Biological Nutrient Removal in Compact Biofilm Systems

João Paulo Bassin

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João Paulo BASSIN

Master of Science in Chemical Engineering, Chemical Engineering
Program – COPPE, Federal University of Rio de Janeiro
geboren te Erechim, Rio Grande do Sul State, Brasil.

Dit proefschrift is goedgekeurd door de promotoren

Prof. dr. dr. h. c. ir. M.C.M. van Loosdrecht

Prof. dr. sc. M.W.C. Dezotti

Copromotor:

dr. ir. R. Kleerebezem

Samenstelling promotiecommissie:

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Biological Nutrient Removal in Compact Biofilm Systems

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To my parents, Gema and Ijair

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List of abbreviations

The following nomenclature was used in this thesis. Notation of parameters used to determine biofilm surface specific detachment rate is shown in Chapter 2; notation for parameters used to calculate the settling velocity of aerobic granules is given in Chapter 3; notation of parameters specifically related to ammonium adsorption kinetics and ammonium adsorption isotherms is presented in Chapter 5.

AGS	Aerobic granular sludge
AOB	Ammonium-oxidizing bacteria
ATP	Adenosine triphosphate
BAF	Biological aerated filters
BAS	Biofilm Airlift Suspension reactor
bp	Base pairs
CANON	Completely autotrophic nitrogen removal over nitrite
cDNA	Complementary DNA
COD	Chemical oxygen demand
DGAOs	Denitrifying glycogen-accumulating organisms
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DO	Dissolved oxygen
DPAOs	Denitrifying polyphosphate-accumulating organisms
EBPR	Enhanced biological phosphorus removal
EGSB	Expanded Granular Sludge Blanket
EPS	Extracellular polymeric substances (exopolysaccharides)
FA	Free ammonia
FISH	Fluorescent in situ hybridization
FNA	Free nitrous acid
GAO	Glycogen-accumulating organisms
HRT	Hydraulic retention time
MBBR	Moving bed biofilm reactor
MBR	Membrane bioreactor
MFCS	Mass flow control system
MLVSS	Mixed liquor volatile suspended solids
NADH	Nicotinamide adenine dinucleotide

NOB	Nitrite-oxidizing bacteria
PAO(s)	Polyphosphate-accumulating organism(s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA(s)	Polyhydroxyalkanoate(s)
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PLC	Programmable logic controller
PP	Polyphosphate
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
RBC	Rotating Biological Contactors
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SBBR	Sequencing batch biofilm reactor
SBR(s)	Sequencing batch reactor(s)
SEM	Scanning electron microscopy
SHARON	Single reactor system for high ammonium removal over nitrite
SND	Simultaneous nitrification and denitrification
SRT	Sludge retention time
SVI	Sludge volume index
SVI_5	Sludge volume index after 5 min of sedimentation
SVI_{30}	Sludge volume index after 30 min of sedimentation
TCA	Tricarboxylic acid
t-RFLP	Terminal restriction fragment length polymorphism
TSS	Total suspended solids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant
XRD	X-ray diffraction analysis

Eutrophication
Photo credit: M. Lamiot

CHAPTER 1

Introduction and thesis outline

Chapter 1

Content

1.1. Background information

1.1.1. Impact of nutrients on the environment

The discharge of untreated wastewater into fresh or marine water bodies can lead to harmful and irreversible effects on water ecology and may present serious health risks for human populations. Historically, wastewater treatment involved mainly the removal of dissolved organic matter and suspended solids. In recent years, there has been a rising concern about water pollution and a strong effort has been made to reduce the amount of nutrients discharged in water basins. Nitrogen and phosphorus are the most important nutrients present in wastewaters. Their source is often attributed to the use of fertilizers in agriculture, cleaning detergents and other synthetic chemicals. Partly treated and untreated sewage from cities and industrial wastewaters are also sources of anthropic nutrient input.

High concentrations of nitrogen and phosphorus can potentially cause negative effects on aquatic environments. One of the main problems caused by the excessive availability of these nutrients in aquatic resources is related to eutrophication. In this natural process occurring in both fresh and marine stratified water bodies, an overgrowth of algae (algal blooms) and other aquatic plants is observed, disrupting the normal ecosystem characteristics and causing several problems to the aquatic environment concerned. Human activity can speed up the eutrophication process in the environment, increasing the input of nutrients beyond its natural capacity to assimilate them and consequently causing a cascade of problems. The relatively high organic matter content present in the eutrophic waters makes their treatment more complex and costly. Moreover, the use of disinfectants in the drinking water production process can promote the production of carcinogenic bioproducts, such as chlorinated compounds. Figure 1.1 represents water bodies in which a high level of eutrophication is observed.

The algae and aquatic plants grown on nutrients may use all the oxygen available in the aquatic environment, resulting in the death of many aquatic organisms. The algal bloom may also block sunlight from photosynthetic plants situated under the surface of the water, which may cause their death. Furthermore, the production of toxins by some species of algae, such as toxic cyanobacteria (Hardman et al., 1993), can also harm the organisms present in the environment. Consequently, problems along the food chain are inevitable. The use of eutrophic waters in which cyanobacteria are present to

produce drinking water is very risky since the removal of toxins produced by these algae can be very complex.

(a) Dianchi Lake, Yunnan Province, China (Source: Greenpeace China)

(b) Barr Lake, Colorado, USA. (Cortesy: Adam Kaningher)

Figure 1.1: Eutrophication taking place in water basins.

Another problem posed by eutrophication is the increased bacterial population necessary to degrade the dead algae, which are often located at the bottom of the water body. Bacteria require oxygen for the algae decomposition. As a result, a significant drop in oxygen levels and consequent suffocation of aquatic organisms may occur, especially in poorly mixed waters. Moreover, the decomposed material is usually carried by inflowing waters into lakes and reservoirs, contributing to gradually fill these water bodies. Low dissolved oxygen concentrations also provoke the release of many compounds normally bound to bottom sediments, including various forms of phosphate, reinforcing the eutrophication process. Odour problems that affect recreational use of rivers and lakes are also some of the consequences of excessive availability of nutrients. Figure 1.2 shows a schematic representation of the typical scenario leading to eutrophication and the link between the important factors involved in this process.

The ratio of nitrogen to phosphorus compounds is an important factor determining which of the two elements will be the limiting factor and therefore which one has to be controlled in order to reduce the overgrowth of algae and higher forms of plant life. In general, phosphorus tends to be the limiting nutrient for phytoplankton in fresh waters (e.g., rivers and lakes), whereas in large marine areas, nitrogen has been considered to be the limiting factor (Volterra et al., 2002).

It is important to remark that additional factors related to the causes of eutrophication and the responses to inputs of nutrients vary for different aquatic systems. Lakes and reservoirs behave differently than rivers and streams, whereas all of

these water bodies differ from estuaries and coastal waters. Moreover, all systems exhibit high variation due to natural successional processes. Therefore, the eutrophication impacts of human interventions on receiving waters and the mechanisms of these impacts are difficult to be assessed (Correll et al., 1999).

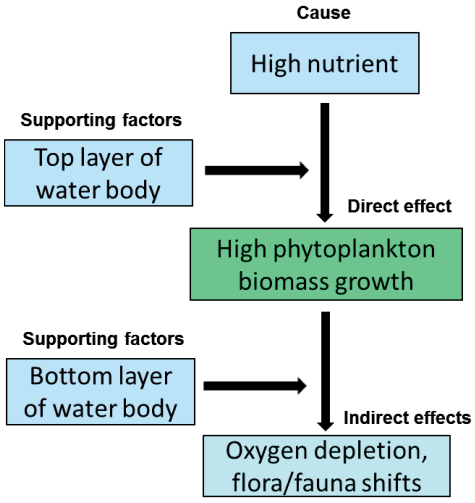


Figure 1.2: Main scenario of the eutrophication process and important factors involved in this process (adapted from Volterra et al., 2002).

Besides the problem of eutrophication, large concentrations of nitrogen compounds can be harmful for life. Free ammonia is toxic to aquatic life, causing massive mortality of fish and other organisms. Nitrite and nitrate are linked to methemoglobinemia, a fatal blood disorder in which the ability to release oxygen to tissues is reduced. This infant's disease is known as "blue baby syndrome". An additional problem brought up by nitrite is the formation of carcinogenic nitrosamines by the interaction of nitrite with organic nitrogen compounds (e.g., amines) (Mulliken and Jolly, 1916), especially under acidic conditions.

In the United States, for instance, around 25% of all water body impairments are caused by excessive presence of nutrients and its consequences (oxygen depletion, rapid algae growth, harmful algal blooms, turbidity, and toxic ammonia) (EPA, 2007). The removal of nutrients such as nitrogen and phosphorus from wastewaters is therefore of major concern, justifying the stringent effluent limits for nitrogen and phosphorus that are being applied worldwide by environmental agencies.

In order to accomplish the removal of these nutrients, biological nutrient removal processes have been widely applied because of their high effectiveness and low cost compared to physical-chemical processes. Essentially, biological nitrogen and phosphorus removal involves an array of biochemical processes carried out in an appropriate sequence of aerobic, anoxic and anaerobic conditions, which are described in the following sections.

1.1.2. Biological nitrogen removal

Nitrogen can exist at various oxidation states in nature. In wastewaters, total nitrogen mainly comprises ammonia, nitrate and particulate/soluble organic nitrogen. The principal conversions taking place in the biological nitrogen removal systems are nitrification and denitrification. Other related reactions include the hydrolysis of organic nitrogen to ammonia (ammonification) and nitrogen uptake for biomass growth.

Nitrification

In the nitrification, initial step of the biological nitrogen removal process, ammonia is oxidized to nitrite and subsequently to nitrate using oxygen as electron acceptor. The first conversion is performed by ammonia-oxidizing bacteria (AOB), whereas the second reaction is carried out by nitrite-oxidizing bacteria (NOB). Some AOB genera are *Nitrosomonas, Nitrosococcus, Nitrosovibrio, Nitrosospira,* and *Nitrosolobus*, while *Nitrobacter*, *Nitrospira, Nitrospina, Nitrococcus, and Nitrocystis* are examples of NOB genera (Rittmann and McCarty, 2001; Metcalf and Eddy, 2003).

The stoichiometric reactions of the oxidation of ammonium and nitrite including their growth (catabolism and anabolism) are described in equations (1.1) and (1.2), respectively (Wiesmann and Libra, 1999).

$$NH_4^+ + 1.3O_2 + 1.98HCO_3^- \rightarrow 0.0182C_5H_7O_2N + 0.98NO_2^- + 1.04H_2O + 1.89H_2CO_3 \quad (1.1)$$

$$NO_2^- + 0.005NH_4^+ + 0.48O_2 + 0.005HCO_3^- + 0.02H_2CO_3 \rightarrow 0.005C_5H_7O_2N + NO_3^- + 0.015H_2O \quad (1.2)$$

As shown in equation (1.1), 2 moles of alkalinity are consumed per mole of ammonium oxidized. Carbonate buffer neutralize the production of protons during

ammonium oxidation through CO_2 stripping. If the amount of buffer needed is not available in the wastewater and not externally supplied, the pH of the system drops and the ammonium oxidation rate significantly decreases. According to equations (1.1) and 1.2, ammonium oxidation requires more oxygen than nitrite oxidation. Moreover, the amount of cells produced per mole of nitrogenous compounds oxidized is higher for ammonium oxidation.

Both AOB and NOB are autotrophic microorganisms, using CO_2 as carbon source and inorganic nitrogen compounds (ammonium and nitrite) for energy production. The growth rate and the sludge yield of nitrifying bacteria are very low compared to heterotrophic bacteria performing chemical oxygen demand (COD) removal and denitrification. Therefore, these slow growing organisms require relatively long sludge retention time (SRT) to be maintained in the bioreactor.

Nitrifying bacteria are also very susceptible to inhibition by several environmental factors such as pH, dissolved oxygen concentration, temperature and presence of toxic organic compounds. In this context, nitrification is often regarded as the rate limiting step in biological nitrogen removal. Optimum pH and temperature values for nitrification are 7.0 and 30 – 35°C, respectively (Henze et al., 1997; Metcalf and Eddy, 2003). Usually dissolved oxygen (DO) concentrations above 2 mgO_2/L are recommended (Surampalli et al., 1997; Henze et al., 1997). The concentration of ammonium and nitrite also influence the nitrification process. If they are too low, substrate limitation will occur. On the other hand, high concentrations of these substrates and especially their unionized forms (free ammonia and free nitrous acid) inhibit the process (Anthonisen et al., 1976).

Deitrification

In the subsequent step of the conventional biological nitrogen removal process, designated as denitrification, the nitrate generated by nitrification is reduced sequentially to nitrite, nitric oxide, nitrous oxide and nitrogen gas under anoxic conditions (Madigan et al., 1997). Organic carbon is needed for the denitrification reaction, functioning as electron donor for ATP production. Nitrate (or nitrite) acts as electron acceptor instead of oxygen.

Denitrification is often carried out by several heterotrophic bacteria capable of using a wide range of electron donors such as organic acids, carbohydrates, and alcohols (Metcalf and Eddy, 2003). *Pseudomonas, Achromobacter*, *Alcaligenes*, *Bacillus*,

Micrococcus, *Proteus*, *Hiphomicrobium*, *Chromobacterium*, *Halobacterium*, *Moraxella*, *Neisseria*, *Paracoccus*, *Azospirillum*, *Rhodopseudomonas*, *Thiobacillus*, *Vibrio*, *Xanthomonas,* and *Klebsiella* were among the several genera of bacteria reported to carry out the denitrification reaction (Rittman and Langeland, 1985). Denitrification can also be conducted by chemolithoautotrophic bacteria, which use H_2 or reduced sulphate compounds as electrons acceptors (Wiesmann et al., 2007).

Equation (1.3) represents a heterotrophic denitrification reaction in which it is considered that the organisms assimilate ammonium (Henze et al., 2002). If all energy is used for growth, the maximum yield constant of the process is around 0.40 kg biomass/kg organic matter, which is 15% lower than that of heterotrophic conversion (Henze et al., 2002). During denitrification, alkalinity is produced, partially compensating the alkalinity loss in the nitrification step.

$$0.52C_{18}H_{19}O_9N + 3.28NO_3^- + 0.48NH_4^+ + 2.80H^+ \rightarrow C_5H_7O_2N + 1.64N_2 + 4.36CO_2 + 3.8\ H_2O \quad (1.3)$$

The denitrification rate, often expressed as gNO_3^--N/(gMLVSS.d), depends primarily on the availability of readily biodegradable compounds. Usually a COD/NO_x (NO_x=NO_2^- or NO_3^-) ratio of 3:1 is optimal for complete denitrification. The type of organic carbon substrate also affects the denitrification rate. Higher denitrification rates can be obtained for instance with fatty acids, whereas denitrification supported by endogenous decay is usually a slow process. Temperature, pH, dissolved oxygen concentration, carbon/nitrogen ratio and nitrate concentrations are some factors influencing the denitrification performance (Nair et al., 2007; Metcalf and Eddy, 2003). Denitrification can occur in a wide range of pH values, although the highest rates are obtained at pH 7.0 – 7.5 (Halling-Sørensen and Jørgensen, 1993). Low concentrations of oxygen (such as 0.2 mgO_2/L) can already inhibit the denitrification process. Denitrification rate increases with increasing temperature until 35°C is reached (Wiesmann et al., 2007).

In conventional systems, such as activated sludge processes (described in section 1.1.5), nitrification and denitrification are often accomplished in different reactor units. The most used configuration is the pre-denitrification, in which nitrate is reduced to nitrogen gas using the incoming COD as electron donor in the anoxic tank and nitrate is recirculated from the subsequent aerobic tank (where nitrification takes place) to the

anoxic tank. In systems partly penetrated by oxygen, such as biofilms/granules, where both aerobic and anoxic zones are simultaneously present in the same reactor, simultaneous nitrification and denitrification (SND) can occur.

New processes for biological nitrogen removal

Recently, new treatment technologies have been developed to improve nitrogen removal from wastewater and overcome limitations of the conventional process (nitrification and denitrification). The new processes are based on partial nitrification of ammonium to nitrite (nitrite route), which can be accomplished in single reactor system commonly known as SHARON (Single Reactor System for High Ammonium Removal Over Nitrite) (Hellinga et al, 1998; van Dongen et al., 2001).

The SHARON reactor is operated without biomass retention at a relatively high temperature (35°C). These conditions are used to favour ammonium-oxidizing bacteria over nitrite-oxidizing bacteria (van Dongen et al., 2001). Besides the temperature, other variables such as pH, hydraulic retention time, dissolved oxygen and ammonium and nitrite concentrations (especially their unionized forms, i.e., free ammonia and free nitrous acid, respectively) can be controlled to achieve stable partial nitrification (Hellinga et al., 1998). Accurate control to achieve partial nitrification can be rather difficult in large-scale operation.

SHARON systems can be followed by a conventional denitrification process or by the anaerobic ammonium oxidation (anammox) process. The anammox process is based on autotrophic members of the order of Planctomycetes (Strous et al., 1999). These slow-growing organisms with doubling times as long as 11 days obtain energy by carrying out ammonium oxidation with nitrite as electron acceptor, without any addition of external organic carbon source (Jetten et al., 1999; Strous et al., 2002).

The occurrence of partial nitrification/anammox process in the same biofilm-based reactor system operated under oxygen limited conditions is designated as CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) process (Third et al., 2001). The new technological developments for biological nitrogen removal are more suitable for high-strength ammonium wastewaters (e.g., sludge reject water and industrial wastewater with relative high ammonium concentration and low amount of organic carbon). Recent studies are directed towards the possibility of applying the anammox process into the main stream of wastewater treatment systems (Winkler et al., 2012).

New nitrogen conversions are still being discovered in the vast microbial world. Examples are the ammonium oxidation carried out by crenarchaea (Konneke et al., 2005) and genome sequencing of several organisms involved in the nitrogen cycle (Strous et al., 2006).

1.1.3. Biological phosphate removal

Phosphate removal can be accomplished efficiently and economically by the so called enhanced biological phosphorus removal (EBPR) process. The process does not only represent a good way to remove phosphate in an efficient way but also consists of an interesting object of study for microbial ecology research (Brdjanovic, 1998). The organisms responsible for phosphate removal, known as polyphosphate-accumulating organisms (PAOs), have a complex physiology involving the formation and consumption of storage polymers (polyphosphate, glycogen, and polyhydroxyalkanoates) (van Loosdrecht et al., 1997a).

The selection of PAOs is obtained by recirculating the sludge through anaerobic and aerobic conditions. Under anaerobic conditions, PAOs are capable of taking up readily biodegradable carbon sources (e.g., volatile fatty acids (VFA)) and store them as intracellular polymers, such as polyhydroxyalkanoates (PHA). In the case of acetate, it will be stored as polyhydroxybutyrate (PHB). The energy required for VFA uptake and subsequent PHA formation is harvested from the cleavage of polyphosphate and release of phosphate from the cell to the bulk liquid. The reducing power required for PHA formation is produced by the glycolysis of stored glycogen (Mino et al., 1998; Smolders et al., 1994a; Smolders et al., 1994b). The energy for transport of VFA and phosphate across the cell membrane is highly influenced by the pH. Therefore, pH has a strong effect on the ratio between VFA taken up and phosphate release to the bulk liquid (Smolders et al., 1994a).

In the subsequent aerobic phase, if electron acceptor is present in the absence of external substrate, PAOs use the stored PHA as energy source for P uptake, replenishment of the glycogen and polyphosphate storage pools and cell growth. Due to the growth of cells, net P removal from the wastewater can be achieved by removing excess sludge with high polyphosphate content (van Loosdrecht et al., 1997a). The growth at the expense of storage substrate is a competitive advantage shown by PAOs over other heterotrophic bacteria, which are not capable to accumulating VFA without external electron acceptor. If external substrate and electron acceptors are

simultaneously available in the aeration period, the substrate is predominantly converted into PHA instead of being used for growth (Brdjanovic et al., 1997). The use of external substrate for the formation of intracellular stored polymers rather than for growth seems to be a basic characteristic of microorganisms subjected to a feast-famine regime, which is common to occur in wastewater treatment processes (van Loosdrecht et al., 1997b).

An interesting characteristic of the organisms involved in bio-P removal systems is the fact that their growth rate was found to be not directly related to substrate availability. The primary use of the substrate (PHA) available in these organisms is for polyphosphate and glycogen replenishment and for maintenance. Growth results from the difference between PHA consumption rate and PHA consumption for the aforementioned processes (Murnleitner et al., 1997).

Although the majority of phosphate removal systems operate under alternating anaerobic-aerobic conditions, phosphorus removal can also be accomplished in systems running on alternating anaerobic-anoxic environments. These systems, in particular, rely on the activity denitrifying PAOs (DPAOs), which are capable of using nitrite and/or nitrate instead of oxygen as electron acceptor to take up P anoxically. The nitrite and nitrate are generated in an aerobic reactor, where nitrification takes place. In this case, phosphate removal is integrated with nitrogen removal. Phosphate removal coupled to denitrification is often denoted as denitrifying dephosphatation (Kuba et al., 1993; Kerrn-Jespersen and Henze, 1993). If the fraction of phosphate removed under anoxic conditions is maximized, the operational costs can be significantly reduced due to the economy in aeration necessary for aerobic P uptake and carbon requirements needed for denitrification. Furthermore, since ATP production with nitrate is approximately 40% lower than with oxygen (Kuba et al., 1996), less sludge is produced if phosphate is taken up with oxidized nitrogen as electron acceptor. Figure 1.3 shows schematically the anaerobic and aerobic (anoxic) metabolism of PAOs.

The phosphorus content of the sludge in EBPR process can reach 4 – 5% of total solids dry weight, which is much higher than that of conventional activated sludge processes for organic carbon removal (1.5 – 2.0% of sludge dry weight). In lab-scale EBPR enrichment processes, a phosphorus content of 15% of sludge dry weight has been reported (Crocetti et al., 2000).

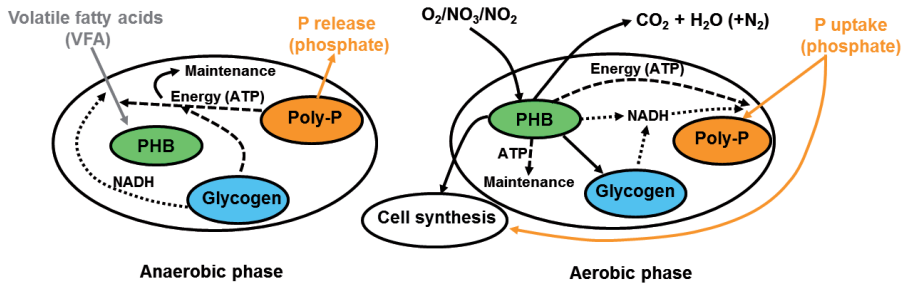


Figure 1.3: Metabolism of polyphosphate-accumulating organisms under anaerobic and aerobic (anoxic) conditions (adapted from van Loosdrecht et al., 1997a). The aerobic (anoxic) metabolism shown in the scheme represents the case when no external substrate is available.

Even though EBPR systems are a sustainable and efficient way to remove phosphate from wastewaters, the stability of this process can be troublesome. One of the main causes of deterioration in performance of EBPR systems is the competition between PAOs and glycogen-accumulating organisms (GAOs). GAOs are usually found in bio-P removal reactors since they also proliferate under alternating anaerobic and aerobic conditions (Crocetti et al., 2002; Saunders et al., 2003), competing with PAOs for the available organic substrate. GAOs primarily gain energy (ATP) and reducing power from the glycogen degradation to uptake VFA under anaerobic conditions and store them as PHA. In the subsequent aerobic phase, PHA is oxidized, leading to replenishment of glycogen and cell growth (Zeng et al., 2002; Zeng et al., 2003a). GAOs do not contribute for phosphate removal since no phosphate release or uptake is involved in their metabolism. Therefore, GAOs are usually considered as undesirable organisms in EBPR systems (Oehmen et al., 2006a; Oehmen et al., 2006b).

Recently, several studies regarding the factors influencing PAO-GAO competition (e.g., pH, carbon source, temperature) have been carried out (Filipe et al., 2001; Oehmen et al., 2005a; Oehmen et al., 2005b; Liu et al., 1997a; Lopez-Vazquez et al., 2008; Whang and Park, 2006). Operational strategies to favour PAOs over GAOs are of extreme importance to enhance the performance and stability of bio-P removal systems.

1.1.4. Biological processes in wastewater treatment

The practical application of microorganisms to treat waste streams in engineered basins is called biological wastewater treatment, consisting of the most efficient and economical way to remove pollutants from wastewaters. Biological removal processes

rely on the ability of microorganisms to take up organic matter (e.g., carbohydrates, fatty acids and proteins) and nutrients (such as nitrogen and phosphorus) present in the wastewater for their growth, maintenance and energy production.

The biological processes are basically the same as those occurring naturally in the environment. However, since the operating parameters such as pH, dissolved oxygen concentration, and temperature are well controlled in engineering systems, the degradation of pollutants by bacteria is significantly enhanced compared to that carried out naturally. All the systems applied for biological treatment rely on biomass retention. Incoming substrate concentrations are usually low, so the amount of biomass produced on the wastewater is also low. To increase the volumetric capacity bacteria are retained in the reactor systems. Conventionally, this is done by creating flocculated sludge which can settle and therefore be separated from the treated wastewater in clarifiers. Since settling of flocs under gravity is a slow process, large clarifiers are needed to achieve proper sedimentation. As a consequence, the footprint of the treatment plant is considerably increased. By immobilising the bacteria as biofilms, the biological treatment system can be made much more compact.

1.1.5. Conventional biological systems for wastewater treatment

In biological wastewater treatment, microorganisms are used to metabolize the pollutants present in the wastewater. This step is usually carried out as a secondary treatment. The primary treatment involves the separation of large and floating particles from the wastewater by means of screens, primary settlers and grit chambers. In this way, the wastewater entering the biological process (secondary treatment) mainly contains dissolved organic matter and nutrients (nitrogen and phosphorus), which are present in both inorganic and organic forms. The most applied system in biological wastewater treatment is the conventional activated sludge process.

Activated sludge process

The activated sludge process was developed in England in 1914 (Ardern and Lockett, 1914) and was called activated sludge since it involves the production of activated mass of microorganisms responsible for the stabilization of waste compounds. Activated sludge systems make use of mixed culture of suspended microorganisms, including bacteria, protozoa and fungi to speed up the decomposition of organic and inorganic compounds.

The process basically involves two main steps: an aeration tank and a secondary clarifier (Figure 1.4). In the aeration basin, air is introduced both to provide oxygen needed for the microorganisms to oxidise the pollutants and to mix the wastewater with the activated sludge. This mixture is often regarded as mixed liquor. After the biological treatment taking place in the aeration tank, the mixed liquor flows to a secondary settling tank (clarifier), where the activated sludge settles and can be separated from the treated effluent (supernatant) by gravity. Part of settled sludge is returned to the aeration tank to keep a viable concentration of biomass inside the reactor to treat the incoming wastes. The clarified effluent can be discharged into receiving waters.

During the biological treatment process, microbes grow on the waste compounds and consequently sludge is produced. Part of the sludge, often regarded as excess, surplus or waste sludge, has to be discharged. The excess sludge can be further digested in a sludge digester and dewatered on sludge drying beds or mechanical devices (e.g., vacuum filter, centrifuge, belt filter press) before its final disposal.

The efficacy of the biological treatment using activated sludge systems depends on the microbial community present in the reactor, which are responsible for the oxidation of pollutants in the waste streams, and also on the biosolid/liquid separation in the clarifier. If the sludge settleability is poor, biomass washout will occur, decreasing the effluent quality.

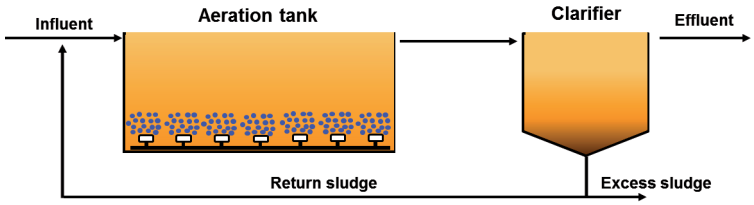


Figure 1.4: Illustration of the main steps involved in the conventional activated sludge process.

In order to achieve biological nutrient removal, several modifications of the activated sludge processes have been proposed. By incorporating anaerobic, anoxic and aerobic zones (multistage treatment processes), not only COD removal and nitrification but also nitrogen and phosphorus removal could be accomplished in the treatment plant. University of Cape Town (UCT) configuration and the Bardenpho process (with two, three or four stages) are common systems applied to remove nutrients from

wastewaters. Basically, the differences between these configurations are the zones (anaerobic/anoxic/aerobic) sequence and the application of recycle streams.

The necessity of several tanks to accomplish the removal of both COD and nutrients (nitrogen and phosphorus) and especially the need for clarifiers significantly increases the footprint of the wastewater treatment plant. This is the reason why compact systems were developed over the years to deal with space limitation and to improve nutrient removal efficiencies.

1.1.6. Compact biological systems for wastewater treatment

The conventional suspended-growth activated sludge-based systems have been widely applied for wastewater treatment. These traditional processes are characterized by low biomass concentration in the reactor (3 – 4 g/L of suspended solids) due to poor settleability of the sludge. Consequently, large surface areas for plant installation are required to reach higher removal efficiency of pollutants.

The exponential growth of population increased the necessity for upgrading the wastewater treatment plants, which are often located in dense urban areas. However, in most cases, the availability of space in urban areas is becoming increasingly scarce. In the meantime, wastewater treatment has been a challenge throughout the years due to stringent effluent regulations posed by environmental agencies, varying influent characteristics and increasing complexity of wastewaters. New technological developments and the enforcement of strict environmental legislations have pushed the development of a new generation of compact wastewater treatment plants showing small footprint requirements and high treatment efficiency, replacing the conventional activated-sludge processes. A common characteristic of all these compact systems is the high biomass retention capability. Since biomass is the biocatalyst for the degradation of all kinds of pollutants in biological treatment systems, retaining high biomass concentration in the reactors means obtaining higher volumetric treatment capacity and lower space requirement. In order to accomplish this, the use of biofilm- or granular-based systems became more popular over the years. Some of the compact systems developed are listed below:

- Upflow Anaerobic Sludge Blanket (UASB) and Expanded Granular Sludge Blanket (EGSB) reactors;
- Biological Aerated Filters (BAF);

- Rotating Biological Contactors (RBC);
- Biofilm Airlift Suspension reactors (BAS);
- Membrane Bioreactor (MBR);
- Sequencing Batch Reactor (SBR);
- Moving Bed Biofilm Reactor (MBBR);
- Aerobic Granular Sludge (AGS) reactor.

A brief explanation of all these processes is described as follows. In this thesis, in particular, the last three compact systems listed above (SBR, MBBR and AGS reactors) were used in different studies regarding important aspects in nutrient removal.

Upflow Anaerobic Sludge Blanket (UASB) and Expanded Granular Sludge Blanket (EGSB) reactors

Anaerobic processes consist of sustainable technology for the treatment of a wide range of different wastewaters (Lettinga, 1995; Lettinga et al., 1995). In anaerobic digestion, organic matter is broken down to simple chemical components in the absence of oxygen in a sequence of reactions: hydrolysis, acidogenesis and methanogenesis.

The treatment processes run in anaerobiosis offer several advantages such as low energy consumption (no aeration needed), production of biogas and lower sludge production (saving on costs associated with sludge disposal) compared to aerobic systems. The sludge bed is composed by microorganisms which naturally form granules (pellets) of 0.5 to 2.0 mm diameter. This granular sludge developed in anaerobic systems, such as UASB (Lettinga et al., 1980) and EGSB (van der Last and Lettinga, 1992) reactors, presents high settling velocity and therefore can be easily kept in the reactor system without being washed out even at high hydraulic loads. Herewith, high organic loading rates (up to 40 kg COD/(m^3·d)) can be applied and compact reactors can be built, reducing the space requirement for plant installation.

In UASB reactors, the wastewater passes upwards through an anaerobic sludge bed (sludge blanket), in which microorganisms come into contact with wastewater substrates. The energy input of these systems is very low, since a good contact between the wastewater and the sludge is provided by agitation and hydraulic turbulence brought up by the biogas produced from anaerobic degradation processes and by a uniform feed distribution at the bottom of the reactor without the need for any mechanical device.

The main constituents of the biogas are CH_4 and CO_2, although small quantities of H_2S, NH_3 and trace amounts of other gases can be released.

Part of the gas produced in the sludge blanket gets attached to the granular sludge (Huishoff Pol et al., 1983). In the upward trajectory through the UASB reactor, the sludge particles strike the bottom of degassing baffles, which release attached gas bubbles and provoke the drop of the granules. To ensure a good separation of the treated wastewater, biomass and biogas, a three-phase (gas-solid-liquid) separator is placed at the top of the reactor, forcing the settling of sludge particles and helping to keep the sludge aggregates in the reactor, which return to the digester compartment. The biogas produced during the anaerobic treatment can be captured in gas collection domes placed in the top of the reactor (Lettinga et al., 1980; Huishoff Pol et al., 1983).

The EGSB reactor is a variant of the UASB concept. The main difference is the higher upward velocity is designed for the wastewater which passes through the sludge bed in the EGSB reactors. The increased flux improves the contact between the sludge and the wastewater and can be achieved by increasing the reactor height and/or by incorporating an effluent recycle. By using effluent recirculation, the upward velocity of the liquid can exceed 5 – 6 m/h, which is much higher than that applied for UASB reactors (0.5 – 1.5 m/h) (de Man et al., 1988).

Even though a high liquid upflow velocity improves the mixing inside the reactor, it can also lead to higher biomass washout. Therefore, a balance should be found between the upflow velocity of the liquid necessary to satisfactorily mix and maintain the sludge hold-up at high levels (Kato et al., 1994). The EGSB is more suitable for the treatment of wastewaters with low COD content (1 – 2 g/L) or wastewaters containing inert or poorly biodegradable suspended particles which should not accumulate in the sludge bed.

Although efficient and economical anaerobic reactors were developed for the treatment of high-strength organic wastewaters (e.g., UASB and EGSB reactors), anaerobic systems are not so efficient for the treatment of diluted municipal wastewaters. Moreover, anaerobic processes present some drawbacks. One of them is the requirement of a further polishing step to meet effluent discharge regulations. Also the removal of nutrients (nitrogen and phosphorus) is marginal and pathogens can be present in the effluent in anaerobic systems. The start-up of the process is much slower compared to aerobic processes and the temperature of the reactor should be relatively high (>20°C).

Biological Aerated Filters (BAF)

BAF combine bacterial degradation of pollutants (soluble organic matter and ammonium) with physical filtration of suspended organic matter by using granular media (Rogalla and Bourbigot, 1990). Biological nutrient removal (nitrogen and phosphorus) can also be accomplished. One important feature of the BAF is the reduced land requirement compared to most conventional systems. These reactors require one-fifth of the area necessary for the construction of conventional activated sludge systems (EPA, 1983). BAF systems are composed of solid, liquid and gas phases. The solid phase consists of the supporting media for biofilm growth; the liquid phase is where the solid material is submerged; and the gas phase is created by aerating the reactor (Mendoza-Espinosa and Stephenson, 1999). The hydrodynamics of BAF systems are influenced by air and liquid input, as well as by the support carrier material (Le Cloirec and Martin, 1984). The hydrodynamics, in turn, affect the development and structure of the biofilm (Wilderer et al., 1995). The absence of subsequent solids separation stage (settlers) avoids problems linked to sludge settling and recirculation.

BAF are usually operated with liquid velocity of 1 to 10 $m^3/(m^2 \cdot h)$ (Stensel et al., 1988; Vedry et al., 1994). However, higher liquid velocities such as 30 $m^3/(m^2 \cdot h)$ have already been applied (Peladan et al., 1997). These reactors can be operated either in upflow or downflow regime, depending on the way the wastewater is supplied to the reactor: from the bottom or top. Upflow systems with concurrent air/wastewater have been claimed to be able to cope with higher inflow rates compared to downflow BAF. The filter can run longer since the formation of air pockets is avoided and the oxygenation is more effective (bubbles do not coalesce and retain their optimum surface-volume ratio) (Mendoza-Espinosa and Stephenson, 1999). In the upflow BAF, odour problems related to air stripping of volatile compounds are reduced since the ambient air is only in contact with the treated wastewater (Iida and Teranishi, 1984).

The depth of the packed media bed of full-scale BAF is usually 2 – 4 m (Stensel et al., 1988; Pujol et al., 1994). The support material used in these reactors can be either denser than water (sunken media, for both downflow or upflow configurations) or less dense than water (floating media, only for upflow configuration). Sunken media can be made of natural products such as shale or phyllosilicates, whereas floating media can be produced from synthetic products such as polystyrene (Ros and Mejac, 1991; Vedry et al., 1994). Periodic backwashing is needed to avoid blocking of BAF systems due to

solids entrapment and microbial film growth, which increases the operating costs of the reactor. Denser media requires higher flow rates when backwashed, implying in higher energy costs.

Rotating biological reactors (RBC)

Rotating biological contacts consist of a series of circular discs mounted on a common shaft placed in a tank in a way that they can slowly rotate at 1 – 2 rpm. The discs are partially submersed (around 40% of the surface area) in a tank where wastewater flows continuously. The discs are usually made of plastic (PVC, polythene, and expanded polystyrene). Biomass grow attached to the plastic disks while they rotate, forming microbial films (biofilms). The biofilm usually presents a very active bacterial population, which is responsible for the degradation of diverse pollutants present in the wastewater. The layer of biological growth depends on the composition of the wastewater, and the biofilm thickness ranges from 1 – 2 mm (Sørensen and Jørgensen, 1993).

When the biofilm attached to the disk is exposed to the air, oxygen is provided for the microorganisms. On the other hand, when the biofilm is submersed in the wastewater, substrates become available for the microorganisms and are metabolized. Excess sludge is removed by shear as the disks rotate. To remove the detached solids, a clarifier can be placed after the RBC (Sørensen and Jørgensen, 1993).

The RBC performance can be enhanced by increasing the number of discs in series. Usually the discs are arranged in compartments with baffles between each compartment. If multiple stages are available, the initial stages receive the highest concentration of organic matter, and a wide diversity of heterotrophs will develop. As the concentration of organic matter decreases in the subsequent stages of the reactor, nitrifying bacteria start to colonize the biofilm. Therefore, not only COD removal but also nitrification can be accomplished in RBC systems (Huang, 1986).

Low power requirements, low operational costs and no need for sludge recycle (attached biomass continuously grow) are some advantages of these systems. However, mechanical problems are common to occur in the rotating system, which requires frequent maintenance.

Biofilm airlift suspension reactors (BAS)

Airlift reactors, in general, consist of a wide range of gas-liquid or gas-liquid-solid pneumatic contacting devices which are characterized by circulation of fluid in a defined pattern through channels appropriately designed for this purpose. The mixture of the reactor is provided by a stream of air. Besides contributing to the reactor agitation, the gas stream facilitates exchange of material between the gas phase and the liquid/solid phases.

Airlift reactors and bubble columns are very similar to each other, and the difference between those two is related to the type of liquid flow, which primarily depends on the geometry of the system. The bubble column is a simple vessel where gas is introduced at the bottom and random mixing is generated by the upward bubbles. In the case of airlift reactors, they are divided in two different zones: the riser, which is usually sparged by a gas, is a channel for gas-liquid upflow; and the downcomer, which is a channel parallel to the riser for the liquid downflow. The different gas hold-up in the gassed and ungassed regions results in different bulk densities of the fluid in these zones, generating a pressure difference which is the driving force for circulation of the fluid in the reactor. The riser and downcomer are linked at the top and at the bottom to form a closed loop. Gas is injected close to the bottom of the riser. The design of the reactor, especially the gas separator, directly influences the fraction of gas that disengages at the top and the fraction that does not disengage, i.e., is entrapped by the downward liquid into the downcomer. This in turn affects the fluid dynamics and hence the performance of the system.

Biofilm airlift biofilm (BAS) reactors are applied in many biotechnological processes, such as biological wastewater treatment (Heijnen et al., 1990; Zhou et al., 2003; Walters et al., 2009). In these systems, the solid phase is usually composed by an inert support (e.g., basalt, sand, activated carbon, and ceramic materials), which supports the growth of microorganisms. Oxygen is transferred from air bubbles to the microbial film, where microorganisms use it for their metabolism. A three phase separator is placed in the top of the reactor. The carrier material used in BAS reactors provides a large surface area for biofilm development, enhancing biomass concentrations and increasing the volumetric conversions in the system.

Some important characteristics of biofilm airlift suspension reactors are simple design and construction, well defined flow patterns and good mixing. However, since these reactors are fed continuously, a complex three-phase separation system is required

to separate the liquid, gas and solid phases. Moreover, in order to accomplish denitrification, nitrate formed during nitrification needs to be circulated in a separate anoxic zone (denitrifying CIRCOX airlift reactor), enhancing the complexity of the reactor. Phosphorus removal is not carried out in BAS systems.

Membrane bioreactors (MBR)

Membrane Bioreactors (MBR) consist of a combination between a conventional activated sludge process (responsible for the biodegradation of waste compounds) and membrane separation technology (responsible for the separation of the treated effluent from the biomass). The integration of biological degradation and membrane filtration offer several advantages. The use of a membrane for biosolid/liquid separation instead of conventional settler allows the complete removal of suspended solids from the effluent, which is free of bacteria and pathogens. This leads to excellent effluent to meet strict discharge requirements and also eliminates problems related to settling properties of the sludge (Chiemchaisri et al., 1992).

Since the biomass is retained inside the reactor, the sludge concentration can be much higher than in conventional wastewater treatment plants. As a consequence, MBR systems are much more compact than conventional activated sludge processes, which significantly reduce the plant footprint. The complete biomass retention inside the reactor also allows for the separation of hydraulic and solids retention times (as in biofilm systems), which favours the control of biological activity and the flexibility in operation. For instance slow-growing organisms, such as nitrifiers, can be kept in the system even at short SRTs (Cicek et al., 2001). Surplus (excess) sludge can be removed from the bioreactor in order to keep the sludge retention time (SRT) at a desired value.

Two main types of MBR systems are available. In the first type, the membrane unit is located inside the reactor and therefore this reactor configuration is called submerged. The driving force across the membrane is achieved by pressurizing the reactor or creating a negative pressure on the permeate side (Buisson et al., 1998). The second type refers to the recirculated (external) MBR, in which a recirculation of the liquid through the membrane unit (located outside the bioreactor) occurs. The driving force for this configuration is provided by the high cross-flow velocity along the membrane surface (Urbain et al., 1998). There are several types of membranes used in MBR installations, including tubular, rotary disk, hollow fiber, organic and inorganic membranes. The pore size can vary from 0.01 to 0.4 μm (Visvanathan et al., 2000).

Some of the main drawbacks encountered in membrane bioreactors are the concentration polarization and other membrane fouling problems, which stop the operation and require cleaning procedures (chemical or mechanical). Chemical cleaning refers to a physical-chemical reaction between the chemical and the foulant. On the other hand, mechanical cleaning is related to the physical removal of suspended solids from the membrane and usually relies on the turbulence caused by aeration, back-flushing and circulation. The excess sludge (waste sludge) removed periodically of MBR systems may show poor settling and filtering properties (Cicek et al., 1999).

The high costs involved in the implementation of the MBR technology limits its application. The development of less expensive, less energy consuming and more efficient membrane modules certainly will contribute to spread the technology worldwide.

Sequencing batch reactor (SBR)

SBR is a variation of the conventional activated sludge process operated discontinuously. In SBR systems, all the metabolic reactions and biosolid/liquid separation occur in a single reactor unit, while in conventional facilities, as shown in section 1.1.5, the different processes occur in separate basins (Metcalf and Eddy, 2003). Therefore, an SBR is actually an activated sludge process which operates in well-defined and continuously repeated time sequence rather than space (Morgenroth and Wilderer, 1998).

The fact that all processes occur in the same tank implies that the footprint of SBRs is much smaller than that of conventional activated sludge processes. This favours the SBR application in areas where space is a limiting factor. The costs of operation of SBR processes are 60% lower than that of the continuous activated sludge process (Ng et al., 1993).

The SBR operation relies on a fill-and-draw principle, being divided into five steps which constitute a SBR cycle (Figure 1.5): filling (static, mixed or aerated), reaction (aerobic or anoxic/anaerobic), settling, effluent withdrawal (removal of supernatant), and idle period (for cycle adjustment, if necessary). The length of an operational cycle usually varies from few hours to one day.

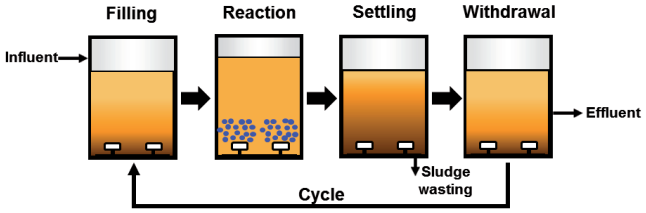


Figure 1.5: Schematic representation of a SBR cycle. The reaction phase shown in the scheme is aerobic; however, it can also be operated under anoxic conditions by using a mechanical stirrer instead of air blowers (adapted from Wilderer et al., 2001).

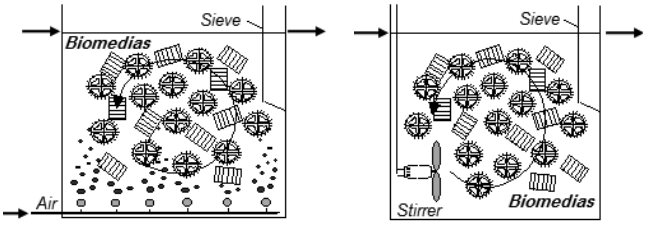
Due to the operational flexibility of SBR systems, their cycle can be modified according to each application in order to increase the process effectiveness. By manipulating the operational cycle (e.g., applying alternating aerobic and anoxic periods), the system can accomplish biological nutrient (nitrogen and phosphorus) removal. The fact that SBR operation relies on time rather than space also allows handling the fluctuation of the flow rates without increasing the footprint of the plant. SBRs have been applied successfully for COD and nutrient (N and P) removal (Hamamoto et al., 1997; Kargi and Uygur, 2004; Debsarkar et al., 2006). Some reports describe the application of SBR for the treatment of high salinity and high-strength nitrogen wastewaters (Woolard and Irvine, 1994; Chang et al., 2000). Improvements accomplished in equipment, such as computer control and aeration devices, enhanced the application of SBR systems, which became a viable alternative for the conventional activated sludge system. Furthermore, SBR technology is also very suitable to achieve aerobic granulation, as will be discussed further.

A modification of the suspended biomass SBR is the sequencing batch biofilm reactor (SBBR), in which the biomass grows attached either to fixed or moving carrier material instead of growing in suspension (Wilderer and McSwain, 2004). The SBBR process has many advantages such as larger surface area for biomass growth and longer sludge age, which favours the development of slow-growing nitrifying bacteria (Jaar and Wilderer, 1992). High biomass concentrations can be kept in the reactor regardless of the settling properties of the sludge and the reactor hydraulic retention time.

Moving bed biofilm reactor (MBBR)

The moving bed biofilm reactor (MBBR) technology was developed in the late 1980's (Ødegaard et al., 1994). This process relies on the use of moving plastic carriers

(supports/biomedias) in which microorganisms form biofilms. In aerobic systems, the aeration is responsible for the movement of the carriers (Figure 1.6a), whereas a mechanical stirrer is often used for this purpose in anaerobic/anoxic systems (Figure 1.6b).



(a) Aerobic reactor (b) Anaerobic/anoxic reactor

Figure 1.6: Moving bed biofilm reactor technology (adapted from Rusten et al., 2006).

The use of moving carriers minimises or even dispenses the need for return sludge flow from the settler to keep sufficient biomass concentration in the reactor (Ødegaard, 2006). Consequently, the footprint of the plant is considerably smaller and the construction costs are decreased. The amount of support materials inside the reactor is often regarded as the carrier filling fraction (ratio between the volume occupied by the supports (fixed bed) and total reactor volume). The filling fraction can be chosen as desired. The biomass concentration can be increased by increasing the amount of support in the reactor. However, in order to achieve good mixing without hydrodynamics problems, carrier filling ratios lower than 70% are recommended (Rusten et al., 2006).

The most common carriers used in MBBR have been developed by AnoxKaldnes®. Carriers with different diameter and shape are commercially available. The density is around 0.95 g/cm^3 for all types (Salvetti et al., 2006; Rusten et al., 1998). Table 1.1 shows some important characteristics of some MBBR carriers. Diverse AnoxKaldnes® carrier materials with biofilm attached are displayed in Figure 1.7.

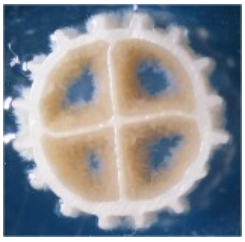
When compared to other biofilm reactors in which biomass is attached to fixed support materials, MBBR systems show lower head loss and no clogging problems (Rusten et al., 2006). Herewith, no backwashing is necessary. The MBBR technology can be applied to upgrade existing wastewater treatment plants. The performance of conventional activated sludge-based systems which often suffer from biomass loss due to poor settleability of the sludge can be improved by introducing MBBR carriers inside

the reactor. In this way, biomass will be present both in suspension and in the biofilm attached to the carriers. If settling problems occur and the sludge return from the settler is affected, at least the fraction which is in the biofilm will be kept in the reactor.

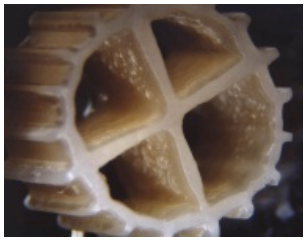
Table 1.1: Characteristics of some supports developed by AnoxKaldnes® (adapted from Rusten et al., 2006, and http://www.anoxkaldnes.com).

	Type of support AnoxKaldnes®						
	K1	K2	K3	Natrix C2	Natrix F3	BiofilmChip M	BiofilmChip P
Nominal diameter (mm)	9.1	15	25	36	64	48	45
Nominal length (mm)	7.2	15	12	30	50	2.2	3.0
Apparent density (kg/m^3)	150	95	100	-	-	-	-
Specific superficial area (m^2/m^3)[a]	500	350	500	220	200	1200	900
Specific superficial area - 60% filling ratio (m^2/m^3)[b]	300	210	300	132	132	720	540

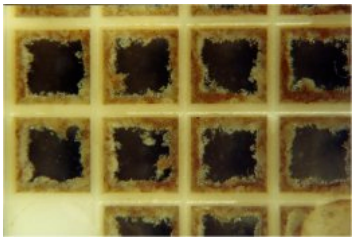
[a] Theoretical total area available for microbial adhesion/reactor volume, completely filled with supports (fixed bed)
[b] Theoretical total area available for microbial adhesion (60% bed)/reactor volume



K1 (Front view)



K1 (Side view)



Biofilm-Chip P (Front View)

Figure 1.7: Different types of AnoxKaldnes® supports containing the biofilm attached. (Source: Courtesy of Veolia).

In aerobic MBBRs, the aeration system plays a double role: provides oxygen for the microorganisms and moves satisfactorily the carriers throughout the reactor volume. This requires a significant air flow, contributing to increase the energy consumption. Moreover, in order to accomplish good mixture of the carriers inside the reactor and avoid stagnant zones, perforated tubes are usually used as aeration devices, especially in pilot- and full-scale MBBR plants. The aeration intensity in MBBR systems also affects directly the detachment of biomass from the carriers. A proper design of the aeration

system is therefore an important criterion to be considered. In order to keep the carriers inside the reactor, a sieve is placed in the reactor outlet, as shown in Figure 1.6.

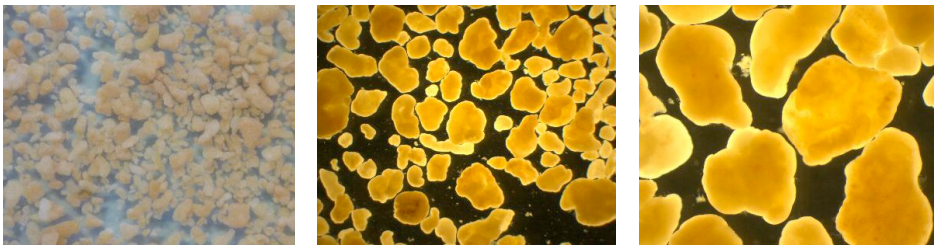
Due to the several advantages offered by the MBBR technology, it has been extensively applied for the treatment of both municipal (Rusten et al., 1995; Zhao et al., 2006; Hu et al., 2009; Kermani et al., 2008) and industrial wastewaters (Ji et al., 2001; Borghei and Hosseini, 2004; Shin et al., 2006; Ratcliffe et al., 2006; Bassin et al., 2011).

Aerobic granular sludge

Granular sludge was initially developed for strictly anaerobic systems in 1980 and the formation of anaerobic granules was carried out in upflow anaerobic sludge blanket (UASB) reactors (Lettinga et al., 1980). The long start-up of this process, high temperature requirement, low nutrient (nitrogen and phosphorus) removal efficiency and the inadequacy of UASB systems to treat wastewaters containing low organic content pushed the development of the aerobic granular sludge technology.

Representing one of the great recent advances in environmental biotechnology, aerobic granular technology consists of a promising and innovative technology for the wastewater treatment segment. Aerobic granules are considered as self-immobilized nearly spherical or elliptical microbial aggregates (special case of biofilm growth with no need for carrier material) containing a high diversity of microorganisms, including both aerobic and facultative bacteria.

Aerobic granular sludge does not coagulate under reduced hydrodynamic shear and its settling velocity is much higher than that of activated sludge (de Kreuk et al., 2005a). The settling velocity of aerobic granules varies from 18 to 90 m/h (Zheng et al., 2005; Xiao et al., 2008). The sludge volume index (SVI), which is another parameter related to the settling properties, is below 80 mL/L (Zheng et al., 2005; Lin et al., 2005). For aerobic granular sludge, in particular, the SVI_5 is the same as SVI_{30}. The diameter of the granules is larger than 0.2 mm, reaching up to 16 mm (Beun et al., 1999; Toh et al., 2003; Zheng et al., 2006; Li et al., 2008). A sieve can be used to classify the granules and to observe the proportion of the granules within the total biomass. The granulation process is completed when the granules correspond to 80% of the total solids present in the reactor (de Kreuk et al., 2005a). The structure of aerobic granular sludge is illustrated in Figure 1.8.



(a) (b) (c)

Figure 1.8: Structure of aerobic granular sludge. Real size (a), 7.5× magnification (b), and 20× magnification (c).

