Investigations on nitrogen transformation processes and stimulation of anaerobic ammonium oxidation activity in an experimental laboratory-scale wetland system

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Erklärung an Eides statt

Hiermit erkläre ich, Diego Paredes Cuervo, dass ich die vorliegende Dissertation selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle (Saale), September 14, 2009 Diego Paredes Cuervo

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1 Introduction

Many technologies, including conventional and natural systems for wastewater treatment are based on the same processes: gravity forces for sedimentation of particles, aerobic and anaerobic biological degradation of organic matter, filtration of suspended solids in river banks, nitrogen uptake by plants, etc. (Crites et al., 2006). All these processes are optimized by the use of energy, chemicals or controlled reactor in the so called conventional technologies. They have been and will be, in many cases, an attractive alternative for wastewater treatment because they provide a compact and an efficient method for controlling or decreasing water pollution. However they have some limitations associated with high commercial energy requirements, high investment and running costs and production, in some cases, of big amounts of byproducts, especially sludge. In the last years near-nature-like systems have become an alternative to conventional systems. In practice near-nature and conventional systems require the same amount of energy for each kilogram of degraded pollutant but the energy source is different. Near-nature-like systems are based on renewable energies that are present in nature: solar radiation, kinetic energy present in the wind, rain water, runoff and ground water, storage of potential energy in biomass and soil, etc. On the other hand, the energy source for conventional systems is coming mainly from fossil fuels. In simple words, near-nature systems are highly intensive in land while conventional systems are highly intensive in commercial energy consumption. A near-nature-like system can require an additional energy source for pumping and piping for waste conveyance but not for maintaining the major treatment responses (Crites et al., 2006).

Near-nature-like systems for wastewater treatment include, among others, land application, stabilization ponds, algal ponds and constructed wetlands (Caicedo, 2005;

Crites et al., 2006; Polprasert, 2007). Land application of wastewater was the first natural technology and became applied in the nineteenth century in different countries (USA and Europe) for waste management (Crites et al., 2006). Stabilization ponds have been used for more than 3000 years and its main aim has been the treatment of domestic wastewater (Caicedo, 2005; Crites et al., 2006). Constructed wetlands are in essence new developments for the treatment of wastewaters and sludge and, in practice, it was originated from research conducted at the Max Planck Institute in West Germany in 1952 (Bastian and Hammer, 1993). Since that different research activities and applications have been done, and from 1985 the implementation of the technology has been accelerating around the world because it is a simple technology with biological complex systems capable of achieving high levels of treatment. In addition, constructed wetlands can be constructed with local materials and local labor, which is a great advantage for developing countries (Kadlec and Wallace, 2009).

Constructed wetlands have been used for the treatment of domestic, animal, industrial and mine wastewater, landfill leachate, polluted groundwater, urban stormwater and field runoff (Kadlec and Wallace, 2009). According with water flow, constructed wetlands are classified in vertical subsurface flow constructed wetlands, horizontal subsurface flow constructed wetlands and free water surface constructed wetlands. In general a lot of reports have been done regarding the removal of organic carbon (expressed ad BOD_5, COD and TOC) (Kadlec et al., 2000; Stottmeister et al., 2003; Langergraber et al., 2006; Kadlec and Wallace, 2009), heavy metals (Kadlec et al., 2000; Stottmeister et al., 2003; Paredes et al., 2007b; Kadlec and Wallace, 2009) and nutrients (Stottmeister et al., 2003; Wießner et al., 2005b; Paredes et al., 2007a; Vymazal, 2007; Kadlec and Wallace, 2009). In this last case, especially in the case of nitrogen, the reported removal rates are contradictory because constructed wetlands

have mainly been applied for domestic wastewater and there are few experiences treating wastewater with high nitrogen loads (Paredes et al., 2007a).

Wastewaters with high nitrogen loads include several industries (fertilizer, fish canning, refinery, slaughterhouses, meat processing, and tannery), agricultural runoff, landfill leachates, dewatering sludge liquor, etc. (Mulder, 2003). Combination of aerobic (nitrification) and anaerobic (denitrification) technologies have been applied as a conventional technology for the removal of the nitrogen in those wastewaters (Henze et al., 2002). Constructed wetlands are also a possible technology but they have some limitations. Intermitted loaded vertical flow systems will produce nitrified effluents but the total nitrogen removal will be low while subsurface horizontal flow constructed wetlands will produce less nitrified effluents but the total nitrogen removal will be higher. In both cases the possible nitrogen removal and transformation processes are nitrification-denitrifition, plant uptake, soil fixation and ammonia volatilization (Kadlec and Wallace, 2009). However the capacity of nitrogen uptake by plant (Brix, 1994; Kadlec et al., 2000) and soil fixation (Kuschk et al., 2003) are limited whereas ammonia volatilization depends on the pH of the wastewater and become important with pH values above 8.5, besides ammonia will be toxic for the plants (Kadlec and Wallace, 2009).

It has been hypothesized that nitrification and denitrification are the main processes for nitrogen transformation and removal in constructed wetlands (Kadlec and Wallace, 2009; Maltais-Landry et al., 2009). If this is true there are two critical points for both processes: the availability of oxygen and organic carbon. In subsurface flow constructed wetlands there are two sources of oxygen: air diffusion and plants. In vertical flow systems the intermittend loading (alternating fill and draw) causes a forced aeration and the air diffusion is higher in comparison with the oxygen input by plants. In subsurface

horizontal flow wetlands the main oxygen contribution is due to the plants. Intermitted vertical flow constructed wetlands (more engineered systems) are more aerated than subsurface horizontal flow constructed wetlands. Even if the ammonium is oxidised to nitrate there will be not enough organic carbon source in the system for denitrification because the oxygen present inside the wetland is primarily used by heterotrophic microbes to remove the organic matter required for denitrification (Richardson and Ferguson, 1992). Plants also brings organic compounds to the system through exudates (Jones et al., 2004) but the amount is limited and it can be important for low loaded ammonium wastewater but it is less important in case of highly loaded ammonium wastewaters.

Some years ago a novel nitrogen transformation process was discovered: the anaerobic oxidation of ammonium – anammox (Mulder et al., 1995). It is an autotrophic process and it does not require any organic carbon source. Nitrite (preferred electron acceptor) and ammonium (electron donor) can be transformed to dinitrogen gas (Jetten et al., 1998). The doubling time of these bacteria has been estimated in 11 days under optimum conditions and they require an anaerobic environment. As a consequence of the very low doubling time, there is a low sludge production and a reactor with high biomass retention is needed (van der Star et al., 2007; Jin et al., 2008b). Some researcher have reported the presence of anammox bacteria in constructed wetlands but their role has not been investigated (Stottmeister et al., 2003; Shipin et al., 2005).

As it was mentioned before, subsurface horizontal flow constructed wetlands are more sensible to the oxygen and organic matter availability for nitrogen removal. Indeed the total nitrogen removal rate is around 0.7 g m^{-2} d^{-1} (Paredes et al., 2007a), which is a relatively low removal rate. Because of their conditions subsurface horizontal flow constructed wetlands could be a potential system for supporting anaerobic oxidation of

ammonium and promoting this process could be an option for increasing the nitrogen removal rates.

1.1 Problem

The rapid population growth, industrial development and the urgent need for food production have generated contamination of receiving water bodies in many places around the world. High investments in wastewater treatments plants during the last years have greatly reduced the organic loading disposed in water bodies, however many of the existing treatment plants are not able to remove the nutrients like nitrogen.

There are several wastewaters containing nitrogen in high concentrations and stricter regulations for nitrogen removal have been introduced in the last years, especially for discharge of into natural water bodies. The conventional technologies used for its removal in most cases are very expensive and they are not affordable for most of the countries with low incomes. Therefore, there is an urgent need to develop and to improve low cost technologies for wastewater treatment that are within the economic and technological capabilities of both developed and developing countries. At the same time these technologies should be reliable and effective in removing a wide range of pollutants.

The use of subsurface horizontal flow constructed wetlands for nitrogen removal is an option that could be used for nitrogen removal but questions such as nitrogen transformation and removal process, the role of anammox bacteria and how to improve nitrogen removal rates are still open.

1.2 Objectives

The objective of this work was to improve the basic knowledge about nitrogen transformation processes in constructed wetlands. For that experiments for promoting the nitrogen removal in laboratory-scale subsurface horizontal flow constructed wetlands have been done by stimulating the anaerobic oxidation of ammonium – anammox. In more detail, the research was focused on the following aspects:

- Presence and activity of anammox bacteria in soils of constructed wetlands and sludges from different wastewater treatment systems.
- Nitrogen removal/transformation processes in experimental laboratory-scale subsurface constructed wetlands.
- Effects on nitrogen removal/transformation processes in experimental laboratory-scale constructed wetlands after inoculation with active anammox biomass.
- Evaluation of partial nitrification in vertical down flow columns as a preliminary step of subsurface horizontal flow wetlands for promoting anammox processes

2 Literature review

2.1 Impacts of nitrogen on the environment and importance of nitrogen removal on wastewater treatment

One of the most affected and altered biogeochemical cycle for human activity is the nitrogen cycle. Nitrogen is present on earth mainly as molecular N_2 in the atmosphere and dissolved in the world's oceans. It becomes reactive and biologically available to plants and algae only by bacterial nitrogen fixation, fixation by lightning and volcanic activity, and fixation from industrial activities including the manufacturing of fertilizers and combustion of fossil fuel. In the last century the world's annual industrial output of nitrogenous fertiliser increased from 10 Mt N in 1960 to about 90 Mt N in 1998 (Mulder, 2003). The global estimate for biological nitrogen fixation is in the range of 200-240 Mt N, which shows that the anthropogenic nitrogen mass flow have a major impact on the global nitrogen cycle (Gijzen and Mulder, 2001). All this fertilizer is required mainly for protein production, and the consumption of protein will ultimately result in the discharge of the protein nitrogen in wastewater. In European countries approximately 18% of fertiliser nitrogen ends up in wastewater in the form of ammonium or organic nitrogen (Mulder, 2003).

Nitrogen compounds are among the principal constituents of concern in wastewater because of their role in eutrophication, their effect on the oxygen content of receiving waters, and their toxicity to aquatic invertebrate and vertebrate species, including human beings. Simultaneously, these compounds are also of interest because of the beneficial role that they can play in augmenting plant growth which in turn stimulates the production of wildlife (Kadlec and Knight, 1996). The most predominant and

important nitrogen species include inorganic and organic compounds. Inorganic compounds cover ammonium, nitrite, nitrate and some gaseous and atmospheric forms like dinitrogen gas, nitrous oxide, nitric oxide and ammonia (Kadlec and Wallace, 2009).

There are several human activities that produce wastewater containing nitrogen with different concentrations. Mulder (2003) suggested a simple classification depending of the nitrogen range. If the total ammonium nitrogen (TAN) is lower than 100 mg N l^{-1}, the wastewater is diluted, but if the TAN is higher than 100 mg N l^{-1}, the wastewater is concentrated. Domestic wastewater is a diluted wastewater while landfill leachates, wastewater from slaughterhouses and tanneries, reject water and piggery manure are typical examples of concentrated wastewater. Biological processes are the main and cheapest alternative for nitrogen removal for both types of wastewater (Siegrist, 1996; Janus and Van der Roest, 1997; Kuai and Verstraete, 1998; van Dongen et al., 2001a; van Dongen et al., 2001b; Hao et al., 2002a, 2002b), however, with TAN concentrations higher than 5000 mg N l^{-1}, physicochemical methods are technically and economically feasible (Mulder, 2003). As other technologies for pollutant removal, biological processes for nitrogen removal are based in natural cycles and microbial activities.

2.2 Microbial catalyzed nitrogen cycle – general aspects.

There are very complex interrelations between different nitrogen species (like ammonium, nitrite and nitrate among others) and different transformation mechanisms take place (Figure 2-1). Organic nitrogen is made up of a variety of compounds including amino acids, amino sugars, urea and uric acid and purines and pyrimidines

(Kadlec and Knight, 1996; Kadlec and Wallace, 2009). By hydrolysis and mineralization, organic nitrogen is converted to ammonium nitrogen. Ammonium is one of the most important nitrogen compounds in surface waters and other ecosystems for three reasons: (1) It is the preferred nutrient form of nitrogen for most plants species and for autotrophic bacteria; (2) it is chemically reduced and therefore can be readily oxidized in natural water, resulting in the consumption and decreasing of dissolved oxygen; and (3) un-ionized ammonia is toxic to many forms of aquatic life already at low concentrations (> 0.2 mg/l) (Kadlec and Knight, 1996; Kadlec and Wallace, 2009).

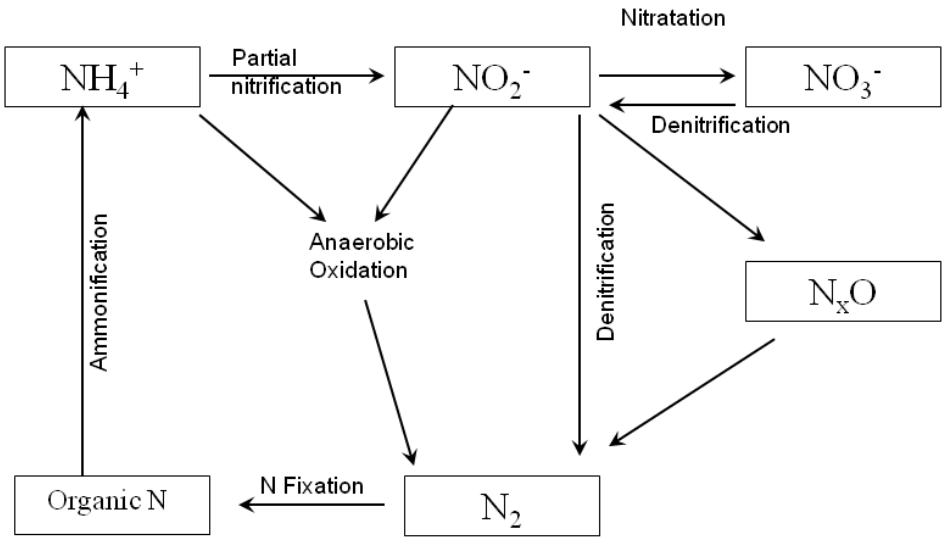

Figure 2-1 Main transformation processes of the nitrogen cycle

The conversion between volatile ammonia (NH3) and ammonium ion (NH4+) strongly depends on pH (Anthonisen et al., 1976). At lower pH and lower temperature values, the conversion decreases significantly. For a normal condition of 25 °C and a pH of 7, un-ionized ammonia is only 0.6% of the total ammonia present. At a pH of 9.5 and a temperature of 30 °C, the percentage of total ammonia present in the un-ionized form increases to 72%.

Under aerobic conditions, ammonium is oxidized by microorganisms to nitrate, with nitrite as an intermediate product. Two different groups of bacteria play a role in the nitrification step: ammonium oxidizers and nitrite oxidizers. In the oxidation of ammonia, nitrite, as an intermediate product, is formed. It has been considered that rarely it can be accumulated in terrestrial and aquatic environments. However, some reports indicate that nitrite can be accumulated in ecosystems. Soil nitrite accumulation was reported for situations where high concentrations of urea, either by chemical fertilizers or by urine, were loaded to the soil (Bremner, 1995). Accumulation of nitrite was also observed in pore waters of some estuarine sediments as well as in some treatment plants at lab-scale (Helder and de Vries, 1983; Hanaki et al., 1990), and it was attributed to a lower affinity for oxygen of nitrite oxidizers than of ammonium oxidizers (Laanbroek and Gerards, 1993). Although on the oxidation level of nitrite denitrification step by electron donors like organic carbon, ammonia, H_2S, etc. (see below) is possible; in treatment plants usually a total oxidation to nitrate is realized. With organic carbon denitrifiers reduce nitrate via nitrite to dinitrogen gas. In treatment plants nitrate is reduced by the organic carbon load of a portion on “untreated” wastewater. Recently, a still unknown pathway was discovered by Mulder et al., (1995): anammox bacteria can use nitrite as an electron acceptor and anaerobically convert ammonium and nitrite to nitrogen gas. In contrast with the traditional nitrification-denitrification route, anammox is an autotrophic process. The microorganisms use bicarbonate as carbon source.

2.3 Selected pathways of the N cycle in relation to new wastewater treatment technologies.

A general overview of the different nitrogen pathways and biochemical conversions for traditional and new processes is presented by simplified equations in Table 2-1 and a description of them is presented below.

Table 2-1 Main nitrogen transformation processes on nitrogen cycle

No.	Process	Biochemical conversion	Reference
1	Nitritification	$NH_4^+ + 1.5\ O_2 + 2\ HCO_3^- \rightarrow NO_2^- + 2\ CO_2 + 3\ H_2O$	(Henze et al., 2002)
2	Nitratation	$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$	(Henze et al., 2002)
1+2	Nitrification	$NH_4^+ + 2\ O_2 + 2\ HCO_3^- \rightarrow NO_3^- + 2\ CO_2 + 3\ H_2O$	(Henze et al., 2002)
3	Denitratation	$2\ NO_3^- + C \rightarrow 2\ NO_2^- + CO_2$	(Henze et al., 2002)
4	Denitrification via nitrite (Denitritification)	$4\ NO_2^- + 3\ C + 2\ H_20 + CO_2 \rightarrow 2\ N_2 + 4\ HCO_3^-$	(Henze et al., 2002)
3+4	Denitrification	$4\ NO_3^- + 5\ C + 2\ H_20 \rightarrow 2\ N_2 + 4\ HCO_3^- + CO_2$	(Henze et al., 2002)
5	Partial nitrification (50% conversion)	$NH_4^+ + 0.75\ O_2 + HCO_3^- \rightarrow 0.5\ NO_2^- + 0.5\ NH_4^+ + CO_2 + 1.5\ H_2O$	(van Dongen et al., 2001b)
6a	Anammox (without cell synthesis)	$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	(van Dongen et al., 2001b)
6b	Anammox (with cell synthesis)	$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.66\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$	(van Dongen et al., 2001b)
1+2+ 3+4	Traditional nitrification denitrification	$4NH_4^+ + 8\ O_2 + 5\ C + 4\ HCO_3^- \rightarrow 2\ N_2 + 9\ CO_2 + 10\ H_2O$	(van Dongen et al., 2001b)
1+6	CANON*	$NH_3 + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$	(Sliekers et al., 2003)
7	OLAND	$NH_4^+ + 0.75\ O_2 \rightarrow 0.5\ N_2 + H^+ + 1.5\ H_2O$	(Verstraete and Philips, 1998)

* Reported as it is cited, however the equation is unbalanced.

2.3.1 Partial nitrification.

Research results in microbial ecology have triggered the development of new nitrogen removal technologies for wastewaters with high ammonium load. Compared with the conventional nitrogen removal process for wastewater, which involves two steps (nitrification and denitrification), the advantages of a technology based on partial nitrification, include a lower oxygen requirement in nitrification (25% less), and lower or none organic carbon consumption if denitrification or anaerobic oxidation of ammonia is the second step (van Dongen et al., 2001a; van Dongen et al., 2001b; Shen et al., 2003; Bernet et al., 2005).

Ammonium is converted to nitrite under aerobic conditions by ammonium-oxidising bacteria. It is an acidifying process that can be partially neutralised by bicarbonate present in the wastewater (Table 2-1, reaction 1). The oxidation of ammonium to nitrite requires 2 mole of bicarbonate for every mole of ammonium. This is essential for the nitrification, because a low content of alkalinity can cause a reduction of the pH and the reaction stops completely. At pH lower than 6.5 nitritification does not take place (van Dongen et al., 2001b). For ammonium oxidisers, NH_3 is the actual substrate rather than NH_4^+, and HNO_2 - no nitrite - is the inhibiting component.

The equilibrium between HNO_2 and NO_2^- is strongly dependent on pH (Anthonisen et al., 1976). Concentrations higher than 0.2 mg HNO_2 l^{-1} inhibit the total nitrification process (Anthonisen et al., 1976). If there is enough oxygen and the environmental conditions are adequate, nitrite oxidising bacteria can develop the second step of nitrification (Table 2-1, reaction 2).

A number of environmental factors influencing the nitrification and some of them, or a combination of them, are the base for the development of partial nitrification

technologies. In practice all of them depend on the inhibition or limitation of the second step of nitrification or nitrate formation.

2.3.1.1 Strategies for controlling partial nitrification

The key step to realize anammox in technical scale is to get a nitrification reactor with a stable nitrite formation. For this, different strategies and approaches have been used (Bernet et al., 2005), including control of temperature, hydraulic retention time, pH control and load of free ammonia (Table 2-2), and control of dissolved oxygen in the reactor.

As shown in the Table 2-2, temperature has different effects on the growth rate of ammonium and nitrite oxidisers. Only at temperatures above 25 °C it is possible that ammonium oxidisers can effectively out-compete the nitrite oxidisers (Brouwer et al., 1996; van Dongen et al., 2001b). If this condition is impaired with a low hydraulic retention time and, also a low cellular retention time, nitrite oxidisers can be selectively washed out (Hellinga et al., 1998). This selective out-competing of nitrite oxidisers is the main concept of the SHARON process (Single reactor High activity Ammonia Removal over Nitrite) (van Dongen et al., 2001b). The SHARON process works at temperatures higher than 26 °C, without biomass retention. It means that the hydraulic retention time is equal to the sludge retention time. The effluent concentration depends only on the growth rate of the bacteria involved and it is independent on the influent concentration (van Dongen et al., 2001b). With high temperatures and no sludge retention, the dilution rate is set in such way that ammonium oxidisers can grow fast enough to stay in the reactor, while the nitrite oxidisers are washed out. The pH has a

strong influence in the system because in the low pH range nitrite oxidisers grow faster than ammonium oxidisers. In this way, the margins in the required hydraulic retention time or dilution rate to maintain ammonium oxidisers and washout the nitrite are much larger at higher pH (Hellinga et al., 1998).

Table 2-2 Effect of pH, temperature, free ammonia and nitrous acid on nitrification process

Factor	Effect	Reference
Temperature T > 15 °C	Ammonium oxidisers grow faster than nitrite oxidisers	(Brouwer et al., 1996; van Dongen et al., 2001b)
T = 25 °C	Ammonium oxidisers can out-compete nitrite oxidiser	(Brouwer et al., 1996; van Dongen et al., 2001b)
pH		
7.0 – 8.0	Optimum range for nitrification	(Jones and Paskins, 1982; Painter and Loveless, 1983; Antoniou et al., 1990)
7.9 – 8.2	Optimum range for ammonium oxidisers (*Nitrosomas*)	(Alleman, 1984)
7.2 – 7.6	Optimum range for nitrite oxidisers (*Nitrobacter*)	(Alleman, 1984)
Free NH_3 (mg l^{-1})		
150	Inhibition of ammonium and nitrite oxidisers	(Anthonisen et al., 1976)
74	Recovery of partial nitrification activity if the activity has been affected	(Xue et al., 2009)
1.0 – 7.0	Inhibition of ammonium oxidisers and nitrite accumulation	(Anthonisen et al., 1976; Abeling and Seyfried, 1992; Fernandez-Polanco et al., 1994; Surmacz-Gòrska et al., 1997; Kim et al., 2003)
Long term	Nitrite oxidisers (pure cultures of *Nitrobacter* and mixed cultures in biofilms) can be adapted to high free ammonia concentration (40 mg l^{-1}) and nitrite accumulation is reduced.	(Wong-Chong and Loehr, 1978; Villaverde et al., 2000; Fux et al., 2004a)
HNO_2 (mg l^{-1})		
> 2.8	Inhibition of ammonium and nitrite oxidisers	(Anthonisen et al., 1976)

Regarding the optimum pH ranges for nitrification (Table 2-2), three different effects that the pH has on nitrifying bacteria have been identified (Villaverde et al., 1997): Activation-deactivation of nitrifying bacteria; nutritional effects, associated with the alkalinity and the species of inorganic carbon, and inhibition through free ammonia and free nitrous acid. Activation-deactivation of nitrifying bacteria is linked to the binding of H^+ or OH^- ions to the weak basic groups of the enzymes, blocking in a reversible way the active sites (Quinlan, 1984). Nutritional effects are associated mainly to the availability of the mineral carbon, which is required as a carbon source for nitrifying autotrophic microorganisms. At low pH, the predominant species CO_2 can be easily removed from water by stripping. On the other hand, with high pH values mineral carbon will mainly be present in the carbonate species, which can barely be assimilated.

At higher pH values, free ammonia increases. In an opposite way, nitrous acid concentration arises at low pH. Both, free ammonia and nitrous acid can inhibit either ammonium or nitrite oxidisers, but nitrite oxidisers are more sensitive than ammonia oxidisers to free ammonia (Table 2-2). However the threshold concentration of free ammonia at which the nitrite oxidation is inhibited increased by time. Wong-Chong and Loehr (1978) observed that pure cultures of *Nitrobacter* acclimated to free ammonia could tolerate concentrations as high as 40 mg l^{-1} NH_3-N, while unadapted ones were inhibited already at concentrations of 3.5 mg l^{-1} NH_3-N. Additional works on biofilms and suspended systems showed that nitrite oxidisers can be adapted to higher free ammonia concentration and, after long time (6 – 12 months) the nitrite accumulation decreases and the nitrate concentration increases (Villaverde et al., 2000; Fux et al., 2004a).

Alkalinity is also an important factor for nitrification. Depending on the alkalinity of the wastewater, the SHARON reactor (see above) can convert a fraction or even the whole load of ammonium into nitrite. If the objective is to use a subsequent anammox process for nitrogen removal, a molar ratio of 1:1 betweeen NH_4^+ and NO_2^- is the optimum. This can be easily achieved controlling the alkalinity in the wastewater. Because the oxidation of 1 mole of ammonia to nitrite consumes 2 moles of bicarbonate, and the reaction stops when the pH is lower than 6.5, a molar ratio 1:1 between ammonium and bicarbonate produces approximately a conversion of 50% of ammonium to nitrite and the rest remains as ammonium. But if a denitrification by nitrite route is used, either a full ammonium oxidation to nitrite is necessary, or an intermittent anaerobic step by the suspension of the aeration and an addition of some carbon source into the reactor is required. In the first case, enough alkalinity is crucial and a second reactor for anammox process is indispensable. In the second case the same SHARON reactor can be used for partial nitrification and denitrification. This second option has the advantage that there are not high requirements of alkalinity because the denitrification produces bicarbonate (Brouwer et al., 1996; Hellinga et al., 1998; van Dongen et al., 2001a; van Dongen et al., 2001b).

Variation of the oxygen concentration in the reactor is also a possibility for enhancing nitrite accumulation. It is based in the differences between the oxygen saturation coefficients of Monod kinetics for ammonium oxidation and nitrite oxidation that are known to be 0.3 and 1.1 mg l^{-1}, respectively (Wiesmann, 1994).

A possible mechanism for inhibition of nitrite oxidation by low oxygen concentration is based in the accumulation of hydroxylamine, which is an intermediate product of the ammonium oxidation. In general, obligately ammonium oxidising bacteria gain their energy by oxidizing NH_4^+ to NO_2^- in a two-step reaction with hydroxylamine (NH_2OH)

as an intermediate. The first step is the oxidation of NH_4^+, catalyzed by ammonia monooxygenase, whereas the second step is the oxidation of hydroxylamine, catalyzed by hydroxylamine oxidoreductase. Under low oxygen concentration and high ammonium concentration hydroxylamine can be accumulated. Hydroxylamine causes inhibition of nitrite oxidisers at values around 250 μM, and more than 2000 μM even inhibit ammonium oxidisers. However, concerning aspects of wastewater treatment, hydroxylamine is typically ignored in nitrification due to an implicit assumption that it will not occur in significant levels (Yang and Alleman, 1992).

Various investigations into controlling partial nitrification through dissolved oxygen have been carried out, including suspended and biofilm reactor systems (Table 2-3). For suspended growth biomass systems under limited oxygen supply, complete and stable conversion of ammonium into nitrite was obtained, independent of the sludge age.

