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Taxonomic and functional diversity of microbial communities in rapid sand filters for groundwater treatment

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Technical University of Denmark

DTU

Taxonomic and functional diversity of microbial communities in rapid sand filters for groundwater treatment

Arda Gülay

DTU Environment
Department of Environmental Engineering

PhD Thesis
October 2014

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

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Preface

This thesis summarizes the results of the above-mentioned PhD project, carried out at the Department of Environmental Engineering of the Technical University of Denmark from January 2011 to January 2014. Professors Barth F. Smets and Hans-Jørgen Albrechtsen supervised the project and the Danish Strategic Research Council supported it financially via the project DW-Biofilter.

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IV **Gülay, A.**, Smets, B. F. An improved method to set significance thresholds for β diversity testing. Submitted to Environmental Microbiology

V **Gülay A**, Musovic S, Albrechtsen H-J, Smets BF. (2013). Neutrophilic iron-oxidizing bacteria: occurrence and relevance in biological drinking water treatment. Water Sci. Technol. Water Supply 13:1295.

VI **Gülay, A.**, Tatari, K., Musovic, S., Mateiu, R. V., Albrechtsen, H-J., Smets, B. F. Internal mineral porosity supports microbial activity in stratified rapid sand filters for drinking water treatment. Accepted in Applied and Environmental Microbiology. DOI : 10.1128/AEM.01959-14

In this online version of the thesis, the papers are not included but they can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk.

In addition, the following publications, not included in this thesis, were also executed during this PhD study:

Papers

a) Pellicer-Nàcher C, Franck S, **Gülay A**, Ruscalleda M, Terada A, Al-Soud WA, et al. (2014). Sequentially aerated membrane biofilm reactors for autotrophic nitrogen removal: microbial community composition and dynamics. Microb. Biotechnol. 7:32–43.

b) Tatari, K., **Gülay, A.,** Thamdrup, B., Albrechtsen, H.-J., Smets, B. F. Use and challenges of specific nitrification inhibitors applied to groundwater treatment biofilters. Submitted to Water Research

c) Musovic S., Tatari K., **Gülay A**., Albrechtsen H-J., Smets B. F. (2014) Microbial abundance, distribution and diversity in Rapid Sand Filters. Manuscript in preparation

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Gülay, A., Musovic, S., Albrechtsen, H-J., & Smets, B. F. 2013. Microbial community structure and a core microbiome in biological rapid sand filters at Danish waterworks. Abstract from 5th International Conference Microbial Ecology and Water Engineering, Michigan, United States.

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“Learn how to see. Realize that everything connects to everything else.”

Leonardo da Vinci

Summary

Groundwaters are common sources for drinking water production in many countries. High-quality groundwaters require a simple treatment to produce drinking water for end-users. Often, rapid sand filters (RSF), proceeded by a proper aeration step, provide sufficient treatment without a need of chlorination. A rapid sand filter consists of a bed of filter material through which the raw water flows vertically by gravitational force and contaminant removal is determined by physical, chemical and biological mechanisms. Biological processes are performed by microorganisms colonizing the filter materials. Although microbes are the main drivers for contaminant removal in rapid sand filters, the microbial communities in charge of these mechanisms as well as their ecology are not fully understood. This thesis therefore focuses on the investigation of microbial communities and their interactions in rapid sand filters. Specifically, we investigated microbial diversity, functional diversity of ammonia, nitrite and iron oxidation, spatial heterogeneity and mineral-microbe relations in rapid sand filters. For all sections, a sequential experimental pipeline was developed and successfully applied in order to test central hypotheses.

Rapid sand filtration is a long-practiced technology, yet the microbial diversity in these ecosystems has been poorly characterized and the ecological patterns governing microbial community structure remain obscure. In addition to this, previous investigations were confined by the methodological and sampling based challenges. We investigated the microbial diversity, characteristics and ecological patterns in rapid sand filters by applying 16S rDNA gene based 454 pyrosequencing to 96 samples from pre- and after filters at five different waterworks and their source groundwaters (Paper I). Our results suggested that rapid sand filters are taxonomically rich systems similar to the activated sludge systems or anaerobic digesters. We found that *Nitrospirae*, *Proteobacteria* and *Acidobacteria* were the dominant phyla in all pre and after filters. We also detected a high abundance of core taxa in investigated waterworks, suggesting that the filter-specific taxa represent a low proportion of the meta-community at each waterworks. Additionally, core taxa were highly abundant in functional groups responsible for ammonia, nitrite, iron, and methane oxidation, indicating the functional importance of the core taxa in rapid sand filters.

Since the purpose of the filters is to remove contaminants, function of the microbial communities is of importance. Our pyrosequencing results revealed unexpected abundances and ratios of functional groups inferred from previous phylogeny-physiology data, which led us to investigate the microbes involved in ammonia, nitrite and iron oxidation. We applied different combinations of cultivation and molecular techniques, including lab-scale column experiments, stable isotope probing, opposing Fe(II)/O_2 gradient enrichments, 454 pyrosequencing, DGGE and cloning (Paper II and III). RNA based stable isotope probing (SIP) showed that active ammonia oxidizing bacteria are not limited to the *Nitrosomonas* lineage. We suggest that mixotrophic or heterotrophic lineages can be involved in ammonia oxidation. Furthermore, SIP experiments also indicated that nitrite oxidation also can be driven by mixotrophic or heterotrophic lineages together with *Nitrospira*. Interestingly, further analysis on SIP results suggested that archaeal ammonia oxidizers cannot compete with bacterial ammonia oxidizers in full scale conditions, and that the *Nitrospira* lineage might be involved in ammonia oxidation. Due to the methodological challenges associated with molecular based detection of iron oxidizers, we applied opposing Fe(II)/O_2 gradient enrichments together with molecular analyses. Our results revealed a new niche for iron oxidizing bacteria different than oxic/anoxic interfaces. Novel iron oxidizers were identified and niche segregation was detected between iron oxidizing guilds isolated from rapid sand filters.

Process heterogeneity within a rapid sand filter has previously been documented and linked to the physical heterogeneity by using a modelling approach. We investigated the spatial heterogeneity of microbial communities within a filter to find out if the process heterogeneity can also be linked to heterogeneity of microbial composition in rapid sand filters (Paper IV). To answer this question, we developed a novel beta diversity significance testing method, which was successfully applied to 16S rRNA pyrosequencing data. We found that rapid sand filters are not spatially homogeneous systems in terms of microbial community composition; therefore we reason that a careful sampling campaign should be applied if one wants to investigate microbial communities or to design any meta-analysis concerning rapid sand filters. Biological heterogeneity was also investigated at the level of iron oxidizing guild using DGGE profiles and PCA plots (Paper V) and confirmed the pyrosequencing based results.

Finally, we investigated the microbial ecology in a rapid sand filter focusing specifically on the relation between mineral and microbial phases (Paper VI). Minerals and microbes interact with each other reciprocally: the mineral proliferation affects microbial proliferation and vice versa. We examined the effect of mineral coating abundance on activity, diversity, abundance, colonization of microbial communities in rapid sand filters using pyrosequencing, E-SEM, SEM, CSLM, BET, and lab-scale column experiments. The spatial distribution and abundance patterns of the microbes showed that the internal porosity positively affected microbial activity by supporting the total and functional microbial densities. The presence of rich EPS and embedded microbes in the internal structure of the mineral coatings suggests a protective role of mineral coatings against hydrodynamic shear forces. Overall, these results suggest a protective and supportive role of mineral coatings for microbes in rapid sand filters.

Taken together, our wide range of investigations indicate that a complex microbial community reside in rapid sand filters with a strong interaction between biotic and abiotic processes. The observations made and factors identified in this thesis, provide a stepping stone to help us construct theories or rules to understand and, ultimately manage, microbial communities in rapid sand filters in order to achieve a more stable, efficient, and reliable contaminant removal.

Dansk sammenfatning

Grundvand er i mange lande en væsentlig kilde til drikkevand. Grundvand af høj kvalitet kræver kun en simple behandling for at producere drikkevand til forbrugerne. Ofte er hurtig sandfiltrering, efter en forudgående beluftning, en tilstrækkelig behandling, uden behov for kloring. Et hurtig sandfilter består af et lag af filtermateriale, som vandet løber igennem drevet af tyngdekraften og hvor uønskede stoffer fjernes ved fysiske, kemiske eller biologiske mekanismer. De biologiske processer udføres af mikroorganismer som koloniserer filtermaterialet. Selvom mikroorganismer er en af de vigtigste drivkræfter i fjernelsen af forureningsstoffer i sandfiltre, er de mikrobielle samfund og deres økologi er endnu ikke helt forstået. Derfor fokuserer denne PhD-afhandling på at undersøge af de mikrobielle samfund og deres interaktion i sandfiltre. Specifikt undersøgte vi mikrobiel diversitet, funktionel diversitet af ammonium-, nitrit- og jern-oxiderende mikroorganismer, rumlige heterogenitet og mineral-mikroorganisme interaktion i sandfiltre. For alle emnerne er der udviklet en sekventiel eksperimentel pipe-line som succesfuldt er anvendt til at teste centrale hypoteser.

Sandfiltrering er en teknologi, der har været anvendt længe, men alligevel er den mikrobielle diversitet i disse økosystemer dårligt beskrevet, og de økologiske mønstre som styrer de mikrobielle samfunds struktur er uafklarede. Ydermere har tidligere undersøgelser været begrænset af metodiske og prøvetagningsmæssige udfordringer. Vi undersøgte mikrobiel diversitet, karakter og økologiske mønstre i sandfiltre ved hjælp af 16S rDNA-genbaseret 454 pyro-sekventering af 96 prøver udtaget fra for- og efterfiltre på fem forskellige vandværker, samt deres ubehandlede grundvand (Publikation I). Vores resultater indikerede at sandfiltre er artsrige systemer, svarende til aktiv slamsystemer eller biogasreaktorer. Vi fandt at *Nitrospirae*, *Proteobacteria* og *Acidobacteria* var dominerende phyla i alle for- og efterfiltre. Vi påviste også en høj forekomst af kerne-taxa blandt de undersøgte vandværker, hvilket tyder på, at de filter-specifikke taxa udgør en mindre andel af hvert vandværks meta-samfund. Desuden udgjorde kerne-taxa en stor andel af de funktionelle grupper, der er ansvarlige for ammonium, nitrit, jern og metan oxidation, hvilket antyder den funktionelle betydning af kerne-taxa i sandfiltre.

Da formålet med filtrene er at fjerne uønskede stoffer, er de mikrobiologiske samfunds funktionalitet vigtig. Vores resultater fra pyrosekventeringen viste nogle uventede forekomster og andel af funktionelle grupper baseret på tidligere fylogeni -fysiologi data, hvilket førte os til at undersøge de mikroorga-

nismer, der er involveret i oxidation af ammonium, nitrit og jern. Vi anvendte forskellige kombinationer af dyrkning og molekylære teknikker, inklusiv laboratorieskala kolonneforsøg, stabile isotop-teknikker, Fe(II)/O_2-gradient berigelser, 454 pyrosekventering, DGGE og kloning (publikation II og III). RNA -aseret stabil isotop-teknik (SIP) viste, at de aktive ammonium-oxiderende bakterier ikke er begrænset til gruppen af *Nitrosomonas*. Vi foreslår, at mixotrofe eller heterotrofe grupper kan være involveret i ammonium-oxidation. Ydermere indikerede SIP-resultaterne også, at nitrit-oxidering også kan være drevet af mixotrofe eller heterotrofe grupper sammen med *Nitrospira*. Interessant nok indikerede yderligere SIP-resultater, at ammonium-oxiderende archaea ikke kan konkurrere med ammonium-oxiderende bakterier under fuldskala-forhold, og at *Nitrospira*-gruppen kan være involveret i ammonium-oxidation. På grund af de metodiske udfordringer forbundet med molekylær-baseret påvisning af jern-oxiderende mikroorganismer, anvendte vi Fe(II)/O_2-gradient-teknik sammen med molekylære analyser. Vores resultater afslørede en ny niche for jern-oxiderende bakterier, som er forskellig fra aerob/anoxisk-grænseflader. Nye jern-oxiderende bakterier blev identificeret og niche-adskillelse blev påvist mellem forskellige jern-oxiderende guilds isoleret fra sandfiltre.

Proces-heterogenitet i et sandfilter er tidligere blevet dokumenteret og knyttet til den fysiske heterogenitet ved hjælp af modellering. Vi undersøgte den rumlige heterogenitet af de mikrobielle samfund i et sandfilter for at afklare, om proces-heterogenitet også kan knyttes til heterogenitet i den mikrobielle sammensætning i sandfiltre (Publikation IV). For at besvare dette spørgsmål, udviklede vi en ny metode til at undersøge for beta-diversitets signifikans, hvilket succesfuldt blev anvendt på 16S rRNA pyrosekventeringsdata. Vi fandt, at sandfiltre ikke er rumligt homogene med hensyn til sammensætning af mikrobielle samfund, og derfor skal prøvetagningskampagner være meget omhyggelige, hvis man ønsker at undersøge mikrobielle samfund eller at designe meta-analyser af sandfiltre. Biologisk heterogenitet blev også undersøgt på jern-oxiderende guild-niveau ved hjælp af DGGE profilering og PCA plots (Publikation V), hvilket bekræftede de pyrosekventeringsbaserede resultater.

Afslutningsvis undersøgte vi den mikrobielle økologi i sandfiltre med specifikt fokus på interaktion mellem mineraler og mikroorganismer (Publikation VI). Mineraler og mikroorganismer påvirker hinanden reciprokt: udfældninger påvirker den mikrobielle vækst og vice versa. Vi undersøgte effekten af omfanget af uorganiske udfældninger og aktivitet, diversitet, forekomst og kolonisering af mikrobielle samfund i sandfiltre ved hjælp af pyrosequencing,

E-SEM, SEM CSLM, BET og laboratorie-skala kolonneforsøg. Den rumlige fordeling og mønstret for mikroorganismernes forekomst viste, at intern porøsitet påvirkede den mikrobielle aktivitet positivt ved at understøtte densiteten af både de funktionelle grupper og det totale antal mikroorganismer. Forekomst of rig EPS og indlejrede mikroorganismer i den interne struktur af mineraludfældninger indikerer, at mineraludfældningerne har en beskyttende og understøttende rolle for mikroorganismer i sandfiltre.

Alt i alt indikerer vores større undersøgelser, at der er et komplekst mikrobielt samfund i sandfiltre med en stærk interaktion mellem biologiske og abiotiske processer. Afhandlingens observationer og identificerede faktorer giver et afsæt for at konstruere teorier eller regler for at forstå - og ultimativt styre – mikrobielle samfund i sandfiltre for at få en mere stabil, effektiv og pålidelig fjernelse af uønskede stoffer.

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Glossary

Biological Replicate: a sample where microbial communities in that sample have a dispersal link with the microbial communities of another sample in situ.

OTU (operational taxonomic unit): the clustering scheme used to categorize individuals (in taxonomic units), in this thesis 97% sequence similarity is used to define OTUs (hence, the clustering unit is also terms $OTU_{0.03}$ referring to the maximum dissimilarity within an OTU).

De novo clustering: the inference of OTUs directly from experimental data (sequences) without any prior information (reference data).

Taxonomic classification: classifying sequence data into known taxonomies.

Community: the sum of the individuals in a sample.

Community composition: the richness, relative abundance, and phylogenetic structure of taxa in a community (Allison & Martiny 2008).

Phylogenetic diversity: a biodiversity measure based on evolutionary relationships between species and represents one of the components of biodiversity (Winter et al. 2013).

Functional diversity: the variety and number of species that fulfil different functional roles in a community or ecosystem (Colwell 2012).

Taxonomic diversity: name and taxonomic classification of species in a community.

Evenness: a measure of the homogeneity of abundances in a sample or a community (Colwell 2012).

Meta community: set of interacting communities which are linked by the dispersal of multiple, potentially interacting species (Battin et al. 2007).

Guild: metabolically related populations, e.g., ammonium oxidizing microorganisms (AOP), iron oxidizing bacteria (IOB) (Friedrich 2011).

Clade: a hypothesis of evolutionary relatedness in which a group of organisms share a single common ancestor (Valentine 2007).

Niche: the particular set of resources and environmental conditions that an individual species exploits (Prosser et al. 2007).

Niche differentiation: the process by which natural selection drives competing species into different uses of resources resulting in different niches (Battin et al. 2007).

Intra β diversity: amount of heterogeneity calculated by comparing subsamples from the same sample – either by comparing samples of different size (e.g. where heterogeneity can be the result of OTU loss) or by comparing random samples of same sample size – this β diversity can affect the inter β diversity estimators, which requires correction.

Inter β diversity: an estimate of the compositional heterogeneity between discrete samples A and B– especially the composition of the community within those samples – which are approximated by the sequence library (or after clustering), the OTU library.

Subsampling: a statistical technique to generate a random representative subset drawn from a sample of a larger sample size.

Rarefaction: is a process to generate the expected number of species in a small collection of n individuals (or n samples) drawn at random from the large pool of N individuals (or N samples) (Gotelli & Colwell 2001).

1. Introduction

Rapid sand filtration is a conventional biotechnological process which is used in numerous full-scale applications in Europe to treat groundwaters. This technique is a part of a treatment chain and it is currently the most widespread filtration system used in drinking water treatment (Crittenden et al. 2012). A conventional groundwater treatment plant often comprises three main process units: aeration, first-stage filtration and second stage filtration. The purpose of the rapid sand filter in water treatment is to remove a variety of contaminants and produce drinking water that meets high quality standards for end-users. A rapid sand filter consists of a bed of filter material through which the influent water flows vertically by the gravitational force (Figure 1). Contaminant removal through filtration is determined by physical (transport, attachment and detachment; Amirtharajah, 1988), chemical (oxidation) and biological mechanisms. Enzyme catalyzed degradation of contaminants is performed by microorganisms that colonize filter materials.

In rapid sand filters, several factors have been detected to affect biological processes, thereby effecting microbial communities. Filter material (Andersson et al. 2001), water temperature (Kihn et al. 2002), backwash regime (Laurent et al. 2008), backwash technique (Kasuga et al. 2007), intra-filter heterogeneity (Lopato et al. 2011), nutrient deficiency (Li et al. 2010), and groundwater taxa inventory (Pinto et al. 2012) were reported as significant factors , either directly or indirectly, affecting microbial communities. These factors can be divided in two categories; (i) filter specific parameters and (ii) environmental factors. While filter specific parameters are mainly affiliated to the filter design such as backwashing technique and frequency, filter material, filter hydraulics, water inlet structure and filter height, environmental factors are affiliated to the physical and chemical processes in a filter, groundwater chemical composition and groundwater microbiology. Most of these factors require a further investigation to experimentally assess their effect on microbial communities in rapid sand filters.

Although implementation of rapid sand filters goes back to 19th century, the design of these filters has always been empirical because very little microbiological information concerning rapid sand filters have as yet been experimentally obtained from which one may predict the development, immigration and dynamics of microbial communities. The lack of knowledge concerning microbial ecology of rapid sand filters has hampered the operation and design of these systems causing process instability and malfunctioning rapid sand filters. Exploration of

structural and functional diversity as well as interactions between microbes and filter environment is therefore crucial and hence is the central focus of this thesis.

In this section, I shortly introduce ecology of groundwaters, because groundwater is the energy source and seed bank for microbial communities in rapid sand filters. Then I provide a review about the ecology of major element cycles occurring in rapid sand filters, followed by methods that I use to assess microbial diversity, and functional diversity. To finalize the introduction, I summarize the hypotheses and aims of the thesis. In the discussion part, I first emphasize the key findings concerning microbial diversity and the core taxa. In the following section, I highlight the functional diversity of ammonium, nitrite and iron oxidizing microorganisms in the rapid sand filters. Third, I present the spatial heterogeneity in rapid sand filters together with a new method to assess it. Finally, I discuss the role of mineral coatings in rapid sand filters.

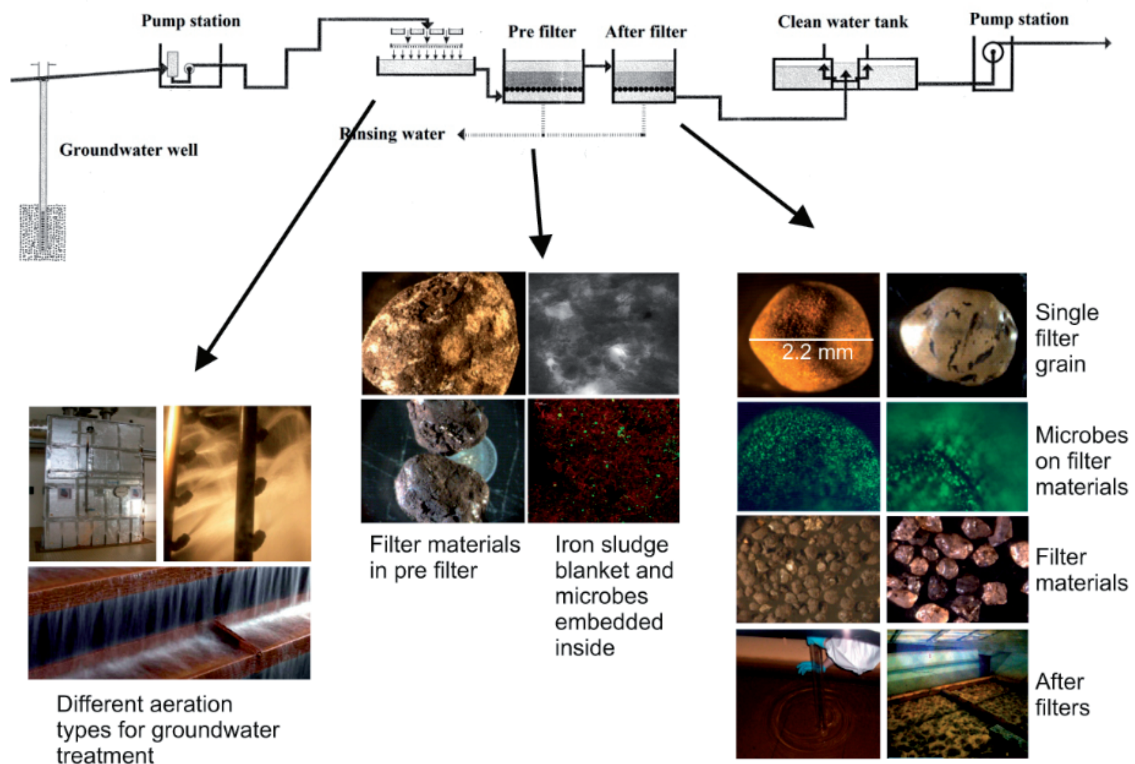


Figure 1 A representative process scheme of a drinking water treatment plant in Islevbro treating groundwater. Images are taken from actual waterworks that were investigated in this study.