Some important characteristics shown by the aerobic granular sludge are (Beun et al., 1999; Liu and Tay, 2004; de Kreuk and van Loosdrecht, 2004; Adav et al., 2008; Shi et al., 2009; Gao et al., 2011):

- Excellent settling properties (settling velocity can reach 50 – 90 m/h), favouring the biosolid/effluent separation;
- Regular and round shape;
- Offers the possibility to retain high amount of biomass in the reactor and achieve long sludge retention times (especially beneficial for nitrifying bacteria);
- Strong and dense microbial structure;
- Presence of both anaerobic and anoxic zones inside the granular structure, allowing for the occurrence of different biological processes in the same reactor unit, such as simultaneous nitrification and denitrification (SND) and partial nitritation of ammonium to nitrite and Anammox reaction;
- Lower operating costs and reduced footprint compared to conventional activated sludge processes.

One of the first researches describing the development of aerobic granular sludge was carried out by Morgenroth et al. (1997) in a sequencing batch reactor (SBR). Subsequently, numerous studies reported the formation of aerobic granules in the same bioreactor type (Beun et al., 1999; Peng et al., 1999; Etterer and Wilderer, 2001; Tay et al., 2001; Liu and Tay, 2002). The granulation process is influenced by several factors, such as the substrate composition (Tay et al., 2001), seed sludge (Liu and Tay, 2004), organic loading rate (Li et al., 2008; Adav et al., 2010), feeding strategy (McSwain et al., 2003; de Kreuk and van Loosdrecht, 2004), reactor configuration (Liu et al., 2005a), settling time (Qin et al., 2004), temperature (de Kreuk et al., 2005b), dissolved oxygen

concentration (McSwain Sturm and Irvine, 2008), and hydrodynamic shear forces provided by the aeration (Tay et al., 2001).

One of the most important selection pressures used to enhance aerobic granulation is the settling time in SBRs. A short settling time can be applied in these sequentially operated systems in order to keep only fast-settling biomass (>15 m/h) and cause the washout of poorly settling suspended biomass. As a consequence, high volumetric loadings can be applied, resulting in a compact reactor configuration. At long settling times, poorly settling sludge cannot be removed effectively and may outcompete granule-forming particles. Consequently, the granulation process can fail (Liu et al., 2005b).

One of the main factors responsible for the density of granules is the actual growth rate of the microorganisms. Slow growing organisms, such as nitrifiers, will produce denser and more stable granules than fast growing organisms, such as heterotrophs (Villaseñor et al., 2000; de Kreuk and van Loosdrecht, 2004). Less stable granules are not desired since they are susceptible to shear stress and can be easily disintegrated. A method to enrich for organisms with low growth rate in systems fed with readily biodegradable substrates is to convert them into intracellular polymers such as polyhydroxyalkanoates (PHA). A good way to accomplish this conversion is by applying a feast-famine regime in a discontinuous system (e.g., SBR). In the feast phase, the substrates which are easily biodegradable are converted to PHA. If this particular phase is run under anaerobic conditions, polyphosphate-accumulating organisms (PAOs) and/or glycogen-accumulating organisms (GAOs) can be selected (de Kreuk and van Loosdrecht, 2004). These organisms have actual growth rate similar to slow-growing nitrifiers (Brdjanovic et al., 1998; Lopez-Vazquez et al., 2009). The development of PAOs in the aerobic granular sludge SBR allows to accomplish phosphate removal from the wastewater. However, if the feast phase is run under aerobic conditions, the development of other fast-growing heterotrophs will occur. Consequently, fluffy granules will potentially appear in the system, harming the biomass settling properties and leading to biomass washout.

The polymers stored intracelullarly can be used for growth and maintenance during the famine period, when limited or no external substrate is available. The growth on PHA is generally slower than the growth on easily biodegradable substrates (Beun et al., 2002a). Herewith, the formation of storage polymers favours the development of stable and dense granules (Beun et al., 2002b).

In aerobic granular sludge systems, COD, nitrogen and phosphorus removal can be simultaneously achieved. Instead of using different compartments as in activated sludge-based systems, the parallel processes occur in the same reactor system but in different zones inside the granules. Figure 1.9 illustrates the main conversions taking place in the aerobic granular sludge when an anaerobic feast phase is applied, followed by a famine aerobic phase. In these operational conditions, the conceptual microbial community of the aerobic granule is mainly composed by PAOs, nitrifying bacteria and denitrifying bacteria. It should be remarked that the microbial composition is directly influenced by the wastewater composition, inoculum and operational conditions.

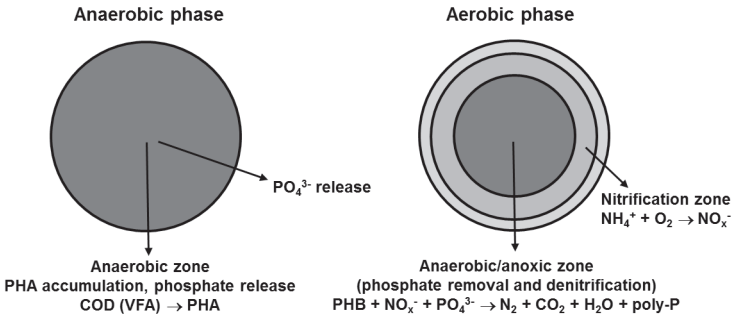


Figure 1.9: Scheme of the main conversions taking place in different layers of the aerobic granular sludge in the anaerobic feast phase (a) and aerobic famine phase (b). The layered structure of each zone in the aerobic phase depends on the DO concentration outside. NO_x^-: NO_2^- and/or NO_3^-.

During the aerobic phase of an aerobic granular sludge SBR, simultaneous nitrification and denitrification (SND) process takes place in different layers of the granular structure. Nitrification is accomplished in the outer aerobic zone of the granule, where autotrophic nitrifying bacteria are usually located. The NO_x^- (NO_2^-/NO_3^-) formed during nitrification can diffuse into the core of granule (anoxic zone) and be denitrifed using PHA stored intracellularly as electron donor. Denitrification is often coupled to anoxic phosphate removal (denitrifying dephosphatation), which is performed by the denitrifying polyphosphate-accumulating organisms (DPAOs). Nitrogen removal efficiency depends on the distribution of the aerobic and anoxic zones, which is linked to the oxygen concentration in bulk (de Kreuk et al., 2005c).

Aerobic granular sludge technology has been applied successfully for COD (Arrojo et al., 2004) and nutrient (nitrogen and phosphorus) removal (de Kreuk et al.,

2005c), removal of toxic substances (Tay et al., 2004) and biosorption of heavy metals (Liu et al., 2002; Liu et al., 2003).

1.1.7. Molecular techniques to study the microbial diversity

Historically, engineers have designed biological treatment systems relying only on chemical and physical analysis, taking into account very few microbiological aspects. The biological reactors were run utilizing the advantages of microbial metabolism without detailed knowledge about the main microorganisms involved in the conversion processes. However, in order to monitor and better control all the biochemical reactions taking place in these systems, it is essential to gain information about the composition and activity of specific bacteria which are playing major role in removing pollutants from the wastewater. Advances in the study of microbial diversity and ecology are crucial for the biological wastewater treatment field, since the design of most wastewater treatment plants are largely based on empirical data (Wagner, 2005), despite the fact that the mechanisms of the biological treatment are fundamentally grounded in microbiology.

Recently, the identification of specific populations of microorganisms in diverse engineered wastewater treatment ecosystems by microbial molecular ecology techniques represents a great breakthrough in environmental engineering evolution over the years. The molecular approaches have enormously increased the insight into the vast diversity of microorganisms in these complex biological systems without the need for cultivation. The molecular biology approaches are based on RNA of small ribosomal subunits (16S rRNA for prokaryotes), which are used as molecular markers. Ribosomal RNA (rRNA) genes are evolutionarily conserved, being very useful to describe phylogenetic relationships between organisms (Woese, 1987).

Several molecular techniques based on the polymerase chain reaction (PCR) amplification and comparison of rRNA sequences have been used to identify microorganisms present in wastewater treatment reactors. Some of them include denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (t-RFLP) and cloning.

DGGE is based on the different mobility of denatured fragments of DNA of the same size (e.g,. amplified rRNA gene fragments with approximately 500 bp) but with distinct sequence of nucleic acids. This generates different banding patterns (fingerprints) in a DGGE gel reflecting the dominant microbial diversity of the studied

sample. Individual DGGE bands can be excised from the gel and sequenced to reveal the identity of the microorganisms (Muyzer et al., 1993; Muyzer and Smalla, 1998). DGGE has been extensively used to observe microbial population shifts and succession of microorganisms over time.

T-RFLP is also an rRNA gene-targeted analysis of microbial community. In this technique, the primers are fluorescently labelled to tag the PCR product. The amplicons are digested with restriction nuclease and the fragments are separated by electrophoresis. The fragments which are labelled can be detected and an electrophoretic fingerprint reflects the microbial community composition (Liu et al., 1997b).

Cloning of PCR products is also used in microbial ecology studies. In this technique, certain genome sequence present in PCR products is inserted in other DNA called vector, which is normally a plasmid. Subsequently, the vector is inserted inside competent cells (e.g., *Escherichia coli*), where it can multiply and produce new plasmids containing the cloned fragment. The plasmid DNA can be extracted, sequenced and a clone library can be created. The phylogenetic affiliation of sequences which were cloned can be determined by means of specific computer software (e.g., ARB).

Another technique applied to detect specific bacteria in mixed cultures is the fluorescent in situ hybridization (FISH) analysis. This technique is based on short sequences of DNA of 15 – 25 bp, often regarded as oligonucleotide probes, labelled with a fluorescent dye. These probes recognize and hybridize in situ with specific sequences of rRNA in fixed cells (DNA - RNA matching), which can be visualized in an epifluorescence microscope. Herewith, specific bacteria can be identified and also quantified in a particular system (Amann et al., 1990). Probes can be developed to detect and identify any taxonomical level, from Domain to individual species. Furthermore, a quantitative analysis can be carried out with FISH, allowing for the determination of the abundance of certain bacteria within the whole microbial population (Wagner et al., 1993).

Recently, more sophisticated molecular methods started to be applied in the wastewater treatment field, such as the DNA microarrays (Kelly et al., 2005). These high throughput technologies provide a way to rapidly examine and screen the microbial composition of any environment (Wilson et al., 2002; Dennis et al., 2003). The combination between molecular methods and process engineering can provide a

complete insight about a biological system, allowing for the improvement of both performance and reliability of the reactor. By applying microbial engineering (as carried out in some studies of this thesis), the competition between some microorganisms present in wastewater treatment systems can be controlled in such a way that a better performance and stable operation can be achieved.

1.2. Background of the thesis

1.2.1. Development of nitrifying biofilms in moving bed biofilm reactors

Moving bed biofilm reactor (MBBR) is a relatively new biofilm technology which is increasingly attracting the attention of engineers engaged in the wastewater treatment sector. MBBR systems incorporate benefits provided by both activated sludge and biofilm processes. This bioreactor technology uses polyethylene carrier elements to provide sites for the attachment of active bacteria (Ødegaard et al., 1994; Ødegaard, 2006). Herewith, in the case of the treatment plant upset, biomass attached to MBBR ring media can be maintained inside the reactor without being washed out. This characteristic is a very important advantage offered by the MBBR technology compared to conventional activated sludge systems, which are easily susceptible of biomass loss due to poor settleability of the suspended sludge.

The development of the biofilm in, for instance, MBBR systems, depends on the operational conditions applied and on several environmental factors. The wastewater composition is a parameter which directly affects the dominant microbial populations within the biological system. The microorganisms present in the bioreactor influences the rate at which biomass accumulates on the plastic carriers. High organic matter concentrations in the influent favour the development of heterotrophs. Heterotrophic bacteria are fast-growing organisms, and easily outcompete slow-growing nitrifiers for both oxygen and space (especially in the case of biofilms) (Figueroa and Silverstein, 1992). Due to the slow growth rate and biomass yield of nitrifying bacteria, a relatively long period is needed to recover nitrification capacity if washout of these organisms occurs. Therefore, their presence in the reactor should be maximized to avoid deterioration of nitrification performance.

In this context, it is reasonable to think that in order to select for nitrifiers and achieve good nitrification performance in biological treatment systems, the presence of heterotrophic population should be minimized as much as possible. However, there are

some circumstances in which heterotrophic bacteria can be advantageous for nitrifiers. The microbial film formed by heterotrophic microorganisms can function as a protection layer for nitrifiers against detachment (Furumai and Rittmann, 1994), creating a suitable environment for the growth and development of autotrophic nitrifying microorganisms. This fact can be taken into consideration when developing operational strategies to enhance the formation of nitrifying biofilms in MBBR systems. Moreover, some previous studies reported that autotrophic nitrifying bacteria hardly form biofilms themselves due to their specific low growth rate and lack of production of extracellular polymeric substances (EPS), responsible for enhancing biomass adhesion to solid surfaces (Tsuneda et al., 2001). As a consequence, the development of nitrifying biofilms on the surface of carrier materials of MBBR systems in which nitrification should be accomplished in wastewaters with limited or no organic carbon can be a very slow process. Furthermore, retaining a significant amount of nitrifying bacteria in autotrophic biofilm reactors might be a difficult task.

Several reports investigating the nitrification process in MBBR are reported in literature (Rusten et al., 2006; Salvetti et al., 2006; Yu et al., 2007; Bassin et al., 2011). No detailed study has been carried out on the application of different operational conditions to enhance the biofilm formation and decrease the time necessary to develop enriched nitrifying biofilms. This thesis was initially intended to address some important questions regarding this issue, as well as to point out some important process parameters to track the development of enriched nitrifying biomass in MBBR systems.

1.2.2. Aerobic granular sludge systems: a network of simultaneous conversions

In conventional biological nutrient removal systems, nitrogen and phosphate removal are usually integrated. The combined enhanced biological phosphorus removal (EBPR) and conventional nitrification/denitrification processes can encounter some problems since organic matter content (usually expressed as chemical oxygen demand (COD)) is usually a limiting factor for phosphorus release/uptake and denitrification. This drawback is especially relevant in the treatment of wastewaters containing low organic carbon/nitrogen (C/N) ratio. The presence of denitrifying PAOs (DPAOs) can overcome the limitation of COD. These organisms are capable of using nitrate and/or nitrite (produced in the nitrification process under aerobic conditions) instead of oxygen as electron acceptor and the intracellular stored polyhydroxyalkanoates (PHA) as electron donor to simultaneously remove phosphorus and nitrogen from wastewater

without the need for an external organic carbon source for denitrification (Kerrn-Jespersen and Henze, 1993; Kuba et al., 1993). Besides using the incoming COD in an efficient way, the use of oxidized nitrogen (e.g., nitrite or nitrate) rather than oxygen as electron acceptor also leads to savings in aeration costs.

Recently, new insights about important organisms present in simultaneous N and P removal systems, such as PAOs capable of denitrification, have been shown in several research studies. He et al. (2006), studying the distribution of different *Candidatus* Accumulibacter phosphatis (henceforth called Accumulibacter) types in lab- and full-scale EBPR systems, detected various clades within the members of this microbial group. The Accumulibacter diversity was further divided into two main types (I and II), using the polyphosphate kinase gene (ppk1) as genetic marker (He et al., 2007; Peterson et al., 2008). A metagenomic analysis of Accumulibacter clade II enriched EBPR sludge demonstrated a lack of respiratory nitrate reductase in this specific microorganism (Garcia Martin et al., 2006). Other investigations showed that Accumulibacter populations established in a lab-scale reactor under nitrite reducing conditions was not capable of reducing nitrate (Guisasola et al., 2009). All these findings related to nitrate reduction capability suggested differences in the metabolism of members of Accumulibacter lineage. Based on experimental evidence, Flowers et al. (2009) have shown that PAO clade I was capable of using nitrate whereas PAO clade II was able to use only nitrite as electron acceptor for anoxic P uptake.

In the last decade, a lot of research studies have been carried out on the development of aerobic granular sludge (AGS) and the factors affecting aerobic granulation (Morgenroth et al., 1997; Beun et al., 1999; Dangcong et al, 1999; Tay et al., 2001). The application of aerobic granules to achieve simultaneous COD, nitrogen and phosphorus removal in sequencing batch reactors is also well described in the literature (Zeng et al., 2003b; Mosquera-Corral et al., 2005; Li et al., 2005; de Kreuk et al., 2005c; Yilmaz et al., 2008; Kishida et al., 2008). All of these studies regarding the operation of AGS systems only describe their general performance in terms of nitrogen and phosphorus removal without specifying the role of specific subpopulations of PAOs (PAO clade I or PAO clade II) on P and N conversions. Also the information about the possible interaction and competition between the main microorganisms involved in those processes (AOB, NOB, PAOs and GAOs) within the different layers of aerobic granules is still limited. In order to address some open questions, a complete characterization of the main process conversions taking place in well-controlled aerobic

granular sludge reactors performing simultaneous N and P removal is an objective of this thesis. An operational strategy to control PAO/GAO competition in order to enhance phosphate removal at tropical temperatures (such as 30°C) is also pointed out.

1.2.3. Microbial diversity and activity of nitrifying bacteria in aerobic granular sludge

The microbial diversity of nitrifying bacteria has been extensively described for conventional activated sludge systems. The most common ammonium-oxidizing bacteria (AOB) in these processes belong to the genus *Nitrosomonas* (Daims et al., 2009), while the most reported nitrite-oxidizing bacteria (NOB) belong to the genus *Nitrospira* (Daims et al., 2001; Gieseke et al., 2003). However, scarce information about nitrifying communities in aerobic granular sludge is reported in literature. Moreover, no comparison between the activity of AOB and NOB has been carried out in AGS systems. In these systems, oxidation of nitrite by NOB and reduction of nitrite by denitrifying bacteria occur simultaneously under normal operating conditions. Therefore, the absolute nitrite-oxidizing capacity is not directly measurable. In this thesis, the nitrifying community structure and the activity of both AOB and NOB in an aerobic granular sludge system was investigated in appropriate batch tests.

An unexpected NOB/AOB ratio higher than 1 (the expected NOB/AOB ratio based on autotrophic ammonium and nitrite oxidation is 0.5 or even lower in systems where simultaneous nitrification and denitrification takes place) was observed by Quantitative PCR (qPCR) only in the aerobic granular sludge system but not in a conventional activated sludge reactor. Furthermore, nitrite oxidation rate was found to be higher than ammonium oxidation rate in the aerobic granules. Based on experimental results and literature review, the potential reasons for the disproportion in the ratio of AOB and NOB and unusual nitrite oxidation capacity observed in granular systems applied in our study are discussed in this research.

1.2.4. Ammonium adsorption to aerobic granular sludge

During operation of lab- and pilot-scale aerobic granular sludge reactors under alternate anaerobic/aerobic phases, ammonium concentrations after anaerobic feeding were found to be lower than expected based on the influent concentration and dilution in the reactor. This fact was associated with a possible ammonium adsorption into the aerobic granules. From the literature, it is known that ammonium can be adsorbed to activated sludge flocs (Nielsen, 1996; Schwitalla et al. 2008) and to biofilms (Wik,

1999; Temmink et al., 2001). However, no information about ammonium adsorption in aerobic granular sludge was available in the literature.

The adsorption of ammonium to the biomass can best be seen as an ion exchange process and the amount of ammonium adsorbed will be related to the compounds functioning as ion exchanger for cations. Potentially, some of these compounds can be the acidic extracellular polymers and the precipitates formed on these polymers present in the sludge. Few investigations focused on identifying those ion exchangers so far. Schwitalla et al. (2008) observed antiparallel desorption of potassium during ammonium adsorption. However, the sources of potassium were not investigated.

In aerobic granular sludge processes, simultaneous nitrification, denitrification, and enhanced biological phosphate removal (EBPR) takes place. The phosphate release by phosphorus-accumulating organisms (PAOs) under anaerobic conditions favours the precipitation of phosphates into diverse minerals (Maurer et al., 1999). Struvite (ammonium magnesium phosphate) formation under anaerobic conditions was firstly suggested by Yilmaz et al. (2008). Subsequently, the presence of struvite-like structure in aerobic granular sludge was indicated by Raman spectrum (Angela et al., 2011).

In our work, pyramidal shaped yellow-brown crystals, similar to K-struvite (potassium magnesium phosphate) as reported by Torzewska et al. (2003), were observed on the surface of the aerobic granules. Therefore, K-struvite was assumed to be a potassium source in the granular biomass, consisting of potential sites for ammonium adsorption. A detailed investigation on the importance and causes of ammonium adsorption to aerobic granular sludge was carried out in this thesis.

1.2.5. Salinity and its effect on the performance and microbial diversity of biological wastewater treatment processes

The production of wastewaters containing high salt concentrations is a common characteristic of seafood processing, pickling, cheese manufacturing, tanning, oil and gas recovery, chemical and pharmaceutical industries. The scarcity of fresh water may obligate some regions to use seawater for toilet flushing, enhancing the presence of salts in the treatment plants (Woolard and Irvine, 1995; Panswad and Anan, 1999). Salt can also be introduced into the sewer system by infiltration of subsurface water in coastal areas through cracks or leaks in sewer pipes.

Salinity has a direct impact on the physiology of microorganisms. Under saline conditions, living organisms need to adapt and adjust structurally (osmoadaptation) and physiologically to defend their cytoplasm against high external osmotic pressure. This process reduces the free water activity inside the cell, which lead to dehydration and growth interruption (Moussa, 2004). This can partly explain why most of the fresh water-based microbial populations are unable to survive at high salinity environments (Madigan et al., 2002). Inorganic salts also affect the structure and settleability of microbial flocs, decreasing the oxygen solubility and its transfer to the liquid phase (Van't Riet and Tramper, 1991). In this context, high salt concentrations have a negative impact on biological wastewater treatment systems and the efficiency of organic matter, nitrogen and phosphorus removal processes in high salinity conditions is expected to decrease (Dalmacija et al., 1996).

Life at high salt concentrations is costly from a bioenergetic point of view. This is especially relevant for nitrifying bacteria, which harvest very small amount of energy from their energy conservation metabolism (Oren, 1999). Several studies reported the reduction in activity of ammonium and nitrite oxidizers at high salt concentrations (Panswad and Anan, 1999; Campos et al., 2002; Moussa et al. 2006). Most of these data are difficult to compare, which can be attributed to the different experimental conditions used, mode of salt addition (either by pulse or gradual dosing) and the microbial culture involved. Gradual adaptation of microorganisms to high salinity levels was described to be an operational strategy to minimize the negative effect of salt on nitrification (Panswad and Anan, 1999; Bassin et al., 2011). However, no study reported on the role of changes in the microbial community structure of nitrifying sludge during adaptation to salt. This particular issue was addressed in this thesis.

The effect of salt on aerobic granular sludge (AGS) is also scarcely reported in literature. This issue was only addressed by Figueroa et al (2008) and Li and Wang (2008). Figueroa et al. (2008) investigated the treatment of wastewater from a fish canning factory containing high salt concentration (up to 30 gNaCl/L) in an aerobic granular sludge sequencing batch reactor. These authors studied the effect of salt on COD and nitrogen removal, although they do not report on the influence of high salinity conditions on phosphorus removal. Li and Wang (2008) investigated the content of both inorganic and organic components in aerobic granules at low (1% w/v) and high (5% w/v) salinity levels based on thermogravimetric analysis. However, no investigation of the salt effect on process conversions was carried out in that study.

None of the aforementioned studies regarding the effect of salt on the aerobic granular sludge process performed a detailed study on the dynamics of the microbial community structure within the granules and on the link between the microbial diversity and activity. This missing information in the literature and the relevance of the effect of salt on biological wastewater treatment processes were the motivation to include in this thesis a study regarding the complete evaluation of the microbial diversity of aerobic granules at increasing salt concentrations and the relation between the process conversions and molecular microbial analysis.

1.3. Scope and outline of the thesis

This thesis has been developed in the field of water pollution, specifically focused on the removal of nutrients from wastewaters by biological processes. Biological nutrient removal is a well-established process in the wastewater treatment segment. However, there are still some interesting engineering and microbiological aspects regarding this issue to be addressed. The research described in this thesis was conducted to fill some gaps in both operational and fundamental knowledge about nutrient removal in compact systems. Moving bed biofilm reactors (MBBR), aerobic granular sludge reactors (AGS) and sequencing batch reactors with suspended biomass were operated in order to enhance and update the knowledge in several aspects of nutrient (nitrogen and phosphorus) removal processes. The driving force for some specific studies has also arisen during the experimental period. The structure of this thesis is displayed in Figure 1.10. An outline of the research topics studied in this thesis is presented as follows.

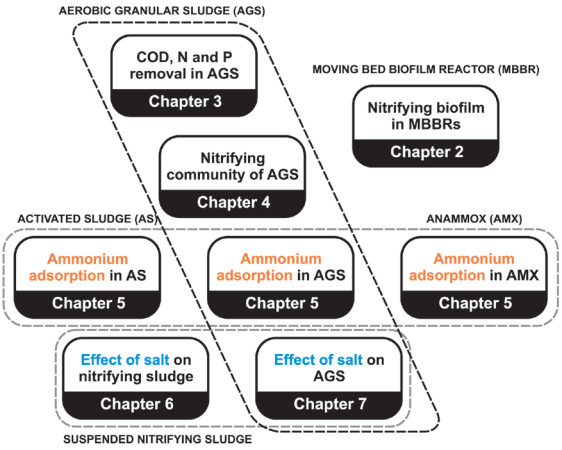


Figure 1.10: Structure of the thesis. Topics inside the central black dashed line indicate studies conducted with aerobic granular sludge. Grey dashed lines indicate topic-related studies carried out with different types of biomass. The names of the corresponding systems are also indicated.

The first aim of this thesis was to study the effect of different operational conditions (e.g., influent composition and feeding regime) on the formation of nitrifying biofilms and on the nitrification process in moving bed biofilm systems. Scanning electron microscopy and FISH analysis were linked to analytical methods to observe the enrichment of the biofilm for nitrifiers. Strategies to develop enriched nitrifying biofilms with minimum time requirement in MBBR systems and the feasibility of applying a MBBR operated in a sequencing batch mode for the treatment of a high-strength ammonium wastewater are discussed as well. This study is described in **Chapter 2**.

Chapter 3 deals with the complete characterization of the main process conversions taking place in aerobic granular sludge reactors operated at different temperatures (20 and 30°C), achieving simultaneous COD, nitrogen, and phosphorus removal. Some aspects such as the effect of dissolved oxygen concentration and temperature on N and P conversions, the role of denitrifying polyphosphate-accumulating organisms to the overall P removal, and the main organisms responsible for denitrification were investigated in this research.

While studying the nitrifying community in an aerobic granular sludge (AGS) reactor operated at 30°C, an unexpected NOB/AOB ratio higher than 1 was observed by means of qPCR. This surprising result was not observed in a conventional activated sludge system studied in parallel. Furthermore, activity batch tests with biomass collected from the AGS reactor have shown that the nitrite oxidation capacity was

higher than the ammonium oxidation capacity. **Chapter 4** describes the possible mechanisms and theories to explain the unexpected observations regarding the NOB/AOB ratio and nitrifying activity measurements.

During operation of lab- and pilot-scale aerobic granular sludge reactors with alternate anaerobic/aerobic phases, ammonium concentrations after anaerobic feeding were found to be lower than expected based on the influent concentration and dilution in the reactor. This fact was associated with a possible ammonium adsorption to the aerobic granules. A detailed study on ammonium adsorption in aerobic granular sludge and its main causes is presented in **Chapter 5**. A comparison between the extent of ammonium adsorption in different types of biomass (aerobic granular sludge, activated sludge, and anammox granules) was also addressed in the particular research. Adsorption kinetics and adsorption isotherms were determined in order to provide a better insight into the ammonium adsorption process and for potential future inclusion in mathematical process models.

In **Chapter 6** and **Chapter 7**, the effect of salt on biological nutrient removal is presented. The salt effect on the microbial diversity, activity, and settling of suspended nitrifying sludge in sequencing batch reactors is described in **Chapter 6**. The role of changes in the microbial community structure during adaptation to high salt concentrations is discussed in this study. **Chapter 7** deals with the effect of elevated salt concentrations on the aerobic granular sludge process. In this study, the salt effect on the main biological processes (COD, nitrogen, and phosphorus removal) within the granules is related to the dynamics of microbial populations. PCR products obtained from genomic DNA and from rRNA after reverse transcription are compared to identify present bacteria as well as metabolically active bacteria. FISH analysis is used to validate the PCR-based results and to quantify the dominant bacterial populations.

The main findings and overall conclusions of this thesis are summarized in **Chapter 8**. In this final chapter, the results obtained in different studies are compared.

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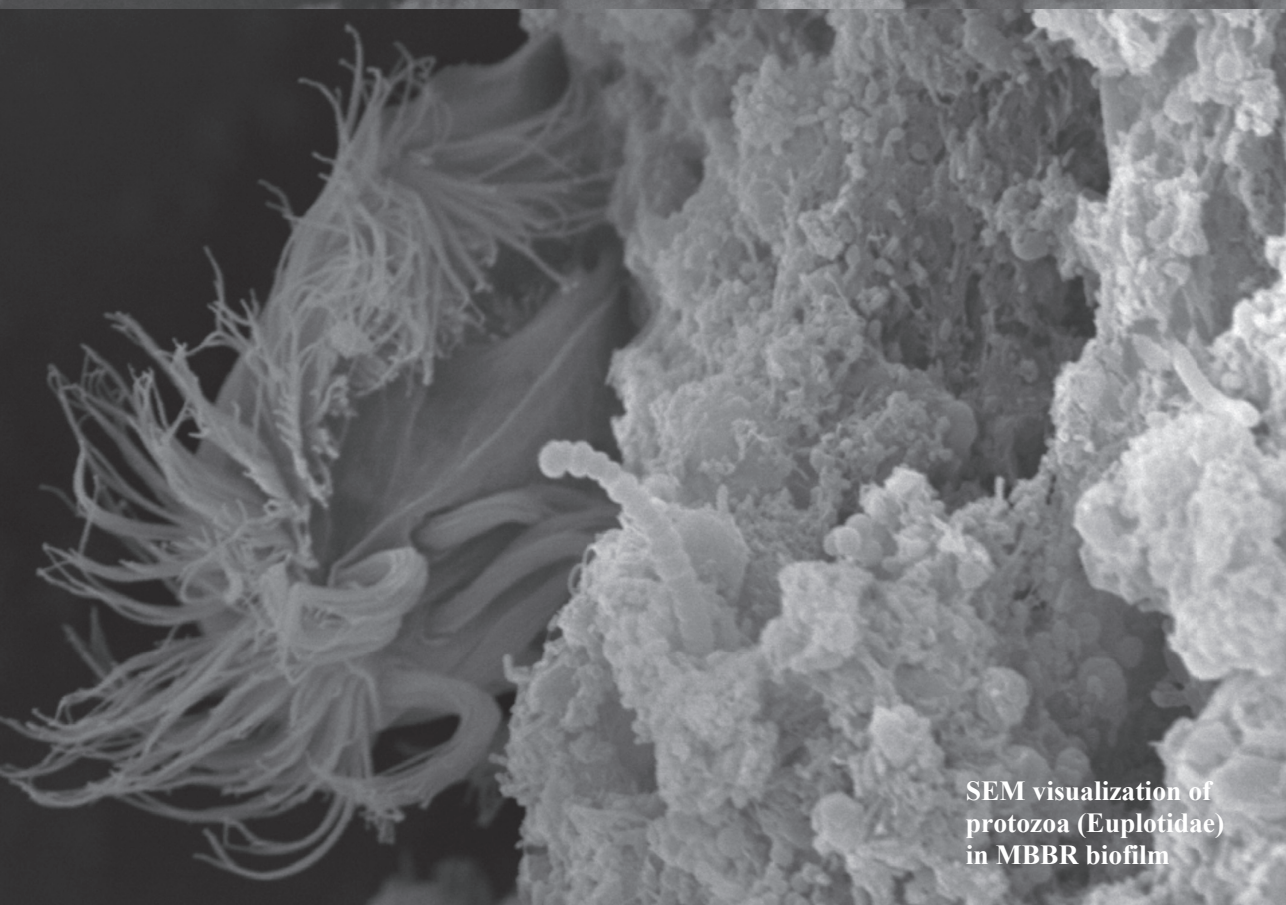
SEM visualization of protozoa (Vorticella) in MBBR biofilm



SEM visualization of protozoa (Euplotidae) in MBBR biofilm

CHAPTER 2

Effect of different operational conditions on biofilm development, nitrification, and nitrifying microbial population in moving bed biofilm reactors

This Chapter is based on two works. Part I is published as: Bassin, J.P., Kleerebezem, R., Rosado, A.S., van Loosdrecht, M.C.M., Dezotti, M. (2012) *Effect of different operational conditions on biofilm development, nitrification, and nitrifying microbial population in moving bed biofilm reactors*. **Environmental Science and Technology** 46(3), 1546-1555. Part II is submitted for publication as: Bassin, J.P., Abbas, B., Kleerebezem, R., Muyzer, G., Rosado, A.S., van Loosdrecht, M.C.M., Dezotti, M. *Characterization of the microbial community of moving-bed biofilm reactors operated under different COD/N ratios*.

Chapter 2

Content

Abstract

In this study, the effect of different operational conditions on biofilm development and nitrification in three moving bed biofilm reactors (MBBRs) was investigated: two reactors were operated in a continuously fed regime and one in sequencing batch mode. The presence of organic carbon reduced the time required to form stable nitrifying biofilms. Subsequent stepwise reduction of influent COD caused a decrease in total polysaccharides and protein content, which was accompanied by a fragmentation of the biofilm, as shown by scanning electron microscopy, and by an enrichment of the biofilm for nitrifiers, as observed by fluorescent in situ hybridization (FISH) analysis. Polysaccharide and protein concentrations proved to be good indicators of biomass development and detachment in MBBR systems. Ammonium- and nitrite-oxidizing bacteria activities were affected when a pulse feeding of 4 gNH_4^+-N/($m^2 \cdot d$) was applied. Free nitrous acid and free ammonia were likely the inhibitors for ammonium- and nitrite-oxidizing bacteria.

2.1. Introduction

Nitrification is considered the rate-limiting step in biological nutrient removal systems (Tsuneda et al., 2003). The stability of nitrification relies on several environmental conditions, such as temperature, pH, and dissolved oxygen concentration. In addition, nitrifying bacteria are very susceptible to inhibition by the presence of organic toxicants (Pagga et al., 2006), heavy metals (Hu et al., 2002), high salinity levels (Moussa et al., 2006), and even high concentrations of substrate (Anthonisen et al., 1976; Seviour and Blackall, 1999). One of the most critical parameters for the nitrification process is the concentration of organic substrate in the wastewater, which directly influences the competition between heterotrophic and autotrophic bacterial populations (Hanaki et al., 1990; Cheng and Chen, 1994). The chemical oxygen demand/nitrogen (COD/N) ratio can affect nitrification performance, especially in biofilm systems, where the competition between heterotrophic and nitrifying bacteria for oxygen and space is even enhanced (Tijhuis et al., 1994a; van Benthum et al., 1997). High COD concentrations favour the development of heterotrophs, which grow much faster than autotrophs and outcompete them for oxygen and nutrients (Figueroa and Silverstein, 1992; Harremoës, 1982). As a result, the autotrophic nitrifiers can be easily overgrown by heterotrophs, which eventually cause the nitrification efficiency to decrease (Lee et al., 2004).

However, the presence of heterotrophic bacteria can be advantageous for nitrifying bacteria in some circumstances. The biofilm layer formed by heterotrophic bacteria can function as a protection for the nitrifying bacteria against detachment (Furumai and Rittmann, 1994). In this way, a suitable environment is created by the heterotrophic bacteria for the autotrophs to grow. Furthermore, autotrophic nitrifying bacteria were reported to hardly form biofilms themselves, due to their specific low growth rate and lack of production of extracellular polymeric substances (EPS) that can enhance adhesion (Tsuneda et al., 2001). Consequently, the development of a nitrifying biofilm on the surface of carrier materials of biofilm reactors when no organic compound is present in the wastewater can be a very slow process (Tsuneda et al., 2001). Therefore, retaining a significant amount of nitrifying bacteria in autotrophic biofilm reactors might be a difficult task.

In recent years, there has been an increasing interest in biofilm processes for wastewater treatment. The use of biofilm-based reactors offers numerous advantages

compared to conventional systems (e.g., activated sludge process), such as less space requirement and easy biosolid-liquid separation. One of the growing biofilm technologies applied in wastewater treatment is the moving bed biofilm reactor (MBBR), which was developed in the late 1980s (Ødegaard et al., 1994). This process relies on the use of moving plastic carriers in which microorganisms form biofilms. In this way, slow-growing organisms such as nitrifying bacteria can be kept in the system without being washed out. This characteristic represents an important advantage compared to the conventional systems, which often suffer from loss of active biomass due to potential process disturbances. The use of moving carriers can also minimize or even dispense the return sludge flow from the settler (Ødegaard, 2006). When compared to other biofilm reactors in which biomass is attached to fixed support materials, MBBR systems show lower head loss and no clogging problems (Rusten et al., 2006).

Due to all these specific features, the moving bed biofilm reactor technology has been applied for the treatment of different wastewaters in lab-scale (Bassin et al., 2011; Yu et al., 2007), pilot-scale (Shin et al., 2006), and full-scale (Johnson et al., 2000) systems. Nevertheless, there is no study regarding the effect of different operational conditions on the development of enriched nitrifying biofilms, crucial for the treatment of wastewaters containing high ammonium concentration. Moreover, no study reported on the use of polysaccharides and protein to monitor trends in the amount of biomass attached to the moving carriers in MBBR systems. In this work, the effect of some parameters (e.g., influent composition and feeding regime, either continuous or sequencing-batch) on the development of nitrifying biofilms and on the nitrification process in different MBBRs was investigated. Some useful methods to develop nitrifying biofilms on the MBBR carrier material with minimum time requirement in moving bed biofilm reactors are pointed out. The physical structure of the biofilm was assessed by scanning electron microscopy (SEM) and the evolution of the nitrifying community was monitored by fluorescent in situ hybridization (FISH) analysis. The feasibility of applying a sequencing batch biofilm reactor for the treatment of wastewater with high ammonium concentration is also addressed in this research.

2.2. Materials and Methods

2.2.1. Moving bed biofilm reactors: configuration and operating conditions

The experiments were conducted in three Plexiglas laboratory-scale moving bed biofilm reactors operated in parallel. Two rectangular reactors (42 cm height, 24 cm width, and 11.8 cm depth) with a useful volume of 5 L, named $MBBR_1$ and $MBBR_2$, were operated in a continuous regime. The other system (50 cm height, 18 cm width, 10 cm depth, and 7 L useful volume), consisted of a sequencing batch biofilm reactor, designated as SBBR. The actuation of pumps and valves in the SBBR was controlled through a programmable logic controller (PLC) connected to a computer for data acquisition. The cycle time was either 48 or 24 h, and the cycle profile comprised the following phases: 3 min feeding, 47 h 54 min aeration (for 48 h cycle) or 23 h 54 min aeration (for 24 h cycle) and 3 min withdraw. No settling phase was needed since the biomass was attached to the plastic carriers. The volume exchange ratio of the SBBR was 85%, resulting in hydraulic residence time of 56 h (for the 48 h cycle) and 28 h (for the 24 h cycle) All the reactors were filled with AMB® plastic carriers, whose specific area for biofilm growth is 500 m^2/m^3 of reactor. These carrier materials have a cylindrical shape with 9 mm height and 15 mm diameter. The amount of support material corresponded to a volume fraction of 40% ($V_{support}/V_{reactor}$).

A settler was placed after the $MBBR_1$ and $MBBR_2$ for collecting liquid samples and also to have an indication of the solids content, which originated from the detachment occurring in the moving carrier elements. Aeration was provided by porous diffusers made from polypropylene, placed in the bottom of each reactor. Besides providing good oxygen transfer into the liquid phase, the air bubble distribution generated by this type of diffuser also allowed proper circulation of the supports in the reactor, avoiding stagnant areas. The dissolved oxygen (DO) concentration was around 6.5 – 7.0 mg/L for the continuously operated systems. For the SBBR, the DO concentration varied along the operational cycle. The temperature was kept at 24 ± 3°C and pH was maintained between 6.5 and 7.5 in all reactors by adding either 1 M NaOH or 1 M HCl. The reactors were fed with synthetic medium (Table 2.1) prepared from inorganic (Campos et al., 1999) and organic components (Holler and Trosch, 2001).

Table 2.1: Inorganic and organic components of the synthetic medium.

Inorganic Components[a]	Concentration (mg/L)[c]	Organic Components[b]	Concentration (mg/L)[c]
NH_4Cl	382	Casein peptone	192
KH_2PO_4	222	Meat extract	132
$MgSO_4$	53	Urea	36
NaCl	889		
$NaHCO_3$	900		

[a] Adapted from the medium described by Campos et al. (1999)
[b] Adapted from the medium described by Holler and Trosch (2001)
[c] Concentrations were varied according to the desired COD and NH_4^+-N concentrations in different experimental runs of the reactors.

The operating conditions of $MBBR_1$, $MBBR_2$ and SBBR are displayed in Table 2.2. Depending on the reactor and on the experimental run, the medium composition was varied in order to obtain the desired COD and ammonium concentrations. A trace elements solution (Vishniac and Santer, 1957) was always added in a proportion of 0.5 mL/L of medium for all the reactors. When the synthetic medium contained organic components, it was stored under refrigeration (4°C) to minimize biodegradation.

Table 2.2: Experimental conditions of $MBBR_1$, $MBBR_2$ and SBBR.

System	Experimental run	Influent COD (mg/L)	Influent NH_4^+-N (mg/L)	HRT[a] or Cycle time[b] (h)	Time of operation (days)
$MBBR_1$ (continuous reactor)	1	0	90 - 100	48[a]	103 (60)[c]
	2	0	140 - 170	48[a]	32
	3	0	180 - 200	48[a]	31
	4	0	180 - 200	36[a]	32
	5	0	180 - 200	24[a]	28
$MBBR_2$ (continuous reactor)	6	400	100 - 200	24[a]	73 (30)[c]
	7	200	100 - 200	24[a]	26
	8	100	100 - 200	24[a]	21
	9	0	100 - 200	24[a]	23
SBBR (sequencing batch reactor)	10	400	150 - 200	56[a]/48[b]	39 (20)[c]
	11	400	150 - 200	28[a]/24[b]	24
	12	200	150 - 200	28[a]/24[b]	16
	13	0	290 - 300	28[a]/24[b]	33
	14	0	550 - 650	28[a]/24[b]	53
	15	0	1200 - 1300	28[a]/24[b]	26

[a] Hydraulic retention time: continuous reactors ($MBBR_1$ and $MBBR_2$)
[b] Cycle time: sequencing batch reactor (SBBR)
[c] Start-up period for biofilm development during run 1 ($MBBR_1$), run 6 ($MBBR_2$), and run 10 (SBBR)

A start-up phase for the initiation of biofilm formation was introduced in the beginning of the experimental runs in all reactors. $MBBR_1$ was always fed only with inorganic substrates (Table 2.1). This reactor was inoculated with suspended biomass from a lab-scale sequencing batch reactor that had been operated with the same

inorganic medium for 2 months in order to obtain an adequate amount of nitrifying bacteria. This operational strategy was performed in $MBBR_1$ to see how the biofilm development proceeds when an enriched nitrifying culture is seeded. The $MBBR_2$ was initially fed with the synthetic medium containing both inorganic and organic components (Table 2.1) and was inoculated with activated sludge from a municipal wastewater treatment plant (ETIG, Rio de Janeiro, Brazil) without a previous acclimation step. The objective in this reactor was to first colonize the biofilm with heterotrophic bacteria containing a low amount of nitrifying bacteria and gradually enrich for nitrifiers by decreasing the organic load (as indicated in Table 2.2). The SBBR was inoculated with a mixture of biomass detached from the plastic carrier materials of $MBBR_1$ (run 2) and $MBBR_2$ (run 6) during normal operation. This procedure was carried out to see if inoculating MBBR-adapted biomass would speed up the biofilm development in SBBR.

2.2.2. Microscopic analysis of the biomass

Characterization of the biomass from all reactors was performed by using scanning electron microscopy (SEM). Representative samples from the end of some experimental phases were collected from all reactors. Initially, a piece of the plastic carrier was cut carefully with a razor blade in order to keep the original biofilm structure. Furthermore, preparation of the sample was performed as follows: fixation with glutaraldehyde 2.5% in cacodylate buffer 0.1 M for 1 h; three washing steps (10 min each) with cacodylate buffer 0.1 M; post-fixation with osmium tetroxide (OsO_4) 1% in cacodylate buffer 0.1 M for 1 h; three washing steps with cacodylate buffer 0.1 M (10 min each); gradual dehydration with successive immersions (10 min each) in increasingly concentrated ethanol solutions (30%, 50%, 70%, 90% and 100%) and critical point drying (Bal-Tec CPD 030, Balzers, Liechtenstein). The dried specimen in the carrier material was attached to supports with silver glue and coated with gold powder in a Balzers FL-9496 metalizer for observation in a JEOL JSM-5310 microscope.

2.2.3. Fluorescent in situ hybridization (FISH)

Biomass samples removed from the carrier materials with a spatula were collected in the end of each operational phase for all reactors. The samples were washed twice with 1× phosphate-buffered saline (PBS) (4.3 mM Na_2HPO_4 + 1.47 mM KH_2PO_4,

137 mM NaCl and 2.7 mM KCl, pH 7.0) and were fixed with paraformaldehyde solution (3% final concentration) for 3 h at 4ºC. Furthermore, the sample was centrifuged at 13000 g for 1 min washed twice in 1× PBS and resuspended in an ethanol-PBS solution (1:1) for storage at -20°C. The hybridization, washing procedures and image acquisition can be found elsewhere (Bassin et al., 2012). Several oligonucleotide probes targeting nitrifying bacteria (ammonium- and nitrite-oxidizing bacteria) and anammox bacteria are listed in Table 2.3.

Table 2.3: Oligonucleotides probes and their targeted microbial groups.

Probe	Sequence (5'-3')	Target group	Mix[a]	Reference
Neu 653	CCCCTCTGCTGCACTCTA	Halotolerant and obligate halophilic *Nitrosomonas*	AOBmix	Wagner et al. (1995)
Nse 1472	ACCCCAGTCATGACCCCC	*Nitrosomonas europaea*, *N. eutropha*, *N. halophila*		Juretschko et al. (1998)
Nso 1225	CGCCATTGTATTACGTGTGA	Ammonia-oxidizers *β-Proteobacteria*		Mobarry et al. (1996)
Nso 190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizers *β-Proteobacteria*		Mobarry et al. (1996)
Nmv	TCCTCAGAGACTACGCGG	*Nitrosococcus mobilis*		Juretschko et al. (1998)
Nit 1035	CCTGT CTCCATGCTCCG	Nitrobacter spp.	NOBmix	Wagner et al. (1996)
Ntspa 662	GGAATTCCGCGCTCCTCT	*Nitrospira*		Daims et al. (2001)
AMX 368	CCTTTCGGGCATTGCGAA	All *Anammox*	Anammox	Schmid et al. (2003)
AMX 820	AAAACCCCTCTACTTAGTGCCC	*Brocadia* and *Kueneia*		Schmid et al. (2000)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	EUBmix	Amann et al. (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*		Daims et al. (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales		Daims et al. (1999)

[a] Combination of probes to detect a certain group of bacteria.
Formamide concentration used during hybridization was 35% for all probes.

2.2.4. Analytical measurements and calculation procedures

Total suspended solids (TSS) and volatile suspended solids (VSS), chemical oxygen demand (COD) and ammonium were measured as proposed by Standard Methods (APHA, 1995). Five representative plastic carriers were taken from each reactor, and the biomass was detached from them for determination of the TSS and

VSS. The total amount of TSS and VSS was calculated on the basis of total number of plastic carriers in the bioreactors. Effluent biomass concentrations, which consisted mainly of detached biofilm biomass, were also determined. Nitrate and nitrite were analysed by ion chromatography (Dionex ICS 90) and an analytical kit provided by Hach Co., respectively. Dissolved oxygen was monitored with a DO meter WTW (Oxi 538), and temperature and pH were measured with a specific electrode (Oakton pH 110 meter) in the continuously fed moving bed biofilm reactors. In the sequencing batch biofilm reactor, DO, temperature, and pH were measured online. In order to have another indicative measurement of the biomass concentration in the reactors, total polysaccharide and protein contents of biomass attached to the plastic carrier materials were determined. Two representative biocarriers from the reactors were placed in a falcon tube. A solution of 1 N NaOH was added to the tubes, which were heated for 5 min at 90 ºC in order to promote complete cell lysis. Polysaccharide determination was performed according to Dubois et al. (1956). Total protein concentration was determined by the Lowry assay (Lowry et al., 1951), by use of the Folin-Ciocalteu reagent. Nitrogen mass balances were performed by consideration of the influent nitrogen (as ammonium) and the effluent nitrogen (as remaining ammonium, nitrite, and nitrate). The amount of ammonium that was nitrified and found back as nitrate was designated as nitrogen recovery in the bulk. Specific ammonium uptakes rate in the cycle tests of the SBBR were obtained by linear regression of the ammonium concentration over time divided by the amount of VSS in the reactor. Free ammonia (FA) and free nitrous acid (FNA) were calculated according to Anthonisen et al. (1976). Since biofilm development is a dynamic process influenced by attachment and detachment of microorganisms to surfaces, the biofilm surface specific detachment rate (k_d) was calculated. Biofilm surface specific detachment rate $k_d \left[\frac{gVSS}{m^2 \cdot d}\right]$ was calculated according to equation (2.1).

$$k_d = \frac{r_x}{A_{total\_biofilm}} \tag{2.1}$$

Where $r_x \left[\frac{gVSS}{d}\right]$ is the biomass (VSS) production rate, calculated according to equation (2.2).

$$r_x = \frac{\left(X_{t+\Delta t} - X_t\right) + \sum_{i=t}^{t+\Delta t} X_i}{ND} \quad (2.2)$$

Where $X_{t+\Delta t}\left[gVSS\right]$ is the total amount of biomass in the reactor at day t+Δt, $X_t\left[gVSS\right]$ is the amount of biomass in the reactor at day t, $X_i\left[gVSS\right]$ is the total amount of biomass washout from the reactor at day i ($t \leq i \leq t+\Delta t$), ND is the number of days between day t and day t+Δt; and $A_{biofilm}\left[m^2\right]$ is the surface area of a single carrier available for biofilm growth is that provided by the manufacture (specific area of 500 m^2/m^3 reactor). Therefore, in 5L ($MBBR_1$ and $MBBR_2$) and 7L (SBBR) of reactor, the $A_{biofilm}$ will be 2.5 and 3.5 m^2, respectively. The total surface area ($A_{total\_biofilm}$) was calculated by multiplying the surface area of the carrier available for biofilm growth (2.5 or 3.5 m^2) by the filling ratio of carriers present in the reactor ($V_{support}/V_{reactor}$ = 0.40). The value obtained for $A_{total\_biofilm}$ is therefore 1.0 (for $MBBR_1$ and $MBBR_2$) or 1.4 m^2 (for SBBR).

2.3. Results

2.3.1. Biofilm development in the moving bed biofilm reactors

In the beginning of the experimental runs, a period of time intended for biofilm formation (designated as start-up phase) was imposed on all three moving bed biofilm reactors. In $MBBR_1$, operated in autotrophic conditions, a period of 2 months was required to establish a stable and thin biofilm on the support material. With the feeding strategy adopted in $MBBR_2$ in which a low organic load (1.93 $gCOD/(m^2 \cdot d)$) was applied to favour the growth of heterotrophic microorganisms, a precursor thin film (similar to that observed in $MBBR_1$) was developed in few days and a relatively thick (compared to $MBBR_1$) and mature biofilm was achieved in 1 month. In SBBR, the same feeding strategy was used as in $MBBR_2$; a thin biofilm layer developed within a few days and the time necessary for developing a thick biofilm in SBBR was even lower than in $MBBR_2$ (around 20 days).

Scanning electron microscopy allowed us to monitor the distribution of the biofilm on plastic carriers in different experimental runs of moving bed biofilm systems.

In the autotrophically run $MBBR_1$, a slight change in biofilm morphology was observed over time. The biofilm structure in $MBBR_1$ during run 3 (Figure 2.1a) is less homogeneous in comparison with the one visualized in run 5 (Figure 2.1b), although it shows bigger biomass clusters in the exopolymeric matrix. Biomass concentration in the autotrophic reactor (displayed in the x-axis of Figure 2.2a) was much lower compared to the other systems ($MBBR_2$ and SBBR), varying from 0.4 gVSS/L (run 1) to 0.7 gVSS/L (run 5). Also the detachment rate was much lower than that of $MBBR_1$ and SBBR (around 0.015 g/(m^2·d) during the whole experimental period).

The gradual decrease in influent COD along the experimental runs in $MBBR_2$ caused a significant reduction of biofilm thickness. The specific surface detachment rate gradually increased in runs 6 – 8 from 0.042 to 0.110 g/(m^2·d), respectively. From run 8 to run 9, this parameter decreased to 0.068 g/(m^2·d). In the meantime, biomass concentration gradually decreased from 2.0 gVSS/L (run 6) to 1.0 gVSS/L (run 9) (x-axis of Figure 2.2b). SEM micrographs showed that the biomass structure significantly changed when influent COD was reduced in $MBBR_2$ (Figures 2.1c,d). In run 6 (Figure 2.1c), when the highest organic load was applied, biomass was evenly distributed throughout the biofilm. However, when influence COD was reduced by half in run 7 (Figure 2.1d), biomass was not attached in some parts of the plastic carrier surface. The microbial film is more concentrated in certain regions of the support material, where dense biomass conglomerates were observed.

A similar trend as occurred in $MBBR_2$ was observed in the biofilm of SBBR, in which biomass was distributed more homogenously in run 11 (Figure 2.1e), when the organic load was the highest applied to that reactor (1.54 gCOD/(m^2·d)). In run 12, when the influent COD was half that of run 11, biomass was not attached in some regions of the plastic carrier, although dense microbial clusters can be observed (Figure 2.1f). The lowest biomass concentrations attached to the support material were observed in runs 13 – 15, when the reactor influent contained no organic carbon source. The specific surface detachment rate increased in runs 11 – 13 from 0.050 to 0.094 g/(m^2·d), respectively. This was accompanied by a gradual reduction in biomass concentration from 2.5 gVSS/L (run 11) to 1.8 gVSS/L (run 13), also evidenced by SEM analysis. For Runs 13 – 15, operated under autotrophic conditions, the detachment rate was very low and remained practically constant at 0.025 g/(m^2·d), suggesting that most of the heterotrophic layer had been removed (detached). Biomass concentration during this period slightly decreased from 1.8 to 1.4 gVSS/L (x-axis of Figure 2.2c).

