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IVAR ZEKKER

Enrichment of anaerobic ammonium oxidizing bacteria for nitrogen removal from digester effluent and anammox process acceleration by intermediate compounds

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LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary of the following papers, referred to in the text by the Roman numerals:

I. Zekker, I.; Rikmann, E.; Tenno, T.; Vabamäe, P.; Tomingas, M.; Menert, A.; Loorits, L.; Tenno, T. (2012). Anammox bacteria enrichment and phylogenic analysis in moving bed biofilm beactors. Environmental Engineering Science, 29(10), 946–950.

II. Zekker, I.; Rikmann, E.; Tenno, T.; Lemmiksoo, V.; Menert, A.; Loorits, L.; Vabamäe, P.; Tomingas, M.; Tenno, T. (2012). Anammox enrichment from reject water on blank biofilm carriers and carriers containing nitrifying biomass: operation of two moving bed biofilm reactors (MBBR). Biodegradation, 23(4), 547–560.

III. Zekker, I.; Kroon, K.; Rikmann, E.; Tenno, T.; Tomingas, M.; Vabamäe, P.; Vlaeminck, S. E.; Tenno, T. (2012). Accelerating effect of hydroxylamine and hydrazine on nitrogen removal rate in moving bed biofilm reactor. Biodegradation, 23(5), 739–749.

IV. Zekker, I.; Rikmann, E.; Tenno, T.; Menert, A.; Lemmiksoo, V.; Saluste, A.; Tenno, T.; Tomingas, M. (2011). Modification of nitrifying biofilm into nitritating one by combination of increased free ammonia concentrations, lowered HRT and dissolved oxygen concentration. Journal of Environmental Sciences, 23(7), 1113–1121.

V. Zekker, I.; Rikmann, E.; Tenno, T.; Saluste, A.; Tomingas, M.; Menert, A.; Loorits, L.; Lemmiksoo, V.; Tenno, T. (2012). Achieving nitritation and anammox enrichment in single moving-bed biofilm reactor treating reject water. Environmental Technology, 33(6), 703–710.

VI. Zekker, I.; Rikmann, E.; Tenno, T.; Kroon, K.; Vabamäe, P.; Salo, E., Loorits, L.; Rubin, S.; Vlaeminck, S.; Tenno, T. (2012). Effect of HCO_3^- concentration on anammox nitrogen removal rate in a moving bed biofilm reactor. Environmental Technology, 33(19–21), 2263–2271.

VII. Zekker, I.; Rikmann, E.; Tenno, T.; Kroon, K.; Vabamäe, P.; Salo, E.; Loorits, L.; Rubin, S.; Vlaeminck, S.; Tenno, T. (2013). Deammonification process start-up after enrichment of anammox microorganisms from reject water in a moving bed biofilm reactor (MBBR). Environmental Technology, accepted. DOI: 10.1080/09593330.2013.803134

Author's contribution

In all papers (I, II, III, IV, V, VI and VII) I was one of the initiators of practical studies, designed and operated the experiment units, participated in data collection (60%) and laboratory analyses (40%), except molecular analysis. I was responsible for most of the data analyses and for the completion of figures in the papers. I also contributed to the submission of the articles, discussions with the referees and the completion of all papers.

LIST OF ABBREVIATIONS

AOB – ammonium oxidizing bacteria
AMO – ammonia monooxygenase
Anammox – anaerobic ammonium oxidation process
Anammox enrichment – anaerobic ammonium oxidation process performing bacteria enrichment
BOD – biological oxygen demand
COD – chemical oxygen demand
Deammonification – partial nitritation of ammonium and the subsequent anaerobic oxidation of residual ammonium by nitrite into nitrogen gas
DGGE – denaturing gel gradient electrophoresis
DIB – deammonification via intermittent aeration in biofilms
DO – dissolved oxygen
FA – free ammonia
FISH – fluorescence *in-situ* hybridization
HAO – hydroxylamine oxidoreductase
Hzo – hydrazine dehydrogenase
Hzs – hydrazine synthase
HRT – hydraulic retention time
MBBR – moving bed biofilm reactor
NirS – nitrite oxidoreductase
NLR – nitrogen loading rate
NOB – nitrite oxidizing bacteria
NRR – nitrogen removal rate
PCR – polymerase chain reaction
SBR – sequencing batch reactor
SHARON – single-reactor system for high-activity ammonium removal over nitrite
TIN – total inorganic nitrogen
TN – total nitrogen
TNRE – total nitrogen removal efficiency
TNRR – total nitrogen removal rate
TNLR – total nitrogen loading rates
TOC – total organic carbon
TSS – total suspended solids
VSS – volatile suspended solids
WWTP – wastewater treatment plant

1. INTRODUCTION

Nitrogen compounds are important pollutants released from different industries and are also present in liquid nitrogen-rich effluents in biogas plants. Treatment of N-rich wastewaters through nitrification-denitrification methods is energy consuming and limited to availability of organic carbon. In the current PhD thesis it was aimed to treat nitrogen-rich reject water by a water treatment process, which is based on anaerobic ammonia oxidation (anammox) conducted by autotrophic bacteria. This is an energy efficient alternative to the process based on heterotrophic denitrification and enables to save 60–90% of total treatment costs. Enrichment of anammox bacteria from reject water onto biofilm carriers in moving bed biofilm reactors (Paper I) is of great practical interest as this reactor type needs less space for the process than other reactor types and as a biofilm reactor is also more tolerant to inhibiting factors such as high and non-constant substrate loading, high nitrite concentrations, low temperatures etc.

Use of a pre-established biofilm containing fast-growing non-anammox (nitrifying) organisms for enhancing anammox organisms' attachment and protection against inhibitory compounds (Paper II) has not been studied before for accelerating the anammox process start-up. It is possible that the anammox process could be also more tolerant to increased substrate concentrations, especially at the beginning of the start-up when additional biofilm as a support surface is used. For example, tolerance to elevated nitrite and dissolved oxygen concentrations could be achieved by pre-established biofilm.

The anammox process has several known and still unknown intermediates, which are formed and further converted to N_2. Addition of optimum amounts of anammox intermediates to two-stage deammonification systems the anammox step could help specifically determine the effect of intermediates on the activity of anammox organisms alone excluding metabolic effects from nitrifying communities (Paper III). External addition of anammox intermediate compounds could be a means of recovery from process inhibition.

Since it is often problematic to start-up nitritation and anammox processes in single-reactor (nitrite and dissolved oxygen maintained could inhibit anammox process, nitrite could be oxidized to nitrate), these processes can be carried out in separate reactors with a less start-up time. After the start-up of a two-stage deammonification process (nitritation and anammox processes in separate reactors), it becomes more defined how to start-up single-stage deammonification when the conditions for nitritation and anammox steps are well studied. High-rate propagation of nitrifying organisms limiting anammox process development could be overcome by intermittent aeration, by elevated free ammonia (FA) as well as by increased HCO_3^- (CO_2) concentrations (Paper VI). These factors could selectively keep ammonium and nitrite to be further oxidized into nitrate, limiting the anammox process as well. FA concentrations around 10 mg N L^{-1} and HCO_3^- concentrations above 1000 mg L^{-1} (respective

CO_2 concentration) already inhibit nitrite oxidizing bacteria, but only little effect on anammox organisms at these concentrations is determined.

In the first part of the study a two-stage deammonification process was developed (Papers I, II and III) by the enrichment of anammox bacteria which need a longer time for propagation than nitrifying communities. After that, a single-stage (nitritation (Paper IV) and anammox in a single reactor) deammonification process was started up (Papers V and VII) more effectively as both processes were already well-defined.

The autotrophic methods developed for nitrogen removal from wastewater can save costs on energy significantly and therefore could be applied for full-scale wastewater treatment systems for the treatment of N-rich wastewaters.

2. OBJECTIVES

The aims of this study were to develop an efficient nitritation-anaerobic ammonium oxidation (anammox) process for reject water (methane tank N-rich effluent) treatment without adding anammox-specific *inoculum* during the start-up period. Direct inoculation of anammox organisms from wastewater can widen the application of the anammox process for various treatment plants without remarkable start-up costs. Autotrophic total nitrogen (TN) removal was purposed to be achieved in the one and two-step deammonification systems through control of different factors (gradual increase of substrate concentrations, optimum HCO_3^- levels) ensuring effluent NO_2^- concentrations at non-inhibitory levels.

It was aimed to determine if there is a positive role of a pre-established nitrifying biofilm as a support surface for anammox bacteria attachment to ensure a more rapid process start-up and higher TN removal rates than it could be achieved with blank carriers for anammox bacteria enrichment.

It was attempted to determine the TN removal rate-increasing effect of the optimum amount of anammox process intermediates added into the reactor system for achieving the highest autotrophic process TN removal rate.

After anammox bacteria enrichment two processes, that is, nitritation and anammox were aimed to be coupled into a single reactor. Optimal conditions for efficient TN removal process operation were expected to be determined on the basis of the monitoring of principal process parameters, such as TN loading, free ammonia and HCO_3^- concentrations.

In the second part of the study different deammonification start-up strategies were compared. Firstly, the anammox process was developed in the anoxic reactor system and then the nitritation step for starting up the single-step deammonification process was added. Secondly, the processes were aimed to be developed in the opposite order: starting the anammox process with nitrifying carriers into which anammox organisms would be developed. Before coupling anammox and nitritation processes in a single reactor, separate development and then coupling of anammox and nitritation processes for the deammonification process start-up was aimed to be achieved as this would shorten start-up time. Also, a more stable process would be developed as mechanisms for both biofilm aerobic and anaerobic N-conversion processes are more specifically defined.

3. LITERATURE OVERVIEW

Various N-rich wastewaters such as landfill leachate and slaughterhouse waste have the characteristics of low C/ N ratios and high NH_4^+ concentrations. The liquid fraction of the effluent from an anaerobic reactor (methane tank) typically has a high NH_4^+ / biodegradable organic carbon ratio as well, which makes water treatment from high N-compounds concentrations costly. For example, reject water from anaerobic digestion of municipal wastewater sludge contains 500–850 mg NH_4^+–N L^{-1} (Szatkowska et al., 2007) and biological oxygen demand (BOD_7) of around 350 mg L^{-1} (Zekker et al., 2011), and the recycling of such a stream to a wastewater treatment plant contributes to an average increase in the total nitrogen load by about 15–20% (Szatkowska et al., 2007), while it makes up to only 2% of the total influent flow. Elimination of N from wastewater is an important issue in avoiding eutrophication. A novel anammox (anaerobic ammonium oxidation) process provides an alternative to the conventional nitrification-denitrification technology. The anammox process reaction uses NH_4^+ as an electron donor and NO_2^- as an electron acceptor, converting chemically bonded nitrogen into N_2 gas. Taking into account carbon fixation and biomass growth, the stoichiometry of the anammox process is as follows (Mulder et al., 1995):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

According to equation (1), 55–60% of NH_4^+ present in wastewater has to be oxidized into NO_2^- in the nitritation phase preceding the anammox process phase. In case of conventional nitrification-denitrification (NO_3^- as the terminal electron acceptor), NH_4^+ has to be oxidized entirely into NO_3^- to achieve nitrogen removal. Thus, the anammox process affords a short-cut in biological nitrogen removal as shown on Figure 1.

Treating the substrate with low organic carbon to the inorganic nitrogen ratio (i. e. reject water), the autotrophic nitrogen removal process is energetically beneficial as there is no need for an external carbon source. Thus, biological treatment of nitrogen-rich wastewaters by the anammox process is one of the most economical processes for nitrogen removal (Fux and Siegrist, 2004). However, the combined nitritation-anammox (deammonification) process has its limits: up to 90% of nitrogen can be removed (emanating from equation (1)), as minor amounts of NO_3^- are formed (Strous et al., 1999). For higher nitrogen removal, a mixed consortium of autotrophic and heterotrophic bacteria is needed, dominated by anammox organisms, but having a minor population of denitrifiers as well.

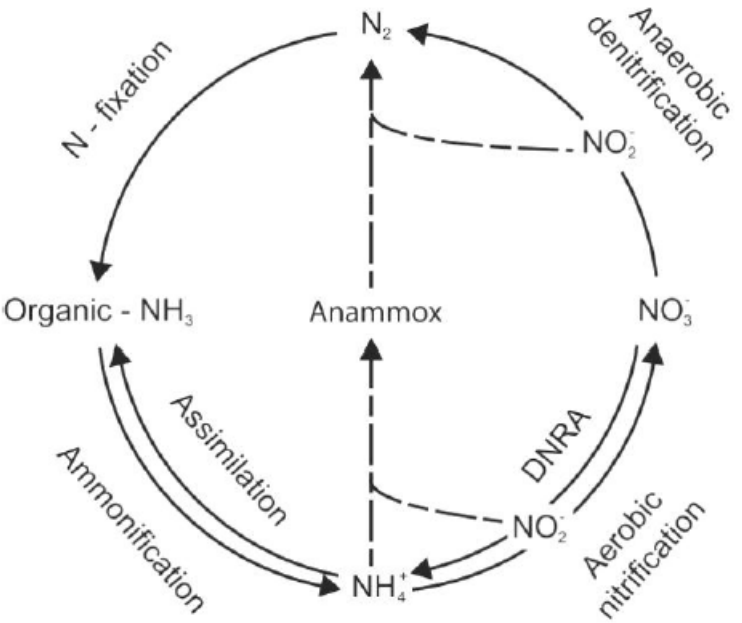

Figure 1. Scheme describing the nitrogen cycle based on processes carried out by autotrophic and heterotrophic bacteria (Bertino, 2010). DNRA– dissimilatory nitrate reduction to ammonium.