1.1 Groundwater ecology

Groundwater is the source water for drinking water production in many countries, especially in Denmark. To understand microbial ecology in rapid sand filters, an understanding of the groundwater chemistry and microbiology is essential.

Groundwater is ultimately the result of rain infiltration into the subsurface. Hydrochemistry of groundwaters therefore depends on the specific geological formations as well as the residence period of the water inside the aquifer (Søgaard & Madsen 2013). In Denmark, limestone aquifers are of crucial importance for the water supply (Figure 2), because they are available all over in Denmark and high quality of water from these aquifers requires only a simple treatment (rapid sand filter) to produce drinking water (Klaus 2003).

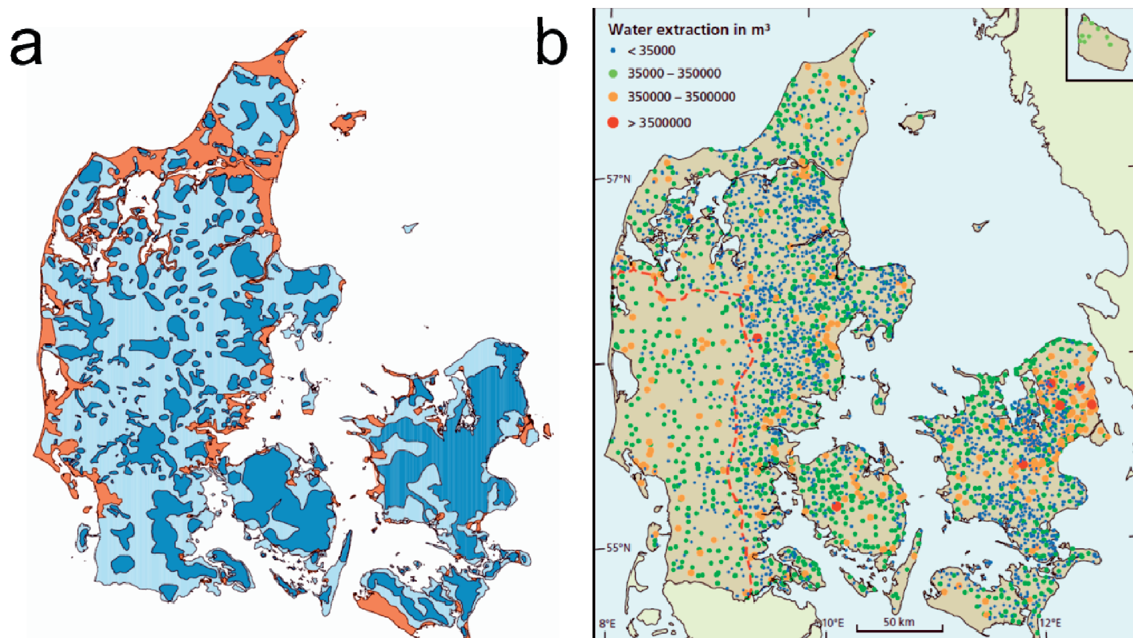


Figure 2 (a) Groundwater quality for drinking water production in Denmark. Particularly valuable (blue), valuable (light blue), less valuable (orange). (b) Groundwater extraction for drinking water production in Denmark (Klaus 2003)

1.1.1 Groundwater chemistry

Groundwater chemical composition and their natural evolution in limestone aquifers are mainly controlled by rainfall chemistry, carbonate dissolution, redox processes (mainly sulphide and organic carbon oxidation), ion exchange (e.g. between Ca and Na) and mixing with saline waters (Klaus 2003). In deep limestone aquifers, oxygen and nitrate are reduced by sulphides and organic carbon in up-gradient parts of the aquifer and ultimately form an-

aerobic conditions together with increased concentrations of sulphate and bicarbonate (Klaus 2003). And at the depths between 30m and 60m below the surface where feed waters of rapid sand filters are abstracted, are in the up-gradient parts of the aquifer, therefore feed waters of rapid sand filters are typically anaerobic. Mineral dissolution and redox reactions (e.g. sulfate reduction, denitrification, methane formation, iron and manganese reduction) in aquifers determine the electron donors (contaminants) for the microbial communities in rapid sand filters (Søgaard & Madsen 2013). The constituents of anaerobic groundwater chemical composition in limestone aquifers are summarized in Table 1.

Table 1. Typical constituents present in Danish groundwater (Søgaard & Madsen 2013).

Group		Constituents
Main components	Cations	Ca^{2+}, Na^{+} , NH_4^{+}, K^{+}, Mg^{2+}, Fe^{2+}, Mn^{2+}
	Anions	HCO_3^{-}, NO_3^{-}, SO_4^{2-}, PO_4^{3-}
Uncharged species		H_4SiO_4
Trace components		Al^{3+}, Ni^{2+}, Zn^{2+}, F^{-}, H_3AsO_3 and others
Gases		CO_2, H_2S, CH_4,O_2
Organic compounds		Humus
Anthropogenic compounds		Pesticides, chlorinated solvents, and others

Although sulphate reduction and methanogenesis, using organic matter as an electron donor, is a common way for production of hydrogen sulphide and methane in groundwaters (Price et al. 1993), these processes are very rare in Danish limestone aquifers due to the very low amounts of organic matter. Previous reports suggested that hydrogen sulphide and methane in the limestone aquifers in Denmark mainly originate from the confining Pleistocene sediments (GEUS 2003; Klaus 2003).

Although chemical constituents of groundwaters are very similar in Denmark, the concentration of these species can vary in composition across distant aquifers (Table 2). However, the variation in chemical composition between Danish limestone aquifers is not big as observed between different type aquifers around Europe.

Table 2 Statistics for major and minor constituents in limestone aquifers around Copenhagen (Klaus 2003)

		n	Min.	Max.	Mean	Median
T (°C)		13	8.9	10.3	9.7	9.8
pH		16	6.9	7.21	7.09	7.11
Eh		15	-110	-41	-75	-77
DO		16	0	0.53	0.05	0.01
SEC		16	543	878	677	633
Ca	(mg/l)	35	55	283	128	114
Mg	(mg/l)	35	10	43	19	19
Na	(mg/l)	35	10	188	29	20
K	(mg/l)	35	2	10	4	4
Cl	(mg/l)	35	12	260	53	47
SO_4	(mg/l)	35	15	433	103	82
HCO_3	(mg/l)	28	133	409	334	336
TOC	(mg/l)	28	0.7	4.6	1.9	2.1
Si	(mg/l)	35	10	15	13	13
Fe	(mg/l)	35	0.01	7.32	1.22	0.66
Mn	(mg/l)	35	0	0.19	0.04	0.02
Sr	(mg/l)	35	0.34	6.48	1.56	0.91
NH_4-N	(mg/l)	16	0	0.29	0.13	0.1
NO_2-N	(mg/l)	9	0.003	0.005	0.003	0.003
NO_3-N	(mg/l)	9	0.2	0.84	0.27	0.2
P	(mg/l)	35	0.02	0.14	0.04	0.02
F	(mg/l)	16	0.25	0.79	0.46	0.43
Br	(mg/l)	15	0.03	0.35	0.1	0.06

1.1.2 Groundwater microbiology

The link between microbial communities in groundwater and rapid sand filter has not yet been investigated. By using pyrosequencing, Pinto et al. (2012) revealed the high level of phylogenetic relatedness between microbial communities in a rapid sand filter and its source water, suggesting the importance of microbial immigration from groundwaters. This observation was confirmed in this thesis with a higher taxonomic resolution of the filter metacommunity. In drinking water treatment, groundwater microbiota may play a more important role than previously thought.

It has been reported that terrestrial underground ecosystems may constitute up to 40% of prokaryotic biomass on earth (Griebler & Lueders 2009). Bacte-

ria, the largest portion of microbial biomass in groundwaters, numbers may vary between 10^2 and 10^6 cells per cm^3 of groundwater (Madsen & Ghiorse 1993; Ghiorse 1988). Although aquifer ecosystem requires a high degree of specialization of the microorganisms due to the lack of light, low organic carbon content and nutrients and low temperatures (Danielopol et al. 2000), previous studies reported a high level of microbial diversity within several phyla, especially *Proteobacteria*, *Nitrospirae*, *Actinobacteria* and *Bacteriodetes* (summarized in Figure 3) (Farnleitner et al. 2005; Griebler & Lueders 2009).

Differences in taxa or gene inventories of groundwaters may cause differences in function and composition of microbial communities in their down-flow rapid sand filters, due to the occurrence of microbial immigration. Microbial evolution is likely follow different ways in different aquifers; because most of deep aquifers are isolated ecosystems from the surface and environmental factors for several thousands or millions of years (Fredrickson & Onstott 2001). In addition, several horizontal gene transfer events have been reported in deep aquifers (Griebler & Lueders 2009); the naphthalene dioxygenase (*nahAc*) gene, for example, was detected in the genome of species in upstream sample of a shallow aquifer, while its origin was detected as a plasmid- borne in downstream samples of the same aquifer (Herrick 1997). Horizontal gene transfer events in deep aquifers may also contribute to the variance of gene banks between different aquifers.

All in all, microbial communities in groundwaters are continuously carried into rapid sand filters; the actual effects of this continuous inoculation on the filter microbiota remains poorly examined.

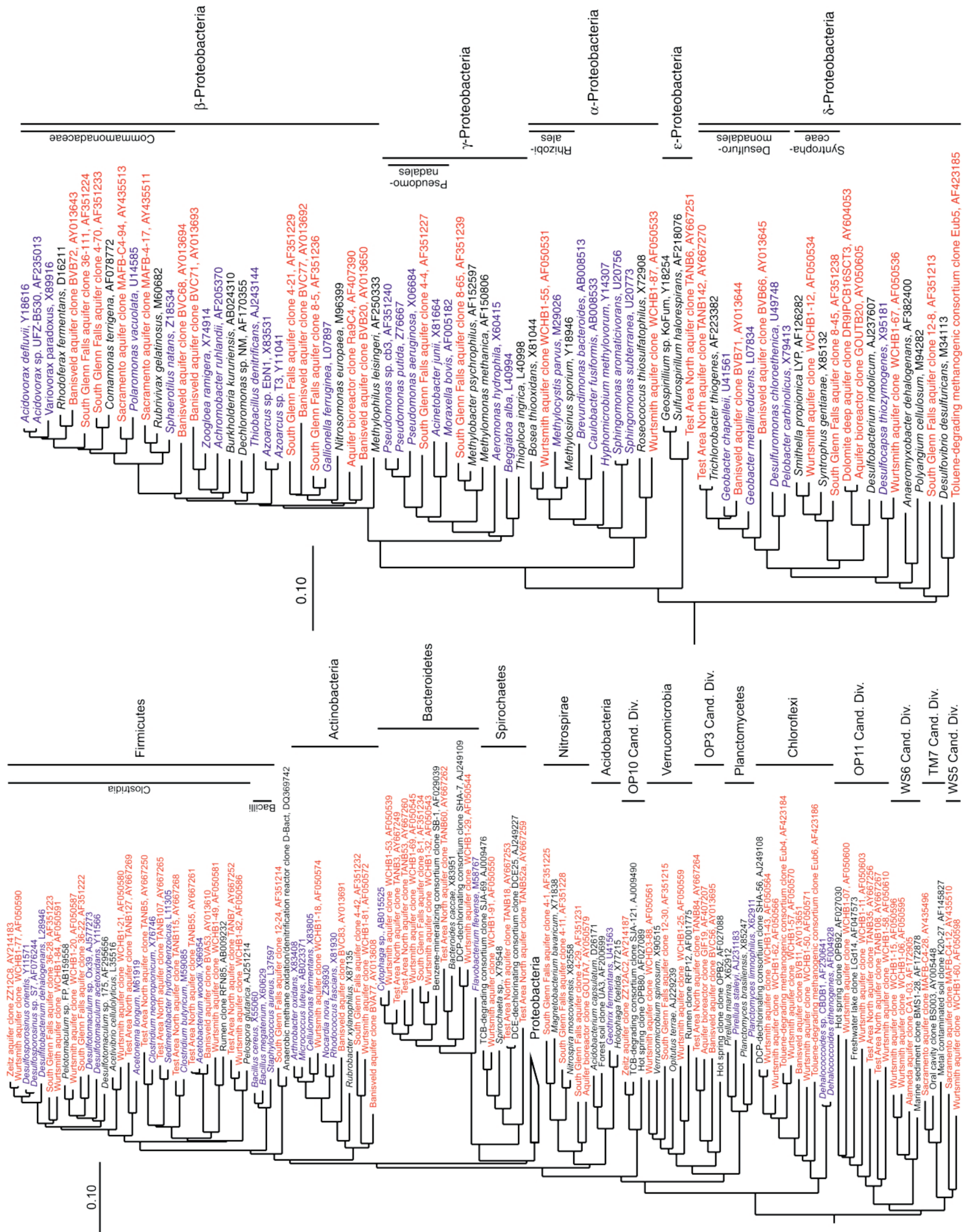


Figure 3 Phylogenetic tree of groundwater *Proteobacteria* (up) and groundwater *Bacteria* other than *Proteobacteria* (down) showing strains actually originating from groundwater environments (marked in blue) and of environmental bacterial 16S rRNA gene sequences retrieved from aquifer samples (marked in red) (Griebler & Lueders 2009).

1.2 Major transformations in rapid sand filters

The quality of drinking water is ensured by the transformation of constituents that have negative effects on human health or the environment, into less negative forms. These transformations are performed by combination of physical, chemical and biological mechanisms. In this section, the major transformations occurring in rapid sand filters are introduced.

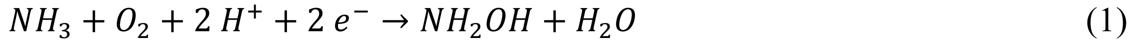
1.2.1 Nitrogen transformation

Among nitrogenous compounds (NH_4^+, NO_3^-, NO_2^- or dissolved organic nitrogen (DON)) in reduced aquifer systems, NH_4^+ is the dominant species, mainly formed by the anaerobic decomposition of organic material (Böhlke et al. 2006) or anthropogenic activities. NH_4^+ flows into the rapid sand filters enables two sequential microbial oxidation processes, during which valence state of nitrogen changes from -3 (NH_3) to +5 (NO_3^-). These processes, nitritation and nitratation constitute the main nitrogen transformations in rapid sand filters and are exclusively performed by microorganisms.

Nitritation

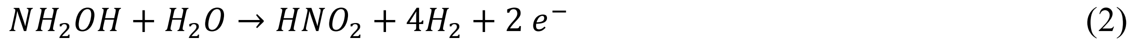
Nitritation is the first step of the nitrification process. First, NH_3 is oxidized into hydroxyl-amine (NH_2OH) with the membrane associated enzyme ammonia monooxygenase (AMO) (Hyman & Wood 1985), and then NH_2OH is oxidized to NO_2^- with the cytoplasmic membrane associated enzyme hydroxylamine oxireductase (HAO) (Bock & Wagner 2006a). The gene encoding ammonia monooxygenase subunit A (*amoA*) has been used phylogenetic marker to detect ammonia oxidizers in many environments (Prosser & Nicol 2012; Klotz & Stein 2008a).

Electron flow during catabolism of ammonia is illustrated in figure 4. The stepwise oxidation of NH_3 to NO_2^- is performed by ammonia oxidizing bacteria (AOB) and archaea (AOA). Alternatively, NH_3 can be oxidized to NO_2^- by aerobic methanotrophs, as they contain particulate methane monooxygenase (pMMO), which has a degree of function homology to ammonia monooxygenase (Holmes et al. 1995; Klotz & Stein 2008b).



$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \rightarrow NH_2OH + H_2O \qquad (1)$$

catalysed by ammonia monooxygenase (AMO)



$$NH_2OH + H_2O \rightarrow HNO_2 + 4H_2 + 2\,e^- \qquad (2)$$

catalysed by hydroxylamine oxireductase (HAO)

In rapid sand filters, nitritation has so far been assumed to be performed by chemolithotrophic microorganisms (van der Wielen et al. 2009) that acquire energy from ammonia oxidation and assimilate carbon from CO_2 via the Calvin cycle (Kowalchuk & Stephen 2001). Strains related to cultured bacterial species *Nitrosomonas europaea* and *Nitrosomonas oligotropha* (White et al. 2012) and cultured archaeal species *Nitrosopumilus maritimus, Candidatus Nitrososphaera gargensis,* and *Candidatus nitrosoarchaeum* (van der Wielen et al. 2009; Bai et al. 2013) have been reported as key lineages for ammonia oxidation in rapid sand filters. In addition to autotrophic nitritation, many *amoA* gene containing organotrophic bacteria have been reported that can oxidize ammonia (Robertson et al. 1988; Daum et al. 1998; Kundu et al. 2014; Lin et al. 2010) as an apparent co-metabolic process (Bock & Wagner 2006b). The contribution of such organotrophic ammonia oxidation to the nitritation process in rapid sand filters is unknown.

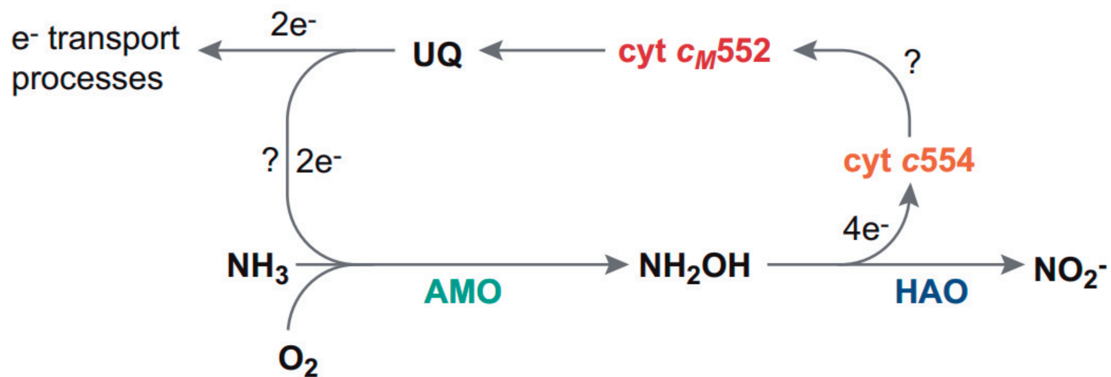
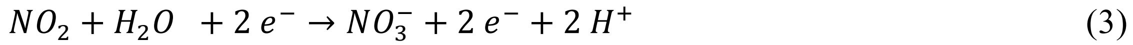


Figure 4 Electron flow during ammonia oxidation in AOB. Questions marks indicate steps that are not experimentally verified (Arp et al. 2007).

Nitratation

In rapid sand filters, the produced NO_2^- from nitritation is converted to NO_3^- with the membrane-bound enzyme nitrite oxidoreductase (NXR). This process is known as nitritation. Nitrite oxidizing bacteria (NOB) rely on the products of AOB and AOA, and belong to a broad set of phylogenetic clades, including *Nitrobacter, Nitrococcus, Nitrospina, Nitrotoga, and Nitrospira.* Although the *nxr* gene is a recently proposed solid phylogenetic marker for NOB, only 16S rRNA

based detection has so far been applied to NOB in rapid sand filters. These very few 16S rRNA based investigations have revealed the presence of *Nitrospira* and speculated that this phylum is solely responsible for nitrite oxidation (de Vet et al. 2009; White et al. 2012).



$$NO_2 + H_2O \ + 2\,e^- \rightarrow NO_3^- + 2\,e^- + 2\,H^+ \quad (3)$$

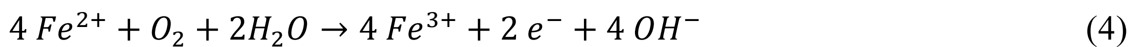
catalysed by nitrite oxireductase (NXR)

1.2.2 Iron transformation

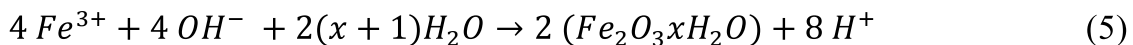
Ferrous iron [Fe(II)] is a common constituent in reduced groundwaters, originating from the weathering of iron minerals such as pyrite. In rapid sand filters, dissolved Fe(II) in the influent can have three destinations under strict aerobic and circumneutral conditions: (i) chemical Fe(II) oxidation; (ii) microbial Fe(II) oxidation; and (iii) Fe(II) adsorption. The chemical processes have been investigated thoroughly, but the microbial Fe(II) oxidation has not.