However, when there was no oxygen limitation, the sludge age became the critical parameter for partial nitrification. Yang and Allemann (1992), working with an enriched nitrifying culture under batch conditions, concluded that a combination between free ammonia inhibition, low oxygen content and hydroxylamine accumulation were the main factors of nitrite build-up.

Using two sequential batch reactors working with different dissolved oxygen, Guo et al. (2009), found out that at low DO (0.4 - 0.8 mg l^{-1}) simultaneous partial nitrification and denitrification took place, and at high DO levels (above 3.0 mg l^{-1}), stable partial nitrification was obtained. In both cases a nitrite to nitrate accumulation ratio of above 95% was achieved.

In the case of biofilm systems (Table 2-3), the results were similar to those obtained with suspended biomass, showing that low dissolved oxygen concentration led to nitrite accumulation, but the suggested mechanisms are different.

Table 2-3 Effects of dissolved oxygen concentration (DO) on nitrification processes

System	DO (mg l^{-1})	Effect	Reference
Suspended growth	0.5	Inhibition of nitrite oxidation and its accumulation	(Hanaki et al., 1990)
	6.0	Full nitrification	(Hanaki et al., 1990)
SBR	>3.0 0.4 – 0.8 4.2 - 4.8	Stable partial nitrification Stable partial nitrification and simultaneous denitrification Maximum partial nitrification with immobilized biomass beads	(Guo et al., 2009) (Yan and Hu, 2009)
Activated sludge	< 0.5	Nitrite and ammonium accumulation	(Ruiz et al., 2003; Ciudad et al., 2005)
	0.7	Nitrite accumulation up to 67% of the applied NH_4^+	(Ruiz et al., 2003; (Ciudad et al., 2005)
	1.0	80% oxidation of NH_4^+, 80% as NO_2^-	(Ciudad et al., 2005)
	1.4	99% oxidation of NH_4^+, 70% as NO_2^-	(Ciudad et al., 2005)
	> 1.7	Full nitrification	(Ruiz et al., 2003)
	2.4	99% oxidation of the applied NH_4^+, 10% as NO_2^-	(Ciudad et al., 2005)
Biofilm airlift reactor	1.0	Stable and 100% nitrite accumulation	(Kim et al., 2003)
	< 1.0	Low ammonium conversion and low NO_2^- and NO_3^- accumulation	(Garrido et al., 1997)
	1.5	50% of ammonium conversion to nitrite	(Garrido et al., 1997)
	> 2.5	Full nitrification. NH_4^+ oxidation depended on applied ammonium load	(Garrido et al., 1997)
Biological aerated filter	2.0 – 5.0	Nitrite accumulation up to 60% of total ammonia conversion	(Joo et al., 2000)
Completely stirred biofilm reactor	0.5	Stable nitrite accumulation (90%) and 100% ammonium removal	(Bernet et al., 2001)
	> 0.5	Increasing dissolved oxygen increases nitrate concentration in the effluent; a further reduction produces nitrite accumulation again	(Bernet et al., 2001)
Moving bed biofilm reactor	3.0	50% of ammonium conversion to nitrite; after 11 months full nitrification took place.	(Fux et al., 2004a)

Tanaka et al. (1981) found out that with an ammonium to oxygen ratio lower than 3.4 (theoretical requirement), oxygen was the limiting factor and nitrite was accumulated. Joo et al. (2000) concluded that the dissolved oxygen within the biofilm, which is generated by the diffusion resistance, selectively limits nitrite oxidisers due to difference in the oxygen saturation constants between ammonium oxidisers and nitrite oxidisers. Bernet et al. (2001), with a completely stirred biofilm reactor, concluded that nitrite oxidizers were always present in the reactor but were outcompeted at low dissolved oxygen concentration.

With a biofilm airlift reactor, Kim et al. (2003) observed stable nitrite accumulation (95%) for more than 200 days by selective inhibition by free ammonia (0.2 mg N l^{-1}) and oxygen limitation (1-2 mg l^{-1}), and, a stable 100% nitrite accumulation was also obtained with only low dissolved oxygen concentration. However, nitrite oxidation activity was recovered as soon as the free ammonia concentration was below the threshold level when dissolved oxygen concentration was not the limiting factor. Further Fluorescence In Situ Hybridization (FISH) analysis showed that densely packed ammonium oxidizers were located outside the biofilm. In contrast, small clusters of nitrite oxidizers were surrounded by ammonium oxidizers and found in the deeper layer of the biofilm. The spatial distributions showed that nitrite oxidizers are more exposed to oxygen limiting conditions than ammonia oxidizers (Kim et al., 2003).

In view of the fact that ammonia oxidizing bacteria (AOB) can subsist with aerobic heterotrophic bacteria, partial nitrification could take place in combination with degradation of organic carbons (Yamamoto et al., 2008), hence the combination of partial nitrification and anammox or denitrification processes could be options for the treatment of wastewater with high organic carbon and ammonium concentration (Yan and Hu, 2009). Immobilization has also been proposed as an efficient method to prevent

the wash out of the biomass and to allow hyperconcentrated cultures, leading to relatively small reactors, and, besides, to provide some protection from adverse temperatures and toxic shocks (Morita et al., 2007). In this way, the use of sodium alginate has been probed for the immobilization of nitrifying biomass with optimal partial nitrification at pH values of 8.0 and slight influence of pH (from 6.0 to 8.5). In the range of 15 ºC to 35 ºC partial nitrification increased compared with free suspended cells systems. Maximum partial nitrification and COD removal was obtained in the DO concentration of 4.2 – 4.6 mg l^{-1} (Morita et al., 2007).

2.3.1.2 Effects of salts on partial nitrification.

High salinity concentrations have negative effects on organic matter, nitrogen and phosphorus removal in the wastewater treatment plants. Both Na^+ and Cl^- are considered inhibitory, even to carbon removal (COD) for activated sludge processes (Abu-ghararah and Sherrard, 1993). The effect of salinity on nitrogen removal, however, is not clear at all, mainly because different experimental conditions were used in the studies, including temperature, pH, presence of inhibitory compounds or factors, the way of salt introduction into the system (pulse or gradual increase), long and short term surveys, reactor configuration and the use of pure or mixed cultures. Table 2-4 shows main effects that salinity has over different used technology for nitrification.

Moussa et al. (2006), opposing the findings of Vredenbregt et al. (1997), concluded that, comparing to nitrite oxidisers, ammonia oxidisers were more sensitive to short and long term salt stress and therefore could be the cause for the inhibition of nitrification processes.

In saline environments the adaptation of microbial cenosis to high salinity values has a significant effect on their species diversity. Using oligonucleotide probes, in the case of ammonium oxidiser bacteria, *Nitrosomonas europaea*, was detected at higher levels in saline environments in comparison with the levels in freshwater systems (Tal et al., 2003; Moussa et al., 2006). High salt concentrations inhibited activity of higher organisms such as protozoa strongly, and the settling characteristics of sludge improves with higher salt concentrations (Moussa et al., 2006).

Table 2-4 Effects of salt on nitrification processes

System	Salinity	Effect	Reference
Activated sludge	10% sea water	No effect on nitrogen removal	(Abu-ghararah and Sherrard, 1993)
	70 g NaCl l^{-1}	55% inhibition on nitrification. Recovery when the salt concentration decreased.	(Panswad and Anan, 1999)
	70 g NaCl l^{-1}	30% inhibition on nitrification with salt adapted sludge.	(Panswad and Anan, 1999)
Sequencing batch reactor	78 g NaCl l^{-1}	No inhibition for adapted sludge.	(Dahl et al., 1997)
	40 g Cl^- l^{-1}	Inhibition of ammonium and nitrite oxidisers	(Moussa et al., 2006.)
Salt adapted sludge in fluidized bed	33 g NaCl l^{-1}	Stable nitrification	(Vredenbregt et al., 1997)
	56 g NaCl l^{-1}	Stable ammonium oxidation and nitrite accumulation if a carrier material is used	(Vredenbregt et al., 1997)
Salt adapted nitrifying activated sludge	13.7 g NaCl l^{-1} 19.9 g $NaNO_3$ l^{-1} 8.3 g Na_2SO_4 l^{-1}	100% full nitrification with applied loads between 1 and 4 g NH_4-N l^{-1} d^{-1} Higher salt concentration caused ammonium (10%) and nitrite (20%) accumulation	(Campos et al., 2002)
SHARON reactor (35°C)	12 g NaCl l^{-1}	Nitrite accumulation increased 30% with salt addition.	(Mosquera-Corral et al., 2005)
	< 50 g NaCl l^{-1}	Stable partial nitrification	(Mosquera-Corral et al., 2005)

2.3.2 Anaerobic oxidation of ammonium.

After some inexplicable high nitrogen removal rates in nitrification-denitrification units with low oxygen and organic carbon concentrations, the first experimental confirmation of anaerobic ammonia oxidation was described (Mulder et al., 1995). The microbial nature of the process was verified and nitrite was shown to be the preferred electron acceptor. Hydroxylamine and hydrazine were identified as important intermediates (Jetten et al., 1999). Experiments showed a very low growth rate (doubling time of more then 11 days) (Strous et al., 1998; Strous et al., 1999b). Therefore, reactors to be used for this novel treatment should have very efficient biomass retention and a long start up period is required. Strouss et al. (1998, 1999b) with a sequencing batch reactor (SBR) could cultivate enough biomass to determine their main physiological parameters. These lithotrophs were identified as the new autotrophic member of the order Planctomycete (Strous et al., 1999a). Anaerobic oxidation of ammonium is carried out by the planctomycetes Candidatus "*Brocadia anammoxidans*", Candidatus "*Kuenenia stuttgartiensis*" and several species of the genus Candidatus "*Scalindua*" (Schmid et al., 2003) and recently, newly discovered mixotrophic anammox bacteria Candidatus *Anammoxglobus propionicus* and *Anammoxglobus* sulphate were also described (Kartal et al., 2007; Liu et al., 2008). Because it is autotrophic, a complete conversion of ammonium to nitrogen gas can take place without the addition of organic matter (van Dongen et al., 2001a; Jetten et al., 2002). Anammox bacteria did not consume ammonia and nitrite in a ratio 1:1 as it could be expected from their catabolism, but in a ratio of 1:1.3 (Table 2-1, reactions 6a and 6b). The excess 0.3 mol of nitrite is oxidized anaerobically to nitrate (Van de Graaf et al., 1996).

These bacteria are very sensitive to oxygen and nitrite. Indeed, oxygen concentration higher than 0.06 mg l^{-1}, nitrite concentrations between 230 mg l^{-1} and 920 mg l^{-1}, and

phosphate concentrations higher than 180 mg l^{-1} inhibit the anammox activity completely but reversibly (Jetten et al., 1999; Jetten et al., 2001). Anammox bacteria are also very sensitive to the presence of some organic carbon sources. For instance, anammox bacteria are very susceptible to alcohols, especially methanol. Even low methanol concentration as 40 mg l^{-1} led to the immediate, complete and irreversible inhibition of anammox process. This aspect should be taken in account because methanol is often used to remove nitrate in postdenitrification or to compensate the reduction of pH in partial nitrification reactors. The formation of formaldehyde by the Anammox enzyme hydroxylamine oxidoreductase was identified as the possible cause for the methanol inhibition. On the other hand, organic acids were converted. Propionate and potentially acetate were shown to be substrates for anammox bacteria. Propionate was oxidized by Anammox bacteria with nitrate and/or nitrite as the electron acceptor, and simultaneously, anaerobic oxidation of ammonium took place. In an anammox enrichment culture fed with propionate for 150 days, the amount of Anammox cells and denitrifiers did not change significantly over time, suggesting that anammox bacteria could compete successfully with heterotrophic denitrifiers for propionate (Güven et al., 2005; Kartal et al., 2007).

Using a UASB reactor with both anammox and anaerobic granular sludges, the activity of anammox process in presence of denitrification process has also been evaluated. The COD concentration was found to be a control variable for processes selection between anammox reaction and denitrification. A gradual reduction of the anammox activity was found with an increase of COD concentration in the range of 100 – 400 mg l^{-1}. At COD concentrations above 237 mg l^{-1} anammox communities were inactivated or eradicated (Chanchoi et al., 2008; Molinuevo et al., 2009) and denitrification became the dominant ammonium removal process.

Anaerobic ammonium oxidation is characterized not only by its slow growth but also by its interaction with other bacteria. Anaerobic ammonium oxidizers depend on a nearby nitrite source. In this context, there are two possibilities for getting high removal rates through Anammox process: two reactors in series, with a partial nitrification reactor as a first step, and a separate unit for the anaerobic oxidation of ammonia as a second step. With this configuration, the two biological processes can be controlled separately (Van Dongen et al., 2001b; Fux and Siegrist, 2004). The second option is to use biofilm systems where classical nitritification is developed by ammonium oxidizers in the outer aerobic layers, and, anaerobic oxidation takes place in the deeper zones of the biofilm. Consequently, the oxygen concentration is a key control parameter for such an application (Helmer-Madhoc et al., 2002; Egli et al., 2003). This process was observed in different parts and termed in different ways: "Aerobic/Anoxic Deammonification" at the University of Hanover (Germany), "Oland" at Ghent University (Belgium) and "Canon" at Delft University (The Netherlands) (Fux and Siegrist, 2004). A variation of the Anammox process was tested recently at lab-scale and was called DEAMOX (DEnitrifying AMmonium OXidation). This process is based on the combination of anammox reaction with autotrophic denitrifying conditions using sulphide as an electron donor for the production of nitrite from nitrate within an anaerobic biofilm (Kalyuzhnyi et al., 2006). The simultaneous ammonium and sulphate removal has been described in an anammox reactor and consisted of ammonium oxidization with sulphate deoxidization: when $(NH_4)_2SO_4$ was used as the sole main substrate, the ammonium and sulphur were successfully removed in the anaerobic reactor, in via of nitrite with solid sulphur and nitrogen gas as the terminal products (Liu et al., 2008).

Anammox bacteria are also present in marine sediments and in marine anoxic water columns (Thamdrup and Dalsgraad, 2002). Local environmental factors have an effect

on the main anammox bacteria characteristics; for example, it was found out that for permanently cold sediments, with a temperature lower than -1 °C, the temperature optimum for anammox was 12 °C and not 37 °C which is the optimum found for the wastewater treatment organisms (Jetten et al., 2001; Rysgaard et al., 2004). In contrast to the anammox processes in wastewater treatment, marine anammox microorganisms depend on other process to get the required nitrite. In marine environments nitrate is much more abundant than nitrite, and anammox process needs an additional reducing step of NO_3^- to NO_2^-. It has been found that in marine sediments, the dissolved oxygen decreases in direction of the deeper layers and in this way, high nitrate concentration can be found in the upper layers, but in deeper layers nitrate reducers produce an accumulation of nitrite and anaerobic ammonium oxidation can take place (Dalsgaard et al., 2005). Because of the low growth rate, it was believed that anammox microorganisms could not contribute significantly to nitrogen cycle in marine environments (Zehr and Ward, 2002). Further surveys showed that anammox process can play a role. So in the deep sediments of Skagerrak (Denmark), at 700 m below the sea level, it has been found the maximum relative importance of anammox in N_2 production, with a value between 67-79% of the total N_2 production, and lower values were found at 380 m and 16 m depth (24% and 2%, respectively). In some cases anammox activity has not been detected (Thamdrup and Dalsgraad, 2002; Engström et al., 2005). Although there is not a perfect relationship and other environmental variables can play a role, it seems to be that the relative significance of anammox in N_2 production is higher at greater depths (Dalsgaard et al., 2005). Also anammox activity and its importance depend on the environment stability. If anammox bacteria grow as slow as it is believed, the process will be significant in nature only in stable environments where there was enough time for bacterial growth and development. In

environments with no biomass retention, because their faster growth rate, denitrifiers will outcompete the anammox bacteria (Dalsgaard et al., 2005).

Besides the finding in marine environments, anammox bacteria have been detected in fresh water sediments (Van Lujin et al., 1998). In theory, the oxic/anoxic layer of sediments is an ideal environment for anaerobic ammonium oxidation, however the associated factors are still poorly understood. In the case of marine environments, water depth seems to be one of the most important factors that triggered the process, however aspects such as sediment mineralization, NO_3^- concentration, microphytobentos activity, seeding of anammox bacteria or local gradients in organic content should be considered as controlling factors for the process in freshwater and marine ecosystems (Jetten et al., 1999; Dalsgaard et al., 2005).

2.4 Consequences of new understandings in nitrogen removal

Depending on the characteristics of the wastewater and economical aspects, different technologies can be used for nitrogen removal, including conventional biological nitrogen removal systems, autotrophic nitrogen removal systems and natural systems. Figure 2-1 shows the flow diagram of most important pathways for nitrogen transformation, including the traditional nitrogen removal process and the novel technologies.

2.4.1 Traditional nitrification/denitrification systems

This technology is the most developed and applied technology for nitrogen removal worldwide. Indeed, it is available in many design variations (Henze et al., 2002; van Loodsdrecht and Salem, 2005). In the process, ammonium nitrogen is transformed to

nitrate via nitrite in a first stage (nitrification). This nitrate is, in a second stage (denitrification), reduced to dinitrogen gas by numerous heterotrophic bacteria in four steps, using the substrates contained in the wastewater as an electron donor. These steps include the reduction of nitrate to nitrite, then its reduction to nitric oxide and nitrous oxide, and finally to nitrogen gas. The overall rate and extension of the denitrification process depends mainly on the biodegradability characteristics of the electron donor used and on the final N/COD ratio in the bioreactor. For these reasons, the denitrification process can be enhanced by adding readily biodegradable carbon sources - such as acetic acid and methanol - or carbon sources presenting high COD/N ratios to the wastewater. However, the addition of these carbon sources is expensive and increases the cost of treating the wastewater. In general, this technology consumes a considerable amount of resources: 4.57 kg O_2 and 2-4 kg COD are required per kg ammonium nitrogen. It means that there is a need to aerate the system for nitrification and supply an external organic carbon source for denitrification (Table 2-1, reactions 3 and 4). Besides, both steps should be developed in different reactors, leading to high costs of construction, operation and maintenance (van Loodsdrecht and Salem, 2005).

2.4.2 Partial nitrification – denitrification systems

The minimization of resources by partial nitrification and denitrification results in a more suitable technology. It is possible to save 25% of energy and 40% of organic carbon source in comparison with the conventional nitrification denitrification process, besides there is only a 30% sludge production in comparison to the traditional process. Although there are several available technologies for denitrification via nitrite, the most successful full scale applications have been obtained with a combination of SHARON process and denitrification. At present, there are six full scale plants working in the

Netherlands and one more is under construction in New York, USA. The applied nitrogen loads in those plants are in the range between 400 and 2500 kg N /d (van Loodsdrecht and Salem, 2005). The system has been applied for several ammonium rich wastewaters, including rejection water of sludge digestion, leachate water of landfill sites, wastewater of composting processes and condensates of sludge drying. The operation sequence of those plants is one or two reactors-based. In the case of one-stage system, a single reactor for nitrification and denitrification is used. The reactions are separated in time with sequencing steps. In the first step the production of nitrite is obtained controlling the hydraulic retention time and temperature in the reactor and, in a further sequence, the air addition is stopped and an organic carbon source is added. In two stages systems, the first reactor is equipped with aerators that provide both oxygen and mixing. The partially treated wastewater then flows to the second reactor where an organic carbon source is added.

2.4.3 Anaerobic ammonium oxidation

Anammox process for nitrogen removal can lead to a reduction of operational costs of up to 90% and a reduction of N_2O emissions (van Loodsdrecht and Salem, 2005). In principle, it is mainly addressed to wastewaters with high ammonium nitrogen loads and little organic material. Different research activities have been done for getting suitable operation conditions and for identifying solutions for critical points of the process: temperature, start up and reactor configuration. Anammox process has been usually applied on the treatment of wastewater with temperatures around 30 ºC looking for optimum conditions; however it can works in the range of 18 to 40 ºC. When the temperature decreases to 15 ºC the system loses stability, and, at 45 ºC irreversible loss of the activity happens due to the biomass lysis. Adaptation of biomass to room

temperatures has been reported with high nitrogen removal rates (Dosta et al., 2008; Vázquez-Padín et al., 2009).

Sludge wash-out has been tested as a strategy for reducing the required time to start up the Anammox process. A time reduction of 100 days has been reported, however it was required 220 days for reaching enough dense bacteria population (Kieling et al., 2007). The reactor configuration has also been evaluated as a strategy for reducing the required starting up time. With a membrane bioreactor anammox activity appeared after 16 days when conventional activated sludge was used as seed. After 2 months, the removal of ammonia and nitrite were both over 90% (Wang et al., 2009). However the use of biomass coming from full-scale anammox reactors as seed is one of the best options (van der Star et al., 2007).

There is a general agreement that a high biomass retention reactor is required for anammox processes and several kind of reactors have been tested at lab and full scale, including sequencing batch reactors (SBR), air/gas lift reactors, fixed bed reactors, rotating biological contactors, and upflow anaerobic sludge blanket (UASB) reactors (Jetten et al., 2001; Dapena-Mora et al., 2004; Schmidt et al., 2004; Jianlong and Jing, 2005; Windey et al., 2005; Dosta et al., 2008; Jin et al., 2008a; Jin et al., 2008b). The activities developed in reactor applications can be divided in two general concepts according with the processes taking place: two reactors systems, and one reactor systems. In two reactor systems, partial nitrification takes place in the first reactor and anammox process is developed in the second one. In one reactor systems both processes, partial nitrification and anammox, happen in the same reactor.

2.4.3.1 Two reactors-based systems.

Anaerobic oxidation of ammonium requires an influent with almost equal amount of ammonium and nitrite. By this, a first partial nitrification step is required. In this step total nitrification should be avoided and the effluent should contain 50% of the ammonium and 50% of nitrite. Different strategies can be used such as was described above. Once the adequate mixture of ammonium and nitrite is obtained in the first reactor the second unit is fed. Different reactors configuration (see Table 2-5) and effluent qualities have been used at lab and at pilot-scales working with different nitrogen loads (Sliekers et al., 2003; Molinuevo et al., 2009). The systems used have very high and efficient biomass retention in order to reduce the effects for the low biomass yield of anammox bacteria. Applied loadings and reported nitrogen removal rates have a big variation depending on the reactor. The highest removal rate (8.9 kg N m^{-3} d^{-1}) was reported by Sliekers et al. (2003) using a gas lift reactor with granular sludge working at lab and pilot-scale.

2.4.3.2 One reactor-based systems.

In theory, a combination of ammonium oxidation and denitrification can easily be obtained in biofilm systems with low oxygen concentrations. For typical denitrification processes the organic carbon could be a limitation because the electron donor for denitrification is more rapidly oxidized than ammonium. If ammonium is the electron donor this problem does not occur (van Loodsdrecht et al., 2004). The process was realised by different research groups and it has been termed in different ways as described above. Rotating biological contactors and moving bed systems have been used for landfill leachate, sludge liquors and synthetic wastewater. The applied nitrogen loading rate ranges between 1.4 and 4.8 kg N m^{-2} d^{-1} and the removal rate was reported

to be higher than 60%. Table 2-6 shows the reported systems and their main characteristics.

Table 2-5 Reactor configuration and nitrogen removal rates in two reactors-based Anammox processes. (nd: no data)

Type of reactor	Wastewater	Nitrogen loading rate (kg N m^{-3} d^{-1})	Nitrogen removal rate (kg N m^{-3} d^{-1})	Reference
Fixed bed reactor	Synthetic	1.3	1.1	(Strous et al., 1997)
	Synthetic	0.07 – 0.55	0.35 – 0.38	(Fux et al., 2004b)
	Sludge liquors	nd	3.5	(Fux et al., 2004b)
Fluidized bed reactor	Synthetic	0.2 – 2.0	1.8	(Strous et al., 1997)
	Sludge liquors	2.5	1.5	(Strous et al., 1997)
	Sludge liquors	0.48 – 2.63	2.5	(Jetten et al., 1997)
Sequencing batch reactor	Sludge liquors	1.0	0.75	(van Dongen et al., 2001b)
	Sludge liquors	2.6	2.4	(Fux et al., 2002)
	Synthetic	1.4	1.1	(Dapena-Mora et al., 2004)
Non-woven strips suspended reactor	Synthetic	nd	1.25	(Fujii et al., 2002)
Air/gas lift reactor	Synthetic	2.3	2.0	(Dapena-Mora et al., 2004)
	Synthetic	10.7	8.9	(Sliekers et al., 2003)
UASB reactor	Synthetic	0.52	0.51	(Schmidt et al., 2004)
	Piggery waste	0.84 – 1.02	0.59 – 0.66	(Ahn et al., 2004)
Upflow reactor	Synthetic	nd	2.9	(Imajo et al., 2004)
	Synthetic	7.0	6.4	(Imajo et al., 2004)

There is a general agreement that one reactor systems are more suitable but more complex than two reactors-based systems. Some mathematical models have been

developed in order to understand and to predict the behaviour of the system for different operating conditions and the effect of ammonium surface load (ASL). The main results of the model showed that ASL is associated with the thickness of the biofilm: a thin biofilm has limited capacity for the activity of anammox process and a stable formation of nitrite is the limiting factor. On the other hand, anammox process can happen in biofilm systems and, time but not ASL is the main key factor for the process: in this way, it was predicted that between 5 and 10 years are required for getting the maximum anammox bacterial population (van Loodsdrecht et al., 2004).

Table 2-6 Reactor configuration and nitrogen removal rates in one reactor-based anammox process. (Nitrogen removal rates are referred to surface area of supporting material)

Type of reactor	Wastewater	Nitrogen loading rate (kg N m^{-2} d^{-1})	Nitrogen removal rate (kg N m^{-2} d^{-1})	Reference
Rotating biological contactor	Leachate	1.4 – 3.2	0.4 – 1.2	(Siegrist et al., 1998)
	Leachate	1.5	0.9	(Hippen et al., 2001)
	Synthetic	2.3	1.55	(Pynaert et al., 2002)
	Synthetic	0.69	0.48	(Hippen et al., 2001)
Moving bed reactor	Sludge liquors	4.8	2.4	(Chen et al., 2009)
	Sludge liquors	4 - 8	2.0	(Seyfried et al., 2001)

Dissolved oxygen concentration is also an important factor and it is linked with the thickness of the biofilm and temperature. With a defined ASL under lower temperature, a thicker biofilm is required hence a higher dissolved oxygen concentration is necessary in the reactor. A thin biofilm requires a lower dissolved oxygen concentration. Higher dissolved oxygen concentrations will cause total nitrification and a lower nitrogen removal rate (Koch et al., 2000; Hao et al., 2002a, 2002b).

2.4.4 Wetlands based systems.

Wetlands are land areas that are wet during part or all of the year. It includes swamps, marshes, bogs, fens and sloughs (Kadlec and Wallace, 2009). Because of their high rate of biological activity, higher than most ecosystems, wetlands can transform different pollutants present in conventional wastewater into harmless by-products or essential nutrients that can be used for additional biological productivity. When this potential was discovered, some natural wetlands were used as a treatment system (Clough et al., 1983), however in the last years modern treatment wetlands are man-made systems (Kadlec and Wallace, 2009). There are several types of wetlands for wastewater treatment depending on vegetation and hydraulic flow (Clough et al., 1983; Kadlec and Wallace, 2009). They contain a shallow bed of inert porous media (crushed rocks, gravel, sand, etc.) which has been planted with aquatic plants. In the case of constructed systems, there are two main types: free water surface constructed wetlands (FWS CWs) and sub-surface constructed wetlands (SS CWs). In FWS CWs, wastewater flows in a shallow water layer over a soil substrate. SS CWs may be either subsurface horizontal flow CWs (SSHF CWs) or sub-surface vertical flow CWs (SSVF CWs). In SSHF CWs, wastewater flows horizontally through the substrate. In SSVF CWs, wastewater is dosed intermittently onto the surface of sand and gravel filters and gradually drains through the filter media before collecting in a drain at the base. CWs may be planted with a mixture of submerged, emergent and, in the case of FWS CWs, floating vegetation. Although the main concept of constructed wetlands is to accept them as natural and passive systems with some limitations like oxygen transfer, more engineered systems have been developed to allow some degree of process control over the system in order to improve treatment efficiency (Wallace et al., 2006; Kadlec and Wallace, 2009). In this way, subsurface vertical flow systems can be included in

engineered systems because they get a higher oxygen transfer through fill and drain strategies. On the other hand, subsurface horizontal flow systems depend mainly of the oxygen transfer by plants.

