I have no special talents. I am just passionately curious

-Albert Einstein

Abstract

Excess of nitrogen in water bodies causes eutrophication. One important source of nitrogen is the effluent from wastewater treatment plants (WWTPs). Nitrogen in wastewater is most commonly removed by nitrification-denitrification. During nitrification-denitrification, aerobic ammonia oxidizing bacteria (AOB) oxidize ammonium to nitrite, which is in turn oxidized to nitrate by their syntrophic partners; aerobic nitrite oxidizing bacteria. Heterotrophic denitrifiers can then convert the nitrate to harmless nitrogen gas. Partial nitritation-anammox (PNA) is an alternative process for nitrogen removal which is today used for treatment of warm and concentrated sidestreams (reject water after anaerobic sludge digestion) at WWTPs, with potential to be used also for the mainstream of wastewater. PNA relies on bacteria capable of anaerobic ammonium oxidation (anammox) using nitrite as electron acceptor. Together with AOB they convert ammonia to nitrogen gas. To increase retention of biomass in bioreactors, bacteria are often grown in biofilms, microbial communities attached to a surface. The overall aim of this thesis was to study nitrifying- and anammox communities in biofilms, using moving bed biofilm reactors as a model system. Reactor performance, microbial community dynamics and biofilm structure of PNA reactors operated a low temperature or low ammonium concentration were studied, showing community stability, but process instabilities. Differences in composition and ribosomal content between reject- and mainstream communities were investigated, showing that both abundance and bacterial activity are important for explaining differences in process rates. Basic question about biofilm ecology were also studied. Here, for the first time, predation of anammox bacteria in biofilms was demonstrated. Furthermore, it was shown how biofilm thickness influences nitrifying communities and biofilm functions, with differences in community composition and ecosystem function. Together these results help to unravel the link between community composition and bioreactor function for anammox and nitrifying biofilms, which can lead to development of new technologies and strategies for N-removal in wastewater.

Keywords: biofilms, AOB, anammox, partial nitritation-anammox, nitrification, wastewater

List of papers

This thesis is based on the following papers, which will be referred in the text by their Roman numerals:

I. Persson F, Sultana R, **Suarez M**, Hermansson M, Plaza E, Wilén B-M. (2014). Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation–anammox at low temperatures. *Bioresource Technology* **154**: 267–273.

II. **Suarez C**, Persson F, Hermansson M. (2015). Predation of nitritation–anammox biofilms used for nitrogen removal from wastewater. *FEMS Microbiology Ecology* **91**: fiv124.

III. Piculell M, **Suarez C**, Li C, Christensson M, Persson F, Wagner M, Hermansson M, Jönsson K, Welander, T. (2016). The inhibitory effects of reject water on nitrifying populations grown at different biofilm thickness. *Water Research* **104**: 292–302.

IV. Persson F, **Suarez C**, Hermansson M, Plaza E, Sultana R, Wilén B-M. (2017). Community structure of partial nitritation-anammox biofilms at decreasing substrate concentrations and low temperature. *Microbial Biotechnology*. **10**: 761-772

V. **Suarez C**, Piculell M, Modin O, Persson F, Hermansson M. (2017). Biofilm thickness matters. Selection of different functions and communities in nitrifying biofilms. *Submitted*

VI. **Suarez C**, Gustavsson D, Persson F, Hermansson M. (2017). Community structure and ribosomal content in reject and mainstream partial nitritation-anammox biofilms. *Manuscript*.

Papers not included

Persson F, **Suarez C**, Hermansson M, Plaza E, Sultana R, Gustavsson D, Wilén B-M. (2015). Microbial community structure of nitritation-anammox biofilms at main stream conditions. *Proceedings of the conference: IWA Nutrient Removal and Recovery 2015: moving innovation into practice*.

Liebana R, Szabo E, Modin O, Persson F, **Suarez C**, Hermansson M, Wilén B-M. (2015). Stability of nitrifying granules exposed to water flux through a coarse pore mesh. *Proceedings of the conference: IWA Nutrient Removal and Recovery 2015: moving innovation into practice*.

Gustavsson DJ., Persson G, **Suarez C**, Persson F. (2017). Four years of piloting mainstream nitritation-anammox. *Nordic Wastewater Conference*. Aarhaus, Denmark.

Abbreviations

- AOA Ammonia Oxidizing Archaea
- AOB Ammonia Oxidizing Bacteria
- AOM Ammonia Oxidizing Microorganisms
- Anammox Anaerobic ammonium oxidation
- CLSM Confocal laser scanning microscopy
- Comammox Complete ammonia oxidizer
- DNRA Dissimilatory nitrate reduction to ammonium
- FISH Fluorescence in situ hybridization
- IFAS Integrated Fixed Film Activated Sludge
- MBBR Moving Bed Biofilm Reactor
- Nr Reactive nitrogen
- N-cycle Nitrogen cycle
- N-Removal Nitrogen removal
- NGS Next Generation Sequencing
- NOB Nitrite Oxidizing Bacteria
- OCT Optical coherence tomography
- PNA Partial Nitritation Anammox
- rDNA 16S ribosomal RNA gene
- rRNA 16S ribosomal RNA
- WWT Wastewater Treatment
- WWTP Wastewater Treatment Plant

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2 Introduction

The industrial fixation of nitrogen gas (N_2) to produce ammonia (NH_3), known as the Haber-Bosh process has been essential for the development of our modern society (Sutton *et al.*, 2011). The use of NH_3 to produce fertilizer allowed a large population growth during the 20th century. However around half the global nitrogen fixation is now done by humans (Fowler *et al.*, 2013). A considerable part of this reactive nitrogen (Nr) eventually leaches into the environment; for example as runoff of ammonia from agricultural fields or as nitrogen in wastewater discharges (Erisman *et al.*, 2011).

Nitrogen together with phosphorus are two of the nutrients limiting productivity in ecosystems. The excess Nr in the environment has led to negative environmental effects influencing global warming (Stocker *et al.*, 2014), as well as reduced soil, water and air quality (Erisman *et al.*, 2011). Runoff of ammonia to water bodies contributes to eutrophication. The social cost of nitrogen leaching is estimated to be in the range of 5-20 €/Kg N, with overall cost of €70–€320 billion per year in Europe (Brink *et al.*, 2011)

Part of the strategy to reduce nitrogen pollution in water bodies is nitrogen removal (N-removal) from wastewater. N-removal in wastewater treatment plants (WWTPs) is achieved through biological process where microorganisms are used to remove ammonium (NH_4^+) from the water and produce harmless N_2. Traditionally this has been done using the process known as nitrification-denitrification. This is an energy intensive process and it is associated with emissions of greenhouses gases such as carbon dioxide (CO_2) and nitrous oxide (N_2O). An alternative to nitrification-denitrification is the anammox process (Lackner *et al.*, 2014), where anammox bacteria are used.

Even though nitrifying bacteria were discovered more than a century ago (Winogradsk, 1890), much is still unknown about microorganisms involved in nitrogen transformations. For instance, anammox bacteria were identified only in 1999 (Strous *et al.*, 1999). New microorganism associated with the nitrogen cycle (N-cycle) are still being discovered (Daims *et al.*, 2015; van Kessel *et al.*, 2015). The study of microorganism involved in the N-cycle has been challenging given the

difficulties of isolating and growing these organism in pure cultures. Furthermore nitrogen transformations in ecosystems (natural or artificial) are often multi-step processes involving several microorganisms. Fortunately the development of molecular techniques has facilitated the study of mixed microbial communities. Nonetheless many questions remain about anammox and nitrifying communities. Challenges also exist for a broader implementation of the anammox process in WWTPs.

2.1 Aim

Our aim was to study anammox and nitrifying biofilms and their associated community in wastewater.

Specific aims of this study were:

1. To study how microbial communities in PNA biofilms are affected by changes in temperature and ammonia concentration (**Paper I and IV**).
2. To study if grazing of anammox bacteria and AOB by protozoa occurs in PNA biofilms (**paper II**).
3. To determine how biofilm thickness can affect community composition, spatial distribution of organism, ecosystem function and response to ecological disturbances (**Paper III and V**).
4. To study how temporary exposure to reject water affects nitrifying communities and processes (**paper III**).
5. To compare differences in ribosomal content and community structure for mainstream and reject PNA biofilms (**paper VI**).

3 The nitrogen cycle and nitrogen metabolism

Life requires nitrogen, which is used in essential cellular processes such as nucleotide- and amino acids synthesis. Despite nitrogen being highly abundant in our atmosphere as N_2, most organisms cannot process the unreactive N_2, and thus requires Nr such as NH_4^+ or nitrite (NO_2^-). Organisms capable of nitrogen fixation can catalyze the reduction of N_2 to ammonium and use it. In addition specialized groups of microorganisms can also use Nr as part of redox reactions, in their cellular respiration processes (Stein and Klotz, 2016). A complex cycle exists in nature involving different microorganisms where nitrogen is transformed into different chemical forms (Figure 1).

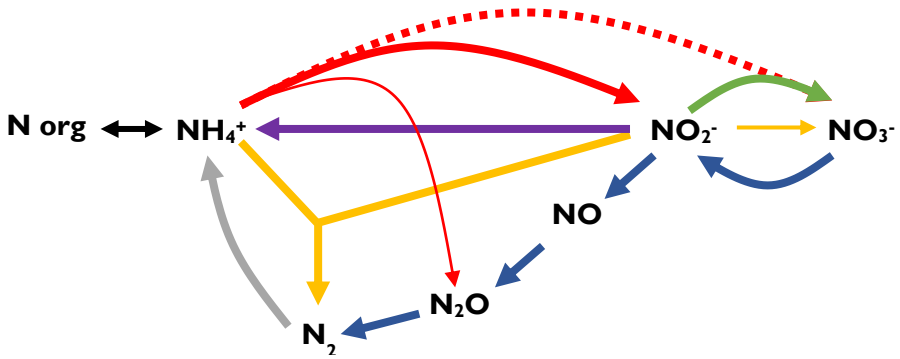

Figure 1 –The nitrogen cycle. Red: ammonia oxidation to nitrite (nitritation), Red-dashed: complete ammonia oxidation to nitrate (comammox). Green: nitrite oxidation to nitrate (nitratation). Yellow: Anammox process. Blue: denitrification. Purple: dissimilatory nitrate reduction to ammonium (DNRA) Grey: nitrogen fixation. Intermediates for nitritation, comammox and anammox are not depicted.

3.1 Nitrification

The oxidation of NH_4^+ to nitrate (NO_3^-) by microorganism is known as **nitrification**, a two-step process where oxygen is used as electron acceptor. In the first step NH_4^+ is oxidized to NO_2^- (**nitritation**) (eq. 1), which is followed by the oxidation of NO_2^- to NO_3^- (**nitratation**) (eq. 2).

1) $NH_3^+ + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O$
2) $NO_2^- + 0.5O_2 \rightarrow NO_3^-$

Ammonia Oxidizing Microorganisms (AOM) are capable of oxidizing NH_4^+ to NO_2^-. Both ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) exist. Known AOB belong to the *betaprotebacteria* (*Nitrosomonas* and *Nitrosospira*) and the *gammaproteobacteria* class (*Nitrosococcus*). All known AOA are members of the phylum *Taumarcheota* (Stahl and de la Torre, 2012). The dominant AOM in wastewater treatment plants appears to be *Nitrosomonas*.

Ammonia is first converted to the intermediate hydroxylamine (NH_2OH) using the enzyme ammonia monoxygenase (AMO) in both AOB and AOA (eq. 3). In AOB the enzyme hydroxylamine oxidoreductase (HAO) is needed for the production of NO_2^- (Bock and Wagner, 2006), however AOA appear to lack this enzyme (Stahl and de la Torre, 2012). The traditional model for nitritation in AOB is that hydroxylamine is converted by HAO into nitrous acid (HNO_2) (eq 4) and therefore nitritation is an acidifying process (Bock and Wagner, 2006). Two electrons of reaction 4 would be used in the respiratory chain with oxygen as terminal electron acceptor (eq 5). However the product of hydroxylamine oxidation by HAO might be nitric oxide (NO) and not NO_2^- (Caranto and Lancaster, 2017). The NO produced by HAO will likely be oxidized to NO_2^- either abiotically or by an unknown enzyme (Caranto and Lancaster, 2017).

3) $NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{AMO} NH_2OH + H_2O$
4) $NH_2OH + H_2O \xrightarrow{HAO} HNO_2 + 4H^+ + 4e^-$
5) $0.5\,O_2 + 2H^+ + 2e^- \rightarrow H_2O$

NO is an essential intermediate for ammonia oxidation in AOA (Kozlowski *et al.*, 2016b; Sauder *et al.*, 2016). A possible mechanism for NO_2^- production in AOA involves the reaction of hydroxylamine and NO by an unknown enzyme (eq 6). The NO would be produced by a copper nitrite reductase (NirK) (eq 7) (Kozlowski *et al.*, 2016b).

