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विद्या ददाति विनयं, (*Vidyā dadāti vinayaṃ,*)

विनयाद्याति पात्रताम्। (*Vinayādyāti pātratām।*)

पात्रत्वाद्धनमाप्नोति, (*Pātratvāddhanamāpnōti,*)

धनाद्धर्मः ततः सुखम्।। (*Dhanāddharma: tata: sukham।।*)

- हितोपदेशः

Hitōpadēśaḥ (beneficial advice), Text 6

(An Indian collection of fables in the Sanskrit language)

Knowledge leads to politeness and sensibility.
From that one attains character, capability and qualification.
From that comes wealth which, if used for good deeds, leads to true peace.
To achieve self-satisfaction by good deeds is the ultimate goal of human life.

Preface

This Ph.D. thesis is based on the research carried out at the Technical University of Denmark, Department of Environmental Engineering as part of the early stage researcher training program Mermaid, ITN-EU-FP7 funded by People Programme (Marie Skłodowska-Curie Actions). The research was performed under the main supervision of Dr. Arnaud Dechesne (Senior Researcher) and co-supervision of Prof. Barth F. Smets and Prof. Hans-Jørgen Albrechtsen.

The thesis is organized into two parts: the first part puts into context the findings of the Ph.D. in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-IV**.

I Dechesne, A.; Musovic, S.; Palomo, A.; **Diwan, V.**; Smets, B. F. Underestimation of Ammonia-Oxidizing Bacteria Abundance by Amplification Bias in AmoA-Targeted QPCR. *Microb. Biotechnol.* 2016, 9 (4), 519–524.

II **Diwan, V.**; Albrechtsen, H-J.; Smets, B. F.; Dechesne, A. Does Universal 16S rRNA Gene Amplicon Sequencing of Environmental Communities Provide an Accurate Description of Nitrifying Guilds? *J. Microbiol. Methods* **2018**, *151*, 28–34.

III **Diwan, V.**, Kinnunen, M., Albrechtsen, H-J., Smets, B. F., Dechesne, A. Identifying selection in rapid sand filter microbial communities: Comparison of differential abundance and model-based approaches. (*Manuscript in preparation).*

IV Wagner, F.B.*, **Diwan, V.***, Dechesne, A., Fowler S. J., Smets, B.F., Albrechtsen, H-J. Copper-induced stimulation of nitrification in biological rapid sand filters for drinking water production by proliferation of *Nitrosomonas* spp. (*Environmental Science and Technology – In Revision).*

* Shared first author

Also, the following co-authored publication, not included in this thesis, was concluded during this Ph.D. study:

- Fowler, S.J., Torresi, E., Dechesne, A., **Diwan V.**, Smets, B.F. Biofilm thickness controls the contribution of stochastic and deterministic processes in microbial community assembly.
  (Manuscript in preparation)

Over the course of this Ph.D., contributions to international conferences were made with the following proceedings and conference papers:

- Fowler, S.J., Torresi, E., Dechesne, A., **Diwan V.**, Smets, B.F. Biofilm thickness controls the contribution of stochastic and deterministic processes in microbial community assembly. ISME, 2018, Leipzig, Germany.
  (Oral Presentation).

- Fowler, S.J., Torresi, E., Dechesne, A., **Diwan V.**, Smets, B.F. Biofilm thickness controls the contribution of stochastic and deterministic processes in microbial community assembly. Biofilms 8 Conference, 2018, Aarhus, Denmark.
  (*Oral Presentation).*

- Wagner, F.B., Nielsen, P.B., **Diwan, V.,** Boe-Hansen, R., Smets, B.F., Dechesne, A., Albrechtsen, H.J. Copper dosing enhances nitrification in biofilters treating groundwater. 2017. Abstract from Water Quality Technology Conference (WQTC) 2017, Portland, United States.
  (*Oral Presentation).*

- Fowler, J.S., Dechesne, A., Wagner, F.B., **Diwan, V.,** Albrechtsen, H.J., Smets, B.F. Niche partitioning within genus *Nitrospira* is affected by environmental copper concentration. 2017. Abstract from ICoN5: 5th International Conference on Nitrification, Vienna, Austria.
  (*Poster Presentation).*

- **Diwan, V.,** Albrechtsen, H.J., Smets, B.F., Dechesne, A. Ecological patterns of nitrifiers in the urban water cycle. The Danish Microbiological Society Annual Congress 2016: Programme & Abstracts. Copenhagen: American Society for Microbiology, 2016. p. 89-89 P80.
  (*Poster Presentation)*

- **Diwan, V.,** Albrechtsen, H.J., Smets, B.F., Dechesne, A. Linking nitrifiers diversity to the flux of their key resources. Microbial ecology and water engineering & biofilms specialist groups (MEWE, 2016). Copenhagen, Denmark: IWA, 2016. p. 204-205.
  (Poster Presentation)

- Musovic, S., Palomo, A., **Diwan, V.,** Dechesne, A., Smets, B.F. qPCR quantification of ammonia-oxidizing bacteria: What should the target be? 2014. Abstract from the Danish Microbiological Society Annual Congress 2014, Copenhagen, Denmark.
  (Poster Presentation)

- **Diwan, V.,** Albrechtsen, H.J., Smets, B.F., Dechesne, A. Functional Gene Approach to Study Nitrifier Diversity, 2014, Nordic Environmental Nucleotide Network (NENUN), University of Helsinki, Finland.
  (Oral Presentation)

Acknowledgments

"Coming together is a beginning, staying together is progress, and working together is a success"

- Henry Ford.

I don't think I can completely express my endless gratitude in words to my main supervisor **"Arnaud Dechesne"**. Looking back at all these years I realize how my research journey and your guidance have grown me intellectually and personally. You ensured that I stay on track and gain scientific skills. You have always nurtured and taught me with great care and stood with me throughout. I had always been immensely impressed with your hardworking attitude, intellect, commitment, calmness, and patience and I tried to learn all these things from you. Thank you so much for keeping the faith and making me learn that hard work and not losing hope is the key to success. You will always be in my best thoughts and as a person who has contributed immensely to my career foundation. Thank you for everything **Arnaud**!

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It was a bit long acknowledgments section but I truly believe:

"No individual can win a game by himself"

– Pele.

Summary

Nitrification is of central importance in engineered systems for drinking water production and wastewater treatment because of its key role in ammonium removal from these systems. Incomplete nitrification can lead to the release of ammonium residues in the finished water that can pose human and environmental health risks. Therefore, efficient drinking water and wastewater nitrifying community are of high importance. Several studies have revealed vast phylogenetic and functional diversity of nitrifier guilds in engineered systems. Nitrifiers are grouped as ammonia-oxidizing prokaryotes (AOP) and nitrite oxidizing bacteria (NOB). AOP further consists of canonical ammonia oxidizing bacteria (AOB), complete ammonia oxidizers (comammox) and ammonia-oxidizing archaea (AOA). Microbial diversity and composition are of central importance in engineered systems because highly diverse communities can contain a greater pool of physiological and genetic traits which provide them the capacity to cope with environmental perturbations and ultimately contribute to better system performance. In addition to the whole microbial community composition, knowing the contribution of guild members along with their compositional and ecophysiological information can allow us to introduce preferred microbial ecotypes as per the engineered system's requirement. Yet, what controls nitrifiers composition in engineered systems is largely unknown. Historically, the emphasis has been put on the role of selection (i.e. fitness difference between and within the guilds) but new developments in community ecology are available that include processes such as immigration and stochastic growth ('neutral' processes). This can be relevant because drinking water and wastewater treatment systems are open systems with influent water that contain nitrifiers.

The overall aim of this Ph.D. project was to assess and implement key molecular and community ecology approaches for describing the abundance and composition of nitrifiers. Then, to identify the determinants of nitrifiers assembly in engineered systems for drinking water production and wastewater treatment.

To quantify canonical AOB, researchers routinely employ qPCR targeting ammonia monooxygenase (*amoA*) gene for canonical AOB or canonical AOB specific 16S rRNA gene. However, these two approaches were not typically compared, and it was unclear whether they were equally good at estimating AOB abundance. In this comparison, inconsistencies were found based on the difference in primer pair selectivity combined with the compositional differences of the canonical AOB in drinking water biofilters. Therefore, we suggested that the primer set selection for canonical AOB quantification should be carefully made as the results can be heavily primer and nitrifier composition dependent. Next, I attempted to develop new primer sets (based on the currently available *amoA* nucleotide sequence data for canonical AOB) that cover most AOB and amplify large enough amplicons to make them suitable for both quantifications (qPCR), and phylogenetic/compositional analysis (amplicon sequencing). Due to the large divergence between AOB genera, I utilized the maximum coverage degenerate primer design method and successfully generated new primer sets targeting *amoA* with better coverage than the ones presently used. But these primer sets showed unspecific amplification in the *in-vitro* analysis therefore, I choose the most suitable primer sets amongst the existing canonical AOB primers for the rest of my work.

Next, I wanted to determine to which degree the universal 16S rRNA gene amplicon sequencing of environmental communities provides an accurate description of nitrifying guilds. I compared universal 16S rRNA gene amplicon sequencing to the functional gene (*amoA* AOB and *nxrB Nitrospira* (NOB)) based targeted sequencing approaches for assessment of nitrifiers relative abundance, diversity, and composition. The universal 16S rRNA gene sequencing approach provided accurate estimates of nitrifier composition and clustered samples consistently with their origin. It also provided relative abundance from the two approaches within ~1.2 orders of magnitude of them, but with a measurable bias that should be considered when comparing estimates from both approaches. The richness and diversity estimations were found to be likely limited by the sequencing depth. Overall, the universal approach works well when guilds of interest are dominant in an en-

vironment (for example, *Nitrospira* in drinking water biofilters) or when the goal is to estimate guild relative abundance.

Further, I compared the neutral community assembly model (which takes into account the frequency of presence and absence of taxa) and differential abundance based approaches in their ability to identify the selected members of the DWTP nitrifying community. Overall, the neutral model always predicted fewer positively selected taxa compared to the differential abundance based approach. The combined abundance of selected members of *Nitrospira* contributed majorly to the total abundance of *Nitrospira* for one drinking water treatment plant (DWTP) but not the other, indicating that the *Nitrospira* community at one DWTP was largely neutrally assembled while at other DWTP neutral processes played a smaller role compared to selection. Highlighting the pros and cons of both methods I suggested that detection of selection in microbial communities should be addressed using a combination of approaches covering both frequency and abundance data of the taxa.

Lastly, the evaluated molecular and community ecology methods were implemented for describing the effect of releasing resource limitation on nitrifying communities in malfunctioning full-scale drinking water biofilters. We showed that releasing copper limitation involved changes in the relative abundance between nitrifying guilds and had no effect on the relative abundance of other microbial guilds or potentially pathogenic microbes. Mainly, the relative abundance of *Nitrosomonas* increased by almost one order of magnitude upon releasing copper limitation. The relative abundance of *Nitrospira* (including comammox *Nitrospira*) also increased, but this was true for only one plant. Also, no changes within nitrifying guild composition were observed which indicated that there were no fitness differences amongst nitrifying guild members. Taken together these findings suggested that it is possible to enhance the biological stability and key process performance in complex microbial communities by influencing the abundance of specific microbial groups through selective nutrient dosing, i.e., by releasing elemental nutrient limitation.

Overall, this Ph.D. project presented a first systematic evaluation and implementation of molecular and community ecology approaches for describing specific microbial (nitrifying) community constituents and processes driving their assembly in complex microbial communities in drinking water and wastewater treatment systems.

Dansk sammenfatning

Nitrifikationsprocessen er central i tekniske systemer til drikke- og spildevandsbehandling på grund af dens nøglerolle i at fjerne ammonium. Ufuldstændig nitrifikation kan resultere i ammonium i det færdigtbehandlede vand, hvilket kan udgøre sundhedsmæssige og miljømæssige risici. Derfor er det vigtigt at have effektive samfund af nitrificerende mikroorganismer, og flere undersøgelser har vist stor fylogenetisk og funktionel diversitet af nitrifikanter i tekniske systemer. Nitrificerende mikroorganismer grupperes som ammonium-oxiderende prokaryoter (AOP) og nitrit-oxiderende bakterier (NOB). AOP opdeles yderligere i kanonisk ammonium-oxiderende bakterier (AOB), fuldstændig ammonium-oxiderende bakterier (comammox) og ammonium-oxiderende arkæer (AOA). Mikrobiel diversitet og sammensætning er af central betydning i tekniske systemer, fordi diverse samfund kan rumme en større pulje af fysiologiske og genetiske træk, som giver dem kapacitet til at håndtere miljøændringer og som kan bidrage til, at systemet fungerer bedre. Udover kendskab til sammensætningen af hele det mikrobielle samfund, giver kendskab til de forskellige gruppers sammensætning og øko-fysiologi mulighed for at kunne tilføre mikrobielle økotyper, der er optimale i forhold til de givne tekniske systemers krav. Det er imidlertid stort set ukendt, hvad der styrer sammensætning af nitrificerende samfund i tekniske systemer. Historisk set har der været fokus på betydningen af ”selektion” (dvs. fitness-forskelle imellem og indenfor mikrobielle grupper), men nye udviklinger i samfundsøkologi omfatter processer som ”indvandring” og ”stokastisk vækst” ("neutrale" processer). Disse kan være relevante, idet både drikke- og spildevandsbehandling er åbne systemer, hvor det tilførte vand indeholder nitrificerende mikroorganismer.

Det overordnede formål med dette ph.d.-projekt var at vurdere og implementere centrale molekylære og mikrobielle samfunds-økologiske fremgangsmåder til at beskrive hyppighed og sammensætning af nitrificerende samfund for at identificere de parametre, der bestemmer deres sammensætning i tekniske syster til drikke- og spildevandsbehandling.

For at kvantificere kanoniske AOB anvendes sædvanligvis qPCR, målrettet enten genet, der koder for ammonium monooxygenase enzymet (*amoA*) eller det specifikke gen for AOB 16S rRNA. Disse to fremgangsmåder sammenholdes imidlertid sjældent, så det var uklart, om de er lige gode til at kvantificere hyppigheden af AOB. En sådan sammenligning, i naerverrende projekt, påviste, at uoverensstemmelserne skyldtes forskel i selektiviteten for primer-par, kombineret med forskelle i sammensætningen af kanonisk AOB i vandværksbiofiltre. Derfor påpegede vi omhyggelighed ved udvælgelse af primersæt til at kvantificere kanoniske AOB, da resultaterne kan afhænge stærkt af den specifikke primer og sammensætningen af nitrificerende samfund. Dernæst forsøgte jeg at udvikle nye primersæt (baseret på de tilgængelige nukleotidsekvenser for amoA for kanoniske AOB, der skulle detektere de fleste AOB), som dækker de fleste AOB og som kopierer tilstrækkeligt store amplicon til at gøre dem egnede til både kvantificering (qPCR) og analyse for fylogeni sammensætning (via amplicon sekventering). På grund af den store forskellighed mellem forskellige AOB-slægter anvendte jeg den primer-design-metode med maksimale dækning, hvilket succesfuldt genererede nye primersæt, der målrettet detekterede amoA med en bedre dækning end de i øjeblikket anvendte. Men disse primersæt viste uspecifikke bindinger i *in vitro* analysen. Derfor udvalgte jeg de mest egnede primersæt blandt allerede eksisterende primersæt for kanoniske AOB i resten af mit arbejde.

Dernæst ønskede jeg at afklare, i hvilket omfang universel 16S rRNA-gen amplikon sekventering af samfund fra miljøet giver en nøjagtig beskrivelse af de nitrificerende grupper. Jeg sammenlignede universal sekventering af 16S rRNA-gen amplikon med sekventeringer målrettet de funktionelle gener (*amoA* AOB og *nxrB* Nitrospira (NOB)) for at vurdere den relative hyppighed, diversitet og sammensætning af nitrificerende samfund. Den universelle sekventering af 16S rRNA-genet gav nøjagtige estimater af sammensætningen af nitrificerende samfund, samt af grupperede prøver i overensstemmelse med deres oprindelse. Den relative hyppighed estimeret med de to tilgange var inden for ~ 1,2 størrelsesordener, men med en målelig bias, som bør tages i betragtning ved sammenligning af estimater fra begge tilgange. Estimater af richness og

diversitet er sandsynligvis begrænset af sekventeringsdybden. Samlet set fungerer den universelle tilgang godt, når de undersøgte mikrobielle grupper dominerer et givent miljø (fx *Nitrospira* i vandværksbiofiltre), eller når målet er at estimere den relative hyppighed af en mikrobiel gruppe.

Endvidere sammenlignede jeg "den neutrale samfundsmodel" ("the neutral community model") (som tager hensyn til hyppigheden af tilstedeværelse og fravær af taxa) og differentierede hyppighedsbaserede fremgangsmåder i deres evne til at identificere de udvalgte mikrobielle medlemmer af de nitrificerende samfund i vandværker. Samlet set, forudsagde "den neutrale model" altid færre positivt udvalgte taxa i forhold til den differentierede hyppighedsbaserede tilgang. Den kombinerede hyppighed af udvalgte medlemmer af *Nitrospira* bidrog overvejende til den samlede hyppighed af *Nitrospira* på et af de undersøgte vandværker, men ikke på det andet undersøgte vandværk. Dette antyder, at det mikrobielle samfund af *Nitrospira* på et vandværk var påvirket af neutral-processer, hvorimod neutralprocesser spillede en mindre rolle i forhold til selektion på andre vandværker. Ved at påpege fordele og ulemper ved begge metoder, foreslog jeg, at påvisning af selektion i mikrobielle samfund bør anvende en kombination af fremgangsmåder, der dækker både hyppighed og mængden af taxa.

Endelig anvendte jeg de evaluerede molekylære og mikrobielle samfunds-økologiske metoder til at beskrive, hvorledes nitrificerende samfund i biofiltre på et fuldskala vandværk med funktionsfejl påvirkes, når ressourcebegrænsning fjernes. Vi påviste, at når kobberbegrænsning fjernes, ændredes den relative hyppighed af nitrificerende grupper uden der var effekt på den relative mængde af andre mikrobielle grupper eller potentielt patogene mikrober.

Den relative hyppighed af *Nitrosomonas* steg med næsten en størrelsesorden, når kobberbegrænsningen blev fjernet. Den relative hyppighed af *Nitrospira* (inklusiv Comammox *Nitrospira*) steg også, men kun på et af anlæggene. Der blev heller ikke observeret ændringer i sammensætningen af de nitrificerende grupper. Dette indikerer, at der ikke var nogen forskelle i fitness blandt de nitrificerende gruppemedlemmer. Tilsammen indikerede resultaterne, at det er muligt at forbedre den biologiske

stabilitet og ydelse af nøgleprocesser i komplekse mikrobielle samfund ved at påvirke hyppigheden af specifikke mikrobielle grupper ved selektiv næringsdosering, dvs. ved at fjerne elementær næringsbegrænsning.

Samlet set præsenterede dette ph.d.-projekt den første systematiske evaluering og implementering af molekylære og mikrobielle samfundsøkologiske måder til at beskrive specifikke mikrobielle (nitrificerende) samfundskomponenter og -processer, der driver deres forekomst i komplekse mikrobielle samfund i drikke- og spildevandsbehandlingssystemer.

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Abbreviations

AMO	Ammonia monooxygenase
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AOP	Ammonia-oxidizing prokaryotes
AR	Anammox reactor
ASV	Amplicon sequence variant
Comammox	Complete ammonia oxidizer
DWTP	Drinking water treatment plant
HAO	Hydroxylamine oxidoreductase
NCM	Neutral community model
NGS	Next Generation Sequencing
NOB	Nitrite-oxidizing bacteria
NR	Nitrifying reactor
NTI	Nearest taxon index
NXR	Nitrite oxidoreductase
qPCR	Quantitative polymerase chain reaction
RSF	Rapid sand filter
WWTP	Wastewater treatment plant

1 Introduction

1.1 Motivation

Nitrogen is an essential element for all organisms as it is required for the biosynthesis of basic cellular components such as proteins and nucleic acids (Cooper, 2000). The transformation of nitrogen by microbes is carried out by six major processes: assimilation, ammonification, nitrification, denitrification, anaerobic ammonium oxidation (anammox) and nitrogen fixation. Amongst these processes nitrification is of central importance in engineered systems used for drinking water production and wastewater treatment because of its key role in ammonium removal from these systems. Incomplete nitrification can lead to the release of ammonium residues in the finished water that can pose human and environmental health risks (Follett and Hatfield, 2001; De Roos *et al.*, 2003; Powlson *et al.*, 2008; Bouchard *et al.*, 1992). Therefore, nitrifying community (ammonia-oxidizing prokaryotes (AOP) and nitrite-oxidizing bacteria (NOB)) that efficiently performs the process of nitrification is of high importance in drinking water and wastewater treatment systems. Yet, sometimes nitrification is incomplete (Lee *et al.*, 2014; Wagner *et al.*, 2018) or accelerating the process of nitrification is required (e.g., accelerating startup of rapid sand filters in drinking water treatment plant; Albers *et al.*, 2018) or it is required to apply nitrification in conditions such as low oxygen and/or low temperature (Liu and Wang, 2013; Liu *et al.*, 2018). Solving these challenges requires microbial resource management i.e. the rational steering of microbial (in this case nitrifying) communities for better systems performance, which is only feasible by understanding their diversity, composition and determinants of their assembly (Verstraete *et al.*, 2007).

Microbial diversity and composition are of central importance in engineered systems because highly diverse communities can contain a greater pool of physiological and genetic traits which provide them the capacity to cope up with environmental perturbations and ultimately contribute to better system performance. In addition to the whole microbial community composition, understanding of microbial ecotypes is also necessary. If we understand the functional contribution of guild members along with their compositional and ecophysiological information then certain microbial types that are beneficial for the engineered system can be introduced to improve the engineered system's performance. Overall, the central question is, 'what controls nitrifiers

composition in engineered systems?' Indeed obtaining such insights requires a deeper understanding of nitrifier ecology and the processes governing their community assembly. This can only be achieved by first obtaining reliable description of nitrifier communities, an area that has been revolutionized by the introduction and development of molecular approaches, before applying community ecology approaches to identify the processes driving community assembly.

Advances in molecular approaches such as next-generation sequencing have now dramatically decreased the experimental costs and democratized sequencing. The generation of novel sequencing data calls for updating the existing primer sets for better coverage and specificity of the target microbial community. Therefore, I first focused on the evaluation of existing PCR primers for key nitrifiers based on latest nucleotide sequence data, followed by new primer development.

Universal 16S rRNA gene-based amplicon sequencing is one of the most widely used molecular approaches in environmental microbiology because it provides an overview of the full microbial community. But it provides only limited compositional information on specific guilds (such as nitrifiers) because of their low contribution to the total sequence pool. Therefore, functional gene targeted sequencing is often used for accessing within guild diversity for nitrifiers. If 16S rRNA gene-based universal amplicon sequencing can provide reliable information about specific microbial guilds in addition to the overview of the whole microbial population then in a single analysis one would be able to generate complete information about the abundance and composition of microbial communities in a given sample. Therefore, I have compared 16S rRNA gene universal amplicon sequencing approach to functional gene targeted sequencing approaches for assessment of nitrifiers relative abundance, diversity, composition and their ability to discriminate samples.

Once appropriate molecular methods have been selected, they can be applied to study the ecology of nitrifiers in full scale water treatments. Several previous studies have described the whole microbial community and nitrifier composition in drinking water and wastewater treatment plants but rarely these systems are compared. The microbial community in these engineered systems gets exposed to contrasting nutrient and environmental conditions and receives immigration flux from sources (groundwater, sewage) that likely differ in their microbial composition. This provides an opportunity to study

the relative role of deterministic (e.g., selection: fitness difference between and within the guilds) and stochastic (e.g., immigration) processes on nitrifier community assembly. Specifically, I focused on selection based on competition for substrates as nitrifiers gets exposed to different availability of nutrients and micronutrients due to changing influent water for treatment in these systems.

Lastly, to identify the contribution of deterministic versus stochastic processes quantitative community ecology tools are required. These tools consist of predictive mathematical models, statistical methods or a combination of both. These methods are increasingly used, they have different assumptions, they use slightly different data input but have not been compared. Therefore, I assessed the neutral community assembly model and differential abundance based approaches for identifying the role of selection in the DWTP nitrifying community.

1.2 Research objectives

The overall aim of this Ph.D. project was to assess and implement key molecular and community ecology approaches for describing the abundance and composition of nitrifiers. Then, to identify the determinants of nitrifiers assembly in engineered systems for drinking water production and wastewater treatment.

The first half of this thesis focuses on existing PCR primer evaluation for nitrifiers, followed by new primer development, and comparison of amplicon sequencing-based approaches for describing nitrifiers **(paper I and II)**. The latter half focuses on the assessment of microbial community ecology approaches for studying the processes shaping nitrifying communities in drinking water productions systems **(paper III)** and implementation of molecular and community ecology methods for quantifying and describing nitrifiers in drinking water productions systems which were stimulated with micronutrient supplementation for improving nitrification **(paper III and IV)**.

More specifically the objectives of this Ph.D. study were to:

1. Assess the coverage and specificity of the most widely used primer sets for quantifying and describing nitrifiers **(paper I)** and design new primer sets with better coverage and specificity.

2. Compare 16S rRNA gene universal amplicon sequencing approach to functional gene targeted sequencing approaches for assessment of nitrifiers relative abundance, diversity, and composition **(paper II).**

3. Compare statistical and model-based approaches for identifying the role of selection in nitrifier community assembly in groundwater-fed biological filters at different waterworks **(paper III).**

4. Identify the effects of releasing resource limitation on the nitrifier community in malfunctioning full-scale drinking water biofilters, using the molecular and community ecology approaches previously assessed **(paper IV).**

2 Background

2.1 Nitrification

The three primary sources of nitrogen fluxes that contribute to the total nitrogen inventory of Earth are terrestrial and marine environments plus anthropogenic activities (intense use of fertilizers in agriculture and combustion of fossil fuels; Gruber and Galloway, 2008). Microbial transformation of nitrogen is carried out in six major processes (assimilation, ammonification, nitrification, denitrification, anammox, and nitrogen fixation) where nitrogen compounds occur in many redox states ranging from -3 (ammonium/ammonia) to +5 (nitrate; Kuypers *et al.*, 2018). In this view of the nitrogen cycle, dinitrogen gas is first fixed to ammonia, which is then assimilated into organic nitrogen (i.e., biomass). In short, ammonification (degradation of organic nitrogen) produces ammonia which is then oxidized to nitrate via nitrification and is ultimately converted back to dinitrogen gas through denitrification or anammox processes (Kuypers *et al.*, 2018).

In the nitrification process, the conversion of ammonia to nitrite (nitritation) is carried out in two steps. First, ammonia is converted to hydroxylamine (NH_2OH) in a reaction catalyzed by a membrane-bound enzyme ammonia monooxygenase (AMO) and then conversion of NH_2OH to nitrite by a periplasmic enzyme hydroxylamine oxidoreductase (HAO). Conversion of nitrite to nitrate (nitratation) is catalyzed by a membrane-bound enzyme nitrite oxidoreductase (NXR). Recent discoveries have shown that both these steps can be carried out by a single group of microbes known as complete ammonia oxidizers (comammox; Daims *et al.*, 2015; van Kessel *et al.*, 2015). Figure 2.1 shows a simplified schematic representation of the nitrification process.

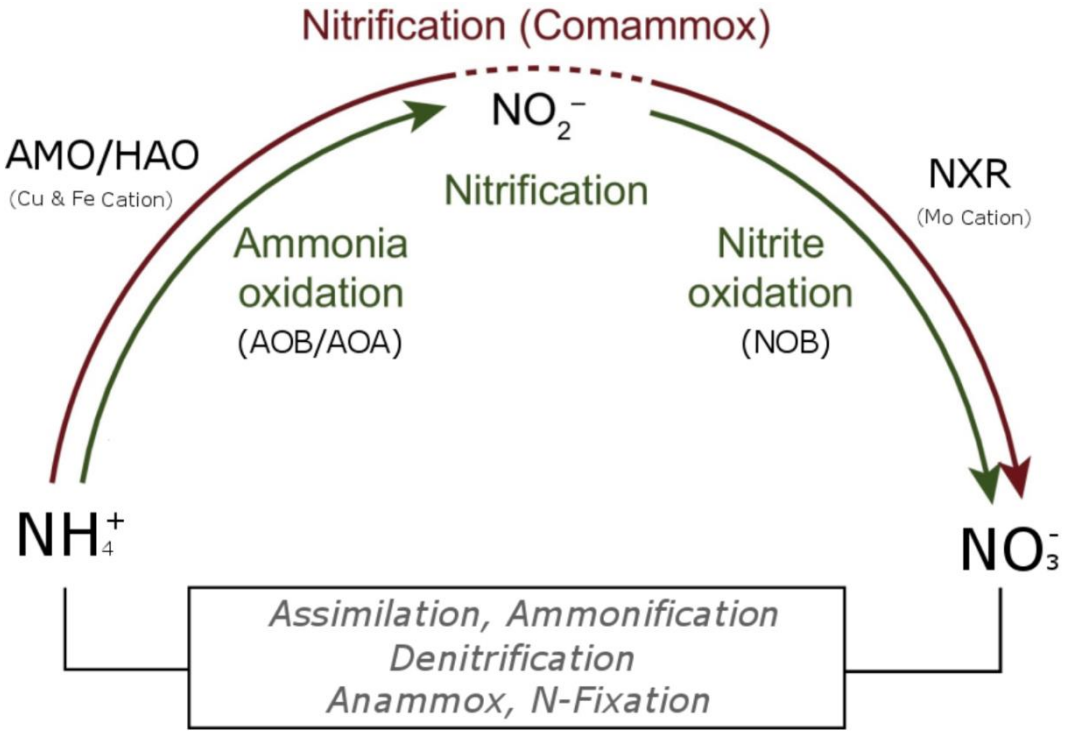

Figure 2.1 Major processes of nitrification including the enzymes required in ammonia and nitrite oxidation plus complete ammonia oxidation (comammox). Enzyme cofactors are bracketed below the respective enzyme abbreviation, AMO (ammonia monooxygenase), HAO (hydroxylamine oxidoreductase), NOR (nitric oxidoreductase; figure modified from Daims *et al.*, 2016).