Constructed wetlands as systems of "biofilm reactors" with biofilm on the soil particles and root surface of the plants, allow the combination of aerobic and anaerobic processes inside the system, and high removal rates of different organic compounds, pathogens and some low-degradable matter can be obtained. For this reason, over the last years different aspects have been investigated, including: effects of soil composition and grain-size distribution, fate of pollutants, engineering aspects, hydraulic flow, oxygen input by plants into the rhizosphere, etc (Stottmeister et al., 2003).

Values of nitrogen removal in CWs vary in a wide range. Some authors report low removal rates (0.1 g m^{-2} d^{-1}) and others report values as high as 0.76 g m^{-2} d^{-1} (Brix, 1994; Kadlec and Knight, 1996; Geller, 1997; Maehlum and Stalnacke, 1999; del Bubba et al., 2000; Mander et al., 2000; Kuschk et al., 2003; Kadlec and Wallace, 2009). In general FWS CWs have lower nitrogen removal rates and SSHF CWs show the highest values. Vertical flow systems are more effective for nitrification but the total nitrogen removal is often near to null. A four year evaluation of nitrogen removal of a stable operating SSHF CW in a moderate climate showed a variation in removal rates depending on the season: the highest removal was in summer (0.70 g m^{-2} d^{-1}) and the lowest value was found during winter (0.15 g m^{-2} d^{-1}). It was suggested that aspects associated with low temperature and inhibition of nitrification and denitrification processes are the cause of the lowest nitrogen removal rate in winter time (Kuschk et al., 2003). Similar results were also found by Maltais-Landry (2009) working with three different plants.

Although there are several processes for nitrogen transformation and removal, the sequential processes of ammonification, nitrification and denitrification has been identified as the major removal mechanism of organic nitrogen in CWs (Kadlec et al., 2000; Kuschk et al., 2003; Kadlec and Wallace, 2009; Maltais-Landry et al., 2009). Other possible mechanisms like ammonia volatilization, plant uptake, and soil adsorption are limited. The potential rate of nutrient uptake by plants is limited by their net productivity (growth rate) and the concentration of nutrients in the plant tissue. It is a general consensus that under optimum conditions the amount of nitrogen removed with the plant biomass does not exceed 10% of the total removed nitrogen (Kadlec et al., 2000). Even if the plants are harvested in secondary treatment systems, the amounts of nutrients that can be removed are generally insignificant in comparison with the loadings into the CWs with the wastewater (Brix, 1994), however it could be more important in treatment systems designed for polishing (Kadlec et al., 2000). The amount of nitrogen that can be removed if the biomass is harvested depends on the plant species, nevertheless the potential rate is roughly in the range 0.27 – 0.68 g m^{-2} d^{-1} (Kadlec et al., 2000). In similar way, nitrogen incorporation into the soil matter (humic matter or adsorption to clay minerals) is also low, with less than 10% of the nitrogen removal load (Osman, 1981). Ammonia volatilization seems to play no important role because pH and temperature in wetlands are usually out of the optimum range for the formation of volatile free ammonia (NH_3). In some cases ammonia volatilization depends of the site-specific conditions. The reduced ammonia volatilization rates for wetlands may be attributed to the plants that avoids and breaks the wind reducing the water-side and air-side mass transfer coefficients (Kadlec and Wallace, 2009). It is expected that emergent free water flow wetlands (FWS CWs) will loss much less ammonia to volatilization than will ponds (Kadlec and Wallace, 2009).

In constructed wetlands there are aerobic and anaerobic zones and nitrification and denitrification process can take place. Plants release oxygen through roots and ammonia oxidisers can use it for oxidizing ammonium to nitrate (nitrification), simultaneously, in anaerobic zones, nitrate is reduced to dinitrogen gas, using in some cases organic compounds released by plants (exudates, dead matter, etc.) as carbon source (denitrification). If the sequential ammonification, nitrification and denitrification process is the main mechanism for nitrogen removal, SSHF CWs have two limiting factors for these two steps: the availability of the oxygen required for nitrification and the organic carbon source required for denitrification (Kuschk et al., 2003). Usually ammonium oxidation in SSHF CWs is limited because sufficient aerobic zones are only provided by the vegetated zones of the bed, and anaerobic processes dominate the bed. For instance, in the case of *Phragmites australis*, which has been reported as one of the plants that can release the highest oxygen rates, the oxygen transport by the helophytes aerenchyme amounts only 5 to 12 g m^{-2} patch area per day (Armstrong et al., 1990), leading to elevated area requirements if high loads of both organic carbon and ammonium are applied. Even in the case that the ammonium is oxidised, there will be not enough organic carbon source for subsequent denitrification. It is believed that oxygen availability inside the wetland is primarily used by heterotrophic microbes to remove organic matter and ammonia oxidisers are oxygen limited (Sun et al., 1998; Stottmeister et al., 2003). On the other hand, denitrification is also sensitive to the presence of oxygen (Christensen et al., 1990; Richardson and Ferguson, 1992), but denitrification activities have been detected in wetland systems with low levels of dissolved oxygen. It has been hypothesized that denitrification occurred in the microscopic anoxic zone of bacterial films (Kadlec and Knight, 1996). However this theoretical model has not been proved yet. In this way, if nitrification-denitrifcation

processes is the main mechanism for nitrogen removal, conventional SSHF CW do not represent a reliable, cost effective system for ammonia removal. They seem to be better suited for denitrification if both nitrified influents and sufficient carbon sources are available at the same time. Because of the poor performance of SSHF CW for ammonia oxidation, vertical systems (SSVF CWs) have been used, however they can remove ammonia nitrogen by full nitrification, but not total nitrogen (Cooper et al., 1996; Langergraber et al., 2006). In some cases a combination of vertical and horizontal flow systems is used for nitrogen removal or, the effluent of vertical flow systems is recycled either septic tanks or anaerobic chambers located at the beginning of the wastewater treatment system.

A nitrogen mass balance evaluation in wetlands (Sun et al., 2005) concluded that besides nitrification-denitrification processes, bacterial biomass assimilation should be considered important when organic carbon source is present. But not only should this nitrogen removal mechanism be included in the possible pathways for nitrogen removal. Constructed wetlands must be considered as biofilm systems (Larsen and Greenway, 2004). The plant roots and the soil particles provide a large surface area, which would certainly encourage the development of the biofilm; however, there has been little research on the role of biofilms in contaminant degradation and nutrient removal. As a biofilm system with low dissolved oxygen and organic carbon source concentrations, it is expected that processes such as partial nitrification and anaerobic oxidation of ammonium play a role in nitrogen removal and stimulating them could improve nitrogen removal rates in wetlands (Stottmeister et al., 2003). Recently, the presence of Anammox bacteria has been reported in surface flow constructed wetlands (Shipin et al., 2005), and an unexplained high removal of nitrogen was attributed to a combination

of partial nitrification and anammox processes happening in a lab-scale SSVF CW (Sun and Austin, 2007).

If Anammox bacteria can be adapted to the environmental conditions existing in SFCWs, the main limitations for nitrogen removal (not enough oxygen and organic carbon) become an advantage for this process, and theoretically, it could be possible to achieve higher nitrogen removal rates, however, knowledge about the role/importance of these microbial nitrogen transformation processes in such a mosaic macro- and micrograd ient system has yet to be investigated (Dong and Sun, 2007; Tao and Wang, 2009; Kadlec and Wallace, 2009). Understanding the basic microbial processes controlling nitrogen removal in biofilters will substantially promote the improvement of their efficiency and will also result a broader application of biofilters like wetland treatment systems as a reliable treatment strategy.

3 Material and Methods

The experiments were executed in four steps. At first (chapter 3.1) anammox activities were tested in sludges/soils from different wastewater treatment systems (like sand from a constructed wetland, sludge from activated sludge system etc.). At second (chapter 3.2) experiments in a definite part of a wetland model system (Planted Fixed Bed Reactor) for evaluating nitrogen turn-over rates with different artificial wastewater compositions were done. In the third series of experiments (chapter 3.3) attempts for artificial accelerated manifestation (inoculation) of anammox activity in these model systems (Planted Fixed Bed Reactor) were executed. Finally laboratory experiments were run to optimize partial nitrification in vertical down flow filters as one of the main prerequisite steps for establishing a full two step process (partial nitrification – anammox) for the treatment of ammonia rich wastewaters (chapter 3.4).

3.1 Preliminary anammox activity tests of sludges/soils from different wastewater treatment systems

The anammox activity of the filter material (sand) of subsurface constructed wetlands (pilot plant at Langenreichenbach, Germany), sludge from the denitrifying basin of a full scale domestic wastewater treatment plant (Langenreichenbach, Germany), and sludge from an activated sludge system for domestic wastewater (treatment plant of Delitzsch, Germany) were evaluated.

The experiments were done under batch condition using 550-ml serum bottles. Different amounts of inoculum and a blank (distilled water) were added to 9 bottles according to Table 3-1, then, all bottles were filled up with nutrient solution, purged with Helium gas for 10 minutes and closed with special rubber stoppers for serum bottles to keep the

content under anaerobic conditions. Finally these bottles were incubated at 30 ºC under dark conditions. Samples (5 ml) were taken twice per week by a syringe with a needle through the rubber stoppers for pH, nitrite, nitrate and ammonium determination.

Without disturbing the sediment layers in the serum bottles the nutrient solutions were changed when the concentration of ammonium or nitrite was near zero. In all cases, after changing the nutrient solutions, the bottles were purged again with helium. This experimental series was run for 150 days.

Table 3-1 Inoculum materials in the bottle experiments for anammox test/enrichment culture ([a]pilot plant Langenreichenbach, [b]treatment plant Delitzsch, [c]treatment plant Langenreichenbach)

Bottles	Inoculum
A	50 ml sand from CW[a]
B	150 ml sand from CW[a]
C	50 ml sludge denitrification step[b]
D	150 ml sludge denitrification step[b]
E	50 ml sand CW[a] + 50 ml sludge denitrification step[b]
F	50 ml sand CW[a] + 150 ml sludge denitrification step[b]
G	200 ml activated sludge[c]
H	500 ml activated sludge[c]
I	Blank (distilled water)

Macronutrient solution for the experiments contained mainly ammonium, nitrite and bicarbonate (Table 3-2). Its composition was defined according with different experiences for anammox enrichment cultures and anammox activity tests (Van de Graaf et al., 1996). Both nitrite and ammonium concentrations in nutrient solutions were 110 mg NO_2 -N l^{-1} and 100 mg NH_4 -N l^{-1}, respectively. pH was adjusted to 7.5 with 1 N H_2SO_4. Later, in those bottles with a clear anammox activity the ammonium and nitrite

concentrations were increased up to 200 mg NH_4 -N l^{-1} and 280 mg NO_2 -N l^{-1} at the end of the experiment.

Table 3-2 Ingredients of the nutrient solution for the anammox test/enrichment culture

Reagents	*mg l^{-1}*
$KHCO_3$	1000
NaH_2PO_4	50
$CaCl_2.2H_2O$	300
$MgSO_4.7H_2O$	200
$(NH_4)_2SO_4$	943 – 1885
$NaNO_2$	542 – 1380
EDTA-Na	6.25
$FeSO_4$	6.25
pH	7.7-8.2
Trace elements solution	1.25 ml l^{-1}

Phosphate was kept below 40 mg l^{-1} to avoid inhibitory effects. Besides the macronutrients, a solution of microelements was added to the bottles. Both solutions (macro and trace nutrients) were prepared with distilled water.

The composition of the microelements solution is shown in Table 3-3 .

Table 3-3 Ingredients of trace element solution for the anammox test/enrichment culture (according to Van de Graaf et al., 1996)

Reagents	*mg l^{-1}*
EDTA	15000
$ZnSO_4.7H_2O$	430
$CoCl_2.6H_2O$	240
$MnCl_2.4H_2O$	990
$CuSO_4.5H_2O$	250
$NaMoO_4.2H_2O$	220
$NiCl_2.6H_2O$	190
$NaSeO_4.10H_2O$	210
H_3BO_4	140

3.2 Nitrogen removal/transformation in experimental laboratory-scale Planted Fixed Bed Reactors

Planted Fixed Bed Reactor

The experiments were done in laboratory-scale Planted Fixed Bed Reactors under conditions of complete mixing by permanent circulation of the pore water (Figure 3-1). The design and the principles of operation of the reactor-system (PFBR planted fixed bed reactor) were previously described in detail (Kappelmeyer et al., 2002; Wießner et al., 2002a; Wießner et al., 2005a). The reactor consisted of a glass vessel 30 cm in diameter and 30 cm tall. A basket of perforated stainless steel 27 cm in diameter and 30 cm tall was placed centrally inside the glass vessel. A pipe of the perforated stainless steel 4 cm in diameter and 30 cm tall was placed centrally inside the basket. The basket was completely filled with gravel around the pipe. Five plants (*Juncus effusus*) were planted in the gravel bed. The glass vessel was closed with a lid containing five holes (diameter of 5 cm) through which the plants in the gravel bed grew. Circulatory flow was arranged by pumping the process water out of the gravel-free pipe into the centre of the gravel bed and back into the reactor through a dosage ring near the bottom of the lid above the gravel-free reactor space between the glass vessel and the gravel-filled perforated steel basket inside in order to permanently mix the fluid. In addition, an inflow from a second pump and an outflow comprising a magnetic valve connected to a fluid level control were integrated into the circulation flow to enable continuous operation via a defined retention time. The free water volume amounted to 10 l and the hydraulic retention time was adjusted to 5 d, in this way the flow rate of each reactor was adjusted to 2 l d^{-1}. The circulation flow was adjusted to 10 times the inflow. This permanent mixing of the process fluid made for hydrodynamic conditions similar to an

ideal mixed vessel inside the rhizosphere (Kappelmeyer et al., 2002; Wießner et al., 2005a).

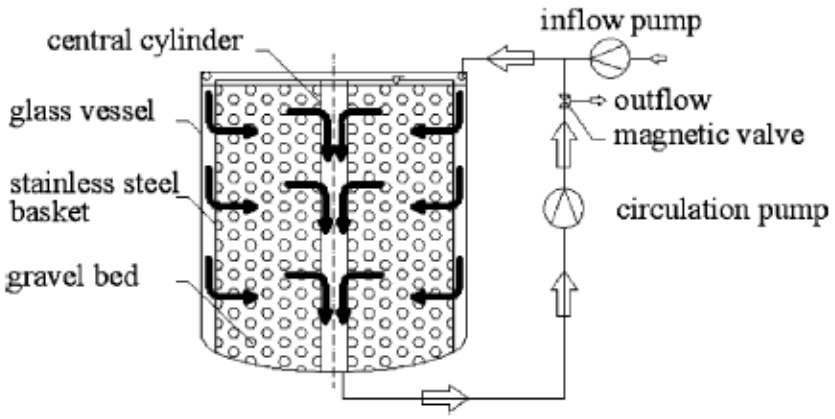

Figure 3-1 Scheme of the Planted Fixed Bed Reactor. (adapted from Kappelmeyer et al., 2002)

Since the internal flow conditions are comparable to an ideal mixed vessel, macro-scale gradients of concentrations, Eh, pH etc. were equalized and the effects of the changes could easily be analyzed. Due to the reactor design, the circulation flow represents the actual concentration of the pore water inside the reactor. Such a system can be seen as a confined part of a constructed wetland and allows better to investigate root-near processes in constructed wetlands.

The pH, redox potential and oxygen concentration were measured in the circulation flow online twice per hour. Plant transpiration was calculated from the inflow and outflow water volumes. Due to the reactor's design, evaporation is negligible; a mean rate of water loss of about 8.4 ml d^{-1} was estimated for an unplanted reactor.

The reactor was placed in a greenhouse and operated under defined environmental conditions to simulate an average summer day in a moderate climate. The temperature

set points were 22 ºC from 6 am to 9 pm to simulate daytime and 16 ºC at night. One lamp (Master SON-PIA 400 W, Phillips, Belgium) per reactor was switched on during daytime as an additional artificial light source whenever the natural light fell below 60 klx. This additional illumination provided approximately only 11 klx at the top of the plants (70 cm), 5 klx at the middle of the plants and 3 klx at ground level (Wießner et al., 2005a) .

Experimental phases

The experiment was divided in three phases. The PFBR was continuously fed with a synthetic wastewater containing nitrogen compounds (ammonium and nitrite), micronutrients and bicarbonate as the mineral carbon source. There was no addition of any organic carbon source. An overview of the different experimental phases is shown in Table 3-4.

Table 3-4. Ammonium and nitrite concentrations of the reactor inflow during the different experimental phases.

Phase	Time (days)	NH_4-N (mg l^{-1})	NO_2-N (mg l^{-1})
I	0 - 160	156	58
II	160 - 210	156	112
III	210 - 380	156	149

Preparation of the synthetic wastewater

The inflow concentrations of the ingredients used are described in Table 3-5. The compounds were dissolved in deionized water and 1.25 ml l^{-1} of trace mineral solution (Table 3-3.) was added.

Table 3-5 Composition of the synthetic wastewater during the first period of the experiments: Phases I, II and III ([a]trace element solution according to Van de Graaf et al., 1996)

Reagent	*mg l^{-1}*
$KHCO_3$	1250
KH_2PO_4	13.6
$CaCl_2.2H_2O$	227.3
$MgSO_4.7H_2O$	136.6
$(NH_4)_2SO_4$	735.8
$NaNO_2$	285.7 – 733.9
EDTA-Na	6.25
$FeSO_4.7H_2O$	11.5
Trace elements solution[a]	1.25 ml l^{-1}

Before starting the experiments the reactor was inoculated with the effluent of a wastewater treatment plant in order to obtain a stable bacterial population and run for three months with a standard synthetic domestic wastewater (DIN-38412-T24, 1981).

3.3 Nitrogen removal/transformation in experimental laboratory-scale Planted Fixed Bed Reactors after inoculation with active anammox biomass

The main objective of this experiment was to identify and to evaluate the role of anammox bacteria in nitrogen transformation processes in PFBRs after inoculation of these reactors with active anammox biomass. In more detail, it should be evaluated the effects that plants, different nitrogen loads and the presence of organic matter have on nitrogen removal in general and, specifically on the anammox process stability in these model systems for CWs.

Experimental set up and phases

Four PFBR, with the same construction as was described above (see chapter 3.2), (three planted with five plants of *Juncus effusus*, one unplanted) were operated in parallel for the investigation of the influence of different factors on nitrogen removal and anammox processes. Reactor 1 (planted) and reactor 2 (unplanted) were used for the investigation of the effects of plants, under different ammonium and nitrite concentrations. In reactor 3 the pathway of partial nitrification-anaerobic ammonium oxidation was evaluated, and in reactor 4 the impact of an organic carbon source at different ammonium and nitrite concentrations on the nitrogen transformation processes was characterized (Table 3-6). Reactor 1 was used previously as was shown in 3.2.

Table 3-6 Influent concentrations, operating conditions and loading rates of the reactors during the different experimental phases carried out after inoculation with anammox biomass.

	Reactor 1 Planted			Reactor 2 Unplanted			Reactor 3 Planted			Reactor 4 Planted		
Phase	IV	V	VI	IV	V	VI	IV	V	VI	IV	V	VI
Concentration (mg l^{-1})												
NH_4^+-N	117	156	156	117	156	156	140	156	259	117	156	156
NO_2^--N	23	46	91	23	46	91	0	0	0	23	46	91
TN	140	202	247	140	202	247	140	156	259	140	202	247
TOC	0	0	0	0	0	0	0	0	0	60	60	60
Loading rate (mg d^{-1})												
NH_4^+-N	239	319	319	239	319	319	286	319	530	239	319	319
NO_2^--N	47	94	186	47	94	186	0	0	0	46	94	186
TN	285	413	505	285	413	505	286	319	530	285	413	505

Before starting the experiments reactors 2, 3 and 4 were inoculated with the effluent of a municipal wastewater treatment plant in order to obtain a stable bacterial cenosis and run for three months with a standard synthetic domestic wastewater (DIN-38412-T24,

1981) and after this all four reactors were inoculated with active anammox biomass. For this 2 l (20 % of the pore volume of the reactor) of an anammox enrichment culture with a specific activity (see Chapter 3.6) of 0.85 g ammonia nitrogen removal per liter per day was injected into all four reactors. The anammox biomass was taken from the anammox reactor of the wastewater treatment plant in Hattingen, Ruhrverband Essen, Germany.

Preparation of the synthetic wastewater

The reactors were fed with a synthetic wastewater containing nitrogen compounds (ammonium and nitrite), micronutrients and bicarbonate as the mineral carbon source. The inflow concentrations of the ingredients used during the different phases of the experiments (Table 3-6) are shown in Table 3-7.

Table 3-7 Composition of the synthetic wastewater ([a]trace element solution according to Van de Graaf et al., 1996)

Reagent	*mg l^{-1}*
$KHCO_3$	2727
KH_2PO_4	13.6
$CaCl_2.2H_2O$	227.3
$MgSO_4.7H_2O$	136.6
NH_4Cl	447.0; 534.9; 596.0; 990.6
$NaNO_2$	113.4; 226.7; 448.5
EDTA-Na	6.25
$FeSO_4.7H_2O$	11.5
Trace element solution[a]	1.25 ml l^{-1}

Furthermore, during all phases to reactor 4 CH_3COONa (200 mg l^{-1}) was added which corresponds a TOC of 60 mg l^{-1}. In order to have a higher buffer capacity and to keep

the pH in the optimum range value for anammox bacteria, the amount of potassium bicarbonate added to the synthetic wastewater was increased in comparison to the experiments done before.

The compounds and trace element solution were dissolved in deionized water. The reactors were placed in a greenhouse and operated under defined environmental conditions to simulate an average summer day in a moderate climate as already described in chapter 3.2 (Wießner et al., 2005a).

3.4 Laboratory-scale experiments on partial nitrification in vertical down flow filters.

Because the anammox process requires nitrite in the influent, this part of the research was focused to identify optimum conditions for partial nitrification in vertical down flow filters and the effects that different size of filter material at different temperatures and salinity have on nitrite accumulation.

Vertical down flow filters and running conditions

The experiments were performed in a laboratory-scale vertical down flow filter-system with different supporting material, salt concentration of the synthetic wastewater and temperature. Nine column filters were used. Each column had a diameter of 12.5 cm, a total height of 65 cm and a filter material height of 60 cm. There was no special aeration system and for controlling the oxygen transfer into the system; all columns worked with a water level of 2/3 of the total height (see Figure 3-2).

Before starting the experiments all reactors were inoculated with the effluent of a wastewater treatment plant (Langenreichenbach, Saxony, Germany) in order to obtain a stable bacterial cenosis and run for three months with synthetic domestic wastewater (DIN-38412-T24, 1981).

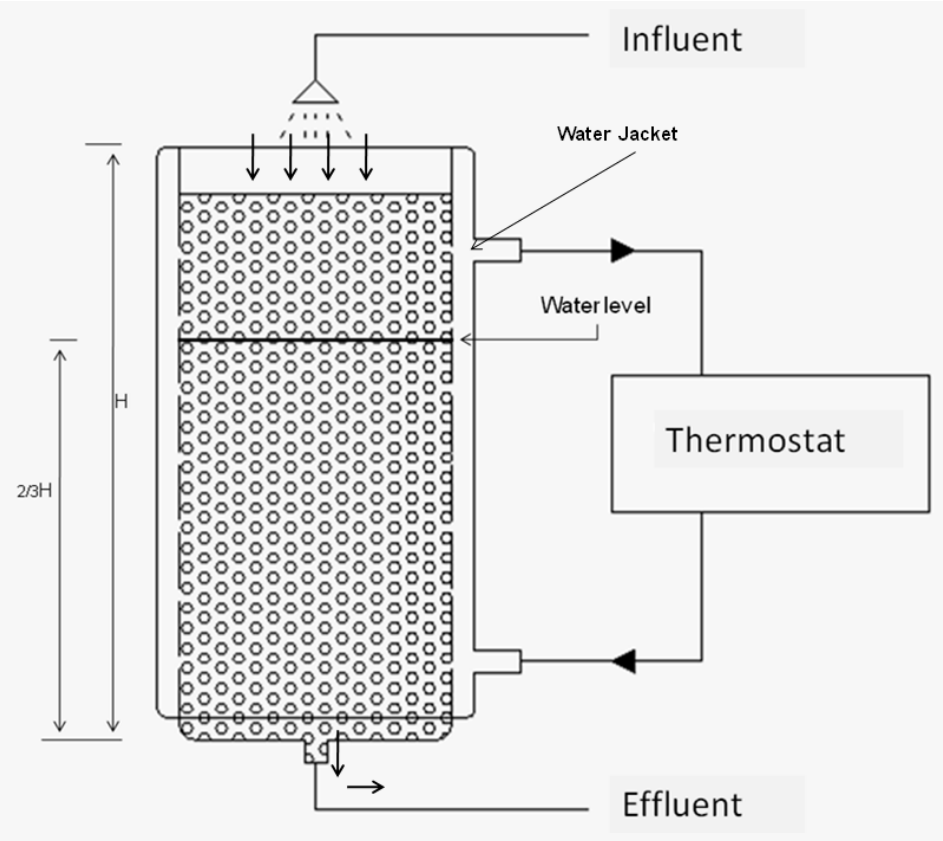

Figure 3-2. Scheme of the vertical down flow column.

Two different sizes of filter materials were used: fine material (sand, D10: 0.10 mm), and medium size material (gravel D10: 2.0 mm / expanded clay D10: 1.8 mm) (sand and gravel from Freudelsperger Beton- und Kiewerk Sprotta GmbH in Sprotta, Saxony, Germany; expanded clay from Masterfoods GmbH, Verden/Aller, Germany). All materials were washed with tap water and distilled water before the experiments. The filters columns had a water jacket to keep constant the experimental temperatures (18 ºC, 26 ºC and 35 ºC). The experimental conditions of the filter columns are shown in

Table 3-8. All filters were fed with a synthetic wastewater with 400 mg NH_4-N l^{-1} and to promote the inhibition of nitrite oxidizer bacteria by low pH, a molar ratio of 1.56:1 between ammonium and bicarbonate was realized during the experiment.

The flow rate in all filter columns was adjusted to 500 ml d^{-1} in order to keep a hydraulic load of 40 m^3 m^{-2} d^{-1}, which allowed a nitrogen load of 27.2 g m^{-3} d^{-1}.

Table 3-8 Experimental conditions of the filer columns for partial nitrification

Column	Filter material size	Temperature (ºC)	Salt concentration (g l^{-1})
1	Medium	18	-
3	Medium	26	-
4	Medium	36	-
5	Small (sand)	18	-
6	Small (sand)	26	-
7	Small (sand)	36	-
8	Medium	18	22.72
9	Medium	26	22.72
10	Medium	36	22.72

Preparation of the synthetic wastewater

The inflow concentrations of the ingredients used for the different conditions of the experiments were (in mg l^{-1}): 1200 NH_4Cl; 3500 $KHCO_3$; 0.0263 $NaH_2PO_4*H_2O$; 0.013 $FeSO_4$ x $7H_2O$.

In those units working under salinity conditions - 66% of the sea's salt concentration (Lyman and Fleming, 1940) - the influent concentrations of the ingredients used were (in g l^{-1}): 15.65 NaCl; 7.09 $MgCl_2*6H_2O$; 2.61 Na_2SO_4; 0.97 $CaCl_2$ x $2H_2O$; 0.44 KCl; 0.123 $NaHCO_3$; 0.064 KBr. The compounds were dissolved in deionized water and 1.5 ml l^{-1} of trace element solution according Van de Graaf et al. (1996) was added.

3.5 Chemical and physicochemical analysis

The TC, TOC and IC were analyzed using a TOC analyzer (Shimadzu, TOC 600, Duisburg, Germany).

BOD_5 was measured using the OxiTop devices (WTW, Weilheim, Germany) following the manufacturer's instructions.

