving to a predominantly physicochemical removal process over time [4,5]. However, characterisation of Mn removal processes at different maturation stages and different depths of the filter is generally unexplored [4,5].

A significant disadvantage of biofiltration is the necessity of a start-up period. During that period, virgin filters mature to fully functional biofilters [1]. When Mn is present, the duration of a start-up varies from weeks to more than a year [6]. Knowledge regarding solutions to shorten the start-up period is of interest to water utilities.

Solutions to shorten the start-up period include the use of alternative filter materials [7–9] and proactive inoculation, e.g., addition of backwash sludge or matured filter material [5,10–13]. However, the effect that those solutions may have on the microbial community of the filter has received less attention [14–16]. Further knowledge could potentially lead to the use of alternative filter material as biostimulators to shorten the start-up period, as well as possible management of the functional roles of the microbial community present in drinking water biofilters.

Another concern regarding Mn removal in drinking water biofilters is the deterioration of the removal process over time [17,18], i.e., increasing concentrations in the biofilter effluent following a period in which compliance is reached. Investigation of strategies to stimulate Mn removal in matured filters is of interest to the drinking water industry.

Previous studies focusing on drinking water biofilters suggest that multiple genera may participate in the biological oxidation of Mn [19–22]. In general, those investigations have characterised the microbial community using filter material samples collected from fully matured filters. The microbial diversity in filter material samples collected during the initial stages of maturation without the use of proactive inoculation is generally underexplored [1]. Characterisation of the microbial community in biofilters during the start-up period or the early stages of maturation can be of interest to identify potential Mn oxidising bacteria (MnOB).

In a time where new knowledge is being generated fast in a conservative industrial space such as the water sector, studies based on applied research are of extreme importance to build trust between academia and industry and to allow a faster input from the research centres to the practical environment. Thus, a strong motivation of this project was also to bring research closer to practice.

1.2 AIMS AND HYPOTHESES

This dissertation aimed to obtain further knowledge of Mn removal in biofilters for drinking water production using anaerobic groundwater. On this basis, specific research objectives were defined:

- To investigate the effect of strategies to shorten the start-up period on the onset of Mn removal and the microbial diversity developed during the early stages of maturation (**PAPER I**, **PAPER II**).
- To study the dominant processes in manganese removal (physicochemical or biological) during the start-up period of virgin filters and normal operations of fully matured filters (**PAPER II**, **PAPER III**).
- To establish relations between Mn removal and microbial diversity during the start-up period of virgin filters and normal operations of matured filters **(PAPER I**, **PAPER II, PAPER IV**).

Based on previous studies [e.g.,4,5,23], it was hypothesised that:

- The filter material type has an impact on the microbial community developed in filters during the early stages of maturation (**PAPER I**).
- The role of biological transformation of Mn is of more importance during the onset of manganese removal in virgin filters than in matured filters (**PAPER II**).

- Mn removal is mostly explained by physicochemical processes across the entire filter depth of matured filters (**PAPER III**).
- Distribution of genera of known MnOB across filter depth correlates with the Mn removal profile of matured filters (**PAPER IV**)

2. STATE-OF-THE-ART

2.1 DRINKING WATER PRODUCTION IN DENMARK

In Europe, approximately 50% of drinking water derives from groundwater sources [24] (Figure 1A). Even though disinfection is vastly used for drinking water production in Europe (88%), approx. 1/5 of drinking water produced from groundwater sources doesn't precede disinfection [24] (Figure 1A). Further, more than 70% of drinking water production by groundwater sources is either untreated or treated by a simple conventional system [24] (Figure 1B).

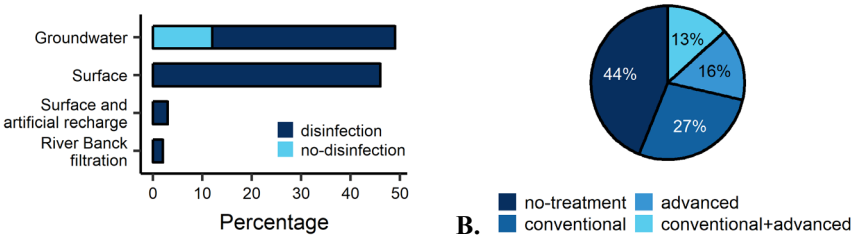


Figure 1 A. Distribution of water sources and use of disinfection for water production in Europe. **B.** Treatment system used for production of drinking water from groundwater in Europe. Data based on the yearly drinking water production of 23 European countries [24].

A conventional drinking water treatment plant (DWTP) using groundwater as source water is often based on a 2-step process: aeration followed by biofiltration (Figure 2). Aeration is accomplished by cascade aeration, diffusor aeration or oxygen (O_2) injection [25]. The main purpose of aeration is to increase the O_2 content in the water to facilitate physicochemical and biological oxidation in the subsequent biofiltration step [26]. During biofiltration, particles are filtered by physical straining, and dissolved compounds are oxidised by chemical or biological processes (e.g., Fe, NH_4 and Mn).

A DWTP can have one or multiple production lines running in parallel. The biofiltration step of a production line can have one or several filtration units running in parallel. The filtration units can have one or two filters running in series. The filters can be open or closed and driven by gravity or pressure (Figure 3A and 3B). For example, Fredensborg DWTP has one production line, starting with cascade aeration, followed by three filtration units (running in parallel) each one composed of two open filters (running in series) driven by gravity (Figure 2).

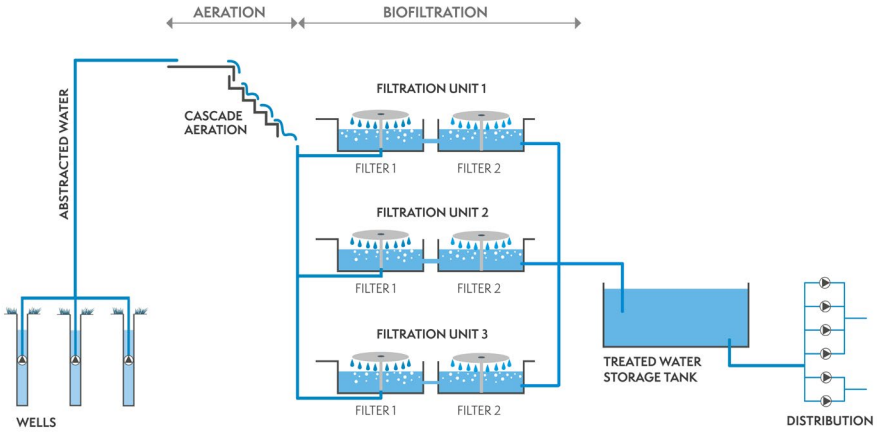
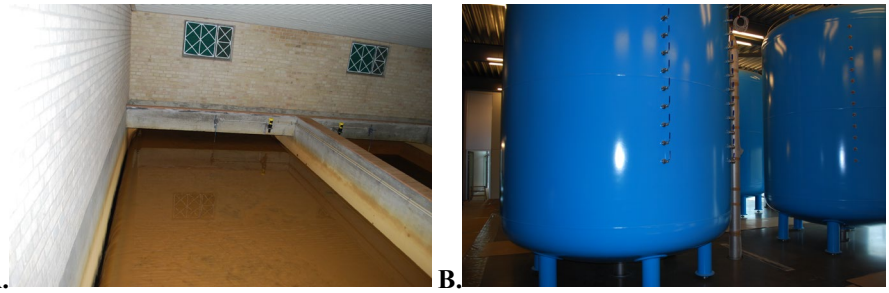


Figure 2 Schematic illustration of Fredensborg DWTP (Skanderborg).



A. **B.**

Figure 3 A. Open gravity filter at Truelsbjerg DWTP (Aarhus, 2013). **B.** Closed pressure filter at Truelsbjerg DWTP (Aarhus, 2014). Photos supplied by Loren Ramsay, VIA University College.

A filter can be composed of a single or double filter material [25]. Quartz is a standard filter material in conventional drinking water treatment systems [26]. However, there are several other filter materials available to the water utilities which have specific properties that can be of value to the water treatment. For example, calcium carbonate (limestone) is known for neutralising aggressive carbon dioxide, whereas Mn oxide is used to promote removal of Mn. Figure 4 illustrates a filtration unit at Truelsbjerg DWTP which is composed of two filters in series: the first filter contains a single filter material (calcium carbonate), and the second filter contains two filter materials (quartz and Mn oxide).

Filtration can be classified as slow or rapid depending on the filtration rate (0.05-0.20 m/h or 3-15 m/h respectively). Slow filtration is affordable and requires minimal operator training, whereas rapid filtration provides higher removal efficiencies and requires a smaller footprint when compared to slow filtration [25].

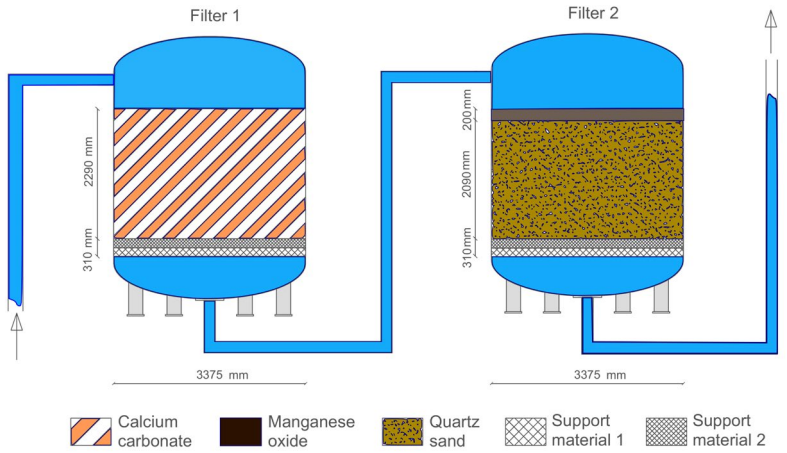


Figure 4 Filtration unit from Truelsbjerg DWTP in 2014. Filter 1 with a single filter material, and Filter 2 with a double filter material. Modified from [27].

In Denmark, almost 100% of drinking water production is based on groundwater sources (Figure 5). Groundwater is abstracted from approx. six thousand boreholes and supplied to DWTPs. The groundwater quality from boreholes supplying Danish DWTPs is available in Table 1. Drinking water production is characterised by conventional treatment systems, with no addition of disinfectants, and rapid filtration with a filtration rate of approximately 3 m/h [28].

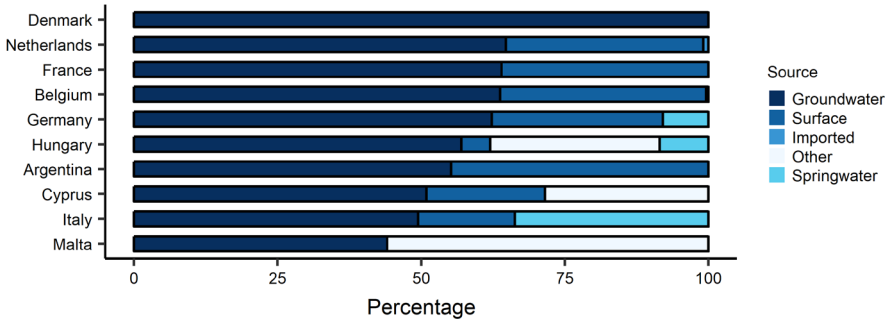


Figure 5 Top 10 countries abstracting groundwater for drinking water production in 2016. Data from IWA Statistics and Economics which includes 34 countries [29].

Table 1 Groundwater quality from boreholes supplying DWTPs in Denmark. Data supplied by Geological Survey of Denmark and Greenland (GEUS, personal communication with Jörg Schullehner) based on the most recent measurements available of water samples collected between 2010 and 2016.

Parameter	Unit	Nu. of boreholes	Percentile				
			10th	25th	50th	75th	95th
Treatment substances							
Oxygen[a] (O_2)	mgL^{-1}	4313	0.1	0.1	0.3	0.8	4.6
Iron (Fe)	mgL^{-1}	6003	0.036	0.370	1.100	2.100	4.500
Manganese (Mn)	mgL^{-1}	5994	<0.005	0.026	0.110	0.200	0.411
Ammonium (NH_4)	mgL^{-1}	5993	0.021	0.074	0.240	0.590	1.400
Methane (CH_4)	mgL^{-1}	3328	<0.010	<0.010	0.017	0.080	3.000
Major ions							
Calcium (Ca)	mgL^{-1}	5992	43	61	82	100	135
Magnesium (Mg)	mgL^{-1}	5992	3.8	6.1	10.0	16.0	30.0
Sodium (Na)	mgL^{-1}	5992	12	15	20	34	100
Potassium (K)	mgL^{-1}	5993	1.280	1.800	2.800	2.800	4.200
Hydrogen carbonate (HCO_3)	mgL^{-1}	5907	133	187	273	353	439
Chloride (Cl)	mgL^{-1}	6006	19	24	34	52	140
Sulphate (SO_4)	mgL^{-1}	5975	5.3	15.4	35.0	64.0	110.0
Nitrate (as NO_3^-)	mgL^{-1}	6007	<0.3	<0.3	<0.5	<0.5	23
Others							
Nitrite (as NO_2^-)	mgL^{-1}	5992	<0.005	<0.005	<0.005	0.008	0.0396
Phosphorous (as P)	mgL^{-1}	5995	0.011	0.021	0.069	0.140	0.270
NVOC (as C)	mgL^{-1}	5994	0.68	0.94	1.5	2.3	4.1
Hydrogen sulphide (H_2S)[a]	mgL^{-1}	277	<0.02	<0.05	<0.05	<0.05	0.31
pH[a]		4037	7	7.2	7.31	7.5	7.88
Conductivity[a]	mSm^{-1}	3951	39	50	63	78	120
Temperature[a]	°C	6039	8.3	8.7	9.1	9.6	10.6

[a] Field measurement.

2.2 THE START-UP PERIOD OF BIOFILTERS

A significant disadvantage of biofiltration is the need for a start-up period, meaning the period during which virgin filter material matures into a functional biofilter. The progress of the start-up is articulated by a set of physical, chemical and biological processes [1,19]. Once the operations commence, an inorganic coating and biofilm start to develop on the surface of the virgin filter material grains. The attachment of both inorganic coating and biofilm is required for the start-up to progress and for the water to reach compliance with drinking water criteria [12,30].

In practice, the commencement of operations of a new filter often requires 4 steps: (1) initial disinfection, (2) exaggerated backwashing to remove fines from the filter material, (3) inoculation of the filter material with microorganisms, and (4) formation of an inorganic coating and biofilm on the individual grains of the filter material [1].

During the start-up period, several concerns arise, the first being that produced water cannot be distributed to the consumers. Also, raw water must be abstracted in larger volumes from a groundwater source, untreated water following the new filters must be discharged, energy must be used for pumping raw water and backwashing the filters, and consumers must have access to treated water from another facility in the meantime [1].

The start-up period can be divided into five conceptual phases based on the concentration of specific substances in the filter's effluent [1,31] (Figure 6). In Phase 1, Fe is removed to the criterion. This phase is often short (may be as short as a day). In Phase 2, no apparent changes in the concentration of the filter's effluent are observed. This is a rather long phase, usually dragging on for weeks. In Phase 3, NH_4 is removed to the criterion. A spike of NO_2 often follows this phase due to the first step of nitrification. In Phase 4, NO_2 is converted to NO_3, and in Phase 5 Mn is removed to the criterion. At the end of Phase 5, and if the microbial quality of the filter's effluent is acceptable, the start-up period is complete.

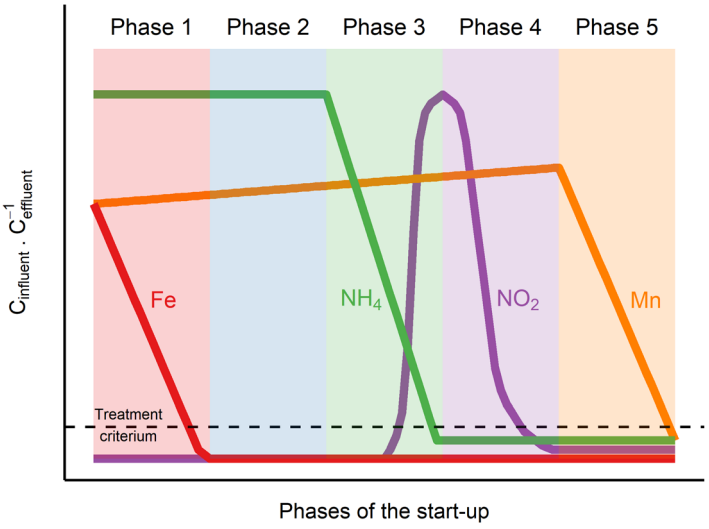


Figure 6 Conceptual phases of the start-up period in drinking water biofilters. Modified from [1].

The European guidelines [32] on the quality of water intended for human consumption define the maximum concentration for treatment substances, e.g., 0.2 mg/L Fe, 0.5 mg/L NH_4 and 0.05 mg/L Mn. However, drinking water criteria are stricter in Denmark, e.g., 0.05 mg/L for NH_4 (Table 2).

Table 2 European directive [32] and Danish guidelines [33] for drinking water intended for human consumption.

Parameter	Unit	European guidelines	Danish criteria
Treatment substances			
Oxygen (O_2)	mgL^{-1}	5	>5
Iron (Fe)	mgL^{-1}	0.2	0.2
Manganese (Mn)	mgL^{-1}	0.05	0.05
Ammonium (NH_4)	mgL^{-1}	0.50	0.05
Major ions			
Sodium (Na)	mgL^{-1}	200	175
Chloride (Cl)	mgL^{-1}	250	250
Sulfate (SO_4)	mgL^{-1}	250	250
Nitrate (as NO_3^-)	mgL^{-1}	50	50
Bacteria			
HPC, 22°C	mL^{-1}	No abnormal change	200
Coliforms	$100mL^{-1}$	0	<1
E. coli	$100mL^{-1}$	0	<1
Others			
Nitrite (as NO_2^-)	mgL^{-1}	0.50	0.1
Arsenic (As)	μgL^{-1}	10	5
NVOC (as C)	mgL^{-1}	No guideline	4
pH		6.5-9.5	7.0-8.5
Conductivity	mSm^{-1}	2500	30-2500
Temperature	°C	No guideline	12

As a final remark, it is important to notice that during the start-up period, virgin filter material matures into a functional biofilter, i.e. a filter that can remove treatment substances to criteria. However, after the start-up period, the maturation of the filter continues until achieving a full maturation stage.

2.3 MANGANESE IN GROUNDWATER AND ASSOCIATED HEALTH CONCERNS

Mn is one of the most abundant metals in Earth's crust, and it can exist in 11 oxidative states [34]. The oxidation states of most significant environmental importance are +II, +III and +IV [35]. Mn(II) is released through the weathering of igneous and metamorphic rock by interactions with surface water and groundwater and then oxidised, forming more than 30 known Mn(III), Mn(IV), or mixed Mn(III, IV) oxide minerals [36].

According to the United States Environmental Protection Agency report [37] based on data from the United States Geological Survey, the 99th percentile level of Mn(II) in groundwater (2900 µg/L) is generally higher than that in surface waters (800 µg/L), but the median level (5 µg/L) is lower than that in surface water (16 µg/L). The reducing conditions found in groundwater favours high Mn levels, e.g., concentrations up to 1300 µg/L have been reported in neutral groundwater, whereas 9600 µg/L can be found in acidic groundwater [38].

A health-based value of 400 µg Mn/l can be derived based on: 1) the upper range value of Mn intake of 11 mg/day identified using dietary surveys at which there are no observed adverse effects, 2) an uncertainty factor of 3 to take into consideration the possible increased bioavailability of Mn in water, 3) a 20% allocation of the tolerable daily intake to drinking water, and 4) the assumption of a 2 L consumption of water per day by a 60 kg adult.

Health concerns associated with the presence of Mn in drinking water are mostly related to neurologic symptoms [39]. Besides, Mn aggravates the health problems caused by arsenic (As), which suggests that Mn in drinking water should be of particular concern in areas where As is problematic [40]. Another study [41] goes further by explicitly suggesting a revision of the health guideline value, acknowledging several investigations debating the calculation of the health guideline value and the health implications of Mn in drinking water for infants and children [42,43].

In Denmark, where drinking water production is based on groundwater sources, the Mn concentration detected in boreholes for drinking water production frequently ranges between 26-200 µg Mn/L (Table 1, Figure 7).

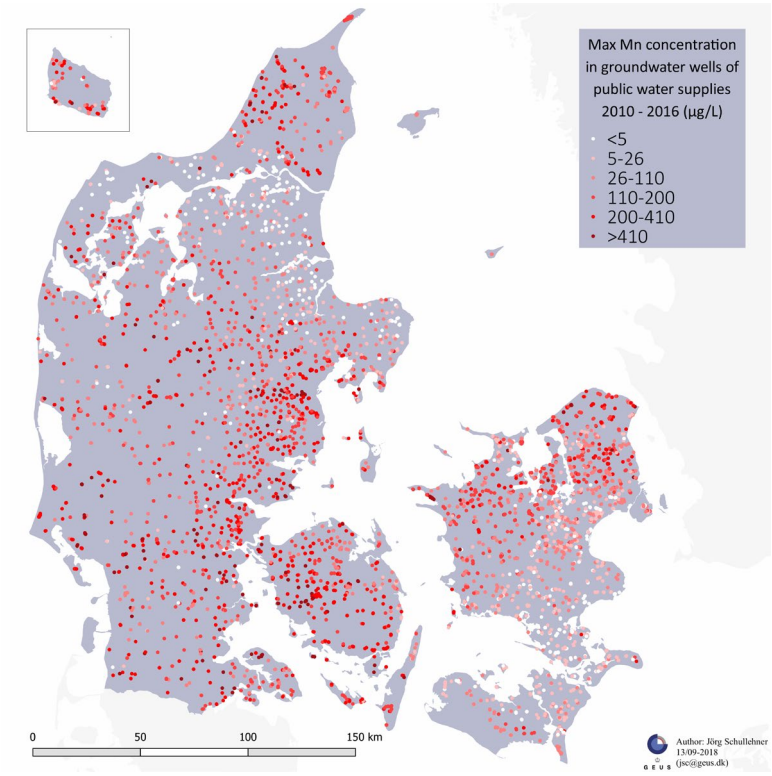


Figure 7 Distribution of the maximum Mn concentration detected in public boreholes for drinking water production from 2010 to 2016. Figure supplied by GEUS.

A recent study [44] investigated the risk of attention deficit hyperactivity disorder (ADHD) as a result of exposure to drinking water with Mn (>5µg/L) in Denmark. The study concluded that exposure to Mn in drinking water during pregnancy or within early life was associated with an increased risk of ADHD. Measurements of Mn concentration in 82.574 water samples collected from the outlet of 3017 DWTPs between 1992 and 2012 showed that Mn was under 5µg/L in most of the samples (67%), but 90% of the DWTPs had at least one episode of Mn levels above 5µg/L. As an example, Figure 8 illustrates Mn above 5µg/L between 2008 and 2012. One should notice that sampling methods and time of sampling concerning the operation of the filter are of extreme importance to the measurements of Mn in water samples. Nevertheless, and considering that deterioration of Mn removal processes has been reported as a common problem in several groundwater-fed DWTPs [5,17,18], the Mn concentration in the effluent of DWTPs should be monitored considering a standard sampling procedure and a consistent synchronisation between the sampling time and the filter operations.

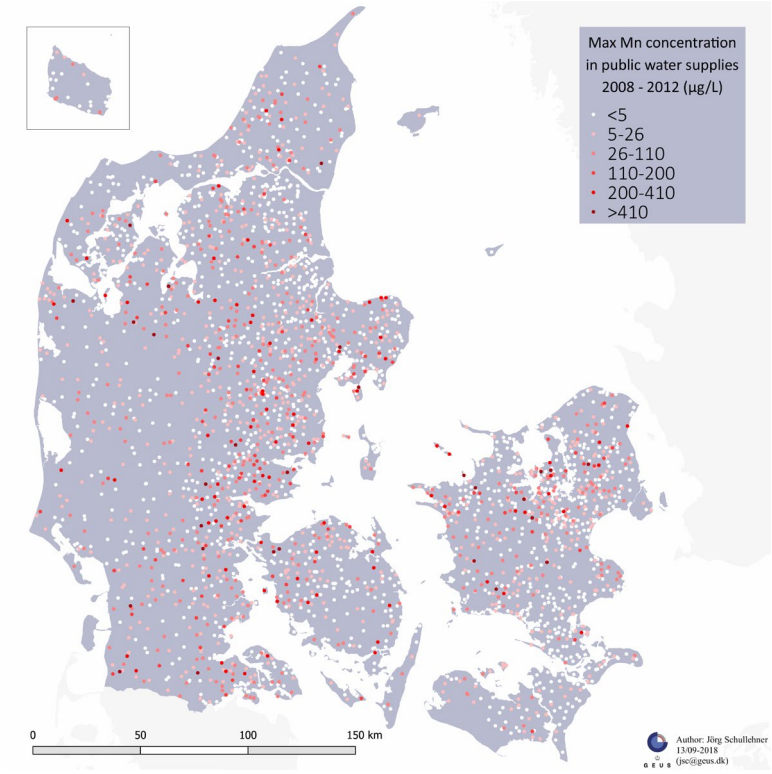


Figure 8 Distribution of the maximum Mn concentration detected in the effluent of public DWTPs from 2008 to 2012. Figure supplied by GEUS.

Over the last 60 years, the World Health Organization has established several guideline values for Mn in drinking water. According to the latest edition [45], Mn is not of health concern at levels commonly causing problems in drinking water. However, undesirable taste to beverages and stains plumbing fixtures and laundry can be caused at Mn concentrations exceeding 100 µg/L [46]. Further, precipitation of Mn by oxidation might result in encrustation problems. For example, the formation of coatings on water pipes has been reported at concentrations as low as 20 µg/L [47]. Hence, Mn in drinking water is mostly limited for aesthetic and taste reasons. Several countries, including Denmark, have set the standard of 50 µg/L Mn (Table 2), above which problems with discolouration may occur [48].

2.4 MANGANESE TRANSFORMATION IN DRINKING WATER BIOFILTERS

The Mn cycle of oxidation states Mn(II), Mn(III) and Mn(IV) is governed by O_2, pH and redox conditions (Figure 9). Mn(II) is thermodynamically stable in the absence of O_2 and at low pH, whereas Mn(III) and Mn(IV) are favoured in the presence of O_2. Mn(III) is thermodynamically unstable in aqueous solutions, as it can easily be reduced to Mn(II) or even undergo disproportion to yield Mn(II)+Mn(IV) [35].

Mn(II) occurs as a cation in solution or as a minor constituent of minerals, e.g., Mn(II, IV) oxides. Mn(III) is primarily found in insoluble form, and Mn(IV) is found in insoluble oxides, oxyhydroxides and hydroxides, in which Mn(III) is often present. Also, Mn(III) and Mn(IV) are found in environmentally common ferromanganese oxide minerals [35].

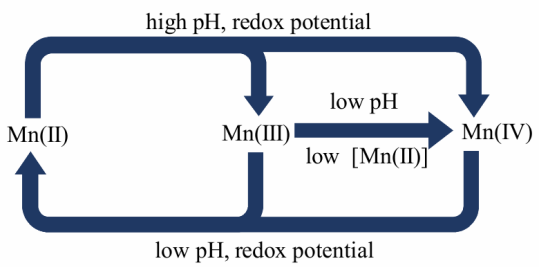


Figure 9 The Mn cycle of oxidation states found in nature, adapted from [35].

In the context of manganese removal in drinking water biofilters, it is essential to remark that Mn oxides have two crucial features: redox potential and mineral structure. Both Mn(III)/Mn(II) and Mn(IV)/Mn(II) couples have high redox potentials, making Mn(III) and Mn(IV) phases some of the stronger oxidants found in the environment [35]. Besides, Mn(III, IV) and Mn(IV) oxides have an open crystal structure, large surface areas with high negative charges and exchangeable charge-balancing cations (e.g., Na^+, K^+, Ca^{2+}, Cu^{2+}, Mn^{2+}). Both these features promote a particularly important autocatalytic behaviour of the Mn oxides coating the filter grains in drinking water filters towards the Mn(II) present in the aerated groundwater entering the filter.

Mn transformation in nature occurs by physicochemical and biological processes (Figure 10). Physicochemical transformation of Mn is described by homogenous and heterogeneous processes [20,49], whereas biological transformation of Mn is characterised by direct and indirect processes [35,50].