The anammox process has a globally important role being an important nitrogen sink of fixed nitrogen as this process contributes around 50% of the N_2 produced in the oceans. The anammox process can be carried out together with dissimilatory nitrate reduction to ammonium and denitrification when there is enough organic matter present.

Anammox organisms belong to phylum *Planctomycetes*. The first of anammox organism determined in a water treatment system based on the anammox process belongs to *Candidatus* Brocadia Anammoxidans. Anammox organisms have a very slow growth rate (doubling time nearly two weeks), which makes the cultivation of these organisms time-consuming. However, as anammox microorganisms have been described to be present in different wastewater treatment systems (Desloover et al., 2011; Mulder et al., 1995) and natural environments (Dalsgaard et al., 2005), specific inoculation of water treatment systems with these organisms is not necessary (is also expensive) (Zekker et al., 2012). With a certain optimum operational strategy (one or two-step process (Jaroszynski and Oleszkiewicz, 2011)) anammox-process-based autotrophic nitrogen removal could be started up in various water treatment plants having a high influent nitrogen concentration.

3.1. Two-step deammonification process

Two-step autotrophic nitrogen removal process involves the separation of ammonium oxidization into nitrite in one aerobic reactor from the other anoxic reactor where produced nitrite and residual ammonium are conversed into N_2 by the anammox process. As the nitritation process (first step of nitrogen elimination) has extensively been studied in special literature (Ganigué et al., 2007; Liang et al., 2011; Zekker et al., 2011), the limiting step of nitrogen removal is usually the anammox process step. The nitritation step in activated sludge processes is often performed through the SHARON-process (single reactor system for high-activity ammonium removal over nitrite) (Hellinga et al., 1998; van Dongen et al., 2001) based on keeping low sludge age and thus limiting NOB growth instead of AOB. When using biofilm systems for nitritation, the SHARON process efficiency is limited as biomass is fixed and wash-out of nitrifying biomass is complicated. Instead of using the SHARON process for deammonification, intermittent aeration, higher levels of free ammonia (FA) and bicarbonate can be applied for efficient partial nitrification and NOB suppression.

The anammox process step has to be studied more and an in-depth analysis of the process intermediate compounds and the factors dictating the efficiency of the process and its limitations has to be done. Anammox process intermediates formation and utilization by anammox bacteria has utmost important role in the completion of autotrophic nitrogen removal.

3.1.1. Anammox process intermediates and enzymes involved

The anammox process has several known (hydrazine, hydroxylamine, nitric oxide) and still not known nitrogen-containing intermediate compounds, which are formed during the autotrophic nitrogen removal process (Figure 2). Production of hydroxylamine by anammox microorganisms has been proven to be carried out by enzyme hydroxylamine oxidoreductase (Schalk et al., 2000), which is located in an anammox-microorganism-specific organelle-anammoxosome. Hydrazine is produced from hydroxylamine by enzyme hydrazine oxidoreductase. Hydrazine conversion into N_2 is carried out by both – an hydrazine-oxidizing enzyme and hydroxylamine oxidoreductase-like enzymes (Kuenen and Jetten, 2001; Strous et al., 2006).

The knowledge of anammox process intermediates is important as it broadens our understanding about the natural nitrogen cycle. From a more practical aspect, use of a proper intermediate of anammox process in needed quantity nitrogen removal in water treatment system could be enhanced. Earlier, the accelerating effect of different combinations (NH_2OH/ N_2H_4) of the intermediates of the anammox process with various ratios on inhibition damaged biofilm systems based on the anammox process has not been studied thoroughly (except one report by (Schalk et al., 1998)).

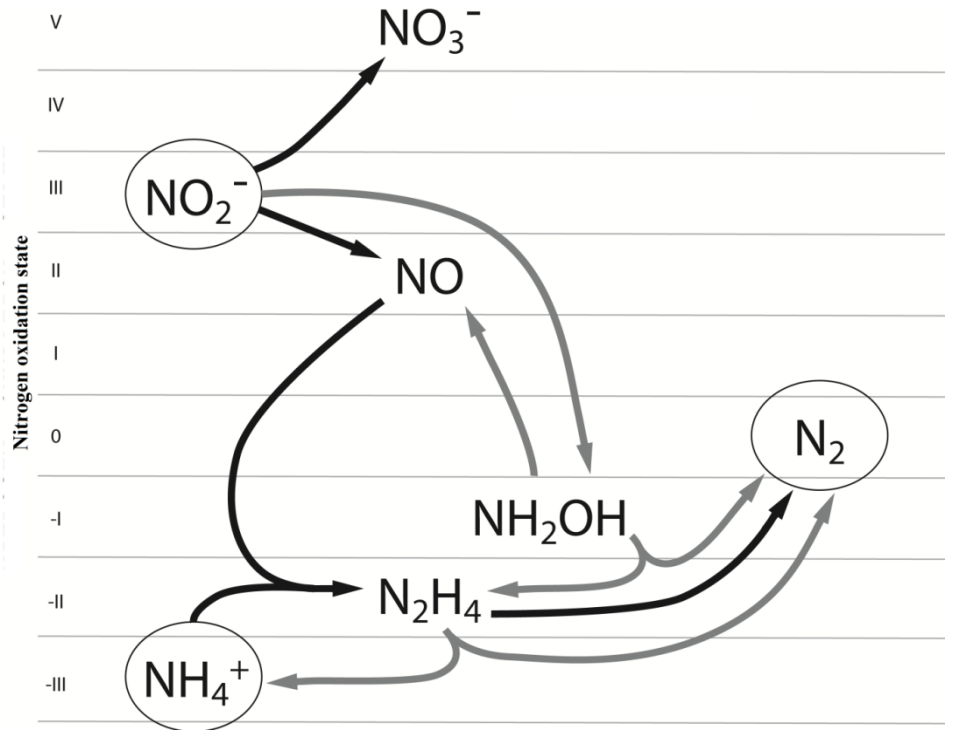

Figure 2. Compendious scheme describing the metabolism of anammox process intermediates based on oxidation state and key reactions according to (Hu et al., 2011; Kuenen and Jetten, 2001; Strous et al., 2006; Zekker et al., 2012b; van der Star, 2008). Involving enzymes: nitrite/ nitrate oxidoreductase, nitrite/ nitric oxide oxidoreductase, hydrazine hydrolase, hydroxylamine oxidoreductase/ hydrazine oxidoreductase (Paper III).

It has been shown that the addition of trace amounts of anammox process intermediates such as hydroxylamine and hydrazine separately can be beneficial for overcoming nitrite inhibition in the sequence batch reactor (SBR) system (Strous et al., 1999). Nitrite utilization rate in granules containing anammox bacteria can according to (Hu et al., 2011), be significantly increased by raising the ratio of hydroxylamine to nitrite from 1/ 1 to 2/1. By comparison, according to (Bettazzi et al., 2010) equal combinations of anammox process intermediate metabolites (N_2H_4 and NH_2OH) together added into sludge containing anammox bacteria can be more beneficial for recovering nitrite inhibition and for accelerating the nitrite removal rate by the anammox process.

Enzymes which are performing the conversion of anammox process intermediates into ammonium are termed as the pentaheme nitrite reductase (NrfA), hybrid cluster proteins (HCP), and the cd1 nitrite reductase (NirS) (Kartal et al., 2007; Schalk et al., 2000; Strous et al., 2006). The presence of

NirS (which encodes an enzyme that converts nitrite exclusively into nitric oxide) in the genome of *Candidatus Kuenenia stuttgartiensis* has been described by (Strous et al., 2006).

Van der Star et al., (2007) and van der Star et al., (2008) proposed two simple kinetic models to explain the sudden accumulation of hydrazine caused by a sudden stop in the conversion of hydrazine, while its production from hydroxylamine still continued. This phenomenon, experimentally observed for the enrichment cultures of *Kuenenia stuttgartiensis* and *Brocadia fulgida*, seems to be a general anammox bacteria characteristic (van der Star et al., 2008). Hu et al., (2011) studied the characteristics of nitrogenous substrate conversion by anammox bacteria enrichment using a speculative anammox process pathway based on Van de Graaf model. In most of the studies hydroxylamine has provoked hydrazine production (Egli et al., 2001; Hu et al., 2011). However, there are some reports that demonstrate the involvement of hydrazine, hydroxylamine or nitric oxide (NO) or all of them as anammox process intermediates (Kartal, 2008). It has also been reported that nitrite is reduced to NO and along with hydrazine are converted into N_2 gas (Strous et al., 2006).

Hydrazine is formed from nitric oxide according to the equation:

$$NH_4^+ + NO + 2H^+ \rightarrow N_2H_4 + H_2O \qquad (\Delta G_0' = -47 \text{ kJ mol}^{-1}) \qquad (2)$$

Hydroxylamine can serve as an electron acceptor in the oxidation of hydrazine (van der Star et al., 2008):

$$NH_2OH + 0.5\ N_2H_4 + H^+ \rightarrow 0.5\ N_2 + NH_4^+ + H_2O \quad (\Delta G_0' = -318 \text{ kJ mol}^{-1}) \quad (3)$$

The anammox process can be recovered in a reasonably short time from the impact of inhibiting amounts of substrates (nitrite in high concentrations) by using intermediate (NH_2OH, N_2H_4) injections after inhibition. While being a product of nitrite reduction itself, hydroxylamine can go into reaction with nitrite (Hu et al., 2011):

$$3NH_2OH + NO_2^- \rightarrow 2N_2 + OH^- + 4H_2O \qquad (\Delta G_0' = -339 \text{ kJ mol}^{-1}) \qquad (4)$$

$$NH_2OH + NO_2^- \rightarrow N_2O + OH^- + H_2O \qquad (\Delta G_0' = -300.5 \text{ kJ mol}^{-1}) \qquad (5)$$

Equation (5) is not anammox-specific reaction as N_2O is formed, which is by-product of denitrification process. Hydrazine, in addition to the disproportionation reaction, can be oxidized using nitrite as an electron acceptor (Schalk et al., 1998):

$$4NO_2^- + 4H^+ + 3N_2H_4 \rightarrow 5N_2 + 8H_2O \qquad (\Delta G_0' = -311 \text{ kJ mol}^{-1}) \qquad (6)$$

The disproportionation reaction proceeds according to the following equation (without using any electron acceptors) (Schalk et al., 1998):

$$3N_2H_4 + 4H^+ \rightarrow 4\ NH_4^+ + N_2 \qquad (\Delta G_0' = -181 kJ\ mol^{-1}) \qquad (7)$$

Based on the nearly-complete sequencing of the genome of anammox organism *Kuenia stuttgartiensis*, (Strous et al., 2006), a new metabolic pathway for the anammox process involving NO as an intermediate has been revealed. It is quite necessary to determine the exact effects of the anammox process intermediate compounds on the autotrophic process firstly in a two-step process where there are fewer variables. In the single-step deammonification process intermediates consumption and production stoichiometry could be affected by nitrifying organisms taking part in hydroxylamine formation.

3.2. Single-stage deammonification

In the single-reactor deammonification process firstly, partial nitrification (ammonium oxidation into nitrite) and subsequently ammonium and simultaneous utilization of nitrite is carried out in one-reactor system. Single-stage systems based on deammonification (nitritation-anammox) processes need less space for the reaction (single reactor instead of two reactors needed for a two-stage process) and are economically feasible for the autotrophic nitrogen removal process and therefore widespread. One-stage deammonification system needs a very stable nitritation process (high nitrite accumulation ratio $(NO_2^-)/(NO_2^- + NO_3^-)$), which can be achieved very well for ammonium-rich wastewaters (i.e. reject water) having high free ammonia (FA) and bicarbonate concentrations and by proper operational conditions. Deammonification via intermittent aeration in biofilm (DIB) technology is a novel modification of the single-stage deammonification system and is an effective single-stage nitrogen removal technology of low construction costs. Low costs are due to biofilm carriers with a high specific surface being applied into a single reactor. The DIB technology involves the treatment of NH_4^+-rich wastewaters in time-based intermittently aerated biofilm reactors.

The formation and consumption of anammox process intermediates in deammonification process anammox step play an important role in autotrophic nitrogen removal. Toxic intermediate compound-hydrazine inhibits more NOB than AOB (Wu et al., 2012) while it accelerates anammox bacteria metabolism. Therefore, those kind of selectively maintained nitrifiers-suppressing parameters (in addition to high FA) can be used as operational control parameters for efficient nitrogen removal with low levels of nitrate produced.