COD (dichromate method) was determined photometrically using Test No. 314 over a range of 15–150 mg l^{-1} (Dr. Bruno Lange GmbH, Düsseldorf, Germany).

Concentrations of acetate, benzoate, ammonia, nitrite and nitrate were analyzed by ion chromatography (DIONEX 100, columns AS4A-SC/AG4A-SC and CS12A/CG12A; Idstein, Germany) using a UV detector for nitrite and nitrate at a wavelength of 215 nm and a conductivity detector for the other ions. The self regenerating suppressors ASRS-Ultra 4 -mm (for anions) and CBES-I 4mm (for ammonia) were used.

Alkalinity was measured by titrimetric procedures according with the standard methods (APHA, 1998).

Dissolved oxygen concentration in reactors and columns was measured on line with needle based and flow-through cell housing optical micro-sensors (Presens, Regensburg, Germany).

The pH and the redox potential (rH) were continuously measured and recorded twice per hour by a microprocessor Standard (pH-ION-Meter pMX 3000/pH, WTW, Weilheim, Germany). The redox potential in the PFBRs was measured by the Pt4805-S7/120 combination Redox, METTLER TOLEDO, and the pH by the pH-electrode Sentix 41. Redox potential values were converted to the potential relative to the normal

hydrogen reference electrode (Eh) taking the sample temperature into account. Once per day or before to make any measurement, pH-meter was calibrated with two different pH buffers according with the manufacturer´s instructions. In similar way the proper functioning of redox electrodes were tested with WTW solution for redox potential (Pt/Ag/AgCl in 1 M KCl, +220 mV/25 °C).

3.6 Anammox activity

Two different tests were developed for checking the anammox activity. The first one was based on the concentration decrease of both ammonia and nitrite under anaerobic conditions and the second was based on the formation of hydrazine by the addition of an excess of hydroxylamine (hydrazinase activity).

3.6.1 Ammonia and nitrite concentration decrease under anaerobic conditions

Because the anammox process is the only one known that can reduce the concentration of ammonia in the presence of nitrite under anaerobic conditions, two different kinds of batch assays were done.

The first one was done in 100 ml serum bottles. For this, 25 ml of sludge/sediments were taken and 75 ml of a solution containing 100 mg NO_2-N l^{-1} and 100 mg NH_4-N l^{-1} was added. The bottles were made anoxic by applying Argon gas, immediately after this the bottles were sealed with rubber stoppers and incubated at 30 ºC under dark conditions.

The second set of assays was done in order to know the potential activity of anammox biomass present in the PFBR. For this, 3 samples of 150 g of the gravel with attached biofilm were taken from each reactor. Each sample was washed 3 times with distilled

water before the addition of ammonium and nitrite (100 and 200 mg l^{-1}, respectively). The medium used for anammox activity had a same composition like the influent (synthetic wastewater) of the PFBRs. The gravel with biofilm with the fresh synthetic wastewater was transferred into 200 ml serum bottles. These bottles with their content were made anoxic by purging them for 10 min with Argon gas and finally sealed with rubber stoppers. The bottles were incubated at similar temperature of the PFBRs (20 °C) and were shaken continuously at 110 rpm.

Anammox activity was defined to be detected if both ammonium and nitrite concentrations simultaneously decreased under anoxic conditions.

3.6.2 Hydrazine formation

Anammox activity can be checked by the formation of hydrazine under an excess of hydroxylamine (Jetten et al., 1998). Continuous flow experiments were done by the addition of a pulse of 1.0425 g hydroxylamine-HCl (NH_2OH HCl) into the reactors. After the addition of hydroxylamine, the reactor was strongly mixed by recirculation of the pore water of the reactor for an hour, then, samples were taken every thirty minutes for hydroxylamine and hydrazine determination. Hydroxylamine and hydrazine determination was done by colorimetric methods according to Watt and Chrisp (1952), Frear and Burrell (1955).

3.7 Microbial community characterization and molecular biology tests

To evaluate the microbial community and the existence of anammox biomass, samples from gravel and pore water were taken at the end of the different experimental phases from each reactor. From these samples DNA was extracted followed by multiplying

fragments by polymerase chain reaction (PCR) and subsequent denaturating gradient gel electrophoresis (DGGE) and fluorescence in-situ hybridization (FISH) analysis.

DNA was extracted directly from samples using the QIAGEN Tissue Kit (QIAGEN, Germany) following the protocol for gram-positive bacteria. The PCR amplification was performed in a Mastercycler (Eppendorf, Germany). The PCR was performed on the extracted DNA samples using primer CTO189f and CTO654r (see Table 3-9 for sequence) with GoTaq® Green Master Mix (Promega, Germany).

The PCR was performed with an initial denaturation at 94 °C for 2 minutes, followed by 25 cycles of 30 seconds at 95 °C, 30 seconds at optimal annealing temperature, and 45 seconds at 72 °C. The PCR products were analyzed on a 1.5% agarose gel and stained with ethidium bromide.

Table 3-9 Primers and probes used in the molecular biology tests for anammox bacteria

Primer or probe	Nucleotide sequence (5'-3')	Reference
CTO 189A/Bf	GGA GRA AAG CAG GGG ATC G	(Kowalchuk et al., 1997)
CTO 198Cf	GGA GGA AAG TAG GGG ATC G	(Kowalchuk et al., 1997)
CTO 654r	CTA GCY TTG TAG TTT CAA ACG C	(Kowalchuk et al., 1997)
amoA-1f	GGG GTT TCT ACT GGT GGT	(Rotthauwe et al., 1997)
amoA-2r	CCC CTC KGS AAA GCC TTC TTC	(Rotthauwe et al., 1997)
46f	GGA TTA GGC ATGC AAG TG	(Schmid et al., 2000)
820r	AAA ACC CCT CTA CTT AGT GCC C	(Schmid et al., 2001)
Amx820	AAA ACC CCT CTA CTT AGT GCC C	(Schmid et al., 2001)
Nso190	CGA TCC CCT GCT TTT CTC C	(Mobarry et al., 1996)
GC-clamp	CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG	(Kowalchuk et al., 1997)

DGGE was carried out as described previously by Kowalchuk et al. (1997), using INGENY phorU (INGENY, The Netherlands) system and 1.5 mm 8 % polyacrylamid gels containing denaturing gradient of 35 to 70 % for separation anammox PCR products. Gels were run 5 h at 200 V in 1 x TAE (Tris-acetate-EDTA) buffer at constant temperature at 60 °C.

For FISH, samples were taken and immediately fixed using 4% paraformamide solution in 1x phosphate-buffered saline (PBS) buffer at 4 °C. After 4 hours samples were washed 3 times with PBS and conserved in a solution of ice-cold PBS and absolute ethanol (1:1) and stored at –20 °C. The hybridization followed a standard protocol (Daims et al., 1999) using the probes Amx820 to assess anaerobic ammonium-oxidizing bacteria, Candidatus `*Brocadia anammoxidans*' and Candidatus `*Kuenenia stuttgartiensis*' (Schmid et al., 2001) and Nso190 to assess betaproteobacterial ammonia-oxidizing bacteria (Mobarry et al., 1996). DAPI (4',6-Diamidino-2-phenylindole-dihydrochloride) was used to apply a positive staining to all organisms. A microscope with filter sets 20, 09 and 02 (Axioscope, Zeiss, Jena Germany) was used for visualization.

3.8 Removal rate calculation

The specific removal rates of the systems were calculated as the difference between the specific inflow and outflow loads (mg m^{-2} d^{-1}); it means the waterloss by plant transpiration and evaporation was considered.

4 Results and Discussion

4.1 Preliminary tests on anammox activity of sludges/soils from different wastewater treatment systems

The experiments aimed to find an appropriated inoculation material or starting material for enrichment with high anammox activity for the further experiments like acceleration of nitrogen turnover in a laboratory-scale model wetland system. For this anammox activity of sand of a horizontal subsurface flow constructed wetland (pilot plant of Langenreichenbach, Germany), sludge from the denitrifying basin of a full scale domestic wastewater treatment plant (Langenreichenbach, Germany), and sludge from an activated sludge system for domestic wastewater (treatment plant of Delitzsch, Germany) were evaluated. The conditions of these experiments were described previously (Table 3-1).

The results of these batch experiments (changes in ammonium and nitrite concentrations) executed in closed bottles are shown in Figure 4 1. With the exception of the control (bottle I) in all other bottles (A-H) at the beginning a relatively high denitrification activity took place. Within less than 5 days nitrite was consumed in all bottles to a concentration below detection limit while the ammonia concentration stayed unchanged. When nitrite concentration was near zero, new solution was added into the bottles allowing an average nitrite concentration of around 100 mg NO_2-N l^{-1}. Depending of the different denitrification activity in the experimental bottles this procedure was repeated several times. After 50 – 75 days the rate of denitrification activity decreased in all bottles (with the exception of the control in bottle I) and both

ammonium and nitrite remained constant for several days, then, a simultaneous nitrite and ammonium concentrations reduction (indicator of anammox activity) was detected.

In general all bottles inoculated with filtering material coming from horizontal subsurface flow constructed wetlands showed, after ninety days (Figure 4-1 B, E and F), a reduction of both ammonium and nitrite concentrations, being necessary the addition of new solution of both compounds when their concentration values were near zero.

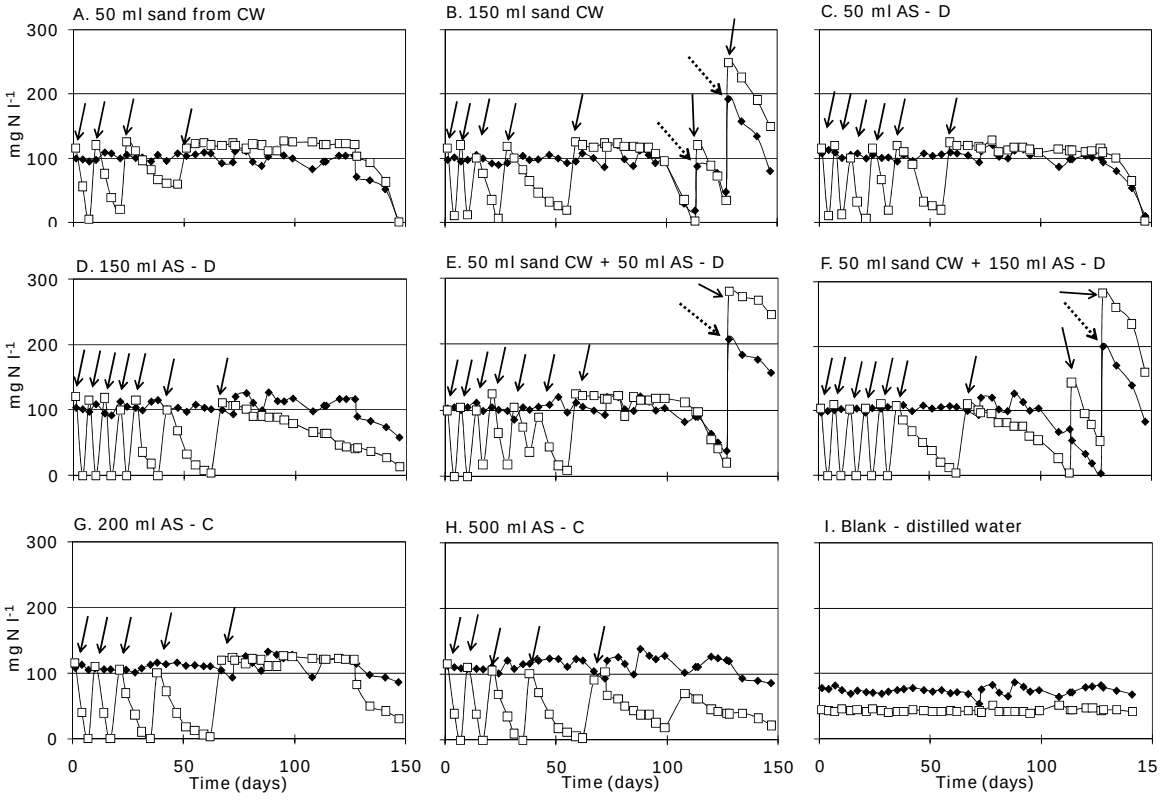

Figure 4-1 Ammonia and nitrite concentrations in dependence on time in the bottle experiments with different inoculum sources or combination of them for enrichment of anammox activity.

(—□—NO_2-N; —♦— NH_4-N; CW: Constructed wetlands, AS – D: Activated sludge from denitrification step; AS – C: Conventional activated sludge) (arrows indicate the recharge of nitrite and ammonium respectively)

Longer periods of time (around 125 days) required the bottles inoculated with sludge coming from the denitrification step of activated sludge system to produce a simultaneous reduction of the concentration of ammonium and nitrite (Figure 4-1 C and D) and no simultaneous reduction was detected in the bottles inoculated with sludge taken from the conventional activated sludge system where no support was provided (Figure 4-1 G and H). In the bottles inoculated with activated sludge only after 130 days a slight decrease of ammonium concentration could be observed. The control bottle only filled with distilled water, nitrite and ammonium solutions (blank) did not present any reduction of both parameters (Figure 4-1– I).

The obtained maximum nitrogen removal rates (MNRR) and required time for getting anammox activity are summarized in Table 4-1. A faster and higher anammox activity (expressed as MNRR) was detected in bottle 2, which corresponds to the filtering material taken in constructed wetlands, however, bottle 3, containing sludge from a denitrification step, reached a similar MNRR in a longer time.

Table 4-1 Time needed for starting anammox activity and maximum nitrogen removal rates in the bottle experiments for anammox enrichment

Bottle No.	Inoculum	Time (days)	MNRR[a] (kg m^{-3} d^{-1})		
			NO_2-N	NH_4-N	Total N
A	50 ml sand from CW	135	0.013	0.009	0.021
B	150 ml sand from CW	99	0.014	0.011	0.025
C	50 ml sludge from denitrification step	135	0.014	0.010	0.023
D	150 ml sludge from denitrification step	128	0.003	0.003	0.006
E	50 ml sand from CW + 50 ml sludge from denitrification step	113	0.007	0.006	0.013
F	50 ml sand from CW + 150 ml sludge from denitrification step	99	0.010	0.008	0.018
G	200 ml activated sludge	ND	ND[b]	ND	ND
H	500 ml activated sludge	ND	ND	ND	ND
I	Blank (distilled water)	ND	ND	ND	ND

[a] Maximum nitrogen removal rates. [b] Not detected

Simultaneously with the reduction of both nitrite and ammonium concentrations, the biomass in the bottles got a brown-red color, which has been associated with anammox biomass.

Discussion

Several sludge sources have been used for anammox enrichment cultures like anaerobically digested cow dung, sludge from activated sludge systems (conventional process, extended aeration, oxidation ditch), anaerobic sludge from UASB reactors treating sewage and sludge from digesters, sludge collected from sewer pipes, membrane filters, denitrifying process systems (conventional and rotating biological contactors) (Sabumon, 2007; Tsushima et al., 2007; Jin et al., 2008b). Positive results of anammox activity have been obtained not only with sludges from wastewater treatment plants. It has been detected in sediments, estuaries and other different habitats. All these findings have a clear implication towards the ubiquity of anammox bacteria (Kartal et al., 2007; Liu et al., 2008; Molinuevo et al., 2009).

The obtained result from the bottle experiment is an indicator that anammox bacteria are also present in subsurface flow constructed wetlands. Actuality several authors have suggested that anammox could be a possible pathway for nitrogen removal processes in constructed wetlands but it has been no proved yet (Stottmeister et al., 2003; Vymazal, 2007; Kadlec and Wallace, 2009), however, anammox bacteria have been found in surface constructed wetlands (Shipin et al., 2005) and a combination of partial nitrification and anammox process has been proposed as a possible pathway for explanation of some high nitrogen removal rates obtained in vertical flow constructed wetlands (Sun and Austin, 2007).

The most complicate aspect for getting a reactor working on the basis of anaerobic ammonium oxidation for nitrogen removal has been the starting up of the system, mainly because the difficulty for obtaining enough anammox biomass. This aspect is primarily due to the slow growth rates of anammox bacteria in fact, the doubling time has been reported to be 11 days (van Dongen et al., 2001a).

An inhibition effect on the growth of the anammox bacteria in the bottles caused by the formation of sulphide by reduction of the sulphate present in the nutrient solution could be excluded. Nitrite was present in surplus and the brown-red color of the biomass and the simultaneous decrease of ammonium and nitrite concentration are indicator that anammox process was present.

The anammox process requires the presence of both ammonium and nitrite, and an extreme long start up time, and perhaps this has been the main limitation for its application in subsurface flow constructed wetlands. On the other hand, anammox process has been mainly applied with high nitrogen loads (Total Nitrogen above 200 mg N l^{-1}), but the main use and experiences for nitrogen removal in constructed wetlands are associated with domestic wastewater which has relatively low nitrogen concentrations, ranging between 15 and 70 mg N l^{-1} (Kadlec et al., 2000; Vymazal, 2007).

Probably this low nitrogen load, the deficiency in nitrite and the extreme slow growth rate of anammox bacteria especially in technical scale systems are the limiting factors for the development of anammox bacteria in constructed wetlands. The deliberate application and stimulation of this anammox activity in full–scale constructed wetlands can probably improve their nitrogen removal rates especially in case of wastewater with a high ammonium load.

4.2 Nitrogen removal in Planted Fixed Bed Reactors (PFBR) as laboratory-scale model systems for subsurface horizontal flow constructed wetlands (SSHF CWs)

For characterization of nitrogen compound turnover under defined environmental conditions experiments were executed in PFBR (see chapter 3.2). The PFBR was feed in a sequence of model wastewaters with a constant ammonium and increasing nitrite concentrations (Phases I, II and III; see Table 3-4).

Figure 4-2 shows the time courses for the nitrogen species concentrations (ammonia, nitrite, nitrate and total nitrogen) within the experimental phases I to III. During the phases there was no or minimal decrease in ammonia concentration. Nitrite concentration decreased within these three phases (I to III), nevertheless there were no stable conditions. Nitrate was formed at a concentration up to approximately 190 mg NO_3-N l^{-1}.

Plants suffered during these three phases (I to III) from toxic effects probably caused by nitrite and their transpiration rate decreased (see Figure 4-3). When the onset of plant death was observed (color change of the stalks from fresh green to brownish) the dying plants were replaced by new ones (marked with dotted lines in Figue 4-3). As a result of this new plantation, nitrite concentration decreased and nitrate concentration increased. Nevertheless, such re-plantations were followed by more or less long periods of stable transpiration until the plant's activity again collapsed.

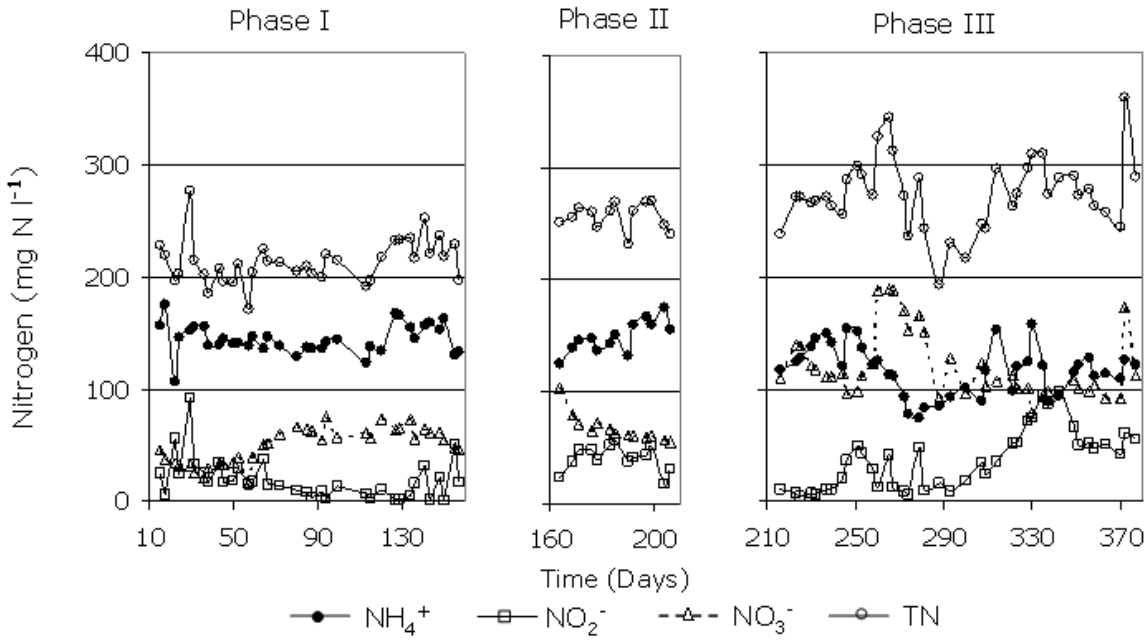

Figure 4-2. Nitrogen species concentrations of the pore water within the PFBR during the different experimental phases. (Influent concentrations: 156 mg NH_4-N l^{-1} and 58 mg NO_2-N l^{-1} for phase I; 156 mg NH_4-N l^{-1} and 112 mg NO_2-N l^{-1} for phase II; 156 mg NH_4-N l^{-1} and 149 mg NO_2-N l^{-1} for phase III).

Higher nitrite concentrations in the influent (phases II and III) did not change the results with respect ammonium removal rates but the plants were even more affected. Overall no stable ammonia removal rates were obtained under these different conditions during these three experimental phases.

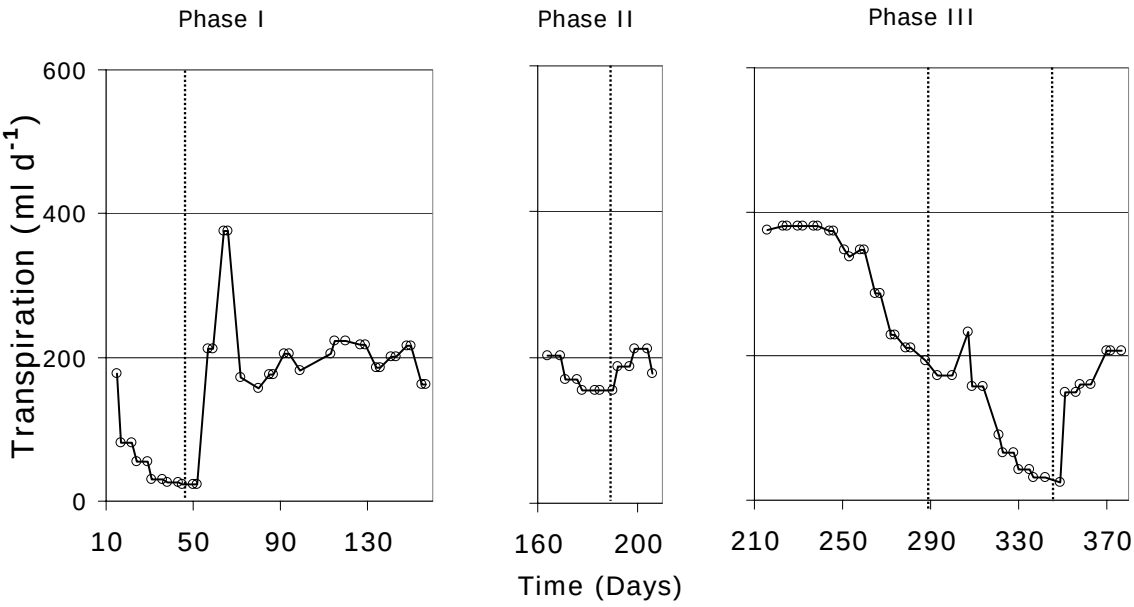

Figure 4-3 Plant transpiration rates during the different experimental phases of the PFBR (dotted lines show the re-plantation times).

There were small variations of the pH and redox potential (Figure 4-4) of the pore water within the PFBR during the different phases of the experiment. Dissolved oxygen concentration was very low (Figure 4-4). The mean oxygen concentrations (+/- standard deviation, µg l^{-1}) within the root-zone of the reactor were for phase I 8.6 +/- 6.2, for phase II 1.2 +/- 0.4, and for phase III 13.9 +/- 23.2. The redox (Eh) was in the range of 330 to 370 mV in these three phases. Although there was no organic carbon source in the influent, the effluent presented some organic carbon (5 to 10 mg l^{-1}). The highest values of total organic carbon (TOC) in the reactor were obtained when the plants were healthy (Figure 4-5).

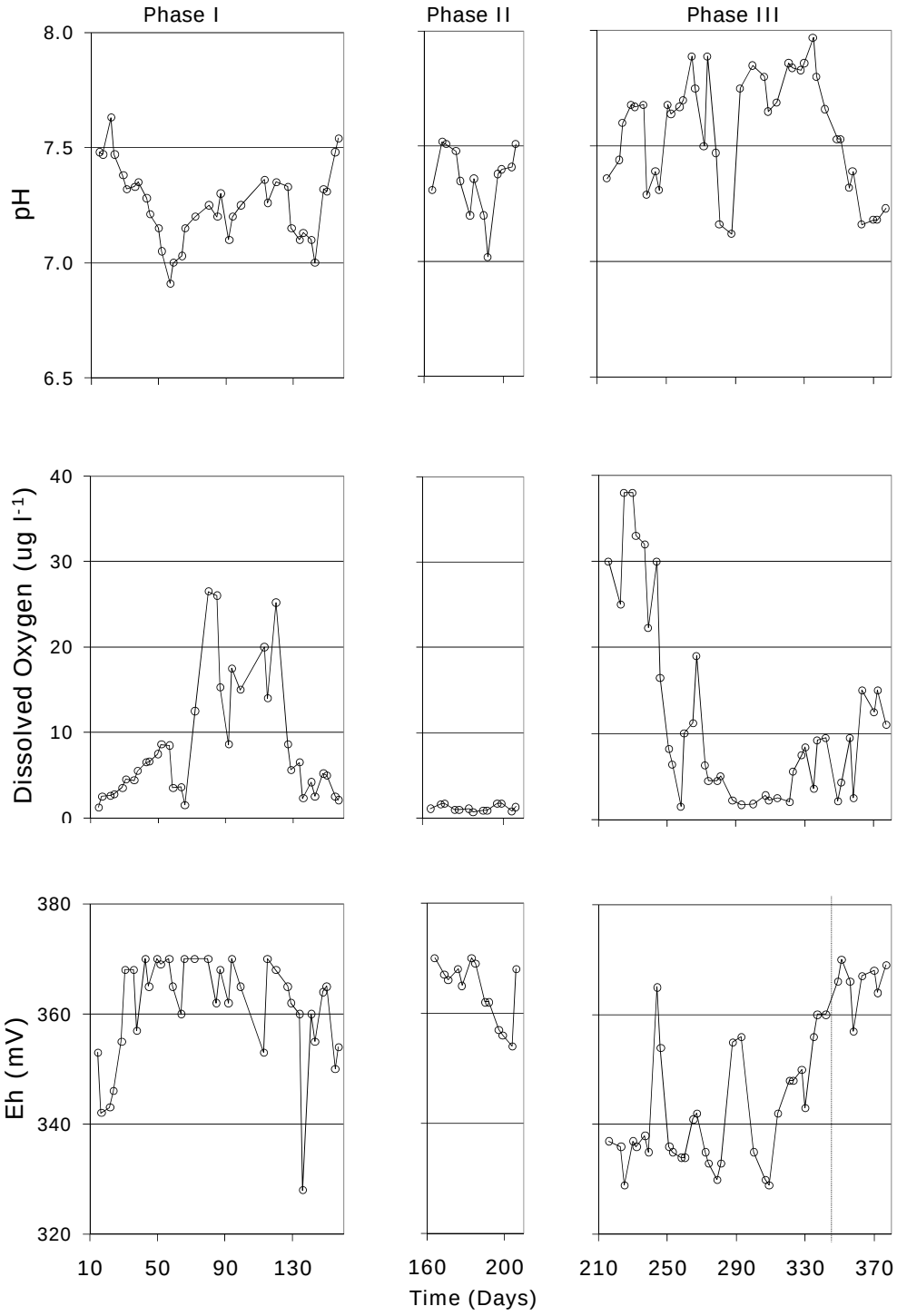

Figure 4-4 Variation of pH, dissolved oxygen concentration and redox potential of the pore water during the different experimental phases of the PFBR

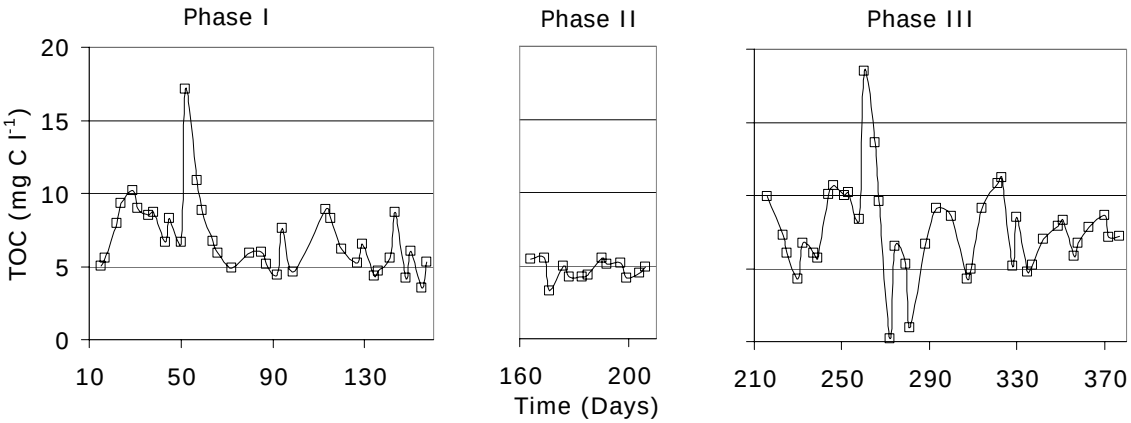

Figure 4-5 Total organic carbon (TOC) concentration of the pore water during the different experimental phases of the PFBR

Discussion

For a similar reactor working with *Juncus effusus* and synthetic domestic wastewater, an average transpiration rate of 532 ml d^{-1} has been reported (Wießner et al., 2005a). The obtained values in the experiments were lower and decreased within the time meaning that some toxic effect took place. Transpiration is controlled by plants and it is affected by environmental conditions. Water flow in plants is a passive process that occurs in response to physical forces, and it is also a stress response (Gruber et al., 2008) and it can be used as an indicator for phytotoxicity (Trapp et al., 2000). In general in all three phases plants were affected and at high concentrations of nitrite the toxic effects were irreversible.