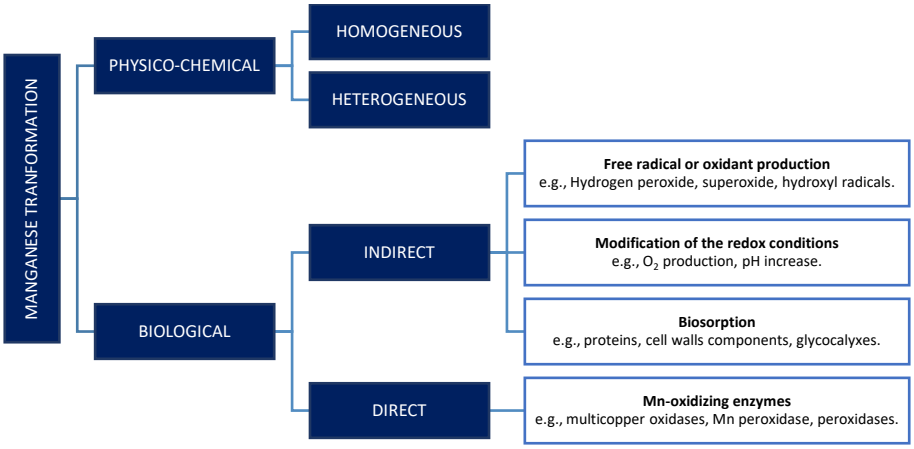


Figure 10 Overview of Mn transformation. Modified from [50]

A homogeneous process is a reaction that occurs in a single phase. The homogeneous oxidation of Mn by O_2 is extremely slow at pH below 9 [51,52]. A previous study [51] on homogeneous oxidation of Mn(II) determined a halftime removal of Mn(II) in the order of years. A following work [53] suggested a reaction constant of 2.0×10^{-2} $(mol/L)^{-2.56}$ s^{-1} (Eq.1). The reaction between Mn(II) and O_2 is around 10^6 times slower than the reaction between Fe(II) and O_2 at neutral pH [54]. Therefore, in drinking water production from groundwater sources, where pH typically ranges between 6 and 8, homogeneous oxidation of Mn is negligible [20].

Homogeneous: $$\frac{d[Mn^{2+}]}{dt} = -k_{Mn,hom}[OH]^{2.56}[Mn^{2+}] \quad \text{(Eq.1)}$$

A heterogeneous process is a reaction in which the reactants are components of two or more phases or in which reactants are transformed at an interface, e.g., on the surface of a solid catalyst. The heterogeneous oxidation of Mn occurs in metal oxide minerals with variable valence present in the coating of the filter grains [20,49,55]. The hydroxyl groups on the metal oxide surface of the grains (Me-OH) attract Mn(II) and promote oxidation. If the metal surface is composed of Mn oxides, the oxidation process is termed autocatalytic. The precipitation and aggregation of Mn(II) oxidation products can occur on particular active sites (Me-OH), or by the autocatalytic oxidation of Mn(II) caused by the initially formed Mn oxides. The metal surface of drinking water biofilter grains often includes Mn and Fe oxides, which results in the co-precipitation of Mn and Fe [54].

The catalytic activity of Mn(II) oxidation in metal oxide mineral surfaces depends on several factors, e.g.,: surface area, hydroxyl groups density, and transport properties of the electronic transfers [56]. The reaction rate of heterogeneous oxidation of Mn(II) is considerably higher than the homogeneous oxidation, e.g., $2.4x10^{-4}$ $(mol/L)^{-1}$ s^{-1} (Eq. 5) [20,49,53,55].

Heterogeneous: $$\frac{d[Mn(II)_{ads}]}{dt} = -k_{Mn,het}[Mn(II)_{ads}][O_2] \quad \text{(Eq.2)}$$

Mn transformation can also occur through biological means (Figure 10). Mn oxidation by microorganisms (bacteria and fungi) is generally fast relative to physicochemical Mn(II) oxidation processes [35]. However, the physiological function of microbial oxidation of Mn is still under investigation, e.g., knowledge regarding why and how microorganisms oxidise Mn [35].

The potential benefits for microorganisms to oxide Mn include energy gain due to thermodynamic favourable Mn oxidation; storage of electron acceptor for later anaerobic respiration; scavenging of micronutrients; and protection against ultraviolet radiation, viral attack or predation [57,58].

Microbial oxidation of Mn can be due to indirect or direct processes (Figure 10). Indirect processes include modification of pH and redox, or release of end-products which can lead to Mn oxidation by physicochemical processes, whereas direct oxidation is conducted by specific macromolecules (polysaccharides or proteins) catalysing the transformation process [59].

Microbial oxidation of Mn via direct process is still under investigation. Previous studies on phylogenetically distinct MnOB (*Leptothrix discophora* SS-1, *Pseudomonas putida* MnB1 and GB-1. and *Bacillus sp.* SG-1) showed that (1) Mn oxidation occurs on an exopolymer matrix surrounding the cell, (2) genes involved in the Mn(II) oxidation share sequence similarity to multicopper oxidase enzymes [60,61]. A previous study [35] discussed several pathways for direct microbial oxidation of Mn and suggested that Mn oxidation occurs as a sequence of two enzymatically mediated one-electron transfer reactions (Eq.6). In the first step, Mn(II) is transformed to Mn(III), and in the second step, Mn(III) is transformed to Mn(IV). The latest step is followed by precipitation of MnO_2 on an exopolymer matrix surrounding the cell.

$$Mn(II) \xrightarrow{Enz.} Mn(III) \xrightarrow{Enz.} Mn(IV) \quad \Rightarrow MnO_2 \quad \text{(Eq.3)}$$

Mn(II) oxidation by either physicochemical or biological processes results in the precipitation of Mn oxides. Mn oxides are generally expressed with the chemical formula MnOx, due to the multiple valence states exhibited by Mn. The valence state of MnOx formed by microbes has been found to be between 3 and 3.5 [20]. Birnessite, a type of MnOx with a valence ranging from 3.5 to 3.9, has been detected in the coating of filter material samples collected from drinking water biofilters, and it is reported to be essential for effective Mn removal [6]. Pyrolusite, the most stable form of MnOx and the main component of Mn oxide filter material used in drinking water filters, has a valence of 4, showing a large adsorption capacity but no autocatalytic oxidative properties. Hence, when adsorption capacity is exhausted, no further Mn oxidation can take place [6]. The intermediate valance is of importance in drinking water biofilters to maintain the autocatalytic behaviour of the MnOx.

2.5 KNOWLEDGE GAP

2.5.1 CHARACTERISATION OF MANGANESE REMOVAL PROCESSES

Manganese removal in drinking water biofilters occurs through physicochemical processes (heterogeneous) and biological processes (direct or indirect). However, the importance of each process for efficient Mn removal is still under investigation [4,30].

Sodium azide (NaN_3) is an inhibitor of respiratory activity in microorganisms. The use of NaN_3 is particularly interesting when investigating the Mn cycle because it does not appear to affect the autocatalytic properties of MnOx [62]. For that reason, NaN_3 has been used for a variety of Mn-related studies, ranging from characterisation of specific MnOB to overall Mn geomicrobiology, passing by studies on marine sediments and drinking water biofilters [4,57,59,63–65].

Particularly interesting to the present study, a previous investigation [4] used NaN_3 to determine the role of physicochemical and biological processes on the removal of Mn by filter material collected from two full-scale biofilters located at the same DWTP with different ages of maturation (three years and fifteen years). Mn removal attained by filter material samples without NaN_3 was assumed to be due to biological and physicochemical processes, whereas Mn removal attained by filter material samples with NaN_3 was assumed to be mainly due to physicochemical processes. Results showed that the effect of NaN_3 on Mn removal was higher in filter material samples with three years of maturation when compared to filter material samples with fifteen years of maturation. The study [4] concluded that physicochemical removal processes become more dominant over time.

The effect of inhibitors on the microbial activity should be interpreted with caution. Rather than a total inhibition of the microbial activity, the effect of inhibitors should be understood as the result of a resistance to the microbial activity. Besides, by inhibiting microbial activity, the amount of Mn available in the liquid phase increases, potentially promoting further removal of Mn by physicochemical processes. Also, the effect of NaN_3 on the biological transformation of Mn is mostly limited to direct pathways (Figure 10). For example, the biosorption capacity of previously formed MnOx on the external layer of the microorganism (indirect biological pathway) is not expected to be affected in the presence of NaN_3. Regardless of the limitations of using a microbial inhibitor, the study [4] gave a reference value to the role of biological Mn removal processes occurring in filters with different ages of maturation.

Heat treatment has also been used as a method to limit microbial activity and determine the removal of Mn attained by physicochemical processes on MnOx surfaces. A previous study [4] used heat treatment to determine the physicochemical removal of Mn by fully matured filter material. The study concluded that the MnOx structure of the filter material was damaged by autoclaving the filter material (121ºC, 30 min) [4]. Another study [66] investigating the effect of temperature treatment on the catalytic activity of MnOx concluded that dehydration at high temperature (120ºC) led to a poorer catalytic performance of the MnOx. Further, an effect on the adsorption of Mn was also observed when using a lower heat treatment (90ºC), due to the conversion of Birnessite (a common MnOx coating matured filter material in drinking water biofilters [6,30]) to Mn_3O_4.

A more recent work [30] characterised the MnOx surface of filter material samples collected from the top layer of a virgin quartz column during the initial two years of maturation. The results showed that Birnessite formed on the quartz surface during the early stages of maturation was mostly of biotic origin but as the maturation of the filter progressed Birnessite was of abiotic origin. The study [30] concluded that biological processes are of more importance during the early stages of maturation.

In addition to the dynamic of Mn removal processes in drinking water biofilters over time (start-up period, early maturation stage and fully maturation stage), Mn removal processes might also vary along the depth of the filter. Some studies have reported a redox increase in filters removing Fe, Mn, and NH_4 along the filter depth [13,67]. Other studies focusing on Mn removal by drinking water biofilters suggest that there is a "Field of biological Mn removal" limited by pH and redox potential [19,68] (Figure 11). Thus, conditions for microbial removal of Mn might be more suitable in the deeper layers of the filters.

Characterisation of Mn removal processes occurring in drinking water biofilters at different maturation stages (from virgin to matured filters) and different depths of the filter (from the top to the bottom of the filter) were addressed in work presented in **PAPER II and III** of this thesis.

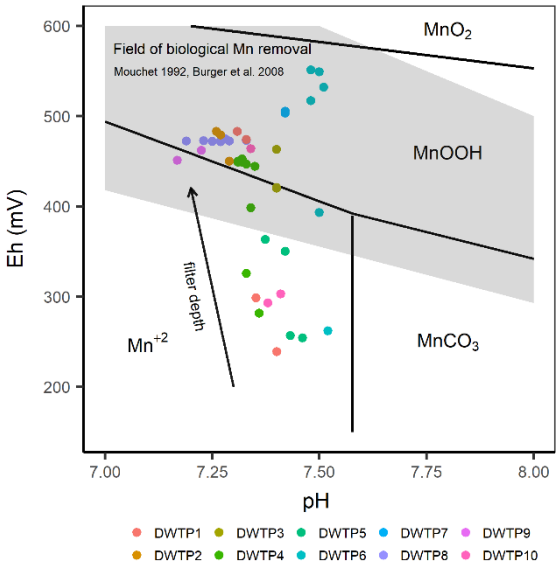


Figure 11 Pourbaix diagram with data points indicating pH and redox in water at each 10 cm depth of the filters. Color distinguishes DWTPs. Grey shading shows the field of biological Mn removal. Pourbaix diagram based on equilibrium constants given by PHREEQC using *wateq4f* database and the following conditions 10 °C, 0.260 mg Mn(II)/L, 273 mg HCO_3/L.

2.5.2 SOLUTIONS TO SHORTEN THE START-UP PERIOD

Mn is of concern during the start-up period as it is the last treatment substance to meet the drinking water criterion (Phase 5, Figure 6). Previous studies indicate that a start-up time of 2-4 months is required in the presence of Fe, Mn and NH_4 [1,12,21,69]. A previous work [6] goes further stating that the duration of the start-up period can vary from weeks to more than a year when Mn is present.

The water industry has been using several methods to bypass the long start-up period of biofilters, the most common being selection of alternative/modified filter materials and use of proactive inoculation methods.

Even though biofilters are often composed of quartz, over the last 20 years several studies have investigated alternative or modified filter materials, e.g., zeolite or Mn coated quartz [7,8,75–79,9,14,55,70–74]. Alternative or modified filter materials are often considered due to their high sorption capacity, which shortens the length of the start-up period. For example, the start-up period of a biofilter for Fe and Mn removal was reduced to half when using chalcedonite instead of quartz [70]. Another reason to select an alternative filter material is that it might represent a cheaper investment or result in lower operational costs. For example, previous studies have investigated the use of polystyrene beads, because their low density could promote savings during backwash operations [9,80–82].

A previous study [83] investigated the effect of matured filter material on the activity, colonisation, diversity, and abundance of nitrifying prokaryotes. The study concluded that mineral coating has a substantial effect on microorganisms in matured drinking water biofilters. However, information on the effect that different virgin filter materials might have on the microbial community developed during the start-up period is limited [14,15].

During the start-up period, the filter material is inoculated with microorganisms by being exposed to the raw water and the water used for backwashing ("inherent inoculation") [1]. Microbes have a strong propensity to attach on a surface [3]. The formation of biofilm on the filter material grains requires a critical initial cell attachment, which is influenced by a multitude of factors, e.g., surface charge, hydrophobicity and surface roughness [84].

A previous study [14] investigated the impact of filter materials (activated carbon, quartz, anthracite and ceramsite) on the concentration of dissolved organic nitrogen and biomass distribution over depth of a column setup. The results showed that biomass in the activated carbon filter was lesser than that in the quartz filter [14]. Furter the study [14] concluded that the concentration of dissolved organic nitrogen could potentially be controlled via the selection of filter material. A more recent study [15] using 16S rRNA gene amplicon sequencing and flow-cytometry showed that quartz and granular activated carbon columns treating the same inlet water were determinant in the selection of the microbial communities formed in drinking water biofilters. Further investigation is of interest to characterise the microbial diversity developed during the start-up period on filter materials preselected to accelerate the onset of Mn removal (e.g., Mn oxide or Mn coated quartz).

The effect of alternative filter materials on the microbial diversity of early matured filters shortly after achieving efficient Mn removal was addressed in work presented in **PAPER I** of this thesis. Further, some practical remarks are made in **PAPER III** regarding the initial selection of alternative filter materials and its removal capacity after the filter has achieved a maturation stage.

Other solutions used by water utilities to shorten the start-up period are based on inoculation of the biofilter by addition of a concentrated source of microorganisms ("proactive inoculation") [1]. Proactive inoculation methods to accelerate the start-up of biofilters include the addition of a concentrated source of microorganisms and bioactive autocatalytic surfaces, e.g., backwash sludge [10,12,13], matured filter material [11,85], mixed bacterial culture [67] or specific bacterial species [86–89].

Information on the effect that proactive inoculation solutions might bring to the microbial community in drinking water biofilters is limited [86]. A study on drinking water biofilters [86] using proactive inoculation by addition of a MnOB (*Pseudomonas* sp. QJX-1) to virgin quartz filters showed that bioaugmented filters had higher overall treatment efficiency and anti-shock load capacity than that of non-bioaugmented filters. Further, 16S rRNA gene amplicon sequencing on filter material samples collected from the bioaugmented and non-bioaugmented filters showed that the inoculation did not affect the microbial diversity developed on the filter material. Addition of specific bacteria to drinking water biofilters might soon become the go-to solution for water utilities to avoid the need for a start-up period of virgin filters. A previous work [10] indicates that proactive inoculation by addition of matured filter material is a common procedure to initiate operations of virgin filters.

Proactive inoculation by addition of matured filter material can provide immediate removal, while potentially promoting the microbial growth on the virgin filter material located in the remaining filter. The matured filter material is available to water utilities, but the transport of matured filter material under controlled conditions can be challenging. Thus, the amount of matured filter material to be moved from an old to a new filter should be kept to a minimum.

A previous study [11] investigated the effect of the addition of matured quartz on the start-up period of a virgin quartz pilot filter for Fe and Mn removal. The results showed that addition of matured quartz (approx. 10% of the volume of the filter) promoted immediate Mn removal and shortened the start-up period from months to 30 days.

A following work [85] compared proactive inoculation by addition of matured quartz and matured anthracite. Results showed that inoculation was successful when supplementing a virgin filter with matured anthracite (approx. 20% of the volume of the filter), avoiding the need for a start-up period completely [85]. In opposite, the addition of matured quartz showed no effect on the length of the start-up period [85]. The justification for the limited effect of inoculation by addition of matured quartz on Mn removal was attributed to changes in the properties of the aged and dried matured quartz [85]. The study suggested that the mature quartz grains lost their adsorption capacity, autocatalytic activity and microbial activity [85].

The effect of proactive inoculation by addition of matured quartz on the onset of Mn removal and the microbial community developed during the start-up period was addressed in work presented in **PAPER II** of this thesis.

2.5.3 MANGANESE REMOVAL EFFICIENCY IN MATURED FILTERS

Mn removal over the filter depth of drinking water biofilters can be described by kinetic models, characterised by an order and a constant [4,12,13,20,49,55,82,89,90]. The Mn concentration profile of drinking water biofilters is often characterised by a first-order model with a constant (k) between 10^{-6} and 10^{-1} min^{-1} [12,13,20,49] (Table 3). The broad range of k-values registered in previous studies might be a consequence of the many factors influencing Mn removal in drinking water biofilters [17,91].

A previous study [17] gathered information on 34 parameters of 100 DWTPs to determine the main operating conditions to attain efficient Mn removal. The study [17] concluded that efficient Mn removal was achieved when: NH_4 removal efficiency > 85%, pH of filtrate > 7.1, Fe loading per filter run < 2.7 kg/m^2; O_2 concentration in filtrate > 1 mg/L; filtration rate < 10.5 m/H and empty bed contact time > 11.5 min.

Another study [91] investigating the Mn removal capacity of filter material samples collected from 14 DWTPs observed a considerable variation of MnOx coating the filter grains (0.01 to 120 mg/g of filter material). Further, the study [91] concluded that the Mn removal capacity of filter material was highly variable when MnOx exceeded 5-10 mg/g of filter material.

Table 3 First order removal constant (k) characterizing Mn removal over depth of drinking water biofilters.

k (min^{-1})	Filter material	Scale	Observations	Reference
0.149	Quartz	Column	50 days, proactive inoculation, addition of nitrifying and backwash sludge (simultaneous at Day 3).	[12]
0.153	Quartz	Column	50 days, proactive inoculation, addition of nitrifying sludge (Day 3) and backwash sludge (Day 13).	[12]
0.174	Polystyrene	Column	Proactive inoculation preceding 8 months of operations.	[20]
10^{-6}-10^{-5}	Anthracite and quartz	Pilot-scale	97-127 days, after the end of the start-up period.	[13]
0.21	Anthracite and quartz	Pilot-scale	159 days, proactive inoculation, addition of backwash sludge (Day 132).	[13]
0.309	Quartz	Pilot-scale	48 days.	**PAPER II**
0.702	Quartz	Pilot-scale	48 days proactive inoculation by addition of matured quartz (Day 0).	**PAPER II**
0.0144	Quartz	Full-scale	23 months.	[49]
0.0001	Quartz	Full-scale	3 months.	[49]
0.120	Quartz	Full-scale	1 month.	[49]
0.0001- 0.120	Anthracite and quartz	Full-scale	3 months.	[49]
0.173	Anthracite and quartz	Full-scale	2 years.	**PAPER III**
0.061	Anthracite and quartz	Full-scale	18 years.	**PAPER III**
0.132	Quartz	Full-scale	7 years.	**PAPER III**
0.028	Anthracite and quartz	Full-scale	33 years.	**PAPER III**
0.079	Anthracite and quartz	Full-scale	18 years.	**PAPER III**
0.052	Anthracite and quartz	Full-scale	30 years.	**PAPER III**
0.021	Anthracite and quartz	Full-scale	10 years.	**PAPER III**
0.025	Anthracite and quartz	Full-scale	6 years.	**PAPER III**
0.032	Calcium carbonate	Full-scale	4 years.	**PAPER III**
0.029	Anthracite and quartz sand	Full-scale	17 years.	**PAPER III**

In addition, Mn removal capacity of matured filters has been reported to deteriorate over time [18], which can lead to an increase in the concentration of Mn in the effluent of matured filters [5,17], Figure 12. Possible reasons for the deterioration of the Mn removal capacity include saturation of adsorption sites in the grain's coating, quality variations on the source water and changes in the operation conditions (e.g., increase of filtration rate). When the deterioration of the Mn removal process compromises the quality of the DWTP effluent, the water utility responsible for the DWTP needs to replace the filter material with either a virgin autocatalytic material (e.g., granular Mn oxide) or a virgin non-autocatalytic material (e.g., quartz). Both solutions result in a cost for filter material disposal/replacement, a risk of microbial contamination and the need for a new start-up period [18].

Biostimulation strategies to enhance the efficiency of drinking water biofilters have been addressed in previous studies [16,92,93] with a particular focus on the enhancement of nitrification. A previous study [92] investigating the effect of P limitation on nitrification in drinking water biofilters observed stimulation of nitrification by addition of phosphoric acid. A recent study [93] concluded that Cu dosing enhanced nitrification of poorly performing full-scale rapid sand biofilters. Hence, the addition of key elements to nutrient-limited matured filters could potentially enhance Mn oxidation facilitated by direct biological processes (Figure 10).

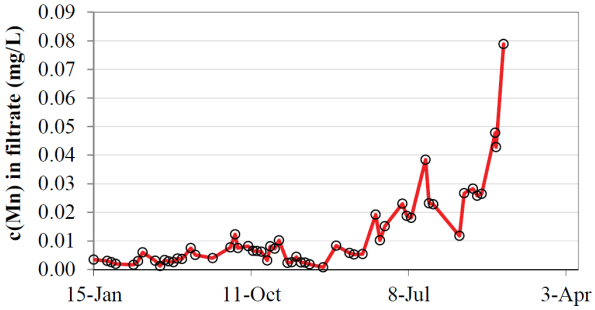


Figure 12 An example of Mn concentration increase in the effluent of a matured full-scale filter from a Dutch DWTP [5].

Additional investigation of Mn removal in drinking water biofilters might provide a further understanding of operating conditions affecting the removal efficiency of Mn. The requirements for efficient removal of Mn in full-scale drinking water were addressed in work presented in **PAPER III**. Further, **PAPER III** includes a preliminary study of the potential use of trace metals and P to enhance Mn removal capacity of fully matured filters.

2.5.4 DISTRIBUTION OF GENERA WITH KNOWN MNOB

As Mn removal is attained by physicochemical and biological processes, characterisation of the microbial community might provide further insight into Mn removal in drinking water biofilters. Advances of analytical and bioinformatic methods have enabled the characterisation and quantification of the prokaryotic communities present in drinking water biofilters. However, identification of MnOB with culture-independent methods is challenging since universal molecular markers are not known [22]. Further, the factors controlling the distribution, activity and biochemical function of MnOB remain unknown [94].

A cultivation study [22] using samples from several points of a drinking water system (source water, DWTP, distribution system) concluded that drinking water treatment strongly selects taxa capable of Mn oxidation. Besides, several genera previously linked to biological oxidation of Mn have been identified in matured filter material samples and along the drinking water distribution system, e.g., *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Metallogenium, Siderocapsa, Siderocystis, Pedomicrobium, Hydrogenophaga*, *Pseudomonas* and *Acinetobacter* [20–22,57,95–100].

A recent study [30] suggested that biological oxidation of Mn is of more importance during the early stages of maturation. Thus, identification of MnOB is of special importance during the start-up of virgin filters. However, studies describing the microbial diversity during the start-up period are in general limited [1,10,12], and particularly rare when focusing on filters running under inherent inoculation conditions [1].

The microbial diversity of filters running under inherent and proactive inoculation conditions during and shortly after achieving efficient Mn removal was included in the work presented in **PAPER I** and **PAPER II** of this thesis.

Even though the collection of filter material samples over filter depth of full-scale filters is challenging, characterisation of the microbial diversity in fully matured filters over depth has gained interest over the past years [2,23,101–103].

A previous study [101] investigated the connection between the microbial diversity and the removal of Fe, Mn and NH_4 over depth of a full-scale filter. The study [101] concluded that the spatial distribution of the microbial community along the depth of the filter corresponded to the stratification of the removal of the primary treatment parameters.

A more recent study [23] characterised the core taxa of the top 60 cm of 3 DWTPs in Denmark using 16S rRNA gene amplicon sequencing. The results showed that sequences within the core taxa were closely related to types with the ability to oxidize NH_4, NO_2, Fe, Mn and CH_4 [23]. Further, the study [23] suggested that environmental conditions in drinking water biofilters are sufficiently similar for the existence of core genera shared across DWTPs. The characterisation of Mn removal (e.g., removal rate, the role of physicochemical and biological processes) and microbial diversity over depth of fully matured full-scale filters at different DWTPs might indicate the most relevant genera for Mn removal in fully matured drinking water biofilters.

Characterisation of Mn removal and spatial distribution of the microbial diversity at 10 DWTPs was included in the work presented in **PAPER III** and **PAPER IV** of this thesis, respectively. Further, **PAPER IV** includes further information on the removal of other treatment parameters (e.g., Fe and NH_4).

3. EXPERIMENTAL APPROACH

3.1.1 OVERVIEW

The experimental work can be organized in 4 dimensions: maturation stage of the filter (start-up period, fully matured), scale of the experimental setup (batch, column, pilot and full-scale), filter material selection (anthracite, calcium carbonate, manganese oxide, polystyrene, quartz), and research focus (manganese removal, microbial community), Figure 13. Each dimension was distributed across the different scientific contributions (**PAPER I-IV**) with the ambition of creating knowledge bridges across the different studies. For example, the maturation stage of the filter was shared between **PAPER I-II** (filters running during the start-up period) and **PAPER III-IV** (filters running in fully matured conditions).

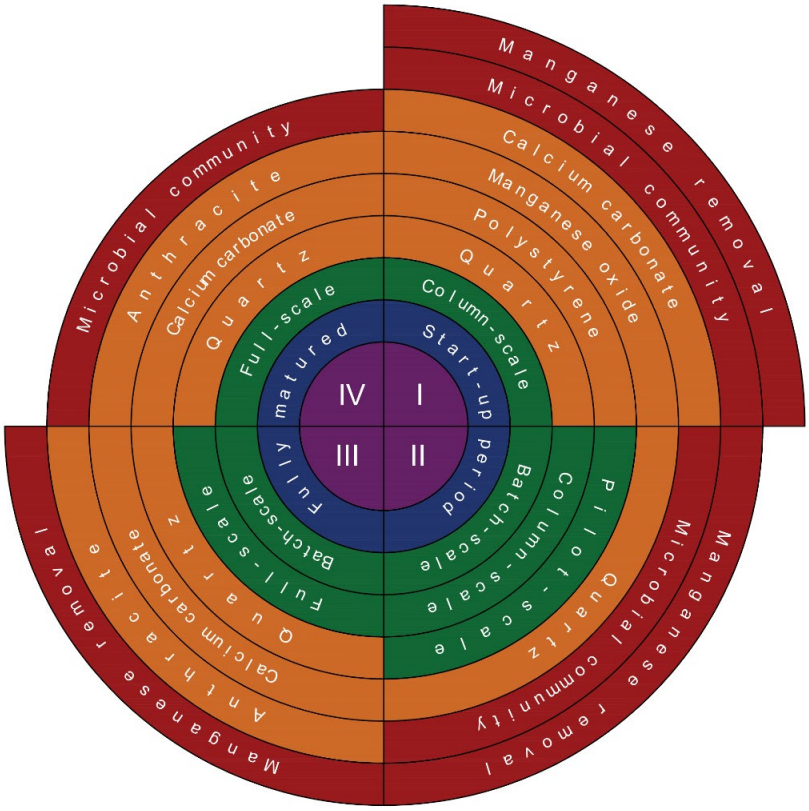


Figure 13 Overview of the experimental work: maturation stage of the filter (blue), experimental setup scale (green), filter material selection (orange), and research focus (red), with regards to **PAPER I-IV** (purple).

3.1.2 BRINGING RESEARCH CLOSER TO PRACTICE

Skanderborg Forsyning A/S has a total of 5 DWTPs, but most of the city is supplied by Stilling and Fredensborg DWTPs. One of the major challenges with regards to drinking water production in Skanderborg city relates to the operations management of Fredensborg DWTP. Skanderborg Forsyning A/S is currently considering renovating Fredensborg DWTP or building a new DWTP capable of dealing with the production demands of the city. In both scenarios, the need for a start-up period is very likely. Thus, the experiments evaluating solutions to shorten the start-up period (Figure 1) were conducted at Fredensborg DWTP.

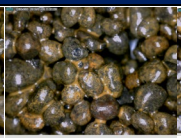
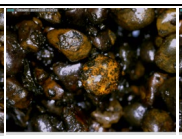
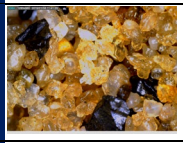
The present study included batch, column and pilot assays, as well as sampling from 10 full-scale DWTPs, Figure 14. Batch and column assays were based on the setup design of previous studies [4,104], whereas pilot-scale assays and access to 10 full-scale DWTPs were a result of cooperation between different external partners.

The pilot-scale filters were designed in collaboration with Silhorko Eurowater A/S and constructed specially for this study (Figure 14C). The design of the pilot-scale filters considered: portability, management of operations (e.g., manual backwash), and collection of both water and filter material samples at 10 cm depth intervals. Another feature of this unique design pilot-scale setup is a 15 cm tube connected to each water sampling tap and oriented along the filter diameter, allowing the collection of water samples from the internal section of the filter.

