Biofilms in Nitrogen Removal

Bacterial Population Dynamics and Spatial Distribution

Robert Almstrand

UNIVERSITY OF GOTHENBURG

AKADEMISK AVHANDLING

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University of Gothenburg, Box 462, SE-405 30 Göteborg, Sweden.

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"Nature, in all its vigour, and at the same time in decline, offers to the imagination something more imposing and picturesque than the sight of this same nature embellished by civilized man´s industry. In wishing to conserve only its beauty, man has managed to destroy its charm, and ruin its exclusive character – the one of always being old, and always new."

A.R.J. de Bruni d´Entrecasteux, 1792

Abstract

Efficient nitrogen removal at wastewater treatment plants (WWTPs) is necessary to avoid eutrophication of recipient waters. The most commonly used approach consists of aerobic nitrification and subsequent anaerobic denitrification resulting in the release of dinitrogen gas into the atmosphere. Nitrification is a two-step process performed by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) often grown in biofilms at WWTPs. An alternative approach is anaerobic ammonium oxidation (anammox) where anammox bacteria convert ammonium and nitrite directly into dinitrogen gas. These groups of bacteria grow very slowly and are sensitive to perturbations, which may result in decreased efficiency or even breakdown of the process. Therefore, the ecology and activity of these bacteria and the structure of the biofilms in which they grow require detailed investigation to improve the understanding of nitrification and to facilitate the design of efficient nitrogen-removal strategies.

To facilitate such studies of relevance for wastewater treatment, a nitrifying pilot-plant was built where environmental conditions and especially ammonium concentrations could be controlled.

In an experiment on model nitrifying trickling filters (NTFs), it was shown that biofilms subjected to intermittent feeding regimes of alternating high and low ammonium concentration in the water, could maintain a higher nitrification potential than biofilms constantly fed with low ammonium water. Such ammonium feed strategies can be used to optimize wastewater treatment performance.

Different AOB populations within the *N. oligotropha* lineage were shown to respond differently to changes in environmental conditions, indicating microdiversity within this lineage which may be of importance for wastewater treatment. This diversity was further investigated through the development of new image analysis methods for analyzing bacterial spatial distribution in biofilms. The diversity within the *N. oligotropha* lineage was also reflected in the positioning of two such populations in the biofilm, where the vertical distribution patterns and relative positions compared to the NOB *Nitrospira* were different.

In combination with a cryosectioning approach for retrieval of intact biofilm from small biofilm carrier compartments, the new image analysis methods showed a three-dimensonal stratification of AOB-anammox biofilms. This may be of importance for mathematical modeling of such biofilms and the design of new biofilm carriers.

Keywords: AOB, NOB, biofilms, image analysis, FISH, *Nitrosomonas,* population dynamics, spatial distribution

Papers included

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

I Lydmark, P., **Almstrand, R.**, Samuelsson, K., Mattsson, A., Sörensson, F., Lindgren, P.-E. and Hermansson, M. Effects of environmental conditions on the nitrifying population dynamics in a pilot wastewater treatment plant. *Environmental microbiology* **9**, 2220-33 (2007).

II **Almstrand, R.**, Lydmark, P., Sörensson, F. and Hermansson, M. Nitrification potential and population dynamics of nitrifying bacterial biofilms in response to controlled shifts of ammonium concentrations in wastewater trickling filters. *Bioresource technology* **102**, 7685-7691 (2011).

III **Almstrand, R.**, Daims, H., Persson, F., Sörensson, F. and Hermansson, M. Spatial distribution and co-aggregation of nitrifying bacteria in cryosectioned biofilms from different wastewater systems. *Submitted manuscript.*

IV **Almstrand, R.**, Daims, H., Ekenberg, M., Christensson, M., Sörensson, F. and Hermansson, M. Three-dimensional stratification of bacterial biofilm populations in moving bed biofilm carriers for the anammox process. *Manuscript*.

V **Almstrand, R.**, Lydmark, P., Lindgren, P.-E., Sörensson, F. and Hermansson, M. Dynamics of specific ammonia-oxidizing bacterial populations and nitrification potential in response to controlled shifts of ammonium concentrations in wastewater. *Submitted manuscript*.

The included papers are attached in their full versions at the end of the thesis, separated by the coloured paper sheets.

Paper not included in this thesis:

Baird,F., **Almstrand, R.**, Herzer, K., Hill, J.E. Characterization of a suspended *Pseudomonas aeruginosa* biofilm cultured under low shear conditions. *Manuscript*.

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Abbreviations

16S rRNA	16S subunit ribosomal RNA
Anammox	Anaerobic ammonium oxidation
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
CLSM	Confocal laser scanning microscopy
Cy3	A cyanine dye
Cy5	A cyanine dye
DAPI	4,6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DSL	Domestic sewage laboratory
EPS	Extracellular polymeric substances
FA	Formamide
FISH	Fluorescence in situ hybridization
FOV	Field of view
$\Delta G^{0'}$	Change in Gibbs free energy (during standard conditions)
MBBR	Moving bed biofilm reactor
MP	Megapixel
NOB	Nitrite oxidizing bacteria
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RNA	Ribonucleic acid
WWTP	Wastewater treatment plant

Aims

The aims of this thesis were:

i) to investigate the response of nitrifying bacterial populations and their activity to shifts in environmental conditions and especially ammonium concentrations, in order to facilitate the design of improved strategies for the nitrification process at wastewater treatment plants

ii) to develop new methods for investigation of structure and bacterial spatial distribution in biofilms

iii) to apply these methods for investigation of diversity and structure of bacterial populations in nitrogen-removing biofilms

Introduction to Nitrogen Removal and Microbial Ecology

Nitrogen

Being the major constituent of the atmosphere and the fifth most abundant substance in the solar system, nitrogen is essential for life as we know it and is literally part of our DNA. Atmospheric nitrogen exists mainly as dinitrogen gas (N_2), an inert molecule requiring considerable energy input before it can be reduced into biochemically available ammonium (NH_4^+). The ability to reduce (fix) N_2 is found only among certain *Bacteria* and *Archaea*, making them essential for the nitrogen supply to the biosphere. Ammonium is either incorporated into organic molecules or transformed in the mainly microbially driven nitrogen cycle (Martinez-Espinosa 2011) which is ultimately closed through the release of N_2 back into the atmosphere by denitrifying or anaerobic ammonium oxidizing microbes (Fig. 1).

During the last 150 years, human activities have significantly affected the global nitrogen cycle (Gruber & Galloway 2008). Today, anthropogenic processes such as the production of fertilizers or fossil fuel combustion are responsible for approximately 50% of the production of fixed nitrogen on Earth (Canfield *et al.,* 2010). In fact, anthropogenically induced changes in the global nitrogen-cycle have by far exceeded the boundaries of what could be considered sustainable on a planetary scale (Rockström *et al.,* 2009). Most of the anthropogenic nitrogen input is used to meet the ever growing demand from agriculture, which led to an 800% increase in the use of nitrogen fertilizer between 1960 and 2000 (Canfield *et al.,* 2010). The efficiency of nitrogen fertilization is however low (Galloway *et al.,* 2008), causing leakage of combined nitrogen to recipient waters. Apart from N_2-release from denitrification, both nitrification and denitrification emit the potent greenhouse gas N_2O (Montzka *et al.,* 2011) which also has a detrimental effect on the ozone layer (Ravishankara *et al.,* 2009). Leakage of nutrients such as nitrogen and phosphorous, which is especially pronounced in areas with high agricultural activity or population density, ultimately leads to nutrient pollution of coastal

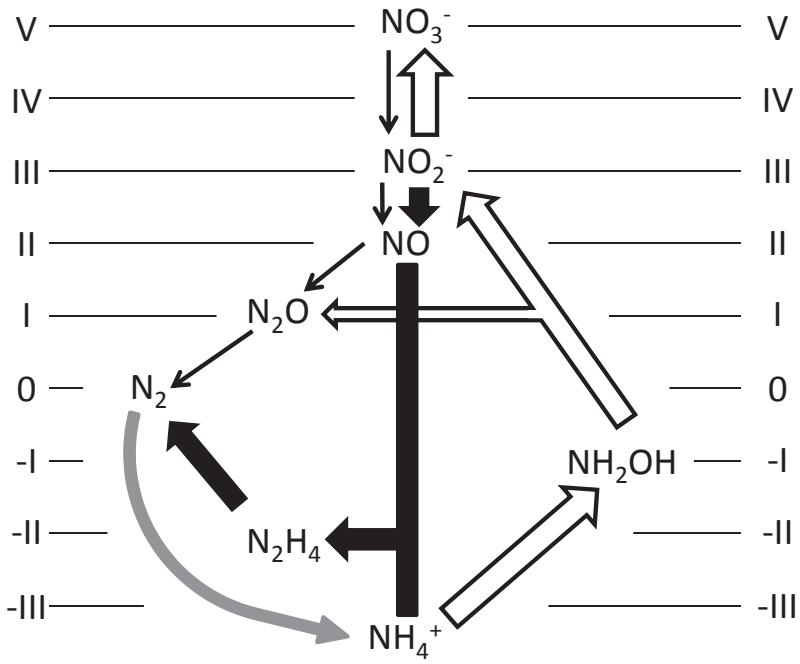


Figure 1. Simplified overview of the biological nitrogen cycle. Reactions of relevance for wastewater treatment are shown as either white arrows (nitrification, aerobic), thin black arrows (denitrification, anaerobic) or thick black arrows (anaerobic ammonium oxidation). Grey arrow indicates nitrogen fixation (anaerobic). Roman numerals show the oxidation state of nitrogen in the corresponding compounds. (AOB) or (NOB) indicates that the reaction is performed by ammonia- or nitrite oxidizing bacteria, respectively. Modified from Canfield et al (2010).

waters. Today, nitrogen pollution is considered to be the primary cause of eutrophication, the nutrient enrichment of water, in most marine coastal systems (Howarth & Marino 2006).

Eutrophication

Eutrophication may cause accelerated primary production and disturbance to ecosystem balance. Therefore, "Zero Eutrophication" was acknowledged by the Swedish Parliament as one of 16 environmental goals to pursue (reviewed in Moksnes *et al.,* 2010). Although seasonal changes in nutrient availability and consumption are normal constituents of coastal and marine ecosystems, anthropogenic nutrient discharge has acutely changed the scale of natural processes,

such as the excessive spring bloom of marine phytoplankton, the main producers of organic carbon in marine ecosystems (Field *et al.,* 1998). When phytoplankton die, they sediment through the water column and will begin to break down, a process facilitated through oxygen-consuming heterotrophic bacteria. If the amount of organic carbon is increased, so is the oxygen consumption.

Due to anthropogenic input of nutrients to coastal oceans, the magnitude and period of the (sometimes toxic, (Hinder *et al.,* 2011)) phytoplankton blooms have increased, leading to more frequent oxygen depletions. In fact, Diaz & Rosenberg (2008) stated that "There is no other variable of such importance to coastal marine ecosystems that has changed so drastically over such a short time as dissolved oxygen" (DO). This is especially pronounced in the bottom waters, where oxygen availability may already be scarce (Conley *et al.,* 2009). In addition, the formation of hypoxic (DO < 2ml l^{-1}) or anoxic (DO below detection limits) waters cause release of phosphorous from sediment (Mortimer 1941), which may further fuel and prolong the ongoing primary production.

Zones with low or no DO have spread exponentially in coastal waters during the last 50 years, (Diaz & Rosenberg 2008) killing bottom-living organisms (Vaquer-Sunyer & Duarte 2008) and causing destruction of fish habitat (Rabalais *et al.,* 2002). Consequently, the anthropogenic discharge of nutrients to recipient waters needs to be significantly and permanently reduced.

Effects of deoxygenation have been extensively investigated in the severely polluted Baltic Sea. Here, a substantial fraction of the bottom waters are today either hypoxic or completely anoxic (Fig. 2) and an alarming increase of hypoxia in the coastal zones has been reported recently (Conley *et al.,* 2011). In addition, hypoxia in the Baltic Sea is a complex issue to pursue. Physical factors, such as inflow of saltwater are important for oxygenation of the deep waters but may also strengthen stratification of the water column. This may reduce mixing of the water and further promote proliferation of oxygen-depleted areas (for review, see Conley *et al.,* 2009). Recently it has also been suggested that loss of large predators such as cod, due to over-fishing or loss of habitat, may alter the ecosystem in a manner that ultimately reduces predation pressure on phytoplankton, thus further increasing sedimentation of organic matter to the sea floor (Granéli & Esplund 2010).

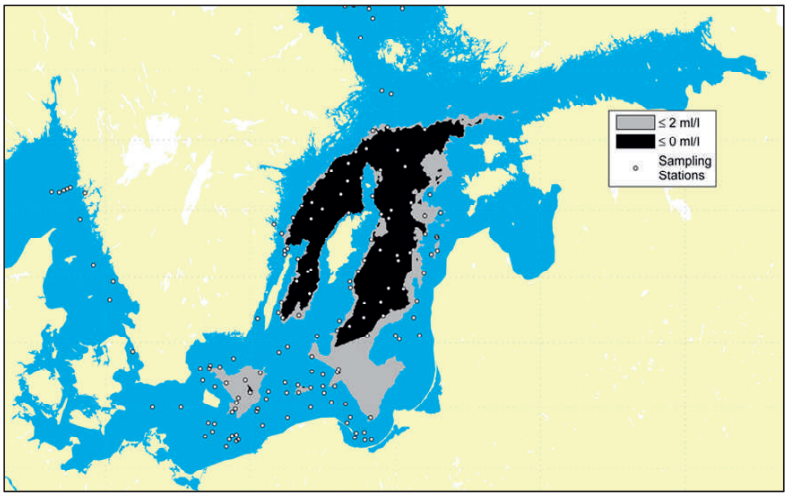


Figure 2. Extent of hypoxic (grey) and anoxic (black) bottom water in the Baltic Sea, autumn 2010. Used with permission from SMHI.

In northern Sweden, phosphorous and nitrogen discharge mainly originates from natural leakage from forests, whereas agriculture and point sources are of greater importance in the southern part of the country (Sonesten 2008). Although diffuse sources of discharge such as leakage from agriculture are rather complicated to deal with, a potential solution may, at least in part, be construction and restoration of wetlands acting as nutrient sinks and thereby preventing runoff to coastal waters (eg Stadmark & Leonardson 2005). Efficient treatment of point sources such as sewage from municipalities and industries is, compared to nutrient reduction from diffuse sources, easier to achieve. At wastewater treatment plants, efficient phosphorus removal can be achieved through chemical precipitation or biologically, through Enhanced Biological Phosphorous Removal (EBPR) (eg Christensson 1997). EBPR is more desirable than chemical precipitation for economical and environmental reasons and is increasingly applied in wastewater treatment, although process stability may still be a problem (Nielsen *et al.,* 2010).

Biological nitrogen removal in wastewater treatment

Nitrogen removal in wastewater treatment is a biological process which can be attained in two ways (Fig. 1). The first and most commonly used approach is a two-step process consisting of aerobic nitrification and subsequent anaerobic

denitrification. The second alternative is combined aerobic and anaerobic ammonium oxidation (anammox) which converts ammonium and nitrite directly into dinitrogen gas.

Nitrification

Nitrification is a two-step process consisting of aerobic oxidation of ammonia (NH_3) to nitrite (NO_2^-) and oxidation of nitrite to nitrate (NO_3^-) by ammonia- and nitrite-oxidizing bacteria, respectively (Winogradsky 1891, Bock & Wagner 2006) (equation (1)):

$$NH_3 \rightarrow NO_2^- \rightarrow NO_3^- \quad (1)$$

Ammonia-oxidation is the first and rate-limiting step of nitrification (Kowalchuk & Stephen 2001), carried out by aerobic chemolithoautotrophic ammonia-oxidizing bacteria (AOB) or archaea (AOA). Most recognized AOB of relevance for wastewater treatment, are either closely related or belong to the phylogenetically well-defined *Nitrosomonas* group of the β-proteobacteria (Koops *et al.,* 2006). AOA on the other hand, are usually not found in significant numbers in Wastewater Treatment Plants (WWTPs) (eg Mussmann *et al.,* 2011), although abundant in nature and of importance for the global nitrogen cycle (Prosser & Nicol 2008, Pester *et al.,* 2011). Due to the slow growth rate and sensitivity of AOB to environmental changes, nitrification has often been regarded as unreliable and failure-prone (e.g. Bellucci *et al.,* 2011). Ammonia is the primary substrate in ammonia-oxidation (Suzuki *et al.,* 1974) and is first converted to hydroxylamine (NH_2OH) through the actions of the enzyme ammonia monooxygenase (AMO) (Hollocher *et al.,* 1981) (equation (2)):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (2)$$

Hydroxylamine is the actual energy source, donating electrons to the respiratory chain in a reaction catalyzed by hydroxylamine oxidoreductase (HAO) (equation (3)):

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad (3)$$

In addition, NO_x play an important role in ammonia oxidation. For instance, it has been shown that supply of NO_2 allows ammonia-oxidation to carry on also in anoxic environments (Schmidt *et al.,* 2001), whereas removal of NO from culture

medium actually inhibit the process (Zart *et al.,* 2000). Interestingly, NO gas have also been shown to trigger biofilm growth for the AOB *Nitrosomonas europaea* (Schmidt *et al.,* 2004a)

The second step of nitrification is the oxidation of nitrite to nitrate which is less thermodynamically favourable ($\Delta G^{0'}$= -74 kJ mol^{-1}, compared to -275 kJ mol^{-1} for ammonia oxidation (Costa *et al.,* 2006)). Nitrite-oxidizing bacteria (NOB) catalyze this reaction via the enzyme nitrite oxidoreductase according to equation (4):

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (4)$$

In wastewater treatment the dominating NOB are most often members of the genus *Nitrospira* within the phylum *Nitrospirae* (Daims *et al.,* 2009), phylogenetically separated from other proteobacterial NOB. Bacteria belonging to the genus *Nitrobacter* within the *Alphaproteobacteria* are more rarely encountered, with the exception of systems with high nitrite concentrations (Daims *et al.,* 2001a, Terada *et al.,* 2010).

The main focus of this thesis is nitrification, which is the main process associated with nitrifying bacteria which are slow-growing organisms with generation times of hours or even days and low growth yield (Bellucci *et al.,* 2011). In fact, higher growth yield, albeit at a lower growth rate, could hypothetically be achieved by a cell combining both ammonia- and nitrite oxidization. Although not yet discovered, existence of the "Commamox" bacteria has been postulated (Costa *et al.,* 2006).