6) $NH_2OH + NO + H_2O \xrightarrow{?} 2NO_2^- + 7H^+ + 5e^-$
7) $NO_2^- + 2H^+ + e^- \xrightarrow{NIR} NO + H_2O$

The NO_2^- produced by AOM can be further oxidized to NO_3^- by nitrite oxidizing bacteria (NOB). This reaction is catalyzed by the enzyme nitrite oxydoreductase (NXR) (eq. 8). With oxygen as electron acceptor, the two electrons of reaction 8 are then used in the respiratory chain.

8) $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$

Nitrobacter (alphaproteobacteria) and *Nitrospira* (*Nitrospirae*) are the traditional NOB in WTTPs. However two additional NOB have recently been discovered: the betaproteobacterium *Nitrotoga*, a cold tolerant NOB that appears to be present in many WWTPs (Lücker *et al.*, 2015) and *Nitrolancea hollandicus* belonging to the phylum *Chloroflexi* (Sorokin *et al.*, 2012). Other known NOB which are associated with marine environments are *Nitrospina* in the *Nitrospinae* phylum (Luecker *et al.*, 2013) and the gammaproteobacteria *Nitrococcus* (Watson and Waterbury, 1971).

The oxidation of NO_2^- to NO_3^- by NOB is dependent in the presence of AOM supplying NO_2^-. AOM likely benefits by the removal of the toxic NO_2^-. Furthermore some *Nitrospira* can convert urea to ammonium, supplying it to urease negative AOM (Koch *et al.*, 2015), which in turn supply NO_2^- to *Nitrospira*. Likewise, *Nitrospira* with the enzyme cyanase, can supply cyanase-negative AOM with ammonium from cyanate (Palatinszky *et al.*, 2015).

Complete oxidation of NH_4^+ to NO_3^- (**comammox**) by a single microorganism is also possible in some members of the genus *Nitrospira* (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Comammox bacteria have been found to be ubiquitous (Pinto *et al.*, 2016; Pjevac *et al.*, 2017), although its relevance to PNA and nitrifying biofilms in WTTPs is still largely unknown. *Nitrospira* is a versatile group of microorganisms, with metabolic functions not restricted to nitrification (Daims *et al.*, 2016), blurring the link between function and identity.

Nitrous oxide (N_2O) a greenhouse gas (Stocker *et al.*, 2014), can be released by organisms involved in nitritation (Wrage *et al.*, 2001; Shaw *et al.*, 2006). Several mechanism are believed to be involved in N_2O production during ammonia oxidation: nitrifier-denitrification in AOB where NO_2^- is used as electron acceptor, abiotic N_2O production from nitrification intermediates, incomplete HAO activity, or conversion of the intermediate NO into N_2O for both AOB and AOA (Wrage *et al.*, 2001; Caranto and Lancaster, 2017; Kozlowski *et al.*, 2016b, 2016a)

3.2 Denitrification

Nitrate and NO_2^- can be reduced to N_2 by a group of heterotrophic microorganism known as denitrifiers. These microorganisms typically use organic carbon as electron donor and NO_3^- or NO_2^- as electron acceptor in anaerobic conditions. **Denitrification** is a process with a broad phylogenetic distribution,

present among many organisms in all the three domains of life (Thamdrup, 2012; Stein and Klotz, 2016).

Denitrification is a multi-step process requiring multiple enzymes (Figure 1). However not all denitrifiers have the complete repertoire of enzymes needed for complete denitrification. This incomplete denitrification is associated with emissions of nitrous oxide (N_2O) (Stein and Klotz, 2016).

3.3 The anammox process

NO_2^- is used an electron acceptor, and NH_4^+ and as electron donor in the process known as anaerobic ammonium oxidation (**anammox**) (Mulder *et al.,* 1995). N_2 is the main product (eq. 12) of the anammox reaction although some NO_3^- is also produced (Kartal *et al.*, 2013). The reaction is carried out by a monophyletic group within *Planctomycetes* (Strous *et al.*, 1999), belonging to the order *Brocadiales*. Five different anammox genera have been identified: *Candidatus* Brocadia (*Brocadia*), *Candidatus* Kuenenia, *Candidatus* Jettenia, *Candidatus* Anammoxoglobus and *Candidatus* Scalindua (Jetten *et al.*, 2010). Although it was believed that anammox bacteria lacked peptidoglycan in their cell walls, its presence was recently shown, confirming that they are gram-negative bacteria (van Teeseling *et al.*, 2015). Inside the cytoplasm (known also as riboplasm) a ribosome free-organelle (the anammoxosome) is located (van Niftrik *et al.*, 2008). The anammoxosome is the location were the anammox catabolism is carried out (Kartal *et al.*, 2013).

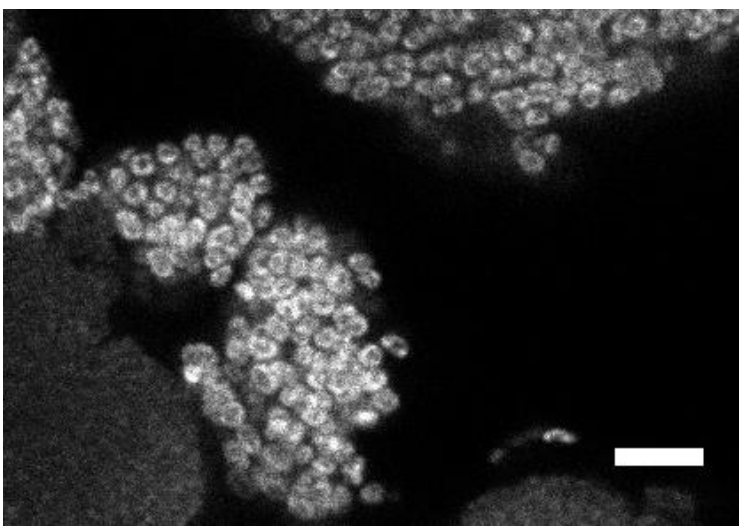

Figure 2 – FISH-CLSM picture of an anammox bacteria aggregate. The central anammoxosome is a free-ribosome organelle, hence is not targeted by the rRNA FISH probes. This gives the donut shape typical of FISH images of anammox bacteria. Scale bar: 5µm.

The anammox metabolism is unique. Either an iron nitrite reductase (NirS) (Strous *et al.*, 2006) or a copper nitrite reductase (NirK) (Hira *et al.*, 2012; Park *et al.*, 2017a) are used by the anammox bacteria to produce the intermediate nitric oxide (NO) from NO_2^- (eq. 9) (Kartal *et al.*, 2011). Hydrazine synthase, HZS uses NH_4^+ and NO as substrates to produce the intermediate hydrazine (N_2H_4) (eq. 10) (Kartal *et al.*, 2011). Finally N_2H_4 is oxidized to N_2 by Hydrazine dehydrogenase (HDH) (also known as hydrazine oxidoreductase, HZO) (eq. 11). The reactions are used for the creation of an electrochemical gradient across the anammoxosome membrane (Kartal *et al.*, 2011). ATP is believed to be produced by an ATP synthase on the anammoxosome membrane (Van Niftrik *et al.*, 2010). Carbon fixation is done by the acetyl-CoA pathway. Electrons lost by intermediate leakages or used for carbon fixation are replenished by oxidation of NO_2^- to NO_3^- by a nitrate reductase. Some members of *Brocadia* appear to lack both NirK and NirS (Ali *et al.*, 2016; Oshiki *et al.*, 2015; Liu *et al.*, 2017; Lawson *et al.*, 2017). It has been proposed that *Brocadia sinica* which lacks both NirK and NirS, might reduce NO_2^- to hydroxylamine, which then will be used by HZS together with NH_4^+ to produce hydrazine (Oshiki *et al.*, 2016).

9) $NO_2^- + 2H^+ + e^- \xrightarrow{\text{NirS/NirK}} NO + H_20$

10) $NH_4^+ + NO + 2H^+ + 3e^- \xrightarrow{\text{HZS}} N_2H_4 + H_20$

11) $N_2H_4 \xrightarrow{\text{HDH}} N_2 + 4H^+ + 4e^-$

12) Overall: $NO_2^- + NH_4^+ \rightarrow N_2 + 2H_20$

Anammox bacteria can also be considered as chemoorganotrophs, since organic electron donors can be coupled to reduction of NO_3^- by the anammox bacteria (Kartal *et al.*, 2007). Furthermore anammox bacteria are able to use NO (Kartal *et al.*, 2010) to oxidize ammonia. The reader is invited to read the review by Kartal *et al.* (2013) for more details on the anammox metabolism.

3.4 Dissimilatory nitrate reduction to ammonium

Another pathway for reduction of NO_3^- is dissimilatory nitrate reduction to ammonium (DNRA) (Stein and Klotz, 2016). It is believed that DNRA is favored

over denitrification at NO_3^- limiting conditions (van den Berg *et al.*, 2015). DNRA has been less studied than denitrification in the context of WWT. However the low Carbon:Nitrogen ratios used for N-removal in WWTPs, suggest that denitrification rather than DNRA might be more relevant in those conditions.

A more complex picture emerges with the linking of the anammox process and DNRA. Some anammox bacteria are capable of doing DNRA coupled with the oxidation of volatile fatty acids (Kartal *et al.*, 2007). Likewise DNRA coupled to iron oxidation has been observed in anammox bacteria (Oshiki *et al.*, 2013). The NH_4^+ produced in DNRA can then be used in the anammox process (Kartal *et al.*, 2007; Oshiki *et al.*, 2013).

4 Removing nitrogen from wastewater

The benefits we obtain from ecosystems are known as ecosystem services, including food, recreation and oxygen among many others (Carpenter, 2005). A Wastewater Treatment Plant is an artificial ecosystem where microbial communities are engineered for providing an ecosystem service: water purification (Graham and Smith, 2004).

The traditional method for N-removal in WWT has been nitrification-denitrification (**Figure 3**-A). Here, first NH_4^+ is converted to NO_3^- by AOB and NOB, in an aerobic process requiring aeration. Secondly, the NO_3^- is transformed to N_2, by heterotrophic denitrifiers. Although nitrification-denitrification is an established technology, the process has large energy requirements for aeration and the addition of methanol as external carbon source for denitrification (post-denitrification) or extensive recycling of wastewater (pre-denitrification) with the energy associated costs of pumping (Kartal *et al.*, 2010). Furthermore, the process is associated with emissions of N_2O and CO_2, contributing to global warming.

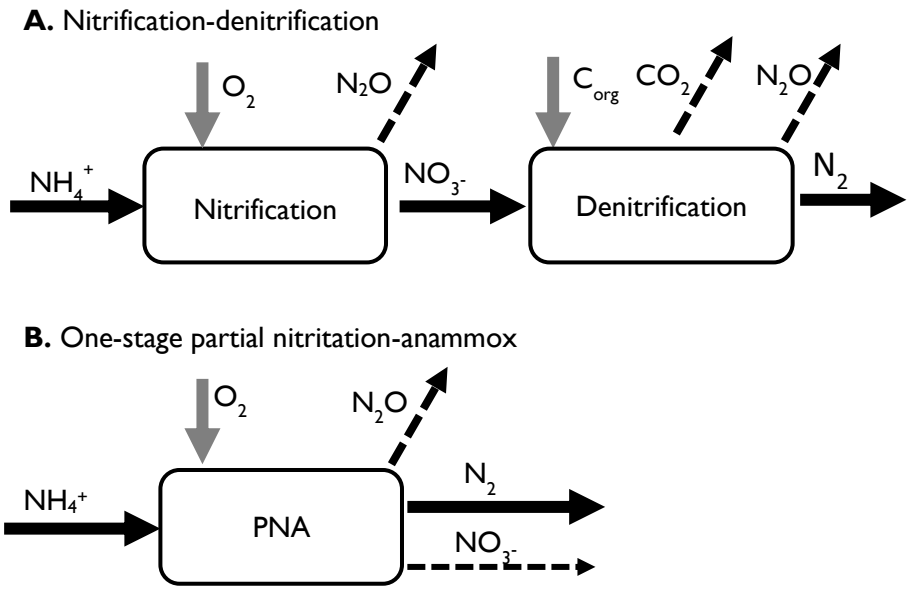

Figure 3 – Two of the main N-removal strategies in WWTP. A) Nitrification-denitrification. B) Partial nitritation-anammox. Boxes represent bioreactors. Major nitrogen fluxes are shown as black solid arrows. The gray lines represent requirements for the process. Undesired byproducts of the biological reactions are shown as black dashed lines.