Chemical Fe(II) oxidation

After aeration, chemical iron oxidation in groundwater becomes feasible due to the existing pH and elevated oxygen concentrations. Chemical iron oxidation is based on the transformation of the soluble Fe(II) in to ferric iron [Fe(III)], as shown in equation 4. Subsequently, Fe(III) undergoes hydrolysis ([Eq. (5)]; Lerk, 1965) and forms various iron hydroxide species that are characterized by very low solubility products.



$$4\,Fe^{2+} + O_2 + 2H_2O \rightarrow 4\,Fe^{3+} + 2\,e^- + 4\,OH^- \quad (4)$$



$$4\,Fe^{3+} + 4\,OH^- \ + 2(x+1)H_2O \rightarrow 2\,(Fe_2O_3xH_2O) + 8\,H^+ \quad (5)$$

Kinetic studies on chemical iron oxidation show that aerobic Fe(II) oxidation strongly depends on the pH and the concentration of oxygen (Equation 6) (Stumm & Lee 1961). Short of pH and oxygen, studies on iron oxidation kinetics have documented that alkalinity (bicarbonate concentration), temperature, organic matter and some elements/ions (Cu^{2+}, Mn^{2+}, Co^{2+} and $H_2PO_4^-$) have significant effects on oxidation kinetics (Sharma 2001). Alkalinity serves as a buffer for pH decrease during iron oxidation, and effects the type of Fe(III)-hydroxides that occur as products of iron oxidation (Sharma 2001). Moreover, a detailed study from Sung and Morgan (1980) showed that a change in water temperature from 5 to 30°C (P_{O2} = 0.20 atm, pH 6.84, I = 110 mM, alkalinity = 9 meq/L) caused a sharp decrease in oxidation half-time from 316 to 4 min due to the change in the degree of water ionization . Lower chemical iron oxidation rates at cold temperatures

can be crucial in the competition for Fe by iron oxidizing microorganisms and chemical oxidation, in that biological oxidation may contribute more to iron oxidation than believed. Moreover, dissolve organic matter (DOC) in groundwater, especially humic substances (Theis & Singer 1974), make complexes with soluble Fe(II), which decrease the chance of Fe(II) oxidation (Knocke et al. 1992); however, DOC concentrations are very low in limestone groundwaters, therefore it is unlikely to see these complexes in rapid sand filters.

$$-d[\mathrm{Fe(II)}]/_{dt} = k_o.pO_2.[Fe(II)].[OH^-]^2 \quad (6)$$

where

[Fe(II)]/dt = rate of iron(II) oxidation (mol l^{-1} min^{-1})

k_o= reaction rate constant=8.0(±2.5)x10^{13} I^2 mol^{-2} atm^{-1} min^{-1} (20.5°C)

$p0_2$ = partial pressure of oxygen (atm) = 0.21 $[0_2]/[0_{2\text{-sat}}]$

$[O_2]$, $[O_{2\text{-sat}}]$ = actual and the saturated concentration of oxygen in water (g/m^3).

[Fe(II)] = concentration of ferrous iron (mol/1).

$[OH^-]$ = concentration of hydroxyl ions (mol/1)

Microbial Fe(II) oxidation

Microorganisms oxidizing Fe(II) have to compete with chemical iron oxidation to capture Fe(II) ions for obtaining energy. Due to the circumneutral pHs and highly oxic conditions, which both favour chemical Fe(II) oxidation, biological iron oxidation is usually not considered important in rapid sand filters. Nonetheless few studies have made circumstantial observations that suggest the contribution of biological iron oxidation in RSFs (Søgaard et al. 2000; Mouchet 1992; Katsoyiannis & Zouboulis 2004). The genera *Gallionella*, *Leptothrix* and *Siderocapsa* are the only iron oxidizing genera documented in rapid sand filters (Katsoyiannis & Zouboulis 2004;. de Vet et al. 2009).

Fe(II) adsorption/oxidation

Beside chemical and microbial oxidation, Fe(II) can be adsorbed onto the surface of the filter media or iron hydroxide precipitates that are present in the rapid sand filter (Sharma et al. 1999). Adsorbed Fe(II) is subsequently oxidized to form additional types of iron hydroxide structures. These newly formed layers create a new adsorption surface for dissolved Fe(II). This

mechanism relies on the iron(II) adsorption capacity of the filter media (Sharma et al. 1999). Importantly, together with sequential iron oxidation, it can lead to formation of mineral coating structure on the surface of the filter materials (Sharma et al. 1998), which can affect both microbiological and physical aspects of the rapid sand filters.

1.2.3 Manganese transformation

Manganese, in the form of soluble Mn(II), is a common constituent in groundwaters. Soluble manganese in aquifers is generally formed by microbially mediated reductive decomposition and dissolution of compounds such as Mn-OOH and MnO_2 under anoxic conditions (Nealson & Saffarini 1994; Buamah 2008). The concentration of dissolved Mn(II) in aquifers is controlled by redox reactions between Mn (II) and Mn (III, IV) and pH. Unlike Fe(II), chemical oxidation of dissolved Mn(II) is not rapid at circumneutral pH (Aziz & Smith 1992), typical of rapid sand filters. Transformation of manganese in rapid sand filters is assumed to occur via two mechanisms: (i) chemical adsorption/oxidation (ii) biological oxidation

Mn(II) adsorption/oxidation

In rapid sand filters, adsorption of Mn(II) onto the MnOx surfaces, followed by auto-catalytic and/or enzyme catalyzed oxidation has been reported as one of the mechanisms to remove Mn(II) (Gouzinis et al. 1998). Surfaces of Mn-(oxyhydr)oxide precipitates was shown to catalyze chemical Mn(II) oxidation through adsorption mechanism (Stumm & Morgan 1996). This mechanism is illustrated in figure 5 and kinetics of manganese oxidation is shown in equation 7 (Stumm & Morgan 1996).

$$-d[\mathrm{Mn(II)}]/_{dt} = kPO_2.[OH^-]^2.[Mn(II)] + k_1.[Mn(II)][MnO_2] \qquad (7)$$

k_o = reaction rate constant (l^2/mol^2.atm.min)

k_1 = reaction rate constant (l^3/mol^3.atm.min)

PO_2 = Partial pressure of oxygen (atm)

Beside Mn-(oxyhydr)oxide, variety of other surfaces, especially Fe-hydroxides can effectively catalyze the oxidation of Mn(II) in rapid sand filters (Junta & Hochella 1994). It should be noted that mineral coating accumulation on the surface of filter material increase the adsorption capacity: 15-year old filter media was found to be less dependent on the microbial activity than the 3-year old filter media (Sahabi et al. 2009).

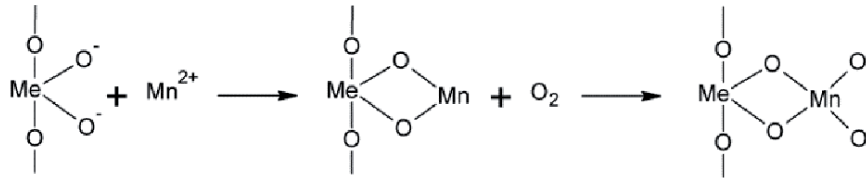


Figure 5 Process scheme for Mn(II) oxidation through adsorption and sequential oxidation. Me symbolizes a transition metal ion (Søgaard & Madsen 2013).

Microbial Mn(II) oxidation

The actual contribution of microbial mediated Mn(II) oxidation to the overall manganese oxidizing activity in rapid sand filters is not known. It was hypothesized that biological Mn(II) oxidation may be crucial for the start-up of the filters, which is subsequently replaced by removal via adsorption/oxidation (Berbenni et al. 2000; Sahabi et al. 2009).

Many studies on rapid sand filtration have reported the contribution of biological Mn(II) oxidation (Hope & Bott 2004; Sawayama et al. 2011; Burger et al. 2008). The bacterial genera involved in Mn(II) oxidation are taxonomically diverse, including *Crenothrix*, *Hyphomicrobium*, *Leptothrix*, *Metallogenium*, *Actinobacteria*, *Siderocapsa, Bacillus* and *Pseudomonas* (Katsoyiannis & Zouboulis 2004; Mouchet 1992; Pacini et al. 2005; Hanert 2006; Cerrato et al. 2010; Tebo et al. 2005).

Specific genes encoding Mn(II) oxidation are unknown because no putative Mn(II) oxidases have so far been purified and no heterologous overexpression pattern of a gene encoding Mn oxidation has been observed to date (Tebo et al. 2005). However, many studies have reported different functional pathways of Mn(II) oxidation, and many genes have been identified that effect bacterial Mn(II) oxidation. (Table 3). Therefore, direct detection and quantification of microbes involved in Mn(II) oxidation using a functional of phylogenetic genetic marker is still not possible.

Table 3 Genes can be involved in Mn(II) oxidation (Tebo et al. 2005)

Gene	Description	Strain
ccmE	c-type cytochrome biogenesis	P. putida MnB1
ccmF	c-type cytochrome biogenesis	P. putida MnB1&GB-1
ccmA	c-type cytochrome biogenesis	P. putida MnB1
sdhABCD	Succinate dehydrogenase complex	P. putida MnB1
aceA	Lipoate acetyltransferase	P. putida MnB1
icd	Isocitrate dh	P. putida MnB1
trpE	Subunit of anthranilate synthetase	P. putida MnB1
xcpA	General secretory pathway	P. putida GB-1
xcpT	General secretory pathway	P. putida GB-1
cumA	MCO	P. putida GB-1
mnxA	No homology	Bacillus sp. SG-1
mnxB	No homology	Bacillus sp. SG-1
mnxC	Homology to scol,	Bacillus sp. SG-1
mnxD	No homology	Bacillus sp. SG-1
mnxE	No homology	Bacillus sp. SG-1
mnxF	No homology	Bacillus sp. SG-1
mnxG	MCO	Bacillus sp. SG-1
mofA	MCO	L. discophora SS-1
mofB	Peptidyl-prolyl-cis-trans isomerase	L. discophora SS-1
mofC	Cytochrome c family protein	L. discophora SS-1

1.2.4 Carbon and sulfur transformations

Methane and hydrogen sulfide are the major form of carbon- and sulfur based electron donors in groundwaters, respectively. In highly reduced aquifers, organic matter may be oxidized to reduce sulfate to hydrogen sulfide after nitrate is depleted and carbon dioxide to methane after sulfate is depleted. In deeper regions of aquifers, methane will diffuse upward after methanogenesis process. However, organic carbon content is generally low in the Danish limestone aquifers therefore formation of methane by methanogenesis and hydrogen sulfide by sulfate reduction appeared to be rare in groundwaters of such aquifers (Klaus 2003). Alternatively, hydrogen sulphide and methane in Danish groundwaters may primarily originate from the confining Pleistocene sediments which contain these gasses (Klaus 2003; Scherer & Wichmann 2000).

As a minor constituent, groundwaters may contain pesticides and their degradation products which originate from anthropogenic activities; however

these constitutes are also rare in limestone aquifers in Denmark (Klaus 2003). Another form of carbon is present in the form of bicarbonate in groundwaters and rapid sand filters. Autotrophs on filter materials transform inorganic carbon to organic carbon via carbon assimilation pathways, which may subsequently be excreted.

In groundwater treatment methane and hydrogen sulfide are generally removed by aeration process which basically based on physical venting of these gasses. An effective aeration process usually results complete removal of these gasses. Although theoretical calculations and measurements suggest a complete removal, microbiological studies reported the presence of methanotrophs in rapid sand filters treating groundwaters (de Vet et al. 2009; Martiny et al. 2005; White et al. 2012). The role of methane oxidizing microorganisms in rapid sand filters requires a further research.

1.3 Materials and methods: Tools to assess taxonomic and functional diversity of microbial communities

A comprehensive understanding of microbial mediated ecosystems is not possible by process monitoring alone. A recent example for this was seen in the study of Canfield and his colleagues (2010). In this study 16S rRNA and metagenomic libraries consistently suggested the activity of both oxidative and reductive sulfur-cycling bacteria in oxygen-free waters off the coast of northern Chile, although no obvious in situ chemical expressions of active sulfur cycle were detected. Further analysis using stable isotopes revealed that sulfur cycling is important in oxygen-minimum zones of the ocean. This example indicates how important microbial identification and quantification are for understanding the biological mechanisms in an ecosystem. In addition, this example also indicates that microbial phylogeny must be linked to microbial physiology to be able to infer microbial abundance and taxonomy patterns; without the knowledge of sulfur oxidizing and reducing bacteria, they could have only been aware of an abundant taxon with unknown function. As for engineered systems, this link should be well established to fully understand the biological mechanism for each specific contaminant degradation process.

Several molecular techniques have emerged to assess different aspects of microbial communities using their genetic markers. These markers are present in all prokaryotic cells. Prokaryotes store the information as DNA, transcribe it into RNA, and use the information to produce proteins which regulate and perform the functions of the cell (Figure 6).

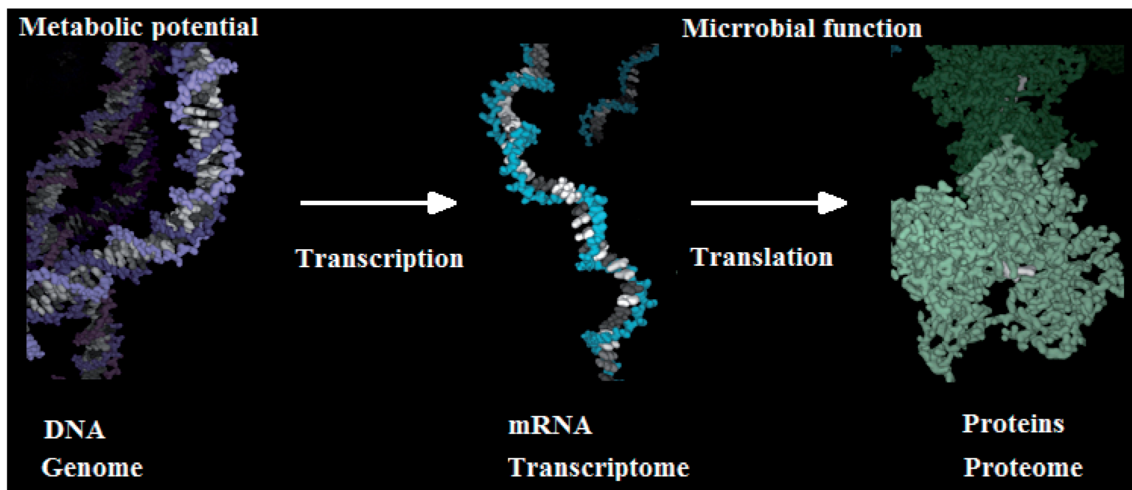


Figure 6 The mechanism that store, carry and transform the genetic information in prokaryotes (modified from Schulz 2011).

DNA, as one of the genetic marker, is stable and easy to extract; molecular analyses on DNA can reveal gene abundance and identity of a particular microbial group. DNA based gene abundance gives an idea about the history of cell replication, because the quantity of a particular gene in a genome can only increase by genome replication. And cell replication may link to energy utilization, because a considerable amount of energy is required for a cell to replicate. On the other hand, DNA does not include information about the actual cell activity and function. And quantification of a particular gene in a sample can be biased due to presence of extracellular DNA in that environment (Recorbet et al. 1993). Moreover, targeted gene number per genome can vary depend on the taxon, which may inflate gene numbers quantified by molecular tools.

RNA has a lower stability compared to DNA, but the level of stability varies among the type of RNA. Messenger RNA (mRNA) is an important RNA component; it reflects the active biological pathways that are regulated at the level of transcription (Dumont et al. 2011). Although the stability is very

short and extraction is challenging, mRNA has been used in many studies (Moran 2009), as a genetic marker.

In addition to mRNA, many studies have used ribosomal RNA (rRNA) to characterize active microbial communities (for example, Vandieken et al. 2012; Glaubitz et al. 2009). While mRNA gives information about the active metabolic pathways, rRNA includes the information of metabolically active microbial communities. Molecular analyses targeting rRNA, especially stable isotope probing (SIP), give more information on functionally active cells than DNA based analyses, because rRNA can also reflect the activity of non-growing cells (Dumont et al. 2011). Although rRNA has longer stability than mRNA in non-stressed conditions, previous reports have revealed shorter degradation times under starvation conditions (Deutscher 2003). All in all, the resolution of the link between microbial taxonomy and function also depends on the biomarker used in the study (Figure 7).

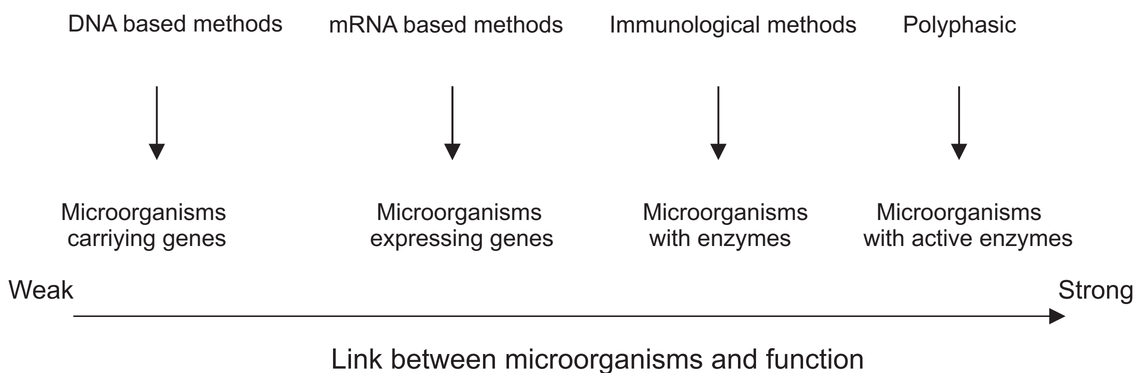


Figure 7 Strength of the possible link between microbial community and function can vary depend on the biomarker used in the study (modified from Philippot & Hallin 2005)

A single molecular technique with a specific genetic marker, is usually not enough to identify cryptic biological mechanisms, unknown taxonomy or unknown metabolic pathways; because identification of a gene is assessed by its evolutionarily relatedness to previously identified genes of a known species. To overcome this, different combinations of cultivation and molecular based tools can be used depending on the hypothesis of research to reveal structural and functional microbial communities. A wide range of molecular techniques (Figure 8) exists in the literature. In following subsections, I will review the approaches applied in this thesis. Furthermore, I will give information on the purposes they were used for.

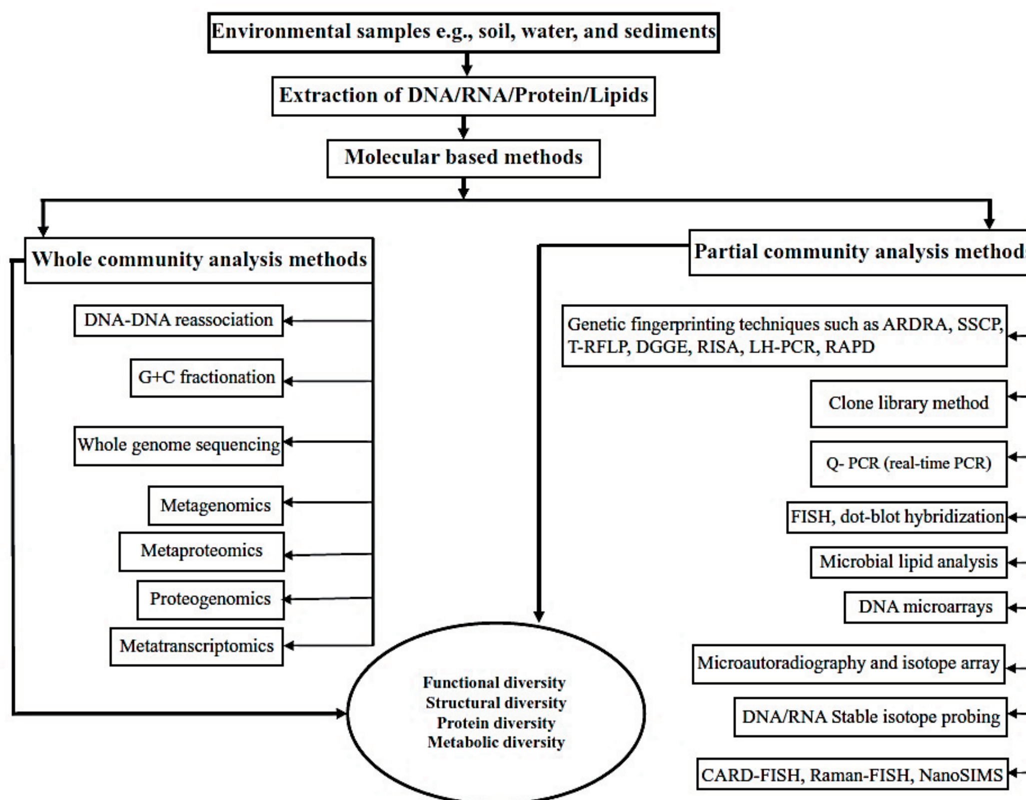


Figure 8 Molecular techniques to characterize the structural and functional diversity of microorganisms (Rastogi & K. Sani 2011)

1.3.1 Opposing Fe(II)/O_2 gradient cultivation

So far, no enzymes and genes specifically involved in iron oxidation at circumneutral pH have been identified; therefore the presence of iron oxidizing microorganisms cannot be assessed in a community using molecular techniques. In addition, the 16S rRNA sequence database of cultivated iron oxidizers is not large enough to detect iron oxidizing microorganisms from complex environmental samples. To identify iron oxidizing microorganisms in an environmental sample, a combination of cultivation and molecular techniques is inevitable.

The Fe(II)/O_2 gradient method (Emerson & Moyer 1997) has been the most common technique to enrich and isolate circumneutral iron oxidizers. Using this method, many mixotrophic, hetetrophic and lithoautotrophic iron oxidizers have been isolated from the genera *Gallionella*, *Sideroxydans*, *Pseudomonas*, *Acidovorax*, *Dechlorospirillum*, *Rhodoferax*, and *Mariprofundus* (reviewed in Emerson et al., 2010). This technique allows isolation of iron oxidizer strains through serial dilutions. This technique can

also be used with 16S rRNA fingerprinting techniques to identify different iron oxidizer guilds in a single enrichment. However, due to the long incubation periods (over 15 days) and cross-feeding of microbial products, heterotrophic microbes without iron oxidizing capability can remain in these enrichments (Yu et al. 2010). These heterotrophs should be identified and excluded from the database.

The concept of the Fe(II)/O_2 gradient method is simple; in an organic carbon deficient medium, counter gradients of oxygen and Fe(II) are formed by supplying FeS in the bottom and air from the top (Figure 9). These opposing fluxes of electron donor (Fe(II)) and electron acceptor (O_2) mimic oxic/anoxic interfaces in nature and allows iron oxidizers to select their optimal gradient position for growth.

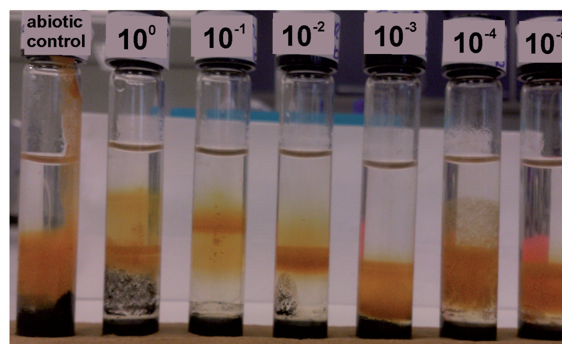


Figure 9 A representative set of Fe(II)/O_2-gradient tubes

In this thesis, this technique was used to enrich and quantify iron oxidizing microorganism together with most probable number (MPN) method. After 15 days incubations, DNA was extracted from gradient tubes and subsequently examined using molecular techniques, including DGGE, cloning and pyrosequencing.