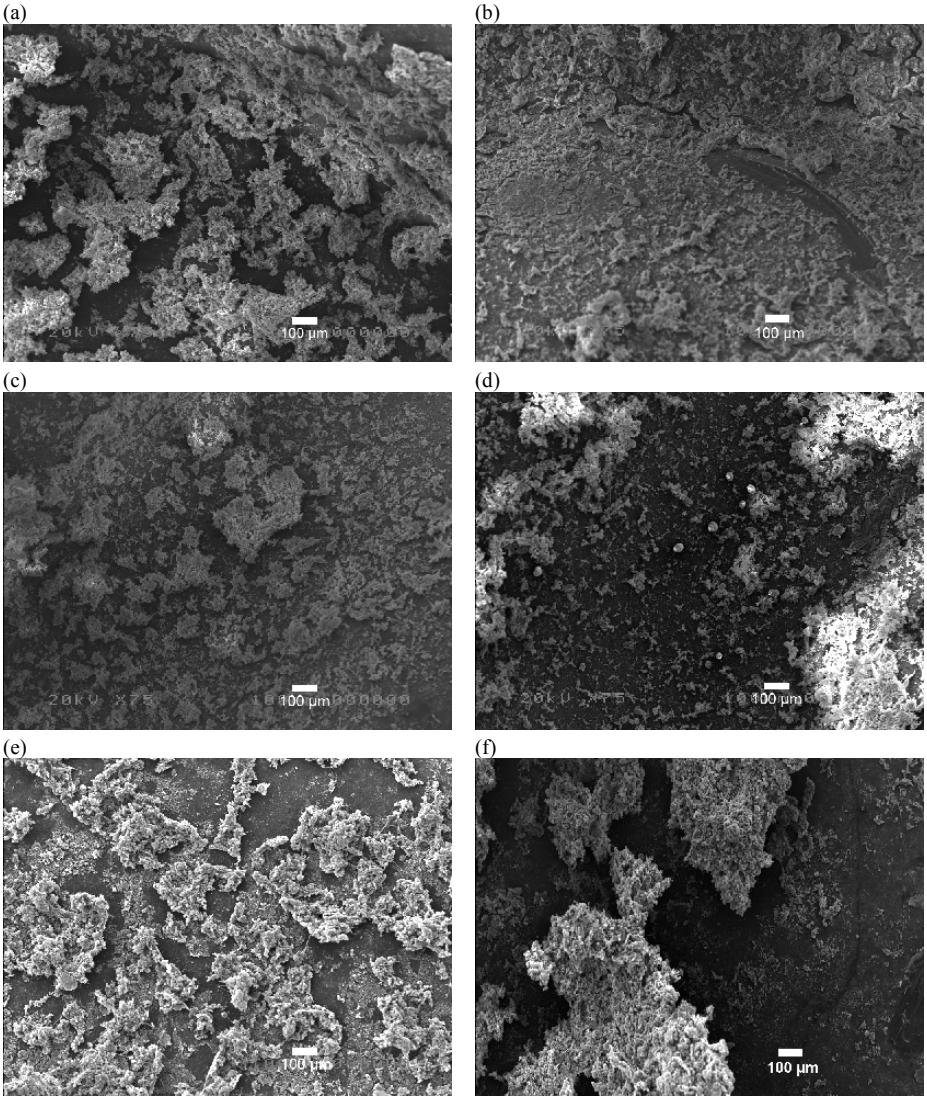


Figure 2.1: SEM micrographs showing the structure of biofilm attached to the carrier material in different experimental runs of all moving bed systems. $MBBR_1$ runs 3 (a) 5 (b), $MBBR_2$ runs 6 (c) and 7 (d), and SBBR runs 11 (e) 12 (f) are shown.

2.3.2. Polysaccharides and Proteins of the biofilm

In moving bed biofilm reactors, determination of the biomass concentration can be rather complicated. The removal of all biofilm from the inner part of the carrier is sometimes a difficult task. To circumvent difficulties encountered during measurement of the biomass concentration by gravimetric methods, total protein and total polysaccharide concentrations were measured in order to provide extra information

about the dynamics of biomass concentrations. The relation between biomass concentration (expressed as VSS) and polysaccharide and protein concentrations for all reactors is displayed in Figure 2.2. In general, polysaccharide/protein and biomass concentrations followed a similar trend.

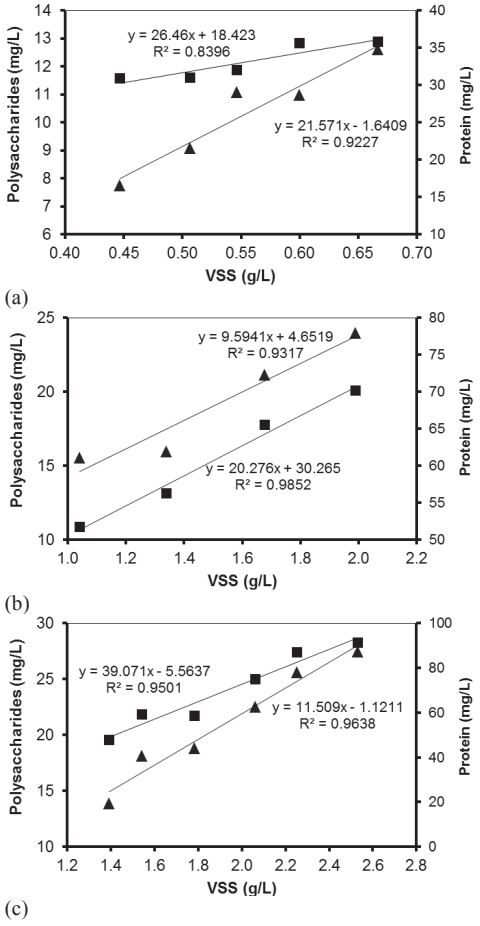


Figure 2.2: Relation between biomass concentration (expressed as VSS) and polysaccharides (▲) and protein (■) concentrations for $MBBR_1$ (a), $MBBR_2$ (c) and SBBR (c).

In $MBBR_1$ (Figure 2.3a), the concentration of polysaccharides slightly increased when the nitrogen load was increased from 0.2 $gN/(m^2 \cdot d)$ (run 1) to 1 $gN/(m^2 \cdot d)$ (run 5). Protein concentration remained practically constant over the three first experimental runs and was increased during the transition from run 3 to 4. The gradual decrease in the influent COD from 400 mg/L (run 6) to 0 (run 9) in $MBBR_2$ was accompanied by a

decrease in the protein concentration (Figure 2.3b). The same trend is valid for the polysaccharide content, although no variation was observed from run 8 to 9. In SBBR, polysaccharide and protein concentrations increased from run 10 to 11 when organic and nitrogen loads were doubled by reducing the cycle time from 48 to 24 h (Figure 2.3c). Subsequently, both polysaccharide and protein concentrations significantly decreased when the influent COD was decreased (from run 11 to 13). A slight decrease was also observed during the long-term operation of the reactor in autotrophic conditions (runs 13-15). Polysaccharide/protein ratio did not vary significantly between reactors, ranging from 0.24 to 0.36.

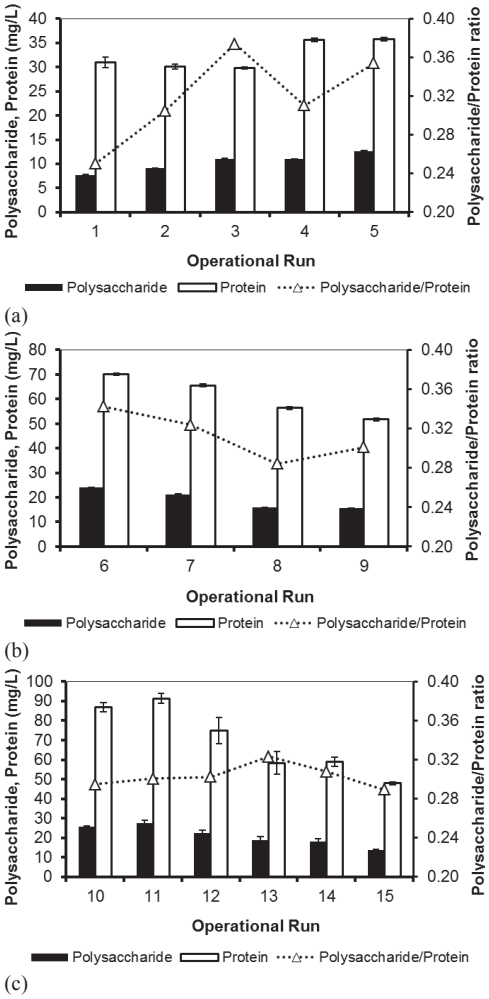


Figure 2.3: Total polysaccharide and protein content of the biofilm: (a) $MBBR_1$; (b) $MBBR_2$; (c) SBBR.

2.3.3. Nitrification performance in the moving bed biofilm reactors

In addition to investigation of biofilm development under different operating conditions, the nitrification performance of the MBBR systems was also evaluated. In the autotrophic $MBBR_1$, ammonium removal efficiency gradually increased during the start-up period, when the development of biomass was occurring. It took around 70 days during run 1 to achieve complete ammonium oxidation. In general, the gradual increase in ammonium surface load from 0.2 to 1.0 $gN/(m^2 \cdot d)$ caused a slight instability only in the beginning of each experimental run when residual ammonium was found in the effluent. However, it was suppressed with further biomass adaptation, and ammonium removal efficiency was kept close to 100% in most of the operational time (Figure 2.4a). Nitrate was formed at close to stoichiometric proportion in relation to ammonium oxidation, and nitrite concentrations were very low (< 1 $mgNO_2^-$-N/L).

In $MBBR_2$, low ammonium removal efficiency was observed during the start-up period in run 6 (Figure 2.4b). However, full ammonium conversion was obtained at the end of this respective experimental run, even though $MBBR_2$ was fed with the highest influent COD (400 mg/L) during this time. Subsequently, with further gradual COD decrease from run 7 until 9, practically no ammonium was detected in effluent. Dissolved nitrogen recovery (indicating the amount of nitrified ammonium found back as nitrate) in the bulk was lower in run 6 (around 77%), reaching around 85% in autotrophic conditions (run 9). The maximum nitrite concentration was 2 $mgNO_2^-$-N/L.

In the moving bed system operated in sequencing batch mode (SBBR), it took around 25 days to achieve full ammonium removal from the beginning of the start-up period intended for biofilm development. Subsequently, ammonium removal efficiency was maintained above 95% until the end of run 13 (Figure 2.4c). When the influent ammonium concentration was increased from 300 to around 600 $mgNH_4^+$-N/L in run 14, ammonium removal efficiency dropped to 60%. However, further biomass acclimation under the conditions of this experimental run allowed recovering full ammonium removal. From the beginning of run 10 until the end of run 13, effluent nitrite concentrations were usually below 1 $mgNO_2^-$-N/L. In the beginning of run 14, effluent nitrite concentration reached 5 $mgNO_2^-$-N/L as a result of process instability caused by doubling the ammonium influent concentration. However, in a few days effluent nitrite concentrations decreased to values below 1 $mgNO_2^-$-N/L. When the influent ammonium concentration was increased from around 550 to 1200 $mgNH_4^+$-N/L

(consequently increasing the nitrogen load from 2 to 4 gN/(m^2·d)) in Run 15, the system was severely affected. Maximum ammonium removal efficiency varied from 30% to 40% and the main nitrification product was not nitrate but nitrite, which accumulated up to 140 mgNO_2^--N/L. Nitrate concentration in the effluent amounted to 40 mgNO_3^--N/L. Run 15 lasted for 1 month and due to the high operation instability on that particular experimental phase, the operation of the reactor was stopped. During the entire operation of SBBR, nitrogen recovery in the bulk was around 70% for runs 10, 11, 12, 14, and 15 and 80% for run 13.

Cycle tests were performed in the end of runs 11-15 in SBBR (Figure 2.5) when a quasi-steady-state operation was reached (i.e., the main biological conversions were stable and not changing from time to time). Specific ammonium uptake rate (q_{NH4}) varied from 9 mgNH_4^+-N/(gVSS·h) (run 15) to 17 mgNH_4^+-N/(gVSS·h) (run 13). In runs 12 and 14, q_{NH4} amounted to around 14 and 13 mgNH_4^+-N/(gVSS·h), respectively. In run 11, when the highest organic load was applied, q_{NH4} was 10 mgNH_4^+-N/(gVSS·h).

As already mentioned in the description of the long-term operation of SBBR, almost no nitrite was present at the end of the cycle in runs 10-14. Nevertheless, nitrite was observed to accumulate along the cycle in all experimental runs. In runs 11 and 12, ammonium uptake was not influenced by the nitrite build-up, which reached a maximum concentration of 50 mgNO_2^--N/L (Figures 2.5a,b). However, during operation in runs 13 and 14, ammonium oxidation rate significantly decreased during the cycle (Figures 2.5c,d), when nitrite concentrations exceeded 50 mgNO_2^--N/L. Nitrite oxidation also seemed to be influenced by high nitrite concentrations, especially in runs 14 and 15, when the highest accumulation of nitrite occurred during the operational cycle. During run 15, in particular, nitrite accumulated up to 150 mgNO_2^--N/L in the end of the cycle, and just part of the nitrite was further oxidized to nitrate. In this experimental run, the nitrite concentration left over from the previous cycle (diluted with the feeding) already amounted up to 30 mgNO_2^--N/L.

A significant drop in DO concentration was observed immediately after feeding in all experimental runs (Figures 2.5a-e). Interestingly, in run 15, when influent ammonium concentration was the highest (1200 mgNH_4^+-N/L), the decrease in DO concentration was lower in comparison with other experimental phases. This fact suggested a possible inhibitory effect on nitrifying bacteria provoked by high initial nitrite concentration (left over from the previous cycle) or by free ammonia. In general,

DO concentrations gradually increased during the course of the cycle when ammonium was being nitrified.

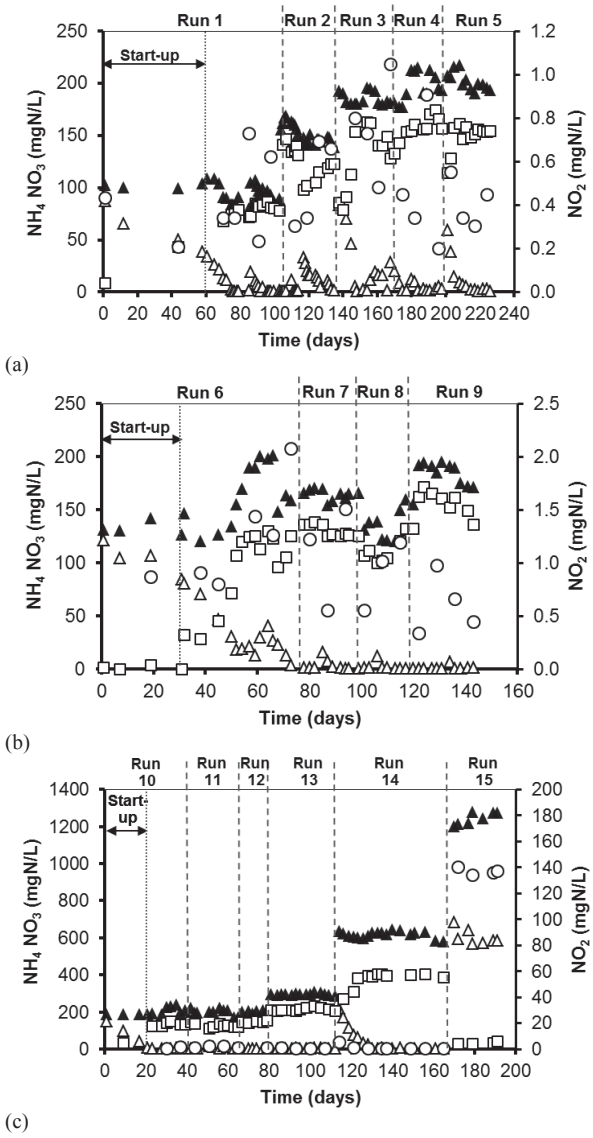


Figure 2.4: Ammonium concentration in the influent (▲) and ammonium (Δ), nitrate (□) and nitrite (○) concentrations in the effluent of $MBBR_1$ (a), $MBBR_2$ (b) and SBBR (c). Start-up phase refers to the period intended for biofilm formation. Ammonium and nitrate concentrations were displayed on the left y-axis, while the nitrite concentration is shown in the right y-axis. The larger scale of the right y-axis for SBBR is due to the higher accumulation of nitrite in this reactor.

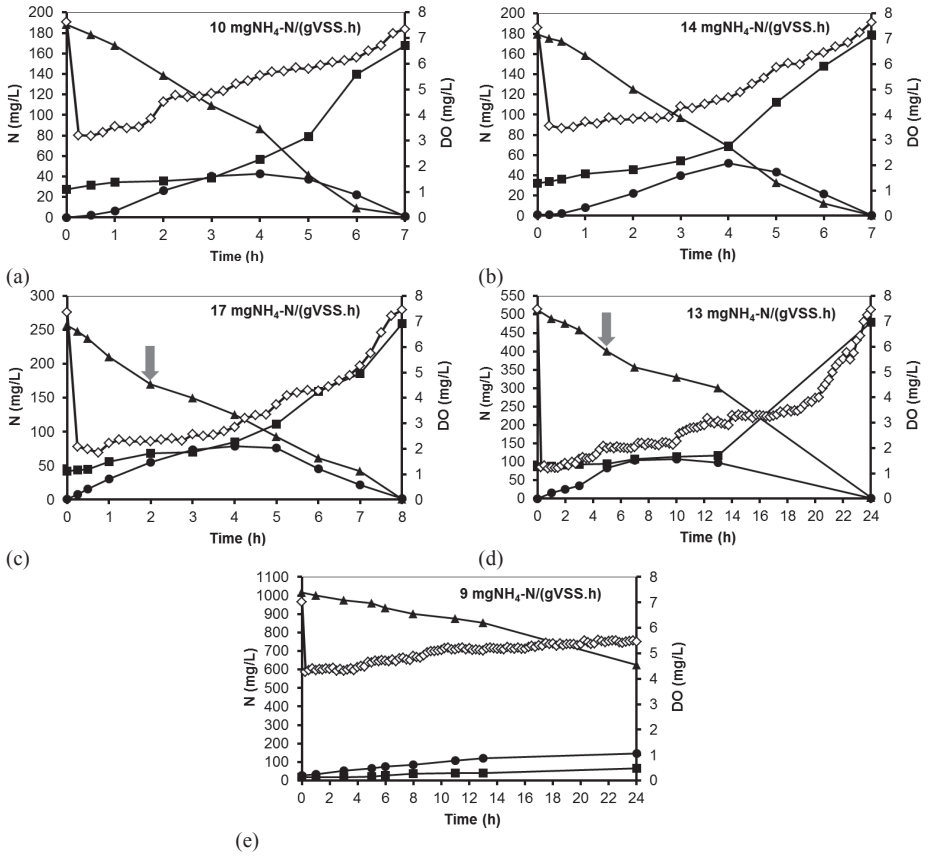


Figure 2.5: Ammonium (▲), nitrate (■), nitrite (●) and dissolved oxygen (◊) profiles obtained in the cycle tests performed in the end of Run 11 (a), Run 12 (b), Run 13 (c), Run 14 (d) and Run 15 (e). Concentrations of ammonium, nitrate and nitrite depicted at time 0 were measured after feeding and dilution of the influent medium with the liquid remaining from the previous cycle (volume exchange ratio of 85%). Ammonium uptake rates obtained in each experimental run are displayed in the top part of each graph. An arrow indicates when ammonium uptake rate decreases along the cycle, when nitrite concentrations exceed 50 mgNO_2^--N/L.

2.3.4. Ammonium- and nitrite-oxidizing bacterial populations assessed by fluorescent in situ hybridization

In order to observe the development of nitrifying bacteria over the experimental phases in all moving bed biofilm systems, a combination of specific oligonucleotide probes (shown in Table 2.3) targeting ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) was used. Representative FISH pictures of the samples collected from the three reactors are shown in Figure 2.6. An increase in the proportion of AOB and NOB among the whole bacterial community was observed over the operation of $MBBR_1$, which was subjected to an increase in the nitrogen load. Since the

operation of this reactor was already started under fully autotrophic conditions, AOB and NOB populations represented a considerable fraction of the whole microbial population, but several non-nitrifying bacteria were present after the start-up phase in run 1 (Figure 2.6a). Nevertheless, the long-term operation without addition of external organic carbon and the increase in nitrogen load contributed to enrich the biofilm for nitrifiers (AOB and NOB), which completely dominated the microbial population in run 5 (Figure 2.6b).

In $MBBR_2$, very few nitrifying bacteria were present in the start-up phase within run 6 (data not shown). However, nitrifiers gradually appeared in this system along run 6 and corresponded to a significant fraction of the whole bacterial community in the end of the respective phase compared to the beginning of the operation (Figure 2.6c). In the meantime, nitrification efficiency increased significantly in this period, as shown in Figure 2.4b. Furthermore, the gradual decrease in the influent COD from 400 mg/L (run 6) to 0 (run 9) also contributed to enrich the biofilm for nitrifiers (Figure 2.6d). However, several other bacteria (neither AOB nor NOB) were still detected in the last experimental run of this reactor, when no organic C-source was provided.

Following a similar trend as observed in the $MBBR_2$, nitrifiers started to dominate the microbial community of the SBBR biofilm as organic load was gradually decreased. As shown in Figure 2.6f, a highly enriched nitrifying biofilm was obtained in run 14, when the influent ammonium concentration was around 500 mgNH_4^+-N/L and the reactor had already been running on pure autotrophic conditions for more than 4 months. A highly enriched nitrifying biofilm was still obtained in run 15, although in this particular phase nitrifiers were possibly inhibited, as will be discussed further.

The non-nitrifying bacteria detected in the microbial community of the moving bed systems even when no organic carbon was supplied are probably heterotrophs growing at the expense of soluble microbial products released from the autotrophs. Even at low influent COD (5 – 10 mg/L), heterotrophic biomass can be formed in a reactor fed with no organic carbon source (Moussa et al., 2005). For instance, organic impurities in the medium and in the air used for aeration can provide such low COD input (Bassin et al., 2012; Moussa et al., 2005).

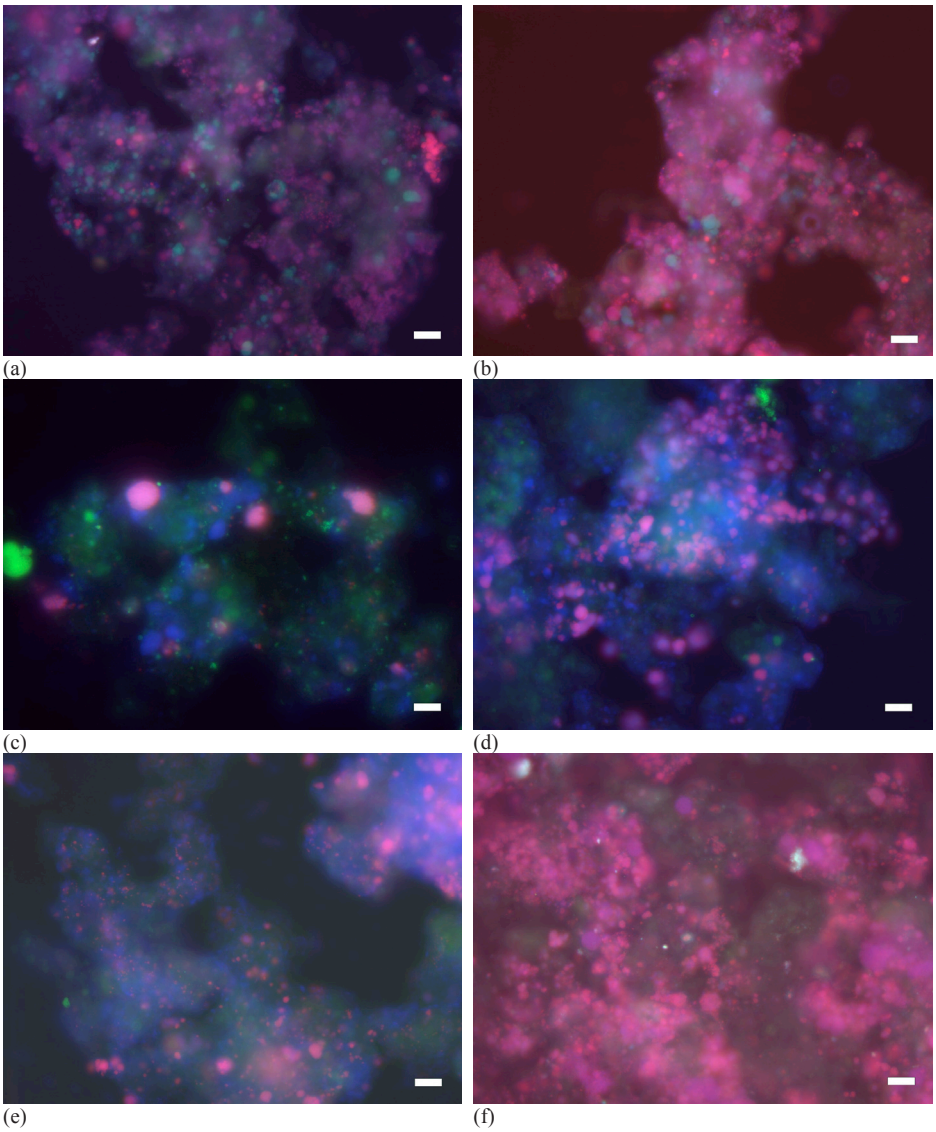
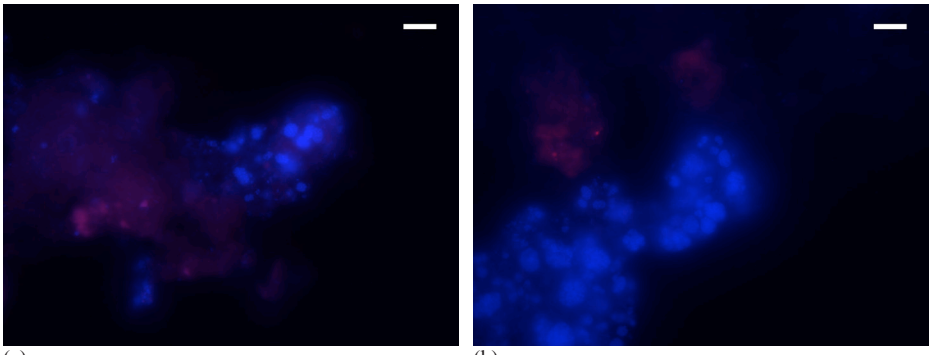


Figure 2.6: AOB (in red) and NOB (in green) populations within the whole bacterial community (in blue): (a) $MBBR_1$, Run 1; (b) $MBBR_1$, Run 5; (c) $MBBR_2$, Run 6; (d) $MBBR_2$, Run 9; (e) SBBR, Run 11; (f) SBBR, Run 14. AOB appear violet due to superposition of AOBmix and EUBmix probes, while NOB appear light green due to the superposition of NOBmix and EUBmix probes. Scale bar indicates 20 µm.

In addition to investigating the AOB and NOB populations within the biofilm of the moving bed biofilm reactors, we also checked for the presence of anammox bacteria. Interestingly, we found some anammox cells only in the biofilm samples from SBBR retrieved in runs 13-15 (Figure 2.7), when high ammonium and nitrite

concentrations were simultaneously present during the operational cycle. Anammox cells increased in number in the last two experimental phases of SBBR (runs 14 and 15).



(a) (b)

Figure 2.7: Anammox bacteria (in red) detected within the whole bacteria community (in blue): (a) Run 14 and (b) Run 15. Preliminary tests have shown that the two probes (AMX 368 and AMX 820) that were used bound to the same cells. Therefore, only one (AMX 368) was used in further FISH analysis. Anammox appear violet due to superposition of AMX 368 and EUBmix probes. Scale bar indicates 10 µm.

2.4. Discussion

2.4.1. Feeding strategies influencing biofilm formation

The time necessary to form stable nitrifying biofilms was observed to be significantly reduced when a heterotrophic start-up phase was applied, in which some organic compounds were fed to the moving bed systems to favour the development of heterotrophs. A thin layer of attached biomass is formed within few days in those systems fed with organic material, which is the precursor of the mature biofilm. The reason for the quicker biofilm formation in the reactors fed with organic material ($MBBR_2$ and SBBR) is related to the fast-growing heterotrophs. As a consequence of higher biomass production, the production of EPS will be enhanced. EPS form a complex matrix of polymers, including polysaccharides, proteins, glycoproteins, phospholipids, nucleic acids and humic acids (McSwain et al., 2005). In the EPS matrix, bacteria and other particulate matter are present (Grotenhuis et al., 1991). In biofilm processes, EPS (especially polysaccharides) act as a fixation matrix, favouring the adhesion of biomass to surfaces and carrier materials (Cammarota and Sant'Anna, 1998). This will facilitate the initial attachment of bacteria by adsorption on the surface of the carrier material and the subsequent growth of microcolonies, which will cover the

carrier surface area available for biofilm deposition (Tijhuis et al., 1994b). Although no specific quantification of exopolymers produced by bacteria was performed in this study, the concentrations of total protein and total polysaccharides within the biofilm were determined in order to have an idea about biomass concentration behaviour and the polysaccharide production in different operational phases of the same reactor and in the different moving bed systems.

The total protein concentrations of the biofilm were found to be much higher in $MBBR_2$ and SBBR compared to the autotrophic $MBBR_1$. This is in line with biomass measurements by conventional methods, showing higher VSS content in the heterotrophic systems. The higher the organic surface loading rate, the thicker the biofilm in the particles. This fact was also reported by Tijhuis et al. (1994b). More biomass was attached to the SBBR carriers compared to the continuously run $MBBR_2$, even at comparable influent organic loads. This is possibly related to the different modes of operation of the two systems. It seems that a sequencing batch regime with pulse feeding is more appropriate for biomass accumulation in the carrier material. The fact that polysaccharide/protein ratio was practically the same and invariable in all the reactors means that the amount of EPS (expressed as polysaccharides) produced by a certain amount of cells (expressed as protein) was roughly the same for all reactors, despite the different microbial populations in each system.

Although not perfect, a reasonable relationship exists between biomass concentration (expressed as VSS) and other parameters used to estimate biomass concentration (polysaccharide and protein concentrations) (Figure 2.2). Therefore, using polysaccharide and protein concentrations can indeed provide, as a first attempt, a qualitative indication of biomass behaviour under different experimental conditions. Furthermore, by performing some biomass measurements and simultaneously monitoring the polysaccharide and protein concentrations, a calibration curve can be obtained that will help to rapidly estimate biomass concentration in MBBR systems. The VSS measurements by conventional methods can be troublesome due to difficulties encountered in removing all biomass from the plastic carriers.

The increase in biofilm surface specific detachment rate and reduction of biomass concentration with decrease in influent COD from runs 6 to 8 in $MBBR_2$ and runs 11 to 13 in SBBR is probably due to the fact that the growth of fast-growing heterotrophic organisms was limited. As a consequence, biomass decay and sloughing of heterotrophs occurred, which were washed out from the reactor. Therefore, the

heterotrophic biofilm layer was gradually reduced, leading to a considerable decrease of total biomass concentration and therefore lower polysaccharide and protein concentrations on the carriers. These results are supported by the SEM observations, which also showed a decrease in biomass attached to the support material as COD was decreased in both heterotrophic reactors ($MBBR_2$ and SBBR). After detachment of the heterotrophic layer, the remaining thin biofilm was enriched for nitrifiers, as demonstrated by FISH analysis. The operational strategy in which COD was gradually reduced proved to be a good method to obtain a biofilm dominated by nitrifying bacteria in less time.

2.4.2. Effect of influent medium composition on nitrification process

A feeding strategy was studied in which some organic compounds fed to $MBBR_2$ and SBBR lowered the time necessary for biofilm development in those systems without causing a detrimental effect on nitrification performance. Actually the application of a heterotrophic start-up phase favoured the accumulation of nitrifying bacteria in the biofilm, which was evident from the lower time necessary to obtain complete ammonium removal compared to the autotrophically run $MBBR_1$ (seeded with enriched nitrifying sludge), under similar influent ammonium concentrations.

The lower ammonium removal efficiency observed in the beginning of run 6 of $MBBR_2$ is related to the fact that only a few nitrifiers were detected in that period. As the biofilm was gradually colonized by nitrifying bacteria, full ammonium removal was obtained even at the highest organic load applied to $MBBR_2$. In the case of SBBR, inoculation with biomass detached from $MBBR_1$ and $MBBR_2$, which already contained a significant amount of nitrifiers, allowed the reactor to reach full ammonium removal in only 20 days. Moreover, gradual reduction of influent COD from run 11 to 13 was accompanied by an increase in specific ammonium uptake rate in SBBR, which is primarily related to enrichment of the biofilm for nitrifiers.

In addition to studying the effect of different influent COD/N ratios on nitrification performance, the effect of ammonium loading rate in this process was also investigated in SBBR. Ammonium oxidation rate was observed to be lower in run 15 (when the biofilm was already completed dominated by nitrifiers) than in run 11 (when the highest organic load was applied and nitrifiers were still colonizing the biofilm). We suspected that substrate inhibition was taking place in the SBBR. Free ammonia (FA) and free nitrous acid (FNA), whose concentrations are directly related to ammonium

and nitrite concentrations, respectively, are reported to cause inhibition of nitrifying bacteria (Anthonisen et al., 1976). The free nitrous acid (FNA) concentration for the highest nitrite concentration observed in runs 13 – 15 was calculated, amounting to 0.02 (run 13), 0.025 (run 14), and 0.034 mgN/L (run 15). According to Anthonisen et al. (1976) the threshold FNA concentration from which both AOB and NOB are inhibited falls within the range between 0.22 – 2.8 mgN/L. The FNA concentrations calculated in this study are lower than that capable of provoking inhibition, reported by Anthonisen et al. (1976) Nevertheless, other studies such as that carried out by Vadivelu et al. (2006) reported lower values for FNA capable of inhibition for both AOB and NOB. Therefore, the inhibitory effect cannot be excluded since the ammonium uptake rate considerably decreased when high nitrite accumulation occurred during the operational cycle.

In SBBR, the higher the ammonium concentration in the beginning of the cycle, the higher the nitrite accumulation observed. This result can be associated with a possible inhibition of NOB by free ammonia (FA). The concentration of FA that can cause inhibition to NOB was reported to be between 0.1 and 1.0 mgNH_3/L (Anthonisen et al., 1976). The FA concentrations calculated in our study were around 5 mgNH_3-N/L (Run 13), 10 mgNH_3-N/L (run 14) and 18 mgNH_3-N/L (run 15), values considerably higher than the reported inhibition level by Anthonisen et al. (1976). Bae et al. (2001) also reported that high accumulation of nitrite (NO_2^-/NO_x^- up to 77%) took place when FA concentration was around 4.7 mg/L. The probable inhibition of NOB by FA and consequent collapse of the second stage of the nitrification process (nitratation) is supported by the results obtained during operation of runs 14 and 15. In run 14, most of the ammonium (around 80%) in the first 10 h of cycle was nitrified only to nitrite, while nitrate was formed in minor proportions. More than 80% of nitrate was formed only after 10 h of cycle. In run 15, in which ammonium loading rate was equal to 4 g NH_4^+-N/(m^2·d), nitrite accumulation was observed over the whole cycle and nitrite was indeed the main nitrification product. As also observed by Park and Bae (2009), NOB were more susceptible to inhibition by FA or FNA. Slawomir et al. (2010), operating a two-stage MBBR system, also observed that nitrite was the predominant product of nitrification at similar ammonium load (3.76 g NH_4^+-N/m^2·d).

Even though the biofilm was completely dominated by nitrifying bacteria, ammonium uptake rate in run 15 was lower compared to the previous experimental runs. In that particular run, FA reached 18 mgNH_3-N/L, which is within the range reported by Anthonisen et al. (1976) to cause inhibition to AOB (10 – 150 mgNH_3/L).

Furthermore, the considerable amount of nitrite present in the beginning of the cycle (left over from the immediate previous cycle) and its gradual accumulation over the cycle likely caused an adverse effect on ammonium oxidation capacity. As pointed out by Park and Bae (2009), FA is the primary inhibitor in the beginning of a batch reaction, while FNA will be predominant later. It is important to remark that the lower decrease in the dissolved oxygen concentration in run 15 after feeding, compared to that observed in runs 13 and 14, suggests that an inhibition effect on both AOB and NOB occurred in run 15, in which the highest FA and FNA concentrations were observed.

The detection of anammox cells in the biofilm of SBBR in runs 13 – 15 was indeed an unexpected result, since that system was operated at a relatively high DO concentration (higher than 1.5 mg O_2/L or 20% air saturation at 24°C). The growth of anammox bacteria could be supported by the simultaneous presence of high concentrations of ammonium and nitrite, which correspond to their substrates (Strous et al., 1999). Moreover, it is likely the anammox bacteria were situated deeper in the biofilm, when the DO concentration was much lower compared to that in the bulk.

The nitrogen recovery in bulk (obtained from the nitrogen mass balance) in the experimental runs in which organic carbon was supplied in the feeding medium (runs 10-12) was lower than that observed in run 13, kept under autotrophic conditions. Two possible reasons can explain this observation: (1) fast-growing heterotrophs would assimilate more nitrogen for growth than autotrophic organisms, resulting in less ammonium available for nitrification; or (2) the influent organic carbon could be used as electron donor by denitrifiers to reduce nitrite/nitrate to nitrogen gas. However, the nitrogen recovery in the bulk observed in runs 14 and 15 (when no organic matter was fed to the reactor, i.e., autotrophic conditions) was similar to those observed when organic-C source was supplied. In fact, this result can partially be related to the presence of anammox bacteria in SBBR, which may also contributed to nitrogen loss from the liquid. Moreover, the fact that nitrogen recovery in the bulk was lower in SBBR compared to the continuously fed systems can be attributed to denitrification on stored intracellular polymers (such as PHB), formed by bacteria under the feast-famine regime of the reactor (Jiang et al., 2011).

2.5. Conclusions

Our results have shown that the application of a heterotrophic start-up phase decreased the time required to develop nitrifying biofilms. The findings of this study can be used in industrial settings, especially when nitrification should be accomplished in wastewaters with limited or no organic carbon. Inoculation of a reactor operated on a pulse-feeding sequencing batch regime with biomass detached from MBBR systems proved to speed up the biofilm development. It was shown that total polysaccharide and protein concentrations can be used as an approach to estimate biomass concentrations in MBBR systems. Nitrification was severely affected in the SBBR when a pulse feeding of 4 $gN/(m^2 \cdot d)$ was applied. Free nitrous acid and free ammonia were likely the inhibitors for ammonium- and nitrite-oxidizing bacteria.

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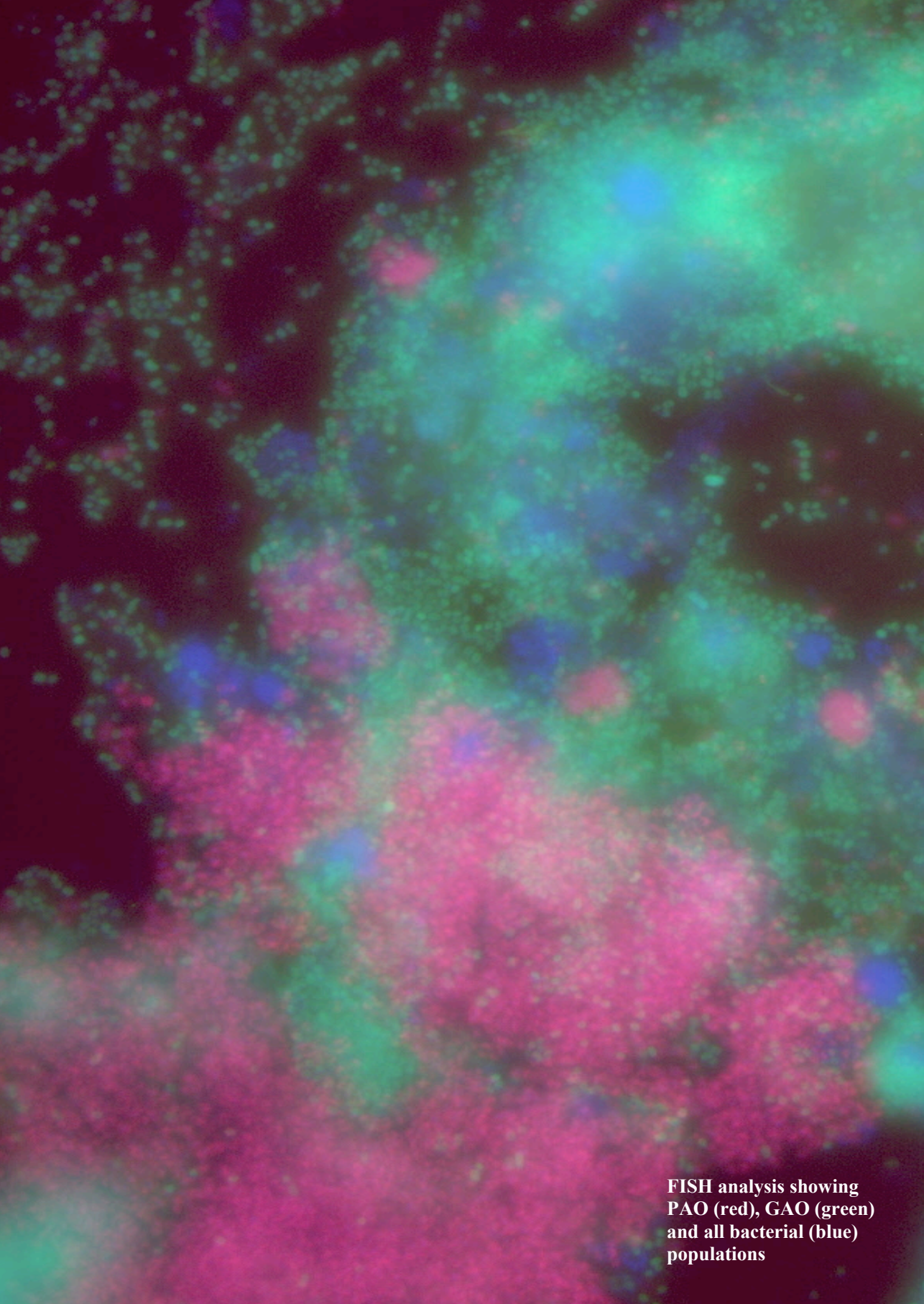


FISH analysis showing PAO (red), GAO (green) and all bacterial (blue) populations

CHAPTER 3

Simultaneous nitrogen and phosphate removal in aerobic granular sludge reactors operated at different temperatures

This Chapter is based on 3 works. Part I is accepted for publication in **Biotechnology and Bioengineering** as: Bassin, J.P., Winkler, M.-K.H., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C.M. (2012) *Improved phosphate removal by selective sludge discharge in aerobic granular sludge reactors*. Part II is accepted for publication in **Water Research** as: Bassin, J.P., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C.M. *Simultaneous nitrogen and phosphate removal in aerobic granular sludge reactors operated at different temperatures*. Part III is submitted as: Bassin, J.P., Kleerebezem, R., van Loosdrecht, M.C.M. *Measuring biomass specific ammonium, nitrite and phosphate uptake rates in aerobic granular sludge*.

Chapter 3

Content

Abstract

The main biological conversions taking place in two lab-scale aerobic granular sludge sequencing batch reactors were evaluated. Reactors were operated at different temperatures (20 and 30°C) and accomplished simultaneous COD, nitrogen and phosphate removal. Nitrogen and phosphate conversions were linked to the microbial community structure as assessed by fluorescent in situ hybridization (FISH) analysis. Samples analysed by FISH indicated a segregation of biomass over the sludge bed: in the upper part, *Candidatus* 'Competibacter phosphatis' (glycogen-accumulating organisms - GAOs) were dominant while in the bottom, *Candidatus* 'Accumulibacter phosphatis' (polyphosphate-accumulating organisms - PAOs) dominated. In order to favour PAOs over GAOs and hence improve phosphate removal at 30°C, the SRT was controlled by discharging biomass mainly from the top (80% of the excess sludge) of the sludge bed, while bottom granules were removed in minor proportions (20% of the excess sludge). With the selective sludge removal proposed, 100% phosphate removal efficiency was obtained in the reactor operated at 30°C. In the reactor operated at 20°C, phosphate removal efficiency remained stable above 90% regardless of the sludge removal procedure for SRT control. Complete nitrification/denitrification and phosphate removal were achieved in both systems. Anoxic tests were performed to evaluate the contribution of anoxic phosphate uptake to the overall phosphate removal and to clarify the denitrification pathway. A considerable fraction of the phosphate removal was coupled to denitrification (denitrifying dephosphatation). From the results obtained in anoxic batch experiments dosing either nitrite or nitrate, denitrification was proposed to proceed mainly via the nitrate pathway. Denitrifying glycogen-accumulating organisms (DGAOs) were observed to be the main organisms responsible for the reduction of nitrate to nitrite. A significant fraction of the nitrite was further reduced to nitrogen gas while being used as electron acceptor by denitrifying polyphosphate-accumulating organisms (PAO clade II) for anoxic phosphate uptake.

3.1. Introduction

Biological nitrogen removal from wastewater is generally accomplished by aerobic nitrification and anoxic denitrification, whereas the removal of phosphate can be achieved through enhanced biological phosphate removal (EBPR) under alternating anaerobic-aerobic conditions. In this environment, the microorganisms responsible for the bio-P removal process, known as polyphosphate-accumulating organisms (PAOs), are selected (van Loosdrecht et al., 1997). However, such conditions also favour the development of a particular group of microorganisms, called glycogen-accumulating organisms (GAOs), which compete with PAOs for the available organic substrate (Zeng et al., 2002). Decrease of phosphate removal performance in EBPR systems is often attributed to the presence of GAOs (Oehmen et al., 2006).

In most wastewater treatment systems, biological nitrogen and phosphate removal processes are usually integrated in a single sludge system. However, the combined EBPR and conventional biological nitrogen removal (nitrification and denitrification) processes can face problems since chemical oxygen demand (COD) is often a limiting factor for phosphate removal and denitrification. The limitation of COD can be overcome when organisms capable of performing simultaneous denitrification and anoxic phosphorous uptake are present in the treatment system (van Loosdrecht et al., 1998). These organisms, referred to as denitrifying polyphosphate-accumulating organisms or DPAOs, have metabolic characteristics similar to those of aerobic PAOs, except for their capacity to use nitrate and/or nitrite (produced in the nitrification process under aerobic conditions) instead of oxygen as electron acceptor to simultaneously remove phosphate and nitrogen from wastewater. These organisms use intracellular polymers stored under anaerobic conditions as electron donor for denitrification and do not require the addition of an external carbon substrate to carry out this process (Kuba et al., 1993; Kerrn-Jespersen and Henze, 1993; Flowers et al., 2009). Besides an efficient use of the incoming COD, the use of oxidized forms of nitrogen (e.g., nitrite or nitrate) rather than oxygen as electron acceptor also leads to aeration energy saving.

In the majority of the studies regarding the operation of EBPR systems, *Candidatus* Accumulibacter phosphatis (henceforth called Accumulibacter) has been considered the most important polyphosphate-accumulating organisms. Recent research efforts exploring the characteristics of PAOs have shown new insights about these

microorganisms. He et al. (2006) demonstrated the existence of various clades within members of the Accumulibacter group in lab- and full-scale EBPR systems. The Accumulibacter diversity was further organized into two main types (I and II), using the polyphosphate kinase gene (ppk1) as genetic marker (He et al., 2007; Peterson et al., 2008). A metagenomic analysis of Accumulibacter clade II enriched EBPR sludge showed a lack of respiratory nitrate reductase in this specific microorganism (Garcia Martin et al., 2006). Further investigations showed that Accumulibacter populations established in a lab-scale reactor under nitrite reducing conditions could not reduce nitrate (Guisasola et al., 2009). All these findings related to nitrate reduction capability suggested differences in the metabolism of members of Accumulibacter lineage. Based on experimental evidences, Flowers et al. (2009) have shown that PAOs belonging to clade I (hereafter referred to as PAOI) were capable of using nitrate whereas PAOs of clade II (hereafter referred to as PAOII) were able to use only nitrite as electron acceptor for anoxic P uptake. Nevertheless, both of them are facultative organisms, capable of using oxygen as electron acceptor under aerobic conditions.

One of the emerging technologies suitable to accomplish simultaneous nitrogen and phosphate removal in a single reactor unit is aerobic granular sludge. This technology is not based on several tanks where aerobic, anoxic and anaerobic zones are separately provided. Instead, different redox conditions are obtained by mass transfer limitations in the biofilm and operation in sequencing batch mode allowing for introduction of periods with and without aeration. Anaerobic feeding of the SBR is required for P-release. In a subsequent aerobic period, nitrification and aerobic P uptake take place in the aerobic layer of the granule whereas denitrification coupled to anoxic phosphate removal occurs in the anoxic zone (de Kreuk et al., 2005). A simplified schematic representation showing some of the conversions occurring in parallel in the aerobic granular sludge structure is displayed in Figure 3.1. The identification of specific conversion routes within these systems is complicated due to simultaneous nitrification, denitrification and P uptake.

Simultaneous nitrogen and phosphate removal in aerobic granular sludge reactors has been reported (de Kreuk et al., 2005; Zeng et al., 2003; Yilmaz et al., 2008; Kishida et al., 2009). These previous studies describe the general performance in terms of nitrogen and phosphate removal without specifying the importance of specific subpopulations of PAOs on phosphate and nitrogen conversions. Moreover, there are

only few reports on simultaneous nitrogen and phosphate removal using aerobic granular sludge under tropical climate conditions, such as 30°C (Winkler et al., 2011a; Ebrahimi et al., 2010).

A comprehensive characterization of the main process conversions taking place in two aerobic granular sludge reactors performing simultaneous nitrogen and phosphate removal and operated at different temperatures (20 and 30°C), was carried out in this study. The effect of the dissolved oxygen concentration and the temperature on nitrogen and phosphate conversions, the contribution of anoxic phosphate uptake to the total phosphate uptake and the evaluation of the role of the main microbial populations involved in the simultaneous nitrogen and phosphate removal system was addressed in this research. Short-term variations of the reactor operating conditions and anoxic batch tests were carried out to better elucidate the pathways involved in the main biological processes taking place in the aerobic granular systems.

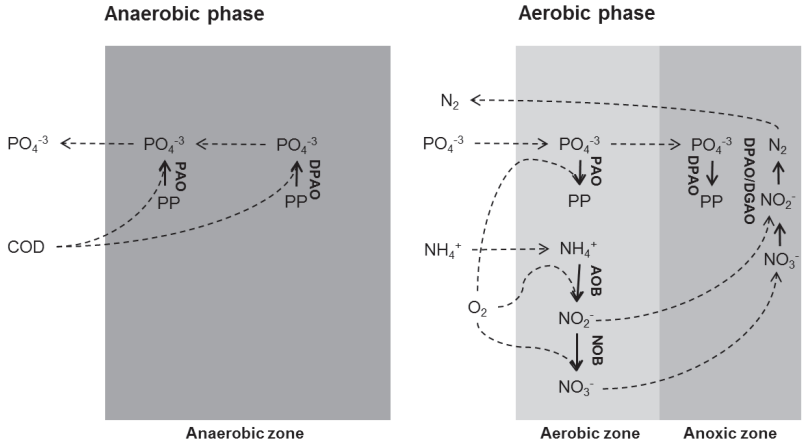


Figure 3.1: Schematic representation of the main process conversions occurring in parallel in the aerobic granular sludge structure. AOB: ammonium-oxidizing bacteria; NOB: nitrite-oxidizing bacteria; PAO: polyphosphate-accumulating organisms; DPAO: denitrifying polyphosphate-accumulating organisms; GAO: glycogen-accumulating organisms; COD: chemical oxygen demand; PP: polyphosphate. Denitrifiers are composed by denitrifying polyphosphate-accumulating organisms (DPAOs) and denitrifying glycogen-accumulating organisms (DGAOs). Accumulation of PHA by PAOs, DPAOs, GAOs and DGAOs during anaerobic phase is not shown in the scheme. During the anaerobic phase, the entire granule is maintained in anaerobic conditions (no biofilm stratification).

3.2. Materials and Methods

3.2.1. Reactor set-up and operating conditions

Experiments were performed in two column-type aerobic granular sludge sequencing batch reactors (SBR_{20} and SBR_{30}). A schematic representation of the experimental set-up is shown in Figure 3.2. Both systems had a working volume of 2.6 L, an internal diameter of 5.6 cm and a total height of 90 cm. SBR_{20} and SBR_{30} were operated, respectively, at room temperature (20 ± 2°C) and at 30°C. For the SBR_{30}, temperature was kept constant at 30°C by pumping water with the desired temperature through tubes, which were placed around the reactor. A thermal insulation was used to prevent cooling of the reactor. Aeration and mixing were supplied through an air diffuser placed in the bottom of the reactors (airflow rate of 4 L/min). pH was controlled at 7.0 ± 0.2 by dosing 1 M NaOH or 1 M HCl. SBR_{20} was seeded with granules from a pilot-scale aerobic granular sludge reactor treating municipal wastewater (WWTP EPE, The Netherlands). In the initial stage of operation of SBR_{20} (start-up phase), dissolved oxygen (DO) concentration was kept close to the air saturation value (9.1 mgO_2/L at 20°C). After 48 days of operation, DO was reduced to 20% air saturation (1.8 mgO_2/L) in order to enhance denitrification. After 104 days of operation, half of the sludge bed (mixed sample) was removed from SBR_{20} to inoculate SBR_{30}, which was initially operated at 1.8 mgO_2/L. From this point forward, the operation of the aerobic granular sludge reactors was divided in different experimental phases, as shown in Table 3.1. Both reactors were operated sequentially in 3h-cycle under alternating anaerobic and aerobic conditions in order to achieve nitrogen and phosphate removal. The cycling profile comprised an anaerobic feeding phase of 60 min from the bottom of the reactor in a plug flow regime through the settled sludge bed, 112 min aeration, 3 min settling and 5 min effluent withdrawal. Effluent was discharged 51 cm above the reactor bottom at a volume exchange ratio of around 57%, resulting in a hydraulic retention time of 5.2 h. A bio controller (Braun DCU4 coupled with MFCS control and data acquisition software) was used to control and operate the SBRs. The DO concentration could be accurately controlled by using two mass flow controllers (one for air and other for nitrogen gas). The synthetic feeding medium consisted of two solutions: (A) $NaCH_3COO{\cdot}3H_2O$ 63 mM, $MgSO_4{\cdot}7H_2O$ 3.6 mM, KCl 4.7 mM and (B) NH_4Cl 35.4 mM, K_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM and 10 mL/L trace element solution (Vishniac and Santer, 1957). Per cycle, 150 mL was dosed from both medium

together with 1200 mL of tap water in order to achieve influent concentrations of 60 mgNH_4^+-N/L, 20 mgPO_4^{3-}-P/L and 400 mg/L of COD. The sludge retention time (SRT) was determined according to Winkler et al. (2011a) and was maintained at around 30 days by periodically removing sludge from the reactor (excess sludge) every two days.