2.2 Nitrifiers in engineered systems

Nitrifiers have been studied for more than a century since their discovery by Winogradsky in the late 19th century. Nitrifiers are primarily grouped as ammonia-oxidizing prokaryotes (AOP) and nitrite-oxidizing bacteria (NOB). AOP consists of canonical ammonia-oxidizing bacteria (AOB), comammox bacteria and ammonia-oxidizing archaea (AOA).

Depending upon the resource availability (ammonia/nitrite) and environmental conditions nitrifying microorganisms are found in many diverse environments across soils, sediments, oceans, inland waters and engineered systems (Prosser, 2007). Several studies have reported that canonical AOB, NOB and comammox bacteria are key nitrifiers in engineered systems, therefore, they are of main focus in below sections (Prosser, 2007; Koops and Pommerening-Röser, 2001; Palomo-González, 2017; Yao and Peng, 2017; Daims *et al.*, 2006a, 2000; Lücker *et al.*, 2015; Ye *et al.*, 2011; Wagner *et al.*, 2002, 1996). Presence and importance of AOA in DWTP and WWTP has

been highlighted in some studies but, in this study they were below detection limit in qPCR and thus assumed to be minor contributors to the nitrification process in the studied systems (Van Der Wielen *et al.*, 2009; Li *et al.*, 2016; Fan *et al.*, 2018; Pan *et al.*, 2018).

2.2.1 Nitrifiers phylogeny and ecological distribution

Ammonia-oxidizing bacteria

AOB are phylogenetically divided into five major genera, *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio*, *Nitrosolobus* (all four belonging to Betaproteobacteria) and *Nitrosococcus* (to Gammaproteobacteria). Figure 2.2 shows the four main betaproteobacterial AOB genera, their respective clusters and briefly summarizes their main ecophysiological and morphological characteristics.

Nitrosomonas based on ecophysiological and morphological characteristics is divided into three major clusters (cluster 6, 7, and 8) including a subcluster 6A. Members of cluster 6 such as *N. oligotropha* and *N. ureae* typically have high substrate (ammonium) affinity range (1.9-4.2 µM), no salt requirements and test positive for urease activity (Koops *et al.*, 2006). The cluster 6 members are typically considered as k-strategist, i.e., adapted to low substrate concentrations for growth, but some studies have reported them from environments with high ammonium concentrations (Limpiyakorn *et al.*, 2007). *N. oligotropha* related strains have also been shown to adapt to low levels of dissolved oxygen (DO; Gieseke *et al.*, 2001). Their preferred habitats include oligotrophic fresh waters, natural soils, and wastewater and drinking water treatment plants (Koops and Pommerening-Röser, 2001; **paper II and IV**).

	Beta Proteobacteria													
	Nitrosomonas											*Nitrosospira*	*Nitrosovibrio*	*Nitrosolobus*
	Cluster 7				Cluster 8		Cluster 6A		Cluster 6			Cluster 3		
	Nitrosomonas europaea	***Nitrosomonas eutropha***	***Nitrosomonas halophila***	***Nitrosococcus mobilis***	***Nitrosomonas communis***	***Nitrosomonas nitrosa***	***Nitrosomonas oligotropha***	***Nitrosomonas ureae***	***Nitrosomonas marina***	***Nitrosomonas aestuarii***	***Nitrosomonas cryotolerans***	***Nitrosospira briensis***	***Nitrosovibrio tenuis***	***Nitrosolobus multiformis***
Cell Shape	Short Rods with pointed ends	Rod to pear shaped with one or both ends pointed	Coccoid	Coccoid or rod shaped	Large rods with rounded ends	Spheres or rods with rounded ends	Spheres or rods with rounded ends	Spheres or rods with rounded ends	Slender rods with rounded ends	Rod shaped	Rod shaped	Tightly closed spirals and vibrio forms	Slender curved rods	Pleomorphic lobate
Cell Size	(0.8-1.1 x 1.0-1.7) µm	(1.0-1.3 x 1.6-2.3) µm	(1.1-1.5 x 1.5-2.2) µm	(1.5-1.7 x 1.5-2.1) µm	(1.0-1.4 x 1.7-2.2) µm	(1.3-1.5 x 1.4-2.2) µm	(0.8-1.2 x 1.1-2.4) µm	(0.8-1.2 x 1.1-2.4) µm	(0.7-0.9 x 1.7-2.2) µm	(1.0-1.3 x 1.4-2.0) µm	(2.0-4.0 x 1.2-2.2) µm	(0.3-0.8 x 1.0-8.0) µm	(0.3-0.4 x 1.1-3.0) µm	(1.0-1.5 x 1.0-2.5) µm
Motility	Not observed	Motile	Tuft of flagella	Tuft of flagella	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Peritrichous flagella	Subpolar flagella	Peritrichous flagella
Salt require-ments	• No obligate	• No obligate	• Obligate	• Obligate	• No obligate	• No obligate	• No obligate	• No obligate	• Obligate	• Obligate	• Obligate	• No obligate	• No obligate	• No obligate
	• Max. Salt conc. 400 mM NaCl	• Max. Salt conc. 400 mM NaCl	• Max. Salt conc. 900 mM NaCl	• Max. Salt conc. 500 mM NaCl	• Max. Salt conc. 250 mM NaCl	• Max. Salt conc. 300 mM NaCl	• Max. Salt conc. 150 mM NaCl	• Max. Salt conc. 200 mM NaCl	• Max. Salt conc. 800 mM NaCl	• Max. Salt conc. 600 mM NaCl	• Max. Salt conc. 550 mM NaCl	• Max. Salt conc. 250 mM NaCl	• Max. Salt conc. 100 mM NaCl	• Max. Salt conc. 200 mM NaCl
Substrate affinity	• 30-61 µM	Max. amm. conc. 600 mN NH4Cl	Max. amm. conc. 400 mN NH4Cl	Max. amm. conc. 250 mN NH4Cl	• 14-43 µM	• 19-46 µM	Max. amm. conc. 50 mN NH4Cl	• 1.9-4.2 µM	• 50-52 µM	Max. amm. conc. 400 mN NH4Cl	• 42-59 µM	Max. amm. conc. 200 mN NH4C	Max. amm. conc. 100 mN NH4Cl	Max. amm. conc. 50 mN NH4Cl
	• Max. amm. conc. 400 mN NH4Cl				• Max. amm. conc. 250 mN NH4Cl	• Max. amm. conc. 100 mN NH4Cl		• Max. amm. conc. 200 mN NH4Cl	• Max. amm. conc. 200 mN NH4Cl		• Max. amm. conc. 400 mN NH4Cl			
Preferred habitat	• Sewage disposal plants	• Sewage disposal plants	• Brackish water	• Eutrophic environment	• Moderate eutrophic pH neutral soils	• Eutrophic freshwater	• Oligotrophic freshwater	• Oligotrophic freshwater	• Marine environment	• Marine environment	• Marine environment	• Natural soils	• Natural soils	• Soils
	• Eutrophic freshwater	• Eutrophic environment		• Aquatic environment	• Freshwater	• Marine environment	• Natural soils	• Natural soils			• Low temperature as low as 5 °C	• Freshwater environment		• Sewage disposal plants
	• Fertilized soils					• Wastewater treatment plants						• Marine environment		

Figure 2.2 Main characteristics of key betaproteobacterial AOBs (Figuree modified from Soliman and Eldyasti, 2018).

N. europaea, N. eutropha, N. halophila, and *N. mobilis* are key cluster 7 members characterized by their low substrate affinity (30-61 µM ammonium), moderate salt requirements, and test negative for urease activity (Stein *et al.*, 2007; Koops and Pommerening-Röser, 2001; Koops *et al.*, 2006). *N. europaea* is one of the most studied AOB and is considered as r-strategist, i.e., adapted to high substrate concentrations for growth (Bollmann *et al.*, 2002). The cluster 7 members are commonly found in eutrophic freshwater, brackish water, and sewage disposal plants (Koops and Pommerening-Röser, 2001).

AOB cluster 8 members (*N. nitrosa and N. communis*) have moderate substrate affinity (14-46 µM) and are commonly found in non-acidic soils, eutrophic freshwaters as well as in municipal and industrial wastewater treatment plants (Lydmark *et al.*, 2006; Limpiyakorn *et al.*, 2005).

The other betaproteobacterial AOB belong to mainly genus *Nitrosospira* (divided into clusters 0, 2, 3 and 4), *Nitrosovibrio* and *Nitrosolobus* (cluster 3) which are typically found in soils and freshwater environments and the members of Gammaproteobacteria AOB genus *Nitrosococcus* (*N. oceani* and *N. halophilus*) are found in habitats with some of the highest salt and ammonium concentrations such as marine environments and salt lakes (Koops and Pommerening-Röser, 2001; Soliman and Eldyasti, 2018).

Nitrite-oxidizing bacteria

NOBs are more phylogenetically diverse than AOB, belonging to seven genera distributed across four bacterial phyla: *Nitrobacter, Nitrococcus,* and *Nitrotoga* from Proteobacteria phylum; marine NOB *Nitrospina* and *Candidatus Nitromaritima* in the Nitrospinae phylum; *Nitrolancea* in the Chloroflexi phylum and *Nitrospira* in the Nitrospirae phylum. *Nitrospira* is the most diverse genus amongst all NOB and is further divided into six phylogenetic lineages, and Lineage II is the most diverse amongst these six lineages Daims *et al.*, 2016). Figure 2.3 shows the phylogenetic affiliation, species diversity, and habitats of NOBs.

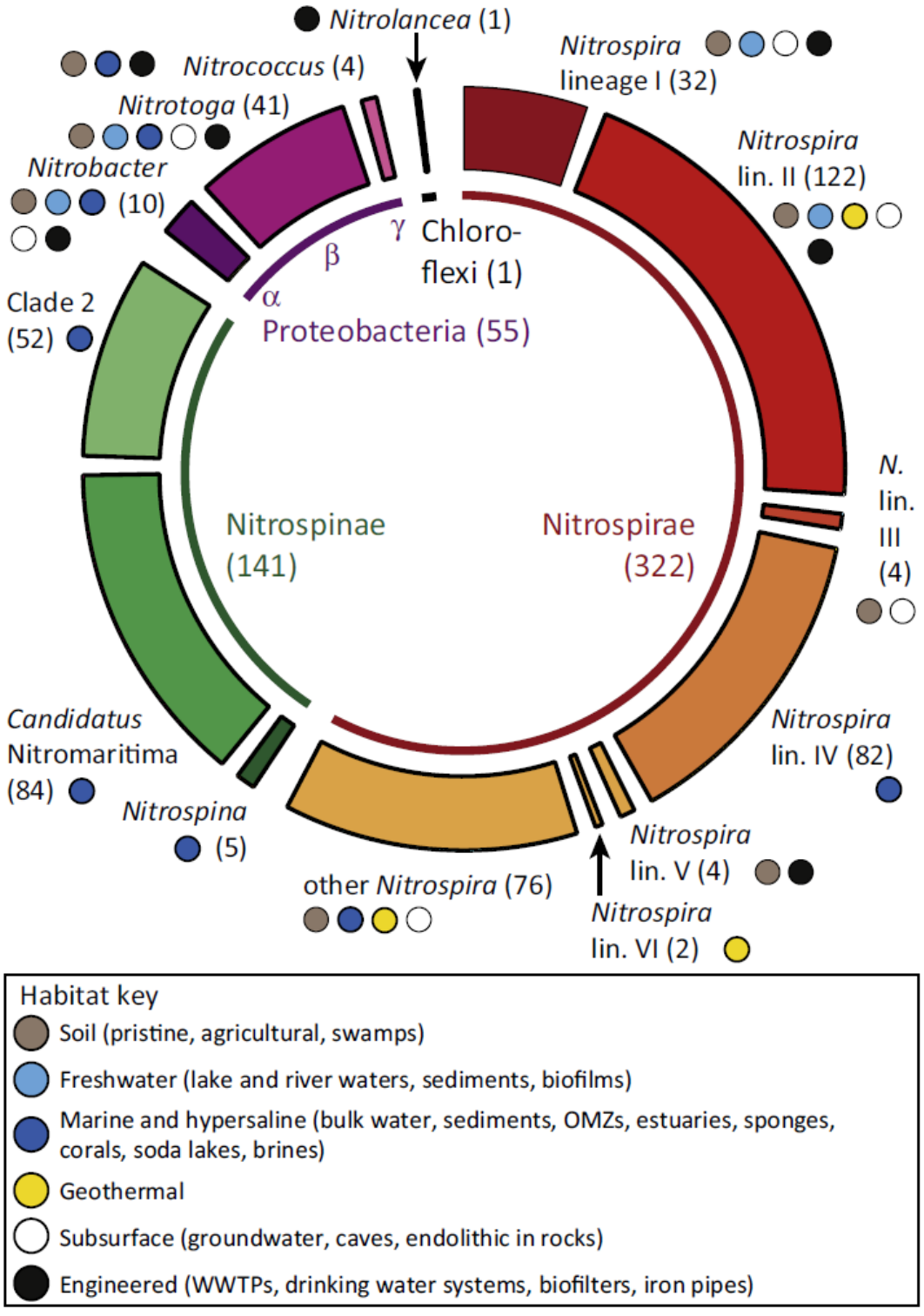

Figure 2.3 The phylogenetic affiliation, species diversity and habitats of nitrite-oxidizing bacteria (NOB). The inner ring indicates bacterial phyla for respective NOBs. The outer ring is drawn proportionally to the species level diversity of each genus based on 16S rRNA gene sequences of known NOB and closely related bacteria

which were clustered in OTUs (98.7% identity) and no. of OTUs are mentioned in parenthesis (Figure adapted from Daims *et al.*, 2016).

The members of genus *Nitrospira* are slow growing aerobic chemolithoautotrophic nitrite-oxidizing organisms (Daims and Wagner, 2018). They typically occur in close collaboration with ammonia oxidizers as they oxidize ammonia to nitrite which is further converted to nitrate by *Nitrospira.* However, *Nitrospira* can also have a reciprocal feeding interaction with ammonia oxidizers by providing them ammonia released from urea/cyanate (Daims and Wagner, 2018). Some *Nitrospira* strains can also utilize H_2 and formate as substrates by using oxygen/nitrate as a terminal electron acceptor for nitrite oxidation (Daims *et al.*, 2016). Due to such metabolic versatility, *Nitrospira* members can inhabit a broad range of environments such as soils, inland waters, groundwater, wastewater treatment **(paper II)** and drinking water systems **(paper II, III and IV)**.

The fundamental ecophysiological differences between NOBs typically result from their nitrite oxidation kinetics; for example, they have different affinity for nitrite (Daims *et al.*, 2016). In wastewater treatment plants (WWTPs) and freshwater, the measured Km (NO_2^-) values of *Nitrospira* strains ranged from ~9 - 27 mM (Nowka *et al.*, 2015; Schramm *et al.*, 1999). The Km (NO_2^-) range of *Nitrobacter* strains measured from sewage, soils, and other habitats were much larger (~49 - 544 mM) compared to *Nitrospira* strains (Nowka *et al.*, 2015). Since *Nitrospira* has a high affinity for nitrite, it has been proposed to be a k-strategist and *Nitrobacter* could be r-strategists because of its preference for high nitrite levels and can have higher growth rate compared to *Nitrospira* in environments with high nitrite levels (Schramm *et al.*, 1999). However, *Nitrobacter* strains have a very broad range for nitrite affinity, therefore; they differ in their ability to adapt to high levels (Nowka *et al.*, 2015). Other NOBs like *Nitrotoga* strains from soil have shown Km (NO_2^-) values of ~58 mM, and those of *Nitrolancea* strains from sewage were as low as ~1 mM (Nowka *et al.*, 2015; Sorokin *et al.*, 2012). Kinetic information for other NOB still need to be obtained, and relevance of other main substrates such as oxygen and carbon dioxide for NOB are to be studied in detail (Daims *et al.*, 2016).

Comammox

The comammox bacteria, which perform complete nitrification, are presently divided in two clades (clade A and clade B), both belonging to *Nitrospira* lineage II (Daims *et al.*, 2015; van Kessel *et al.*, 2015). The key known comammox *Nitrospira* are *Ca. Nitrospira inopinata*, *Ca. Nitrospira nitrosa* and *Ca. Nitrospira nitrificans*. Comammox *Nitrospira* were first discovered from a trickling filter and a biofilm in groundwater borehole (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Metagenomic analysis have now revealed their widespread presence in freshwater, soil, wastewater and drinking water treatment systems (Pinto *et al.*, 2016; Palomo-González, 2017; Daims *et al.*, 2015; van Kessel *et al.*, 2015; Fowler *et al.*, 2018; Palomo *et al.*, 2018; **paper III and IV**). A recent kinetic analysis of *Ca. Nitrospira inopinata* has revealed that it is adapted to slow growth in oligotrophic conditions based on its high ammonium affinity, low maximum ammonium oxidation rate and high yield compared to other nitrifiers (Dimitri Kits *et al.*, 2017). Hitherto, ecophysiological differences between the two clades of comammox are presently largely unknown.

Ammonia-oxidizing archaea

All characterized AOA are affiliated to the phylum Thaumarchaeota (Brochier-Armanet *et al.*, 2008). The three main described species are *Nitrosopumilus maritimus*, *Nitrososphaera viennensis*, and *Nitrososphaera gargensis*. AOA inhabit a varied range of natural environments such as soils (~1–5% of all prokaryotes), marine environments (~20–40% of all marine bacterioplankton), and geothermal habitats (Ochsenreiter *et al.*, 2003; Lehtovirta-Morley *et al.*, 2011; Karner *et al.*, 2001; DeLong *et al.*, 2010; De La Torre *et al.*, 2008; Dodsworth *et al.*, 2011; Urich *et al.*, 2008; Huang *et al.*, 2008). Physiological studies on *N. maritimus* strain SCM1 showed that its stoichiometry of ammonia oxidation is not different from that of AOB and this marine isolate is an extreme oligotroph having a *Km* of 132 nM (at near-neutral pH) and cannot tolerate ammonia at concentrations significantly above ~1 mM (Martens-Habbena *et al.*, 2009). Similar observations have been reported for the moderately thermophilic *N. gargensis* (Richter *et al.*, 2008). On the other hand, *Nitrososphaera viennensis* isolated from garden soil in Vienna, Austria, tolerates higher concentrations of ammonia than *N. maritimus*, with complete conversion of ammonia at ammonia concentrations up to 3 mM (Spang *et al.*, 2011). These observations in combination with in situ studies show that AOA can grow over a wide range of ammonium con-

centrations in comparison to AOB (Pratscher *et al.*, 2011; Verhamme *et al.*, 2011, 2010). Presence of AOA has also been reported from engineered systems used for drinking water production and wastewater treatment (Limpiyakorn *et al.*, 2013; Park *et al.*, 2006; Van Der Wielen *et al.*, 2009; Bai *et al.*, 2012). As mentioned earlier, in this study AOA were below the detection limit in qPCR and thus assumed to be minor contributors to the nitrification process in the studied systems

2.2.2 Factors affecting growth and activity of key nitrifiers

Nitrifiers are generally specialized chemolithoautotrophs that use either ammonia (AOP) or nitrite (NOB) as an energy source and carbon dioxide as a carbon source. However, many studies have shown that different nitrifiers can utilize other energy sources such as urea, cyanate, fructose, and can also grow by aerobic hydrogen oxidation (Hommes *et al.*, 2003; Bock *et al.*, 1995; Koch *et al.*, 2014; Qin *et al.*, 2014; Bock, 1976). Clade A comammox have very low K_M (NH_3) in comparison to AOB and higher yield plus oxygen consumption compared to AOB and canonical *Nitrospira* (Table 2.1).

Table 2.1 Physiological parameters of AOB, canonical and comammox *Nitrospira* (Table adapted from Lawson and Lücker, 2018).

Parameter	Clade A comammox	Clade B comammox	Canonical *Nitrospira*	AOB
K_M (NH_3) [µM]	0.049	n.d.	n.a.	1.9–200
K_M (NO_2^-) [mM]	n.a.	n.a.	9 - 27	n.a.
K_O [µM]	n.d.	n.d.	4.06–16.88	1–40
µmax [h−1]	0.0061	n.d.	0.019–0.058	0.028–0.068
Ybio [mg protein/mol NH_3]	395	n.d.	120–213*	250
Microcolony size [µm]	n.d.	n.d.	1–8	2–16
Oxygen consumption [mol O2:NH_3]	2	2	0.52*	1.5
Refrences: (Dimitri Kits *et al.*, 2017; Manser *et al.*, 2005b; Park *et al.*, 2017; Manser *et al.*, 2005a; Blackburne *et al.*, 2007; Laanbroek and Gerards, 1993; Nowka *et al.*, 2015)				
K_M: Half saturation constant				
K_O: Oxygen half saturation coefficient				
Y_{bio}: Biomass yield				
n.d. = not determined; n.a. = not applicable				
***per mol NO_2^-**				

Apart from the key growth substrates (ammonia and nitrite) and carbon sources, the growth and activity of AOB and NOB can be affected by different abiotic factors such a temperature, pH, alkalinity and DO (Koops and Pommerening-Röser, 2001; Soliman and Eldyasti, 2018).

Several studies have shown that temperature is one of the significant factors affecting nitrification in DWTP and WWTP (Andersson *et al.*, 2001; Pintar and Slawson, 2003; Kapoor and Viraraghavan, 1997; Sudarno *et al.*, 2011; Guo *et al.*, 2010; Bougard *et al.*, 2006; Antoniou *et al.*, 1990). Generally, AOB show higher growth rates than NOB at a temperature of more than 20 °C (Hellinga *et al.*, 1998; Bougard *et al.*, 2006). For example, a study using nitrifying biofilm reactors with synthetic wastewater observed higher ammonia removal efficiency (80 %) because of AOB proliferation in the reactors operated at 25 °C but lower (30 %) in reactors operated at 15 °C (Kim and Lee, 2011).

The proposed optimum pH range for AOB and NOB are 8.2±0.3 and 7.9±0.4 respectively (Park *et al.*, 2007). Different pH set points can directly affect AOB and NOB activities by impacting the enzyme reaction mechanisms, speciation of substrates and gene expression (Van Hulle *et al.*, 2007). For example, an *amoA* based analysis of AOB showed a low abundance of AOB at pH<5.5, whereas, increased AOB abundance was observed with increasing pH (De Boer and Kowalchuk, 2001; Nicol *et al.*, 2008). Also, NOB in WWTPs showed optimal activity at pH 7.9±0.4 which is consistent with the observations from pure cultures of *Nitrospira* and *Nitrobacter* strains that have shown optimal growth at pH 7.6–8.0 and 7.9 respectively (Park *et al.*, 2007; Ehrich *et al.*, 1995; Grunditz and Dalhammar, 2001).

DO concentrations can also have a profound effect on the growth of AOB and NOB. For example, in activated sludge systems where reactors were operated at low DO concentrations (0.37 and 0.16 mg/L) *Nitrosomonas europaea/eutropha* were dominant AOB and *Nitrospira* increased in abundance compared to *Nitrobacter* (Liu and Wang, 2013). Another study by Hanaki *et al.*, 1990 showed that the AOB growth rate was doubled at elevated DO concentrations with no change in growth rate of NOBs, while at low DO concentrations (0.5 mg/L) there was no effect on ammonia oxidation.

Apart from temperature, pH and DO, AOB and NOB growth can be affected or inhibited by several other factors. For example, studies on drinking water biofilter microbial communities have shown that phosphorous (generally required in the form of phosphate (PO_4^{3-})) limitation can limit nitrifiers growth

whereas its addition in trace amounts can increase nitrification (Lee *et al.*, 2014). AOB and NOB growth can also be affected by inorganic carbon limitation. Further, the inhibitory effects of nitric oxide and volatile fatty acids have also been reported on nitrification (Courtens *et al.*, 2015; Takai *et al.*, 1997).

Another critical factor affecting the growth of nitrifiers is the availability of micronutrients and metal cofactors that are essential for key enzymatic functions (Kapoor *et al.*, 2016; Black *et al.*, 2016; Liu *et al.*, 2014). For example, trace metal copper is a cofactor of AMO enzyme in the process of nitritation. Studies have shown that copper supplementation in trace amounts can drastically improve nitrification in biological sand filters (Wagner, 2017). The impact of copper supplementation on the nitrifying community is also a part of this Ph.D. study **(paper IV)**. Another study has shown that nitrifier activity in potable water systems was inhibited by the presence of copper (Cu) and zinc (Zn) at >100 ug/L and phosphate at <5 ug/L and inorganic carbon in higher amounts than 0.1 mg C/mg oxidized nitrogen can reduce the toxicity of Cu/Zn and increase phosphate availability to nitrifiers (Zhang and Edwards, 2010).

2.3 Nitrification in engineered systems

2.3.1 Nitrification in drinking water treatment systems

Biological filtration is one of the key components for the successful treatment of water for human consumption. In short, the process involves microbial removal of compounds/particles from the raw water by passing it through a granular or porous media (Basu *et al.*, 2016; Prevost, 1991). Most commonly, surface water and groundwater are used for drinking water production. Although reactive forms of nitrogen can be present in both water sources, surface water is commonly characterized by higher organic matter content and groundwater with higher mineral content, and little or no oxygen (Sophocleous, 2002). Amongst various filtration methods, granular media based biological filtration is commonly preferred because of its economic feasibility (Lopato, 2011).

In Denmark, where this Ph.D. study was conducted, drinking water supply is entirely based on groundwater (Hansen and Thomsen, 2017; Sorensen and Møller, 2013). Figure 2.4 shows a simplified workflow of a Danish drinking water treatment plant treating groundwater for potable water production.

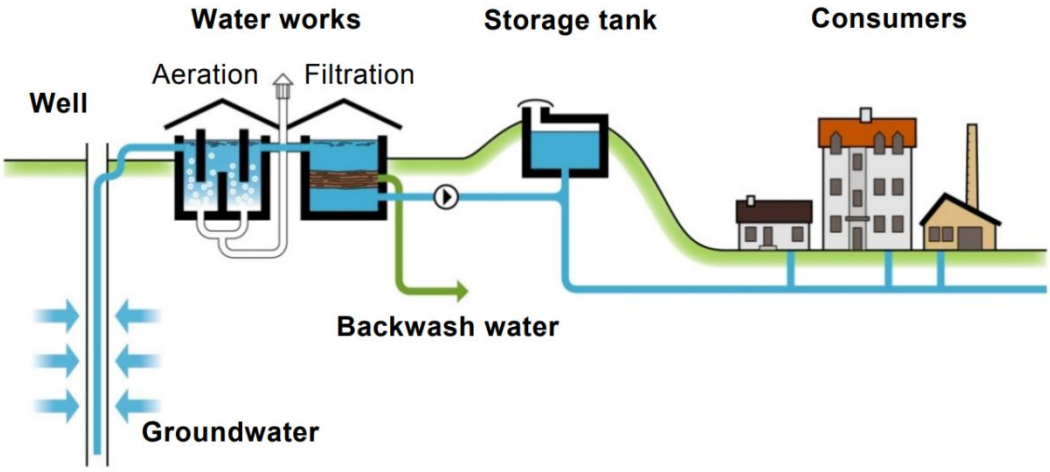

Figure 2.4 Typical groundwater based potable water production workflow (Figure adapted from VandCenterSyd, 2013).

The anaerobic groundwater from aquifers contains compounds such as ammonium (NH_4^+), manganese (Mn^{2+}), dissolved iron (Fe^{2+}), methane (CH_4) and hydrogen sulphide (H_2S; Sogaard and Madsen, 2013). These compounds need to be removed in the DWTPs before the water is ready for distribution to the consumers. Since the groundwater aquifers have a relatively high water quality, its treatment comprises of only aeration and filtration (primary/secondary or both) steps. At the aeration step, unwanted volatile compounds such as H_2S and CH_4 are removed via stripping and oxidation of Fe^{2+} and Mn^{2+} makes them precipitate as metal hydroxides (Van Der Hoek *et al.*, 2014). In the granular media filtration step (e.g., rapid sand filtration), the remaining fractions of metal hydroxides and dissolved gases are removed along with oxidation of dissolved compounds like NH_4^+ (Lopato, 2011). The oxidation of Fe^{2+} and Mn^{2+} can be mediated either chemically or biologically via iron- and manganese oxidizing bacteria and $NH4^+$ is biologically removed by nitrifiers (Tekerlekopoulou *et al.*, 2013).

Rapid sand filter (RSF) is the most common type of granular media filter used in Danish drinking water production facilities. The word 'rapid' refers to the water filtration rate which is typically ~3 m/h (Basu *et al.*, 2016). These filters are comprised of a top filter bed (or 'active layer') commonly made up of quartz grains varying from 0.8-1.4 mm in size (Lopato, 2011). The nitrifying microorganisms are organized within a biofilm around the filter media thus making it an active layer responsible for biological removal of contaminants (Gülay *et al.*, 2014). Samples from the active layer of the RSFs

across various DWTPs in Denmark were used for studying nitrifying microorganisms in all sub-studies of this Ph.D. study **(paper I-IV)**.