But not only nitrite could produce a toxic effect on the plants. Higher sulphide concentration of 15 mg l^{-1} in wastewater produces toxicity to the plants (*Juncus effusus*) (Gonzalias et al., 2007), however, although the synthetic wastewater contained sulphate, sulphide formation can be discarded because the relatively high redox potential observed in the reactor (Figure 4.4).

The mass balance calculation based on the results of Figure 4-2 and 4-3 showed that the mean total nitrogen removal rates were 39.1 mg N d^{-1} (0.56 g N m^{-2} d^{-1} or 1.96 g N m^{-3} d^{-1}), 69.5 mg N d^{-1} (0.99 g N m^{-2} d^{-1} or 3.48 g N m^{-3} d^{-1}) for phases I and II, respectively (Figure 4-6). Apparently, in phase III there was a higher total nitrogen removal, however there were unstable conditions. The mean ammonium removal rates obtained in phases I and II (45 and 49 mg N d^{-1}, respectively) were similar to those reported by Wießner et al. (2005a) working with a synthetic domestic wastewater and using a similar reactor. He recorded an ammonia nitrogen removal rate of 49.5 mg N d^{-1} (0.71 g N m^{-2} d^{-1} or 2.34 g N m^{-3} d^{-1}).

Under conditions of domestic sewage treatment it is assumed that ammonia nitrogen is removed via the route of nitrification-denitrification. In subsurface flow wetlands, where direct oxygen diffusion through the upper soil layer is limited, the oxygen transport is done by the helophytes aerenchyme into the rhizosphere. The amount of oxygen released depends on the redox state of the rhizosphere, the illumination intensity and also on the plant species (Wießner et al., 2002a; Wießner et al., 2002b). *Phragmites australis* and *Typha latifolia* has been identified as the plants with the highest potential to release oxygen while *Juncus effusus* release around 50% less oxygen (Armstrong et al., 1990; Wießner et al., 2002b). Under these moderate oxidizing conditions of the pore water within the PFBR (see Figure 4-4) ammonia oxidation can be assumed. So it is generally accepted that at oxygen concentration lower than 0.2 mg l^{-1} microbial ammonium oxidation is faster than microbial nitrite oxidation (Wiesmann, 1994; Ciudad et al., 2005; Guo et al., 2009), but the obtained results with such low oxygen concentrations of the pore water within the PFBR, showed that both ammonium oxidation and nitrite oxidation (Figure 4.6) happen simultaneously. However transfer and turnover rates remain speculations.

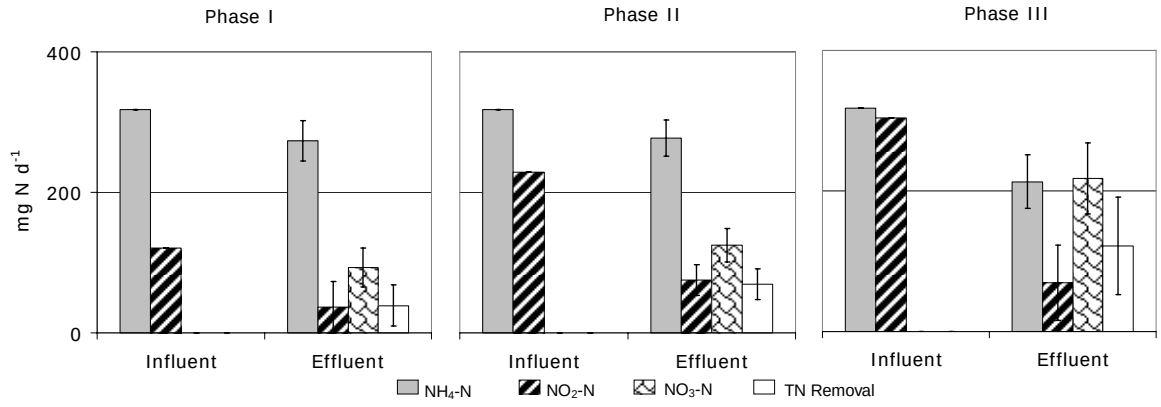

Figure 4-6 Loading rates of nitrogen species of the reactor influent and effluent and total nitrogen removal rates during phases I, II and III of the experiments.

Plants also have a carbon input into the rhizosphere through their rhizodeposition products (Farrar et al., 2003; Jones et al., 2004). The estimated TOC of the pore water within the PFBR (see Figure 4-5) could be either the result of carbon release by the plants or the presence of some dead bacterial biomass material.

However the amounts of oxygen and organic carbon released by the plants are not high enough that an ideal bi-phasal conversion of the nitrogen compounds of the model wastewater under the conditions of the PFBR via total oxidation up to nitrate and further denitrification of the nitrate by plant's rhizodeposition products can be imagined. So in general there are two limiting factors for nitrification-denitrification processes in the system: the availability of dissolved oxygen required for nitrification, and the accessibility of an organic carbon source required for denitrification.

At present it is not well understood which nitrogen transformation processes take place in especially such PFBR as well as in subsurface flow constructed wetlands in general.

It is still not clear if the ammonia is oxidized completely to nitrate or only to nitrite. In the case of denitrification via the nitrite route, several following potential electron donors are possible: the organic carbon compounds from the wastewater or the rhizodeposition products, some other compounds such as biogenic H_2S are very likely formed in anaerobic micro-niches, or even ammonia by itself can function as reducing agents for nitrite reduction (anaerobic ammonia oxidation) (Paredes et al., 2007a). In the specific case of sulphate reduction and sulphide production, the effects on plants and microbial activity have been already discussed (Gonzalias et al., 2007), Sulphide concentrations higher than 2 mg l^{-1} cause instabilities in sulphide and nitrogen removal processes in wetlands. Higher sulphide concentration than 10 mg l^{-1} causes plant toxicity and values higher than 15 mg l^{-1} exceed the tolerance capacity of *Juncus effusus* and made it inapplicable for wetland treatment (Gonzalias et al., 2007). The existing moderate oxidizing conditions in the PFBR (Figure 4-4) could discard the possibility of sulphide generation via sulphate reduction. Even if in some micro-niches low redox potential (-150 mV) were reached and sulphate reduction took place, by abiotic oxidation in the oxic zones sulphide could be oxidized to different sulphur species with a higher sulphur valency than -2 (Gonzalias et al., 2007).

Despite of the model wastewater composition with high concentrations of nitrite and ammonia, suitable for the anammox process, the anaerobic ammonium oxidation route was probably of less importance for nitrogen removal in the three experimental phases of the PFBR. Furthermore, with respect to the “traditional” pathways via ammonia oxidation and the reduction of either the nitrite or nitrate, the system was limited by both oxygen and organic carbon. In preliminary experiments using FISH (Fluorescence In Situ Hybridization) probes, anammox bacteria have already been observed in the reactor (data not shown), but only in very low numbers. Of course, it is assumed that the

anammox activity would feedback positively on increase itself through bacterial proliferation, but that would take many years (Hao et al., 2002a) because of their extreme slow growth rate.

4.3 Inoculation and adaptation of anammox activity in the PFBR - influence of plants and organic matter on nitrogen removal

After the characterization of nitrogen compound turnover under defined environmental conditions in the PFBR where the PFBR was fed in a sequence of model wastewaters with a constant ammonium and increasing nitrite concentrations (phase I, II and III; see chapter 4-2) it aroused the question if inoculation with active enrichment culture of anammox bacteria could accelerate the process of establishing a stable anammox bacteria population within the PFBR, even for longer periods.

As described in chapter 3.3.1 (Materials and Methods), in this experiment three PFBR and one unplanted fixed bed reactor were used; the running conditions for the different experimental phases (IV – VI) are listed in Table 3-6.

Figure 4-7 shows the development of the porewater concentration of the nitrogen species (ammonium, nitrite, nitrate and total nitrogen) for studied planted and unplanted reactors in the experimental phases IV to VI.

In general, ammonium nitrogen was transformed in all reactors during the different experimental phases. Neither nitrate nor nitrite was detected in high concentrations in reactor 1 (planted subsurface-flow reactor), however, high variation of ammonium and nitrate was observed in the reactor 2 (unplanted reactor). When the applied loads of ammonium and nitrite were increased in phase V, the ammonium concentration in the porewater had an increment (Figure 4-7), but it decreased after some time. A higher

applied load of nitrite (phase VI) produced similar effects. Very low values of nitrite and nitrate concentrations were detected at these phases in the planted reactor 1 and an increment of nitrate and ammonium concentrations was observed in the unplanted reactor 2 in phase IV.

The reactor 3, only fed with ammonium in the influent, showed high values of ammonium and nitrate in the effluent (Figure 4-7). Subsequent increment of ammonium load increased the concentration of both parameters (Phases V and VI). Nitrite was also detected in the effluent but in small values.

The reactor 4, working with similar conditions like rector 1 but with addition of organic carbon source, showed low and nitrate concentrations in the effluent. Successive increment of ammonium and nitrite loads in the influent (Phases V and VI) produced an increase of ammonium concentration in the effluent, but the nitrate and nitrite concentrations decreased over time. The addition of organic carbon caused a less variation in the concentration of nitrogen species in the effluent in comparison to the other reactors.

The plants of the reactors showed no toxic effects (expressed as a decrease of transpiration rate) during the experimental phase IV (Figure 4-8). Plants in case of reactor 1, fed with a mixture of ammonium and nitrite (NO_2-N 23 mg N l^{-1}, NH_4-N 117 mg N l^{-1}), and reactor 3 with only ammonium in the influent (NH_4-N 140 mg N l^{-1}) showed a high and increasing transpiration rate, reaching values above 50% of the influent flow rate (2 l d^{-1}). When the nitrite concentration was increased (phases V and VI) in reactor 1, toxic effects took place and the transpiration rate decreased. The toxic effects were stronger with higher nitrite and ammonium concentrations, especially in reactor 1 during phase VI (NO_2-N 91 mg N l^{-1}, NH_4-N 156 mg N l^{-1}) and the plants

died. In reactor 3, the transpiration rate was reduced at influent ammonium concentration of 269 mg N l^{-1}, however the values were still high (Figure 4-8). Plants of reactor 4 had a relatively constant transpiration rate along the time during the three phases.

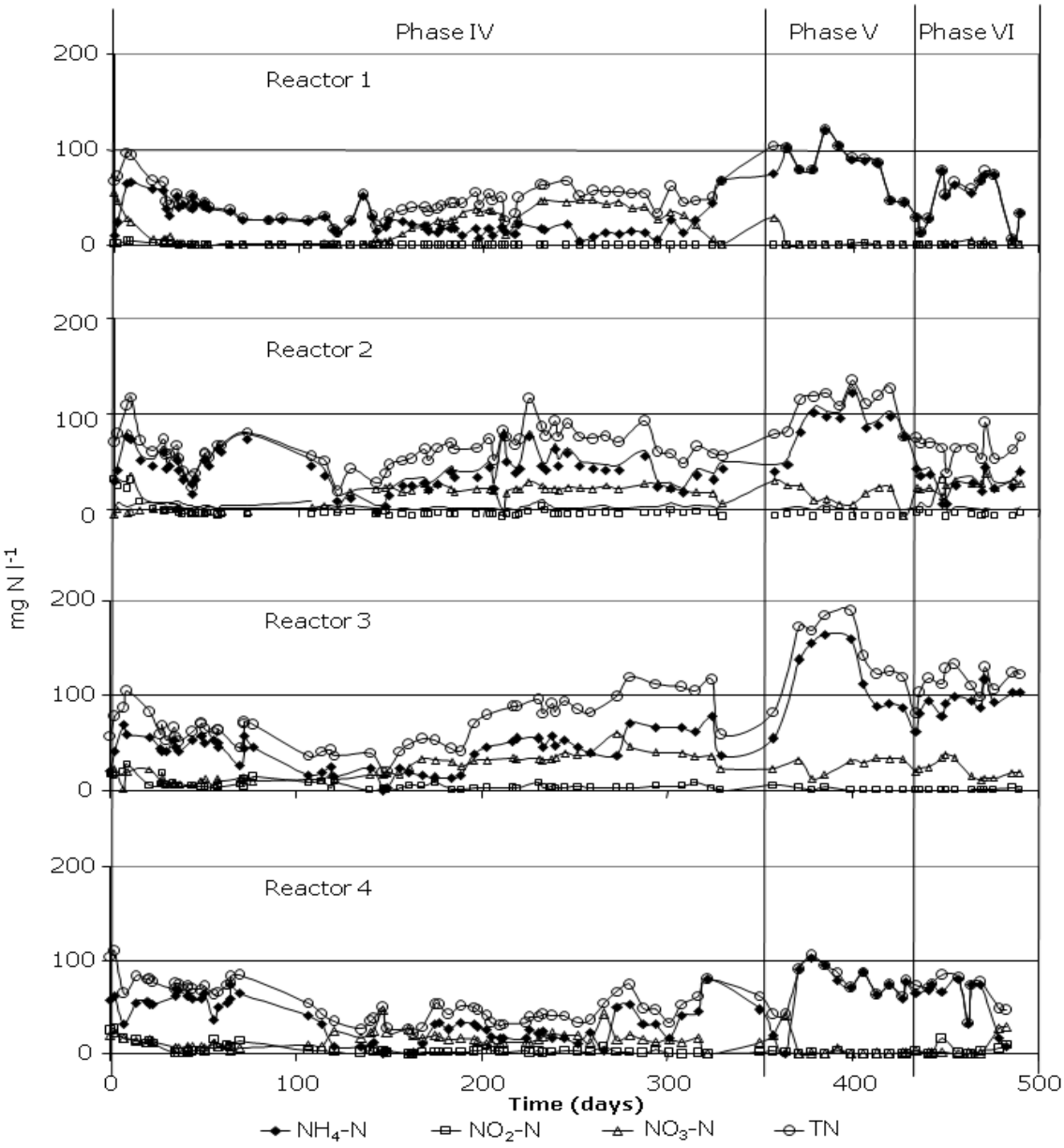

Figure 4-7 Nitrogen species concentrations in the porewater of the PFBRs during the different experimental phases (the compositions of the reactor inflow is listed in Table 3-6)

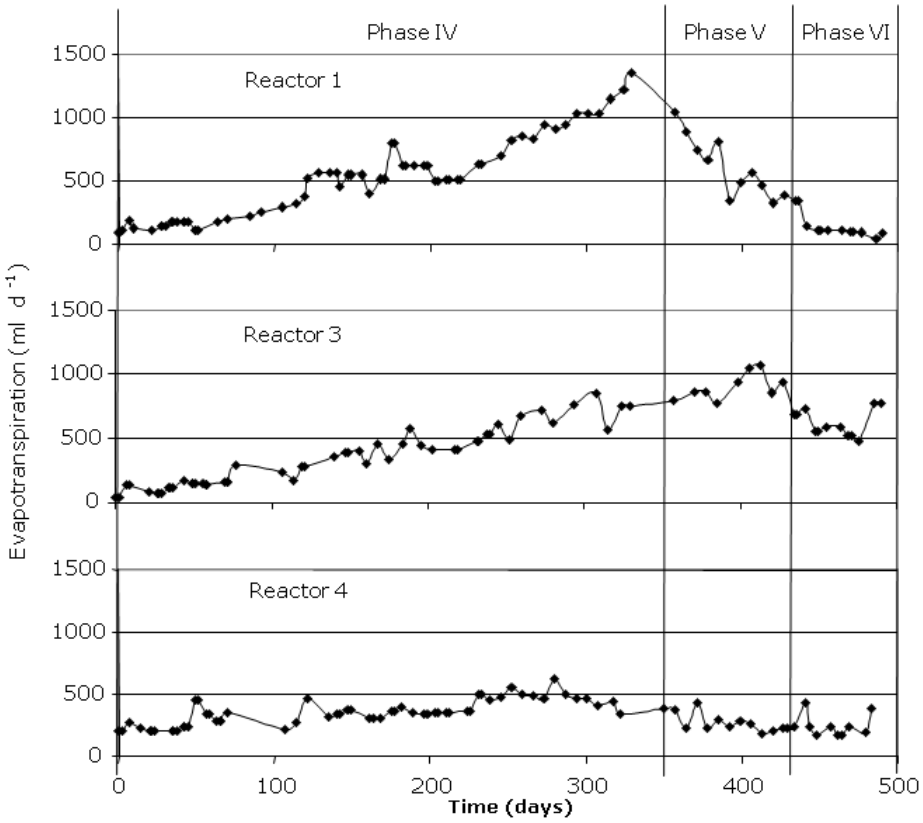

Figure 4-8 Plant transpiration during the different experimental phases, (evaporation rate of the unplanted reactor was about 8.4 ml d^{-1}).

The pH and redox potential values (Eh) within each reactor for the different experimental phases are shown in Table 4-2. In general the pH values were in the range between 6.5 and 7.6, with an average value around 7.3. The redox potentials were different for each reactor, and the reactor with organic carbon addition showed the lowest redox values. Reactor 1 and reactor 2 showed high differences in redox values between phases IV and V. Lower redox values were obtained in phase V, probably due to the increase of the ammonium concentration in the influent. In reactor 1 a toxic effect on the plants was probably caused by the increase of the applied nitrite load which caused their death with a subsequent release of organic carbon, reduction of the oxygen content and reduction of the redox potential up to -200 mV. In this point some sulphide

could be formed by sulphate reduction and the plants were likely more affected. In phase VI the redox increased again, perhaps because there was possibly not any more enough organic matter to keep this redox value on the same low level further more, however almost 90 % of the plants died mainly because nitrite toxicity. Sulphide toxicity is discarded because the oxidizing conditions of the reactor 1 (redox potential of 130 mV), however optimum redox conditions for sulphate reduction were present in reactor 4 (-345 to -377 mV) but plant transpiration remained constant along the experiments which is an indicator that there was no affectation on the plants.

Table 4-2 pH and redox values (Eh) of the porewater of the reactor during the different experimental phases.

	pH Values			Redox Values (Eh, mV)		
	Phase IV	Phase V	Phase VI	Phase IV	Phase V	Phase VI
Reactor 1	7.4 ± 0.1	7.1 ± 0.5	7.2 ± 0.2	326 ± 38	-200 ± 65	130 ± 212
Reactor 2	7.5 ± 0.1	7.5 ± 0.1	7.8 ± 0.1	326 ± 85	194 ± 43	180 ± 28
Reactor 3	7.1 ± 0.2	7.0 ± 0.2	7.5 ± 0.2	358 ± 82	354 ± 26	303 ± 21
Reactor 4	7.0 ± 0.3	7.6 ± 0.2	7.9 ± 0.2	-377 ± 128	-357 ± 138	-345 ± 122

Considering the plants transpiration values, the total and ammonium nitrogen removal were estimated for each reactor with time (Figure 4-9). For all reactors, with the exception of reactor 3, an increase of the nitrogen load (either ammonium or nitrite) caused an increase of the ammonium and total nitrogen removal rates. In the case of reactor 3, supplied with ammonium as the only nitrogen source in the influent, high total nitrogen and ammonium removal rates were also obtained in phase VI, however the oxygen input calculation was not enough to explain the high nitrogen removal.

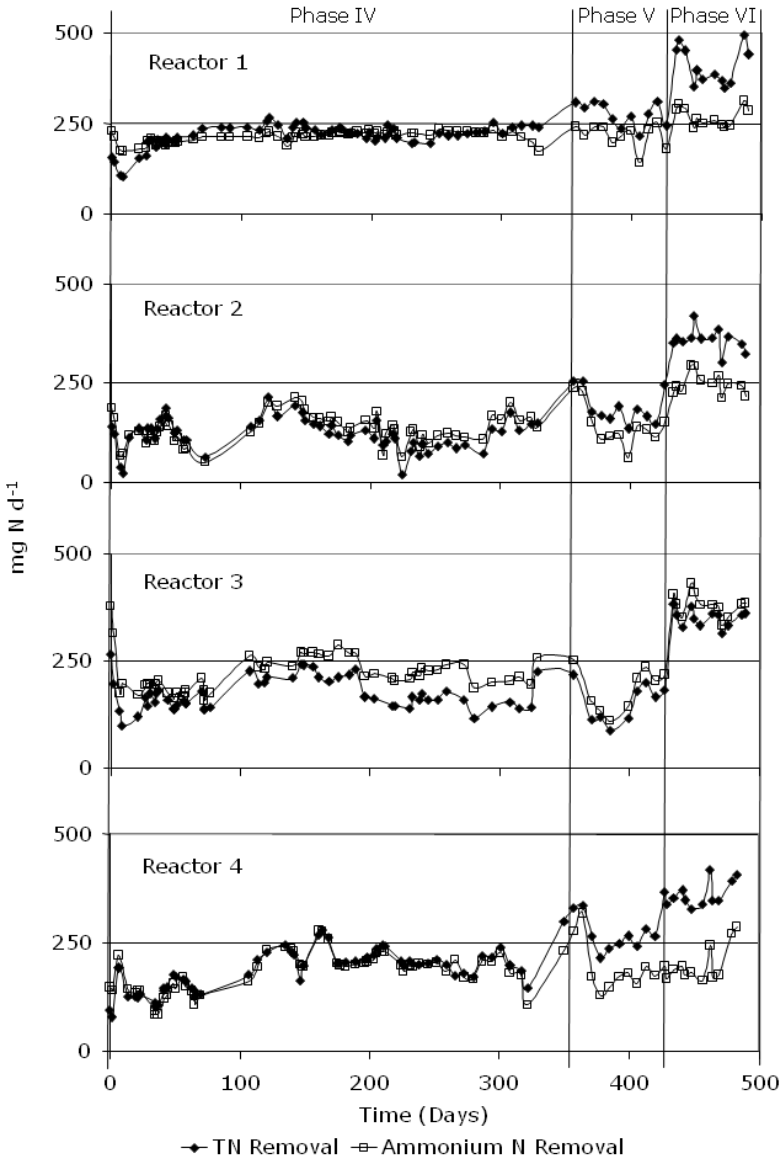

Figure 4-9 Total nitrogen (N) and ammonium nitrogen removal rates for the reactors during the different experimental phases.

The mean values and standard deviation of total nitrogen and ammonium nitrogen removal rates obtained (see chapter 3.8) for each reactor during the different experimental phases are shown in Table 4-3. A significant difference ($p < 0.05$) for total nitrogen removal between planted and unplanted systems, working with different ammonium and nitrite applied loads without any organic carbon source addition was

found for all three phases. A similar situation was found for ammonium removal for the phases IV and V. In the last phase, no significant difference was found between planted and unplanted systems for ammonium nitrogen removal rate, perhaps because there were no healthy plants left in reactor 1.

Table 4-3 Mean and standard deviation of total nitrogen and ammonium nitrogen removal rates obtained during the different experimental phases.

	Total N removal (g N m^{-2} d^{-1})			NH_4^+-N removal (g N m^{-2} d^{-1})		
	Phase IV	Phase V	Phase VI	Phase IV	Phase V	Phase VI
Reactor 1	3.1 ± 0.1	3.9 ± 0.1	5.8 ± 0.2	3.0 ± 0.03	3.1 ± 0.1	3.8 ± 0.1
Reactor 2	1.7 ± 0.1	2.7 ± 0.2	5.1 ± 0.1	1.9 ± 0.1	2.0 ± 0.2	3.5 ± 0.1
Reactor 3	2.3 ± 0.1	2.2 ± 0.2	5.0 ± 0.1	2.7 ± 0.1	2.6 ± 0.2	5.4 ± 0.1
Reactor 4	2.6 ± 0.1	3.9 ± 0.2	5.2 ± 0.2	2.6 ± 0.1	2.8 ± 0.2	2.9 ± 0.2

Similar analysis could be done between planted systems working with and without addition of organic carbon source, however, the effects of the addition of an organic carbon source did not have the same impact as the presence of plants. For phases IV and VI the reactor working without organic carbon source addition showed slightly higher both total nitrogen and ammonium nitrogen removal rates in comparison with the reactor working with organic carbon addition. In terms of percentage, the total nitrogen and ammonium removal rates ranged between 45 and 85%, however it is not possible to make a further analysis or comparison because the applied loads are different for each phase.

The total organic carbon content in the effluent of reactors 1, 2 and 3, which worked without organic carbon source addition, ranged between 5 and 10 mg l^{-1}. In the case of reactor 4, which was fed with acetate (60 mg l^{-1} TOC), the TOC concentration in the effluent ranged between 10 and 15 mg l^{-1} in all three phases.

During all phases of the experiments the alkalinity consumed in each reactor was measured and it was correlated with the total nitrogen removal (see Figure 4-10). It is well known that nitrification consumes alkalinity, and denitrification and anaerobic ammonium oxidation processes generate alkalinity. For phase IV (117 mg l^{-1} NH_4^+-N and 23 mg l^{-1} NO_2^--N) no significant difference was found in alkalinity consumption between planted reactors working without organic matter addition (Reactor 1) and with organic matter addition (Reactor 4). The alkalinity consumption was around 4 mg $CaCO_3$ per mg of total nitrogen removed. Higher ammonium and nitrite concentrations in the influent during phase VI (156 mg l^{-1} NH_4^+-N and 91 mg l^{-1} NO_2^--N) caused lower alkalinity consumption, ranging between 1 and 2 mg $CaCO_3$ per mg of total nitrogen removed.

The unplanted reactor had slightly higher alkalinity consumption in comparison with planted reactors fed with ammonium and nitrite, but lower than the planted reactor fed with only ammonium (reactor 3). It is clear that nitrification is an important factor for nitrogen removal and alkalinity consumption in that reactor, however a lower value of the theoretical rate (7.1 mg $CaCO_3$ mg^{-1} N) indicates that some other processes that can produce alkalinity are also taking place.