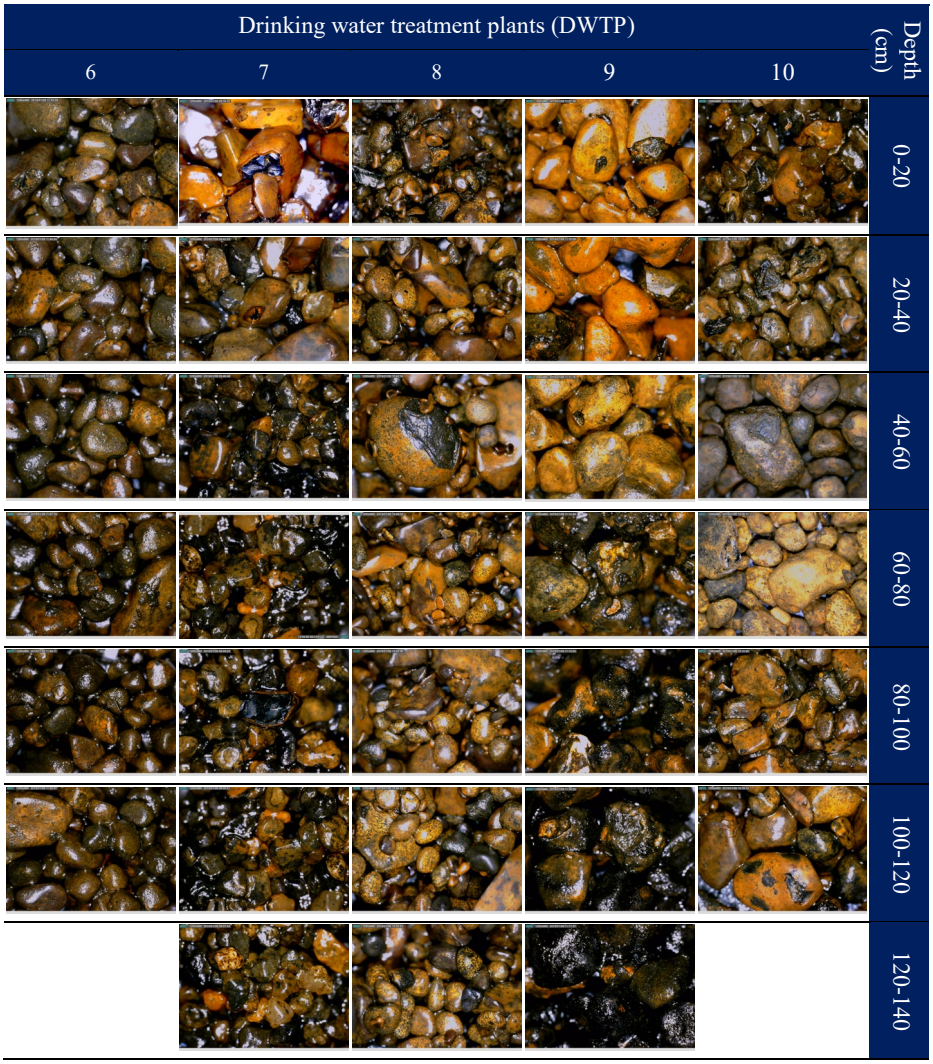
The access to 10 full-scale DWTPs was based on a collaboration with the MUDP project “Smart Redesign of Drinking Water Production”. The project gathered several partners including two Danish water utilities (Aarhus Vand and Vand Center Syd). The water utilities responsible for the DWTPs assisted in all sampling events (Figure 15D, Table 4).and shared information on the operational conditions (e.g., backwash procedures, flow, the age of filters maturation) leading to a more informed interpretation of the data and opening the possibility for practical conclusions.

Figure 14 Photographs of **A.** batch-scale assay **B.** column-scale assay **C.** pilot-scale assay designed in collaboration with Silhorko-Eurowater A/S **D.** sampling from a full-scale filter at a DWTP.

Table 4 Filter material sampled from 10 full-scale DWTPs

Depth (cm)	Drinking water treatment plants (DWTP)				
	1	2	3	4	5
0-20					
20-40					
40-60					
60-80					
80-100					
100-120					
120-140					

collected at each 20 cm depth intervals.



4. IMPACT OF THE RESEARCH

Mn removal processes are dynamic over time. Filter material samples collected from a pilot-scale drinking water biofilter showed that Mn removal was dominated by biological processes during the start-up period and the early stages of maturation (**PAPER II**). In contrast, filter material samples collected from 10 fully matured DWTPs showed that physicochemical processes represented on average approx. 75% of the Mn removal (**PAPER III**). The Mn removal profile over depth of a full-scale filter showed that Mn and NH_4 are removed at different depths when comparing removal profiles collected during early maturation and fully maturation periods. In an early maturation stage (approx. 70 days) Mn was removed at a greater depth than NH_4. However, after four years of operations, the matured filter removed Mn at a lesser depth than NH_4. This can be explained by a continuous spread of Mn oxides throughout the filter during backwash operations, promoting physicochemical removal of Mn at a lesser depth of the filter, before NH_4 removal commences (**PAPER III**). Future studies should be conducted to evaluate the effect of operating conditions (e.g., backwash process, filtration rate) on the Mn removal efficiency of drinking water biofilters running at different maturation stages. Further, results suggest that the design of drinking water biofilters should be interchangeable over time to follow the maturation stage of the filter.

Solutions to shorten the start-up period can affect the diversity of the microbial community. Strategies to shorten the start-up period are divided into two main groups: alternative filter material and proactive inoculation. Both strategies showed to shorten the start-up period: an alternative filter material (Mn oxide) avoided the need for a start-up (**PAPER I**) and proactive inoculation by addition of matured quartz (approx. 20% of the filter volume) reduced the start-up period by ten days (**PAPER II**). However, the strategies showed to have a different effect on the microbial community of the filters. When using alternative filter material (quartz, calcium carbonate, polystyrene, Mn oxide), the microbial community diversity formed on the materials coating was strongly influenced by the filter material type (**PAPER I**). In opposite, proactive inoculation by addition of matured quartz to a virgin quartz filter showed a limited effect on the diversity of the microbial community developed in initially virgin quartz filter (**PAPER II**). On the one hand, management of bacterial communities during the start-up period may be possible by selecting specific filter materials to enhance growth and activity of specific microbes (**PAPER I**). On the other hand, the use of proactive inoculation might bring more controlled conditions to the management of microbial communities during the start-up period (**PAPER II**).

Initial filter material selection becomes of less importance over time. Immediate manganese removal was achieved by virgin Mn oxide, whereas virgin quartz took 48 days (**PAPER I**). In contrast, Mn removal capacity of filter material samples collected at 20 cm depth intervals of 10 fully matured drinking water biofilters was independent of filter material type (quartz sand, anthracite, calcium carbonate), **PAPER III**. Results suggest that filter material selection might be important during the start-up period but become less relevant after the maturation of the filters with regards to removal efficiency of Mn (**PAPER I, PAPER III**). This information is of interest to the industry for assessing the filter design and determining the return of investments when selecting alternative filter material.

***Hydrogenophaga*, *Pseudomonas*, *Hyphomicrobium*, and *Pedomicrobium* might be of importance for the onset of Mn removal.** Filter material collected during and shortly after the start-up period of Mn feed filters showed that *Hydrogenophaga*, *Pseudomonas*, *Hyphomicrobium* and *Pedomicrobium* were the most abundant genera known to include MnOB (**PAPER I**, **PAPER II**). Biological processes dominate the Mn removal during and shortly after the start-up period of initially virgin filters. Future studies should investigate the role of those genera for the onset of Mn removal. Further, future studies focusing on methods to shorten start-up period of new filters for Mn removal should include the use of proactive inoculation by addition of species from those genera.

Microbial diversity in fully matured filters show a difference over depth but mostly among different DWTPs. Spatial distribution of the microbial communities on filter material samples collected along the filter depth of 10 DWTPs corresponded to the stratification of the removal of Fe and NH_4 (**PAPER IV**). In opposite, genera known to include MnOB showed limited correlation with the Mn removal profiles registered along the depth of the fully matured filters (**PAPER IV**). Characterisation of the Mn removal processes by addition of NaN_3 also suggested that the presence of MnOB varied along the filter depth of the ten fully matured filters with no stratification pattern (**PAPER III**). Further, the addition of P and trace metals showed a limited overall effect on the Mn removal capacity of the matured filter material samples (**PAPER III**). The groundwater supplied to each DWTP showed a small variation of the primary treatment elements concentration (Fe, Mn, NH_4). Preliminary results indicate that trace elements present in the groundwater might have a substantial impact on the microbial diversity found in matured drinking water biofilters.

This study adds practical knowledge to the water sector, not only to optimise the existing traditional treatment systems but also to generate discussions on how drinking water will be produced in the future. Particularly to Skanderborg Forsyning A/S the results from the present study contributed to a revaluation of operating conditions of their DWTPs, further understanding on filter material selection and potential solutions to shorten the start-up period. Besides, Skanderborg Forsyning A/S has hired a microbiologist to join the operations technical team and significantly increased the number of research projects in collaboration with universities, industry partners and other water utilities.

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PAPER I

MANGANESE OXIDATION AND BACTERIAL DIVERSITY ON DIFFERENT FILTER MEDIA COATINGS DURING THE START-UP OF DRINKING WATER BIOFILTERS.

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INÊS L. BREDA, LOREN RAMSAY & PETER ROSLEV

Manganese oxidation and bacterial diversity on different filter media coatings during the start-up of drinking water biofilters

I. L. Breda, L. Ramsay and P. Roslev

ABSTRACT

Manganese removal is a typical concern in drinking water production. Biofiltration may be used when treating groundwater sources but the onset of manganese removal in virgin biofilters can vary considerably. The aim of this study was to investigate the effect of different filter media on manganese oxidation and bacterial diversity in biofilters during the start-up. The onset of manganese oxidation in four virgin granular filter media (quartz, calcium carbonate, polystyrene, and manganese oxide) and one matured medium (quartz) was followed during the start-up. Immediate manganese removal was achieved by manganese oxide, while 48, 57 and 72 days were required by virgin quartz, calcium carbonate and polystyrene, respectively. The bacterial community was investigated using DAPI staining, quantitative polymerase chain reaction (qPCR), 16S rRNA gene pyrosequencing, and bacterial enrichments. Bacterial abundance was greatest on polystyrene and matured quartz. Molecular community analysis and bacterial enrichments suggested the presence of manganese oxidizing bacteria on all media coatings after the start-up period. Virgin quartz and calcium carbonate showed similar bacterial communities whereas manganese oxide and polystyrene were distinct. This investigation suggests that when inoculating different filter media with an identical water source, the bacterial diversity and onset of manganese oxidation during start-up is strongly influenced by the filter media type.

Key words | bacterial diversity, bench-scale, drinking water, filter media, manganese oxidizing bacteria, start-up

I. L. Breda (corresponding author)
P. Roslev
Department of Chemistry and Bioscience,
Aalborg University,
Fredrik Bajers Vej 7H, 9220 Aalborg,
Denmark
E-mail: *ilb@bio.aau.dk*

I. L. Breda
Group of Project and Development,
Skanderborg Forsyningsvirksomhed A/S,
Døjsøvej 1, 8660 Skanderborg,
Denmark

L. Ramsay
Research Group for Energy and Environment,
VIA University College,
Chr. M. Østergaards Vej 4, 8700 Horsens,
Denmark

INTRODUCTION

Manganese is a typical concern in drinking water production (Tekerlekopoulou *et al.* 2013) and should be limited for health and aesthetic reasons (WHO 2011). In northern Europe, drinking water treatment is commonly based on aeration and biofiltration (Mouchet 1992). Biofilters are defined as granular filters coated with both inorganics and biofilm capable of treating water to national drinking water criteria (Tekerlekopoulou *et al.* 2013).

The period during which virgin filter media matures into a fully functional biofilter is designated as the start-up period. Start-up of drinking water biofilters hinges on a set of interconnected physical, chemical and biological processes (Mouchet 1992). When manganese is present, the duration of a start-up varies from weeks to more than a year (Tekerlekopoulou *et al.* 2013). During this time, produced water cannot be distributed to the consumers. Costs of labor, water and energy during the start-up are a major concern for water utilities. Further, frequent manganese breakthrough after some years of operation may require filter media replacement and consequently a new start-up period (Buamah *et al.* 2009). Knowledge regarding solutions to shorten the start-up period is of interest for water utilities.

Abiotic manganese oxidation is described by homogeneous and heterogeneous processes (Katsoyiannis &

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Zouboulis 2004). Homogeneous oxidation of manganese by oxygen is slow at pH below 9 (Stumm & Morgan 1996). Thus, homogeneous oxidation of manganese in drinking water treatment using a groundwater source, where typically pH ranges between 6 and 8, is mostly mediated biologically (Tekerlekopoulou *et al.* 2013). Heterogeneous oxidation of manganese is based on the autocatalytic activity of solid manganese oxides present in the coating of the filter medium grains (Katsoyiannis & Zouboulis 2004). Sahabi *et al.* (2009) conclude that manganese removal in matured biofilters is mainly based on autocatalytic action, whereas investigations by Bruins (2016) suggest that manganese removal in a non-coated virgin medium is initiated biologically, evolving to a predominantly physicochemical removal process over time. Thus, biological oxidation of manganese may be of particular importance during the start-up period.

Recent advances of analytical and bioinformatic methods have enabled characterization and quantification of the prokaryotic communities present in biofilters. Previous studies have found several genera related to manganese oxidation on matured biofilter media. These include *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Metallogenium*, *Siderocapsa*, *Siderocystis*, *Pedomicrobium*, *Hydrogenophaga*, and some species of *Pseudomonas* and *Acinetobacter* (Mouchet 1992; Ehrlich 1996; Larsen *et al.* 1999; Katsoyiannis & Zouboulis 2004; Pacini *et al.* 2005; Tebo *et al.* 2005; Das *et al.* 2011; Abu Hasan *et al.* 2012; Beukes & Schmidt 2012; Marcus *et al.* 2017; Su *et al.* 2016). This suggests that multiple genera may participate in the biological oxidation of manganese. However, the bacterial diversity in drinking water biofilters during the initial stages of manganese removal is generally underexplored.

During the start-up period, filter media provide a surface for the attachment of microorganisms. The use of alternative filter media in drinking water filters has been investigated in several studies (Qiu *et al.* 2010; Grace *et al.* 2015; Schöntag *et al.* 2015). However, the effect that these alternative filter media may have on the bacterial community structure has received little attention. More knowledge could potentially lead to the use of alternative filter media as biostimulators to shorten the start-up period, as well as possible management of the functional roles of the bacterial community present in biofilters. Hence, the aim of this study is to investigate the effect of different filter media on the start-up period of manganese oxidation and on the diversity of bacterial communities present in the biofilters at the end of the start-up period.

MATERIALS AND METHODS

Setup configuration

Fredensborg waterworks (Skanderborg, Denmark) treats anaerobic groundwater by aeration and two stage biofiltration. The matured quartz filter medium located in the second stage biofilter had been in operation for 47 years without being replaced. The experiment was conducted in a bench-scale setup using virgin and matured filter media. An illustration of the experimental setup is available in the supplementary materials (S1), available with the online version of this paper.

Source water for the experiment was prepared by mixing non-chlorinated treated water from the storage tank of the waterworks with a concentrated solution of $MnCl_2$. Treated water from the waterworks has the advantage of ensuring concentrations of all treatment parameters below national drinking water criteria while maintaining stable physical and biological conditions. Further, direct interference from waterworks processes are also avoided (e.g. backwash and changes in production rates). The concentrated manganese solution was prepared using distilled water and $MnCl_2{\cdot}4H_2O$ (Emsure ACS), which was then pumped to a mixing vase using a diaphragm pump (Digital DDC, Grundfos) and mixed with the treated water using a magnetic mixer (MR 1000, Heidolph), resulting in a manganese concentration of 0.281 ± 0.018 mg L^{-1} before distribution to the filter columns (Table 1).

The source water was distributed by gravity to 15 polyethylene filter columns, each with a diameter of 5 cm and a 10 cm layer of granular medium. The columns were operated in downflow mode with a filtration rate of 3.5 m/h and an empty bed contact time of 1.8 min. No backwash was used during the experiment.

Five granular filter media were selected for the experiment and placed in triplicate filter columns. The media were: virgin quartz, calcium carbonate, polystyrene, manganese oxide, and matured quartz collected from the top layer

Table 1 | Quality of the source water

Parameter	Unit	Average	Std. dev.
Manganese	mg/L	0.281	0.018
Iron	mg/L	0.027	0.01
Ammonium	mg/L	<0.02	–
Nitrite	mg/L	<0.001	–
pH[a]	–	7.7	0.1
Oxygen[a]	mg/L	11.1	0.1
Redox[a,b]	mV	298	58
Temperature[a]	°C	10.7	0.2
Turbidity	NTU	<0.1	–
Hydrogen carbonate	mg/L	278	15
NVOC	mg/L	1.0	0.1

[a]Field measurements.
[b]Redox measurements increased over time.

of the second stage filter of the waterworks (Table 2). The latter served as a positive control since this filter medium successfully removes manganese in the full-scale waterworks.

Before the experiment commenced, all filter media were submitted to pretreatment to remove fines and disinfect the media. A sample of approximately 1.5 kg of each filter medium was dry-sieved (630 μm), then washed ten times in a 2-L blue cap bottle with distilled water to remove fines. Each medium was then subdivided using a rotary sample divider (DR100 and PT100, Retsch GmbH, Germany), washed with 65 °C distilled water and incubated for a period of 12 h at 67 °C. A disinfection procedure of heat treatment at 75 °C for 12 h was also applied to all tubing, filter columns and sampling valves.

Sampling and chemical analysis

Filter column inlet and outlet samples were collected two to three times a week during the experiment. Outlet samples were collected from each filter column after a contact time of 20 minutes by pausing the flow. Both inlet and outlet samples were collected manually, filtered immediately (0.45 μm) and analyzed within two hours for manganese according to manufacturer's instructions (kit LCK304 and DR3900 spectrophotometer, Hach, Denmark).

Sampling for microbiological analysis

Samples of source water (4 L) were collected in the middle and end of the experiment and filtered using sterile 0.20 μm membrane filters (Advantec). The filters were stored at −21 °C for subsequent molecular analysis. Filter media from the 15 columns were collected at the termination of the experiment to characterize the bacterial community. Media samples for DAPI staining were fixed by submerging in a 3% formaldehyde solution and stored at 4 °C in the dark. Media samples for molecular analysis were stored at −21 °C until extraction.

DAPI staining and cell counting

Formaldehyde-fixed filter media samples (5 g) were sonicated, vortexed, and aliquots of the supernatant were transferred to sterile test tubes. Homogenization was obtained by pulling/pushing several times through a syringe needle. The supernatant was then transferred to a sterile test

Table 2 | Characterization of filter media

Parameter	Matured quartz	Virgin quartz	Calcium carbonate	Polystyrene beads	Manganese oxide
Main composition day 0	–	100% SiO_2	96.8% $CaCO_3$	100%	80% MnO_2
Grain size (mm, 10–90%)	1.03–1.86	1.10–1.76	1.64–5.08	0.97–1.22	1.75–3.44
Sphericity (4π·area/perimeter^2)	0.90	0.87	0.85	0.97	0.82
Particle density (kg/L)	2.50	2.60	2.56	1.00	3.56
Porosity (%)	42.60	40.63	46.93	39.84	51.66
Supplier	Fredensborg waterworks	Dansk Kvarts Ind. Denmark	Faxe Kalk, Denmark	Bewi Styrochem, Finland	Unitex, Poland
Cost (euros/L)	–	0.34	0.55	0.06	5.7

tube and diluted with filtered sterilized tap water. Cells were filtered onto 0.2 μm black polycarbonate filters, and stained for 15 min with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) at a concentration of 20 mg/L. DAPI stained cells were counted using an epi-fluorescence microscope at 630X or 1,000X magnification.

DNA extraction, qPCR amplification, 16S rRNA gene amplicon sequencing and library preparation

DNA was extracted from water and filter media samples using the PowerWater DNA Isolation Kit (MOBIO) and the FastDNA spin kit for soil (MP Biomedicals), respectively; 4 L of water and 1.5 mL of filter medium samples were used for DNA extraction. Full description of the methodology used for quantitative polymerase chain reaction (qPCR) amplification, 16rRNA amplicon sequencing and library preparation can be found in the supplementary materials (S2), available online. Rarefaction curves for all individual samples were determined to ensure exhaustive sequencing of the diversity in the sample.

Manganese oxidizing bacteria enrichment

Filter media homogenates (0.1 mL) were serially diluted and manganese oxidizing bacteria (MnOB) were enriched in MnOB broth and on MnOB agar containing: peptone from casein (0.5 g/L), peptone from soybean (0.5 g/L), meat extract (0.5 g/L), yeast extract (0.5 g/L), glucose (0.1 g/L), soluble starch (0.1 g/L), HEPES (10 mM), $MnCl_2$ (10 mM), and 2.0% agar (agar plates only). Initial enrichment was carried out for 2 weeks at 20 °C. Manganese oxidation was subsequently compared in liquid cultures with and without heat treatment to inactivate MnOB (121 °C for 20 min). Manganese oxidation was detected after 24 hours incubation of 0.1 mL culture in 10 mL MnOB broth. Oxidized manganese was detected in 0.5 mL subsamples by adding 0.1 mL 0.04% Leucoberbelin blue I in 45 mM acetic acid. Leucoberbelin blue I reacts with oxidized Mn but not Mn (II) to form a blue color (Krumbein & Altmann 1973). Production of oxidized manganese was quantified as an increase in absorbance measured at 620 nm (Beukes & Schmidt 2012) using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific).

Bioinformatics and statistical analysis

Results from 16S rRNA gene amplicon sequencing were analyzed in R (R Core Team 2017) through Rstudio IDE using the ampvis package v.2.0.0 (Albertsen *et al.* 2015). To compare the bacterial communities in the different filter media samples, multivariate statistics based on principal component analysis (PCA) was carried out using amp_ordinate function with Hellinger transformed OTU counts. Distribution of principal components axes in percentage of total inertia are available in the supplementary materials (S4), available online.

RESULTS AND DISCUSSION

Manganese removal by different filter media

Manganese removal in the 15 filter columns was monitored for a period of 75 days (Figure 1). The start-up period was considered finished when the manganese concentration of each medium's outlet reached 10% of the manganese concentration in the source water.

On day 75, all columns removed manganese to concentrations below the method detection limit (0.005 mg/L). Virgin quartz, calcium carbonate and polystyrene share a common onset of manganese removal after approximately 28 days (Figure 1). However, the length of the maturation

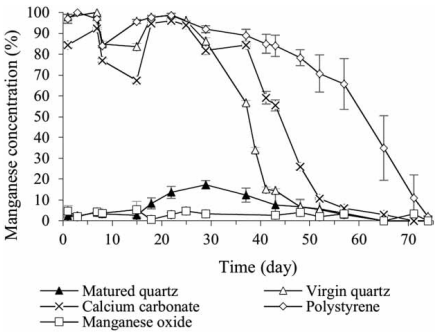

Figure 1 | Manganese concentration in the column outlets over time as a percentage of manganese concentration in the source water (0.281 mg/L). Each datum point represents the average of three filter columns and the error bars represent standard deviations.

process was somewhat different. The start-up period ended at day 48 for virgin quartz, day 57 for calcium carbonate and day 72 for polystyrene. A similar pattern of manganese start-up by virgin quartz has been reported by Bruins (2016).

Investigations on the potential use of polystyrene as filter media for treatment of surface water conclude that energy savings can be achieved during backwash (Schöntag *et al.* 2015). In our study using polystyrene as a biofilter, this medium required a longer start-up period which will likely result in higher water and energy consumption during the start of a full-scale waterworks.

Matured quartz showed efficient removal of manganese (>90%) at the beginning of the experiment (day 1 to day 21). The manganese concentration in the outlet increased slightly during the following 8 days, returning to efficient manganese removal from thereon (Figure 1). Minor fluctuations in manganese removal have also been observed for filter media in other studies (Tekerlekopoulou *et al.* 2013; Bruins 2016). The exact reason for these fluctuations is not known but may be related to varying roles of abiotic and biotic removal mechanisms. These results emphasize the complex nature of manganese oxidation processes (Ehrlich 1996; Tebo *et al.* 2005; Das *et al.* 2011).

Virgin manganese oxide media showed efficient manganese removal from day 1, which is consistent with its abiotic autocatalytic capacity to remove manganese. Collectively, the results suggest that the start-up length and stability of manganese removal is dependent not only on the initial filter media properties but also on the maturing process of the biofilter.

qPCR and DAPI

DAPI counts and qPCR measurements at day 75 with broad range primers showed that the abundance of bacteria was greatest on polystyrene and matured quartz media compared with virgin quartz, calcium carbonate and manganese oxide (Table 3). The apparent differences between media with low bacterial abundance (e.g. virgin quartz) and material with high bacterial abundance (e.g. matured quartz) was >10-fold. Larger relative differences were generally observed with qPCR compared with DAPI staining. Variations in abundance determined with the two methods may be explained by differences in extraction procedures and detection principles (DNA vs. intact cells). In general, the abundance of bacteria determined with the two cultivation independent methods in the present study is in the same range as related studies of microorganisms in drinking water biofilters from groundwater sources (Nitzsche *et al.* 2015; Gülay *et al.* 2016).

Table 3 | qPCR and DAPI numbers for filter media at day 75

Filter medium	qPCR (copies/g)	DAPI (cells/g)
Matured quartz	1.9×10^8	1.8×10^7
Virgin quartz	2.0×10^6	1.1×10^6
Calcium carbonate	4.0×10^4	1.6×10^6
Polystyrene	3.4×10^8	3.6×10^7
Manganese oxide	3.9×10^6	8.2×10^5

Each number represents the average of three filter columns.

16S rRNA amplicon sequencing analysis

The bacterial diversity of the source water (day 44 and day 75) and the filter media coating (day 75) was investigated using 16S rRNA gene amplicon sequencing analysis. The microbial diversity present on the coating of the filter medium at the phylum level indicated a clear dominance of *Proteobacteria* phylum but significant differences in *Nitrospira* phylum abundance between matured quartz and initially virgin media (Figure 2).

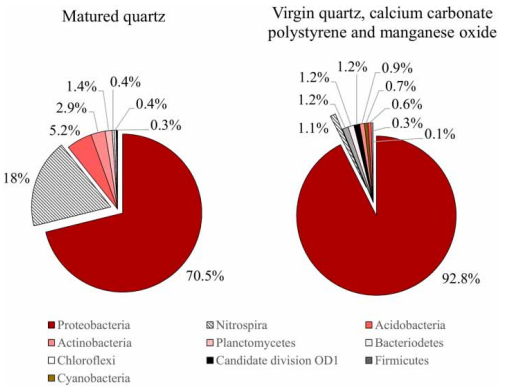

Figure 2 | Read abundance (%) at the phylum level on the coating of matured quartz and virgin quartz, calcium carbonate, polystyrene and manganese oxide at day 75. Data per medium available in the supplementary materials (S3), available with the online version of this paper.

At the genus level (Figure 3), the bacterial community of the source water included families and genera previously reported in matured drinking water biofilters, such as: *Comamonadaceae*, *Gallionellaceae*, *Hydrogenophaga*, *Sulfuricurvum*, *Nitrospira* and *Gallionella* (Zhu *et al.* 2010; Lühriq *et al.* 2015; Bruins 2016). The most dominant genus present on the coating of each media was *Nitrospira* (matured quartz), *Novosphingobium* (virgin quartz and calcium carbonate), *Sulfuritalea* (polystyrene) and *Pseudomonas* (manganese oxide).

Matured quartz maintained its original bacterial community fingerprint (dominance of *Nitrospira*) even after 75 days of contact with the nitrite and ammonium-free source water used in this experiment (Table 1). A recent study on rapid gravity filter microbial communities suggested a novel physiology of *Nitrospira* spp., which included a potential capability to oxidize manganese (Palomo *et al.* 2016).

Abundant bacteria found in the media coating include genera with known MnOB (Figure 3), such as *Pedomicrobium*, *Hyphomicrobium, Ralstonia, Pseudomonas, Hydrogenophaga* and *Leptothrix* as part of the *Comamonadaceae* family (Mouchet 1992; Ehrlich 1996; Larsen *et al.* 1999; Katsoyiannis & Zouboulis 2004; Pacini *et al.* 2005; Tebo *et al.* 2005; Das *et al.* 2011; Abu Hasan *et al.* 2012; Marcus *et al.* 2017). These genera were part of the bacterial community of all five filter media, with exception of *Pseudomonas* and *Pedomicrobium* which were not detected on polystyrene, and *Ralstonia* which was not detected on polystyrene and matured quartz. The low abundance of *Hyphomicrobium* and *Comamonadaceae* and absence of *Ralstonia*, *Pseudomonas* and *Pedomicrobium* in the polystyrene samples coincide with a long start-up of this medium.