2.3.2 Nitrification in wastewater treatment

The WWTPs are generally designed to remove ammonium by converting it into N_2. In conventional WWTP, nitrification is used to convert ammonia into nitrate which is further converted into dinitrogen gas by denitrification process (Olsson and Newell, 1999). The nitrification process in WWTP is predominantly implemented as activated sludge or biofilm-based system. The inorganic nitrogen received through influent wastewater is first converted to nitrite by ammonia-oxidizing bacteria or archaea (AOB or AOA) which is further converted to nitrate by NOBs in the presence of oxygen (Grady and Lim, 1980). Anaerobically this nitrate is then converted to N_2 by the denitrifying microorganism. The coupling of nitrification and denitrification requires alternation between oxic and anoxic phases and supply for organic carbon sources for maintaining denitrification performance, making the overall operation expensive (Leitão *et al.*, 2006). A significant advancement in this direction was the discovery of anammox microorganisms. Anammox microorganisms can oxidize ammonia in the absence of oxygen thereby reducing the oxygen demand for ammonium removal by up to 60% and also reduces the cost of supplying organic matter for denitrification (Gonzalez-Martinez *et al.*, 2018). The leftover organic matter can be used for anaerobic digestion for methane which can be ultimately used for energy production (Lettinga, 1995).

In this Ph.D. study, activated sludge samples from Danish and Swedish WWTPs and an anammox reactor from a Swedish WWTP were used along with DWTP samples for comparing amplicon sequencing-based approaches for assessment of nitrifiers **(paper II)**.

2.3.3 Nitrifiers in DWTP and WWTP

Drinking water and wastewater treatment systems provide very different environments for nitrifiers growth. These environmental conditions primarily govern the nitrifier types that inhabit these environments.

RSFs in DWTPs are typically characterized by low temperatures and low ammonium concentrations (~0.5-2 mg/l) which are favorable for AOB cluster 6 and 7 members and *Nitrospira* lineage II members. Several previous studies has shown the dominance of AOB strains closely related to cultured members of *Nitrosomonas* (*N. europaea* and *N. oligotropha*) from cluster 7 and 6 as

primary ammonia (Gülay *et al.*, 2016; Tatari *et al.*, 2017; Gülay *et al.*, 2016b; Thapa Chhetri *et al.*, 2013; Bai *et al.*, 2013; Gülay, 2014; Van Der Wielen *et al.*, 2009). Apart from AOB, AOA strains related to *Nitrosopumilus maritimus, Candidatus Nitrososphaera gergensis,* and *Candidatus nitrosoarchaeum* and other organotrophic bacteria that can oxidize ammonia as a co-metabolic process has also been reported from RSF (Van Der Wielen *et al.*, 2009). For nitrite oxidation, *Nitrospira* primarily those belonging to lineage II have been reported as main NOB performing nitratation in RSF (Palomo-González, 2017; Gülay, 2014). The members of *Nitrospira* have been repeatedly detected in high abundances compared to the respective canonical AOB especially in drinking water biofilters and distribution systems (Gülay *et al.*, 2016; **papers II, III and IV)**. Recent studies have shown that the high abundances of *Nitrospira* previously reported in the RSF were majorly contributed by comammox *Nitrospira* suggesting their key contribution in nitrification in RSF (Fowler *et al.*, 2018; Palomo-González, 2017; Palomo *et al.*, 2016; Pinto *et al.*, 2016; **paper III and IV**).

The activated sludge systems in municipal WWTP have relatively very high concentrations of ammonium (~20-75 mg/l) compared to DWTP (Henze *et al.*, 2002). The members of *Nitrosomonas* are the most abundant AOB in activated sludge systems due to their relatively higher growth rate than other AOB (Wittebolle *et al.*, 2008). The high abundance of *Nitrosomonas* related strains has been observed in a wide variety of activated sludge systems such as full-scale municipal WWTP, sequencing batch and continuously stirred tank reactors, activated sludge bioreactors, nitrification tanks (*N. europaea and N. eutropha*) and aeration basins of industrial (*N. nitrosa*) and municipal (*N. oligotropha*) WWTPs (Adamczyk *et al.*, 2003; Dionisi *et al.*, 1999; Prosser, 1990; Wells *et al.*, 2009; Mobarry *et al.*, 1996; Wittebolle *et al.*, 2008; **Paper II**). In addition to AOB, other studies have reported AOA in different types of activated sludge systems (Bai *et al.*, 2012; Khardenavis *et al.*, 2007). The most abundant NOB representatives found in activated sludge systems are from genus *Nitrospira* belonging to lineages I (*Nitrospira defluvii;* Wittebolle *et al.*, 2008). Studies have also shown that NOB clusters with AOB and form microcolony aggregates in the activated sludge flocs and syntrophically perform the process of nitrification (Daims *et al.*, 2006b). In addition to canonical *Nitrospira*, comammox *Nitrospira* has also been reported from WWTP (Annavajhala *et al.*, 2018; Gonzalez-Martinez *et al.*, 2016).

Overall, a wide range of nitrifier compositions are observed in different drinking water and wastewater treatment systems (Soliman and Eldyasti, 2018; Koops and Pommerening-Röser, 2001; Kuypers *et al.*, 2018; Prosser, 2007; Lawson and Lücker, 2018). These varied nitrifier compositions exist mainly because nitrifiers are exposed to diverse environmental conditions (seasonal variations) and these systems receive different input (variable influent water for treatment). On a broader scale, i.e., at the community level, it is still not completely understood how different nitrifiers at the guild and within guild level react to these diverse environmental conditions that they get exposed to in the engineered systems. In the next segment, I have presented the ecological rules that try to explain how (microbial) communities are assembled.

2.4 Community assembly processes

Microbial diversity, composition, and activity of guild members in the community are of particular relevance to the maintenance and enhancement of engineered biological system performance. It has been proposed that engineering microbial diversity and composition is possible if we can incorporate theoretical community ecology into process design (Curtis *et al.*, 2003; see table 2.2 for terminology). The next sections provide a brief background on some of the key ecological theories and related process with emphasis on studying microbial community composition and assembly.

Table 2.2 Key terminology and definitions from community ecology used in this work (Table adapted from Kinnunen, 2017).

Term	Definition	Adapted from
Diversity	The evenness and types of taxa within a local community	(Shade, 2016)
Composition	An aspect of diversity that accounts for the number and abundance of unique taxa and their identities, which can be taxonomic or operational.	(Shade, 2016)
Community assembly	The sum of all processes that shape the composition of a microbial community, including dispersal, selection, drift, and speciation	(Vellend, 2010)
Metacommunity	A set of local communities that are linked by dispersal of multiple interacting species	(Leibold *et al.*, 2004)
Local community	The individuals of all species that potentially interact within a single patch or local area of habitat	(Leibold *et al.*, 2004)

2.4.1 Niche and Neutral theories

The two main ecological theories that have received the most attention while interpreting the composition of microbial communities are the niche theory and the unified neutral theory of community assembly (Hutchinson, 1957; Hubbell, 2001). The niche theory emphasizes the deterministic factors/processes such as interspecies interactions (e.g., competition, predation, mutualism and trade-offs), species traits, and environmental conditions (e.g., pH, temperature, etc.) in governing community structure (Holt, 2009; Chesson, 2000). In contrast, the neutral theory assumes that community structure is independent of deterministic factors and is governed by stochastic processes such as birth, death, colonization, extinction and speciation/diversification (Chave, 2004).

Niche theory

The basic assumption of niche theory is that species within an environment differ in their niches. Niche is an n-dimensional hypervolume comprising of a set of biotic and abiotic conditions for species to persist (Hutchinson, 1957). A niche for a species is determined by species traits that enable them to obtain resources, compete with other members and adapt to adverse environmental conditions. Although niche theory has been widely used in the development of deterministic ecological theories and has been the most fundamental concepts in ecology, it still faces several challenges in explaining patterns of community structure. Niche, by definition, comprises of numerous dimensions, therefore, defining it is a highly challenging task. Therefore, most community assembly studies focus on parameters that are easily measurable when defining niche (e.g., resource availability and substrate affinity of community members or their environmental conditions required for growth). (Waldrop *et al.*, 2006; Litchman *et al.*, 2015; Larson *et al.*, 2016; Ma *et al.*, 2015).

Neutral theory

The neutral theory considers the niche differences to be irrelevant in the context of community assembly (Hubbell, 2001). It is worth noticing that the community can have a slightly different meaning for macro and microorganisms. Hubbell's neutral community assembly theory was formulated for plant communities (i.e., group of organisms sharing the same basic type of metabolism or, in Hutchinson's terminology, sharing many dimensions of their niches). In microbial ecology, we often use communities to encompass all the microbes irrespective of their types even though some

might have (almost) completely different niches. Hubbell's neutral theory hypothesizes functional equivalence for all species and individuals in a community. It considers that stochastic processes control species dynamics and not their competitive abilities. According to neutral theory, community assembly is governed by continuous cycles of birth and deaths, the chance of colonization (dispersal), and random changes in organism abundance (ecological drift). Thus, if community assembly is neutrally governed, then any void in the community can be randomly filled by an individual from within or outside the community (Burke *et al.*, 2011). Neutral theory fundamentally challenges the core concepts of niche theory that all species differ ecologically depending upon their niche and that species abundance and distribution (SAD i.e. the distribution of abundances of all species within a sample or community) are governed by their environments (Matthews and Whittaker, 2014; Chesson, 1991; Matthews and Whittaker, 2015). Due to the neutral theory's surprisingly simple approach, it has been highly debated since its publication (Matthews and Whittaker, 2014).

Despite its simplicity, neutral theory has successfully predicted SADs and species-area relationships (SAR i.e. pattern of increase in number of observed species with increasing sampled area of observation) for numerous communities (Kneitel and Chase, 2004; Preston, 1960; Gewin, 2006; Gravel *et al.*, 2006; Adler *et al.*, 2007; Cencini *et al.*, 2012). It has also been widely used for investigating microbial community assembly (Nemergut *et al.*, 2013; Kinnunen, 2017; Yan *et al.*, 2016; Venkataraman *et al.*, 2015; Jizhong Zhou, 2017). Collectively, these observations suggest an essential role for stochastic processes in explaining community patterns, in some cases even better than niche-based processes (Hubbell, 2005).

The debate of the relative contribution of niche versus neutral processes in community assembly has now led the researchers to realize that both deterministic and stochastic processes are not mutually exclusive. Various studies have shown that both processes are rather complementary and work in unison for community assembly (Stegen *et al.*, 2016; Hubbell, 2005; Graham *et al.*, 2016; Adler *et al.*, 2007; Gravel *et al.*, 2006; Graham *et al.*, 2017). Considering the importance of both deterministic and stochastic processes in community assembly, numerous theoretical models have been developed (Tilman, 2004; Vergnon *et al.*, 2012; Adler *et al.*, 2007; Gravel *et al.*, 2006).

2.4.2 Vellend's Conceptual synthesis in community ecology

To delineate the rather complicated network of theories and concepts in community ecology, Vellend, 2010 proposed a conceptual framework for community assembly. Vellend's inspiration originated from the evolutionary processes (selection, drift, mutation and gene flow) concept in population genetics, which classically studies (Meta) populations. For simplicity, an allele in population genetics can be theoretically considered as an equivalent to a species in community ecology. Vellend suggested that all ecological processes can be grouped into four fundamental processes: selection, ecological drift, speciation and dispersal (Vellend, 2010). Instead of 'speciation' Nemergut *et al.*, 2013b suggested using 'diversification' because changes in community structure due to evolutionary processes can be caused even without the creation of new species.

Vellend's conceptual framework is advantageous because it unifies niche (deterministic; e.g., selection) and neutral perspectives (stochastic; e.g., drift). Three of the four processes (dispersal, drift and speciation/diversification) suggested by Vellend are of the main focus in neutral theory (Hubbell, 2001). Additionally, the framework acknowledges the importance of evolutionary processes such as speciation/diversification in community assembly and provides a unified ground under which different communities from across habitats can be compared (Stegen *et al.*, 2015). Below, is the brief description of the four main processes of Vellend's conceptual framework and their associated stochastic (neutral) and deterministic (niche) components (Figure 2.5).

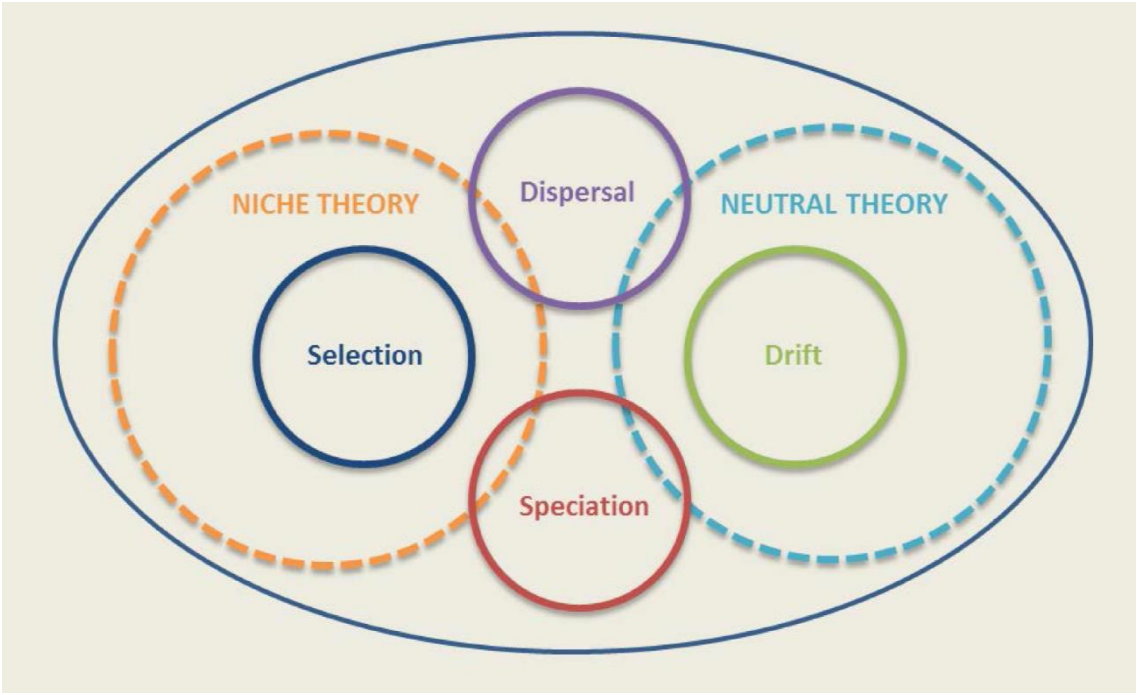

Figure 2.5 The four main community ecology processes proposed by Vellend and their theoretical association with niche and neutral theories (Figure adapted from Kinnunen, 2017).

Selection

Selection is a deterministic fitness difference between individuals of different species that can influence community structure (Vellend, 2010). Selection amongst individuals of different species can be due to abiotic conditions (e.g., temperature, pH, salinity, etc.) or biotic interactions (e.g., competition, mutualism, predation, etc.) causing variation in their reproductive success (Nemergut *et al.*, 2013; Stegen *et al.*, 2013; Hanson *et al.*, 2012). Thus, selection is undoubtedly the closest to the niche theory (Figure 2.1; Vellend *et al.*, 2014; Chase and Myers, 2011).

In this work, I have approached selection in microbial communities from two directions. First, I have compared the metacommunity with the local/system community and considered that a big deviation in the composition is indicative of non-neutrality. For example, a significant increase in the relative abundance of the species is indicative of positive selection. If the species abundance is significantly decreased then it is considered as negative selection **(paper III and IV;** further discussed in chapter 5 and 6**)**. A significant increase/decrease in relative abundance is assumed to be a result of fitness differences amongst guild members. In the second approach, since resources

are important in the fundamental definition of niche, I have assumed that modification of resources (i.e. releasing resource limitations) will be reflected in the observed community composition/abundance (Hibbing *et al.*, 2010; **Paper IV)**. This interpretation of the second approach calls for a description of some basic concepts about resource availability, limiting resources and selection based on the competition of limiting resources.

Resource availability, competition and limiting resources

Resource availability has been addressed as one of the main factors affecting the ecological dynamics of community members (Tilman *et al.*, 1982). Liebig's law of the minimum states that under steady-state conditions the essential resource available in amounts most closely approaching the critical minimum needed by a given organism will tend to be the limiting one (Odum, 1953). In other words, the growth of an individual is not dictated by all the available resources but by the scarcest (limiting) resource. Different nutrients can be limiting for the guild members and if a nutrient is limiting in an environment adding it in higher amounts should increase the growth and eventually the abundance of the guild member.

Jacques Monod presented the first empirical demonstration of the relation between limiting nutrient concentration, and bacterial growth (Monod, 1949; **Box 1**; Prosser *et al.*, 2007).

Box 1: Monod growth kinetics

Monod growth kinetics describes how the concentration of a growth-limiting substrate influences the specific growth rate of microorganisms. This description is similar to the Michaelis–Menten equation for enzyme kinetics. The growth kinetics can be determined mainly by two growth parameters a) the maximum specific growth rate which is achieved at high i.e. non-limiting substrate concentration and b) affinity constant i.e. the concentration of substrate at which the specific growth rate is half of the maximum specific growth rate.

Resource competition and its effect on community composition stem back to the earlier work done by MacArthur, 1972 on patterns in the distribution of species. Based on MacArthur's work and using Monod kinetics, Tilman examined the competition between two different types of populations, as a function of limiting resource ratios. Tilman's resource-ratio theory (RRT) which is one of the main ecological theory for explaining community structure

based on resource competition states that the availability and demand for nutrients (resources) and their consumption rate will determine the prevalence of a species in the community (Tilman, 1982). If there are more than one resources limiting then the stable coexistence of competing species is possible by means of trade-offs, or they can get outcompeted under nutrient limitations by other species who can best utilize the resource in its lowest available form. This ability to efficiently consume limiting nutrients will over time reflect in selecting certain species and could further shape the community structure. A trade-off is basically when a species can have the benefit of performing a function well by compromising on another function, for example, a trade-off can be differential utilization of resources (Kneitel and Chase, 2004). Trade-offs can also be an element of niche differentiation amongst species (Chase and Leibold, 2003). Niche differentiation refers to a process by which natural selection drives competing species to coexist by differentiating their patterns of resource utilization differential resistance to predation etc.

One of the main predictions of RRT is "High species diversity and evenness occur when there are higher number of limiting resources, while resource enrichment leads to decreased species richness and evenness." This prediction is discussed in chapter 6 based on **paper IV** observations.

I acknowledge that the RRT provides a conceptual foundation for predicting the outcomes of microbial competition, but it does not encapsulate the mechanisms (e.g., motility, antibiotic production, and coordinated behavior) by which certain microbe can tip the competitive balance, resulting in outcomes that significantly differ from those predicted by resource abundance alone.

Dispersal in community ecology is the movement of an organism across space, considering the immigration in and emigration out of the community (Kinnunen, 2017). Dispersal has features of both niche and neutral theory as it can depend on both deterministic and stochastic processes (Finlay, 2002; Vellend *et al.*, 2014; Hanson *et al.*, 2012). For example, dispersal of seeds would be stochastic if it is dependent on a stochastic event such as dominating winds. Dispersal can be neutral if all species have the same basic probability to disperse and in this case dispersal of a species would be a function of its relative abundance. On the other hand, the rate of dispersal can be entirely different for different species based on their traits and active status (e.g., spore/dormancy) thus here dispersal would be deterministic (Jizhong Zhou, 2017).

Ecological drift is a process by which changes in the relative abundance of species in the community over time are caused by the random process of birth and death (Kinnunen, 2017; Jizhong Zhou, 2017). Ecological drift is probably closest to the assumptions of the neutral theory. Various studies have shown that drift can alter and shape community structure and is more important when selection is weak, and the local community size is (Chave, 2004; Chase and Myers, 2011).

Speciation/Diversification is a process where the generation of new genetic variants causes an increase in diversity (Vellend, 2010; Jizhong Zhou, 2017). In the context of this Ph.D. study, the effect of diversification is considered negligible because we assumed that the rate of diversification will often be small compared to the dispersal rate as in DWTP the amount of nitrifiers coming in and out of the studied system is large.

3 Molecular approaches for microbial communities

High-throughput molecular technologies can be grouped into two major categories: open and closed formats (Zhou et al., 2015). “Open format” refers to the technologies whose potential experimental results cannot be anticipated before performing the analysis, and thus, the experimental outcome is considered open. The main characteristic of open format technologies is they typically do not require a priori sequence information from the community of interest. This category includes techniques like next-generation genomic sequencing (or metagenomics) and mass spectrometry-based proteomics and metabolomics approaches. “Closed format” refers to the detection technologies whose range of potential experimental results is defined before performing the analysis, and thus, the experimental outcome is considered closed. The defining feature of these technologies is that they require a priori sequence information. Next generation targeted amplicon sequencing, DNA and protein microarrays, as well as quantitative polymerase chain reaction (qPCR), are some of the closed-format technologies (Zhou et al., 2015). These ‘closed-format’ methods typically depend on the ability of a primer set for unbiased amplification of all (or most) of the target sequences without amplifying non-target sequences. The most commonly used marker gene in microbiological research is the 16S rRNA gene for prokaryotes. The rRNA gene has different regions; some are highly conserved across all phylogenetic domains while other regions are variable between related species, and this variability allows for inferring phylogenetic information. To aid the identification of sequences recovered from environmental samples, databases of 16S rRNA sequences have been developed and are continually expanding. Conserved regions of the 16S rRNA gene can be targeted using universal and specific primer sets. Universal or specific primer sets are designed based on the current information from nucleotide databases and require occasional validations as new sequences from the environment are added. Universal primer sets are those that are designed to target most members of the microbial community such as universal primers that target the V3-V4 variable region of the 16S rRNA gene for most bacteria (Berg-Lyons *et al.*, 2010; Wang and Qian, 2009). Whereas specific primers are designed to target specific microbial guilds for example, 16S rRNA gene-specific primers for targeting AOB and NOB or those targeting conserved signature functional genes such as *amoA* for AOP and *nxrB* for NOB (Degrange and Bardin, 1995; Kowalchuk

et al., 1997; Hermansson and Lindgren, 2001; Graham *et al.*, 2007; Pester *et al.*, 2014).

The important technological advances in the field of molecular microbial ecology are shown in Figure 3.1 and the ones relevant to this study related to quantifying and describing microbial community composition are discussed in subsequent sections.

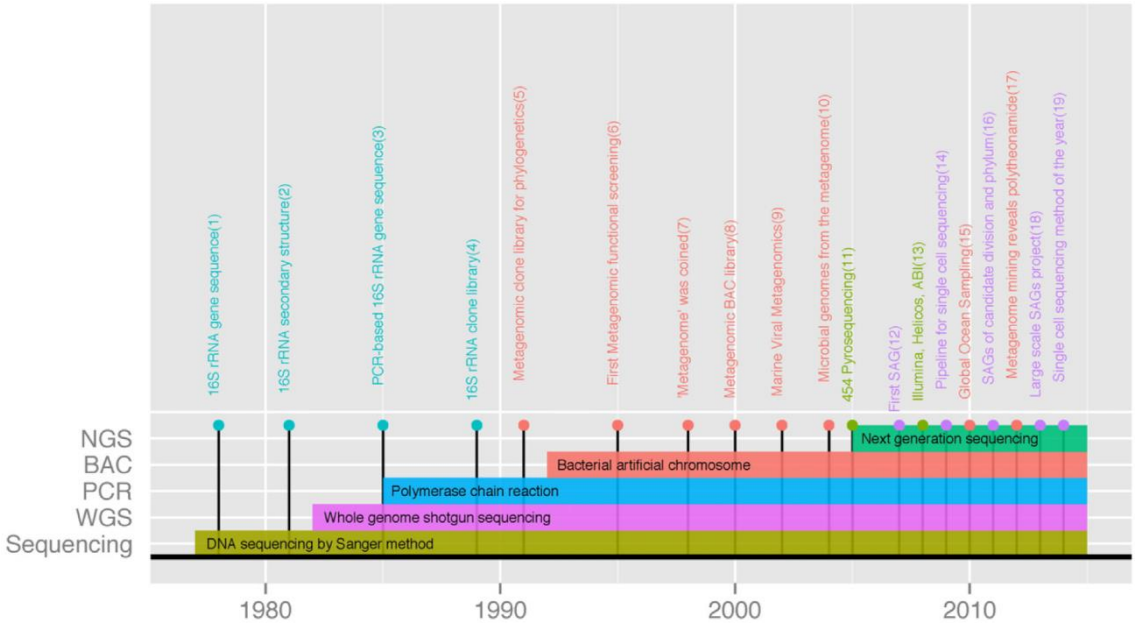

Figure 3.1 Timeline plot of important events in microbial ecology. Events are color labeled as blue for 16S rRNA methods, red for metagenomics, green for next-generation sequencing, and indigo for single-cell genomics (Figure adapted from Rashid and Stingl, 2015).

3.1 PCR and sequencing-based approaches

Some of the most used PCR based techniques for detecting and quantifying microorganisms are multiplex-PCR, qPCR and Digital PCR. Quantitative PCR is a sensitive tool to quantify microorganisms in environmental samples based on quantifying the number of target gene copies present in a sample. Quantitative PCR monitors the amount of PCR product obtained during the exponential phase of the PCR reaction by quantifying a fluorescent reporter. The amount of detected reporter is then correlated with the initial amount of target template allowing the quantification of the target organism. Several studies have shown the applicability of PCR based methods for enumeration of microbial abundance (Pester *et al.*, 2014; Fowler *et al.*, 2018; Ramanathan *et al.*, 2017; Hatzenpichler, 2011; Sipos *et al.*, 2007). However, PCR based

techniques have some drawbacks such as they can produce misleading results due to contamination and/or un-specificity of primers, therefore, sometimes these techniques are difficult to use on complex microbiological samples.

3.2 PCR primer analysis for nitrifiers

3.2.1 Canonical AOB quantification ability of traditional primers

To study bacterial functional groups primer sets may either target a relevant functional gene or if the function is performed by one or a few taxa the phylogenetic 16S rRNA gene can be used. This is the case for AOB which carry a functional *amoA*. To quantify canonical AOB, researchers routinely employ qPCR targeting *amoA* for canonical AOB or canonical AOB specific 16S rRNA gene. However, as these two approaches are not typically compared, it is unclear whether they are equally good at estimating AOB abundance.

In the **paper I**, I attempted this comparison using biomass from prefilter and after filters (RSF) at three full-scale municipal DWTPs in Denmark. RSFs are known to be habited with canonical AOBs as they mediate ammonium removal in these filters along with other (Tatari *et al.*, 2017; Gülay *et al.*, 2016; Wagner, 2017). I evaluated two commonly applied AOB specific primer sets (CTO189a/b/c – RT1r for 16S rRNA gene and amoA1f - amoA2r for *amoA*) for coverage and specificity by *in silico* and experimental analysis on DNA extracted from biomass obtained from prefilters and RSFs (Rotthauwe *et al.*, 1997; Kowalchuk *et al.*, 1997; Hermansson and Lindgren, 2001).

The AOB abundance based on the AOB specific 16S rRNA genes ranged from ca. 3×10^6 to ca. 3×10^8 copies/g drained wet sand across RSF units and waterworks (Figure 3.2). At two (Islevbro and Sjælsø) out of three waterworks, the abundance of *amoA* was consistently approximately 50-fold lower than that of the 16S rRNA gene (Figure 3.2). Even though the number of copies per genome for each gene differs, the 50 fold difference in abundance is too high to be explained by that alone.

The phylogenetic affiliation of clones from two of the three waterworks that showed maximum and minimum deviation amongst the two approaches indicated that most clones from one waterwork (Islevbro) were tentatively assigned to *Nitrosomonas* cluster 6A (*N. oligotropha* lineage), irrespective of the primer set **(paper I, SI)**. At another waterwork (Langerød), in contrast, the identity of the dominant AOB lineage changed depending on the molecular approach: the 16S rRNA gene-based approach identified AOB belonging to cluster 6A as dominant, while the *amoA* based approach only retrieved se-

quences from the *Nitrosomonas europaea/eutropha* lineage (cluster 7, **(paper I, SI)**. While the difference between the compositions retrieved by the two primer sets could also originate from cloning bias, I further explored the role of amplification bias.

The *in silico* analysis for 16S rRNA primer set revealed a high coverage for both forward and reverse primers across all clusters, while analysis of *amoA* primer sets revealed that the average number of mismatches were twice for cluster 6A members compared to other clusters **(paper I, SI**). From *in silico* analysis, I concluded that the *amoA* primer set would be, on an average, less efficient at amplifying cluster 6 sequences than cluster 7 ones when both are present in the microbial community.