Another strategy for N-removal is Partial Nitritation Anammox (PNA). Here half of the NH_4^+ is oxidized only to NO_2^- by AOB, thus reducing aeration costs; the remaining NH_4^+ and the NO_2^- are converted to N_2 by anammox bacteria, which also eliminates the organic carbon requirements (equation 13). PNA can be configured as two consecutive bioreactors (two-stage) separating the nitritation and anammox processes, or as a single reactor (one-stage) where both processes are combined (Figure 3-B).

$$13)\ 2\,NH_4^+ + 1.5\,O_2 \rightarrow\ N_2 + 2H^+\ 3H_20$$

In theory up to an 89% of nitrogen removal can be achieved with PNA, with 11% being converted to NO_3^- during anammox metabolism (Kartal *et al.*, 2013; Strous *et al.*, 1998), but see Lotti *et al.* (2014c). PNA is used in several WTTPs for reject water treatment (Lackner *et al.*, 2014), i.e. water from anaerobic sludge digestion with high ammonium concentration and high temperature. A problem with anammox systems is the slow growth of the anammox bacteria (Strous *et al.*, 1999). Hence retention of anammox biomass in the bioreactor becomes very important, which can be achieved by providing conditions that favors biofilm formation.

5 Biofilms and bioreactors

Many bacteria have two lifestyles, either as free-living planktonic bacteria or living in communities attached to a substrate know as biofilms. Biofilms are microbial communities attached to each other and/or a surface and surrounded by an extracellular matrix (Flemming *et al.*, 2016). These are complex communities where redox gradients can be found and complex ecological interactions are observed (Stewart and Franklin, 2008).

Autotrophs like nitrifiers and anammox bacteria are relative slow growing bacteria, which could lead to a biomass washout from the bioreactor and eventual process loss. However these bacteria can form biofilms and this ability is useful for wastewater treatment. By enhancing biofilm formation, biomass can be retained, increasing process stability. Several biofilm strategies exist for bioreactors, among them granules, trickling filters, rotating biological contactors and MBBRs (moving bed biofilm bioreactors). In MBBRs small plastics carriers are used in the bioreactor, which are retained. The carries offer a protected area where the biofilm can growth (Figure 4).

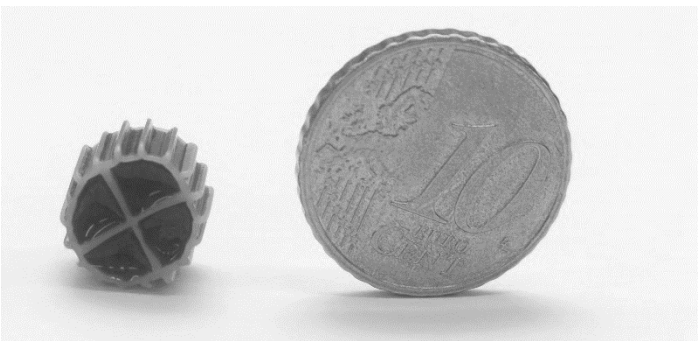

Figure 4 - K1 carrier (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden) with a PNA biofilm. A 10 euro cent coin is shown for size comparison.

5.1 Gradients in biofilms

Diffusion is limited in biofilms, and thus oxygen in biofilms is quickly consumed close to the water phase of the biofilm by aerobic microorganism, (Stewart, 2003). Microsensor measurements (Mašić *et al.*, 2010; Schramm *et al.*, 1996; Gieseke *et al.*, 2003) and mathematical modelling (**paper V**, Mašić *et al.*, 2010) has shown an oxygen gradient trough biofilms, with anoxic regions at the bottom of the biofilm.

Several factors affect the oxygen gradient in a biofilm, including the amount of aerobic bacteria, density of the biofilm, oxygen concentration in the water phase (Schramm *et al.*, 1996) and thickness of the boundary layer (Mašić *et al.*, 2010; De Beer *et al.*, 1996). The boundary layer is the region next to the biofilm-water interphase where flow is slower, its thickness being affected by flow velocity (De Beer *et al.*, 1996). Since biofilm carriers move freely through the bioreactor, flow velocity and thus thickness of the boundary layer are likely not to be constant.

The microbial community is responsible for the oxygen gradients in the biofilm, but the community itself is also affected by those oxygen gradients in the biofilm. Microsensor measurements combined with FISH in cryosections have shown that in nitrifying biofilms *Nitrosomonas* are preferentially located in the oxic regions of the biofilm (Schramm *et al.*, 1996), while *Nitrospira* are more abundant in deeper layers of the biofilm (Lydmark *et al.*, 2006; Schramm *et al.*, 2000; Okabe *et al.*, 1999). Anammox bacteria have also been observed in nitrifying biofilms (Lydmark *et al.*, 2006; Egli *et al.*, 2003). Thus the presence of anoxic regions in the biofilm, allows the growth of anaerobic microorganisms, which might use a different electron acceptor that oxygen (Stewart and Franklin, 2008). Stratification of populations in the biofilms (Figure 5) was indeed noticed in all studied biofilms (**paper I, II, IV, V**).

Different microbial populations are thus located in different regions of the biofilm. Since they perform different biochemical reactions, this means that functions in the biofilm are linked to position in the biofilm. This can be used to predict emergent properties of the biofilm or even to go a step forward and design processes such as partial-nitritation anammox.

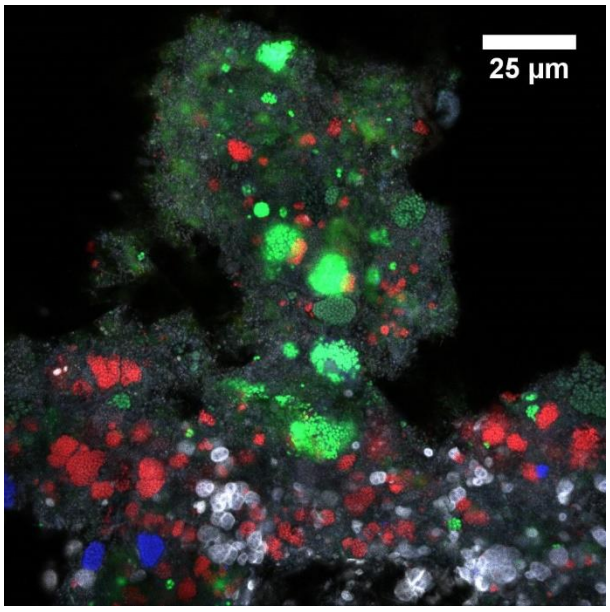

Figure 5 – FISH-CLSM picture of a 400µm thick nitrifying biofilm (Z400 carrier) showing stratification of populations. The water-biofilm interface is on the upper side. Only the upper part of the biofilm is shown. Green: *Nitrosomonas,* Red: *Nitrospira*, Yellow: *Nitrotoga*, Blue: *Brocadia*. White: nucleic acids stained by SYTO40.

5.1.1 Biofilm architecture in PNA

Dissolved oxygen (DO) in one-stage PNA reactors is intentionally low. The aim is that AOB growing in the oxic layers next to the water phase, will consume oxygen and create anoxic regions where anammox can thrive (Figure 6) (Almstrand *et al.*, 2014; Vlaeminck *et al.*, 2010). Anammox are obligate anaerobes, being temporary inhibited by oxygen (Strous *et al.*, 1997). AOB thus can be considered as the syntrophic partner for anammox bacteria providing both conditions and resources needed for anammox growth.

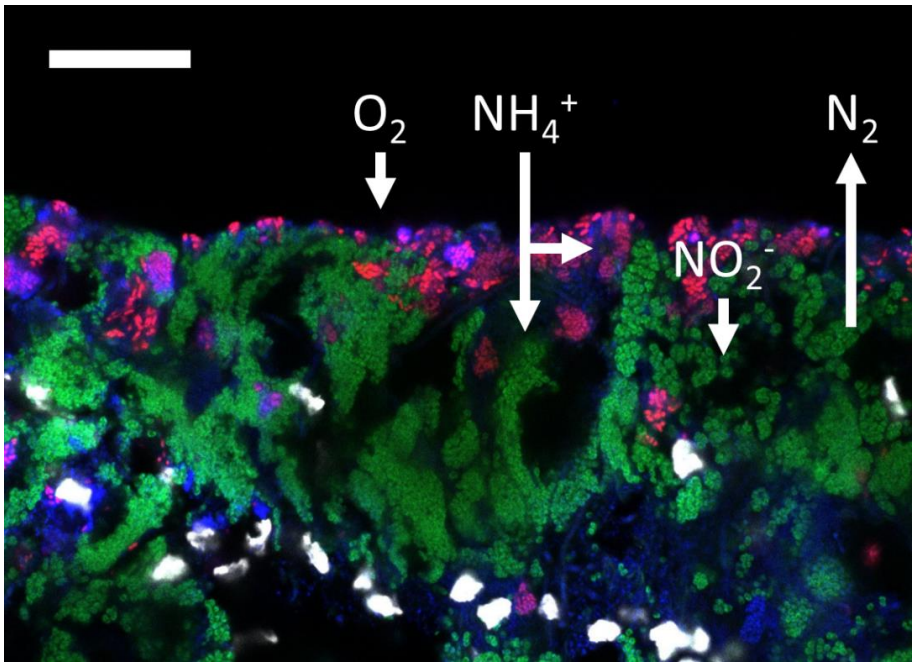

Figure 6 – FISH-CLSM picture showing biofilm stratification in the LTA PNA reactor. Bulk-water is on the top. Oxygen is consumed by AOB (Purple), which oxidize ammonia to nitrite. Green: Anammox bacteria. Purple: AOB. White: Protozoa. Blue: DNA (DAPI). Scale bar: 25μm.

5.2 Reactors and biofilm carriers used in this project

Microbial communities were studied in five large pilot or full-scale bioreactors for N-removal treating real wastewater. The reactors had different configurations (one-stage-PNA-MBBR, IFAS or fully nitrifying MBBR) (Table 1) and were feed with different influent water, with either mainstream wastewater or reject water from anaerobic sludge digestion (Table 2). Unlike PNA-MBBRs or fully nitrifying MBBRs, AOB in IFAS reactors are mostly in the activated sludge phase, while anammox bacteria grown in the biofilm carriers.

Table 1 – List of bioreactors used in this study.

Reactor	Type	Study	Carriers
LTA	One-stage PNA MBBR	I, II, IV	K1
IFAS	IFAS	II	K3
Reject	One-stage PNA MBBR	II, VI	K1
Mainstream	One-stage PNA MBBR	II, VI	K1
NIT	Nitrifying MBBR	III, V	Z50, Z400

LTA (Low Temperature and Ammonium), was a 200L pilot PNA MBBR situated at the Centre for municipal wastewater purification (Hammarby Sjöstadsverk research facility, Stockholm, Sweden). The MMBR was 40% filled with K1 carriers (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden). During the study in **Paper I** the MBBR received reject water from anaerobic sludge digestion. Temperature in the reactor was lowered stepwise in **Paper I** from 19°C to 10°C. Samples for **paper II** were taken during that period. For the duration of **study IV** the MBBR received diluted reject water. Temperature was kept constant at 13°C through this latter study, but influent concentration was lowered from 500 to 45 mg-N l^{-1}.

Samples from a full-scale Integrated Fixed Film Activated Sludge (IFAS) reactor were taken for **study II**. The reactor was filled with 50% K3 carriers (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden). The IFAS reactor was located at the Sjölunda WTTP (Malmö, Sweden) and operated by Veolia Water Technologies- Anoxkaldnes (Lund, Sweden). The reactor is described in detail in Veuillet *et al.* (2014).

Three pilot PNA MBBRs filled with K1 carriers were located at the Sjölunda WTTP (Malmö, Sweden). An MBBR received reject water from anaerobic sludge digestion. Two consecutive MBBRs were feed with mainstream water from a high-rate activated-sludge plant. The reject water MBBR (Reject) and the first of the two mainstream MBBRs (Mainstream) were studied in **paper II** and **VI**. The pilot PNA MBBRs are described in detail in Gustavsson *et al.* (2014).

A 500L nitrifying MBBR was located at Sjölunda WTTP and operated by Veolia Water Technologies AB –Anoxkaldnes (Lund, Sweden). The MBBR was feed with effluent from high-rate activated-sludge. It was filled with a mixture of Z50 and Z400 carriers (Veolia Water Technologies AB - Anoxkaldnes, Lund, Sweden).

Biofilm thickness can be controlled in Z-carriers (Piculell *et al.*, 2016b), and that property was used to study the effect of biofilm thickness in strategies for NOB inhibition (**paper III**) and the microbial community (**paper V**).

Table 2 – Summary of the papers in this thesis.