The top three most abundant genera detected on the manganese oxide coating included genera with known MnOB (*Pseudomonas, Hydrogenophaga and Leptothrix* as part of the *Comamonadaceae* family). Even though the main

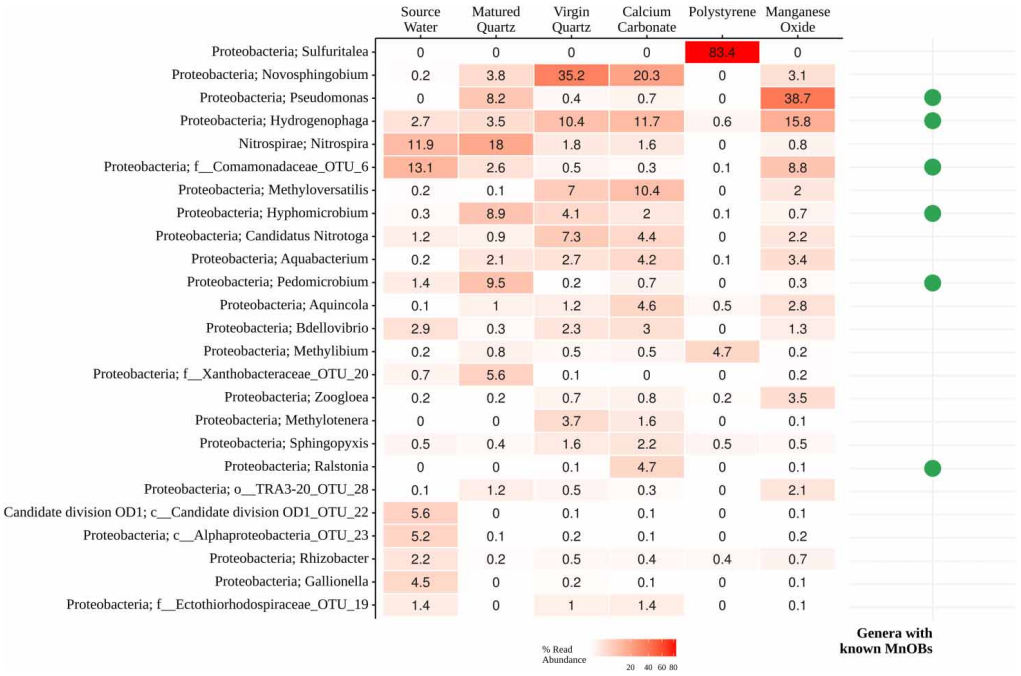

Figure 3 | Heatmap of the 25 most abundant genera present in filter media coatings at day 75 (average of three filter columns). The most abundant genera in the source water are shown for comparison (average of day 44 and day 75). Shadings are based on read abundance (%) and green dots indicate genera with known MnOB. Standard deviations available in the supplementary materials (S3), available with the online version of this paper.

manganese removal on this medium is likely based on an abiotic autocatalytic process, the presence of genera with known MnOB on this medium may suggest that MnOB are taking advantage of the morphology of the manganese oxide medium (80% MnO_2) or of the autocatalytic oxidation process occurring at the surface of the medium grains. However, the underlying biochemical mechanisms behind bacterial oxidation of manganese are yet to be fully understood (Tebo *et al.* 2005). Further investigations on the potential use of manganese oxide medium to initiate the microbial growth in the virgin media biofilters might be of interest to the industry.

Sphingomonadaceae genera were detected as part of the 25 most abundant genera of filter media samples with 0.57 ± 0.05, 0.767 ± 0.330, 0.333 ± 0.249, 1.000 ± 0.510 and 0.233 ± 0.047 percent for matured quartz, virgin quartz, calcium carbonate, polystyrene and manganese oxide media, respectively. *Sphingomonadaceae* has previously been reported in drinking water systems, and it has been suggested that *Sphingomonas* spp. plays a unique role assisting the initial formation and spatiotemporal development of microbial biofilms (Bereschenko *et al.* 2010; Lührig *et al.* 2015; Bruins 2016).

MnOB enrichment

MnOB were enriched from all filter media samples to confirm the presence of culturable manganese oxidizers at the end of the experiment (day 75). The Leucoberbelin blue assay for detection of oxidized manganese (Krumbein & Altmann 1973) was performed after enrichment of MnOB from filter media samples taken from all columns. Control samples and subsamples with autoclaved inoculum showed no color formation after 24 h in MnOB broth while color was displayed in subsamples containing live inoculum. Spectrophotometric measurements with a signal/background ratio >1.5 were considered positive (data not shown). Differences in absorbance between autoclaved enrichments and live enrichments confirmed that live bacteria capable of oxidizing manganese were present on filter coatings from all filter media at the end of the start-up periods. The concentration of culturable heterotrophic MnOB corresponded to $\geq 10^1$ presumptive MnOB/g. This is likely a minimum estimate because a significant number of viable but not immediately culturable MnOB may also be present in the source material.

Principal component analysis

Figure 4 illustrates a PCA plot where each point represents the bacterial community present in the coating of the filter medium of each column at the end of the experiment based on all taxa. The replicates of each medium share a clear clustering, which indicates comparable bacterial composition of the biofilm developed on the coating of the three replicates.

The PCA identifies four main clustering areas (Figure 4). The bacterial community present on the matured quartz was rather similar to the one found in the source water whereas virgin quartz and calcium carbonate shared strong similarities in their bacterial community composition. Interestingly, these two media also share a similar manganese removal pattern (Figure 1). Contrarily, manganese oxide and polystyrene have distinct clustering areas when compared with the other media. For example, the distinct clustering of polystyrene was strongly affected by the large relative abundance of *Sulfuritalea*, while manganese oxide and virgin quartz/calcium carbonate clustering areas were influenced by the relative abundance of *Pseudomonas* and *Novosphingobium*. These results suggest that when inoculating different filter media with an identical water source, the bacterial community formed in the filter media coatings during the start-up period is influenced by the media type.

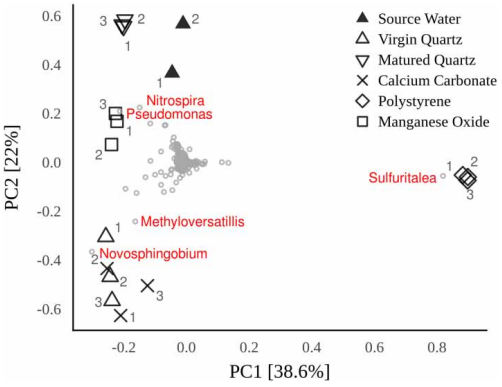

Figure 4 | PCA of bacterial communities present in the source water (day 44 and day 75) and on the coating of each filter medium (day 75). Numbers 1 to 3 represent medium replicates. Grey dots illustrate the taxa detected in all samples. Red labels identify selected key taxa. Please refer to the online version of this paper to see this figure in color: http://dx.doi.org/10.2166/aqua.2017.084.

Characterization and comparisons of the microbial diversity in biofilters treating groundwater has been reported in relatively few studies using 16S rRNA amplicon sequencing (e.g. quartz sand by Gülay *et al.* 2016; Palomo *et al.* 2016 and Nitzsche *et al.* 2015; and anthracite/sand by White *et al.* 2012). Gülay *et al.* (2016) found similarities among the core microbial communities at five different Danish drinking water treatment plants, all of them using quartz as filter medium to treat groundwater. This is in alignment with our study which indicates a cluster of microbial communities found in the quartz sand columns (Figure 2). Similar to Gülay *et al.* (2016), our matured quartz microbiome was dominated by different *Proteobacteria* and a high relative abundance of *Nitrospira*. White *et al.* (2012) investigated the microbial community of a drinking water biofilter with anthracite and quartz and also observed a significant presence of *Nitrospira*. However, the role of filter media for microbial community composition and metabolic activity is not well understood for many alternative media suggested for treating groundwater. Even though all our different filter media completely removed the manganese by day 75, the results of the present study indicate that the potential role of biotic manganese oxidation likely differs among the different filter media. Hence, biostimulation of MnOB by selection of proper media may be a possibility for the industry to enhance performance of drinking water biofilters for manganese removal during start-up.

Further, Figure 4 shows that during the maturation process, there is a tendency of different genera to develop in the different filter media. Biological inoculation using MnOB to accelerate the start-up of biofilters is of interest and is being investigated (Burger *et al.* 2008; McKee *et al.* 2016). Preselection of MnOB species to inoculate biofilters could potentially be based on the predisposition of the biofilter towards specific genera.

CONCLUSIONS

Manganese removal and bacterial diversity of five different filter media were investigated during a 75-day start-up period. This investigation concludes the following:

- The start-up period for virgin quartz, calcium carbonate and polystyrene ended at day 48, day 57 and day 72, respectively. In contrast, no start-up period was needed for efficient manganese removal by virgin manganese oxide media. All media completely removed manganese by day 75. Quartz and calcium carbonate showed comparable start-up length and bacterial communities. Polystyrene required a longer start-up period compared with virgin quartz and calcium carbonate, and fewer genera with known MnOB were detected in the bacterial community of this medium.
- Matured quartz maintained its original bacterial community fingerprint (dominance of *Nitrospira*) after 75 days of exposure to source water in which ammonia and nitrite were virtually absent.
- Culturable MnOB capable of oxidizing manganese were detected on all filter media at the end of the start-up period. Manganese oxide medium included genera with known MnOB even though the main oxidation process is likely based on abiotic processes.
- When inoculating different filter media with an identical water source, the bacterial communities formed during the start-up period are strongly influenced by the filter media type. Hence, management of bacterial communities may be possible by selecting specific filter media to enhance growth and activity of specific bacteria.

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SUPPLEMENTARY MATERIAL

S1. Schematic illustration of the experimental setup

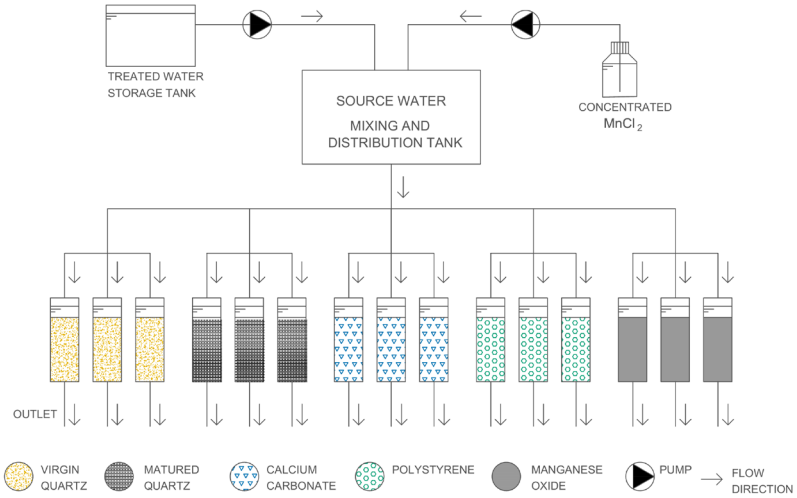

Figure S1 | Experimental setup placed at the waterworks: 15 gravity filter columns.

S2. Full description of the molecular methods

qPCR

The abundance of 16S rRNA genes per ng of isolated DNA was estimated based on the broad-range qPCR probe and primer set (Nadkarni *et al.* 2002). A linearized plasmid containing the qPCR amplicon was used to create the standard curve as previously described (Karst *et al.* 2016). Briefly, the forward (5'-TCCTACGGGAGGCAGCAGT-3') and reverse (5'-GACTACCAGGGTATCTAATCCTGTT-3') primers (Nadkarni *et al.* 2002) were used to amplify the qPCR amplicon of from *E. coli* MG1655 using the AccuPrime Pfx DNA polymerase (Thermo Scientific). The PCR was carried out according to the manufacturers recommendations with the following PCR program: PCR activation (94 °C, 2 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s) and extension (68 °C, 90 s) and a final extension (68 °C, 5 min). The PCR product was purified on an E-gel CloneWell gel (Thermo Scientific), and cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Thermo Scientific) according to the manufactures recommendations. The obtained plasmid was subsequently linearized with FastDigest NcoI (Fermentas), and blunted using the Klenow fragment (Fermentas). The concentration of the amplicon stock was determined using the Qubit HS dsDNA assay kit (Life Technologies), and the copy number then calculated based on the molecular weight of the linearized plasmid.

The amplicon stock was diluted to 10^8 copies/μL in 10 mM tris buffer (pH 8.5) and stored as aliquots at -18 °C. qPCR were carried out in technical duplicates using the Mx3005P qPCR system (Stratagene), and the EXPRESS qPCR Supermix (Life Technologies). Reactions of 20 μL were prepared according to manufacturer's instruction using 50 nM ROX, 500 nM of each primer, 200 nM hydrolysis probe ((6-FAM)-5'- CGTATTACCGCGGCTGCTGGCAC- 3'-(BHQ-1)), and 5 μL template DNA. The qPCR reaction conditions were as follows: UDG incubation (50 °C, 2 min) and PCR activation (95 °C, 2 min) followed by 45 cycles of denaturation (95 °C, 15 s) and combined annealing and extension (60 °C, 1 min). Amplicon standards with concentrations ranging from 10^1-10^7 copies/μL were included for all qPCR runs and used for quantification. A clear logarithmic correlation was found between amplicon concentration and the Cq value (R^2=0.99) and the efficiency of the qPCR was acceptable (105.8%). The lower level of detection for water and filter medium samples was approximately 100 copies per mL or gram, respectively. All primers and probes were HPLC purified (DNA Technology, Denmark).

16S rRNA amplicon library preparation: Bacteria V3-4 Amplicon PCR

Bacteria V3-4 amplicon libraries were prepared by a custom protocol adapted from an Illumina protocol (Illumina, 2015). 2 μL of DNA sample was used as template for PCR amplification. Each PCR reaction (25 μL) contained dNTPs (400 μM of each), MgSO4 (2.5 mM), Platinum® Taq DNA polymerase HF (0.5U), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA) and tailed primermix (400 nM of each forward and reverse). PCR was run with following program: Initial enaturation at 95°C for 2 min, 35 cycles of amplification (95°C for 20 s, 50°C for 30 s, 72 °C for 60 s), and a final elongation at 72 °C for 5 min. The forward and reverse tailed primers were designed according to (Illumina, 2015) and contain primer parts targeting bacteria V3-4 16S fragments. V3-4 primers (Herlemann *et al.* 2011): 5'-CCTACGGGNGGCWGCAG (341F) and 5'- GACTACHVGGGTATCTAATCC (805R). The primer tails enable attachment of Illumina Nextera adaptors for sequencing in a subsequent PCR. The amplicon libraries were purified with Agencourt Ampure XP Bead (Beckman Coulter, USA) following the vendor recommended protocol, except for a bead to sample ratio of 4:5. The DNA was eluted in 15 μL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Quant-iT DNA Assay Kit, high sensitivity (Thermo Fisher Scientific, USA), and the quality of a subset of samples was validated with a Tapestation 2200, using D1000 ScreenTapes (Agilent, USA).

Library PCR

Sequencing libraries were prepared from the purified bacteria V3-4 amplicon libraries using a second PCR. Each PCR reaction (25 μL) contained 1x PCRBIO HiFi buffer (PCRBiosystems, UK), PCRBIO HiFi Polymerase (1U) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse), and 2 μL of amplicon library template (< 5 ng/µL). PCR was run with following program: Initial denaturation at 95 °C for 2 min, 8 cycles of amplification (95 °C for 20 s, 55 °C for 30 s, 72 °C for 60 s) and a final elongation at 72 °C for 5 min. The sequencing libraries were purified with Agencourt Ampure XP Bead (Beckman Coulter, USA) follwoing the vendor

recommended protocol, except for a bead to sample ratio of 1:1. The DNA was eluted in 15 μL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Quant-iT DNA Assay Kit, high sensitivity (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000 screentapes (Agilent, USA) was used to check the product size and purity of a subset of sequencing libraries.

DNA sequencing
The purified sequencing libraries were pooled in equimolar concentrations and diluted to 4 nM or lower. The samples were paired end sequenced (2x301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. 20% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples.

16S rRNA amplicon bioinformatic processing
As the amplicons generated using both primers are within the borders of what is possible to merge, only the first 275 bases of read 1 was used. The reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger *et al.* 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN: 275. The trimmed reads were dereplicated and formatted for use in the UPARSE workflow (Edgar 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97. Taxonomy was assigned using the RDP classifier (Wang *et al.* 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso *et al.* 2010), using the MiDAS database v.1.20 (McIlroy *et al.* 2015). The results were analysed in R (R Core Team 2015) through the Rstudio IDE using the ampvis package v.1.9.1 (Albertsen *et al.* 2015).

S3. 16S rRNA gene amplicon sequencing analysis

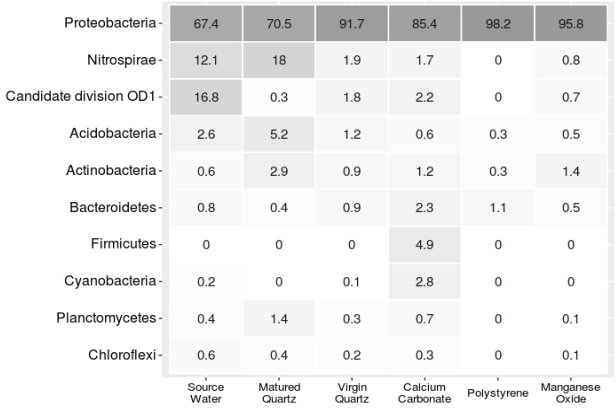

Figure S3 | Microbial diversity at phylum level on the source water (average of Day 44 and Day 75) and on the coating of the different filter media (Day 75 average of 3 columns per medium).

Table S3 | List of the 25 most abundant genera present at Day 44 and Day 75 in source water and at Day 75 in filter media coatings. Abundance is listed in % of all genera in each sample (mean ± standard deviation). Grey shading indicates the most abundant genera in each sample with darker color indicating the dominant genera.

Genus name	Source water	Matured quartz	Virgin quartz	Calcium Carbonate	Polystyrene	Manganese Oxide
Proteobacteria; Sulfuritalea	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	83.4±4.6	0.0±0.0
Proteobacteria; Novosphingobium	0.2±0.2	3.8±0.3	35.2±6.4	20.3±8.9	0.0±0.0	3.0±1.0
Proteobacteria; Pseudomonas	0.0±0.0	8.2±0.5	0.4±0.2	0.7±0.1	0.0±0.0	38.7±3.7
Proteobacteria; Hydrogenophaga	2.7±2.7	3.5±0.7	10.4±1.0	11.7±2.3	0.6±0.1	15.8±2.2
Nitrospirae; Nitrospira	11.9±7.3	18.0±1.3	1.8±1.1	1.5±0.3	0.0±0.0	0.7±0.2
Proteobacteria; f__Comamonadaceae_OTU_6	13.1±13.0	2.7±0.1	0.5±0.2	0.3±0.1	0.1±0.0	8.9±0.9
Proteobacteria; Methyloversatilis	0.2±0.2	0.1±0.0	7.0±2.7	10.4±2.0	0.0±0.0	2.0±0.2
Proteobacteria; Candidatus Nitrotoga	1.2±0.5	0.9±0.0	7.4±1.5	4.4±1.1	0.0±0.0	2.1±0.5
Proteobacteria; Hyphomicrobium	0.3±0.0	8.8±0.7	4.0±0.8	1.7±1.1	0.1±0.0	0.7±0.1
Proteobacteria; Aquabacterium	0.2±0.2	2.1±0.0	2.7±0.9	4.2±0.4	0.1±0.1	3.4±0.5
Proteobacteria; Pedomicrobium	1.4±0.1	9.5±0.5	0.2±0.1	0.5±0.6	0.0±0.0	0.3±0.1
Proteobacteria; Aquincola	0.1±0.1	1.0±0.4	1.2±0.3	4.6±3.2	0.5±0.1	2.8±1.0
Proteobacteria; Bdellovibrio	2.4±1.5	5.7±0.4	2.3±0.1	2.3±1.6	0.0±0.0	1.2±0.3
Proteobacteria; Methylibium	0.2±0.1	0.8±0.0	0.5±0.1	0.5±0.4	4.7±1.8	0.2±0.0
Proteobacteria; f__Xanthobacteraceae_OTU_20	0.7±0.1	5.7±0.4	0.1±0.1	0.0±0.0	0.0±0.0	0.2±0.0
Proteobacteria; Zoogloea	0.2±0.2	0.2±0.0	0.7±0.0	0.8±0.3	0.2±0.1	3.5±0.6
Proteobacteria; Sphingopyxis	0.5±0.5	0.4±0.0	1.5±0.3	2.1±0.3	0.5±0.2	0.5±0.2
Proteobacteria; Methylotenera	0.0±0.0	0.0±0.0	3.8±0.3	1.5±1.0	0.0±0.0	0.2±0.0
Proteobacteria; Ralstonia	0.0±0.0	0.0±0.0	0.2±0.0	4.7±3.1	0.0±0.0	0.1±0.0
Proteobacteria; o__TRA3−20_OTU_28	0.1±0.0	1.2±0.0	0.5±0.3	0.3±0.2	0.0±0.0	2.1±0.3
Candidate division; c__Candidate division_OTU_22	5.7±4.3	0.0±0.0	0.1±0.0	0.1±0.1	0.0±0.0	0.1±0.0
Proteobacteria; c__Alphaproteobacteria_OTU_23	5.2±3.9	0.1±0.0	0.2±0.1	0.1±0.2	0.0±0.0	0.2±0.0
Proteobacteria; Rhizobacter	2.3±2.3	0.2±0.0	0.5±0.0	0.4±0.3	0.4±0.3	0.7±0.1
Proteobacteria; Gallionella	4.5±1.1	0.1±0.0	0.2±0.1	0.0±0.0	0.0±0.0	0.1±0.0
Proteobacteria; f__Ectothiorhodospiraceae_OTU_19	1.5±0.9	0.0±0.0	0.8±0.6	1.3±1.0	0.0±0.0	0.1±0.0

S4. PCA analysis

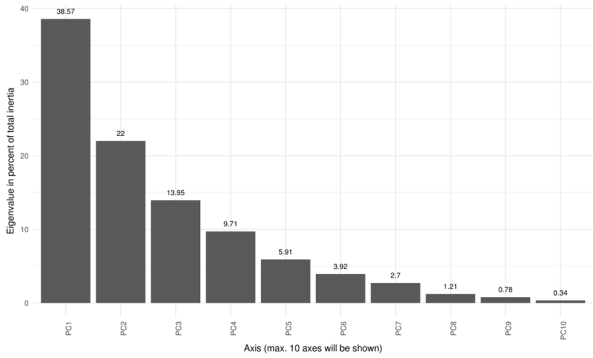

Figure S4 | Eigenvalue in percent of total inertia for 10 PC-axis.

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PAPER II

MANGANESE REMOVAL PROCESSES DURING START-UP OF INOCULATED AND NON-INOCULATED DRINKING WATER BIOFILTERS

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INÊS L. BREDA, DITTE A. SØBORG,
LOREN RAMSAY & PETER ROSLEV

Manganese removal processes during start-up of inoculated and non-inoculated drinking water biofilters

I. L. Breda, D. A. Søborg, L. Ramsay and P. Roslev

ABSTRACT

Manganese removal in drinking water biofilters is facilitated by biological and physico-chemical processes, but knowledge regarding the relative role of these mechanisms during start-up is very limited. The aim of this study was to identify the dominant process for manganese removal occurring during the start-up period of sand filters with and without inoculation by addition of matured sand collected from an operating groundwater-based waterworks. Inoculation with matured filter sand is frequently used to accelerate the start-up in virgin biofilters and to rapidly obtain compliant water quality. The non-inoculated filter took 41 days to comply with manganese quality criteria, whereas the inoculated filter with 20% matured sand showed removal from Day 1 and compliance from Day 25. By Day 48, the inoculated filter showed two times higher manganese removal rates and manganese oxides deposits. Using sodium azide as an inhibitor of microbial activity, it was found that manganese removal in the non-inoculated filter was dominated by biological processes, whereas physico-chemical processes were of more importance in the inoculated filter (Day 35, 39 and 48). 16S rDNA sequencing of the microbiota collected during filter maturation indicated a limited immediate effect of inoculation on the microbial community developed on the remaining filter material.

Key words | groundwater, inoculation, manganese removal processes, sodium azide, start-up

I. L. Breda (corresponding author)
P. Roslev
Department of Chemistry and Bioscience,
Aalborg University,
Fredrik Bajers Vej 7H, 9220 Aalborg,
Denmark
E-mail: *ilb@bio.aau.dk*

I. L. Breda
Group of Project and Development,
Skanderborg Forsyningsvirksomhed A/S,
Døjsøvej 1, 8660 Skanderborg,
Denmark

D. A. Søborg
L. Ramsay
Research Group for Energy and Environment,
VIA University College,
Chr. M. Østergaards Vej 4, 8700 Horsens,
Denmark

INTRODUCTION

Biofilters are often used in production of drinking water from groundwater sources. However, a major disadvantage of biofiltration is the necessity of a start-up period to mature virgin filter media. When manganese is present in source water, the start-up period can last from weeks to more than a year (Tekerlekopoulou *et al.* 2013).

Proactive inoculation methods to accelerate the start-up of biofilters include the addition of a concentrated source of microorganisms and/or autocatalytic surfaces, e.g. backwash sludge (Štembal *et al.* 2004; Cai *et al.* 2015; Dangeti *et al.* 2017), matured filter sand (Zeng *et al.* 2010; Bruins 2016), mixed bacterial culture (Tekerlekopoulou & Vayenas 2008) or specific bacterial species (Qin *et al.* 2009; Bai *et al.* 2016; Li *et al.* 2016; McKee *et al.* 2016). The most common methods used by the drinking water industry are based on the addition of backwash sludge or matured filter sand (Štembal *et al.* 2004). Amendment with matured sand provides immediate removal, while potentially promoting the microbial growth on the virgin sand in the remaining filter.

Manganese removal in biofilters is based on physico-chemical and biological processes (Mouchet 1992). Recent studies suggest that after initial sorption, manganese removal by a non-coated virgin medium is initiated biologically, evolving to a predominantly physico-chemical removal process over time due to the development of an autocatalytic coating of manganese on the filter grains (Sahabi *et al.* 2009; Bruins 2016). This recent knowledge could be of help in reducing typically long start-up periods by creating conditions that are favorable for the growth of

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manganese oxidizing bacteria (MnOB) and other microorganisms involved in manganese oxidation.

Previous studies have investigated the importance of biological manganese oxidation in drinking water biofilters using various methods to inhibit biological removal (Vandenabeele *et al.* 1992; Gounot 1994; Olańczuk-Neyman & Bray 2000; Sahabi *et al.* 2009). However, further investigations are required to understand the contribution of different mechanisms of manganese removal during the start-up period.

The aim of this study was to identify the dominant process in manganese removal (physico-chemical and biological) during the start-up of a virgin sand pilot biofilter with and without inoculation by addition of matured sand. In addition, the aim of this study was to shed light on the effect of proactive inoculation by addition of a layer of matured filter sand on the microbial community developed in the adjacent virgin layers of the filter.

MATERIALS AND METHODS

Pilot scale set-up

Treated groundwater from the storage tank of a Danish drinking water treatment plant was used as source water (Fredensborg waterworks, Skanderborg, Denmark). The treated water contains a natural background of drinking water microorganisms as disinfection is not used at this and most other waterworks in Denmark. The unchlorinated treated water was continuously spiked with a concentrated solution of $MnCl_2 \cdot 4H_2O$ (Emsure ACS), using a diaphragm pump (Digital DDC, Grundfos), and distributed to two pressurized 0.3 m^3 filter tanks (Type NS20, Silhorko Eurowater). The filters were placed at the water treatment plant and operated at a temperature of 11 °C (Table 1, Figure 1).

Each filter has a diameter of 30 cm and a 1 m layer of granular quartz sand. The non-inoculated filter was filled solely with virgin quartz sand (Dansk Kvarts Industri), and the inoculated filter with two intercalated layers of matured sand in the virgin sand (Figure 1, Table 2). The matured sand used for inoculation of the pilot filter was collected from the top layer of a second stage biofilter of Fredensborg waterworks, which had been removing manganese for the last 47 years. Throughout the experiment, no visual mixing was observed between the two-filter media used in the inoculated filter (virgin and matured sand).

Before the start of operation, the filter tanks and the virgin sand medium were disinfected overnight with 2% H_2O_2 according to the manufacturer's standard procedures

Table 1 | Source water quality

Parameter	Unit	Average	Std. dev.
Manganese	mg/L	0.281	0.018
Iron	mg/L	0.019	0.01
Ammonium	mg/L	<0.02	–
Nitrite	mg/L	<0.001	–
Hydrogen carbonate	mg/L	276	0.5
pH	–	7.95	0.05
Oxygen	mg/L	10.8	0.05
Temperature	°C	10.7	0.2
NVOC[a]	mg/L	1.0	0.1

[a]Non-volatile organic carbon.

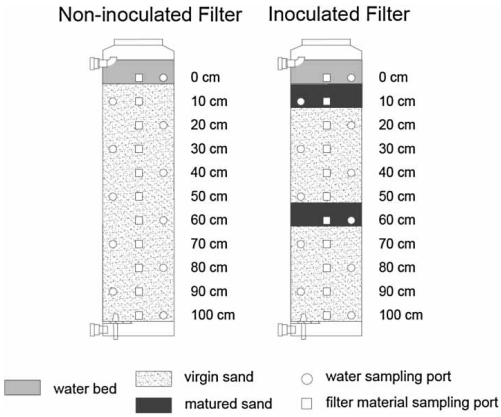

Figure 1 | Filter tanks and sampling depths for water and filter medium.

Table 2 | Filter medium properties

Parameter	Matured sand	Virgin sand
Grain size (mm, 10–90%)	1.03–1.86	1.10–1.76
Sphericity	0.90	0.87
Particle density (kg/L)	2.50	2.60
Porosity (%)	42.6	40.6

which included successive backwashes to remove fines and excess H_2O_2. Matured sand was added subsequently to ensure that its microbial community was not affected during disinfection. The filters were operated in downflow mode with a filtration rate of 5 m/h and an empty bed contact time of 12 min. No backwash was used after the start of operation.