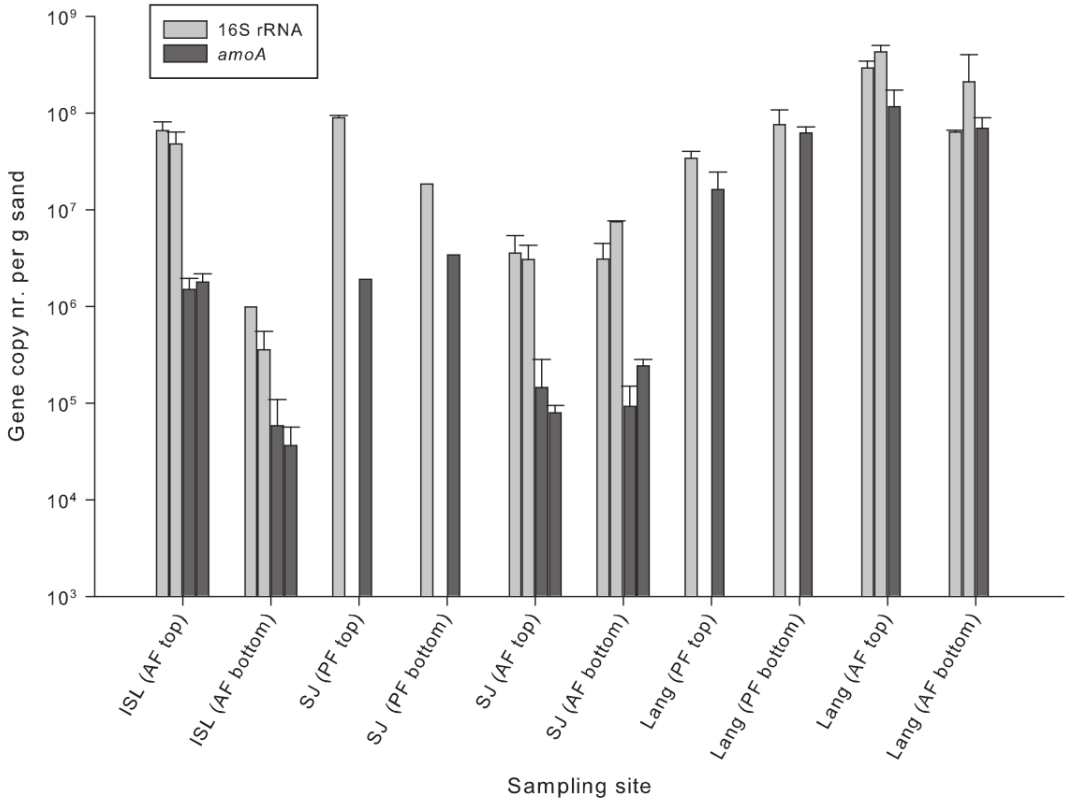

Figure 3.2 QPCR enumeration of AOB in the top (0–5 cm) and bottom (40–50 cm) layers of RSF units (pre-filter (PF) and after-filter (AF)) at three Danish waterworks: Islevbro (ISL), Sjælsø (SJ), and Langerød (Lang; paper I).

Overall I found that the difference in primer pair selectivity, combined with the compositional differences of the AOB guild across sand filter communities, had a major effect on the quantification outcome of both primer sets (Dechesne *et al.*, 2016).

3.2.2 Primer coverage and specificity analysis for nitrifiers

To test the coverage of the traditional primer sets used for detecting key nitrifiers (AOB/AOA/NOB), I first performed an *in silico* coverage analysis. Since this study was done before the discovery of comammox *Nitrospira*, they were not included in this analysis.

AOB

The *in silico* coverage analysis revealed that the traditional, *amoA* AOB primers (amoA1f and amoA2r; Rotthauwe *et al.*, 1997) had overall high amplicon length ~491 bases and low coverage (~43%) compared to their updated version amoA1F-mod and GenAOBR (Meinhardt *et al.*, 2015) which has ~70 bases amplicon length and ~68% coverage (Appendix I; Table 1).

Indeed the updated version of *amoA* AOB primers (amoA1F-mod and GenAOBR) had better coverage compared to the older version (amoA1f and amoA2r), and they are suitable for AOB quantification but, due to their small amplicon length, they are not the best choice for obtaining phylogenetic and compositional information using amplicon sequencing. Therefore, my goal here was to obtain a single primer set that had higher specificity and coverage than the existing *amoA* AOB primers along with an amplicon length (~300 bases) that is suitable for both AOB quantification (qPCR) and phylogenetic plus composition estimation (sequencing).

I designed 6 new primer sets targeting *amoA* of AOB from a combination of 2 forward (amoA-F1: GGGG**H**TT**Y**TACTGGTGGT and amoA-F4: CTGGGG**H**TT**Y**TACTGGTG) and 3 reverse primers (amoA-R1: CTGCAC-**M**GC**N**TTCT**W**CTA, amoA-R2; **R**T**M**TCCATGCT**S**ATGTT**Y** and amoA-R3: **YR**T**M**TCCATGCT**S**ATGTT). These primer sets showed higher coverage (~70%) compared to amoA1f and amoA2r primers (~43%) and amplicon length ranging from 315-365 bases which were better compared to amoA1F-mod and GenAOBR primers (~70 bases; Appendix I; Table 1). To test the specificity of these primers on environmental samples, a gradient PCR was performed. The melting temperature (Tm) in gradient PCR ranged from 42.1 °C to 52.8 °C depending upon Tm of each primer combination. Unfortunately, all these primer pairs were found to be unspecific yielding multiple bands in the gradient PCR run. Further details on primer designing and coverage analysis are in Appendix I.

Overall, I observed that there is no conserved nucleotide region amongst different AOB clade members (*Nitrosomonas* and *Nitrosospira* are too divergent) long enough to design higher coverage *amoA* targeting primers than the ones presently used. Therefore, the existing AOB primer sets are hard to update with higher coverage primers that can be used for both qPCR and amplicon sequencing considering the currently available nucleotide sequence data.

In conclusion, as suggested in **Paper I** and by Meinhardt *et al.*, 2015 when exploring AOB in unknown communities one should consider using multiple primer sets and the abundance estimates should be checked with other methods (e.g., qFISH). When high consensus regions are lacking in the target gene for primer design, other approaches exploiting the potential of tagged highly degenerate primer sets (THDP) can be used. For example, Wang *et al.*, 2017 suggested an improved THDP-PCR protocol for community analysis of methane- and ammonia oxidizers. This approach consists of a copper-containing membrane-bound monooxygenase (CuMMO) gene-specific PCR followed by secondary PCR with a tag on a single primer.

AOA and NOB

The coverage analysis of three main primer sets targeting *amoA* of AOA showed that CrenamoA23f and CrenamoA616r from Tourna *et al.*, 2008 had the highest coverage (~63%; Appendix I; Table 2) and an amplicon length that can be used for quantification, phylogenetic and compositional analysis. Further, attempts were also made to design one set of primer for all NOBs but failed due to high differences amongst NOB genera. Therefore, separate primers for *Nitrospira* (nxrB169f/nxrB638r) and *Nitrobacter* (NxrB-1F/NxrB-1R) genus were considered for qPCR and sequencing (Vanparys *et al.*, 2007; Pester *et al.*, 2014).

3.3 Comparison of universal and targeted amplicon sequencing approaches

Although universal amplicon sequencing approach using 16S rRNA gene is useful for phylogenetic and compositional analysis, it is not the best option for obtaining functional information about the community because it does not provide any enzymatic functional details about the target microorganism (Poretsky *et al.*, 2014; Scholz *et al.*, 2012). Functional information can be typically obtained by functional gene assays such as functional gene metagenomics or microarrays (Scholz *et al.*, 2012).

Two other common drawbacks of 16S rRNA gene universal amplicon sequencing are: First, it provides only limited compositional information on non-dominant guilds because of their low contribution to the total sequence pool. Therefore, for accessing within guild diversity often functional genes targeted sequencing is used. Targeting functional genes is also advantageous because they have a higher rate of evolution compared to the 16S rRNA gene. Therefore, they could provide better phylogenetic resolution. The second issue is related to its ability to provide reliable estimates of the relative abundance for specific guilds in the microbial community. This quantitative information is typically obtained by qPCR based analysis. If 16S rRNA gene-universal amplicon sequencing can provide reliable information about specific microbial guilds in addition to the overview of the full microbial population then in a single analysis one would be able to generate complete information about the abundance and composition of microbial communities in a given sample. I addressed these issues in **paper II,** and the findings are presented below.

In **paper II,** I compared bacterial 16S rRNA gene amplicon sequencing (further referred to as 'universal approach') to a guild-targeted approach (using functional genes *amoA* and *nxrB*) in their abilities to infer relative abundance, diversity, richness, and composition of AOB and NOB. I investigated biomass extracted from three nitrifying and one anammox wastewater treatment bioreactors and four biological RSFs used for potable water production. At both types of plants, nitrifiers are known to range from 1% to 10% in abundance relative to the whole microbial community (Tatari *et al.*, 2017; Wagner *et al.*, 2002; Gülay *et al.*, 2016)

Relative abundance

The relative abundances obtained from the universal approach for AOB were always lower than the 16S rRNA gene- qPCR (Figure 3.4 A). For *Nitrospira*, compared to the universal approach, the *nxrB* qPCR yielded slightly higher relative abundance estimates in all WWTP but lower for all DWTP (Figure 3.4 A). For both the guilds, despite the inconsistency observed between the targeted and the universal approaches, the universal approach based estimates were always within ~1.2 orders of magnitude of the targeted approaches. Major axis regression analysis of the estimates from both approaches suggested that for AOB there was a direct proportionality between the approaches and *Nitrospira* quantification based on universal amplicon sequencing increased more rapidly than *nxrB* qPCR with *Nitrospira* relative abundance (Figure 3.4 B). Overall, I showed that the universal approach certainly provides useful quantitative information for AOB and *Nitrospira* but the highlighted biases should be considered when comparing estimates from universal and targeted approaches for these guilds.

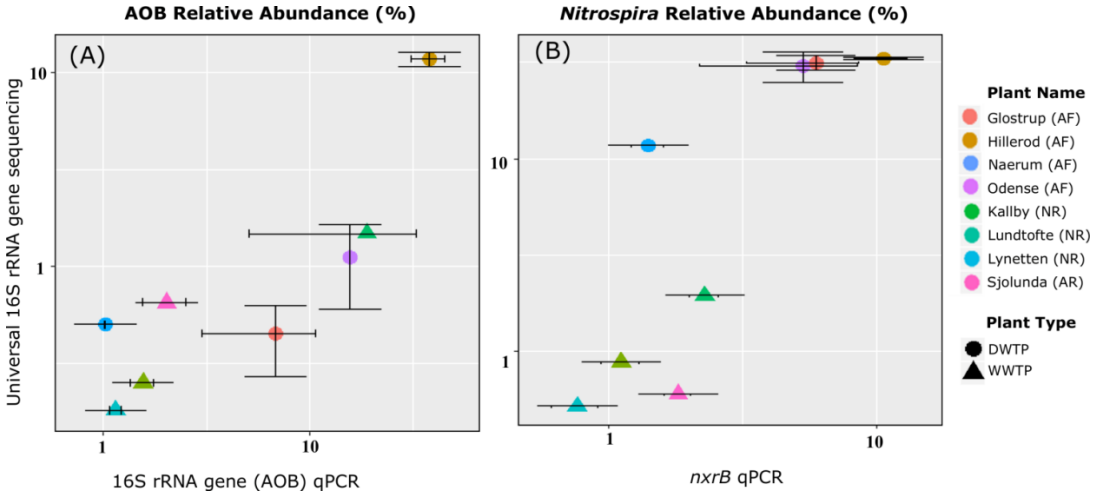

Figure 3.4 Comparison of the relative abundance of AOB (A) and Nitrospira (B) based on the universal (y-axis) and targeted (x-axis) approach. Universal 16S rRNA gene- amplicon sequencing was compared with qPCR (target: 16S rRNA gene for AOB in panel A and nxrB for Nitrospira in panel B) for four after filters (AF) from DWTP, three nitrifying reactors (NR) and one anammox reactor from WWTP. The error bars represent the standard deviation for each plant. (paper II)

Alpha diversity

While comparing alpha diversity, for both guilds and irrespective of the metric used, the targeted approach always resulted in higher alpha diversity val-

ues compared to the universal approach (Figure 3.5). I attributed this observation to three things 1) the functional gene libraries contained approximately ~24 fold (AOB) and ~6 fold (*Nitrospira*) more sequences than retrieved from the universal sequencing, 2) diversity estimates can be highly reliant on the depth of sequencing (Caporaso et al., 2011; Gihring et al., 2012; Smith and Peay, 2014), and 3) the rate of evolution of functional genes (*amoA*, *nxrB*, *rpoB* etc.) is known to be higher than that of ribosomal genes (16S rRNA gene), which can make them more phylogenetically resolutive (Pester *et al.*, 2014, 2012).

Phylogenetic diversity for AOB and observed richness for *Nitrospira* were the only diversity indices with a significantly positive correlation between both approaches (p-value 0.009; Figure 3.5 C and p-value 0.01; Figure 3.5 E).

Because of the frequent inconsistency between targeted and universal approach, I suggest that the guild-targeted approaches should be preferred to estimate the diversity of AOB and *Nitrospira*.

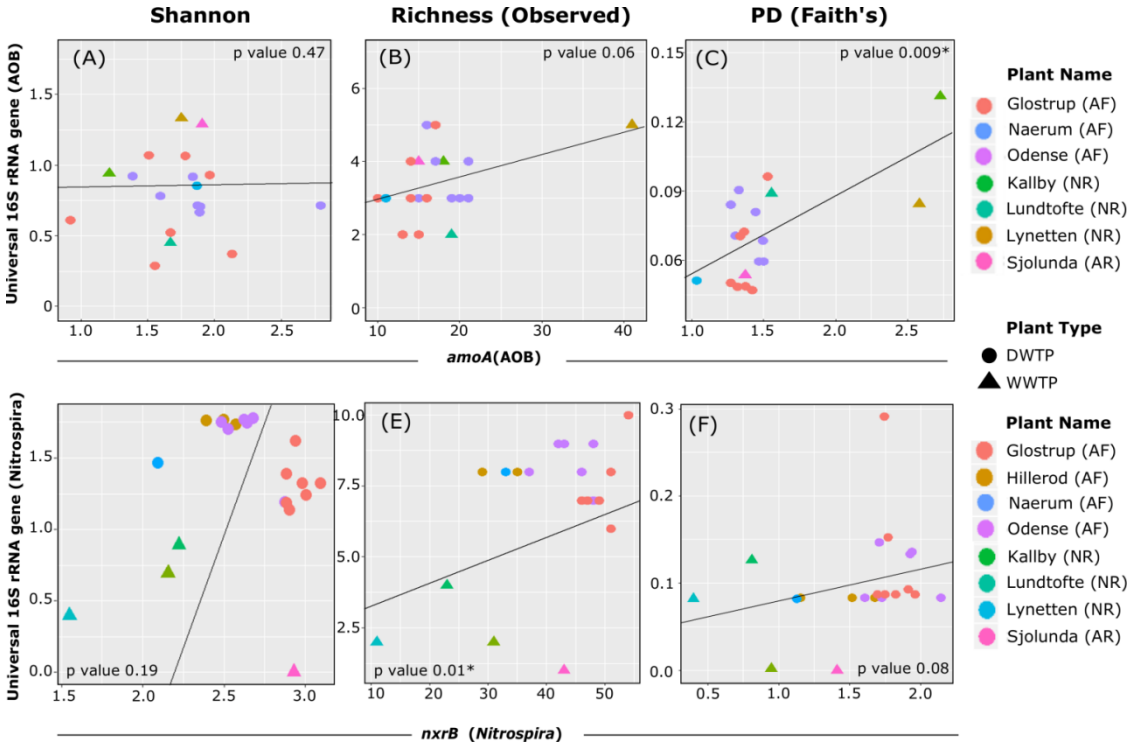

Figure 3.5 Comparison of alpha diversity (Shannon; A and D), observed richness (B and E) and phylogenetic diversity (Faith's PD; C and F) of AOB and *Nitrospira* based on universal (16S rRNA gene) and targeted (*amoA* -top and *nxrB* -bottom) approach. The black line is the major axis regression (Paper II).

Composition

While estimating AOB composition, I found that both approaches identified the same dominant cluster (cluster 6) in five out of seven sites. Only for one plant (Sjolunda) did both approaches provide the same picture of the cluster-level composition. For all DWTP and one WWTP, the universal approach only showed the presence of cluster 6; whereas the targeted approach indicated the presence of additional clusters: cluster 7 in all these plants, plus cluster 0, 2, 3 and 8 in one WWTP (Kallby; (Figure 3.6). These observations relate with the low relative abundance of AOB observed earlier by universal 16S rRNA gene sequencing (Figure 3.4) as clusters other than cluster 6 were largely unmapped for four out of seven sites by the universal approach due to their very low relative fraction to the total community. Analysis at the subcluster taxonomic resolution revealed that all samples where cluster 6 was predominant consisted mainly of subcluster 6A members (represented by *Nitrosomonas oligotropha* and *Nitrosomonas* sp. ls79A3; Figure 3.7). As I showed in the **paper I**, *amoA* AOB based primers (from Rotthauwe *et al.*, 1997), underestimate AOB abundance when subcluster 6A members are dominant.

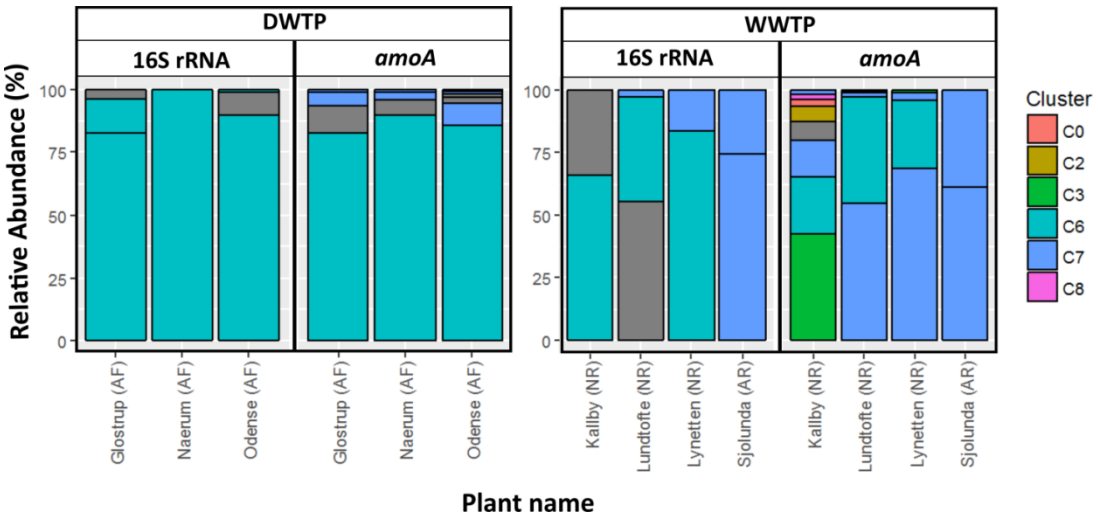

Figure 3.6 AOB composition obtained from the universal (16S rRNA gene amplicon sequencing) and targeted (*amoA* amplicon sequencing) approaches for the two types of water treatment plants (paper II).

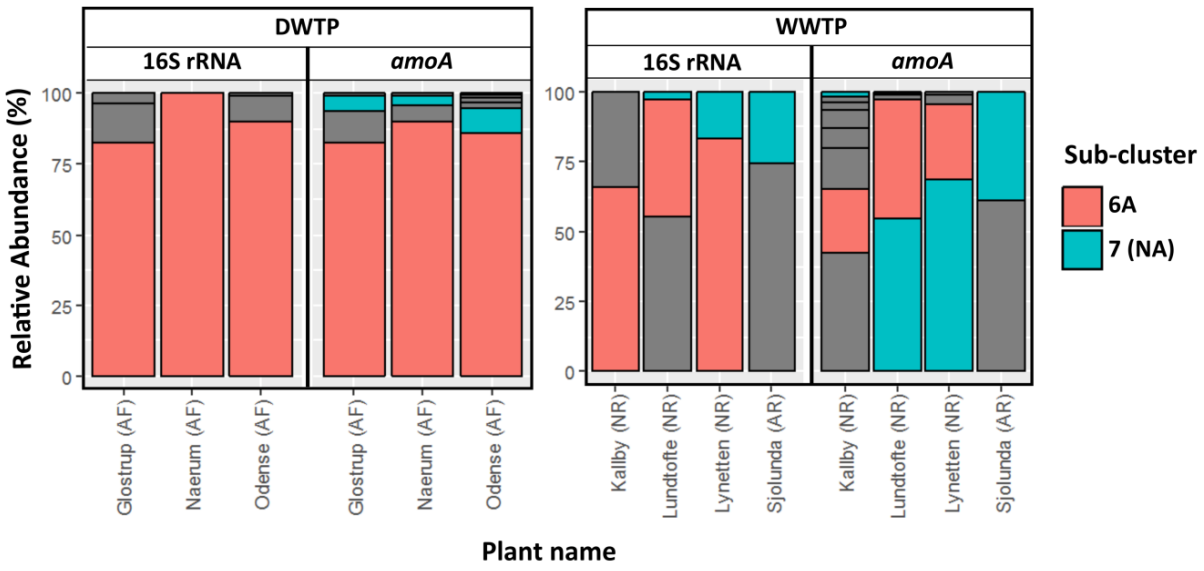

Figure 3.7 AOB composition obtained from the universal (16S rRNA gene sequencing) and targeted (*amoA* sequencing) approach (paper II).

Nitrospira composition inferred from both approaches was largely similar for all sites (Figure 3.8). Both approaches identified strong compositional differences between DWTP and WWTP, the former being dominated by lineage 2 and the later by lineage 1 (Figure 3.8).

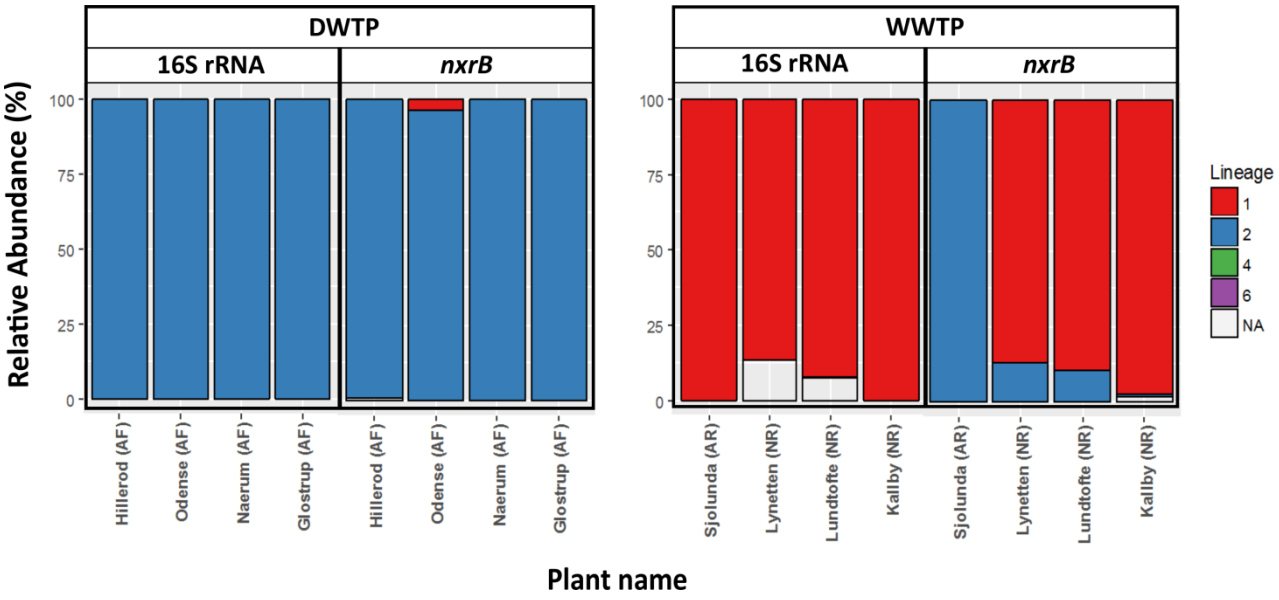

Figure 3.8 *Nitrospira* composition obtained from the universal (16S rRNA gene sequencing) and targeted (*nxrB* sequencing) approach. (Paper II).

Overall, by the composition analysis, I showed that the universal approach was successful in identifying the dominant clusters/lineages within AOB and *Nitrospira* for most plants. Therefore, I concluded that, when the focus is on dominant guild members, the universal approach can be preferred for estimating the composition of these guilds.

Composition based sample clustering

In the K-medoids sample clustering analysis based on AOB and *Nitrospira* composition, I found that for AOB, the universal approach separated the samples into seven clusters corresponding to the seven plants (misplacing only one replicate; Figure 3.9 B). On the other hand, *amoA* incorrectly distributed the DWTP samples into four clusters (cluster 4, 5, 6 and 7), with one cluster (cluster 5) comprising samples from all three plants (Figure 3.9 A). Also, samples from two WWTP were placed in the same cluster (cluster 2; figure 3.8 A) whereas; they were separated by the 16S rRNA gene (cluster 2 and 4; Figure 3.9 B). Therefore, it was evident that the targeted approach did not provide better clustering than the universal approach for AOB. For *Nitrospira*, the universal and targeted approach clustered samples similarly with some minor variations (Figure 3.9 C and D) and overall gave similar clustering outputs.

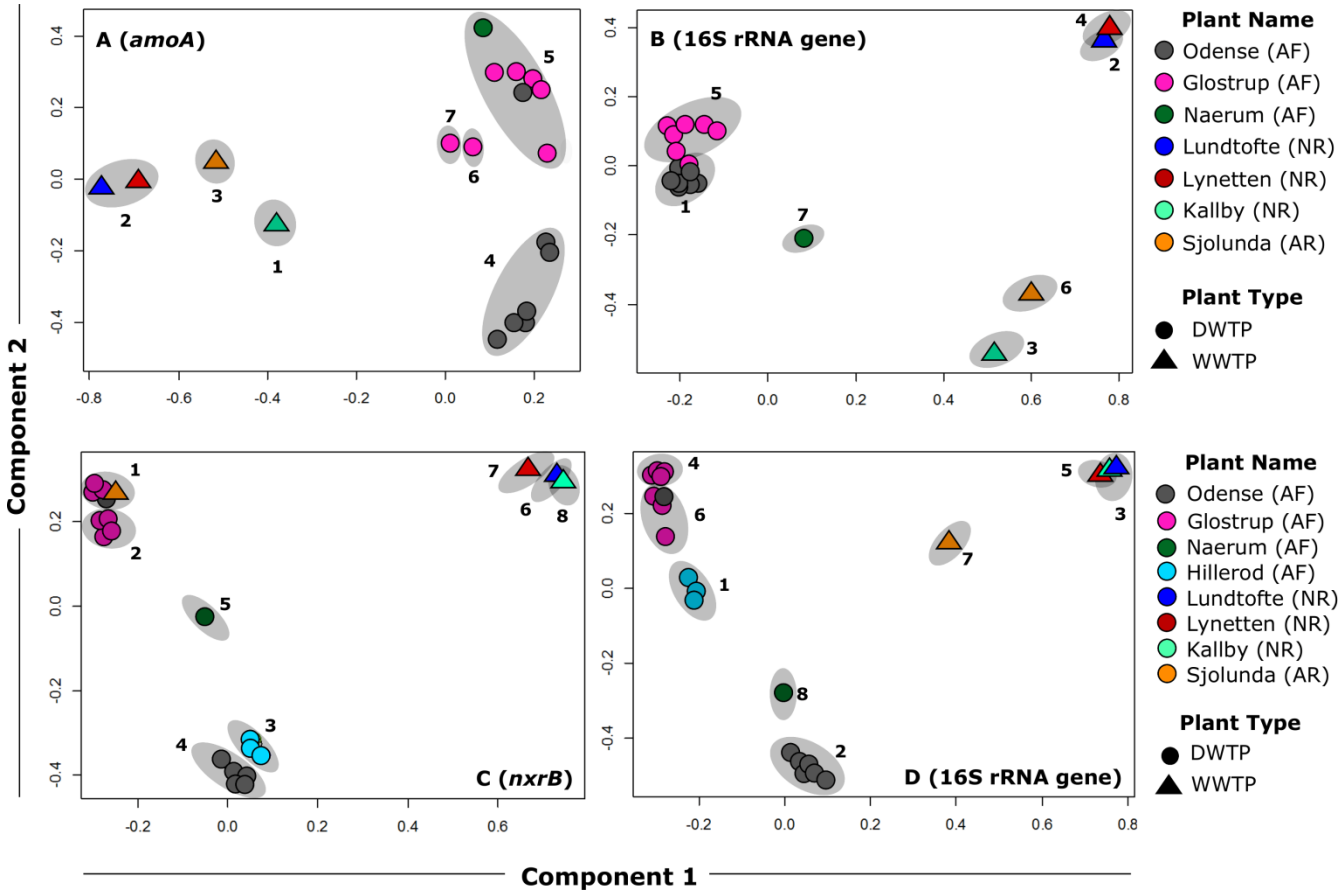

Figure 3.9 Clustering analysis of samples after PCoA ordination of their compositions of AOB (panels A & B) and *Nitrospira* (panels C & D) obtained from the targeted and universal approach. Shaded ellipses followed by cluster number represents the clusters. Each solid circle and triangle is a sample, with color coding according to the plant. (Paper II).

In conclusion, the universal amplicon sequencing provided accurate estimates of nitrifier composition and clustered the samples based on these compositions consistently with sample origin. It also provided estimates of the relative abundance of the guilds correlated with those obtained from the targeted approaches within ~1.2 orders of magnitude of them. But the relative abundance estimates had a measurable bias that should be considered when comparing estimates from both approaches. Lastly, the diversity and richness estimations using the universal approach were likely limited by the sequencing depth; therefore, we suggest preferring targeted approaches for assessing nitrifiers diversity and richness or using sequencing depth larger than those currently typically practiced.