The slow metabolism of nitrifying bacteria is disfavourable when subjected to competition for ammonia or oxygen by heterotrophic bacteria (Verhagen & Laanbroek 1991). Consequently, organic carbon should be kept low to facilitate nitrification, especially since it has been observed that heterotrophic bacteria may benefit from nitrification possibly through provision of additional electron donors due to nitrification (Gieseke *et al.,* 2005, Rittmann *et al.,* 1994). In contrast to its usefulness in wastewater treatment, nitrification can actually also pose a threat to public health via nitrate contamination of groundwater and release of metals into drinking water due to nitrification-mediated pH reduction (Zhang *et al.,* 2009). Additional metabolical features have also been observed for these organisms. For instance, several studies have observed and characterized "nitrifier denitrification" (as reviewed in Klotz & Stein 2008) and AOB and NOB have been shown to harbor nitrite reductase genes (eg Schmidt *et al.,* 2004b, Lücker *et al.,* 2010).

Denitrification

Maintaining respiration under anoxic conditions calls for the ability to use substances other than oxygen as terminal electron acceptors in the respiratory chain. This is achieved in the process of denitrification, where the inorganic nitrogen compounds nitrate, nitrite, nitric oxide and nitrous oxide are consecutively reduced to dinitrogen gas which is released into the atmosphere, (equation (5)):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (5)$$

The entire process or parts of it can be used for anaerobic respiration and not all denitrifying microbes have the complete set of enzymes needed to catalyze the entire pathway (Shapleigh 2006). The ability to denitrify is widespread among bacteria and archaea, and has also been observed in some nitrifying bacteria (eg Bock *et al.,* 1995). The denitrifying community at WWTPs can consequently be phylogenetically diverse (eg Morgan- Sagastume *et al.,* 2008). Being a heterotrophic process, external carbon, often in the form of ethanol or methanol is added during the wastewater treatment process to ensure efficient denitrification (Isaacs *et al.,* 1994).

Anaerobic ammonium oxidation (anammox)

The anammox process, carried out by a monophyletic group of *Planctomycete* bacteria is the anaerobic conversion of ammonium (NH_4^+) and nitrite into dinitrogen gas (equation (6)):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (6)$$
$$(\Delta G^{0'} = -357 \text{ kJ mol}^{-1})$$

This overall reaction is comprised of three enzymatic reactions (equations (7-9)) catalyzed by nitrite reductase, hydrazine synthase and hydrazine dehydrogenase, respectively (Kartal *et al.,* 2011):

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (7)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (8)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (9)$$

In addition, nitrate is produced from nitrite via a nitrite reductase. The anammox process has a large potential for wastewater treatment, and may in the future at least

partially replace conventional nitrogen removal. Anammox metabolism requires input of nitrite, which can be acquired by combining anammox with aerobic ammonia oxidation. Although this requires partial aeration, which constitutes an economical burden for treatment plants, the oxygen demand of the process would still be much smaller than that for nitrification and addition of organic carbon is not needed. Hence, the economical incentives for running full-scale anammox-mediated nitrogen removal are substantial, potentially reducing operational costs with as much as 90% (Strous & Jetten 2004). Nitrogen removal through the anammox process has so far been hampered by the slow growth of anammox bacteria, typically having generation times of ~11-12 days (Strous *et al.,* 1998, Third *et al.,* 2005) or even longer (van de Graaf *et al.,* 1996). Consequently, the anammox process has so far mostly been limited to wastewater with elevated temperature and ammonium content (Kartal *et al.,* 2010) and there are still relatively few WWTPs using the anammox process at full scale.

Investigated systems for biological nitrogen removal

Rya WWTP

Rya WWTP in Gothenburg, Sweden is one of the largest WWTP´s in the Nordic countries receiving municipal and industrial wastewater from 865000 person equivalents in 2010 (Davidsson 2010). Phosphate removal is performed through precipitation and particle separation, whereas organic matter is removed biologically in anoxic and aerated activated sludge basins. For nitrogen removal, approximately half of the effluent water is mixed with ammonium-rich reject water and returned to the anoxic, denitrifying activated sludge basins after passing through nitrifying trickling filters (NTFs). The NTFs consist of corrugated plastic material (Figs. 3e and 4e) with a high surface to volume ratio ($230 m^2/m^3$) where nitrification takes place in nitrifying biofilms that are formed on the plastic material (Persson *et al.,* 2002, Lydmark *et al.,* 2006). A reason for using biofilm-based systems, such as NTFs or Moving Bed Biofilm Reactors (MBBRs) for nitrification is to increase process stability. In fact, AOB cells have shown higher substrate affinity and more rapid recovery from starvation when growing in biofilms, compared to planctonic or unattached growth (Batchelor *et al.,* 1997, Bollmann *et al.,* 2005).

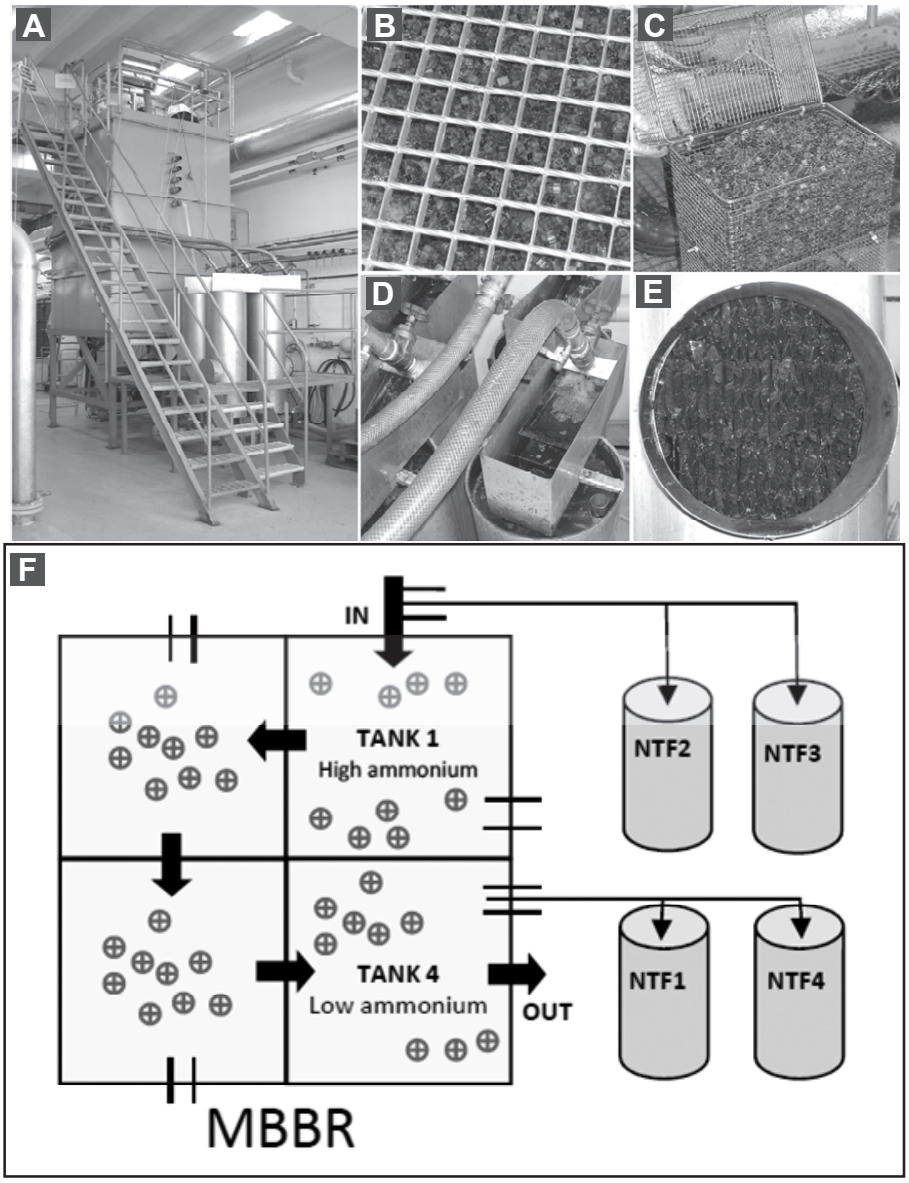


Figure 3. The pilot plant at the Rya WWTP. (A) overview of the pilot plant with the tank compartment and the four NTFs. (B) K1 carriers in tank 2. (C) K1 carriers in a of the cage used in Paper V. (D) The shovel devices mounted on top of each NTF. (E) Open NTF sampling door, showing the trickling filter media (black). (F) Schematic outline of the pilot-plant. See text for details. Images A-E used with permission from Lydmark (2006).

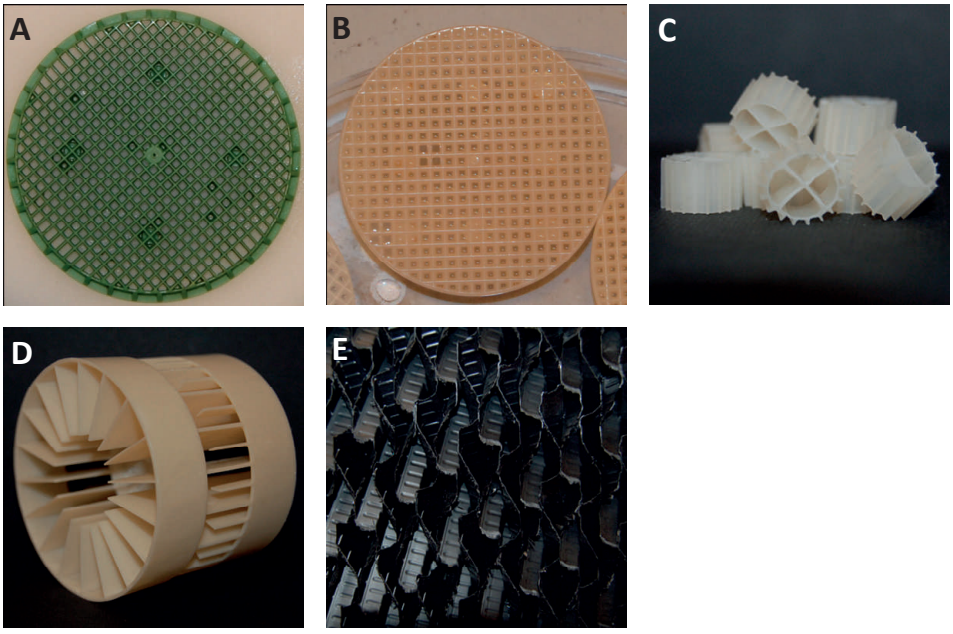
To further improve nitrogen removal, a post-denitrifying MBBR system was recently added to the plant to ensure that the requirement for nitrogen discharge, currently an annual average of <10 mg l^{-1}, is fulfilled (Davidsson 2010). Similarly, the introduction of a new large disc-filter system will enable separation of smaller particles from the water, thus enhancing phosphorous removal and ensuring that the effluent phosphorous levels will stay below the newly imposed discharge limits of 0,3 mg P l^{-1} (Davidsson 2010).

On a local scale, it has been debated, as pointed out by (Selmer & Rydberg 1993), whether or not nitrogen removal of wastewater in the Gothenburg region would have a significant effect on the recipient waters, considering that the major release of nitrogen to the Göta Älv estuary originates from the river itself, mainly as nitrate. In a recent report it was however concluded that the record-low levels of chlorophyll observed in the estuary during the summer of 2010 were, at least in part, owed to the reduced levels of ammonium, the biologically most readily utilized form of inorganic nitrogen, in the effluent from Rya WWTP (Rydberg 2010).

Samples from the full-scale NTF´s originally retrieved by (Lydmark *et al.,* 2006) were analyzed in Paper III. In addition, a population-density screening of *Lumbricillus sp.* oligochaetes, feeding on bacteria in the biofilm, was performed (see pages 38-39).

The nitrifying pilot-plant at Rya WWTP

Between 2003 and 2006, a nitrifying pilot-plant was connected to the treatment process at Rya WWTP (Fig. 3). Receiving the same water as the full-scale NTF´s, the pilot-plant consisted of two subsystems designed for studying the effect of changes in environmental factors on activity and community composition of nitrifying populations without disrupting nitrification efficiency of the full-scale plant. The first part of the pilot-plant consisted of a 12,8m^3 MBBR divided into four sequential tank-compartments filled with suspended biofilm carriers of either the AnoxKaldnes Biofilm Chip M or K1 types (Fig. 4a and c), together with a number of larger carriers (Fig. 4d). The tanks were connected in series and separated by grids allowing the water to flow through the tank but preventing exchange of carriers between them. This created an ammonium gradient between the tanks due to ongoing nitrification. In addition, a second step of four small NTFs was constructed. These filters could be fed with the same water as incoming



NTF Plastic Crossflow Material (Munthers, Sweden)	Area:230 m^2 m^{-3}				
MBBR Carriers (AnoxKaldnes, Sweden)	K1	Chip M	Minichip	Minichip	Large MBBR Carriers
Length	7,0 mm	2,2 mm	2 mm	3 mm	50 mm
Diameter	9,0 mm	48 mm	30 mm	30 mm	62 mm
Protected surface	500 m^2 m^{-3}	7,5 x 10^{-3} m^2	2,7253 x 10^{-3} m^2	4,0879 x 10^{-3} m^2	

Figure 4. MBBR biofilm carrier models and support material used in this thesis. (A) Biofilm-chip M (Paper I). (B) Minichip (Paper IV). (C) K1 (Papers I & V) . (D) Large MBBR carriers (Paper III). (E) NTF plastic crossflow material (Papers II, III & V).

to tank 1 or water from either of the tanks. This way, also the ammonium concentration fed to the NTFs could be controlled. To mimic the water distribution over the full-scale biofilms, the water was distributed over the model NTFs in short pulses.

The design of the plant, together with continuous monitoring of physical parameters made a variety of experimental approaches possible. In Paper I and V, *in situ* studies of ammonium-concentration as a structuring force for the nitrifying communities in the MBBR subsystem were performed. In Paper I, total biomass and cell-specific activity was estimated as well. In Paper II, the effect of controlled

variations in substrate supply on nitrification potential and nitrifier abundance was investigated in the model NTFs. Finally, in Paper III, stratification and co-aggregation of individual nitrifying populations in biofilms from both subsystems was analyzed. By using the same wastewater as was fed to the full-scale NTFs, the relevance of the observations made in the pilot-plant for the full-scale process was assured and has resulted in applications for the nitrification process at Rya WWTP (Paper II).

AOB-anammox MBBR

In Paper IV, biofilm samples were taken from a labscale MBBR reactor (7 liters) fed with synthetic medium. Here, nitrogen removal was obtained through aerobic and anaerobic ammonium oxidation. Biofilms of different age, growing inside AnoxKaldnes Minichip (Fig. 4b) carrier compartments, were analyzed with respect to their three-dimensional structure and stratification of different bacterial groups (Paper IV).

Microbial ecology in biological nitrogen removal

The great plate count anomaly and the dawn of microbial ecology

Proper identification of microbial cells is a vital part of microbiology and for centuries, it was also one of the most difficult objectives to accomplish with accuracy. Before the arrival of molecular techniques, microbiologists were limited to observing morphological differences, such as shape and size, or physiological traits, such as the ability to grow on certain media, when trying to distinguish between microbes. Because of the small size and high similarity between prokaryotic cells, morphological properties are generally not enough for identification and are of no use when trying to assess the evolutionary relationships between bacteria (eg Fox *et al.,* 1980, Woese 1987). Culture-based methods and reagents are still of diagnostic importance in, for example, clinical microbiology, for distinguishing between cells based on their physiological properties. However, these methods do not necessarily reflect a genetic relationship (eg Fox *et al.,* 1980) since the trait in question may be wide-spread over phylogenetically diverse groups that including bacteria also lacking the ability. In addition, the culturable fraction in a mixed sample is usually very small. In fact, less than one percent of the total bacterial community can generally be cultivated from environmental samples. Consequently, there is no

definite correlation between culturability and actual diversity in a complex sample, a fact known as the great plate count anomaly (Staley & Konopka 1985).

However, twenty years after the death of Sergei Winogradsky, the father of microbial ecology (Dworkin & Gutnick 2011), the arrival of molecular techniques, such as DNA-sequencing (eg Sanger *et al.,* 1973, 1977) and PCR (Saiki *et al.,* 1985), provided unprecedented possibilities for identification and phylogenetic classification of microbes. The increase in available DNA sequence data also enabled the design of PCR-primers targeting genes involved in bacterial metabolism that could be used as funtional markers, such as the *amoA* gene in ammonia-oxidizing bacteria (Rotthauwe *et al.,* 1997), coding for the alpha subunit of the enzyme ammonia monooxygenase (McTavish *et al.,* 1993).