Paper	Bioreactor	Summary of the experiment	Process	Feed
I	LTA	Change of temperature from 19°C to 10°C	PNA	Reject water from anaerobic sludge digestion
II	LTA, IFAS, Reject, Mainstream	Screening for potential predation of *Brocadia* and *Nitrosomonas* in PNA bioreactors.	PNA,	**LTA, Reject; IFAS:** Reject water from anaerobic sludge digestion. **Mainstream:** Effluent from high-loaded activated-sludge
III	NIT	Exposure to reject water in biofilms with different thickness	Nitrification	Effluent from high-rate activated-sludge
IV	LTA	Change of ammonia concentration from 500 to 45 mg-N l^{-1},	PNA	Reject water from anaerobic sludge digestion, diluted with tap water.
V	NIT	Impact of biofilm thickness on microbial communities.	Nitrification	Effluent from high-rate activated-sludge
VI	Reject, Mainstream	Comparing rDNA and rRNA abundance on reject-fed and mainstream-fed bioreactors	PNA	**Reject:** Reject water from Anaerobic sludge digestion. **Mainstream:** Effluent from high-loaded activated-sludge

6 How do we know who is there?

Who are they? What do they do? These are some of the questions that are faced by microbial ecologists. Molecular methods are the key to solve these question. Methods such as sequencing, Fluorescence in situ hybridization (FISH) and quantitative PCR (qPCR) are often used with 16S rRNA as the target gene. An advantage of using 16S rRNA when studying many N-cycle organisms is that ecological coherence is often observed among them; *i.e.* the process is restricted to few taxa; the exceptions being denitrification and DNRA. This means that 16S rRNA gene sequences can often be used as marker for the presence of N-cycle organism. However detection of other, functional key process genes is still useful, both as phylogenetic and functional markers.

6.1 FISH

Presence of microorganisms in an environmental sample can be assessed with fluorescence in situ hybridization. Here oligonucleotide probes are labeled with a fluorophore to target specific sequences, often in the small ribosomal subunit, either 16S or 18S (Manz *et al.*, 1992). Labeled microorganism can be visualized by Confocal Laser Scanning Microscope (CLSM).

Several populations can be observed simultaneously by using fluorophores with different excitation/emission wavelengths. Three different populations were routinely studied in a CLSM by using Fluorescein or Alexa488, Cy3 and Cy5 fluorophores, excited by 488nm, 555nm and 638nm lasers respectively. Samples were also counterstained with DAPI or SYTO40 (405nm laser).

Double labeling of oligonucleotides, known as DOPE-FISH (Stoecker *et al.*, 2010) can be used to visualize up to six different taxa in a sample (Behnam *et al.*, 2012), by using two different fluorophores in a single oligonucleotide probe. This is known as multicolor-FISH and it was used in **Paper V** to visualize four different populations.

We also combine FISH with biofilm cryosections, to obtain spatial information about the physical location of the target microorganism (For example see Figure

6). Furthermore quantitative information can be obtained by digital image analysis. Examples are quantitative FISH, where abundance of different groups is measured as a fraction of all targeted cell.

FISH is however limited to the detection of cells with ribosome numbers above a certain threshold (Hoshino *et al.*, 2008). Furthermore, similar to PCR, detection of taxa is limited to sequences targeted by the oligonucleotide. Most microbial community studies have focused on bacterial members of the community, ignoring organism in *Archaea* and *Eukarya*, but see **II** for an example where *Eukarya* are targeted. Other factors that might impair detection with FISH are limited probe permeability and possible secondary structures in the rRNA. This means that only a part of the community is detected with FISH (Figure 7).

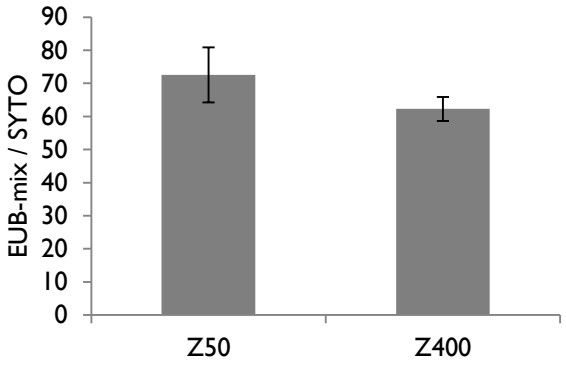

Figure 7 – Fraction of biomass in the NIT reactor that were detected using universal bacteria FISH probes. Total biomass was stained with STYO40. Bacteria were detected with the probes EUB338, EUB338-II, EUB338-III and EUB338-IV. Error bars indicate 95% confidence interval. N=30.

6.2 qPCR

Abundance of the target organism in environmental samples can be assessed by quantitative PCR (qPCR). This has been used in papers **I** and **IV** for measuring time series of replicate samples, since qPCR allows high throughput. Like other PCR approaches, qPCR results are influenced by the method of DNA extraction applied and the PCR primers used.

6.3 Sequencing

A major goal of microbial ecology studies is to know the identity of the studied microorganism: here sequencing of 16S rRNA or functional genes is an obvious approach. Sequencing provides further benefits; new primers and FISH probes can be designed when the sequences of the target organism are known (such as for *Brocadia* in **paper I**). Two different approaches for sequencing were used in our group: clone libraries and high throughput amplicon sequencing.

6.3.1 Clone libraries

Clone libraries and Sanger sequencing have been the traditional sequencing method for many years. However this approach has several limitations, a low number of sequences are obtained and the process is time consuming. Next generation sequencing (NGS) like Illumina have largely replace Sanger sequencing for microbial profiling using 16S rRNA gene (rDNA). Sanger sequencing is still useful though, reads are much longer than those obtained by several NGS methods, this allows us to tell apart closely related organism, as done in **Paper I**.

6.3.2 High throughput amplicon sequencing

A higher number of sequences can be obtained with high throughput amplicon sequencing, with Illumina being the major system used in microbial ecology. MiSeq (Illumina) has become the standard for rDNA profiling. However, MiSeq reads are short, with some increase in read length being obtained by Paired-end sequencing (up to 2x300bp). The read length limitation, means that hypervariable regions in the rDNA are preferred when using MiSeq, with primers targeting the conserved flanking regions. The V4 region is commonly used for bacteria (Caporaso *et al.*, 2011). With high throughput amplicon sequencing, thousands of reads are obtained, which allows quantification of the different taxa present in the community. An additional advantage of MiSeq is Multiplex sequencing, i.e. multiple samples can be analyzed in a single MiSeq run by using barcode sequences (also known as metabarcoding) (Kozich *et al.*, 2013), reducing sequencing costs.

In addition to the limited read length of Illumina, another issue with rDNA gene sequencing is that current detection of microorganism is biased by the "universal" primers used in PCR. A considerable fraction of the bacterial community cannot be detected with primers commonly used for sequencing (Brown *et al.*, 2015). An additional drawback of community analysis using the 16S rRNA gene (rDNA) sequences, is that it might not be representative of the actual abundance of microbial populations. For example multiple copy numbers of the 16S rRNA gene

might inflate the relative abundance of a taxa estimated by 16S sequencing (Větrovský and Baldrian, 2013). Furthermore extracellular DNA in the biofilm might contain 16S rRNA gene fragments leading to overestimation of taxa (Albertsen *et al.*, 2015). An additional complication is that microorganism can be growing, active, dormant or deceased (Blazewicz *et al.*, 2013). Sequencing of rDNA does not allow us to distinguish among those metabolic states.

7 Microbial communities in nitrifying and PNA biofilms

Some of the ecosystem functions used in wastewater treatment are carbon, nitrogen and phosphorus removal, among others. This can be achieved by selecting and growing microorganisms that can perform that function *i.e.* the members of the *functional group*. Nitrogen removal for example might involve the use of bioreactors which contains AOB to perform nitritation, a first step in the removal of nitrogen (Ahn, 2006). Providing an environment where AOB are supplied with oxygen and ammonia creates an *ecological niche* were AOB can thrive.

The assembly of microorganism interacting with each other and living together are known as a *microbial community* (Konopka, 2009). Biofilm carriers moves freely through the bioreactor and hence it can be argued that an MBBR is a *metacommunity* (Leibold *et al.*, 2004), with each carrier representing a *patch*. The communities in an MBBR are linked through dispersal either by biofilm detachments events or carriers randomly bumping into each other. Since dispersal is likely to be equal between carriers, it can be considered a *spatially implicit system* (Leibold *et al.*, 2004). MBBRs are open systems with a constant seeding of microorganism in the influent.

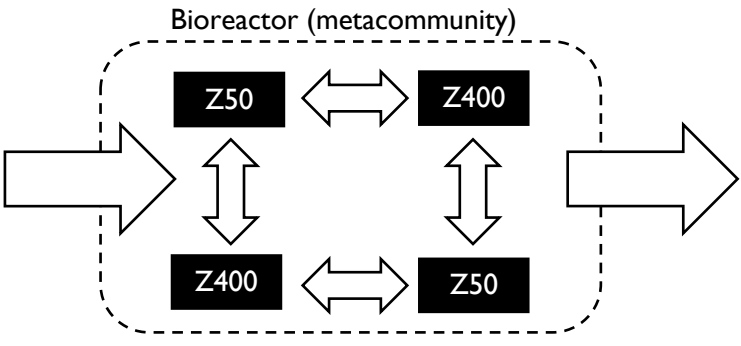

Figure 8 – The NIT MBBR from **paper III** and **V** as metacommunity. Arrows represent possible flows of microorganisms. The biofilm carriers can move freely through the bioreactor.

One of the main concerns in WWTPs is achieving high efficiency and functional stability and to preserve *ecological functions*. Further, for an ecologists, it is appealing that a bioreactor is a locality with defined boundaries; bioreactors could be studied as biological islands (Curtis *et al.*, 2003). Even more, the relative fast growth rate of microbes in WWTP compared to macro-organisms, facilitate ecological studies. Although bioreactors are complex ecosystems, conditions are more controlled that other natural environments (Briones and Raskin, 2003). Therefore WWTP could be used for ecological research, concerning microbial ecology and even general microbial theory (Graham and Smith, 2004).

We have studied the microbial community of PNA and nitrifying biofilms. We believe that our results are not only of interest to the wastewater community, but it also relevant to research in biofilms and microbial ecology.

7.1 Knock, knock! Who is there?

Functional identity is important for N-removal processes. Anammox bacteria and AOB are needed for PNA systems, while nitrifying biofilms had been traditionally described as a community of AOB and NOB, which together perform the oxidation of ammonia to nitrate. The presence of these organisms is essential if a specific ecosystem function is desired. Likewise presence of undesired organisms, like NOB in PNA systems could lead to different ecosystem functions.

Hence studying community composition is important from both a process and biological perspective. Key questions that could be asked are: Do we see "desired taxa" in the studied bioreactors? Are there any undesired taxa? (Table 3). Can reactor performance be linked to presence of certain organism? Can we identify which conditions favor or disfavor the growth of certain taxa? Do other taxa present in the bioreactor have some effect in the populations of key-taxa or in process performance?

Table 3 – Desired and undesired functional groups in PNA and NIT bioreactors.

	Desired		Undesired	Observed		
	PNA	**NIT**	**PNA**	**PNA**	**NIT (thin)**	**NIT (thick)**
Anammox	X			X		X
AOB	X	X		X	X	X
NOB		X	X	X	X	X
Comammox*				X	X	X

*Unpublished results. See section 11.4

7.1.1 Nitrifiers in nitrifying biofilms

Using both qFISH and rDNA sequencing the nitrifier community in a nitrifying reactor was determined to be composed by *Nitrosomonas*, *Nitrospira* and *Nitrotoga* (Table 4) (**Paper IV** and **V**). *Nitrospira* and *Nitrotoga* are the most abundant NOB in WWTPs (Daims *et al.*, 2001; Juretschko *et al.*, 1998; Saunders *et al.*, 2016), where they have been observed to sometimes co-exist (Lücker *et al.*, 2015). However, nitrifiers are not the only members of nitrifying biofilms. For example, sometimes anammox bacteria are found in these systems blurring the distinction between PNA and nitrifying biofilms.

7.1.2 AOB and anammox bacteria in PNA biofilms

As in nitrifying biofilms, *Nitrosomonas* is the dominant AOB in PNA biofilms. Relative abundance of *Nitrosomonas* was much lower than that of anammox bacteria in the studied PNA reactors (Table 4). The low DO used in PNA systems to limit NOB growth and avoid anammox inhibition, also limits AOB growth. Nevertheless despite their low abundance, their effect in the reactor is disproportionately high, with ammonia oxidation being the limiting-rate step in the PNA process. Thus AOB inhibition might lead to process failure (Vázquez-Padín *et al.*, 2010). Low abundant taxa, with a large impact in ecosystem function, like nitrifiers are considered keystone-species (Lynch and Neufeld, 2015).

Although the anammox process is present trough five different genera, *Brocadia* is often the dominant anammox in PNA biofilms while *Kuenenia* is often observed in suspended samples (Zheng *et al.*, 2016). 16S rDNA sequencing showed that the LTA, Mainstream and Reject reactors were dominated by *Brocadia* sp. 40 (**Paper I, III and VI**), while the IFAS reactor was dominated by *Brocadia fulgida* (unpublished results). Indeed *B.* sp. 40 and *B. fulgida* appears to be the dominant anammox bacteria in PNA reactors operating at different conditions (Gilbert *et*

al., 2014b; Park *et al.*, 2010). See section 7.3 for details of the observed *Brocadia* populations in the studied PNA reactors.