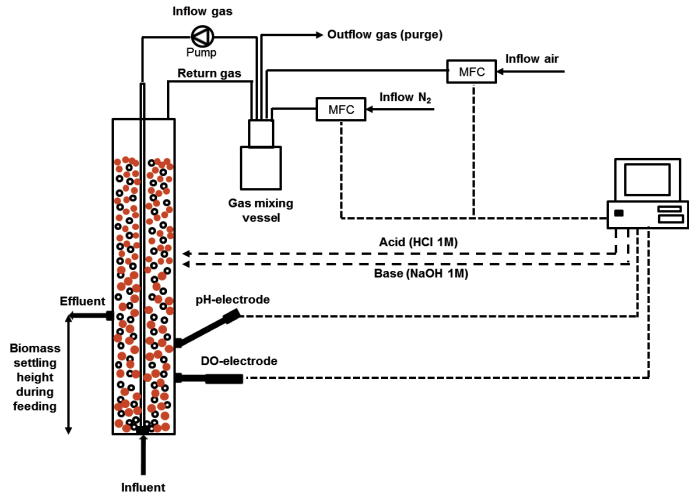


Figure 3.2: Schematic representation of the experimental set-up. DO: dissolved oxygen; MFC: mass flow controller. Brown filled spheres represent the aerobic granular sludge and white filled spheres indicate the gas bubbles.

As shown in Table 3.1, excess sludge was removed during aeration phase (mixed sample) during phases A1 and A2 in SBR_{20} and SBR_{30}, respectively. Subsequently, in Phase B1 (SBR_{20}) and Phases B2a and B2b (SBR_{30}), 80% (on volume basis) of excess sludge was removed from the top and 20% from the bottom of the sludge bed (in order to favour PAOs over GAOs). In Phases C1 and C2, sludge was removed only from the PAO-rich bottom of the sludge bed and influent phosphate concentration was decreased from 20 to 2 mgP/L to provoke the washout of PAOs. Moreover, the supernatant concentrated in phosphate, released by PAOs during the anaerobic feeding, was replaced by a mineral solution (similar to the reactor influent but without acetate and phosphate compounds) immediately after the start of the aeration phase. This procedure was repeated for several days in order to speed up the washout of PAOs which would not be able to recover the poly-P content of cells. Nitrite, which was reported to inhibit PAOs (Saito et al., 2004), was also added in the beginning of aeration in some cycles in order to reach 20 mgNO_2^--N/L. The washout of PAOs was performed

to simplify the system complexity, obtaining only one main type of denitrifying organism (denitrifying GAOs) in the reactor, and hence clarify the denitrification pathway. The removal of PAOs was also carried out to observe the link between the key factors influencing biomass segregation within the reactor sludge bed (i.e. chemical precipitation, poly-P and ash content of biomass).

Typical cycle tests were conducted when a pseudo-steady-state condition was achieved at different operational phases in both SBRs. Samples were collected every 10 - 20 min only during aeration phase, when the reactor content was mixed. The first sample was taken 2 min after aeration period has started to allow sufficient mixing. Ammonium and phosphate uptake rate were determined by linear regression of their concentrations over time divided by the volatile suspended solids (VSS) concentration in the reactor. The denitrification rate under normal operation was estimated based on the difference between the ammonium uptake rate and the nitrite and nitrate production rate during nitrification.

Table 3.1: Experimental phases of aerobic granular sludge sequencing batch reactors.

Reactor	Experimental Phase[a]	Dissolved oxygen (mg/L)	Excess sludge for SRT control[b]	Operational time (days)
SBR_{20} (20°C)	Phase A1	1.8	Mixed sample	39
	Phase B1	1.8	80% from top 20% from bottom	131
	Phase C1	1.8	100% from bottom	77
SBR_{30} (30°C)	Phase A2	1.8	Mixed sample	39
	Phase B2a	1.8	80% from top 20% from bottom	119
	Phase B2b	1.3	80% from top 20% from bottom	12
	Phase C2	1.3	100% from bottom	77

[a] A, B and C indicate the experimental phase; 1 or 2 refer to SBR_{20} and SBR_{30}, respectively.
[b] Information on how biomass was removed for controlling the SRT. Biomass was removed during aeration (mixed sample) or during anaerobic feeding (either 80% from top and 20% from bottom or 100% from bottom). Top and bottom refers to the upper and down part of sludge bed, respectively.

3.2.2. Additional experiments

Batch experiments under anoxic conditions

Batch experiments were conducted to determine the biomass-specific anoxic phosphate uptake rates with different electron acceptors (nitrite or nitrate). These tests

were performed during Phases B1 and C1 (SBR_{20}) and during Phases B2a and C2 (SBR_{30}). Granules were taken from the reactors immediately after the feeding phase, i.e., after accumulation of intracellular PHA (by PAOs and GAOs) and release of phosphate by PAOs to the bulk liquid. Granules were sieved and washed with tap water. Equal amounts of granules (based on the wet weight) were introduced in different 250-mL flasks filled with a 0.1 M Tris-HCl buffer (pH 7.0) containing the same minerals of the synthetic medium fed to reactor (except acetate and phosphate). The flasks containing biomass from SBR_{20} and SBR_{30} were maintained at the same operating temperature of the respective reactors, i.e., 20°C and 30°C. Nitrogen gas was supplied to each flask through porous diffusers in order to maintain anaerobic conditions. In the beginning of the experiment (first 13 min), nothing was added to flasks to verify whether secondary phosphate release without electron acceptor (for maintenance purposes) could occur. At 15 min from the start of the experiment, a pulse of a concentrated phosphate solution (6.25 gPO_4^{3-}-P/L) was added to each flask to obtain a final concentration of around 25 mgP/L. At 30 min, a pulse of either a concentrated nitrite or a nitrate solution (stock solutions of 7.5 gN/L) was dosed to obtain a final concentration of 30 mgN/L. From this point forward, samples were taken regularly every 10 to 20 min for determination of PO_4^{3-}-P, NO_2^--N and NO_3^--N concentrations. Since only liquid sample was removed from each flask in the defined intervals of time, the amount of biomass per volume of liquid increased over the experiment. Measured concentrations and biomass-specific rates were corrected for the volume of liquid taken from the system due to sampling. The denitrification rate was expressed either as nitrite or nitrate reduction rate, and was obtained from linear regression of the nitrite or nitrate concentration over time divided by the VSS concentration.

Additional cycle tests

Besides the typical cyclic studies performed under normal operating conditions, cycle tests were also carried out under either fully anoxic or fully aerobic conditions, achieved by bubbling either nitrogen gas or air through the porous diffuser, respectively. The anoxic cycle tests were conducted irregularly during different experimental phases of both aerobic granular sludge systems. In these experiments, either nitrite or nitrate was gradually added based on the denitrification rate observed during normal operational conditions. Two types of tests were carried out: one dosing

nitrite or nitrate equal to the denitrification rate and other dosing them at twice as the regular denitrification rate obtained under normal operating conditions. Biomass-specific nitrite and nitrate reduction rates were obtained by subtracting the nitrite or nitrate dosage rate from the nitrite or nitrate accumulation rate observed during the experiment divided by the VSS concentration.

3.2.3. Determination of granules physical properties

Average diameter of the granules from both top and bottom of the sludge bed (here further referred as top and bottom granules, respectively) was determined by using an image-analyser. Biomass density was measured with a pycnometer (Winkler et al., 2011a). Oxygen penetration depth and aerobic volume fraction available for nitrification were calculated according to Winkler et al. (2011b). The surface area of the granules was calculated based on the average diameter of the top and bottom granules. In order to calculate theoretical settling behaviour, the average density and diameter of both top and bottom granules were taken into account. If the Reynolds number of the particle was lower than 1, Stokes' law was used to calculate the settling velocity $v_s = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{\nu_w}$. The settling velocity for particles with Reynolds higher than 1 were determined by calculating the Archimedes number ($Ar = \frac{\rho_p - \rho_w}{\rho_w} \cdot d_p^3 \cdot \frac{g}{\nu_w}$) and hence defining the omega number from an Omega-Archimedes diagram. The settling velocity was then calculated by using the equation $\Omega = \frac{v_s^3 \cdot \rho_w}{\nu_w \cdot g \cdot (\rho_p - \rho_w)}$. Definitions for equations are given in the Appendix.

3.2.4. Analytical measurements

Ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) were measured with standard cuvette tests (Hach Lange) or by flow injection analysis (QuikChem 8500, Lachat Instruments, Inc.). Phosphate (PO_4^{3-}-P) was determined by Hach Lange cuvette tests. Biomass concentration was determined according to Standard Methods (APHA, 1998). The analytical procedure for polyhydroxyalkanoates (PHA) determination can be found elsewhere (Johnson et al., 2009).

3.2.5. Fluorescent in situ hybridization (FISH)

FISH analysis was conducted to assess the evolution of microbial populations in the granular sludge SBRs. Granules were collected from the top and bottom of the settled sludge bed of the aerobic granular sludge reactors (during the anaerobic feeding phase) once a week for the whole experimental period. The top and bottom biomass were crushed separately, washed twice with 1× phosphate-buffered saline (PBS) and immediately fixed with 4% (w/v) paraformaldehyde in PBS solution for 3 h at 4ºC. After fixation, cells were centrifuged at 13000 g for 1 min, washed twice in 1× PBS and resuspended in an ethanol-PBS solution (1:1) for storage at -20°C. Hybridization was carried out according to Bassin et al. (2011a). The images were exported as .jpg format from the Zeiss microscopy imaging software (AxioVision version 4.7). The rRNA-targeted oligonucleotide probes labelled with three different fluorescent dyes (Cy3, Fluos and Cy5) are listed in Table 3.2.

Table 3.2: Oligonucleotides probes and their targeted microbial groups.

Probe	Sequence (5'-3')	Target group	Mix	Reference
PAO 462	CCGTCATCTACWCAGGGTATTAAC	PAO cluster[a]	PAOmix[e]	Crocetti et al. (2000)
PAO 651	CCCTCTGCCAAACTCCAG	PAO cluster[a]		Crocetti et al. (2000)
PAO 846	GTTAGCTACGGCACTAAAAGG	PAO cluster[a]		Crocetti et al. (2000)
Acc-I-444	CCCAAGCAATTTCTTCCCC	Clade IA[b]	PAOI/PAOII[f]	Flowers et al. (2009)
Acc-II-444	CCCGTGCAATTTCTTCCCC	Clade IIA[c]		Flowers et al. (2009)
GAO Q431	TCCCCGCCTAAAGGGCTT	Competibacter phosphatis[d]	GAOmix[g]	Crocetti et al. (2002)
GAO Q989	TTCCCCGGATGTCAAGGC	Competibacter phosphatis[d]		Crocetti et al. (2002)
Nso 1225	CGC CAT TGT ATT ACG TGT GA	*Nitrosomonas*	AOBmix[h]	Mobarry et al. (1996)
Nso 190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizers *β*-proteobacteria		Mobarry et al. (1996)
Neu 653	CCC CTC TGC TGC ACT CTA	Most halophilic and halotolerant *Nitrosomonas spp*.		Wagner et al. (1995)
Ntspa 662	GGA ATT CCG CGC TCC TCT	Genus *Nitrospira*	NOBmix[i]	Daims et al. (2001)
Nit 1035	CCT GTG CTC CAT GCT CCG	*Nitrobacter spp*.		Wagner et al. (1996)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	EUBmix[j]	Amann et al. (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*		Daims et al. (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales		Daims et al. (1999)

[a] Closely related to Rhodocyclus (*Candidatus* Accumulibacter phosphatis)
[b] Probe Acc-I-444 also targets some (but not all) member of other Type I clades.
[c] Probe Acc-II-444 also targets some (but not all) members of clades IIC and IID. Clades were defined previously (Peterson et al., 2008).
[d] *Candidatus* Competibacter phosphatis
[e] The PAOmix combination (PAO 462, PAO 651 and PAO 846) was used to target for *Candidatus* Accumulibacter (Oehmen et al., 2007).
[f] For the differentiation of clades of PAO (clade I and clade II, capable and not capable of reducing nitrate, respectively), specific probes developed by Flowers et al. (2009) were used.
[g] GAO phenotype bacteria was also targeted by combinations of the probes GAOQ 431 and GAOQ 989, designated as GAOmix.
[h] Ammonium-oxidizing bacteria were detected with a combination of probes Nso 1225, Nso 190 and Neu 653, designated as AOBmix.
[i] Nitrite-oxidizing bacteria were detected with a combination of probes Ntspa 662 and Nit 1035, designated as NOBmix.
[j] General bacteria community was detected with a combination of probes EUB338 I, EUB338 II and EUB338 III, designated as EUBmix. Bacteria which were not targeted with any of the specific probes were considered ordinary heterotrophic bacteria.

3.3. Results

3.3.1. Start-up operation

During the first 48 days of the start-up operation of SBR_{20} (data not shown), the DO concentration was fixed at 100% air saturation (9.1 mgO_2/L at 20°C). During this period, phosphate release gradually increased from 20 to 50 mgP/L and full ammonium removal was established. During this start-up period, phosphate and nitrogen removal reached 90% and 60%, respectively. In order to increase the nitrogen removal efficiency by increasing the anoxic zone of the granules, DO was decreased to 1.8 mgO_2/L. After the DO decrease, around 6.0 mgN/L of ammonium started to accumulate in the effluent for 15 days. Nevertheless, prolonged operation at 1.8 mgO_2/L enabled the establishment of full ammonium removal. Moreover, overall nitrogen removal increased and ranged between 80 and 90% until the end of the start-up phase of SBR_{20}, which lasted 104 days. The considerable decrease in DO affected the phosphate release by PAOs, which dropped from 50 to 30 mgP/L, and the phosphate removal, which decreased from 80 to 30%. Longer biomass adaptation to the lower DO allowed reaching a stable operation with phosphate removal of 90% and a phosphate release of 65 mgP/L.

3.3.2. Long-term operation of the aerobic granular sludge reactors

After the start-up period, half of the sludge bed (mixed sample) was removed from SBR_{20} to inoculate SBR_{30} and Phases A1 (SBR_{20}) and A2 (SBR_{30}) started (time 0 for both systems). The measured profiles of nutrient components (phosphate and nitrogen compounds) obtained in the long-term operation of both SBRs from the time they started to be operated in parallel are shown in Figure 3.3 and Figure 3.4, respectively. As can be seen from the beginning of Phase A1, immediately after removal of half of the biomass of SBR_{20}, phosphate removal and phosphate release were not affected in this reactor (Figure 3.3a). On the other hand, even though the SBR_{30} was inoculated with granules from SBR_{20}, which showed good P removal, the start-up period of SBR_{30} (Phase A2) was characterized by a low phosphate removal (around 50%). Moreover, phosphate release in this particular reactor was lower than 20 mgP/L in the beginning of Phase A2 (Figure 3.3b). The results clearly suggested that the growth and activity of PAOs were severely affected by the temperature increase from 20 to 30°C.

The removal of half of the biomass in SBR_{20} caused a significant decrease in the nitrification capacity, which lead to poor nitrogen removal in the first days of Phase A1 (Figure 3.4a). It took around 25 days to recover complete ammonium conversion, and, consequently, full nitrogen removal was obtained in the end of Phase A1. Similar results were obtained in SBR_{30} (Figure 3.4b), although in this system it took a bit longer to achieve full nitrification and nitrogen removal (33 days).

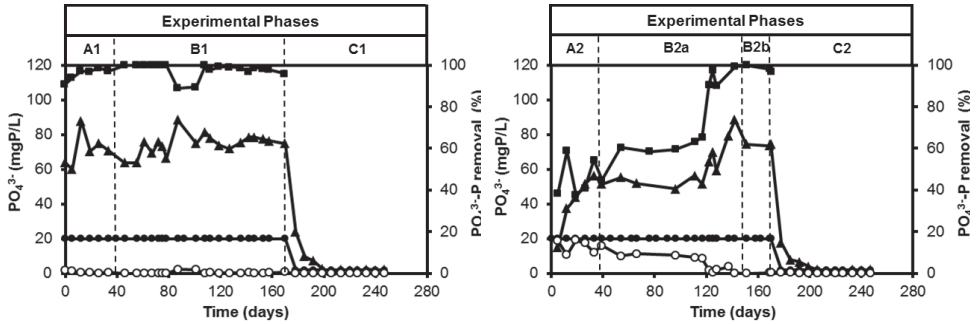


Figure 3.3: Phosphate concentrations in the influent (●) and effluent (○), phosphate release during anaerobic feeding (▲) and phosphate removal (■) in the long term operation of the SBR_{20} (a) and SBR_{30} (b). The phosphate removal efficiency was not considered in Phase C1 (SBR_{20}) and Phase C2 (SBR_{30}) when influent phosphate concentration was only 2 mgPO_4^{3-}-P/L.

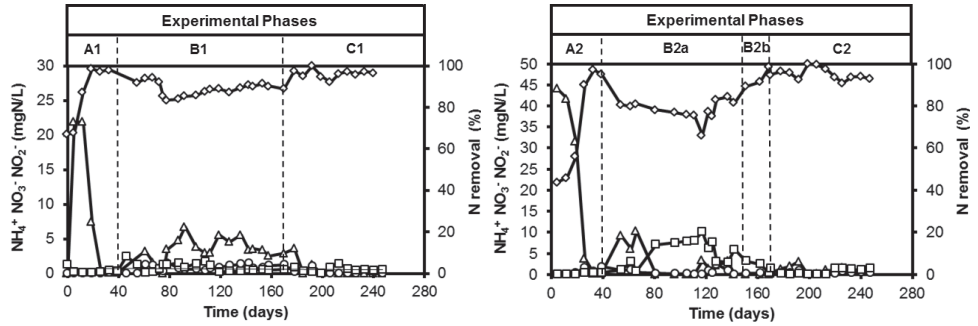
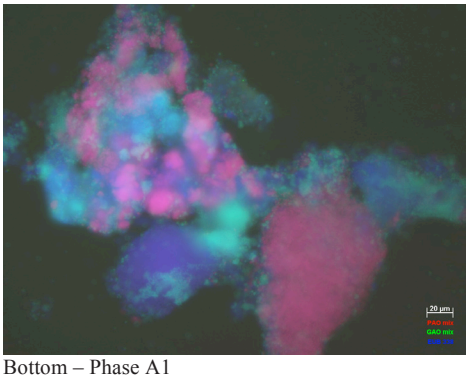


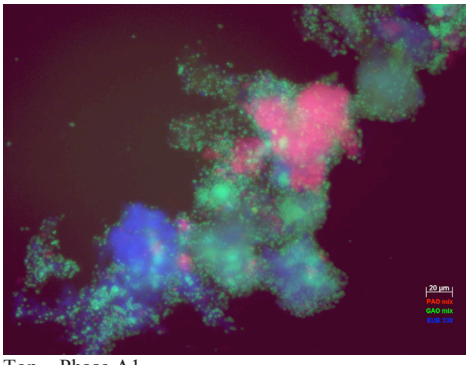
Figure 3.4: Ammonium (Δ), nitrite (○) and nitrate (□) concentrations in the effluent and nitrogen removal (◊) in the long-term operation of the SBR_{20} (a) and SBR_{30} (b). Ammonium influent concentration was set at 60 mgN/L.

FISH results (Figure 3.5 and Figure 3.6) showed a stratification of microbial community structure over the sludge bed during Phases A1 (SBR_{20}) and A2 (SBR_{30}). In the top of the sludge bed in both SBRs, GAOs clearly dominated over PAOs. Conversely, in the bottom of the sludge bed, PAOs were dominant. A similar trend was observed in previous research at 30°C (Winkler et al., 2011a). Therefore, in order to

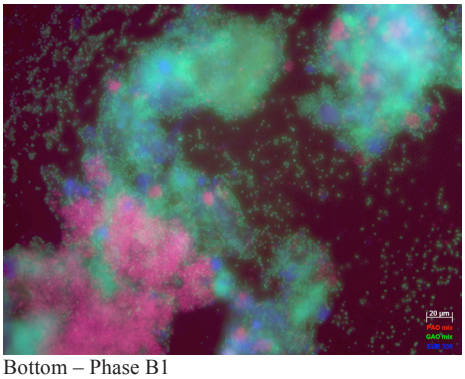
favour PAOs over GAOs and achieve better and stable phosphate removal, particularly in SBR_{30}, excess sludge for SRT control started to be removed mainly from the GAO-rich top of the sludge bed in Phase B2a, while bottom granules were removed in minor proportions. In order to have the same operational conditions in both reactors, the selective sludge removal mainly from top of the sludge bed was also implemented in SBR_{20} in Phase B1, although phosphate removal in this particular reactor was around 100% in the end of Phase A1.



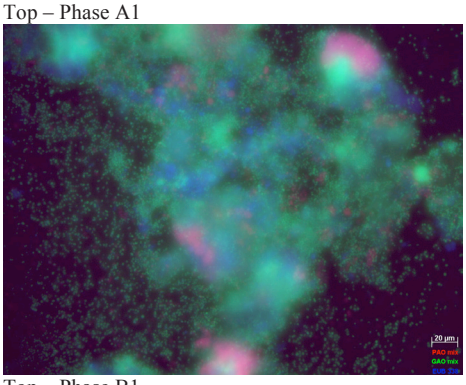
Bottom – Phase A1



Top – Phase A1

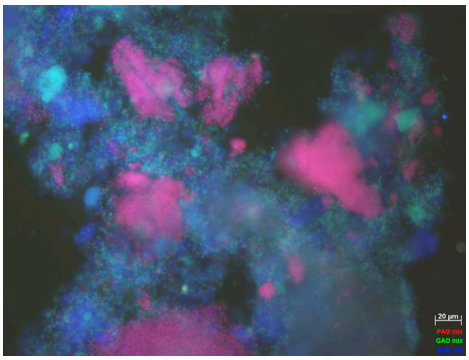


Bottom – Phase B1

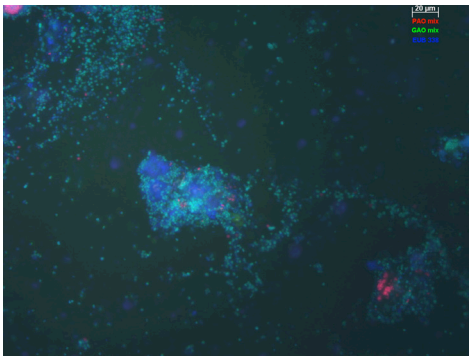


Top – Phase B1

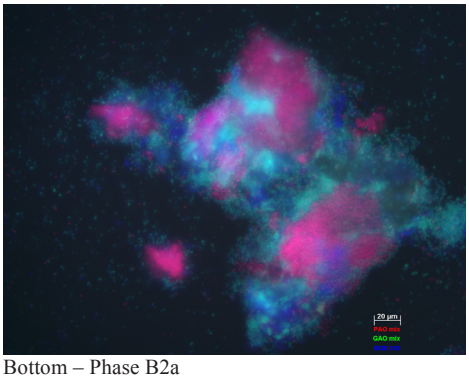
Figure 3.5: Fluorescent in situ hybridization analysis of the PAO/GAO populations among all the bacteria present in the SBR_{20} during phases A1 and B1. Combinations of specific probes for PAOs (PAOmix, shown in red), GAOs (GAOmix, shown in green) and general bacteria EUB338 (EUBmix; shown in blue) were used. PAOs appear violet due to superposition of the red-labelled PAOmix and the blue-labelled EUBmix probes, while GAOs appear turquoise due to the superposition of green-labelled GAOmix and the blue-labelled EUBmix probes.



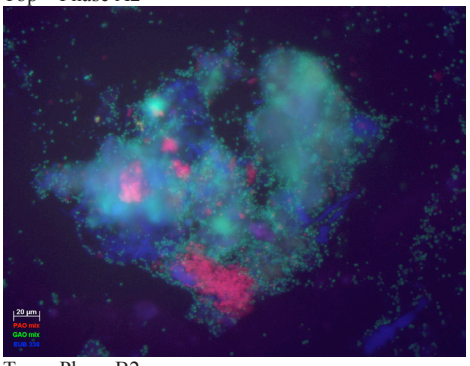
Bottom – Phase A2



Top – Phase A2



Bottom – Phase B2a

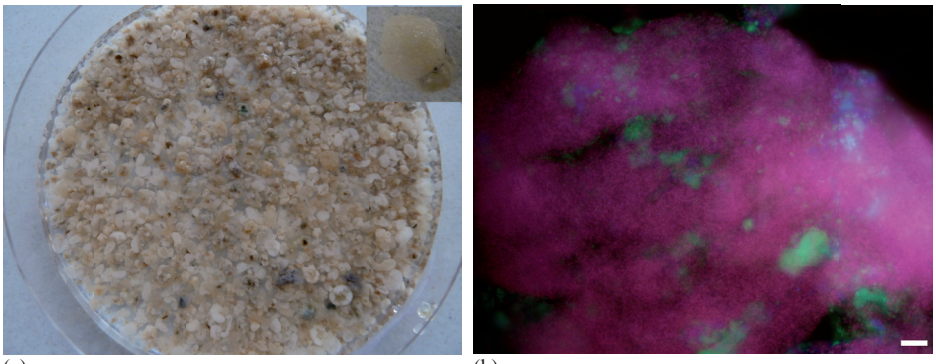


Top – Phase B2a

Figure 3.6: Fluorescent in situ hybridization analysis of the PAO/GAO populations among all the bacteria present in the SBR_{30} during phases A2 and B2a. Combinations of specific probes for PAO (PAOmix, shown in red), GAO (GAOmix, shown in green) and general bacteria EUB338 (EUBmix; shown in blue) were used. PAOs appear violet due to superposition of the red-labelled PAOmix and the blue-labelled EUBmix probes, while GAOs appear turquoise due to the superposition of green-labelled GAOmix and the blue-labelled EUBmix probes.

With the selective sludge removal, phosphate removal efficiency higher than 90% was obtained in the reactor operated at 30°C after 80 days of operation in Phase B2a. Concomitantly, big white granules, strongly dominated by PAOs (Figure 3.7), appeared in this system. The ash content of the white granules was around 40%, which is higher compared to other granule types in the reactor (presenting around 20% ash content). From day 120, phosphate removal was always kept above 90%. Phosphate release increased during Phase B2a and reached a stable value of approximately 80 mgP/L at the end of the respective phase. In SBR_{20}, phosphate removal and phosphate release were stable during the whole Phase B1 and amounted above 95% and 60 mgP/L, respectively. As shown in Figures 3.5 and 3.6, the stratification in the microbial population over the sludge bed (more GAOs at the top and more PAOs at the bottom of the sludge blanket) continued in Phase B1 (SBR_{20}) and Phase B2a (SBR_{30}).

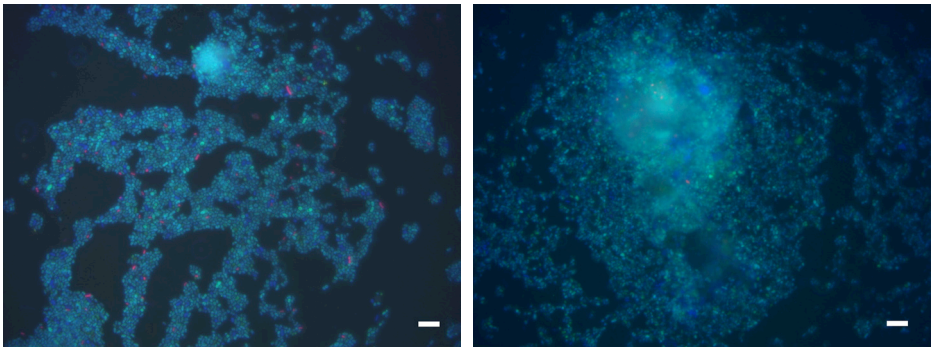
Nitrogen removal was also kept constant and around 90% during Phase B1, although ammonium (around 3 mgN/L) started to be detected in the effluent along the operation in this particular phase. In SBR_{30}, nitrate was not completely denitrified and nitrogen removal amounted to around 80% during most of Phase B2a. In order to achieve better nitrogen removal without causing problems to the nitrification step, DO concentration was slightly decreased from 1.8 to 1.3 mgO_2/L in Phase B2b. The respective phase was not applied to SBR_{20}. Complete nitrification was still obtained and practically full denitrification (more than 95%) was achieved in this phase. Phosphate removal was not influenced by the reduced DO concentration in SBR_{30}.



(a) (b)

Figure 3.7: White granules (shown in detail in the top right of the image) (a) and fluorescent in situ hybridization analysis of the PAO/GAO populations among all the bacteria present in the white granules (b) collected from SBR_{30} during Phase B2a. Combinations of specific probes for PAO (PAOmix, shown in red), GAO (GAOmix, shown in green) and general bacteria EUB338 (EUBmix; shown in blue) were used. Scale bar indicates 20 µm.

In Phase C1 (SBR_{20}) and Phase C2 (SBR_{30}), when the excess sludge for SRT control started to be removed only from the bottom of the sludge bed, the amount of phosphate released during the anaerobic period gradually decreased and after 20 days of operation in those respective phases, almost no phosphate release was observed in both reactors (Figure 3.3). Few cells of PAOs were still detected in SBR_{20} and SBR_{30} after 20 days of operation in Phases C1 and C2, respectively (Figure 3.8), when more than 95% of the microbial population corresponded to GAOs. Full nitrification and more than 95% nitrogen removal were obtained in both GAO-dominated systems (Figure 3.4). In SBR_{30}, the colour of the granules changed back from white to its original colour (brownish-black) and the ash content of the biomass in both reactors gradually decreased from 30% to 10%.



(a) (b)

Figure 3.8: Fluorescent in situ hybridization analysis of the PAO/GAO populations among all the bacteria present in the (a) SBR_{20} (Phase C1) and (b) SBR_{30} (Phase C2). Combinations of specific probes for PAO (PAOmix, shown in red), GAO (GAOmix, shown in green) and general bacteria EUB338 (EUBmix; shown in blue) were used. Scale bar indicates 10 µm.

3.3.3. Cycle tests under normal reactor operating conditions

Cycle tests performed when a pseudo-steady-state condition was achieved at different operational phases in SBR_{20} and SBR_{30} are displayed in Figure 3.9a and 3.9b, respectively. The results obtained in this set of experiments are summarized in Table 3.3. During anaerobic feeding from the bottom of the reactors, all organic carbon (acetate) was completely consumed, which was accompanied by the consumption of glycogen, production of PHA (Polyhydroxybutyrate – PHB + Polyhydroxyvalerate – PHV) (data not shown) and phosphate release by PAOs. As illustrated in Figure 3.9, ammonium concentration after anaerobic feeding was lower than expected based on the influent concentration and the dilution in the reactor (around 34 mgN/L, as depicted at the time 0). This fact was attributed to ammonium adsorption, phenomenon investigated in a separate study (Bassin et al., 2011b; Lin et al., 2012). In the subsequent aerobic stage, ammonium was nitrified and nitrite/nitrate were simultaneously denitrified in the anoxic core of the granules. Meanwhile, glycogen was replenished and PHA was oxidized (data not shown) and phosphate was taken up both aerobically and anoxically.

According to the cycle tests performed in SBR_{20}, the profiles of phosphate, ammonium, nitrite and nitrate in the steady-state operation in Phases A1 and B1 were very similar (Figure 3.9a). In SBR_{30}, the strategy in which excess sludge for SRT control was mainly removed from the top of the sludge bed to favour PAOs over GAOs

in Phase B2a caused a considerable increase in the specific P uptake (Table 3.3). Specific ammonium uptake rates were a bit higher for SBR_{30} compared to those of SBR_{20}. As shown in Figure 3.9b, ammonium was depleted around 20 min before the end of a SBR_{30} cycle in the steady-state operation in Phase B2a and a considerable amount of nitrate (around 12 mgN/L) was detected in the effluent. The decrease in DO concentration from 1.8 to 1.3 mgO_2/L in Phase B2b intended to increase the anoxic zone and therefore the denitrification capability caused a slight decrease in the nitrification rate (Table 3.3). On the other hand, denitrification rate increased and consequently, lower nitrate accumulation was observed in the effluent.

PHA was also quantified along the cycle tests. PHB was the main form of PHA (80%), although polyhydroxyvalerate (PHV) was also detected in small proportions (20%). Taking into account just the main fraction of PHA (i.e. PHB), its content was observed to decrease from around 6.0 to 4.5% of the total biomass dry weight during the aerobic period of an operational cycle for both SBRs. SRT control in both aerobic granular sludge reactors resulted in biomass concentrations between 10 and 14 gVSS/L.

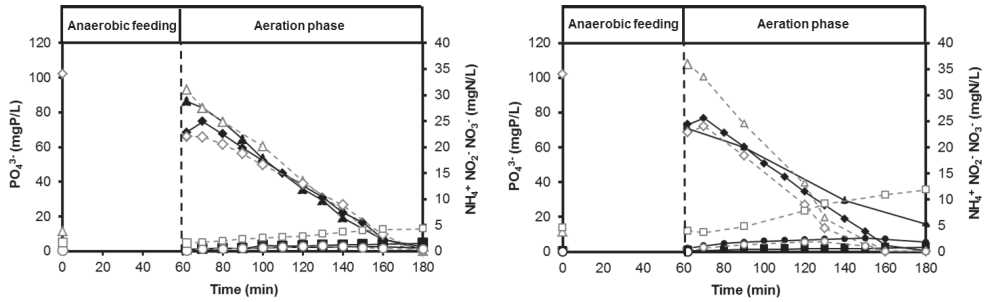


Figure 3.9: Cycle tests performed in the SBR_{20} (a) and SBR_{30} (b) during different operational phases: Phosphate (▲), Ammonium (♦), nitrite (●) and nitrate (■) in phases A1 (SBR_{20}) and A2 (SBR_{30}); Phosphate (Δ), Ammonium (◊), nitrite (○) and nitrate (□) in phases B1 (SBR_{20}) and B2a (SBR_{30}). The starting ammonium and phosphate concentrations depicted at time 0 were calculated based on the influent concentration (60 $mgNH_4^+$-N/L and 20 $mgPO_4^{3-}$-P/L) and the dilution in the reactor. Nitrite and nitrate concentrations at time 0 were calculated based on their concentrations in the end of the cycle and the dilution in the reactor.

Table 3.3: Results obtained in the cycle tests conducted under normal operating conditions in both aerobic granular sludge systems.

Reactor	Phase[a]	Phosphate uptake rate ($mgPO_4^{3-}$-P/(gVSS·h))	Ammonium uptake rate ($mgNH_4^{+}$-N/(gVSS·h))	Denitrification rate ($mgNO_x$-N/(gVSS·h))[b]
SBR_{20}	A1	3.5	1.0	1.0
	B1	3.9	0.9	1.2
SBR_{30}	A2	2.3	1.3	0.9
	B2a	5.9	1.5	1.3
	B2b	4.8	1.2	1.5

[a] Indicates the phase during which the cycle test was conducted.
[b] $NO_x = NO_2^-$ or NO_3^-

3.3.4. Batch experiments under anoxic conditions and cycle tests under either anoxic or aerobic conditions

During normal operation of the reactors, aerobic and anoxic P uptake occur simultaneously and therefore it is not possible to identify directly the amount of phosphate taken up either aerobically or anoxically. Therefore, batch experiments and cycle tests were performed under fully anoxic conditions in order to determine the maximum anoxic P uptake capacity. This is important for instance to estimate the potential significance of anoxic removal of phosphate linked to nitrogen removal by DPAOs (denitrifying dephosphatation). The anoxic batch tests were also conducted with the objective to identify the denitrification pathway, i.e., to observe if denitrification was run mainly over nitrite or nitrate under normal operating conditions and also to have an idea about the main organisms responsible for nitrite and/or nitrate reduction in the reactor (either DPAOs or DGAOs). Table 3.4 summarizes the results obtained in this set of experiments performed with biomass from both reactors. The concentration profiles are displayed in Figures 3.10 (SBR_{20}) and 3.11 (SBR_{30}).

Table 3.4: Denitrification rate (nitrite/nitrate reduction rate), anoxic phosphate uptake rate and anoxic phosphate uptake per N removed (P/N) in the batch experiments under anoxic conditions.

	Phase[a]	Nitrite/nitrate reduction rate ($mgNO_x^-$-N/(gVSS·h))[b]		Phosphate uptake rate ($mgPO_4^{3-}$-P/(gVSS·h))		P/N ratio	
Electron acceptor		NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
SBR_{20}	B1	2.0	6.3	1.2	2.7	0.6	0.4
	C1	2.5	5.6	0.2	0.17	0.1	0.03
SBR_{30}	B2a	2.8	4.9	1.4	1.6	0.5	0.3
	C2	2.5	5.9	0.2	0.2	0.1	0.04

[a] Indicates the phase from which biomass was collected
[b] $NO_x^- = NO_2^-$ or NO_3^-

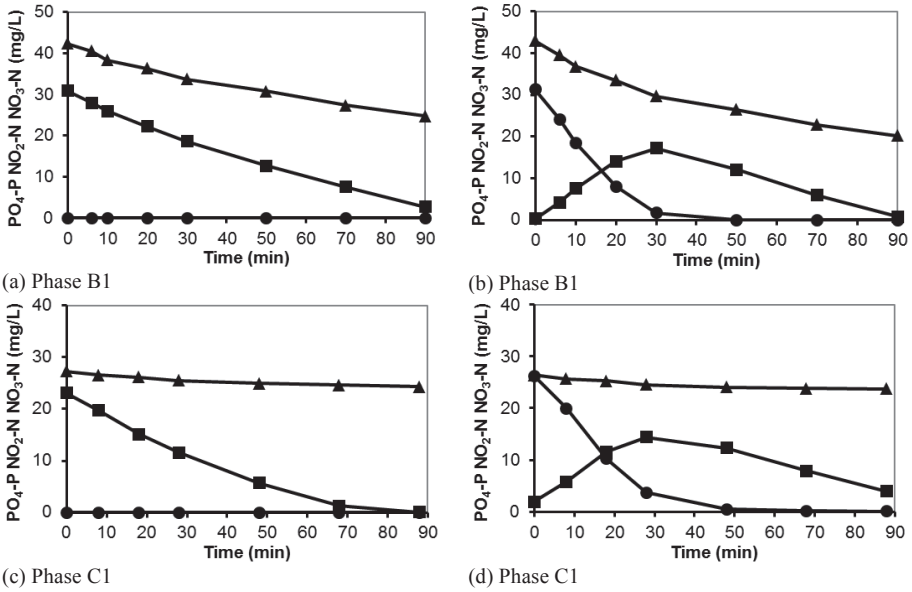


Figure 3.10: Anoxic batch experiments with biomass from SBR_{20}. Nitrite-dosing experiments (a) and (c); nitrate-dosing experiments (b) and (d). Phosphate (▲), nitrite (■) and nitrate (●) concentrations were measured every 5 – 20 min.

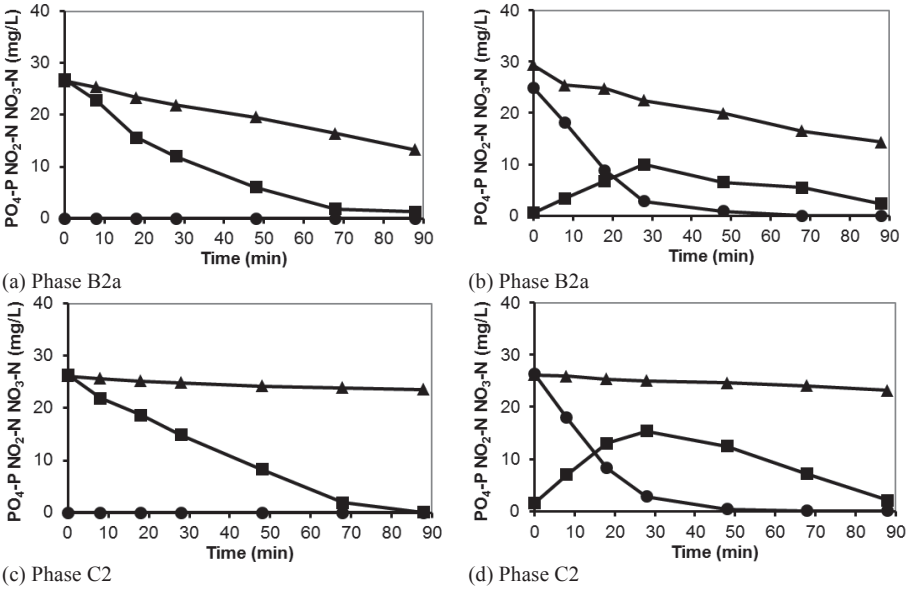


Figure 3.11: Anoxic batch experiments with biomass from SBR_{30}. Nitrite-dosing experiments (a) and (c); nitrate-dosing experiments (b) and (d). Phosphate (▲), nitrite (■) and nitrate (●) concentrations were measured every 5 – 20 min.

In the experiments with biomass taken from SBR_{20} during Phase B1, the phosphate uptake rate with nitrate was higher than with nitrite (Table 3.4). For the SBR_{30}, the P uptake rate obtained in the experiment using biomass collected during Phase B2a was similar for nitrite and nitrate as electron acceptor. Anoxic phosphate uptake per N reduced (P/N ratio) was very similar in the experiments carried out with biomass from SBR_{20} and SBR_{30} during Phases B1 and B2a, respectively. Nitrate reduction rate was observed to be always higher than nitrite reduction rate in the experiments using biomass from both SBRs. This suggests that denitrification proceeded predominantly via nitrate instead of nitrite, even though PAOII (without nitrate reduction capability) was the only PAO type in SBR_{30} and the dominant PAO type in SBR_{20} at the time of the experiments.

To investigate the denitrification potential of GAOs, the same type of experiments was performed when PAOs were washed out from both SBRs (Phase C1 in SBR_{20} and Phase C2 in SBR_{30}). As expected, phosphate uptake both with nitrite and nitrate was observed to be negligible (Table 3.4), since only few cells of PAOs were still detected by FISH in those respective phases (Figure 3.8). The nitrate reduction rate was significantly higher (more than 2 times higher) than nitrite reduction rate, similarly to the results observed in the anoxic batch experiments in which biomass composed by both PAOs and GAOs was used. This also suggests that simultaneous nitrification/denitrification proceeded mainly via nitrate in the GAO-dominated systems.

A similar type of experiments dosing either nitrite or nitrate was performed in the reactors (anoxic cycle tests). During aeration (mixing) phase, the reactor was not supplied with air but only with nitrogen gas to assure anoxic conditions. The anoxic cycle tests were carried out when PAOs and GAOs coexisted in both SBRs (Phase B1 in SBR_{20} and Phase B2a in SBR_{30}) and also when GAOs represented more than 95% of the microbial population (Phase C1 in SBR_{20} and Phase C2 in SBR_{30}). Nitrite or nitrate was not dosed as a pulse but in a way that resembles better the operation of the reactor, i.e., was added gradually based on the denitrification rate observed in the aerobic period of the reactor under normal operating conditions. Extra experiments in which electron acceptor were dosed at twice as the regular denitrification rate were also performed. The results are shown in Table 3.5 (concentration profiles are shown in Figure 3.12).

In SBR_{20}, in which PAOII was dominant and PAOI was present in minor proportions (Phase B1), anoxic P uptake with nitrate was higher than with nitrite. However, in the anoxic experiments performed in SBR_{30} during Phase B2a, phosphate uptake was the same regardless the electron acceptor dosed. When dosing nitrite at a rate two times higher than the nitrite reduction rate observed under normal operating conditions, very low accumulation of nitrite was observed (data not shown). In this particular test, P uptake rate was observed to increase in both reactors. This result, combined with the fact that intracellular PHB was still available at the end of the anoxic cycle tests, suggests that P uptake under anoxic conditions was limited by nitrite supply in the test in which nitrite dosage equalled to the denitrification rate.

The same type of experiments was performed after PAOs were washed out from both reactors (Phase C1 in SBR_{20} and Phase C2 in SBR_{30}). As expected, phosphate uptake rate with either nitrite or nitrate as electron acceptor was very low in the experiments with GAO-dominated biomass (around 0.2 $mgPO_4^{3-}$-P/(gVSS·h) (Table 3.5). When nitrite or nitrate was dosed according to the denitrification rate obtained under normal operating conditions, no accumulation of these electron acceptors was observed since they were completely denitrified. When nitrite or nitrate was dosed at twice as the regular denitrification rate, no difference between nitrite and nitrate reduction rate was observed and very low accumulation of nitrite and nitrate was detected for both SBR_{20} and SBR_{30} experiments.

Table 3.5: Nitrite/nitrate dosage rate, nitrite/nitrate reduction rate, anoxic phosphate uptake rate and anoxic phosphate uptake per N removed (P/N) obtained in the anoxic cycle tests in both SBRs when PAOs and GAOs coexisted (Phase B1 in SBR_{20} and Phase B2a in SBR_{30}) and when PAOs were washed out (Phase C1 in SBR_{20} and Phase C2 in SBR_{30}).

	NO_2^- or NO_3^- dosage rate ($mgNO_x^-$-N/(gVSS·h))	NO_2^- or NO_3^- reduction rate ($mgNO_x^-$-N/(gVSS·h))		Phosphate uptake rate ($mgPO_4^{3-}$-P/(gVSS·h))		P/N ratio	
Electron acceptor		NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
SBR_{20}	1.3[a]	1.2	1.2	2.2	3.0	1.7	2.5
(Phase B1)	2.6[b]	1.9	-	3.5	-	1.8	-
SBR_{20}	1.5[a]	1.4	1.4	0.2	0.2	0.2	0.2
(Phase C1)	3.0[b]	2.6	2.6	0.3	0.3	0.1	0.1
SBR_{30}	1.5[a]	1.3	1.2	3.4	3.4	2.5	2.7
(Phase B2a)	3.0[b]	2.5	-	3.8	-	1.5	-
SBR_{30}	1.3[a]	1.2	1.2	0.2	0.2	0.2	0.2
(Phase C2)	2.6[b]	2.5	2.5	0.2	0.2	0.1	0.1

[a] Dosage equal to denitrification rate observed during normal operational conditions
[b] Dosage equal to two times the denitrification rate observed during normal operational conditions. No experiment dosing NO_3^- at two times the denitrification rate was performed in Phase B1 (SBR_{20}) and Phase B2a (SBR_{30}).

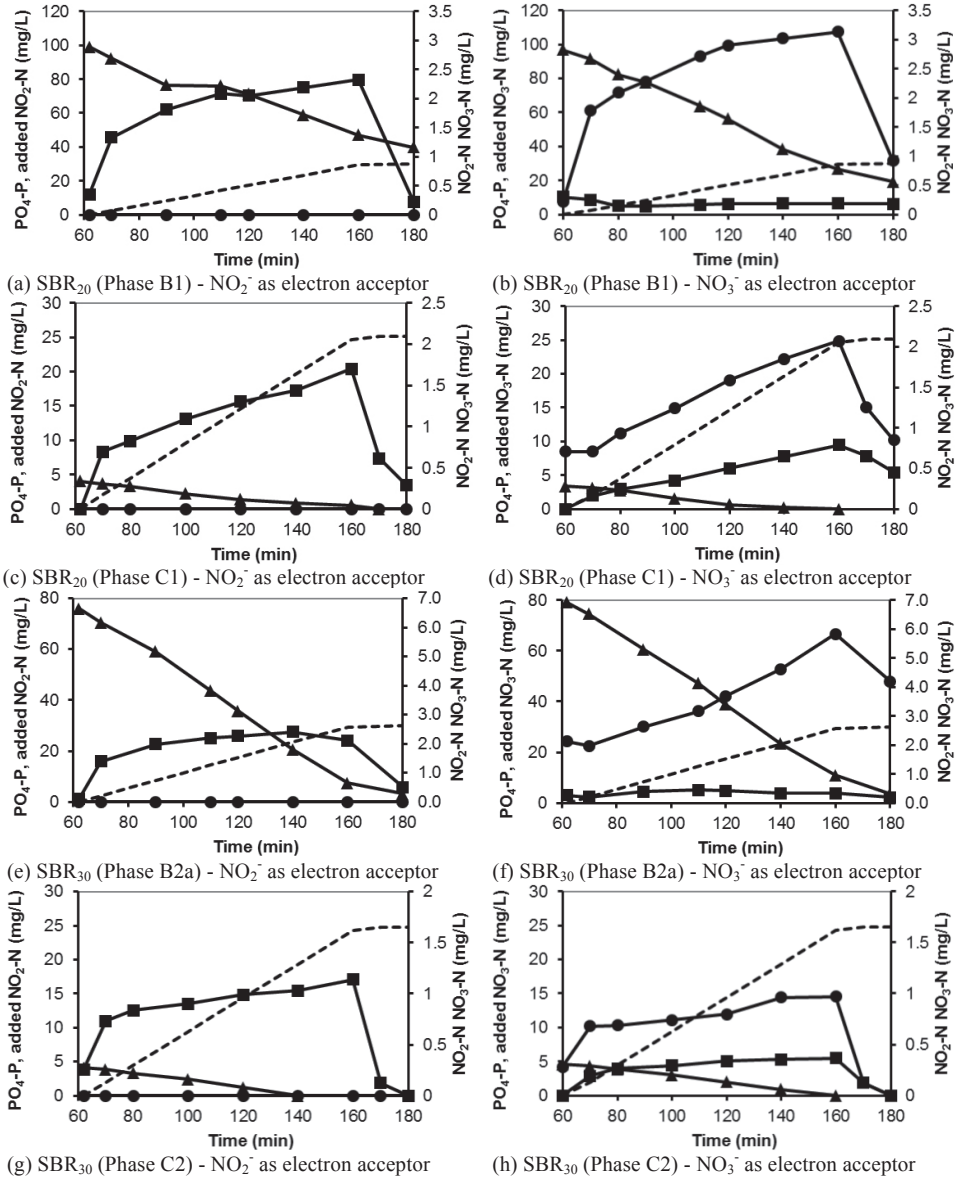


Figure 3.12: Cycle tests under anoxic conditions performed in SBR_{20} (Phase B1 and C1) and SBR_{30} (Phase B2a and C2). Phosphate (▲) in the left y-axis, nitrite (■) and nitrate (●) measured every 5 - 20 min in the right y-axis. Dashed lines represent the addition of nitrite or nitrate (left y-axis). The experiments in which nitrite or nitrate were dosed as twice as the denitrification rate observed during normal operating conditions are not shown.

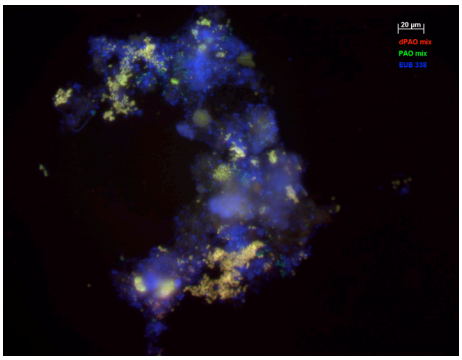
Besides the experiments under anoxic conditions, cycle tests were performed in each system under fully aerated conditions (100% air saturation) when the steady-state operation during Phases B1 (SBR_{20}) and Phases B2b (SBR_{30}) was reached (data not

shown). Phosphate uptake rates obtained in obtained in SBR_{20} and SBR_{30} were approximately 9.6 and 7.9 $mgPO_4^{3-}$-P/(gVSS·h), respectively, while ammonium uptake rate increased to 1.6 and 2.2 $mgNH_4^+$-N/(gVSS·L), respectively. Denitrification rates considerably decreased compared to normal operation at low DO and amounted to 0.7 and 0.4 $mgNO_x^-$/(gVSS·h) (NO_x^- = NO_2^- and NO_3^-) for SBR_{20} and SBR_{30}, respectively.

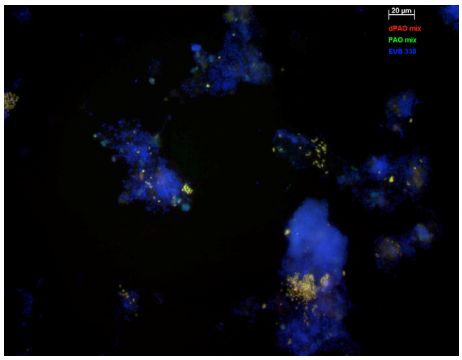
3.3.5. General characterization of microbial community analysis by FISH

Several specific oligonucleotide probes (Table 3.2) were used for FISH analysis to observe dominant microbial populations in SBR_{20} and SBR_{30} along the experimental phases. Results obtained with FISH analysis indicated that the majority of bacteria present in the both SBRs in all operational phases either belonged to PAO or GAO group (Figures 3.5 and 3.6). The presence of ordinary heterotrophs was minimal, since full acetate uptake by PAOs and GAOs under the anaerobic conditions was obtained.

The distribution of PAOs and GAOs did not change very much in SBR_{20} from Phase A1 to Phase B1, although the fraction of PAOs increased considerably along the operation of SBR_{30} from Phase A2 to Phase B2a. The increase of PAOs in SBR_{30} is especially strong after removing excess sludge mainly from the GAO-rich top of sludge bed. A complete overlap between the mixture of PAOI and PAOII populations and the total PAO population was observed (Figure 3.13). This result indicated that the whole PAO population in both systems was composed by PAOI and/or PAOII subgroups. During the whole operation of SBR_{20}, the PAO population was composed by both PAOI and PAOII subgroups, although PAOII was dominant, especially in Phase B1 (Figure 3.14). In SBR_{30}, the presence of PAOI was only detected in Phase A2, in which this reactor was inoculated with biomass from SBR_{20}, whereas PAOII was the only PAO subgroup present during Phase B2a and B2b (Figure 3.15). In Phase C1 (SBR_{20}) and Phase C2 (SBR_{30}), when PAOs were removed from both systems, GAOs represented more than 95% of the microbial population (Figure 3.8). Ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) represented a small fraction of the whole bacterial community over the whole experimental period (Figure 3.16).

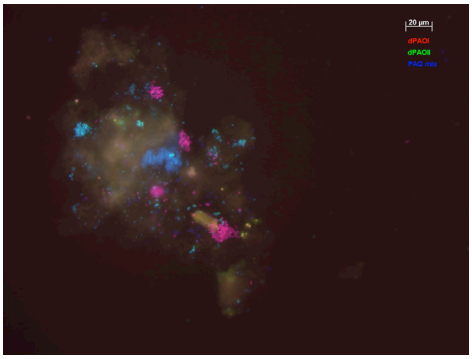


SBR_{20} (room temperature)

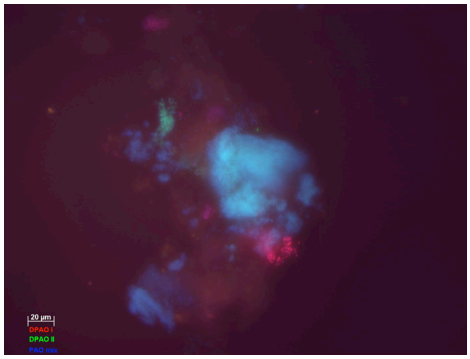


SBR_{30} (30°C)

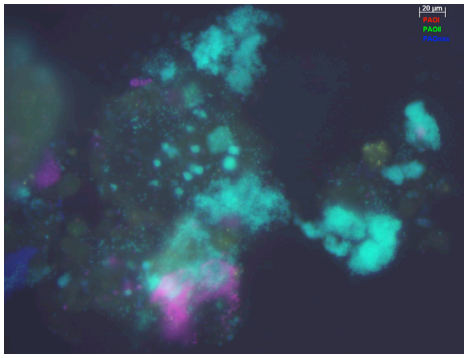
Figure 3.13: Fluorescent in situ hybridization analysis of the PAOI+PAOII populations among the whole PAO community in Phases B1 (SBR_{20}) and Phase B2a (SBR_{30}). PAOI+PAOII shown in red; PAOmix shown in green; EUBmix shown in blue. Yellow colour is due to the superposition of red (PAOI+PAOII) and green (PAOmix).