It soon became clear that the genes encoding the ribosomal subunits, such as the 16S, were especially well suited for phylogenetic analysis (eg Woese & Fox 1977). Ribosomes are the sites where mRNA translation and protein synthesis occur in the cell. They are present in all living cells and contain both evolutionarily conserved and variable regions, allowing for discrimination between closely related species as well as more distantly related groups of bacteria. The early use of molecular methods for analyzing 16S rRNA and its gene in mixed communities and natural samples (e.g. DeLong *et al.,* 1989, Giovannoni *et al.,* 1990) has been described as the beginning of molecular microbial ecology (Case *et al.,* 2007), revolutionizing the way we look at microbial diversity and evolution (eg Olsen *et al.,* 1994). Subsequently, a variety of molecular methods for the identification and quantification of bacteria in complex samples were developed, leading to the conceptual approach known as the full-cycle 16S rRNA approach (Wilderer *et al.,* 2002) (Fig. 5). Since then, it has been shown that 16S rRNA analysis perhaps is not the "gold standard", it was considered to be. It has been concluded that many bacteria have several copies of the 16S rRNA gene and that intragenomic heterogeneity exists in some of these species. This in turn affects the interpretation of results gained from methods based on DNA amplification (von Wintzingerode *et al.,* 1997) such as Denaturing Gradient Gel Electrophoresis (DGGE), as observed by Dahllöf and co-workers (2000). Accordingly, this would also mean that different versions of ribosomes (ribotypes) may exist in the same cell, potentially affecting 16S-based *in situ* techniques. As an alternative, the gene coding for the RNA polymerase beta subunit (*rpoB*) could be used instead (e.g. Mollet 1997, Dahllöf *et al.,* 2000). Being present only in one copy per cell, analysis of this gene would

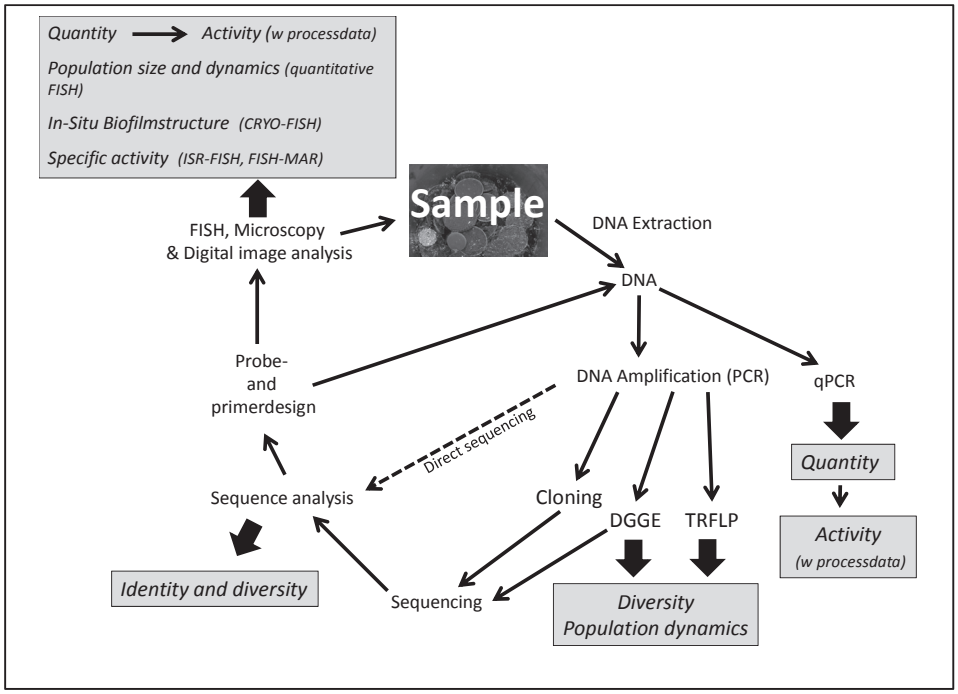


Figure 5. Schematic outline of the full-cycle 16S rRNA approach. See text for details.

certainly circumvent some of the mentioned problems. With only one probe-target site available, the *rpoB* gene, this approach would however present severe limitations for *in situ* analysis. For instance, *in situ* hybridization techniques require, depending on the type of sample, ~400-1400 target molecules per cell for proper detectability (Hoshino *et al.,* 2008). Recent methodological developments could possibly circumvent this (Case *et al.,* 2007), although not without limitations. At the time of writing however, all known genome sequences from nitrifying- and anammox bacteria include only one 16S rRNA gene copy (Strous *et al.,* 2006, Rastogi *et al.,* 2009, Lücker *et al.,* 2010, Yuichi *et al.,* 2011). A possible explanation for this may be their low growth rate (Krawiec and Riley 1990). In this respect, 16S rRNA based methods are appropriate for the analysis of these groups of bacteria.

An ongoing task for microbial ecology is to define and implement ecological theories that are of use for understanding and predicting microbial ecosystem processes (Prosser *et al.,* 2007). Such theories have in many cases already been formulated and applied in, for instance the biology of plants and animals to couple disturbance to diversity (Connell 1978) and diversity to ecosystem services (eg

Worm *et al.,* 2006), estimate population densities (eg Sheldon & Kerr 1972), assess the importance of inactive populations (Edwards & Crawley 1999) or spatial factors. Some of these existing theories are applicable also to the ecology of microbes albeit sometimes with some modification. In the case of island biogeography for instance, treeholes (Bell *et al.,* 2005), machine tanks (van der Gast *et al.,* 2005) or wastewater reactors may represent the islands. In other cases, microbial ecology has been in the forefront due to its suitability for replication and testing of hypotheses within short time-frames (eg Naeem & Li 1997, Wittebolle *et al.,* 2009a). However, when estimating the diversity of an ecosystem, an appropriate definition of the variable analyzed, such as "species", is needed. This is still an obstacle in microbial ecology.

The microbial species concept

It is beyond the scope of this thesis to participate in the ongoing discussion on how microbial species should be defined, for reviews see instead (Staley 2006, Konstantinidis *et al.,* 2006, Schleifer 2009, Achtman & Wagner 2008, Koeppel *et al.,* 2008, Doolittle & Papke 2006, Doolittle & Zhaxybayeva 2009). The reason for the debate however, raises a number of questions which may be appropriate to keep in mind when using the term "species".

16S rRNA gene is probably too conserved to generally provide adequate resolution at the species level (eg Schleifer 2009) and *rpoB* may actually be better suited as a universal phylogenetic marker gene (Case *et al.,* 2007). One could however question whether it is appropriate to discuss *Bacteria* and *Archaea* in terms of species based on sequence information from only one gene. But, how many and what genes could be regarded as an actual evolutionary backbone if this is considered and do bacterial species even exist?

A pragmatic approach is to consistently use a definition of relevance for the questions asked and ecosystems analyzed. If the investigation is to be made on a certain function in an ecosystem, identifying species may even be irrelevant, unless the function has already been coupled to species identity. If the function in question, such as denitrification is polyphyletic but present only in some members of the phyla, identification based on 16S rRNA gene sequence data may not link identity with ability, let alone provide information on the diversity or evolution of the function (eg Jones *et al.,* 2008). Instead, analysis of the functional genes would

make sense if the aim is to determine the origin, evolution, spatial distribution or environmental impact of the microbial process in question.

For nitrifying and anammox bacteria of relevance for wastewater treatment, the situation seems less complicated. Most of the bacteria that share the relevant trait belong to the same or very few phylogenetic groups. Hence, 16S rRNA approaches can generally be utilized to represent the function without significant loss of information. As an example, it has been shown for AOB that phylogenetic analysis of the 16S rRNA gene and *amoA* sequences generate similar results (Purkhold *et al.,* 2000, Aakra *et al.,* 2001a). This was also the case when the 16S rRNA gene and the more variable 16S-23S rRNA intergenic spacer region were compared (Aakra *et al.,* 2001b). Reevaluation may however be needed if ammonia oxidizing archaea (AOA) are shown to be abundant and have a significant impact on the nitrification process in wastewater treatment. Thus far, however, available evidence suggests that this is not the case (Mussmann *et al.,* 2011).

But what if two closely related co-existing AOB, with high sequence similarity at the 16S level, respond differently to changes in the environment, as in Paper I, or consistently are found at different depths or locations in a biofilm, as in Paper III? Is it then appropriate to designate them as the same species? An important task in microbial ecology in general and wastewater treatment systems in particular, is to characterize such diversity regardless of taxonomic rank and improve the resolution to the level of significance in the current context.

For the purpose of simplicity, the term "bacterial populations" will be used together with existing species designations throughout this thesis. Facilitating communication is, after all, a reason *per se* to categorize life.

Wastewater treatment and microbial ecology - a mutualistic relationship

Microbial ecology is of great importance to wastewater treatment. If we do not know who is there, what they do and how they do it, there will be only limited possibility to predict how changes in the ecosystem will affect process performance (eg Graham *et al.,* 2007). Thus, *in situ* studies of nitrifying microbial communities and their ecology are crucial for understanding the nitrification process in wastewater treatment (eg Schramm *et al.,* 1999, Graham *et al.,* 2007) By combining ecological investigations on the dynamics of the nitrifying community with activity measurements on the macroscale, such as nitrification rates, new process strategies may be developed (Paper II).

Vice versa, wastewater treatment is of great importance to the field of microbial ecology. The systems are often well-defined in terms of environmental factors and ecosystem functions, such as nitrification, can often be measured effectively. Both mutualism (such as between AOB and NOB) and competition (between populations requiring the same substrate) can be investigated. In biofilm-based systems, spatial distribution patterns and substrate gradient formation can bring further important insights into the ecophysiology of the organisms. Hence, wastewater microbiology can be regarded as a discipline within microbial ecology (Daims *et al.,* 2006a).

Diversity and Ecology of Nitrifying and Anammox Bacteria

The betaproteobacterial ammonia-oxidizing bacteria

Aerobic ammonia-oxidizing bacteria are widespread in nature and encountered in various aquatic habitats such as marine (Koops *et al.,* 2006), estuarine (Bollmann & Laanbroek 2002) and freshwater (Koops & Pommerening-Röser 2001), as well as in terrestrial environments (eg Wessén *et al.,* 2011). The distribution of ammonia-oxidizing bacteria in the environment is regulated by a number of environmental factors such as substrate affinity and tolerance (Bollmann and Laanbroek 2001, Limpiyakorn *et al.,* 2006, Yuichi *et al.,* 2011). Salt requirement and tolerance (Koops *et al.,* 1991, Bollmann & Laanbroek 2002), oxygen availability (Bollmann & Laanbroek 2002, Geets *et al.,* 2006, Park & Noguera 2004, 2007, Bellucci *et al.,* 2011), nitrite concentration (Yu & Chandran 2010), pH and temperature (Koops *et al.,* 2006) are other factors affecting the distribution of these bacteria. Although both β- and γ-proteobacterial ammonia-oxidizers exist, only the former are generally regarded as important in wastewater treatment (Koops *et al.,* 2006). These bacteria typically grow as microcolonies in biofilms (Fig. 6) and belong to the genus *Nitrosomonas*, although members of *Nitrosospira* may be favoured under certain conditions. The latter are further divided into separate lineages or subclusters (Fig. 7). The lineages of AOB that were detected in the systems analyzed in this thesis are presented below.

The Nitrosomonas oligotropha lineage (Cluster 6a)

Bacteria affiliated with this lineage are often encountered in municipal WWTPs (Koops *et al.,* 2006, Papers I-III, V), rivers, lakes and soils (Koops & Pommerening-Röser 2001), suggesting physiological versatility within this lineage (Gieseke *et al.,* 2001). The *Nitrosomonas oligotropha* lineage includes two described species: *Nitrosomonas oligotropha* and *Nitrosomonas ureae* (Koops *et al.,* 1991) which are both urease-positive and salt sensitive (Koops *et al.,* 2006). In addition, intra-lineage diversity in adaptation to substrate concentrations has been reported

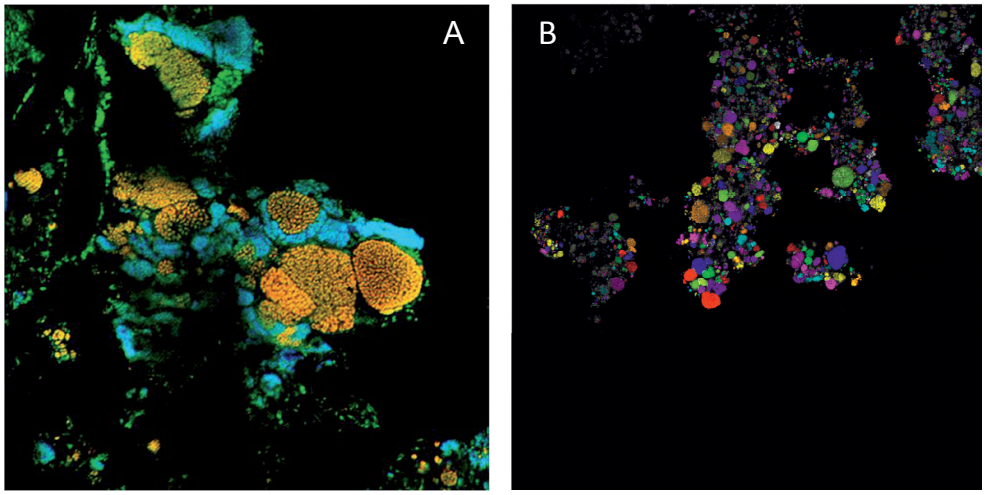


Figure 6. FISH micrographs of nitrifier microcolonies. (A) Homogenized biofilm showing nitrifier microcolonies hybridized with the AOB probe mix (yellow) and NOB probe Ntspa662 (blue). Other bacteria are targeted by the EUB 338 probe mix only (green). (B) Colour-coded AOB microcolonies from the AOB-anammox biofilms in Paper IV, as distinguished by a semi-automatic approach using the digital image analysis software Daime. Figure (A) was originally published in Paper II.

(Limpiyakorn *et al.,* 2006, 2007, Paper I & III). The members of this lineage are considered to be the K-strategists among the AOB, adapted for growth at low substrate concentrations (Bollmann & Laanbroek 2001, Limpiyakorn *et al.,* 2007) but having a rather long reactivation period after starvation (Bollmann *et al.,* 2002). Together with high substrate affinity (1.9-4.2 μM NH_3) (Koops & Pommerening-Röser 2001), also sensitivity to high ammonia concentrations have been reported for members of this lineage (Limpiyakorn *et al.,* 2006, Yuichi *et al.,* 2011). The effect of oxygen limitation on *N. oligotropha*-related cells is somewhat unclear. Gieseke *et al.,* (2001) observed a *N. oligotropha* distribution in biofilms that suggested adaptation to low levels of DO, whereas Park & Noguera (2004) published somewhat contradictory results. It is however quite possible that the two investigations analyzed strains with different response to DO levels and that there exist a range of phenotypes within this lineage. Moreover, some strains of *N. oligotropha* have been observed to produce large amounts of EPS, which may explain why considerable heavy metal resistance was observed for these cells (Stehr *et al.,* 1995).

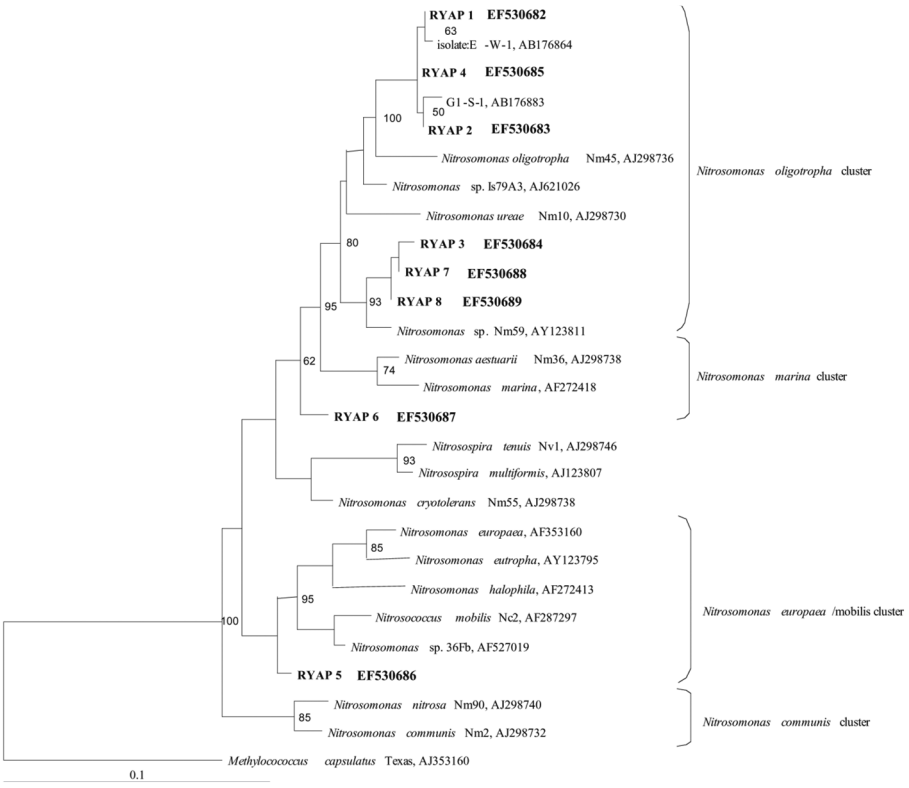


Figure 7. Phylogenetic tree of ammonia-oxidizing bacterial partial 16S rRNA gene sequences. DGGE sequences from the Rya WWTP pilot-plant are shown in bold and compared to reference sequences from the GenBank database. For details on the construction of the trees, see Paper I. Only bootstrap values ≥50 are shown. Scale bar represents the number of nucleotide substitutions per position. The image was originally published in Paper I.

The Nitrosomonas europaea/Nitrosococcus mobilis lineage (Cluster 7)

Four species within this lineage have been described. These share several traits, such as tolerance for high concentrations of ammonia and salt and lack urease activity which may be disadvantageous in low-nitrogen environments (Juretschko *et al.,* 1998, Koops *et al.,* 2006, Stein *et al.,* 2007). *N. europaea* och *Nitrosomonas eutropha* and *Nitrosococcus mobilis* are often found in wastewater treatment plants (Koops *et al.,* 2006) and especially *N. europaea*, which is perhaps the most investigated AOB, has been described as an r-strategist with a comparatively high growth rate and

capability of fast recovery after starvation (Bollmann *et al.,* 2002). Both *N. eutropha* and *N. europaea* have the ability to denitrify (Bock *et al.,* 1995, Schmidt *et al.,* 2004b) and *N. europaea* has accordingly been shown to cope well with low levels of DO and high levels of nitrite (Yu & Chandran 2010). Some taxonomic confusion may arise from the misnomer *Nitrosococcus mobilis*, since this bacteria is closely related to the other nitrosomonads of Cluster 7 and not to members of the γ-proteobacterial genus *Nitrosococcus*. Recently, it was therefore suggested that *Nitrosococcus mobilis* should instead be validated as *Nitrosomonas mobilis* (Campbell *et al.,* 2011).

An interesting feature of the sequenced genomes of *N. europaea* and *N. eutropha* is the different sets of genes coupled to iron uptake and metabolism. Iron (Fe) is an important co-factor in electron transfer and due to their numerous cytochromes and haem-containing enzymes, ammonia-oxidizing bacteria have very high Fe requirements (Wei *et al.,* 2006). However, whereas *N. eutropha* probably can synthesize its own siderophores for Fe scavenging (Stein *et al.,* 2007), the *N. europaea* genome has only one gene for siderophore synthesis despite having a large set of receptors for these molecules (Chain *et al.,* 2003). Consequently it has been suggested that *N. europaea* utilizes a variety of Fe uptake mechanisms, including uptake of siderophores synthesized by other organisms (Wei *et al.,* 2006, Chain *et al.,* 2003).

As the name implies, the fourth species within this lineage, *Nitrosomonas halophila*, has high salt requirements and also displayed extreme alkali tolerance when isolated from soda lakes (Sorokin *et al.,* 2001).

The Nitrosomonas communis lineage (Cluster 8)

Cells related to *Nitrosomonas nitrosa* or *Nitrosomonas communis* have been detected in both municipal (eg Lydmark *et al.,* 2006, Siripong & Rittmann 2007) and industrial WWTPs (Layton *et al.,* 2005). *N. communis* was originally isolated from soil (Koops *et al.,* 1991) and has been hypothesized to require rather high ammonia concentrations, due to relatively low affinity for ammonia and lack of urease activity (Koops *et al.,* 2006). Although isolated from eutrophic environments (Koops *et al.,* 1991), *N. nitrosa* have a comparatively low maximum ammonia tolerance which, together with its ability to utilize urea may make it more suited for less eutrophicated environments than *N. communis* and has, despite apparently low salt tolerance, been isolated also from the marine environment (Koops *et al.,* 2006).