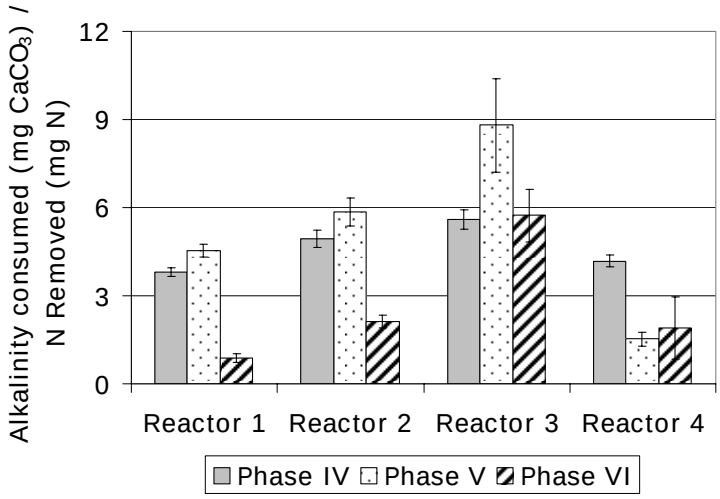

Figure 4-10 Ratio between alkalinity consumption (mg $CaCO_3$) and total nitrogen removal (mg N) for all reactors during the different experimental phases (Reactor influent composition see Table 3-6).

The conversion of hydroxylamine to hydrazine is an indicator that anammox activity is present (Jetten et al., 1998). Figure 4-11 shows the test results within experimental phase IV. In all four reactors, after the hydroxylamine addition, hydrazine was formed. Highest activity was observed in reactor 4 which had also the lowest redox potential of all four tested reactors (see Table 4-2).

The results of the serum bottle batch tests for anammox activity are summarized in Table 4-4. By this test 150 g of the gravel of the reactors were placed under anaerobic conditions into 200 ml serum bottles with nutrient solution containing nitrite and ammonium as described in the chapter Materials and Methods. The results were expressed as N removed per day per kg of gravel, so, with the total weight of the gravel used in the reactors (20 kg) it was possible to estimate the potential nitrogen removal by anammox process in each reactor during the different experimental phases.

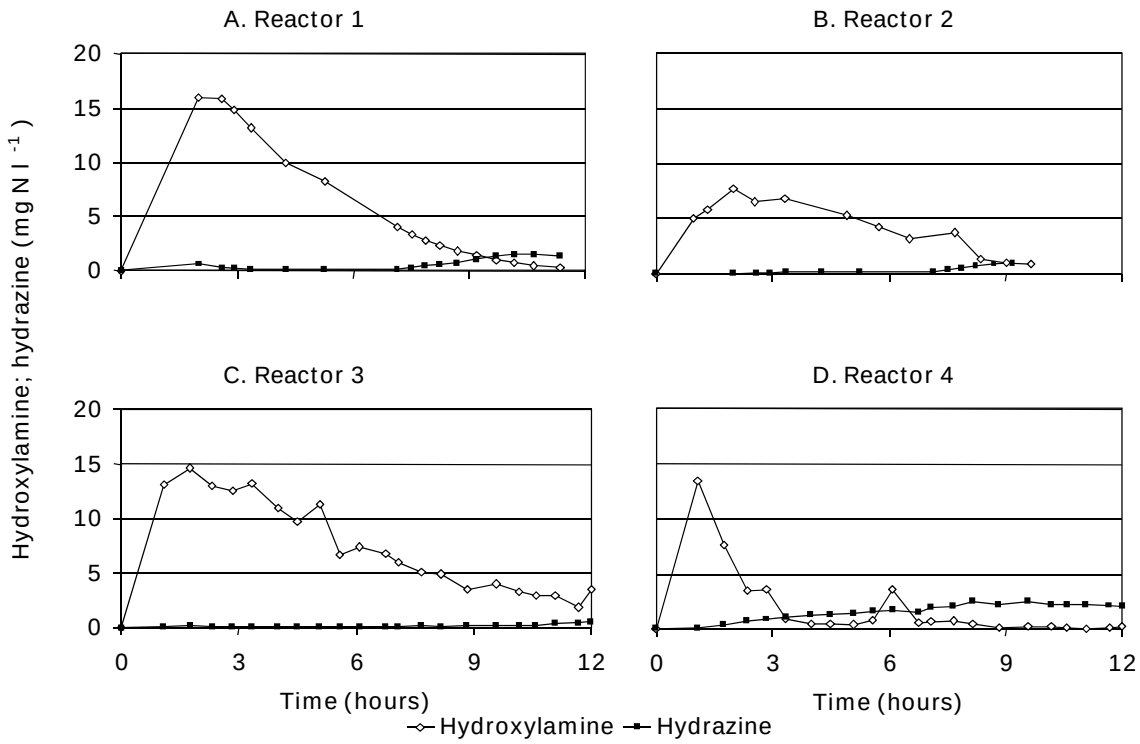

Figure 4-11 Hydroxylamine and hydrazine concentrations after the pulse addition of hydroxylamine in all four reactors during experimental phase IV

Table 4-4 Anammox activity and potential nitrogen removal by anammox process for the reactors during the different experimental phases.

Reactor	Anammox activity (mg N d^{-1} kg^{-1} gravel)			Potential N removal by anammox (g N m^{-2} d^{-1})		
	Phase IV	Phase V	Phase VI	Phase IV	Phase V	Phase VI
1 (Planted)	8.3	17.6	19.1	2.4	5.0	5.5
2 (Unplanted)	9.9	11.9	15.4	2.8	3.4	4.4
3 (Planted)	4.7	4.6	5.5	1.3	1.3	1.6
4 (Planted)	6.9	16.1	17.1	2.0	4.6	4.9

The existence of ammonium oxidizing bacteria belonging to β–proteobacteria was detected by a specific PCR using the primer CTO189f and CTO654r (see Table 3-9 for primer sequence). These results together with these of amoA specific PCR showed positive reaction in all reactors. Beside the FISH analysis to quantify anammox bacteria, a DGGE analysis was performed, showing that there was high bacterial diversity in the reactors (data not shown).

A nested PCR was performed to amplify specifically anammox bacteria using the primer Pla46f and Amx820r followed by a second PCR inside the amplicon using ana_f2 linked at the 5' end with GC-clamp and ana_r1. The gel showed only one strong band corresponding to the same melting domain. This leads to the assumption of the dominance of only one main anammox species inside the reactors.

The FISH analysis of biomass in the pore water and of biofilm samples on the gravel showed that anammox bacteria were present during all experimental phases (Table 4-5). Because the anammox bacteria were strongly associated in aggregates and applied attempts to separate them into single cells failed, it was impossible to count them in an accurate way. In phase IV, only reactor 2 (unplanted) showed a relatively high number and its number was higher in comparison to the ammonium oxidizers. The planted reactors (1, 3 and 4) had a similar or even lower number of anammox bacteria in relation to the nitrifiers. At higher nitrite inflow concentration, the relative amount of Anammox bacteria increased in the planted reactors 1 and 4, but number of nitrifiers remained at the same level.

Table 4-5 Relative abundance of ammonium oxidizers and anammox bacteria during the different experimental phases of the reactors. (reactor plantation and influent composition see Table 3-6)

Reactors		Phase IV				Phase V				Phase VI			
		Reactor				Reactor				Reactor			
		1	2	3	4	1	2	3	4	1	2	3	4
Nitrifiers	Biofilm	+	+	+	+	+	+	+	+	+	+	+	+
	Pore Water	+	+	+	+	+	+	-	-	+	+	-	-
Anammox	Biofilm	+	++	-	+	++	++	+	++	++	++	+	++
	Pore Water	+	+++	+	+	+++	++	++	++	++	++	++	++

Note: - not detected, + present, ++ present in relatively high number, +++ abundant

Discussion

The nitrogen removal rates obtained in the four reactors inoculated with active anammox biomass were, during all the three different experimental phases, higher than values reported by different authors working with similar but not especially inoculated reactors (Wießner et al., 2005b).

As it was shown in chapter 4.2, a similar reactor, which was not especially inoculated, a nitrogen removal rate ranging between 39.1 and 69.5 mg N d^{-1} (0.56 – 0.99 g N m^{-2} d^{-1}) for different applied load ratios of ammonium and nitrite was obtained.

The inoculation of active anammox biomass increased the total nitrogen and ammonium removal rates to values up to 400 mg N d^{-1} (equivalent to 5.7 g N m^{-2} d^{-1}), which is almost 10 times higher than values reported for SSHF CWs, (Kadlec et al., 2000; Kuschk et al., 2003). It is obvious that the presence of plants caused a higher removal rate. According with the data from Table 4-3, phase IV, around 1.4 g N m^{-2} d^{-1} (0.1 g N d^{-1}) more nitrogen was removed in the planted reactor (reactor 1) in comparison with the

unplanted system (reactor 2), however plant uptake was not the main reason. A typical value for nitrogen uptake by *Juncus effusus* has been estimated for optimum conditions in 0.22 g N m^{-2} d^{-1} (Kadlec et al., 2000) and the maximum value at growing season is suggested to be 0.33 g N m^{-2} d^{-1} (Kadlec and Wallace, 2009). These amounts are of great importance for very lightly loaded wetlands, but of no importance for heavily loaded wetlands. So, the main mechanism for nitrogen removal must be different.

With high nitrite influent concentration, the role of the plants became less important, mainly because of the toxic effects of nitrite (see Table 4-3 reactors 1 and 2, phases V and VI). Under those conditions it seems that microorganisms played the main role for total nitrogen and ammonium removal. An oxygen balance was done taking into account an oxygen diffusion rate of 7 g m^{-2} d^{-1} and an oxygen release rate of 0.25 mg O_2 $hour^{-1}$ $plant^{-1}$ which have been reported previously for such kind of reactor and *Juncus effusus* as a wetland plant (Wießner et al., 2002a, 2002b). The results of such balances (data not shown) indicated that there was not enough oxygen input for oxidation of all the removed ammonium. It can be assumed that some other processes than total nitrification-denitrification took place. Possible pathways for explaining the high ammonium removal rates include the conventional nitrification-denitrification process, and especially partial nitrification, denitrification via nitrite and anaerobic oxidation of ammonium.

“Conventional” complete nitrification to nitrate and denitrification process can happen in wetlands as has been described by different authors (Kadlec et al., 2000; Kuschk et al., 2003; Wießner et al., 2005b; Vymazal, 2007). But, low concentration of dissolved oxygen, the main condition for getting partial nitrification (Pollice et al., 2002; Ruiz et al., 2003; Ciudad et al., 2005), is common in horizontal subsurface flow wetlands and was also the situation in the model systems described in this paper.

In the reactors the dissolved oxygen ranged between 0 and 100 μg l^{-1} and was therefore much lower regarding the maximum reported values required for nitrite accumulation (< 1.4 mg l^{-1}) (Pollice et al., 2002; Ciudad et al., 2005; Guo et al., 2009). Strict anaerobic micro-zones, which are characteristic for subsurface flow constructed wetlands and supposedly also in the reactors, could be the basis for keeping alive the anammox bacteria in those systems working with relatively high redox potential (reactors 1, 2 and 3) in the pore water.

In the case of "conventional" complete nitrification and denitrification process and assuming carbohydrates for the organic compounds in the denitrification step, the following equations can be assumed:

Nitrification:

$$NH_4^+ + 2\ O_2 + 2\ HCO_3^- \rightarrow NO_3^- + 2\ CO_2 + 3\ H_2O \qquad (1)$$

Denitrification:

$$5\ C + 2\ H_2O + 4\ NO_3^- \rightarrow 2\ N_2 + 4\ HCO_3^- + CO_2 \qquad (2)$$

Nitrification-denitrification

$$4\ NH_4^+ + 8\ O_2 + 4\ HCO_3^- + 5\ C \rightarrow 2\ N_2 + 9\ CO_2 + 10\ H_2O \qquad (3)$$

These simple equations show that 1.25 mole carbon of a hydrocarbon (CH_2O) are required for each mole of ammonium removed by this "conventional" nitrification-denitrification process, which corresponds to 0.83 mg carbon per mg NH_4^+ removed. In

similar way the equations for nitritification and denitrification via nitrite can be defined and a requirement of 0.5 mg carbon per mg NH_4^+ removed can be calculated:

Nitritification:

$$NH_4^+ + 1.5\ O_2 + 2\ HCO_3^- \rightarrow NO_2^- + 2\ CO_2 + 3\ H_2O \quad (4)$$

Denitrification via nitrite:

$$3\ C + 2\ H_2O + 4\ NO_2^- + CO_2 \rightarrow 2\ N_2 + 4\ HCO_3^- \quad (5)$$

Nitritification-denitrification via nitrite:

$$4\ NH_4^+ + 6\ O_2 + 4\ HCO_3^- + 3\ C \rightarrow 2\ N_2 + 7\ CO_2 + 10\ H_2O \quad (6)$$

If nitrification-denitrification is the only pathway for nitrogen removal in the reactors, it is possible to estimate the required organic carbon for each unit (see Table 4-6). In our experiments, reactor 4 was the only one that was fed with the same amount of organic carbon source (acetate with an atomic ratio like a hydrocarbon, equivalent to 60 mg l^{-1} TOC, or 120 mg TOC d^{-1}) during all the different experimental phases, and the organic carbon required for denitrification is higher than that amount added, even if the organic carbon added is not used by other non-denitrifying heterotrophic microorganisms. However denitrification can also happen with nitrite, in this case, less organic carbon is required (Table 4-6). Plants also release organic carbon into the rhizosphere through their rhizodeposition products (dead plant matter and root exudates), which could also be used as electron donors.

Table 4-6 Theoretical organic carbon need (calculated as hydrocarbon) for "complete" nitrification-denitrification and nitritification-denitrification via nitrite pathways for ammonium removal during the different experimental phases of the reactors.

	Organic carbon requirements (mg C d^{-1})					
	Nitrification-denitrification route			Nitritification-denitrification via nitrite		
	Phase I	Phase II	Phase III	Phase I	Phase II	Phase III
Reactor 1	175.8	180.8	224.2	105.9	108.9	135.0
Reactor 2	100.8	118.3	206.7	60.7	71.3	124.5
Reactor 3	158.3	154.2	310.5	95.4	92.9	187.0
Reactor 4	150.7	163.0	168.2	90.8	98.2	101.3

In those planted systems, if denitrification happened, the organic carbon could come from the plants. The amount of the carbon inputs has been associated with plant growth and it is affected by several factors including the presence of biotic and abiotic stresses (e.g. nutrient deficiency, hypoxia, pathogen attack, and drought) among others. In the same way the composition of the exudates changes in accordance with the existing environmental factors in the roots (Jones et al., 2004).

How large is the carbon flux from the roots is a question that is not totally clear, however for soil-based plants, it has been estimated that approximately 5 – 10% of the net carbon fixed by photosynthesis is lost by root exudation (Jones et al., 2004), and some experiments performed in hydroponic cultures showed that only between 0.5 – 1.5% of fixed carbon is lost (Farrar et al., 2003). Carbon fixation by wetland plants depends on different aspects including plant species, season of the year, nutrient availability, etc. (Brix et al., 2001). It has been estimated that *Phragmites australis* and *Juncus effusus* can fix, in summer season, around 9.6 g C m^{-2} d^{-1} and 5.7 g C m^{-2} d^{-1}, respectively (Brix et al., 2001; Stanley et al., 2003). Considering the planted surface

area of the reactor, the carbon fixed by *Juncus* could be estimated in 399 mg C d^{-1}, meaning that a maximum of 39 mg C d^{-1} (assuming a release of 10% of the total carbon fixed) was available for denitrification, which is quite low compared to the required amounts for complete denitrification (Table 4-6). It is clear that, for those planted reactors working without organic carbon addition, complete denitrification could take place but it was not the main mechanism. Nitrogen removal by plant uptake was also not the main mechanism. For *Juncus effusus* a maximum nitrogen plant uptake value of 0.219 g N m^{-2} d^{-1} (800 kg N ha^{-1} a^{-1}) (Kadlec et al., 2000) has been reported, which is less than 10 % of the total nitrogen removal rates obtained in the planted reactors (see Table 4-3). Because the unplanted reactor 2 without addition of any organic carbon source showed also high nitrogen removal rates, it can be concluded that the main mechanism for explaining the high nitrogen removal rates in all four reactors obtained during the experiments was the anaerobic ammonia oxidation. So, conventional denitrification and plant uptake could take place but played a minor role.

The presence of organic carbon has a big influence on the nitrogen removal processes. In one hand it is required for denitrification processes but, on the other hand it can function also as a competitor for the anaerobic oxidation of ammonium. Anammox have been described as strictly autotrophic, fixing CO_2 with nitrite as the electron donor, leading to the anaerobic production of nitrate (Van de Graaf et al., 1996; Strous et al., 1999a). However, some surveys done with enrichment cultures showed that anammox bacteria could compete successfully with heterotrophic denitrifiers for propionate and, the presence of acetate did not change the stoichiometry of the anammox process (Güven et al., 2005). This is an important aspect for the anammox processes in SSHF CWs working with both organic carbon and high nitrogen loads. Under anaerobic conditions, that are typical in subsurface flow constructed wetlands, the organic carbon

degradation could produce fatty acids. Also it has been probed that anammox and denitrification processes can take place simultaneously in the presence of organic carbon. COD concentration was found to be a control variable for process selection: COD concentrations higher than 100 mg l^{-1} causes a gradual reduction of anammox activity and COD values of 242 and 300 mg l^{-1} anammox communities were inactivated or eradicated (Chamchoi et al., 2008; Molinuevo et al., 2009) and denitrification was the main removal mechanism.

The results of alkalinity measurements also support the strong indication of the anammox process in the reactors for nitrogen removal, especially in the last phases. Nitrification and nitritification processes have the same alkalinity consumption because further bicarbonate is not required for nitrite oxidation to nitrate. If ammonium is oxidized to nitrite or nitrate, the alkalinity consumption should be 7.14 mg $CaCO_3$ per mg of N removed (Azevedo et al., 1995). Denitrification process produces half of the alkalinity consumed by nitrification (Equations 2 and 5), so the overall process nitrification/nitritification–denitrification either via nitrite or nitrate will consume 3.57 mg CaCO3 per mg of NH_4-N removed. On the other hand, if the anammox process is the main and only mechanism for nitrogen removal, and nitrite is already present, alkalinity consumption should be around 0.22 mg $CaCO_3$ per mg of N removed. With the exception of reactor 3, which was fed only with ammonium, the detected values in our experimental reactors in the phase IV (Figure 4-10) were slighly higher to the reported data for CANON systems (3.1 to 3.4 mg $CaCO_3$ mg^{-1} N removed) and complete nitrification-denitrification process (Ahn et al., 2004).

Regarding the planted reactor fed with just ammonium, the alkalinity consumption was not so high like the demand for nitrification, but higher than the reported consumption

for nitrification-denitrification process including CANON. This suggests that perhaps partial nitrification and anammox process happened simultaneously.

The results obtained during the different experimental phases, showed that higher nitrite concentrations in the influent of the systems (phase VI) decreased the alkalinity consumption, meaning that, in the case of our model SFCW systems, anaerobic oxidation of ammonium became the main pathway for the nitrogen removal process coupled with partial nitrification at high nitrite and ammonium concentrations (1:1.7 for NO_2-N : NH_4-N). Lower ammonium and nitrite concentrations and low nitrite to ammonium ratios in the influent (1:5 for NO_2-N : NH_4-N) did not affect the anammox bacteria, but their role seems to be less important.

The assay of hydrazine formation from hydroxylamine is a strong indicator for anammox activity. Anammox bacteria are the only known bacteria that are able to produce hydrazine as a metabolite (Jetten et al., 1998). During the experiments with the addition of hydroxylamine into the reactors, hydrazine was produced and the reactor with stronger reducing condition got the highest hydrazine concentration (Reactor IV with addition of organic carbon source), however not all hydroxylamine was transformed to hydrazine or at least it was not detected perhaps because of the high metabolic turnover rates.

The findings from molecular biology tests fit with the obtained results of the anammox activity tests. Anammox bacteria were found in both biofilm and pore water, and their relative amount increased with higher nitrite loads in the influent. In the case of those reactors fed with a mixture of ammonia and nitrite, the anammox activity increased with higher nitrite concentrations. However it was lower and remained more or less constant in the planted reactor working with only ammonium. An increase of ammonium load in

this reactor did not increase the anammox activity. The addition of organic carbon, especially acetate, apparently did not have a big influence on anammox activity; this result is congruous with some reports. Guven, et al. (2005) reported that anammox bacteria were inhibited by methanol while organic acids were converted by them and concluded that anammox bacteria have a more versatile metabolism than previously assumed.

Nevertheless the addition of organic carbon source influenced the redox potential. In the reactors without addition of organic carbon source (reactor 1 and 2) the redox potential in the pore water ranged at a relatively high level between 100-330 mV. But in reactor 4 (with additional organic carbon source) the redox potential was considerably lower (Eh ≈ -350 mV) and low concentrations of sulphide (< 1 mg l^{-1}) were detected (data not shown) and not toxic effects were expected on plants. The toxicity for *Juncus effusus* has been reported to be higher than 10 mg l^{-1} (Gonzalias et al., 2007). In general, the presence of sulphur compounds and their interactions with the nitrogen cycle and its application for nitrogen removal processes in wetlands is an interesting topic that should be studied in more detail, because sulphide could be toxic for microorganisms and plants, furthermore sulphur compounds also play an important, not yet well known role, in the microbial processes within the rhizosphere (Wießner et al., 2005b, Gonzalias et al., 2007).

It seems that oxidative and reductive pathways for nitrogen removal were happening simultaneously in the reactors and the interactions between them were very complex. For instance, partial nitrification could produce nitrite that together with the added nitrite can be metabolised by the anammox bacteria, but also the produced and added nitrite can be used for denitrification directly via nitrite.

It is quite difficult to predict the contribution of each pathway to the total nitrogen removal, and even the use of labelled ammonium and nitrite could not help to clarify this aspect. Several assays with $^{15}NH_4$ were done during the different phases, but it was impossible to differentiate which amount of nitrogen was removed by nitrification-denitrification or anammox pathways (data not shown). Via nitrification, the labelled ammonium was converted to $^{15}NO_2^-$ and mixed with the unlabelled NO_2^-. At the end, with the exception of $^{28}N_2$, which could be produced by the nitrification-denitrification process of the unlabelled ammonium only or for denitrification via nitrite, $^{29}N_2$ and $^{30}N_2$ could be produced by the nitrification-denitrification pathway via nitrate or nitrite, and it could be also produced by anaerobic oxidation of ammonium.

The potential anammox activity (in separate batch tests) can give an idea about the importance of this pathway, but it cannot be used for an accurate estimation of its contribution to the total nitrogen removal. For instance, in the case of reactor 3, fed with ammonium only and without addition of organic carbon source, the total nitrogen removal in experimental phase 1 was 3.1 g N m^{-2} d^{-1} (Table 4-3) while the obtained potential nitrogen removal rate by anaerobic ammonium oxidation was 2.4 g N m^{-2} d^{-1} (Table 4-4). If we consider that for the anammox activity, both nitrite and ammonium are needed, and the consumption of ammonium and nitrite follows a molar ratio of 1 to 1.3 (Jetten et al., 2001), it can be concluded that partial nitrification and anammox were the main mechanisms for nitrogen removal in this reactor. It means, nitrite should be formed in any way and then it can be metabolized by anammox bacteria. For the unplanted reactor 2 during the phase I the potential nitrogen removal estimated by potential anammox activity was higher than the value obtained in the reactor. In this case, probably nitrite was the limiting factor. Indeed, in subsequent phases, with higher nitrite concentrations in the influent, the nitrogen removal rate increased and during the

last phase III it was higher in comparison to the estimated potential nitrogen removal by anammox.

4.4 Stability of partial nitrification in vertical down flow columns at laboratory-scale

Partial oxidation of ammonium to nitrite is one main prerequisite for establishing nitrogen removal from wastewater via the anammox pathway. Because in SSHF CW the oxygen input is limited it arouses the interest to investigate the possibility in a simple pre-step treatment to realize partial nitrification in an ammonium rich model wastewater.

By that the use of partial nitrification pre-treatment step should be evaluated. For that purpose different options for the inhibition of nitrite oxidation, including water level (as a way for controlling oxygen input), temperature and salinity, were tested in vertical down flow filters working with two different materials: gravel and sand (see chapter 3.4). As it was already mentioned in chapters 2.3.1.1 and 2.3.1.2, there are different options for controlling partial nitrification. Low dissolved oxygen, pH and temperature have been the most used strategies, however they have been mainly applied in suspended biomass systems and few experiments have been carried out in fixed biomass systems.

Before to start the description of the obtained results, it is important to remark that the experiments were designed with the following assumptions:

- If there is not enough alkalinity, the pH will drop down and an inhibition of nitrite and ammonium oxidizing bacteria will be produced. At the end a mixture of ammonium and nitrite will be obtained.

- The oxygen input into the system can be controlled by controlling the water or saturation level in the column. Thus, a third part of the column is water free and it works in a similar way like a trickling filter. The remaining 2/3 parts of the column are full of water and it works as an anaerobic filter.

- Temperature plays an important role in the partial nitrification process and it is already a key factor for some developed technologies based on suspended biomass and low hydraulic and biomass retention times (SHARON process). It is expected that it also plays a rol in fixed biofilms systems where the biomass retention time is different and higher in comparison to the hydraulic retention time.

- Not plants are used because high nitrite accumulation could be obtained and plants will die of toxic effects.

- Some ammonium-rich wastewaters contain high salt concentrations, so, the salinity could also affect the partial nitrification process.

According with the results, in general only few variants of columns showed low long term stability of partial nitrification. In principle, temperature has a direct impact on the process. Despite the fact that high temperature causes for ammonium oxidizing bacteria a higher growth rate than for nitrite oxidizing bacteria in ideal mixed reactor systems without biomass fixation, the attached biomass based systems, like the ones used in our experiments, showed a different tendency. In columns working with sand as a filter

material (Figure 4-12), those units with a temperature of 26ºC and 36ºC showed a full nitrification after 100 days and 200 days, respectively. However, the column at 18 ºC showed a stable nitrite yield during all time.

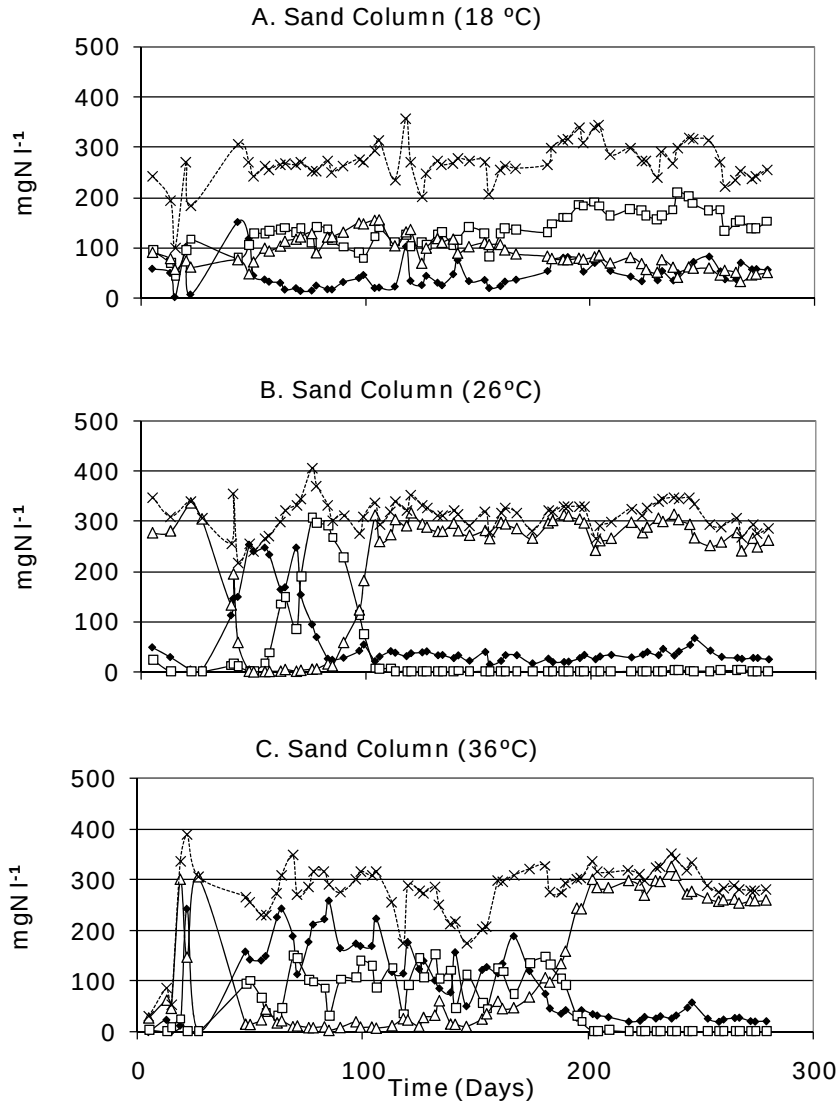

Figure 4-12 Nitrogen species in vertical down filter effluents working with sand as filter material at different temperatures

—●—NH_4-N —□—NO_2-N —Δ— NO_3-N ·····x···· Total N

Making a mass balance for all ionic nitrogen species in the effluents within the first 100 days of the experiment, it is possible to estimate that the column at 18 ºC had a nitrite production higher than 30% (regarding the applied nitrogen), however, in the last 100

days the effluent of this column showed 20% of the applied nitrogen was transformed to nitrate, 60% was converted to nitrite and the remaining 20% kept as ammonium (Figure 4-13A).