Water and filter medium sampling

Inlet and outlet samples (10 mL) from the filters were manually collected each couple of days for a period of 72 days, filtered (0.22 μm) and analyzed immediately for total dissolved manganese. In addition, water samples were collected from both filters at 10 cm depth intervals (profile) once a week following the same procedure. Inlet water (4 L) was filtered (0.20 μm membrane filters, Advantec) and stored at −21 °C for subsequent microbial diversity analysis.

Before the start of operations (Day 0) virgin sand and matured sand samples were collected to quantify the manganese coating the grains, to investigate the manganese removal rate and to analyze the microbial diversity. During operations (Day 35, 37 and 48) filter media samples were collected from both filters to quantify the manganese coating the grains (depth 10, 20 and 30 cm), to investigate the manganese removal rate (depth 10, 20 and 60 cm) and to analyze the microbial diversity (depth 10, 20, 30, 60 and 80). Sampling days during operations were selected with focus on the period in which the non-inoculated filter started to remove manganese. A previous investigation conducted by Breda *et al.* (2017) in column scale using the same source water and filter materials as in the present study indicated that manganese removal would start shortly after 30 days. Sampling depths were defined to represent the most active sections of the filters. One month after the end of the start-up period of the non-inoculated filter (Day 72) filter media from the top 10 cm of each filter were collected to investigate the manganese removal rate.

Manganese measurements in water and medium samples

Total dissolved manganese was measured according to the manufacturer's instructions (kit LCK304, lower level of detection of 0.005 mg/L, DR3900 spectrophotometer, Hach, Denmark).

The coating of filter medium samples (0.5 g in duplicates) was extracted in a 50 mL mixture of 4 M HCl and 2 g/L oxalic acid ($C_2H_2O_4$) as described by De Vet *et al.* (2009). The manganese concentration in the decanted acid solution was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES) according to Standard Methods (American Public Health Association 1975).

Batch assay with and without NaN_3 for determining manganese removal rates

Filter medium samples (1 g) were placed in four serum bottles with 25 mL deionized water. Half of the bottles were incubated for 1 h with NaN_3 (25 mM, Merck KGaA) to inhibit microbial respiration (Tebo *et al.* 2005). All bottles were then spiked with $MnCl_2 \cdot 4H_2O$ (Emsure ACS) to obtain an initial concentration of 0.3 mg/L. Water samples (1.5 mL) were collected each 5 min for 20 min, filtered (0.22 μm) and analyzed for total dissolved manganese (kit LCK304, Hach, Denmark using a Multiskan FC Microplate Photometer, Thermo Fisher Scientific). All bottles were continually mixed on an orbital shaker (150 rpm) at room temperature during the experiment. Control bottles without filter medium, with and without NaN_3, were included to account for any precipitation or sorption of manganese to glass surfaces of the incubation bottles.

Column assay with and without NaN_3 for determining manganese removal rates

Filter medium samples (100 g in quadruplicate) from the top 10 cm of each filter were collected one month after the end of the start-up period (Day 72). Immediately after sampling, two replicates of the medium samples from each filter were pretreated by immersion in a 25 mM NaN_3 solution (Merck KGaA). An eight-column assay with four columns with NaN_3 and four columns without NaN_3 was constructed using the same filtration rate as the pilot filters. Source water consisted of treated water from the waterworks spiked with $MnCl_2 \cdot 4H_2O$ (Emsure ACS) to obtain an initial concentration of 0.3 mg Mn/L. To ensure a continuous inhibition of microbial respiration, 25 mM NaN_3 was added to the

source water feeding the columns with NaN_3 pretreatment. The manganese removal capacity of the filter columns was followed for 3 h. Water samples were collected each hour from the outlet of each column, filtered (0.22 μm) and analyzed for total dissolved manganese as described above.

DNA extraction and 16S rDNA amplicon sequencing and library preparation

DNA was extracted from filter medium samples using the FastDNA spin kit for soil (MP Biomedicals) and from water samples using the PowerWater DNA Isolation Kit (MOBIO). A full description of the methodology used for quantitative polymerase chain reaction (qPCR) amplification, 16S rDNA amplicon sequencing and library preparation can be found in the supplementary material. Rarefaction curves for all individual samples were determined to ensure exhaustive sequencing of the diversity in the sample.

Bioinformatics and statistical analysis

Statistical analysis including the non-parametric Mann-Whitney U test was performed in R through Rstudio IDE (R Core Team 2017). A nominal *p*-value less than 0.05 was considered to be of statistical significance. Results from 16S rDNA amplicon sequencing were analyzed using the ampvis package v.2.0.0 (Albertsen *et al.* 2015). Multivariate statistics based on Principle Component Analysis (PCA) were carried out to compare the bacterial communities in the different filter sand samples, using the amp_ordinate function with Hellinger transformed operational taxonomic unit (OTU) counts (Albertsen *et al.* 2015).

RESULTS AND DISCUSSION

Manganese removal in inoculated and non-inoculated filters during the start-up period

Manganese removal was not detected in the non-inoculated pilot filter (virgin sand) during the first 30 days of the start-up period with exception of initial adsorption at Day 0 (Figure 2). Compliance with the drinking water criterion of 0.05 mg/L was observed on Day 41 (Figure 2). Similar removal patterns by initially non-coated virgin filter media in columns treating solely manganese have been reported previously (Bruins 2016; Breda *et al.* 2017).

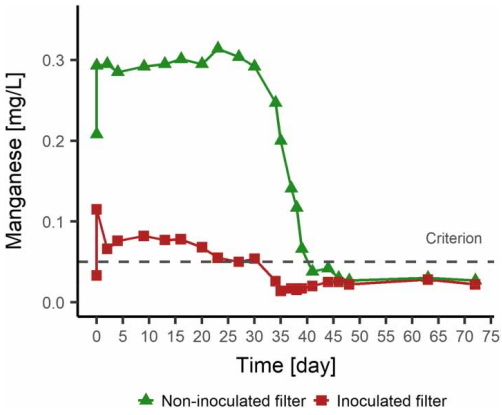

Figure 2 | Manganese concentration over time at the outlet of the pilot biofilters with and without inoculation.

The inoculated pilot filter with 20% matured sand showed significant removal (approx. 65–80%) from Day 1. Compliance for the inoculated filter was achieved after 25 days of operation (Figure 2). After 48 days, manganese removal in the inoculated and non-inoculated filters was comparable (approx. 90% removal).

The investigation by Bruins (2016) showed no difference in manganese removal efficiency during the start-up period of a non-inoculated and an inoculated (7.5% matured manganese coated sand) full-scale filter using groundwater as source water. The absence of immediate manganese removal by the inoculated filter was assumed to be caused by the loss of the autocatalytic and biological activity of MnOx and MnOB, respectively coating the medium during storage in open air for several months. In contrast, results of this study indicate that inoculation with fresh matured manganese coated sand accelerates the onset of manganese removal and that the long start-up period for manganese can be substantially reduced by initially supplementing the filter vessel with approx. 20% fresh matured manganese coated sand.

The non-inoculated filter (Figure 3(a)) initially showed no detectable manganese removal (Day 6 and 20) followed

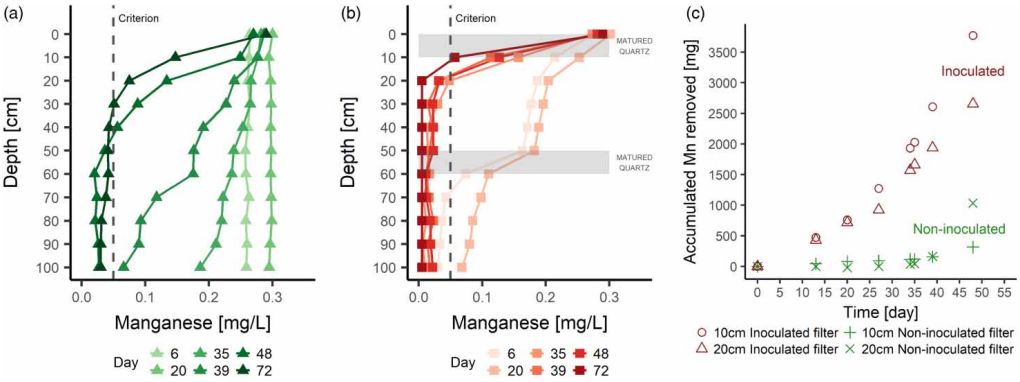

Figure 3 | Manganese concentration profile over time in (a) the non-inoculated filter (b) the inoculated filter. (c) Total manganese removed by both non-inoculated and inoculated filter at depth 10 and 20 cm over time.

by slight manganese removal at all depths (Day 35 and 39). In contrast, the initial manganese concentration profile for the inoculated filter (Day 6, Figure 3(b)) shows a step pattern which clearly indicates the location of the mature sand layers (10 and 60 cm, Figure 1). At these depths, an especially high removal of manganese occurred. Slight manganese removal also occurred in the layers directly under the mature sand.

Manganese removal in the non-inoculated filter continued to increase throughout the investigation period (Figure 3(a)). On Day 72, the removal in the non-inoculated filter, however, was still less efficient on a volumetric basis than the removal in the inoculated filter (i.e. approximately 30 cm required to reach compliance in the non-inoculated filter as opposed to 10 cm in the inoculated filter).

Manganese removal at the upper third of the inoculated filter was greatly increased by Day 35 (Figure 3(b)). At this time, nearly all manganese was removed at a depth of only 20 cm, meaning that the deeper layer of mature sand (20–60 cm) no longer contributed substantially to manganese removal. The top mature sand layer of the inoculated filter (0–10 cm) showed an increase in manganese removal over time (Figure 3(b)). The greatest manganese removal rate was observed at Day 72 in the inoculated filter from 0–10 cm depth. In this interval, the removal rate for an initial manganese concentration of 0.3 mg/L was approximately 0.2 mg/L/min. Similar manganese removal rates were reported by Dangeti *et al.* (2017) after inoculation of a pilot-scale biofilter with backwash sludge.

The accumulated amount of manganese removed at each depth of each filter was calculated based on the water samples from the manganese profiles (Figure 3(c)). Interestingly, the amount of manganese removed from the 10–20 cm layer in the inoculated filter (initially virgin sand) was similar to the amount removed from the 0–10 cm layer (initially mature sand). This suggests that manganese removal capacity was transferred from the mature sand to the virgin sand layer directly underneath. It should be noted that removed manganese at 0–10 and 10–20 cm layers diverged near the end of the experiment, not because the 0–10 cm layer was more efficient, but due to the 10–20 cm layer receiving lower manganese concentrations.

Manganese coating in inoculated and non-inoculated filters during the start-up period

The manganese coating on the initially virgin sand grains showed an increase over time, whereas the manganese coating on the mature sand at 10 cm depth of the inoculated filter remained in the order of 10 mg/g medium (Figure 4).

By Day 39, the manganese coating the grains at 20 cm depth of the inoculated filter was 1% of the manganese coating at 10 cm depth (Figure 4). Despite this difference, the manganese removal profile of the inoculated filter at Day 39 showed

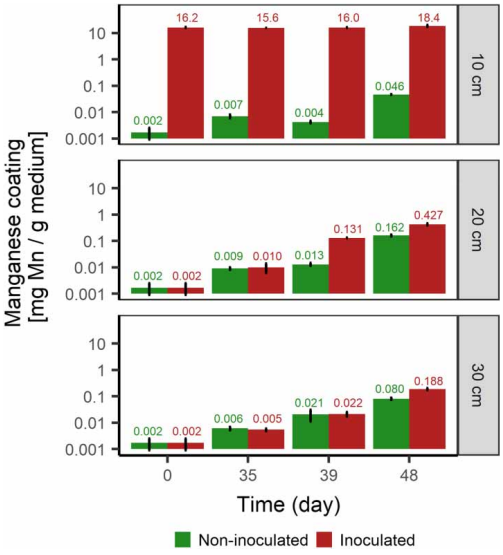

Figure 4 | Manganese coating development in duplicates at depth 10, 20 and 30 cm at Day 35, 39 and 48 from the non-inoculated filter (green) and from the inoculated filter (red). Numbers are the average of the replicates and error lines indicate standard deviation between replicates. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wqrj.2018.016.

that comparable amounts of manganese were removed by those layers (Figure 3(b)). These results suggest that accumulation of approx. 1% (≈0.1 mg/g) of the manganese coating the matured filter sand was sufficient for the initially virgin sand to achieve a performance comparable to the fully matured sand. These results indicate that freshly precipitated manganese oxide on the initially virgin filter is more efficient than precipitates on the initially matured grains.

At Day 48, the amount of manganese coating on the initially virgin medium at 20 cm and 30 cm depth of the inoculated filter was twice as high as the non-inoculated filter (Figure 4). The same two-fold difference was observed when comparing the total manganese removed by the 10–20 cm layer of each filter by Day 48 (20 cm depth, Figure 3(c)).

Manganese removal with and without NaN_3 in batch and column assays

NaN_3 is an inhibitor of respiratory activity in microorganisms while it does not appear to affect autocatalytic properties of MnOx coatings (Rosson *et al.* 1984). NaN_3 addition was used in the current study to compare manganese removal processes related to physico-chemical and biological mechanisms. Manganese removal observed in medium samples with NaN_3 was assumed to be mainly due to physico-chemical processes, and the difference between the manganese removal observed in medium samples with and without NaN_3 was assumed to be mainly due to biological processes (Figure 5). To identify the dominant process in manganese removal, the ratio between apparent physico-chemical and biological removal rates was calculated. When the ratio was <1, most manganese removal was attributed to biological processes, and when the ratio was >1 most manganese removal was attributed to physico-chemical processes.

Medium samples collected from the non-inoculated filter during the start-up period of manganese removal indicated that the manganese removal was attained by both biological and physico-chemical processes (Day 35 and 39, Figure 5(a)). During that period, the ratio between physico-chemical and biological removal rates was 1 on average and showed no statistical difference over depth ($p > 0.05$ after Mann-Whitney test). In contrast, physico-chemical removal mechanisms appeared to dominate after the start-up period at the deeper biofilter layers (depth 20 and 30 cm) with an average ratio of 15, whereas biological mechanisms remained important for manganese removal at 10 cm depth of the filter with an average ratio of 0.5 (Day 48, Figure 5(a)).

The manganese removal rate of the non-inoculated filter at Day 48 due to physico-chemical processes was three times higher at 20 cm than at 10 cm depth (Figure 5(a)). Similarly, the amount of manganese coating on the grains of the non-inoculated filter at Day 48 was 3.5 times higher at 20 cm than at 10 cm depth (0.162 mg Mn/g medium and 0.046 mg Mn/g medium respectively, Figure 4).

In contrast to the non-inoculated filter, no time dependent change was observed in the manganese removal processes occurring in the inoculated filter. Filter medium samples from all depths showed that the manganese removal from Day 35 to Day 48 was mostly due to physico-chemical processes (Figure 5(b)). Further, the ratio between physico-chemical and biological removal rates showed no statistical difference over depth ($p > 0.05$ after Mann-Whitney test).

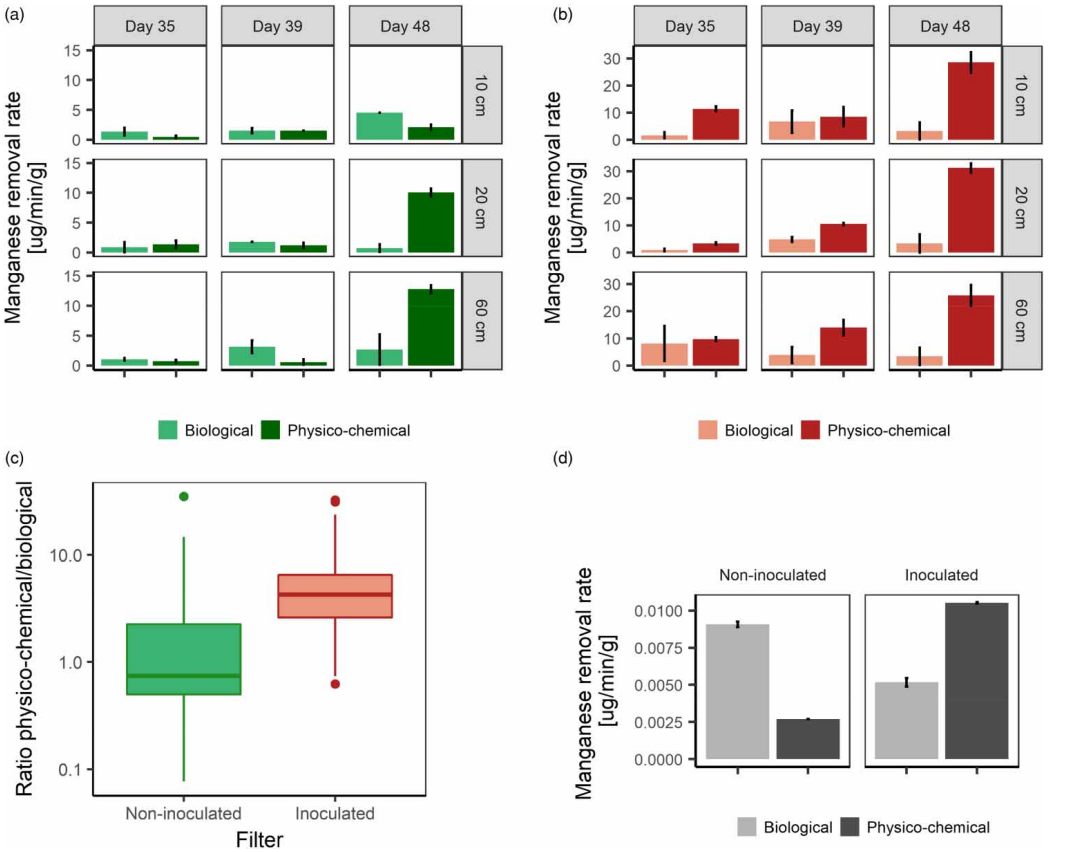

Figure 5 | Manganese removal rates in batch assay associated to biological and physico-chemical processes in filter medium samples in duplicates collected from depth 10, 20 and 60 cm on Day 35, 39 and 48 from (a) the non-inoculated filter and (b) the inoculated filter. (c) Boxplot of the ratio between physico-chemical and biological processes occurring at Day 35, 39 and 48 of both filters ($n = 18$). (d) Manganese removal rates in column assay associated to biological and physico-chemical processes in filter medium samples collected from 0–10 cm layer of both pilot filters at Day 72. Error lines indicate standard deviation between replicates.

The initially matured sand layers in the inoculated filter more than doubled their manganese removal rates over time from Day 35 to 48. This increase in manganese removal at 10 cm depth (initially matured sand) of the inoculated filter was also observed in the manganese profiles (Figure 3(b)). From Day 39, the initially virgin sand layer at 20 cm depth of the inoculated filter showed similar manganese removal rates as media samples from the initially matured sand layers (0–10 cm and 50–60 cm, Figure 5(b)). This similar performance in manganese removal at 10 cm and 20 cm depth (initially matured sand and initially virgin sand, respectively) was also observed in the manganese profiles of the inoculated filter (Figure 3(b)).

Overall, the sum of the physico-chemical and biological manganese removal rates in media samples from the inoculated filter was on average two times higher than the total manganese removal rates from the non-inoculated filter (Figure 5(a) and 5(b)). A two-fold difference between the filters was also observed at 20 cm depth by Day 48 in the total manganese removal of the pilot filters (Figure 3(c)) and in the MnOx coating (Figure 4).

The ratio between physico-chemical and biological removal rates of all medium samples was statistically different between the filters ($p < 1 \times 10^{-11}$ after Mann-Whitney test), with a 0.8 median for the non-inoculated filter indicating a more active role of biological processes, and a median of 4.3 for the inoculated filter suggesting a more active role of physico-chemical processes (Figure 5(c)).

A column assay with and without NaN_3 addition was conducted using filter medium collected at Day 72 from the inoculated and non-inoculated filters at 10 cm depth (Figure 5(c)). The results indicated that the contribution of biological processes continued to be most pronounced on samples from the top 10 cm of the non-inoculated filter (Figure 5(c)). In total the manganese removal rates observed in each column were similar to the manganese removal rates from the top 10 cm of each filter at Day 72 (0.009 μg/min/g medium in the non-inoculated filter and 0.014 μg/min/g medium in the inoculated filter).

Fully matured samples collected from the second stage filter of Fredensborg waterworks (Figure S1) showed a physico-chemical to biological manganese removal ratio of 5.6, indicating a limited contribution of biological means to manganese removal in matured biofilters (47 years old). This suggests that the contribution of biological mechanisms in the removal of manganese continues to be less prominent in fully matured biofilters compared to filters during start-up. Bruins (2016) showed a clear difference in structure between biologically and physico-chemically formed MnOx (Birnessite) in matured filters. Currently, the importance that biogenic manganese oxides might have in the long-term efficiency of manganese removal in matured biofilters is not known.

In related work, Vandenabeele *et al.* (1992) investigated the manganese removal capacity of matured sand (for decades removing manganese) in a PYM-medium (using peptone, yeast extract and manganese sulfate) with and without addition of NaN_3 (15 mM). The results showed that addition of NaN_3 reduced the manganese removal by 50% after 5 days of incubation but had no effect on the manganese removal under 24 h of incubation. In another study, Olańczuk-Neyman & Bray (2000) investigated the role of physico-chemical and biological processes in manganese removal from groundwater using NaN_3 (15 mM) on matured sand (no reference to maturation age but reported to remove manganese successfully). The results suggested that the removal of manganese on matured sand occurred mostly due to autocatalytic oxidation by the previously formed manganese oxides (by physico-chemical and biological processes). In a more recent work, Sahabi *et al.* (2009) investigated biotic and abiotic manganese removal in fully matured filter medium samples with and without NaN_3 (10 mM). The results showed that biological processes had a 50% contribution to manganese removal in a 3-year-old matured anthracite medium but no significant role in a 15-year-old mature anthracite medium (Sahabi *et al.* 2009). Hence, the results obtained in the current study complement previous investigations of matured filters, suggesting that the physico-chemical processes in manganese removal increase with filter medium age.

Bacterial diversity in inoculated and non-inoculated filters during the start-up period

Despite reducing the duration of the start-up period and enhancing the manganese removal capacity of the filter (Figure 2 and 5), the effect of initially matured sand layers on the microbial community formed on initially virgin sand layers is unknown for this inoculation method. To better understand this, source water samples and filter medium samples collected from several depths of both pilot-scale filters were analyzed by 16S rDNA amplicon sequencing.

The relative abundance of the top 20 most abundant bacterial genera of all samples is included in the supplementary materials. A PCA based on all taxa detected in each sample identifies two main clustering areas: initially virgin sand and initially matured sand (Figure 6). The microbial community of medium samples of initially virgin sand showed changes in diversity over time, e.g. three medium samples of initially virgin sand collected from the non-inoculated filter at Day 35 and 39 showed a closer proximity to the microbial diversity of the source water (Figure 6). In contrast, the microbial diversity on initially matured sand samples showed a clear clustering over time that was distinct when compared to the bacterial communities developed on the initially virgin sand. Overall, the microbial community of the medium samples clustered according to the filter medium type (initially

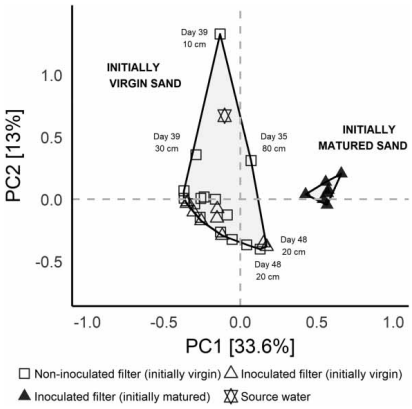

Figure 6 | PCA of bacterial community present in the source water and in filter medium samples collected at depth 10, 20, 30, 60 and 80 cm from both filters at Day 0, 35, 39 and 48. Each point represents the bacterial community present in the source water and in the medium samples collected from the non-inoculated filter and the inoculated filter.

virgin or matured), suggesting that the initially matured sand layers located at depth 10 and 60 cm of the inoculated filter had limited effect on the microbial community developed on initially virgin sand layers during the 48 days of the experiment (Figure 6). Hence, significantly longer time is likely required to obtain fully matured microbial communities.

Bai *et al.* (2016) investigated the microbial diversity after start-up of bioaugmented and non-bioaugmented columns removing Mn(II), Fe(II), As(III) and Sb(III) using a manganese oxidizing bacterium (*Pseudomonas* sp. QJX-1). Results showed higher overall treatment efficiency by the bioaugmented columns but no significant difference between the bacterial community of bioaugmented and non-bioaugmented columns after 120 days. Our results complement the previous ones by indicating limited immediate effect of inoculation on the microbial community when using a microbial consortium inoculation method with addition of matured sand.

CONCLUSIONS

Manganese removal and microbial community development of a non-coated virgin sand pilot filter with and without inoculation with 20% matured sand was monitored for a period of 72 days. Based on findings in this study, the following is concluded:

- The non-inoculated filter took 35 days to initiate significant manganese removal and 41 days to comply with manganese water quality criteria. The inoculated filter showed significant initial removal from Day 0 and compliance from Day 25.
- During the first 48 days of operations, similar amounts of manganese were removed in the inoculated filter by the top layer of matured sand and the following layer of virgin sand. By Day 48, the inoculated filter showed two times higher manganese removal rates and manganese in the coating of filter media.
- From the onset of manganese removal to compliance, both physico-chemical and biological processes contributed to the manganese removal in the non-inoculated filter. One week after compliance, biological mechanisms remained important for manganese removal at the top 10 cm of the non-inoculated filter, whereas physico-chemical processes were of more importance at deeper filter layers.
- The major manganese removal processes occurring in the filters were statistically different. The non-inoculated filter was dominated by biological processes, whereas physico-chemical processes were of more importance in the inoculated filter. Inoculation appeared to mainly enhance the physico-chemical manganese removal potential during start-up.
- The use of proactive inoculation by addition of matured filter sand contributes to a shorter start-up period of biofilters with limited effect on the microbial community developed in the adjacent layers of the filter.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

S1. Manganese removal rate under NaN_3 of matured sand collected from the filter at the waterworks

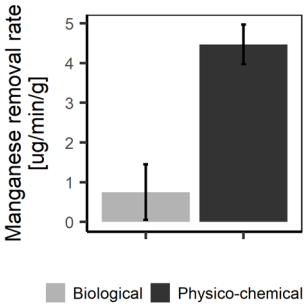


Figure S1 | Manganese removal rates in batch assay associated to biological and physico-chemical processes in fully matured filter medium samples collected from the second filter at Fredensborg waterworks (*n*=10). Average of 10 replicates. Error lines indicate standard deviation between replicates in all plots.

S2. DNA extraction, qPCR amplification, 16S rDNA amplicon sequencing and library preparation

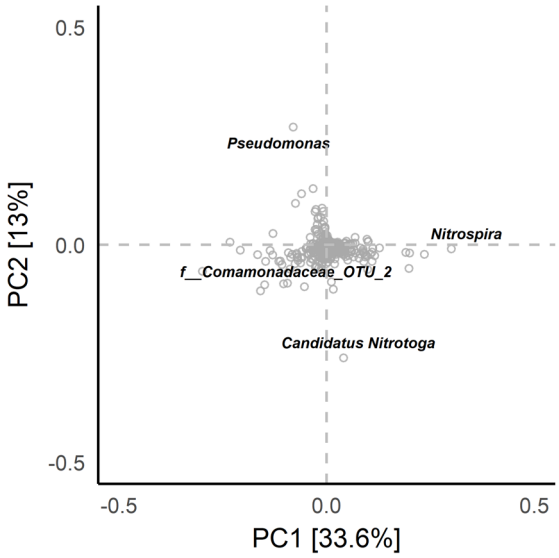
Polymerase chain reaction (PCR)

Bacterial V3-V4 16S rRNA gene sequencing libraries were prepared by a custom protocol based on an Illumina protocol (Illumina 2015). Up to 10 ng of extracted DNA was used as template for PCR amplification of the 16S gene fragments. Each PCR reaction (25 µL) contained dNTPs (100 µM of each), MgSO4 (1.5 mM), Platinum Taq DNA polymerase HF (0.5 U/reaction), Platinum High Fidelity buffer (1X) (Thermo Fisher Scientific, USA) and tailed primermix (400 nM of each forward and reverse primer). PCR was conducted with the following program: Initial denaturation at 95°C for 2 min, 35 cycles of amplification (95°C for 20 s, 50°C for 30 s, 72°C for 60 s) and a final elongation at 72°C for 5 min. Duplicate PCR reactions were performed for each sample and the duplicates were pooled after PCR. The forward and reverse tailed primers were designed according to Illumina (2015) and contain primers targeting the bacterial 16S rRNA gene V3-4 regions (Herlemann *et al.* 2011): 5'-CCTACGGGNGGCWGCAG (341F) and 5'-GACTACHVGGGTATCTAATCC (805R). The primer tails enable attachment of Illumina Nextera adaptors necessary for sequencing in a subsequent PCR. The resulting amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Bead (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 µL of nuclease free water (Qiagen,

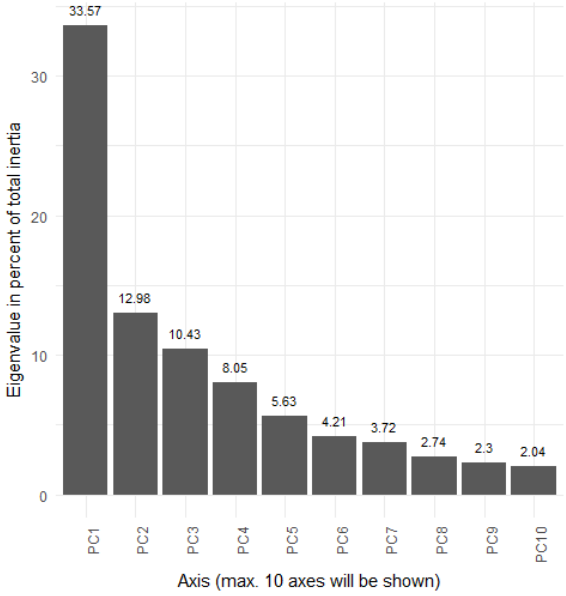
Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.
Sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 µL) contained PCRBIO HiFi buffer (1x), PCRBIO HiFi Polymerase (1 U/reaction) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. PCR was conducted with the following program: Initial denaturation at 95°C for 2 min, 8 cycles of amplification (95°C for 20 s, 55°C for 30 s, 72°C for 60 s) and a final elongation at 72°C for 5 min. The resulting sequencing libraries were handled as described previously.