The DIB technology has found full-scale application in the Hattingen wastewater treatment plant (Germany) in which during operation at 30 °C, nitrogen removal efficiency and the rate of more than 80% and 1 kg m^{-3} d^{-1},

respectively have been achieved (Gaul et al., 2005). In one-stage nitrogen removal technologies, a rank of more than 0.5 kg m^{-3} d^{-1} for nitrogen removal has been reported (Rosenwinkel and Cornelius, 2005).

The DIB technology start-up and anammox bacteria enrichment on carriers containing active nitrifying bacteria have been thought to be difficult due to a need for the development of nitritating biofilm instead of nitrifying one in the first place. High NO_2^- accumulation ratio (NO_2^-/ (NO_2^-+NO_3^-)) for carrying out an effective anammox process in this system is achieved by the duration of aeration time during the whole aeration and non-aeration cycle (Tokutomi et al., 2010). For wastewaters with high NH_4^+ content, FA levels may be sufficiently high for NOB suppression in a single-stage deammonification process. Based on different reports, FA concentrations of 10–17 mg NH_3–N L^{-1} inhibitory for NOB (Cema et al., 2006; Tang et al., 2010) have not inhibited anammox bacteria which tolerate higher FA concentrations than NOB. Earlier, free ammonia concentrations at 1.7–8.3 mg N L^{-1} have been reported to be inhibitory to anammox bacteria (Jaroszynski and Oleszkiewicz, 2011; Jung et al., 2007), which has not been confirmed by (Waki et al., 2007) observing inhibition of the anammox process at a higher and wider FA concentration range– 18–90 mg N L^{-1}.

Feeding regime can affect stable nitrite formation for the anammox process as well. Spiked addition of FA (Li et al., 2012) into the reactor can more effectively diminish NOB activity than continuous feeding with high NH_4^+ concentration as NOB can be adapted to free ammonia (Chung et al., 2005). Elevated HCO_3^- concentrations (present in landfill leachate and reject water) can keep low NO_3^- levels for an effective anammox process as the nitritation process can be selectively accelerated by bicarbonate addition (Tokutomi et al., 2010).

High NO_2^- accumulation ratio (> 90%) can be established by nitrite oxidizing bacteria (NOB) wash-out through a combination of lowered hydraulic retention time (HRT) (Hellinga et al., 1998), intermittent aeration (Mota et al., 2005) and elevated free ammonia (FA – dissolved non-ionized NH_3) concentrations (Aslan and Dahab, 2008).

Among molecular methods the FISH analyses have been used for detecting the clusters of aerobic ammonium oxidizers and anammox organisms in biomass, making it possible to detect the most suitable biomass architecture for an efficient N-removal process (Vlaeminck et al., 2010). In addition to PCR and DGGE analyses quantitative PCR have been performed for determining the presence of ammonium oxidizers presence in water treatment systems by 16S RNA gene-based methods (Junier et al., 2010), whereas by functional genes encoding ammonia monooxygenase subunits *amo*A and *amo*B, nitrite oxidoreducatase (NirS), hydrazine dehydrogenase (hzo) and hydrazine synthase (hzs) nitrogen-converting organisms quantity have also been detected. However, pyrosequencing for the detection of a higher number of different anammox bacterial strains, still needs to be improved.

4. MATERIAL AND METHODS

4.1. Anammox process start-up and sampling

For continuous reactor operation of anammox, nitrification, nitritation and deammonification processes cylindrical plexiglass reactors with a maximum of 20 L liquid volume were used (Figure 3). The reactors were equipped with a water jacket maintained at a constant temperature (26.0±0.5°C) by an Assistant 3180 (Assistant, Germany) water bath thermostat. The reactors were filled with carriers with a high specific surface (800 m^2m^{-3}) made of polyethylene (Bioflow 9, Aquamyc, RVT Process Equipment GmbH, Germany). The systems were either continuously or intermittently fed by diluted/undiluted anaerobic tank reject water (NH_4^+ source) taken from the Tallinn Wastewater Treatment Plant (WWTP) for single-step deammonification (reactors R_1 and R_2) and in a two-step deammonification process (reactors MBBR1 and MBBR2) NO_2^- was added synthetically. For single-step deammonification systems intermittent aeration was applied. Further information about technological setup is available in papers I–VII.

Earlier, anammox organisms have been determined to be present in many aerobic and anaerobic wastewater treatment units, but their presence in the N-rich effluent of the methane tank (reject water) and potential use for anammox process start-up has not been shown. So, reject water was chosen for the influent of nitritation in one and in two-stage deammonification-process-based systems.

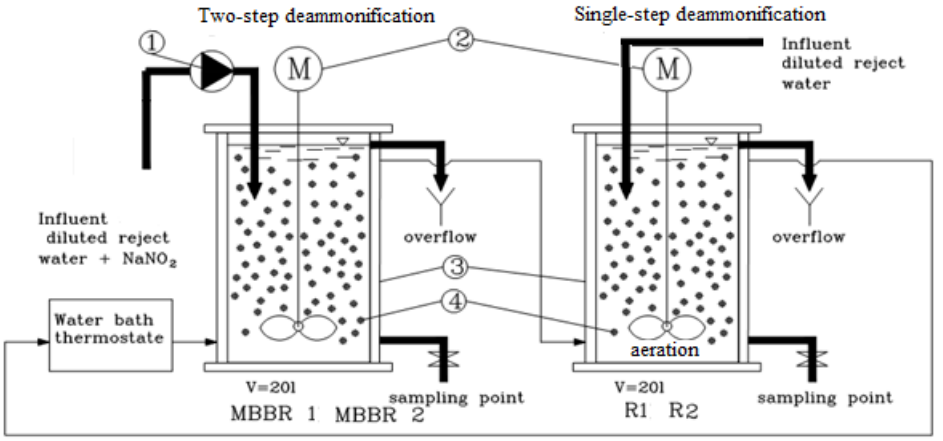

Figure 3. Setup of moving bed biofilm reactors based on two-step (left – MBBR1, MBBR2) and single-step deammonification process (right – R_1 and R_2). 1 – influent pump, 2 – mechanical mixers, 3 – water-jacket, 4 – biofilm carriers.

The influent and effluent samples were collected 1–2 times per week. Biomass samples were collected with a 2 month-interval and stored in a freezer at –20 ºC.

As according to (van de Graaf et al., 1996) a decrease of 30–50% in anammox bacteria activity due to light has been reported, the reactors were covered from visible light with dark plastic material.

4.2. Batch experiments

Batch assays (duplicate/triplicate batch tests) were conducted in order to study the effect of various compound combinations on 200 biofilm carriers with a mature biofilm based on the anammox (nitritation-anammox) process. For other biomass tests, biomass with VSS concentration 1–2 g L^{-1} was used. The carriers with biomass were washed with tap water for 4–5 times before using them in experiments. NH_4Cl, $NaNO_2$ and $NaHCO_3$ were used as nitrogen and carbon sources. 3 mL of an acidic solution of microelements in addition to 3 mL of an alkaline solution of microelements were dispensed into the substrate of batch experiments along with a 40 mL solution of macroelements made according to (Zhang et al., 2010). Stock solutions of TN concentration of around 45–85 mg N L^{-1} were prepared for the tests. Negative control measurements with no biomass added into the substrate were also performed. An Assistant 3180 (Assistant, Germany) water bath thermostat maintained the temperature at 25 (±0.5 °C).

Before the start of the reaction, the liquid phase of the batch reactors was flushed with N_2 or Ar for about 15 minutes to eliminate oxygen from the liquid and gas phase, also favouring biomass acclimatization to the substrate. Then the batch reactors were sealed with butyl rubber stoppers. Sampling was performed with the aid of overpressure of N_2 or Ar created at one end of the three-necked reactor. The batch reactor was stirred by a magnetic bar at around 200 rpm. The pH value was maintained consistently at 8.0–8.5 by a HCO_3^- buffer system formed. Concentrations of nitrogen compounds were monitored every 2 hours during an 8-h period.

Effects of N_2H_4 and NH_2OH concentrations on the TN removal rate of anammox process were estimated. NH_2OH and N_2H_4 were added in the form of hydroxylamine hydrochloride ($NH_2OH{\times}HCl$) and hydrazine sulfate ($N_2H_4{\times}H_2SO_4$), respectively.

4.3. Chemical analysis

Samples of reactor influent and effluent were analysed for NH_4^+–N, NO_2^-–N, NO_3^-–N, total and volatile suspended solids (TSS and VSS, respectively) concentrations according to (Greenberg et al., 1992). The TSS concentration of biofilm was measured gravimetrically 3×20 carriers before and after biomass

removal. pH was measured with a pH meter (Evikon, Estonia) and DO controlled by a (Elke sensor, Estonia) DO controller.

Hydrazine (N_2H_4) was determined by using a Hach Lange DR2800 type spectrophotometer. Prior to the application of the Hach Lange HydraVer 2 reagent (containing *p*-dimethylaminobenzaldehyde), a 0.5% solution of sulfamic acid was added to the sample in order to eliminate interference from NO_2^- and NO_3^-, as described by (Georgea et al., 2008). Hydroxylamine (NH_2OH) was measured with the spectrophotometer at a wavelength of 705 nm according to the method reported by (Frear, 1955).

4.4. Data analysis

Data and statistical analyses were performed by the MS Excel 2010 Analysis ToolPak. Homogeneity of group variances and the difference between group means were checked using the f-test and the two-way t-test, respectively. The level of significance was set at $\alpha < 0.05$.

Linear regressions of the changes of substrates concentrations in time were derived in order to determine the conversion rates of TN and other substrates. The conversion rate per test was determined as the maximum rate, excluding the values obtained when the substrate was depleted. The data with coefficient of determination (R^2) higher than 0.9 were used for TN removal rate calculations. To calculate the biomass-specific conversion rate (mg N g^{-1} TSS h^{-1}), the maximum volumetric conversion rates were divided by added biomass concentration.

4.5. Polymerase chain reaction (PCR) methodology and sequencing

Pla46f / Amx368r primers were used for targeting anammox bacteria. Nitrifying bacterial strains were identified by PCR-DGGE using the eubacterial primer sets GC-BacV3f / 907r and 8F/357R (Zekker et al., 2012).

The sequences obtained are from a comparison with the database (National Center for Biotechnology Information). The sequences acquired were compared to the available database sequences via a Basic Local Alignment Search Tool (BLAST) search from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Molecular analysis of biomasses taken from the reactors were done at the Chair of Biotechnology, Tallinn University of Technology.

4.6. Fluorescent *in-situ* hybridization (FISH)

Fluorescent *in-situ* hybridization (FISH) analyses was performed to detect anammox bacteria strains (genera *"Candidatus* Brocadia and Kuenenia") (Schmid et al., 2001) as specified by (Amann et al., 1990). By using a Cy3-labelled Amx820 probe at 35% formamide solution, anammox bacteria genera *Candidatus* Brocadia anammoxidans cells were detected. The probed and stained biomass was examined using a Carl Zeiss Axioskop 2 Plus an epifluorescence microscope (Jena, Germany) equipped with differential interference contrast (DIC), and the scales were added using ImageJ freeware. Biomass was harvested from the MBBR, fixed in a 4% paraformaldehyde solution. The samples were counterstained with the DNA stain 4',6-diamidino-2-phenylindole (DAPI) (from (Zekker et al., 2012)).

5. RESULTS AND DISCUSSION

5.1. Two-step deammonification start-up

Deammonification process was developed for the elimination of N from reject water by autotrophic bacteria through energetically effective technology. Two-step deammonification processes were started up for the treatment of real wastewater at submesophilic temperature in different moving bed biofilm reactor systems containing high specific-surface blank biofilm carriers or carriers with nitrifying biofilm. Two-step demmonification processes were developed before one-stage processes to determine all needed parameters for the anammox process alone after combining it with nitritation in a single reactor.

5.1.1. Start-up of anammox process in MBBR from scratch (Paper I)

Successful start-up of the anaerobic autotrophic ammonium oxidation process was made without using specific inoculation material or strict process control of dissolved oxygen, pH and alkalinity in a moving bed biofilm reactor. Reject water and synthetically added nitrite solution were used as a feeding medium for a continuously fed MBBR. Reject water contained some solid fractions inside which anammox bacteria could be attached and transferred into the anaerobic zone of biofilm carriers. In a full-scale wastewater treatment plant it is not economical to purchase the bacteria needed for the start-up and add the anammox bacteria-specific biomass into the system having different conditions as than those during biomass growth. It means, lower FA and bicarbonate concentrations can be applied during biomass cultivation when compared with those compounds being present in higher concentrations in real wastewater. So, the anammox process for the treatment of biogas plant effluent should be started from scratch at each wastewater treatment plant on-site, by developing and adapting optimum biomass consortium to each specific wastewater.

A biofilm reactor type was chosen for anammox bacteria enrichment as it is a temperature and inhibitor-tolerant system. Other positive sides why biofilm configurations were preferred over activated sludge were: biofilm systems have a better stability towards fluctuations in the influent, settling performance is not needed and sludge recycling costs are low (Odegaard, 2006).