The Nitrosospira (Cluster 0-4) and Nitrosomonas marina (Cluster 6b) lineages

Although not detected in any of the work included in this thesis, members of these lineages, and especially *Nitrosospira*, have frequently been observed in WWTPs (Koops *et al.,* 2006 and references therein). *Nitrosospira briensis* has some features similar to members of the *N. oligotropha* lineage, namely a high substrate affinity (1.8 μM NH_3 in biofilms) (Bollmann *et al.,* 2005) and slow reactivation respons after prolonged periods of starvation (Laanbroek & Bär-Gilissen 2002). Recently, it was for the first time shown how prophage induction affects AOB performance (Choi *et al.,* 2010). The investigation, which was conducted on *Nitrosospira multiformis*, may pave the way for more investigations on phage infection in nitrifying systems.

The nitrite-oxidizing bacteria

Nitrite-oxidizing bacteria are a rather diverse group of bacteria (Teske *et al.,* 1994). NOB are widespread in a variety of habitats in terrestrial and aquatic (eg Watson *et al.,* 1986) environments, even including hot springs (Lebedeva *et al.,* 2005, Lebedeva *et al.,* 2011). Because of their mutualistic relationship, NOB are often found together with AOB (Abeliovich 2006 and references therein) as for example in WWTPs. Although the cold-adapted *Nitrotoga* (Alawi *et al.,* 2007) has been detected once in activated sludge (Alawi *et al.,* 2009), NOB of relevance for wastewater treatment are principally *Nitrobacter* of the *Alphaproteobacteria* and *Nitrospira* of the phylum *Nitrospira* (Ehrich *et al.,* 1995) of which the latter is the most commonly encountered in wastewater treatment samples (Daims *et al.,* 2001a, Daims *et al.,* 2006b). *Nitrospira* is considered to be the K-strategist of the two, being favoured when nitrite concentration is low, probably due to a higher substrate affinity than *Nitrobacter* (Schramm *et al.,* 1999, Wagner *et al.,* 2002, Spieck *et al.,* 2006). *Nitrobacter* on the other hand seems to prefer higher nitrite concentrations and have a higher growth rate, thus representing the r-strategist of the two (Kim & Kim 2006). In the biofilms analyzed in this thesis, only *Nitrospira* cells were detected, growing in characteristic microcolonies in association with AOB (Fig. 6a).

The genus Nitrospira

Nitrospira are slow growing, recalcitrant organisms that are difficult to obtain in pure culture (eg Spieck *et al.,* 2006, Lebedeva *et al.,* 2008) and today, a total of six sublineages have so far been suggested for this genus (Daims *et al.,* 2001a, Lebedeva *et al.,* 2011). The first report of isolated *Nitrospira* cells was published by Watson and colleagues (1986) who isolated *Nitrospira marina* of sublineage IV from the marine environment. Other characterized species include *Nitrospira calida*, described as moderately thermophilic, a property seemingly characteristic of sublineage VI members (Lebedeva *et al.,* 2011). Similarily, Candidatus *Nitrospira bockiana* of sublineage V, thrives at relatively high temperatures (Lebedeva *et al.,* 2008). The most important NOBs for wastewater treatment are however found in sublineage I (Daims *et al.,* 2001a) which has been shown to thrive in higher nitrite concentrations than sublineage II cells (Daims *et al.,* 2006b, Maixner *et al.,* 2006), including the nitrite sensitive *Nitrospira moscoviensis* (Ehrich *et al.,* 1995). Niche differentiation between sublineage I and II was also observed in the pilot-plant where sublineage I dominated over the small population affiliated with sublineage II, which seemed to prefer the low substrate environment in MBBR tank 4 (Paper I). Enrichment cultures of the candidate species *N. defluvii* of sublineage I revealed ampicillin resistance at concentrations inhibiting growth of *Nitrobacter* and heterotrophic bacteria, thus facilitating isolation (Spieck *et al.,* 2006). *Nitrospira defluvii* was recently the first *Nitrospira* genome to be completely sequenced, revealing substantial metabolic differences compared to other NOB (Lücker *et al.,* 2010), and also suggesting potential mixotropohy for *N. defluvii*, in accordance with Daims *et al.,* (2001a). In addition, an unexpected evolutionary link to the anaerobic ammonia oxidizer *Kuenenia stuttgartiensis* was discovered. This anammox bacterium harbored the highest number of closest protein homologues of *Nitrospira defluvii,* including that of the nitrite oxidoreductase. In fact, microaerophily would actually explain many observations of the biofilm spatial distribution patterns in biofilms for this NOB (Okabe *et al.,* 1999, Schramm *et al.,* 2000).

Anammox bacteria

Since the first evidence that the postulated existence (Broda 1977) of anammox bacteria was true (Mulder *et al.,* 1995), it has become clear that the global impact on the nitrogen cycle by this monophyletic group of planctomycete bacteria is

considerable (Kuypers *et al.,* 2005, Op den Camp *et al.,* 2006). Currently, five anammox genera have been suggested: *Brocadia* (Strous *et al.,* 1999), *Kuenenia* (Schmid *et al.,* 2000), *Scalindua* (Schmid *et al.,* 2003), *Anammoxoglobus* (Kartal *et al.,* 2007) and *Jettenia* (Quan *et al.,* 2008). Originally discovered in wastewater treatment systems, anammox bacteria are widespread in freshwater and marine environments and thrive in sediments, biofilms and stratified waterbodies (Jetten *et al.,* 2010). Recently, *Kuenenia sp*. was isolated from deep sea hydrothermal vents (Byrne *et al.,* 2009) and *Scalindua*-related cells were found in the high-temperature environment of a geothermal subterranean oil reservoir (Li *et al.,* 2010). Interesting physiological features include a compartmentalized metabolism where the catabolic anammox reactions take place in the anammoxosome (Lindsay *et al.,* 2001, Jetten *et al.,* 2001) which is in principle a bacterial cell organelle for energy metabolism (Neumann *et al.,* 2011).

All known anammox grow very slowly due to a low maximum substrate conversion rate, have low K_s values for ammonium and nitrite (<5 μM) and are reversibly inhibited by oxygen, although the sensitivity varies considerably between species (Oshiki *et al.,* 2011).

In Paper IV, anammox bacteria related to *Brocadia fulgida*, were detected and analyzed with respect to their spatial distribution in MBBR carrier compartments.

Life and Death in Nitrifying Biofilms

Spatial distribution in biofilms – competition and collaboration

A bacterial biofilm can be defined as a multicellular community attached to a surface and embedded in a matrix of extracellular material. The advantages of growing in biofilms are many. By growing attached to a surface, bacteria can stay indefinitely in a favourable environment under conditions where external forces, such as flowing water, would otherwise sweep them away. In biofilm communities, bacteria are more resistant to antibiotics (eg Nickel et al 1985) and may also avoid predation through microcolony formation (Matz et al 2005).

Biofilms containing multiple functional groups competing for resources or collaborating under mutualistic forms are by definition exceptionally complex and even more so if each functional group contains several populations, each occupying its own ecological niche. Using combinations of FISH, microscopy and microelectrodes, spatial distribution patterns of nitrifiers and formation of substrate gradients in biofilms under different conditions have been shown repeatedly (Schramm *et al.,* 1998, Okabe *et al.,* 1999, Gieseke *et al.,* 2001, Gieseke *et al.,* 2003). Substrate gradients in the biofilm are formed due to biological activity and influence the distribution pattern of microbial populations, resulting in considerable stratification of AOB and NOB (Okabe *et al.,* 1999, Schramm *et al.,* 1999, Paper III) and their activity (Gieseke *et al.,* 2005). In addition, population-specific distributional differences within AOB communities in biofilms have been demonstrated, leading to the conclusion that spatial localization patterns may provide important insights into the interactions and ecology of nitrifying bacteria (eg Schramm *et al.,* 1999, Gieseke *et al.,* 2003, Lydmark 2006, Paper III).

Some of these spatial distribution patterns are predictable. For instance, if the reactor in question is at least moderately aerated and contain biofilms where AOB and anammox coexist, one can expect them to be rather tightly associated, due to their mutualistic relationship. The biofilms would probably also be stratified because of the opposite oxygen demands of the two groups of bacteria. Consequently, AOB can be expected in the aerated region of a biofilm (usually the upper parts, especially if the biofilm is thick) and anammox in the anoxic regions,

below the AOB (eg Tsushima *et al.,* 2007, Vlaeminck *et al.,* 2010, Vazquez-Padin *et al.,* 2010, Paper IV). At the elevated ammonium concentrations usually associated with the anammox process in wastewater treatment, the presence of AOB with high growth rate, such as *N. europaea* would not be surprising especially since they have the ability to cope with low oxygen levels (Yu & Chandran 2010) and denitrify (Bock *et al.,* 1995 Schmidt *et al.,* 2004b). Similarily, biofilm stratification can be expected because of the mutualistic co-aggregation between AOB and NOB (Mobarry *et al.,* 1996, Juretschko *et al.,* 1998, Schramm *et al.,* 1996) (Fig. 6a). Furthermore, when certain members of both sublineage I and II *Nitrospira* co-exist, the latter one can be expected at a location where nitrite levels are low (Maixner *et al.,* 2006).

However, predicting the spatial distribution for a single population in a biofilm is rather difficult. For instance, although the vertical distribution of *Nitrospira* sublineage I cells have been reported as concentrated to the deeper layers of different biofilms (eg Okabe *et al.,* 1999, Paper III), they have in other studies been found concentrated to the surface (eg Gieseke *et al.,* 2001, Paper III). Seemingly inconsistent distribution patterns have also been shown for *Nitrobacter* (eg Terada *et al.,* 2010).

Physiochemical conditions in the surrounding bulk environment, such as electron donor- and acceptor availability, temperature, pH, alkalinity as well the hydrodynamic properties of the system in question, together provide the prerequisites and set the borders for bacterial growth. However, the community composition *per se* influence the spatial distribution of the bacterial populations (eg Okabe *et al.,* 2004, Wu *et al.,* 2009, Terada *et al.,* 2010) as different bacteria have different substrate affinity, growth rate, yield and tolerance or strategies to cope with inhibiting substances and conditions (eg Bollmann *et al.,* 2002, Park & Noguera 2004, Koops *et al.,* 2006, Yuichi *et al.,* 2011, Oshiki *et al.,* 2011). The populations also interact with each other through competition, collaboration, parasitism (for example siderophore snatching by *N. europaea*), exchange of genetic material (Sørensen *et al.,* 2005) and quorum sensing (De Clippeleir *et al.,* 2011). Literally on top of this, predation and viral infections have the potential of altering the biofilm and community structure (see below). In addition, temporal variations of environmental conditions and biotic interactions may create a niche separation in time (Gieseke *et al.,* 2001) which in turn could impact community diversity and

spatial distribution patterns (Connell 1978, Gieseke *et al.,* 2003, Nielsen *et al.,* 2010).

Consequently, extrapolating spatial distribution patterns of individual species based on one or a few studies into a detailed conceptual model may not be feasible. In addition, most, if not all, studies define the nitrifying populations based on hybridization patterns of FISH-probes that may well include multiple populations or entire lineages, thereby obscuring any functional differentiation within these defined populations. Since DNA-sequencing and design of more specific probes have begun to reveal high diversity and functional differentiation within the phylogenetic lineages of nitrifying bacteria (eg Maixner *et al.,* 2006), one of the most intriguing questions, as pointed out by Maixner and colleagues (2006) is whether all phylotypes, based on 16S rRNA gene analysis, in an environment represent differentiated phenotypes. To answer this question, the phylogenetic resolution needs to be further increased along with the development and combination of increasingly sensitive tools for analyzing spatial abundance, distribution, interaction and activity.

Meanwhile, at the current level of methodological availability, a suitable approach for gaining further insights into bacterial ecophysiology through spatial distribution analysis may be to investigate and perhaps even continuously re-analyze existing samples from biofilms with high species diversity at the best phylogenetic resolution possible.

Microcolony growth

One of the inherent properties of nitrifying bacteria in biofilms is their ability to form microcolonies of varying size, shape and density (eg Schramm *et al.,* 1996, Schramm *et al.,* 1999, Gieseke *et al.,* 2003, Okabe *et al.,* 2004, Hallin *et al.,* 2005, Papers I-IV, Fig. 6). These microcolonies are often structurally stable and difficult to disintegrate without killing the bacteria (Larsen *et al.,* 2008, Frank Persson, persson-al communication) and it was recently shown that production of extracellular DNA (eDNA) provides structural strength to the microcolonies which contained high amounts of these molecules (Dominiak *et al.,* 2011). Okabe *et al.,* (2004) measured how nitrifier microcolony average size varied with depth and organic carbon availability in the medium. They observed that AOB microcolony size was rather constant throughout the biofilm when organic carbon was not added. In contrast, size distribution was significantly stratified in the biofilm

residing in a reactor with a C/N ratio of 1, probably an effect of heterotrophic bacteria outcompeting autotrophic nitrifiers in the surface layers of the biofilm, where AOB microcolony size was the smallest. Gieseke *et al.,* (2003) also observed hetereogenous size distribution of AOB microcolonies and reported that *Nitrosococcus mobilis* microcolonies were smaller in less densely populated biofilm regions, whereas larger ones were often found together with AOB from the *Nitrosomonas europaea/eutropha* lineage (cluster 7). In addition, Okabe *et al.,* (2004) observed that microcolonies of two different groups of AOB, belonging to *Nitrosomonas* and *Nitrosospira* respectively, differed in their areal cell density and it was speculated that the looser colonies of *Nitrosospira* would facilitate oxygen and ammonium diffusion which could partly compensate for a lower growth rate. Such loose microcolony structures have also been observed in *Nitrosococcus mobilis* (Gieseke *et al.,* 2003) and *Nitrospira* (Daims *et al.,* 2001a). Microcolony disintegration in *Nitrospira* has been reported as an effect of nitrate accumulation in the system (Spieck *et al.,* 2006). Spieck and colleagues (2006) hypothesized that switching from microcolony to planctonic growth would be a straightforward way of escaping detrimental changes in environmental conditions. Furthermore, it was shown for the AOB *N. europaea* that NO gas functions as a signal for switching between planctonic and biofilm growth (Schmidt *et al.,* 2004a). Thus, several observations indicate that nitrifier microcolony size distribution and density reflects the ecophysiology of the organisms and the conditions prevailing in their environment. Further discussion on this topic is found in the "Conclusions and Outlook" section in this thesis.

Predation

One important reason to form microcolonies is protection against protozoan grazing (Matz *et al.,* 2004, Matz et al, 2005, Johnson 2008). Protozoa constitutes the major group of bacteria consumers in many environments (Fenchel 1987) and grazing actually triggers microcolony formation among certain species of bacteria, thereby reaching a joint size large enough to be inedible for the predator (Matz *et al.,* 2005).

In wastewater treatment systems, protozoan and metazoan predation has been shown to influence the nitrification process (eg Lee & Welander 1994) and has also been suggested to affect nitrifying community structure due to differential vulnerability to grazing between fast- and slow-growing bacteria (Yu *et al.,* 2011).

Large predators, such as larvae and oligochaetes are, due to their size, less likely to be negatively affected by bacterial microcolony formation. These larger organisms may however not only have a detrimental effect on wastewater biofilms, since burrowing may possibly oxygenate deeper layers of the biofilm and perhaps also disseminate nitrifier microcolonies through transport on or even inside the predator. In addition, the importance of clogging prevention of biobeds such as NTFs (Mattsson & Gingsjö 2003), led Palka & Spaul (1970) to suggest that activity of larger predators such as the enchytraeid worm *Lumbricillus lineatus* (Annelida: Clitellata: Enchytraeidae) (10-15 mm in length) may actually be beneficial to certain wastewater treatment systems.

A *Lumbricillus sp*. worm was most likely also the dominating macrofaunal predator in both the full-scale and pilot-plant NTFs at Rya WWTP. Although sequencing and database comparison of the mitochondrial cytochrome c oxidase subunit I (COI) did not provide a decisive match to any taxonomically identified species of *Lumbricillus*, this worm was identical to specimens that have been found to thrive in both fresh and brackish waters in other regions of southern Sweden (Christer Erséus, unpublished data). *Lumbricillus spp.* are generally favoured in oxygenated environments with high nutrient availability (Christer Erséus, personal communication). This is consistent with their existence in the NTF, where they feed on the organic material of the bacterial biofilm.

Screening of worm distribution on sampled NTF plastic material in the 0.5m level of the full-scale NTF revealed a heterogeneous pattern. Abundance varied between <0.1 – 1.9 specimen cm^{-2} corresponding to a biomass varying from <1 to 25% of total biomass dry-weight. These values were however originating from only two sampling times and *Lumbricillus* abundance is most likely subjected to heavy seasonal variations. In addition, predator-prey relationships in nitrifying biofilms may be complex with an as yet partially undetermined impact on the nitrification process. Nonetheless, the patchy distribution of *Lumbricillus* suggests that predator pressure is spatially heterogeneous in the NTF and may, at least locally, suppress biofilm growth. This is supported by the increased nitrifying activity observed after intense flushing of the full-scale NTFs in order to prevent clogging and remove larger organisms (Mattsson & Gingsjö 2003).

Bacteriophage infection

One of the potentially most important but yet almost completely overlooked aspects of nitrifying biofilm function and ecology is bacteriophage (phage) infections of nitrifying bacteria. Keeping in mind that nitrification often has been described as a failure prone process (eg Bellucci *et al.,* 2011) and subjected to sudden community collapse, it may seem rather strange that effects imposed on nitrifying bacteria by the probably most numerous biological entities on earth (Bergh *et al.,* 1989, Chibani-Chennoufi *et al.,* 2004, Suttle *et al.,* 2007) have hardly been investigated at all.

Whether or not viruses are actually living organisms have been extensively debated (Raoult & Forterre 2008, Moreira & Lopez-Garcia 2009). Either way, they have a tremendous impact on life as we know it and for every living cell, there probably exists at least one virus capable of infecting it (Fuhrman 1999). Accordingly, they constitute a key regulating factor of microorganisms in, for instance, marine environments (Bergh *et al.,* 1989). Although there are several viral life cycles (Weinbauer 2004), phage infection starts with adsorption and release of nucleic acids into the bacterial cell. From here, the nucleic acids may be expressed as part of the lytic cycle where phage genome replication and assembly is followed by cell lysis, leading to death of the host cell and proliferation of the phage into the environment. Alternatively, the injected nucleic acids are integrated as a prophage into the host genome and are hence replicated as an integral part of the chromosome during cell division. Whenever the prophage is induced, the lytic cycle is initiated. The host range of phages in the environment varies considerably (eg Holmfeldt *et al.,* 2007) from extremely narrow, where only one specific bacterial strain is susceptible to infection (eg Eydal *et al.,* 2009) to broad host-range phages, observed also in wastewater samples (Jensen *et al.,* 1998), capable of infecting several different bacterial species. Furthermore, it has been shown that phages are indeed important also in biofilms. Rice and collegues (2009) reported that a prophage in *Pseudomonas aeruginosa* was of significant importance for the biofilm life cycle of this bacterium and also suggested that phage particles could contribute to physical stability of biofilms. Vice versa, phage treatment has also been suggested as a means of biofilm control or destruction (Sutherland *et al.,* 2004).