Table 4: Relative average read abundance (%) of anammox bacteria and nitrifiers in the studied reactors. Data from study III, V and VI. Notice that Greengenes was used for classification in LTA library, while SILVA was used for classification of sequences in all other reactors. Paper number is shown inside the parenthesis. The NOB *Nitrobacter* was sometimes detected with qPCR or FISH, but classification at genus level among members of the *Bradyrhizobiaceae* was not possible using the V4 region of the rDNA.

		PNA			Nitrification	
Group	**Genus**	**LTA (IV)**	**Reject (VI)**	**Mainstream (VI)**	**Z400 (V)**	**Z50 (V)**
Anammox	*Brocadia*	54.7	38.3	12.5	6.0	0.08
AOB	*Nitrosomonas*	0.2	0.4	0.2	1.4	7.3
NOB	*Nitrospira*	0.4	0.1	0.6	2.9	10.8
NOB	*Nitrotoga*	0.07	0.002	0.003	0.1	3.2
NOB	*Nitrolancea*	0	0	0.007	0.001	0.003

7.1.3 Nitrite oxidizers in PNA biofilms

NOB are also commonly observed in PNA biofilms, and unlike in the nitrification-denitrification process, here they are considered undesirable. NOB compete with anammox bacteria for nitrite and with AOB for oxygen. Furthermore nitrate produced by NOB remains in the system and will lead to incomplete N-removal.

Nitrospira appears to be the dominant NOB in PNA biofilms. However *Nitrobacter* and *Nitrotoga* were also detected in the LTA reactor during **study IV**. Excess nitrate production was observed during the study. This was the first detection of *Nitrotoga* in a PNA system. Likewise low abundant *Nitrotoga* and *Nitrolancea* were also present in the Reject and Mainstream MBBRs (Table 4) (**paper VI**). Detection of *Nitrotoga* is interesting. Current strategies for NOB suppression in PNA reactors are based on the assumption that NOB are *Nitrospira* or *Nitrobacter*. However little is known about *Nitrotoga* and hence it is possible that current NOB suppression strategies will not work against *Nitrotoga* in low temperature PNA reactors. For discussion on NOB inhibition in WWT see section 11.

7.1.4 Ammonia Oxidizing Archaea?

In theory AOA could perform the same process as AOB in PNA and nitrifying bioreactors. AOA appear to be dominant in soil (Leininger *et al.*, 2006) and marine environments (Wuchter *et al.*, 2006). However the high ammonia concentration in WWTP might favor AOB (Martens-Habbena *et al.*, 2009). Nevertheless AOA are often observed in WWTP, and sometimes are more abundant than AOB (Bai *et al.*, 2012), specially at low ammonia concentrations (Sauder *et al.*, 2012).

Using MiSeq, reads of *Thaumarchaeota* were not detected in **study IV**. However the primers 515F and 806R have low in silico coverage among the *Thaumarchaeota*, with only 0.7% of the *Thaumarchaeota* sequences in SILVA ssu128 being targeted as estimated with TestPrime (Klindworth *et al.*, 2013). A higher coverage among *Archaea* can be obtained with primer 515F' (Hugerth *et al.*, 2014), with 91% of the *Thaumarchaeota* sequences in SILVA ssu128 being targeted. Despite the increased coverage by the primers used here, *Thaumarchaeota* was not detected in **study V**, and only a few reads were observed in **study VI** (data not shown). Detection of *Thaumarchaeota* amoA by PCR also failed (data not shown). Overall this suggest that AOA might not be important in the studied bioreactors. Although possible bias related to the DNA extraction protocol exist.

7.1.5 Who else is there?

Microbial communities in PNA and nitrifying biofilms are not limited to those of nitrifiers and anammox bacteria. Despite the dissimilar conditions of the different reactors, *Plantomycetes*, *Proteobacteria*, *Chloroflexi*, *Bacteroidetes* and *Acidobacteria*, where found to comprise the majority of the reads (Figure 9). PNA communities are often dominated by *Proteobacteria*, *Chloroflexi* and *Bacteroidetes* (Gilbert *et al.*, 2014b; Pereira *et al.*, 2014). Activated sludge communities are often dominated by members of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Zhang *et al.*, 2012), as well as in nitrifying reactors (Ye *et al.*, 2011). *Proteobacteria* and *Bacteroidetes* are also highly abundant in stream biofilms (Besemer *et al.*, 2012; Wilhelm *et al.*, 2013; Battin *et al.*, 2016). The presence of the same phyla among these systems suggest that members of these phyla, might show ecological coherence at high taxonomic ranks (Philippot *et al.*, 2010), likely represented by taxa adapted to live in biofilms.

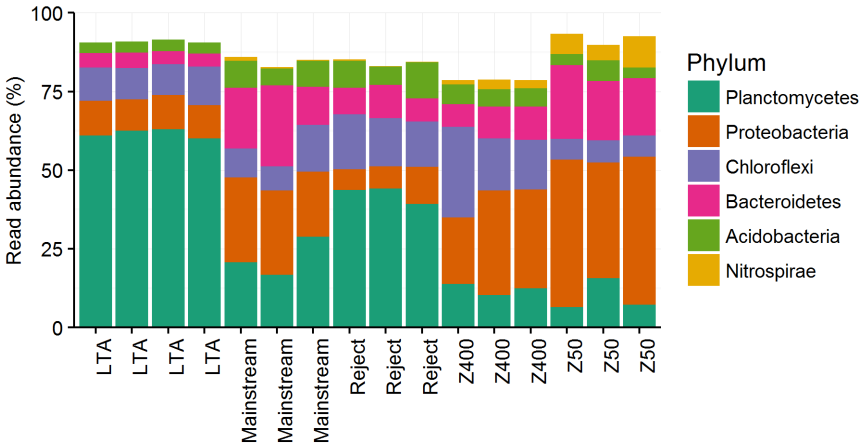

Figure 9: Read abundance of the top phyla in the studied reactors. Each column represents a sample. Data from study III, V and VI. For study V only data from 3 Z50 and 3 Z400 carriers is shown.

Chloroflexi are filamentous bacteria commonly observed in WWTP (Björnsson *et al.*, 2002); filamentous bacteria like *Chloroflexi* and *Bacteroidetes* are believed to have an important role structural role on biofilms but can also contribute to undesirable conditions such as foaming and bulking in activated sludge systems. *Chloroflexi* might survive on organic material from anammox cells (Kindaichi *et al.*, 2012). In addition members of *Chloroflexi*, *Bacteroidetes* and *Acidobacteria* might be capable of doing DNRA or denitrification (Speth *et al.*, 2016; Lawson *et al.*, 2017). Some taxa detected in the PNA and nitrifying communities are likely capable of denitrification like *Thauera*, *Sulfuritalea*, *Denitratrisoma* and *Competibacter*.

7.1.6 Spatial location of populations is important

Although it is useful to study microbial communities in the macro-scale, i.e. how reactors differ; populations in biofilms are not homogenously distributed in the biofilm, neither are conditions similar through the biofilm (Stewart and Franklin, 2008; Lydmark *et al.*, 2006, **paper I, II, III, V**). Populations located in different regions of the biofilm will have access to different substrates, and in turn will be involved in different biochemical reactions affecting overall ecosystem functions of the biofilm. Thus to link community composition with function, it is necessary to study ecological interactions at a micro-scale.

7.2 Predation in PNA biofilms

Nitrification and anammox are both processes with low functional redundancy, *i.e.* because of the narrow phylogenetic distribution of the trait, a disturbance affecting one of the groups will cause a loss of function. In other words, if *Nitrosomonas* or *Brocadia* die or get washed out, there is no one else to replace them. The very low anammox growth rate also means long recovery times if anammox bacteria are lost during a disturbance. Hence it is of interest to understand causes of mortality of these two groups.

A major cause of bacterial mortality is predation, which might have important effects on productivity. For example loss of biomass by predation might lead to lower nitrogen removal rates or even process failure. Several defense mechanism against predation exist, including the production of biofilms (Matz and Kjelleberg, 2005; Matz *et al.*, 2004; Weitere *et al.*, 2005). However bacteria in biofilms are not completely protected against predation by other bacteria (Kadouri and O'Toole, 2005) or protozoa (Huws *et al.*, 2005).

A WWTP biofilm, like an activated sludge floc, granule or carrier in an MBBR is a complex community, including both primary producers and predators. Thus grazing of bacteria, including nitrifiers is known to occur. For example *Nitrospira* grazing by other bacteria in activated sludge was suggested by Dolinšek *et al.* (2013) based on stable isotope probing and FISH. Likewise swarming of *Bdellovibrio* cells around *Nitrosomonas* colonies has been observed in granules (Liebana, 2017). Protozoa have been known for a long time to be present in WWTP. Their impact in community composition, biofilm structure (Böhme *et al.*, 2009; Derlon *et al.*, 2012) and ecosystem function (Lee and Welander, 1994) is complex.

Predation of autotrophic bacteria by ciliates in Ammonia-Oxidizing Activated Sludge was suggested by Moreno *et al.* (2010) based on $^{13}CO_2$ labeling experiments. Anammox bacteria are highly abundant in PNA biofilms and thus our aim in **paper II** was to study if grazing of anammox bacteria by protozoa occurs in PNA biofilms. Although both protozoa and anammox are present in the bioreactor that does not imply that predation occurs. Anammox bacteria in one-stage PNA reactors live in the anoxic regions of the biofilm, and thus it is possible that they might be protected against predation.

Although in general it is difficult to study predation in multi-species biofilms, PNA biofilms are challenging since deep layers of the biofilm cannot be directly

observed with traditional microscopy. We used FISH-CLSM in **paper II** to target both protozoa and prey. Grazing was defined as bacteria present inside the food vacuoles of protozoa (Gunderson and Goss, 1997; Jezbera *et al.*, 2005). In addition using cryosections allowed us to show predation in anoxic layers of a biofilm for the first time.

Protozoa were present in all studied reactors (LTA, Reject, Mainstream, and IFAS). A grazing event was observed in the LTA reactor (Figure 10) during the same period corresponding to **study I**. The grazing event was a short duration event, with grazing fronts being observed only at 19°C and 16°, further grazing was not observed during the subsequent **study IV** (data not shown). Anammox and AOB cells were seen inside protozoa and protozoa were also observed inside the AOB aggregates. A decline in the number of AOB was measured with qPCR during the grazing event (**Paper I**). However it cannot be certainly attributed to the grazing event. The importance of grazing in PNA biofilm is still unknown. Nevertheless predation should not be over-looked as a process that can influence reactor performance.

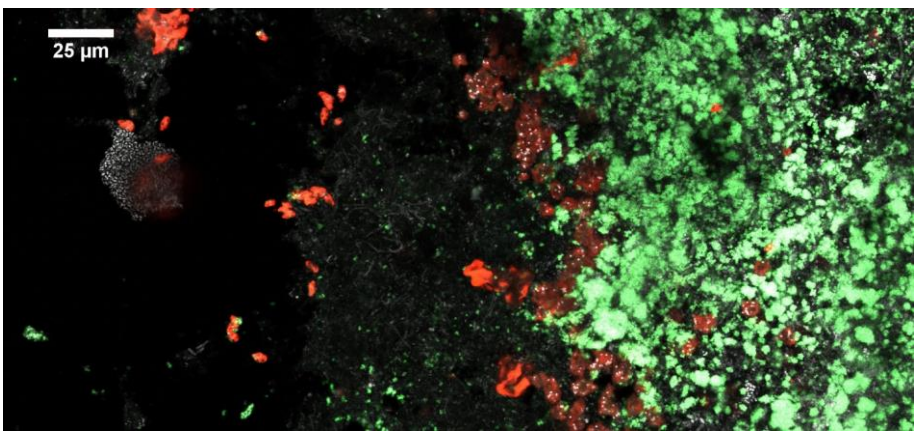

Figure 10 – FISH-CLSM picture of the LTA biofilm. Water-biofilm interface is in the left side. Red: Eukaryotes. Green: Anammox bacteria. Gray: DAPI.

Other possible cause of anammox mortality could be phage activity, which has not been studied. However CRISPR-CAS regions (genetic signatures that indicate that a phage, or some other foreign DNA has invaded the cell) are found in anammox genomes, suggesting that anammox might be attacked by viruses.

7.3 A tale of two anammox bacteria

The LTA, Mainstream and Reject reactors were dominated by *B.* sp. 40, but another less abundant *Brocadia* was also observed (**Paper I, II and VI**), which henceforth we refer to as *Brocadia* C10 (clone C10 in the Paper I clone library). A similar microdiversity and co-existence of closely related species has been noticed for *Nitrospira* in activated sludge (Gruber-Dorninger *et al.*, 2015).

We were able to visualize the two *Brocadia* populations with FISH, using FISH probes Ban162 and Bfu613 originally designed for *Brocadia anammoxidans* and *B. fulgida* respectively. The probe Ban162 also targets *B.* sp. 40, while Bfu613 also targets the closely related *B.* C10 (Figure 11). The target sequences differ by one single mismatch, hence unlabeled competitors were needed (**Paper I and II**).