DNA sequencing
The purified sequencing libraries were pooled in equimolar concentrations and diluted to 4 nM. The samples were paired end sequenced (2x300bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. A 20% Phix control library was spiked in to overcome the low complexity issue often observed with amplicon samples.

Bioinformatic processing
Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger *et al*. 2014). The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoc & Salzberg 2011). The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar 2013). The dereplicated reads were clustered and OUT abundances estimated using the id 0.97. Taxonomy was assigned using the RDP (Ribosomal Database Project) classifier (Wang *et al*. 2007) as implemented in QIIME (Caporaso *et al*. 2010), using the MiDAS database v.1.23 (McIlroy *et al*. 2017), which is a curated database based on the SILVA database, release 123 (Quast *et al*. 2013). The results were analyzed in R (R Core Team 2017) through the Rstudio IDE using the ampvis package v.2.3.0 (Albertsen *et al*. 2015).



A



B

Figure S2 | A. PCA loadings of the top 4 most abundant bacteria. **B.** Distribution of eigenvalues for the first 10 principal components axis.

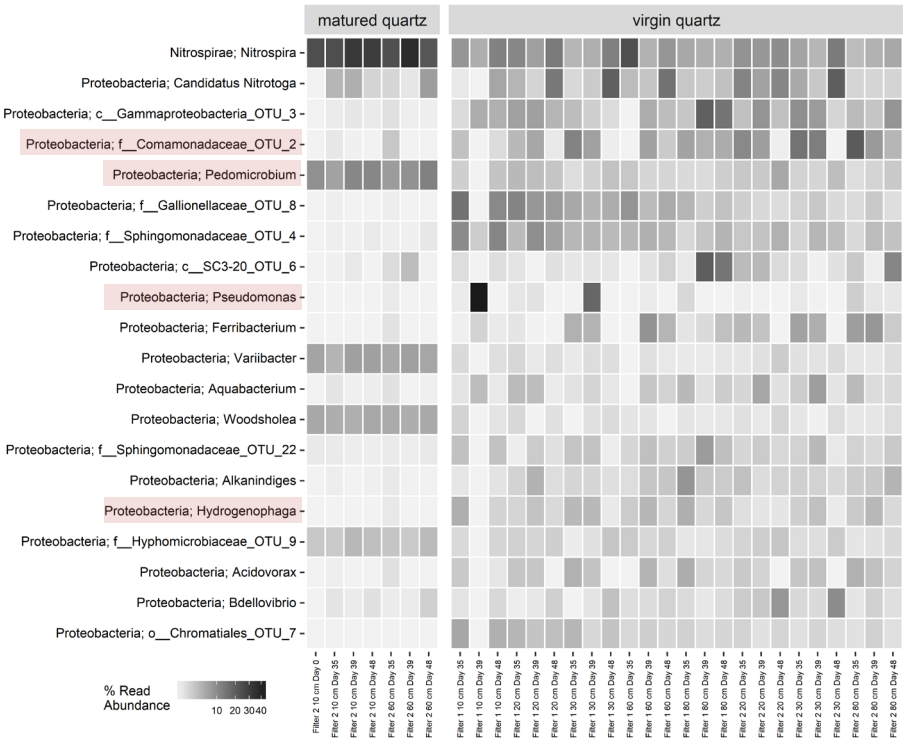


Figure S3 | Most abundant taxa in all samples collected at depths 10, 20, 30, 60 and 80 cm from Filter 1 (Non-inoculated) and Filter 2 (Inoculated) at Day 0, 35, 39 and 48. Red shading identifies genus that contain species which have been identified as potential manganese removers.

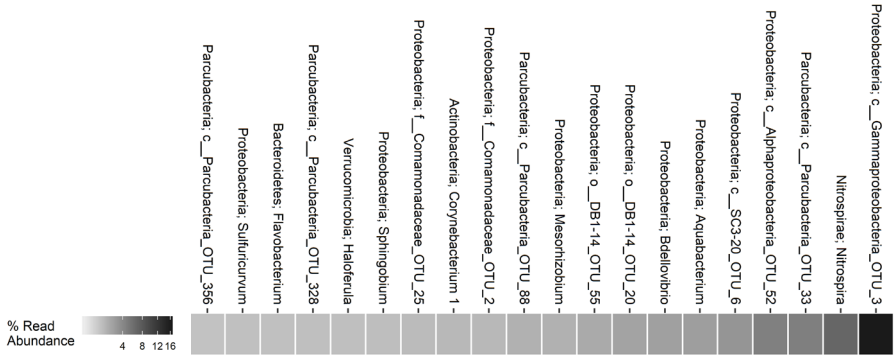


Figure S4 | Most abundant taxa in the source water.

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PAPER III

MANGANESE REMOVAL PROCESSES OCCURRING AT 10 DRINKING WATER TREATMENT PLANTS USING GROUNDWATER

Submitted to Water Quality Research Journal

INÊS L. BREDA, LOREN RAMSAY, DITTE A. SØBORG,
RENI DIMITROVA & PETER ROSLEV

Manganese removal processes at 10 groundwater fed full-scale drinking water treatment plants

Short title: Mn removal in 10 full-scale drinking water filters

I.L. Breda[a,b#], L. Ramsay[c], D.A. Søborg[c], R. Dimitrova[a], and P. Roslev[a]

[a]Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220 Aalborg, Denmark
[b]Project and Development Group, Skanderborg Forsyningsvirksomhed A/S, Døjsøvej 1, 8660 Skanderborg, Denmark
[c]Research Group for Energy and Environment, VIA University College, Chr. M. Østergaards Vej 4, 8700 Horsens, Denmark

#Corresponding author: E-mail: ilb@bio.aau.dk Phone: +45 87 55 42 93

ABSTRACT

Manganese (Mn) removal in drinking water filters is facilitated by biological and physico-chemical processes. However, there is limited information about the dominant processes for Mn removal in full-scale matured filters with different filter materials over filter depth. Water and filter material samples were collected from 10 full-scale drinking water treatment plants (DWTPs) to characterize the Mn removal processes, evaluate the potential use of enhancers, and gain further insight on operational conditions of matured filters for efficient Mn removal.

The first-order Mn removal constant at the DWTPs varied from 10^{-2} to 10^{-1} min^{-1}. The amount of Mn coating on the filter material grains showed a strong correlation with the amount of iron, calcium and total coating, but no correlation with concentration of ATP. Inhibition of biological activity showed that Mn removal in matured filters was dominated by physico-chemical processes (59-97%). Addition of phosphorous and trace metals showed limited effect on Mn removal capacity, indicating that enhancement of Mn removal in matured filters is possible but challenging. There was limited effect of filter material type (quartz, calcium carbonate, anthracite) on Mn removal in matured filters, which can be relevant information for the industry when assessing filter designs and determining returns of investments.

Keywords: ammonium, phosphorous, trace metals, sodium azide, filter material, removal capacity.

INTRODUCTION

Drinking water treatment from groundwater sources in Denmark is based on aeration and biofiltration (single or double stage). Bacteria play an essential role for removal of ammonium (NH_4), while iron (Fe) may be removed chemically following aeration. Removal of manganese (Mn) involves both biological and physico-chemical processes, but knowledge regarding the role of these mechanisms is very limited in fully matured, full-scale filters (Sahabi *et al.* 2009, Bruins *et al.* 2015, Breda *et al.* 2018).

Recent studies suggest that after a short initial sorption phase, Mn removal by a non-coated virgin filter material is initially biological, evolving to a predominantly physico-chemical removal process over time (Sahabi *et al.* 2009, Bruins et al. 2015, Breda *et al.* 2018). Sahabi (2009) characterized the Mn removal processes in a drinking water treatment plant (DWTP) using filter material samples collected from a specific depth of 6 full-scale matured filters with 3 and 15 years of maturation. Bruins (2015) characterized the origin of Mn oxides coating filter material samples collected from the top layer of a virgin quartz filter column during the initial 2 years of maturation. To the authors' knowledge, characterization of the Mn removal processes occurring at different depths of matured filters using different filter materials and located at different full-scale drinking water treatment plants (DWTPs) has not been reported.

Inadequate Mn removal in previously well-functioning, fully matured filters has been reported in previous studies (Bruins *et al.* 2014). Review of operational conditions for efficient Mn removal in matured filters and investigation of possible performance enhancers could potentially help utilities combat Mn breakthrough and/or increase the resilience to variations in flow and concentration in functional filters.

Enhancement strategies promoting NH_4 removal capacity of matured filters have been investigated in previous studies, especially addition of trace metals (TM) and phosphorous (P) to enhance nitrification (de Vet *et al.* 2012, Wagner *et al.* 2017). Hence, addition of key elements to nutrient limited matured filters could potentially enhance Mn oxidation facilitated by biological processes.

Even though biofiltration is common in drinking water treatment, several aspects of filter performance and optimization are sometimes referred to as a "black box" (Zhu et al. 2010, Tatari et al. 2014, Zhang et al. 2018a). The present study contributes to the overall knowledge of drinking water filters by collecting both water and filter material samples over depth at 10 full-scale DWTPs with distinct source water

qualities.The aim of this study was to characterize the Mn removal processes (biological or physico-chemical) occurring at different filter depths, and to evaluate the potential use of trace metals and phosphorous to enhance the Mn removal capacity of matured filters. In addition, this study provides practical advice that can be used by water utilities to manage operations and obtain efficient Mn removal, such as role of filter material coating and importance of filter material selection.

MATERIALS AND METHODS

Sampling at 10 DWTPs

This study investigated 10 DWTPs treating groundwater in Denmark: 5 located in the city of Aarhus and 5 located in the city of Odense (Dalum, Elsted, Holmehaven, Hovedværket, Kasted, Lindved, Lunde, Stavtrup, Truelsbjerg and Østerby DWTP, labeled DWTP1-10). Location and multivariate analysis on the groundwater quality feeding each DWTP is available in the Supplementary Materials S1. Mn was efficiently removed to below the national criterion of 0.05 mg/L (BEK 1068, 2018) at all DWTPs over the last 20 years.

The removal of iron (Fe), NH_4 and Mn by the DWTPs included in this study occurs in the first filter. In the present investigation, water and filter material samples were collected from the first filter of each DWTP before backwash, i.e. at the end of the ripening cycle of the filter.

Water samples (100 mL) were collected at the filter's inlet, every 10 cm within the filter bed and at filter's outlet. Collection of filter bed water samples was accomplished by a multiple-depth sampling device with separate screens every 10 cm which was lowered into the filter bed during backwash and left in the filter until the following backwash. Water samples were withdrawn from the screens with a multichannel peristaltic pump (Cole Parmer, USA). Samples for analysis of chemical parameters were collected after pH, redox, oxygen (O_2) and temperature levels were stable in the withdrawn water. Inline measurements of pH, redox and temperature were recorded using a digital pH electrode (SENTIX940), a redox sensor (ORP-T900), an optical dissolved O_2 sensor (FDO 925) and a Multi 3430 meter (WTW GmbH, Germany). Redox measurements were corrected to temperature conditions of 10°C. Filtered (0.45 um) and non-filtered water samples were analyzed for Fe and Mn (DIN EN ISO 17294-2: 2017-01, Agrolab, Germany), and non-filtered water samples were analyzed for NH_4 (DIN ISO 15923-1: 2014-07, Agrolab, Germany). Treated water (15 L) was collected at each DWTP to use in batch experiments (described later in this section).

Filter material was sampled after draining of the filters overnight. Filter material samples (approx. 500g) were collected from the filter bed over 20 cm depth intervals. This was accomplished with a sampling device constructed with a 50 mm PVC pipe lowered into the sand bed and a shop vac to lift the sand through the pipe and into a 500 mL blue-cap sampling flask, after separating sand and air/water in a hydrocyclone. After the pipe was lowered 20 cm, the flask was replaced with a new one, and the pipe was lowered another 20 cm.

Characterization of matured filter material

The filter material total coating mass was calculated by subtracting the mass of the grains before and after acid digestion and drying. Acid digestion was conducted at room temperature in triplicates using approx. 2g of dried filter material in a 50 mL mixture of 4 M hydrochloric acid (HCl, Merck KGaA) and 2 g/L oxalic acid ($C_2H_2O_4$, Merck KGaA) as described by De Vet *et al.* 2009. The filter material type (e.g. sand, anthracite) was classified visually after acid digestion. The filter material coating composition (Fe, Mn, Ca, P) was determined by Eurofins (Denmark), after acid digestion, following Standard Methods 3120 (APHA/AWWA/WEF, 1989).

Batch experiment to assess the effect of NaN_3 and NH_4 on the Mn removal capacity

A batch experiment was conducted to investigate the Mn removal capacity in the presence of NaN_3 and NH_4 using filter material from each DWTP collected over 20 cm depth intervals. Filter material (5 g) was placed in 9 serum bottles with 50 mL treated water (collected at the same DWTP as the filter material). Three of the bottles were pretreated by spiking with a 25 mM solution of NaN_3 (Merck KGaA) to inhibit biological activity. All bottles were then placed on a shaker (100 rpm) and incubated overnight at 16°C. After the incubation period, the bottles were spiked with Mn ($MnCl_2 \cdot 4H_2O$, Emsure ACS) and NH_4 (NH_4Cl, Bie&Berntsen) resulting in triplicates of three different types of additions: i. 1 mg/L Mn (control), ii. 1 mg/L Mn and 1 mg/L NH_4, and iii. 1 mg/L Mn and 25 mM NaN_3. Aliquots (2.5 mL) were collected after 30 and 60 minutes, filtered (0.22 µm), and analyzed for Mn according to the manufacturer's instructions (kit LCK304, Hach, and a Multiskan FC Microplate Photometer, Thermo Fisher Scientific). NH_4 was analyzed using an autoanalyzer (Technicon TRAACS 800). The bottles were shaken (100 rpm) at 16°C during the experiment.

Batch experiment to assess the effect of P and TM on the Mn removal capacity

A batch experiment was conducted to investigate the Mn removal capacity in the presence of P, TM and both P and TM, using filter material from each DWTP collected at the top 20 cm, middle 20 cm layer and bottom 20 cm. Filter material (5g) was placed in serum bottles with 50 mL of treated water spiked with 1 mg/L Mn and 1 mg/L NH_4. Triplicate bottles were spiked with a concentrate of P (1 M P using $NaHPO_4$ and KH_2PO_4 buffered at 6.8-7.0 pH), TM (1.7 µg/L Zn, 2.4 µg/L Mo, 1.4 µg/L Mn, 2.5 µg/L B, 1.5 µg/L Co, 1.5 µg/L Ni, 6.4 µg/L Cu), and both P and TM. A remaining set of triplicate bottles were used as control. All bottles were then placed on a rotation table (100 rpm) and incubated for 7 days at 16°C. The solutions of each bottle were renewed every 3 days to ensure the availability of substrate and nutrients. After the incubation period, the bottles were spiked with a concentrate of Mn ($MnCl_2 \cdot 4H_2O$, Emsure ACS) and NH_4 (NH_4Cl, Bie&Berntsen) resulting in an initial concentration of 1mg/L Mn and 1mg/L NH_4. Aliquots (2.5 mL) were collected after 1h and 1h30, filtered (0.22 µm) and analyzed for Mn and NH_4 as described previously. The bottles were shaken (100 rpm) at 16°C during the experiment.

First-order removal constant and removal capacity

The removal constant was calculated for each full-scale filter at each 10 cm depth interval assuming first-order removal, as follows:

$$\frac{d[C_{out}]}{dt} = -k[C_{in}] \quad \Rightarrow \quad k = -\frac{\ln\left({}^{[C_{out}]}/_{[C_{in}]}\right)}{t} \qquad (1)$$

where: k is the first-order removal constant (min^{-1}), $[C_{in}]$ and $[C_{out}]$ are the total concentration of C in water samples collected at the top and bottom of each depth interval (mg/L), and t is the empty bed contact time (EBCT) at each depth interval (min), calculated as:

$$EBCT = \frac{depth\ interval}{v} \qquad (2)$$

where: $depth\ interval$ is the distance between sampling ports placed across the full-scale filter depth (0.01 m) and v is the filtration rate of the full-scale filter (m/min).

The removal capacity R_c of the filter material (µg/min/g) was determined through the batch assays (previously described). The constant k (min^{-1}) was determined as the slope of a first-order regression of C (mg/L) over time t (min) by replacing C_{in} and C_{out} with $C_{initial}$ and C_{final}, respectively in equation 1. R_c was then calculated by multiplying k (min^{-1}) by the quotient of the initial mass of the substrate m_{sub} (µg) and the mass of the filter material sample used in the batch m_{fm} (g), as follows:

$$R_c = k \frac{m_{sub}}{m_{fm}} \quad (3)$$

Statistics

Data analysis was performed in R (R Core Team 2017). The difference between conditions in batch assays was evaluated using the non-parametric Mann-Whitney test. Correlations between variables were examined with a scatterplot and calculation of the Pearson correlation coefficient. Shapiro-test was used to evaluate the normality of Mn R_c of each DWTP. ANOVA was used to investigate the effect of filter material type on Mn removal capacity, and filter material age on Mn coating.

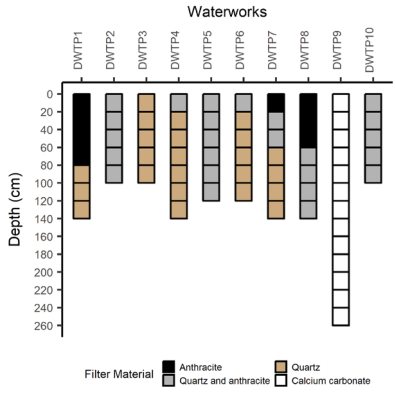
RESULTS AND DISCUSSION

First-order removal constant at each DWTP

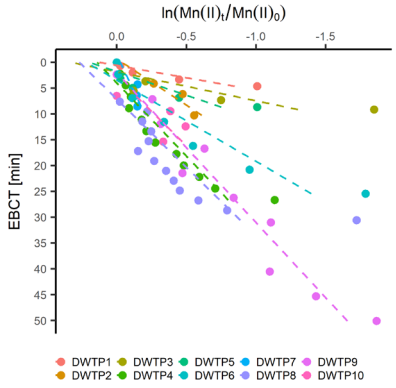
Drinking water filters are often composed of single or dual filter material. In the present study, 2 filters were composed of a single filter material (DWTP3 with sand and DWTP9 with calcium carbonate), and 8 filters were composed of dual filter materials (sand and anthracite). Some of those filters showed a complete mix of filter material throughout the filter depth (DWTP2, DWTP5, DWTP10), while others showed a strong stratification (DWTP1, DWTP8, Figure 1A).

Water samples collected from each 10 cm depth intervals of the filters at the 10 DWTPs were used to calculate *k*. Figure 1B shows approximate linearity between $\ln([Mn(II)_t]/[Mn(II)_0])$ and EBCT for each DWTP suggesting that Mn removal can be approximated by a first-order removal. The first-order removal constant *k* (min^{-1}) was determined for each DWTP as the slope from the linear regression between $\ln([Mn(II)_t]/[Mn(II)_0])$ and EBCT and varied from 0.02 to 0.17 min^{-1} between DWTPs (Figure 1C). Similar values (0.0001 to 0.120 min^{-1} and 0.17 min^{-1}) were reported by Vries (2017) for heterogeneous Mn removal at 5 DWTPs, and by Katsoyiannis and Zouboulis (2004) in a column filter treating groundwater, respectively.

The pH and redox measurements in water samples at each 10 cm depth from the 10 DWTPs showed an increase in redox potential with filter depth (Figure 1D). A similar increase of redox potential along the filter depth in filters removing Fe, Mn and NH_4 has been reported previously (Tekerlekopoulou *et al.* 2008).



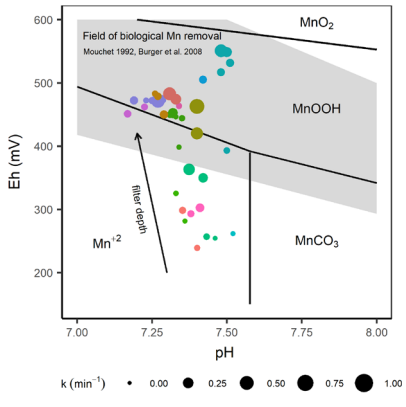
A.



B.

DWTP	k (min^{-1})	$t_{1/2}$ (min)	SE	R^2
1	0.173	4.01	0.028	0.903
2	0.061	11.4	0.006	0.956
3	0.132	5.25	0.035	0.777
4	0.028	24.8	0.003	0.901
5	0.079	8.77	0.019	0.805
6	0.052	13.3	0.007	0.898
7	0.021	33.0	0.003	0.929
8	0.025	27.7	0.004	0.744
9	0.032	21.4	0.002	0.980
10	0.029	23.9	0.007	0.844

C.



D.

Figure 1 | A. Filter material distribution over depth at each DWTP. **B**. Analysis of linearity between ln([Mn(II)$_t$]/[Mn(II)$_0$]) and EBCT in water samples with Mn concentration above 0.05 mg/L. **C.** First-order removal constant *k* at each DWTPs, $t_{1/2}$ is the half-life, *SE* and the R^2 are the standard error and multiple R-squared of the linear regression, respectively. **D.** Pourbaix diagram with data points indicating pH and redox in water at each 10 cm depth of the filters until the Mn criterion was met. Color distinguishes DWTPs. Size distinguishes removal rates at each 10 cm depth of the filter. Grey shading shows the field of biological Mn removal (Mouchet 1996, Burger *et al.* 2008). Pourbaix diagram based on equilibrium constants given by PHREEQC using *wateq4f* database (Parkhurst and Appelo, 1999) and the following conditions 10 °C, 0.260 mg Mn(II)/L, 275 mg HCO_3/L.

Some studies in Mn removal by drinking water filters suggest that there is a "Field of biological Mn removal" limited by pH and redox potential (shaded area in Figure 1D, Mouchet 1992, Burger *et al.* 2008, Dangeti *et al.* 2016). The pH and redox at most depths investigated in the present study fall within this field, suggesting that the filters investigated in this study were running under pH and redox values suitable for biological removal of Mn. Further, higher values of removal constants were registered within the "Field of biological Mn removal" than outside of this area, indicating that biological conditions for Mn removal could potentially contribute to higher values of *k*.

Characterization of filter material coating with relation to physico-chemical Mn removal

Mn present on the coating of filter material grains collected over depth at each DWTP varied from 0.58 to 67 g/kg. A large variation in Mn from 0.01 to over 120 g/kg was also reported by Islam *et al.* (2010) on filter material samples collected at 14 DWTPs.

The Mn coating present in filter material from DWTPs under 10 years of operation was statistically lower than from the ones running for over 10 years ($p<0.05$ after ANOVA). This can be caused by an accumulation of Mn on the filter grains over time. A previous investigation using backwash samples from DWTP8 indicated that over 90% of Mn entering the filter was accumulated on the filter grains (Breda *et al.* 2016). Although the autocatalytic properties of Mn oxides present in the coating of the grains contribute to the Mn removal, the amount of Mn in a thick coating does not represent the amount of Mn in contact with the water phase. Thus, a higher amount of Mn in the coating does not necessarily mean higher Mn removal efficiency.

The coating was dominated by Fe and Mn, with a Fe/Mn ratio median of 2 ranging from 0.7 to 2.9 (10^{th}-90^{th} percentile, respectively). The correlation coefficients between elements present in the coating of the filter grains were determined using the Pearson method (Figure 2A). Mn present in the coating showed a strong positive correlation with total coating, Fe, Ca, and P but no correlation with ATP (Figure 2A-B). The lack of correlation between ATP and Mn does not preclude that biological activity has no effect on the presence of Mn in the coating of the grains, since ATP is influenced by many microorganisms in addition to Mn oxidizing bacteria (MnOBs).

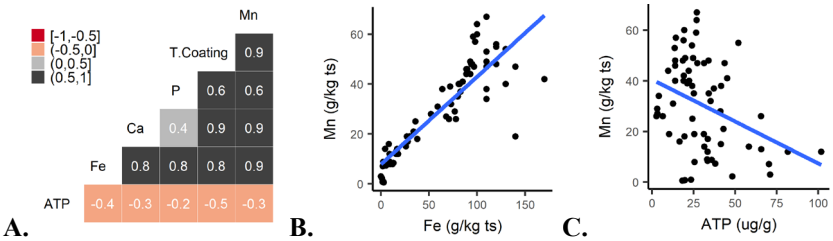


Figure 2 | A. Pearson correlation between selected filter material properties from filter medium samples collected at 20 cm depth intervals of each filter. **B.** Correlation plot between amount of Mn and amount of Fe. **C.** Correlation plot between amount of Mn and concentration of ATP.

Effect of the biological inhibitor NaN_3 on the Mn R_c

The Mn R_c was determined in batch assays using filter material collected over 20 cm depth intervals of each filter. Even though there was a change in filter material type through depth at some of the DWTPs (Figure 1A), the filter material type showed no significant effect on the Mn R_c ($p > 0.05$ after ANOVA). Water utilities often select a dual or alternative filter material to improve the operation conditions of the filters, e.g. reduction of cost and/or improvement of removal efficiency. But results from the present study question the value of type of filter material with regards to the Mn R_c of matured filters. One should consider that the R_c of virgin filter materials varies with the filter material type. For example, the Mn R_c of a virgin Mn sand is approx. 30 times superior to the one from virgin sand (Supplementary Materials S2). Thus, selection of filter material type can be important during the initial stages of the filter maturation, more specifically during the start-up period. In contrast, the present investigation suggests that there is no effect of filter material type on Mn R_c after a maturation time of 3 years. This information is of interest to the industry for assessing the filter design and determining the return of investments.

NaN_3 is an inhibitor of respiratory activity in microorganisms, while it does not appear to affect autocatalytic properties of Mn oxide coatings (Rosson *et al.* 1984). This was observed in an additional assay, in which NaN_3 showed limited effect on the Mn R_c of virgin quartz sand and virgin Mn sand (Supplementary Materials S3). To characterize the Mn removal processes occurring at functional full-scale filters, the effect of NaN_3 on the Mn R_c was investigated using the filter material samples collected over filter depth. Mn removal attained by filter material samples without NaN_3 was assumed to be due to biological and physico-chemical processes, whereas Mn removal attained by filter material samples with NaN_3 was assumed to be mainly due to physico-chemical processes alone.

In the present study, the Mn R_c was statistically lower in the presence of NaN_3 ($p<0.05$, Mann-Whitney test). Mn removal by physico-chemical processes dominated in most filters, representing approx. 77% of the Mn R_c of the matured filter material (Figure 3A). The effect of NaN_3 for each DWTP (average of triplicates over depth) ranged from 7 to 30% (DWTP5< DWTP1< DWTP8< DWTP9< DWTP2< DWTP4< DWTP10< DWTP6< DWTP7< DWTP3). As all the 10 DWTP were efficiently removing Mn, the results suggest that dominance of biological removal of Mn is not required to attain efficient removal of Mn in full-scale filters with over 3 years of maturation.

The effect of NaN_3 on the Mn R_c of samples collected over 20 cm depth intervals ranged from 3% to 41% (10^{th}-90^{th} percentile, respectively), with no correlation between filter depth and effect of NaN_3 at any DWTP (correlation factor of 0.3 after Pearson).

Sahabi *et al.* (2014) concluded that Mn removal in filter material matured for more than 3 years is mostly due to physico-chemical processes, and Bruins *et al.* (2015) concluded that Birnessite (Mn oxide) formation became a predominantly physico-chemical process with the progress of filter maturation and development of the filter material coating. Even though the highest removal rates were registered within the "Field of biological Mn removal" (Figure 1D), and previous investigations suggesting that MnOBs are prevalent in drinking water systems (Marcus *et al.* 2016), batch tests indicate that physico-chemical processes dominate the Mn removal in the DWTPs (Figure 3A).