Until recently, no direct proof of phage infection of nitrifying or anammox bacteria had been published. Complete genome sequencing of the AOB *Nitrosomonas eutropha* and *Nitrosospira multiformis* revealed genes coding for phage-

related proteins (Stein *et al.,* 2007, Norton *et al.,* 2008). Moreover, a region of clustered regularly interspaced short palindromic repeats (CRISPR) providing resistance against phages (Barrangou *et al.,* 2007) was recently discovered in the genome of the NOB *Nitrospira defluvii* (Lücker *et al.,* 2010). Vlaeminck (2010) and colleagues presented images of phages replicating in AOB cells growing in granular aggregates. These granules also contained anammox cells, apparently unsusceptible to the observed virus particles. Later that year, Choi *et al.,* (2010) successfully induced a prophage in *N. multiformis*, showing for the first time that phage-mediated cell lysis could indeed be of importance for ammonia oxidation efficiency in wastewater treatment.

Methods for studying microbial diversity

The full-cycle 16S rRNA approach

Rather than being a method *per se*, the full-cycle 16S rRNA approach is a strategy for analyzing microbial diversity and activity in complex environmental samples, such as wastewater biofilms. This is achieved through the combination of different molecular methods and microscopy (Fig. 5). A full cycle is not always needed, since each included method will provide important information on its own. Usually, an appropriate first step is the extraction of DNA from the sample followed by PCR amplification of the 16S rRNA gene. From here, the construction of clone libraries for gene sequencing and/or analysis using DNA-fingerprinting methods is possible. Alternatively, quantitative PCR (Q-PCR) can be used for measuring the abundance of specific genes in the extracted DNA or, in combination with reverse transcriptase (RT-Q-PCR), activity in terms of gene expression. Q-PCR techniques are used extensively and have contributed significantly to the field of microbial ecology (for review see Smith & Osborn (2009)).

DNA fingerprinting techniques

For analysis of microbial diversity in environmental samples, DNA-fingerprinting methods provide a time-efficient alternative to conventional cloning and sequencing. Two of the most widely used techniques are terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.,* 1997) and denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.,* 1993).

For community analysis using T-RFLP, the 16S rRNA gene is amplified from extracted DNA using at least one fluorescently labeled oligonucleotide primer per primer pair. The amplicons are cut with restriction enzymes, resulting in fluorescent restriction fragments of varying lengths due to DNA sequence differences. Analysis of restriction fragments will thereafter assess diversity and changes in the microbial community structure. Together with multivariate statistical analysis, such alterations may be coupled to environmental changes (eg

Wessén *et al.,* 2011) or susceptibility to toxic compounds (Persson *et al.,* 2011). However, the short and variable length of the restriction fragments does not allow for further phylogenetic analysis. Hence, parallel construction of 16S rRNA gene clone libraries is necessary for accurate identification of the species present in the sample.

DGGE is a method used for separating 16S rRNA gene amplicons of equal length, based on small sequence differences. DNA is amplified using modified primers adding a 40bp GC-clamp to the PCR product. The amplicons are thereafter applied onto a denaturing gradient gel. Differences in base-pair composition result in melting temperature differences within the molecule and whenever a region of the DNA melts, the molecule will halt in the gel. This will result in spatial separation of bands containing the different amplified DNA molecules in the gel. The number of bands reflects the genetic diversity in the sample, which can be further analyzed through retrieval and sequencing of the DNA fragments (eg van der Gast *et al.,* 2005, Paper I). The technique is sensitive, as it can detect populations comprising less than one percent of the total bacterial community (Muyzer *et al.,* 1993).

DGGE was used in Paper I for investigating the diversity of partial 16 rRNA gene and *amoA* sequences in a nitrifying moving bed biofilm reactor (MBBR) (Paper I). Phylogenetic trees were constructed from DGGE gel band sequences from both genes. In Paper III, the construction and sequencing of 16S rRNA gene clone libraries enabled the construction of both AOB and NOB trees, which together with *in situ* spatial distribution analysis provided insight into the microdiversity of *N. oligotropha* and its spatial relationship with *Nitrospira*.

Fluorescence In Situ Hybridization (FISH)

Fluorescent cell-staining in combination with microscopy and manual cell-counting was for a long time the standard method for enumeration of bacteria, and is still of great importance to the field. Application of staining agents such as 4',6-diamidino-2-phenylindole (DAPI) for staining DNA or the protein stain fluorescein isothiocyanate (FITC) to a sample, allows for immediate visualization and quantification of cells. Live/Dead stains or activity stains, such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Rodriguez *et al.,* 1992), provide information, although somewhat limited (Nielsen *et al.,* 2003a), on their metabolical state and compounds staining EPS-components may provide important structural

information of biofilms (eg Baird *et al.,* 2012) (Fig. 8). However, these agents do not discriminate between species, thus making identification complicated (if not impossible) unless the samples analyzed are defined mixed cultures where community composition is already known and cells can be distinguished from each other based on well-characterized morphological properties. Hence, for samples with unknown species composition, such as environmental samples, an alternative approach for identification must be utilized.

Similar to 16S rRNA gene-targeting oligonucleotide primers for PCR, oligonucleotide probes can be designed to target the corresponding gene product, the 16S ribosomal RNA. Via fluorescent labeling of the oligonucleotides, with photostable fluorescent dyes such as carbocyanine or Alexa dyes (Panchuk-Voloshina *et al.,* 1999), ribosomes can be targeted *in situ* through hybridization, allowing identification of cells based on their genetic properties in the original sample. This is the fundamental principle of FISH, thus closing the 16S-analysis cycle. In many bacteria, RNA synthesis decrease rapidly during starvation (eg Nyström *et al.,* 1992) and ribosome content is therefore often correlated with cellular activity, making FISH probe signal intensity proportional to growth rate (eg DeLong *et al.,* 1989). It was however shown that AOB maintain their ribosome levels even under starvation (Johnstone & Jones 1988, Morgenroth *et al.,* 2000) or inhibition (Wagner *et al.,* 1995). Observations leading to the same conclusion for NOB (Daims *et al.,* 2001a) and anammox (Schmid *et al.,* 2001) have also been made. Hence, probe signal alone will not necessarily reflect the metabolical state of these bacteria.

One of the great advantages of FISH is the possibility to target several microbial populations simultaneously (eg Amann *et al.,* 1996, PaperII, III, IV and V). This method has made a significant contribution to microbial ecology since its introduction and first applications on environmental and wastewater samples in the early 1990´s (Amann *et al.,* 1990a, Manz *et al.,* 1994). It is becoming an increasingly important tool also in clinical microbiology (Moter *et al.,* 2010, Fröjd *et al.,* 2011). In combination with Confocal Laser Scanning Microscopy (CLSM) and digital image analysis, FISH has been the methodological backbone of all papers included in this thesis for population dynamics studies (Papers I and II) and *in situ* spatial distribution analysis (Papers III and IV).

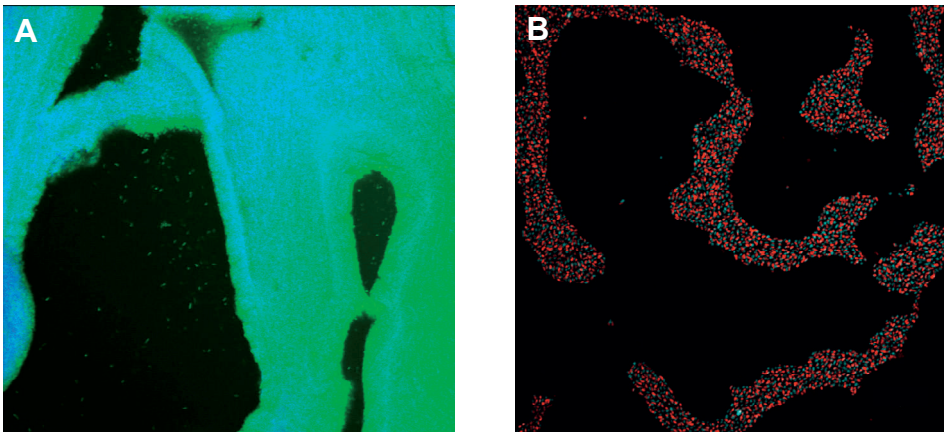


Figure 8. Examples of bacterial staining agents. (A) *Pseudomonas aeruginosa* biofilm simultaneously stained with DAPI (cyan) and FITC (green). (B) DAPI (blue) and CTC (red) stained *P. aeruginosa* cell suspension. The red signal from the CTC-stain originates from the formation of formazan crystals, indicative of actively respiring cells. Image (A) courtesy of Fiona Baird and the author. Image (B) courtesy of Fiona Baird.

Many of the technical aspects of FISH are reviewed by Moter & Göbel (2000) and Bouvier & del Giorgio (2003). Briefly, the technique requires initial fixation to permit proper probe penetration into cells. A proper fixative should also preserve cell morphology, making paraformaldehyde (PFA) suitable for fixation of cells with gram-negative cell wall (Amann *et al.,* 1990b), such as nitrifying and anammox bacteria. After an initial dehydration step in an ethanol-series, hybridization is performed through application of a solution including the following components: i) the fluorescently labeled oligonucleotide probe(s) and, if needed, unlabeled competitor probes preventing unspecific signal from sequences very similar to the target (Manz *et al.,* 1992), ii) formamide (FA) which controls hybridization stringency by lowering the melting temperature of nucleic acids (Casey & Davidson 1977, Stahl and Amann 1991), thus enabling the use of a fixed hybridization-temperature for FISH (usually 46°C), iii) sodium dodecyl sulfate (SDS) which denatures the ribosomes, thus eliminating the effect of ribosomal tertiary structure on probe accessibility (Behrens *et al.,* 2003). The optimal formamide concentration is determined for each probe experimentally (Manz *et al.,* 1992) and evaluated through a probe dissociation profile where probe signal intensity is plotted against increasing FA-concentrations (Daims *et al.,* 1999). Since the optimal FA-concentration differs between probes, analysis using multiple probes on the same

sample may require separate serial hybridizations, always starting with the probe requiring the highest concentration of FA. This particular order is important because a subsequent stringent washing step, wich gets rid of excess- and unspecifically bound probes, will otherwise wash out the hybridized probes from the first FISH. The hybridized specimen is thereafter mounted in antifading agent and analyzed using fluorescence microscopy and digital image analysis.

Empirical evaluation of FISH-probes

Over the years, a substantial number of 16S rRNA-targeted oligonucleotide probes have been designed to target different groups of bacteria. As an example, over 2600 probe sequences have been submitted to the online resource probeBase (Loy *et al.,* 2003, 2007). Out of these, approximately 200 sequences are designed to target microbes described as relevant for wastewater treatment, such as nitrifying and anammox bacteria (eg Amann *et al.,* 1990b, Mobarry *et al.,* 1996, Daims *et al.,* 2001a, Kartal *et al.,* 2008). However, the increase in available 16S sequence data, continually reveals new information that may call for revision of certain probes and their specificity (eg Purkhold *et al.,* 2000). One example is the lack of a single probe targeting all known AOB, since sequence comparison have shown that even broad-target probes have mismatches towards some of the supposed target cells (eg Hallin *et al.,* 2005, Paper I). However, a properly designed AOB probe mix will provide the relevant coverage of this functional guild (Daims *et al.,* 2009, Paper II and III). Moreover, empirical observations have shown discrepancies between the supposed specificity of certain probes and the data obtained from corresponding experiments (eg Daims *et al.,* 2009). Some probes have been published with erroneous sequences, sometimes with no erratum available, thus seriously impairing their use in new studies or, even worse, leading to incorrect interpretation of experimental data.

Hence, there are a number of pitfalls to avoid and options to consider, some of which are discussed below, when searching for appropriate FISH probes targeting nitrifying and anammox bacteria.

Nsm156: Screening with this probe, targeting members of *Nitrosomonas spp*. and *Nitrosococcus mobilis*, on MBBR samples, raised some questions regarding probe specificity. A subsequent comparison using the online-tool probeCheck (Loy *et al.,* 2008), comparing the sequence to the 16S/18S rRNA database SILVA (Pruesse *et al.,* 2007), returned a number of perfect hits for non-AOB organisms. In

addition, Terada *et al.,* (2010) reported only weak hybridization signals for this probe when applied on *Nitrosomonas*-containing biofilm samples.

Nso1225: One of the broader AOB-specific FISH probes available which has been used extensively. In the original publication (Mobarry *et al.,* 1996), the Nso1225 probe sequence is incorrect. The correct sequence was later published in an erratum (Mobarry *et al.,* 1997) and is also to be found in probeBase. Nso1225 has been observed to produce a seemingly unexplainable dim and weak fluorescence signal (Daims *et al.,* 2009, Terada *et al.,* 2010). A possible reason for this can be found when analyzing probe dissociation curves in (Schramm *et al.,* 2002). In this paper, the authors developed a novel method, Clone-FISH, for the evaluation of new FISH probes. As a proof of principle, probe dissociation curves were constructed for Nso1225 when applied to both a *N. europaea* culture as well as matching *in vivo* transcripts of 16S rRNA gene clones. Both curves indicated a considerable drop in signal intensity between 30 and 35% FA. The latter FA concentration is currently the standard for this probe, thereby risking loss of fluorescence signal from target populations. Hence, reevaluation of optimal hybridization conditions may be warranted for Nso1225.

Nso190: Initially designed to encompass most known β-AOB sequences (Mobarry *et al.,* 1996). The authors identified single base-pair mismatches towards two *Nitrosomonas* sp. and suggested the probe to be used together with Nso1225 in order to target the entire β subclass of AOB. The increase in available 16S-sequences led to later revision of probe specificity, as additional mismatches between the probe and certain strains of AOB were revealed (eg Purkhold *et al.,* 2000, Utåker and Nes 1998). Nevertheless, Nso190 has been used as a universal β-AOB probe in several investigations. To circumvent limitations in probe coverage, hybridization stringency has often been reduced by changing the FA concentration in the hybridization buffer from the originally suggested 55% to different concentrations ranging from 20-50% (e.g. Konuma *et al.,* 2001, Biesterfeld *et al.,* 2001, Persson *et al.,* 2002, Pynaert *et al.,* 2003, Kindaichi *et al.,* 2004, Satoh *et al.,* 2006, Hallin *et al.,* 2005, Lydmark *et al.,* 2006, Kindaichi *et al.,* 2006). It was however reported that at FA concentrations of 25-30% or lower, non-AOB cells having two mismatches at the target site, hybridized with the probe (Konuma *et al.,* 2001). Therefore, changes in hybridization conditions should be undertaken with care and appropriate controls are necessary to avoid unspecific probe binding. As of today, the best solution for obtaining close to complete AOB-coverage is the use of

a mix of AOB probes. In order to maximize both coverage and stringency, such mixes usually do not include Nso190 (eg Paper II, III and Daims *et al.,* 2009), although exceptions exist (eg Terada *et al.,* 2010).

Cluster6a192: Designed to target the *N.oligotropha* lineage (Cluster 6a), it is one of the most suitable probes for targeting this group of bacteria. However, in Paper III, sequence data show that mismatches exist in the target site of cells within this lineage. Nonetheless, it is suitable for use in a 35% FA AOB probe-mix. To avoid targeting non-*N.oligotropha* AOB, it should be used together with an unlabelled competitor, at least when not included in an AOB-mix. When analyzed with probeCheck, the published competitor does not completely match any of the intended targets (*N. eutropha*) or any other available sequence. This is also the case for the alternative competitor sequence published in Daims *et al.,* (2009). However, the competitor sequence available in probeBase generates 85 perfect hits and would thus be preferred. Unfortunately, one of the erroneous competitor-sequences was consistently used in the work of this thesis for analyses involving Cluster6a192. However, DNA-sequencing (Paper III) of samples from the pilot-plant did not produce sequences targeting any of the competitors and cross-hybridization with probe Neu, targeting *N. eutropha* for which the original competitor was designed, showed no overlap with Cluster6a192. Finally, a potential risk of targeting *N. communis* was ruled out in Adamczyk *et al.,* (2003), where no signal was obtained from such cells hybridized with Cluster6a192, even when FA-concentration was lowered to only 20%.

Neu: Target organisms include *N. europaea*, *N. eutropha* and *N. halophila* with an optimal formamide concentration of 40% (Wagner *et al.,* 1995). However, at 30% FA, the probe dissociation curve showed no detectable signal from non-AOB cells known to have one mismatch towards the probe sequence. Hence, hybridization at 35% FA is acceptable for this probe, making it suitable for an AOB-probe mix, such as in Paper II and III.

Nsv443: Ambiguous results from using this probe have been reported (Daims *et al.,* 2009).

Table 1. AOB probe-mixes suggested in the literature.

Probe	Purkhold et al (2000)	Daims et al (2009)	Paper II & III	Terada et al (2010)	Specificity according to probeBase and/or original article.
Nso1225	+	+	+	-	Betaproteobacterial ammonia-oxidizing bacteria
Nso190	-	-	-	+	Betaproteobacterial ammonia-oxidizing bacteria
Nsv443	+	-	-	-	*Nitrosospira spp*.
Nsm156	+	-	-	-	*Nitrosomonas spp*., *Nitrosococcus mobilis*
NEU	-	+	+	-	Most halophilic and halotolerant *Nitrosomonas spp*.
CTE	-	+	+	-	*Comamonas spp*., *Acidovorax spp*., *Hydrogenophaga spp*., *Aquaspirillum spp*.
NmV	-	+	-	-	*Nitrosococcus mobilis* ("*Nitrosomonas*") lineage.
Cluster6a192	-	+	+	+	*N. oligotropha* lineage (Cluster 6a)
c6a192	-	+	-		Used as unlabeled competitor to Cluster6a192. Targets *N. eutropha*
Noli191	-	-	+	-	*N. oligotropha*/ *N.ureae*
cNoli	-	-	+	-	Various bacteria
Nmo218	-	-	-	+	*N. oligotropha* lineage (Cluster 6a)

(+)= Probe included. (-) = Probe not included. Probes CTE, c6a192 and cNoli were included as unlabeled competitors only.