Before Phase A1

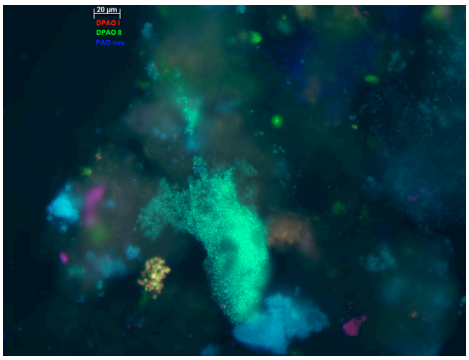


Phase A1

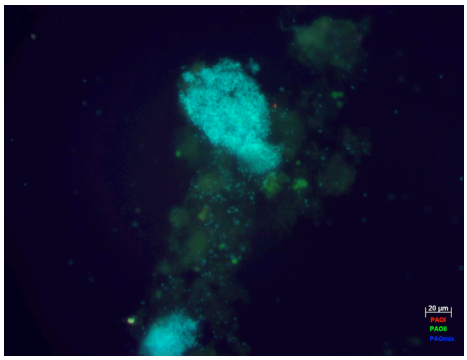


Phase B1

Figure 3.14: Fluorescent in situ analysis hybridization of the PAOI/PAOII populations among all PAO in the start-up phase of SBR_{20} (prior to inoculation of SBR_{30}) and during phases A1 and B1. PAOI (Acc-I-444) shown in red; PAOII (Acc-II-444) shown in green; PAOmix shown in blue.

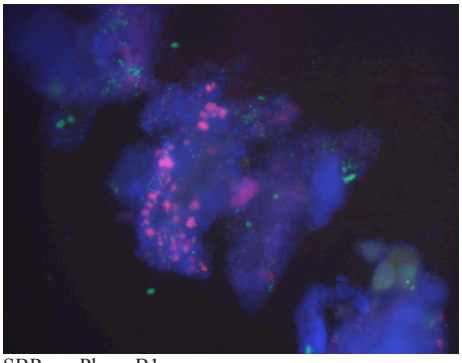


Phase A2

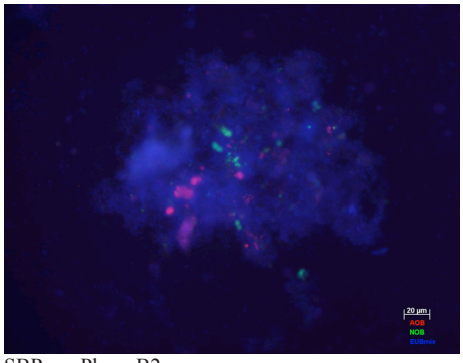


Phase B2a

Figure 3.15: Fluorescent in situ hybridization analysis of the PAOI/PAOII populations among all PAO in the SBR_{30} during phases A2 and B2a. The PAO community composition in Phase B2b (data not shown) is the same of Phase B2a. PAOI (Acc-I-444) shown in red; PAOII (Acc-II-444) shown in green; PAOmix shown in blue.



SBR_{20} – Phase B1



SBR_{30} – Phase B2a

Figure 3.16: Fraction of AOB (AOBmix, shown in red) and NOB (NOBmix, shown in green) within the whole bacterial community (EUBmix, shown in blue) in the granules.

3.3.6. Physical properties of aerobic granules

Biomass density, average diameter and settling velocity of both top and bottom granules are displayed in Table 3.6. In general, both the diameter and the density of top and bottom granules increased from Phase A1 to Phase B1 in SBR_{20} and from Phase A2 to Phase B2a in SBR_{30}. The increase of these parameters was more noticeable in the bottom biomass. However, when PAOs were washed out from SBR_{20} and SBR_{30} in Phases C1 and C2, respectively, the density of both top and bottom granules considerably decreased. The calculated settling velocity (based either on Stokes law or Archimedes number) of bottom granules was considerably higher than top granules due to their higher diameter and density.

Table 3.6: Density, average diameter and settling velocity of both top and bottom granules from SBR_{20} and SBR_{30}.

	SBR_{20}						SBR_{30}					
	Phase A1		Phase B1		Phase C1		Phase A2		Phase B2		Phase C2	
Parameter	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
Density (g/L)	1003±2	1008±4	1004±3	1010±5	1001±2	1004±5	1004±2	1005±3	1004±1	1008±4	1001±2	1003±2
Average diameter (mm)	0.6±0.1	0.7±0.1	0.7±0.2	1.0±0.3	0.6±0.3	0.9±0.2	0.7±0.1	0.7±0.2	0.6±0.2	0.9±0.3	0.5±0.1	0.8±0.1
Reynolds[1]	0.6	1.8	1.1	6.3	0.3	2.3	2.4	2.7	1.5	7.6	0.6	3.2
Archimedes' number	n.c.	32.4	19.1	114.0	n.c.	41	40.5	49.0	17.4	137.4	n.c	57.3
Calculated settling velocity (m/h)	3.3	20.2	15.1	50.6	1.9	37.1	39.4	41.6	15.4	48.4	3.2	39.0

[1] For Re<1, Stokes law was considered to calculate the settling velocity; for Re>1, settling velocity was calculated from the Archimedes number, as described in the Materials and Methods Section.
n.c. Not calculated.

From Phase A1 to Phase B1, the total granules surface area decreased from 5.1 to 4.2 m^2 and the volume fraction of the granules available for nitrification decreased from 16.5 to 13.6%. For the SBR_{30}, the volume fraction for nitrification remained the same (15%). In this reactor, however, the granules surface area slightly increased from Phase A2 (4.5 m^2) to Phase B2a (5.2 m^2), which is possibly associated with the higher ammonium uptake rate obtained in the later phase.

3.4. Discussion

3.4.1. Factors controlling PAO/GAO competition

GAOs are usually associated with the deterioration of bio-P removal systems, since they compete with PAOs for COD but do not contribute to phosphate removal (Oehmen et al., 2006). High temperatures are reported to favour the growth of GAOs over PAOs (Lopez-Vazquez et al., 2009). Indeed, phosphate removal in the reactor operated at 20°C was higher than in the reactor operated at 30°C. The ratio between phosphate release and COD uptake (P-release/COD uptake) can be used to estimate the amount of organic carbon (COD) taken either by PAOs or GAOs under anaerobic conditions (Schuler and Jenkins, 2003). This ratio is expected to be around 0.5 P-mol/C-mol in a highly enriched PAO culture and zero in a pure GAO culture (Brdjanovic et al., 1997). The lower P-release/COD uptake ratio in the 30°C reactor (0.22±0.08 P-mol/C-mol) compared to the reactor at 20°C (0.37±0.05 P-mol/C-mol) indicate a lower contribution of PAOs to the anaerobic substrate uptake at 30°C. Moreover, the P-release/COD uptake ratio observed in both reactors indicates that a significant amount of acetate was uptaken by non-PAO bacteria.

The segregation of the biomass over the sludge bed (PAOs concentrated at the bottom and GAOs at the top) observed during the operation of both reactors opened a possibility to influence the PAO-GAO competition. The selective removal of excess sludge mainly from the GAO-rich top part of the 30°C reactor sludge bed in Phase B2a allowed obtaining complete phosphate removal and an increase of phosphate release/COD uptake ratio up to 0.4 P-mol/C-mol, which is comparable to that obtained in the SBR_{20}. Also the appearance of white granules in SBR_{30} in which PAOs corresponded to more than 90% of the microbial population may also explain why higher P uptake rates and phosphate release/COD uptake ratio started to be obtained in SBR_{30} than in SBR_{20}.

The strategy used to remove excess sludge in order to influence the competition between PAOs and GAOs, suggested by Winkler et al. (2011a), proved again to be a good method to favour the development and growth of PAOs at unfavourable conditions for the bio-P removal process (e.g., high temperatures), especially in reactors showing biomass segregation. The selective sludge removal proposed also enhances PAO activity since the feeding is performed from the bottom of the reactor, where PAOs are located. Consequently, these organisms get more substrate than GAOs. However, in our previous study, no sludge was removed from the PAO-rich bottom of the sludge bed for SRT control. This will lead to a very high SRT of bottom granules and PAOs will get completely filled with polyphosphate due the decreased effective sludge yield, limiting further P removal. In the research we conducted previously, this fact was neglected leading to an open question for implementation of the strategy applied. We pointed out that eventually it would be necessary to remove part of the bottom sludge to avoid excessively high SRT values and to remove poly-P from the system. High SRT of bottom granules will cause a big fraction of inert material (ash) in this particular biomass, which is not desirable. First of all, high ash content would lead to significantly heavy granules and probably cause mixing problems during aeration. It is also well possible that the granules containing high ash would become very big and the surface area available for aerobic organisms (e.g., aerobic PAOs, AOB and NOB) would decrease.

Taking into account all these factors, the selective removal of sludge from specific parts of the sludge bed was modified in the current research from that applied in our previous work (Winkler et al., 2011a). Therefore, even though sludge was removed mainly from the GAO-rich top of the sludge bed, in this study we also removed a small fraction of the excess sludge (20%) from the PAO-rich bottom sludge. This procedure of renewal of bottom biomass, besides not causing a negative impact on phosphate removal, allowed obtaining lower amount of ash in the biomass compared to our previous study and avoided the deterioration of phosphate removal in the long-term operation of both SBRs.

Even though the importance of selective sludge removal was confirmed in the current research to be a good method to improve and obtain stable phosphate removal at 30°C, we observed that at lower temperatures, such as 20°C, the selective removal sludge from specific parts of the sludge blanket to favour PAOs over GAOs is not

crucial, as indicated by the results obtained during the operation of SBR_{20}. In that system, full phosphate removal was obtained even when excess sludge was removed during aeration phase, in which biomass was equally distributed throughout the reactor. Therefore, the strategy adopted to control microbial population in our study is especially relevant under operational conditions in which the development of GAOs is favoured compared to that of PAOs, such as high temperatures. It is important to remark that we have chosen to remove 80% of the excess sludge from the top of the sludge bed and 20% from the bottom of the sludge blanket to favour PAOs over GAOs. For full scale applications, it is likely that the ratio of top and bottom sludge has to be controlled based on process performance. This ratio might depend for instance on seasonal variations. This will have to be further tested in full scale and pilot scale research.

Despite the fact that GAOs are usually considered undesirable organisms in the EBPR process, in our experiments full phosphate removal was obtained with a mixed culture in which PAOs and GAOs coexisted. From a process point of view, GAOs also contribute for COD removal and may play an important role in the denitrification, as will be discussed below.

3.4.2. Understanding the link between the main factors influencing biomass segregation within the reactor sludge bed

A possible explanation for the occurrence of biomass segregation can be the different settling velocities shown by bottom (dominated by PAOs) and top granules (dominated by GAOs). In the granules in which PAOs are dominant, higher poly-P would be present in the biomass compared to the GAO-dominated granules, since no P uptake or P release is involved in the metabolism of GAOs. In a first attempt, the significant amount of poly-P accumulated by PAO-rich bottom granules during the aerobic phase was assumed to be responsible for their higher ash content and density compared to GAO-rich granules, making those granules heavier and hence contributing to improve their settling properties. As a consequence, PAO-dominated granules settled faster than GAO-rich granules, and thereby accumulate in the bottom fraction of the sludge bed (Winkler et al., 2011a). Additionally, heavier poly-P containing granules, present at the bottom of the sludge bed, were more exposed to substrate (acetate) fed from the bottom of the reactor. This can also enhance the dominance of PAOs over GAOs, driven by the selective sludge removal mainly from the top of the sludge bed.

The appearance of strongly PAO-dominated white granules in the reactor operated at 30°C was also associated to the amount of poly-P present in the biomass. The development of these granules is possibly a consequence of the sludge removal procedure adopted. Since most of the sludge was removed from the GAO-rich upper part of sludge bed, bottom sludge could be highly enriched for PAOs. Eventually, in some of the granules, the fraction of PAOs and therefore the amount of poly-P was higher than in others. This could enhance chemical precipitation of PO_4^{3-} within the granules (Carlsson et al., 1997; Maurer et al., 1999). As a consequence of chemical precipitation, the colour of the granules may have turned white and the ash content in this specific biomass may have increased (to around 40%). However, the colour changes of the granules observed in our study (from dark colour to white) is not a proof, although it gives an indication of higher precipitation in some granules. The substantial increase in phosphate release observed during the operation of SBR_{30} in Phase B2a could possibly enhance chemical precipitation within the granules and therefore contributed for the appearance of white granules.

In order to better understand the link between the main factors influencing biomass segregation and therefore explain why PAOs and GAOs occupy different positions within the sludge bed, we decided to perform extra experiments to support the observations pointed out in this work and in our previous research (Winkler et al., 2011a). By provoking the washout of PAOs in both reactors, we could observe how the ash content, poly-P content and chemical precipitation within the granules change from an operational stage in which PAOs and GAOs coexisted to other when GAOs are prevalent (corresponding to more than 95% of all bacterial community). In the GAO-dominated systems, we observed that not only the ash but also the density of the granules considerably decreased. As a consequence, the settling velocity of the granules was substantially reduced when PAOs were removed from the system. This implies that PAO/GAO segregation over the sludge bed is primarily associated with the different poly-P and hence different ash content/density of the PAO- and GAO-dominated granules, confirming our previous expectations.

The biomass segregation observed in this study seems to be a trend in sequencing batch reactors operated in a similar way. When a plug flow feeding regime from the bottom of the reactor is applied, bottom biomass would have more availability of substrate and the chance of having this type of segregation (more PAOs at the bottom

and more GAOs at the top of the sludge bed) is even higher. The probability of having the segregation is especially relevant at high temperatures (e.g., 30°C).

3.4.3. Nitrification/denitrification and phosphate uptake under aerobic/anoxic conditions

After biomass acclimation, full ammonium removal could be achieved even at lower DO at both temperatures evaluated. The fact that ammonium started to be detected in the effluent after 40 days of operation in SBR_{20} during Phase B1, without any change in operating conditions, can be attributed to the decrease in the surface area and consequently the aerobic volume fraction of the granules available for nitrification. On the other hand, the increase in ammonium uptake rate from Phase A2 to Phase B2a in SBR_{30} is possibly related to the increase in the granular surface area.

The anoxic phosphate uptake rate obtained in the anoxic cycle tests for sludge characterization represented around 60 - 70% of that obtained in a normal cycle test performed in the same period. This finding suggests that a considerable amount of phosphate was taken up anoxically by DPAOs under normal operation of the SBRs. The relevance of anoxic dephosphatation for the overall phosphate removal was even larger when nitrite was added at twice as the regular denitrification rate in the anoxic batch tests. The P uptake rate amounted to 90% (SBR_{20}) and 70% (SBR_{30}) of that during a regular aerated cycle kept on 1.8 mgO_2/L. It is important to remark that the actual fraction of anoxic phosphate removal in the aerated cycle will be lower than these amounts.

The fraction of PAOs which are actively performing denitrifying dephosphatation in aerobic granular sludge reactors depends on the distribution of aerobic and anoxic zones within the granules structure and the availability of electron acceptors such as nitrite and nitrate. The low DO applied in both SBRs possibly stimulated the development of DPAOs by enhancing the anoxic zone within the granules. It is interesting to mention that during the start-up period of SBR_{20} (data not shown), when DO corresponded to 100% air saturation (9.1 mgO_2/L at 20°C), other PAOs which were neither PAOI nor PAOII (i.e. with uncertain denitrifying dephosphatation capability), were detected in the microbial community by FISH analysis. At low DO (1.8 mgO_2/L), only PAOI and PAOII composed the PAO population, reinforcing the hypothesis that the reduction of DO concentration and

consequent increase in the anoxic zone within the granules enhanced the development of DPAOs (PAOI and/or PAOII).

Anoxic P uptake rates are generally reported to be lower than aerobic P uptake (Hu et al., 2002; Chung et al., 2006). Indeed, the cycle tests performed under fully aerobic conditions (DO fixed at 100% air saturation in the respective operating temperature) during operation of both SBRs (Phase B1 in SBR_{20} and Phase B2b in SBR_{30}) have shown much higher phosphate uptake rate than those observed under normal operation at low DO or under fully anoxic conditions. At high DO, the oxygen penetration depth within the granules considerably increases, which in turn causes a decrease in the granule anoxic layer and in the denitrification rate. Since all PAOs were PAOI and/or PAOII in both reactors at the time of the experiment and therefore being able to reduce nitrite and/or nitrate, the higher P uptake obtained at high DO compared to that observed at low DO suggests that even PAOs which were normally using only nitrite and/or nitrate as electron acceptor in the anoxic zones of the granules can immediately take up phosphate aerobically when the oxygen penetration depth increases due to an increase in DO concentration.

Both systems were observed to have a higher denitrification capacity than observed under normal conditions when nitrate/nitrite availability is limited by the nitrification rate. This overcapacity provides operational freedom to increase the DO in order to enhance nitrification, without deteriorating denitrification. Evaluating the maximum denitrification capacity of the sludge in separate experiments is a useful tool for process control and provides extra information which can be used to optimize reactor operating conditions and performance.

When the results obtained in anoxic cycle tests are compared with those obtained in the anoxic batch experiments, a considerable difference is observed. The phosphate uptake rate and P/N ratio observed in the anoxic batch tests were much lower than that observed in the anoxic cycle test. This may relate to an inhibitory effect of high nitrite concentrations in the anoxic batch experiments (not present in the anoxic cycles). Some studies reported that nitrite inhibition on the anoxic phosphate uptake started at approximately 3 to 5 mgNO_2^--N/L (Meinhold et al., 1999; Saito et al., 2004). In the nitrite addition experiments, initial nitrite concentration was around 30 mgN/L and in the nitrate addition experiments, nitrite was observed to accumulate during the experiment up to 20 mgN/L. Therefore, the anoxic phosphate uptake rates observed in

the anoxic cycle tests in the reactor reflect better the maximum anoxic P removal capability of the granular biomass. The anoxic tests in which nitrite or nitrate was added as a pulse (anoxic batch experiments) are important to observe if denitrification have run mainly over nitrite or nitrate pathway by comparing the reduction rates of these two electron acceptors. This information cannot be obtained from the anoxic cycle tests in which the electron acceptors were dosed gradually, since in this case the conversion was partly limited by the dosing rate. The nitrate reduction rate in the anoxic batch experiments was much higher than the nitrite reduction rate (Table 3.4). In the cycle tests performed under normal operating conditions, the accumulation of nitrite was much lower than nitrate accumulation. These observations point to a nitrogen removal with mainly nitrate as intermediate between nitrification and denitrification.

3.4.4. PAOI/PAOII and DGAOs: implications on nitrogen and phosphate conversions

From the results obtained in the anoxic batch experiments and in the cycle tests carried out under normal operating conditions, denitrification was assumed to occur mainly through the nitrate pathway. Ideally, PAOI could be selected to simultaneously reduce nitrate and perform anoxic dephosphatation. However, in both systems, PAOII (incapable of using nitrate as electron acceptor) and not PAOI was the dominant PAO subgroup. In SBR_{30}, in particular, only PAOII were present at the time when the anoxic batch tests were carried out. This observation, together with the fact that phosphate uptake rate obtained in the anoxic cycle test performed in SBR_{30} was the same regardless the electron acceptor used (nitrite or nitrate), suggest that other flanking organisms were reducing nitrate to nitrite which could be used by PAOII for anoxic phosphate uptake.

Many ordinary heterotrophs can perform denitrification by using nitrite and/or nitrate as electron acceptors. In this study however, all acetate supplied to the systems was completely consumed during the anaerobic feeding period. Therefore, since no soluble COD was present during the aeration phase when the denitrification takes place in the anoxic zones of the granules, the presence of ordinary heterotrophs was marginal (as observed by FISH analysis) and therefore their contribution in the denitrification process can be neglected. The only microorganisms which could actively develop denitrification were those which accumulated intracellular PHB during the anaerobic feeding (i.e. DPAOs or DGAOs). The absence of PAOI in SBR_{30}, which can use both

nitrite and nitrate as electron acceptor for anoxic P uptake and the complete dominance of PAOII (no nitrate reduction capability) in both reactors, suggested that DGAOs were likely the main candidates responsible for the reduction of nitrate to nitrite.

The anoxic cycle tests in which nitrite was dosed at twice as the regular denitrification rate also suggest that DGAOs were playing a big role in the denitrification in both SBRs. Even though the nitrite dosage was double of that of the previous anoxic cycle tests, P/N ratio remained practically the same in SBR_{20} and even decreased in SBR_{30}. Moreover, the anoxic batch tests with biomass from SBR_{30} (only PAOII present) have shown that the nitrate reduction rate was much higher than the nitrite reduction rate even though anoxic phosphate uptake rates were roughly the same with both electron acceptors. This confirms that a major percentage of denitrification was not coupled with anoxic phosphate uptake and suggests that denitrifying GAOs (DGAOs) were involved in the denitrification pathway. However, no firm conclusion could be drawn at this point since PAOs and GAOs coexisted in both systems. Investigations regarding the denitrifying capability of GAOs are scarcely reported in literature and need more in depth analysis (Zeng et al., 2003).

By washing out PAOs from the system (Phase C1 in SBR_{20} and Phase C2 in SBR_{30}) a complete dominance of GAOs was established in both systems. The observation that full denitrification was still obtained under normal operating conditions suggests that DGAOs occupied a niche previously owned by PAOII (reduction of nitrite to nitrogen gas). Furthermore, the fact that nitrate reduction continued to be higher than nitrite reduction in the anoxic batch tests suggests that DGAOs were actually the main organisms responsible for reducing nitrate to nitrite, which could be used by PAOII (only PAO type present in SBR_{30} and the dominant PAO type in SBR_{20}) for anoxic phosphate uptake in previous experimental phases. This points to a symbiotic relationship between GAO and PAOII where GAO reduce nitrate and in a lower rate nitrite, making the nitrite available for the PAOII population (Figure 3.17). The potential dominance of PAOII in the conversion of nitrite to nitrogen gas was supported by the anoxic cycle tests. We observed a high ratio between the maximum anoxic P uptake obtained in the anoxic cycle tests and the total P uptake obtained under normal operating conditions. However, DGAOs would have also participated to some extent in the conversion of nitrite to nitrogen gas.

Despite of the fact that all experiments carried out in this study suggested that the main pathway of nitrogen removal in the simultaneous nitrification/denitrification system is via nitrate, part of the nitrite could also be directly denitrified to nitrogen gas by PAOII (and at lower extent by DGAOs), without being oxidized to nitrate by NOB. For the optimisation of granular sludge processes it would be advantageous to find a method to stimulate this conversion relative to the nitrate based nitrification-denitrification.

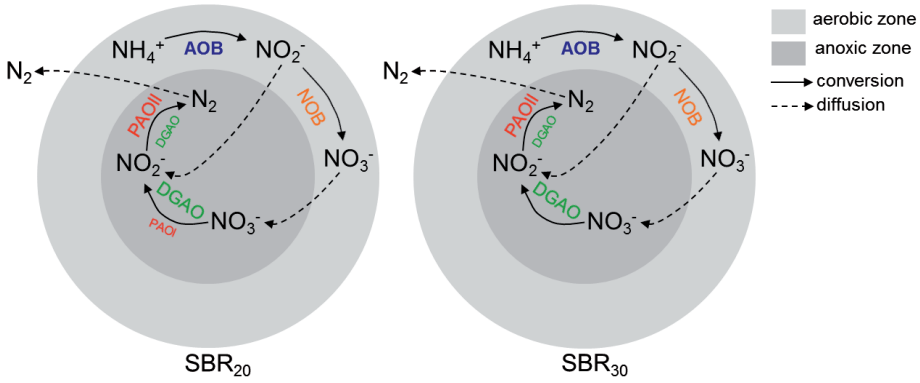


Figure 3.17: Schematic representation of the nitrification/denitrification route in the aerobic granular sludge structure for both SBR_{20} and SBR_{30}. The microorganisms involved in the different conversions are indicated in different colours: AOB (blue), NOB (orange), PAO (red), and GAO (green).

3.5. Conclusions

Full nitrification/denitrification and phosphate removal were characterised in two sequencing batch aerobic granular sludge reactors operated at different temperatures (20 and 30°C) and at low dissolved oxygen (DO) concentrations (less than 2 mgO_2/L). Segregation of biomass offered a possibility to control PAO-GAO competition. The selective removal of biomass mainly from the GAO-rich part of the sludge bed is especially relevant at high temperatures, such as 30°C, unfavourable for the phosphate removal process. The removal of sludge from the PAO-rich part of the sludge bed in minor proportions did not negatively affect phosphate removal and allowed to obtain biomass with a lower ash content. Our results indicate that high ash content and density positively correlated with the presence of PAO-dominated granules

and therefore with high biomass poly-P content and P removal efficiency. The low DO applied favoured the development of denitrifying PAOs, which enhanced phosphate removal coupled to denitrification (denitrifying dephosphatation). Denitrification proceeded mainly over the nitrate route and DGAOs were the dominant organisms reducing nitrate to nitrite, which was reduced to nitrogen gas concomitant with anoxic phosphate uptake by PAOII.

Appendix

$$Ar = \frac{\rho_p - \rho_w}{\rho_w} \cdot d_p^3 \cdot \frac{g}{\nu_w}$$

$$\Omega = \frac{v_s^3 \cdot \rho_w}{\nu_w \cdot g \cdot (\rho_p - \rho_w)}$$

$$v_s = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{\nu_w}$$

v_s = sedimentation velocity of a single particle $\left[\frac{m}{s}\right]$

d_p = particle diameter $[m]$

ρ_p = density of particle $\left[\frac{kg}{m^3}\right]$

ρ_w = density of the fluid $\left[\frac{kg}{m^3}\right]$

g = gravitational constant 9.81 $\left[\frac{m}{s^2}\right]$

ν_w = kinematic viscosity water $\left[\frac{m^2}{s}\right]$

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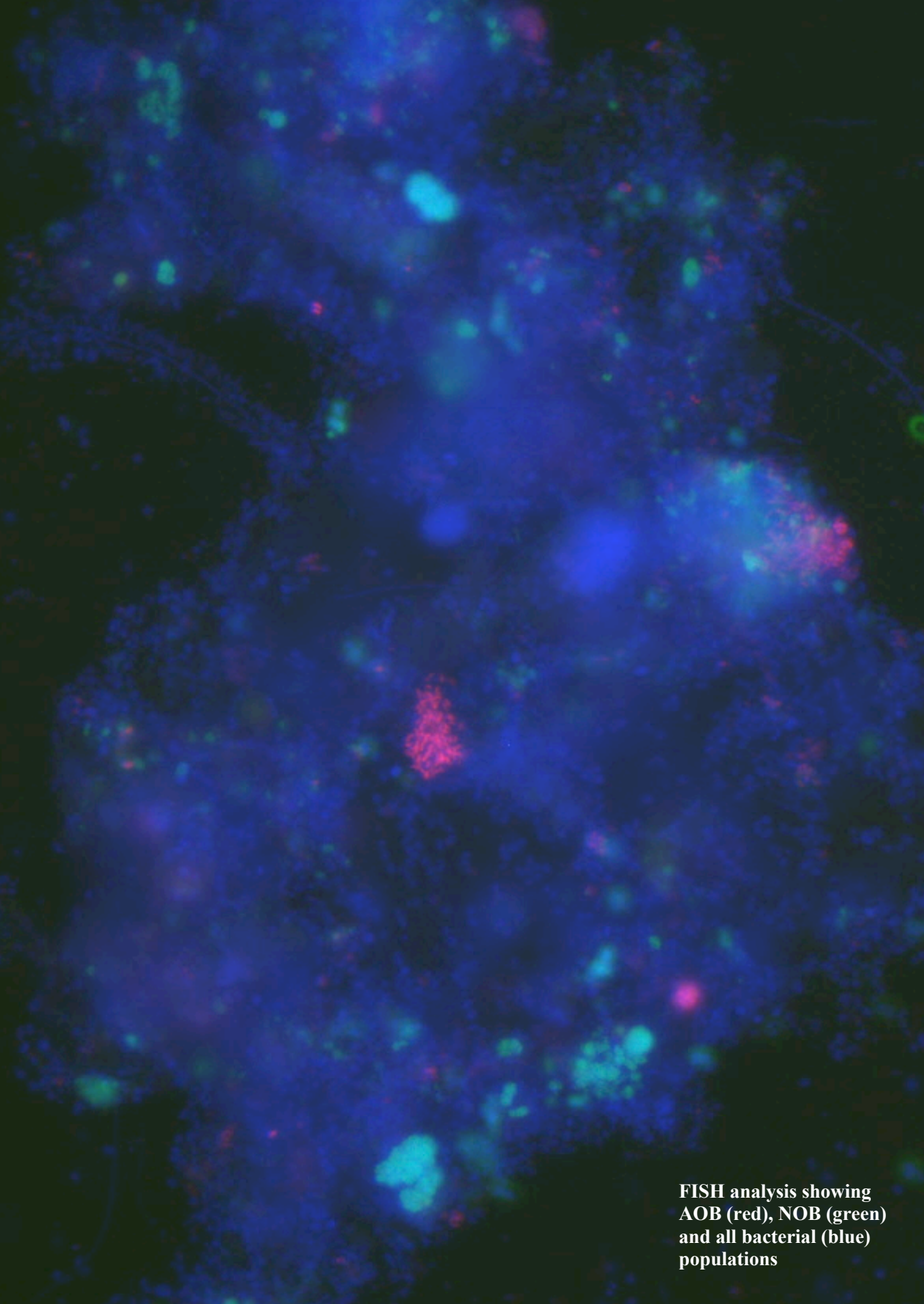

FISH analysis showing AOB (red), NOB (green) and all bacterial (blue) populations

CHAPTER 4

Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge

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* These authors contributed equally to this work

Chapter 4

Content

Abstract

In this study, the nitrifying microbial community (ammonium-oxidizing bacteria - AOB and nitrite-oxidizing bacteria - NOB) was analysed within three different aerobic granular sludge treatment systems as well as within one flocculent sludge system. Granular samples were taken from one pilot plant ran on municipal wastewater as well as from two lab-scale reactors. Fluorescent in situ hybridization (FISH) and quantitative polymerase chain reaction (qPCR) showed that *Nitrobacter* was the dominate NOB in acetate fed aerobic granules. In the conventional system, both *Nitrospira* and *Nitrobacter* were present in similar amounts. Remarkably, the NOB/AOB ratio in aerobic granular sludge was elevated but not in the conventional treatment plant suggesting that the growth of *Nitrobacter* within aerobic granular sludge, in particular, was partly uncoupled from the lithotrophic nitrite supply from AOB. This was supported by activity measurements which showed an approximately 3 fold higher nitrite oxidation capacity than ammonium oxidation capacity. Based on these findings, two hypotheses were considered: either *Nitrobacter* grew mixotrophically by acetate-dependent dissimilatory nitrate reduction ('ping-pong' effect) or a nitrite oxidation/nitrate reduction loop (*nitrite loop*) occurred in which denitrifiers reduced nitrate to nitrite supplying additional nitrite for the NOB apart from the AOB.

4.1. Introduction

Nitrification is accomplished in a two-step process: oxidation of ammonium to nitrite by ammonium-oxidizing bacteria (AOB) and/or archaea (AOA) and oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). Both AOB and NOB are known to grow lithoautotrophically without the need for any organic compound. AOB belong to four genera in the Betaproteobacteria and Gammaproteobacteria and use oxygen as electron acceptor to oxidize ammonium in two steps by using AMO and HAO enzymes (Chain et al., 2003). NOB belong to five genera within different classes of the Proteobacteria and depend on AOB that generate their substrate (nitrite). NOB generate only two electrons by the oxidation of nitrite to nitrate, which is 3 times lower than the amount of electrons generated by AOB during oxidation of ammonium to nitrite. Due to ammonium activation by the AMO enzyme in the AOB metabolism, two electrons are not available for energy generation and therefore the biomass yield of NOB is expected to be around 2 times lower than that of AOB per unit of nitrogen. This implies a theoretical NOB/AOB ratio of 0.5 (Aleem, 1966; Ferguson, 1982; Hagopian and Riley, 1998; Hooper et al., 1997). The total numbers in NOB compared to AOB is expected to be even further lowered in systems where simultaneous nitrification/denitrification (SND) is taking place. Both nitrite and nitrate can be used as electron acceptor by denitrifying bacteria to generate nitrogen gas. If denitrification takes place mainly over nitrite, NOB would have to compete for nitrite with denitrifying organisms. In this case, it is likely that the NOB/AOB ratio will be even lower than 0.5. This trend will be true unless when the metabolism of NOB is changed in such a way that their biomass yield increases. This is possible if the growth of NOB does not only rely on the nitrite provided autotrophically by AOB, but also on other substrates (e.g., organic compounds), which suggests a mixotrophic metabolism of NOB. *Nitrobacter* is known to be capable of utilizing organic compounds and can grow by dissimilatory nitrate reduction (Freitag, et al. 1987; Smith and Hoare, 1968; Spieck and Bock, 2005; Steinmüller and Bock, 1976; Watson et al. 1989). Freitag et al. (1987) showed that a *Nitrobacter* biofilm culture performed oxidation of nitrite to nitrate on the surface of a silicon tubing from which oxygen diffused into the biofilm. The nitrate produced diffused away to the anoxic fraction of the biofilm where it was used by *Nitrobacter* as electron acceptor in the presence of an organic electron donor and converted back to nitrite. Due to this 'ping-pong' effect, the biomass yield increased 3 times compared to

fully autotrophic cultures kept under aerobic conditions. Such an effect may also play an important role in biofilm systems in which multiple processes occur at the same time within different layers of the biofilm.

The microbial diversity of nitrifying bacteria has been extensively described for conventional activated sludge-based wastewater treatment systems. In these processes, the most common AOB found belong to the genus *Nitrosomonas* (Daims et al. 2009), while *Nitrospira* has been reported to be the most important NOB (Daims et al. 2001; Gieseke et al. 2003). Although the information about nitrifying bacteria in conventional systems is abundant, the information on the community of nitrifiers in aerobic granular sludge is sparse. To investigate the nitrifying community composition in granular biofilm, we decided to test the proportions between *Nitrospira*, *Nitrobacter* and *Nitrosomonas* by qPCR and FISH as well as to measure the microbial conversion rates (such as nitrite and ammonium oxidation rates) within different biofilm and flocculent sludge systems.

4.2. Materials and Methods

4.2.1. Reactor set-up and operating conditions

A lab-scale aerobic granular sludge sequencing batch reactor (SBR) of 2.6 L was operated at 30°C. Temperature was held constant by means of a thermostat and the reactor was protected against cooling with a thermal isolation placed around the reactor. The operational cycle lasted 3 h and was divided into the following phases: 60 min anaerobic feeding from bottom of the reactor in a plug flow regime; 112 min aeration period; 3 min settling; and 5 min effluent withdrawal. Aeration and mixing were achieved through an air diffuser placed in the bottom of the reactors (airflow rate of 4 L/min). pH was kept at 7.0±0.2 pH units by dosing 1 M NaOH or 1 M HCl. Dissolved oxygen (DO) concentration was controlled at around 2.0 mgO_2/L by using two mass flow controllers (one for air and other for nitrogen gas). The volume exchange ratio and the hydraulic retention time (HRT) were 57% and 5.2 h, respectively. Sludge retention time (SRT) was maintained around 30 days by periodically removing sludge from the reactor. The SRT calculation can be found elsewhere (Winkler et al., 2011a). The activation of influent/effluent and pH pumps was controlled by a bio controller (Braun DCU4 coupled with MFCS control and data acquisition software). The synthetic feeding medium consisted of two solutions: (1) $NaCH_3COO \cdot 3H_2O$ 63 mM,

$MgSO_4.7H_2O$ 3.6 mM, KCl 4.7 mM and (2) NH_4Cl 35.4 mM, K_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM and 10 mL/L trace element solution (Vishniac and Santer 1957). Per cycle, 150 mL was dosed from both solutions together with 1200 mL of tap water in order to achieve influent concentrations of 400 mg/L of chemical oxygen demand (COD), 60 mgNH_4^+-N/L and 20 mgP-PO_4^{3-}/L. Cycle tests were conducted under normal operational conditions and samples were taken every 10 to 20 min during the aeration-mixing period to obtain the phosphate, ammonium, nitrite and nitrate profiles over an operational cycle.

4.2.2. Anammox/nitration reactor

An autotrophic partial nitritation/Anammox granular reactor, based on complete autotrophic nitrogen removal over nitrite (CANON), was operated as described in previous research (Winkler et al. 2011b). The cycle consisted of 60 min anaerobic feeding period followed by a 112 min of aeration, 3 min settling period and 5 min effluent withdrawal. Mixing was performed with nitrogen gas during the first 60 minutes to keep anaerobic conditions. pH was kept at 7. The DO concentration was controlled at 1.0 mgO_2/L in order to suppress the growth of NOB (Hao et al., 2005).

4.2.3. Pilot-scale aerobic granular sludge and activated sludge systems

The pilot-scale aerobic granular sludge reactor and the activated sludge reactor, treating the same wastewater, were operated in parallel in a domestic wastewater treatment plant (Epe, The Netherlands). The pilot-scale granular sludge reactor (1.5 m^3) was operated in a similar way as the lab-scale reactor. pH values were kept at around 7. The influent COD and influent ammonium corresponded to approximately 600 mg/L and 50 – 100 mgN/L, respectively.

4.2.4. Batch experiments under fully aerobic conditions

In order to compare the maximum ammonium and nitrite oxidation rates, batch tests were performed under fully aerated condition (DO kept at 100% air saturation) with granular sludge from the lab-scale reactor. Representative biomass was taken at the end of the operational cycle (t=180 min) and was pre-aerated for two hours to assure that all ammonium was completely oxidized. Subsequently, the granules were washed with tap water whose temperature was adjusted to 30°C (operating temperature of the reactor), sieved and gently crushed to prevent the occurrence of denitrification in the

anoxic zones of the granules, which would interfere in the nitrite oxidation rate measurements. The crushed granules were distributed equally (based on the wet weight) to 250-mL flasks. Air was supplied to the batch flasks, which were filled with 50 mM Tris HCl buffer (pH 7.0) and placed in a water bath kept at 30°C. A pulse of either ammonium or nitrite solution was added in the beginning of the experiment in order to achieve initial concentrations of 50 mgN/L and 20 mgN/L, respectively. Samples were collected every 5 to 20 min and measured for ammonium, nitrite and nitrate. Biomass-specific rates were obtained by linear regression of either ammonium or nitrite concentrations over time divided by the constant concentration of volatile suspended solids (VSS) in the batch flask. The experiment was repeated several times over a period of 2 months.

4.2.5. Batch experiments under fully anoxic conditions

Batch experiments were performed on biomass from the lab-scale reactor under anoxic conditions. Granules were collected from the aerobic granular sludge reactor after anaerobic feeding phase, i.e. after accumulation of Polyhydroxybutyrate (PHB) by polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs). Denitrifying PAOs and GAOs were actually the main active denitrifiers in the reactor, since no external organic carbon source was available during aeration period to be used as electron donor by other heterotrophic bacteria to accomplish denitrification in the anoxic zones of the granules. Granules were sieved and washed with tap water at 30°C. Equal amounts of granules (based on the wet weight) were introduced in different 250-mL flasks filled with a Tris-HCl buffer (pH 7.0) containing the same compounds of the synthetic medium fed to the reactor (except acetate and phosphate). Nitrogen gas was supplied to each flask through porous diffusers in order to keep anaerobic conditions. A pulse of nitrate was given to obtain a final concentration of 30 mgN/L. From this point, samples were taken regularly every 10 to 20 min for determination of nitrate concentrations. Specific nitrate reduction rates could then be obtained by linear regression of nitrate concentration over time divided by the constant amount of VSS in the batch flask.

4.2.6. Anoxic cycle tests

Anoxic cycle tests were also performed in the lab-scale reactor under fully anoxic conditions. In these tests, no air was supplied to the reactor and anoxic conditions were achieved by supplying only nitrogen gas through the porous diffuser. Nitrate was gradually added according to the denitrification rate, which was estimated based on the difference between the ammonium uptake rate and the nitrite/nitrate production rate obtained in the cycle tests performed under normal operation conditions (DO of approximately 2.0 mgO_2/L). Specific nitrate reduction rates were obtained by linear regression of the nitrate uptake in time divided by the amount of VSS in the reactor.

4.2.7. Analytical measurements

Ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) were measured by means of a flow injection analyzer (QuikChem 8500, Lachat Instruments, Inc.). Phosphate (PO_4^{3-}-P) was quantified by Hach Lange cuvette tests (LCK 350). Biomass concentration, in terms of volatile suspended solids (VSS), was determined as described in previous research (Winkler et al. 2011a).

4.2.8. Sample collection for qPCR

Samples were taken over a period of 1 year from four different reactors: two lab-scale aerobic granular sludge reactors (one operated as a conventional granular system and other as an autotrophic partial-nitritation/Anammox (CANON) reactor (Winkler et al. 2011b), a pilot-scale aerobic granular sludge sequencing-batch reactor and an activated sludge reactor. All samples were checked with qPCR for their AOB/NOB ratios.

4.2.9. Quantitative PCR (qPCR)

Primers and PCR conditions are listed in Table 4.1. Primers were checked in the ARB database as well as in the Ribosomal Database Project (RDP). All samples were measured in triplicates. DNA extraction was conducted with the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, USA). First, a regular PCR was performed followed by a purification step with the QIAquick PCR Purification Kit (250) (Germany, QIAGEN). The PCR product was used for a qPCR procedure with a variable primer concentration and 25µM iQ SYBR® Green Supermix (BIO-RAD Laboratories, USA)

(Table 4.1). iCycler iQ™ Multi-Color Real-Time PCR Detection System (BIO-RAD Laboratories, USA) was used to run the qPCR assays. All primers were optimized with a gradient qPCR. The resulting conditions, primer concentrations as well as the DNA used as a standard for the qPCR are listed in Table 4.1. A Picogreen protocol was used to determine the amount of DNA template in order to normalize all C_T values to 5 ng DNA by using a Pico Green dsDNA Quantitation Kit (Molecular Probes Inc., USA). ΔC_T was calculated by the following equation $\Delta C_T = C_{T(ref)} - C_{T(target)}$ (Zhang and Bao, 2009). For determination of the ratios of a specific organism (e.g., target = AOB to the total community (ref)), the following equation was applied: $ratio_{target/ref} = 2^{\Delta C_T}$. For construction of a ratio between NOB and AOB the calculated ratio of AOB was divided by the calculated ratio of NOB.

Table 4.1: Primers, qPCR conditions, primer concentrations and references.

Primer	Primer con. [µM]	qPCR conditions	Standard	Reference
CTO189F A/B & CTO189F C CTO654R	0.2	94°/2' [94°/0.30' 61°/1' 72°/0.45'] x30 52°/10' 10°/∞	*Nitrosomonas europaea*	(Kowalchuk et al., 1997)
NTSPA1026F NTSPA1026R	0.2	94°/1' [94°/0.5' 45°/0.2' 72°/0.3'80°/0.25'] x40 72°/10' 10°/∞	*Nitrospira defluvii*	(Juretschko et al., 1998)
NTS232F/ NTS 1200R	0.2	95/5m, (95/40s, 55-48/30s, 72/60s)*35, 72/10min, 12/∞	*Nitrospira defluvii*	(Degrange et al., 1997; Lim et al., 2008)
FGPS872/ FGPS 1269	0.2	95/3m, (95/60s, 50/60s, 72/60s)*35, 72/3min, 12/∞	*Nitrobacter*	
AMX1066R AMX818F	0.3	95°/10' [94°/0.15' 61°/0.1' 68°/0.25' 80°/0.25']x40 52°/10' 10°/∞	Slude from WWTP[1]	(Tsushima et al., 2007)
Bac341F	0.355	95°/6' [95°/0.30' 55°/0.4' 52°/0.4'80°/0.25'] x40	Sludge from WWTP[2]	(Muyzer et al., 1993)
Bac905r		52°/5'10°/∞	Sludge from WWTP[2]	(Weisburg et al., 1991)

Primers used and their concentrations as well as the standard used. qPCR conditions show temperature and cycle length for denaturing, annealing, elongation and cooling steps.
[1] Anammox enriched sludge from Anammox reactor in Rotterdam, The Netherlands
[2] Sludge from the aerobic granular sludge pilot plant in Epe, The Netherlands

4.2.10. Fluorescent in situ hybridization (FISH)

Granules from the lab- and pilot-scale aerobic granular sludge reactors were fixed in 4% paraformaldehyde. Granules where embedded in a tissue freezing medium (Tissue Freezing medium, Leica Microsystems), hardened by freezing (-20°C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 25 µm thin slices. Dried slices were kept on a microscopic glass slide and FISH was performed to

observe the distribution of PAOs, GAOs, AOB and NOB (Fluos) populations within the granular sludge in the same manner as reported by Winkler et al. (2011b). Sequences are listed in Table 4.2. Samples were analysed with an epifluorescence microscope (Axioplan 2, Zeiss) equipped with filters suited for Cy3, Cy5 as well as Fluos.

Table 4.2: Oligonucleotide probes, target microorganisms and references used in this study.

Probes	Sequence (from ‘5 to ‘3)		Specificity	Reference
Ntspa662	GGAATTCCGCGCTCCTCT	NOB mix	*Nitrospira-like organisms*	(Daims et al., 2001)
NIT1035	CCTGTGCTCCATGCTCCG		*Nitrobacter*	(Wagner et al., 1996)
NSO190	CGATCCCCTGCTTTTCTCC	AOB mix	All AOB	(Mobarry et al., 1996)
NSO1225	CGCCATTGTATTACGTGTGA		All AOB	(Mobarry et al., 1996)
PAO 462	CCGTCATCTACWCAGGGTATTAAC	PAO mix	Most *Accumulibacter*	(Crocetti et al., 2000)
PAO 651	CCC TCTGCCAAACTCCAG		Most *Accumulibacter*	(Crocetti et al., 2000)
PAO 846	GTTAGCTACGGACTAAAAGG		Most *Accumulibacter*	(Crocetti et al., 2000)

Probes from NOBs were tagged with the fluorescent dye Fluos (green) Anammox with Cy3 (red) and AOBs with Cy5 (blue). For analysis probes of one target group were mixed

4.2.11. Biomass yields

The estimated autotrophic community composition was based on the biomass yields on ammonium (AOB) or nitrite (NOB) ($Y_{X/N}$). For mixotrophic growth of NOB on acetate, a biomass yield from heterotrophs was assumed ($Y_{X/HAC}$) (Table 4.3). The theoretical amount of COD needed to increase the NOB/AOB ratio (ratio including autotrophic NOB) to 3 was calculated to be 37 mg/L. For the conversion from COD to VSS, a factor of 1.4 was used (Scherer et al., 1983).

Table 4.3: Biomass yields for AOB, NOB and an assumed heterotrophic biomass yield for mixotrophic NOB and the corresponding biomass concentration and relative community composition according to consumed substrate (37mg/L of COD; 60mgNH_4^+-N/L). Calculations were based assuming a) autotrophic growth of nitrifiers and b) mixotrophic growth of NOB on COD of 37 mg/L. $Y_{x/Hac}[Cmol/Cmol]$; $Y_{x/N}[Cmol/Nmol]$

	$Y_{x/Hac}$	$Y_{x/N}$	mgVSS/L	Community composition (%)		Reference for yields
AOB	-	0.13	8	67[a]	25[b]	(Blackburne et al., 2007b)
NOB	-	0.07	4	33[a]	12[b]	(Blackburne et al., 2007a)
Mixotropic NOB	0.4	-	21	-	63[b]	(Beun et al., 2001)
NOB/AOB ratio				**0.5**	**3**	

4.3. Results

4.3.1. Reactor cycle measurement

Figure 4.1 shows a typical cycle test performed during the operation of the lab-scale reactor, when a steady-state condition was reached. The first sample was collected 2 min after the aeration phase has started (t=62min) to allow sufficient mixing. During the anaerobic feeding period, all acetate is converted to intracellular polymers (e.g., Polyhydroxybutyrate (PHB)) by PAOs or GAOs leaving no dissolved organic carbon for the subsequent aerobic period (data not shown). During anaerobic feeding, phosphate is released by PAOs and is subsequently taken up by the same microorganisms during the aerobic period. After the feeding period, PAOs and GAOs degrade internally stored PHB and use the generated energy for growth ((Smolders et al., 1994). Ammonium concentration after anaerobic feeding was observed to be lower than expected based on the influent concentration and the dilution in the reactor. This fact is due to ammonium adsorption, phenomenon that was investigated in a separate study (Bassin et al., 2011). Ammonium removal was practically constant and amounted to around 100% during the whole experimental period. Nitrite concentrations were very low along the operational cycle (lower than 0.3 mgN/L), although an accumulation of nitrate was observed in the end of the cycle. The nitrate remaining after effluent discharge was present in the subsequent cycle although diluted with the following feed.

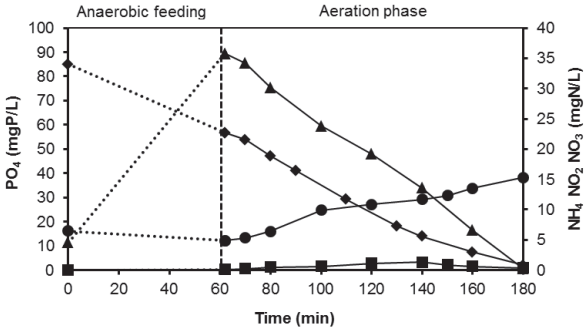
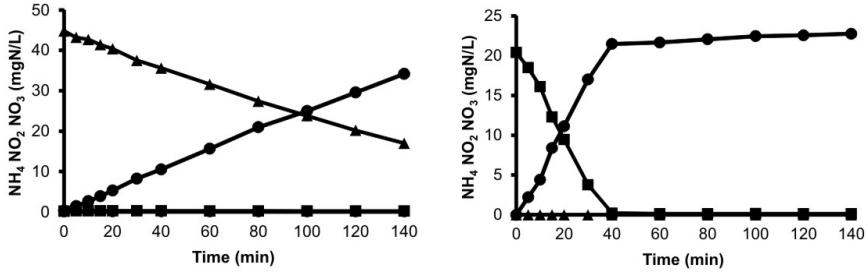


Figure 4.1: Typical cycle test showing the profiles of (♦) ammonium, (●) nitrate and (■) nitrite (right y-axis) and (▲) phosphate (left y-axis). The starting ammonium and phosphate concentrations depicted at time 0 were calculated based on the influent concentration (60 mgNH_4^+-N/L and 20 mgPO_4^{3-}-P/L) and the dilution in the reactor. Nitrite and nitrate concentrations at time 0 were calculated based on their concentrations in the end of the cycle and the dilution in the reactor. The dashed line displayed from the beginning until the end of the feeding period does not represent data points. They were inserted to better visualize the amount of phosphate release and ammonium adsorption observed during the anaerobic phase.

4.3.2. Batch experiments under fully aerobic conditions

Batch experiments with crushed granules (to eliminate oxygen diffusion limitation) under fully aerobic conditions (DO kept at 100% air saturation) were performed to compare the maximum ammonium and nitrite oxidation activities. The ammonium, nitrite and nitrate profiles of the aerobic tests with ammonium and nitrite dosage are illustrated in Figure 4.2a and Figure 4.2b, respectively. Results from this set of experiments using the same biomass revealed an approximately 3 fold higher nitrite uptake rate (6.0 mgNO_2^--N/(gVSS·h)) than the ammonium uptake rate (2.3 mgNH_4^+-N/(gVSS·h)). Since denitrification was prevented by using crushed granules (therefore eliminating the anoxic zones within the granules), all nitrite consumption was due to nitrite-oxidizing activity. Repeated experiments showed a good reproducibility. The results shown in Figure 4.2 are in line with FISH and q-PCR observations, which showed a higher NOB/AOB ratio in aerobic granular biomass compared to the calculated theoretical ratio of 0.5, assuming autotrophic nitrifying growth (Table 4.3).



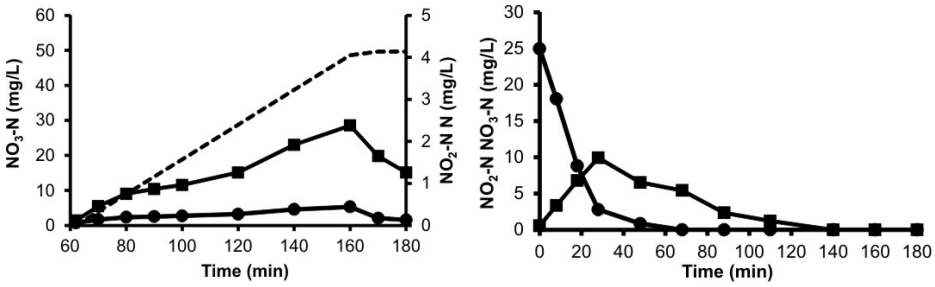
(a) Ammonium oxidation (b) Nitrite oxidation

Figure 4.2: Aerobic batch tests dosing either ammonium (a) or nitrite (b). Legend description: ammonium (▲), nitrite (■) and nitrate (●).

4.3.3. Batch experiments and cycle tests under fully anoxic conditions

During normal reactor operation, nitrification and denitrification processes occur simultaneously. Therefore, it is difficult to distinguish the amount of nitrite oxidized by NOB and the amount of nitrite reduced by denitrifiers. In order to have a better insight into the denitrification potential in the granular sludge reactor, two types of experiments were carried out under anoxic conditions: anoxic cycle test in the aerobic granular sludge reactor with a continuous supply of nitrate instead of oxygen during the aeration (mixing) phase (Figure 4.3a) and a batch experiment in which nitrate was added as a

pulse in the beginning of the test (Figure 4.3b). The anoxic cycle test has shown that almost no nitrite accumulated over the whole experiment, result similar to that observed under normal reactor operating conditions (see cycle test in Figure 4.1). In the batch experiment in which nitrate was supplied as a pulse (high concentration in the beginning of the test), nitrate reduction was accompanied by nitrite accumulation. After nitrate depletion, the accumulated nitrite was reduced.



(a) Continuous feeding (b) Fed batch

Figure 4.3: Anoxic experiments dosing nitrate in a continuous feeding regime (a) and pulse feeding regime (b). Legend description: nitrite (■), nitrate (●). The dashed line shows the calculated amount of nitrate added.