Biological removal of Mn may still be of importance in maintaining the filter function over time, since Mn oxides of biogenic origin show higher autocatalytic properties (Bruins *et al.* 2015). Inadequate Mn removal in previously well-functioning fully-matured filters might be related to the absence of biogenic Mn oxides, resulting in limited biological removal of Mn. Classification of Mn removal processes over depth in filters experiencing Mn breakthrough could provide further insight into the causes for such events.

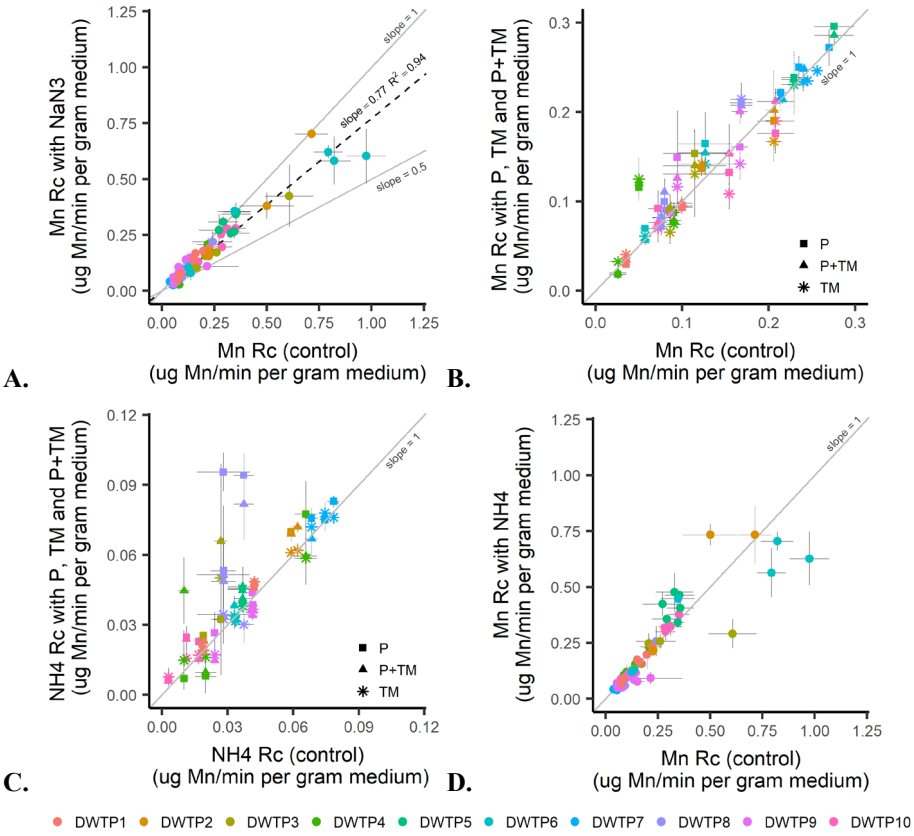


Figure 3 | A. Mn R_c with NaN_3 versus Mn R_c without NaN_3 (control). Dashed black line represents linear regression. **B.** Plot of Mn R_c with P, TM and P+TM versus Mn R_c without potential enhancers (control). **C.** Plot of NH_4 R_c with P, TM and P+TM versus NH_4 R_c without potential enhancers (control). **D.** Plot of Mn R_c with NH_4 versus Mn R_c without NH_4 (control). Color distinguishes DWTPs. Error-bars show the standard deviation of triplicates.

Effect of potential enhancers P, TM, and P+TM on the Mn *Rc*

To investigate the effect of potential enhancers on the Mn R_c of fully matured filters, filter material samples collected from the top, middle and bottom 20 cm of the filters at each DWTP were used in a batch assay to determine the Mn R_c with and without the addition of P, TM, and P+TM. Concentration profiles of water samples from individual DWTPs showed that Mn and NH_4 are removed around the same filter depths. For that reason, the batch assay used a spike of both NH_4 and Mn to investigate the effect of the referred compounds on the R_c of the filter material.

Collectively the results indicated no statistical difference in Mn R_c with and without potential enhancers ($p>0.05$, Mann-Whitney test). However, the Mn R_c increased in the presence of the potential enhancers (P, TM and P+TM) in filter material samples collected at 120-140 cm filter depth from DWTP4 and DWTP8, by a factor of 2.4 and 1.2, respectively (Figure 3B). These results can be relevant for the water utilities managing DWTP4 and DWTP8, as the respective Mn removal constant k at both DWTPs were among the lowest (Figure 1C).

Regarding the overall effect of the potential enhancers on NH_4, results showed that the NH_4 R_c was statistically greater when P and P+TM were added ($p<0.05$), but not for TM alone ($p>0.05$). Previous studies have reported performance enhancement in NH_4 removal when individually dosing P and TM (de Vet *et al.* 2012, Wagner *et al.* 2017, Zhang *et al.* 2018b). Wagner *et al.* (2017) concluded that Cu dosing enhanced nitrification of poorly performing full-scale rapid sand filters while remarking that pilot column filters could not always successfully reproduce full-scale filter behavior.

DWTPs showing a positive effect of the potential enhancers on the Mn R_c were not the same as the ones showing a positive effect on the NH_4 R_c. Further, enhancers showed a stronger effect on NH_4 R_c than on Mn R_c. Nonetheless, and despite of Mn removal being mostly dominated by physico-chemical processes (Figure 3A), our results suggest that there might be possibilities to increase the Mn R_c of matured filters through enhancement strategies.

Operational conditions

Bruins *et al.* (2014) carried out a multivariate statistical analysis using PCA on 34 parameters of 100 DWTPs treating groundwater followed by univariate statistics to determine limiting operational conditions for efficient Mn removal. The study concluded that efficient removal of Mn correlated with 6 parameters: NH_4 removal efficiency, Fe loading per filter run, pH of the filtrate, EBCT, filtration rate and oxygen in the filtrate. These 6 operational parameters for the 10 DWTPs investigated in the present study and corresponding Mn removal efficiencies are summarized in Table 1.

Table 1 | Operational conditions of the 10 filters.

Operational conditions	Bruins *et. al* 2014	DWTP									
		1	2	3	4	5	6	7	8	9	10
Manganese removal [%]	-	99	97	92	80	99	99	98	99	99	99
Ammonium removal [%]	> 85	97	98	67	45	97	99	85	85	87	65
Iron loading per filter run [kg/m^2]	< 2.7	0.2	0.2	0.3	0.3	0.1	0.2	0.4	0.7	0.2	0.8
pH of filtrate	> 7.1	7.4	7.5	7.4	7.4	7.5	7.3	7.4	7.3	7.3	7.4
Empty bed contact time [min]	> 11	19	23	15	31	27	55	27.8	34	69	48
Filtration rate [m/h]	< 10.5	4.5	2.9	3.3	2.7	3.2	1.3	2.8	3.1	2.5	2.0
Oxygen in filtrate [mg/L]	> 1	9.9	10.7	9.9	9.4	11.1	9.3	9.5	8.1	6.7	10.8

Efficient Mn removal was achieved in all DWTPs included in this study, and nearly all operational conditions were within the operational conditions determined by Bruins *et al.* (2014). However, the operational condition > 85% NH_4 removal was not met at DWTP3, DWTP4 and DWTP10. For DWTP3, a low NH_4 removal efficiency (67%, Table 1) was observed even though this DWTP had the second highest *k* for Mn (Figure 1B and 1C). This suggests that Mn removal efficiency is not always dependent on the compliance of the 85% NH_4 removal minimum established by Bruins *et al.* (2014).

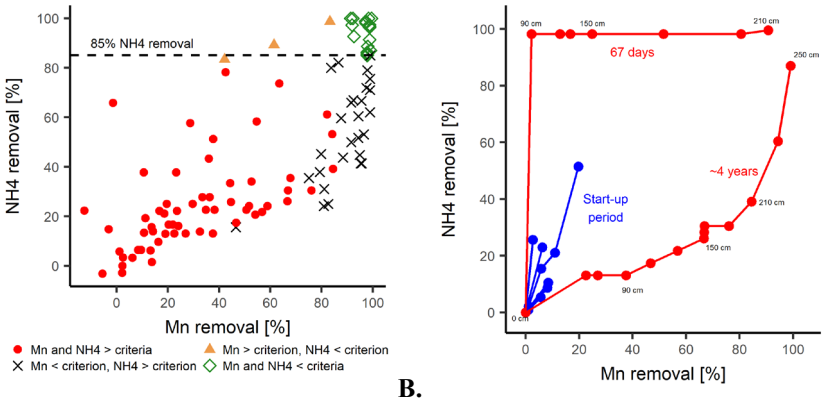
In the present study, the concentration of Mn and NH_4 measured in water samples collected over filter depth clearly showed that the Mn criterion was often met before the 85% NH_4 removal was reached (Figure 4A). More specifically, half of the observations with Mn below criterion are found in samples with 60% NH_4 removal (Figure 4A). In addition to the observations, *in situ* batch tests using matured filter material collected over 20 cm depth intervals of the filters at the 10 DWTPs showed that 1 mg/L NH_4 had no significant detrimental effect on the Mn R_c ($p>0.05$ after Mann-Whitney test, Figure 3D).

Both negative and positive effects of simultaneous NH_4 and Mn removal have been reported (Vandenabeele *et al.* 1995, Lin *et al.* 2012). Further, previous research has demonstrated that the presence of nitrite can have an inhibitory effect on microbial oxidation of dissolved Mn (Brandhuber *et al.* 2013).

Recent studies have indicated that the contribution of biological processes for the removal of Mn varies with the age of the filter (maturation time). To the authors' knowledge, age of filter maturation was not included as a parameter in the PCA conducted by Bruins *et al.* (2014). It can be hypothesized that the degree of importance of the 6 operational parameters varies with the filters' ages.

A comprehensive description of the start-up period after construction of DWTP9 was conducted by Ramsay *et al.* (2018) including measurements of Mn and NH_4 in water samples collected over the filter depth during the start-up period and shortly after conclusion of the start-up period. According to the observations in this study, NH_4 complied with the national criterion before commencement of Mn removal (Figure 4B, 67 days). On the contrary, the present study shows that after 4 years of maturation, the same filter removes Mn at a lesser depth than NH_4 (Figure 4B). This can be explained by a sequence of events. Complete nitrification is required for the onset of Mn removal during the start-up period of a virgin filter. Thus, by the end of the start-up period, Mn removal often takes place in the bottom section of the filter, preceding NH_4 removal. Shortly after the start-up period, Mn continues to be removed in the bottom section of the filter, and Mn oxides start to coat the filter grains. During normal operations, the filters run in a sequence of ripening cycles and backwash procedures. The backwash promotes a transport of filter grains and deposits from the bottom section of the filter to the upper section of the filter. By the end of the backwash, Mn oxides initially accumulated in the bottom section of the filter are spread and attached to grains across the filter depth. As the ripening cycle restarts, Mn in the water phase starts to be removed across the filter depth due to the autocatalytic properties of Mn oxides. Consequently, the amount of Mn oxides coating the grains increases promoting further autocatalytic removal of Mn across the filter depth. This study indicates that the shift in the location of Mn removal from bottom to upper sections of the filter can lead to a change in the spatial order of the main removal processes occurring over the filter depth: from an initially stratification of Fe, NH_4 and Mn removal to Fe, Mn and NH_4 removal.

On a practical note, it is important to clarify that the end of the start-up period is not the end of the filter's maturation (Ramsay *et al.* 2018). During the start-up period, virgin filter material matures into a functional filter that can remove substances to drinking water criteria. However, the maturation of the filter continues, changing not only in the category of the removal processes occurring in the filter (e.g. Mn removal from biological to mostly physico-chemical), but also the stratification of the removal of specific substances over the filter depth (e.g. Mn and NH_4 removal, Figure 4B).



A. **B.**

Figure 4 | A. Mn versus NH_4 removal (percentage) from water samples collected at every 10 cm depth of the 10 DWTPs in relation to inlet concentrations at each DWTPs. Color and shape distinguish criterion conditions for Mn and NH_4 (national criteria of 0.05 mg/L for both Mn and NH_4). **B.** Mn versus NH_4 removal (percentage) over time (Day 11, 27, 35, 39, 43, 67 and approx. 4 years) calculated based on the concentration of each substance in water samples collected over 20 cm depth intervals in relation to the inlet concentrations at DWTP9. All data was retrieved from Ramsay *et al.* 2018 with exception of observations at 4 years.

CONCLUSIONS

After characterization of Mn removal at 10 full-scale DWTPs efficiently removing Mn, this study concludes that:

- The first-order removal constant for Mn in 10 fully matured DWTPs varied from 10^{-2} to 10^{-1} min^{-1}.
- Mn coating the filter material grains showed a strong correlation with the amount of Fe, Ca, P and total coating, but no correlation with the concentration of ATP.
- Mn removal was mostly mediated by physico-chemical processes in the matured filters. Addition of a known biological inhibitor (NaN_3) resulted in an approx. 23% reduction of the Mn removal capacity, ranging from 3% to 41% (10^{th}-90^{th} percentile, respectively) in filter material collected over filter depth at the different DWTPs. Collectively the results suggested that dominance of biological removal of Mn is not required to attain efficient removal of Mn.

- Addition of P and TM showed limited overall effect on the Mn removal capacity of matured filters. However, for a specific depth at two DWTPs, an increase in the Mn removal capacity was observed, suggesting that enhancement of Mn removal in matured filters is possible but challenging.
- In addition, this investigation provides practical guidance to the design and operation of drinking water filters for efficient Mn removal. Results showed a limited detrimental effect of NH_4 and a limited effect of filter material type (quartz sand, anthracite, calcium carbonate) on the Mn removal capacity of matured filters. Further, results indicate a switch in the spatial stratification of Mn and NH_4 removal processes over filter depth as the filter ages.

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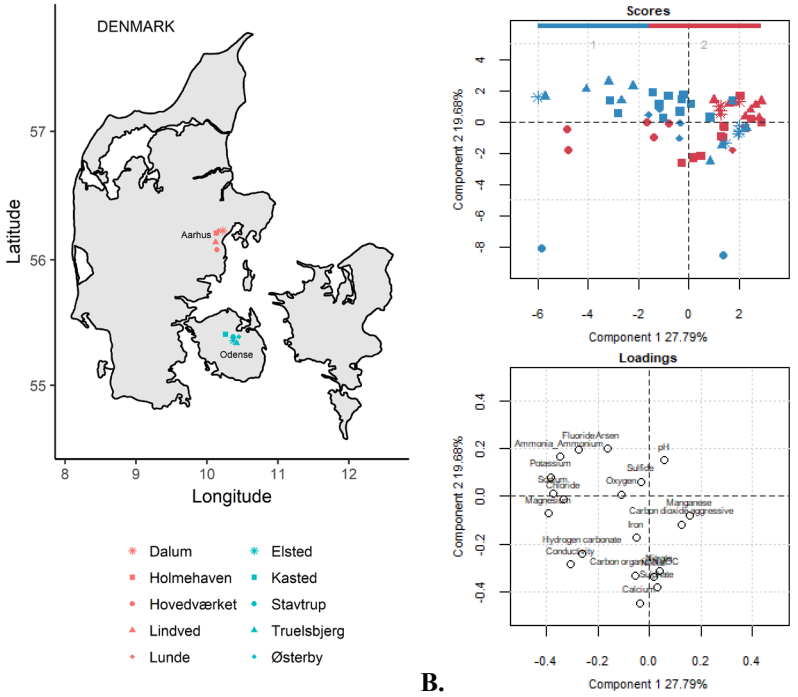
SUPLEMENTARY MATERIALS

S1. Location and groundwater quality feed to the 10 DWTPs included in this study

The location of each DWTPs is represented in Figure S1A. The groundwater quality (20 parameters) of the wells feeding each DWTP was retrieved from the Danish national geological database Jupiter (http://www.geus.dk/jupiter/ index-dk.htm) and illustrated in a Principal Component Analysis (PCA), Figure S1B. Considering the 3 main treatment parameters (iron, ammonium and manganese), wells from Aarhus (red) showed to have higher concentrations of manganese, whereas wells from Odense (blue) showed to have higher a concentration of ammonium (Figure S1B, Table S1). This difference was also observed in the water entering the filters at each DWTP, e.g. manganese concentration entering the filters at DWTPs located in Aarhus was on average 0.324 mg/L and in Odense 0.201 mg/L.



A. **B.**

Figure S1. A. Geografical location of the 10 DWTPs included in the study. Shape distinguishes DWTPs and color distinguishes city. **B.** PCA scores (top) and loadings (bottom) of groundwater quality from wells feeding each of the 10 DWTPs. Each dot represents the water quality of each well. Shape distinguishes DWTPs and color distinguishes city.

Table S1. Loadings for PC1 and PC2

Variable	PC1	PC2
pH	0.0555	0.1503
Conductivity	-0.3072	-0.2850
Calcium	-0.0369	-0.4462
Magnesium	-0.3917	-0.0735
Sodium	-0.3735	0.0110
Potassium	-0.3827	0.0795
Hydrogen carbonate	-0.2601	-0.2407
Chloride	-0.3320	-0.0130
Sulphate	0.0283	-0.3824
Nitrate	0.0382	-0.3152
Oxygen	-0.1106	0.0071
Ammonia_ammonium	-0.3479	0.1637
Iron	-0.0504	-0.1735
Manganese	0.1548	-0.0829
Carbon organic NVOC	-0.0575	-0.3314
Carbon dioxide aggressive	0.1239	-0.1175
Sulfide	-0.0334	0.0618
Fluoride	-0.2748	0.1949
Nikkel	0.0168	-0.3361
Arsen	-0.1617	0.1984

S2. Removal capacity of virgin sand and Mn sand

Filter material (5g for virgin sand and 2.5 g for Mn sand) was placed in serum bottles with 50 mL of treated water (Fredersborg DWTP, Skanderborg, Denmark). The bottles were skaking (100 rpm) and incubated overnight at 16°C. After the incubation period, the bottles were spiked with to an initial concentration of 0.25 mg/L and 1 mg /L Mn ($MnCl_2 \cdot 4H_2O$, Emsure ACS). Water samples (2.5 mL) were collected after 30 and 90 minutes, filtered (0.22 µm), and analyzed for Mn according to manufacturer's instructions (kit LCK304, Hach, and a Multiskan FC Microplate Photometer, Thermo Fisher Scientific). The bottles were skaking (100 rpm) at 16°C during the experiment.

The removal capacity of virgin sand and Mn sand was determined as the constant of a first-order removal. Results showed that the removal capacity Mn sand was approx. 30 times superior to the one from virgin sand.

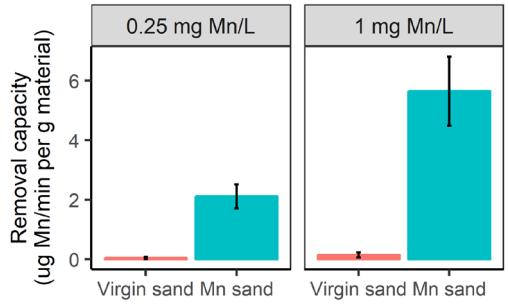


Figure S2. Removal capacity of virgin sand and Mn sand at two different initial concentrations of Mn (0.25 mg/L and 1 mg/L).

S3. Effect of sodium azide (NaN_3) on the removal capacity of virgin sand and Mn sand

The effect of NaN_3 on the sorption and autocatalytic properties of Mn oxides present on the coating of the filter grains was investigated in a batch assay, using virgin sand (Dansk Kvarts Ind. Denmark) and virgin Mn sand (Unitex, Poland) as filter material. Filter material (5g for virgin sand and 2.5g for Mn sand) was placed in serum bottles with 50 mL of treated water (Fredersborg DWTP, Skanderborg, Denmark). The bottles were spiked with a concentrate of NaN_3 (Merck KGaA) in triplicates (0, 10 and 25 mM). The bottles were shaking (100 rpm) and incubated overnight at 16°C. After the incubation period, the bottles were spiked with a Mn concentrate ($MnCl_2 \cdot 4H_2O$, Emsure ACS) to an initial concentration of 1 mg Mn/L. Water samples (2.5 mL) were collected after 30 and 90 minutes, filtered (0.22 µm), and analyzed for Mn according to manufacturer's instructions (kit LCK304, Hach, and a Multiskan FC Microplate Photometer, Thermo Fisher Scientific). The bottles were shaking (100 rpm) at 16°C during the experiment.

The removal capacity of virgin sand and Mn sand was determined assuming first-order removal. Results showed no significant difference in the Mn removal capacity between the different NaN_3 concentrations ($p>0.05$ after Mann-Whitney test), suggesting that NaN_3 had limited effect on the physico-chemical properties of the filter material.

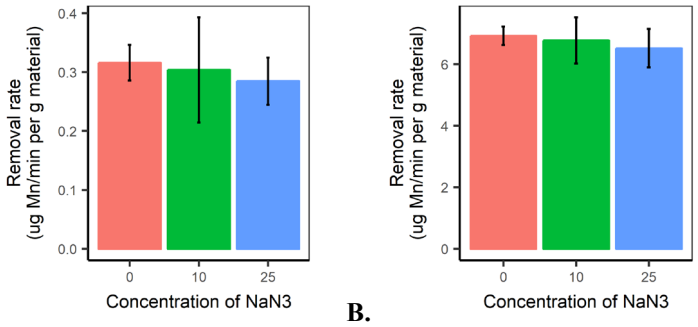


Figure S3. Mn removal capacity of quartz sand (**A**) and Mn sand (**B**) with NaN_3 concentrations of 0, 10, and 25 mM. Each bar represents the Mn removal capacity average of triplicates and the error bars show the standard deviation of triplicates. Colors distinguish concentrations of NaN_3.

PAPER IV

MICROBIAL DIVERSITY AND DISTRIBUTION IN 10 GROUNDWATER-FED FULL-SCALE DRINKING WATER BIOFILTERS IN DENMARK

In preparation

DITTE A. SØBORG, INÊS L. BREDA & PETER ROSLEV

Microbial diversity and distribution in 10 groundwater fed full-scale drinking water biofilters in Denmark.

D.A. Søborg [a#], I.L. Breda [b,c] and P. Roslev[b]

[a]Research Group for Energy and Environment, VIA University College, Chr. M. Østergaards Vej 4, 8700 Horsens, Denmark
[b]Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220 Aalborg, Denmark
[c]Group of Project and Development, Skanderborg Forsyningsvirksomhed A/S, Døjsøvej 1, 8660 Skanderborg, Denmark

#Corresponding author: E-mail: dans@via.dk Phone: +45 87 55 42 90

ABSTRACT

Studies of microbial diversity and distribution in drinking water biofilters treating groundwater are often limited by few samples within and across drinking water treatment plants (DWTPs). Here, microbial communities through the entire active depth of 10 groundwater fed full-scale drinking water biofilters in Denmark were analyzed. ATP concentration varied from 10^3 to 10^5 ug per g of filter material with distinct differences in stratification patterns among the DWTPs. 16S RNA gene sequencing revealed dominance by Proteobacteria, Nitrospirae, Planctomycetes, and Acidobacteria, which represented 82.6-93.6% of the abundance across the 10 examined biofilters. Core taxa of 22 genera (> 0.1% abundance) was shared between the biofilters of the 10 DWTPs. *Nitrospira*, *Methyloglobulus* and *Hyphomicrobium* were the most abundant genera in the biofilters with an average of 17%, 11% and 5% of read abundance. Bacterial genera with known importance for removal processes of iron, manganese and ammonium were present at all depths of the biofilters with high removal rates of the individual compounds.

Keywords: biofilters, microbial diversity, spatial distribution, core taxa.

INTRODUCTION

In Denmark, a simple treatment process consisting of an aeration step followed by rapid sand filtration in open (gravity) or closed (pressure) biofilters has been used for decades to produce drinking water from groundwater. This simple treatment process is enough to remove parameters such as iron (Fe), ammonium (NH_4^+) and manganese (Mn) from the groundwater and produce drinking water that in general is free of pathogens, is biologically stable, and does not require any form of disinfection.

The biofilters used at most Danish drinking water treatment plants (DWTPs) are based on one or several filter media, which are covered with an inorganic coating, and biofilm that play a key role in the treatment. The microbial community of the biofilm is influenced by the groundwater chemistry of the raw water and the filter material (Albers et al., 2015). The biofilters continuously receive aerated raw water from aquifers that are characterized by low temperature, variable or no oxygen, low levels of organic carbon and various concentrations of Fe, NH_4^+ and Mn as well as other electron donors (Danielopol, Pospisil and Rouch, 2000; Gülay et al., 2016).

Studies on diversity and distribution of microbial communities in groundwater fed biofilters have only recently started to be explored. Recent studies have identified bacterial genera with functional importance for the treatment processes. These include iron oxidizing bacteria (IOB), e.g. *Gallionella*, *Leptotrix,* and *Bacillus*, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), e.g. *Nitrosomonas*, *Nitrospira, Acinetobacter*, and *Comamonas* (Gülay et al., 2016), as well as manganese oxidizing bacteria (MnOB), e.g. *Crenothrix*, *Gallionella*, *Hyphomicrobium*, *Leptothrix*, *Metalloaenium*, *Pseudomonas*, and *Siderocapsa* (Yang et al., 2014; Li et al., 2013; Gülay et al., 2016; Cheng et al., 2017; Abu Hasan et al., 2012). Further, archaea such as ammonium oxidizing archaea (AOA), e.g. Candidatus *Nitrososphaera* has also been identified in groundwater fed biofilters (Cheng et al., 2017; de Vet et al., 2009).

The studies on microbial diversity, abundance and distribution in drinking water biofilters are often limited by not being performed in full-scale or by being based on few sampling sites within and across different biofilters and DWTPs. This makes it difficult to identify taxa that are shared between biofilters or DWTP and taxa that are specific to a given biofilter (Gülay et al., 2016). Further, relationships between operation conditions and the microbial community in a biofilter is lacking (Cheng et al., 2017). Another limitation is few samples through depths and little knowledge on relationships between microbial diversity and removal depths of treatment parameters. This makes identification of spatial variation difficult (Gülay et al., 2016). Further, while NH_4^+ removal is known exclusively to rely on microbial processes, such comparisons may also shed more light on the role of IOB and MnOB in Fe and Mn removal, respectively.

In the present study, microbial diversity and distribution was investigated in 10 groundwater fed full-scale drinking water biofilters. The study documented different process parameters and included water samples for every 10 cm and filter medium samples for every 20 cm of the entire active layers of the biofilters.

Thereby, this study was able to examine shared taxa between different DWTPs as well as correlate microbial diversity with different process parameters such as groundwater quality, flow and differences in removal depths of Fe, NH_4^+ and Mn.

MATERIALS AND METHODS

Waterworks and biofilters included in this study

Biofilters of 10 Danish DWTPs were sampled over the entire active depths of the filters. Nine DWTPs used single filtration in rapid gravity filters, while one DWTP used rapid pressure sand filters and double filtration. In the last case, only the pre-filter was sampled as all drinking water quality requirements were met for water leaving this filter. The DWTPs included: Dalum, Elsted, Holmehaven, Hovedværket, Kasted, Lindved, Lunde, Stavtrup, Truelsbjerg, and Østerby, named DWTP1-10 from this point. Location and composition of each DWTP is available in the supplementary materials.

Filter media samples

Filter media were collected from each DWTP at the end of the ripening period of the filters just before backwash. Filters were drained overnight before sampling and filter media samples (approx. 500g) were collected from the filter bed at 20 cm depth intervals down to a depth of 140 cm. This was accomplished with a sampling device constructed with a 50 mm PVC pipe lowered into the sand bed, and a shop vac to lift the sand through the pipe and into a 500 mL blue-cap sampling flask, after separating sand and air/water in a hydrocyclone. After the pipe was lowered 20 cm, the flask was replaced with a new, and the pipe lowered another 20 cm.

Water samples

Water samples were as filter media samples collected from each DWTP just before backwash. Water samples (100 mL filtered, 0.45 µm, and non-filtered water samples for Fe and Mn, 500 mL non-filtered water samples for NH_4) were collected at the filter's inlet, for every 10 cm within the filter bed and at filter's outlet. Collection of filter bed water samples was accomplished by a multiple-depth sampling device with separate screens for every 10 cm to a depth of 150 cm, which was lowered into the filter bed during backwash and left in the filter until the following backwash. Water samples were withdrawn from the screens with a multichannel peristaltic pump (Cole Parmer, USA). Samples were only taken after pH, redox, oxygen (O_2) and temperature levels were stable for the water withdrawn.

Water analyses of field parameters and Fe, NH_4^+ and Mn

Inline measurements of pH, redox and temperature were recorded using a digital pH electrode (SENTIX940), a redox sensor (ORP-T900), an optical dissolved O_2 sensor (FDO 925) and a Multi 3430 meter (WTW GmbH, Germany). Redox measurements were corrected to temperature conditions of 10°C. Water samples were analyzed for Fe and Mn (DIN EN ISO 17294-2: 2017-01), and NH_4 (DIN ISO 15923-1: 2014-07) by Agrolab, Germany.

Analysis of filter media coating

The coating of the filter media from different depth in the biofilters were analyzed as described previously (PAPER III).

ATP

ATP (adenosine triphosphate) of 2 g filter media was measured in triplicates using the deposit and surface analysis (DSA) kit and the PhotonMaster™ (both LuminUltra Technologies Ltd., Canada) according to the manufacturer's instructions.