The two-step deammonification (nitritation and anammox process steps separated between different reactors) MBBR operation was divided into three periods with a total duration of 450 days (Figure 4). The MBBR showed a high average total nitrogen removal rate (TNRR) (0.5 kg N m^{-3} d^{-1}) and total nitrogen removal efficiency (TNRE) (80%) after a considerably long adaptation period of anammox organisms maintaining a HRT of 0.5–1 days. Our TNRR was similar to the one achieved by the other authors' working on anammox process-based MBBR (Cema et al., 2006), but lower than the rate achieved by

some authors works (Gaul et al., 2005; Rosenwinkel and Cornelius, 2005) on the MBBR.

When TN loading is increased more rapidly in comparison with TN removal, nitrogenous compounds (especially NO_2^-) accumulate into the system causing inhibition to anammox bacteria. An inhibition episode occurred during the high-loading period in our MBBR too, after which TN removal was recovered by decreasing the TN loading rate (Figure 4).

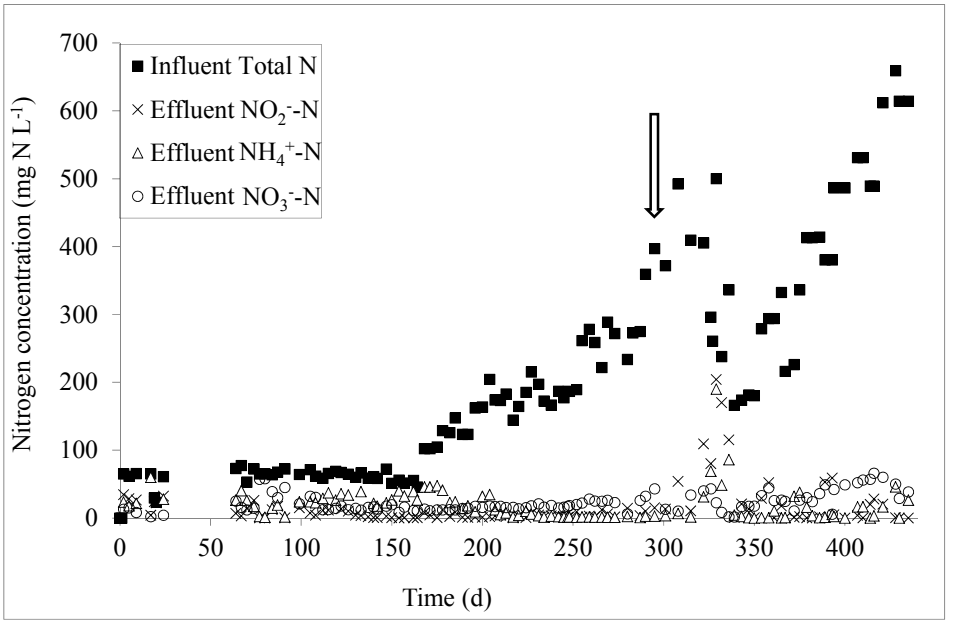

Figure 4. Changes in the concentrations of nitrogen compounds in the influent and effluent in moving bed biofilm reactor (MBBR) system (Paper I).

Within a period of the first 109 days the TNRR reached 0.02 kg m^{-3} d^{-1} and a TNRR of 0.5 kg m^{-3} d^{-1} was achieved by day 181. The maximum TNRR achieved after the 308th day of operation was around 1 kg N m^{-3} d^{-1} (Figure 5), showing a successful start-up.

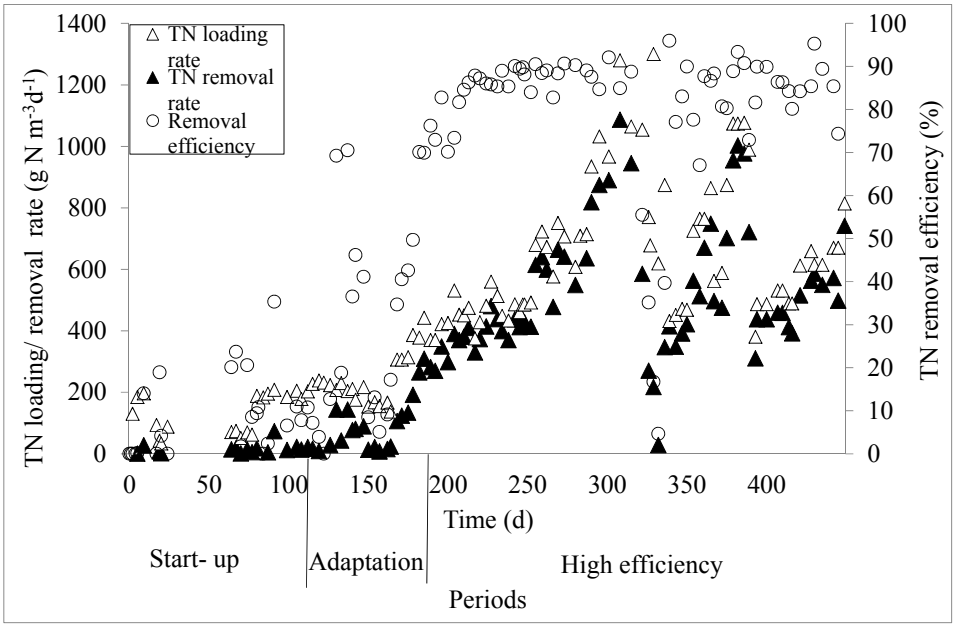

Figure 5. Total nitrogen (TN) removal efficiencies, total nitrogen removal rates (TNRR), total nitrogen loading rates (TNLR) in MBBR system. Periods (start-up, adaptation and high efficiency period) of reactor operation are separated by vertical lines (Paper I).

For the anammox process developed in our system the average ratio of the production of NO_3^-, removal of NO_2^- and removal of NH_4^+ was 0.11/ 1.14/ 1, respectively. By comparison, a respective stoichiometrical ratio for the conventional anammox process presented by (Jetten et al., 1997) was 0.26/ 1.32/ 1. Lower ratio of NO_3^- formed determined by us can be due to some heterotrophic denitrifying activity present in the reactor, which actually is improving water quality in terms of lowered NO_3^- concentrations. Anammox microorganisms with sequence similarity of 98% with *Candidatus Brocadia fulgida* also confirmed the autotrophic ammonium oxidation process in the developed biofilm (See figure. 7).

5.1.2. Anammox process start-up on carriers with and without nitrifying biofilm (Paper II)

Pre-grown nitrifying biofilm on carriers was meant to have a positive effect on the shortening of the start-up of anammox process when compared with using blank carriers. For anammox bacteria enrichment using carriers with nitrifying biomass (MBBR1) in one and in the other reactor (MBBR2) using blank carriers were compared with the aim of choosing the best carrier type for the

anammox process start-up. Nitrite and reject water in anammox process stoichiometrical ratio were fed into both systems maintaining a HRT of 0.5–1 days within three periods of operation. Nitrifying biomass, which is available from aerobic tanks of wastewater treatment plants can also act as starting material (in terms of nitritation) and protecting matrix for the single-stage deammonification operation.

In the reactor containing nitrifying biofilm carriers when compared with the reactor containing blank carriers, slightly higher TN removal rates were achieved with less time (Figure 5a, 5b).

During the adaptation period average TN removal rates between the reactors differed significantly (around 2 times, p-value <0.05) in favour of the reactor containing nitrifying biofilm carriers. The TN removal rate increased from 130 g N m^{-3} d^{-1} to 290 g N m^{-3} d^{-1} in MBBR2, which circumstance this favours the concept of start-up of the reactor using pre-grown biomass on carriers.

In the high efficiency period the reactor containing nitrifying biofilm carriers had a better tolerance to high TN loading (with high influent nitrite concentration of 288 mg N L^{-1}) compared with the system having blank carriers. During the period of constant TN loading, a decrease in the TN removal rate (from 890 g N m^{-3} d^{-1} to 260 g N m^{-3} d^{-1}) occurred for the system containing nitrifying biofilm carriers. It was recovered within four days (TN removal rate of 880 g N m^{-3} d^{-1} was achieved again, Figure 6a). When the reactors were operated at the same TNLR and nitrite concentrations the reactor with blank carriers showed a major drop in the TN removal rate to 29 g N m^{-3} d^{-1}, also having a longer recovery time of a week. TN removal rate of 410 g N m^{-3} d^{-1} was achieved again after a week (Figure 6b). For both reactors the system recovered from inhibition by using a higher influent NH_4^+–N/ NO_2^-–N ratio (around 1.2) along with a lower HCO_3^- concentration of 750 mg HCO_3^- L^{-1}.

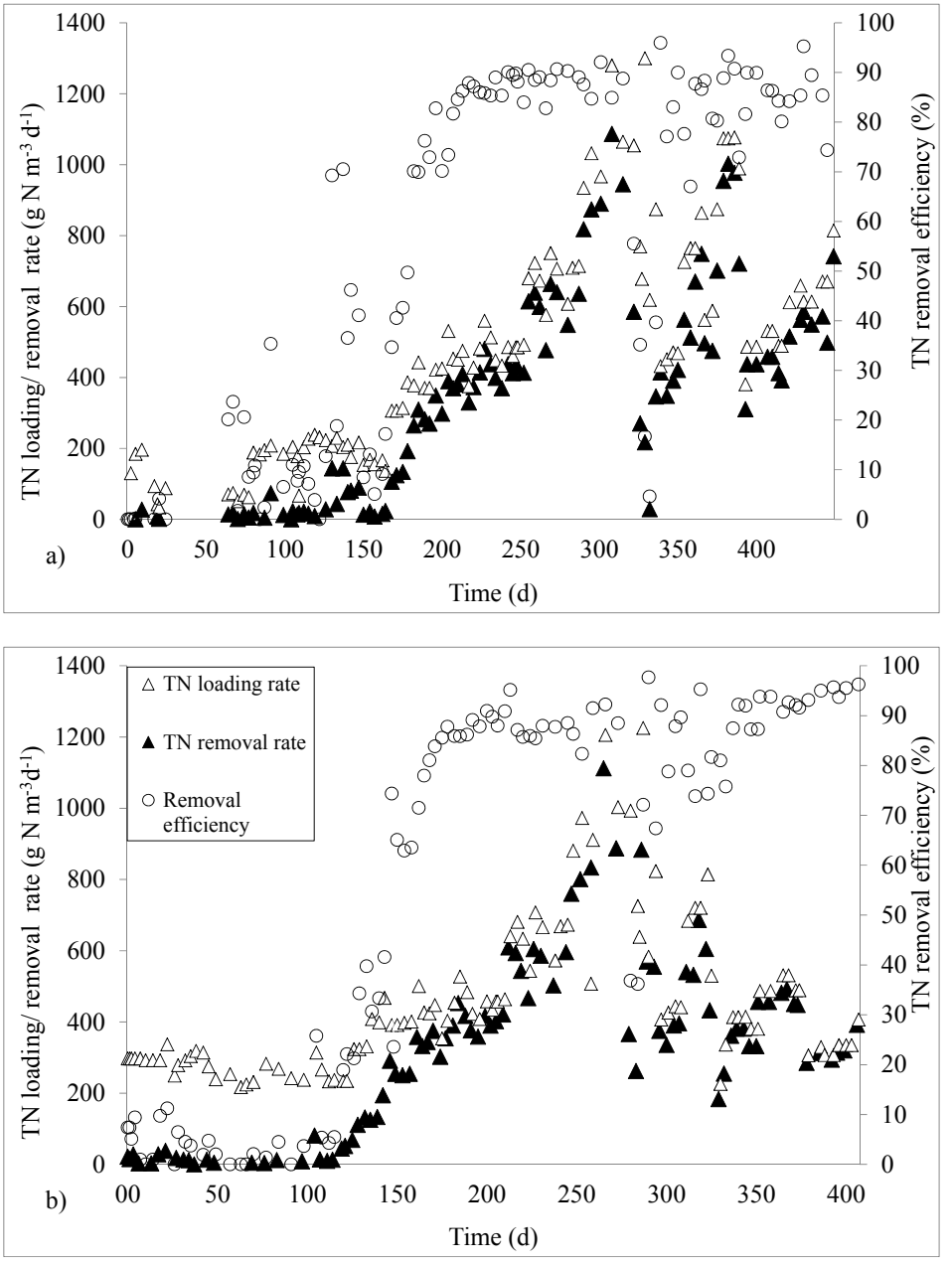

Figure 6. Total nitrogen loading rates (NLR-hollow triangle), total nitrogen removal rates (NRR-filled triangle) and total nitrogen (TN) removal efficiencies (hollow circle) in **a)** MBBR1 and **b)** MBBR2 system (Paper II).

5.1.3. Microbiology of reactors with and without carriers with pre-established biofilm

PCR and DGGE analyses performed after 6 and 7 months of the anammox process in MBBR1 and MBBR2, using anammox bacteria DNA-specific primers Pla46f/ Amx368r, resulted in the detection of sequences which were similar (85%) to DNA sequences belonging to uncultured *Planctomycetales bacterium clone P4* (GenBank ID: DQ304521.2) (Figure 7).