1.3.2 Denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE of ribosomal DNA was introduced into microbial ecology by Muyzer et al. (1993). In principle, DNA fragments of the same size but different base-pair sequence are subject to electrophoresis on an acrylamide gel with a linearly increasing gradient of denaturing agents (a mixture of formamide and urea). During electrophoresis, the DNA fragments melt in unique segments. When the melting-domains, which are sequence-specific, reach their melting temperature, the partly denatured double-stranded DNA

amplicons are formed which stop at different points during migration in the gel.

DGGE has been widely used in microbial ecology to detect and compare microbial communities in complex environmental samples (for example Wang et al. 2009; Mühling et al. 2008; Dowd et al. 2008). To detect a taxon with typical DGGE, its relative abundance needs to exceed 1% (Muyzer et al. 1993). In rapid sand filters, it has been used with 16S rRNA targeting universal primers to identify dominant members of the microbial community (de Vet et al. 2009) in a full scale rapid sand filter.

In this thesis, I used DGGE technique to assess dominant iron oxidizing communities as well as microbial diversity in opposing Fe(II)/O_2 gradient tubes. In addition, using DGGE and liquid incubations, we developed a screening technique to distinguish metabolisms of iron oxidizing guilds (Paper III).

1.3.3 Cloning and sequencing

The cloning technique includes the steps in the order of cloning, screening and sequencing. In the cloning step, the extracted DNA samples, firstly, are subjected to PCR to amplify the target genes of organisms. Later, this amplified DNA fragments can be cloned into a plasmid vector or a bacteriophage. After the vector-plus-insert is transformed into an *E. coli* strain the *E. coli* strain is grown on selective agar plates and screened for the presence of cloned inserts in the cells. Like DGGE, this technique has been widely used in microbial ecology. In rapid sand filters, it has been used with 16S rRNA and amoA targeting primers to identify dominant members of the microbial and ammonia oxidizing communities in a full scale and a pilot scale rapid sand filters, respectively (White et al. 2012; Feng et al. 2012).

In this thesis, this technique was applied to obtain full 16S rRNA sequences of iron oxidizing microorganisms in liquid incubations amended with Fe(II) (Paper III).

1.3.4 Pyrosequencing

Sanger sequencing has provided new insights for the identification and quantification of microbial communities in complex environments. In Sanger sequencing, fragments need to be isolated prior to sequencing (i.e. DGGE or cloning), which limits the number of detectable microorganisms in a meta-community. An earlier study investigating soil ecology estimated the microbial diversity as between 2000-10.000 species per gram soil sample at

the strain taxonomic level (Torsvik et al. 1990; Schloss & Handelsman 2006) using sanger sequencing. However, in the era of next-generation sequencing technology, it has been reported that the estimated microbial diversity in soils ranges between 25972 and 43652 (at the strain level based on Chao1 estimator ,Roesch et al. 2007). It is clear that previous diversity estimates were underestimates of microbial diversity, which is mainly caused by insufficient sequencing depth of classical molecular methods to detect rare microbial taxa.

Pyrosequencing is one of the next-generation sequencing techniques (Nyrén 2007), and removes the pre-isolation step for fragments (i.e. DGGE or cloning). The isolation of each DNA fragment is performed on micron beads which allow isolated fragments to be copied in millions in order to get a light signal. Pyrosequencing is depend on the pyrophosphate (PPi) molecule released when a dNTPs base incorporate into a DNA chain. Released pyrophosphate then enables enzymatic production of ATP. By the enzyme luciferase, released ATP provides the energy required to convert luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. Light colour and intensity are captured with a camera. However, too strong or too weak light signals can lead to an over- or under-call for the corresponding nucleotide type (Balzer et al. 2011). This effect, so called "noise" should be corrected for diversity estimations and abundance calculations (Quince et al. 2011).

In this thesis, I used pyrosequencing in all of my papers to (i) estimate 16S rRNA based alpha and beta diversity of microbial communities, (ii) characterize the microbial communities and known functional groups, (iii) detect the relative abundances of particular full-length 16S rRNA genes that were obtained from cultivation based experiments, and (iv) identify the ^{13}C–labeled microbial communities in rapid sand filters.

1.3.5 Stable isotope probing (SIP)

To understand the biological mechanism of a particular process, a first step is to identify the microorganisms that are involved in that process. In an engineered environment, knowing the taxa involved in a particular function can provide information about the life style and metabolic strategies of these taxa, which is important for design and operation of the system. SIP technique allows identification of microorganisms and/or metabolic pathways that are actively involved in the assimilation specific compounds. It is essential that the compounds can be provided in a form containing heavy isotopes such as ^{15}N, ^{2}H or ^{13}C (Radajewski

et al. 2000). This technique has been used to identify active sulphate reducers, methanotrophs, iron reducers and cellulose degraders fed with different ^{13}C-labeled substrates (i.e ^{13}C-cellulose, ^{13}C-methane, ^{13}C-acetate) (Parkes et al. 1998; Chatzinotas et al. 2013). For autotrophic microorganisms assimilating in-organic carbon (i.e ammonia oxidizers), SIP is usually performed using ^{13}C-labeled CO_2 or CO_3^{2-} as carbon source (Glaubitz et al. 2009; Pratscher et al. 2011).

SIP is based on density separation of labeled (heavy) from unlabeled nucleic acid (DNA or RNA) ones. Separated heavy nucleic acid can be characterized by a combination of fingerprinting and sequencing techniques or directly by next-generation sequencing techniques (Figure 10).

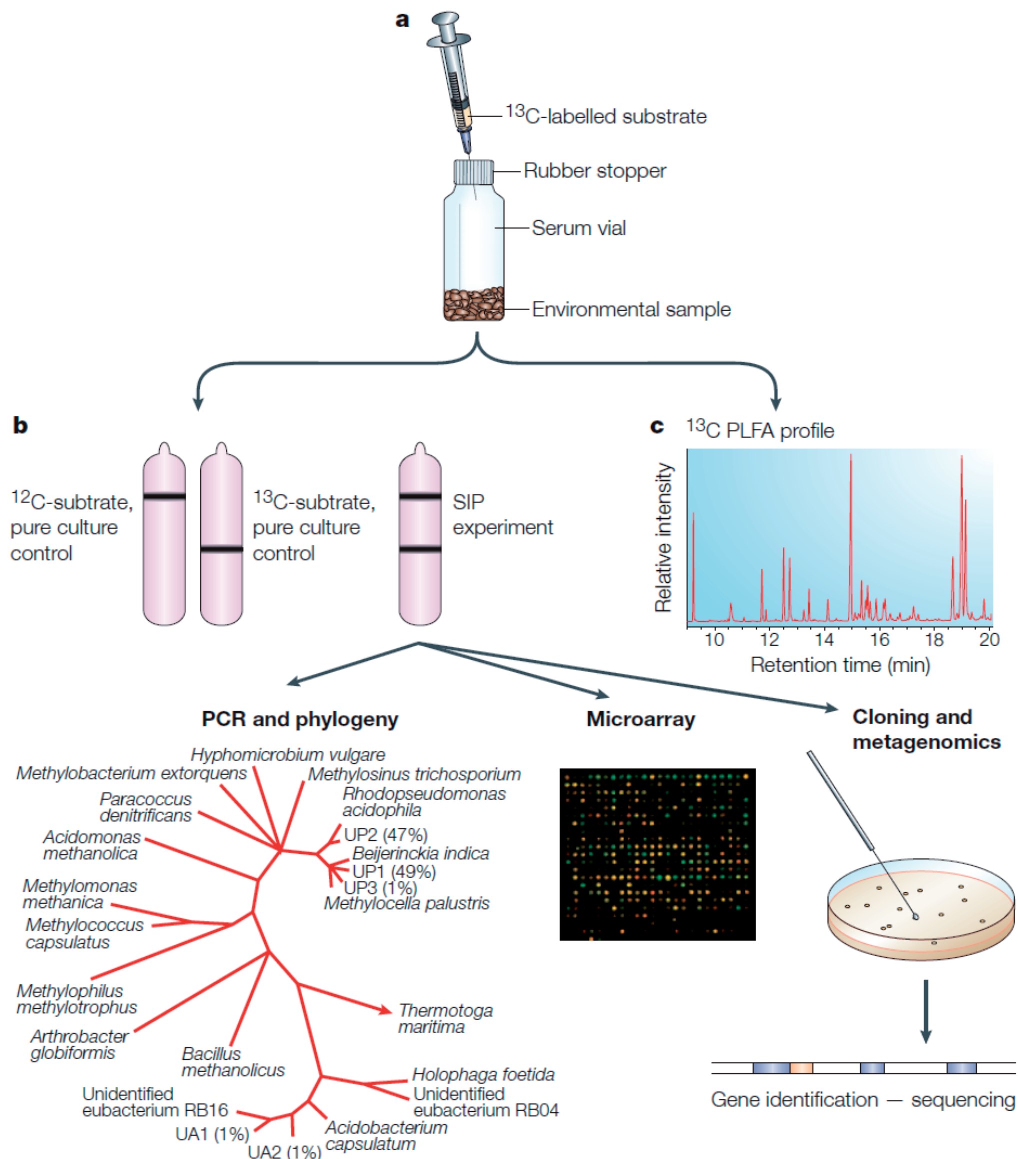


Figure 10 DNA-based stable isotope probing (SIP) (Dumont & Murrell 2005)

1.4 Aims and hypotheses of the PhD thesis

It is well established that microorganisms contribute to the removal of the major contaminants in rapid sand filters. Previous rapid sand filter studies have primarily focused on processes with little investigation devoted on characterizing the density, composition and variation of the microbial community components.

The aim of this thesis to reveal the large pool of microbial members present in rapid sand filters alongside their occurrence patterns at different waterworks, identify the microbes involved in specific contaminant cycles, characterize the spatial heterogeneity of microbial communities, and examine the relationship between microbial communities and activities and the mineral precipitates.

The following hypotheses were tested in this thesis:

- The microbial communities in rapid sand filters at different waterworks are filter-specific, with few shared taxa present between filters and between waterworks
- No significant taxonomic difference exist between the top and bottom filter layers
- Pre-filters (first stage filters) are taxonomically rich environments like after-filters (second stage filters), but have a significantly different microbial community structure.
- The *Nitrosomonas* lineage is not the sole taxon responsible for ammonium oxidation in rapid sand filters.
- The *Nitrospira* phylum is not the sole taxon responsible for nitrite oxidation in rapid sand filters.
- Nitrospira is involved in ammonia oxidation in rapid sand filters.
- Ammonia oxidizing archaea do not coexist with ammonium oxidizing bacteria in rapid sand filters,
- Iron oxidizing microorganisms are present and active in waterworks receiving low Fe(II) concentrations.
- Taxa different from *Gallionella* and *Leptothrix* are involved in Fe(II) oxidation at circumneutral pH in rapid sand filters
- Rapid sand filters are not spatially homogeneous in terms of microbial community structure and composition.

- Mineral coating deposits on the surface of filter grains, support microbial density and activity in rapid sand filters.

To test these hypotheses a field study comprising 5 waterworks, a cultivation based experiment and two lab-scale column based experiments were conducted. A comprehensive sampling was performed at 5 waterworks including source waters, pre filters, after filters and subsequently 16S rRNA targeted pyrosequencing was performed on these samples (Paper I). Shared taxa were identified for all process units, together with their alpha diversities. Physiology of cultured closest relatives were used to predict the metabolism of identified shared taxa.

To identify and quantify the microorganisms involved in the Fe(II) oxidation at different waterworks a combination of cultivation and molecular based techniques were applied; opposing Fe(II)/O_2 enrichment and selective liquid incubations were performed together with molecular techniques such as DGGE, cloning and pyrosequencing (Paper III).

Unexpected relative abundances of ammonia and nitrite oxidizing bacteria based on 16S rDNA data led us to try and identify microbial communities in the nitrification process using column experiments, RNA-SIP and pyrosequencing (Paper II).

Previous studies have reported the different nitrification rates within a filter (Lopato et al. 2011, 2013); we investigated the existence of spatial heterogeneity in a filter with respect to microbial community structure. For this purpose, I developed a new beta-significance detection method and used it on pyrosequencing libraries (Paper IV). Significant beta diversity values between sampling points within a filter were also tested using a specific functional group: iron oxidizing bacteria (Paper V). PCA plots from DGGE profiles of gradient tubes were used to assess spatial heterogeneity of IOB guild composition within a rapid sand filter.

Finally, by examining filter materials retrieved from a single filter and filters of different waterworks, I identified a strong correlation between mineral coating mass and microbial mass in rapid sand filters. On the basis of these preliminary observations, we implemented an experimental set-up to reveal if these mineral coatings support microbial growth and may have protective roles against environmental insults (Paper VI).

2. Microbial diversity in rapid sand filters (Paper I)

Rapid sand filters are microbe-mediated engineered systems which aim to produce high quality drinking water by removing different contaminants from groundwaters. Transformation of contaminants is driven by different functional microbial groups in these filters as well as chemical and physical processes, such as oxidation, aggregation, and precipitation. Since the purpose of the filters is to remove contaminants, the function of the microbial communities is of importance. A specific function can be facilitated by different taxa, and the potential function of an identified taxon can typically not be predicted unless its physiology was previously established (Dumont & Murrell 2005). Therefore, in engineered systems which are designed to carry out similar functions, microbial communities can tentatively be compared in terms of their identity and abundance in order to determine if contaminant bioremoval performance similarities or differences between the systems are driven by metabolic similarity or differences between microbial communities at those systems (Werner et al. 2011). By comparing taxa abundance between microbial communities deriving from ecosystems receiving with similar substrates and conditions, the metabolic similarity of microbial communities can be compared (Rivière et al. 2009) only if physiology and phylogeny relation of these taxa has previously been established.

We applied 16S rDNA based pyrosequencing to 96 samples from pre- and after filters of five different waterworks including the groundwater sources (Paper I). Our aim was to characterize microbial communities in these waterworks and reveal similarities in terms of evenness, richness, taxonomy, and predicted metabolism of microbial communities. Selected waterworks are similar with regards to the treatment chain (aeration, pre- and after filter), type of groundwater compounds and backwashing frequency, however, they differ in detailed chemical composition groundwater, original filter bed grain material, and hydraulic flow rates through the filter.

In the following subsections, I will introduce the taxonomy of microbial communities in rapid sand filters, the comparisons of alpha diversity between waterworks and the surprising abundance of shared taxa between the investigated waterworks. Extended findings and material methods can be found in Paper I.

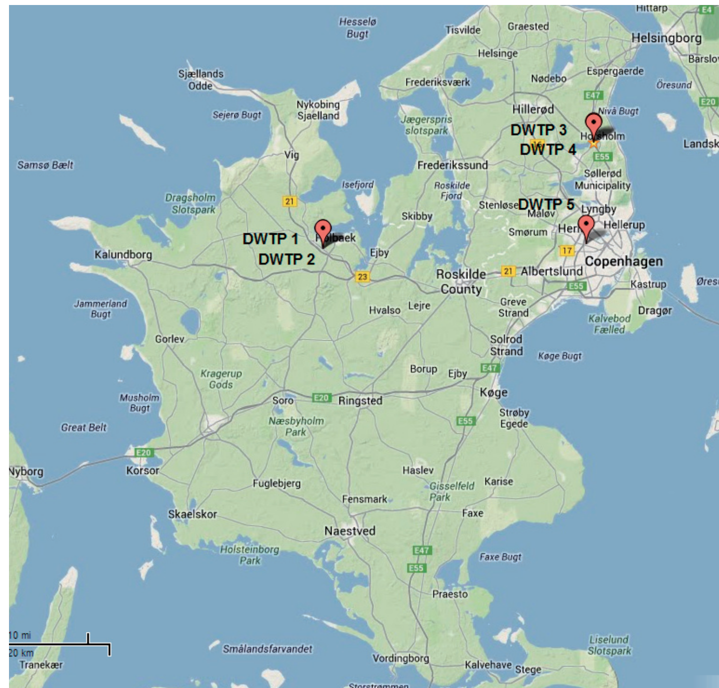


Figure 11 Geographical locations of investigated waterworks. The picture was obtained with Google Maps.

2.1. Microbial communities and their characteristics

Rapid sand filters have received far less attention for microbial investigation than other engineered biosystems such as activated sludge, anammox, or nitrification systems. Microbial community members in waterworks receiving groundwaters were previously investigated with 16S rDNA based cloning and DGGE techniques (White et al. 2012; de Vet et al. 2009). By applying pyrosequencing, we found that the level of microbial diversity in rapid sand filters is comparable with that found in activated sludge systems and anaerobic digesters, although they receive much lower carbon and energy inputs. The Chao1 richness estimations in after filters revealed richness values 13 times higher (157±31 vs. 2142±1312) than those found in a cloning based study (White et al. 2012). Community richness estimates of groundwaters were close to those in after filter units (1944±746), whereas pre filter units showed the lowest richness with 1150±490 $OTU_{0.03}$s. Diversity pattern (Shannon index or Simpson index) followed the richness patterns, but evenness showed a completely reverse pattern (Figure 12). The change in microbial diversity and evenness patterns going from groundwater to pre-filters are consistent and interesting; the decrease in microbial diversity could be explained by strong environmental selection, e.g. in the DO level (<0.1 to >8 mg/l), resulting in massive loss of taxa from groundwater. The difference in evenness was mainly caused by the extremely high abundance

of a few $OTU_{0.03}$s in pre filters; potentially the best-adapted taxa to the new environmental conditions. Furthermore, the highly abundant $OTU_{0.03}$s in all pre-filters are taxonomically identical, which strengthens the notion of strong environmental selection versus random selection.

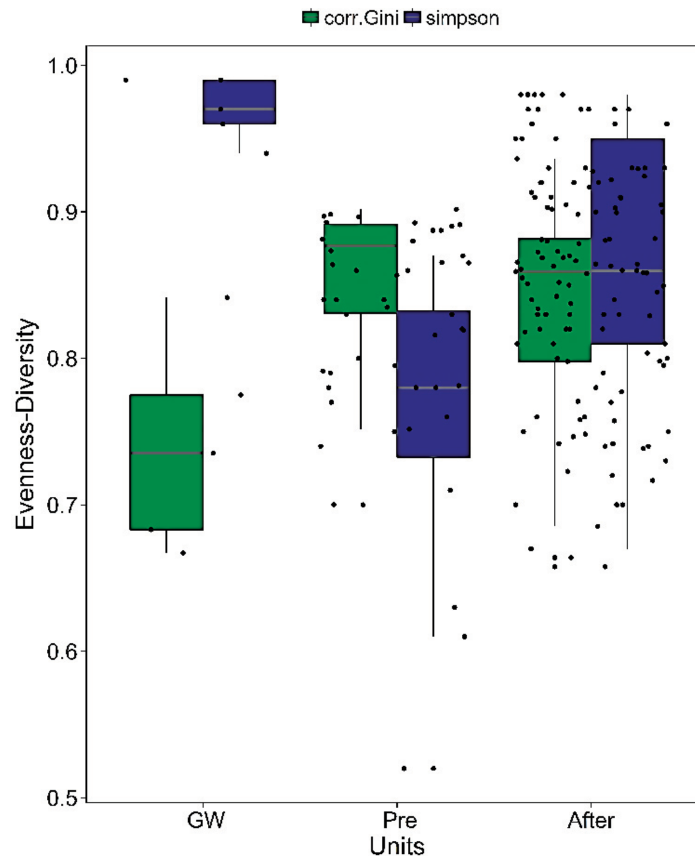


Figure 12 Diversity and evenness of microbial communities within the different process units of studied waterworks. Dots represent data points for the corresponding box-plot. The gini coefficient measures the normalized area between the Lorenz curve and the line of perfect evenness. It gives a single value describing the degree of evenness, which ranges between zero and one. The higher the Gini coefficient, the more uneven a community is. Simpson's Diversity Index is a measure of diversity ranging between zero and one. It considers the number of species present, as well as the relative abundance of each species. The higher the Simpson Diversity Index, the more diverse a community is.

Taxonomic classification (Figure 13) revealed that the dominant phyla in pre-filters consist of the *Proteobacteria* (58%±28), *Nitrospirae* (27%±25), *Acidobacteria* (3.6%±1.7), *Actinobacteria* (3.6%±3), *Candidate division OD1* (1.8%±1) and *Planctomycetes* (1.7%±1). Specifically, the pre-filter communities were dominated (30.3%±24) by the genus *Methylococcales* (Gamma-proteobacteria), well-known methane oxidizers. The second most dominant taxon in pre filters

was the genus *Nitrospira* (27%±23), which are key nitrifiers in many natural and engineered ecosystems (Pester et al. 2013). In the after filters, the phyla of *Nitrospira* (44%±23)*, Acidobacteria* (9.2%±5), *Planctomycetes* (3.4%±2), *Actinobacteria* (2.7%±1), *Chloroflexi* (2.3%±1.8) and *Gemmatimonadetes* (1.3%±1.2) were dominant taxa together with *Proteobacteria* (33.8%±17). These results, and especially the high *Nitrospira* abundance, are consistent with the results from other studies; White et al.(2012) detected *Nitrospira* in 50.4% of the total clones retrieved from filter samples taken from a full-scale rapid sand filter treating anaerobic groundwaters. Similarly, *Nitrospira* phylum was found as the thickest DGGE bands among bacterial 16S rRNA amplicons retrieved from a full-scale rapid sand filter de Vet et al. 2009). Martiny et al, (2005) studied model drinking water distribution system fed with RSF effluent and found that 5-27% in biofilm and 30-40% in bulk water clones were *Nitrospira*. It is important to note that the enormous *Nitrospira* abundance, observed in our and in previous reports cannot be satisfactorily explained by the influent nitrite concentrations (<0.01 mg/lt) or the abundance of ammonium oxidizers prokaryotes (3.2%±3) in the filters. For example, Martiny et al. (2005) could not predict the type of metabolism in Nitrospira phylum due to the deep branching in the phylogenetic tree and they stated that the functional role of Nitrospira phylum is unknown in their experimental system. The observation on the abundance of the *Nitrospira* phylum in rapid sand filters requires further investigation.