4 Comparison of nitrifier composition between selected DWTP and WWTP

Many studies have described the whole microbial community and nitrifiers composition in DWTPs and WWTPs but rarely these systems are compared (Holinger *et al.*, 2014; Berry *et al.*, 2006; Pinto *et al.*, 2012; Chao *et al.*, 2013; Xu *et al.*, 2018; Vaz-Moreira *et al.*, 2013; Meerbergen *et al.*, 2017). As described earlier, drinking and wastewater treatment plants expose nitrifiers to a broad range of resource concentrations and environmental conditions. This makes them interesting for studying the relative role of deterministic (e.g., selection) and stochastic (e.g., immigration) processes on nitrifier community assembly in these systems. In this substudy, I compared the composition of nitrifiers and total microbial community composition amongst total eight DWTP and WWTP.

The principal coordinate analysis (PCoA) based on the 16S rRNA gene total bacterial community composition analyzed from influent, and reactor samples at different DWTP and WWTP revealed a clear difference of bacterial communities between the two types of plants (Figure 4.1). For the WWTPs, the reactor communities were similar to that of the influent as these samples clustered together, whereas the influent and RSF community at DWTPs were different in composition indicated by separate clustering of the two types of samples. These observations indicate a larger role of immigration from influent to the reactor community at WWTPs and of selection in the RSF community at DWTPs. An interplant difference in the total community was also observed for DWTPs (Figure 4.1). The influent community at DWTPs seemed closely related to the WWTPs community as the influent samples from all DWTPs clustered with the WWTPs samples but these samples were separated at the third axis (Figure 4.1). To see if these patterns were also reflected at the nitrifier guild level, I further analyzed the AOB and *Nitrospira* composition in these systems.

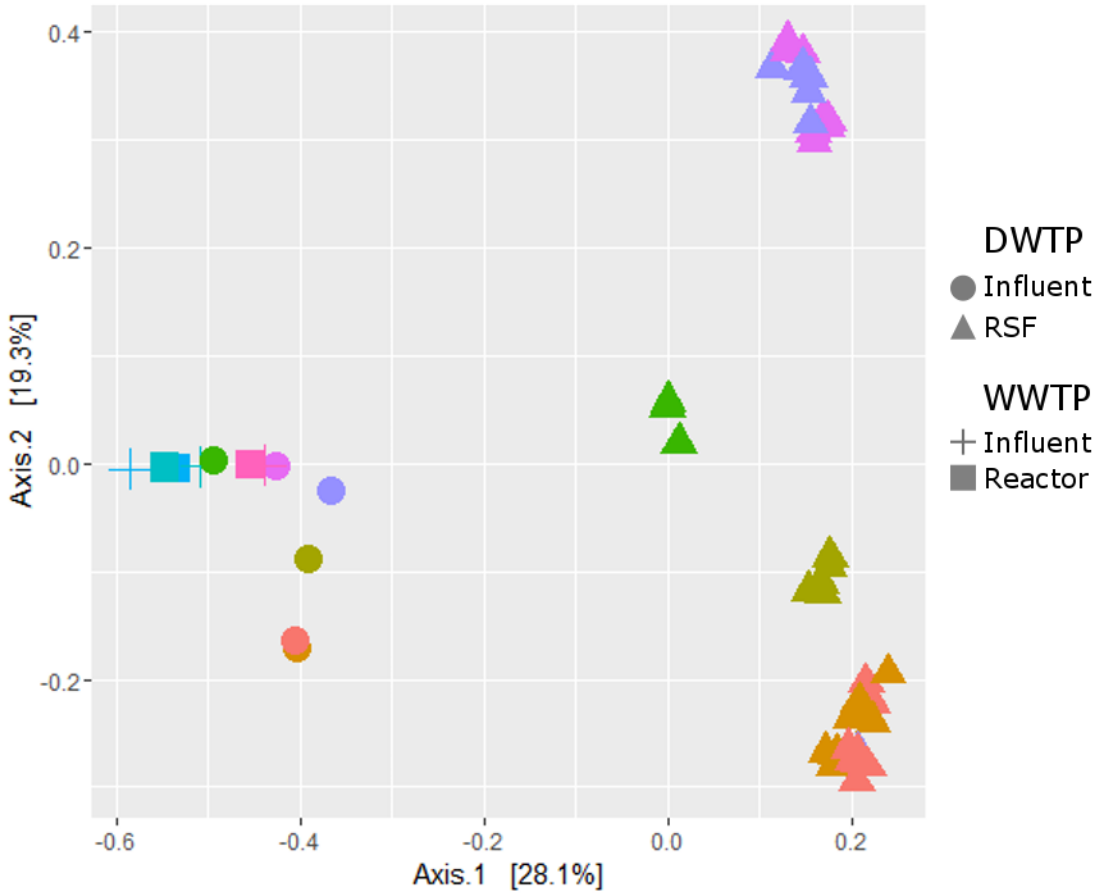

Figure 4.1 PCoA based on the universal 16S rRNA gene total bacteria composition for DWTP and WWTP. The colors represent DWTP and WWTP.

The *amoA* AOB composition comparison amongst DWTP and WWTP showed tree main things 1) Cluster 0, 2 and 3 (*Nitrosospira*) members were specific to WWTP, 2) Cluster 7 and 8 (*Nitrosomonas*) members mainly belonged to WWTPs and 3) Cluster 6 (*Nitrosomonas*) members were evenly distributed across different DWTPs (Figure 4.2). These observations indicate the selection of different AOB types in DWTPs and WWTPs depending upon the available resource and environmental conditions along with immigration for WWTPs (table 4.1). As described before, cluster 6 members prefer oligotrophic environments which in this case were representative of DWTPs, whereas, cluster 7 members can tolerate a much higher level of resource conditions typical at WWTPs (table 4.1). Further, the *nxrB Nitrospira* composition comparison showed that lineage 1 members were almost exclusively associated with WWTPs and lineage 2 members were mostly found in DWTPs (Figure 4.3). Members of lineage 3 were exclusive to two DWTPs.

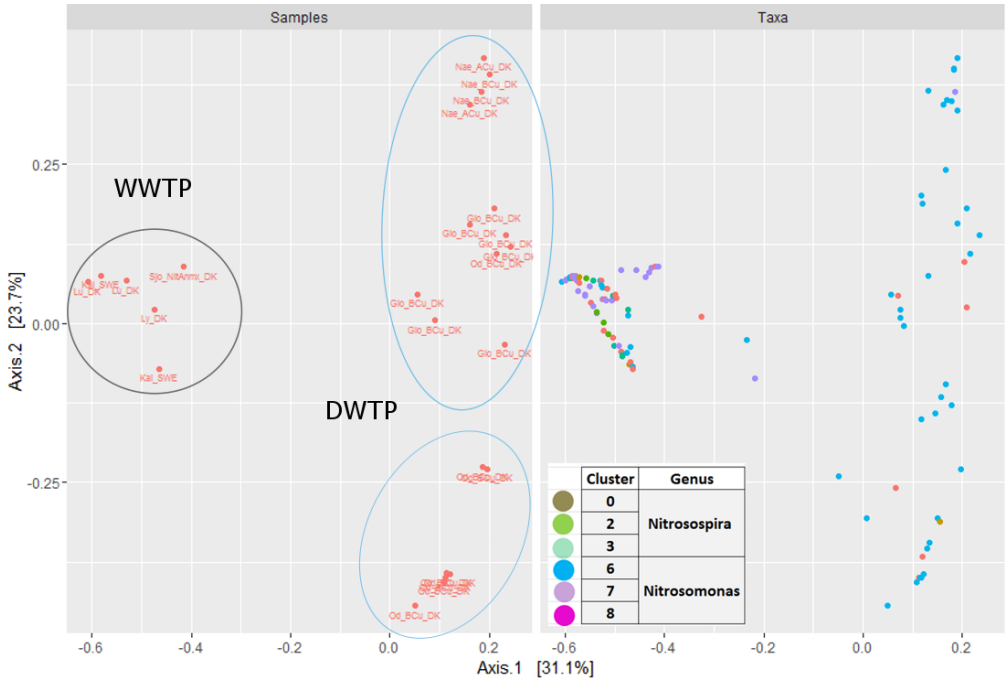

Figure 4.2 PCoA biplot based on the *amoA* AOB composition in the analyzed DWTP and WWTP. The left panel shows the clustering of different DWTP, and WWTP samples and the right panel shows the AOB unique sequence variants observed in different samples.

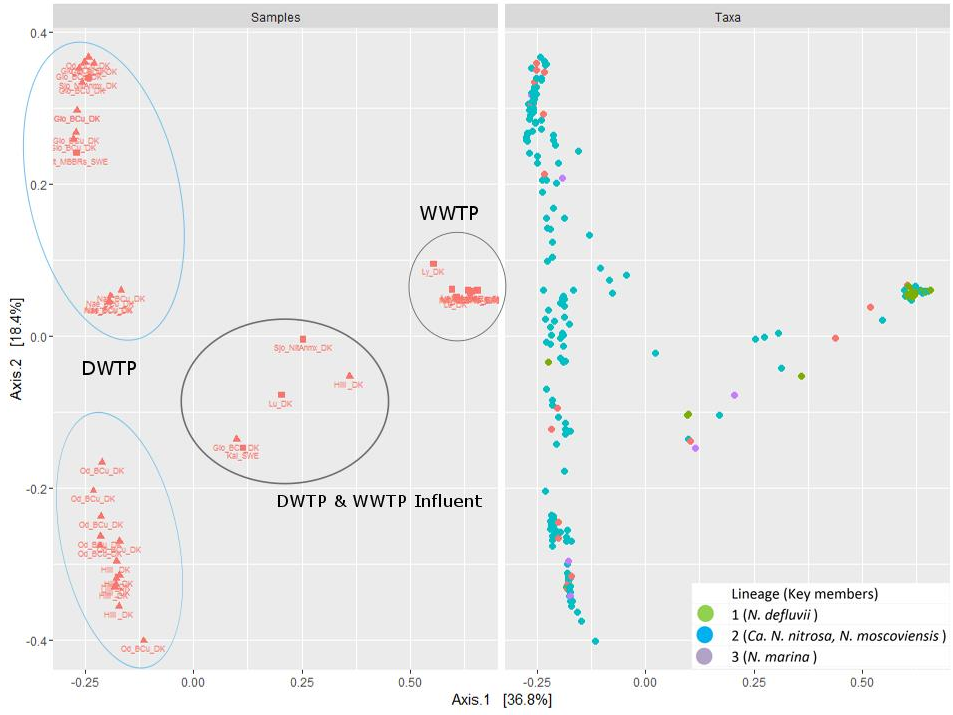

Figure 4.3 PCoA biplot based on the *nxrB Nitrospira* composition in the analyzed DWTP and WWTP. Other details are similar to figure 4.2.

All potential nutrient and micronutrients differ strongly between DWTP and WWTP (table 4.1). Therefore, the overall differences in nitrifiers between DWTP and WWTP mainly come from ammonium or nitrite concentration differences between these plants and/or due to concentrations of other compounds. For example, some metals such as copper (Cu) and molybdenum (Mo) which are essential cofactors for nitrifiers are in very low concentrations in DWTP which could cause selection of certain nitrifier types in DWTP (table 4.1; further discussed in chapter 6).

Table 4.1 Minimum and maximum range of different parameters measured in the influent of the sampled DWTP and WWTP.

Parameter*	DWTP		WWTP	
	Min	Max	Min	Max
Ammonium	0.36	0.53	26.11	31.87
Nitrite	0.001	0.006	1.51	10.93
Nitrate	0.05	0.05	0.05	23.06
Phosphate	0.01	0.07	1.40	6.49
Chloride	11.60	57.30	81.00	602.00
Sulphate	1.63	14.84	5.13	32.80
Cu	0.00007	0.00049	0.002	0.021
Mo	0.00008	0.00207	0.001	0.012
P	0.004	0.088	4.25	19.65
Fe	0.07	1.34	0.07	0.38
K	4.36	5.36	18.28	100.17
Mg	13.11	40.72	4.46	41.57
Mn	0.03	0.31	0.01	0.08
Na	36.80	42.96	69.07	348.70
Zn	0.001	0.003	0.02	0.06
Co	0.00001	0.00003	0.000	0.006
Ni	0.00006	0.00032	0.002	0.008
Ca	78.66	113.08	41.26	149.63
Temp. (°C)	9.50	11.50	14.60	16.60
pH	7.34	7.80	7.03	8.32
TAL (meqv/l)	5.14	7.24	4.67	6.41
DO	8.92	11.04	1.60	7.70
NVOC	1.53	3.05	38.03	95.52

*all readings except temperature, pH and total alkalinity (TAL) are in mg/l.
NVOC = Nonvolatile organic carbon.

5 Community ecology approaches

Several model-based and statistical approaches are available to observe the community patterns and infer which ecological processes are responsible for community assembly. These approaches can be broadly divided into two major categories as multivariate statistics-based analysis and neutral theory-based process models (Jizhong Zhou, 2017). The neutral model-based approaches can be used to study community patterns and infer the role of stochastic processes (immigration and drift) in community assembly. The role of deterministic process in community assembly (such as selection) can be estimated either by observing its consistent difference from stochastic processes (e.g., immigration) using neutral model and/or by relating it with the environmental factors (i.e., the niche) by multivariate statistics-based approaches. The below sections briefly describe some of the key community ecology approaches while keeping the focus on selection estimation.

5.1 Multivariate statistics-based analysis

Numerous multivariate statistical approaches have been developed for examining and estimating the relative role of selection and dispersal limitation (Morrison-Whittle and Goddard, 2015; Peres-Neto *et al.*, 2012; Or *et al.*, 1996; Borcard and Legendre, 2002). Typically the multivariate statistics can be applied in two ways. First, direct comparison of community composition differences between or within different environments by using permutational multivariate analysis of variance (PERMANOVA or Adonis), or analysis of multivariate dispersions (PERMDISP) and analysis of similarities can be used (Anderson *et al.*, 2006; Clarke, 1993; Anderson, 2001). Second, by correlating community composition and environmental variables using a Mantel test, multiple regression on (dis)similarity matrices (MRM), redundancy analysis (RDA) or canonical correspondence analysis (CCA; for examples, see references Anthony et al., 2015; Dumbrell et al., 2010; Ordonez and Svenning, 2015; Vannette and Fukami, 2014). Although multivariate approaches are widely used, in general, they always face the problem of unmeasured environmental factors because it is practically impossible to precisely measure all environmental variables (Jizhong Zhou, 2017).

5.2 Neutral theory-based process models

There are many neutral theory-based process models that are used to test the mechanisms of species coexistence and biodiversity maintenance in ecological communities. These models offer a simple mechanistic explanation of the observed SADs (Matthews and Whittaker, 2014). The most commonly used model is Hubbell's neutral community assembly model. This model states that the local community dynamics is saturated where each death is immediately replaced by a new individual and speciation/immigration enables diversity maintenance in the metacommunity. While, the local communities interact with the metacommunity through the process of migration (Hubbell, 2001). This model has three main parameters a) abundance of the local community (J), b) the rate of immigration/dispersal (m), and c) the fundamental diversity number (θ) which is dependent on the abundance of the metacommunity, (J_M) and the rate of speciation (v; Figure 5.1).

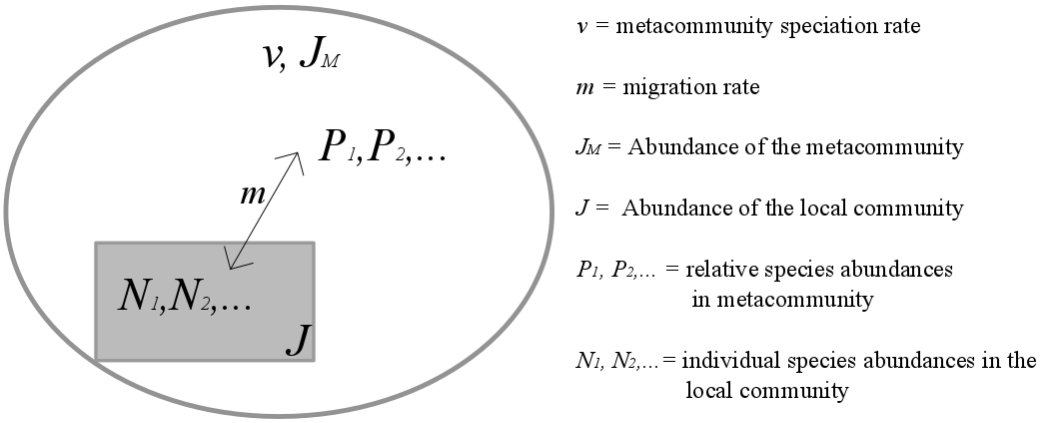

Figure 5.1 Graphical representations of Hubbell's neutral community assembly model (Hubbell, 2001; Chave, 2004).

Estimating the above mentioned neutral model parameters from ecological data is theoretically possible, but practically it is problematic. For example, estimating the abundance of the metacommunity is difficult and the rates of immigration and speciation are nearly impossible to measure directly (Matthews and Whittaker, 2014; Gotelli and McGill, 2006). Therefore, commonly the neutral model parameters are only indirectly estimated by fitting the model to observed community structure data (Jizhong Zhou, 2017).

Since the focus of this Ph.D. study was on microorganisms, therefore, I have used the *'near neutral model for prokaryotes'* developed by Sloan et al. 2006 that fits the observed species abundance-frequency relationship with a beta

distribution derived from the neutral model. Basically the *'near neutral model for prokaryotes'* (hereon referred to as NCM) predicts the frequencies with which each taxon should occur in the local community (in this Ph.D. study it is the RSF community) based on their abundance in the metacommunity (i.e., influent water) assuming only neutral processes such as random births and deaths and stochastic immigration from the metacommunity. NCM also provides information about the selected and counter selected taxa in the community. For example, if the taxa are found at higher detection frequencies (i.e., over-represented) in the local community than predicted by the model based on their abundance in the metacommunity, then they would be referred to as selected or positively selected in the community. Similarly, if the taxa are found at lower detection frequencies (i.e., under-represented), then they would be referred as negatively selected. This approach has been used to estimate the contribution of selection in microbial community assembly (Vignola *et al.*, 2018; Kinnunen *et al.*, 2017; Morris *et al.*, 2013; **paper III**). For ease of understanding, the NCM elements mentioned above are graphically represented in figure 5.2.

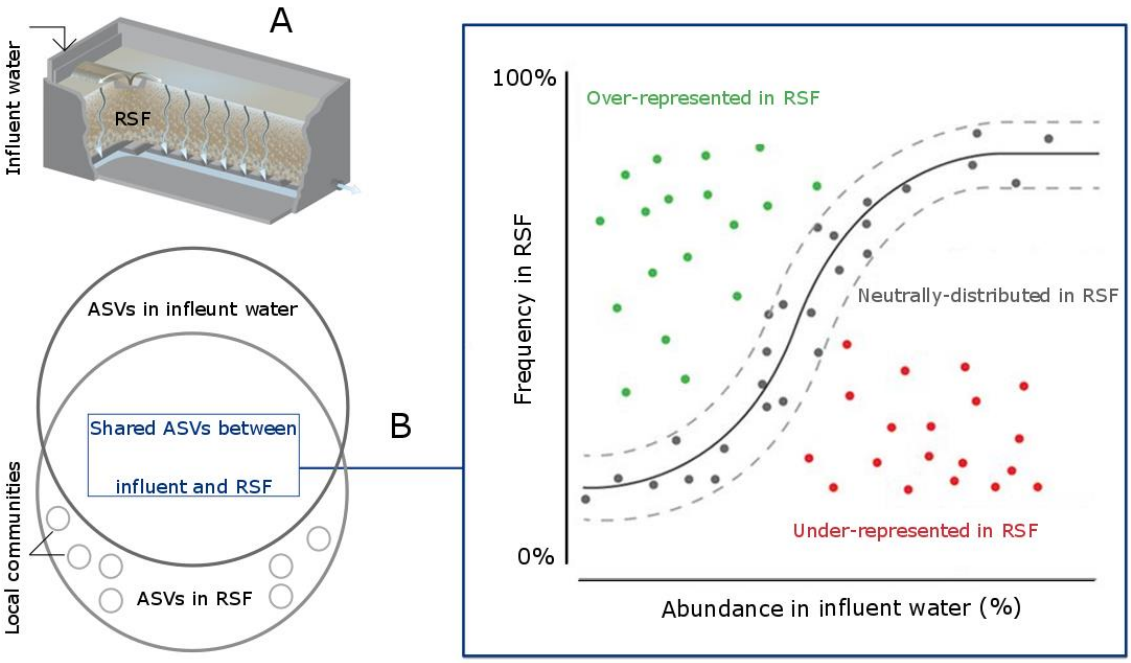

Figure 5.2 (A) Graphical representation of a typical granular media rapid sand filter (RSF) including the flow of influent (aerated raw ground) water. (B) Implementation of a neutral model for analysis of DWTP microbial community structure using the shared amplicon sequence variants (ASVs) between the influent water and RSF. ASVs in multiple RSF communities are represented as empty grey circles (bottom left). The solid black line represents the best fit of the neutral model generated using a probability distribution. The 95% confidence interval is represented by the dashed lines

around the best fit. ASVs represented as grey dots are likely present in RSF because of neutral processes such as dispersal/immigration from the influent water and ecological drift within the RSF. ASVs deviating from the neutral model are represented either as over-represented/positively-selected (green dots) or under-represented/negatively-selected (red dots) in the RSF.

Metacommunity estimation in NCM relies on having multiple samples from the site of interest such as water, sand, sludge samples etc. Either multiple samples can be collected at a single time point or single samples at several time points. In either case, the taxa abundance in the metacommunity is at the base of NCM which makes the metacommunity estimation of central importance. The metacommunity estimation can be done in two different ways, and I have applied both in this study. The first method implies a direct description of the metacommunity by sampling the actual source (influent water) metacommunity (Figure 5.2). But, observations of the actual source metacommunity are tricky if the influent is constantly changing (for example in DWTP). Therefore, this approach has a risk of providing only a snapshot of the source metacommunity especially in this case where the source is constant flowing influent water community. The second method is advantageous in cases where the source metacommunity is prone to perturbations. Here the source metacommunity is estimated by averaging the composition of replicate local communities (from here on called as 'averaged source metacommunity') assembled from the same metacommunity.

Although NCM is a powerful statistical model, this implementation of NCM comes with a limitation that it primarily predicts taxon detection frequencies and does not include the information about their actual abundance in the local communities. For example, if taxa have high abundance in the metacommunity they will be predicted as highly frequent in the local community by NCM, but their abundance might be very low in the local community. In this case these taxa in reality would be negatively selected in the local community but will fit neutrality according to NCM because NCM does not take into account the actual abundance of taxa in the local community. In the context of detection of positively or negatively selected taxa, this would be a false negative for the NCM. To overcome this limitation I have used differential abundance based statistical analysis that compares the abundance of each taxon between the source and the local community. Differential abundance based approaches statistically test whether, for a given taxon, an observed difference in abundance between the source and the local community is significant, i.e. whether

it is greater than expected just by chance (Paulson *et al.*, 2013; Sohn *et al.*, 2015; Anders and Huber, 2010). I utilized this type of analysis to compare source and local communities in DWTP, assuming that taxa under positive selection would have a higher abundance in the local community (**paper III and IV**). I acknowledge that this type of differential abundance analysis for assessing selected members of the community also has some drawbacks which are discussed later in this chapter and **paper III**.

5.3 Phylogenetic composition based analysis

The phylogenetic composition can also be utilized for detecting selection. For example, mean nearest taxon distance (MNTD) and the nearest taxon index (NTI) can give information about how phylogenetically close the taxa are in the community. If the community has highly related taxa, then it indicates that environmental selection has overcome stochastic assembly (Stegen *et al.*, 2012; Vignola *et al.*, 2018). Although this type of analysis is widely used, it only utilizes the phylogenetic composition information and similar to NCM does not include information about the actual abundance of the taxa (Swenson, 2011; Moroenyane *et al.*, 2016; Lin *et al.*, 2012). Although newer methods exist (the so-called 'abundance informed ecophylogenetic matrices') that provides an option for including the abundance information along with the phylogenetic composition these methods also do not clearly state how one can identify which taxa are selected (Cadotte *et al.*, 2010; Swenson, 2011).

In this Ph.D. study, selection and the methods used to estimate the effect of selection on DWTP microbial communities are approached on two different lines. Firstly the raw/influent water community was compared to the RSF community without inducing any deterministic abiotic changes on the microbial communities (paper III and Section 5.2). This approach was used for analyzing the extent of selection on RSF microbial communities versus the effect of immigration by the influent water communities. Also, it served as a ground for comparing NCM and differential abundance based approaches in their ability to estimate the effect of selection on the DWTP nitrifying community. In the second approach, the effect of releasing single resource limitation was studied on the RSF community composition and is further discussed in the light of the competition for limiting resources (paper IV and Chapter 6). In this approach, only the RSF microbial communities were analyzed and compared.

5.4 Identifying selection in nitrifying communities

5.4.1 Selection of Nitrospira in RSF microbial community

Several recent studies have shown high abundances of *Nitrospira* (~10-40% in relative abundance) and mainly of complete ammonia-oxidizing groups of *Nitrospira* (Comammox) in the RSF (Fowler et al., 2018; Diwan et al., 2018; Tatari *et al.*, 2017). Gülay *et al.*, 2016 in a survey of Danish drinking water treatment plants (DWTP) measured community composition in raw water and RSF and observed a drastic increase in *Nitrospira* abundance from raw water to RSF (Figure 5.3).

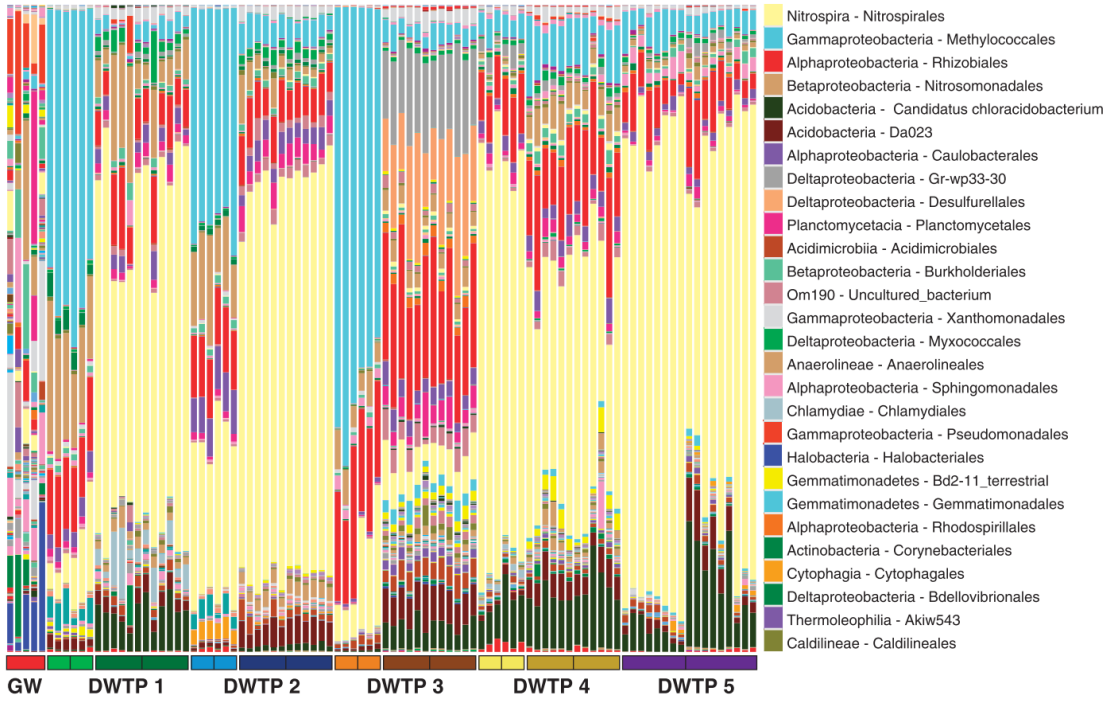

Figure 5.3 Relative abundance and order-level taxonomic classification of 16S rRNA amplicons in raw groundwater and RSF of five DWTPs in Denmark (Figure adapted from Gülay *et al.*, 2016).

In sub-studies of this Ph.D. study, I confirmed that *Nitrospira* (canonical and comammox) was the most abundant genus amongst all nitrifiers in the analyzed RSF **(paper II, III and IV)**. When influent water community was compared to RSF community at two full-scale municipal DWTPs at two different time points, *Nitrospira* increased drastically between influent and RSF, canonical AOB were in low abundance compared to *Nitrospira*, and other NOB and AOA were below detection limit (Figure 5.4). These observations indicated positive selection of *Nitrospira* (and *Nitrosomonas*) over other non-

nitrifying bacteria. Further, if selection is one of the driving forces then which *Nitrospira* types are positively selected?