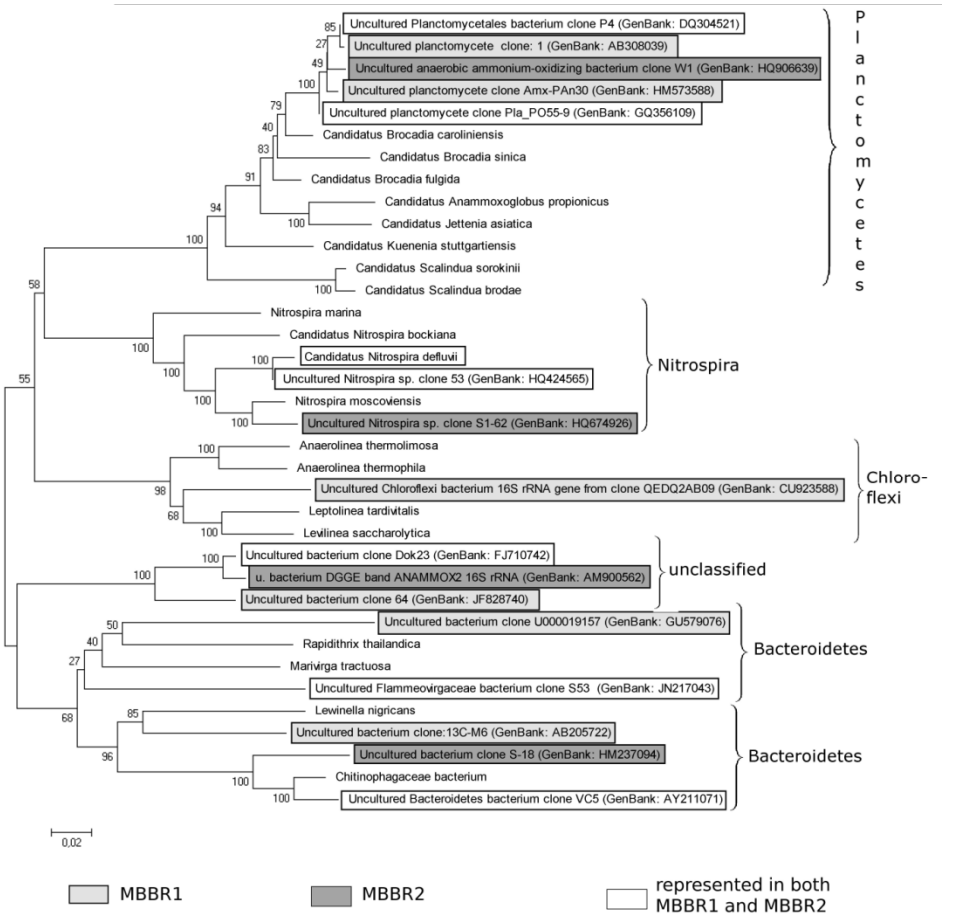

Figure 7. Phylogenetic neighbour-joining tree, reflecting the relationships between some known anammox bacteria (*Planctomycetales*) and the identified sequences in MBBR1, MBBR2 and in both reactors, based on 16S rRNA genes amplified, using *Planctomycetales*-specific primers and several eubacterial primers. Numbers at the nodes are percentages of bootstrap values. Branch lengths correspond to sequence differences as indicated by the scale bar. The GenBank accession numbers are indicated.

From both reactors also uncultured *Nitrospira* and *Nitrospira* defluvii cells belonging to NOB were determined. These organisms presence could be due to presence of abundant nitrite concentration and low (~0.2 mg L^{-1}) DO concentration. Other organisms belonging to bacteroidetes were determined by PCR and DGGE from both reactors as well as several unclassified (anammox) organisms. Only from the MBBR1 anammox organisms uncultured *planctomycete clone: 1* and uncultured *planctomycete clone Amx PAn30* were determined.

Also, similar sequences to uncultured anaerobic *ammonium-oxidizing bacterium clone W1* 16S ribosomal RNA gene, partial sequence (Genbank ID: HQ906639.1) were detected in MBBR2.

The *Planctomycetales* microorganisms were detected before and after applying substrates in high or even inhibiting concentrations with increased TN loading rate. It shows the biofilm systems tolerance to abrupt changes in substrate levels. Also, no important shifts of bacteria composition having significant effect on TN removal performance were determined during reactor operation.

5.1.4. Accelerating effects of anammox intermediates of anammox organisms cultivated in two-step deammonification moving bed biofilm reactor (MBBR) (Paper III)

In practical applications the knowledge of the behaviour of anammox process intermediates in the nitrogen removal process is essential to sustain the stability of the anammox process by adding small amounts of an intermediate into the inhibition-damaged system. Toxic substances (i. e. NO_2^- and NH_4^+ at high concentrations) could be utilized faster by intensification of processes carried out in anammox bacteria cells by means of using optimal amounts of intermediates (i.e. hydrazine). This fact makes it possible that nitrogen removal is carried out along with a shorter metabolic pathway until the elimination of inhibition.

The role of different hydroxylamine and hydrazine concentrations in acceleration of the total nitrogen (TN) removal rate of the biofilm system grown on blank carriers containing anammox bacteria was determined. Also, it was intended to find out optimum intermediate concentrations (combinations of them), which bring along the highest TN removal rate in the continuous reactor operation (Figure 8) and in batch assays.

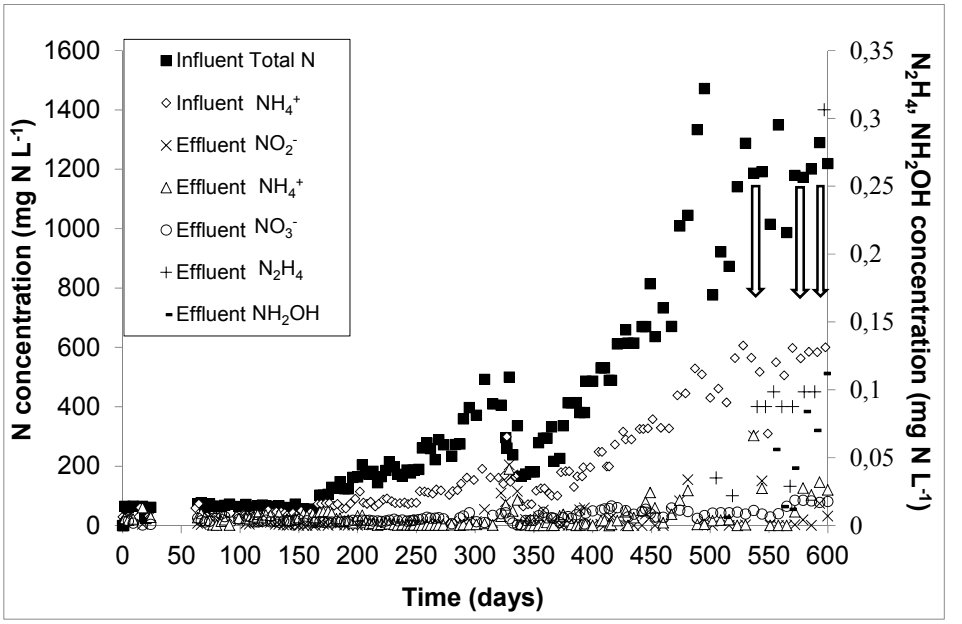

Figure 8. Changes in the influent and effluent concentration of nitrogen compounds (including intermediates); injections of intermediates (NH_2OH and N_2H_4 at concentrations (1.31 and 1.27 mg N L^{-1}, respectively) during inhibiting episodes are shown by arrows (Paper III).

In our continuous MBBR1 system experiments with intermediates, after inhibiting episodes in the reactor containing carriers initially without biomass during the start-up period, addition of both intermediates–NH_2OH and N_2H_4 into the reactor helped to sustain anammox process activity in the similar range as before inhibition. Both intermediates together were preferred over N_2H_4 addition alone to restore the activities of the inhibition-damaged hydrazine-oxidizing enzyme and hydroxylamine oxidoreductase (Kuenen and Jetten, 2001). The two-step conversion of intermediates (NH_2OH to N_2H_4 and N_2H_4 to N_2) would sustain them longer in the process, enabling a short and effective overcoming from inhibition. According to (Bettazzi et al., 2010), spiked injections of NH_2OH (6.36 mg N L^{-1} in total) gave a 20% permanent recovery from the complete NO_2^- inhibition. Our experiments showed that after the system had been inhibited, addition of NH_2OH and N_2H_4 in lower concentrations (1.31 and 1.27 mg N L^{-1}, respectively) resulted in an increase in TN removal efficiency of around 20% in the continuously fed reactor (Figure 8). Direct biological reactions between NH_2OH, N_2H_4 and NO_2^- can be significant factors contributing to the ameliorating effects observed when anammox process intermediates are added in case of inhibition caused by a high NO_2^- concentration.

Anammox process in batch experiments with biofilm carriers taken from the previously described reactor were studied in detail by measuring the effect of different quantities of intermediates on anammox TN removal rate. The optimum amount of anammox process intermediate – N_2H_4 increasing TN removal rate in batch experiments was 4.38 mg N L^{-1}. Different amounts of N_2H_4 than this as well as different N_2H_4 and NH_2OH concentration combinations resulted in lower TN removal rates (Figure 9).

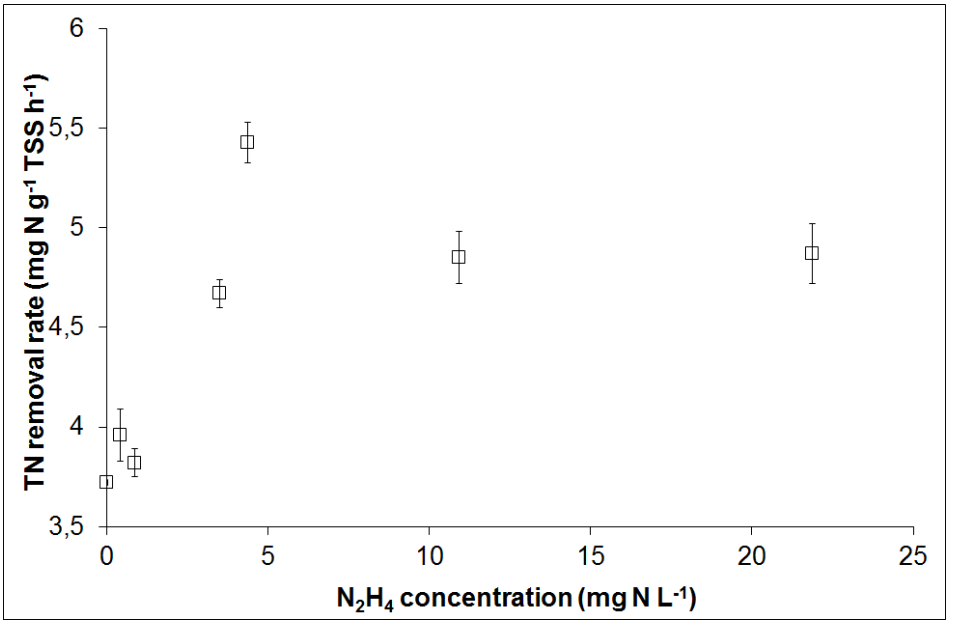

Figure 9. Dependence of TN removal rates on different N_2H_4 concentrations injected (Paper III).

Hydrazine disproportionation rate (non-biological) was significantly slower (p-value < 0.05) than biological hydrazine consumption in batch incubation without nitrite and ammonium (Zekker et al., 2012b), confirming that the anammox process was responsible for the conversion of the intermediate.

In batch experiments higher TN removal rates were achieved by the addition of N_2H_4 as compared with the addition of NH_2OH and N_2H_4 together in the same quantity (Figure 10), despite the circumstance that both reactions should kinetically be of equally low negative free energy (– 311 kJ mol^{-1} and –318 kJ mol^{-1}, respectively). Better TN removal rates with N_2H_4 addition could be assumed to be due to the lower reduction state of N in the intermediate and a better preference of this specific intermediate to microorganisms present in the biofilm.