4.3.4. Relative quantification of AOB and NOB by qPCR

To study the nitrifying population within the aerobic granular sludge, a comparative study between the amount of AOB and NOB was performed by means of qPCR. Ratios between NOB and AOB were constructed for samples collected from an autotrophic CANON/Anammox reactor, a conventional treatment plant, an aerobic granular sludge pilot plant and a lab-scale aerobic granular sludge reactor (Figure 4.4). Both assays from aerobic granular sludge samples showed high NOB/AOB ratios (3 – 4), a moderate ratio in the conventional treatment plant (0.2±0.1) and low ratios in the CANON reactor (0.004±0.002). *Nitrobacter* was the main NOB present in aerobic granular sludge. In the conventional treatment plant, both *Nitrospira* and *Nitrobacter* coexisted in roughly equal amounts.

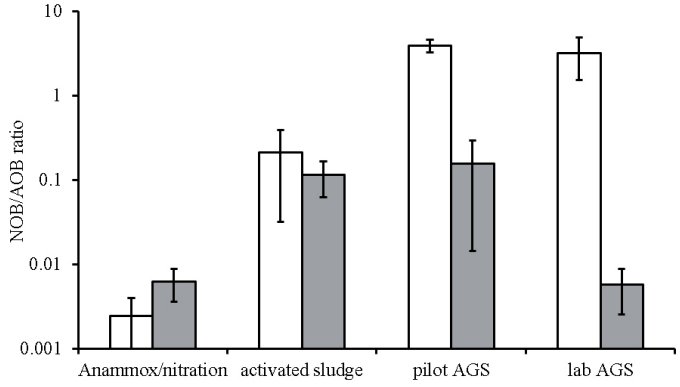


Figure 4.4: Average of NOB/AOB ratios build on the basis 16S rRNA gene analysis of *Nitrobacter*/AOB ☐ and *Nitrospira*/AOB ▬ of (left to right) autotrophic partial nitritation/Anammox reactor, activated sludge, aerobic granular sludge pilot plant, and aerobic granular sludge lab reactor. Error bars indicate maximum and minimum observed ratios.

4.3.5. Nitrifying microbial population assessed by FISH

In wastewater treatment systems, nitrifiers are expected to account for 1 – 3% of the total community composition due to their low growth yield. Therefore, FISH was also conducted with PAOs and GAOs to have a reference for the heterotrophic community (Figure 4.5b). The FISH pictures showed the same trend as the qPCR results, i.e., an elevated number of NOB. Moreover, it also showed that NOB grew deeper in the biofilm when compared to AOB (Figure 4.5a).

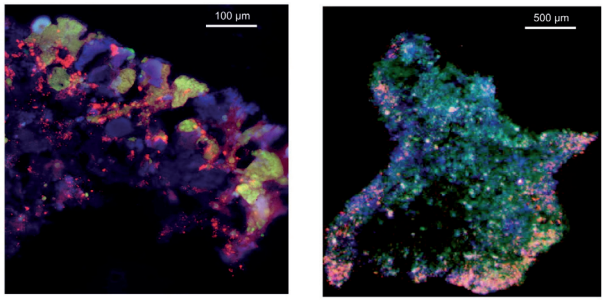


Figure 4.5: Microscopic FISH image on sliced granule showing AOB (green), NOB (red) and PAO (blue) (a) as well as nitrifiers (mix of AOB and NOB) (red), PAO (blue) and GAO (green) (b).

4.3.6. Biomass yields

In order to calculate how much COD is needed to increase the NOB/AOB ratio to 3, we assumed that NOB grew mixotrophically on organic acids with nitrate as

electron acceptor and we considered a biomass yield similar to general heterotrophs. Calculations showed that a theoretical COD of 37 mg/L is needed to elevate the expected autotrophic NOB/AOB ratio of 0.5 by a factor 6 (Table 4.3). This amount is less than 10% of the total COD (as acetate) fed into the system (400 mg/L).

4.4. Discussion

4.4.1. Introduction

Our research demonstrated an elevated NOB/AOB ratio (higher than the expected ratio of 0.5 based on autotrophic ammonium and nitrite oxidation) in heterotrophic aerobic granular sludge from a pilot plant and a lab-scale reactor using FISH, qPCR analysis and activity batch tests. In contrast, qPCR measurements on samples from the conventional treatment plant operated in parallel with the pilot-scale aerobic granular sludge reactor did not show elevated NOB levels. Moreover, the negative control samples collected from a CANON reactor revealed expectedly low NOB/AOB ratios (i.e. ca. 100-fold lower than AOB). A qPCR on ammonium-oxidizing Archaea was conducted but no signal could be detected (data not shown). *Nitrobacter* and not *Nitrospira* was the dominant NOB in aerobic granular sludge pilot plant and lab scale reactor. This is interesting since *Nitrospira* has been identified as dominant NOB in activated sludge systems (Daims et al., 2001; Gieseke et al., 2003). Based on these experimental results, we propose two possible mechanisms to explain our unexpected observations regarding the NOB/AOB ratio. The first assumption refers to the 'pingpong' effect (Figure 4.6a). In this process, excess nitrate is reduced by mixotrophic *Nitrobacter* using acetate as electron donor and carbon source and therefore the growth of *Nitrobacter* is uncoupled from the direct nitrite supply of the AOB. The second theory is referred to as the 'nitrite loop', in which a nitrite oxidation/nitrate reduction loop takes place in the granules (Figure 4.6b). If denitrification would be incomplete (i.e. from nitrate to nitrite only), accumulated nitrite could be reused by NOB, which would partly uncouple their growth from AOB. Both theories can be relevant especially in biofilm systems, in which simultaneous oxidation of nitrite to nitrate (by NOB) and reduction of nitrate to nitrite (by denitrifiers or mixotrophic NOB) can occur.

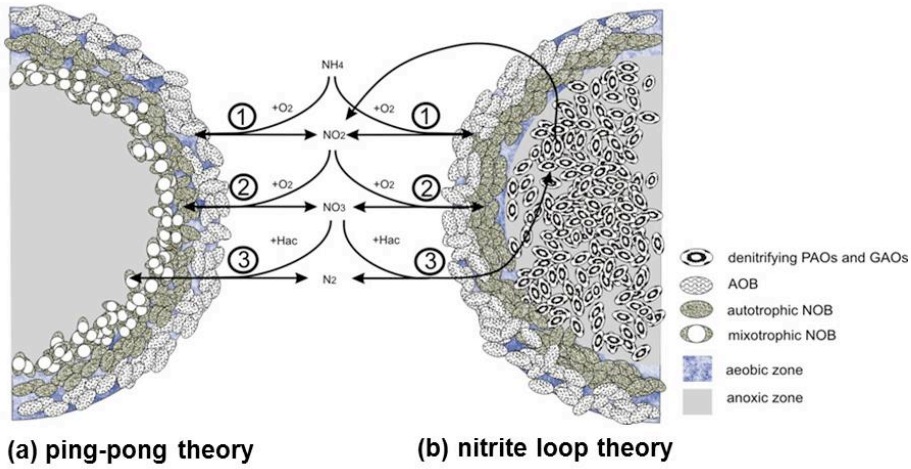


Figure 4.6: Schematic view of the ping pong theory (a) and nitrite loop theory (b). Both theories have step 1) oxidation of ammonium to nitrite via ammonium-oxidizing bacteria (AOB) and step 2) oxidation of nitrite by autotrophic nitrite-oxidizing bacteria (NOB) in common. In the case of the 'ping-pong' effect a) the third step assumes that organotrophic NOB take up acetate as PHB and hence outcompete general heterotrophs for organic acids. *Nitrobacter* can then grow on acetate and hence increase their cell yield. In the case of the nitrite loop (b) it is assumed that denitrifying PAOs and GAOs incompletely reduce nitrate with acetate only until nitrite by which it can be reoxidized by NOB, which in turn can grow uncoupled from the nitrite supply of AOB.

4.4.2. 'Ping-pong' theory

Nitrospira is suggested to outcompete *Nitrobacter* at low nitrite concentrations due to the lower nitrite half-saturation constant of the former microorganisms (Blackburne et al., 2007a). Very low concentrations of nitrite were detected during normal operation of the lab- and pilot-scale aerobic granular sludge reactors indicating that conditions are more likely to be in favour of *Nitrospira*. However, the aerobic granular sludge system was dominated by *Nitrobacter* (Figure 4.4). Numerous researches have shown experimentally as well as by mathematical modelling that oxygen penetration is restricted to the outer rim (<100 µm) of the granule (Nielsen et al., 2005; Picioreanu et al., 2004). Conversely, according to our FISH pictures, *Nitrobacter* grew up to 300 µm deep. AOB were only present in the outer layer until approximately 100 µm. This suggests that NOB might have used another electron acceptor than oxygen for growth. In our experimental setup, all acetate supplied was stored as PHB by PAOs and GAOs during anaerobic feeding. However, during the feeding period with acetate, remaining nitrate from the previous cycle is available in the granules which may allow for heterotrophic denitrification by the NOB. It can also not be excluded that *Nitrobacter* present in the anoxic regions grows heterotrophically on

soluble microbial products or decay products from the active biomass. Based on our calculations assuming that mixotrophic NOB would grow on organic acids with a biomass yield typical for heterotrophs, we found that a COD of only 37 mg/L is needed to increase the NOB/AOB ratio to the observed one of approximately 3 (Table 4.3). In a previous research in which a single *Nitrobacter* culture was studied, aerobic oxidation of nitrite to nitrate was shown to occur on the surface of a silicon loop while produced nitrate diffused away to the anoxic parts of the biofilm (Freitag et al., 1987). In the anoxic environment, nitrate was used as electron acceptor with a provided organic electron donor and was converted back to nitrite. During this 'ping-pong' effect, *Nitrobacter* carry out dissimilatory nitrate reduction and was reported to store PHB (Freitag et al., 1987).

The accumulation of reserve polymers (e.g., PHB and poly-P) have been experimentally proven in pure *Nitrobacter* culture experiments (Bock, 1976; Freitag et al., 1987; Pope et al., 1969; Van Gool et al., 1971; Watson et al., 1989). Additionally, *Nitrobacter* has the genes *NarJ* and *NarI* confirming their capability of dissimilatory nitrate reduction (Starkenburg et al., 2006; Starkenburg et al., 2008). From an ecological perspective, it is advantageous for bacteria to rapidly store organic carbon as energy rich PHA and use it for growth when no external substrate is available (i.e. under starvation conditions). This particular metabolism is often observed in sequencing batch processes, in which microorganisms are subjected to alternating phases of high and low substrate availability, designated as feast and famine regimes, respectively (Johnson et al., 2010; Jiang et al., 2011). The activated sludge process is mainly based on continuously fed systems, whereas aerobic granular sludge is primarily operated in SBRs, which could indeed stimulate the organisms (e.g., *Nitrobacter*) to store intracellular polymers to balance their growth (van Loosdrecht et al., 1997). In the aerobic granular sludge systems used in this research, the fast uptake of acetate could give *Nitrobacter* an extra competitive advantage. *Nitrosomonas europea* (AOB) has also all enzymes of the tricarboxylic acid (TCA) cycle but does not synthesize PHB as storage products and there is also no experimental evidence that *Nitrosomonas* can generate PHB from organic acids (Abeliovich and Vonshak 1992; Chain et al. 2003). *Nitrobacter* could hence increase its proportion in a microbial community relative to *Nitrosomonas* and even general heterotrophs by using organic acids and storing reserve polymers. We suspect that if *Nitrobacter* used its mixotrophic capability, it is more probably due to its

ability to grow by heterotrophic nitrate reduction in anoxic environment rather than by an aerobic metabolism.

4.4.3. Nitrite loop theory

To support the "nitrite loop" theory, nitrite needs to become available in the biofilm or even in the bulk, which in turn can be reused by NOB. Such accumulation was not measurable during normal reactor operation (Figure 4.1). However, in a batch test in which we dosed twice the amount of nitrate denitrified under normal operation (Figure 4.3a), an accumulation of nitrite was observed. In the denitrification pathway, seven enzymes catalyse the facultative respiratory pathway, in which nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), are reduced to nitrogen gas (N_2) (Philippot, 2002; Zumft, 1997). Earlier studies have shown that the reduction of soluble NO_2^- to gaseous NO by nitrite reductases (*NirK* or *NirS*) can be the rate limiting step in denitrification in certain types of bacteria (Firestone et al., 1979). The measured nitrite accumulation designates that additional nitrite might become available within the biofilm during reactor operation hence enabling the nitrite loop. In a biofilm system, all oxidation and reduction processes occur within multi-structural layers harbouring all microorganisms. Since nitrification and denitrification processes occur simultaneously, it is troublesome to measure them separately. In conventional wastewaters treatment systems, the nitrite loop is unlikely to occur, since nitrification and denitrification processes are usually carried out in different compartments. In pre-denitrification systems, the anoxic compartment is rich in organic carbon whereas the aerobic compartment has only little organic carbon. This is supported by the qPCR results showing lower NOB/AOB ratios for conventional treatment plant as opposed to higher NOB/AOB ratios observed in both pilot- and lab-scale aerobic granular sludge reactors (Figure 4.4).

4.5. Conclusions

In this study, several methodologies (FISH, qPCR and activity tests) were used to investigate and understand the unexpected elevated NOB/AOB ratio in aerobic granular sludge systems. We observed that the NOB population in the granular systems was dominated by *Nitrobacter*. Two hypotheses were proposed: either *Nitrobacter* have grown mixotrophically by acetate-dependent dissimilatory nitrate reduction ('ping-

pong' effect) or a nitrite oxidation/nitrate reduction loop (*nitrite loop*) have happened in which denitrifying bacteria reduced nitrate to nitrite providing additional nitrite for the NOB apart from the AOB. The disproportion of the amount of AOB and NOB in granular sludge should be investigated further to confirm the hypothesis made in this work.

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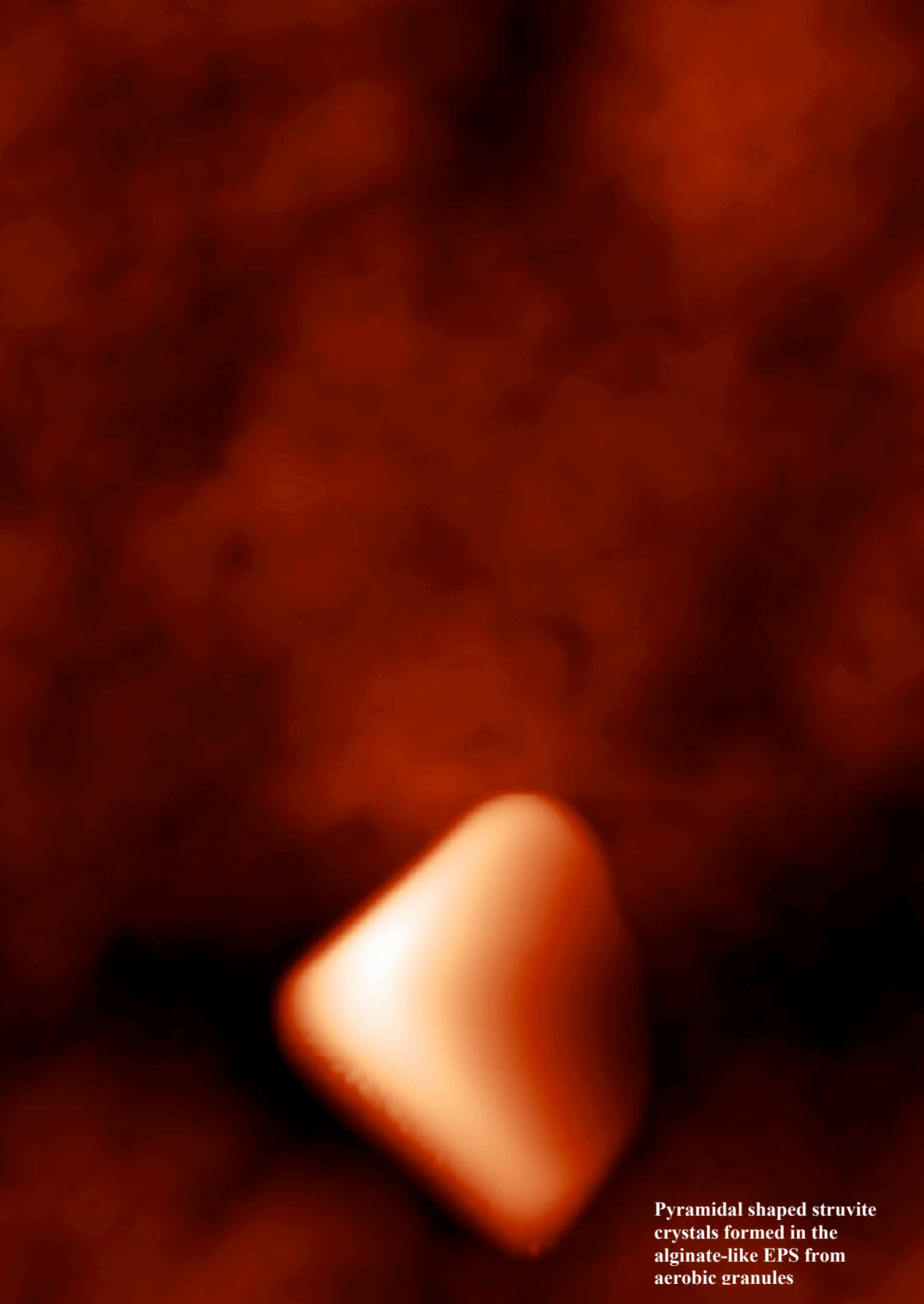


Pyramidal shaped struvite crystals formed in the alginate-like EPS from aerobic granules

CHAPTER 5

Ammonium adsorption in aerobic granular sludge, activated sludge and anammox granules

This Chapter is based on 2 works. Part I is published as: Bassin, J.P., Pronk, M., Kraan, R., Kleerebezem, R., van Loosdrecht, M.C.M. (2011) *Ammonium adsorption in aerobic granular sludge, activated sludge and anammox granules*. **Water Research**, 45(16), 5257-5265. Part II is published as: Lin*, Y.M., Bassin*, J.P., van Loosdrecht, M.C.M. (2012) *The contribution of exopolysaccharides induced struvites accumulation to ammonium adsorption in aerobic granular sludge*. **Water Research** 46(4), 986-992.

* These authors contributed equally to this work

Chapter 5

Content

Abstract

The ammonium adsorption properties of aerobic granular sludge, activated sludge and anammox granules have been investigated. During operation of a pilot-scale aerobic granular sludge reactor, a positive relation between the influent ammonium concentration and the ammonium adsorbed was observed. Aerobic granular sludge exhibited much higher adsorption capacity compared to activated sludge and anammox granules. At an equilibrium ammonium concentration of 30 mgN/L, adsorption obtained with activated sludge and anammox granules was around 0.2 mgNH_4^+-N/gVSS, while aerobic granular sludge from lab- and pilot-scale reactors exhibited an adsorption of 1.7 mgNH_4^+-N/gVSS and 0.9 mgNH_4^+-N/gVSS, respectively. Potassium release accompanied ammonium adsorption to granules from a lab-scale reactor, indicating an ion exchange process. The existence of potassium magnesium phosphate (K-struvite) as one of potassium sources in the granular sludge was studied by X-ray diffraction analysis (XRD). Artificially prepared K-struvite was indeed shown to adsorb ammonium. Alginate-like exopolysaccharides were isolated and their inducement for struvite formation was investigated as well. Potassium magnesium phosphate proved to be a major factor for ammonium adsorption on the granular sludge. Struvites (potassium/ammonium magnesium phosphate) accumulate in aerobic granular sludge due to inducing of precipitation by alginate-like exopolysaccharides. The results indicate that adsorption or better ion-exchange of ammonium should be incorporated into models for nitrification/denitrification, certainly when aerobic granular sludge is used.

Nomenclature

C_{eq}: Equilibrium ammonium concentration (mgN/L)
C_{inf}: Influent ammonium concentration (mgN/L)
Γ_{ads}: Ammonium adsorbed (mgN/gVSS)
Γ_{ads}^{max}: Maximum adsorption constant (mgN/gVSS)
Γ_{ads}^{30}: Ammonium adsorbed at a C_{eq} concentration of 30 mgNH_4^+-N/L (mgN/gVSS)
K: Half saturation constant (mgN/L)
X: Total biomass concentration (gVSS/L)
k_{ads}: Adsorption rate constant (L/(gVSS·h))
VER: Volume Exchange Ratio
K-struvite: potassium magnesium phosphate
Struvite: ammonium magnesium phosphate

5.1. Introduction

The increasing amounts of nitrogen compounds in water and wastewater contribute to the occurrence of eutrophication of surface waters. Nitrogen removal is generally accomplished by nitrification and denitrification, two important processes involved in wastewater treatment successfully applied for many decades. Nitrification is the microbial oxidation of NH_4^+ to NO_2^- and further to NO_3^-. The last compound is reduced to nitrogen gas during denitrification in a multi-step reaction ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$).

Calculation of nitrogen conversion and mass balances in full scale treatment systems or batch activity tests is a complex task due to the numerous parallel conversions involved in nitrogen removal processes. For instance, regularly the measured production of NO_2^- and NO_3^- by nitrification is higher than the NH_4^+ removed from solution. This is generally attributed to simultaneous nitrification of the ammonium generated by ammonification, biomass decay or to analytical problems (Nielsen, 1996). There are however indications in literature suggesting that other phenomena should be taken into account to track the flow of the nitrogen compounds. Among them, adsorption of ammonium to biomass seems to be an important process. The extracellular polymeric substances (EPS) and microbial cell surfaces carry a negative electric charge (Wilkinson, 1958). Therefore, the EPS matrix can function as an ion exchanger for cations (e.g., Ca^{2+}, Mg^{2+} and NH_4^+) and heavy metals. The binding of heavy metals (Liu et al., 2001; Fukushi et al., 2006; Guibaud et al., 2003; Comte et al., 2006) and some cations such as Ca^{2+} and Mg^{2+} (Dupraz et al., 2004; Dupraz et al., 2009) to EPS has been well studied. However, only a limited number of references point to ammonium adsorption in activated sludge systems or biofilms. Nielsen (1996) studied the extent of adsorption of NH_4^+ to activated sludge from full scale wastewater treatment plants (WWTP) where nitrification and denitrification were occurring. In that study, the percentage of ammonium adsorbed to the sludge flocs was observed to be between 20 – 25% at dissolved ammonium concentrations of 1 to 6 mgNH_4^+-N/L. When the bulk concentration was around 15 mgNH_4^+-N/L, the equivalent of 2 mgNH_4^+-N/L was absorbed. The maximum adsorption capacity reported was in the range of 0.3 – 0.4 mgNH_4^+-N/gVSS. Wik (1999) estimated an ammonium adsorption of 2.7 mgNH_4^+-N/m^2 in a trickling filter at an influent ammonium concentration of 15 mgN/L. During the treatment of municipal wastewater by the BIOFIX-process, Temmink et al. (2001)

observed that 9 or 21% of the influent ammonium load was adsorbed by the biofilm when the influent ammonium concentration was 52 ± 20 mg/L or 37 ± 20 mg/L, respectively. Schwitalla et al. (2008) found that the adsorption to activated sludge flocs was within a range of 0.07 to 0.20 mgNH_4^+-N/gVSS. Neglecting the ammonium adsorption could therefore in cases lead to underestimations of 10 – 25% of the ammonium available for nitrification.

In the experiments with lab- and pilot-scale aerobic granular sludge reactors with alternate anaerobic/aerobic phases carried out in this study, ammonium concentrations after anaerobic feeding were found to be lower than expected based on the influent concentration and dilution in the reactor. This fact was associated with a possible ammonium adsorption phenomenon to the aerobic granules. Since ammonium adsorption can best be seen as an ion exchange process, the amount of ammonium adsorbed will be directly related to the compounds functioning as ion exchanger for cations. The acidic extracellular polymers present in the sludge and the precipitates formed on these polymers could be the potential ion exchangers. Few investigations focused on identifying those ion exchangers so far. Schwitalla et al. (2008) observed antiparallel desorption (i.e. ion exchange) of potassium during ammonium adsorption. However, the sources of potassium were not investigated.

Particularly in the aerobic granular sludge process, nitrification, denitrification and enhanced biological phosphorus removal (EBPR) processes often take place simultaneously in a single reactor unit, which is frequently operated in a sequencing batch regime. The considerable amount of phosphate released during the anaerobic phase by phosphorus accumulating organisms (PAOs) favours the precipitation of phosphates into diverse minerals (Maurer et al., 1999). Struvite (ammonium magnesium phosphate) formation during the anaerobic phase was firstly suggested by Yilmaz et al. (2008). Subsequently, Raman spectrum indicated the existence of struvite-like structures in aerobic granular sludge (Angela et al., 2011). In our research, pyramidal shaped yellow-brown crystals, which are similar to K-struvite (potassium magnesium phosphate), as reported by Torzewska et al. (2003), were observed on the surface of aerobic granules. Thus, it is reasonable to assume that K-struvite is a potassium source in aerobic granular sludge, providing potential sites for ammonium adsorption.

Concerning the causes for K-struvite accumulation, investigations on urinary calculi demonstrated that exopolysaccharides (EPS) play an initial important role in the formation of calculi, which have struvite as a main component (McLean et al., 1989;

Ghannoum and O'Toole, 2004). In this context, the formation of K-struvite in aerobic granular sludge is hypothesized to be induced by exopolysaccharides, and this K-struvite in turn contributes to ammonium adsorption.

In this research, we decided to investigate the ammonium adsorption properties of aerobic granular sludge in comparison with activated sludge and anammox granular sludge. Adsorption kinetics and adsorption isotherms were determined in order to provide a better insight in the ammonium adsorption process and for potential future inclusion in mathematical process models. In order to investigate the causes of ammonium adsorption, particularly in the aerobic granules, the presence of K-struvite and its capability to adsorb ammonium were evaluated by X-ray diffraction analysis (XRD) and batch tests on lab-synthesized K-struvite, respectively. The alginate-like exopolysaccharides were isolated from aerobic granular sludge and the relation between the isolated EPS and the formation of struvite was also investigated.

5.2. Materials and Methods

5.2.1. Lab- and pilot-scale aerobic granular sludge reactors

Two lab-scale aerobic granular sludge (AGS) sequencing batch reactors with a working volume of 2.6 L, internal diameter of 5.6 cm and height of 90 cm, were operated at different temperatures (20 and 30ºC). The AGS reactor operated at 20°C was inoculated with aerobic granular biomass from a Nereda® pilot-reactor in Epe WWTP, The Netherlands. The other AGS system, operated at 30°C, was inoculated from biomass from the 20°C reactor. The cycle time of both reactors was 3 h and was divided in four phases: 60 min anaerobic feeding from the bottom of the reactor in a plug flow regime through the settled bed, 112 min aeration, 3 min settling and 5 min effluent withdrawal. The volume exchange ratio (VER) was 0.57, resulting in a hydraulic retention time of 5.2 h. Dissolved oxygen (DO) concentration during aeration phase was kept constant at 20% air saturation by mixing air and nitrogen in the inlet gas by using two mass flow controllers. The mixed gas was introduced by a porous diffuser placed at the bottom of the reactor in order to obtain a flow of 4 L/min. The reactors were fed with synthetic wastewater with the following composition: Medium (1) NaAc 63 mM, $MgSO_4 \cdot 7H_2O$ 3.6 mM, KCl 4.7 mM and Medium (2) NH_4Cl 35.4 mM, K_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM and 10 ml/L trace element solution (Vishniac and Santer, 1957). In each cycle, 150 mL from both medium together with 1,200 mL of tap

water was mixed. This resulted in COD of 400 mg/L and ammonium concentration of 60 mgN/L in the feeding medium. The initial ammonium concentration for each cycle was considered to be the expected concentration after anaerobic feeding (i.e. 34 mgN/L), taking into account the dilution of the feeding medium with the remaining liquid in the reactor (volume exchange ratio of 0.57) and assuming that all ammonium was depleted during the previous cycle. The reactor operated at 20ºC was previously fed with the same wastewater containing different salt concentrations (0 – 30 gNaCl/L). The pilot-scale aerobic granular sludge reactor had a volume of around 1.5 m^3. Domestic sewage and wastewater from a slaughterhouse contributed to 75% and 25% of the incoming COD, respectively. Influent COD and ammonium concentrations were around 600 mg/L and 50 – 100 mgNH_4^+-N/L, respectively. The cycle profile was largely comparable to that of the lab-scale reactors.

5.2.2. Ammonium adsorption batch tests

Adsorption tests were carried out using granular sludge that was collected from lab- and pilot-scale sequencing batch reactors at the end of their operational cycle. Anammox granules were collected from Dokhaven WWTP (Rotterdam, The Netherlands). Activated sludge was taken from the outflow of the nitrification tank from two WWTP (Harnaschpolder (biological P removal) and Kralingseveer (chemical P removal), The Netherlands). Aerobic granular sludge and activated sludge were aerated for 1 h before the batch tests to minimize residual ammonium that could be present. For the anammox granules, nitrogen gas was supplied instead of compressed air. Two types of adsorption batch experiments were conducted: one varying the ammonium concentration and keeping biomass concentration constant and the other varying biomass concentration and keeping the initial ammonium concentration the same. In the experiments with a constant biomass concentration, 250 mL flasks were filled with a fixed amount of biomass (either aerobic granules, activated sludge or anammox granules) and with 0.1 M Tris-HCl buffer (pH 7). At the start of the experiment, pulses of a concentrated ammonium solution were added to the flasks in order to have different initial ammonium concentrations. For the second type of experiment (same initial ammonium concentration), the flasks were filled with different amounts of biomass and with 0.1 M Tri-HCl buffer (pH 7). A pulse of concentrated ammonium solution was added to have a similar final concentration in each flask. Nitrogen gas was supplied for to all flasks to ensure anaerobic conditions. Liquid samples were taken in different time

intervals in order to have an overview of the ammonium adsorption kinetics and maximum adsorption capacity of aerobic granules, activated sludge and anammox granules. For experiment with aerobic granular sludge from the lab-scale reactor, the samples were also measured for potassium to better understand the mechanisms of the adsorption.

5.2.3. Ammonium desorption batch tests

Desorption batch tests were conducted to observe the reversibility of ammonium adsorption. Firstly, a normal adsorption test was performed as described in section 5.2.2, in which a certain amount of biomass (8.4 gVSS/L) was used. A pulse of a concentrated ammonium solution was added in the beginning of the experiment to reach 50 mgN/L. The equilibrium ammonium concentration was measured after the test and the amount of ammonium adsorbed in the granules ($mgNH_4^+$-N/gVSS) was estimated based on the removed ammonium. These granules with known amount of adsorbed ammonium were sieved (to remove all the bulk liquid) and transferred to batch flasks (250 mL) filled with 0.1 M Tris-HCl buffer (pH 7). After 80 min the ammonium concentration was measured and the amount of ammonium remaining adsorbed was estimated. The theoretical ammonium concentration expected in the liquid phase after desorption was calculated from the previous obtained adsorption isotherm with the granular sludge used in the desorption tests.

5.2.4. X-ray powder diffraction (XRD)

Aerobic granular sludge from the lab-scale reactor was sampled at the end of aerobic phase, rinsed with a volume of mili-Q water, dried and incinerated in an oven at 500˚C for 2 hours in order to remove the organic fraction. X-ray powder diffraction patterns were recorded in a Bragg-Brentano geometry of a Bruker D5005 diffractometer equipped with Huber incident-beam monochromator and Braun PSD detector. Data collection was carried out at room temperature using monochromatic Cu Kα1 radiation (λ = 0.154056 nm) in the 2θ region between 5° and 90°, step size 0.038 degrees 2θ. Samples of about 20 milligrams were deposited on a Si <510> wafer and were rotated during measurement. Data evaluation was performed with the Bruker program EVA.

5.2.5. Ammonium adsorption by lab-synthesized K-struvite

Ammonium adsorption by synthetic K-struvite was tested to confirm the hypothesis that K-struvite was playing a significant role in ammonium adsorption observed in the lab-scale granular sludge. A similar ammonium adsorption experiment of that described in item 5.2.2 was performed with synthetic K-struvite instead of aerobic granules. In this particular test, K-struvite was prepared by mixing 3.3 g/L $MgCl_2 \cdot 6H_2O$ with 2.2 g/L KH_2PO_4 at pH 9, as described by Wilsenach et al. (2007). Precipitates were recovered and washed with mili-Q water. Subsequently, the synthetic K-struvite was placed in 100 mL NH_4Cl solution (10 mgNH_4^+-N/L) for ammonium adsorption test. pH was adjusted to 9 (in which K-struvite is chemically stable) in the beginning of the experiment. An experiment (blank) was also conducted without K-struvite to observe if stripping of ammonium occurred at the pH of the test (pH 9). The adsorption due to K-struvite was obtained by subtracting the ammonium adsorption obtained in the batch experiment with K-struvite and that obtained in the experiment without K-struvite (blank).

5.2.6. Alginate-like EPS isolation and their capability to adsorb ammonium

Alginate-like EPS were isolated from the biomass collected in the lab-scale AGS reactor according to Lin et al. (2010). The isolated EPS (0.5 g) was first dissolved in 15 mL of NaOH solution (0.05M) and pH was then adjusted to 7.0 by 0.05 M HCl. The EPS solution was placed inside a dialysis tubing (3500 MWCO) against demineralized water for 48 hours to remove loosely bound ions. Demineralized water was changed every 8 hours. The concentration of K^+, Ca^{2+}, Mg^{2+}, NH_4^+ and PO_4^{3-} ions was determined before and after dialysis. After removing loosely bound ions, the EPS solution was placed inside a dialysis tubing (3500 MWCO) against NH_4Cl solution (10 mgNH_4^+-N/L) for 3 hours. Ammonium and potassium concentrations of the EPS solution and bulk were quantified at both the beginning and end point.

To visualize the crystals formed after ammonium adsorption, solution of EPS (2 uL) at the end of ammonium adsorption was deposited onto mica surfaces for 10 s, and then quickly removed with a pipette. Subsequently, the mica surfaces were air dried (1 h) in a dust-free enclosure, scanned with a Digital Instruments Multimode atomic force microscope (Veeco nanoscopy iva dimension 3100, Veeco Inc.).

5.2.7. Calculation procedures and analytical measurements

The importance of adsorption in the aerobic granular sludge reactors, expressed as percentage, was calculated based on the expected ammonium concentration at the end of anaerobic feeding without adsorption and the measured ammonium concentration at the end of the feeding phase. In order to be able to detect even small differences in the ammonium concentrations in the adsorption batch tests, ammonium was measured by a flow-injection analysis system (QuikChem 8500, Lachat Instruments, Inc.). The detection limit was 0.1 mgNH_4^+-N/L. Biomass concentration was determined according to Standard Methods (APHA, 1998).

K^+, Ca^{2+}, Mg^{2+} and PO_4^{3-} ions were measured by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). For the total phosphorus measurement, aerobic granules from the lab-scale reactor were sampled at the end of aerobic phase, rinsed with a volume of mili-Q water and digested by persulfate method (APHA, 1998). Total phosphorus was determined as phosphate after digestion. As struvite is soluble in hydrochloric acid (HCl), the determination of HCl soluble phosphates was performed by collecting granules at the end of the aerobic phase, which were extracted by 0.5 M HCl solution for 10 min to get phosphate fixed in struvite (Fukumoto et al., 2011). The concentration of K^+, Ca^{2+}, Mg^{2+}, NH_4^+ and PO_4^{3-} ions in the supernatant after extraction was quantified.

5.2.8. Modelling ammonium adsorption

A mathematical tool was developed to predict ammonium adsorption in an aerobic granular sludge reactor as a function of the expected ammonium concentration at the end of the feeding phase, which was estimated based on the ammonium influent concentration (C_{inf}) and the dilution in the reactor taking into account the volume exchange ratio (VER). Ammonium adsorption can be described by an ammonium mass balance (equation (5.1)), and a Langmuir adsorption isotherm (equation (5.2)):

$$C_{\inf} \cdot VER = \Gamma_{ads} \cdot X + C_{eq} \tag{5.1}$$

$$\Gamma_{ads} = \frac{C_{eq}}{\left(C_{eq} + K\right)} \cdot \Gamma_{ads}^{\max} \tag{5.2}$$

The equations consist of three known variables ($C_{\inf}$, VER and X), two unknown variables (C_{eq} and Γ_{ads}) and two parameters ($\Gamma_{ads}^{\max}$ and K). Parameter values characterizing the adsorption capacity of the biomass were estimated by fitting the measured C_{eq}-values to the modelled values that can be obtained from a quadratic solution of equation (5.1) and (5.2). For easy comparison of the adsorption capacity of different types of biomass, a characteristic value of the adsorption capacity at an equilibrium concentration of 30 mgN/L is defined and calculated from equation (5.1) and (5.2) (Γ_{ads}^{30}, mgN/g VSS).

Ammonium adsorption kinetics were characterized using a simple model that assumes that the biomass-specific ammonium adsorption is first order in the driving force for adsorption (equation (5.3)):

$$\frac{dC(t)}{dt} = k_{ads} \cdot X \cdot \left(C(t) - C_{eq}\right) \tag{5.3}$$

In this equation k_{ads} is the biomass-specific kinetic constant for ammonium adsorption (L/(gVSS·h)), and $C(t)$ is the time dependent ammonium concentration in the liquid. Integration of this equation allows for description of $C(t)$ as a function of time (equation (5.4)):

$$C(t) = C_{eq} + \left(C_0 - C_{eq}\right) \cdot e^{k_{ads} \cdot X \cdot t} \tag{5.4}$$

Where C_0 is the initial liquid concentration of ammonium. Values for k_{ads} were estimated by minimizing the sum of the square of the errors between measured and calculated liquid concentrations of ammonium.

5.3. Results

5.3.1. Lab-scale reactor operation

A typical cycle test performed in the lab-scale aerobic granular sludge reactor operated at 20°C is shown in Figure 5.1. The first sample was collected 2 min after the aeration phase has started (t=62min), i.e., after complete homogenization. In the

anaerobic feeding period, all acetate is converted to intracellular polymers (e.g., Polyhydroxybutyrate (PHB)) and no external organic carbon source is available in the subsequent aerobic period (data not shown). Phosphate is released by PAOs. Ammonium concentration after anaerobic feeding was lower than expected based on the influent concentration and the dilution in the reactor. This fact was attributed to ammonium adsorption to granular sludge, phenomenon investigated in this study. In the subsequent aerobic phase, phosphate is taken up by PAOs in around 90 min. Ammonium is converted to nitrite by ammonium-oxidizing bacteria (AOB) and nitrite is subsequently converted to nitrate by nitrite-oxidizing bacteria (NOB). Denitrification occurred simultaneously, and nitrite/nitrate formed during nitrification was converted to nitrogen gas. Very low concentrations of nitrite were detected along the operational cycle (lower than 0.5 $mgNO_2^-$-N/L). A slight accumulation of 5.0 $mgNO_3^-$-N/L was observed in the end of the cycle. Nevertheless, nitrogen removal was maintained above 95%. The amount of nitrate remaining in the bulk in one cycle would be present in the subsequent cycle, although diluted with the next feed.

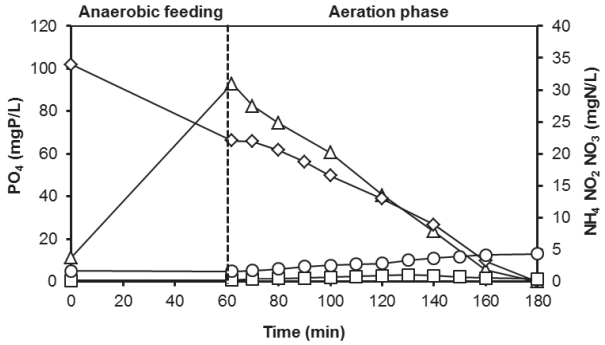


Figure 5.1: Typical cycle measurement showing the profiles of (◊) ammonium, (○) nitrate and (□) nitrite (right y-axis) and (Δ) phosphate (left y-axis) during the anaerobic feeding (before the dotted line) and aeration phase (after the dotted line). The starting ammonium and phosphate concentrations depicted at time 0 were calculated based on the influent concentration (60 $mgNH_4^+$-N/L and 20 $mgPO_4^{3-}$-P/L) and the dilution in the reactor (volume exchange ratio of 57%). Nitrite and nitrate concentrations at time 0 were calculated based on their concentrations in the end of the operational cycle and the dilution in the reactor with the feed.

5.3.2. Adsorption in pilot-scale aerobic granular sludge reactor

During operation of the pilot-scale aerobic granular sludge reactor, we observed a positive relation between the influent ammonium concentration and the ammonium adsorbed. Based on the equilibrium ammonium concentration at the end of the anaerobic feeding phase (C_{eq}) and the ammonium concentration that was expected

(represented as C_{inf}*VER) based on the influent ammonium concentration (C_{inf}) and the dilution in the reactor considering the volume exchange ratio (VER), the amount of adsorbed ammonium (Γ_{ads}*VSS) was estimated (Figure 5.2). Dotted lines in Figure 5.2 represent the model fitted to the data and will be discussed in the Discussion section. In general, the higher the influent ammonium concentration (and therefore the expected ammonium at the end of the anaerobic feeding), the greater the amount of ammonium adsorbed. The biomass concentration in the pilot-scale reactor was roughly constant and equal to 8 gVSS/L. Around 18 – 24% of the expected ammonium concentration at the end of the feeding phase was adsorbed when the ammonium concentration in the incoming wastewater ranged from 50 to 100 mgNH_4^+-N/L. Since some ammonification may have taken place in the anaerobic period, the data in Figure 5.2 do not provide a true adsorption isotherm.

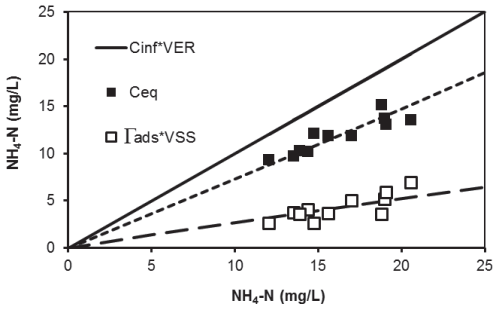


Figure 5.2: Amount of ammonium adsorbed (Γ_{ads}*VSS) and ammonium concentration in the equilibrium (C_{eq}) at different expected ammonium concentration after anaerobic feeding (C_{inf}*VER). The expected ammonium concentration was estimated based on the influent ammonium concentration and the dilution in the reactor considering the volume exchange ratio (VER). Dotted lines represent the model fitted to the experimental data.

5.3.3. Adsorption in lab-scale aerobic granular sludge reactor

In two lab-scale reactors operated at different temperatures (20 and 30ºC) and fed with the same medium, the adsorption of ammonium was quite similar to the pilot scale system (Γ_{ads}^{30}~1 mgNH_4^+-N/gVSS). The adsorption varied from 23 to 36% (for 20ºC reactor) and from 27 to 37% (for 30ºC reactor) of the ammonium concentration expected after anaerobic feeding (34 mgNH_4^+-N) in the aerobic granular sludge reactors. Biomass concentration was kept roughly constant in both reactors (around 12 gVSS/L). The temperature seems not to influence the adsorption in granules in the range studied (20 – 30°C). During operation of the aerobic granular sludge lab-scale reactors, the amount of ammonium adsorbed during anaerobic feeding was observed to

be reversely proportional to the ammonium concentration remaining in the end of the previous cycle. Therefore, when incomplete nitrification occurred, a smaller amount of influent ammonium was adsorbed. This observation emphasises the importance of nitrification for the extent of adsorption.

In the reactor fed with synthetic wastewater containing salt (NaCl), it was observed that the amount of ammonium adsorbed considerably decreased when the salt concentration was increased (Figure 5.3). At 10 gNaCl/L, the ammonium adsorption was approximately half of that obtained when no extra salt was added to the reactor. Moreover, no adsorption was observed at 30 gNaCl/L.

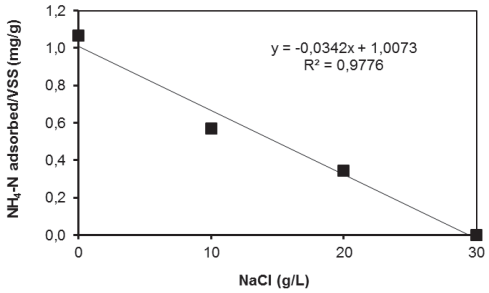


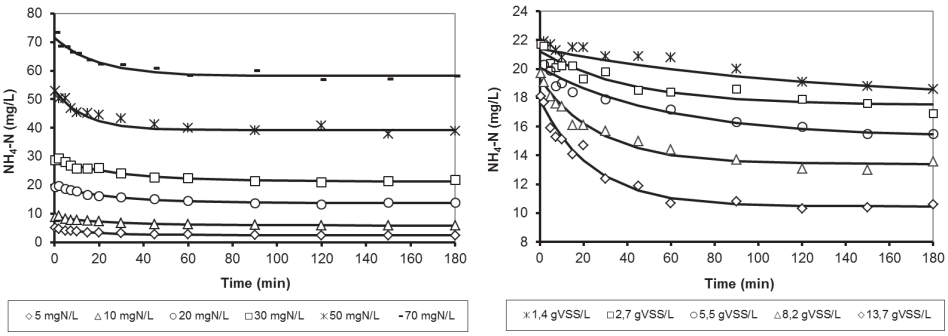
Figure 5.3: Ammonium adsorption in a lab-scale aerobic granular sludge reactor operated at different salt concentrations.

5.3.4. Adsorption batch tests

Determination of adsorption kinetics

Experiments were conducted in order to determine the kinetics of the adsorption taking place in aerobic granular sludge. No adsorption kinetics study was performed for activated sludge, since the experiments clearly showed that adsorption was very fast (within 5 minutes). Adsorption rates with granular sludge are significantly lower probably due to mass transfer limitations in the biofilm. The data from the adsorption kinetics experiments at variable biomass or initial ammonium concentrations are shown in Figure 5.4. In both experiments, we observed a rapid ammonium adsorption in the beginning of the experiment. The adsorption rate gradually decreased until the equilibrium concentration (C_{eq}) was reached. In general, C_{eq} was reached within 60 min of experiment. The anaerobic feeding phase in the aerobic granular sludge reactors have a similar length, suggesting that in this period equilibrium adsorption is reached in the reactors.

A kinetic model described in the Materials and Methods section was used to characterize the kinetic properties of the adsorption process. The average biomass-specific kinetic constant for ammonium adsorption obtained in the experiments with variable biomass and initial ammonium concentrations were comparable and amounted 0.33±0.06 L/(gVSS·h) and 0.31±0.14 L/(gVSS·h), respectively.



(a) (b)

Figure 5.4: Experimental data and model fitted to the adsorption kinetics for the experiment keeping biomass (a) and initial ammonium (b) concentrations constant.

Determination of adsorption isotherms

The ammonium adsorption isotherms obtained in the experiments with activated sludge, in which either ammonium concentration was varied and the amount of biomass was kept constant or the other way around are shown in Figure 5.5. The experimental data where fitted to a Langmuir adsorption isotherm. Parameters such as maximum adsorption capacity (Γ_{ads}^{max}) and the half saturation constant (K) are not reported here since measurements were conducted at ammonium concentrations that were too low for identification of the maximum adsorption capacity. At ammonium concentrations lower than 40 mgN/L, the amount of ammonium adsorbed was directly proportional to the equilibrium concentration, independently of the activated sludge used. Ammonium adsorption obtained at 30 mgNH_4^+-N/L (Γ_{ads}^{30}) for the activated sludge collected from Harnaschpolder and Kralingseveer WWTP was around 0.18 and 0.16 mgNH_4-N/gVSS, respectively. The sludges of Harnaschpolder and Kralingseveer showed a similar ammonium adsorption behaviour. Since typical sludge concentrations are lower than 4 gVSS/L in activated sludge systems, less than 2% of the ammonium will be present in an adsorbed form. This amount can, as is usually done, be neglected.

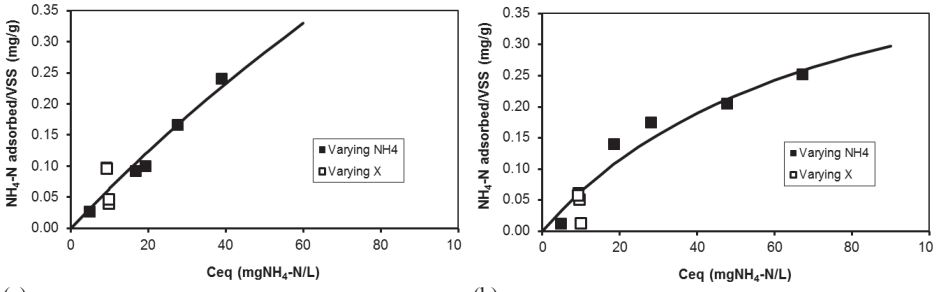


Figure 5.5: Ammonium adsorption isotherms in activated sludge from Harnaschpolder (a) and from Kralingseveer (b) obtained in the batch experiments either keeping biomass concentration constant and varying ammonium concentration (■) or keeping initial ammonium concentration constant and varying biomass concentration (□).

The adsorption of ammonium to aerobic granular sludge from both lab- and pilot-scale reactors is illustrated in Figure 5.6. As for activated sludge, at lower ammonium concentrations the amount of ammonium adsorbed is linearly proportional to the adsorbed amount. The Γ_{ads}^{30}-values were equal to 1.7 $mgNH_4^+$-N/gVSS (1.5 $mgNH_4^+$-N/gTSS) and 0.9 $mgNH_4^+$-N/gVSS for the lab-scale and pilot-scale reactor granules. Clearly these values are an order of magnitude higher than for activated sludge. At sludge concentrations of 8 gVSS/L (10 gTSS/L) and higher in aerobic granular sludge reactors, this means that a very significant fraction of ammonium is adsorbed to the granular sludge. A Langmuir-type isotherm was fitted through the experimental data (non-linear regression). Values obtained for the maximum adsorption constant (Γ_{ads}^{max}) were 10 and 1.65 mgN/VSS for lab-scale granules and pilot-scale granules, respectively. Half saturation constants (K) amounted to 175 and 28 mgN/L for lab-scale granules and pilot-scale granules, respectively.

For comparison, adsorption tests with anammox granular sludge were conducted as well (Figure 5.7). The Γ_{ads}^{30} was around 0.20 $mgNH_4^+$-N/gVSS. Anammox granules showed therefore also a low ammonium adsorption capacity. However, due to much higher biomass content in these anammox granular sludge reactors (up to 20-30 gVSS/L), the total mass of adsorbed ammonium can be significant in a reactor system.

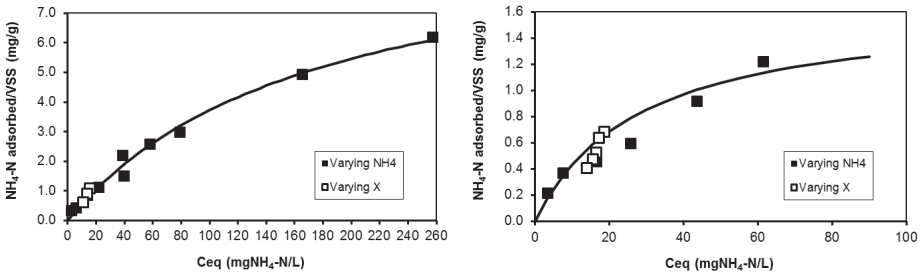


Figure 5.6: Ammonium adsorption isotherms in aerobic granular sludge from lab-scale reactor (a) and pilot-scale reactor (b) obtained in the batch experiments either keeping biomass concentration constant and varying ammonium concentration (■) or keeping ammonium concentration constant and varying biomass concentration (□). Γ_{max}: 10 mgN/gVSS (7 mgN/gTSS); K: 175 mgN/L (considering VSS) and K: 140 mgN/L (considering TSS) for lab-scale granules; Γ_{max}: 1.65 mgN/gVSS; K: 28 mgN/L for pilot-scale granules.

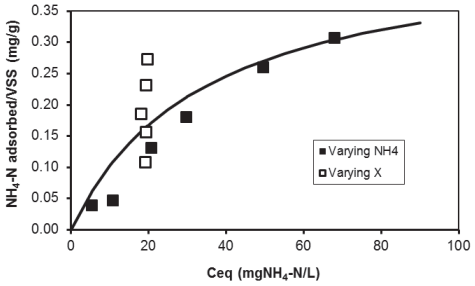


Figure 5.7: Ammonium adsorption isotherms in anammox sludge from a pilot-scale reactor obtained in the batch experiments either keeping biomass concentration constant and varying ammonium concentration (■) or keeping ammonium concentration constant and varying biomass concentration (□). Γ_{max}: 0.46 mgN/gVSS; K: 35 mgN/L.

At the end of ammonium adsorption experiment with biomass from the lab-scale aerobic granular sludge reactor operated at 20°C, large amount of K was found in the bulk liquid, which was released from the granular biomass (data not shown). During the experiment, no external organic carbon source is available in the system; therefore neither polyphosphate release nor formation of intracellular polymers (namely polyhydroxyalkanoates) by PAOs will occur. Therefore, potassium release is intrinsically related to ion exchange instead of phosphate release.

5.3.5. Desorption batch tests

The reversibility of the ammonium adsorption process was investigated by performing desorption batch experiments. First, ammonium was allowed to adsorb to the biomass by incubation at an initial concentration of ammonium of 50 mgN/L. After

adsorption, 37 mgN/L remained, giving an adsorbed amount of 1.8 mgN/gVSS. In the subsequent desorption test, the granules were incubated in ammonium free medium. The ammonium concentration in the bulk after desorption reached 8.5 mgN/L. Based on the corresponding ammonium adsorption isotherm, it was possible to estimate the ammonium concentration in the bulk assuming full desorption. From this test was concluded that more than 90% of the adsorbed ammonium could be desorbed, indicating full reversibility of the adsorption.

5.3.6. K-struvite identification in aerobic granular sludge

The observation of yellow-brown crystals accumulated on the surface of aerobic granular sludge (Figure 5.8) and their pyramidal shape suggested the presence of struvite crystals (Torzewska et al., 2003). In order to confirm the presence of struvite, XRD analysis was performed. By comparison with ICDD database, most of the peaks in the XRD spectrum of granules (Figure 5.9) revealed similarity with potassium magnesium phosphate (dehydrated form of K-struvite) and potassium zinc phosphate (dehydrated form of Zn struvite-like structure).