Our study also demonstrated that the groundwater microbial communities can be related to the downflow communities. On average 75.4%±20 of the dominant $OTU_{0.03}$s in pre-filters was detected in the groundwaters, be it as rare members. For the dominant $OTU_{0.03}$s in all after-filters, the average OTU overlap was found to be 99%±2 with pre-filter $OTU_{0.03}$s. This result clearly shows that the groundwater microbiota provide a seed bank for downflow filter units.

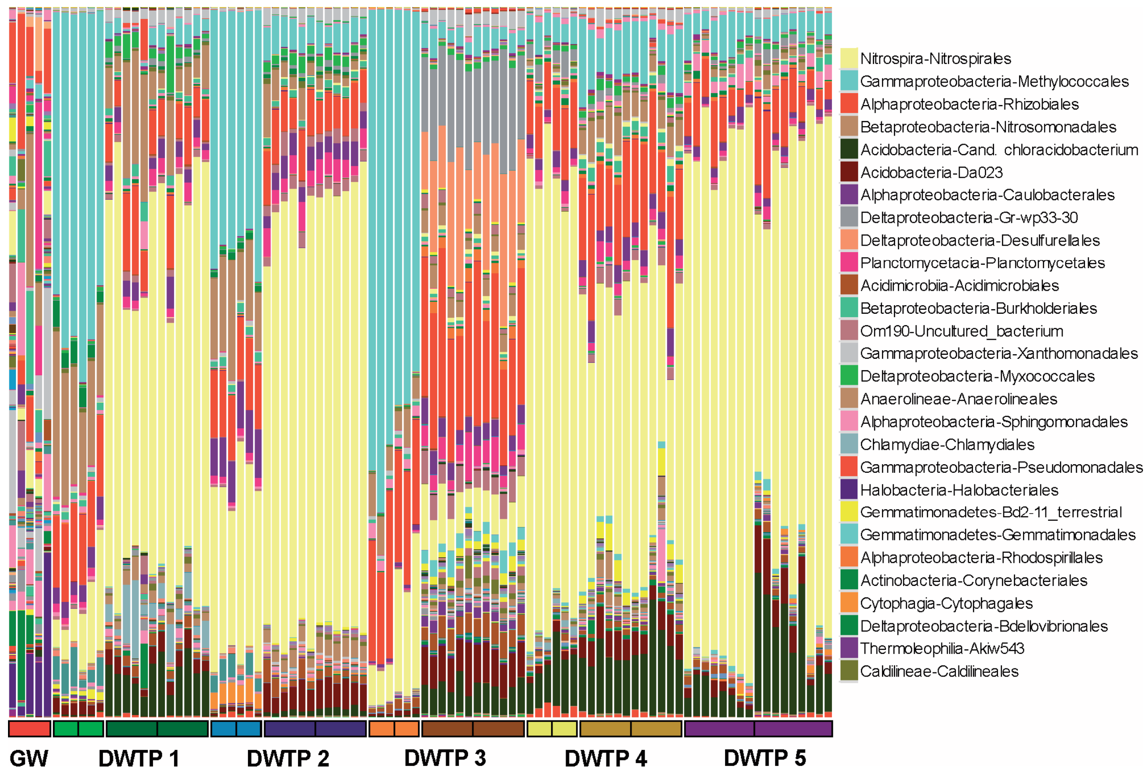


Figure 13 Order-level taxonomic classifications of 16S rRNA amplicons across all waterworks against the Silva108 prokaryotic reference database. For each DWTP, different rapid sand pre-filters (brighter boxes) and after-filters (darker boxes) are plotted along the x-axis. The first and second box, in each color, refers to the results from the top and composite depth sample of the filter, respectively. Taxa abundance is expressed in percentage. Groundwater microbial communities (GW) are shown in first five columns.

2.2. The core taxa

Commonly occurring microorganisms between functionally similar environments could indicate that these species have a functional importance in the metacommunity. In our study, we investigated the presence of shared taxa among the 5 waterworks as well as the abundance of these core taxa in each waterworks community. At the genus level, a total of 30 (6.2%) and 29 (3.7%) taxa were found as core taxa in pre- and after filters, respectively. Although the number of shared taxa was very few, their relative abundance in each waterworks was strikingly high (87%±5 in pre- and 75%±18 in after-filters respectively). The high abundance of few core taxa in all waterworks indicates important points: (i) the differences in operation and design of the rapid sand filters at investigated waterworks were insignificant to disturb the core taxa, (ii) the major biological reactions in rapid sand filters may be driven by same taxa across filters and waterworks.

In addition, the relative abundance of the core taxa was slightly higher in pre filters than in after filters although chemical compositions of influent waters of pre filters are more dissimilar to each other than the influent waters of after filters. This may suggest that different factors may drive core community abundance than chemical groundwater composition.

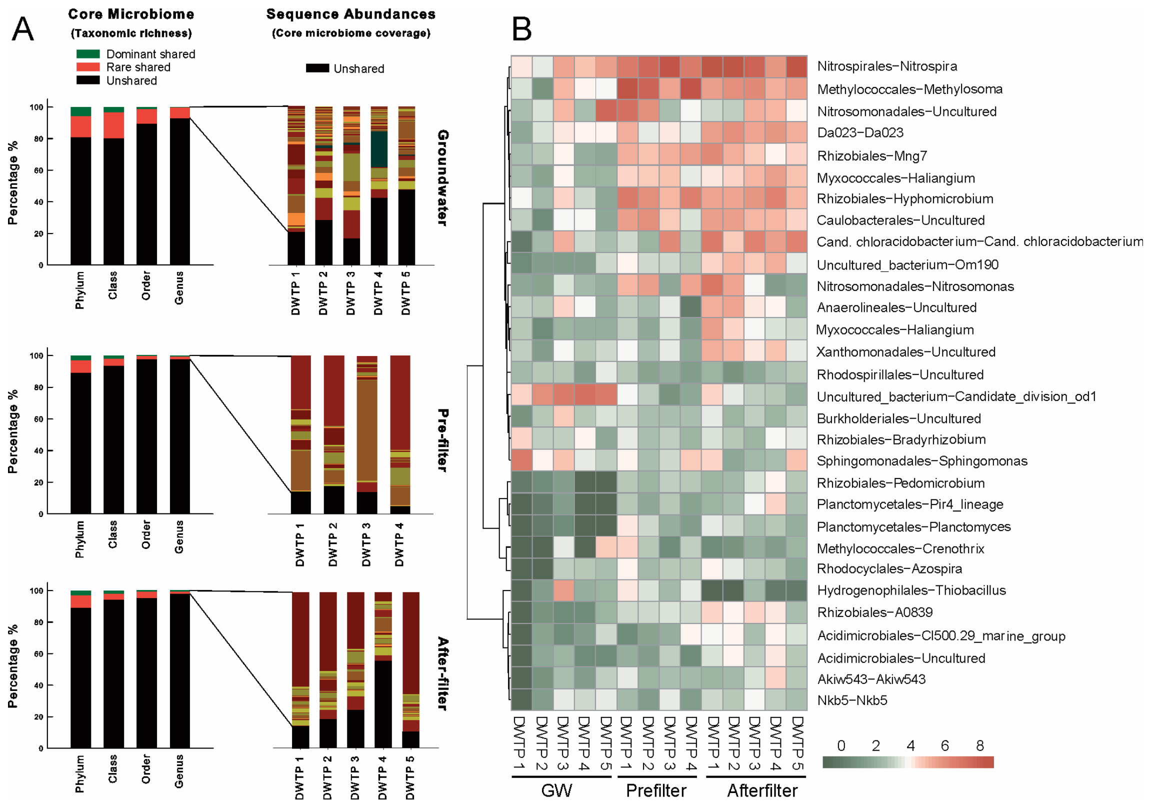


Figure 14 Richness, abundance, and taxonomic identity of taxa shared across all waterworks. (A) Taxonomic richness and sequence coverage of shared taxa in groundwater, pre-filter and after-filter units. (B) State of shared taxa members (genus level) from up to downflow units based on their absolute abundance. Absolute sequence abundances were log transformed and coloured red to black to present higher to lower abundance. Heatmap boxes without any sequence were assigned 10-6 (darkest black).

2.3. Metabolic potential of the core taxa

The observation of core taxa, beckons the question of “are the core members functionally important in rapid sand filters?”. From known metabolic properties of isolated taxonomically placed microbial strains s, we can infer potential functions of the core taxa in pre- and after filter units. Primary metabolisms like ammonium, nitrite, methane, manganese and iron oxidation, all essential functions for contaminant removal in RSF, were readily detected among the core taxa. Phylotypes that could be putatively assigned to these metabolisms dominated the core communities in pre- (79%±2) and after-filters (66%±0.5), suggesting that the core taxa may responsible for the key-processes in rapid sand filters. Many phylotypes were closely related to *Nitrosomonas oligotropha* as, a known ammonium oxidizer, both in pre- and after filters. With the exception of $OTU_{0.03}$s that grouped closely with *Ca. Nitrospira defluvii* and *Nitrospira moscoviensis* in the pre-filter and *Nitrospira marina*, *Nitrospira Calida* and *Ca. Nitrospira defluvii* in the after-filter core taxa, most of the nitrite-oxidizing phylotypes were affiliated with uncultured representatives of the *Nitrospira* genus. The closest cultured relative to methane oxidizing phylotypes in pre filters was *Crenothrix polyspora*. In after filter core taxa, phylotypes related to *Methylovulum miyakonense* and uncultured representatives of the *Methylovurum* clade were detected as abundant putative methane oxidizing bacteria. No archaeal sequences were found in the core taxa.

3. Functional microbial diversity in rapid sand filters (Paper II-III)

Microbial communities involved in a specific function often cannot be inferred from taxonomic identity alone. Although there are several evolutionarily clustered groups that are remarkably coherent in physiological attributes (Stahl et al. 2006), for example, nitrifiers (Teske et al. 1994; Head et al. 1993), methanotrophs (Hanson et al. 1996), members of some functional groups such as iron oxidizers, manganese oxidizers are widely distributed over different phyla and most of them are ecologically incoherent, in that they do not share general life strategies or traits. A good example may be the ecological differences between *Gallionella* and *Pseudomonas* lineages, which are known to be iron oxidizing microorganisms. Although any isolated strain closely related to the *Gallionella* lineage can be inferred as iron oxidizer, this is not the case for *Pseudomonas* lineage. High ecological diversity in *Pseudomonas* linage prevents physiological inferences from its phylogeny. In addition, adaptations and gene transfer events may allow different taxa to be involved in a specific process than those which can be inferred from the known physiologies.

Therefore, we investigated the microbial members involved in nitrification and Fe(II) oxidation by a combination of cultivation-based and non-cultivation molecular-based techniques. We focused on iron and ammonia oxidation processes because performance problems at waterworks are often related to ammonia and iron removal. In addition, our 16S rRNA pyrosequencing results (Paper I) revealed a surprising ratio between AOB/NOB abundances and enormous abundance and diversity of *Nitrospirae* phylum, which needed further investigation. Extended findings and material methods can be found in Paper II for ammonia and nitrite oxidizing microorganisms and in Paper III for iron oxidizing microorganisms.

3.1. Ammonia oxidizing bacteria and archaea

Although nitritation is a critical process in rapid sand filters, the direct experimental confirmation of the taxa involved in nitritation has, to our knowledge, not been performed to date. Based on amoA abundances in community in DNA, the genera *Nitrosomonas* (from bacterial domain; van der Wielen et al. 2009; White et al. 2012; Feng et al. 2012), *Nitrosoarchaeum* and *Nitrososphaera* (from archaeal domain; Bai et al. 2013) have been inferred as key lineages for ammonia oxidation in rapid sand filters. There were, however, several methodological

limitations in these studies: The identified amoA genes may not reflect the active AOB, in that the amount of microbes generating active enzymes is usually much lower than the amount of microbes that can generate them in the environment (Philippot & Hallin 2005). Detection of ammonia oxidizers may be biased by the use of amoA genes, as it is well known that ammonia monooxygenase can catalyze the oxidation of several compounds, beyond ammonia, such as methane, carbon monoxide and various hydrocarbons (Hooper et al. 1997). Therefore, we applied a RNA based stable isotope probing SIP approach together with selective growth experiments using inhibitors (Paper II).

Using stable isotopes of N, does not provide a useful approach for identifying nitrifiers, as most of the end products are non-assimilated (Pratscher et al. 2011). Instead we used ^{13}C provided in the form of bicarbonate, recognizing that nitrifiers are mostly autotrophic and capable of carbon fixation.

We performed four different ^{13}C-bicarbonate incorporation experiments, supplemented with different inhibitors to identify active microbial members performing different processes (summarized in Table 3). The fresh filter material used in column experiments of this study was obtained from a conventional drinking water treatment plant in Islevbro, Denmark. All columns were operated with 100% ^{13}C-labeled or unlabeled bicarbonate for 15 days. To mimic the full scale conditions at the waterworks, treated water from the investigated after-filter was used as influent medium for the column experiments.

Table 4 Summary of experimental set-up, ^{13}C incorporation and process values, and pyrosequenced samples

Runs		N source	C source (^{12}C-^{13}C)	Inhibitor	^{13}C / ^{12}C [(a)]	NH_4^+ [(b)] removal	NO_2^- [(b)] re-moval	NO_3^- [(b)] accretion	Pyrosequencing		
									Total DNA	Total RNA	SIP frac-tionation
Run 1 Full-scale RSF inoculum 1	Col.1	NH_4^+	$H^{13}CO_3^-$	-	279	99%±1	100%±0.3	66%±12	+	+	+
	Col.2	NH_4^+	HCO_3^-	-	-	98%±3	100%±0.2	70%±13	+	+	+
	Col.3	NH_4^+	HCO_3^-	Allylthiourea (ATU)	-	16%±14	100%±4.9	0%	+	+	+
	Col.4	NH_4^+	$H^{13}CO_3^-$	Allylthiourea (ATU)	54	18%±30	99%±4.5	0%	+	+	+
Run 2 Full-scale RSF inoculum 2	Col.5	NO_2^-	$H^{13}CO_3^-$	-	89	-	88%±1.0	67%±7	+	+	+
	Col.6	NO_2^-	HCO_3^-	-	-	-	92%±3.0	54%±23	+	+	+
	Col.7	NH_4^+	HCO_3^-	Chlorate (ClO_3^-)	-	11%±4.8	54.5%±38	18%±31	+	+	+
	Col.8	NH_4^+	$H^{13}CO_3^-$	Chlorate (ClO_3^-)	63	7%±6.2	26.4%±83	0%	+	+	+

[(a)] ^{13}C and ^{12}C ratio after 15 day lab-column run

[(b)] Removal and accumulation rates were calculated by averaging daily NH_4^+ and NO_2^- measurements

First and second columns were fed with only 1 mgNH_4/l of ammonia to achieve complete nitrification. Col.3 and Col.4 were fed with 1 mg/l of ammonia and 100 μM ATU to detect archaeal ammonia oxidation. In Col.5 and Col.6, only 1 mg/l of nitrite was added as substrate to select for nitratation process. Col.7 and Col.8 were fed with 1 mg/l of ammonia and 1 mM ClO_3^- to select for nitritation process.

At the end of the experiments, RNA and DNA were extracted from the biomass of each column and subjected to equilibrium density centrifugation to separate light (unlabeled) and heavy (labeled) RNA. Gradients of density-resolved RNA were fractionated and the cesium trifluoroacetate buoyant density of each fraction was determined. Selected fractions, after RNA precipitation, were conducted to reverse transcription of 16S rRNA, and then subjected to tag-based pyrosequencing. Labeled $OTU_{0.03}$s in SIP experiments were selected by comparing sequence abundances of $OTU_{0.03}$s in heavy and light fractions of the RNA from $^{13}CO_3$ fed columns as well as heavy fractions of the control columns. Extended descriptions for bioinformatics are presented in Paper II.

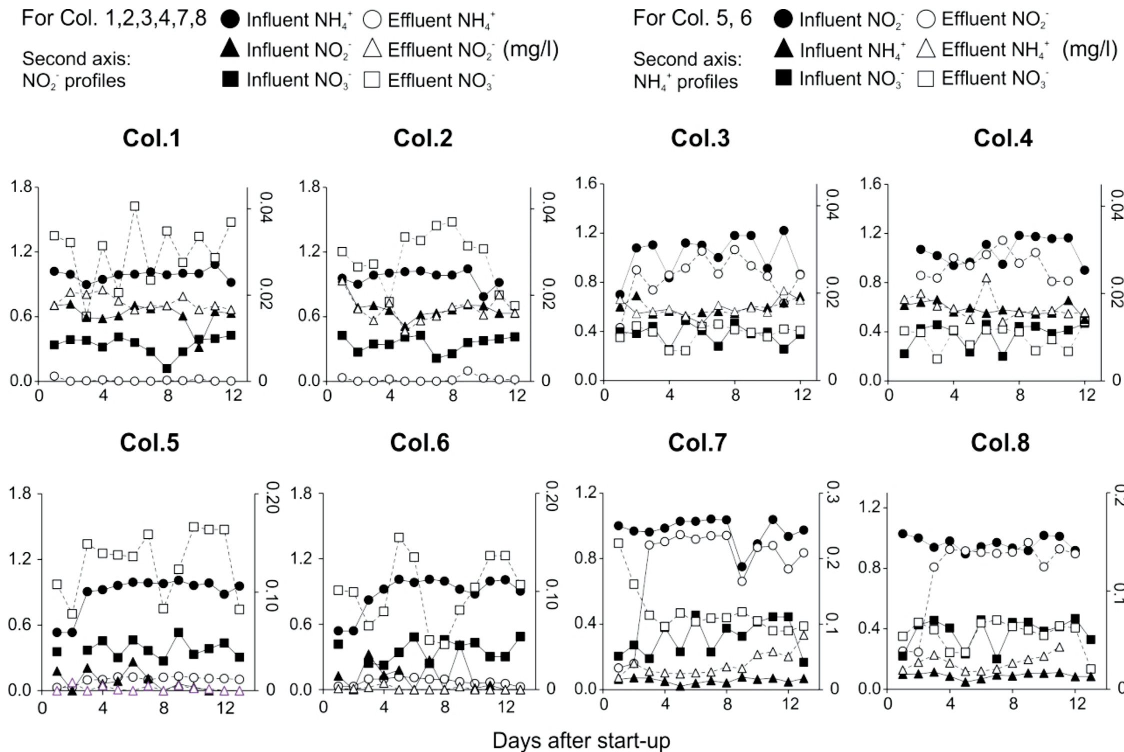


Figure 15 Concentrations of influent and effluent NH_4^+, NO_2^- and NO_3^- in control columns ($^{12}CO_3$) and $^{13}CO_3$ fed columns

Parallel columns fed with ^{13}C and ^{12}C carbonate showed very similar ammonia and nitrite oxidation patterns during 15 days of operation (Figure 15). Complete nitrification was observed in columns fed with only ammonia (Col 1-2; Figure 1), whereas no ammonia oxidation was detected in columns amended with ATU (Col 3-4; Figure 1). Ammonia oxidation levels were confirmed by nitrate accumulation patterns, in that no nitrate accumulation was observed in columns fed with ATU. As expected, high nitrate accumulation and nitrite consumption were detected in columns fed with nitrite, suggesting high activity of nitrite oxidizers. Although ClO_3^- was used to inhibit only nitrite oxidation in columns 7 and 8, both ammonia and nitrite oxidation were inhibited. It appears that ClO_3^- and its reduced product ClO_2^- allowed partial nitritation (9%±2.8), but completely inhibited the nitratation process and caused nitrite to accumulate. These inhibition results are consistent with prior studies suggesting that ClO_3^- had a strong negative effect on nitrification and possibly other processes (Hynes & Knowles 1983; Bauhus et al. 1996). Ammonium and nitrate concentrations fit into nitrogen balances for the three N-species in all column experiments, excluding any additional nitrogen cycling path.

In Col. 1 where complete nitrification was observed, ^{13}C-labeled taxa were distributed among *alpha, beta, gamma*, and *Bacteriodetes* (Figure 16; Col.1) phyla. Specifically, *Herbaspirillum*, *Nitrosomonas*, *Comamonas*, *Xanthomonadaceae*, *Acidovorax*, *Janthinobacterium*, *Halomonas*, *Pseudomonas, Methylobacterium* and *Shewanella* related $OTU_{0.03}$s were identified as ^{13}C-labeled taxa (Figure 16; Col.1). Most of these taxa are known as heterotrophs (*Herbaspirillum*, *Xanthomonadaceae*, *Pedobacter* and *Janthinobacterium*) and have previously not been associated with nitritation. Although ammonia oxidation, classically, has been considered to be performed by chemolithoautotrophs, the contribution of heterotrophs to nitrification by many pure cultures (Robertson et al. 1988; Daum et al. 1998; Kundu et al. 2014; Lin et al. 2010) and in environments (Tortoso 1990; Bauhus et al. 1996) have been reported. The ability of heterotrophic nitrifiers to assimilate CO_2 has not been investigated, but CO_2 fixation by heterotrophic organisms through carboxylation reactions is known and reported thoroughly (Hesselsoe et al. 2005). To carefully interpret our results we used a framework which is summarized in Table 5.

Table 5 Putative CO_2 fixing phenotypes in chemostat columns fed with different energy sources and inhibitors in this study

Pheno type	Carbon source	Energy source	Enzyme	NH_4^+ (Col.1-2)	NH_4^+ (Col.3-4)	NH_4^+ (Col.7-8)	NO_2^- (Col.5-6)
				-	ATU	ClO_3^-	-
1	CO_3	NH_4^+	amoA, amoB, amoC	+	-	+	-
2	CO_3	NO_2^-	norA, norB	+	-	-	+
3	CO_3	SMP_{AOP}	PC, PEP, coA carboxylase	+	-	+	-
4	CO_3	SMP_{NOP}	PC, PEP, coA carboxylase	+	-	-	+
5	CO_3	SMP_{other}	PC, PEP, coA carboxylase	+	+	+	+

amo: Ammonia monooxygenase; nor: Nitrite oxidoreductase; PC: Pyruvate carboxylase; PEP: phosphoenolpyruvate carboxylase; coA: Co enzymeA carboxylase

Due to the lack of nitrification activity in column fed with ATU and NH_4^+ (Col.4), ^{13}C labeled taxa, *Sphingomonas*, *Caulobacter*, *Pseudomonas*, *Env.OPS 17* and *Piscinibacter* lineages, were considered as background communities utilizing SMP_{other} (Table 5) from the influent medium or available organic carbon on the filter material.