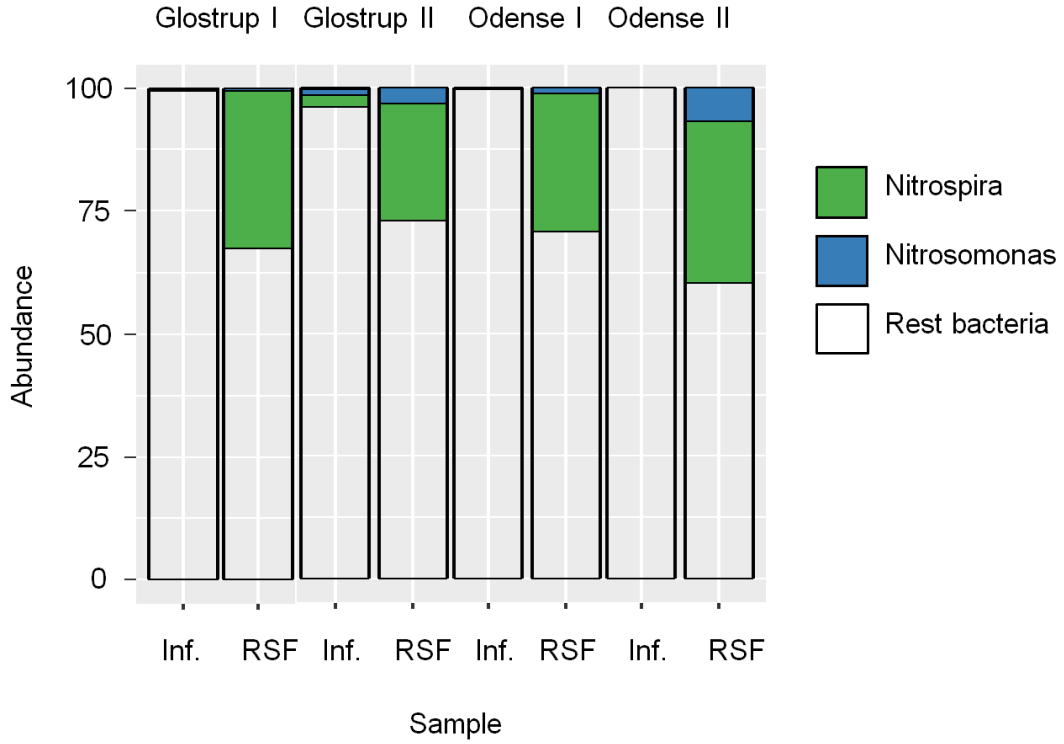

Figure 5.4 Relative abundance of nitrifier genera to the rest of the influent water and RSF microbial community. Data from two DWTPs (Glostrup and Odense) from two different time points (suffixed as I and II) based on 16S rRNA gene sequencing.

To answer this question, I used previously described NCM and differential abundance based statistical approaches. As the focus here was on a specific microbial guild, i.e. *Nitrospira* and assembly within *Nitrospira* therefore, its composition was estimated using *nxrB* targeted amplicon sequencing. The *Nitrospira* community compositional differences between influent and RSF samples were clearly identified by the NMDS analysis (Figure 5.5). The *Nitrospira* composition was significantly different ($p = 0.001$) between plants and between influent and RSF samples for both DWTP ($p = 0.001$; Figure 5.5).

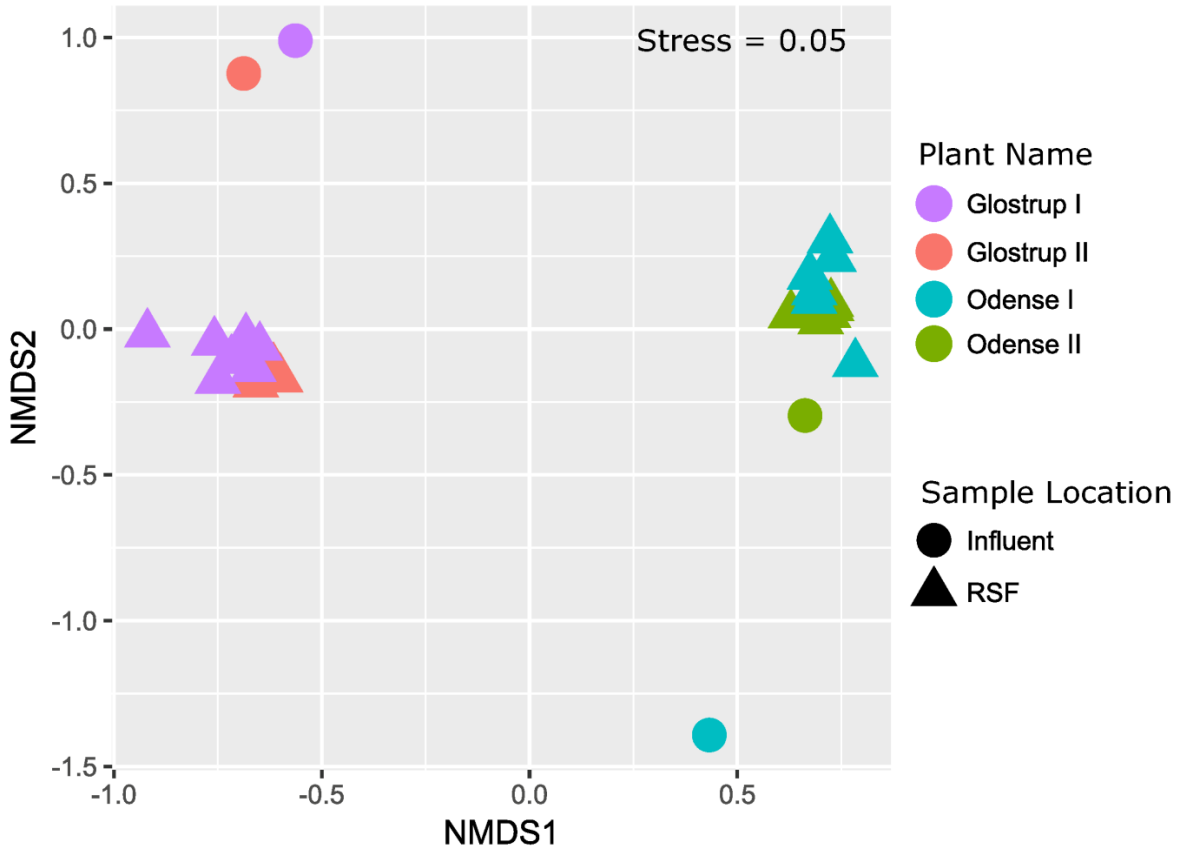

Figure 5.5 NMDS analysis of *Nitrospira* composition from *nxrB* amplicon sequencing (paper III).

The *nxrB Nitrospira* amplicon sequencing revealed that all (but one) samples were dominated by Lineage 2 *Nitrospira* and more than 80% of the RSF sequences assigned to *Nitrospira* lineage 2 corresponded to putative clade A or clade B comammox *Nitrospira* (Figure 5.6). Similar observations have been reported in a recent survey of multiple Danish DWTPs by Fowler et al. 2017. Between influent water and RSF, both DWTPs showed an overall increase in comammox clade A and B and a decrease in other lineage 2 members, lineage 1 members and unidentified *Nitrospira* (Figure 5.6). Further, comammox *Nitrospira* abundance relative to total *Nitrospira* was largely similar at both Glostrup and Odense RSF with clade A comammox always more abundant than clade B in Glostrup RSF and lower in Odense RSF.

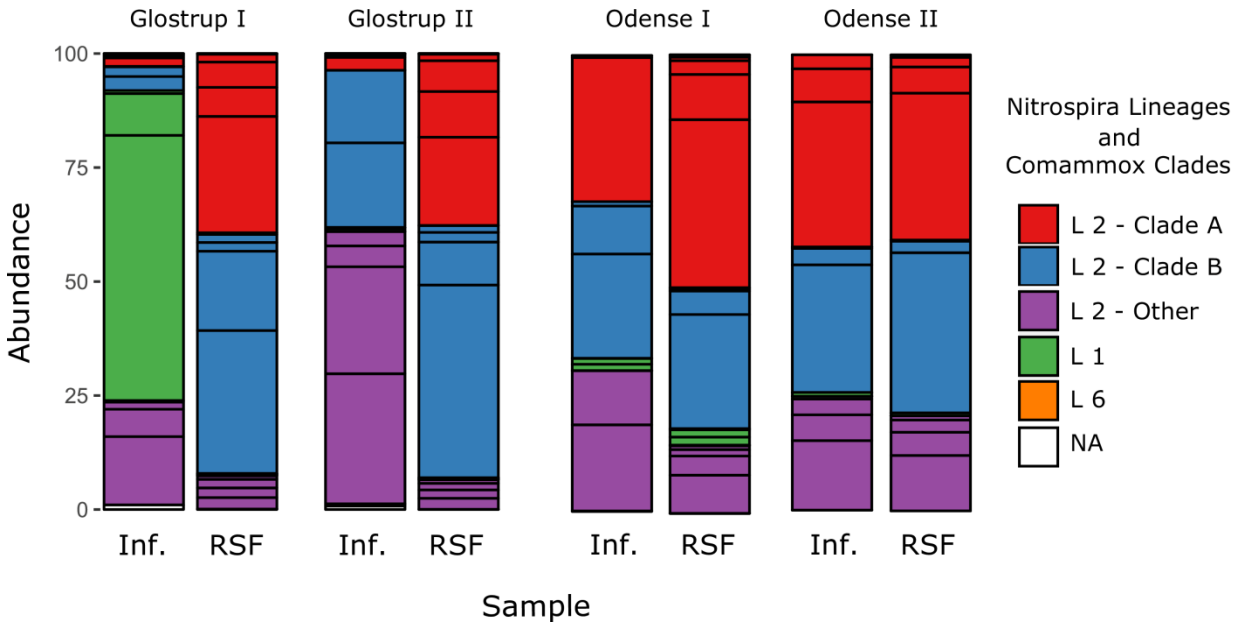

Figure 5.6 Relative abundance of *Nitrospira* spp. ASVs by lineages and comammox clades based on *nxrB Nitrospira* sequencing. (paper III)

Based on the NCM analysis I found that the majority of *Nitrospira* community in the analyzed DWTPs is shaped by neutral forces with a smaller contribution of selection (Table 5.1). This was true in both cases, a) when the source metacommunity was described directly by sampling the actual influent water and RSF community, and b) when the averaged source metacommunity was used.

Further, I found that the majority of neutrally assembled *Nitrospira* were affiliated to comammox ASVs contributing up to 66% and 75% of total *Nitrospira* abundance in Glostrup and Odense DWTPs respectively.

Table 5.1 Comparison of NCM fit between the source (influent water) and the local (biological sand filter) community using different types of source metacommunity and testing the null hypothesis (H0) that there is no correlation between model prediction and observed data, i.e., H0: ρ=0 (paper III).

		No. of *nxrB Nitrospira* ASVs (relative Abundance)						
DWTP	**Source meta-community#**	**Shared**	**Neutral**	**Positively selected**	**Negatively selected**	**ρ***	**p-value**	**N_Tm ****
Glostrup	influent water	60	33 (70.7%)	11 (20.6%)	16 (0.5%)	0.40	0.001	142.5
	Averaged Metacommunity	119	105 (98.9%)	3 (0.03%)	11 (1%)	0.87	$2.2*10^{-16}$	339
Odense	influent water	60	47 (94.3%)	6 (0.2%)	7 (2.2%)	0.77	$6*10^{-13}$	872.5
	Averaged Metacommunity	127	103 (94.3%)	10 (0.2%)	14 (5.4%)	0.86	$2.2*10^{-16}$	180

*Spearman's Rank correlation coefficient

**Biofilm community size multiplied by the immigration rate

The actual influent water source metacommunity was sampled along with the local community, and the averaged source metacommunity was calculated by averaging the taxa in the local community

On the other hand, for one DWTP (Glostrup) the differential abundance based analysis indicated ~13 fold average increase in the positively selected taxa (30 out of total 124 ASVs) and ~9 fold decrease on an average (10 ASVs) in abundance of negatively selected taxa from influent water to RSF community **(paper III)**. These positively selected ASVs collectively formed ~90% of the *Nitrospira* abundance in the biological sand filter; whereas, the negatively selected ASVs only formed ~2.6% in abundance **(paper III)**. The affiliation of positively selected ASVs showed that in relative change 7 and 9 ASVs of comammox clade A and B were positively selected respectively and formed a majority (~83% combined) of *Nitrospira* abundance in the RSF at Glostrup DWTP **(paper III)**.

At the other DWTP (Odense) 19 ASVs (out of total 294) were found to be positively selected ~5.5 fold on an average (all adjusted p values < 1.65E-02) contributing only ~6.7% of *Nitrospira* abundance in the RSF **(paper III)**. The positively selected comammox clade A (5 ASVs) and B (3 ASVs) contributed ~2.7 and ~1% in abundance respectively **(paper III)**.

In the comparison of NCM and differential abundance based approaches, I found that they did not provide similar predictions regarding selected members of the community. However, for both DWTPs the numbers of predicted positively selected ASVs were low from NCM compared to the differential abundance based approach (Figure 5.7 and 5.8).

For both approaches, the combined abundance of selected ASVs contributed majorly to the total abundance of *Nitrospira* for Glostrup whereas for Odense it did not, showing a clear enrichment of selected ASVs in Glostrup (Figure 5.7 and 5.8). Overall based on these estimates the *Nitrospira* community at Odense seemed to be entirely neutrally assembled and at Glostrup neutral processes played a smaller role compared to selection. Further, we do not know why the relative strength of selection is so different at both DWTPs as they do not differ in design or water treatment train. The only major difference was their geographically different locations further impacting them regarding the groundwater received by the DWTPs for treatment.

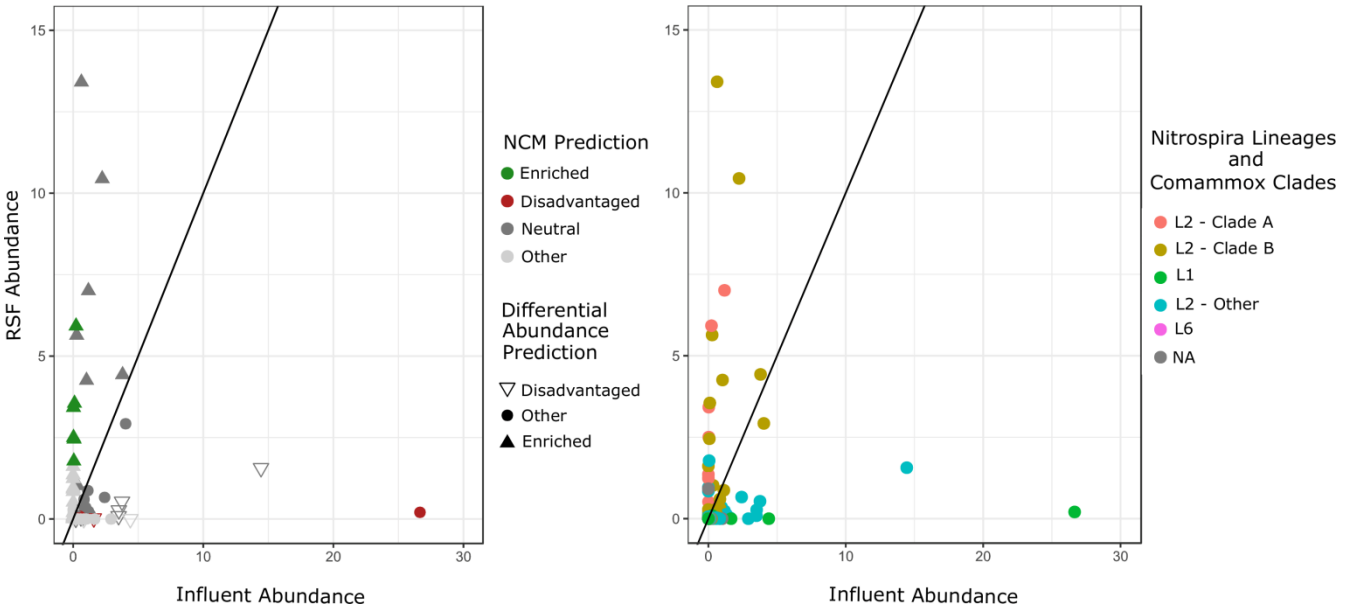

Figure 5.7 Comparison of NCM and differential abundance based analysis for Glostrup. For all ASVs their abundance in the influent and biological sand filter (RSF) is compared. ASVs are colored as per the prediction from NCM and shaped according to predictions from the differential abundance based analysis (left panel). The ASVs not considered in the analysis are marked as other for both the analysis. The taxonomic affiliation of each ASV is highlighted by coloring as per Nitrospira lineages and comammox clades (right panel). The black line represents the 1:1 identity (paper III).

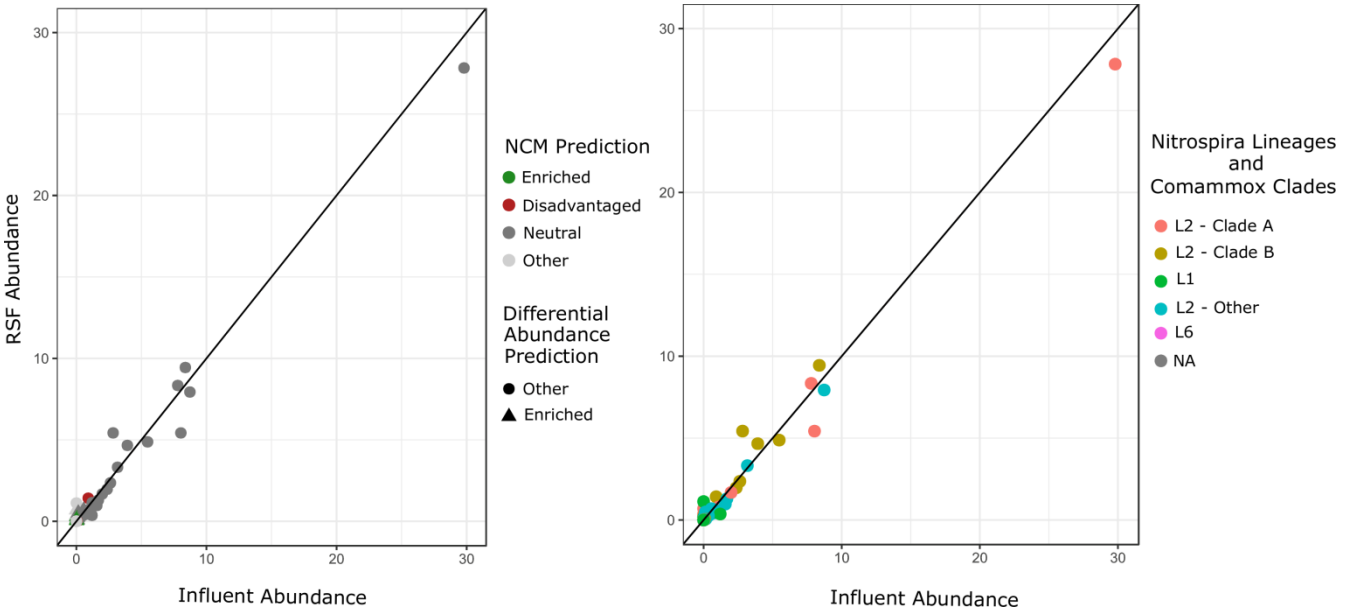

Figure 5.8 Comparison of NCM and differential abundance based analysis for Odense. Other details are similar to figure 4.5 (paper III).

From a methodology perspective, the differences in identification of selected *Nitrospira* ASVs by NCM and differential abundance approach suggested that the abundance data provides a more powerful test for identifying selected members of the community as some of the enriched taxa in the local community were detected as neutrally assembled by the NCM (grey triangles - figure 5.7). Amongst these enriched taxa in the differential abundance analysis (grey triangles - figure 5.7) some of them are likely false negative in the context of selection detection and just differentially abundant due to neutral demographic processes because the differential abundance merely tests for difference in abundance without taking into account the stochasticity of assembly. Yet, some of the highly differentially abundant taxa (such as those above 10% abundance in the figure 5.7, grey triangles) can be confidently considered selected because such high differences in abundance cannot be explained by drift alone.

6 Effect of the release of nutrient limitation on nitrifier community

In the previous study, we saw the positive selection of *Nitrospira* types in the RSF microbial community at two DWTPs. Although the causes of this selection were not directly understood as RSF microbial communities were not exposed to any external deterministic changes (apart from the natural differences in groundwater and RSF environment) and DWTPs did not differ in design or water treatment train. I further investigated the effect of selection to see whether modifying resource conditions translate into detectable selection on RSF microbial community composition including the effect on within guild diversity. As mentioned earlier this investigation was performed in light of one of the RRT predictions that "High species diversity and evenness occur when there are higher number of limiting resources, while resource enrichment leads to decreased species richness and evenness."

6.1 Effect on nitrifier community composition upon releasing Cu limitation

Recent studies have shown that copper deficiency can limit nitrification in RSF and it can be remediated (to the effluent concentration of <0.01 mg NH4+-N/L within less than 3 weeks) through dosing of copper to RSF in trace (µg/L) amounts (Wagner *et al.*, 2016, 2018). Copper is a cofactor for the AMO enzyme, which catalyzes ammonia oxidation to hydroxylamine in AOB. Although previous studies reported the effect of dosing copper on nitrification performance in RSF comprehensively, it remains unknown whether the stimulation is reflected in a shift of abundance or composition of nitrifiers. These investigations were performed at two municipal DWTP (Nærum and Holmehave[1]) where copper was dosed to enhance nitrification.

The ammonium removal responded promptly to copper dosing in the two DWTP, with increased removal rates within only a few days for both DWTPs **(paper IV)**. The improved nitrification was noted in terms of the filter effluent concentrations of <0.01 mg NH4+-N/L that was achieved after 22 days of

[1] 'Holmehave' is the same drinking water treat plant mentioned as 'Odense' in the previous studies.

dosing for Nærum and 21 days for Holmehave DWTP. During the dosing, the test filters were exposed to average influent dosing concentrations of 0.34 ± 0.22 µg Cu/L (n=11) for Nærum and 0.85 ± 0.24 µg Cu/L (n=8) for Holmehave. The reference filter was used as a control, which did not receive copper dosing and here the ammonium removal did not change during the investigation at the Holmehave DWTP **(paper IV)**. Also, other filter functions such as the removal of iron and manganese were not impaired by the dosing at either plant **(paper IV)**.

6.1.1 Changes in absolute cell densities

After dosing began, AOB absolute densities increased by 52 % in the filter receiving copper at Holmehave, and by 239 % at Nærum, based on qPCR of the 16S rRNA gene (Figure 6.1). Overall, qPCR analyses showed that copper dosing increased the abundance of canonical AOB at both investigated sites, in stark contrast to the reference filter which was not dosed with copper. For comammox *Nitrospira*, at one site, the positive effect was less pronounced, while their abundance decreased at the other DWTP.

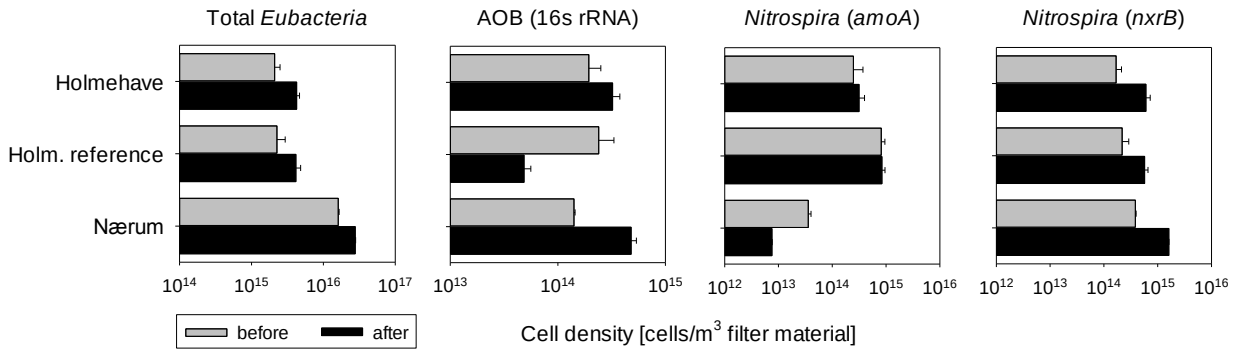

Figure 6.1 Cell densities measured by qPCR before dosing of copper and 67 or 116 days after the start of dosing to the test filters at Holmehave and Nærum DWTPs. The reference filter at Holmehave (Holm. reference) did not receive copper dosing. (Paper IV)

6.1.2 Changes in microbial community composition

The compositional analysis based on universal 16S rRNA gene amplicon sequencing confirmed the effect of copper dosing on the groups targeted by our qPCR assays and showed that the copper dosing did not provoke a drastic change in the microbial composition of the filters (Figure 6.2). *Nitrospira* was the dominant genus in both plants (Figure 6.2, top). In the control filter, the relative abundance of both *Nitrosomonas* and *Nitrospira* genera remained unchanged (Figure 6.2, center panel). However, in the test filters, the relative abundance of *Nitrosomonas* increased by almost one order of magnitude in

both filters with copper dosing (Figure 6.2, bottom). The relative abundance of *Nitrospira* (Figure 6.2, top) also increased, but this was only statistically significant (p-value of 0.014) for Nærum. In the test filters, although the increase in relative abundance of *Nitrosomonas* sequence reads was much higher than for *Nitrospira*, their increase in absolute abundance was similar (~+7% of the total reads for both genera at Holmehave) or larger for *Nitrospira* (~+7% vs +2% at Nærum) because *Nitrospira* was initially >20 times more abundant than *Nitrosomonas*. Besides *Nitrosomonas* and *Nitrospira*, very few genera amongst the 50 most abundant were significantly affected by copper dosing **(paper IV)**.

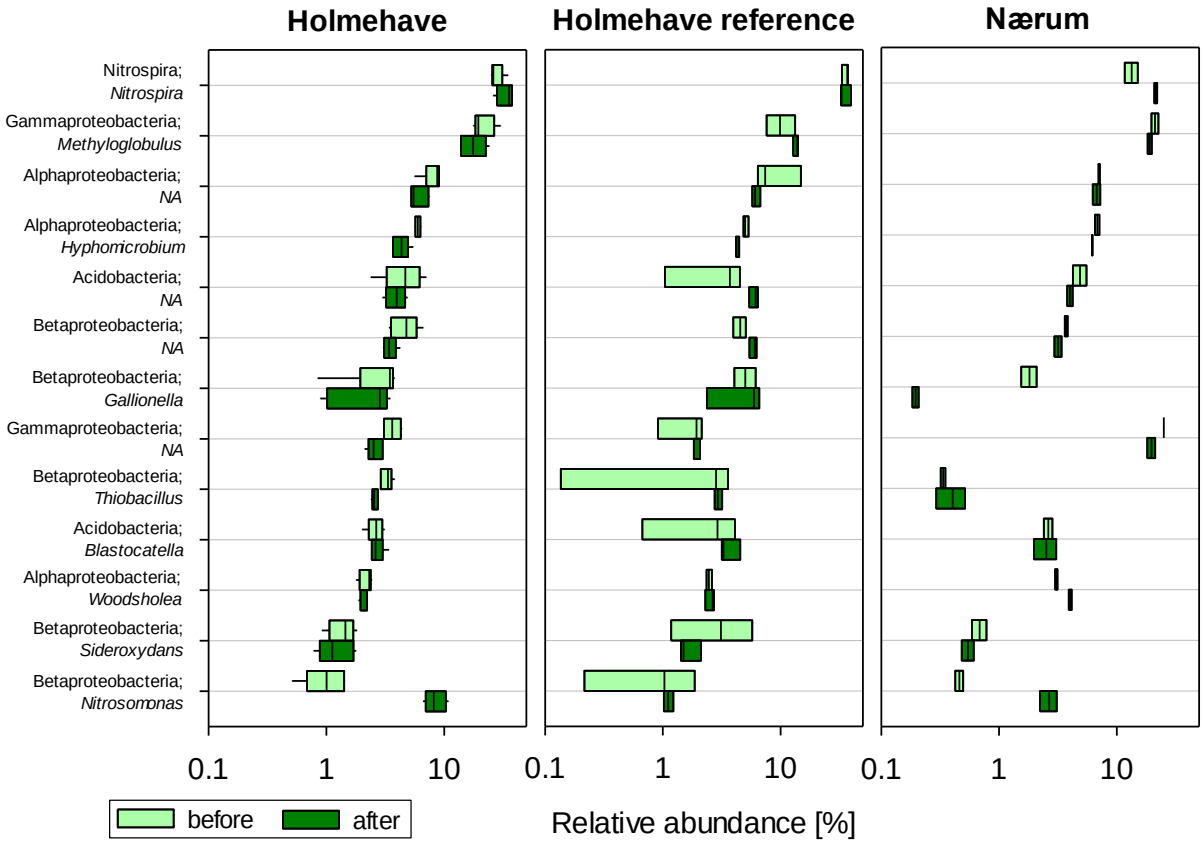

Figure 6.2 Taxonomic distribution of 16S rRNA gene sequences in the copper-dosed and reference filters. The relative abundance of the 13 most abundant genera are presented. The sequences are grouped according to their assigned genus (labeled as "class; genus," NA indicates unidentified genera). Box plots are computed from 5 biological replicates for Holmehave, 3 for Holmehave reference, and 2 for Nærum treatment plants. (Paper IV)

In the within guild analysis at the sequence variant level, the copper-induced stimulation of nitrification did not markedly modify the composition within

Nitrosomonas and *Nitrospira*, suggesting that this did not constitute a strong differential selective pressure at the genus level (Figure 6.3). The composition in the two plants for these two genera was very similar down to the sequence variant level, with most of the dominant variants being shared. This probably contributes to their consistent response to copper supplementation.