It should be pointed out that samples for XRD analysis were preheated at 500˚C to remove the organic fraction of the biomass and reduce the significant noise caused by it (Angela et al., 2011). From the cycle test depicted in Figure 5.1, the phosphate release and uptake by PAOs can be seen. As the samples were collected at the end of the aerobic phase, polyphosphate should be accumulated inside the cells. After the organic part of the biomass was removed, polyphosphate would remain in the ash. Polyphosphate is a linear polymer of orthophosphate monomers joined by an ester bond with potassium and magnesium as the counter ions (Prescott et al., 1990). It degrades to orthophosphate only by hydrolysis (Serafim et al, 2002). The heating step at 500˚C does not change the form of polyphosphate from a polymer to a crystal of potassium magnesium phosphate (Isupov, 2002), which can be detected by XRD analysis. Therefore, XRD results confirmed the existence of K-struvite in aerobic granular sludge.

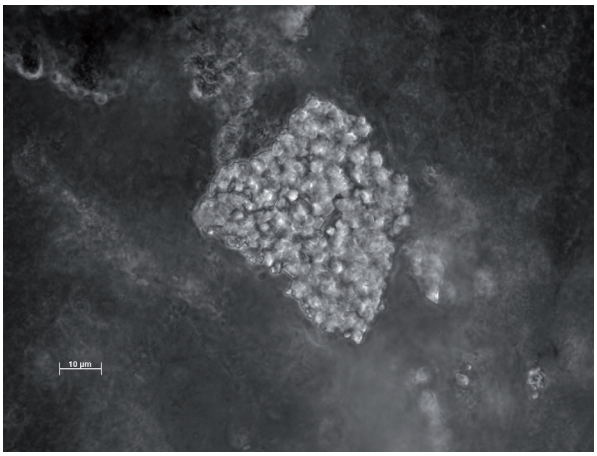


Figure 5.8: Pyramidal shape crystals on the surface of aerobic granular sludge, indicating the presence of struvite crystals.

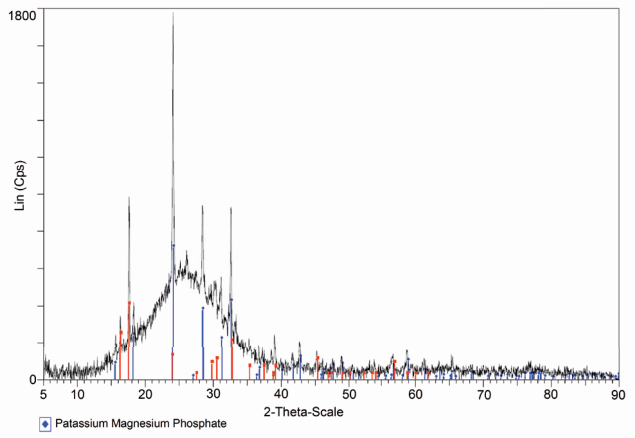


Figure 5.9: X-ray powder diffraction (XRD) spectrum of aerobic granular sludge preheated at 500°C.

5.3.7. Ammonium adsorption by lab-synthesized K-struvite

As the existence of K-struvite was confirmed by XRD analysis, its contribution on ammonium adsorption was evaluated in a batch experiment using K-struvite artificially synthesized in the laboratory. The ammonium adsorption capacity of K-struvite was 3.9 mgNH_4^+-N/g (initial ammonium concentration 10.8 mgN/L). After the adsorption process was finished and the equilibrium was reached (40 min), potassium was detected in the bulk. The molar ratio between ammonium adsorbed and potassium released was almost 1:1.

5.3.8. Alginate-like EPS isolation and their capability to adsorb ammonium

To figure out the causes for K-struvite accumulation in the granular biomass from the lab-scale reactors, the alginate-like EPS was isolated from granules. The extraction yield was 125 ± 14 mgEPS/gVSS. The isolation process was intended to transform the EPS into their sodium salt. It is interesting to find that these EPS still bind ions (e.g., Ca^{2+}, K^{+}, Mg^{2+} and NH_4^{+}) other than Na^{+} after the isolation process. The bindings are extremely strong enough to resist both the isolation process and dialysis against demineralized water (Table 5.1).

Table 5.1: Some elements combined with alginate-like EPS before and after dialysis against demineralized water.

Elements (mg/g EPS)	Ca	Fe	K	Mg	PO_4-P	NH_4-N
Before dialysis	0.35	-	5.59	3.29	7.35	0.31
After dialysis	0.17	-	2.82	1.74	5.76	0.20

To check the ammonium adsorbing capacity of the EPS, an NH_4Cl solution was utilized to dialyze against instead of the demineralized water. The cut-off of the dialysis tubing is 3500 Da. Therefore, only those particles which are smaller than the pore size of the dialysis bag (e.g., salts such as NH_4Cl) can pass freely through it. If only mass transfer happened due to concentration gradient of NH_4Cl between the bulk and EPS solution inside the dialysis tubing, ammonium concentrations inside and outside the dialysis tubing should be identical when the mass transfer balance is reached. However, the ammonium concentration inside the dialysis tubing was significantly higher than outside (Table 5.2), which indicated that complexion of ammonium on EPS occurred. Pyramidal shaped struvite crystals formed inside the dialysis tubing were clearly observed under atomic force microscope (Figure 5.10). In addition, potassium was also released from EPS, exchanging with ammonium. Therefore, the alginate-like EPS is indeed capable to induce struvite-like structure formation. Whether it is K-struvite or ammonium struvite depends on the availability of potassium and ammonium ions in the bulk. Their ammonium adsorption capacity was 0.36 mg/g (at initial ammonium concentration of 10.4 mgN/L), which is about 60% of the adsorption capacity of the granular sludge at the same equilibrium ammonium concentration (Figure 5.6).

Table 5.2: Ammonium adsorption by the alginate-like EPS.

Time	Inside the dialysis tubing			Outside the dialysis tubing		
	NH_4-N (mg/L)	K (mg/L)	Volume (ml)	NH_4-N (mg/L)	K (mg/L)	Volume (ml)
T=0	6.82	95.4	15	10.41	-	80
T=3h	18.99	68.6	15	8.11	5.14	80

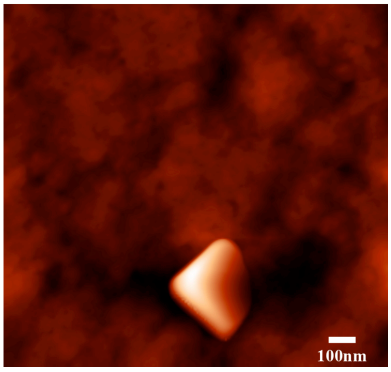


Figure 5.10: Pyramidal shaped crystals formed inside the dialysis bag when the alginate-like EPS dialyzed against NH_4Cl solution.

5.3.9. Total phosphorus and phosphorus fixed by struvite-like precipitates

Formation of stuvite results to intercellular phosphate accumulation. Since struvite is soluble in acid, extraction method with HCl was used to quantify phosphorus fixed in the struvite present in granular sludge. As shown in Table 5.3, the phosphates which were soluble in HCl contained significant amount of K^+ and Mg^{2+} ions and trace amounts of Ca^{2+}, Fe^{3+} and NH_4^+. These struvite fixed phosphates amounted to 286 mg/gTSS. The ash content of granules was 344 ± 40 mg/gTSS. Therefore, struvite-like precipitates corresponds to the majority of the inorganic fraction (ash) of the aerobic granular sludge, which is in agreement with the results obtained in XRD analysis.

The struvite-like precipitates are possibly playing an important role in the phosphorus removal process. The mean value of total phosphorus of the biomass was 93 mg/gTSS, while struvite-fixed phosphorus already reached 72 mg/gTSS. De Kreuk et al. (2005, 2007) claimed that phosphate precipitation in EBPR aerobic granular sludge could double phosphorus accumulation in the biomass. Indeed, this work demonstrated that the formation of phosphate precipitation in aerobic granular sludge, mentioned by de Kreuk et al. (2005, 2007), could be induced by exopolysaccharides.

Table 5.3: Elements present in the biomass solubilized by 0.5 M HCl solution.

Elements	Ca	Fe	K	Mg	PO_4-P	NH_4-N
mg/g biomass	3.32 ± 0.15	0.18± 0.02	32.7±1.4	28.5±2.2	72.4±4.7	0.25±0.04

5.4. Discussion

From the literature it is known that ammonium can be adsorbed to activated sludge flocs (Nielsen, 1996; Schwitalla et al. 2008) and to biofilms (Wik, 1999; Temmink et al., 2001). In the lab- and pilot-scale aerobic granular sludge reactors with alternate anaerobic/aerobic phases, the ammonium concentration after the anaerobic feeding was observed to be lower than expected based on the influent concentration and dilution in the reactor, which suggested the occurrence of a possible ammonium adsorption phenomenon inside the granules. Table 5.4 summarizes the different investigations on ammonium adsorption obtained from literature and from this research. Some results are difficult to interpret in a proper way due to the lack of information. For instance, the ammonium adsorption found by Temmink et al. (2001) was higher when the influent ammonium concentration was lower, which was possibly caused by different biomass concentrations in the reactor. Unfortunately, the biomass amount or biofilm amount was not mentioned in the publication. The same is true for the work of Wik (1999), who only mentioned the ammonium adsorption per m^2 or m^3 of biofilm, without a reference to the biofilm thickness. Also in general the actual equilibrium concentrations at a certain amount of ammonium adsorbed is not presented.

During operation of a pilot-scale aerobic granular sludge reactor, a positive relation between the influent ammonium concentration and the ammonium adsorbed was observed. This is consistent with the results obtained from adsorption batch experiments that showed higher adsorption when C_{eq} was higher. Temperature variation in the lab scale reactors of 10°C did not influence the extent of adsorption, which is in line with expectations for adsorption processes. The results obtained in this study also showed that salt (NaCl) concentrations are also a key factor for the amount of ammonium that will be adsorbed. The fact that the adsorption significantly decreased as the salt concentration was increased can be explained by the competition between Na^+ and NH_4^+ for exchanging with other ions. The adsorption of the ammonium to the

biomass is essentially an ion exchange process and the presence of other cations will directly influence the amount of ammonium adsorbed. Since an adsorption saturation behaviour was observed at high ammonium concentration, a Langmuir isotherm was used to describe the adsorption process, although an ion exchange model would likely be more appropriate. Values obtained with a Langmuir model for ammonium can only be used for estimating adsorption at the medium composition for which the isotherm is determined.

Table 5.4: Ammonium adsorption in different wastewater treatment systems.

Author (s)	System	Adsorption %	mgN/gVSS at 30 mgN/L	Influent NH_4^+ (mgN/L)
Nielsen (1996)	Activated sludge	NA	0.3-0.4[d]	NA
Wik (1999)	Trickling filter	NA	2.7[e]	~16
Temmink et al. (2001)	BIOFIX-process	9 – 21% [a]	NA	30-50
Schwitalla et al. (2008)	Activated sludge	NA	0.07-0.20	NA
Valdivia et al. (2007)	Biofilm SBR	14 – 27% [b]	NA	22
This research	Activated sludge	NA	0.16-0.18	20 - 50
This research	Pilot-scale AGS	18 - 24% [c]	0.9	50 – 100
This research	Lab-scale AGS (T=20ºC)	23 – 36% [c]	1.7	60
This research	Lab-scale AGS (T=30ºC)	27 – 37% [c]	NA	60
This research	Lab-scale AGS (0 to 30 gNaCl/L)	0 – 30% [c]	NA	60

[a] Percentage is relative to ammonium influent load
[b] Percentage is relative to ammonium influent concentration
[c] Percentage is relative to the expected ammonium concentration after anaerobic feeding
[d] Total adsorption capacity at an ammonium concentration of approximately 5 mgN/L
[e] mgN/m^2 of biofilm
NA: not available

The adsorption isotherms obtained in the batch experiments clearly demonstrated that the adsorption in aerobic granular sludge is considerable higher than the one achieved with activated sludge and anammox granules. The estimated Γ_{ads}^{30}-values for activated sludge and anammox granules were 0.16 and 0.20 $mgNH_4^+$-N/gVSS, respectively. The adsorption to aerobic granular sludge was characterized by Γ_{ads}^{30}-values for lab- and pilot-scale granules of 1.6 and 0.8 $mgNH_4^+$-N/gVSS, respectively. From the comparison between anammox granules and aerobic granular sludge it is clear that granular sludge as such does not lead to a higher ammonium adsorption capacity. The pilot-scale granular sludge as well as the sludge from Harnaschpolder were grown on wastewater that employed biological P removal conditions. From the comparison it is therefore clear that the higher adsorption to granular sludge is not directly related to the presence of phosphate accumulating organisms in the sludge. In the adsorption batch tests where biomass was varied and

initial ammonium concentration was kept constant, we observed that the ammonium concentration in equilibrium hardly varied, especially in the experiments using activated sludge and anammox granules. This observation can be partly related to the substantially low amount of ammonia adsorbed at low biomass concentrations which increases the measurement error. Still, the higher adsorption per unit of biomass at lower biomass concentrations is to date not fully understood. Actually these experiments at low biomass concentrations do not reflect the biomass content in the biological systems from where sludge samples were collected.

The fact that ammonium adsorption in granular sludge is considerably higher than that observed for activated sludge and anammox granules reinforce the importance of taking this ion exchange process into consideration especially when working with aerobic granules. For mass balancing over a treatment plant this is no real problem. Under stationary conditions, adsorption does not make a difference in the ammonium effluent concentrations. However, when doing kinetic studies in batch experiments or evaluating conversions from dynamic changes in for instance sequencing batch reactor (SBR) processes or plug flow systems, a significant error can be introduced in the calculations when ammonium adsorption is neglected.

Since the adsorption strongly depends on the type of EPS produced and currently this cannot be predicted from basic principles, adsorption of ammonium has to be considered in each experimental evaluation of nitrogen conversion processes. For example the adsorption of ammonium to the moving bed biofilm in the BIOFIX process made the process in practise not feasible due to excessive release of ammonium in the anoxic phase of the process (Temmink et al., 2001). Also the ammonium dynamics in the trickling filters described by Wik et al. (1999) was heavily influenced by the ammonium adsorption to the biofilm matrix. Yilmaz et al. (2008) observed a decrease of 30 mgNH_4^+-N/L during anaerobic feeding of an aerobic granular sludge reactor, but they neglected the occurrence of ammonium adsorption due to the relatively long time to reach the equilibrium concentration. The assumption of these authors was referred to the publication of Nielsen (1996), who worked with activated sludge. As indicated by the experiments performed in this study, adsorption in granules takes longer and is not a fast process like in activated sludge. Therefore, ammonium adsorption should not have been neglected.

5.4.1. Modeling ammonium adsorption

Ammonium adsorption was well described using a Langmuir isotherm. Due to the low ammonium concentrations applied, the maximum adsorption capacity (Γ_{ads}^{max}) could only be estimated for the aerobic granular sludges. The biomass-specific adsorption characteristics as determined in the batch experiment were extrapolated to the granular sludge bioreactor conditions to validate the impact of ammonium adsorption on the ammonium concentration after feeding. Figure 5.2 demonstrates that using the Γ_{ads}^{max} and K values obtained in the batch experiments and the biomass concentration (X, 8 gVSS/L) and the influent ammonium concentration (C_{inf}) in the pilot could very well be used to predict the ammonium concentration after feeding. The data clearly suggest that biomass characterization in terms of Γ_{ads}^{max} and K values is strictly required to describe nitrogen conversion processes in dynamic processes, like SBRs.

Thus, knowing the influent ammonium concentration (and therefore the ammonium expected after feeding in aerobic granular sludge SBRs) and the biomass concentration, it is possible to predict the amount of ammonium that will be adsorbed and the amount that will remain in the bulk solution. The developed approach is valid when no ammonium is present at the end of a SBR cycle (complete nitrification). In the case where ammonium is not completely removed during the cycle, the amount of ammonium adsorbed would be lower and depends on the residual ammonium concentration in the bulk. We are assuming that granules have maximum adsorption capacity when nitrification is complete. This assumption is based on the desorption tests performed in this study, which showed that > 90% of the ammonium adsorbed in the biomass can be desorbed and will therefore be available for nitrification. However, it is possible that even when ammonium is completely depleted in the liquid phase, a fraction of the adsorbed ammonium remains inside the granules. From the experiments of Nielsen (1995), using activated sludge, it was observed that 0.5 – 0.6 mgNH_4^+-N/L was still adsorbed to the sludge flocs even when the dissolved ammonium was almost completely removed by microbial oxidation. Nielsen (1995) also pointed out that ammonium desorption rate can be quite slow, based on the experiment where the biomass containing 0.5 mgNH_4^+-N/L adsorbed was vigorously shaken in a flask for 50 min for ammonium desorption and further oxidation, and still 0.3 mgNH_4^+-N/L was adsorbed. Therefore, desorption kinetics seems to be important to predict the amount of

ammonium that can be exchanged and further oxidized, and should be studied in more details.

5.4.2. Exopolysaccharides induced struvite formation in aerobic granules

K-struvite was found in aerobic granular sludge from a reactor performing enhanced biological phosphorus removal. The accumulation of K-struvite was attributed to the inducement of alginate-like EPS. The alginate-like EPS were acidic due to the presence of uronic acids monomers. These negatively charged groups enable the EPS to attract cations (e.g., Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) via electrostatic interactions. Alginates has higher affinity to Ca^{2+}, but lower affinity to Mg^{2+}, making Ca^{2+} participate in the formation of gel structure, while Mg^{2+} only use as the counter ions like Na^{+} and K^{+} in the gel. The weak binding of Mg^{2+} and K^{+} enabled them to combine with other ionic groups. Thus, the EPS matrix functions as a chelator for cations and as a template for crystal nucleation (Dupraz and Visscher, 2005). In EBPR process, phosphates with potassium and magnesium as counter ions are released by PAOs during the anaerobic feeding phase. The entrapment by alginate-like EPS of potassium and magnesium ions produces a spatial-temporal gradient of concentration that enhances supersaturation conditions for the development of struvite-like structure. The anionic nature and diverse structures of acidic exopolysaccharides among bacteria are well documented (Clapham et al., 1990). Many researchers have shown that these anionic polymers bind a number of metal cations through electrostatic interactions (Mclean et al., 1990). The nature of the EPS matrix determines which ion is combined and, consequently, which mineral phase is formed. To date, specific accumulation of K-struvite by alginate-like EPS in aerobic granules has not been reported. This is the first work to show that alginate-like EPS can induce the growth of a mineral.

Ammonium adsorption in aerobic granular sludge from the lab-scale reactor was found to be considerably higher when compared to that observed with granules from the pilot-scale reactor. In both systems the amount of alginate-like EPS was similar. Likely, the extra ammonium adsorption was that there is much more K-struvite formed in the granular sludge from lab-scale reactor, which has higher amounts of phosphate, potassium and magnesium release in the anaerobic period.

5.4.3. Ammonium-potassium exchange

K-struvite accumulated in aerobic granules was proved to be a source for ammonium adsorption. The main mechanism lies in ionic exchange between potassium and ammonium. The radii of potassium and ammonium ions are almost identical (1.52 vs 1.51 Å) (Huheey and Keiter, 1993). K-struvite ($KMgPO_4 \cdot 6H_2O$) and struvite ($NH_4MgPO_4 \cdot 6H_2O$) are isostructural, with the existence of a complete isomorphous series from 100% K^+ to 100% NH_4^+ struvite (Mathew and Schroeder, 1979). The ion replacement of K^+ to NH_4^+ (or vice versa) is possible. So either $KMgPO_4 \cdot 6H_2O$ or $NH_4MgPO_4 \cdot 6H_2O$ can be accumulated in aerobic granular sludge depending on the variations of the concentrations of potassium ion and ammonium ion in the bulk. Since ammonium was transformed to dinitrogen gas by simultaneous nitrification/denitrification process, $KMgPO_4 \cdot 6H_2O$ (K-struvite) is the main struvite type in the aerobic granules.

The fact that Schwitalla et al. (2008) observed antiparallel desorption of potassium during ammonium adsorption is also be evidence that K-struvite could be the source of potassium in their biomass. Future investigation is needed to investigate in a deeper way the relation between ammonium adsorption and potassium release.

5.5. Conclusions

Adsorption tests have shown that ammonium adsorption in aerobic granular sludge can be considerably higher than that occurring in activated sludge and anammox granules. Kinetic experiments with granules showed furthermore that adsorption in granules is much slower than for activated sludge. Ammonium adsorption cannot be neglected in granular sludge bioreactor systems that are characterized by strongly variable ammonium concentrations as a function of place (plug flow systems) or time (batch systems). A method for description of ammonium adsorption in computational models for nitrification/denitrification in biofilm and granular sludge systems was proposed.

Potassium magnesium phosphate (K-struvite) accumulated in aerobic granular sludge performing simultaneous nitrification/denitrification and enhanced biological phosphorus removal processes. K-struvite was found to be a potassium source for ion-exchange with ammonium, resulting in high ammonium adsorption to the granular sludge.

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DGGE banding profile of the samples from SBRs sujected to increasing salinity

CHAPTER 6

Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors

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Chapter 6

Content

Abstract

The effect of salinity on the activity of nitrifying bacteria, floc characteristics, and microbial community structure assessed by fluorescent in situ hybridization (FISH) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) techniques was investigated. Two sequencing batch reactors (SBR_1 and SBR_2) treating synthetic wastewater were subjected to increasing salt concentrations. In SBR_1, four salt concentrations (5, 10, 15, and 20 gNaCl/L) were tested, while in SBR_2, only two salt concentrations (10 and 20 gNaCl/L) were applied in a more shock-wise manner. The two different salt adaptation strategies caused different changes in microbial community structure, but did not change the nitrification performance, suggesting that regardless of the different nitrifying bacterial community present in the reactor, the nitrification process can be maintained stable within the salt range tested. Specific ammonium oxidation rates were more affected when salt increase was performed more rapidly and dropped 50% and 60% at 20 gNaCl/L for SBR_1 and SBR_2, respectively. A gradual increase in NaCl concentration had a positive effect on the settling properties (i.e., reduction of sludge volume index), although it caused a higher amount of suspended solids in the effluent. Higher organisms (e.g., protozoa, nematodes, and rotifers) as well as filamentous bacteria could not withstand the high salt concentrations.

6.1. Introduction

Nitrification is a key process in biological nitrogen removal. Commonly, it is considered as the rate-limiting step of the overall biological wastewater treatment process due to the low growth rate of the organisms involved. Moreover, in general, the autotrophic nitrifying bacteria have been reported to be more sensitive for the presence of inhibitory organic compounds (Juliastuti et al., 2003), heavy metals (Hu et al. 2002; Juliastuti et al., 2003; You et al., 2009), and high substrate concentrations (Anthonisen et al., 1976).

Nitrification is also susceptible to inhibition by salt, which has been considered an instability factor in many wastewater treatment plants, especially in industrial settings (Moussa et al., 2006). High salt concentrations can be found in wastewaters generated by chemical, pharmaceutical and petroleum industries, fish canning, seafood processing, meatpacking, tannery, and cheese factories (Dahl et al., 1997). The use of seawater for toilet flushing can also contribute to increasing salt levels in sewage (Panswad and Anan, 1999). Salinity is known to affect the metabolic activity of nitrifying bacteria, reducing microbial growth and ammonium oxidation rates (Moussa et al., 2006). Besides that, the structure and settling properties of sludge flocs, as well as the maximum oxygen solubility and its transfer to the liquid phase, are affected by salt (Van't Riet and Tramper, 1991). In this context, the detrimental effect of salt on nitrification has been the subject of several investigations (Panswad and Anan, 1999; Dahl et al., 1997; Moussa et al., 2006; Campos et al., 2002). In many cases, the results presented in the literature are difficult to compare and show contradictory results. The configuration of the system and the instability of the experimental conditions (in relation to pH, temperature, and presence of inhibitory compounds) are among the factors that directly influence the extent of salt effect on the nitrification process. In addition, the way how the microbial community is adapted to increasing salt concentrations (either by gradual or pulse dosing) may considerably change the results obtained.

One operational strategy that can be applied to minimize the effect caused by salt on the nitrification process is to gradually adapt the microorganisms to high salinity levels. This procedure was successfully applied in a previous research project (Bassin et al., 2011a), during operation of a moving bed biofilm reactor, when the salt concentration was progressively increased from 0 to 13 gNaCl/L over a period of 6

months. Panswad and Anan (1999) observed that the effect of a gradual increase in the salt concentration (from 0 to 30 gNaCl/L) on the chemical oxygen demand (COD) and N removal was less severe for the anaerobic/anoxic/aerobic system inoculated with salt-adapted biomass in comparison to one containing non-adapted biomass. The sludge acclimated to salt also showed a shorter recovery period after a salt shock loading of 70 gNaCl/L. Other studies regarding gradual microbial adaptation to salt showed that, depending on the salt concentration, the long-term effect of salt can be more pronounced than the short-term effect (Moussa et al., 2006).

These studies do not report on the role of changes in the microbial community structure during adaptation. So, the goal of this research was to evaluate how different salt adaptation strategies will influence the microbial diversity, activity, and settling of the activated sludge. For this purpose, a combination of denaturing gradient gel electrophoresis (DGGE) and sequence analysis of polymerase chain reaction (PCR)-amplified 16S ribosomal RNA (rRNA) gene fragments was used. Fluorescent in situ hybridization (FISH) was applied to validate the PCR-based results and to observe the dominant bacterial populations. All the results from the molecular approaches were related to the nitrification process performance.

6.2. Materials and Methods

6.2.1. Experimental set-up and operational conditions

Two identical lab-scale sequencing batch reactors (SBR_1 and SBR_2), with a volume of 4 L, were operated in parallel in a 24-h cycle, which consisted of the following phases: (1) feeding for 3 min, (2) aeration for 23 h and 7 min, (3) settling for 45 min, and (4) effluent withdrawal for 5 min. The volume exchange ratio was fixed at 75%. Both reactors were inoculated with activated sludge from a municipal wastewater treatment plant (ETIG - Rio de Janeiro) and fed with synthetic wastewater consisting of: 305 mg/L NH_4Cl, 222 mg/L KH_2PO_4, 53 mg/L $MgSO_4$, 889 mg/L NaCl, and 900 mg/L $NaHCO_3$. A trace element solution (Vishniac and Santer, 1957) was added to the culture medium in a proportion of 0.5 mL/L medium. The medium was also supplemented with different salt (NaCl) concentrations. The salt concentration was increased from 0 to 20 g/L in both reactors, although in a different manner in each system. The operational phases of SBR_1 and SBR_2 are indicated in Table 6.1. As can be seen, the operational phases of both reactors were similar, although phases II and IV were skipped in SBR_2.

The experimental conditions were chosen in such a way to allow a more gradual adaptation to salt in SBR_1 (four-step salt increase) in comparison with SBR_2 (two-step salt increase). Air was supplied by diffusers placed in the bottom of each reactor. Dissolved oxygen (DO) level was kept above 6 mg/L and the pH was maintained between 6.8 and 7.5 by using either 1M NaOH or 1M HCl. All experiments were conducted at room temperature (24 ± 2°C).

Table 6.1: Operational phases of SBR_1 and SBR_2 according to the salt concentration.

	Operational Phase	NaCl (g/L)	Time of operation (days)
SBR_1	I	0	24
	II	5	17
	III	10	16
	IV	15	16
	V	20	35
SBR_2	I	0	41
	III	10	32
	V	20	35

6.2.2. Analytical methods and calculation procedures

Ammonium, biomass concentrations determined as total suspended solids (TSS), the fraction corresponding to the biomass as volatile suspended solids (VSS), and sludge volumetric index (SVI) were determined by standard methods (APHA, 1995). Quantification of nitrate and nitrite was performed by ion chromatography (Dionex ICS 90) and an analytical kit provided by Hach Co., respectively. Dissolved oxygen was monitored with a DO meter (WTW Oxi-538). pH and temperature were measured with a specific electrode (Oakton pH 110 meter).

Ammonium removal efficiency was calculated on influent-effluent basis, and 100% efficiency was hence obtained when no ammonium was detectable in the effluent. The specific ammonium oxidation rate (q_{NH4}) was determined by linear regression of the concentration of ammonium over time divided by the VSS concentration and was expressed as a fraction of the maximum specific ammonium oxidation rate (q_{max}) observed during the operation of the reactors.

6.2.3. DNA extraction and PCR amplification

DNA was extracted from SBR_1 and SBR_2 samples at the end of phases I, III, and V. Samples were taken in duplicate from different parts of each reactor to confirm reproducibility of the results. No samples were taken at the end of phase II and IV of

SBR_1, since there were no corresponding phases in SBR_2. Extraction was performed using the FastDNA® SPIN Kit (Qbiogene, Carlsbad, CA, USA), following the manufacturer's recommendations. The extracted DNA was evaluated on 1% (w/v) agarose gel and stored at -20ºC until further use. Around 10 ng of genomic DNA was used as template for PCR amplification of the 16S rRNA gene, which was performed with universal primers for the domain bacteria: BAC341F (containing a 40-bp GC clamp) and BAC907RM (M=A/C) (Schäefer and Muyzer, 2001). The standard thermal profile used included a pre-cooling phase of the thermocycler at 4°C for 1.5 min and initial denaturation at 95°C for 5 min, followed by 32 cycles of denaturing at 95°C for 30 s, primer annealing at 57°C for 40 s and elongation at 72°C for 40 s. After the last cycle, a final elongation at 72°C for 30 min took place and the amplification ended at 12°C. The primer set *amoA*-1F-GC and *amoA*-2R (Hornek, et al. 2006) was used for amplification of the gene encoding ammonia monooxygenase of ammonia-oxidizing bacteria (*amoA* gene). The following conditions were chosen for the amplification of *amoA* gene: 4 min at 94°C (initial denaturation), then 35 cycles consisting of 30 s at 94°C (denaturation), 40 s at 60°C (annealing), and 40 s at 72°C (elongation) and 30 min at 72°C (final extension). PCR products of 16S rRNA and *amoA* genes were quantified in a 1% (w/v) agarose gel.

6.2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the Bio-Rad DCode System (Bio-Rad, Richmond, USA). Electrophoresis was run in 1-mm-thick gels, containing either 6% or 8% polyacrylamide for 16S rRNA and *amoA* gene PCR products, respectively. The denaturing gradients of the gels varied from 20% to 70% for 16S rRNA fragments and from 10% to 50% for *amoA* fragments (100% denaturants is defined as 7 M urea and 40% (v/v) deionized formamide). Gels were submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4) for 16S rRNA fragments, or 0.5× TAE buffer for the *amoA* gene fragments. Around 250 ng of the GC-clamped PCR products was added to each gel lane. Electrophoresis of 16S rRNA PCR products lasted for 16 h at a constant voltage of 100 V and a temperature of 60°C. For the *amoA* gene PCR products, the electrophoresis was run for 5 h at 200 V and a temperature of 55°C. After electrophoresis, the gels were stained for 30 min with a 5-mL 1× TAE solution containing SYBR green nucleic acid stain (Molecular Probes, Eugene, OR, USA) in the dark and visualized in a Safe Imager Blue-Light Transilluminator (Invitrogen, Carlsbad,

CA). The gel images were captured with the GeneSnap system (Syngene, Cambridge, UK).

Individual bands from DGGE gels of both 16S rRNA and *amoA* genes were excised using sterile razor blades, eluted in 1× Tris-HCl buffer, and stored overnight at 4°C. The same PCR programs as described previously were followed for the DNA re-amplification with non-GC-clamped primers, in which a volume of 1 µL of the DNA eluted from the DGGE band was used as template. DNA sequencing analysis was carried out by the commercial company Macrogen (South Korea). The obtained 16S rRNA and *amoA* gene sequences were compared to sequences stored in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/blast). In a further step, the sequences were imported into the ARB software (http://www.arb-home.de), aligned by using the ARB automatic aligner. The alignment was further verified and corrected manually. Phylogenetic trees were generated by performing neighbour-joining algorithm. The DGGE banding pattern of different samples was analysed and compared using the GelCompar II software package (Applied Maths, Belgium). Cluster analysis, displayed as a dendrogram, was done using the unweighted pair group method with mathematical averages (UPGMA) and calculation of the pair-wise similarities was based on the Dice correlation coefficient.

The sequences were deposited in GenBank under the following accession numbers: JF710450–JF710456 and JF710458–JF710469 (for 16S rRNA sequences) and JF710470–JF710480 (for *amoA* sequences).

6.2.5. Fluorescent in situ hybridization (FISH)

Sludge samples were taken from both the sequencing batch reactors at the end of phases I, III and V. The samples were washed twice with 1× phosphate-buffered saline (PBS) (4.3 mM Na_2HPO_4 + 1.47 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.0) and were fixed with freshly prepared 4% (w/v) paraformaldehyde for 3 h at 4ºC. Subsequently, the samples were centrifuged at 13000 g for 1 min, washed twice in 1× PBS, and resuspended in an ethanol-PBS solution (1:1) for storage at -20°C. In the hybridization step, fixed samples were spread on gelatin-coated microscope slides and were placed in the oven at 46ºC for drying. The slides containing the dried cells then were dehydrated in three steps (3 min for each step) with 50, 80, and 96% (v/v) ethanol. After dehydration, 10 µL of a hybridization buffer solution containing 0.9 M NaCl, 0.02

M Tris-HCl, 35% (v/v) formamide for all probes and 0.02% (w/v) sodium dodecyl sulfate (SDS) and including fluorescently labelled oligonucleotide probes (0.5 pmol for Cy3/Cy5-labelled and 0.83 pmol for fluorescein-labelled probes), were added to the cells. The hybridization was carried out in a humid chamber for at least 1.5 h of incubation at 46°C. A subsequent washing step to remove unbound oligonucleotides was performed by immersing the gelatin-coated slides in a buffer containing 20 mM Tris-HCl (pH 8), 0.01% (w/v) sodium dodecyl sulfate, 0.08 mM NaCl, and 0.005 mM EDTA for 10 min at 48°C. The wells of the slides were rinsed with Milli-Q water, dried by compressed air, and embedded in 2 µL of Vectashield H-1000 mounting oil for fluorescence (Vector Laboratories, Burlingame, CA). Slides were covered with cover slips and observed with an epifluorescence microscope (Axioplan 2, Zeiss). Image acquisition was performed with a Leica D350F camera. The hybridization experiments were performed using different fluorochromes for each probe to validate the results. The images were exported as .jpg format from the Zeiss microscopy imaging software (AxioVision version 4.7). The rRNA-targeted oligonucleotide probes, labelled with three different fluorescent dyes (Cy3, Fluos, and Cy5), are listed in Table 6.2.

Table 6.2: Oligonucleotides probes and their targeted microbial groups.

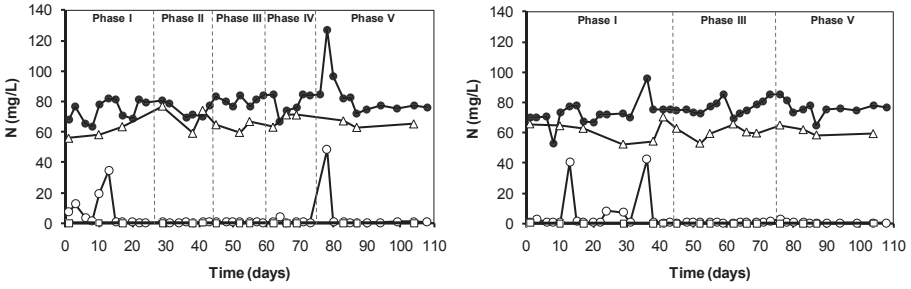
Probe	Sequence (5'-3')	Target group	Mix[a]	Reference
Neu 653	CCC CTC TGC TGC ACT CTA	Halotolerant and obligate halophilic *Nitrosomonas*	AOBmix	Wagner et al. (1995)
Nse 1472	ACCCCAGTCATGACCCCC	*Nitrosomonas europaea*, *N. eutropha*, *N. halophila*		Juretschko et al. (1998)
Nso 1225	CGCCATTGTATTACGTGTGA	Ammonia-oxidizers β-Proteobacteria		Mobarry et al. (1996)
Nso 190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizers β-Proteobacteria	-	Mobarry et al. (1996)
Nit 1035	CCT GTG CTC CAT GCT CCG	*Nitrobacter* spp.	NOBmix	Wagner et al. (1996)
Ntspa 662	GGA ATT CCG CGC TCC TCT	*Nitrospira*		Daims et al. (2001)
Nmv	TCCTCAGAGACTACGCGG	*Nitrosococcus mobilis*	-	Juretschko et al. (1998)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	EUBmix	Amann et al. (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*		Daims et al. (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales		Daims et al. (1999)

[a] Combination of probes to detect a certain group of bacteria

6.3. Results

6.3.1. Nitrification

The influent ammonium concentration was kept around 80 mgN/L and the effluent concentration was usually lower than 1 mgN/L during all experimental phases for both reactors (Figure 6.1). Some small disturbances occurred due to problems related to pH adjustment or to an accidental high ammonium load. The average ammonium removal efficiency was 95% and 96% for SBR_1 and SBR_2, respectively. The nitrite concentration was always below 0.5 mgN/L. According to the amount of ammonium oxidized and nitrate/nitrite produced, a nitrogen balance was performed. The soluble nitrogen recovery amounted approximately 88% for SBR_1 and 86% for SBR_2, independent of the actual salt concentration. Stripping of ammonia can be neglected, since the pH values were always lower than 7.5 and a low ammonium concentration was present in the reactors.



(a) (b)

Figure 6.1: Ammonium concentration in the influent (●) and ammonium (○), nitrate (Δ) and nitrite (□) concentrations in the effluent of SBR_1 (a) and SBR_2 (b) during the whole experimental period.

Cycle measurements were performed at the end of each operational phase for both sequencing batch systems. Ammonium profiles are shown in Figure 6.2. The specific ammonium oxidation rate (q_{NH4}) obtained during operation at different salt concentrations was calculated and expressed as a fraction of the maximum specific ammonium oxidation rate observed at non-elevated salt concentrations (q_{max}). The actual biomass loading rate in the system was approximately 2 mgN/(gVSS·h). The results are shown in Figure 6.3. In SBR_1, in which salt adaptation was performed more slowly, the maximum specific ammonium oxidation rate was approximately 14 mgN/(gVSS·h), value obtained at 5 g/L (phase II). The q_{NH4} obtained in phase I (no salt addition) and phase III was a bit lower and amounted to around 13 mgN/(gVSS·h).

When the NaCl content was increased from 10 to 15 g/L (transition from phase III to phase IV), the q_{NH4} considerably decreased and reached only 70% of the q_{max}. The lowest specific ammonium oxidation activity was observed in Phase V (20 gNaCl/L) and was equivalent to 50% of q_{max}. The salt acclimation procedure in SBR_2 caused a stronger impact on the specific ammonium oxidation rate compared to the results obtained during operation of SBR_1. For SBR_2, the maximum specific ammonium oxidation rate was observed in phase I (13.5 mgN/(gVSS·h)), when no salt was present in the synthetic medium fed to the reactor. When the NaCl concentration was increased directly from 0 (phase I) to 10 g/L (phase III), q_{NH4} decreased by 20%. This activity was reduced by 60% at the end of phase V. Results from cycle measurements also showed that no accumulation of nitrite occurred during all the experimental phases of both reactors. This implies that the specific nitrite oxidation rate (q_{NO2}) was roughly equal to the specific ammonium oxidation rate (q_{NH4}).

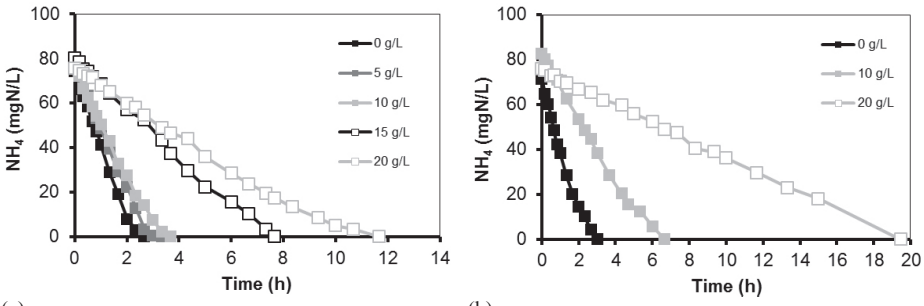


Figure 6.2: Ammonium profiles obtained in the cycle measurements in SBR_1 (a) and SBR_2 (b) during different operational phases.

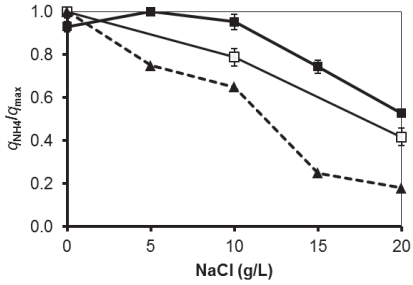


Figure 6.3: Specific ammonium oxidation rate (q_{NH4}) as a fraction of the maximum ammonium oxidation rate (q_{max}) obtained during the operation of SBR_1 (■) and SBR_2 (□) under different salt concentrations. Data from Moussa et al. (2006) are shown in dotted lines.

6.3.2. Biomass concentration and sludge properties

Total suspended solids (TSS) and volatile suspended solids (VSS) of SBR_1 and SBR_2 along the whole experimental period are shown in Table 6.3. During phase I, biomass concentration tended to be constant in both reactors. When the salt concentration was increased from 0 to 5 g/L in SBR_1, a decrease in the biomass concentration was observed, which can be attributed to a higher washout of cells as indicated by the increased turbidity of the supernatant. When phase III was started, a considerable washout of solids was observed, particularly in SBR_2, where the NaCl content was increased directly from 10 to 20 g/L. In SBR_1, the increase of salt from 5 (phase II) to 10 g/L (phase III) did not cause a big difference in VSS concentration. Conversely, with the transition from phase III (10 g/L) to phase IV (15 g/L), the concentration of biomass in the SBR_1 supernatant considerably increased, which lead to more suspended solids in the effluent and, consequently, to a decrease in the VSS in the reactor. In phase V, just a slight decrease in biomass concentration was observed in both reactors, tending to reach a constant value. In general, the decrease in solids concentration was more pronounced in SBR_2 in comparison with SBR_1, especially when considering phase III of that reactor. Consequently, the sludge retention time (SRT) decreased more in SBR_2, which reached values of around 20 days at the end of the operation. The percentage of volatile suspended solids among the total suspended solids was also reduced with the increment of salt in both reactors, indicating the accumulation of inorganic matter in the activated sludge. Biomass yield did not change very much with increasing salinity and amounted to around 0.02 $gVSS/gNH_4^+$-N removed.

The sludge volume index (SVI) was monitored during phases I-V (Table 6.3). A decrease in the SVI was observed with an increase in salinity levels in both reactors. In SBR_1, the SVI decreased gradually from 110 mL/g (phase I) to 70 mL/g (phase IV). From phase IV to phase V no variation was observed. In SBR_2, the reduction of the SVI was slightly higher as compared to that observed in SBR_1. The SVI dropped from 110 mL/g (phase I) to 60 mL/g (phase V).

Higher organisms, such as protozoa, nematodes, and rotifers were found in the sludge at the beginning of the operation of both SBRs. When the salt concentration was increased from 5 to 10 g/L during the transition from phase II to phase III in SBR_1, these organisms started to disappear. At the end of phase III, only a small number of them could be found. In SBR_2, these organisms completely disappeared during the passage from phase I to phase III. The osmotic pressure seems to play a big role in the

higher organisms survival (Moussa et al., 2006), and the slower adaptation to salt in SBR_1 enhanced their tolerance to increased salinity levels. Filamentous bacteria were also found in phases I and II of SBR_1 and in phase I of SBR_2. When the salt concentration was equal or above 10 g/L, these microorganisms were not detected anymore.

Table 6.3: Average values of total suspended solids (TSS), volatile suspended solids (VSS), sludge retention time (SRT) and sludge volume index (SVI) in different operational phases of SBR_1 and SBR_2.

Operational Phases	SBR_1					SBR_2		
	I	II	III	IV	V	I	III	V
$TSS_{reactor}$ (g/L)	2.9	2.5	2.3	1.9	1.8	2.6	1.8	1.6
$VSS_{reactor}$ (g/L)	2.4	2.0	1.8	1.1	1.1	2.1	1.0	0.7
SRT (days)	85	68	55	36	34	85	28	27
SVI (mL/g)	110	91	80	70	70	110	68	60

6.3.3. Microbial population dynamics revealed by PCR-DGGE

Dynamics of general bacterial community

The composition of the microbial community in the sequencing batch reactors was investigated through PCR-DGGE analysis. Figure 6.4 shows the typical DGGE fingerprints of both reactors at the end of phases I, III, and V. A dendrogram representing the percent similarity of the different DGGE fingerprints is also presented. SBR_1 and SBR_2 initially contained a very similar community, but there was a strong divergence upon increasing the salt concentration. The more shock-wise salt adaptation strategy performed in SBR_2 caused a more pronounced change in the microbial community compared to the change observed in SBR_1. Duplicates of each sample were assayed to check the reproducibility of the results. The similarity between all the duplicates was above 97%. Since each duplicate sample was taken from a different place in the reactors, no microbial population gradient was present inside the reactors due to the perfect mixture in the liquid phase. The DGGE profiles indicate the presence of a diverse microbial community. A total of 27 bands were excised from the DGGE gel and sequenced. From those, six bands (i.e., bands B13, B16, B20, B22, B24, and B25) gave ambiguous sequencing results and were neglected in the further phylogenetic analysis.

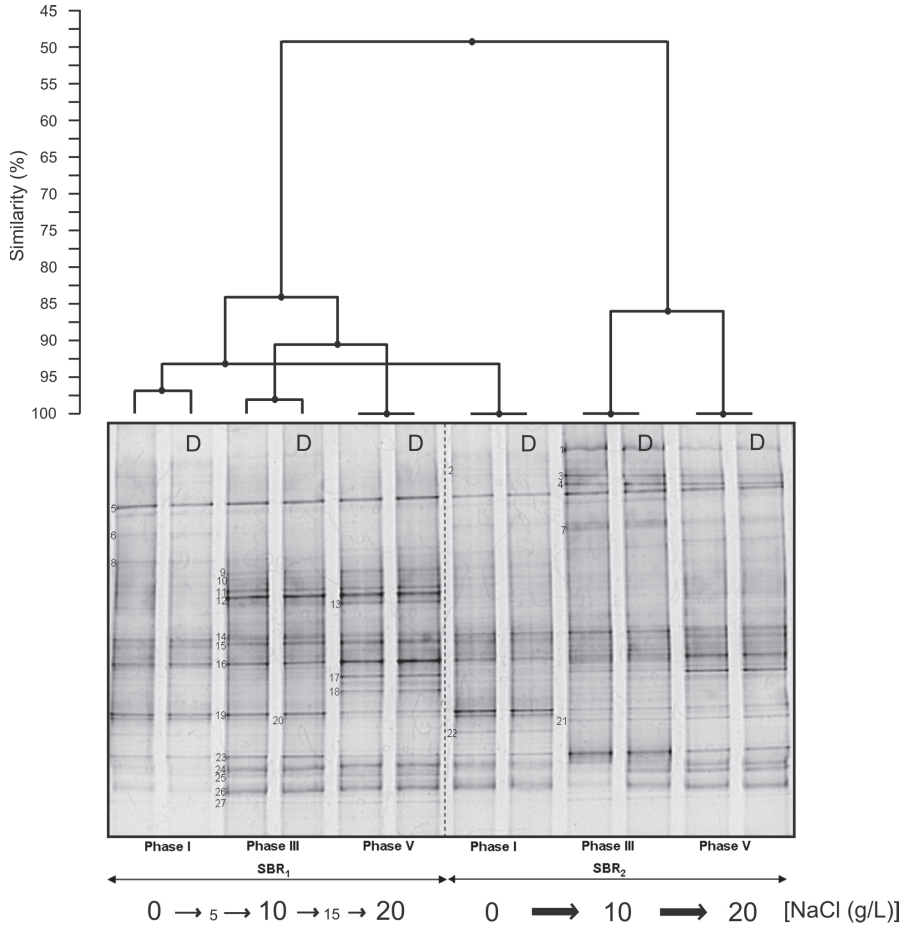


Figure 6.4: DGGE gel banding profile of microbial community of the sequencing batch reactors exposed to increasing salt concentrations. Cluster analysis showing the similarities between different DGGE fingerprints is displayed graphically as a dendrogram. D indicates the duplicate of each sample.

Figure 6.5 illustrates a phylogenetic tree based on partial sequences of the 16S rRNA gene. The microorganisms found belong to several phyla, such as Proteobacteria (α-, ß- and γ-subclass), Bacteroidetes, Chloroflexi, Firmicutes, and Actinobacteria. At the end of phase I, the community fingerprints of SBR_1 and SBR_2 were very similar, as indicated by the cluster analysis (similarity of 93%). This seems logical, since both reactors were operated at the same conditions in phase I (no salt addition). The majority of the excised bands were already found in the sample taken at the end of the first experimental phase, with the exception of bands B1, B4, B7, B17, B18, and B21, which were retrieved in further phases. Among the bands of phase I, ammonia-oxidizing bacteria (AOB) were detected in bands B9 – B12, all of them belonging to the

Nitrosomonas group. Particularly, bands B9 and B11 gave identical sequencing results. Nitrite-oxidizing bacteria were found in bands B14, B15, and B26. Band B14 showed high sequence similarity with *Nitrobacter vulgaris* and bands B15 and B26 were closely related to *Nitrobacter hamburgensis*. Even though the reactors were fed only with autotrophic medium without any organic carbon source, some heterotrophic bacteria were present in the microbial community in phase I. These microorganisms were found in bands B3, B5, B6, B8, B19, B23, and B27. From those, bands B3, B5, and B6 clustered well with members of the Bacteroidetes phylum. Comparative sequence analysis showed that band B8 belongs to α-Proteobacteria. Bands B19 and B23 represented bacteria belonging to the Firmicutes and Chloroflexi, respectively. Band B27 was closely related to an uncultured Gemmatimonadetes bacterium, isolated from an enhanced biological phosphate removal process.

The banding pattern shown in phase III revealed clear differences between the microbial composition of SBR_1 and SBR_2, which is likely due to the different salt adaptation strategy adopted in each reactor. In SBR_1, it was observed that bands B2, B3, and B6 have disappeared from phase I to phase II. Conversely, the intensity of band B12 and bands B23 – B27 became higher when the salt concentration was increased from 0 (phase I) to 5 g/L (phase II) and then to 10 g/L (phase III). The banding pattern similarity between phases I and III of SBR_1 was around 85%. With the increase of salt concentration directly from 0 (phase I) to 10 g/L (phase III), the microbial population in SBR_2 shifted in a different manner than observed in SBR_1. A significant variation in the microbial community was observed from phase I to phase III, as indicated by the relatively low percentage of similarity (50%) between the fingerprints of these two experimental phases. It was observed that new bands (B1, B4, B7 and B21) appeared. With the exception of band 21, in all other bands, microorganisms belonging to the Bacteroidetes group were retrieved. Bands B1 and B4, in particular, showed high sequence similarity to members of the family Chitinophagaceae. The closest relative to band B21 among the culture bacteria was *Nitrospira moscoviensis*. The ammonia oxidizers related to the *Nitrosomonas* group retrieved in bands B9 – B12 were not detected anymore, the intensity of bands B3 (Bacteroidetes) and B23 (Chloroflexi) increased, and the intensity of the band B19 decreased.

According to the SBR_1 banding profile, the differences between the microbial community structure in phase III and phase V are minimal. According to cluster analysis, more than 90% of similarity between the microbial fingerprints of these two

phases was observed. The only differences were the appearance of bands B17 (closely related to *Denitromonas* sp.) and B18 (closely related to *Arthrobacter soli*) and the decrease in the intensity of band B19. The transition from phase III to phase V in SBR_2 was also accompanied by small variations in the bacterial fingerprint, particularly referred to the appearance of band B17 and the increase in the intensity of bands B8, B15, and B16. According to the cluster analysis, the similarity between phases III and V for SBR_2 was approximately 85%.

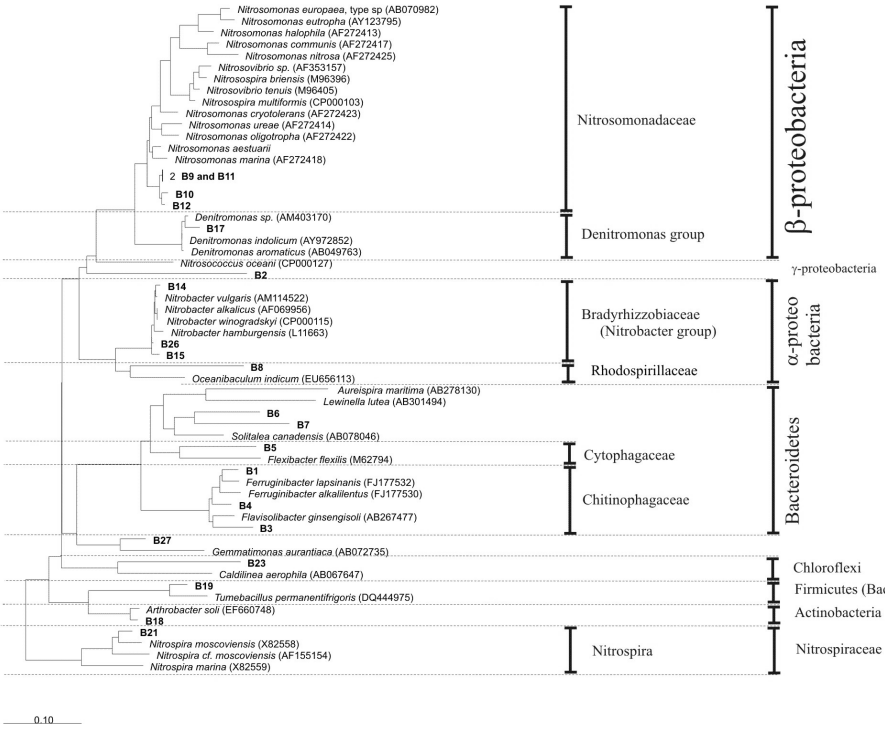


Figure 6.5: Neighbour-joining tree of the sequences retrieved from the 16S rRNA gene DGGE analysis. Sequences determined in this work are printed in bold. The bar indicates 10% sequence difference. The sequence of *Nitrosopumilus maritimus* (Archaea) was used as an outgroup, but was pruned from the tree.

Dynamics of ammonia-oxidizing bacteria (AOB)

In order to get a better insight into the AOB dynamics in the course of the experiment and to obtain sufficient sequence information from DGGE bands for phylogenetic analysis of AOB community, a study was carried out using DGGE analysis of PCR-amplified *amoA* gene fragments (Figure 6.6). According to the 16S rRNA gene DGGE gel, the duplicate samples were practically identical. Therefore, only

one sample representative of each experimental phase was taken into consideration in the DGGE analysis of *amoA* gene fragments.

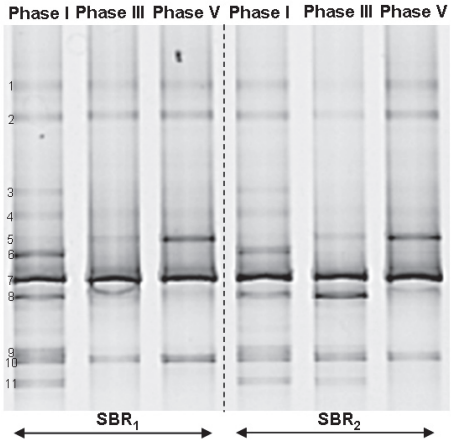


Figure 6.6: DGGE banding patterns showing the ammonia-oxidizing bacteria (AOB) composition over the experimental phases.