To acquire more information on labeled taxa in all columns, we identified phylogenetic (16S rRNA gene) relations between labeled $OTU_{0.03}$s and the cultured strains comprising *amoA* or *nxr* genes (Figure 17). We found that taxa comprising amoA gene in their genomes are evolutionarily closely related to the labeled $OTU_{0.03}$s belonging to *Comamonas*, *Xanthomonadaceae*, *Acidovorax*, *Halomonas*, *Pseudomonas, Methylobacterium* lineages. Altogether, results suggest that these taxa could be involved in ammonia oxidation in rapid sand filters.

In ClO_3^- and ammonium fed column, only partial ammonia oxidation was observed (Figure 15). Taxa closely related to uncultured representatives of, *Halomonas*, *Janthinobacterium, Herbaspirillum, Acinetobacter, Thaumarchaetoa* and *Nitrospira* lineages were obtained as ^{13}C-labeled communities (Figure 16; Col.8). Loss of *Nitrosomonas* activity in this column was most likely due to the release of ClO_2^- by ClO_3^- reduction (Belser & Mays 1980), because *Nitrosomonas* activity was reported to be sensitive to toxic effects of ClO_2^- (Hynes & Knowles 1983). Unlike *Nitrosomonas*, archaeal ammonia oxidizers affiliated to *Thaumarchaetoa* were highly active in heavy RNA fractions of Col.8. Their high ribosomal activity in spite of the toxic ClO_2^- effect is likely due to the presence of chlorite dismutase (*cld*) gene which encodes enzymes that degrade ClO_2^- to chloride (Cl_2) and oxygen (O_2). This gene lacks in AOB genomes, but it is present in all sequenced AOA genomes, including the *AR1, AR2, N. maritimus, Ca. "Na. koreensis"*, *Thaumarchaetoa, Ca. "Na. limnia", Ca. "N. gargensis", and Ca. "C. symbiosum"* genomes (Park et al. 2014). High activity of *Thaumarchaetoa* in only selective conditions suggests their inability to compete with AOB under full-scale conditions.

Furthermore, high 16S rRNA activity of *Nitrospira* related taxa in heavy fractions together with very high abundance of ^{13}C-labeled *Nitrospira* $OTU_{0.03}$s in both RNA (15%) and DNA (21%) of samples retrieved from full scale filter, surprisingly suggest that *Nitrospira* phylum may be involved in ammonia oxidation. Similar to the AOA, sequenced genomes of *Nitrospira* comprise *cld* gene which could remove the toxic effects of ClO_2^- for *Nitrospira* (Maixner et al. 2008). It is possible that *Nitrospira* feed on SMP_{AOB} produced by ammonia oxidizers; how-

ever, two ^{13}C-labeled $OTU_{0.03}$s affiliated to *Nitrospira* were the most active taxa in the Col.8, therefore no higher ammonia oxidizer activity exists to support *Nitrospira* activity.

Overall, our experiments suggest that the *Nitrosomonas genus* is not the sole taxon involved in ammonia oxidation in rapid sand filters. Functional gene targeted taxa identification (DNA-SIP) may provide additional information and clarify the effects of ^{13}C cross-feeding or carboxylase based CO_2 assimilation.

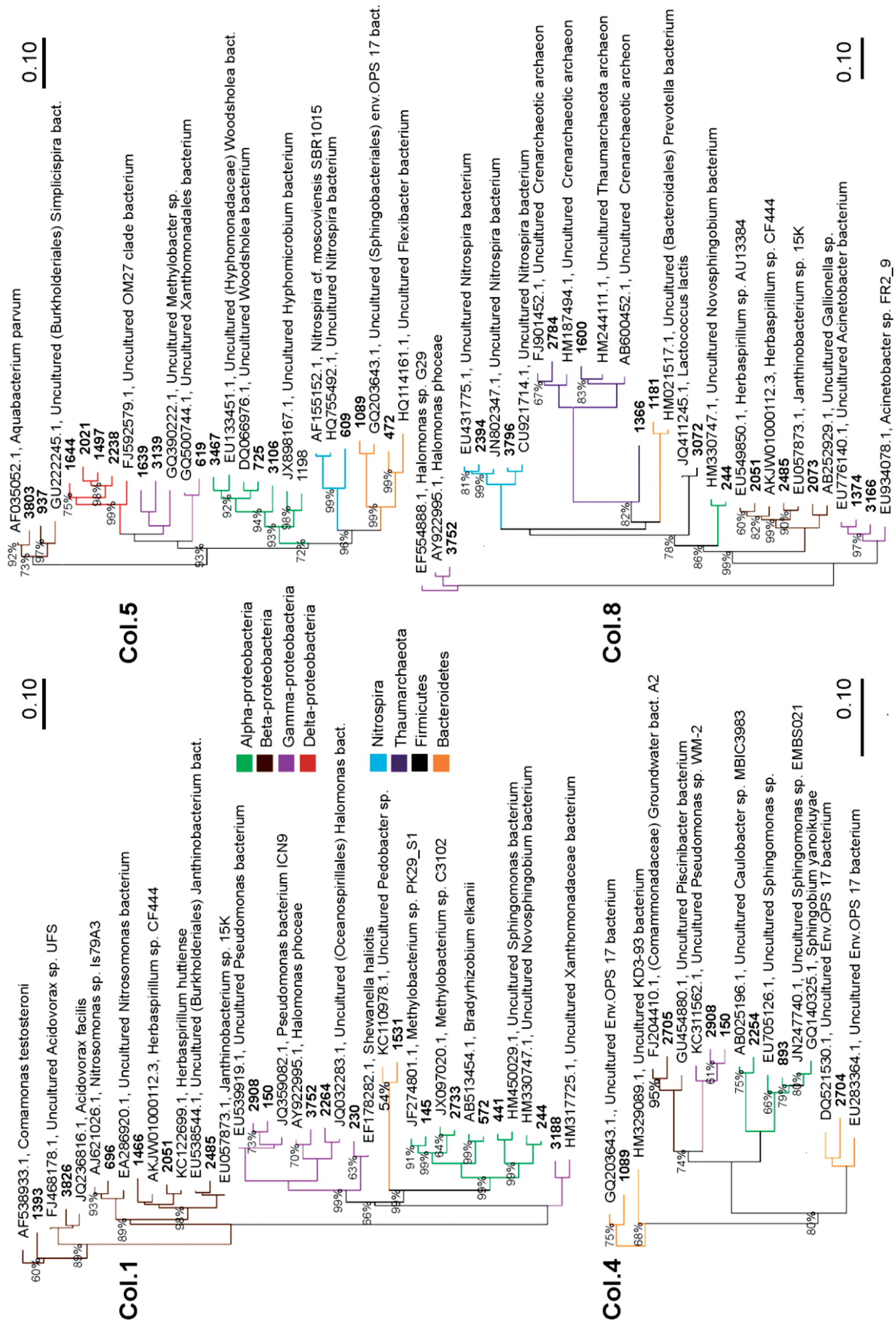


Figure 16 Phylogenetic trees of $OTU_{0.03}$s (> 70 sequences) retrieved from 'heavy' RNA from stable-isotope probing (SIP) column runs with ^{13}C-bicarbonate.

3.2. Nitrite oxidizing bacteria

Nitrite oxidizing bacteria rely on the products of ammonia oxidizing microorganisms in rapid sand filters. The *Nitrospira* lineage has been reported as the sole nitrite oxidizer in rapid sand filters from 16S rDNA based clone libraries; *Nitrobacter*, *Nitrococcus* or *Nitrospina* have not been detected(W W J M de Vet et al. 2009; White et al. 2012). Our pyrosequencing results (Paper I) confirm these observations, but we additionally identified a sequence of *Candidatus Nitrotoga artica,* a cold-adapted nitrite-oxidizing bacterium (Alawi et al. 2007) in rapid sand filters. Surprisingly, we found an enormous diversity of *Nitrospira* in all waterworks, at 15 times higher sequence abundance than all known ammonia oxidizers detected in the same samples. To further reveal the microorganisms involved in nitrite oxidation, we performed SIP experiments as described in section 1.1 (Paper II).

Col.5 process results showed nearly complete oxidation of influent nitrite (Figure 12; Col.5). After 15 days of exposure to NO_2^- and ^{13}C-bicarbonate in the labs-scale column, the following taxa were selectively enriched in the heavy SIP fractions (Figure 16; Col.5): *OM27* clade, *Woodsholea*, *Methylobacter*, *Aquabacterium*, *Xanthomonadales*, *Nitrospira* and *Hyphomicrobium*. The *nxr* gene has been detected in the cultured strains of *Methylobacter* and *Hyphomicrobium* lineages (Figure 17; Col.5). Noticeably, $OTU_{0.03}$s affiliated to methane oxidizing bacteria, *Methylobacter*, were consistently detected in heavy RNA fractions of the columns fed with NH_4^+ (Col.1) and NO_2^- (Col.5), suggesting that *Methylobacter* may be a nitrite oxidizer in rapid sand filters. *Nitrospira* was found as the sole known nitrite oxidizer among ^{13}C-labeled microbial communities. *OM27* clade, *Woodsholea, Aquabacterium*, and *Xanthomonadales* lineages have previously not been associated with nitratation process and no sequenced genomes for *OM27* clade, *Woodsholea* and *Aquabacterium* lineages exist. These lineages require further investigation to reveal whether they assimilated $^{13}CO_3$ through ^{13}C-cross feeding, carboxylation pathway or Calvin-Benson cycle. The relative abundance of labelled taxa involved in CO_2 fixation coupled with nitrite oxidation varied between 5% and 6.4% in the RNA of samples from full-scale filters.

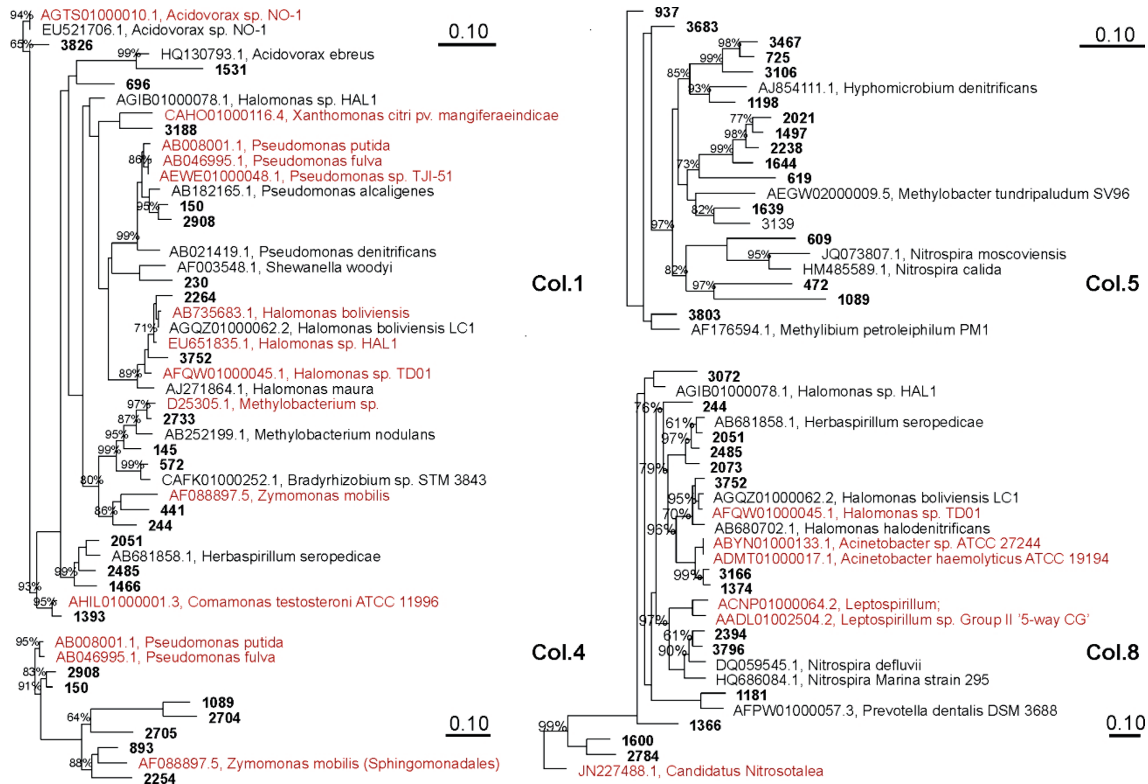


Figure 17 16S rRNA based phylogenetic trees of ^{13}C-labelled $OTU_{0.03}$s (> 70 sequences) and strains that comprise amoA (red) and nxr (black) genes.

3.3. Iron oxidizing bacteria

Gallionella, *Leptothrix* and *Sideroxydans* have been identified as abundant lineages of iron oxidizing bacteria in rapid sand filters; these observations have been based on microscopic examinations or 16S rDNA based clone libraries (Søgaard et al. 2000; Vet et al. 2012). To extend our knowledge on the diversity and abundance of iron oxidizing lineages in RSFs, a combination of opposing Fe(II)/O_2 enrichments, liquid batch incubations (Figure 18), qPCR, DGGE, cloning and pyrosequencing approaches were performed (Paper III). Our results demonstrated the iron oxidizing metabolism of 95 phylotypes while 2 new families were identified containing four iron oxidizing genera.

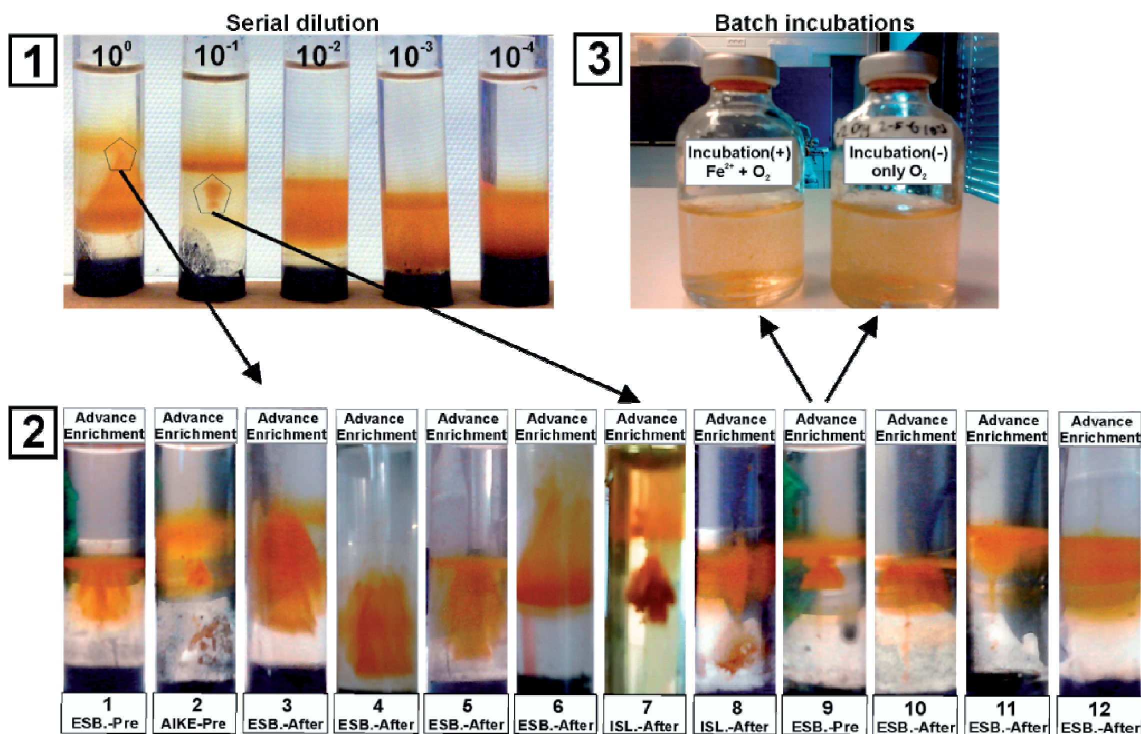


Figure 18 Sequential steps to select and confirm Fe(II) dependent growth of IOB enrichments.1.Microsampling (10 µm) at distinct locations of the IOB enrichment band in the original Fe(II)/O_2 gradient tube. 2. Growing subsampled enrichment in new Fe(II)/O_2 gradient tubes. 3. Verifying Fe(II) dependent growth in liquid cultures by comparing batches with and without Fe(II) amendment.

Among these phylotypes, many were closest related with uncultured representatives of genus *Gallionella*, in accordance with previous results from rapid sand filters (cloning results in Vet et al., 2012). Our results also strongly suggested that the family *Pseudomonadales* contain iron oxidizing types in investigated rapid sand filter, both in the genus *Acinetobacter* and *Pseudomonas* (Figure 19). A hetetrophic iron oxidation metabolism has previously been described to *Pseudomonas* and *Acinetobacter* strains identified in a volcanic seamount (Sudek et al. 2009) and in groundwater wells (Szewzyk et al. 2011). Our results further revealed that the members of the *Undibacterium* and *Herbaspirillum* genera in the *Oxalobacteraceae* family and of the *Curvibacter* genus in the *Comamonadaceae* family are iron oxidizers in rapid sand filters (Figure 19). Although the Fe(II)/O_2 gradient tubes enrichments resulted in very few isolates in the *Acidovorax*, *Sideroxydans* and *Leptothrix* lineages, these lineages are well represented in the pyrosequencing libraries.

Neutrophilic iron oxidizers are typically known as gradient species, because they thrive in the oxic/anoxic interfaces where they can compete with the chemical oxidation of iron (Sobolev & Roden 2001). Our results suggest a new niche for iron oxidizing bacteria at neutral pH, which comprise highly oxic conditions. Further, it appears that the genus *Gallionella* and *Sideroxydans* are the main contributors to biological iron oxidation at waterworks that receiving high Fe(II) concentrations; in these waterworks, AIKE and Esbjerg, Fe(II) concentrations were measured as 1.8 and 12 mg/l Fe. In rapid sand filters receiving lower iron concentrations (1.06 mg/l in Islevbro waterworks), and that are subject to intense aeration, genera *Pseudomonas*, *Rhodobacter*, *Dechloromonas*, *Curvibacter*, *Undibacterium* and *Herbaspirillum* are also identified as additional iron oxidizers. Taxonomic richness and abundance of each lineage at all waterworks was reported in Paper III.

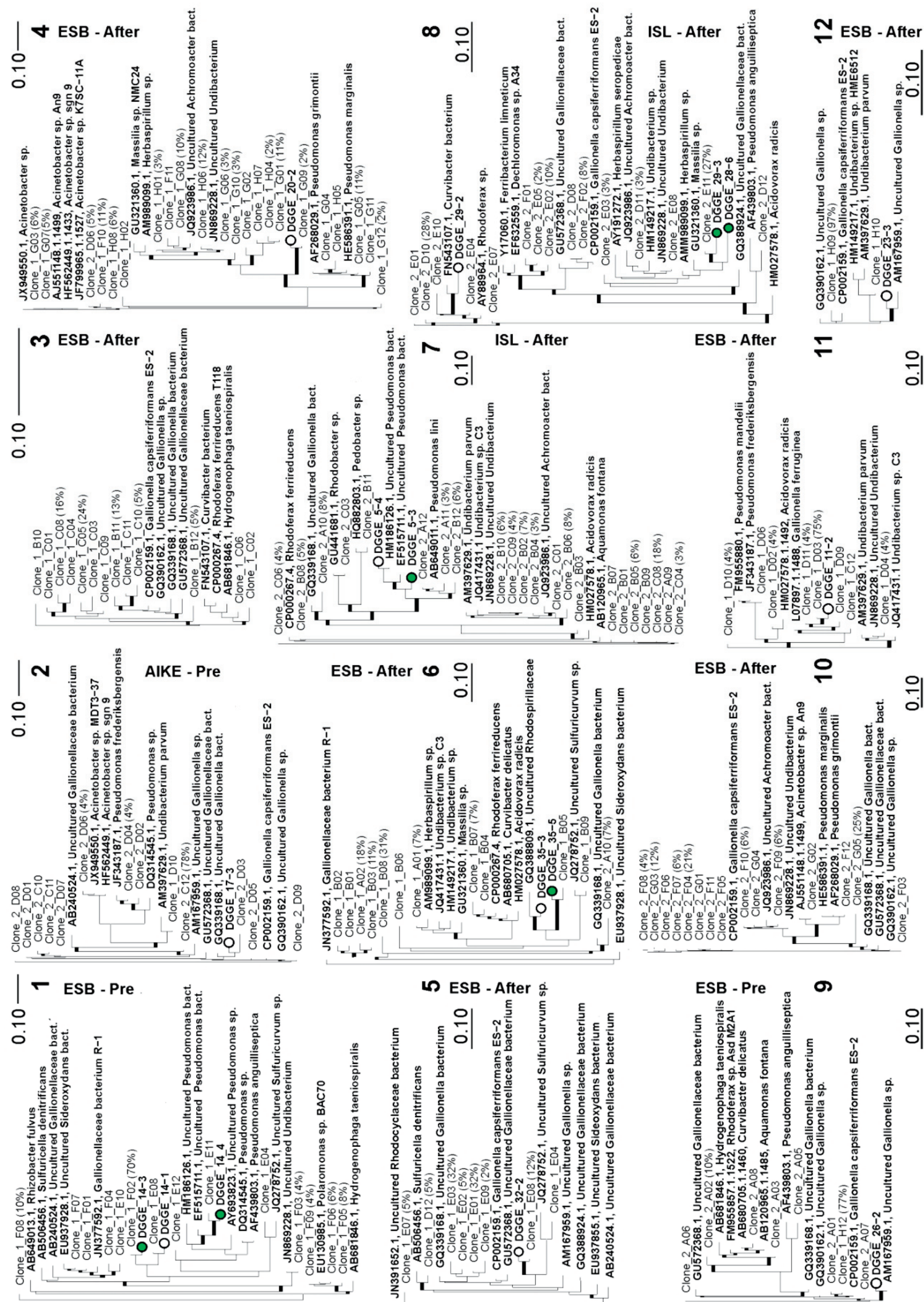


Figure 19 Phylogenetic trees of bacterial 16S rRNA gene sequences retrieved from incubations with ferrous iron addition after 12 days incubation. The tree was constructed using neighbor-joining method in ARB software from full length sequences obtained by cloning

and partial (170 bp) sequences obtained from DGGE band excision. Percentages in parenthesis correspond to the relative abundance of clones with identical ARDRA profiles; remaining clones represented as single OTU in corresponding ARDRA profiles. Thick branches on phylogenetic trees indicate high parsimony bootstrap support (≥ 70%) based on 1000 iterations. Scale bars indicate 10% sequence difference White and green dots are sequences from DGGE bands that were solely detected in incubations with Fe(II) and detected in both incubations with and without Fe(II), respectively. ISL, ESB, and AIKE represent the waterworks Islevbro, Esbjerg, and AIKE, respectively and pre and after represent pre- and after filters.