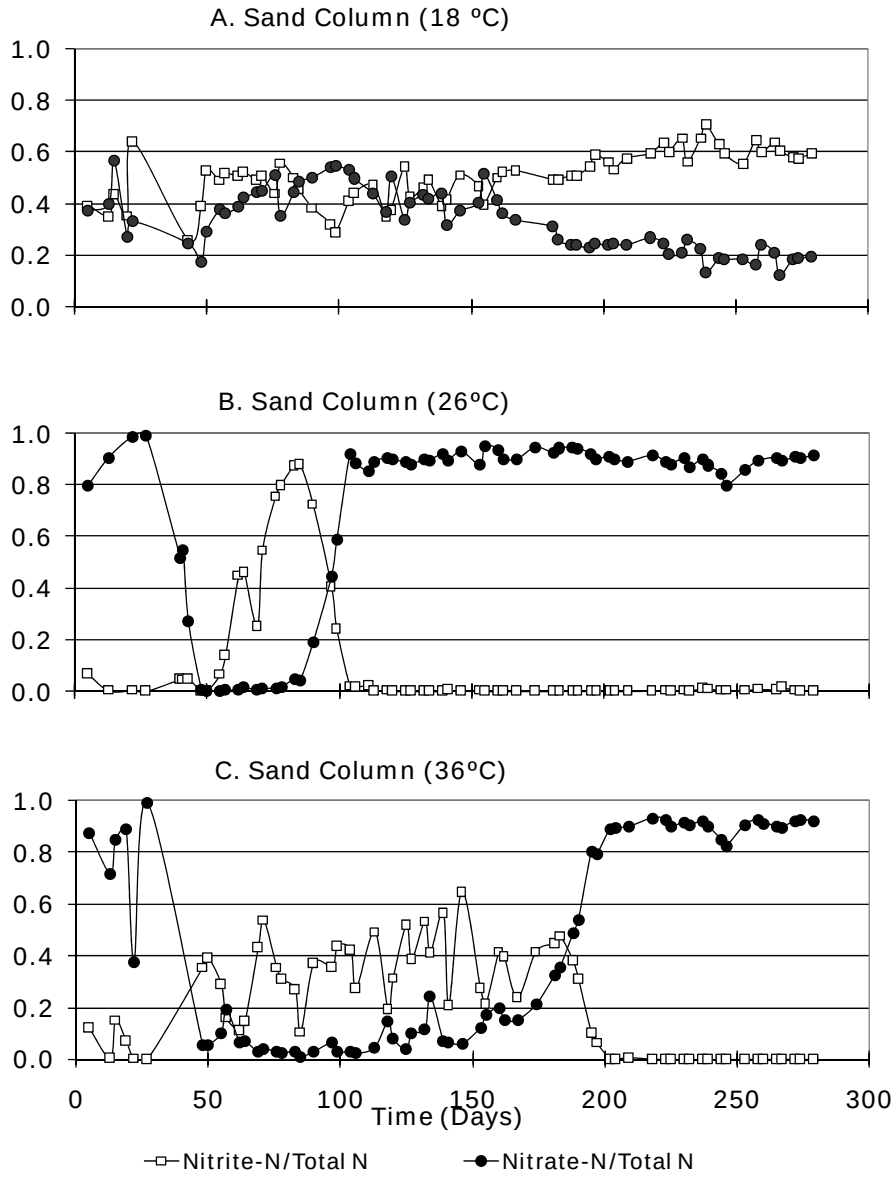

Figure 4-13 Ratio of nitrite/Total nitrogen and nitrate/Total nitrogen for effluents of the columns working with sand as filter material at different temperatures

At 26 ºC within the first 100 days of the experimental period there was some nitrite production but after 100 days full nitrification happened (Figure 4-13 B). At 36 ºC a similar situation was found, nitrite production up to 200 days and full nitrification

afterwards happened (Figure 4-13 C). At the end, those columns working at 26 ºC and 36 ºC, 10% of the applied nitrogen remained as ammonia and the other 90% was totally oxidised to nitrate.

The pH behaved according to the reaction took place within the columns, thus the effluents with higher ammonium conversion to nitrate showed lower pH values. The pH of the influent of the columns was very close to 8.5 (Figure 4-14). The column at 18ºC had an effluent with pH values ranging between 6.0 and 7.5, while columns working at 26 ºC and 36 ºC (Figure 4-14) pH values reached below 4.0. The lowest values of pH were obtained after 150 days.

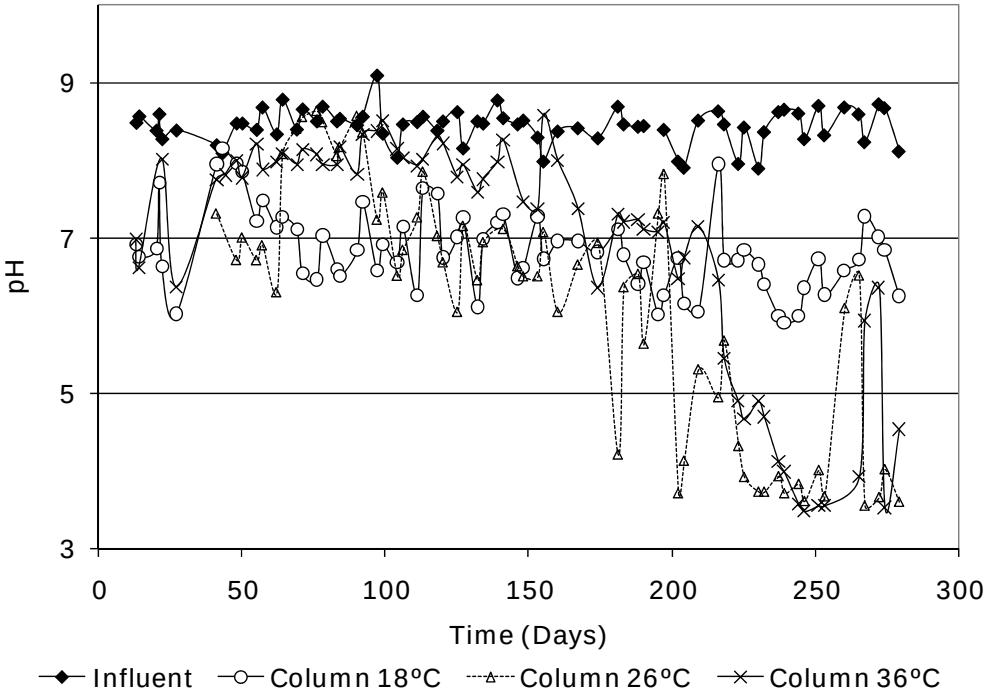

Figure 4-14 pH values of influent and effluents of the columns with sand as filter material at different temperatures

In a similar way to the columns with sand, the columns with bigger filter material (either gravel or expanded clay) showed after 100 days long-term stability at 26 ºC and

36 ºC for complete nitrification and a relatively stable partial nitrification at 18 ºC (Figure 4-15).

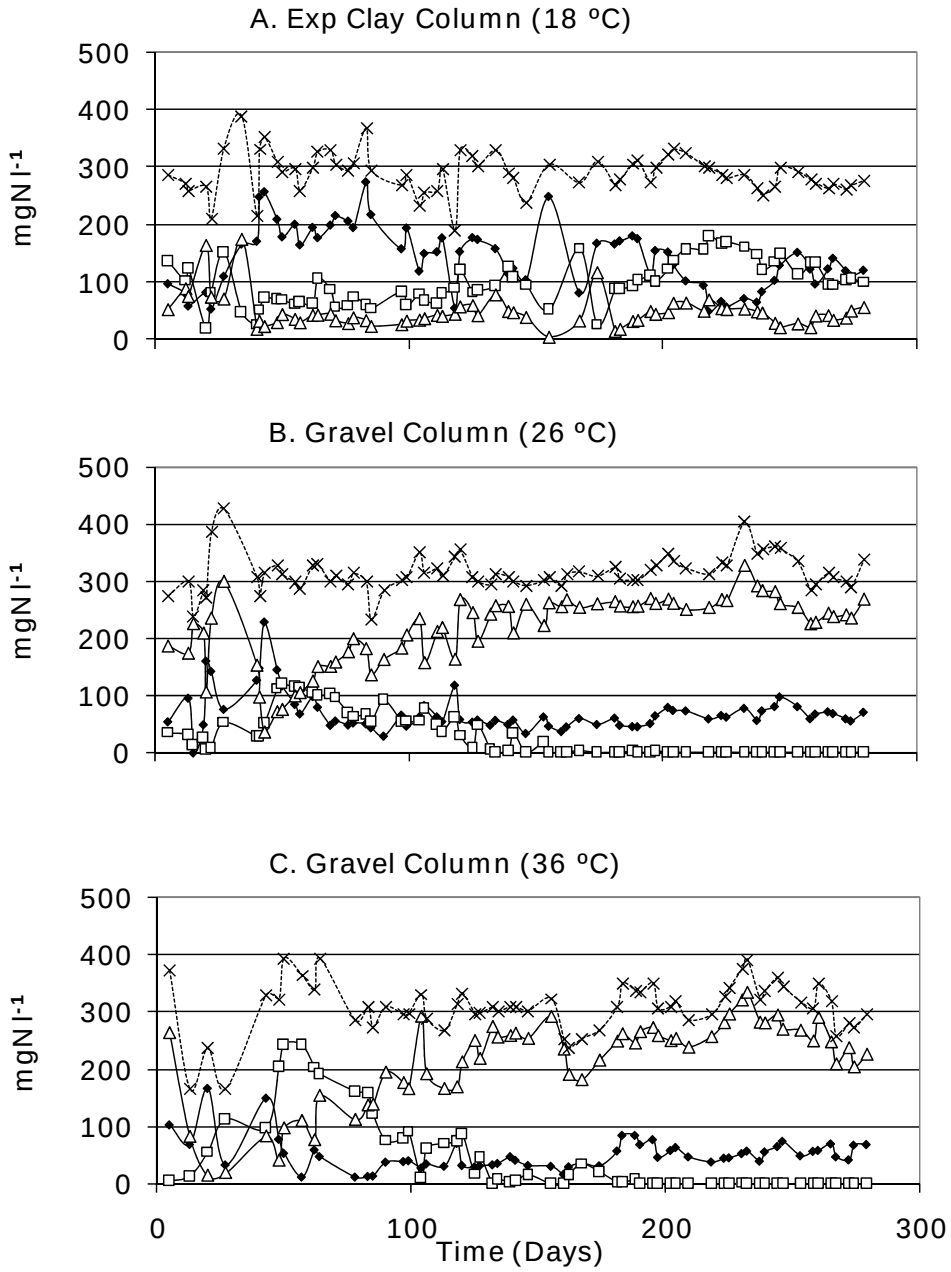

Figure 4-15 Nitrogen species in the effluents of the vertical down filters with gravel-expanded clay as filter material at different temperatures

—●—NH_4-N —□—NO_2-N —Δ— NO_3-N ·····x···· Total N

Considering the nitrogen mass balance, at 18 ºC, 36% of the applied ammonium was partially oxidised to nitrite, 16% was oxidised to nitrate and 48% of the applied nitrogen remained as ammonium, (Figure 4-16 A).

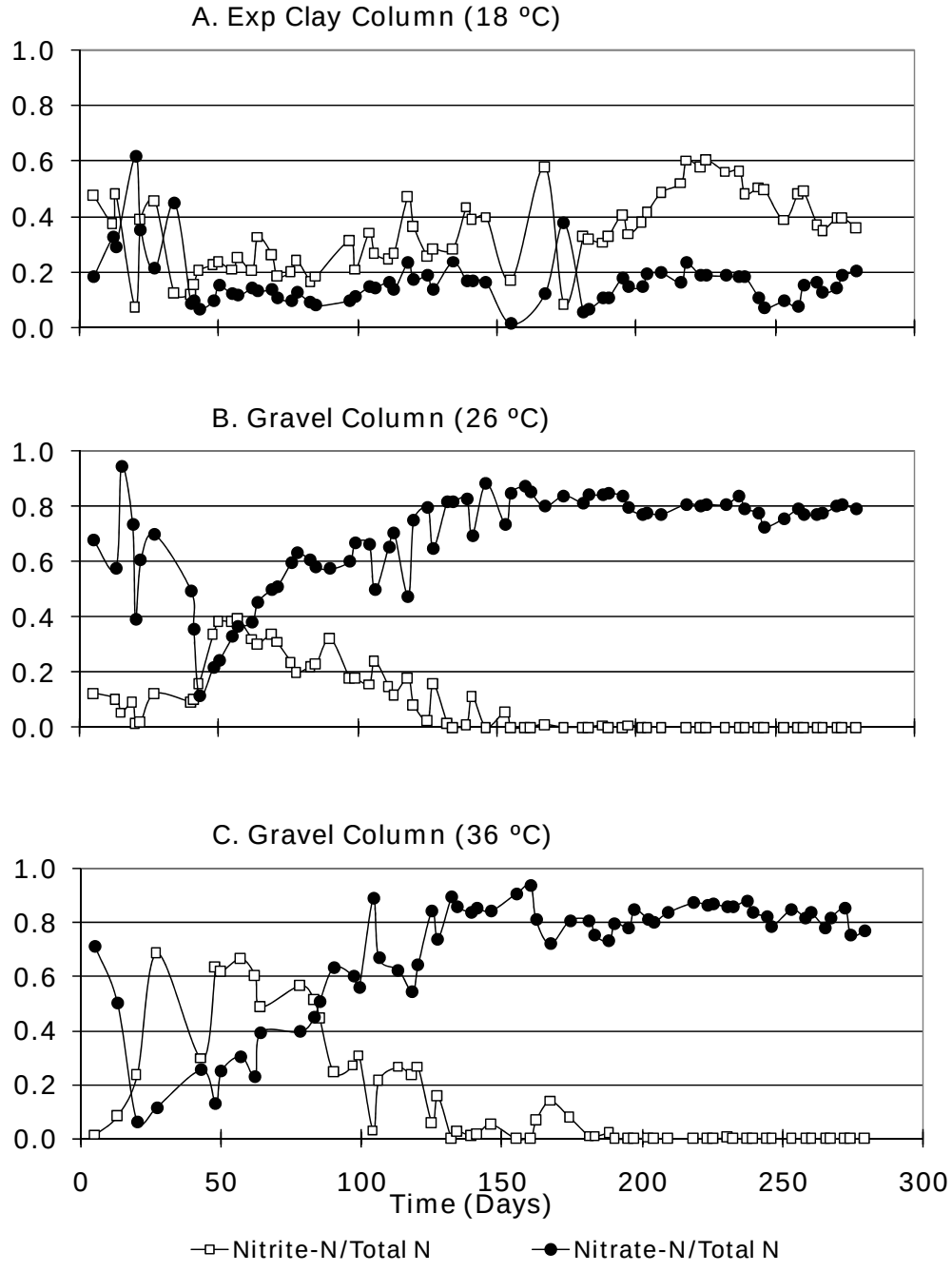

Figure 4-16 Ratio of nitrite/Total nitrogen and nitrate/Total nitrogen for columns effluents with gravel as filter material at different temperatures

In the columns at 26 ºC and 36 ºC (Figure 4-16 B and C) there was some partial nitrification in the first 120 days, however, full nitrification gradually increased and took place after 120 days. In the last 100 days of the experiments, 20% of the applied nitrogen remained as ammonia and the rest (80%) was totally oxidised to nitrate.

The pH values of the columns with bigger sized filter material showed also a similar tendency like the obtained values with sand columns. At 18 ºC the pH of the effluent decreased a little in comparison with the pH of the influent (Figure 4-17), however at higher temperatures the pH of the effluent decreased along the time and reached values below 5.0 for the column at 26 ºC, and around 5.0 for the column at 36 ºC (Figure 4-17). Again, lower pH values were obtained in case of higher ammonium conversion to nitrate.

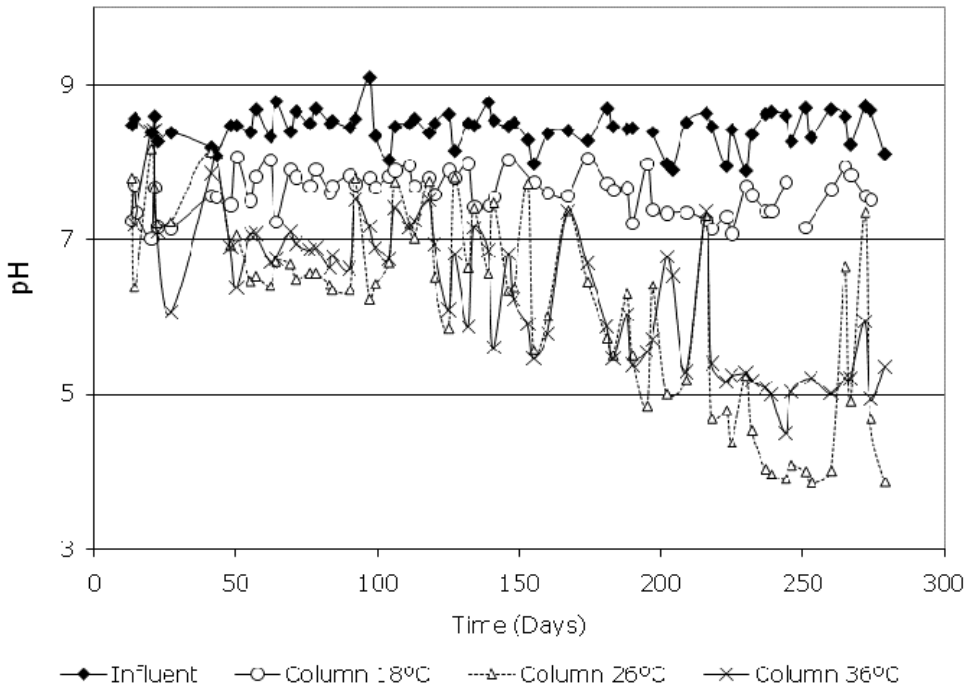

Figure 4-17 pH values of influent and effluents of the vertical columns with gravel as filter material at different temperatures

The effects of temperature on the stability of partial nitrification is not so clear when there is salt (22.72 g l^{-1}) in the influent as can be observed in the Figure 4-18. Both columns at 18 ºC and 36 ºC showed an effluent with relative stable nitrite concentration, but the system at 26 ºC showed high nitrate concentrations after 10 days of starting the experiments.

The obtained results from the nitrogen mass balance (Figure 4-19) showed that at 18ºC and presence of salt in the concentration value shown above, 24% of nitrogen was converted to nitrite and 31% was totally oxidised to nitrate, the rest of the applied nitrogen remained as ammonium (45%),. At 26 ºC the conversion increased and only 8% of the applied nitrogen remained as ammonium and 92% was oxidised to nitrate. No significant nitrite accumulation was detected. On the other hand, at 36 ºC, after few days high nitrite production took place (around 60% of the applied ammonium was converted to nitrite), however it decreased after 100 days. Afterwards, 25% of the applied nitrogen was partially oxidised to nitrite, 58% of the nitrogen was transformed to nitrate and 17% remained as ammonium.

The pH gradually decreased in the columns with salt at 26 ºC and 36 ºC, however the column at 18 ºC kept similar pH values to those found in the influent. Higher ammonium conversions to nitrite and nitrate produced lower pH values (Figure 4-20).

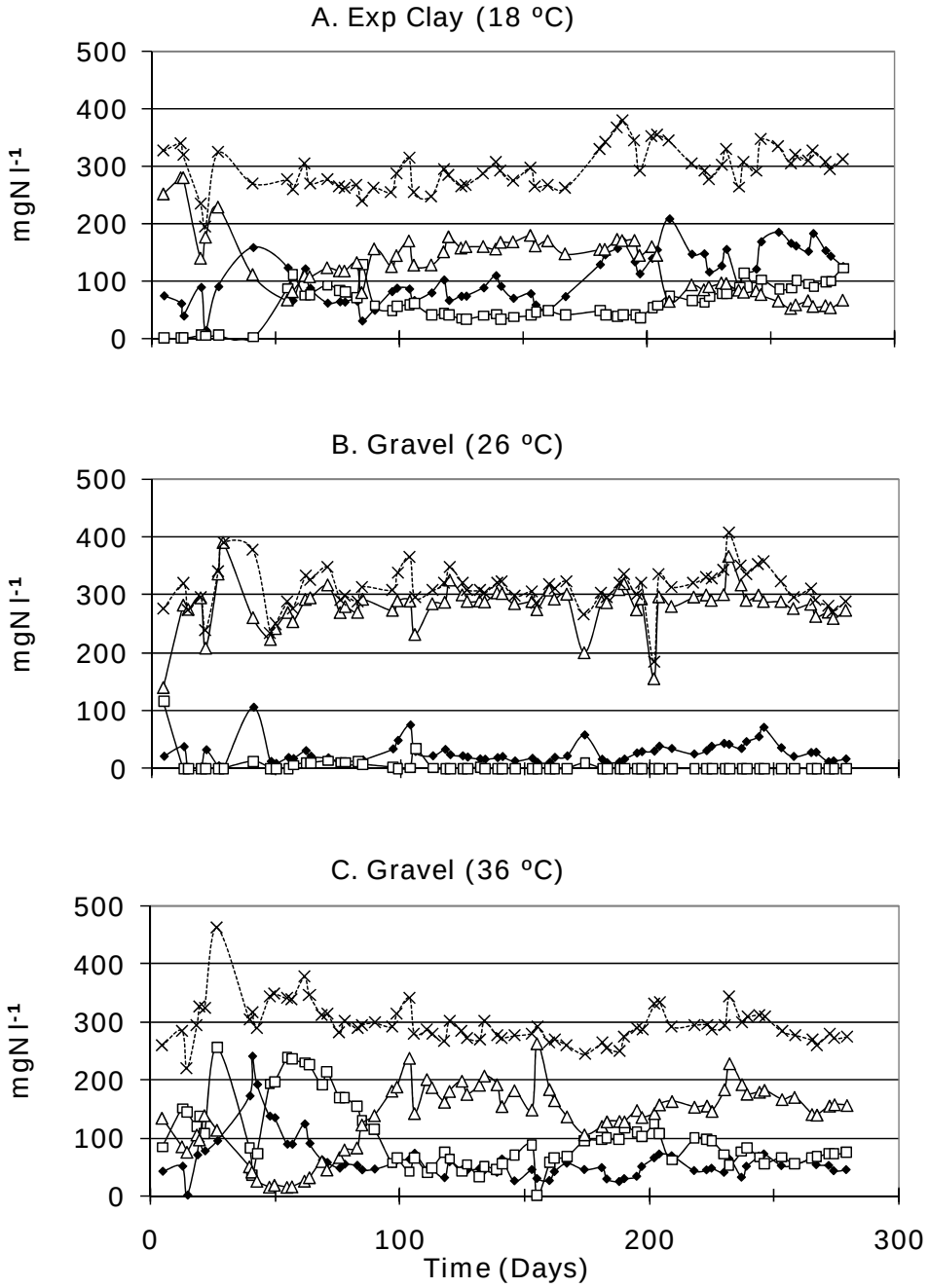

Figure 4-18 Nitrogen conversions in vertical filters working with addition of salt (22.72 g salt l^{-1}) and gravel as filter material at different temperatures

—●—NH_4-N —□—NO_2-N —Δ— NO_3-N ·····x···· Total N

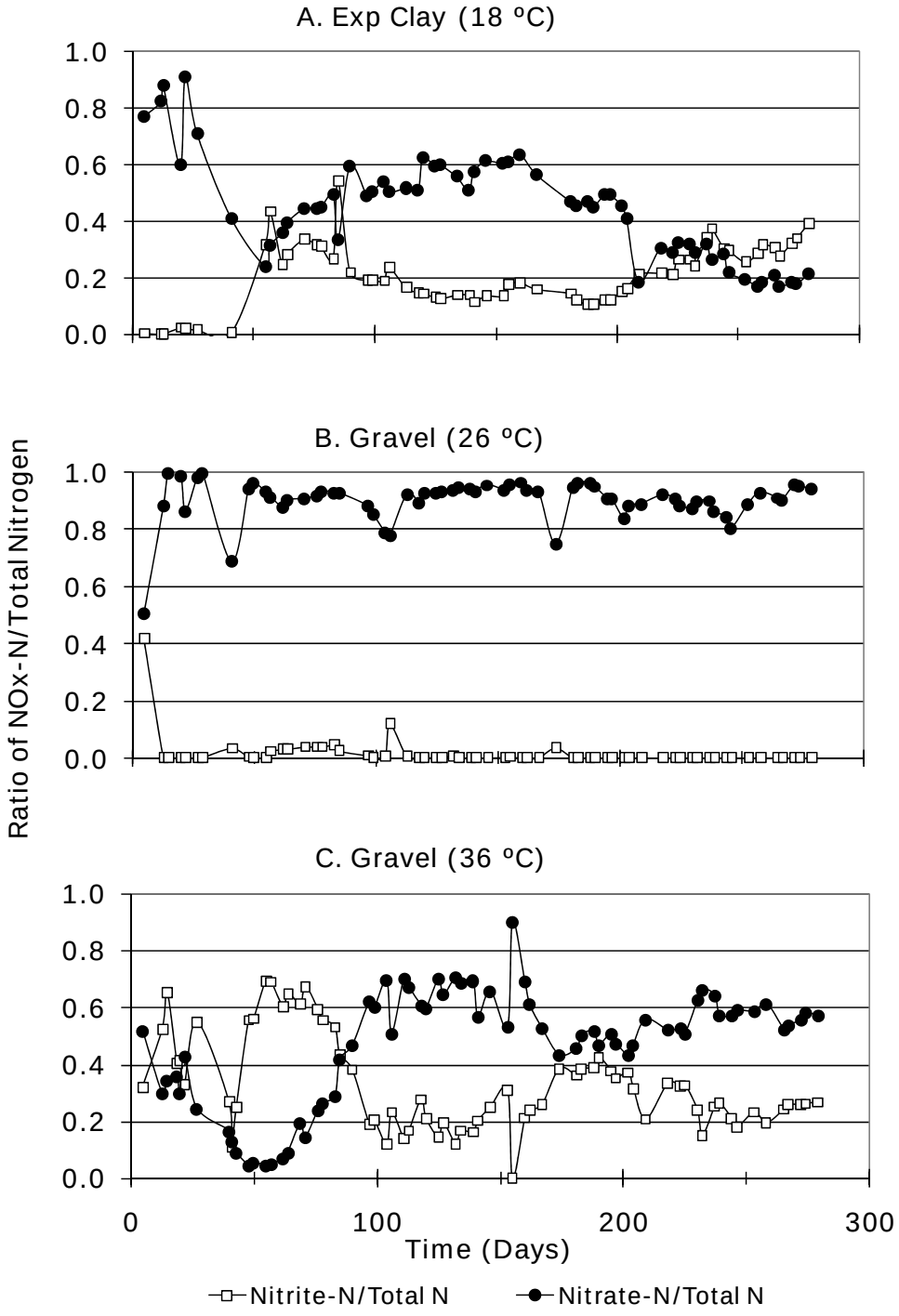

Figure 4-19 Ratio of nitrite/Total nitrogen and nitrate/Total nitrogen for effluents of the columns with addition of salt (22.72 g salt l^{-1}) and gravel or expanded clay as filter material at different temperatures

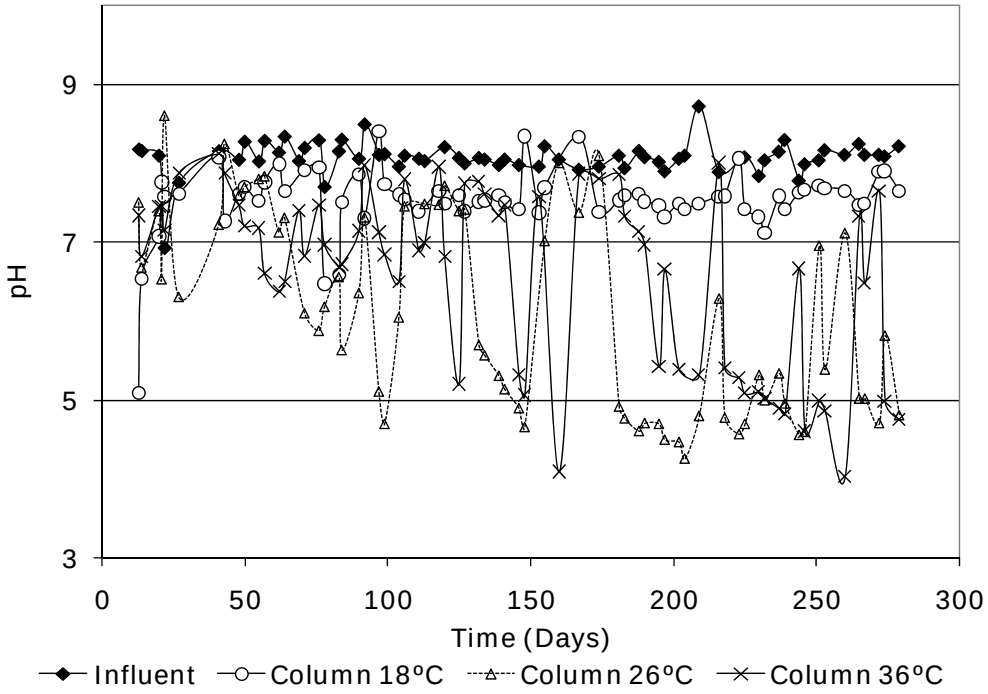

Figure 4-20 pH values of influent and effluents of the vertical down-flow columns with addition of salt (22.72 g salt l^{-1}) and gravel/expanded clay as filter material at different temperatures

Discussion

It is clear that temperature and size of the filter material have an influence on stability of partial nitrification for the experimental conditions of vertical down filters with ⅔ of the column working under water saturation. In general higher ammonium oxidation was obtained at higher temperatures and no stable nitrite formation was obtained at 26 ºC and 36 ºC. The decrease of pH was not a stable strategy for stoping nitrite oxidation. After almost 100 days of a relative stable nitritification in most of the columns, nitrite concentrations in the effluents of the columns decreased and nitrate was formed. One of the main reasons of this is that for nitrite oxidation no further alkanity requirement is

needed and also microbes can be adapted by time to the adverse conditions. In fact, adaptation and acclimation to different concentrations of nitrite and ammonium have been reported (Fux et al., 2004a; Villaverde et al., 2000).