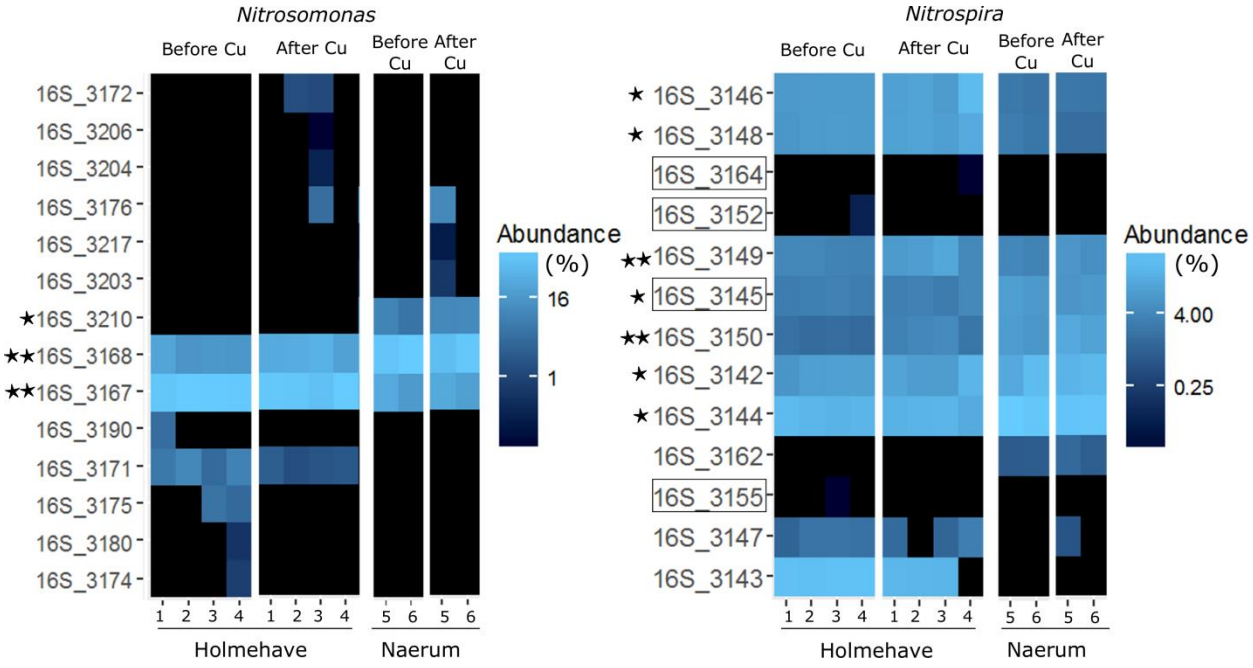

Figure 6.3 Abundance of sequence variants assigned to *Nitrosomonas* (left) and *Nitrospira* (right) relative to the total number of *Nitrosomonas* or *Nitrospira* sequence variants before and after copper addition at the test filters at Holmehave (columns 1-4) and Nærum (columns 5 and 6). In the right panel, boxed sequence variant indicate putative canonical *Nitrospira* while the rest are putative comammox *Nitrospira*. (Paper IV)

The increase in relative abundance of *Nitrosomonas* and largely of that of *Nitrospira* in the test filters upon releasing copper limitation indicates their selection over other community members.

It should be noted that this effect can only be attributed to the increase in copper availability to the microorganisms as all other filter parameters were unchanged during the operation **(paper IV)**. At the genus level, *Nitrospira* growing more than *Nitrosomonas* indicates towards the fitness differences amongst members of both genera possibly in this case due to competition for copper acquisition. Further, inside *Nitrospira* and *Nitrosomonas* the composi-

tion did not change which shows that there were no fitness differences amongst members of these genera and indicates that within guild composition is largely neutrally assembled. These observations are in contrast with the observations mentioned in the previous chapter where I observed neutral assembly at the guild level (i.e. *Nitrospira*) and selection within the guild (i.e. of *Nitrospira* types). This could be because when there is no micronutrient limitation certain *Nitrospira* types outcompetes other types in the RSF environment i.e. by better utilization of niche space or other key resources.

One of the predictions from RRT states that high species diversity and evenness occur when low to an intermediate amount of resources are supplied, while resource enrichment leads to decreased species richness and evenness. However, in this case, the enrichment of copper to biological filter showed no change in composition within *Nitrospira* and *Nitrosomonas*. One possible reason for this could be that the method, i.e. 16S rRNA gene- total community amplicon sequencing due to its low resolutive power could not accurately unveil the within guild diversity which possibly underestimated the richness within *Nitrosomonas* and *Nitrospira* **(paper II and IV)**. This issue could be overcome by utilizing functional genes (e.g., *amoA* and *nxrB*) based amplicon sequencing.

Overall, through this substudy, I showed that copper-induced stimulation of nitrification in biological rapid sand filters involved changes within the nitrifier genera. Copper was indeed limiting in the analyzed DWTPs as its supplementation in trace amounts positively affected the nitrifier abundance and specifically of *Nitrosomonas*. Also, copper dosing did not markedly affect the relative abundance of other microbial guilds or potentially pathogenic microorganisms in the filters which is highly valuable information from a practical perspective. Taken together, these findings suggest that it is possible to enhance treatment performance by influencing the abundance of specific microbial groups through selective nutrient dosing or in other words by lifting key elemental resource limitation.

Lastly, this study also served as a ground for implementing molecular, and community ecology methods described earlier (qPCR, 16S rRNA gene universal amplicon sequencing, differential abundance based analysis) in their ability to quantify and describe specific microbial community constituents and further strengthened the observations from **papers I-III** in an applied scenario.

7 Conclusions

The fundamental understanding of the processes governing microbial community assembly and maintaining their diversity is necessary for better management of biological systems with potential biotechnological applications. To obtain these insights various molecular and community ecology approaches can be used. In this thesis, I focused on assessment and implementation of key molecular and microbial community ecology methods for describing the ecology of nitrifiers in engineered systems employed for drinking water production and wastewater treatment. Overall this work has provided the first systematic evaluation and insights on the appropriate selection, and implementation of qPCR and amplicon sequencing-based approaches for quantifying and describing specific microbial guilds and identifying the effect of key ecological process on nitrifier community assembly in engineered systems. The main findings of this work are as follows:

Primer design specificity and coverage analysis

- The investigation of the consistency of two molecular approaches for quantifying canonical AOB by targeting phylogenetic (16S rRNA) and functional (*amoA*) genes revealed that the difference in primer pair selectivity combined with the compositional differences of the canonical AOB guilds across RSF communities had a major effect on the quantification outcome of these approaches. The primer set selection for canonical AOB quantification should be carefully done as the results can be heavily primer and nitrifier composition dependent.
- Using maximum coverage degenerate primer design method, new primer sets targeting *amoA* of AOB were designed that showed better coverage in the *in silico* analysis than the ones presently used but yielded unspecific amplification in the *in-vitro* analysis. Based on the currently available *amoA* sequence data, it was observed that there is no longer enough conserved nucleotide region amongst different canonical AOB clade members (as *amoA* of *Nitrosomonas* and *Nitrosospira* is too divergent) to design higher coverage primers that can amplify long enough amplicons to be suitable for obtaining quantitative, phylogenetic and compositional information about canonical AOB.

Comparison of universal and targeted amplicon sequencing approaches for describing nitrifying guilds

- The universal amplicon sequencing-based on the 16S rRNA gene provided, in a single analysis, useful information on the abundance and composition of diverse guilds in complex DWTP and WWTP microbial communities. In comparison to the functional gene (*amoA* AOB and *nxrB Nitrospira*) based targeted sequencing, the universal amplicon sequencing provided accurate estimates of the nitrifier composition and clustering of samples based on these compositions which were consistent with sample origin.
- The relative abundance estimates of the nitrifying guilds from the universal amplicon sequencing correlated with those obtained from the targeted sequencing approach but with measurable biases that should be considered while comparing estimates from both approaches. The diversity and richness estimations from the universal amplicon sequencing were likely limited by the sequencing depth; therefore nitrifier diversity and richness estimation using targeted functional gene approaches or sequencing depths larger than those typically practices should be preferred.

Comparison of neutral-model and differential abundance based approaches for identifying selection in nitrifiers

- The neutral model and differential abundance based approaches were different in the identification of positively and negatively selected members of the guild. The neutral model always predicted fewer taxa as positively selected compared to the differential abundance based approach. I acknowledge that the differential abundance based analysis merely tests for difference in abundance without taking into account the stochasticity of community assembly making the approach prone to detecting false negative taxa in the context of selection which could be merely differentially abundant due to neutral demographic processes.
- I highlighted the fact that despite being a comprehensive statistical model-based approach, NCM only utilizes the frequency of presence data and overlooks the abundance of taxa in the metacommunity. The NCM output was largely impacted by the way metacommunity was measured. For example, the derivation of influent community as averaged source metacommunity approach showed even stronger contribution of stochastic process in RSF microbial community compared to the approach where the influent community was derived using comprehensive measurements.

- The differences amongst neutral model and differential abundance based approaches in their ability to identify selection suggested that detection of selection in complex microbial communities should be carefully addressed using a combination of approaches covering both frequency and abundance data of the taxa.

Effect of releasing nutrient limitation on nitrifying community

- The copper-induced stimulation of nitrification in biological rapid sand filters involved an increase in the relative abundance of some of the nitrifying guilds without evidently affecting the relative abundance of other microbial guilds or potentially pathogenic microorganisms in the RSF.
- Releasing the copper limitation in RSF did not affect the composition within nitrifying guilds. These findings indicate that it is possible to enhance biological stability and key process performance in complex microbial communities by influencing the abundance of specific microbial groups through selective nutrient dosing.

Nitrifier ecology in DWTP and WWTP

- The AOB composition comparison between DWTP and WWTP showed that Cluster 0, 2, 3, 7, and 8 members mainly inhabited WWTP while cluster 6 members were found mainly in DWTP. Unlike other cluster members, both types of plants shared the taxa from *Nitrosomonas* subcluster 6A. While for *Nitrospira* the DWTP were dominated by lineage 2 members and WWTP by lineage 1 members. These observations further strengthened the argument that nitrifiers have preferred niches and are selected for specific environments based on the type/amount of resource exposure.
- The *Nitrosomonas* and *Nitrospira* composition in the three studied DWTPs was very similar down to the sequence variant level, with most of the dominant sequence variants being shared amongst the DWTPs. This indicates selection of specific nitrifier taxa at individual sequence variant level performing the same function at different DWTPs.
- At the RSFs releasing copper limitation resulted in the selection of certain nitrifying groups (*Nitrospira* and *Nitrosomonas*) at the guild level whereas no effect on the within guild nitrifier composition indicated neutral assembly.
- At the well-functioning, not-micronutrient limited RSFs; the nitrifiers at the guild level (i.e. *Nitrospira*) were neutrally assembled whereas certain

within guild nitrifiers (i.e. *Nitrospira* types) were selected in those RSF. This could be because in the absence of micronutrient limitation certain *Nitrospira* types outcompete other types in the RSF environment either by better utilization of niche space and/or key resources.

8 Perspectives

I first explored the potential of qPCR and amplicon sequencing-based approaches for quantifying and describing specific nitrifying guilds. Both these molecular methods come with some limitations. First, these methods are amplification-based therefore they are highly dependent on appropriate primer set selection. I tried to develop new primer sets using the highly degenerate primer design strategy for maximum coverage and specificity compared to the existing primers. This strategy was not successful as there was unspecific amplification in the in vitro analysis by the newly designed primers. In future, other strategies such as tagged highly degenerate primer (THDP) PCR can be used which allows for amplification of specific microbial guilds in the first stage followed by a secondary PCR with a tag on a single primer. This approach has the potential for minimizing the unspecific amplification. Second, any amplification based molecular method suffers from the amplification bias problem which can be resolved by using PCR-independent methods such as qFISH or direct 16S rRNA gene sequencing from bacterial communities. Lastly, both these approaches used in this study are DNA based which is good to estimate the abundance and/composition of the target guild but does not provide valuable functional information on the guild members. In the future, rRNA based techniques such as real-time quantitative PCR (RT-qPCR) can be used for functional profiling of nitrifiers. Furthermore, if the interest is in identifying the functional and metabolic potential of the whole microbial community then metagenomics coupled with meta-transcriptomics can also be used.

While estimating the effect of selection and identification of selected microbial ecotypes I found that it is important to consider the abundance of taxa while estimating their selection because the abundance information indicates towards the extent of the role guild members play in an environment. To do so, here I used the differential abundance based approach in addition to the NCM. In future efforts can be drawn towards extending the NCM that can include the experimentally determined abundance of the taxa in addition to their frequency of presence or absence information.

I found that specific nitrifying members (mainly comammox) were selected in the studied DWTPs. In the future, these selected ecotypes can be studied in depth using whole genome sequencing to identify their full potential and role in nitrification. Additionally, lab scale culture experiments can be done to study the physiological parameters that can help in achieving the highest ac-

tivity of these microbes in a given environment (such as RSF). These studies in future can aid the ultimate goal of creating custom inoculum for the RSF for improved performance.

In the DWTP releasing copper limitation resulted in a higher abundance of certain nitrifiers in the RSF and in turn improved the RSF performance. In the future detailed analysis of other substrates and micronutrients required by nitrifiers and the effect of their limitation on nitrifiers composition can be studied.

This study was performed at few DWTP and WWTP from Denmark and Sweden therefore; in the future, the study can be extended to diverse plants across different geographical locations receiving different types of water for treatment. This would be interesting to see if the methodological approach taken in this study works the same way under different conditions and more importantly to observe if similar nitrifiers are selected in other environments as well.

9 References

Adamczyk J, Hesselsoe M, Iversen N, Horn M, Lehner A, Nielsen PH, *et al.* (2003). The Isotope Array , a New Tool That Employs Substrate-Mediated Labeling of rRNA for Determination of Microbial Community Structure and Function. *Appl Environ Microbiol* **69**: 6875–6887.

Adler PB, HilleRislambers J, Levine JM. (2007). A niche for neutrality. *Ecol Lett* **10**: 95–104.

Albers CN, Ellegaard-Jensen L, Hansen LH, Sørensen SR. (2018). Bioaugmentation of rapid sand filters by microbiome priming with a nitrifying consortium will optimize production of drinking water from groundwater. *Water Res* **129**: 1–10.

Anders S, Huber W. (2010). Differential expression analysis for sequence count data. *Genome Biol* **11**: 1–12.

Anderson MJ. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol* **26**: 32–46.

Anderson MJ, Ellingsen KE, McArdle BH. (2006). Multivariate dispersion as a measure of beta diversity. *Ecol Lett* **9**: 683–693.

Andersson A, Laurent P, Kihn A, Prévost M, Servais P. (2001). Impact of temperature on nitrification in biological activated carbon (BAC) filters used for drinking water treatment. *Water Res* **35**: 2923–2934.

Annavajhala MK, Kapoor V, Santo-Domingo J, Chandran K. (2018). Comammox Functionality Identified in Diverse Engineered Biological Wastewater Treatment Systems. *Environ Sci Technol Lett* **5**: 110–116.

Anthony SJ, Islam A, Johnson C, Navarrete-Macias I, Liang E, Jain K, *et al.* (2015). Non-random patterns in viral diversity. *Nat Commun* **6**: 1–7.

Antoniou P, Hamilton J, Koopman B, Jain R, Holloway B, Lyberatos G, *et al.* (1990). Effect of temperature and ph on the effective maximum specific growth rate of nitrifying bacteria. *Water Res* **24**: 97–101.

Bai Y, Liu R, Liang J, Qu J. (2013). Integrated Metagenomic and Physiochemical Analyses to Evaluate the Potential Role of Microbes in the Sand Filter of a Drinking Water Treatment System Ibekwe AM (ed). *PLoS One* **8**: e61011.

Bai Y, Sun Q, Wen D, Tang X. (2012). Abundance of ammonia-oxidizing bacteria and archaea in industrial and domestic wastewater treatment systems. *FEMS Microbiol Ecol* **80**: 323–330.

Basu OD, Dhawan S, Black K. (2016). Applications of biofiltration in drinking water treatment - a review. *J Chem Technol Biotechnol* **91**: 585–595.

Berg-Lyons D, Knight R, Lozupone CA, Turnbaugh PJ, Caporaso JG, Lauber CL, *et al.* (2010). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci* **108**: 4516–4522.

Berry D, Xi C, Raskin L. (2006). Microbial ecology of drinking water distribution systems. *Curr Opin Biotechnol* **17**: 297–302.

Black A, Hsu PCL, Hamonts KE, Clough TJ, Condron LM. (2016). Influence of copper on

expression of nirS, norB and nosZ and the transcription and activity of NIR, NOR and N2OR in the denitrifying soil bacteria Pseudomonas stutzeri. *Microb Biotechnol* **9**: 381–388.

Blackburne R, Vadivelu VM, Yuan Z, Keller J. (2007). Kinetic characterisation of an enriched Nitrospira culture with comparison to Nitrobacter. *Water Res* **41**: 3033–3042.

Bock E. (1976). Growth of Nitrobacter in the presence of organic matter - II. Chemoorganotrophic growth of Nitrobacter agilis. *Arch Microbiol* **108**: 305–312.

Bock E, Schmidt I, Stüven R, Zart D. (1995). Nitrogen loss caused by denitrifying Nitrosomonas cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. *Arch Microbiol* **163**: 16–20.

De Boer W, Kowalchuk GA. (2001). Nitrification in acid soils: Micro-organisms and mechanisms. *Soil Biol Biochem* **33**: 853–866.

Bollmann A, Bär-Gilissen MJ, Laanbroek HJ. (2002). Growth at low ammonium concentrations and starvation response as potential factors involved in niche differentiation among ammonia-oxidizing bacteria. *Appl Environ Microbiol* **68**: 4751–4757.

Borcard D, Legendre P. (2002). All-scale spatial analysis of ecological data by means of principal coordinates of neighbour matrices. *Ecol Modell* **153**: 51–68.

Bouchard DC, Williams MK, Surampalli RY. (1992). Nitrate contamination of groundwater: sources and potential health effects. *J / Am Water Work Assoc* **84**: 85–90.

Bougard D, Bernet N, Chèneby D, Delgenès JP. (2006). Nitrification of a high-strength wastewater in an inverse turbulent bed reactor: Effect of temperature on nitrite accumulation. *Process Biochem* **41**: 106–113.

Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P. (2008). Mesophilic crenarchaeota: Proposal for a third archaeal phylum, the Thaumarchaeota. *Nat Rev Microbiol* **6**: 245–252.

Burke C, Steinberg P, Rusch D, Kjelleberg S, Thomas T. (2011). Bacterial community assembly based on functional genes rather than species. *Proc Natl Acad Sci* **108**: 14288–14293.

Cadotte MW, Jonathan Davies T, Regetz J, Kembel SW, Cleland E, Oakley TH. (2010). Phylogenetic diversity metrics for ecological communities: Integrating species richness, abundance and evolutionary history. *Ecol Lett* **13**: 96–105.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, *et al.* (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* **108 Suppl**: 4516–22.

Cencini M, Pigolotti S, Muñoz MA. (2012). What ecological factors shape species-area curves in neutral models? Cornell SJ (ed). *PLoS One* **7**: e38232.

Chao Y, Ma L, Yang Y, Ju F, Zhang XX, Wu WM, *et al.* (2013). Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. *Sci Rep* **3**: 3550.

Chase JM, Leibold MA. (2003). Ecological niches : linking classical and contemporary approaches. University of Chicago Press.

Chase JM, Myers JA. (2011). Disentangling the importance of ecological niches from

stochastic processes across scales. *Philos Trans R Soc B Biol Sci* **366**: 2351–2363.

Chave J. (2004). Neutral theory and community ecology. *Ecol Lett* **7**: 241–253.

Chesson P. (1991). A need for niches? *Trends Ecol Evol* **6**: 26–28.

Chesson P. (2000). Mechanisms of Maintenance of Species Diversity. *Annu Rev Ecol Syst* **31**: 343–366.

Clarke KR. (1993). Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**: 117–143.

Cooper GM. (2000). The Molecular Composition of Cells. In: *The cell - a molecular approach*. Sinauer Associates, p 689.

Courtens ENP, De Clippeleir H, Vlaeminck SE, Jordaens R, Park H, Chandran K, *et al.* (2015). Nitric oxide preferentially inhibits nitrite oxidizing communities with high affinity for nitrite. *J Biotechnol* **193**: 120–122.

Curtis TP, Head IM, Graham DW. (2003). Theoretical Ecology for Engineering Biology. *Environ Sci Technol* **37**: 64A–70A.

Daims H, Lebedeva E V., Pjevac P, Han P, Herbold C, Albertsen M, *et al.* (2015). Complete nitrification by Nitrospira bacteria. *Nature* **528**: 504–509.

Daims H, Lücker S, Wagner M. (2016). A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends Microbiol* **24**: 699–712.

Daims H, Maixner F, Lücker S, Stoecker K, Hace K, Wagner M. (2006a). Ecophysiology and niche differentiation of Nitrospira-like bacteria, the key nitrite oxidizers in wastewater treatment plants. *Water Sci Technol* **54**: 21–27.

Daims H, Nielsen PH, Nielsen JL, Juretschko S, Wagner M. (2000). Novel Nitrospira-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: Diversity and in situ physiology. In: Vol. 41. *Water Science and Technology*. IWA Publishing, pp 85–90.

Daims H, Taylor MW, Wagner M. (2006b). Wastewater treatment: a model system for microbial ecology. *Trends Biotechnol* **24**: 483–489.

Daims H, Wagner M. (2018). Nitrospira. *Trends Microbiol* **26**: 462–463.

Dechesne A, Musovic S, Palomo A, Diwan V, Smets BF. (2016). Underestimation of ammonia-oxidizing bacteria abundance by amplification bias in amoA-targeted qPCR. *Microb Biotechnol* **9**: 519–524.

Degrange V, Bardin R. (1995). Detection and counting of Nitrobacter populations in soil by PCR. *Appl Environ Microbiol* **61**: 2093–2098.

DeLong EF, Karl DM, Karner MB, Church MJ, Preston CM, Ducklow HW. (2010). Abundance and distribution of planktonic Archaea and Bacteria in the waters west of the Antarctic Peninsula. *Limnol Oceanogr* **48**: 1893–1902.

Dimitri Kits K, Sedlacek CJ, Lebedeva E V, Han P, Bulaev A, Pjevac P, *et al.* (2017). Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* **549**: 269–272.

Dionisi HM, Layton a C, Robinson KG, Brown JR, Gregory IR, Parker JJ, *et al.* (1999). Quantification of Nitrosomonas oligotropha and Nitrospira spp. using competitive

polymerase chain reaction in bench-scale wastewater treatment reactors operating at different solids retention times. *Water Environ Res* **74**: 462–9.

Dodsworth JA, Hungate BA, Hedlund BP. (2011). Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea. *Environ Microbiol* **13**: 2371–2386.

Dumbrell AJ, Nelson M, Helgason T, Dytham C, Fitter AH. (2010). Relative roles of niche and neutral processes in structuring a soil microbial community. *ISME J* **4**: 337–345.

Ehrich S, Behrens D, Lebedeva E, Ludwig W, Bock E. (1995). A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, Nitrospira moscoviensis sp. nov. and its phylogenetic relationship. *Arch Microbiol* **164**: 16–23.

Fan XY, Gao JF, Pan KL, Li DC, Dai HH, Li X. (2018). Temporal heterogeneity and temperature response of active ammonia-oxidizing microorganisms in winter in full-scale wastewater treatment plants. Chemical Engineering Journal, November 1. e-pub ahead of print, doi: 10.1016/j.cej.2018.10.234.

Finlay BJ. (2002). Global dispersal of free-living microbial eukaryote species. *Science (80-)* **296**: 1061–1063.

Follett RF, Hatfield JL. (2001). Nitrogen in the Environment: Sources, Problems, and Management. *Sci World J* **1**: 920–926.

Fowler SJ, Palomo A, Dechesne A, Mines PD, Smets BF. (2018). Comammox Nitrospira are abundant ammonia oxidizers in diverse groundwater-fed rapid sand filter communities. *Environ Microbiol* **20**: 1002–1015.

Gewin V. (2006). Beyond neutrality - Ecology finds its niche. *PLoS Biol* **4**: 1306–1310.

Gieseke A, Purkhold U, Wagner M, Amann R, Schramm A. (2001). Community Structure and Activity Dynamics of Nitrifying Bacteria in a Phosphate-Removing Biofilm. *Appl Environ Microbiol* **67**: 1351–1362.

Gihring TM, Green SJ, Schadt CW. (2012). Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. *Environ Microbiol* **14**: 285–290.

Gonzalez-Martinez A, Muñoz-Palazon B, Rodriguez-Sanchez A, Gonzalez-Lopez J. (2018). New concepts in anammox processes for wastewater nitrogen removal: recent advances and future prospects. *FEMS Microbiol Lett* **365**. e-pub ahead of print, doi: 10.1093/femsle/fny031.

Gonzalez-Martinez A, Rodriguez-Sanchez A, van Loosdrecht MCM, Gonzalez-Lopez J, Vahala R. (2016). Detection of comammox bacteria in full-scale wastewater treatment bioreactors using tag-454-pyrosequencing. *Environ Sci Pollut Res* **23**: 25501–25511.

Gotelli NJ, McGill BJ. (2006). Null versus neutral models: What's the difference? *Ecography (Cop)* **29**: 793–800.

Grady CPL, Lim HC. (1980). Biological wastewater treatment : theory and applications. John Wiley & Sons Ltd.

Graham DW, Knapp CW, Van Vleck ES, Bloor K, Lane TB, Graham CE. (2007). Experimental demonstration of chaotic instability in biological nitrification. *ISME J* **1**: 385–93.

Graham EB, Crump AR, Resch CT, Fansler S, Arntzen E, Kennedy DW, *et al.* (2016). Coupling Spatiotemporal Community Assembly Processes to Changes in Microbial Metabolism. *Front Microbiol* **7**: 1949.

Graham EB, Crump AR, Resch CT, Fansler S, Arntzen E, Kennedy DW, *et al.* (2017). Deterministic influences exceed dispersal effects on hydrologically-connected microbiomes. *Environ Microbiol* **19**: 1552–1567.

Gravel D, Canham CD, Beaudet M, Messier C. (2006). Reconciling niche and neutrality: The continuum hypothesis. *Ecol Lett* **9**: 399–409.

Gruber N, Galloway JN. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature* **451**: 293–296.

Grunditz C, Dalhammar G. (2001). Development of nitrification inhibition assays using pure cultures of Nitrosomonas and Nitrobacter. *Water Res* **35**: 433–440.

Gülay A. (2014). Taxonomic and functional diversity of microbial communities in rapid sand filters for groundwater treatment. http://orbit.dtu.dk/files/99974327/Arda_G_lay_PhD_Thesis_WWW_Version.pdf (Accessed August 28, 2018).

Gülay A, Musovic S, Albrechtsen H-J, Al-Soud WA, Sørensen SJ, Smets BF. (2016). Ecological patterns, diversity and core taxa of microbial communities in groundwater-fed rapid gravity filters. *ISME J* **10**: 2209–2222.

Gülay A, Tatari K, Musovic S, Mateiu R V., Albrechtsen HJ, Smets BF. (2014). Internal porosity of mineral coating supports microbial activity in rapid sand filters for groundwater treatment. *Appl Environ Microbiol* **80**: 7010–7020.

Guo J, Peng Y, Huang H, Wang S, Ge S, Zhang J, *et al.* (2010). Short- and long-term effects of temperature on partial nitrification in a sequencing batch reactor treating domestic wastewater. *J Hazard Mater* **179**: 471–479.

Hanaki K, Wantawin C, Ohgaki S. (1990). Nitrification at low levels of dissolved oxygen with and without organic loading in a suspended-growth reactor. *Water Res* **24**: 297–302.

Hansen M, Thomsen CT. (2017). An integrated public information system for geology, groundwater and drinking water in Denmark. *Geol Surv Denmark Greenl Bull* **38**: 69–72.

Hanson CA, Fuhrman JA, Horner-Devine MC, Martiny JBH. (2012). Beyond biogeographic patterns: Processes shaping the microbial landscape. *Nat Rev Microbiol* **10**: 497–506.

Hatzenpichler R. (2011). Identification and characterization of novel ammonia-oxidizing archaea and bacteria. http://othes.univie.ac.at/15145/.

Hellinga C, Schellen AAJC, Mulder JW, Van Loosdrecht MCM, Heijnen JJ. (1998). The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. In: Vol. 37. *Water Science and Technology*. No longer published by Elsevier, pp 135–142.

Henze M, Harremoës P, Jansen J la C, Arvin E. (2002). Wastewater Treatment - Biological and Chemical Processes. Springer.

Hermansson A, Lindgren P. (2001). Quantification of Ammonia-Oxidizing Bacteria in Arable Soil by Real-Time PCR Quantification. *Appl Environ Microbiol* **67**: 972–976.

Hibbing ME, Fuqua C, Parsek MR, Peterson SB. (2010). Bacterial competition: Surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **8**: 15–25.

Van Der Hoek JP, Bertelkamp C, Verliefde Bertelkamp ARD, Singhal N. (2014). Drinking water treatment technologies in Europe: State of the art - Challenges - Research needs. *J Water Supply Res Technol - AQUA* **63**: 124–130.

Holinger EP, Ross KA, Robertson CE, Stevens MJ, Harris JK, Pace NR. (2014). Molecular analysis of point-of-use municipal drinking water microbiology. *Water Res* **49**: 225–235.

Holt RD. (2009). Bringing the Hutchinsonian niche into the 21st century: Ecological and evolutionary perspectives. *Proc Natl Acad Sci* **106**: 19659–19665.

Hommes NG, Sayavedra-Soto LA, Arp DJ. (2003). Chemolithoorganotrophic Growth of Nitrosomonas europaea on Fructose. *J Bacteriol* **185**: 6809–6814.