AOB probe-mix: Since there is no probe today that targets all known AOB, a mixture of probes is generally required to obtain close to full coverage of this group. Consequently an AOB probe-mix was designed and applied in Paper II and III. Today, a number of probe-mix variants exist differing slightly from each other (eg. PaperII, Purkhold *et al.,* 2000, Daims *et al.,* 2009, Terada *et al.,* 2010). Some of these suggested mixes are shown in Table 1.

Eub338 (I-IV): Accurate estimates of the total bacterial population are imperative for FISH on environmental samples when quantifying the relative or absolute numbers of specific bacterial populations. Furthermore it provides a control against unspecific binding, since there should always be a fluorescence overlap with Eub338 for the population-targeting probe signal to be treated as specific. Initially, a single probe, Eub338, was designed to meet these demands (Amann *et al.,* 1990b). Later it became apparent that this probe alone was not enough for covering all eubacterial phyla. Among others, cells belonging to *Planctomycetales* such as the anammox bacteria, were missed, leading to the design of two new Eub338 variants (Eub338-II and -III) targeting the missing groups (Daims *et al.,* 1999) and used together with original Eub338-I in an equimolar

probemix. A few years later, it became apparent that not all anammox bacteria were sufficiently targeted by this mix. Consequently, a fourth probe, Eub338-IV, was added (Schmid *et al.,* 2005)

NON338: Used as a negative control for nonspecific binding (Wallner *et al.,* 1993). The probe is complementary to Eub338-I.

CLSM and Digital Image Analysis of FISH Micrographs

For quantitative analysis of wastewater or biofilm samples, standard epifluorescence microscopy is severely affected by out-of-focus fluorescence, making quantification of biofilm structure or population composition difficult and inaccurate. This was overcome through the use of Confocal Laser Scanning Microscopy (CLSM) for analyzing microbial biofilms (eg Lawrence *et al.,* 1991). CLSM effectively blocks out-of-focus light via an adjustable pinhole. The thin focal depth, together with the powerful light-source provided by the lasers, allow optical sectioning along the z-axis in a sample.

Nitrifying bacteria in biofilms generally are heterogeneously distributed and grow as microcolonies of varying size and shape (Fig. 6), each made up of hundreds or even thousands of cells. Consequently, statistically reliable manual cell counts are virtually impossible to obtain within reasonable time-frames. A more time-efficient and reliable option is the combined use of microscopy and digital image analysis (Daims & Wagner 2007). The relative abundance, or biovolume fraction, of individual populations present in a sample can be calculated digitally from CLSM micrographs and expressed as the areal percentage of the total number of pixels obtained with a bacterial reference stain, usually the Eub338 probe mix (Amann *et al.,* 1990b, Daims *et al.,* 1999). This enables the use of FISH as a quantitative method for estimating population densities as well as absolute cell numbers (Schramm *et al.,* 1999, Daims *et al.,* 2001b), Hallin *et al.,* 2005) or biovolumes (eg Kuehn *et al.,* 1998) of specific populations. Together with process data, cell-specific nitrification can be calculated (Paper I) and the relative abundance of the nitrifying functional guilds can be correlated with nitrification potential (Biesterfeld *et al.,* 2003, Paper II)

Image analysis software suitable for microbial population or biofilm quantification include Daime (Daims *et al.,* 2006c), COMSTAT (Heydorn *et al.,* 2000) and PHLIP (Mueller *et al.,* 2006). Daime was used for image analysis in all papers. In addition, COMSTAT was used in Paper I for quantification of total

bacterial biovolume. Photoshop CS4 Extended (Adobe Systems, San Jose, CA, USA) was used for superimposition of sequential FISH images in Paper III as well as manual assembly and preparatory intensity thresholding in Paper IV.

A picture can tell a thousand lies – Image analysis ethics

Digital image analysis has undoubtedly become a very important tool in modern microbial ecology (eg Nature Reviews Microbiology 2006), enabling quantification of cell numbers and their spatial distribution patterns in complex biological samples. Image processing software can however easily be misused for inappropriate manipulation of images which ultimately may lead to publication of fake results (e.g. Kennedy 2006). An introduction to image analysis ethics have been published by Rossner and Yamada (2004).

Post-acquisitional editing of scientific images should be undertaken with outmost care. An appropriate and generally accepted guideline states that "No specific feature within an image may be enhanced, obscured, removed or introduced" (Rossner and Yamada 2004). Although increasing brightness and contrast of selected parts or individual objects within an image would clearly violate this guideline, such changes may be acceptable for demonstrational purposes if applied on the *entire* image in a way that does not remove or obscure information, as has been done on the micrograph images included in this thesis. When applied, this should be clearly stated and any quantitave image analysis processes should be undertaken on the original unedited images (eg Paper III). However, in order to properly distinguish signal from background, operations such as intensity thresholding or noise reduction are often needed. Similarly autofluorescent artefacts may be present and need to be excluded, to avoid biased measurements of e.g. biovolume fractions. Such manipulations must consequently be applied with caution and presented in detail to avoid erroneous interpretation of image information. Details on image processing procedures are found in the experimental procedures sections for each paper included in this thesis and detailed guidelines for use of the Daime software can be found at http://www.microbial-ecology.net/daime/daime-faq.asp.

Moreover, image acquisition must also be undertaken with care to avoid flawed results and conclusions. When quantifying population abundances, each field of view (FOV) must be randomly chosen to ensure that the estimate is representative and the CLSM settings must be chosen carefully and used consistently to obtain

reliable results (eg Sekar *et al.,* 2002). Minor changes in CLSM settings may however be needed to ensure that objects present in more than one channel are of the same size and shape, i.e. that they are congruent. Such minor adjustments were needed for acquisition of congruent images during sequential FISH in Paper III. In Paper IV, substantial variation in the signal to noise ratio of the different hybridizations required more adjustments in the CLSM settings in order to obtain comparable images for the subsequent stratification analysis. To facilitate visualization of entire Minichip compartments in Paper IV, individual biofilm micrographs were manually assembled which required acquisition of images with higher resolution (1024*1024 pixels) than normally used for analyzing homogenized biomass samples (512*512 pixels) (Paper I & II). This high level of detail also made possible manual setting of global intensity threshold levels for each channel in each assembled image in order to overcome most of the variations in signal strength. In fact, signal strength in FISHed cryosectioned biofilms were generally weaker than in homogenized biomass from the same samples hybridized with the same set of probes. One explanation could be that FISH-probes may access target cells more easily in homogenized biomass, compared to dense undisrupted biofilms.

FISH a la Carte: Evolution and future of FISH

Perhaps the most intriguing characteristic of FISH is versatility. Novel applications and improvements are being published on a yearly basis, much due to the suitability of combining FISH with a variety of other techniques. It is therefore likely that FISH will continue to evolve as new methodological approaches are introduced in microbial ecology. Some of the species of FISH are described below:

DOPE-FISH, *CARD-FISH* and *Gene-FISH*: The detection limits for conventional FISH is significantly lower for pure cultures compared to environmental samples such as activated sludge (Hoshino *et al.,* 2008). This makes cells with low ribosome content or probe targets other than ribosomal RNA, sometimes hard to detect using a single FISH-probe on environmental samples. Therefore, alternative protocols have been developed to improve detectability. Double labeling of oligonucleotide probes (DOPE)-FISH is a straightforward way of increasing signal strength by labeling both the 3´- and 5´-ends of the probe, which also increased *in situ* probe accessibility when Cy3 was used (Stoecker *et al.,* 2010). It should however be noted that 3´-labeling with Cy5 may be more sensitive

to degradation during synthesis, thus resulting in lower yield compared to Cy3 (Holger Lommel, Thermo Fisher Scientific, personal communication). Compared to conventional FISH, the sensitivity of CAtalyzed Reporter Deposistion-FISH (CARD-FISH) (Pernthaler *et al.,* 2002) was up to 41 times higher, depending on sample type (Hoshino *et al.,* 2008). The protocol is however time-consuming and will not necessarily detect cells with only one probe target site. In addition, endogenous peroxidase activity may result in unspecific signal, thus requiring sample-specific optimization of the protocol (Pavlekovic *et al.,* 2009). The use of CARD-FISH for targeting mRNA for the *rpoB* gene as an alternative to 16S have been suggested (Case *et al.,* 2007) and would be feasible (Pernthaler & Amann 2004), although mRNA leakage and degradation due to the nature of the CARD-FISH protocol need consideration (Hoshino *et al.,* 2008). However, a promising new method, GeneFISH, was recently developed for linking gene presence with cell identification through the combination of CARD-FISH and gene detection in environmental samples (Moraru *et al.,* 2010).

Clone-FISH and *mathFISH* may both become important tools for the evaluation and design of new FISH probes. Using Clone-FISH, FISH-probes may be validated through hybridization with *in vivo* transcribed rRNA from vector inserts (Schramm *et al.,* 2002), whereas mathFISH is a novel web-based tool for evaluating potential FISH-probes *in silico* (Yilmaz *et al.,* 2011).

PNA-FISH is the application of peptide nucleic acid (PNA) probes for *in situ* hybridization. Such probes may offer increased hybridization sensitivity and the effect of PNA-FISH hybridization protocols on cell integrity is lower (Worden & Chisholm 2000).

Several FISH-applications for linking structure and function of microbial cells have been developed. *FISH-MAR* (Lee *et al.,* 1999, Daims *et al.,* 2001a) utilizes microautoradiography (MAR) for analyzing uptake of radiolabeled substrates which, in combination with FISH can be used to visualize *in situ* activity of defined microbial populations. Further development of the technique showed that absolute uptake of radiolabeled substrate could be quantified, using the *Quantitative FISH-MAR* protocol (Nielsen *et al.,* 2003b). The FISH-MAR methods are reviewed by Wagner *et al.,* (2006). Uptake of the stable isotope ^{13}C can be analyzed through the combination of Raman spectroscopy and FISH (*Raman-FISH*, Huang *et al.,* 2007). The resolution of this method allows analysis at the single-cell level, making it suitable also for complex multispecies environmental samples. An alternative

approach, not involving radio-labeled substrates is *ISR-FISH* (Schmid *et al.,* 2001) where mRNA of the intergenic spacer region (ISR) between the 16S and 23S genes is targeted through simultaneous application of several probes. Transcription of these genes *in vivo* results in a single mRNA molecule which is followed by cleavage and degradation of the intergenic spacer transcript. Hence, only transcriptionally active cells will generate a positive hybridization signal. However, the ISR sequence is variable and separate probe-sets need to be designed even for very closely related cells.

Finally, Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH) (Valm *et al.,* 2011) Is a powerful new method combining FISH with multispectral imaging, potentially allowing hundreds of microbial populations to be visualized simultaneously.

Spatial distribution analysis in bacterial biofilms

Spatial distribution analysis in complex samples such as biofilms is dependent on digital image processing to obtain statistically reliable results (eg Daims & Wagner 2011). However, time-consuming manual image-processing required to quantify biofilm stratification (e.g. Lydmark 2006) may limit data collection and thereby the flexibility of the experimental setup and statistical evaluation. Recent software developments for digital image analysis have improved analysis of microbial spatial relationships (eg Daims *et al.,* 2006c) and provided valuable insights into the ecophysiology of nitrifiers (eg Maixner *et al.,* 2006). However, the examination of co-aggregation patterns of defined microbial populations could not be performed on directionally dependent (anisotropic) biofilms until recently (Daims & Wagner 2011).

In Paper III, new methods for *in situ* investigation and quantification of vertical distribution and co-aggregation patterns of defined biofilm populations were presented. It consisted of sequential FISH in combination with a novel image analysis tool (the automated Slicer) for examining biofilm stratification. Applied on cryosectioned biofilms from the nitrifying pilot-plant and on samples from the full-scale NTF at Rya WWTP, these methods were used together with the new "inflate" algorithm for co-aggregation analysis of microbial populations in anisotropic samples (Daims & Wagner 2011).

Sequential FISH

With a hierarchical, partly overlapping set of probes and different fluorochrome labeling, seven populations were detected simultaneously by Amann *et al.* (1996). In fact, when combined with a standard three-laser CLSM, the limiting factors for the number of populations that may be visualized simultaneously are often not probe-availability but the number of lasers and fluorescence detectors, together with their excitable and detectable spectral ranges. The development of Sequential FISH in Paper III further increased the maximum amount of populations that may be analyzed simultaneously in biofilm samples using a standard three-laser CLSM. This approach is preferably used on samples with a pronounced topography, such as cryosectioned biofilms, since it requires repeated sequential image acquisitions at the same location in the sample. Through the application of partially overlapping probes in a first round of FISH but omitting the Eub338 probe mix, the full capacity of the confocal microscope can be used to acquire images of the specific microbial populations (Fig. 9a). A subsequent hybridization, adding only the Eub-mix is then followed by a second image acquisition at the same position in the biofilm (Fig. 9b). Images from the two hybridizations can thereafter be superimposed and checked for congruency to ensure that all corresponding objects are of the same size and shape (Fig. 9c). Thereafter, analysis of relative abundance or spatial distribution can be performed using digital image analysis software, such as Daime.

The automated Slicer

Vertical distribution analysis of biofilm populations requires separation of the biofilm layers to be analyzed. This can be achieved through image analysis techniques, such as the manual image-editing method in Lydmark *et al.,* (2006). However, manual image editing is very time-consuming and inflexible, which is why the automated Slicer was developed in Paper III. The Slicer is an automated tool for *in silico* generation of biofilm slices for vertical distribution analysis of microbial populations in biofilms. It will be implemented in the 2.0 version of the Daime image analysis software and utilizes an edge-detection algorithm for recognizing the biofilm surface, resulting in a line with the same contour. The line is subsequently projected throughout the biofilm in user-defined intervals thereby creating virtual slices (Fig. 10a). Abundance of microbial populations can thereafter be quantified for each slice (depth), resulting in a vertical distribution pattern of the

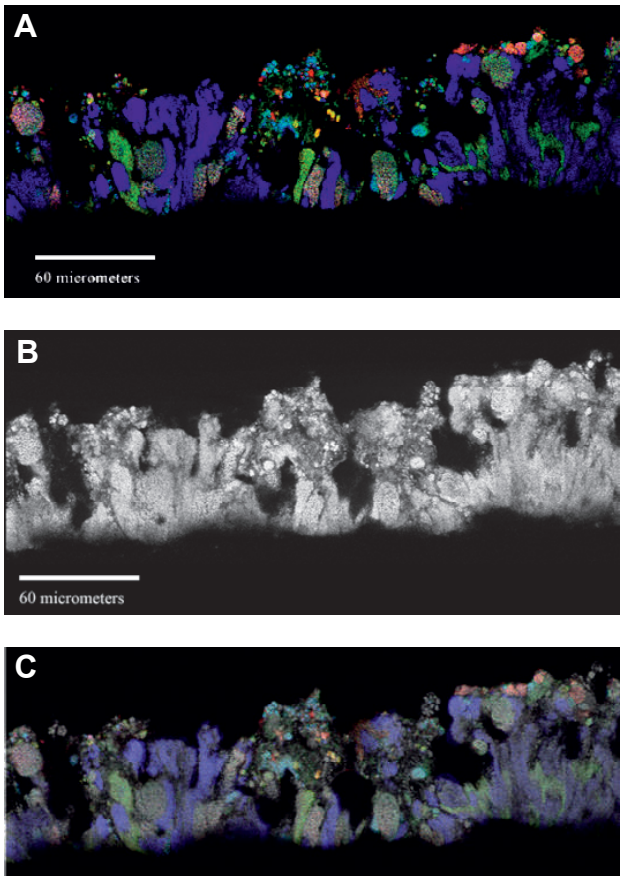


Figure 9 Sequential FISH. (A) Application of partially overlapping probes in a first round of FISH, utilizes the full capacity of the CLSM for acquiring images of specific microbial populations. (B) The following hybridization adds only the Eub-mix and a second image acquisition is performed at the same position in the biofilm. (C) Images from the two hybridizations are superimposed and checked for congruency. For details on the probe hybridization patterns, see Paper III.

populations in question. To ensure statistically reliable results, several biofilm FOVs need to be analyzed. Therefore, the Slicer also supports batch processing of images. In addition, the Slicer is multidirectional and may therefore be used to analyze biofilms either top-down or bottom-up. It can also analyze biofilms with different substratum shapes, such as the compartments of the Minichip MBBR

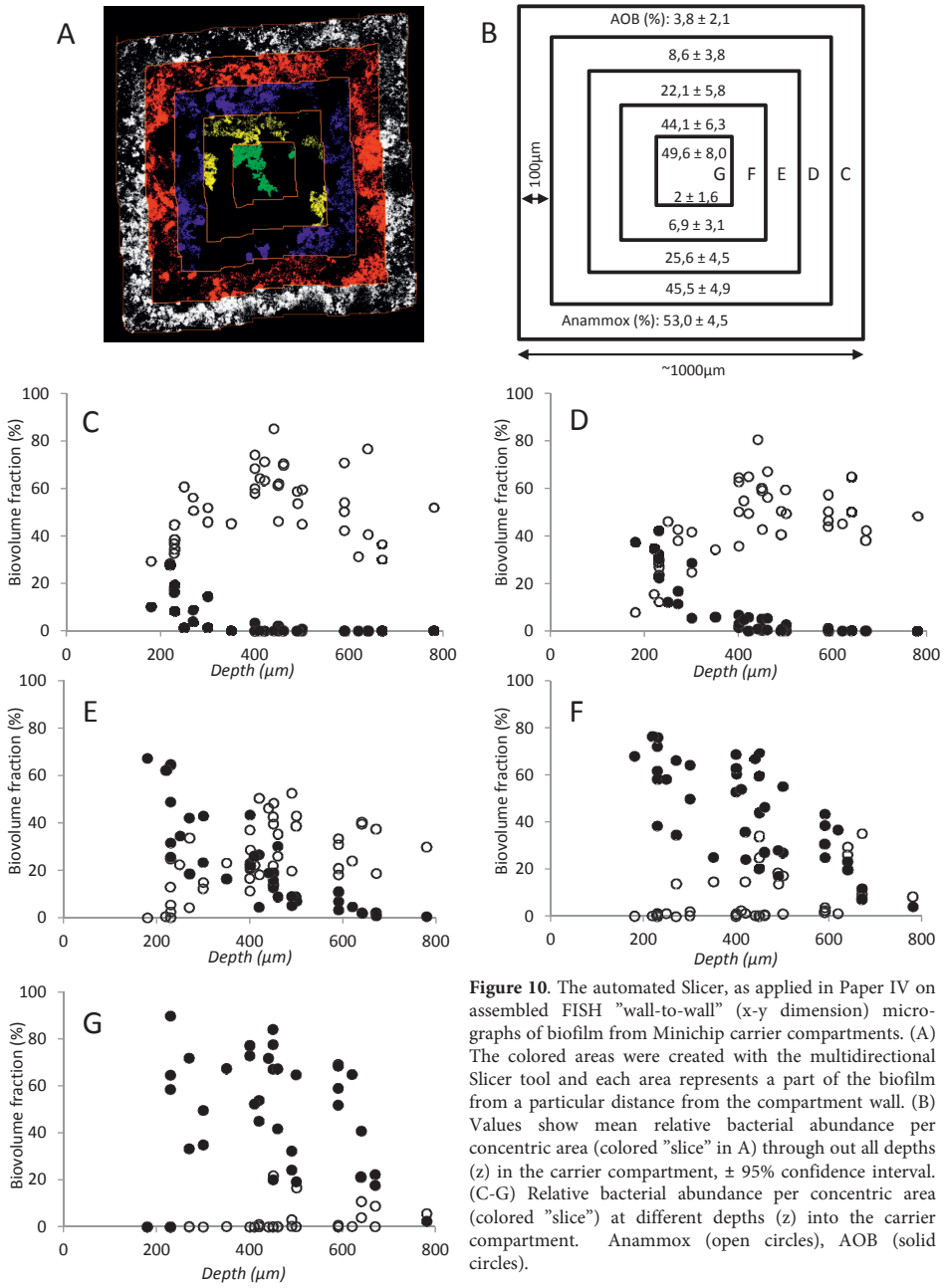


Figure 10. The automated Slicer, as applied in Paper IV on assembled FISH "wall-to-wall" (x-y dimension) micrographs of biofilm from Minichip carrier compartments. (A) The colored areas were created with the multidirectional Slicer tool and each area represents a part of the biofilm from a particular distance from the compartment wall. (B) Values show mean relative bacterial abundance per concentric area (colored "slice" in A) through out all depths (z) in the carrier compartment, ± 95% confidence interval. (C-G) Relative bacterial abundance per concentric area (colored "slice") at different depths (z) into the carrier compartment. Anammox (open circles), AOB (solid circles).

carriers in Paper IV where the Slicer was used to analyze the three-dimensional spatial distribution of AOB and anammox cells (Fig. 10b-g).