As it was already mentioned above, different strategies and approaches have been used for getting a stable partial nitrification. Perhaps the most studied process has been the so called SHARON process, which is based on the higher growth rate of ammonium oxidising bacteria in comparison to the growth rate of nitrite oxidising bacteria at temperatures above 26 ºC (Brouwer et al., 1996; Hellinga C. et al., 1998; van Dongen et al., 2001b). However, this process is based on suspended biomass growth without biomass retention (hydraulic retention time is similar to the retention time of the bacterial cells). In the case of the columns there was biomass retention; partly the bacteria grew as attached biomass on the filter material. So, higher temperatures caused higher ammonium oxidation but also higher nitrite oxidation.

Perhaps the most critical and important parameter for controlling partial nitrification in biofilm based systems, where there is no control of the biomass retention time, is the dissolved oxygen (Guo et al., 2009; Yan and Hu, 2009). The strategy used in the experiments to have 2/3 volume parts of the columns submerged or saturated allowed to keep a dissolved oxygen concentration below 0.3 mg l^{-1} in the saturated part of the column, however little control could be done in the water-free part of the column. The main reactions happen in the upper part of the filters.

The mean conversion values obtained from the different columns, expressed as ratio of nitrogen species over total nitrogen are shown in the Figure 4-21. The best ratio of NH_4-N:NO_2-N:NO_3-N for anammox process was obtained in the column at 18ºC with gravel (0.41:0.43:0.15 respectively).

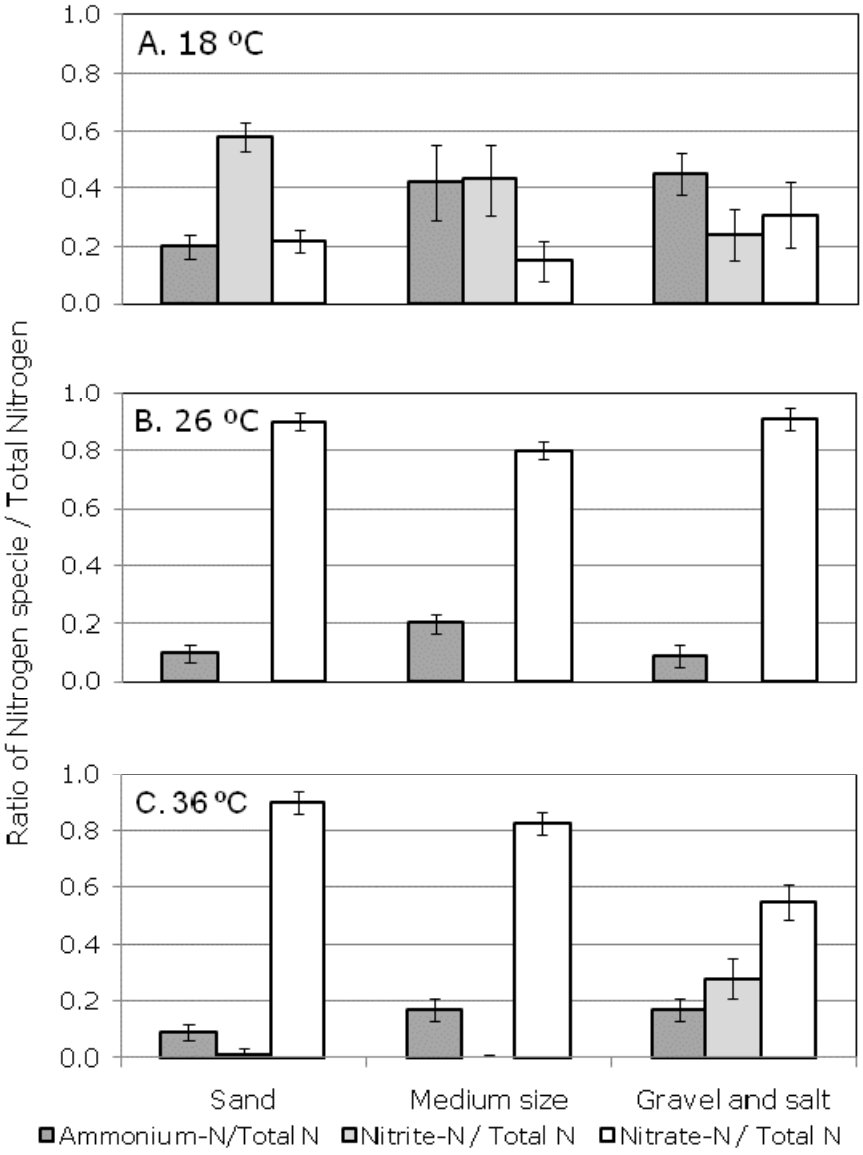

Figure 4-21 Ratio of nitrogen species / Total nitrogen of the column effluents with different filter material size at different temperatures

According to the stoichiometry for anammox process, around 55 – 60% of the ammonium nitrogen must be oxidised to nitrite nitrogen in a first aerobic reactor if a combination of partial nitrification and anaerobic oxidation of ammonium processes can be used for nitrogen removal (Fux et al., 2004a).

In the column experiments it was not possible to have a total control of the nitrite oxidation to nitrate. Indeed, at 18 ºC, 15% of the ammonium was oxidised to nitrate, however at the highest temperature (36 °C) the conversion to nitrate was higher than 80%.

In general it seems to be very unpredictable to realize stable partial nitrification in biofilm systems like in down flow filters as used in this study. While in bioreactors like completely stirred reactor almost no concentration gradients exist and by this different parameters are easy to estimate. Biofilm reactors and especially filters are characterised by concentration gradients in flow direction and within the biofilms. Because the biofilm growth is a slow process even after long running times changes can be expected. By this the realisation of stable partial nitrifications in simple biofilm reactors stays still an interesting task for further research.

5 Summary and Conclusions

5.1 Preliminary tests on anammox activity of sludges/soils from different wastewater treatment systems

Anammox bacteria are present in several kinds of anaerobic environments and they are more tolerant to oxygen and other stress factors then it was believed in the past. The obtained results from the bottle experiments show that they are also present in soils of subsurface flow CWs and confirm the speculations made by Stottmeister et al. (2003), Shipin et al. (2005) and Kadlec and Wallace (2009).

With appropriate environmental conditions inside the experimental bottles, the soil samples from CWs showed anammox activity after an incubation time of 99 days; they needed a little bit less time in comparison to sludges taken from other wastewater treatment plants. Because the main experiences with nitrogen removal in SSHF CWs have been related with domestic wastewaters, which are characterized with relatively low nitrogen loads, there are not suitable optimum conditions for growth of anammox bacteria in high cell density. However, the role of anammox bacteria in nitrogen removal and transformation processes in CWs especially with a high ammonium load is not yet clear and also an open topic is how to give or to keep the appropriate environmental conditions in CWs in order to stimulate anammox process.

Further research should be focused on the starting-up of CWs aimed to enhance anammox process.

5.2 Nitrogen removal processes in horizontal subsurface flow constructed wetlands

The use of PFBR allows a better understanding of the different transformation and removal processes without the interference of macrogradients in SSHF CW. High nitrite and ammonium loads to the PFBR caused stress and toxicity to the plants, expressed as a reduction of their transpiration rate. A gradual increase of the nitrite concentration (from 58 mg NO_2-N l^{-1} to 112 mg NO_2-N l^{-1}) did not increase significantly the total nitrogen removal rate. Nevertheless, similar values to those reported by different authors (0.56 g m^{-2} d^{-1} to 0.99 g m^{-2} d^{-1}) (Kuschk, et al., 2003; Wiessner et al., 2005b) were reached. With a higher nitrite concentration (149 mg NO_2-N l^{-1}) the system got unstable and plants died. Even re-plantation did not keep stable plant growth and transpiration. Main nitrogen removal mechanisms were associated with nitrification and denitrication processes. Because of the low oxygen concentration in the reactor (less than 0.2 mg l^{-1}) and considering that microbial ammonium oxidation is faster than microbial nitrite oxidation under low oxygen levels (Wiesmann, 1994; Ciudad et al., 2005; Guo et al., 2009), it can be presumed that ammonium oxidation happened at faster rate than the further nitrite oxidation to nitrate. For the second step (denitrification) it can be assumed that it happened via nitrite instead of nitrate. But it remains a speculation what was the electron donor: organic carbon compounds from rhizodeposition products (there was no organic carbon addition in the synthetic wastewater) or ammonia itself. Also sulphide could be a possible electron donor.

The composition of the wastewater was suitable for anammox process and the results for FISH probes showed the presence of anammox bacteria in very small amounts after 380 days, suggesting that the anaerobic ammonium oxidation route was probably of less

importance in the first three experimental phases. The results also imply that for getting a high enough number of anammox bacteria in subsurface constructed wetlands many years will be required supporting the concept that also especially time and not only load is the key factor for having a stable anammox population in biofilm based systems (Hao et al., 2002a).

The experiments also gave some key information about how to start a full-scale constructed wetland with high ammonium loads. Because ammonium oxidizing bacteria grow faster than nitrite oxidizer, nitrite accumulation could take place and plants could be affected and probably would die. But usually in such wetland systems no accumulation of nitrite or nitrate is observed. This means that in general the oxygen availability is an important bottle neck for the nitrogen transformation processes.

Nevertheless the question arouse if an inoculation with active anammox biomass could be a good strategy to accelerate the start up and improve the nitrogen removal rate of SSHF CWs.

5.3 Effects of the inoculation of active anammox biomass in subsurface horizontal flow constructed wetlands

The inoculation with active anammox biomass in PFBR in comparison to non-inoculated PFBR had a positive impact on different operational parameters. With nitrite and ammonium concentrations of 23 mgl^{-1} NO_2-N and 117 mgl^{-1} NH_4-N, respectively, plants transpiration increased (up to 60% of the volume influent) as a consequence of a higher microbial activity and less availability of nitrite and therefore less toxicity to the plants. Further increase of nitrite and ammonium concentrations reduced the

transpiration rate because anammox biomass could not cope this increased load at the given time and toxic effects to the plants were detected again. However, the total and ammonium nitrogen removal rates were higher (5.8 and 3.8 g N m^{-2} d^{-1} for total nitrogen and ammonium, respectively) than those obtained by other authors using the same type of reactor (Wieβner et al., 2005a and b). Comparisons with a similar system working with the same conditions but without plants showed that plants play an important role. The planted reactors removed higher nitrogen loads. Nevertheless, it can be concluded that at high nitrite and ammonium concentrations microbial transformation processes were more important than the role of the plants.

Anaerobic oxidation of ammonium and nitrification-denitrification has been defined as the main nitrogen transformation processes in the experiments. Nitrogen plant uptake is also a possible removal process but it is limited to a maximum of 0.33 g N m^{-2} d^{-1} for *Juncus effusus* (Kadlec and Wallace, 2009), thus, nitrogen plant uptake is an important pathway for very low nitrogen loaded wetlands but of minor importance for heavily loaded wetlands. The very low concentrations of dissolved oxygen in the reactors (only up to 100 μg l^{-1}) cause anoxic or strict anaerobic micro-zones, which are typical for SSHF CWs. These anoxic/anaerobic micro-zones are the basis for keeping alive the anammox bacteria in a relatively high redox potential environment, but also it is a limiting factor for ammonia oxidation and further nitrite oxidation. Because the required oxygen for ammonium and nitrite oxidation is higher than the estimated value of oxygen input into the system and, in a similar way, because the available organic carbon for denitrification is quite low in comparison with the carbon input from the plants, complete nitrification-denitrifation processes cannot explain the high nitrogen removal rates. A further comparison with the unplanted reactor, where there is no oxygen input

with the exception of air diffusion, and considering that both reactors had not addition of any organic carbon source, and, both showed high nitrogen removal rates, it can be concluded that anaerobic ammonium oxidation was the main mechanism for nitrogen removal. Alkalinity consumption decreased with higher nitrite concentrations in the influent. High nitrite concentration in the influent produced similar alkalinity consumption values to those reported for the anammox process and, lower nitrite concentration in the influent turns out alkalinity consumption values close to those reported for CANON process. It means that anaerobic oxidation of ammonium became the main pathway for the nitrogen removal process coupled with partial nitrification at high nitrite and ammonium concentrations and also high nitrite to ammonium ratios (1:1.7 for NO_2-N to NH_4-N). Lower ammonium and nitrite concentrations and low nitrite to ammonium ratios in the influent (1:5 for NO_2-N to NH_4-N) did not affect the anammox bacteria, but their role seemed to be less important.

The addition of organic carbon (acetate, TOC of 60 m gl^{-1}) to the PFBR inoculated with anammox biomass had no significant effect on anammox activity and it was proved that anammox process could take place in the presence of organic matter. The results confirm the findings obtained by Güven et al. (2005), Chamchoi et al. (2008) and Molinuevo et al. (2009) regarding the low influence of organic matter on anammox activity and corroborate the fact that anammox bacteria are more versatile than it was thought some years ago. The obtained total and ammonium nitrogen removal rates were similar to the obtained values without addition of organic matter with the advantage that the reactor behaved in a more stable way in terms of plant transpiration although it was lower in comparison with reactor 3.

The nitrogen species in the influent of the inoculated PFBR had also some effects on the total and ammonium removal rates and nitrogen transformation processes. Lower values of ammonium and total nitrogen removal rates were obtained in the PFBR fed with only ammonium in comparison with the PFRB fed with ammonium and nitrite, however, obtained values are quite higher to those reported for similar reactors working without inoculation. No toxic effects were detected in the reactor and plant transpiration kept stable during the three phases.

5.4 Stability of anammox biomass

After almost 500 days of the inoculation of the PFBRs the anammox bacteria were still present in the reactors. The results from FISH probes showed that they were present in biofilm attached to the gravel and also in the pore water. Their amount increased with higher nitrite concentrations in the influent and remained relatively constant, during the three phases, in the reactor fed with only ammonium. The highest activity of anammox was detected at highest nitrite concentrations and remained more or less constant for the PFBR fed with only ammonium. It can be concluded that after inoculation anammox bacteria will keep stable their number and activity if high nitrogen loads are applied. The presence of nitrite in the system will increase the number and activity of anammox bacteria.

5.5 Stability of partial nitrification in vertical down flow columns

The presence of nitrite in treatment system is one main prerequisite for establishing the anammox pathway for nitrogen removal from wastewater. One possibility for enhancing anammox pathway in horizontal subsurface flow CWs could be the use of vertical

down flow filters as a partial nitrification reactor as a first pretreatment step. Medium and fine sized filter materials were tested and the effect of temperature was also evaluated. Higher ammonium oxidation was obtained at higher temperatures but partial nitrification at 26 ºC and 36 ºC was not stable. The best result, in terms of ratio of NH_4-N to NO_2-N: NO_3-N, was obtained with medium sized material (gravel) at 18 °C with values of 0.41:0.43:0.15, respectively, which is close to the theoretical ratio for anammox process (0.40:0.60:0.00 for NH_4-N:NO_2-N:NO_3-N, respectively), however 15% of the applied ammonium load was totally oxidized to nitrate.

The control of partial nitrification in biofilm systems is a very complex task because they are characterized by concentration gradients (in the flow direction and within the biofilm) and high cell retention time. Even after long running times of the systems changes can be expected. So, this problem remains a challenging task for further research.

5.6 Concluding remarks

The results showed that the inoculation of active anammox biomass was successful for improving nitrogen removal in experimental laboratory-scale SSHF CWs. By this it was possible to increase nitrogen removal rates from 0.56 g N m^{-2} d^{-1} up to 5.8 g N m^{-2} d^{-1}. After inoculation anaerobic ammonium oxidation was the main nitrogen transformation and removal process. Partial nitrification, denitrification and plant uptake played a minor role. At low ammonium loads applied in the system nitrification-denitrification became the main mechanism for nitrogen removal and anammox played a minor role.

Higher nitrogen removal rates obtained through anammox processes will have a direct impact on size and investment costs for wastewater treatment systems based on SSHF CWs because less area will be needed, however it requires further research in order to scale up the results.

There are several methodologies for designing SSHF CWs including first order kinetics, area based methods, loading charts, etc., but most of them are based on the high oxygen demand for nitrification processes. The obtained results in this research open a door for improving and enhancing the nitrogen removal in subsurface wetlands with a lower oxygen requirement process: the anaerobic oxidation of ammonium. This process will cope with the main disadvantage that has been identified with SSHF CWs, the low oxygen transfer rate.

Because the low oxygen transfer rate in SSHF CWs, a combination of vertical flow and SSHF CWs can be used for nitrogen removal. The first one is more engineered, aerobic and normally requires pumps or some mechanical devices to apply intermitent load into the system which allows a forced aeration. The second one (SSHF CW), less aerobic, can be defined and constructed for denitrification process, however in most of the cases organic carbon source becomes a limitation.

Promoting anammox process in SSHF CWs will handle the limitations of aeration and carbon source in one single unit with minimum technical aspects.

Future research should be addressed to applied and basic topics. In applied research guidelines like how to start up full scale systems, how to inoculate them with active anammox biomass, how to enhance appropiated environmental conditions for establishment anammox process in SSHF CWs, effects of different temperature, soil matrix and plants should be developed. In basic research, nitrogen transformation processes and interactions of nitrogen and sulphur cycles are examples of still open questions.

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7 Appendix

Appendix A: Abbreviation directory.

Appendix B: Picture of anammox cells found out in PFBR 1 after Phase III of the experiments.

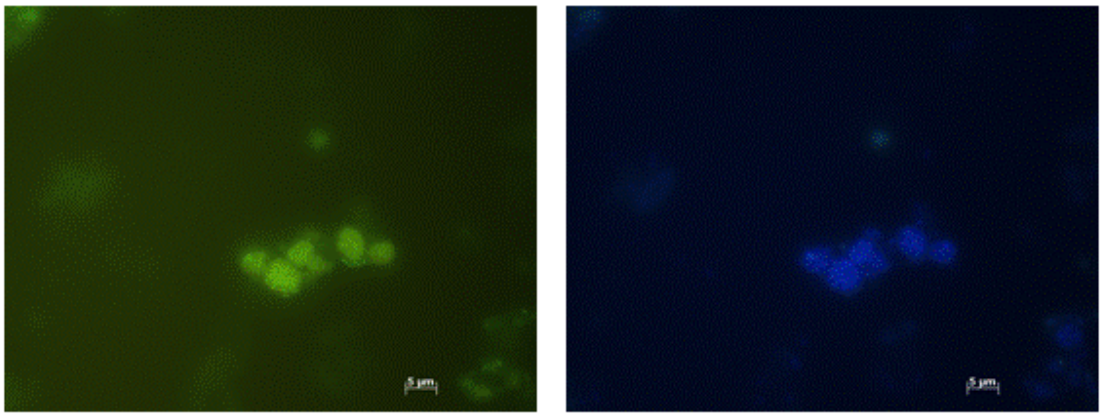
Appendix C: Selected images of FISH analyses after inoculation of PFBRs with anammox biomass.

Appendix A: Abbreviations directory

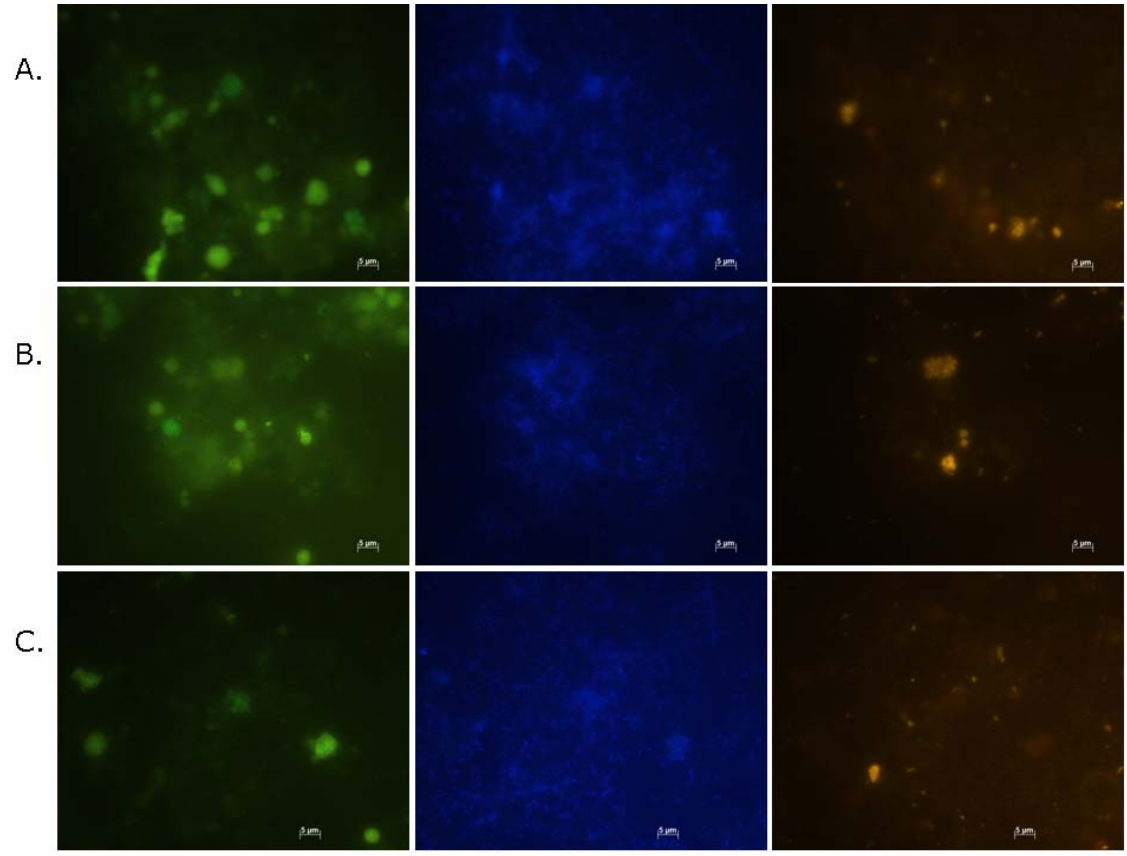
a	year
Anammox	anaerobic ammonium oxidation
AS	activated sludge
ASL	ammonium surface load
BOD_5	5-day biochemical oxygen demand
CANON	completely autotrophic nitrogen removal over nitrite
COD	chemical oxygen demand
CW	constructed wetland
d	day
D_{10}:	diameter, in mm, than 10% of sand pass through
D_{60}:	diameter, in mm, than 60% of sand pass through
DEAMOX	denitrifying ammonium oxidation
DGGE	denaturating gradient gel electrophoresis
DIN	german institute for standardization
DNA	deoxyribonucleic acid
DO	dissolved oxygen concentration
EDTA	ethylenediamine tetraacetic acid
Eh	normal hydrogen reference electrode redox potential
et al.	and others, (Latin: et alteri)
FISH	fluorescence in-situ hybridization
FWS CW	free water surface constructed wetland
ha	hectare
H	height
IC	inorganic carbon
m	meter
Mt	metric ton
MNRR	maximum nitrogen removal rates
Nd	no data
OLAND	oxygen-limited autotrophic nitrification-denitrification
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFBR	planted fixed bed reactor
rH	redox potential
rpm	revolutions per minute
SBR	sequencing batch reactor
SF CW	surface flow constructed wetland

SHARON	Single reactor High activity Ammonia Removal over Nitrite
SS CW	sub-surface constructed wetland
SSHF CW	subsurface horizontal flow constructed wetland
SSVF CW	sub-surface vertical flow constructed wetland
TAN	total ammonium nitrogen
TC	total carbon
TN	total nitrogen
TOC	total organic carbon
UASB	upflow anaerobic sludge blanket
UV	ultraviolet

Appendix B: Picture of anammox cells found out in PFBR 1 after Phase III of the experiments. Pictures left side Amx820 probe (Anammox bacteria cells appear green); Pictures right side DAPI staining.



Appendix C: Selected images of FISH analyses after inoculation of PFBRs with anammox biomass. A. Reactor 1, Phase V; B. Reactor 2 Phase IV; C. Reactor 3 Phase IV. Pictures left side Amx820 probe (Anammox bacteria cells appear green); Pictures in the center DAPI staining; Picture right side Nso 190 (Ammonia-oxidizing bacteria cells appear reddish).



Curriculum Vitae

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