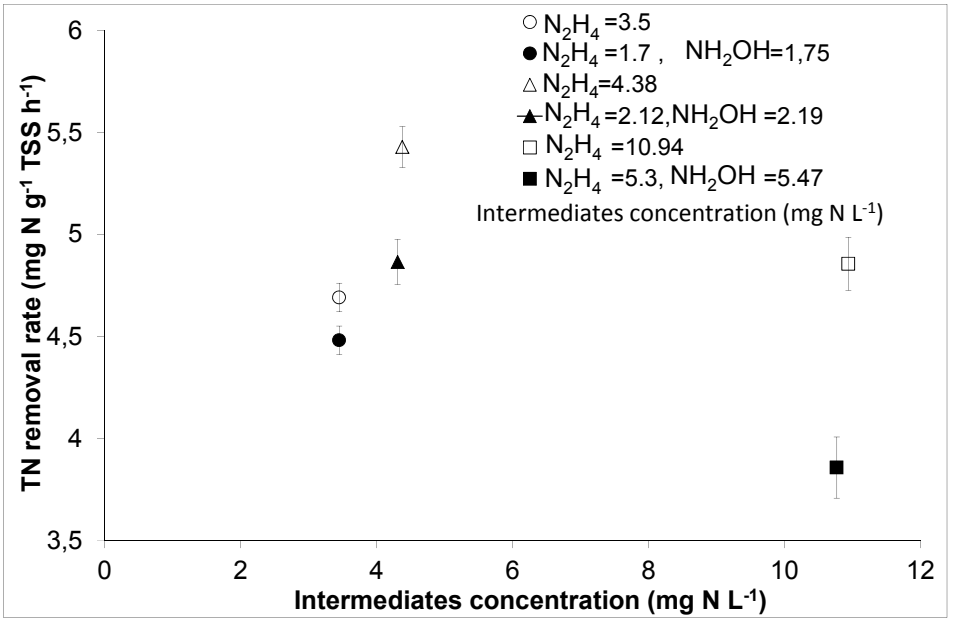

Figure 10. TN removal rate with different N_2H_4 and NH_2OH + N_2H_4 concentration combinations added (Paper III).

In batch experiments incubated with anammox bacteria cultures and hydrazine it was observed that 3 mol N_2H_4 was converted to 4 mol NH_4^+ similarly to that depicted in Eq. (7). The assumption whether one enzyme (R2 subunit of ribonucleotide reductase from *Escherichia coli*) is catalyzing conversion of hydrazine into dinitrogen gas and ammonium (Han et al., 1996) is responsible for the conversion of hydrazine into dinitrogen gas and ammonium or whether two different enzymes are involved needs further investigation. It is possible that an enzyme similar to the one observed in the R2 subunit or an enzyme similar to that of hydroxylamine oxidoreductase (HAO) from N. Europaea is carrying out hydrazine conversion in an anammox bacteria cell (Schalk et al., 1998).

5.1.5. Nitritating, nitrifying and anammox bacteria communities determined by PCR-DGGE in the reactor boosted with intermediates

The nested PCR and the following DGGE performed with anammox bacteria-specific primers resulted in the detection of 323 bp DNA sequence belonging to uncultured *Planctomycetales bacterium clone P4* (99% sequence similarity to GenBank ID: DQ304521, *Candidatus Brocadia fulgida*) (after a six months of anammox bacteria enrichment process on blank biofilm carriers). *P4* had the

same sequence as described by (Quan et al., 2008). Phylogenetical analysis also showed the presence of other bacteria strains in the biofilm similar to sequences available in the GenBank. One of the strains was related (not closely) to *Nitrosomonas europaea* (84 %)-strains of aerobic ammonium-oxidizing bacteria, which is commonly present in oxygen-limited reactors relying on partial nitritation (Vlaeminck et al., 2010). The biofilm also harboured the strains of nitrite-oxidizing bacteria *Candidatus Nitrospira defluvii* and uncultured *Nitrospira sp. clone S1–62*. Interestingly, *Nitrospira* strains could thrive under very low oxygen conditions in the MBBR based on the deammonification process (Mota et al., 2005).

5.1.6. Results of Fluorescent *in-situ* hybridization (FISH)

Biomass developed on blank carriers and on the carriers with nitrifying biomass had both anammox organisms belonging to *"Candidatus* Brocadia and Kuenenia". The FISH analysis revealed that anammox microorganisms were located densely in clusters in the biofilm (Figure 11). Although anammox bacteria cells have been described to grow in clusters mostly on the upper surface of biofilm support material (Tal et al., 2006), anammox bacteria cells seemed to be located throughout the biofilm in our case. Breakage of the clusters during the operation of an anammox process may be responsible for a lower production of anammox process intermediates, reducing biomass growth (Dapena-Mora et al., 2004).

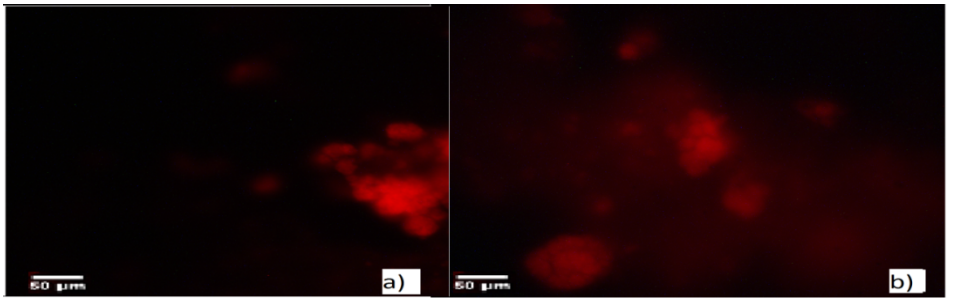

Figure 11. Representative micrograph set of MBBRs' anammox bacteria biofilm with FISH staining displaying anammox bacteria with Cy3-labelled Amx820 of **a)** from carriers having no biomass in the start-up and **b)** from carriers with nitrifying biomass present in the start-up. Scale-bar 50 µm (Paper II).

From the studies on the two-step deammonification process start-up suitable conditions for the process development of real N-rich wastewater (reject water) treatment without a specific inoculation procedure were determined. Using pre-grown biofilm has some benefits for a more rapid anammox start-up. The role of optimum anammox process intermediates in anammox-process step acceleration was proven in this thesis.

5.2. Single-reactor deammonification studies

Based on the successful start-up of a two-stage deammonification reactor, a single-stage deammonification start-up was established. Stable nitritation with low nitrate accumulation is one of the basic needs for a properly working one-stage deammonification system. In this thesis establishment of an one-stage deammonification system from biofilm with nitrifying activity (VI) and from biofilm with pre-established anammox bacteria nitrifying biomass was developed (VII). The latter sequence of processes was done for the first time in the speciality literature by us (Paper VII).

5.2.1. Nitrifying biofilm conversion to nitritating one (Paper IV)

In order to start-up a single-stage N-removing water treatment system based on a deammonification (nitritation-anammox) process, nitritating biomass (with a minimum amount of nitrifiers) has to be grown. In the biofilm system it is more difficult to achieve nitritation based on the SHARON process by a low HRT-based strategy, which is possible in systems working by unattached biomass.

Nitrifying biomass (with high nitrate accumulation) was converted to nitritating one in a MBBR by means of a combination of lowered HRT, DO concentrations and elevated FA levels (up to 6.5 mg N L^{-1}) within around a month's period. Selective inhibition of NOB by FA for achieving high nitritation rates of high-surfaced biocarriers biomass taken from the same continuously fed MBBR was also determined at various higher FA concentrations (5, 34 and 70 mg N L^{-1}) in batch tests. The maximum nitrite accumulation ratio (96.6%) evaluated as NO_2^-–N/ (NO_2^-–N + NO_3^-–N) was achieved for a FA concentration of 70 mg N L^{-1} at 36 °C. Suitability of the developed biomass as a nitrite-producing biofilm for the anammox process was determined by a 30 times higher specific nitrite formation rate compared to specific nitrite oxidation rate (Paper IV).

5.2.2. Deammonification process start-up with nitrifying biofilm carriers (Paper V)

After the conversion of biofilm with high nitrifying efficiency into a biofilm with high nitritating (Paper (IV) efficiency, the MBBR biofilm was converted into biofilm performing a deammonification (nitritating-anammox) process in a moving bed biofilm reactor at 26.5 (±0.5) °C (Paper V). Based on operation strategies the reactor operation was divided into VII periods (depicted on Figure 12, Paper V). For an efficient deammonification process two key strategies, namely, increased FA and HCO_3^- concentrations as the limiting factors for the nitrification process and NOB growth helped to maintain nitrite as a substrate for anammox bacteria. By an intermittently maintained higher FA

concentration (FA increased to 6 mg N L^{-1}), an effective nitritation process was not sustained simultaneously with the anammox process (significant NO_3^- accumulation occurred at the end of period VI, days 160–210). Additionally (in period VII), higher influent HCO_3^- concentrations were applied (Figures 12, 13). After the influent HCO_3^- concentration was increased from range 700–750 (derived from diluted reject water) to 1200–2350 mg L^{-1} (by $NaHCO_3$ addition), the effluent NO_3^- concentration decreased from around 30 mg N L^{-1} to below 10 mg N L^{-1}. It brought along NO_2^- accumulation ratio of over 90% (Figure 13). Nitrite oxidizing bacteria (NOB) were more effectively suppressed by an enhanced HCO_3^- (or concentration of CO_2 being in equilibrium with HCO_3^-) concentration from 1200 to 2350 mg L^{-1} combined with an elevated FA concentration. This was different from solely FA-based process control where NOB recovery from inhibition took place. TN removal rates with only enhanced HCO_3^- concentration-based and only FA-based strategies were 0.3 kg N m^{-3} d^{-1} and 0.2 kg N m^{-3} d^{-1}, respectively (Paper V).

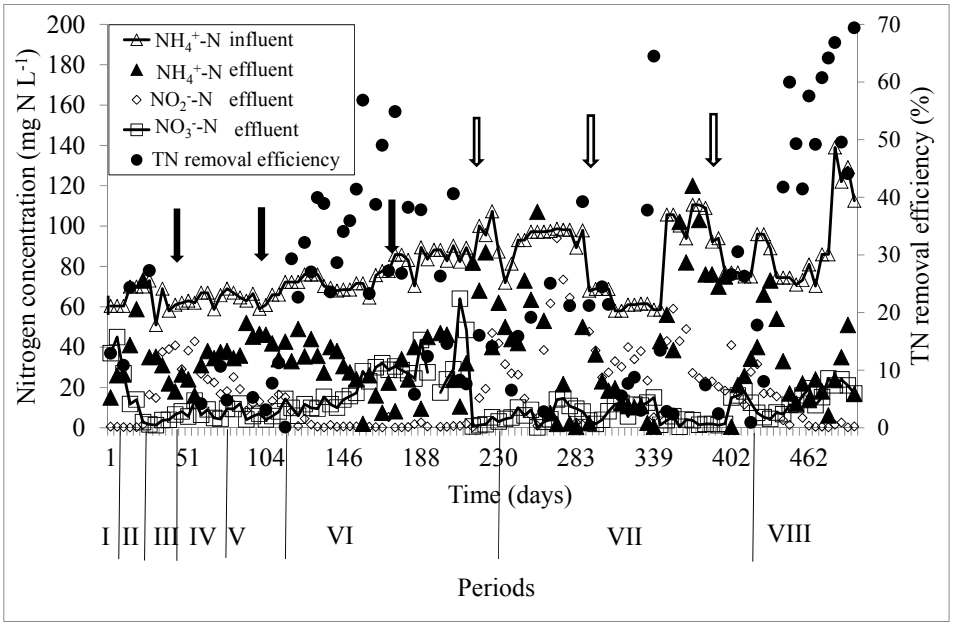

Figure 12. Concentrations of nitrogen compounds' and TN removal efficiencies in the influent and effluent during the operation of nitritation-anammox process-based system (primary x-axis) in different periods (I–VIII, borders between periods are shown by vertical lines in the secondary x-axis) (Paper V). Arrows filled-incresed free ammonia concentration. Arrows empty-increased free ammonia concentations coupled with increased bicarbonate concentrations.

In our study, in periods VI, VII and VIII an increasing HCO_3^- concentration selectively held NOB activity low. In phases with low NO_2^- accumulation ratio the anammox process proceeded effectively with the help of increased HCO_3^- concentrations (Figure 13). This has also been previously reported by (Tokutomi et al., 2010) who achieved over 90% NO_2^- accumulation ratio (NO_2^-/ (NO_2^- + NO_3^-)) with the effluent HCO_3^- concentration of 500 mg L^{-1}. According to these authors an increasing effluent HCO_3^- concentration from 500 to 1270 mg L^{-1} helped to reduce a sudden increase of NOB activity since after an increase in bicarbonate concentration effluent NO_3^- concentrations decreased from 200 mg N L^{-1} to below 50 N mg L^{-1}.

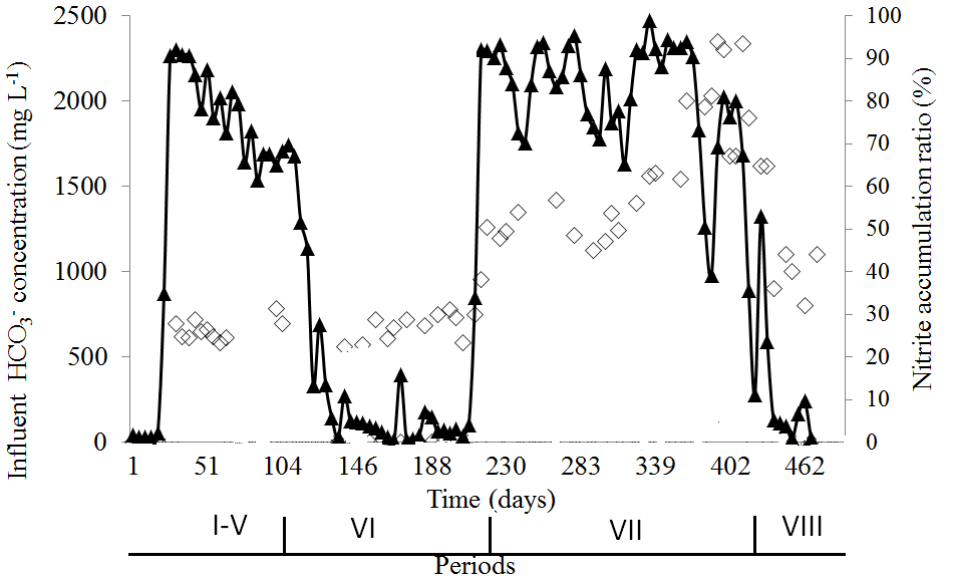

Figure 13. Changes in influent HCO_3^- concentrations (empty triangles) and effluent NO_2^- accumulation ratios (NO_2^-/ (NO_2^- + NO_3^-)) (full triangles) during MBBR operation (Paper V).