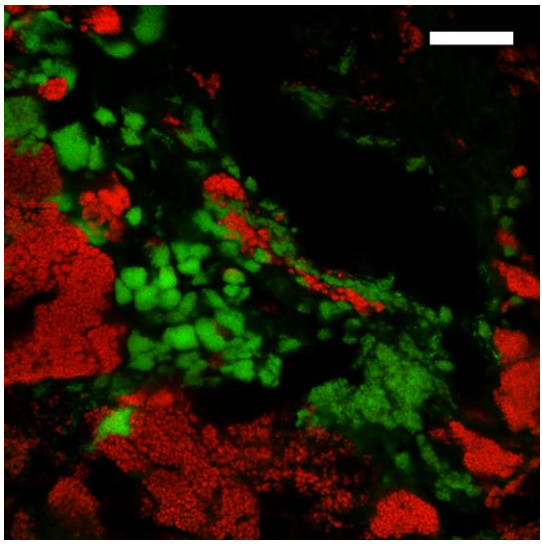

Figure 11 - Subpopulations of *Brocadia* in the LTA reactor, targeted by FISH probes Ban162 (Green) and Bfu613 (Red). The detection of these subpopulations is based on singe mismatches, hence unlabeled competitors were used. Scale Bar: 25µm.

Anammox populations in PNA biofilm appear to be stable. One of the aims of **paper I** was to see if lower temperatures in a PNA reactor could lead to the selection of a cold-tolerant anammox bacteria. Hence temperature was lowered from 19°C to 10°C in the LTA MBBR. Later in **paper IV**, influent ammonium concentration was lowered from 500 to 45 mg-N l^{-1}. Both *B.* sp. 40 and *B.* C10 were present during the entire duration of both studies.

B. C10 in the LTN reactor was restricted to the upper layer of the biofilm (Figure 9) (**paper I, and II**), with *B.* sp. 40 present throughout the biofilm depth (**paper II**). This suggests that different ecophysiological properties of the two *Brocadia* populations, which might allow co-existence of the two populations. Similar changes in the relative abundance of anammox populations were seen along a salinity gradient in an estuary (Dale *et al.*, 2009)

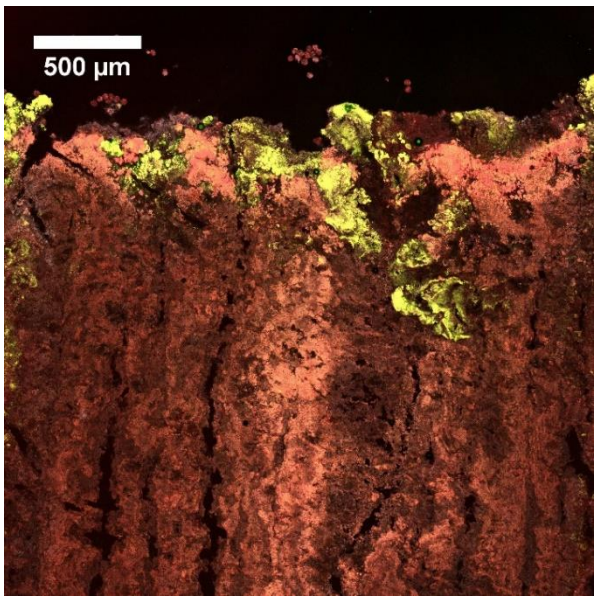

Figure 12 – FISH-CLSM picture showing the location of *Brocadia* C10 in the biofilm. Bulk-water is on the top. Red: *Brocadia* sp. 40. Yellow (red+green): *Brocadia* C10. Grey: All bacteria

The LTA, Mainstream and Reject MBBRs were originally seeded from the same source, Himmerfjärden WWTP. Hence it is possible that the presence of these two *Brocadia* populations is a result of the shared history of the reactors. The Himmerfjärden PNA MBBR was originally described to be dominated by *B. anammoxidans*, however that identification was based on FISH using the Ban162 probe (Szatkowska *et al.*, 2007). The Ban162 and Bfu613 FISH probes cannot differentiate *B. anammoxidans*, *B.* sp. 40, *B. fulgida, Brocadia caroliniensis* and *B.* C10 if competitor probes are not used (unpublished results). Thus, it is possible that Himmerfjärden PNA and other reactors where those FISH probes where used for identification were actually dominated by *B.* sp. 40.

8 Biofilm thickness matters

Functions in biofilms are related to spatial distribution of organisms, thus not surprisingly, a link between biofilm thickness and ecosystem function appears to exist. For example it has been observed in PNA granular systems, that small granules are efficient in nitrification, while bigger granules can perform the anammox process (Nielsen *et al.*, 2005; Vlaeminck *et al.*, 2010).

It is difficult to control biofilm thickness in a bioreactor. Several factors are known to influence thickness, including shear forces, community composition and nutrient load. A strategy to control biofilm thickness would be to limit the height of the biofilm exposed to shear forces. This can be done in MBBRs using Z-carries (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden), where the protected area of the biofilm is defined by grid-height in the biofilm carrier (Piculell *et al.*, 2016b). Biofilms growing outside the protected area thus are susceptible to shear forces and scraping off by other carriers in the reactor.

We wondered how biofilm thickness could affect community composition, spatial distribution of organisms, ecosystem functions and response to ecological disturbances. We evaluated this by growing thin and thick biofilms together in the same nitrifying reactor using a mixture of Z400 and Z50 carriers, with grid height of 400 and 50μm respectively (**paper III and V**). An average biofilm thickness of 45 and 379 μm for Z50 and Z400 respectively was measured with optical coherence tomography (OCT) (**paper III**).

8.1 Microbial community and biofilm thickness

We consider the MBBR a metacommunity, with Z50 and Z400 carriers being linked by dispersal (Figure 8), but having different local conditions (biofilm thickness). Our aim in **paper V** was to study if community composition and ecosystem function in mature biofilms were affected by biofilm thickness, or if niche processes could be overriden by regional factors such as migration between carriers or immigration from the incoming water (mass-effects) (Leibold *et al.*, 2004).

We observed both higher richness and evenness in the Z400 biofilms than in the Z50 biofilms. The differences in evenness can be explained by the fact that Z50 biofilm is being dominated by nitrifiers, with lower abundance of this group in the Z400 biofilm. Among those OTUs with differential abundance between the biofilms 79% had higher abundance in the Z400 biofilm including *Brocadia*.

Mathematical modelling of oxygen profiles in the biofilm suggested that the Z400 biofilm have large anoxic regions, explaining the presence of *Brocadia* and other anaerobic taxa in that biofilm. Likewise, since most of the Z50 will be mostly oxic, conditions there might favor the growth of aerobic organism like *Nitrosomonas*. Thus despite that both biofilm were being present in the same bioreactor, different communities developed suggesting that species-sorting is an important mechanism explaining community assembly in biofilms.

8.1.1 Nitrifiers and biofilm thickness

Nitrospira in nitrifying biofilms are usually located below *Nitrosomonas*, in the oxic-anoxic interface of the biofilm (Lydmark *et al.*, 2006; Schramm *et al.*, 2000; Okabe *et al.*, 1999). Hence, it is possible that *Nitrospira* growth could be disfavored in thin biofilms. Populations were stratified in Z400 as shown in other studies, but that was not true for Z50, were *Nitrosomonas* and *Nitrospira* appear to growth side by side. A lower *Nitrospira*/*Nitrosomonas* ratio was noticed in Z50 (Table 5) suggesting that *Nitrospira* growth is favored in thick biofilms, although its growth is not completely repressed in thin biofilms.

One of the most puzzling results was the NOB *Nitrotoga* being mainly restricted to the Z50 biofilm (Table 5). *Nitrotoga* can outcompete *Nitrospira* at slightly lower pH (Hüpeden *et al.*, 2016). Since oxidation of ammonia is an acidifying process, it is possible that the higher nitrification rates in Z50 might have created conditions where *Nitrotoga* could co-exist with *Nitrospira*. It is possible that *Nitrotoga* contributed to nitrification in the biofilm, with the Z50 biofilm having higher NO_3^- production rates than the Z400 biofilm.

Table 5 – Ratios of nitrifiers between Z50 and Z400, measured with both MiSeq and qFISH

	Nitrospira/ Nitrosomonas	***Nitrospira/ Nitrotoga***	**AOB/ NOB**
MiSeq – Z50	1.4	3.4	0.5
MiSeq – Z400	2.1	19.6	0.4
qFISH – Z50	0.9	16.5	1.0
qFISH – Z400	1.7	103.5	0.6

8.2 Unexpected differences

Differences between thin and thick biofilms were not restricted to nitrifiers and anammox bacteria. Potential denitrifiers were in general more abundant in the Z400, although some OTUs in *Haliangium* and *Hyphomicrobium* had higher relative abundance in Z50 OTUs (Figure 13). Although *Nitrospira* and *Nitrosomonas* were in general more abundant in Z50, some OTUs in these genera were more abundant in Z400. Sorting of these and other OTUs between the two biofilms, suggest that species-sorting might be important for community assembly of nitrifying biofilms. That sorting can have surprising consequences as seen by the fact that for members of the predatory bacteria *Bdellovibrio*, some OTUs were more abundant in Z400, while others were more abundant in Z50. This suggests that sorting of predatory bacteria could be due to different prey abundance in the two biofilms.

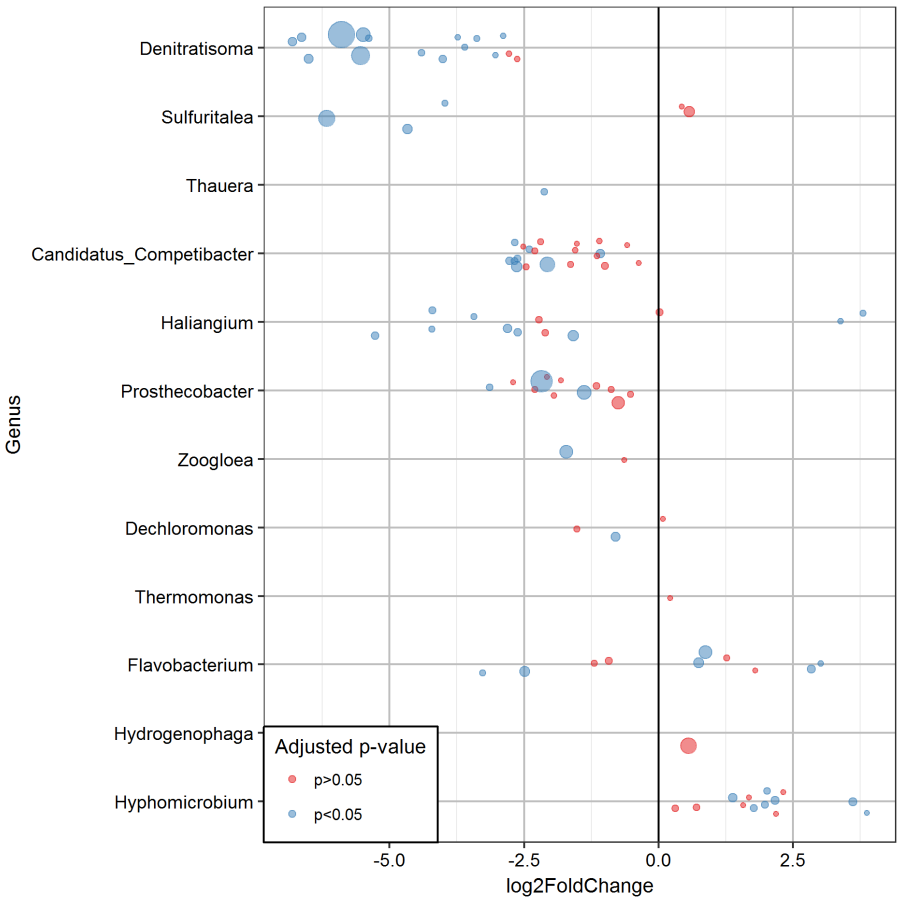

Figure 13 - Log2fold (DESeq2) changes for putative denitrifiers OTUs. Genus classification is shown. Each circle represents an OTU. The size of the circle is proportional to the total sequence read abundance for the OTU. A negative log2 fold change indicates that OTUs are more abundant in Z400 biofilm, while a positive log2 fold change indicates OTUs more abundant in Z50 biofilms. OTUs with a NA p(adj) value (DESeq2) are not shown.

8.3 Linking community to ecosystem function.

In **paper V** nitrification measured as NO_3^- production normalized by area was higher in Z50 than Z400. Differences between Z50 and Z400 were more evident when rates were normalized by biomass. Torresi *et al.* (2016) also observed higher nitrification rates in thinner biofilms. This results differ from Piculell *et al.* (2016b) where lower NO_3^- production was observed in thinner biofilms, although the experimental conditions in that study were different and community composition was not known.