4. Biological heterogeneity in rapid sand filters (Paper IV-V)

The aim of rapid sand filtration is stable and efficient contaminant removal. The factors causing instabilities or inefficiencies in RSF performance must be identified to improve filter design and operation. Lopato et al. (2013) observed that process performance within a filter varies spatially, which can cause lower than expected overall filter performance (Lopato et al. 2011). We investigated whether this process heterogeneity can be related to the heterogeneous distribution of microbial communities within a filter. One way to investigate community heterogeneity is to measure beta (β) diversity together with β significance testing. There is, however, no consensus regarding appropriate β significance testing methods for NGS datasets and how to overcome bias associated with sampling depth (rarefaction bias, explained in Paper IV).

To measure the spatial heterogeneity of microbial communities within a filter and overcome these challenges, I developed a rigorous sequential procedure using the notion of rarefaction (Olszewski 2004) to standardize pairwise comparisons, and the notion of a meta-community (Leibold et al. 2004) (Paper IV). This procedure allowed me to determine a threshold value above which a β diversity must lie to be called significant. The procedure was summarized in figure 21.

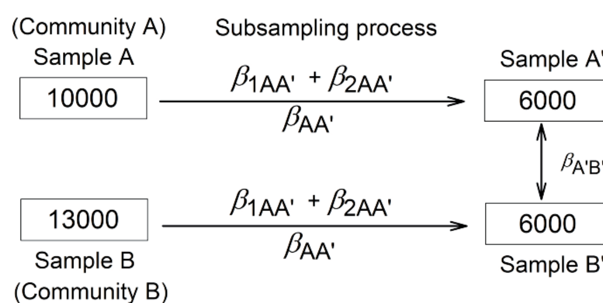


Figure 20 Components of observed β diversity between sample A and B, calculated from rarefied samples A' and B'. Numbers inside the boxes refer to the number of individuals.

The procedure is mainly based on (i) the notion of meta-community and (ii) the error originating from rarefaction. Constructing representative meta-communities from local communities, as introduced by Hubbell`s (2001), has

been used to explain evolutionary and ecological processes shaping microbial diversity patterns (Palacios et al. 2008). We approximate the meta-community as the combination of all replicate communities.

The rarefaction usually used to standardize the sample size adds a potential error into sample comparison, which should be removed from the β diversity estimation in order to estimate the true difference between the samples. When sample is rarefied (A to A' and B to B' in Figure 20), two errors are introduced ([Eq.(5)]): (i) $\beta_{1AA'}$ which is the β diversity calculated between random subsamples at a defined sample size (two random same-size subsamples are not identical) and (ii) $\beta_{2AA'}$ which is the β diversity between a sample and its subsample arising from taxon loss (in case, OTU loss) associated with the decrease in sample size. $\beta_{1AA'}$ and $\beta_{1BB'}$ can be calculated from the mean β diversity of multiple equally sized subsamples from sample A and B. The inter-sample β diversity, β_{AB}, can then be corrected by subtracting the average value of $\beta_{1AA'}$ and $\beta_{1BB'}$ ([Eq. (5) and (6)]). We propose the β diversity associated with random subsampling of the meta-community ($\beta_{1AA'}$ called β_{1MC} in Figure 21) as a significance threshold against any pairwise β diversity between replicates.

$$\beta_{AA'} = \beta_{1AA'} + \beta_{2AA'} \tag{5}$$

$$\text{corrected } \beta_{A'B'} = \text{observed } \beta_{A'} - \left(\frac{\beta_{1AA'} + \beta_{1BB'}}{2}\right) \tag{6}$$

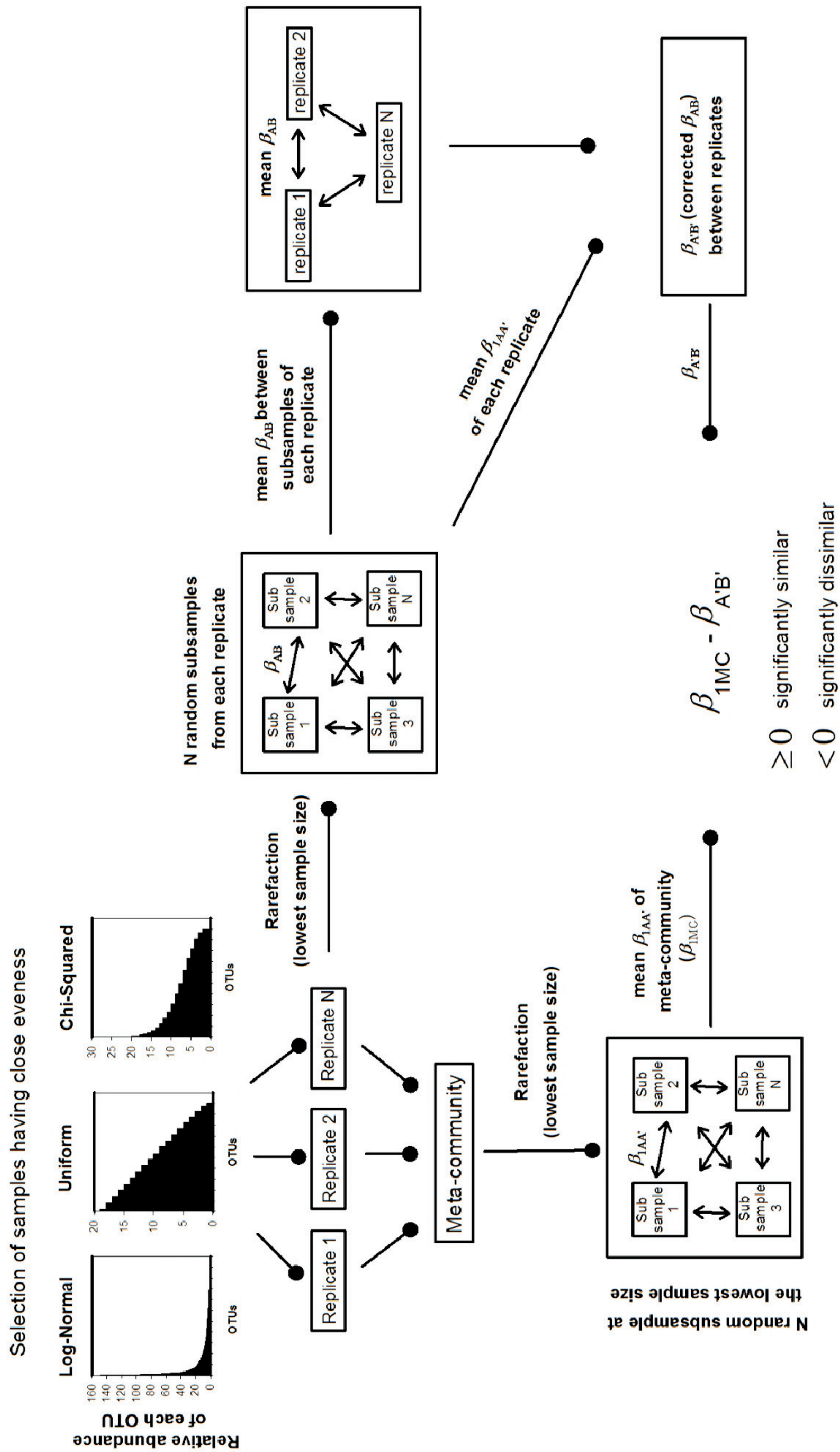


Figure 21 Schematic explanation of the β diversity significance assessment technique using the meta-community concept. 10 subsamples are recommended for $\beta_{1AA'}$ and $\beta_{2AA'}$ assessment.

We applied this method to examine dissimilarity between communities from replicate samples within an individual RSF and between different RSFs at one waterworks. Significant β diversity values would indicate sustained heterogeneity in environmental conditions – leading to significantly different microbial communities. All investigated filters were at the same waterworks and receive, on average, the same groundwater feed throughout their operation. By using both phylogenetic and compositional dissimilarity measures together with our significance method, we conclude that even over the small spatial scales examined, microbial communities in individual RSFs are significantly different (Figure 22): these differences stem in large part from the rare taxa, as seen by others (Youssef et al. 2010). Our analysis cannot identify whether heterogeneity in process performance (as observed by Lopato et al. 2013) and microbial community differences within a filter are causally related However, previous studies have reported that rare taxa may carry functional importance at the community level (Pester et al. 2010). The environmental factors causing differences in microbial communities over these small scales remain obscure.

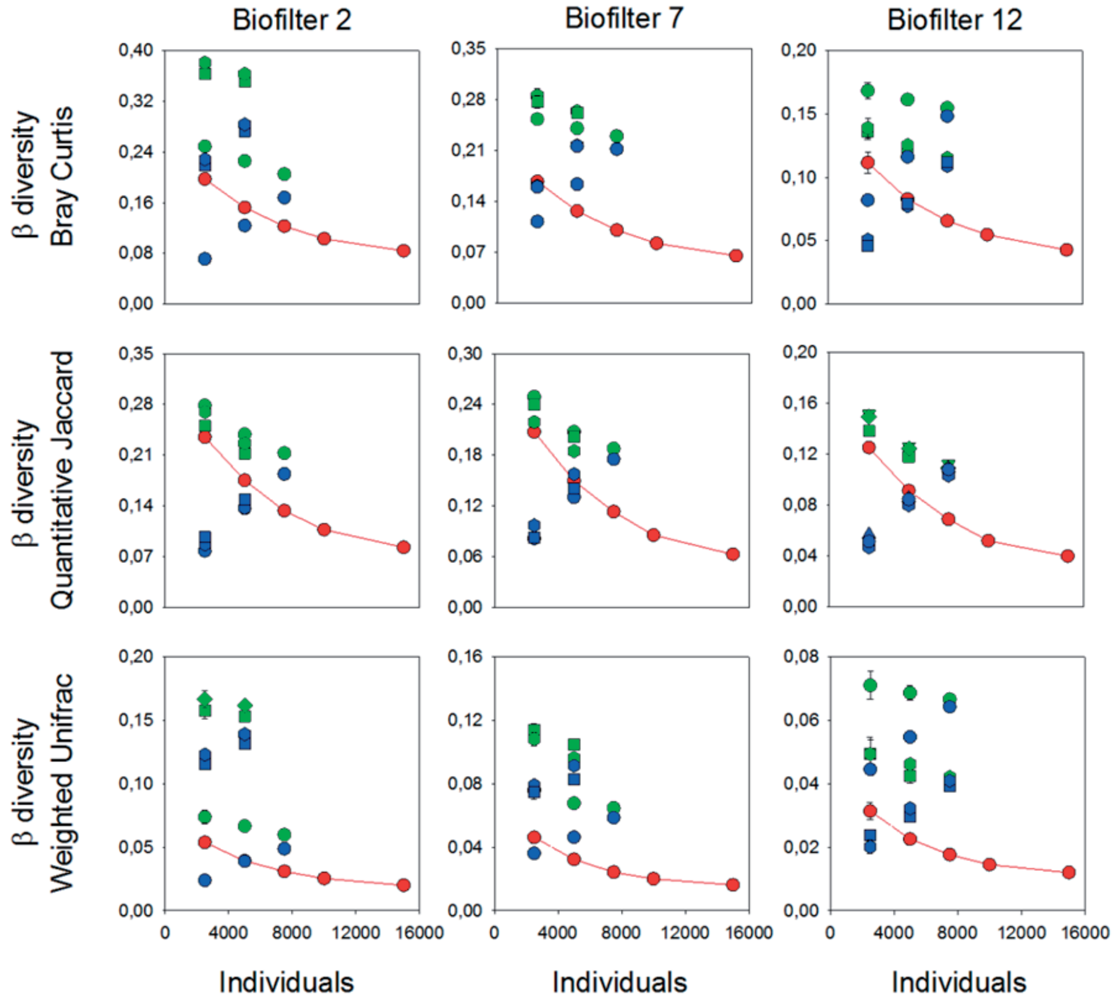


Figure 22 β diversity analysis applied to triplicate samples from triplicate biofilters using taxon based (Bray-Curtis and Jaccard) and phylogeny based indices (Weighted UniFrac), at different sampling depths. (Replicate 1 vs. Replicate 2: ○, Replicate 1 vs. Replicate 3: □, Replicate 2 vs. Replicate 3: ⬡). β_{AB} (green) was corrected (blue) per Eq.(2) using $\beta_{1AA'}$ of each sample at different subsampling depths. The meta-community was created by combining all individuals of triplicate libraries, and β_{1MC} (red) was calculated at consistent sampling depths.

Spatial heterogeneity was further investigated at the level of the iron oxidizing bacterial functional guild (Paper V). A combination of opposing Fe(II)/O_2 technique and DGGE was used to detect compositional heterogeneity of iron oxidizing bacteria within a filter. Our results from PCA plots confirmed the results from the pyrosequencing effort. The spatial heterogeneity of iron oxidizing guild composition as well as its density indicate strong spatial variations within a single RSF. In sum, we demonstrate that rapid sand filters cannot be considered as well mixed homogeneous environments.

Although significant dissimilarities originated from the rare taxa have been detected within individual after filters, abundant taxa are similar within an after-filter and between after filters at different waterworks. Indeed, this leads to the presence of abundant core taxa among waterworks, as reported in Paper I. Our phylogeny based dissimilarity analysis showed that at the level of OTU of 97% similarity, taxa in pre- and after filters at different waterworks clustered separately from each other while taxa in the samples of the same waterworks clustered closely, suggesting a filter-specific microbial community structure formation (panel A; Figure 23). However, when the dissimilarity patterns were assessed with combined abundance and phylogenetic information (Weighted UniFrac), we observed identical dissimilarities across and within waterworks, especially in the after filters (panel B; Figure 23), indicating that dominant taxa in all waterworks are phylogenetically similar confirming the core taxa results. Overall, the filter-specific taxa at all waterworks are phylogenetically distinct to each other; they comprise high $OTU_{0.03}$s richness but low sequence abundance at all waterworks.

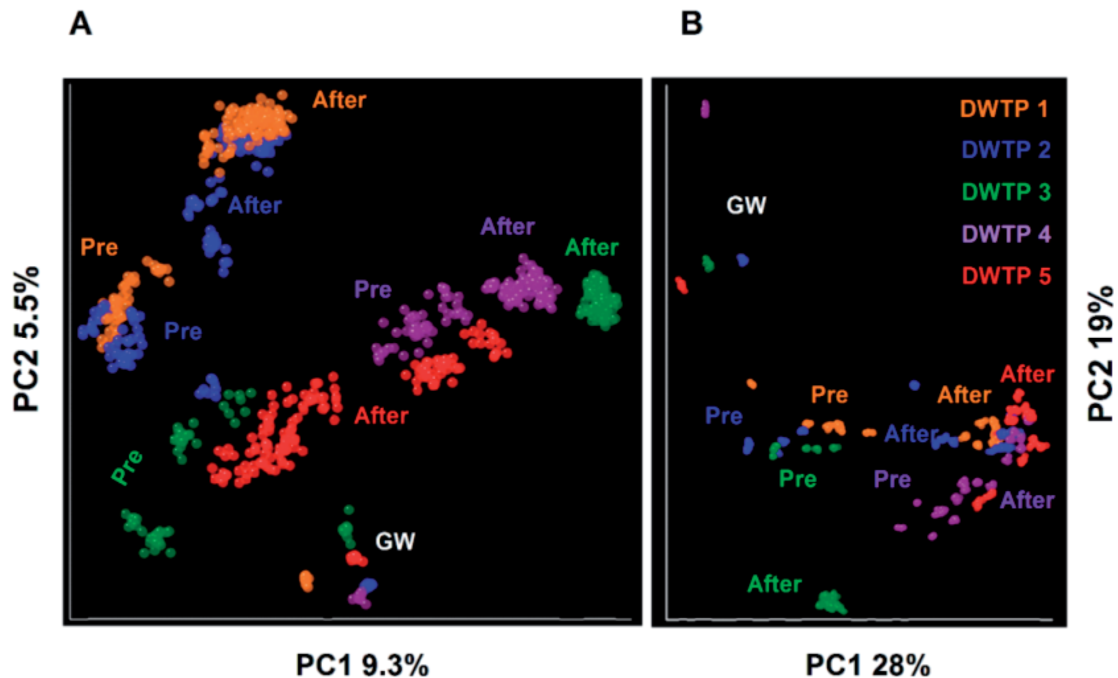


Figure 23 Dissimilarity patterns of pre and after filters at investigated waterworks and their source waters. (A) Variation in microbial community structure was assessed using phylogeny based "Unweighted UniFrac" algorithm and combined abundance and phylogeny based (B) "Weighted Unifrac" algorithm. 8 subsamples were taken at the depth of 4000 sequences and plotted with PcoA ordination. Colors represent the investigated waterworks.

In addition, using pyrosequencing libraries, we found that the degree of spatial heterogeneity in community composition within a pre filter is significantly lower than between pre filters at a waterworks. On the other hand, similar degrees of heterogeneity were found within an after filter and between replicate after filters at waterworks (Figure 24). This suggests that pre filters are more homogeneous than after filters. As expected, microbial compositions in different groundwaters showed the highest dissimilarity to each other, potentially due to the long-term isolation of these aquifer environments. Our results further suggested that weighted phylogenetic dissimilarity between process units at different waterworks (inter β diversity) were strongly correlated with influent chemical composition, by which the degree of inter phylogenetic dissimilarity decreases from upflow to downflow units as dissimilarity between influent chemical composition decreases from upflow units to downflow units.

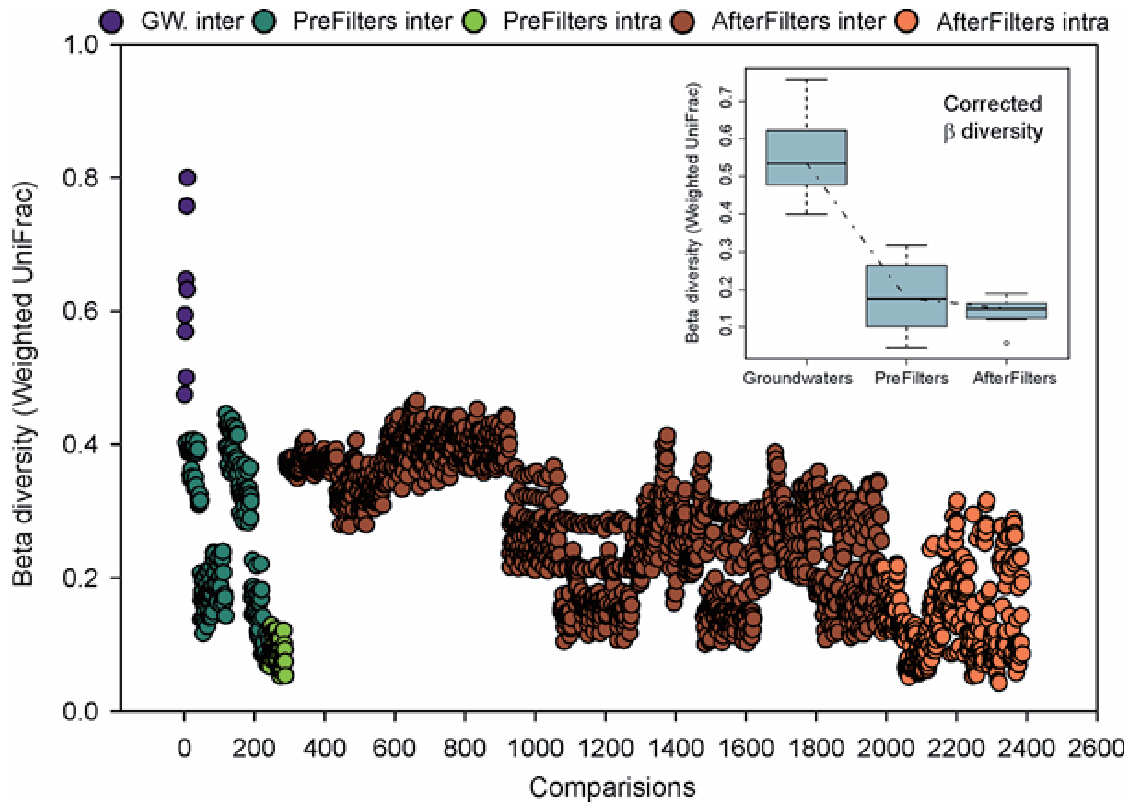


Figure 24 Patterns in β diversity within and between groundwaters (GW), pre filters and after filters at 5 different waterworks. β diversity was computed using Weighted UniFrac algorithm from replicate samples within the same process units at a waterworks (intra) and replicate samples of the same process units at different waterworks (inter). The corrected inter β diversity between replicate samples of the same process unit at different waterworks is shown as a small panel on the top right corner.

The spatial biological heterogeneity within pre- and after filters at all waterworks were further investigated using Weighted UniFrac (Figure 25). Boxplots confirmed that phylogenetic dissimilarity between replicates from pre filters is significantly lower than from after filters. In pre filters, spatial heterogeneity was found to be higher on Z axis than X-Y plane of both top and bottom layers in all waterworks. In after filters of all waterworks, the spatial heterogeneity was similar in both Z axis and X-Y plane, suggesting homogeneous distribution of significant heterogeneity within an after filter. Overall, these results suggest that any microbiological research concerning rapid sand filters should consider taking replicate samples from both horizontal plane and Z-axis to represent meta-community.