5.2.3. Nitrogen converting bacteria present in single-stage deammonification MBBR

After nitrifying biofilm conversion to nitritating one, anammox microorganism strains (*Planctomycetales bacterium clone P4* (99% sequence similarity to GenBank ID: DQ304521, Candidatus *Brocadia fulgida*)) were enriched from reject water on carriers of single-reactor deammonification plant performing the nitritation-anammox process (Figure 14). AOB strains, with 100% similarity to *Nitrosomonas spp.* were determined. Among nitrifying organisms *Nitrobacter spp.* strain (present until 36 days of operation) was replaced with other NOB strains– *Nitrospira spp.* clone 53 (GenBank: HQ424565, sequence similarity 82%)), which can thrive under a lower DO concentration range than *Nitrobacter*

spp.. Nitrospira spp. is less influenced by aeration duration in aeration-non-aeration cycles (Mota et al., 2005) and presumably tolerate higher FA concentrations than *Nitrobacter spp.. Nitrospira spp.* in abundance was observed to limit nitrogen removal efficiency of the deammonification system.

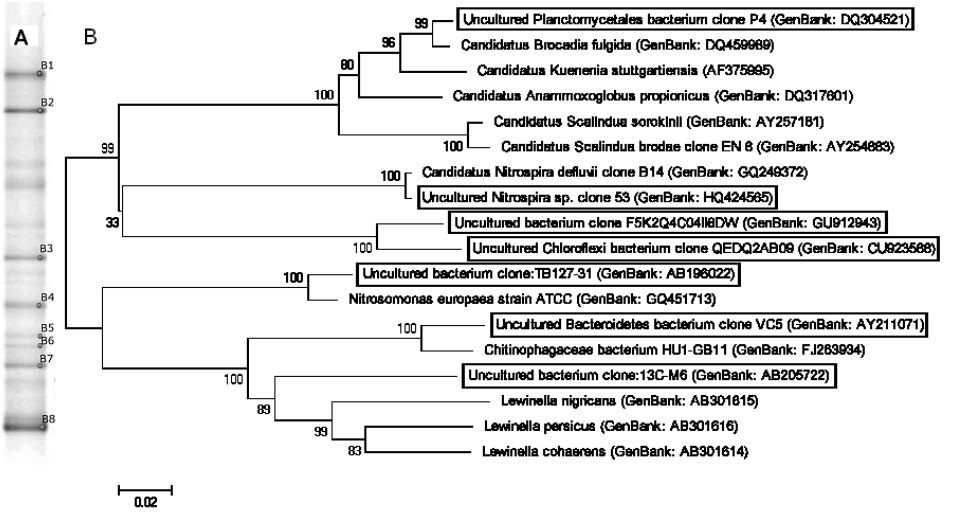

Figure 14. (A) Profiles of DGGE bands of detected bacteria marked as: B1– uncultured Bacteroidetes bacterium clone VC5; B2– uncultured bacterium clone TB127–31; B3– uncultured bacterium clone F5K2Q4C04II6DW; B4 –uncultured *Nitrospira* sp. clone 53; B5–B8– *Candidatus* Nitrospira defluvii. Uncultured *Planctomycetales bacterium* clone *P4* (GenBank ID: DQ304521.2) was determined, using anammox bacteria-specific primers Pla46f/Amx667r. **(B)** Phylogenetic tree showing bacterial diversity in the system and similarity to identified sequences based on 16S rRNA genes amplified. Numbers at the nodes are percentages of bootstrap values. Branch lengths corresponding to sequence differences are indicated by the scale bar. The GenBank accession numbers are also given (Paper V).

5.2.4. Reverse order deammonification start-up: nitrifying biomass development onto anammox bacteria-based biomass (Paper VII)

Usually, deammonification start-up is achieved with firstly developing of nitritating cultures and subsequently developing of the anammox process. In this study two biofilm reactors (R_1 and R_2) based on deammonification process were started up with reject water feeding at temperatures 26 (±0.5) °C. Into an anaerobic zone of MBBR system biofilm having pre-established nitrifying biofilm (R_1), anaerobic ammonium oxidizers were developed. In the second MBBR (R_2) firstly, anammox organisms were enriched and deammonification process was established by a subsequent integration of nitrifiers. So, the second deammonification system was successfully started up with real Tallinn WWTP reject water (influent NH_4^+ concentration around 800 mg N L^{-1}) in the reverse order as done previously.

Table. NOB outcompetition strategies in two reactors (R_1 and R_2) through FA spiking times and corresponding concentrations of anammox process intermediates during continuous reactor operation (from paper VII). Nd – not determined. ± – standard deviation. [DO] – dissolved oxygen concentration. [FA] – free ammonium concentration. FA spiking times – times FA spiked per day. TSS – total suspended solids concentration per carrier. [N_2H_4] – effluent hydrazine concentration. [NH_2OH] – effluent hydroxylamine concentration.

Parameters in R_1						**Parameters in R_2**					
Time (days)	**[DO] range (mg L^{-1})**	**[FA] (mg N L^{-1})/ FA spiking times (d^{-1})**	**[TSS] (mg $carrier^{-1}$)**	**[N_2H_4] (mg N L^{-1})**	**[NH_2OH] (mg N L^{-1})**	**Time (days)**	**[DO] range (mg L^{-1})**	**[FA] (mg N L^{-1}) / FA spiking times (d^{-1})**	**[TSS] (mg $carrier^{-1}$)**	**[N_2H_4] (mg N L^{-1})**	**[NH_2OH] (mg N L^{-1})**
0–55	2– 4.5	42/ 2	4.3 (±0.8)	nd	nd	0–379	0.1–1.0	7.5/ 40	1.6 (±0.3)	nd	nd
56–313	0–2.3	8/ 2	4.3 (±0.8)	0.067 (±0.082)	0.02 (±0.014)	380–616	0.1–1.2	10/ 20	2.47 (±0.2)– 3.865 (±0.09)	0.028 (±0.014)	0.018 (±0.017)
314–433	0–2.3	5/ 2	nd	0.094 (±0.041)	0.034 (±0.043)	617–812	0.1–1.1	19/ 12	5.38 (±0.31)	0.028 (±0.014)	0.026 (±0.027)
434–500	0–0.3	11/ 5	3.38 (±0.2)	0.055 (±0.015)	0.05 (±0.047)	813–879	0.1–1.1	11/ 20	7.01 (±0.2)	0.028 (±0.014)	0.05 (±0.05)
501–560	0.2–1.1	11/ 7	3.74 (±0.25)	0.022 (±0.011)	0.028 (±0.026)	880–911	0.7–1.1	11/ 20	7.42 (±0.25)	0.019 (±0.003)	0.028 (±0.026)
561–670	0.5–0.9	21/ 12	6.3 (±0.2)	0.027 (±0.026)	0.025 (±0.025)	912–970	0.6–0.9	11/ 24	11.9 (±0.2)	0.036 (±0.036)	0.05 (±0.033)

After the R_1 system start-up the TN removal rate increased from 0 to 0.5 g N m^{-2} d^{-1} (efficiency of 70%) already within 16 days. The start-up of the anammox process followed by the development of nitritating biomass in R_1 rendered a faster and better start-up in comparison with the strategy applied in R_2, and should be therefore economically favoured (Figure 15). A higher maximum N-removal rate (1 g N m^{-2} d^{-1}) with lower NOB activity was determined for the nitritation-anammox process in reactor R_1 started from anammox biomass, as compared with the second system started from nitrifying biofilm (R_2), having a poorer maximum N-removal rate (0.5 g N m^{-2} d^{-1}) and higher effluent NO_3^- concentration (Figure 15). Surface specific TN removal rate (g N m^{-2} d^{-1}) was used instead of volumetric rate (g N m^{-3} d^{-1}) due to thicker biofilm developed during the longer time interval and need for more exact determination of TN removal rate for specific amount of carriers applied when compared with other studies. Thick biofilm applied has higher inert fraction which has to be taken into account when calculating specific TN removal rate.

Based on chemical parameters measured during the R_1 operation, NOB were more effectively and permanently suppressed than in the other MBBR (R_2) (Figure 15). Also, higher residual effluent hydrazine concentrations produced by anammox bacteria were determined in the first MBBR (R_1) than in the second MBBR (R_2). These determined higher hydrazine levels in the effluent also had a role in maintaining a more efficient TN removal rate of deammonification process (Table). At the same time hydrazine selectively suppressed NOB, but had a minor effect on AOB activity (nitrite accumulated after 300 days). A similar selective NOB suppressing (but not AOB suppressing) effect has been found by (Wu et al., 2012) in soil nitrification.

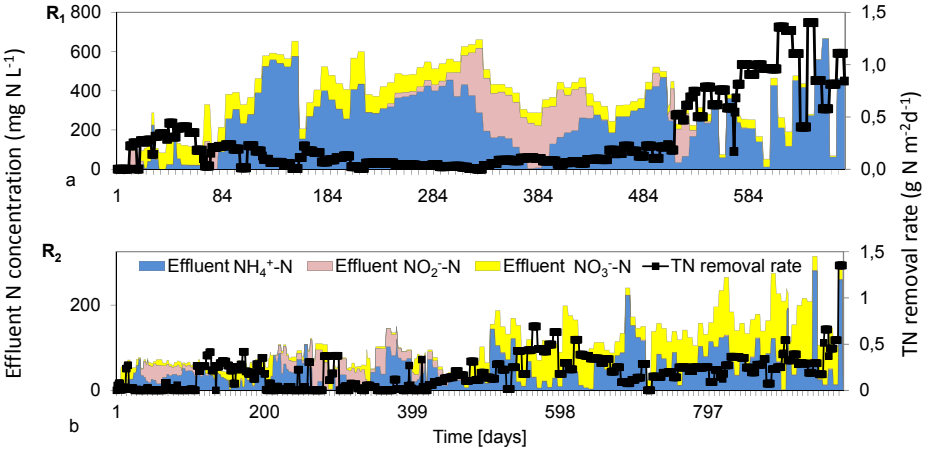

Figure 15. Effluent N-compounds' concentrations and TN removal rates in two deammonification MBBRs (R_1 and R_2) during long-term operation (Paper VII).

Higher TN removal and low NOB activity was determined for less FA spiking frequency <12 times per day when compared with >20 times-per-day spiking (Figure 15, Table). TN removal rate of the deammonification process increased in R_1 to significantly higher (p-value < 0.05) values than in R_2 due to a more evenly spread feeding pattern correlating with an increasing N_2H_4 concentration from 50 to 150 μg N L^{-1}.

In combination with the spike feeding strategy in reactors R_1 and R_2, increased levels of N_2H_4 decreased the effluent NO_3^- levels making this parameter as a unique one for controlling NO_3^- levels (Figure 16) and for increasing the autotrophic TN removal rate. Permanent outcompetition of NOB can be achieved firstly by the development of anammox bacteria and then adding a nitritating bacteria-based process which finding is of great interest for stable full-scale deammonification process start-up.

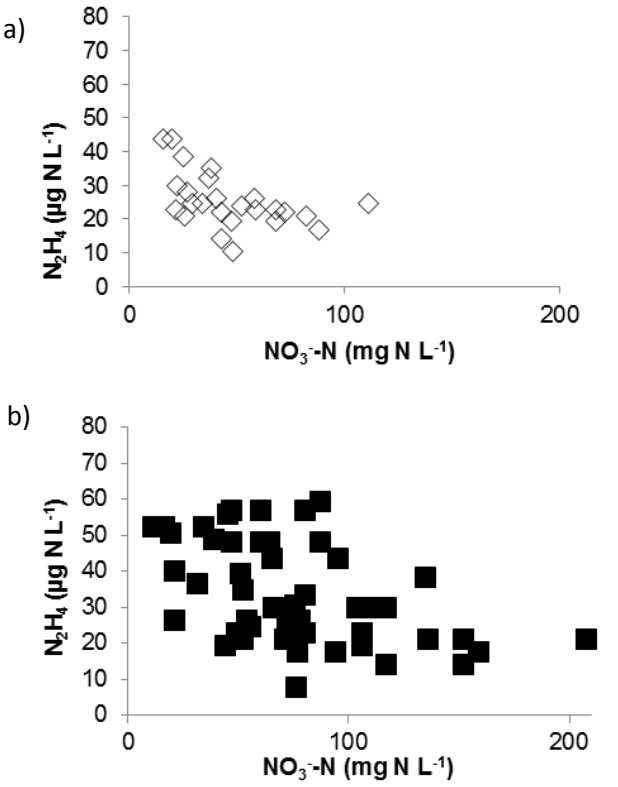

Figure 16. Relationship between effluent N_2H_4 concentrations and achieved effluent NO_3^- concentration in two deammonification MBBRs (a) – R_1 and b) – R_2) (Paper VII).