Table 6 – Nitrification rates in Z400 and Z50 as measured NO_3^- production normalized by area or biomass. For details see materials and methods in paper V.

	Z400	**Z50**
NO_3^- production ($gN\text{-}NO_3^-/m^2$, day)	0.68-0.72	1.35-1.51
NO_3^- production ($gN\text{-}NO_3^-$ /g total solids, day)	0.05-0.05	0.41-0.46

Overall these results agree with Torresi *et al.* (2016), confirming that biofilm thickness has an impact on ecosystem function, either on nitrogen transformations (**paper V**) or on removal of micropollutants (Torresi *et al.*, 2016). Nitrification rates clearly increased in thinner biofilms, but some micro-pollutants were removed at higher rates in thin and others in thick biofilms. Thus, while a positive relationship between biofilm thickness and evenness (Torresi *et al.*, 2016) appears to exist, a mechanistic explanation of why biofilm thickness affects biofilm function was lacking.

Measuring relative abundances of populations is interesting when studying how community members in the biofilm are affected by the different thickness. However, a link between relative abundance and ecosystem function is meaningless, since both biofilms differ in thickness and thus biomass. A better comparison can be obtained if the amount of biomass is taken into account. In **paper III** qFISH data was normalized with biovolume data from optical coherence tomography, suggesting higher abundance of nitrifiers in the Z400 biofilm. In **paper V** read abundance was normalized by biofilm mass measured as total solids, and suggested that the amount of *Nitrosomonas* and *Nitrospira* appears to be similar in both biofilms, while *Brocadia* is more abundant than nitrifiers. Those results appear to contradict nitrogen transformation rates observed, with higher nitrification rates in Z50. However, another element to be taken into consideration is position of microorganism in the biofilm. Nitrifiers in the Z50 might have better access to oxygen and NH_4^+, while a large number of nitrifiers in the Z400 are buried in the biofilm limiting access to these resources.

8.4 Identity matters

Although higher richness (Bell *et al.*, 2005) and evenness (Wittebolle *et al.*, 2009) are believed to be associated to higher productivity, the relationship between diversity and function is not straightforward (Hillebrand *et al.*, 2008; Knelman and Nemergut, 2014) and might not even exist for natural communities (Roger *et al.*, 2016). Species identity rather than diversity might be more important for

specialized process (Peter *et al.*, 2011) like nitritation, nitratation and anammox which are restricted to few taxa. Anoxic NO_2^- removal was favored in the Z400 biofilm, which likely can be explained by the higher abundance of anammox bacteria and several denitrifiers. Thus higher diversity lead to the emergence of new functions in the biofilm, as observed for micropollutant removal by Torresi *et al.* (2016). However, nitrification was favored in the less diverse Z50 biofilm. Hence a higher evenness can lead to a dilution effect, with lower abundance of specialized taxa (Knelman and Nemergut, 2014; Hillebrand *et al.*, 2008). Other less specialized processes like denitrification and BOD removal might benefit of a higher evenness and thus higher functional redundancy.

9 Microbial activity

Bacteria in a microbial community might be growing, active, dormant or deceased (Blazewicz *et al.*, 2013). A better link between identity and function could be achieved if active taxa are identified. This cannot be done with rDNA sequencing.

A complementary alternative to rDNA sequencing, is 16S RNA ribosome (rRNA) sequencing, since ribosomal content could be considered as a proxy for potential activity (Blazewicz *et al.*, 2013; Schaechter *et al.*, 1958). During starvation many bacteria degrade their ribosomes (Fegatella *et al.*, 1998; Deutscher, 2003). Likewise ribosome number also decreases in anammox bacteria during inhibition (Schmid *et al.*, 2001). However, the link between ribosomal content and activity is not straightforward. Some bacteria might increase ribosomal content before dormancy (Sukenik *et al.*, 2012). Additionally, other bacteria might keep their ribosomes during starvation (Kramer and Singleton, 1992), among them *Nitrosomonas* (Wagner *et al.*, 1995). For mixotrophic bacteria like anammox bacteria and *Nitrospira*, it is possible that cells are metabolically active; however, they could be involved in alternate metabolic pathways not directly related to anammox process and nitrification respectively.

Although sequencing of only rRNA has been done (Rosselli *et al.*, 2016), the usefulness of that approach is limited. Another approach is to normalize rRNA content per cell by using rRNA:rDNA ratios (Blazewicz *et al.*, 2013). We compared rRNA and rDNA from a reject and mainstream PNA reactors in **paper VI**.

Ratios of rRNA:rDNA have been used to classify bacteria in mixed microbial communities as active or dormant (Jones and Lennon, 2010) for diverse environments such as biofilms (Wilhelm *et al.*, 2013), ocean (Campbell *et al.*, 2011), estuaries (Campbell and Kirchman, 2013), atmosphere (Klein *et al.*, 2016), enhanced biological phosphorus removal plants (Lawson *et al.*, 2015), and even among protists (Debroas *et al.*, 2015). Bacteria with rRNA:rDNA ratios above one are classified as active, or dormant for ratios below one (Figure 14).

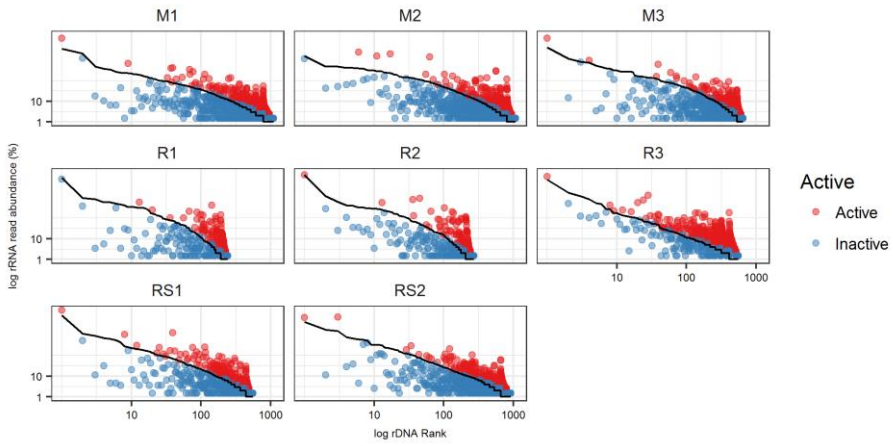

Figure 14 – OTUs classified as active or dormant in paper VI using the approach in Jones and Lennon (2010). **Solid line:** Rank abundance curve of rDNA for each sample. **Points:** rRNA read abundance of an OTU. For many OTUs rDNA and rRNA abundance are different, hence rRNA OTUs don't match the rDNA rank abundance curve. OTUs with rRNA:rDNA ratios above one (above the curve) have been traditionally classified as active, or dormant for ratios below one (below the curve).

In addition to the limitations of using rRNA as activity measurement (Blazewicz *et al.*, 2013), the approach that classifies a cell with a certain rRNA:rDNA ratio as active or dormant can also be misleading (Steven *et al.*, 2017). rRNA content is not uniform across taxa, for example Steven *et al.* (2017) observed that bacteria in the TM7 phylum from forest floor communities had mostly a rRNA:rDNA ratio below one. Small bacteria can have only a limited number of ribosomes, for example see Luef *et al.* (2015). In **paper VI**, rRNA:rDNA ratios of less than one were observed for *Acidobacteria*, *Bacteroidetes* and *Chlorobi*. On the other hand most *Firmicutes* had rRNA:rDNA ratios above one (Figure 15). Furthermore despite anammox process being observed for both reactors, sometimes rRNA:rDNA ratio below one were observed for the dominant *Brocadia* OTU.

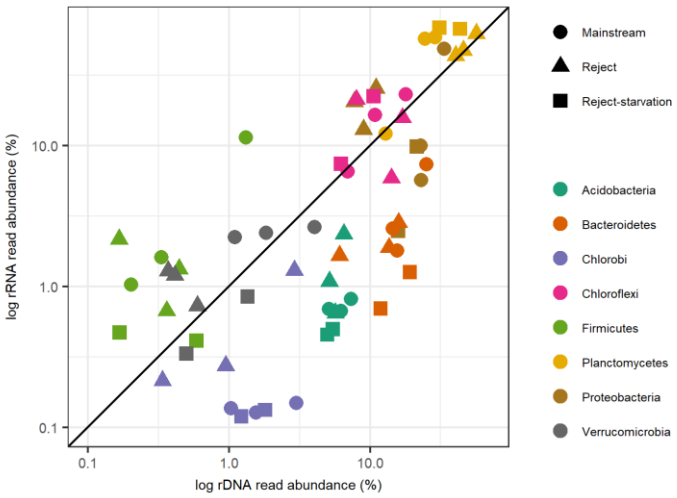

Figure 15 – Correlation of rDNA and rRNA at the phylum level. An rRNA:rDNA ratio of 1 is shown by the black diagonal line. Each symbol represents a taxa in different biofilm carriers

Since in practice it might be difficult to classify bacteria in mixed communities and with different life strategies as active or dormant, instead we compared the rRNA:rDNA ratios between the two reactors in **paper VI**, one fed with warm Reject water and one with cold, Mainstream wastewater. We also measured the impact of a disturbance, starvation. A positive relation between growth rate and changes in rRNA content is observed for several bacteria (Kemp *et al.*, 1993; Schaechter *et al.*, 1958). Hence, differences in rRNA:rDNA ratios between reactors and treatments could be consider a proxy for differences in growth rate.

When OTUs present in both rDNA and rRNA libraries of both reactors are only included, a "core-community" of 65 OTUs was observed, including *Brocadia*, *Nitrosomonas* and *Nitrospira*. *Brocadia* was more abundant in Reject, and *Nitrospira* was more abundant in Mainstream (Table 4). In addition one of the two *Nitrosomonas* OTUs had higher rRNA:rDNA ratio in Reject. This suggest that higher N-removal rates in Reject might be a result of the higher abundance of *Brocadia* and higher activity of *Nitrosomonas*.

Comparison of rRNA:rDNA ratios between reactors was hindered by several OTUs not being present in some of the samples. For example several denitrifiers were often restricted to Mainstream. It is also possible that for some diverse functional groups like denitrifiers, core-communities don't exist. So even if an

OTU is not present in all samples, other OTUs with similar ecological traits might exist and ecosystem function is present. Despite this, some trends are noticed. Potential denitrifiers appear to be often more abundant in Mainstream than in Reject, however higher rRNA:rDNA ratios are observed in Reject when the taxa are present there (Figure 16). This might be explained by higher temperatures in Reject.

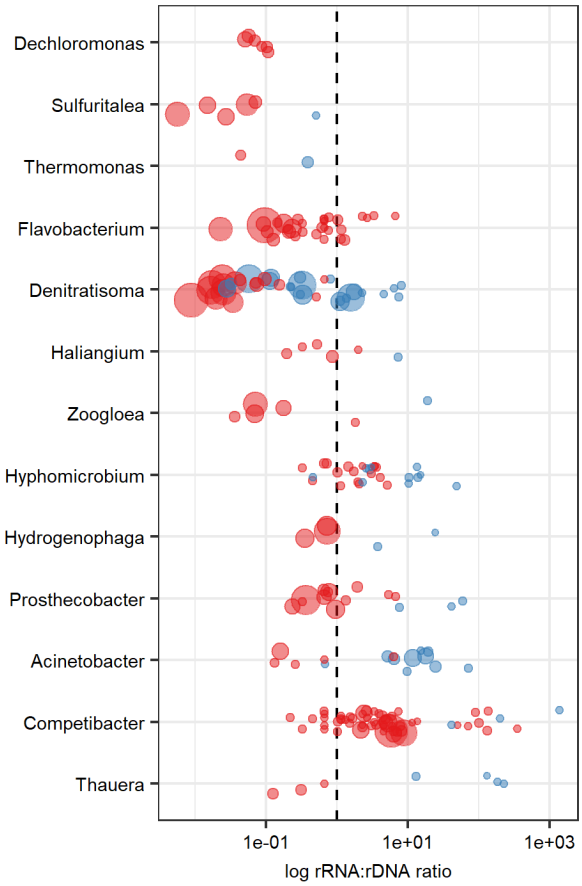

Figure 16 – Ratios of rRNA:rDNA for potential denitrifiers in each genus. Each circle represents an OTU in that genus, thus is possible that several OTUs might be assigned to a same genera. Data of all 3 Reject (Blue) and 3 Mainstream (Red) samples has been pooled together. However ratios are only shown for OTUs with both rDNA and rRNA reads, hence for some taxa like *Decholoromona*s in Reject it is not possible to calculate rRNA:rDNA ratios. The size of the circle is proportional to the rDNA read abundance.