Co-aggregation analysis

In Paper III, the recently developed "inflate" algorithm (Daims & Wagner 2011), was applied on stratified biofilms for the first time. Briefly, the algorithm measures co-aggregation patterns between individual populations by counting the number of pixels in the population to be analyzed and thereafter virtually "inflates" the second population in defined intervals in all directions. For each iteration of inflation, overlapping pixels are counted. Populations that co-aggregate closely will consequently show a more pronounced overlap than expected from a randomly distributed virtual control population (Fig. 11). Vice versa, avoidance can be shown if the overlap is significantly lower than expected.

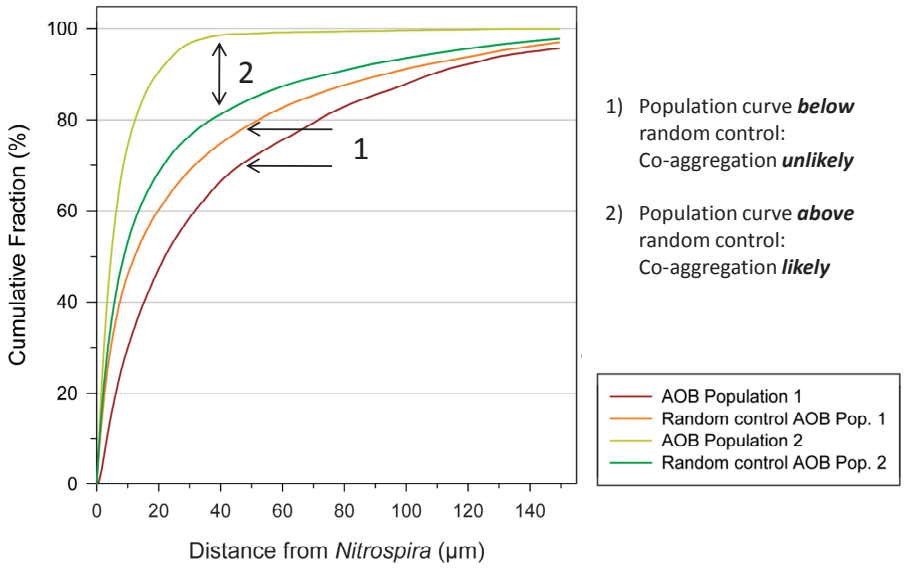


Figure 11. Principle of co-aggregation analysis of AOB and NOB using the "Inflate" algorithm (Daims & Wagner 2011) on biofilm micrographs. Image courtesy of Holger Daims.

In Situ Investigations of Nitrogen-Removing Biofilms

Community composition and functional stability

Inoculum composition

It has been hypothesized that wastewater treatment efficiency, stability and startup may be improved if the system is augmented with the right mix of bacterial functional groups (Plaza *et al.,* (2001), Leu & Stenstrom (2010)). Hence, the importance of inoculum composition for microbial community structure and functioning is an interesting aspect with potential application in wastewater treatment. Wittebolle *et al.,* (2009b) and Terada *et al.,* (2010) applied inoculums from different sources to lab-scale reactors and observed how different bacterial communities arose as a function of inoculum differences. Both studies concluded that function (nitrification efficiency) of the communities were comparable even though the phylogenetic composition differed and it was concluded that AOB diversity *per se* is the primary objective to accomplish in bioaugmentation attempts (Wittebolle *et al.,* 2009b). However, as pointed out by Daims and colleagues (2001c), an important factor that must be taken into account when extrapolating inoculum effects at the laboratory scale to bioaugmentation strategies in full-scale treatment plants, is the presence and importance of indigenous bacterial populations. The diversity of these populations results from selection during the constant seeding and invasion of microorganisms from the environmental metacommunities (Curtis & Sloan 2004). Consequently, the extrapolation of laboratory-scale investigations to full-scale WWT bioaugmentation is not straightforward. Thus, in order to draw conclusions that are readily applicable to wastewater treatment, investigations on the effect of diversity or population dynamics on wastewater treatment should ideally be conducted under conditions that are as similar to the full-scale treatment as possible. Interestingly, on-site bioaugmentation strategies have actually been shown to, at least temporarily, improve process efficiency (Plaza *et al.,* (2001), Leu & Stenstrom (2010)).

Diversity and functional stability

Several investigations have indicated and suggested that biodiversity is positively correlated with ecosystem functioning and stability (Daims *et al.,* (2001c), McCann (2000), Naeem & Li (1997), Wittebolle *et al.,* (2009b)), and vice versa (Worm *et al.,* 2006). This in turn can be attributed to the diversity but also composition of the communities performing certain functions in the ecosystem (Bell *et al.,* 2005). If a high enough level of species richness and evenness is maintained within a functional guild, the community may better respond to sudden changes in environmental conditions, thereby providing resilience to the function in question (Wittebolle *et al.,* 2009a).

It has been hypothesized that ecosystems that are frequently encountered with moderate disturbances may better retain diversity and functional stability than ecosystems in equilibrium (Connell 1978). This intermediate disturbance hypothesis relies on the intuition that moderate disturbance, defined by Pickett and White (1985) as "…any relatively discrete event in time that disrupts ecosystem, community, or population structure and changes resources, substrate availability, or the physical environment", will reduce competitive dominance (Miller *et al.,* 2011). This might be of great importance for wastewater treatment processes, where the functional bacterial communities may be constantly subjectected to variations in the environment within both a diurnal as well as a seasonal time-scale. Gieseke *et al.,* (2001) observed co-existence of several AOB in biofilms and suggested that spatial distribution and temporal differentiation in activity allowed co-existence of these populations. The relationship between diversity and disturbance can be complex and variable (Miller *et al.,* 2011). Nonetheless, the applicability of the intermediate disturbance hypothesis to wastewater treatment was recently supported by Nielsen *et al.,* (2010) who published a conceptual ecosystem model for EBPR and suggested that variations in starvation times and substrate concentrations would promote both community diversity and functional stability.

An efficient and resilient nitrifying community is desired for a well-functioning and stable nitrification process. Since the composition of nitrifying communities and their activity are shaped by environmental factors (eg Gieseke *et al.,* 2003), understanding of how nitrifying bacteria respond to such changes *in situ*, may improve nitrification at WWTPs. Consequently, investigations of specific nitrifying bacteria have revealed valuable insights into the autoecology and competitiveness of several AOB and NOB species and their response to changes in temperature,

dissolved oxygen, substrate concentration etc. Historically, many of these investigations were performed under controlled conditions in laboratory reactors and/or on a very limited number of strains (eg Bollmann *et al.,* 2002, 2005). Although valuable for our understanding of these bacteria, the observations can not necessarily be extrapolated to the variable conditions in WWT reactors or be regarded as valid even for closely related strains, if the experiments do not take into account intra-species niche differentiation (Gieseke *et al.,* 2005). To ensure its relevance for wastewater treatment, such studies need to be performed *in situ* at WWTPs on indigenous nitrifying communities receiving authentic wastewater. This was the rationale behind constructing the nitrifying pilot-plant at Rya WWTP (Papers I-III).

AOB community composition and dynamics in the Rya WWTP pilot-plant

In total, at least six AOB populations, in total representing 3-32% of the bacterial community (Papers I and II), were detected in the pilot-plant using FISH, DGGE and sequencing of 16S rRNA gene clone libraries. Out of these, at least three were affiliated with the *N. oligotropha* cluster (6a). These were generally dominating the AOB community in investigated samples from the pilot-plant. Two of the three *N. oligotropha* populations were distinguishable with FISH. In addition, cells related to *N. communis* were abundant in all MBBR tanks in Paper I and cells belonging to the *N. europaea*/*Nc. mobilis* cluster (7) were permanently present in the pilot-plant although mostly in low numbers. *N. europaea* is usually considered as an AOB r-strategist, being favoured by high substrate concentrations due to its comparatively high growth rate and also responds rapidly to increased ammonium levels after starvation (Bollmann *et al.,* 2002). Accordingly, it was shown in Paper I that the relative abundance of this population decreased with declining ammonium concentrations. However, DGGE (Paper I) and 16S rRNA gene sequence data (Paper III) from the MBBR tank 1 indicated that the exact identity of this population was unresolved, although targeted by probe Nse1472. No signal was obtained with probe NmV targeting *Nc. mobilis* (Paper I). An up-shift in ammonium concentrations via transfer of carriers from tank 4 to tank 1 resulted in an increase of this population. However, it did not reach levels comparable to that of *N. oligotropha* population I, until the temperature of the incoming water increased (Paper V). However, this delayed response which was potentially coupled

to temperature, showed that the fluctuating conditions residing in the pilot-plant at least temporarily provided a favourable environment for this population.

Although an increased resolution of the AOB community perhaps would show an even greater diversity in the pilot-plant than observed in Papers I-III, it was concluded that several AOB populations did co-exist and that their different patterns of response to environmental changes indicate a well-adapted and potentially resilient ammonia-oxidizing community.

Niche differentiation within the Nitrosomonas oligotropha cluster 6a

The AOB studied in most detail in this thesis belong to the *N. oligotropha* lineage (cluster 6a) and are referred to as *N. oligotropha* population I and II as defined by their respective FISH-probe hybridization patterns. Population I was targeted by both probes Nmo218 and 6a192 and population II was targeted by probe 6a192 but not Nmo218. In Papers I, III and V, differences in the respective spatial distribution patterns and responses to shifts in environmental conditions and especially ammonium concentrations, suggested niche differentiation between the two populations. A plausible reason for this would be differences in growth rate and substrate affinity. A lower growth rate but higher substrate affinity of population II compared to I would explain its higher relative abundance in MBBR tanks 3 and 4 where ammonium levels were lower than in tank 1 where population I was dominant (Paper I).

This may also in part explain the vertical distribution patterns of the two populations observed in Paper III (Fig. 12): In the full-scale NTF, where the mean influent ammonium concentration was 14.3 ± 3.2 mg NH_4^+ l^{-1} during the period of 2 months prior to sampling (Lydmark *et al.,* 2006), population I was large and clearly concentrated to the surface region of the biofilm, whereas population II was present only in very low numbers. NTF2 in the pilot-plant on the other hand, had a mean ammonium concentration of approximately half of that of the full-scale NTF during the 2.5 months preceding the sampling. Here, *N. oligotropha* population I was still more abundant in the region closest to the surface albeit in lower numbers than in the full-scale NTF. Population II was also present in NTF2, although in lower numbers than population I (Paper V) and also more evenly distributed throughout the biofilm, once again suggesting an ability to cope with lower substrate or oxygen concentrations deeper in the biofilm, beneath population I. Ammonium concentration in MBBR tank 1, was 7.6 ± 1.1 mg NH_4^+ l^{-1} (Paper

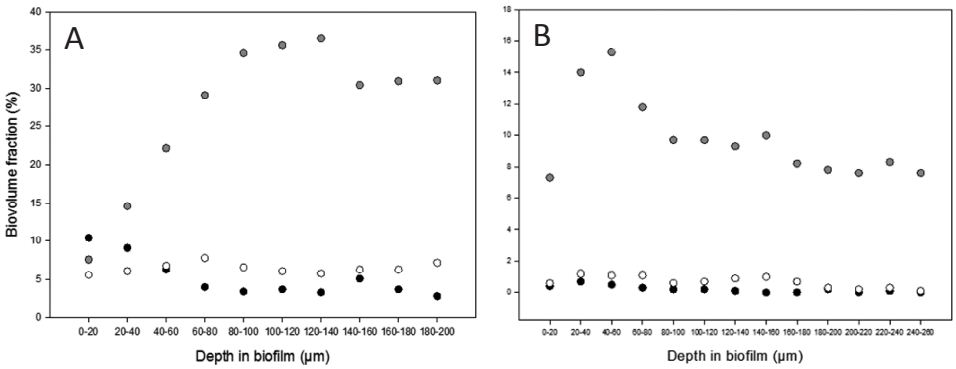


Figure 12. Vertical distribution of *Nitrospira* (grey circles), *N. oligotropha* I (black) and II (white) from the pilot-plant NTF 2 (A) and MBBR T1 (B), expressed as biovolume fraction per depth. For detailed analysis, please refer to Paper III. Image courtesy of Holger Daims.

V) which was even lower than that of NTF2. Both populations were present in low numbers but *N. oligotropha* population II was the more abundant. No pronounced stratification was observed for any of the two populations (Paper III and Fig. 12b). These spatial distribution patterns are in line with the observed niche differentiation in Paper I, suggesting that *N. oligotropha* population I outcompetes population II at elevated ammonium levels and vice versa.

Knowing that substrate gradients are formed throughout biofilms due to biological activity, one would expect the small amount of ammonia provided after down-shifts in the MBBR or NTF experiments, to be completely consumed in the top layers, leaving nothing to the AOB deeper down in the biofilm. So, even though population II seems to compete well when substrate availability is low, its position deeper into a thick biofilm makes it vulnerable to down-shifts, as seen in NTF2 (Paper V and Fig. 13). This hypothesis is supported by the thin biofilms observed in the starved NTF 1 (Fig. 14b), showing that thicker nitrifying biofilms would not be supported under these limiting conditions.

Since the physiological properties, such as substrate affinity or growth rate are unknown for the two *N. oligotropha* populations, the suggestions above need more experimental data in order to be confirmed or rejected. Nonetheless, these observations clearly show that intra-species diversity within *N. oligotropha* may be reflected in both population dynamics patterns as well as biofilm spatial distribution.

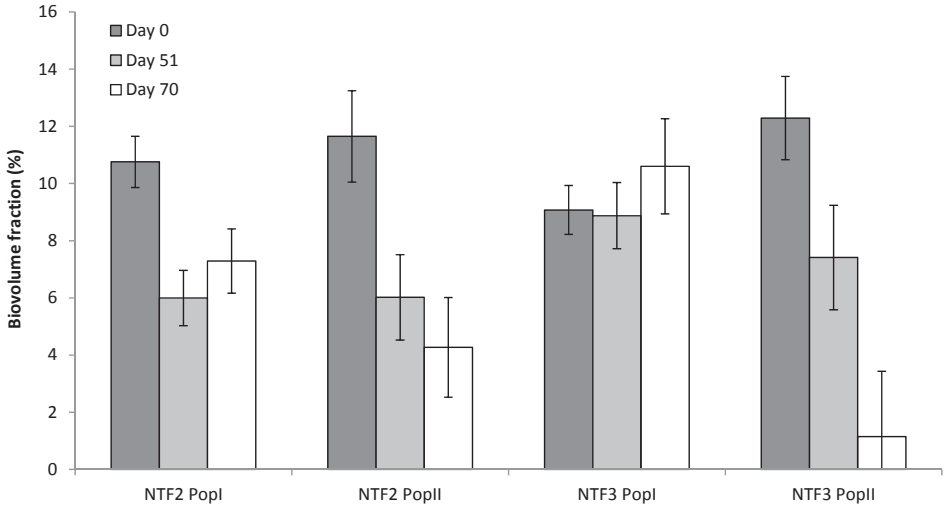


Figure 13. Population dynamics of *N. oligotropha* in the pilot-plant NTF 2 & 3 during a period of 70 days, expresssed as relative abundance (%). NTF 2 was continuously fed with ammonium-rich water, whereas NTF 3 was shifted from ammonium-rich water to starvation conditions on day 0. Error bars = 95% confidence interval. Figure adapted from paper V.

Further evidence for differences in the autoecology within the *N. oligotropha* lineage was provided by Limpiyakorn *et al.,* (2007) who, in concordance with the results in Paper I, showed and related differences in the autoecology of *N. oligotropha*-related cells to ammonium concentration. Comparison of the phylogenetic trees in (Limpiyakorn *et al.,* 2007) and Paper III, indicates that none of the analyzed *N. oligotropha* populations was present in both studies, leading to the conclusion that the diversity within this lineage is substantial. Wan *et al.,* (2011) characterized an AOB community almost entirely consisting of *N. oligotropha* related cells and therefore described it as remarkably simple and potentially sensitive to perturbations. However, based on *amoA* sequence data it was concluded that at least 10 distinct operational taxonomic units were present within this community, suggesting substantial *N. oligotropha* intra-lineage diversity. The investigations included in this thesis have shown that the ecophysiology of closely related AOB may differ significantly. Thus, if intra-species niche partitioning is significant, functional redundancy and high community resilience could be preserved even in nitrifying systems with limited numbers of species present.