Huang Z, Inskeep WP, Gao L, Song Z, Bagwell C, Ye Q, *et al.* (2008). Global Occurrence of Archaeal amoA Genes in Terrestrial Hot Springs. *Appl Environ Microbiol* **74**: 6417–6426.

Hubbell SP. (2005). Neutral theory in community ecology and the hypothesis of functional equivalence. *Funct Ecol* **19**: 166–172.

Hubbell SP. (2001). The Unified Neutral Theory of Biodiversity and Biogeography. 32nd ed. Princeton University Press.

Van Hulle SW, Volcke EI, Teruel JL, Donckels B, van Loosdrecht MC, Vanrolleghem PA. (2007). Influence of temperature and pH on the kinetics of the Sharon nitritation process. *J Chem Technol Biotechnol* **82**: 471–480.

Hutchinson GE. (1957). Population studies: animal ecology and demography - Concluding Remarks. *Cold Spring Harb Symp Quant Biol* **22**: 415–427.

Jizhong Zhou DN. (2017). Stochastic Community Assembly: Does It Matter in Microbial Ecology? *Am Soc Microbiol* **81**: 1–32.

Jongseol Kim, Bora Lee. (2011). Effect of temperature on nitrogen removal and microbial community composition in nitrifying biofilm reactors. In: Vol. 1. *Proceedings of 2011 6th International Forum on Strategic Technology*. IEEE, pp 476–479.

Kapoor A, Viraraghavan T. (1997). Nitrate Removal From Drinking Water—Review. *J Environ Eng* **123**: 371–380.

Kapoor V, Li X, Chandran K, Impellitteri CA, Domingo JWS. (2016). Use of functional gene expression and respirometry to study wastewater nitrification activity after exposure to low doses of copper. *Environ Sci Pollut Res* **23**: 6443–6450.

Karner MB, Delong EF, Karl DM. (2001). Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**: 507–510.

van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJM, Kartal B, *et al.* (2015). Complete nitrification by a single microorganism. *Nature* **528**: 555–559.

Khardenavis AA, Kapley A, Purohit HJ. (2007). Simultaneous nitrification and denitrification by diverse Diaphorobacter sp. *Appl Microbiol Biotechnol* **77**: 403–409.

Kinnunen M. (2017). Invasion in microbial communities: Role of community composition and assembly processes. http://orbit.dtu.dk/files/138319055/Thesis_online_version_Marta_Kinnunen.pdf (Accessed

August 8, 2018).

Kinnunen M, Gülay A, Albrechtsen HJ, Dechesne A, Smets BF. (2017). Nitrotoga is selected over Nitrospira in newly assembled biofilm communities from a tap water source community at increased nitrite loading. *Environ Microbiol* **19**: 2785–2793.

Kneitel JM, Chase JM. (2004). Trade-offs in community ecology: Linking spatial scales and species coexistence. *Ecol Lett* **7**: 69–80.

Koch H, Galushko A, Albertsen M, Schintlmeister A, Gruber-Dorninger C, Lücker S, *et al.* (2014). Microbial metabolism: Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. *Science (80-)* **345**: 1052–1054.

Koops H-P, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M. (2006). The Lithoautotrophic Ammonia-Oxidizing Bacteria. In: *The Prokaryotes*. pp 778–811.

Koops HP, Pommerening-Röser A. (2001). Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiol Ecol* **37**: 1–9.

Kowalchuk GA, Stephen JR, De Boer W, Prosser JIMI, Embley TM, Woldendorp JANW, *et al.* (1997). Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol* **63**: 1489–1497.

Kuypers MMM, Marchant HK, Kartal B. (2018). The microbial nitrogen-cycling network. *Nat Rev Microbiol* **16**: 263–276.

De La Torre JR, Walker CB, Ingalls AE, Könneke M, Stahl DA. (2008). Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* **10**: 810–818.

Laanbroek HJ, Gerards S. (1993). Competition for limiting amounts of oxygen between Nitrosomonas europaea and Nitrobacter winogradskyi grown in mixed continuous cultures. *Arch Microbiol* **159**: 453–459.

Larson CA, Adumatioge L, Passy SI. (2016). The number of limiting resources in the environment controls the temporal diversity patterns in the algal benthos. *Microb Ecol* **72**: 64–69.

Lawson CE, Lücker S. (2018). Complete ammonia oxidation: an important control on nitrification in engineered ecosystems? *Curr Opin Biotechnol* **50**: 158–165.

Lee C, Odell ;, Binning P, John ;, Albrechtsen H-J;, Smets BF;, *et al.* (2014). Processes effecting nitrification performance in biological rapid sand filters. http://orbit.dtu.dk/files/100177270/Carson_Odell_Lee_PhD_Thesis_WWW_Version.pdf (Accessed August 28, 2018).

Lehtovirta-Morley LE, Nicol GW, Stoecker K, Prosser JI, Vilcinskas A. (2011). Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proc Natl Acad Sci* **108**: 15892–15897.

Leibold MA, Holyoak M, Mouquet N, Amarasekare P, Chase JM, Hoopes MF, *et al.* (2004). The metacommunity concept: A framework for multi-scale community ecology. *Ecol Lett* **7**: 601–613.

Leitão RC, Van Haandel AC, Zeeman G, Lettinga G. (2006). The effects of operational and environmental variations on anaerobic wastewater treatment systems: A review.

Bioresour Technol **97**: 1105–1118.

Lettinga G. (1995). Anaerobic digestion and wastewater treatment systems. *Antonie Van Leeuwenhoek* **67**: 3–28.

Li Y, Ding K, Wen X, Zhang B, Shen B, Yang Y. (2016). A novel ammonia-oxidizing archaeon from wastewater treatment plant: Its enrichment, physiological and genomic characteristics. *Sci Rep* **6**: 23747.

Limpiyakorn T, Fürhacker M, Haberl R, Chodanon T, Srithep P, Sonthiphand P. (2013). amoA-encoding archaea in wastewater treatment plants: a review. *Appl Microbiol Biotechnol* **97**: 1425–39.

Limpiyakorn T, Kurisu F, Sakamoto Y, Yagi O. (2007). Effects of ammonium and nitrite on communities and populations of ammonia-oxidizing bacteria in laboratory-scale continuous-flow reactors. *FEMS Microbiol Ecol* **60**: 501–512.

Limpiyakorn T, Shinohara Y, Kurisu F, Yagi O. (2005). Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. *FEMS Microbiol Ecol* **54**: 205–217.

Lin X, McKinley J, Resch CT, Kaluzny R, Lauber CL, Fredrickson J, *et al.* (2012). Spatial and temporal dynamics of the microbial community in the Hanford unconfined aquifer. *ISME J* **6**: 1665–1676.

Litchman E, de Tezanos Pinto P, Edwards KF, Klausmeier CA, Kremer CT, Thomas MK. (2015). Global biogeochemical impacts of phytoplankton: A trait-based perspective Austin A (ed). *J Ecol* **103**: 1384–1396.

Liu G, Wang J. (2013). Long-term low DO enriches and shifts nitrifier community in activated sludge. *Environ Sci Technol* **47**: 5109–5117.

Liu W, Tang JH, Xia GQ. (2014). Effects of Heavy Metals on the Characteristics of Microorganisms in Activated Sludge: A Review. *Adv Mater Res* **1051**: 600–604.

Liu X, Kim M, Nakhla G. (2018). Performance and Kinetics of Nitrification of Low Ammonia Wastewater at Low Temperature. *Water Environ Res* **90**: 498–509.

Lopato L. (2011). Granular filters for water treatment: heterogeneity and diagnostic tools. http://orbit.dtu.dk/fedora/objects/orbit:86115/datastreams/file_5612186/content (Accessed August 20, 2018).

Lücker S, Schwarz J, Gruber-Dorninger C, Spieck E, Wagner M, Daims H. (2015). Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. *ISME J* **9**: 708–720.

Lydmark P, Lind M, Sörensson F, Hermansson M. (2006). Vertical distribution of nitrifying populations in bacterial biofilms from a full-scale nitrifying trickling filter. *Environ Microbiol* **8**: 2036–2049.

Ma C, Liu M, Wang H, Chen C, Fan W, Griffiths B, *et al.* (2015). Resource utilization capability of bacteria predicts their invasion potential in soil. *Soil Biol Biochem* **81**: 287–290.

MacArthur RH. (1972). Geographical ecology : patterns in the distribution of species. 6th ed. Princeton University Press.

Manser R, Gujer W, Siegrist H. (2005a). Consequences of mass transfer effects on the

kinetics of nitrifiers. *Water Res* **39**: 4633–4642.

Manser R, Gujer W, Siegrist H. (2005b). Membrane bioreactor versus conventional activated sludge system: Population dynamics of nitrifiers. *Water Sci Technol* **52**: 417–425.

Martens-Habbena W, Berube PM, Urakawa H, De La Torre JR, Stahl DA. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* **461**: 976–979.

Matthews TJ, Whittaker RJ. (2014). Neutral theory and the species abundance distribution: Recent developments and prospects for unifying niche and neutral perspectives. *Ecol Evol* **4**: 2263–2277.

Matthews TJ, Whittaker RJ. (2015). On the species abundance distribution in applied ecology and biodiversity management Fuller R (ed). *J Appl Ecol* **52**: 443–454.

Meerbergen K, Van Geel M, Waud M, Willems KA, Dewil R, Van Impe J, *et al.* (2017). Assessing the composition of microbial communities in textile wastewater treatment plants in comparison with municipal wastewater treatment plants. *Microbiologyopen* **6**: e00413.

Meinhardt KA, Bertagnolli A, Pannu MW, Strand SE, Brown SL, Stahl DA. (2015). Evaluation of revised polymerase chain reaction primers for more inclusive quantification of ammonia-oxidizing archaea and bacteria. *Environ Microbiol Rep* **7**: 354–363.

Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria . These include : Phylogenetic Probes for Analyzing Abundance and Spatial Organization of Nitrifying Bacteria. *Appl Environ Microbiol* **62**: 2156–2162.

Monod J. (1949). The Growth of Bacterial Cultures. *Annu Rev Microbiol* **3**: 371–394.

Moroenyane I, Chimphango SBM, Wang J, Kim HK, Adams JM. (2016). Deterministic assembly processes govern bacterial community structure in the Fynbos, South Africa. *Microb Ecol* **72**: 313–323.

Morris A, Beck JM, Schloss PD, Campbell TB, Crothers K, Curtis JL, *et al.* (2013). Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med* **187**: 1067–1075.

Morrison-Whittle P, Goddard MR. (2015). Quantifying the relative roles of selective and neutral processes in defining eukaryotic microbial communities. *ISME J* **9**: 2003–2011.

Nemergut DR, Schmidt SK, Fukami T, O'Neill SP, Bilinski TM, Stanish LF, *et al.* (2013). Patterns and Processes of Microbial Community Assembly. *Microbiol Mol Biol Rev* **77**: 342–356.

Nicol GW, Leininger S, Schleper C, Prosser JI. (2008). The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ Microbiol* **10**: 2966–2978.

Nowka B, Daims H, Spieck E. (2015). Comparison of Oxidation Kinetics of Nitrite-Oxidizing Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation Voordouw G (ed). *Appl Environ Microbiol* **81**: 745–753.

Ochsenreiter T, Selezi D, Quaiser A, Bonch-Osmolovskaya L, Schleper C. (2003). Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol* **5**: 787–797.

Odum EP. (1953). Fundamentals of ecology. In: *Philadelphia: W. B. Saunders Company*. Wiley-Blackwell, p 383.

Olsson G, Newell B. (1999). Wastewater Treatment Systems: Modelling, Diagnosis and Control. IWA Pub.

Legendre P, Borcard D, Peres-Neto P.R. (1996). Analyzing or Explaining Beta Diversity? Comment. *Ecology* **89**: 3227–3232.

Ordonez A, Svenning JC. (2015). Geographic patterns in functional diversity deficits are linked to glacial-interglacial climate stability and accessibility. *Glob Ecol Biogeogr* **24**: 826–837.

Palomo-González A. (2017). Discovery and description of complete ammonium oxidizers in groundwater-fed rapid sand filters. http://orbit.dtu.dk/files/133577209/Thesis_online_version_Alejandro_Palomo.pdf.

Palomo A, Jane Fowler S, Gülay A, Rasmussen S, Sicheritz-Ponten T, Smets BF. (2016). Metagenomic analysis of rapid gravity sand filter microbial communities suggests novel physiology of Nitrospira spp. *ISME J* **10**: 2569–2581.

Palomo A, Pedersen AG, Fowler SJ, Dechesne A, Sicheritz-Pontén T, Smets BF. (2018). Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox Nitrospira. *ISME J* **12**: 1779–1793.

Pan KL, Gao JF, Li HY, Fan XY, Li DC, Jiang H. (2018). Ammonia-oxidizing bacteria dominate ammonia oxidation in a full-scale wastewater treatment plant revealed by DNA-based stable isotope probing. *Bioresour Technol* **256**: 152–159.

Park HD, Wells GF, Bae H, Griddle CS, Francis CA. (2006). Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Appl Environ Microbiol* **72**: 5643–5647.

Park MR, Park H, Chandran K. (2017). Molecular and Kinetic Characterization of Planktonic Nitrospira spp. Selectively Enriched from Activated Sludge. *Environ Sci Technol* **51**: 2720–2728.

Park S, Bae W, Chung J, Baek SC. (2007). Empirical model of the pH dependence of the maximum specific nitrification rate. *Process Biochem* **42**: 1671–1676.

Paulson JN, Colin Stine O, Bravo HC, Pop M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* **10**: 1200–1202.

Peres-Neto P-R, Leibold M, Dray S. (2012). Assessing the effects of spatial contingency and environmental filtering on metacommunity phylogenetics. *Ecology* **93**: 14–30.

Pester M, Maixner F, Berry D, Rattei T, Koch H, Lücker S, *et al.* (2014). NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing Nitrospira. *Environ Microbiol* **16**: 3055–3071.

Pester M, Rattei T, Flechl S, Gröngröft A, Richter A, Overmann J, *et al.* (2012). amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions. *Environ Microbiol* **14**: 525–39.

Pintar KD., Slawson RM. (2003). Effect of temperature and disinfection strategies on ammonia-oxidizing bacteria in a bench-scale drinking water distribution system. *Water Res* **37**: 1805–1817.

Pinto AJ, Marcus DN, Ijaz UZ, Bautista-de lose Santos QM, Dick GJ, Raskin L. (2016). Metagenomic Evidence for the Presence of Comammox Nitrospira -Like Bacteria in a Drinking Water System. *mSphere* **1**: e00054-15.

Pinto AJ, Xi C, Raskin L. (2012). Bacterial community structure in the drinking water microbiome is governed by filtration processes. *Environ Sci Technol* **46**: 8851–8859.

Poretsky R, Rodriguez-R LM, Luo C, Tsementzi D, Konstantinidis KT. (2014). Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS One* **9**: e93827.

Powlson DS, Addiscott TM, Benjamin N, Cassman KG, de Kok TM, van Grinsven H, *et al.* (2008). When Does Nitrate Become a Risk for Humans? *J Environ Qual* **37**: 291.

Pratscher J, Dumont MG, Conrad R. (2011). Ammonia oxidation coupled to CO2 fixation by archaea and bacteria in an agricultural soil. *Proc Natl Acad Sci* **108**: 4170–4175.

Preston FW. (1960). Time and Space and the Variation of Species. *Ecology* **41**: 611–627.

Prevost M. (1991). BIOLOGICAL TREATMENT OF DRINKING WATER A REVIEW. *Sci Tech L'eau* **24**: 311+319.

Prosser JI. (1990). Autotrophic Nitrification in Bacteria. *Adv Microb Physiol* **30**: 125–181.

Prosser JI. (2007). The Ecology of Nitrifying Bacteria. Elsevier B.V. **2**: 223-243.

Prosser JI, Bohannan BJM, Curtis TP, Ellis RJ, Firestone MK, Freckleton RP, *et al.* (2007). The role of ecological theory in microbial ecology. *Nat Rev Microbiol* **5**: 384–392.

Qin W, Amin SA, Martens-Habbena W, Walker CB, Urakawa H, Devol AH, *et al.* (2014). Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation. *Proc Natl Acad Sci* **111**: 12504–12509.

Ramanathan B, Boddicker AM, Roane TM, Mosier AC. (2017). Nitrifier Gene Abundance and Diversity in Sediments Impacted by Acid Mine Drainage. *Front Microbiol* **8**: 1–16.

Rashid M, Stingl U. (2015). Contemporary molecular tools in microbial ecology and their application to advancing biotechnology. *Biotechnol Adv* **33**: 1755–1773.

Richter A, Hatzenpichler R, Lebedeva E V., Wagner M, Daims H, Stoecker K, *et al.* (2008). A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci* **105**: 2134–2139.

De Roos AJ, Ward MH, Lynch CF, Cantor KP. (2003). Nitrate in public water supplies and the risk of colon and rectum cancers. *Epidemiology* **14**: 640–649.

Rotthauwe JH, Witzel KP, Liesack W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker Molecular fine-scale analysis of natural ammonia-oxidizing populations. **63**: 4704–4712.

Scholz MB, Lo CC, Chain PSG. (2012). Next generation sequencing and bioinformatic bottlenecks: The current state of metagenomic data analysis. *Curr Opin Biotechnol* **23**: 9–15.

Schramm A, De Beer D, Van Den Heuvel JC, Ottengraf S, Amann R. (1999). Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. *Appl Environ Microbiol* **65**: 3690–3696.

Shade A. (2016). Diversity is the question, not the answer. *ISME J* **4**: e2287v1.

Sipos R, Székely AJ, Palatinszky M, Révész S, Márialigeti K, Nikolausz M. (2007). Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. *FEMS Microbiol Ecol* **60**: 341–350.

Sloan WT, Lunn M, Woodcock S, Head IM, Nee S, Curtis TP. (2006). Quantifying the roles of immigration and chance in shaping prokaryote community structure. *Environ Microbiol* **8**: 732–740.

Smith DP, Peay KG. (2014). Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. *PLoS One* **9**. e-pub ahead of print, doi: 10.1371/journal.pone.0090234.

Sogaard EG, Madsen HT. (2013). Groundwater Chemistry and Treatment: Application to Danish Waterworks. InTech.

Sohn MB, Du R, An L. (2015). A robust approach for identifying differentially abundant features in metagenomic samples. *Bioinformatics* **31**: 2269–2275.

Soliman M, Eldyasti A. (2018). Ammonia-Oxidizing Bacteria (AOB): opportunities and applications—a review. *Rev Environ Sci Biotechnol* **17**: 285–321.

Sophocleous M. (2002). Interactions between groundwater and surface water: The state of the science. *Hydrogeol J* **10**: 52–67.

Sorensen BL, Møller RR. (2013). Evaluation of total groundwater abstraction from public waterworks in Denmark using principal component analysis. *Geol Surv Denmark Greenl Bull* 37–40.

Sorokin DY, Lücker S, Vejmelkova D, Kostrikina NA, Kleerebezem R, Rijpstra WIC, *et al.* (2012). Nitrification expanded: Discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. *ISME J* **6**: 2245–2256.

Spang A, Stieglmeier M, Schintlmeister A, Richter A, Wagner M, Engel M, *et al.* (2011). Nitrososphaera viennensis, an ammonia oxidizing archaeon from soil. *Proc Natl Acad Sci* **108**: 8420–8425.

Stegen JC, Fredrickson JK, Wilkins MJ, Konopka AE, Nelson WC, Arntzen E V., *et al.* (2016). Groundwater–surface water mixing shifts ecological assembly processes and stimulates organic carbon turnover. *Nat Commun* **7**: 11237.

Stegen JC, Lin X, Fredrickson JK, Chen X, Kennedy DW, Murray CJ, *et al.* (2013). Quantifying community assembly processes and identifying features that impose them. *ISME J* **7**: 2069–2079.

Stegen JC, Lin X, Fredrickson JK, Konopka AE. (2015). Estimating and mapping ecological processes influencing microbial community assembly. *Front Microbiol* **6**: 370.

Stegen JC, Lin X, Konopka AE, Fredrickson JK. (2012). Stochastic and deterministic assembly processes in subsurface microbial communities. *ISME J* **6**: 1653–1664.

Stein LY, Arp DJ, Berube PM, Chain PSG, Hauser L, Jetten MSM, *et al.* (2007). Whole-genome analysis of the ammonia-oxidizing bacterium, Nitrosomonas eutropha C91: Implications for niche adaptation. *Environ Microbiol* **9**: 2993–3007.

Sudarno U, Winter J, Gallert C. (2011). Effect of varying salinity, temperature, ammonia and nitrous acid concentrations on nitrification of saline wastewater in fixed-bed reactors.

Bioresour Technol **102**: 5665–5673.

Swenson NG. (2011). Phylogenetic beta diversity metrics, trait evolution and inferring the functional beta diversity of communities Hector A (ed). *PLoS One* **6**: e21264.

Takai T, Hirata A, Yamauchi K, Inamori Y. (1997). Effects of temperature and volatile fatty acids on nitrification-denitrification activity in small-scale anaerobic-aerobic recirculation biofilm process. In: Vol. 35. *Water Science and Technology*. No longer published by Elsevier, pp 101–108.

Tatari K, Musovic S, Gülay A, Dechesne A, Albrechtsen HJ, Smets BF. (2017). Density and distribution of nitrifying guilds in rapid sand filters for drinking water production: Dominance of Nitrospira spp. *Water Res* **127**: 239–248.

Tekerlekopoulou AG, Pavlou S, Vayenas D V. (2013). Removal of ammonium, iron and manganese from potable water in biofiltration units: A review. *J Chem Technol Biotechnol* **88**: 751–773.

Thapa Chhetri R, Suzuki I, Takezaki J, Tabusa H, Takeda M, Koizumi J. (2013). Bacterial Diversity in Biological Filtration Plant for the Removal of Iron and Manganese from Groundwater. https://www.jstage.jst.go.jp/article/jwet/11/1/11_33/_pdf (Accessed August 28, 2018).

Tilman D. (2004). Niche tradeoffs, neutrality, and community structure: a stochastic theory of resource competition, invasion, and community assembly. *Proc Natl Acad Sci U S A* **101**: 10854–61.

Tilman D. (1982). Resource competition and community structure. Princeton University Press.

Tilman D, Kilham SS, Kilham P. (1982). Phytoplankton Community Ecology: The Role of Limiting Nutrients. *Annu Rev Ecol Syst* **13**: 349–372.

Tourna M, Freitag TE, Nicol GW, Prosser JI. (2008). Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environ Microbiol* **10**: 1357–64.

Urich T, Schleper C, Daims H, Schwark L, Reigstad LJ, Richter A. (2008). Nitrification in terrestrial hot springs of Iceland and Kamchatka. *FEMS Microbiol Ecol* **64**: 167–174.

VandCenterSyd. (2013). VandCenter Syd. *VandCenter Syd*. http://www.vandcenter.dk/viden/drikkevand (Accessed August 20, 2018).

Vannette RL, Fukami T. (2014). Historical contingency in species interactions: Towards niche-based predictions. *Ecol Lett* **17**: 115–124.

Vanparys B, Spieck E, Heylen K, Wittebolle L, Geets J, Boon N, *et al.* (2007). The phylogeny of the genus Nitrobacter based on comparative rep-PCR, 16S rRNA and nitrite oxidoreductase gene sequence analysis. *Syst Appl Microbiol* **30**: 297–308.

Vaz-Moreira I, Egas C, Nunes OC, Manaia CM. (2013). Bacterial diversity from the source to the tap: A comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. *FEMS Microbiol Ecol* **83**: 361–374.

Vellend M. (2010). Conceptual Synthesis in Community Ecology. *Q Rev Biol* **85**: 183–206.

Vellend M, Srivastava DS, Anderson KM, Brown CD, Jankowski JE, Kleynhans EJ, *et al.* (2014). Assessing the relative importance of neutral stochasticity in ecological

communities. *Oikos* **123**: 1420–1430.

Venkataraman A, Bassis CM, Beck JM, Young VB, Curtis JL, Huffnagle GB, *et al.* (2015). Application of a neutral community model to assess structuring of the human lung microbiome. *MBio* **6**: 1–9.

Vergnon R, van Nes EH, Scheffer M. (2012). Emergent neutrality leads to multimodal species abundance distributions. *Nat Commun* **3**: 663.

Verhamme DT, Prosser JI, He J-Z, Zhang L-M, Nicol GW, Offre PR. (2010). Autotrophic ammonia oxidation by soil thaumarchaea. *Proc Natl Acad Sci* **107**: 17240–17245.

Verhamme DT, Prosser JI, Nicol GW. (2011). Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *ISME J* **5**: 1067–1071.

Verstraete W, Wittebolle L, Heylen K, Vanparys B, de Vos P, van de Wiele T, *et al.* (2007). Microbial Resource Management: The Road To Go for Environmental Biotechnology. *Eng Life Sci* **7**: 117–126.

Vignola M, Werner D, Wade MJ, Meynet P, Davenport RJ. (2018). Medium shapes the microbial community of water filters with implications for effluent quality. *Water Res* **129**: 499–508.

Wagner FB. (2017). Importance of copper for nitrification in biological rapid sand filters for drinking water production. http://orbit.dtu.dk/files/140496805/WWW_Version.pdf (Accessed August 20, 2018).

Wagner FB, Nielsen PB, Boe-Hansen R, Albrechtsen HJ. (2016). Copper deficiency can limit nitrification in biological rapid sand filters for drinking water production. *Water Res* **95**: 280–288.

Wagner FB, Nielsen PB, Boe-Hansen R, Albrechtsen HJ. (2018). Remediation of incomplete nitrification and capacity increase of biofilters at different drinking water treatment plants through copper dosing. *Water Res* **132**: 42–51.

Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, Daims H. (2002). Microbial community composition and function in wastewater treatment plants. *Antonie Van Leeuwenhoek* **81**: 665–680.

Wagner M, Rath G, Koops HP, Flood J, Amann R. (1996). In situ analysis of nitrifying bacteria in sewage treatment plants. In: Vol. 34. *Water Science and Technology*. No longer published by Elsevier, pp 237–244.

Waldrop MP, Zak DR, Blackwood CB, Curtis CD, Tilman D. (2006). Resource availability controls fungal diversity across a plant diversity gradient. *Ecol Lett* **9**: 1127–1135.

Wang JG, Xia F, Zeleke J, Zou B, Rhee SK, Quan ZX. (2017). An improved protocol with a highly degenerate primer targeting copper-containing membrane-bound monooxygenase genes for community analysis of methane- and ammonia-oxidizing bacteria Laanbroek R (ed). *FEMS Microbiol Ecol* **93**: fiw244.

Wang Y, Qian PY. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies Field D (ed). *PLoS One* **4**: e7401.

Wells GF, Park HD, Yeung CH, Eggleston B, Francis CA, Criddle CS. (2009). Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor:

Betaproteobacterial dynamics and low relative abundance of Crenarchaea. *Environ Microbiol* **11**: 2310–2328.

Van Der Wielen PWJJ, Voost S, Van Der Kooij D. (2009). Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems. *Appl Environ Microbiol* **75**: 4687–4695.

Wittebolle L, Vervaeren H, Verstraete W, Boon N. (2008). Quantifying community dynamics of nitrifiers in functionally stable reactors. *Appl Environ Microbiol* **74**: 286–293.

Xu S, Yao J, Ainiwaer M, Hong Y, Zhang Y. (2018). Analysis of Bacterial Community Structure of Activated Sludge from Wastewater Treatment Plants in Winter. *Biomed Res Int* **2018**: 1–8.

Yan Q, Li J, Yu Y, Wang J, He Z, Van Nostrand JD, *et al.* (2016). Environmental filtering decreases with fish development for the assembly of gut microbiota. *Environ Microbiol* **18**: 4739–4754.

Yao Q, Peng DC. (2017). Nitrite oxidizing bacteria (NOB) dominating in nitrifying community in full-scale biological nutrient removal wastewater treatment plants. *AMB Express* **7**: 25.

Ye L, Shao MF, Zhang T, Tong AHY, Lok S. (2011). Analysis of the bacterial community in a laboratory-scale nitrification reactor and a wastewater treatment plant by 454-pyrosequencing. *Water Res* **45**: 4390–4398.

Zhang Y, Edwards M. (2010). Nutrients and metals effects on nitrification in drinking water systems. *J / Am Water Work Assoc* **102**: 56–66.

10 Papers

I Dechesne, A.; Musovic, S.; Palomo, A.; **Diwan, V.**; Smets, B. F. Underestimation of Ammonia-Oxidizing Bacteria Abundance by Amplification Bias in AmoA-Targeted QPCR. *Microb. Biotechnol.* 2016, 9 (4), 519–524.

II **Diwan, V.**; Albrechtsen, H-J.; Smets, B. F.; Dechesne, A. Does Universal 16S rRNA Gene Amplicon Sequencing of Environmental Communities Provide an Accurate Description of Nitrifying Guilds? *J. Microbiol. Methods* 2018, *151*, 28–34.

III **Diwan, V.**, Kinnunen, M., Albrechtsen, H-J., Smets, B. F., Dechesne, A. Identifying selection in rapid sand filter microbial communities: Comparison of differential abundance and model-based approaches. *Manuscript in preparation*

IV Wagner, F.B.*, **Diwan, V.***, Dechesne, A., Fowler S. J., Smets, B.F., Albrechtsen, H-J. Copper-induced stimulation of nitrification in biological rapid sand filters for drinking water production by proliferation of *Nitrosomonas* spp. (*Environmental Science and Technology – In Revision).*

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TEXT FOR WWW-VERSION (without papers)

In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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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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