Among putative denitrifiers *Competibacter* had high rRNA:rDNA ratios, while *Denitratisoma*, *Dechloromonas* and *Sulfuritalea* had ratios below one (Figure 16). *Competibacte*r a glycogen accumulating bacteria often observed in WTTP, had average rRNA:rDNA ratio of 43.4. Some *Competibacter* strains can use nitrite as electron acceptor (Kong *et al.*, 2006) , with *Competibacter denitrificans* having genes for denitrification (McIlroy *et al.*, 2014). Thus *Competibacter* might play an important role in the nitrogen cycle for the MBBRs.

Intragenomic variation in copies of the rDNA is sometimes observed in bacteria (Pei *et al.*, 2010), which might explain low rRNA:rDNA ratios. *Rhodocyclaceae* a family known to harbor several denitrifiers, like *Thauera*, *Denitratisoma* and *Zoogloea* had often rRNA:rDNA ratios below one. However in *rrn*DB 5.2 (Stoddard *et al.*, 2015), genomes among *Rhodocyclaceae* are shown to have between 2 and 5 rDNA copies, suggesting that actual rRNA:rDNA ratios for *Rhodocyclaceae* might be higher.

In conclusion, a more complete picture of the link between community composition and ecosystem function can be obtained by studying both rDNA and rRNA abundance.

10 Mainstream PNA

PNA is successfully used for treatment of warm and nitrogen rich wastewater, such as the water from sludge treatment (Lackner *et al.*, 2014). The next step is treatment of mainstream wastewater (with low temperature and ammonium concentration) with PNA (Cao *et al.*, 2017). Replacing nitrification-denitrification systems with PNA may greatly reduce operation cost in WWTP (Kartal *et al.*, 2010). However several challenges exist: A higher COD/N ratio is found in the mainstream and anammox bacteria might be outcompeted by denitrifiers. Activity of both AOB and anammox must be maintained in these conditions. Additionally, retention of biomass is critical, due to the slow growth rate of the anammox bacteria. Furthermore NOB activity needs to be suppressed (Xu *et al.*, 2015), see section 11. Anammox activity is known to be negatively affected by the reduced temperature in the mainstream (compared with the reject stream) (Lotti *et al.*, 2015b). However anammox bacteria have been found in natural environments with low temperature and low substrate availability such as the oxygen minimum zones in the ocean (Lam and Kuypers, 2011) and hence mainstream PNA should be possible.

10.1 PNA at low temperature

The viability of PNA at low temperature has been studied, by reducing the temperature in various reactors (De Clippeleir *et al.*, 2013; Gilbert *et al.*, 2015; Lotti *et al.*, 2014a). In **paper I** the reactor performance was stable from 19-13°C, with a loss of function at 10°C. Loss of the anammox process below 15°C appears to be common (Laureni *et al.*, 2016; Lotti *et al.*, 2014b; Gilbert *et al.*, 2014b).

Despite the challenging conditions in the reactor, anammox bacteria were the dominant members of the bacterial community on **study I** and **IV**. This agrees with the results by Gilbert *et al.* (2015), where anammox community in MBBRs was also stable at low temperatures. Nitrite accumulation at low temperatures is sometimes observed, suggesting anammox or NOB inhibition (De Clippeleir *et al.*, 2013; Gilbert *et al.*, 2015, **paper I**).

Since nitritation is the limiting step in PNA systems, low AOB activity will also lead to low nitrogen removal (Lotti *et al.*, 2015a). Furthermore presence of NOB and their associated nitrate production has been also observed in low temperature PNA (Gilbert *et al.*, 2015; Gustavsson *et al.*, 2017). Although NOB belonging to genus *Nitrospira* were observed in low abundance during **study I and IV**, a considerable fractions of the oxidized ammonium was converted to nitrate. This suggest either a disproportionately large effect of NOB, being key species, or the presence of unknown NOB.

Although anammox bacteria are kept in the system, the anammox process appears to be partially replaced by nitrite oxidation during mainstream conditions, with the community becoming a nitrate, rather than a N_2 producer (**paper I, IV**). A somehow similar result is the co-existence of *Brocadia* and *Nitrospira* in the Z400 biofilm (**paper V**), which is not reflected in N_2-producing capabilities. Although co-existence of NOB and anammox bacteria is interesting from an ecological point of view, it is completely undesirable from a process perspective.

11 NOB inhibition

From a process perspective NOB are undesired in PNA systems. When treating warm and nitrogen rich wastewater, *Nitrosomonas* and *Brocadia* appear to be capable of outcompeting *Nitrospira*. However suppression of NOB has been difficult to achieve in mainstream conditions.

11.1 Oxygen limitation, does it work?

Dissolved oxygen (DO) limitation is often used a strategy to suppress NOB (Xu *et al.*, 2015). The AOB *Nitrosomonas* has a higher affinity for oxygen than the NOB *Nitrobacter* (Laanbroek and Gerards, 1993). Another strategy for NOB suppression is intermittent aeration (Xu *et al.*, 2015); as a lag phase is observed for NOB in response to changes from anoxic to aerobic conditions. (Gilbert *et al.*, 2014a). Also maintenance of residual ammonium in the reactor has shown to be important so that the AOB never are lacking substrate (Pérez *et al.*, 2014). A drawback with the low DO strategy is that low oxygen concentrations are believed to lead to increased emission of N_2O during nitritation (Kampschreur *et al.*, 2009); hence NOB strategies based on DO limitation might cause an increase in N_2O emissions. Another drawback of DO limitation is lower N-removal rates because of lower AOB activity.

However many NOB are versatile organisms not limited to using oxygen and nitrite. Denitrification can be used by several NOB and hydrogen instead of NO_2^- can be used as electron donor in *Nitrospira moscoviensis* (Koch *et al.*, 2014). *N. moscoviensis* can also survive in anoxic conditions by oxidizing formate and reducing NO_3^- (Koch *et al.*, 2015). Additionally, most reactors are dominated by *Nitrospira* rather than *Nitrobacter*, the former has a higher affinity for oxygen (Gilbert *et al.*, 2014a; Park *et al.*, 2017b). In addition higher affinity for oxygen in *Nitrospira* than AOB has been reported (Regmi *et al.*, 2014). Therefore it is possible that current strategies for NOB suppression based on oxygen and nitrite competition with AOB and anammox respectively might not be entirely successful. *Nitrospira* appears to lack defense against oxidative stress (Lücker *et al.*, 2010). This suggest that high DO might actually be a better strategy for NOB inhibition in systems where *Nitrospira* is the dominant NOB (Malovanyy *et al.*, 2015; Regmi *et al.*, 2014; Piculell *et al.*, 2016b), although inhibition of *Nitrospira*

might lead to *Nitrobacter* or *Nitrotoga* dominance. The possible advantage of *Nitrotoga* at lower temperatures and lower pH was discussed in section 8.1.1.

11.2 NOB inhibition in nitritation reactors

Partial nitrification (nitritation) can be also used as part of a two-stage anammox process, with partial nitritation in the first stage and anammox in the second one. Another system requiring partial nitritation is nitritation–denitritation, where ammonia is oxidized only to nitrite, followed by denitrification. The aim in both systems is to ensure nitrite accumulation, which can only be achieved with NOB (or comammox) inhibition.

In a two-stage PNA system, if partial nitritation is achieved, then the second stage can operate at anoxic conditions, thus avoiding NOB growth in the second stage. Isanta *et al.* (2015) achieved NOB inhibition with excess ammonia, although community was dominated by *Nitrobacter* rather than *Nitrospira*. Other strategies for NOB inhibition could be exposure to free ammonia (FA) or free nitrous acid (FNA), which appears to inhibit both AOB and NOB (Kim *et al.*, 2006). High concentrations of FA or FNA cannot be reached in mainstream. An alternative could be temporal exposure to reject water (Piculell, 2016). This can be done by moving biomass carriers between reactors as done for the PNA MBBRs in Sjölunda WTTP (Gustavsson *et al.*, 2014, 2017) or by temporally switching the feed to reject water (Piculell *et al.*, 2016a).

11.3 Biofilm thickness and NOB inhibition

In thick biofilms such as MBBRs or granules, anoxic regions in the biofilm will develop even at high DO (**paper V**) allowing growth of *Nitrospira* (paper **III** and **V**), and limiting the usefulness of high DO for *Nitrospira* inhibition. A better NOB inhibition might be achieved in thin biofilms (Piculell *et al.*, 2016b). In **paper V**, thin biofilms appear to have partially limited *Nitrospira* growth, seen as lower *Nitrospira*/*Nitrosomonas* ratio in Z50; however, growth of *Nitrotoga* was observed making NOB/AOB ratios similar in both Z50 and Z400 (Table 5).

Intermittent reject exposure in thin biofilms (200μm) was previously used for successful nitrite production in nitrifying biofilms (Piculell *et al.*, 2016a). The effect of different thickness was further studied in **paper III** with thinner (50μm) and

thicker biofilms (400μm). Overall nitrification was negatively affected by reject exposure, with nitrite accumulation being observed in Z50, but less in Z400. A possible cause of nitrite accumulation in the Z50 biofilms was the high FA concentrations in the bulk water, with the thick Z400 biofilm protecting *Nitrospira* from FA inhibition. It is also possible that low pH and nitrite production by increased AOB activity in the Z50 biofilm could have caused high concentrations of FNA inside the Z50 biofilm. FNA is a known inhibitor activity of bacteria including *Nitrosomonas*, *Nitrobacter* and *Nitrospira*, with NOB being more sensitive than AOB (Zhou *et al.*, 2011). For example Wang *et al.* (2016) achieved partial nitritation by using both FNA exposure and DO limitation in a community composed of both *Nitrospira* and *Nitrobacter*.

For reactors dominated by only one type of NOB like *Nitrospira,* functional stability, *i.e.* stable NO_3^- production, would be expected to be closely linked to community stability, *i.e.* presence of NOB (Shade *et al.*, 2012). Thus, a disturbance that affects the single NOB in the reactor would have a direct effect on the function. According to the model by Allison and Martiny (2008) any disturbance to which the microorganisms are neither resistant nor resilient would lead to process failure and require a slow recover. If that is true for a PNA or partial-nitritation community, suppression might be successful.

However, diversity among NOB is not often considered. According to the *insurance hypothesis* a higher biodiversity would ensure that ecosystem functioning is maintained when affected by disturbances (Yachi and Loreau, 1999). This is based on the assumption that disturbances often have unequal effects across different species. The complete inhibition of NOB we sought might have been hampered by the unexpected functional diversity, represented by the presence of both *Nitrospira* and *Nitrotoga*. In addition, diversity among NOB is not restricted to the presence of different genera like *Nitrospira*, *Nitrobacter*, *Nitrotoga* or others. Diversity and co-existence of different *Nitrospira* has been observed (Gruber-Dorninger *et al.*, 2015), and might exist also in other NOB. Thus, suppression of one NOB might not affect other NOB in the reactors.

Nevertheless temporal loss of the dominant NOB after the disturbance, would entail a drop in NO_3^- production, since the other NOB are less abundant. The minor NOB populations must increase (grow) to maintain the process (Allison and Martiny, 2008). NOB suppression strategies might have an initial partial success with the inhibition of the dominant NOB. However with time there is

risk of selecting NOB resistant to the disturbance, especially in open system like a WTTP being constantly seed by new bacteria.

11.4 Comammox?

A more complex picture of *Nitrospira* has emerged with the discovery of comammox. Long before being discovered, comammox were predicted to thrive in biofilms (Costa *et al.*, 2006), and thus might be present in PNA biofilms. These *Nitrospira* might be considered undesirable in PNA systems since partial-nitritation is avoided. However coaggregation of anammox bacteria and comammox has been observed, which suggest a possible non-competitive interaction (van Kessel *et al.*, 2015).

Comammox were recently detected in all the five reactors from this project (Unpublished results), by PCR of the *Nitrospira* amoA (Pjevac *et al.*, 2017). However when this thesis was printed, their abundance and their possible effect on bioreactor function was still unknown

12 Future perspectives

Some question were answered during this project, sometimes with expected results, and sometimes completely unexpected observations were made. However as is common in science, the answers raised many new questions. Some of these questions are:

Why is the anammox process lost at low temperature, despite anammox bacteria being retained in the system? This is a key question for the development of mainstream PNA.

NOB inhibition is critical for development of new technologies in N-removal. Much research has been done on the topic, however success may have been hampered by a possible functional diversity among NOB and likely heterogeneity in environmental conditions. From a biological perspective NOB inhibition could be studied as an ecological stability issue. For example we do not know how *Nitrotoga* or mixed NOB communities will be affected by NOB inhibition strategies like DO control or reject exposure.

Many WWTPs appear to be dominated by one or s few anammox bacteria, but we also discovered anammox microdiversity. How important is this for process performance and process stability? How common is anammox microdiversity?

How common is predation in PNA and nitrifying biofilms? What is the impact of predation in these communities?

Is higher ribosomal content in anammox bacteria and nitrifiers linked to higher process rates? If yes, estimations of ribosomal content could be used to get a more detailed picture of their activity, perhaps even on a per cell basis, which in turn could tell us how stratified biofilms work.

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