DNA extraction from filter media

DNA extraction was performed using the standard protocol for FastDNA Spin kit for Soil (MP Biomedicals, USA) with the following exceptions. 500 µL of sample, 480 µL Sodium Phosphate Buffer and 120 µL MT Buffer were added to a Lysing Matrix E tube. Bead beating was performed at 6 m/s for 4x40s (Albertsen et al., 2015). Gel electrophoresis using Tapestation 2200 and Genomic DNA screentapes (Agilent, USA) was used to validate product size and purity of a subset of DNA extracts. DNA concentration was measured using Qubit dsDNA HS/BR Assay kit (Thermo Fisher Scientific, USA).

Bacterial community analysis targeting 16S V3-4 rRNA

Library preparation

Bacteria 16S rRNA gene region V3-4 sequencing libraries were prepared by a custom protocol based on an Illumina protocol (Illumina, 2014). Up to 10 ng of extracted DNA was used as template for PCR amplification of the Bacteria 16S rRNA gene region V3-4 amplicons. Each PCR reaction (25 µL) contained dNTPs (100 M of each), $MgSO_4$ (1.5 mM), Platinum Taq DNA polymerase HF (0.5 U/reaction), Platinum High Fidelity buffer (1X) (Thermo Fisher Scientific, USA) and tailed primer mix (400 nM of each forward and reverse primer). PCR was conducted with the following program: Initial denaturation at 95 ºC for 2 min, 35 cycles of amplification (95 ºC for 20 s, 50 ºC for 30 s, 72 ºC for 60 s) and a final elongation at 72 ºC for 5 min.

Duplicate PCR reactions were performed for each sample and the duplicates were pooled after PCR. The forward and reverse tailed primers were designed according to (Illumina, 2014) and contain primers targeting the Bacteria 16S rRNA gene region V3-4: [341F] CCTACGGGNGGCWGCAG and [805R] GACTACHVGGGTATCTAATCC (Herlemann et al., 2011). The primer tails enable attachment of Illumina Nextera adaptors necessary for sequencing in a subsequent PCR. The resulting amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 µL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

Sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 µL) contained PCRBIO HiFi buffer (1x), PCRBIO HiFi Polymerase (1 U/reaction) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. PCR was conducted with the following program: Initial denaturation at 95 ºC for 2 min, 8 cycles of amplification (95 ºC for 20 s, 55 ºC for 30 s, 72 ºC for 60 s) and a final elongation at 72 ºC for 5 min. The resulting sequencing libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 µL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

DNA sequencing

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. The samples were paired-end sequenced (2x300 bp) on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. >10% PhiX control library was spiked in to overcome low complexity issues often observed with amplicon samples.

Bioinformatic processing

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger, Lohse and Usadel, 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN: 275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoč and Salzberg, 2011) with the settings -m 10 -M 250. The trimmed reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97 –maxaccepts 0 -maxrejects 0. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using –confidence 0.8 and the MiDAS database v. 1.23 (McIlroy et al., 2017), which is a curated database based on the SILVA database, release 123 (Quast et al., 2013). The results were analysed in R v. 3.5.0 (R Core Team (2017) R: A language and environment for statistical computing) through the Rstudio IDE using the ampvis package v.2.3.19 (Albertsen et al., 2015).

Nucleic acid sequences

Raw sequences were deposited to GenBank.

RESULTS AND DISCUSSION

Biofilter performance of Fe, NH_4^+ and Mn

The performance of the biofilters from the ten DWTPs concerning Fe, NH_4^+ and Mn removal differed (Figure 1). However all DWTPs removed the compounds to compliance with Danish drinking water criteria within the filter depth (0.02, 0.05, and 0.05 mg/L for Fe, NH_4^+ and Mn, respectively).

Fe was always the first parameter with depth to meet compliance (Figure 1). At 30 cm filter depth, Fe concentrations in water samples met the drinking water criterion for 8 out of 10 DWTPs. Fe concentrations complied for the remaining two DWTPs (DWTP 3 and 8) after 40 and 50 cm filter depth, respectively.

For 9 out of 10 DWTPs, Mn was the next parameter with depth to meet compliance with the drinking water criterion (Figure 1). The removal depth varied between DWTPs from 45 cm for DWTP1 and 200 cm for DWTP9. Most of the DWTPs removed Mn to compliance at about 60 cm depth.

NH_4^+ was removed to compliance with the drinking water criterion in the filter layers just below Mn in 9 out of 10 DWTPs (Figure 1). NH_4^+ was removed to compliance at depths from 60 cm in DWTP1 to 250 cm for DWTP9 with varying removal depths in between these levels for the remaining DWTPs. DWTP6 showed a distinct removal pattern compared to the other DWTPs as NH_4^+ was removed to compliance in higher layers of the biofilter than Mn (40 cm for NH_4^+ as compared to 70 cm for Mn).

ATP

Figure 2 shows the ATP concentration over 20 cm depth intervals of each biofilter. ATP concentration varied from 10^3 to 10^5 ug per g of filter material. Rather different stratification of ATP was observed among the 10 DWTP biofilters. Decreasing ATP concentration with filter depth was observed in some filters (e.g. DWTP 1 and 10), whereas others show a more homogenous spatial distribution across filter depth (e.g. DWTP5 and 8). Relatively high concentrations of ATP was observed at the bottom of the biofilter at DWTP9 (Figure 2) which coincided with attenuated manganese and ammonium removal (Figure 1), and distinct stratification of microbial diversity (Figure 6 and 7).

Reads and OTUs

In average, 42698±9467 reads were obtained considering all samples of the ten DWTP after quality check and bioinformatics processing. The total number of Operational Taxonomic Units (OTUs) considering all DWTPs, identified based on 97% sequence identity, was 2903. The average number of OTUs for the individual DWTPs was 1281, varying from 785 OTUs for DWTP8 to 1459 OTUs for DWTP7.

Rarefaction curve

All DWTPs were sequences deeply. As seen in the Rarefaction plot in Figure 3, a plateau was reached, and sequencing of more samples would not add much additional diversity for most DWTPs.

Richness, evenness, and diversity

The average Shannon-Weaver diversity index for OTUs sharing >97% sequence similarity was 4.2±0.7. The highest diversity was observed for DWTP5 with a Shannon-Weaver Index of 4.7±0.1.

Diversity and core taxa

A total of 12 phyla were classified as core taxa based on being present in all DWTPs with abundances of more than 0.1%. At the genera level, 22 genera (0.1%) was shared between DWTPs.

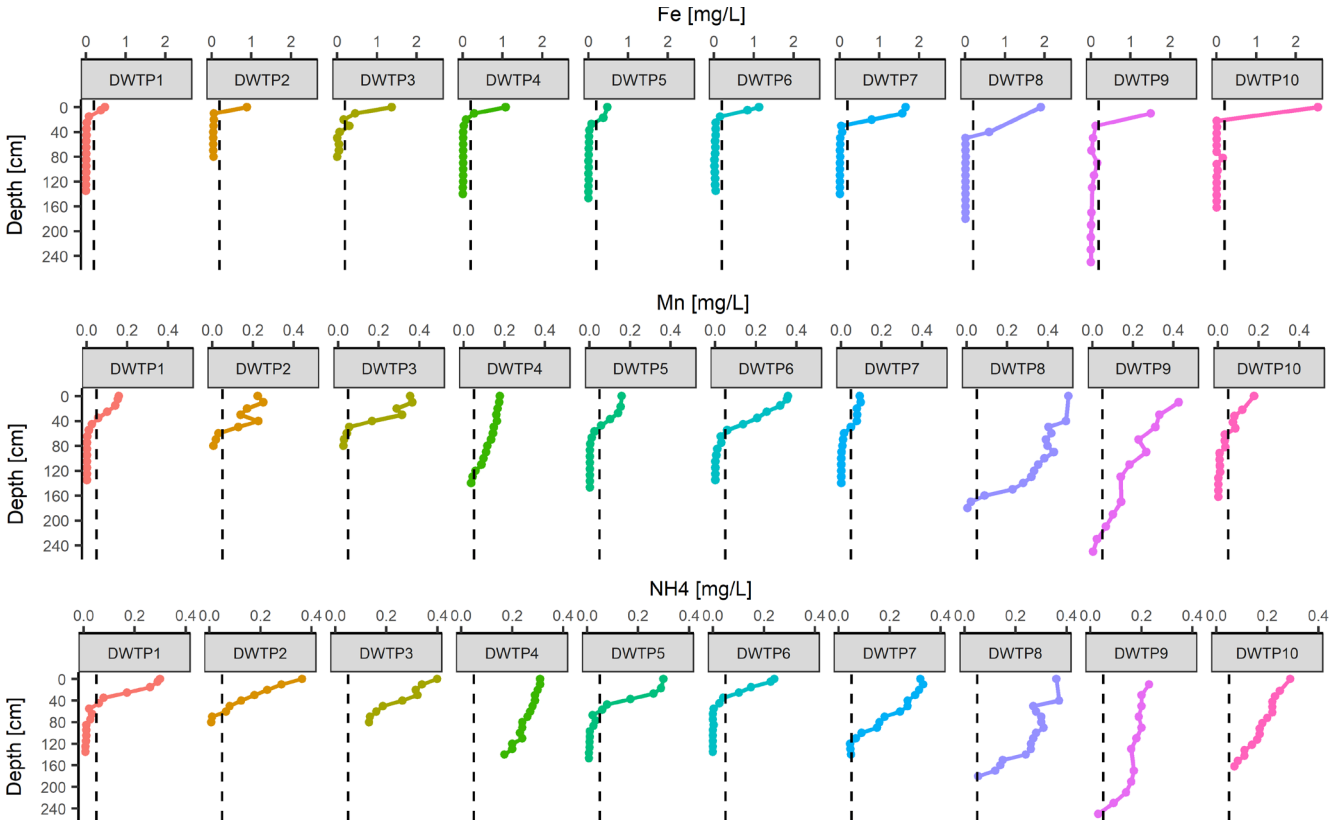
The heatmap in Figure 4 shows that the DWTPs were dominated by Proteobacteria, Nitrospirae, Planctomycetes, and Acidobacteria, which represented 82.6-93.6% of the abundance across the ten examined biofilters. Overall, Proteobacteria was the dominant phyla, accounting for approximately 50% of the total reads.

The heatmap in Figure 5 shows that the DWTPs were dominated by *Nitrospira*, *Methyloglobulus* and *Hyphomicrobium* with an average of 17%, 11% and 5% of read abundance. The genus *Nitrospira* include hydrogen and nitrite oxidizing bacteria and Commamox organisms. The genus *Methyloglobulus* include methane oxidizing bacteria related to Type I methanotrophs.

Differences in microbial diversity was shown by a Principal Component Analysis that included all depth for each DWTP (Figure 6). The 10 DWTPs clustered separately and spatial heterogeneity was observed which was most pronounced for DWTP1, 4, 6 and 9. An example of taxa variation with depth is shown for DWTP9 in Figure 6 and 7. For example, a decrease in IOBs through depth was observed, represented by OTU8 belonging to the family *Gallionellaceae*. In contrast, *Nitrospira* showed a distinct increase in relative abundance with filter depth (Figure 7).

Figure 9, 10 and 11 show boxplots with abundance of genera previously linked to Fe, NH_4 and Mn oxidation, respectively.

Figure 1. Concentration profiles of Fe, Mn and NH_4 at each waterworks. Dashed line indicates the Danish water criterion for each parameter.



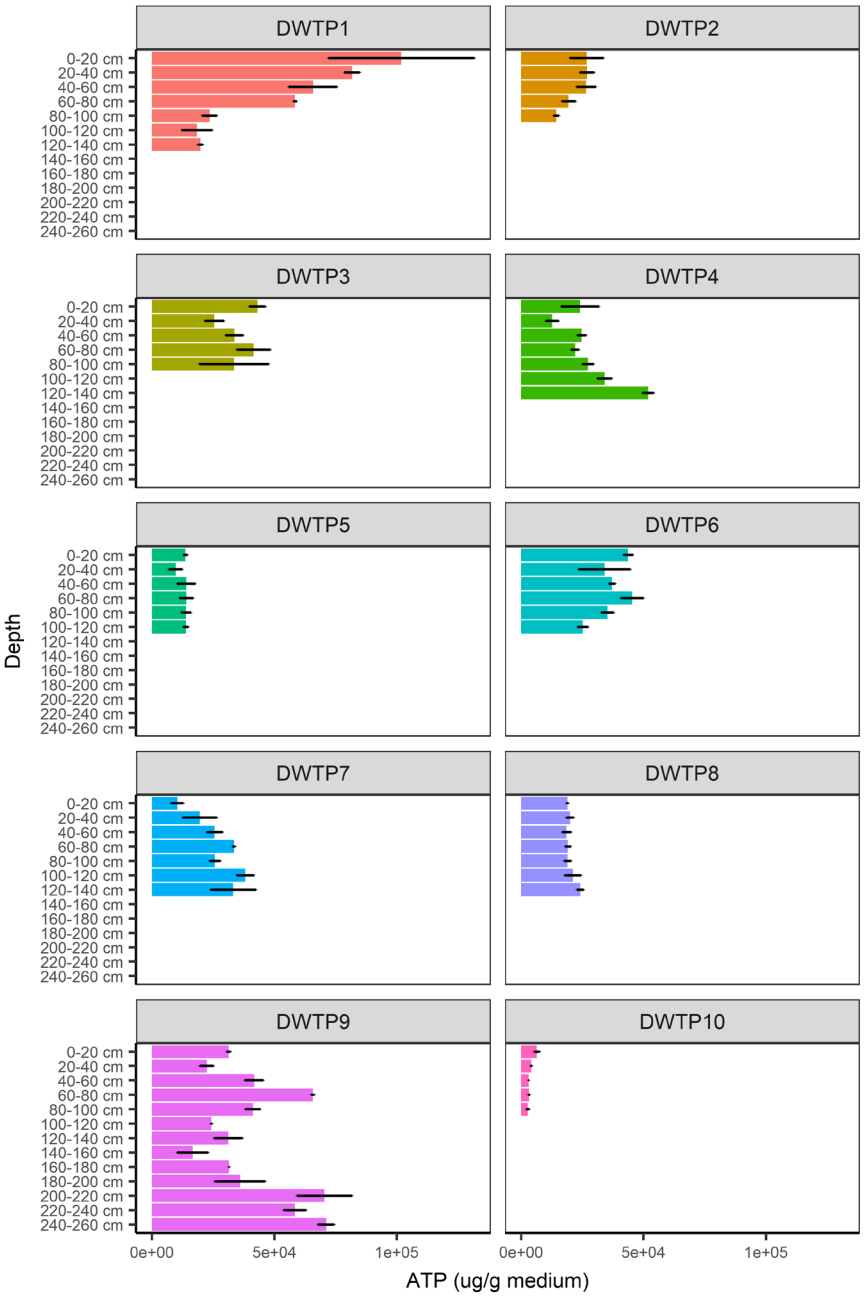


Figure 2. ATP concentration over depth of the filter at each DWTP. Numbers are the average of triplicates and error lines indicate standard deviation between replicates.

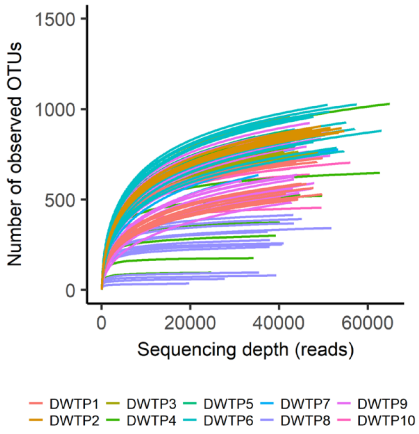


Figure 3. Rarefaction curves.

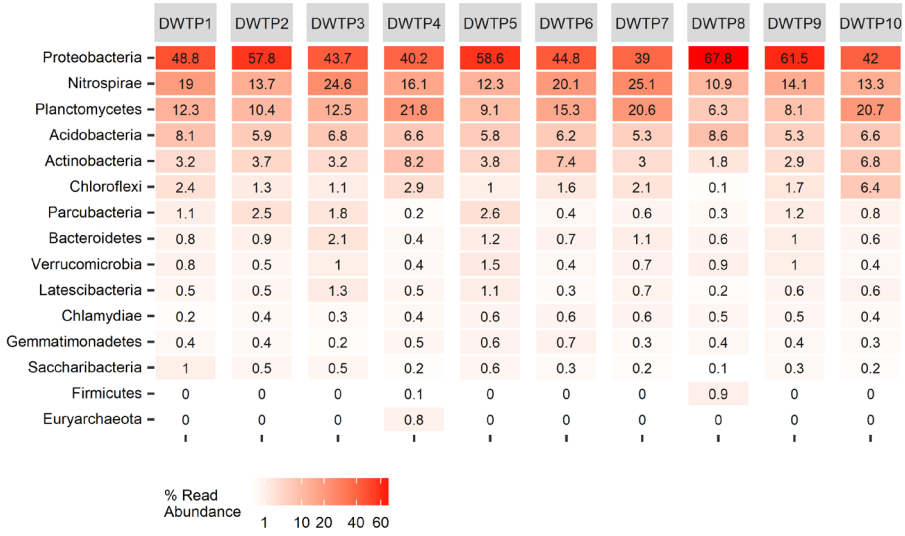


Figure 4. Heatmap of the 50 most abundant phyla present in filter material coatings at each DWTP (average from samples collected at each 20 cm depth interval).

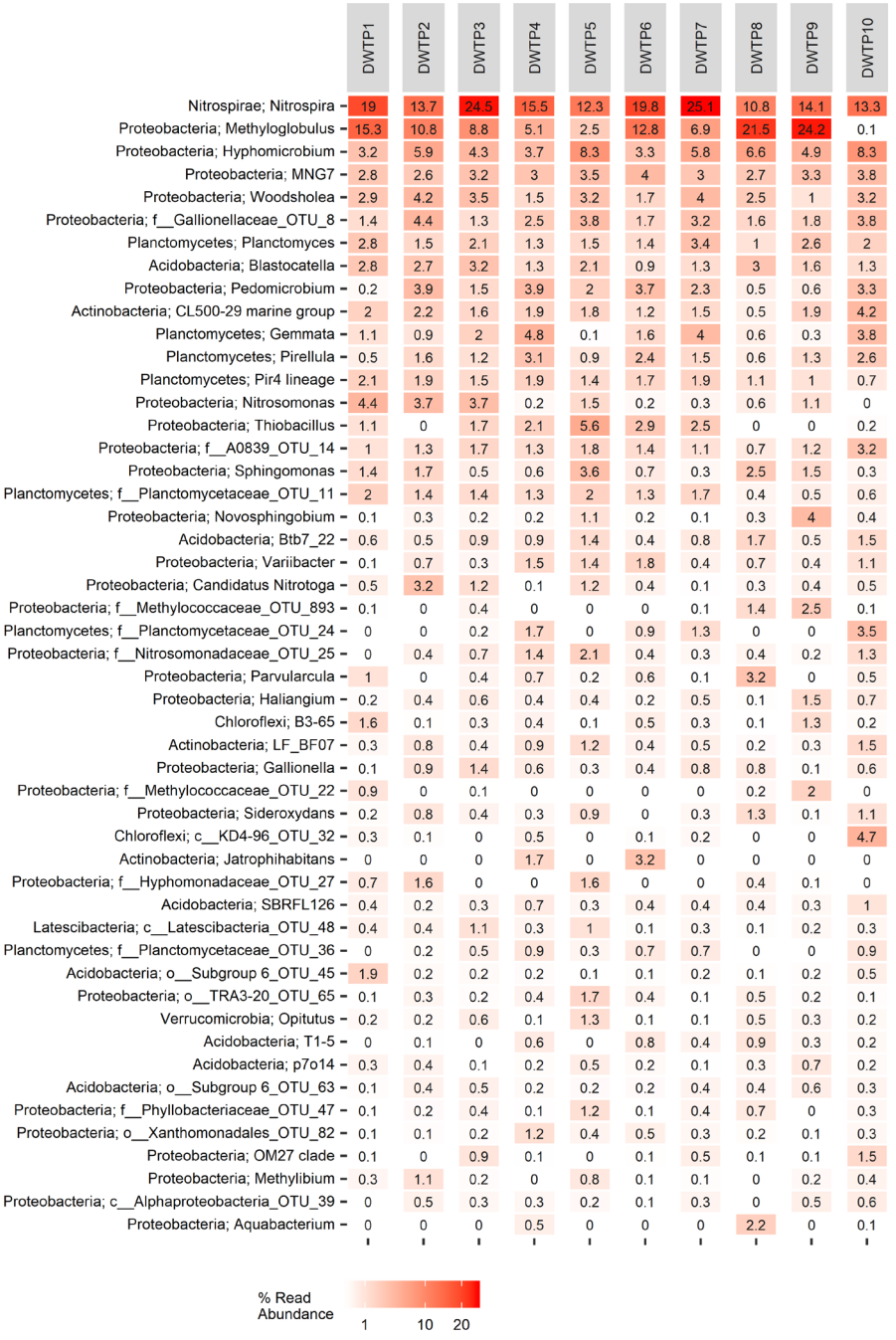


Figure 5. Heatmap of the 50 most abundant genera present in filter material coatings at each DWTP (average from samples collected at each 20 cm depth interval).

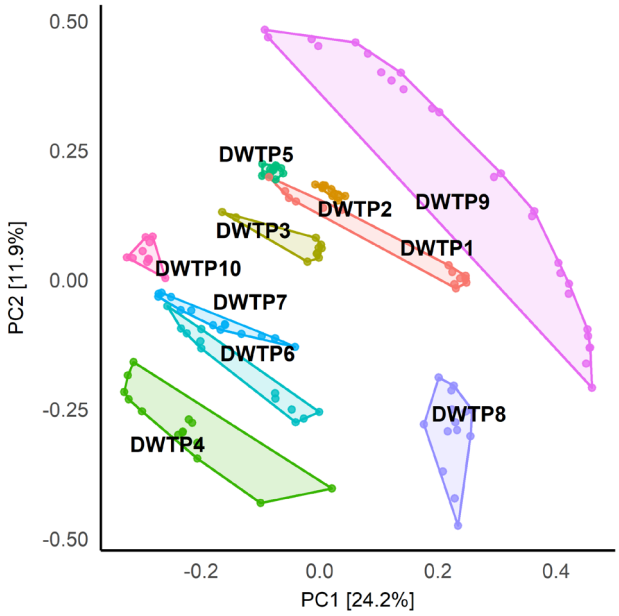


Figure 6. Principal component analysis (PCA) of microbial community in filter material samples collected at each 20 cm depth interval of each DWTP filter.

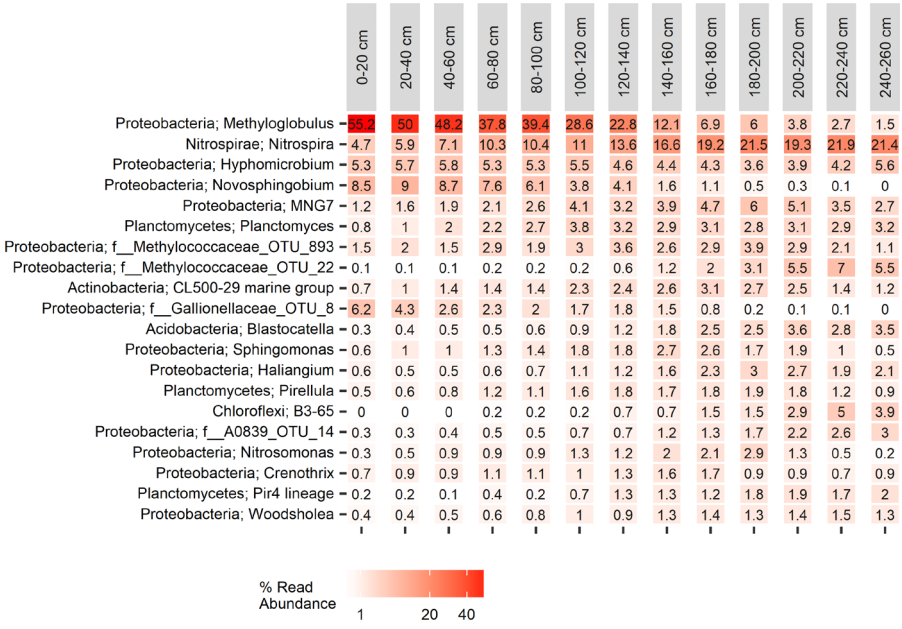


Figure 7. Example of taxa variation over filter depth at DWTP9.

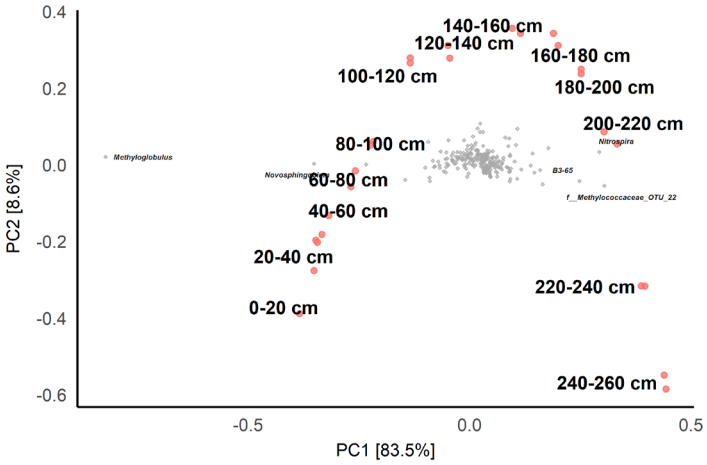


Figure 8. Example of PCA of microbial community in filter material samples collected at each 20 cm depth interval of DWTP9 filter and respective loadings.

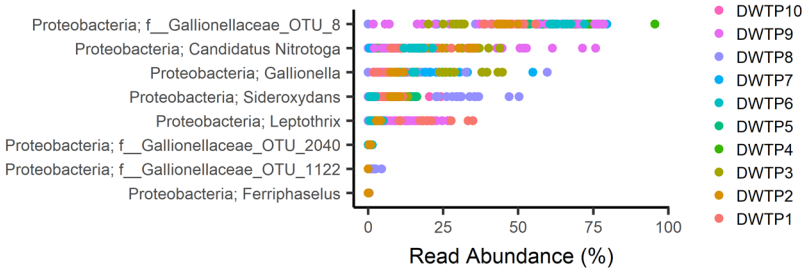


Figure 9. Boxplot of abundance of genera previously linked to iron oxidation in filter material samples collected at each 20 cm depth interval of each DWTP filter.

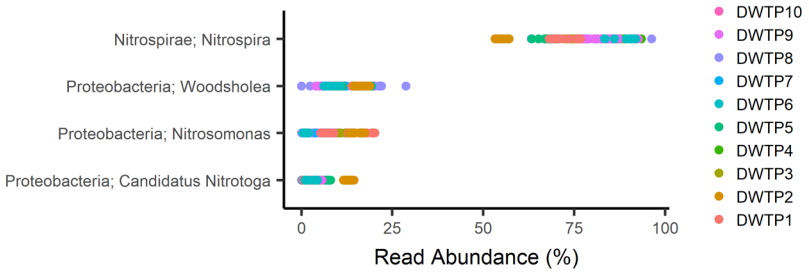


Figure 10. Boxplot of abundance of genera previously linked to ammonium oxidation in filter material samples collected at each 20 cm depth interval of each DWTP filter.

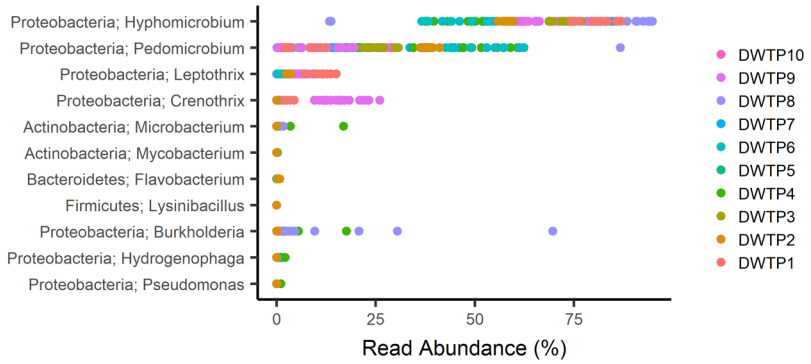


Figure 11. Boxplot of abundance of genera previously linked to manganese oxidation in filter material samples collected at each 20 cm depth interval of each DWTP filter.

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SUPPLEMENTARY MATERIALS

Location and composition of the Waterworks

DWTP	Waterworks	Coordinates	Filter number	Filter material
DWTP1	Dalum	55.36ºN, 10.37ºW	2	Anthracite and sand
DWTP2	Elsted	56.23ºN, 10.22ºW	1	Anthracite and sand
DWTP3	Holmehaven	55.41ºN, 10.26ºW	2	Sand
DWTP4	Hovedværket	55.39ºN, 10.37ºW	8	Anthracite and sand
DWTP5	Kasted	56.21ºN, 10.13ºW	2	Anthracite and sand
DWTP6	Lindved	55.34ºN, 10.42ºW	2	Anthracite and sand
DWTP7	Lunde	55.39ºN, 10.45ºW	1	Anthracite and sand
DWTP8	Stavtrup	56.14ºN, 10.12ºW	1	Anthracite and sand
DWTP9	Truelsbjerg	56.23ºN, 10.16ºW	1	Calcium carbonate
DWTP10	Østerby	56.08ºN, 10.14ºW	6	Anthracite and sand

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