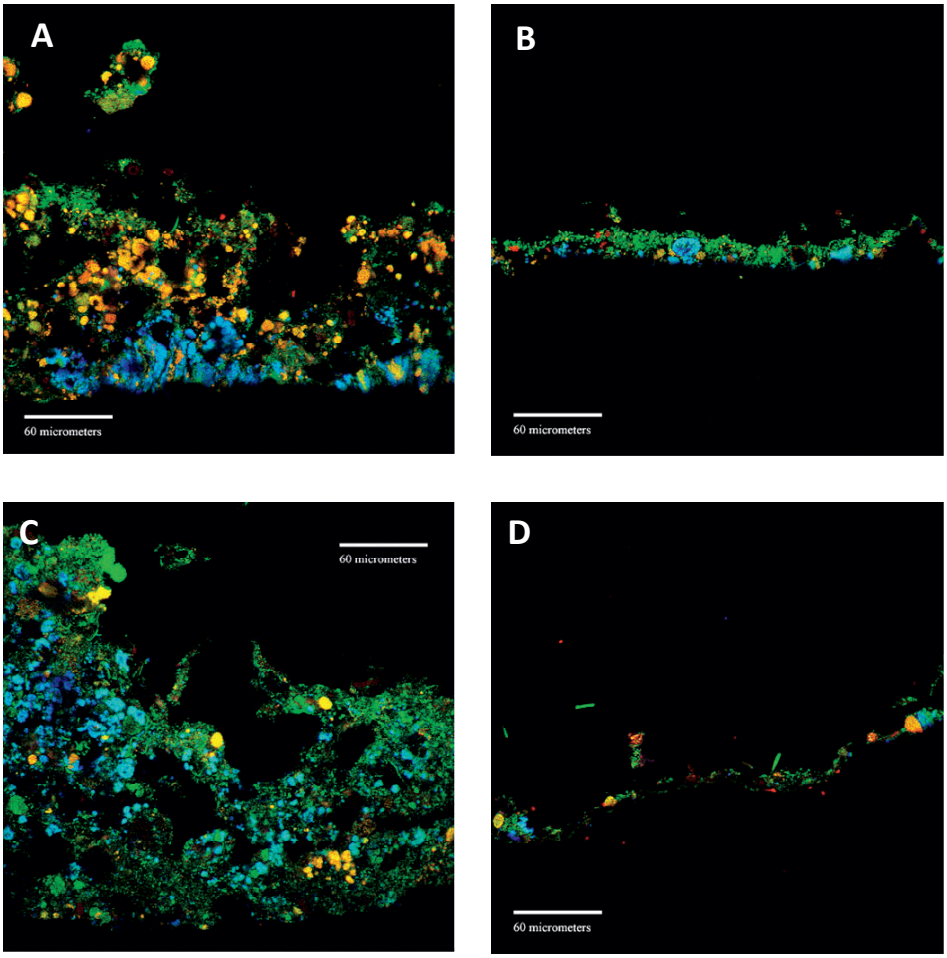


Figure 14. FISH micrographs of cryosectioned nitrifying biofilms from the pilot-plant hybridized with the AOB probe mix (yellow) and NOB probe Ntspa662 (blue). Other bacteria are targeted by the EUB 338 probe mix only (green). Note the different thickness of the biofilms fed with water high in ammonium (A&C) compared to the biofilms from starvation conditions (B&D). (A) Biofilm from NTF 2. (B) Biofilm from NTF 1. (C) Biofilm from MBBR tank 1. (D) Biofilm from MBBR tank 4. Scale bar = 60 µm.

Population dynamics and spatial distribution of Nitrospira

Nitrospira-related cells were the only detected nitrite-oxidizing bacteria in the pilot-plant, making up 5-40% of the total bacterial community (Papers I and II). Out of the six sublineages identified for these bacteria, sublineage I is the most important in wastewater treatment (Daims *et al.,* 2001a). Sublineage I *Nitrospira* also

dominated the NOB community in the pilot-plant and were detected both with FISH and 16S rRNA gene sequencing (Paper I and III). Members of sublineage II were detected, albeit in low numbers and only with FISH (Paper I and III). Sublineage II *Nitrospira* has been shown to prefer lower nitrite concentrations than sublineage I (Maixner *et al.,* 2006), which was also reflected in their distribution in the pilot-plant (Paper I). In the MBBR, sublineage II cells were present in low numbers (2-6% of total bacteria) and the highest abundance was observed in the low-substrate environment of MBBR Tank 4. A similar pattern was observed in the NTFs, were sublineage II made up less than 0,1% of the bacterial community in NTF 2, receiving high-ammonium water. In partially starved NTFs 1 & 4 on the other hand, approximately 3% of the biofilm consisted of sublineage II.

Vertical distribution patterns were determined for sublineage I *Nitrospira* only, due to the low amount of sublineage II cells. Sublineage I *Nitrospira* vertical distribution differed between the analyzed biofilms (Paper III). In the pilot-plant trickling filter NTF2, *Nitrospira* were significantly more abundant in the deeper layers of the biofilm (Fig. 12a), which is in agreement with earlier investigations (Okabe *et al.,* 1999, Schramm *et al.,* 2000). However, in the full-scale NTF biofilm, *Nitrospira* cells were concentrated to the upper biofilm region which was in contrast to previous findings (Lydmark *et al.,* 2006). The concentration to the upper biofilm region was confirmed through re-analysis of the actual CLSM images from Lydmark *et al.,* (2006), with the new automated "Slicer". The different result was probably due to the higher resolution of the new method. In the MBBR biofilm, less stratification was observed for *Nitrospira* (Fig. 12b), possibly owing to the differences in biofilm structure which may have been caused by the lower substrate levels in the MBBR compared to the NTFs.

The vertical distribution pattern of Nitrospira may also have been influenced by the distribution of AOB, since a strong co-aggregation pattern within short distances of 1-20μm was shown between *N. oligotropha* population II and *Nitrospira* (Paper III). This was in contrast to the co-aggregation pattern for *N. oligotropha* I, which was only present at larger distances. Possibly, *Nitrospira* cells in these biofilms were dependent on nitrite produced in close vicinity and since the microaerophilic *Nitrospira* (Park & Noguera 2008, Lücker *et al.,* 2010) probably thrived in the deeper parts of the biofilm, just like *N. oligotropha* II, this might explain their spatial relationship. In the thinner biofilms of the full-scale NTF on the other hand, *Nitrospira* actually co-aggregated with the dominant *N. oligotropha*

I, suggesting that nitrite production by AOB is indeed the main driving force behind these patterns. Still, other factors may however also cause such relationships. For instance, unexpected co-aggregation between the AOB *N. europaea* and *N. mobilis* was reported by Gieseke and colleagues (2003) who suggested that partial co-operation could exist even though the bacteria competed for the same substrate. Alternatively, parasitism, such as uptake of iron-binding siderophores synthesized by other bacteria could provide an explanation (Chain *et al.,* 2003). Consequently, nitrification alone is not necessarily the only reason for AOB-NOB aggregation in biofilms.

Activity of nitrifying communities: implications for wastewater treatment

Activity of the nitrifying communities present in the pilot-plant was investigated in Paper I and II. In Paper I, nitrification rates based on ammonium consumption and nitrate production in the MBBR tanks were measured. During the experimental period of nine months, a substantial seasonal variation in incoming ammonium concentration was observed. Ammonium concentrations in the MBBR tanks varied accordingly and during summer, only tank 1 had concentrations that were constantly above the detection limit and stable nitrification rates. However, during spring and fall, when ammonium concentrations increased, the highest rate per carrier area was observed in tank 2. An explanation for this may be oxygen limitation in tank 1 during periods of high substrate concentrations, possibly due to competition with heterotrophic bacteria. Heterotrophs are known to have higher affinity for oxygen than nitrifiers (Belser 1979), Geets *et al.,* 2006) and may also be supported by nitrifier production of soluble microbial products (Rittmann *et al.,* 1994, Kindaichi *et al.,* 2004).

The nitrification rates observed in tank 2 suggested that biofilms in this system had a higher nitrification potential than observed in tank 1, despite the constant feed of water low in ammonium. Moreover, nitrification rates in tank 2-4 seemed to increase rapidly when substrate conditions improved, suggesting that a high nitrification potential could potentially be maintained in systems subjected to alternating ammonium concentrations. Implementations of such strategies have been suggested previously for NTFs (Andersson *et al.,* 1994, Wik 2003). When tested in a full-scale NTF, it was observed that 10 days of starvation resulted in severe break-down of the nitrification rate and another 10 days were thereafter needed to regain similar nitrification rates (Boller & Gujer 1986). Consequently, in

order to maintain function, nitrifying biofilms should not starve for too long (Wik 2003). In Paper II, this was investigated in detail by deliberate changes in the ammonium concentration of water fed to the four model NTFs. One of these model NTFs in the pilot-plant, when subjected to intermittent feast-famine conditions following several months of starvation, reached and stabilized at potential nitrification levels comparable to that of a control NTF constantly fed with high-substrate wastewater. This required 42 days, with a 3 days high/4 days low weekly ammonium feed schedule. On the contrary, a parallel NTF receiving 1 day high/6 days low ammonium feed remained at very low potential nitrification levels during the rest of the experiment. A screening of biofilm thickness from the pilot-plant NTFs showed that the biomass in the continuous high-ammonia NTF was considerably higher than in the continuously starving NTF (Fig. 14a-b). Thus, long-term feed regimes are reflected in total biomass and biofilm thickness. Because the pilot-plant received the same water as the full-scale NTFs, these findings have influenced into the nitrification process strategy at Rya WWTP.

The high nitrification potential observed under non-stationary conditions in the pilot-plant also suggests that alternating feed strategies may not only retain nitrification capacity under periods of low ammonium loads, but could possibly also result in a more active biofilm. Biofilm thickness and community composition are likely important factors to investigate in relation to nitrification potential. More specific measurements of biofilm activity, using for example FISH-MAR, would also be needed to address these questions in future experiments.

Spatial distribution of microbial populations in AOB-anammox biofilms

In Paper IV, the spatial distribution patterns of AOB and anammox bacteria were investigated using a novel cryosectioning approach for biofilm retrieval within MBBR Minichip carrier compartments. When combined with FISH, CLSM and the multidirectional Slicer tool for digital image analysis (Paper III), three-dimensional stratification of the two populations was observed. AOB was always concentrated to the surface region of the biofilm and decreased in relative abundance in the deeper parts of the compartment. Anammox on the other hand were generally found closer to the compartment surface although the thickness of the anammox layer gradually increased with depth (Fig. 10). Due to their sensitivity towards oxygen (eg Schmid *et al.,* 2001), anammox bacteria are usually found deeper in the biofilms compared to AOB (eg Vlaeminck *et al.,* 2010,

Vázquez-Padín *et al.,* 2010). Therefore, the observation of a similar anammox distribution in MBBR Minichip carrier compartments was not surprising. However, the previously unreported three-dimensional stratification of AOB and anammox bacteria in these biofilms may have implications for mathematical modeling of the anammox process in similar systems. Although three-dimensional biofilm models exist (eg Alpkvist *et al.,* 2006), mathematical biofilm models, and especially those developed for MBBR systems with biofilm chip carriers, are generally one- or two-dimensional (eg Alpkvist *et al.,* 2007, Masić *et al.,* 2010) and therefore do not take into account the population distribution patterns observed in Paper IV. Such distribution is likely to affect the formation of substrate gradients within carrier compartments and should thus be of importance for optimizing the design of MBBR carriers.

In addition, replication of heterogeneous biofilms poses a number of problems, such as requirement of running several reactors in parallel under identical conditions (Lewandowski *et al.,* 2004, Lewandowski *et al.,* 2007). This could be circumvented via the use of Biofilm chip carriers and the cryosectioning protocol for biofilm retrieval since the Minichip carriers used in Paper IV each contain >300 compartments with protected surface for biofilm growth (Fig. 4). Abrasion caused by the constant motion and collisions of the carriers inside the MBBR reactor, prevents biofilm from forming outside the protected surface. Consequently there is no exchange between the compartments except via the reactor medium. The biofilms could therefore be regarded as independent (Alpkvist *et al.,* 2007) and treated identically. Microsensor measurements of, for example, oxygen gradients inside these compartments, may however not be conducted in such a system but would require fixation of the carriers in a flow chamber (Masić *et al.,* 2010) where the hydrodynamic properties are most likely different from an MBBR.

Conclusions and Outlook

Diversity of nitrifying bacteria in the pilot-plant at Rya WWTP

The pilot-plant studies revealed a rather diverse nitrifying community with at least five AOB and two NOB populations present at all times (Papers I and III). Ecophysiological differences of AOB within the *N. oligotropha* lineage were observed in Papers I, III and V. The development of new tools for analyzing biofilms (Paper III) allowed more detailed studies and showed that these differences were not only reflected in the population dynamics but also the spatial distribution patterns of two N. oligotropha-related populations. Different adaptations to environmental conditions and especially ammonium concentrations were suggested as the main forcing factors behind this niche differentiation. In addition, the diversity within the AOB guild may be even greater, as suggested by the clone libraries in Paper III, suggesting that increased resolution through the design of more specific FISH-probes might be needed to obtain a comprehensive understanding of the functional diversity of this group of bacteria.

Activity of nitrifying biofilms and applications for WWTPs

In Paper II, it was shown that nitrification potential could be maintained during periods of low ammonium loads if biofilms were subjected to alternating feed strategies. By performing the investigation in a pilot-plant receiving authentic wastewater, the results were applicable to the full-scale NTF at Rya WWTP. An interesting extension of similar future investigations would be to combine the nitrification potential measurements with more specific activity measurements, such as FISH-MAR, and spatial distribution analysis using the automated Slicer which would allow estimation of the active fraction of nitrifying bacteria and its spatial distribution in the biofilm.

Three-dimensional structure and stratification of AOB-anammox biofilms

Using a cryosectioning approach combined with FISH and digital image analysis, intact biofilms from MBBR Minichip biofilm carrier compartments were examined with regard to their structure and population distribution in three dimensions in

Paper IV. High-resolution composite FISH-images of entire biofilm sections in the xy-dimension ("wall to wall") from different depths revealed stratification in all dimensions in mature biofilm, probably due to the formation of oxygen and substrate gradients. So far, mathematical modelling of similar carriers has not taken such biofilm distribution into account. Hence, the findings in Paper IV, may be of importance for future modelling efforts. In addition, since each of the approximately 300 compartments in each carrier can be regarded as independent, compartmentalization of biofilms in eg Minichip carriers provides a suitable option for biofilm replication.

Re-evaluation of probes and meta image-analysis

During the work presented in this thesis, a number of observations suggested that re-evaluation of some FISH-probes and their competitiors is necessary. In addition, the design of new and more specific probes will continuously improve the resolution of the nitrifying community when analyzed with FISH. Together with new tools for spatial distribution analysis, such as the automated Slicer (Paper III) and the inflate algorithm (Daims & Wagner 2011), re-analysis of previously cryosectioned biofilm samples and/or micrographs from earlier studies would, in principle, be possible. Such a "meta"-approach could potentially provide new information on biofilm spatial distribution patterns in a very time-efficient manner.

Microcolony size distribution

As microcolony size distribution may reflect the conditions prevailing in a biofilm, analyses as the ones undertaken by Okabe *et al.,* (2004) would indeed be interesting and possible to perform in the high-resolution images of the AOB-anammox biofilms analyzed in Paper IV. Such an analysis would however need some optimization in order to identify the most appropriate segmentation approach for recognizing each microcolony as a separate object (Fig. 6b), thus allowing automatic measurement of its area, shape etc. Three-dimensional distribution patterns of microcolony properties may thereafter be acquired. As nitrifier microcolonies are an important structural component of the biofilms they grow in, this may potentially provide new information on biofilm structure that would be of relevance for mathematical modeling of these processes. One interesting question is to what extent the population dynamics of nitrogen removing bacteria are a result

from changes in the abundance of microcolonies compared to changes in average microcolony size.

Effect of bacteriophages and predator-mediated proliferation of nitrifying bacteria

Investigations on the interactions between nitrifying bacteria and other organisms are of importance for understanding the ecology of nitrifying biofilms. Bacteriophages are certainly abundant in wastewater treatment plants and nitrifying bacteria are susceptible to these viruses. Hence, more investigations on the importance of bacteriophages for bacterial community structure and function in biological nitrogen removal are needed. Similarily, other aspects of predation than only biofilm consumption may be of importance to the nitrification process. For instance, the role of predator-mediated dissemination of nitrifying bacteria in biofilms has not yet been investigated.

Populärvetenskaplig sammanfattning

Utsläpp av avloppsvatten i hav, sjöar och floder tillför näringsämnen i form av kväve och fosfor till dessa miljöer. Detta orsakar onormalt stora algblomningar, vilket i sin tur resulterar i allvarliga störningar av växt- och djurlivet. För att förhindra detta renas avloppsvatten från fosfor och kväve i avloppsreningsverk innan det släpps ut. Kvävereningen är en biologisk process, där olika grupper av bakterier utnyttjas för att i ett antal steg omvandla kväveföreningar till kvävgas som avgår till atmosfären.

Ett av dessa steg är nitrifikation, vilken utförs av nitrifierande bakterier. Nitrifikation är en viktig men känslig process, därför behöver förståelsen för de nitrifierande bakterierna och hur de påverkas av miljön de växer i förbättras. De bakterier som undersökts i denna avhandling växte i bakteriesamhällen på ytor (biofilmer).

Då avloppsvatten hela tiden varierar i sammansättning är det viktigt att genomföra studier i en miljö som är så lik riktiga avloppsreningsverk som möjligt. Därför konstruerades en experimentanläggning på Ryaverket i Göteborg. En studie som presenteras här visade att växelvis tillförsel av låga och höga halter av näring, i form av ammoniumkväve, gjorde att de bakteriella biofilmerna behöll en bättre nitrifikationsförmåga än om de istället fick en jämn låg halt. Genom att använda en sådan växelvis matningsstrategi kan reningsverk bättre klara av svängningar mellan låga och höga kvävekoncentrationer i avloppsvattnet.

Genom mikroskopi kombinerat med cellspecifik inmärkning och digital bildanalys observerades det hur bakteriesamhället i biofilmerna förändrades på grund av olika näringshalter. Olika arter av bakterier gynnas eller missgynnas nämligen i olika utsträckning av förändringar i deras livsmiljö. Dessutom påverkades grupper av bakterier som normalt anses tilhöra samma art (*Nitrosomonas oligotropha*) olika av dessa förändringar. Därför är även skillnaderna *inom* arter av nitrifierande bakterier viktiga för kväverening och bör undersökas i detalj.

Genom att utveckla nya metoder för att digitalt analysera mikroskopibilder av biofilmer, visades det att olika bakterier även satt på olika ställen i biofilmerna. Detta kan i sin tur påverka biofilmers funktion.

Kompletterande studier från en alternativ kvävereningsprocess (anaerob ammoniumoxidation) visade dessutom att bakteriers positionsmönster i en biofilm kan variera i tre dimensioner vilket kan få betydelse för matematisk modellering och utveckling av nya biofilmsbaserade lösningar för biologisk kväverening.

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