5.2.5. Comparison of microorganisms determined by DGGE of single-step deammonification systems with pre-established anammox bacteria and with nitrifying bacteria biofilm

Nitrogen converting bacteria (including anammox bacteria) identified from two deammonification reactors after 500 days of operation showed a difference in terms of the composition of nitrifying communities. Namely, *Nitrobacter spp.* were outcompeted by a long-term NH_4^+ (FA) maintainance in R_1 (*Nitrospira spp.* was still present), whereas *Nitrobacter spp.* was present in R_2 at the beginning of operation at lower NH_4^+ levels in the effluent (Figure 17). *Nitrospira spp*. is known as low oxygen-tolerant NOB, which growth is dependent on the lengths of aerobic/ anoxic cycles of aeration (Mota et al., 2005).

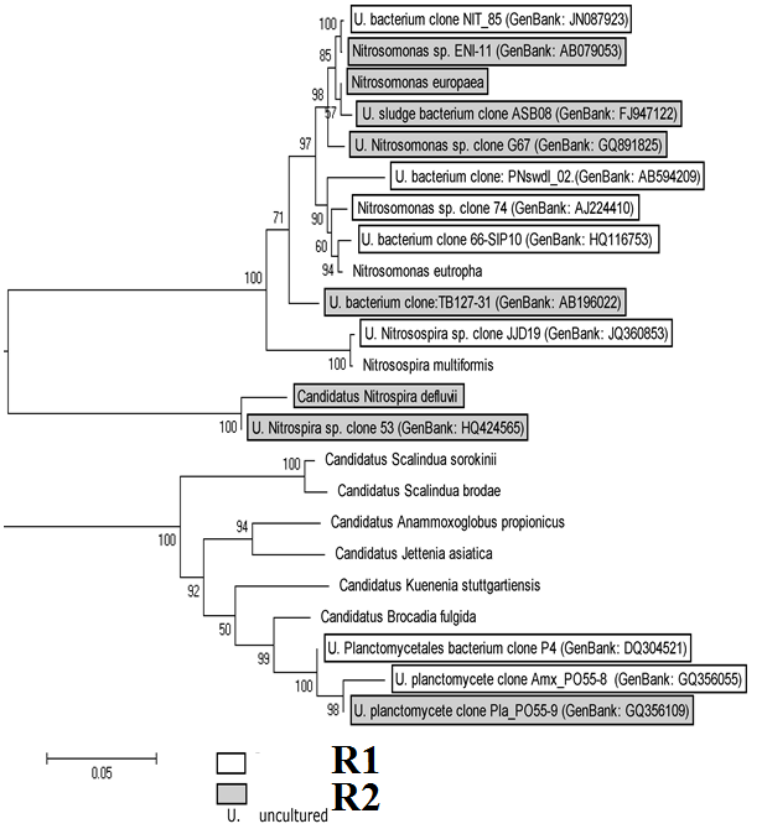

Figure 17. Phylogenetic tree showing N-converting bacteria diversity in two deammonification MBBR (R_1 and R_2) systems and the similarity of identified sequences based on 16S rRNA genes determined by DGGE after 500 days of operation. Branch lengths corresponding to sequence differences are indicated by the scale bar. Numbers at the nodes are percentages of bootstrap values. The GenBank accession numbers are indicated (Paper VII).

In both systems anammox organisms (however of slightly different strains) were present along with nitrifying organisms showing proper conditions present for aerobic and anaerobic bacteria at maintained conditions.

5.2.6. FISH analysis of single-step deammonification systems with different pre-established biofilms

The FISH analysis showed the presence of similar dense anammox bacteria clusters in both (R_1 and R_2) reactors' biofilms showing a successful enrichment of anammox bacteria with no visible effect of different applied FA concentrations on the denseness of anammox bacteria clusters in biofilm (Figure 18).

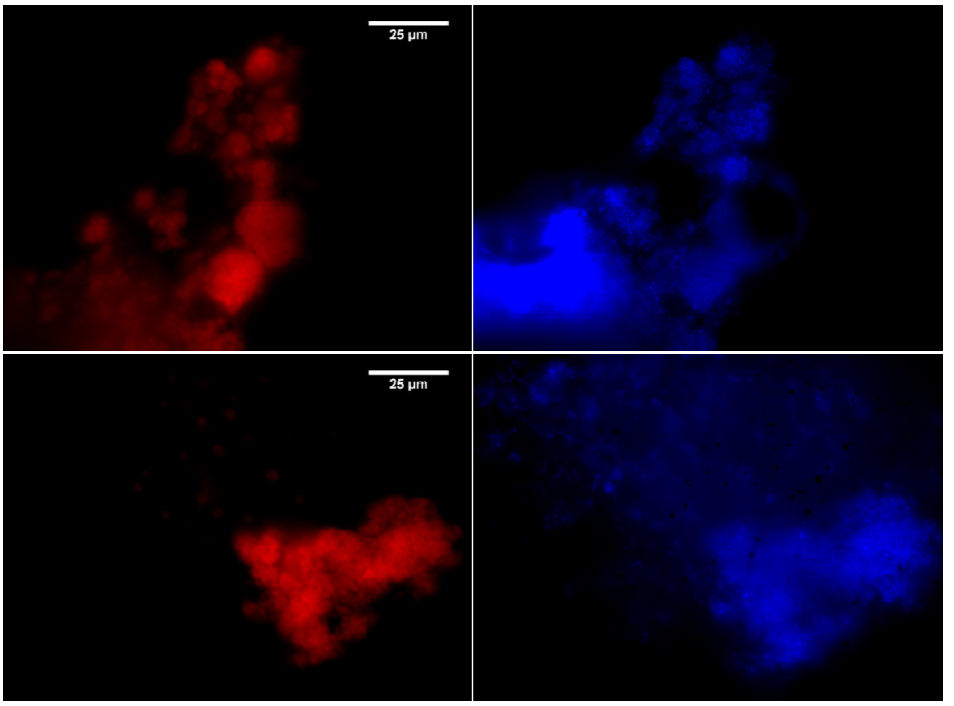

Figure 18. Micrographs of biofilms from R_1 (top) and R_2 (bottom), with FISH-stained anammox bacteria appearing in red (left) and in blue (right). Scale bar 25 µm (Paper VII).

Efficient one and two-stage nitritation-anammox-systems-based processes were started up for the treatment of reject water. Start-up with pre-established biomass has some advantages over the process start-up from zero. Molecular analysis confirmed the presence of different N-converting organisms, whose composition was directed by changes in the technological setup and by the application of the substrate at elevated concentration levels.

6. CONCLUSIONS

Autotrophic nitrogen removal technologies have been developed to save energy on aeration and on addition of organic carbon sources. In this PhD dissertation the technology of anaerobic ammonium oxidizing (anammox) bacteria enrichment and the performance of a moving bed biofilm reactor for the treatment of nitrogen-rich wastewater was studied in detail. Reject water the anaerobic digester effluent of the Tallinn Wastewater Treatment Plant was used as a substrate and also as a source of anammox bacteria. The anammox organisms were enriched onto the biofilm carriers' by selective environmental conditions and substrate loading. Satisfactory autotrophic total nitrogen (TN) removal rates (1 kg N m^{-3} d^{-1}) were achieved for the treatment of digester effluent showing a potential for a full-scale application of the developed technology (Paper I).

Pre-established biofilm on carriers had a slight positive effect on the anammox start-up and, therefore, could be preferentially used for start-up of full scale biofilm anammox in wastewater treatment plants. However, similar TN removal rates were achieved for blank carriers and for carriers with nitrifying biofilm during the start-up, adaptation and stationary phases of the operation of anammox-process-performing reactors. The anammox process started up with carriers having nitrifying biomass on it showed a somewhat better stability against increased nitrite concentrations (Paper II). Rapid overcoming from nitrite inhibition or tolerance to higher nitrite concentrations is important for sustaining a stable TN removal rate in anammox process operated at high substrate loading.

The role of anammox process intermediate compounds on biofilm containing anammox bacteria was assessed (Paper III). We have demonstrated that addition of anammox process intermediate compounds into a flow-through reactor and into activity test solutions accelerated the anammox process after the application of inhibiting amounts of nitrite. The acceleration of post-inhibition recovery of the anammox process by optimum (small) amounts of intermediate metabolites (hydrazine and hydroxylamine) on the anammox process is a matter of great interest as it can be used in case of inhibition in water treatment systems.

After the start-up of two-stage deammonification systems, the nitritation process was developed from the nitrifying process by limiting the activity of nitrite oxidizing bacteria to minimum. A suitable technique for that was application of a combination of low HRT, increased free ammonia and intermittent aeration (Paper IV). When nitritating and anammox bacteria enrichment in separate single moving bed biofilm reactors treating reject water were achieved, a single reactor deammonification process was developed (Papers V and VII). The deammonification process in a single reactor encountered problems when nitrite was further oxidized into nitrate in a form mainly unavailable to anammox organisms. Nitrite oxidation into nitrate was avoided

by means of applying increased free ammonia (FA) (Paper V) concentration and also by increased HCO_3^- (CO_2) concentrations (Paper VI).

The methods developed for the start-up of one and two-step deammonification processes and for anammox bacteria enrichment and inhibition recovery can be applied for a full-scale cost-saving treatment.

7. SUMMARY IN ESTONIAN

Anaeroobset ammoniumi oksüdatsiooniprotsessi läbiviivate bakterite rikastamine anaeroobse kääriti väljavoolu lämmastikurikka vädu käitlemiseks ning anammox protsessi kiirendamine protsessi vaheühenditega

Lämmastikurikaste reovete käitlemine tavapäraste nitrifikatsiooni-denitrifikatsiooni meetoditega on energiamahukas. Antud doktoritöös uuriti alternatiivset viisi lämmastikuärastuse läbiviimiseks autotroofsete bakterikultuuride abil. Dissertatsiooni raames rikastati anaeroobset ammooniumi oksüdatsiooni (anammox) protsessi läbiviivaid baktereid setteveest biokilekandjatele ning lämmastikuärastust teostati liikuvate biokilekandjatega süsteemis. Erinevates biokilesüsteemides (maksimaalne lämmastikuärastuse kiirus 1 kg N m^{-3} d^{-1}) käivitati efektiivne kahe-etapiline ning üheetapiline autotroofne lämmastikuärastuse protsess anaeroobse kääriti separeerimisel allesjääva lämmastikurikka vädu käitlemiseks (Artikkel I).

Katsetes, kus võrreldi erinevate biokilekandjatega s.o. nitrifitseeriva biokilega ja haljastel kandjatel toimuvat lämmastikuärastuse protsessi käivitamist, saavutati sarnased lämmastikuärastuse kiirused start-up'i, protsessi adaptatsiooni ja protsessi statsionaarses faasis. Samas oli nitrifitseeriva biokilega süsteemis käivitatud anammox protsess vastupidavam kõrgemate nitriti kontsentratsioonide suhtes (Artikkel II).

Dissertatsioonis hinnati ka anammox protsessi vaheühendite mõju nitriti poolt inhibeeritud ja inhibeerimata anammox protsessil põhineva biokilesüsteemi kandjate lämmastikuärastuse efektiivsusele (Artikkel III). Lämmastikuärastuse protsessi kiirendamine optimaalsete vaheühendite (hüdrasiini ja hüdroksüülamiini) sisalduste abil on olulise tähtsusega. Antud vaheühendite lisamine võimaldab ületada nitriti poolt pärsitud anammox-süsteemides inhibitsiooni, samal ajal kiirendades lämmastikuärastust.

Pärast kaheetapilise (nitritatsioon ja anammox protsessid eraldi mahutites) anammox protsessi käivitamist liikuvate biokilekandjatega süsteemis saavutati üheetapilistes lämmastikuärastuse süsteemides efektiivne nitritatsiooni (Artikkel IV) ning deammonifikatsiooni protsess (Artikkel V ja VII) kõrgendatud vaba ammoniaagi (FA) (Artikkel V) ning kõrgendatud HCO_3^- (CO_2) kontsentratsiooni tingimustes (Artikkel VI). Antud faktorid inhibeerisid selektiivselt nitriti edasist oksüdeerimist nitraadiks, mille tõttu nitrit oli substraadina anammox protsessi jaoks kättesaadav, tagades protsessis efektiivse lämmastikuärastuse.

Antud meetoditel käivitatud autotroofne lämmastikuärastuse protsess on võimalik senisest energiaefektiivsemal moel tööle seada ka täismahulistel reoveepuhastuse seadmetel lämmastikurikaste reovete käitlemiseks.

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