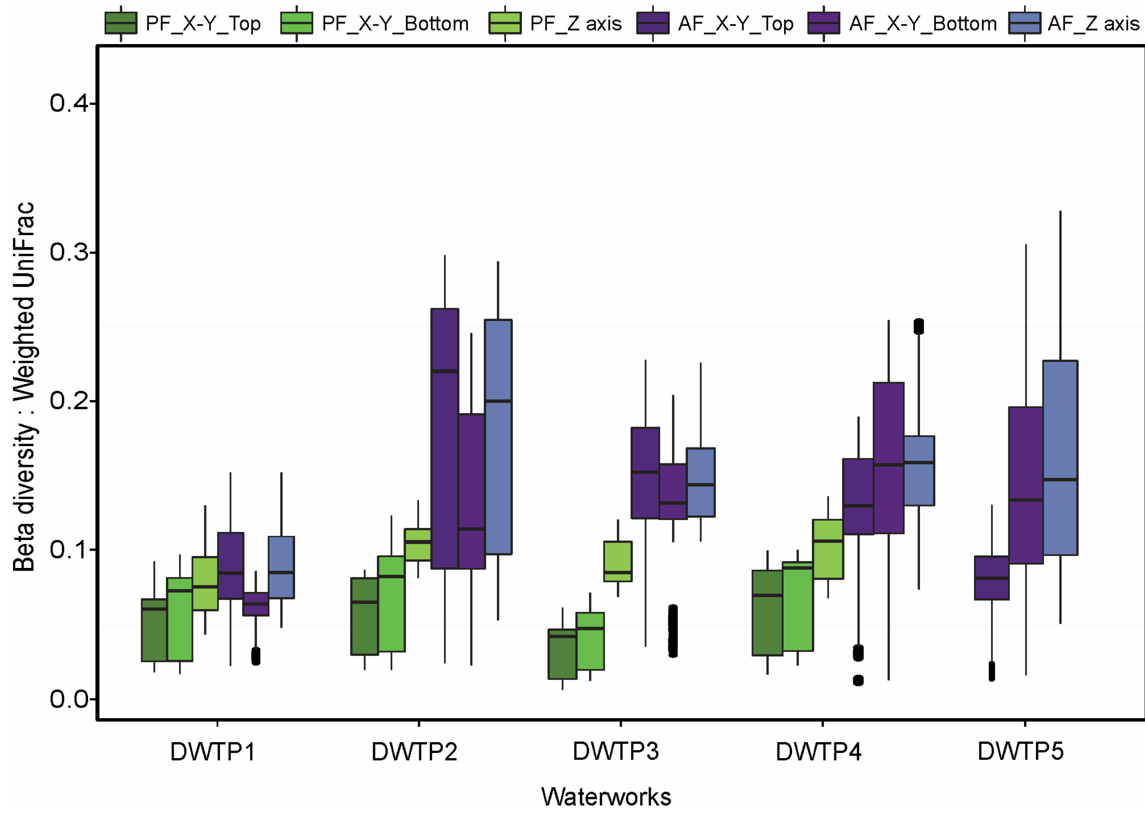


Figure 25 Spatial biological heterogeneity within the pre and after filters of investigated waterworks. Samples were taken from the 3 spatially distant locations on horizontal plane of top 10cm layer as well as composite bottom layer of a rapid sand filter. Dissimilarity values in boxplots were combined from different filters of the same unit and assessed using Weighted UniFrac algorithm.

5. Mineral-microbe interactions in rapid sand filters (Paper VI)

5.1. Supportive and protective role of mineral coatings in rapid sand filters

As microbes proliferate and form structured communities such as biofilms, they are often close associated with inorganic or mineral phases. Microbes and mineral phases can interact in two-ways: mineral structures can affect the dynamics of microbiological structures but microbiological dynamics can also affects the evolution of mineral structures (Caldwell & Caldwell 2004; Ehrlich 1999; Fortin et al. 1997). In groundwater treatment mineral phases can be of utmost importance, supporting oxidation or adsorption of specific contaminants. Among these contaminants, Fe(II) is a dominant and common constituents in groundwaters (Søgaard & Madsen 2013). Fe(II) is readily oxidized to Fe(III) to form various metal oxyhydroxides (MetOOH) of low solubility (Teunissen et al. 2008) in rapid sand filters. These hydroxides can aggregate or coalesce with the filter grain materials yielding a mineral coating. Mineral coatings change the surface and thus the physical characteristic of the initial filter material (Hu et al. 2004; Sahabi et al. 2009; Chang 1997; Lo 1996). In preliminary investigations, I observed that the quantity of the extracted mineral coating highly correlated with the extracted DNA from filter grain materials within and across filters at different waterworks (Figure 26). de Vet also indicated that a link may exist between the mineral coating mass and degree nitrification in full scale filters (de Vet 2011). Hence, I hypothesized “mineral coating supports microbial density and activity in rapid sand filters”. To test this hypothesis, we investigated the relationship between mineral coating and activity, diversity, abundance, and colonization of microbial communities in rapid sand filters (Paper VI).

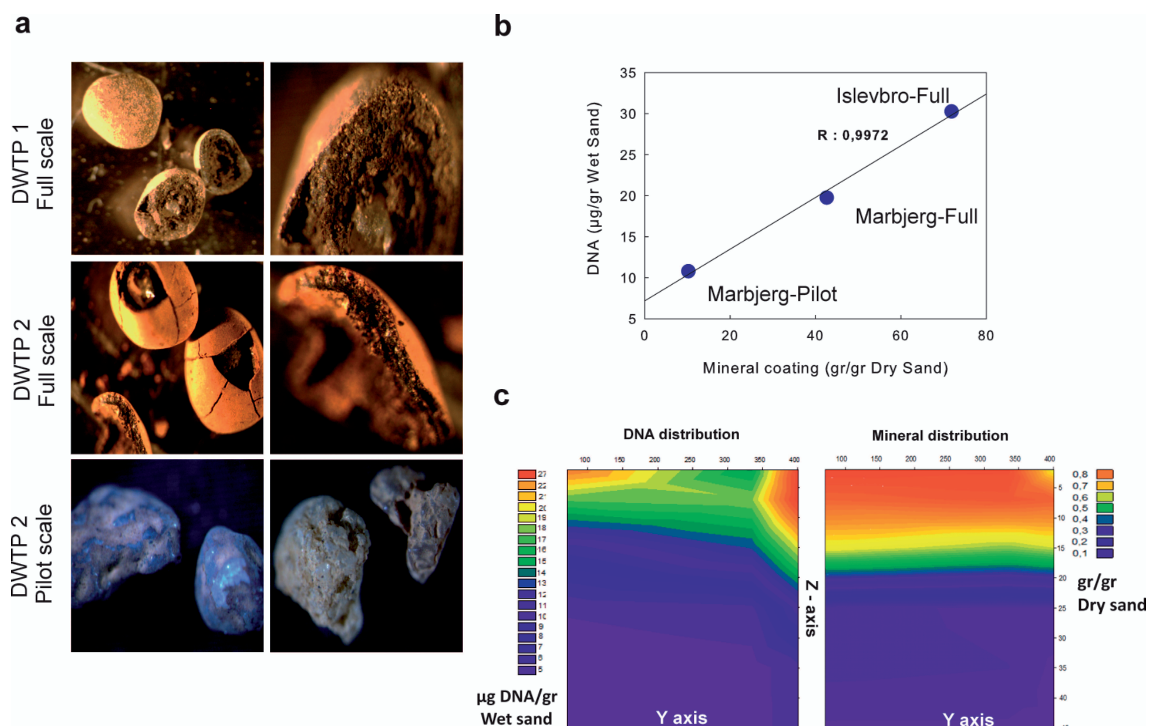


Figure 26 Preliminary investigation: extractable DNA and mineral coating mass from rapid sand filter materials at different water works. Mineral coating on filter materials from the after-filters at Islevbro (full-scale) and Marbjerg (full-scale and pilot-scale) waterworks (Panel A). Correlation between extracted coating mass and extracted DNA mass across waterworks (Panel B) and across samples of a single full-scale filter (n=24) (Panel C).

Four differently sized filter material fractions were retrieved from a single rapid sand filter (Islevbro water works). We applied several diagnostic tools on these fractions to describe both the physical and microbiological properties, including pyrosequencing, E-SEM, SEM, CSLM, BET, and lab-scale column activity experiments. Surface area measurements and electron micrographs indicated that the mineral coatings have an internal porosity, vastly extending the surface area of the uncoated filter grains (Figure 27). The internal porosity positively correlated with microbial activity, as well as total and ammonium oxidizing microbial densities. We can conclude that the internal mineral porosity provides solid-liquid interfaces that extend the surfaces area which supports the microbial community (Figure 28). Furthermore, E-SEM micrographs indicate the presence of a rich EPS- like matrix and embedded microbial cells inside the mineral coatings. The mineral coatings and their internal porosity – housing microbial cell and EPS, may thus protect and stabilized microbial communities from against removal by shifts in hydrody-

namic shear as might be experienced during backwashing. (; Kasuga et al., 2007; de Vet et al., 2011).

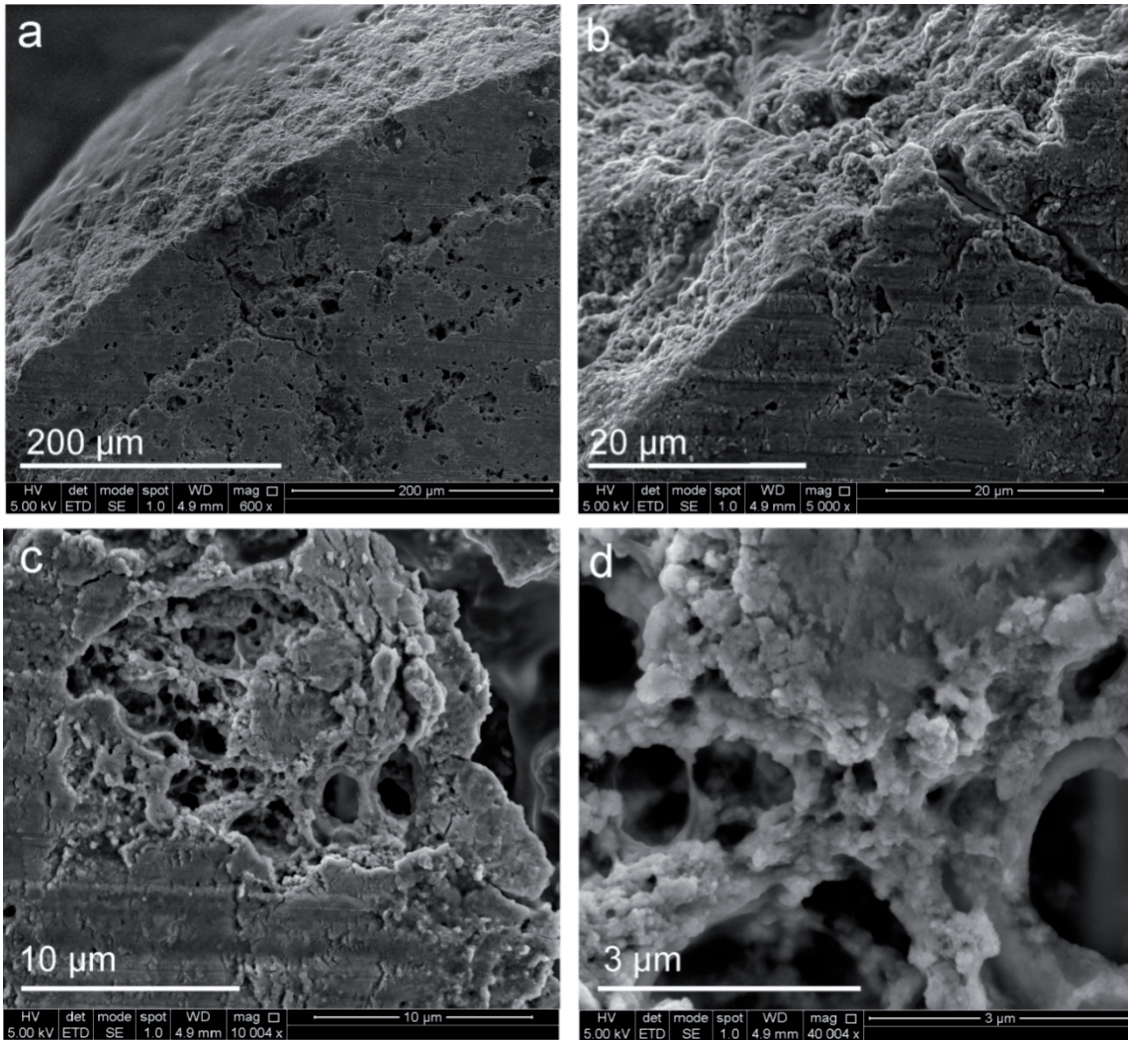


Figure 27 SEM micrographs of cryosection across the mineral coating of T2.0-2.4 grains. (a-b) External and internal view of the mineral coating. (c) Internal pore. (d) Encrusted organics and individual pore structures.

It appears that mineral coating accumulation did not affect the taxonomy and phylogeny of microbial communities and nitrifiers. Weighted and unweighted UniFrac statistics on 16S rDNA based pyrosequencing libraries from all grain fractions estimated insignificant taxonomic dissimilarities between each grain fraction. However, a better demonstration of function (ammonia oxidation) and taxonomy can be established using RNA instead of DNA, as a biomarker.

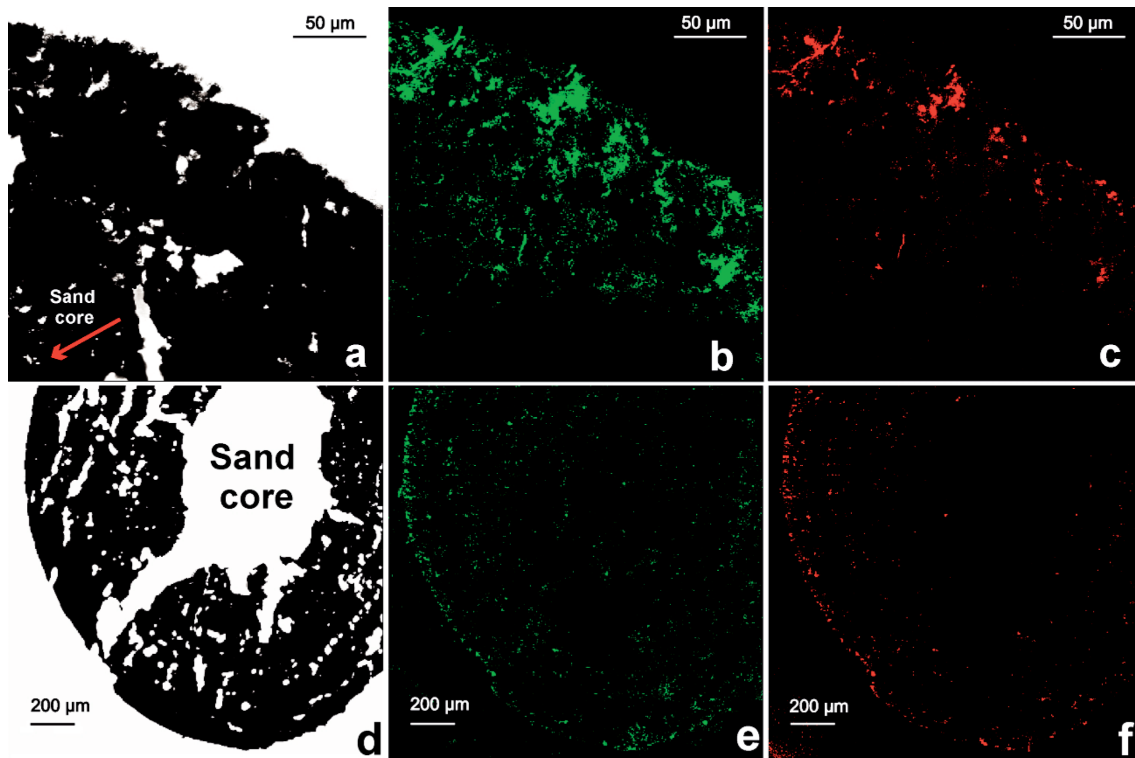


Figure 28 Confocal laser scanning micrographs of cryosectioned mineral coating layer from T2.0-2.4 grains, showing the spatial distribution of microorganisms.

6. Conclusions

The focus of this thesis has been on the microbial ecology of rapid sand filters. Specifically, my investigation has dealt with four interconnected topics: (i) microbial diversity, (ii) functional diversity, (iii) spatial heterogeneity and (iv) the interaction between mineral and microbial phases. Each topic has addressed different features of microbial communities in rapid sand filters, yet they all have served a single purpose: to better understand how microbial community composition, structure, abundance, and location might link with contaminant removal in RSFs. For all sections, a logical set experiment was developed and successfully applied to test the posed hypotheses. The major findings of each section can be summarized as follows:

*Microbial diversity in rapid sand filters (***Paper *I****)*

- Microbial communities in rapid sand filters are taxonomically rich similar to communities in activated sludge systems and anaerobic digesters.
- Microbial communities in pre-filters are taxonomically as rich as those in after-filters, but they differ significantly in composition.
- In the pre filters, the phyla *Proteobacteria, Nitrospirae, Acidobacteria, Actinobacteria, Candidate division OD1* and *Planctomycetes* were dominant taxa
- In the after filters, the phyla *Nitrospira, Acidobacteria*, *Planctomycetes*, *Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes* were dominant together with *Proteobacteria.*
- Core taxa shared across all rapid sand filters were identified. These taxa comprise few linages, but their relative abundance was high at all waterworks.
- Rare members of pre filter core taxa were found as dominant members of the after filter core taxa in all waterworks.
- A high number of $OTU_{0.03}$s in the core taxa of the pre- and after-filters were phylogenetically close to taxa with known physiologies such as those performing ammonium, nitrite, iron, manganese and methane oxidation.
- Groundwater seed pre filters and pre filter seed after filters. Taxa inventory of groundwaters appears important for downflow units.
- No archaeal ammonia oxidizers were detected at the top filter layers.

*Functional diversity associated with ammonia, nitrite and iron oxidation (***Paper II-III***)*

- In rapid sand filters, ammonia oxidation is not only driven by members of the *Nitrosomonas* lineage.
- Archaeal ammonia oxidizers cannot compete bacterial ammonia oxidizers in full scale conditions in the top filter layers.
- The genus *Nitrospira* may be involved in ammonia oxidation in rapid sand filters
- *Herbaspirillum, Comamonas, Xanthomonadaceae, Acidovorax, Janthinobacterium, Halomonas, Methylobacterium* and *Shewanella* might be involved in ammonia oxidation in rapid sand filters.
- In rapid sand filters, nitrite oxidation is not only driven by members of the *Nitrospira* lineage.
- *OM27* clade, *Woodsholea*, *Methylobacter*, *Aquabacterium*, *Xanthomonadales*, and *Hyphomicrobium* might be involved in nitrite oxidation in rapid sand filters.
- Strains belonging to the *Herbaspirillum, Curvibacter, Pseudomonas, Actinobacter* and *Undibacterium* genera *were* identified as likely iron oxidizing bacteria in RSFs that are not operated for microbial iron oxidation
- The *Pseudomonas* lineage contributes the iron oxidation process in rapid sand filters.
- *Gallionella* and *Sideroxydans* sequences are the dominant lineages found in waterworks that are operated for intentional biological iron removal (low degree of aeration, high influent Fe(II) concentrations), whereas the members of the genus *Pseudomonas*, *Rhodobacter*, *Dechloromonas*, *Curvibacter*, *Undibacterium* and *Herbaspirillum* are most abundant at typical waterworks (high degree of aeration, low influent Fe(II) concentrations).
- An experimental approach was developed to identify taxa with iron oxidizing metabolism.

*Spatial heterogeneity of microbial composition in rapid sand filters (***Paper** ***IV-V)***

- Microbial community composition of rapid sand filters varies at small spatial scales.
- A novel beta diversity significance testing method was developed to compare 16S rRNA tag based libraries.
- Rare microbial community members cause spatial heterogeneity of microbial communities in rapid sand filters.
- Spatial heterogeneity also exists at the level of guilds as revealed for iron oxidizing bacteria in rapid sand filters.
- Spatial heterogeneity is significant and similar at both X-Y plane and Z-axis of an after-filter.
- Pre filters are spatially more homogeneous environments than after filters.
- While rare taxa comprise filter-specific microbiota in rapid sand filters, the same abundant taxa are present in all waterworks.

*Mineral microbe interactions in rapid sand filters (**Paper VI**)*

- Mineral coatings on the original filter material create a porous matrix
- The internal porosity of the mineral coatings increases volumetric microbial activity and density, as demonstrated by increased volumetric ammonium removal rates.
- Microbial structures and an abundant EPS matrix can easily be visualized inside the porous mineral structure.
- Total community diversity and nitrifier diversity are not affected by the degree of mineral coating on filter grains.
- Mineral coating has a positive effect on contaminant removal in rapid sand filters.

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I **Gülay A.**, Musovic, S., Albrechtsen H.-J., Waleed A. A., Sørensen S. J., Smets, B. F., 2014. Microbial diversity and identification of core taxa in rapid sand filters treating groundwaters. Submitted to ISME journal

II **Gülay, A.**, Tatari, K., Musovic, S., Albrechtsen, H.-J., Thamdrup B., Sørensen S. J., Smets, B. F., Identification of key microbial players involved in nitrification coupled to CO_2 assimilation in rapid sand filters using RNA-SIP and 454 pyrosequencing. Manuscript in preparation

III **Gülay, A.**, Musovic, S., Albrechtsen, H.-J., Smets, B. F. Diversity, abundance and niche of iron oxidizers in rapid sand filters: Identification of novel iron oxidizers. Submitted to Environmental Microbiology.

IV **Gülay, A.**, Smets, B. F. An improved method to set significance thresholds for β diversity testing. Submitted to Environmental Microbiology

V **Gülay A**, Musovic S, Albrechtsen H-J, Smets BF. (2013). Neutrophilic iron-oxidizing bacteria: occurrence and relevance in biological drinking water treatment. Water Sci. Technol. Water Supply 13:1295.

VI **Gülay, A.**, Tatari, K., Musovic, S., Mateiu, R. V., Albrechtsen, H-J., Smets, B. F. Internal mineral porosity supports microbial activity in stratified rapid sand filters for drinking water treatment. Accepted in Applied and Environmental Microbiology, DOI : 10.1128/AEM.01959-14.

In this online version of the thesis, the papers are not included but they can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from:

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
Water Resources Engineering, Urban Water Engineering,
Residual Resource Engineering and Environmental Chemistry & Microbiology.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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