Eleven dominant bands were selected, excised, and sequenced in order to reveal the identity of the microorganisms involved. The phylogenetic affiliation of the dominant bands was analysed and depicted in a phylogenetic tree, shown in Figure 6.7. As observed for the 16S rRNA gene DGGE, the microbial community fingerprint in phase I (no salt addition) was the same in SBR_1 and SBR_2. Furthermore, DGGE analysis showed that all the main bands representing the diverse community of AOB were already detected in the steady-state operation of phase I in both reactors. With the increasing salt concentration from 0 (phase I) to 5 g/L (phase II) and subsequently to 10 g/L (phase III) in the SBR_1, bands B3, B6, B8, B9, and B11 have completely disappeared. For the SBR_2, a similar behaviour was observed with the transition from phase I directly to phase III, although some differences were observed: band B4 was not detected anymore, band B8 did not disappear and its intensity was even increased, and bands B9 and B11 were still present at the end of phase III.

The same bands of phase III were detected in phase V in SBR_1. For SBR_2, the only modification in the banding pattern from phase III to phase V refers to the disappearance of bands B8, B9, and B11. For both reactors, it was observed that the intensity of band B5 increased along the different phases, and bands B1, B2, B7, and B10 remained stable over all experimental phases.

The phylogenetic analysis showed that all AOB belonged to the *Nitrosomonas* group. Sequencing results showed that bands B1 – B4, B6, B7, and B9 were closely related to *Nitrosomonas europaea*. The intensity of band B5 (closely related to *Nitrosomonas* sp.) was strongly enhanced at the highest salt concentration. Bands B8, B10, and B11 showed high sequence similarity with *Nitrosomonas eutropha*. Although the banding profile changed differently from phase I to phase III in SBR_1 and SBR_2, the AOB community in both systems was very similar at the end of the experiment (phase V). It is clear that the changes in microbial diversity observed with the *amoA* gene are less than with the 16S rRNA gene, indicating that the impact of salt on the ammonium-oxidizing bacteria was small.

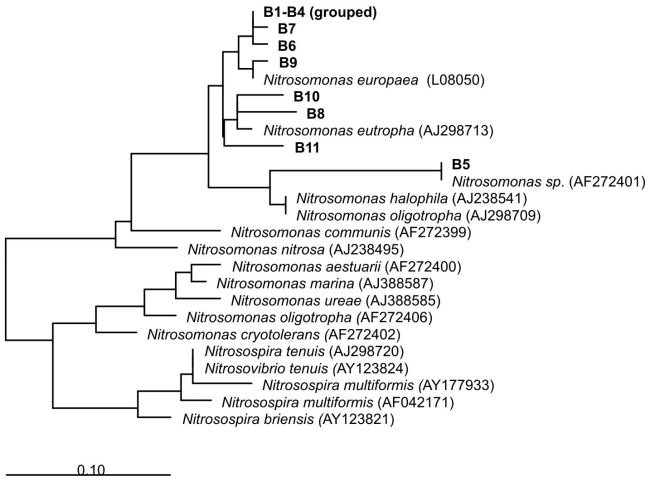


Figure 6.7: Maximum likelihood phylogeny of bacterial *amoA* sequences from DNA retrieved from the DGGE gel. The bar indicates 10% sequence difference. The sequence of *Nitrosococcus halophilus* was used as an outgroup, but was pruned from the tree.

6.3.4. AOB and NOB populations assessed by FISH analysis

FISH analysis was performed using a combination of specific oligonucleotide probes to detect AOB and nitrite-oxidizing bacteria (NOB). Figure 6.8 shows representative FISH pictures obtained from both SBR samples taken at the end of phases I and V. It was observed that the proportion of nitrifying bacteria among the whole bacterial community was similar for both SBRs at the end of phase I, when both systems were operated at the same conditions (no salt addition). FISH results showed that the proportion of AOB and NOB in the whole bacterial community increased when salt concentration was increased from 0 to 10 gNaCl/L in SBR_1, remaining relatively

constant when the salt concentration was further increased to 20 g/L. In SBR_2, the number of AOB cells decreased as the salt concentration was increased, although no significant variations were observed for the NOB population. In SBR_1, the number of AOB and NOB cells was comparable, while in SBR_2 the NOB cells were detected in a higher number in comparison with AOB.

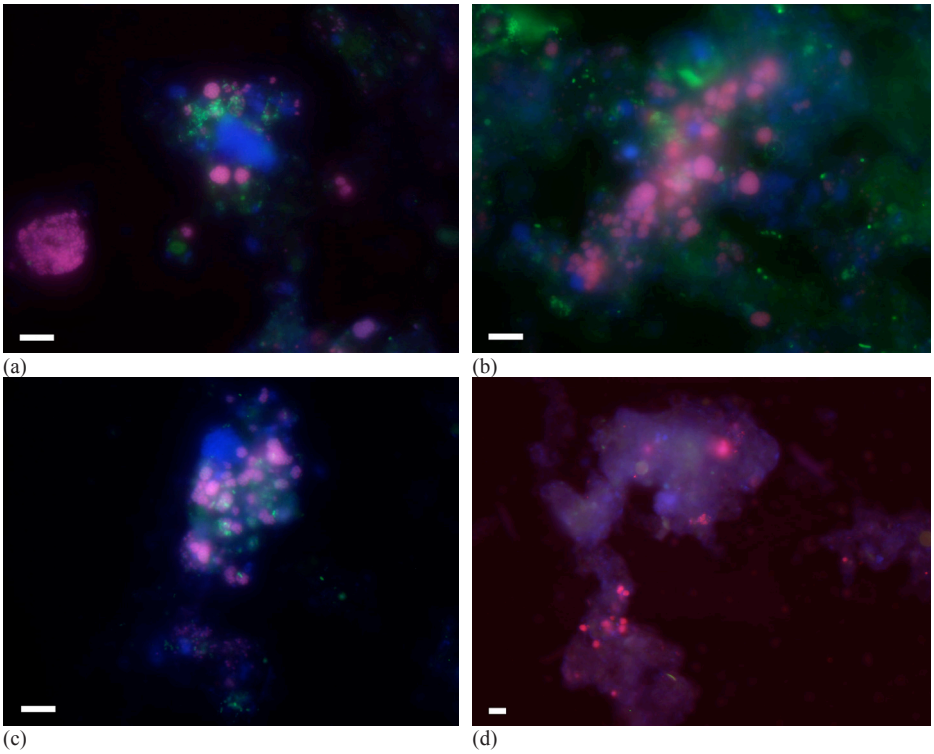


Figure 6.8: AOB (in red) and NOB (in green) populations within the whole bacterial community (in blue): a) SBR_1, phase I; b) SBR_1, phase V; c) SBR_2, phase I; d) SBR_2 phase V. AOB appear violet due to superposition of the red-labelled AOBmix and the blue-labelled EUBmix probes, while NOB appear turquoise due to the superposition of green-labelled NOBmix and the blue-labelled EUBmix probes. Scale bar indicates 5 μm.

6.4. Discussion

6.4.1. Effect of salt on biomass concentration and sludge properties

The results have shown that total suspended solids (TSS) and volatile suspended solids (VSS) decreased gradually as the salinity was increased from Phase I to V in both reactors, and the more rapid salt adaptation procedure in SBR_2 caused a higher washout of cells as compared to SBR_1. Moreover, the sludge was enriched in inorganic material,

which was evidenced by a decrease in the VSS/TSS ratio, especially at the highest salt concentration (20 gNaCl/L).

An increase of the supernatant turbidity, which resulted in higher washout of biomass, was also noticed. The fact that biomass yield remained practically constant over all the experiment runs in both reactors suggested that elevated biomass washout due to increasing salt concentrations was the main cause of lower sludge content in the reactor. Elevated salt concentrations are known to increase water density, which can potentially cause the washout of small and poor settling sludge flocs. According to Woolard and Irvine (1995), biological systems subjected to salt shock loads respond with increased effluent suspended solids and loss of volatile suspended solids, similar to that observed in this work. Panswad and Anan (1999) also observed a decrease in TSS when the salt concentration was increased. These authors observed a reduction of 60% of TSS with an increasing in salinity from 0 to 30 g/L of salt. In this study, the decrease in TSS was a bit lower (40% and 45% for SBR_1 and SBR_2, respectively), but the maximum salt concentration tested was only 20 gNaCl/L.

The effect of salt on the physical properties of activated sludge is a major concern in order to maintain good biomass retention in the reactor. Sludge volumetric index is an important parameter in activated sludge to evaluate the settling ability of the flocs. In this study, the presence of increasing salt concentrations had no negative effect on the sludge volume index. A gradual decrease of SVI was observed when the salt concentration was increased. This contrasts with an increased turbidity of the supernatant, mainly caused by the poor settling properties of smaller aggregates or dispersed organisms that were washed out from the reactor. According to Moussa et al. (2006), due to increased salinity levels and consequently increase in water density, lighter flocs will be washed out (increasing effluent turbidity), while dense flocs will remain in the reactor (decreasing SVI). The quicker salt adaptation in SBR_2 caused a slightly higher decrease in the SVI than the one observed in SBR_1. Moreover, the decrease in solids concentration was more pronounced in SBR_2 in comparison with its counterpart, especially when considering phase III of that reactor, when salt was increased directly from 0 to 10 g/L. This result can be explained by the fact that gradual increase in salinity will stimulate the selection of dense flocs with minimum washout (as observed in SBR_1), while sudden salt increase (as performed in SBR_2) enhances water density, which can cause excessive washout of biomass (Moussa et al. 2006). Several investigations have been done considering the effect of salinity on SVI. In

accordance to our experiments, Campos et al. (2002) and Moussa et al. (2006) also showed a decrease in SVI with increasing salt concentrations. Moon et al. (2002) pointed out that increasing salt concentrations incite physical modifications in aggregates and also observed an increase in the SVI immediately after the salt concentration was increased.

Microscopic observations revealed that higher organisms, such as protozoa, nematodes, and rotifers, gradually disappear as salinity levels were increased. In addition, the more gradual four-step salt adaptation in SBR_1 enhanced their tolerance to increased salinity levels when compared to the two-step salt adaptation in SBR_2. Moussa et al. (2006) also observed that rotifers, protozoa, and nematodes present in a nitrifying sludge could not survive when the salinity (in terms of chloride) was above 5 g Cl^-/L (around 8 gNaCl/L). Salvadó et al. (2001) reported that an increase in salt concentration from 3 to 10 gNaCl/L gradually affected the protozoa and metazoan communities. Higher organisms, such as protozoa, are considered important predators which particularly feed on suspended bacteria and other organic suspended particles in wastewater treatment systems (Curds et al., 1968; Wheale and Williamson, 1980). As a consequence, they indirectly influence the formation of sludge flocs, favouring the biosolid/liquid separation. Due to their important clarification ability, the gradual disappearance of higher organisms when salt concentration was increased could contribute to the higher turbidity of the supernatant and consequent higher biomass washout observed at high salinity levels. The results of this study also indicated that filamentous bacteria, which are commonly related to sludge bulking problems (Pipes, 1978), could not withstand salt concentrations above 10 gNaCl/L in both SBRs. This can explain the fact that higher SVI values, directly reflecting poorer sludge settling characteristics, were observed when the salt concentration was below 10 g/L. On the other hand, filamentous bacteria are also important, providing a superstructure for activated sludge flocs that could enhance mechanical integrity beyond the cell interactions required for flocculation (Urbain et al. 1993). Therefore, a lack of filamentous organisms could also have some implications on the supernatant turbidity. The complete absence of these organisms from 10 gNaCl/L in both SBR_1 and SBR_2 possibly increased effluent turbidity, leading to higher biomass washout and, consequently, higher loss of biomass. Reduction of protozoa and filamentous organisms required for proper flocculation due to high salt levels was also reported by Woolard (1993) and Ludzack and Noran (1965).

6.4.2. Different salt adaptation strategies: implications for nitrification process and microbial community structure

The gradual salt adaptation process performed in both sequencing batch reactors allowed to obtain stable ammonium removal efficiency close to 100% in all experimental phases of both reactors. However, although the functionality of both systems remained the same, DGGE analysis of 16S rRNA fragments (Figure 6.4) showed a significant variation in the ammonia-oxidizing bacteria community, especially in the SBR_2. Most of the bands whose sequences fell within the *Nitrosomonas* group (B9 – B12) were practically not detected in phase III of that reactor, although ammonium removal efficiency was not affected along phases I – V. Moreover, FISH analysis (Figure 6.8) using specific probes targeting *Nitrosomonas* genus showed that these organisms were still detected in SBR_2 even at the highest salt concentration tested (20 gNaCl/L), although in a reduced number compared to the operational phase when no salt was added to the reactor influent medium. The reason why AOB were almost not detected anymore in SBR_2 particularly at the end of phases III and V, is that their relative abundance was below the detection limit of DGGE using universal 16S rRNA gene-targeted primers.

The fact that AOB were hardly detected in the 16S rRNA DGGE analysis is of course an unexpected result, since the reactor was fed with inorganic medium during the whole operational period. Moreover, this fact was not observed in SBR_1, submitted to the same process conditions, with the exception of the different salt increase procedure adopted. Therefore, the microbiological results suggest that the two-step salt adaptation which occurred in SBR_2 was more severe to the AOB compared to the four-step salt adaptation performed in SBR_1, a fact supported by FISH analysis results and in agreement with the lower ammonium oxidation activities obtained in SBR_2 when compared to those obtained in SBR_1, especially when salinity was increased from 10 to 20 gNaCl/L.

It is interesting to compare these results with the ones obtained during operation of an aerobic granular sludge reactor, subjected to salt increase up to 33 gNaCl/L (Bassin et al., 2011b). For example, the shift in ammonia-oxidizing bacteria community within the granules due to salt increase was considerably lower compared to that observed in SBR_1 and SBR_2. The granular sludge reactor, operated for simultaneous

nitrogen and phosphorus removal, was also fed with synthetic medium containing ammonium as nitrogen source, although it also contained acetate as carbon source.

The low concentrations of salt had a positive effect on the ammonium oxidation activity, particularly when considering the increase of salt from 0 to 5 g/L in SBR_1. At concentrations higher than 10 gNaCl/L, the specific ammonium oxidation activity significantly decreased. This result, combined with the decrease in SRT when the salinity was gradually increased, resulted in the substantial decrease in volumetric conversion rate.

Uygur and Kargi (2004) also observed that ammonium removal rate was not significantly affected by salt content when its concentration was lower than 10 g/L. However, these authors observed that this rate decreased sharply when salt concentration exceeded 10 g/L, which similar to the results obtained in our experiments. A considerable decrease in the ammonium oxidation rate with salt increase was also observed by Moussa et al. (2006), whose results are also shown in Figure 6.3.

To have a better overview of the AOB community as salt concentration was increased, a functional gene (*amoA*) playing a key role in the ammonia-oxidizing process was used. It was demonstrated that the AOB community was quite diverse in both reactors. Moreover, the different acclimation to salt performed in SBR_1 and SBR_2 had only a slight influence on the AOB community.

Closely related to *Nitrosomonas* sp., the microorganism whose sequence was retrieved in band B5 seemed to have high affinity to salt, while others closely related *Nitrosomonas eutropha* (bands B8 and B11) and to *Nitrosomonas europaea* (band B9) seemed to be more affected by the longer exposition time to salt which occurred in SBR_1 than by more rapid changes in salinity levels in SBR_2. It should be remarked that the *amoA* gene analysis does not reflect a quantitative evaluation, but a qualitative one. In this sense, although the AOB diversity was higher in SBR_2 at 10 g/L (phase III), nothing can be said about their quantity.

It should be pointed out that the microbial population structure was changing faster than can be expected based on SRT. This can be observed in the DGGE analysis of both 16S rRNA and *amoA* fragments. For instance, the disappearance of several organisms in SBR_1 was observed from the end of phase I to the end of phase III (33 days of operation), despite an SRT of 85 days. Often reported in the literature, accumulation of nitrite due to the salt inhibition of NOB (Ye et al., 2009; Jin et al., 2007) was not observed in the cycle measurements performed in this study, even at the

highest salt concentration tested (20 gNaCl/L). Indeed, when considering the 16S rRNA DGGE profile, the bands representing NOB (B14, B15, and B26) were present along the whole experiment. Besides that, the intensity of bands B15 and B26 was even enhanced when salinity levels were increased. Moreover, a microorganism closely related to *Nitrospira moscoviensis* (band B21) showed high affinity for salt, since it was detected only in phases III and V of SBR_2. Results obtained from FISH analysis also showed that NOB were not affected by increasing salt concentration and the NOB proportion of the whole bacterial community was even increased in the reactors.

Although the nitrifying sludge in both SBRs was cultured in inorganic medium, not only autotrophic AOB and NOB were detected through PCR-DGGE analysis, but also several heterotrophic bacteria. Heterotrophic bacteria could possibly have grown on soluble products released from nitrifying bacteria. Chemolithoautotrophic nitrifiers fix and reduce inorganic carbon (e.g., CO_2) for cell synthesis (Brock and Madigan, 1991) and produce and release soluble organic products into solution from substrate metabolism and decaying biomass (Rittman et al., 1994). Therefore, these microorganisms also interact with the exchange of organic materials. Okabe et al. (1999) also verified the coexistence of nitrifying and heterotrophic bacteria in a nitrifying biofilm fed only with inorganic synthetic medium. Rittmann and Brunner (1984) observed that an active heterotrophic population was maintained in a biofilm reactor when the organic compounds were removed from the feed. These authors postulated that the active nitrifying bacteria produced a continuous flow of organic substrates for the heterotrophs. Rittmann et al. (1994) have done some experiments with *Nitrobacter* sp. and *Nitrosomonas europaea* and demonstrated their capability to produce soluble organic products that can be used by heterotrophic bacteria. Nevertheless, they also mentioned that just a small heterotrophic population was maintained in the autotrophic system. It remains unknown whether the diversity of heterotrophic populations growing in pure autotrophic systems can possibly be related to different usage of various organic compounds produced by autotrophs. A lack of information regarding the ecophysiological interaction between nitrifiers and heterotrophs limits further analysis. A mathematical model to describe the interaction between nitrifiers and heterotrophs in biological treatment systems was proposed by Moussa et al. (2005). Their results showed that the influent COD is responsible for around 40% of the total formed heterotrophic biomass and 60% result from biomass decay. Moreover, even at a low COD input (5 – 10 mg/L), heterotrophic biomass could

be formed in an autotrophic reactor. Moussa et al. (2005) mentioned that such low values of COD can be indirectly introduced to the system by organic impurities in the medium and in the air used for aeration. The long starvation period (up to 20 h in some cycles at 0 and 5 gNaCl/L) due to lack of substrate (totally consumed) could also favour the presence of a significant diversity of heterotrophic bacteria in both systems, enhancing the turnover of microbial population. In the absence of nitrogenous substrate (ammonium), nitrifying biomass decay could have happened, which in turn favoured the heterotrophic growth at the expense of decaying nitrifying bacteria. Moreover, the considerable starvation period could also influence the low growth yield obtained (around 0.02 gVSS/gN), which is much lower compared to that obtained in autotrophic activated sludge systems reported by Blackburne et al. (2007) and Brauer et al. (1992), who measured 0.14 and 0.4 gVSS/gN, respectively.

Nitrospira-like organisms play a big role in other ecosystems, such as soil and drinking water distribution systems, and seem to outcompete *Nitrobacter* under substrate limitation (Wagner and Loy, 2002; Dionisi, et al. 2002). Schramm et al. (1999) hypothesized that species of *Nitrospira* are K-strategists, thriving at low nitrite concentrations, while *Nitrobacter* is considered as an r-strategist, which means that they can compete only in environments containing significant amounts of nitrite. This hypothesis is supported by the fact that *Nitrospira* and *Nitrobacter* coexisted at higher nitrite concentrations (Daims et al., 2001). However, in our case, even at really low nitrite concentrations, both *Nitrobacter* sp. (band B14) and *Nitrobacter vulgaris* (band B26) coexisted with *Nitrospira* sp. (band B21), particularly in SBR_2. The results of this work are different from those obtained by Moussa et al. (2006), who observed that *Nitrospira* sp. was the dominant nitrite oxidizer up to 10 g Cl^-/L (16.5 gNaCl/L), whereas they could not be detected above that concentration.

Among the heterotrophs found in the bacterial community of the sludge, a microorganism closely related *Arthrobacter soli* (band B18) was detected. Verstraete and Alexander (1972) found out that *Arthrobacter* sp. is an organism involved in heterotrophic nitrification, capable of oxidizing ammonium to hydroxylamine, hydroxamic acid, and nitrite. Several organic and inorganic substances can be used as nitrogen source for heterotrophic nitrification, and organic carbon source is necessary for growth of the microorganisms and for nitrification of inorganic N sources (Focht and Verstraete, 1977). In our study, the occurrence of heterotrophic nitrification cannot be neglected due to the presence of organic C source derived from the soluble organic

products released by active nitrifying bacteria and possibly from organic impurities within the culture medium or the air used for aeration.

As described in the Results section, soluble nitrogen recovery amounted approximately 88% for SBR_1 and 86% for SBR_2, which generates a nitrogen loss from solution of 12% and 14% in SBR_1 and SBR_2, respectively. The nitrogen loss from solution observed during the course of the operation can be attributed to other parallel processes, such as N_2O or NO formation by nitrifying bacteria (Kampschreur et al. 2007). Moreover, the sludge produced will also contain nitrogen. Taking into account the amount of sludge (VSS) produced per day and considering a standard nitrogen content of biomass of 10%, it was estimated that the amount of nitrogen used for growth was around 5 – 8% of the influent ammonium nitrogen.

6.5. Conclusions

The results of this work reinforced the fact that activated sludge from a conventional domestic wastewater treatment can be used as a source of nitrifying bacteria which can be adapted to significant salt concentrations through gradual acclimation. The two different salt adaptation methods applied in this study allowed to reach good nitrification performance and caused different shifts in microbial community structure. The findings of this work suggest that independent of the different nitrifying bacterial community present in each reactor, the nitrification process can be maintained stable within the salt range tested. Ammonium oxidation activity was more affected when salt adaptation was performed more rapidly. Settling properties of the sludge improved at higher salt content, although higher organisms (protozoa, nematodes, and rotifers) and filamentous bacteria could not withstand high salinity.

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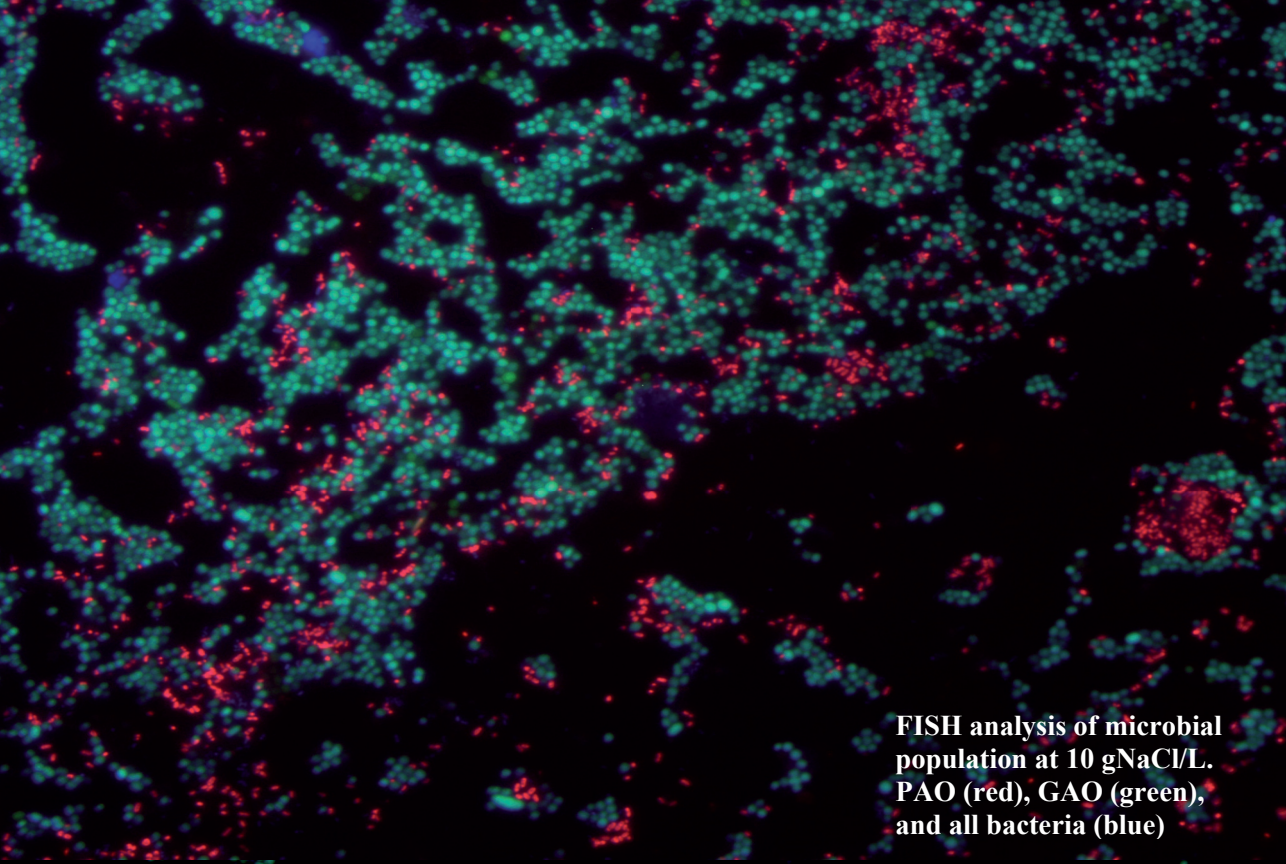
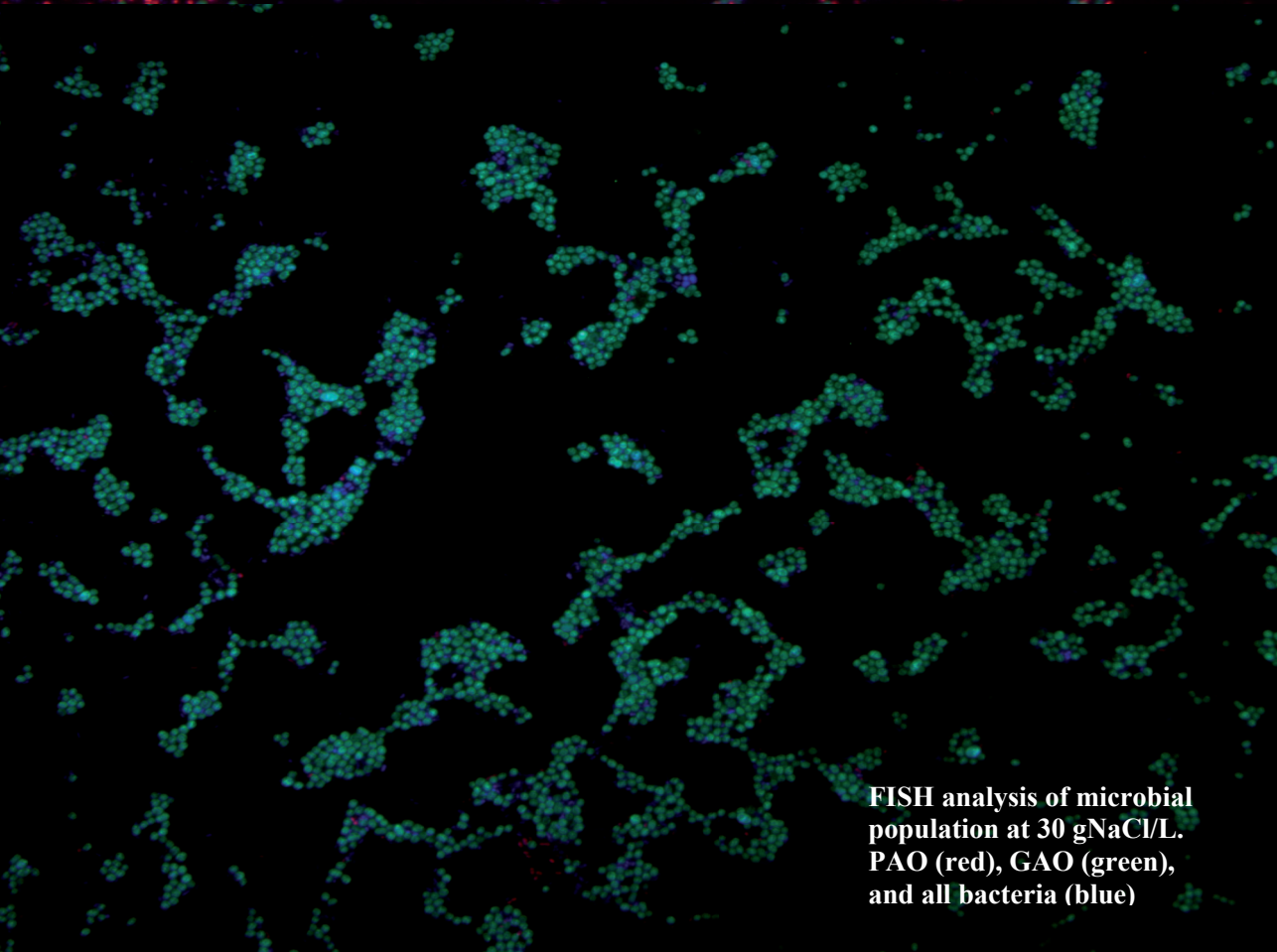


FISH analysis of microbial population at 10 gNaCl/L. PAO (red), GAO (green), and all bacteria (blue)



FISH analysis of microbial population at 30 gNaCl/L. PAO (red), GAO (green), and all bacteria (blue)

CHAPTER 7

Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with the microbial community structure

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Chapter 7

Content

Abstract

The long- and short-term effects of salt on biological nitrogen and phosphorus removal processes were studied in an aerobic granular sludge reactor. The microbial community structure was investigated by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) on 16S rRNA and *amoA* genes. PCR products obtained from genomic DNA and from rRNA after reverse transcription were compared to determine the presence of bacteria as well as the metabolically active fraction of bacteria. Fluorescent in situ hybridization (FISH) was used to validate the PCR-based results and to quantify the dominant bacterial populations. The results demonstrated that ammonium removal efficiency was not affected by salt concentrations up to 33 gNaCl/L. Conversely, a high accumulation of nitrite was observed above 22 gNaCl/L, which coincided with the disappearance of *Nitrospira* sp. Phosphorus removal was severely affected by gradual salt increase. No P release or uptake was observed at steady-state operation at 33 gNaCl/L, exactly when the polyphosphate-accumulating organisms (PAOs), *Candidatus* Accumulibacter phosphatis, were no longer detected by PCR-DGGE or by FISH. Batch experiments confirmed that P removal could still occur at 30 gNaCl/L, but the long exposure of the biomass to this salinity level was detrimental for PAOs, which were outcompeted by glycogen-accumulating organisms (GAOs) in the bioreactor. GAOs became the dominant microorganisms at increasing salt concentrations, especially at 33 gNaCl/L. In the comparative analysis of the diversity (DNA-derived pattern) and the activity (cDNA-derived pattern) of the microbial population, the highly metabolically active microorganisms were observed to be those related to ammonium (*Nitrosomonas* sp.) and phosphate removal (*Candidatus* accumulibacter).

7.1. Introduction

Numerous wastewaters, like those generated in seafood canning, pickling, and cheese processing industries, can contain significant amounts of inorganic dissolved salts (Dahl et al., 1997). The use of seawater for toilet flushing in ships, offshore installations, and regions with problems related to water supply also introduces salts into freshwater. Wastewaters with a high salt content require the activity of salt-tolerant microorganisms which may not be present in high numbers in microbial inocula from sewage treatment plants. Salt ions exert high osmotic pressure on the microorganisms, and most of the freshwater-based microbial populations are unable to survive at these high osmotic pressures and either die or become dormant in these conditions (Madigan et al., 2002). Besides that, inorganic salts can affect the structure and settling properties of microbial flocs as well as the maximum solubility of oxygen and its transfer to the liquid phase, which can lead to oxygen limitation. Consequently, high salt concentrations have a negative influence on existing biological wastewater treatment plants, affecting organic matter, nitrogen, and phosphorus removal (Dalmacija et al., 1996). So far, most studies on chemical oxygen demand (COD), N, and P removal from saline wastewaters have shown that inhibition takes places when salt concentrations exceed 1% (w/v) (Panswad et al., 1999; Uygur and Kargi, 2004; Wu et al., 2008).

Currently, there is an increasing interest in the use of aerobic granular sludge (AGS) in wastewater treatment. The AGS process has numerous advantages compared to the activated sludge process, such as excellent settling properties, good biomass retention, strong compact structure, and capability to withstand shocks of both organic and toxic compounds (Beun et al., 1999). Moreover, the granulation process takes place without reliance on surfaces for biofilm growth, which makes artificial carriers unnecessary. Developed in the last decade (Heijnen et al., 1998), an impressive number of studies have been performed to explore the potentials of the AGS technology in the treatment of high-strength organic wastewaters (Chen et al., 2008; Schwarzenbeck et al., 2005; Moy et al., 2002), toxic aromatic pollutants (Liu et al., 2009; Zhu et al., 2008, Jiang et al., 2002), heavy metals (Xu and Liu, 2008; Yao et al., 2008; Liu et al., 2002), and textile dyes (Sun et al., 2008). Additionally, AGS has been extensively applied for nutrient removal (de Kreuk and van Loosdrecht, 2004; de Kreuk et al., 2005; Mosquera-Corral, 2005; Qin and Liu, 2006). Only a few studies can be found in the literature related to the effect of salt on aerobic granules. Figueroa et al. (2008) investigated the

treatment of fish canning effluents containing high salt concentration (up to 30 gNaCl/L) in an aerobic granular sludge sequencing batch reactor and observed that AGS biomass could withstand the saline conditions. Once the aerobic granules were formed, the presence of salt did not cause a detrimental effect on the operation of the reactor. Based on thermogravimetric analysis, Li and Wang (2008) investigated the content of both inorganic and organic components in aerobic granules structure at low (1% w/v) and high (5% w/v) salinity levels. These authors observed that the surface of the granules was more regular and smoother, and granules were found to grow faster and larger at high salinity levels. Also, the porosity of aerobic granules was found to be reduced at salt concentrations as high as 5% (w/v).

However, none of the aforementioned studies however performed a detailed study on the dynamics of the microbial community structure in the granules. A comprehensive evaluation of the microbiology and ecology of aerobic granules at increasing salt concentrations has not been conducted to date. The major aim of our study was to evaluate the effect of increasing salt concentrations on the main biological processes taking place in an aerobic granular sludge reactor and relate those observations to the dynamics of microbial populations. For this purpose, we used PCR-DGGE on 16S rRNA and *amoA* genes. We compared PCR products obtained from genomic DNA and from rRNA after reverse transcription to identify bacteria present and metabolically active. Moreover, we used fluorescent *in situ* hybridization (FISH) with specific probes for the 16S rRNA of specific subpopulations to validate the PCR-based results and to quantify the dominant bacterial populations. This is one of the first molecular ecology-based characterizations of metabolically active microorganisms of a simultaneous nitrogen and phosphorus removal process.

7.2. Materials and Methods

7.2.1. Reactor set-up and operating conditions

A sequencing batch reactor with a working volume of 2.7 L, an internal diameter of 5.6 cm, and a total height of 90 cm was used in our experiments. The reactor was inoculated with aerobic granular biomass from a Nereda® pilot-reactor in Epe WWTP, The Netherlands. Air was introduced with a fine bubble aerator located at the bottom of the reactor (4 L/min). The dissolved oxygen (DO) concentration and the pH were measured continuously. DO was kept at more than 90% air saturation, and the pH was

controlled at 7.0 ± 0.2 by dosing 1 M NaOH or 1 M HCl. The temperature was maintained at 20°C. To achieve nitrogen and phosphate removal, the reactor was operated in successive cycles of 3 h under alternating anaerobic and aerobic conditions, comprising four phases: 60 min anaerobic feeding from the bottom of the reactor in a plug flow regime through the settled bed, 112 min aeration, 3 min settling and 5 min effluent withdrawal. Effluent was discharged from a port close to the middle of the reactor, and the volume exchange ratio was around 56%. Herewith, the hydraulic retention time (HRT) was 5.3 h. The synthetic wastewater had the following composition: solution A, sodium acetate (NaAc) 63 mM, $MgSO_4 \cdot 7H_2O$ 3.6 mM, KCl 4.7 mM; solution B, NH_4Cl 35.4 mM, K_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM, and 10 ml/L trace element solution (Vishniac and Santer, 1957). 150 mL per cycle was dosed from both medium together with 1200 mL of tap water. The system was operated for 449 days and its operation was divided in four phases, which represented different saline conditions (Table 7.1). Sludge retention time (SRT) was maintained at approximately 30 days by periodically removing sludge from the reactor.

Table 7.1: Operational phases according to the salt concentration.

Phase	NaCl (g/L)	Time of operation (days)
I	0	15
II	11	317
III	22	71
IV	33	46

7.2.2. Batch experiments

Several batch experiments were performed to assess the short-term effect of salt on nitrogen and phosphorus removal. For this set of experiments, granules were taken from the reactor in the last day of operation of each operational phase (Table 7.1 shows the duration of each phase). Biomass was taken from the reactor immediately after the feeding phase. An incubation step with acetate under anaerobic conditions (established by sparging nitrogen into the solution) was performed to enhance the release of residual phosphate potentially present inside the cells and accumulation of an extra polyhydroxyalkanoates by polyphosphate-accumulating organisms (PAOs). The granules were then placed in a sieve and washed with tap water. Equal amounts of granules (based on the wet weight) were introduced in different 250-mL flasks filled with a Tris-HCl buffer (pH 7.0) containing the same minerals of the synthetic medium

fed to reactor (except acetate) and different NaCl concentrations (varying from 0 to 40 g/L). The flasks were aerated through air diffusers, and the batch experiments lasted for 2 hours. Samples were collected every 5 to 20 min, and only liquid sample was removed from each flask in the defined intervals of time, meaning that the amount of biomass per volume of liquid increased over the experiment. In order to avoid problems related to volume correction for the calculation of biomass concentration, the determination of biomass-specific rates did not rely on the concentration of biomass but on the total amount of biomass, which was constant during the batch tests. Similarly, instead of concentrations, the total amounts (in mg) of PO_4^{3-}-P and NH_4^+-N present at certain times of the experiment were considered, which was obtained by summing the amount of PO_4^{3-}-P and NH_4^+-N remaining in the flask and the amount removed by sampling. Biomass-specific rates then could be obtained by dividing the total amount of PO_4^{3-}-P and NH_4^+-N at a specific time by the constant amount of volatile suspended solids (VSS).

7.2.3. Analytical measurements

Ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) were measured through a flow injection analyzer (QuikChem 8500, Lachat Instruments, Inc.). Phosphate (PO_4^{3-}-P) was quantified by Hach Lange cuvette tests (LCK 350). Biomass concentrations were determined according to standard methods (APHA, 1998).

7.2.4. Nucleic Acids Extraction

Samples for genomic DNA and RNA extraction were collected from the reactor in different weeks of operation from phases II to IV. No sample was collected during phase I. In total, five or three samples of each experimental phase were taken into consideration for further DNA- and RNA-based analysis, respectively. Biomass was centrifuged, and the same amount of biomass was introduced in the DNA/RNA extraction kit tubes. DNA and RNA extraction were performed, respectively, by using the UltraClean Microbial DNA isolation kit and UltraClean Microbial RNA isolation (MO BIO, Carlsbad, CA), following the manufacturer's instructions. The extracted DNA and RNA products were evaluated on 1% (w/v) agarose gel and stored at -20 ºC until further use. About 10 ng of the extracted DNA was used as the template for the PCR in which specific primers for either the bacterial 16S rRNA gene (rDNA) or *amoA*

gene (encoding the site of ammonia monooxygenase of ammonia-oxidizing bacteria) were used.

7.2.5. Reverse transcription of RNA and PCR amplification

Reverse transcription of isolated RNA into cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad, CA), according to the manufacturer's protocol. The reverse transcription procedure was carried out for two samples of each experimental phase (except phase I). 1 μL (80 to 100 ng) of the RNA template was used in this step. The set of primers used for 16S rRNA gene amplification was BAC341f (containing a 40-bp GC clamp) and BAC907rM (M=A/C) (Schäfer and Muyzer, 2001). Both genomic DNA and cDNA (0.5 μL each) were used as templates for the amplification reactions. The PCR program for the 16S rRNA gene included a pre-cooling phase of the thermocycler at 4°C for 1.5 min and initial denaturation at 95 °C for 5 min, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 57°C for 40 s, and elongation at 72°C for 40 s. After the last cycle, a final elongation at 72°C for 30 min took place, and the amplification ended at 12°C. For the *amoA* gene amplification, the primers used were *amoA*-1F-GC and *amoA*-2R (Hornek et al., 2006). The cycling regime for amplification of the *amoA* gene was 4 min at 94°C (initial denaturation) and then 35 cycles consisting of 30s at 94°C (denaturation), 40 s at 60°C (annealing), and 40 s at 72°C (elongation), with a final extension for 30 min at 72°C. The quality of the PCR products of both genes (16S rRNA and *amoA*) was evaluated on a 1% (w/v) agarose gel.

7.2.6. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the Bio-Rad DCode System (Bio-Rad, Richmond, CA). Electrophoresis was run in 1-mm-thick gels, containing either 6% polyacrylamide (for 16S rRNA PCR products) or 8% polyacrylamide (for *amoA* gene PCR products). The gels were prepared with denaturing gradients ranging from 20 to 70% for the 16S rRNA fragments and from 10 to 50% for the *amoA* fragments (100% is defined as 7 M urea and 40% (v/v) deionized formamide), and they were submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1mM EDTA, pH 7.4) for 16S rRNA fragments or 0.5× TAE buffer for the *amoA* fragments. Approximately 250 ng of the GC-clamped PCR products were added to individual lanes on the gel. The electrophoresis of 16S rRNA PCR products was run for 16 h at a constant voltage of 100 V and a temperature

of 60°C. For the *amoA* gene PCR products, the electrophoresis lasted for 5 h at 200 V and a temperature of 55°C. After electrophoresis, the gels were stained for about 30 min with a 5 mL 1× TAE solution containing Sybr Green nucleic acid stain (Molecular Probes, Eugene, OR) in the dark and visualized in a Safe Imager Blue-Light Transilluminator (Invitrogen, Carlsbad, CA). Images were acquired with GeneSnap system (Syngene, Cambridge, UK).

7.2.7. Band isolation, DNA sequencing and phylogenetic analysis

In order to obtain a substantially pure PCR product for DNA sequencing, individual bands from DGGE gels of both 16S rRNA and *amoA* genes were carefully excised using sterile razor blades, placed in 1.5-mL microcentrifuge tubes containing 40 µL of 1× Tris-HCl buffer, and stored for 48 h at 4°C. A volume of 1 µL of the DNA eluted from the DGGE band was used for reamplification with non-GC-clamped primers by following the same PCR programs described above. DNA sequencing analysis was carried out by a commercial company (Macrogen, South Korea). The obtained 16S rRNA and *amoA* genes sequences were compared to those stored in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/blast). Subsequently, they were imported into the ARB software (http://www.arb-home.de) and aligned by using the ARB automatic aligner. The alignment was further verified and corrected manually. A phylogenetic tree was generated by performing neighbour-joining algorithm.

The 16S rRNA sequences determined in the course of this work were deposited in GenBank under the following accession numbers: JF276761 to JF276773.

7.2.8. Fluorescent in situ hybridization (FISH)

Granule samples collected from the reactor during phases II - IV for FISH analysis were crushed, washed with 1× phosphate-buffered saline (PBS), and immediately fixed with 4% (w/v) paraformaldehyde in PBS solution for 3 h at 4 ºC. After fixation, cells were centrifuged at 13000 g for 1 min, washed twice in 1× PBS, and resuspended in an ethanol-PBS solution (1:1) for storage at -20°C. In the hybridization step, fixed samples were spread on gelatin-coated microscope slides and were placed in the oven at 46 ºC for drying. The slides containing the dried cells were then dehydrated in three steps (3 min each step) with 50, 80 and 96% (v/v) ethanol. After dehydration, 10 µl of a hybridization buffer solution containing of 0.9 M NaCl,

0.02 M Tris/HCl, 35% (v/v) formamide for all probes and 0.02% (w/v) sodium dodecyl sulfate (SDS) and including fluorescently labelled oligonucleotide probes (0.5 pmol for Cy3/Cy5 and 0.83 pmol for fluorescein labelled probes) were added to the cells. The hybridization was carried out in a humid chamber for at least 1.5 h incubation period at 46°C. A subsequent washing step to remove unbound oligonucleotides was performed by immersing the gelatin-coated slides in a buffer containing 20 mM Tris-HCl (pH 8), 0.01% (w/v) sodium dodecyl sulfate, 0.08 mM NaCl and 0.005 mM EDTA for 10 min at 48°C. The wells of the slides were rinsed with Milli-Q water, dried by compressed air and embedded in 2 µl of Vectashield H-1000 mounting oil for fluorescence (Vector Laboratories, Burlingame, CA). Slides were observed with an epifluorescence microscope (Axioplan 2, Zeiss) and image acquisition was performed with a Leica D350F camera. The hybridization experiments were performed using different fluorochromes for each probe to validate the results. The images were exported as .jpg format from the Zeiss microscopy imaging software (AxioVision version 4.7). The quantification of probe-targeted bacterial cells labelled with different dyes (Cy3 and Fluos) and the determination of the fraction of positive signal from each probe relative to the signal visualized with general bacterial probes (Cy5) was performed with quantitative imaging software (Leica QWin). At least 10 recorded images from the samples representing an experimental run of the reactor were taken into account for the quantification analysis. The rRNA-targeted oligonucleotide probes, labelled with three different fluorescent dyes (Cy3, Fluos, and Cy5) are listed in Table 7.2. According to Oehmen et al. (2007), the PAOmix combination (PAO462, PAO651, and PAO846) is the most sensitive and specific for *Candidatus* Accumulibacter from the currently available probes for these bacteria. In order to differentiate clades of PAO (clade I and clade II, capable and not capable of reducing nitrate, respectively), specific probes developed by Flowers et al. (2009) were used. GAO phenotype bacteria was also targeted by combinations of the probes GAOQ431 and GAOQ989 and designated GAOmix.

Table 7.2: Oligonucleotides probes and their targeted microbial groups.

Probe	Sequence (5'-3')	Target group	Mix	Reference
PAO 462	CCGTCATCTACWCAGGGTATTAAC	PAO cluster[a]	PAOmix	Crocetti et al. (2000)
PAO 651	CCCTCTGCCAAACTCCAG	PAO cluster[a]		Crocetti et al. (2000)
PAO 846	GTTAGCTACGGCACTAAAAGG	PAO cluster[a]		Crocetti et al. (2000)
Acc-I-444	CCCAAGCAATTTCTTCCCC	Clade IA[b]		Flowers et al. (2009)
Acc-II-444	CCCGTGCAATTTCTTCCCC	Clade IIA[c]		Flowers et al. (2009)
GAO Q431	TCCCCGCCTAAAGGGCTT	Competibacter phosphatis[d]	GAOmix	Crocetti et al. (2002)
GAO Q989	TTCCCCGGATGTCAAGGC	Competibacter phosphatis[d]		Crocetti et al. (2002)
Nso 190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizers *β*-proteobacteria	-	Mobarry et al. (1996)
Ntspa 662	GGA ATT CCG CGC TCC TCT	Genus *Nitrospira*	-	Daims et al. (2001)
Nit 1035	CCT GTG CTC CAT GCT CCG	*Nitrobacter spp*.	-	Wagner et al. (1996)
Neu 653	CCC CTC TGC TGC ACT CTA	Most halophilic and halotolerant *Nitrosomonas spp*.	-	Wagner et al. (1995)
Nso 1225	CGC CAT TGT ATT ACG TGT GA	*Nitrosomonas*	-	Mobarry et al. (1996)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	EUBmix	Amann et al. (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*		Daims et al. (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales		Daims et al. (1999)

[a] Closely related to Rhodocyclus (*Candidatus* Accumulibacter phosphatis)
[b] Probe Acc-I-444 also targets some (but not all) member of other Type I clades.
[c] Probe Acc-II-444 also targets some (but not all) members of clades IIC and IID. Clades were defined previously (He et al., 2007; Peterson et al., 2008).
[d] *Candidatus* Competibacter phosphatis

7.3. Results

7.3.1. Long-term and short-term effect of salt on nitrification/denitrification and phosphate removal

Figure 7.1 shows the ammonium, nitrate, and nitrite concentration profiles obtained in the cycle measurements performed at the end of phases I to IV, when a pseudo-steady-state condition was achieved. The first measured sample was taken 2 min after the aeration phase had started (for mixing purposes). As can be seen, the ammonium concentration after anaerobic feeding was lower than expected based on the influent concentration and the dilution in the reactor. This fact is due to ammonium adsorption, phenomenon that was investigated in a separate study (Bassin et al., 2011). Moreover, the nitrite and nitrate

concentrations depicted at time 0 were calculated based on their concentrations at the end of the cycle and the dilution in the reactor. Due to the plug flow feeding regime of the reactor, both nitrite and nitrate were pushed up during the feeding phase, avoiding the growth of ordinary heterotrophs which will use the influent COD to denitrify these nitrogen compounds. The ammonium removal efficiency was not affected by salt in the tested concentrations (up to 33 gNaCl/L). Ammonium was depleted within 60 min of aeration at all salt concentrations. Nitrate was the main product of nitrification during phases I to III. During phase III, intermediate nitrite accumulated but subsequently was either oxidized to nitrate by nitrite-oxidizing bacteria (NOB) or denitrified to N_2 by denitrifying polyphosphate-accumulating organisms (DPAOs) or glycogen-accumulating organisms (DGAOs). During operation at 33 gNaCl/L (phase IV), a significant amount of nitrite (up to 20 mgN/L) accumulated, which indicated that NOB were more sensitive than ammonia-oxidized bacteria (AOB) to the highest salinity level tested. In contrast to ammonium removal, phosphate removal was severely affected by an increased salt concentration. Both phosphate release during anaerobic feeding and phosphate uptake in the subsequent aeration phase decreased with the increase in NaCl concentration from 11 to 22 g/L. At 11 and 22 gNaCl/L, a decrease in P uptake rate can clearly be noticed during the course of an operational cycle, exactly during the period when high nitrite concentrations accumulated, as shown in Figures 7.1b and 7.1c. Furthermore, when nitrite concentrations started to decrease, the P uptake increased again. The P release and uptake stopped completely when a pseudo-steady-state operation was reached at 33 gNaCl/L. In this operational phase, nitrite accumulated and was not further nitrified by NOB. Furthermore, denitrification by denitrifying GAOs (PAOs were not present anymore) was almost negligible.

Batch experiments were conducted to evaluate the short-term effect of salt on the nitrification/denitrification and phosphate removal potential of the biomass at different salt concentrations (0, 5, 10, 15, 20, 30, and 40 gNaCl/L). Specific ammonium and phosphate uptake rates obtained for each salt concentration tested were expressed as a fraction of the maximum value obtained (Figure 7.2). In the test using biomass collected at the end of phase I, not acclimated to salt (0 g/L NaCl), maximum P uptake was obtained when no extra salt was present (Figure 7.2a). Even at low salt concentrations (5 gNaCl/L), the specific P uptake rate was only around 25% of the maximum rate. From 10 to 40 gNaCl/L, no P uptake was observed. On the contrary, additional release of phosphate was observed. For the biomass collected at the end of phase II and phase III (adapted to 11 and 22

gNaCl/L, respectively), maximum P uptake was observed at 10 g/L of salt. The specific PO_4^{3-}-P uptake rate remained practically constant from 0 to 10 gNaCl/L in the test with biomass acclimated to 11 g/L. At higher salt concentrations (30 g/L), the P uptake decreased to almost zero. For the biomass acclimated to 22 g/L of NaCl, specific rates obtained at 0, 5 and 15 g/L were roughly the same, and a significant decrease was observed from 15 to 40 gNaCl/L. In the test with biomass collected at the end of phase IV (adapted to 33 g/L), no phosphate uptake was observed at all salt concentrations and minor P release was observed.

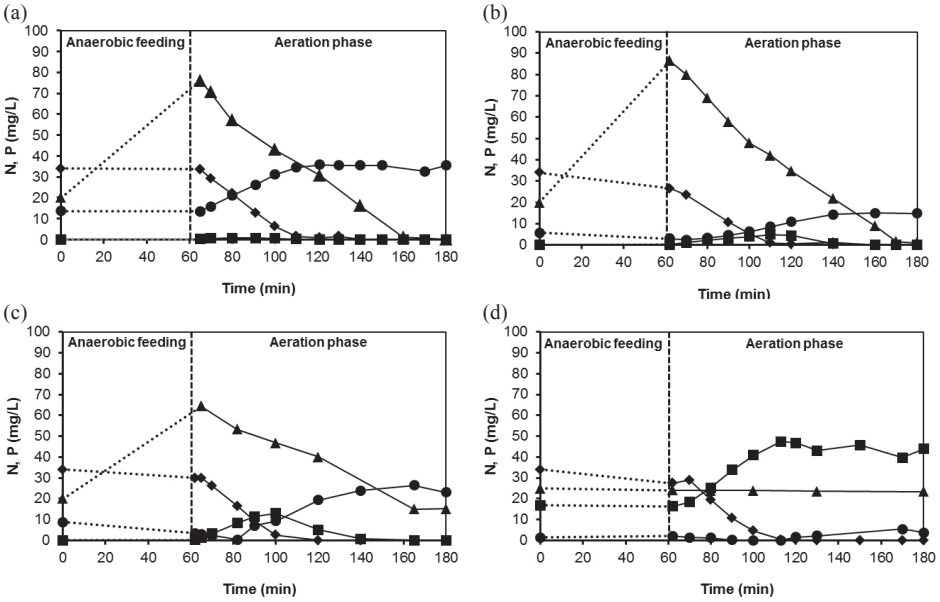

Figure 7.1: Ammonium (♦), nitrate (●), nitrite (■), and phosphate (▲) profiles acquired in the cycle measurements performed at the end of phase I (no salt) (a), phase II (11 gNaCl/L) (b), phase III (22 gNaCl/L) (c), and phase IV (33 gNaCl/L) (d). The starting ammonium and phosphate concentrations depicted at time 0 were calculated based on the influent concentration (60 mgNH_4^+-N/L and 20 mgPO_4^{3-}-P/L) and the dilution in the reactor. Nitrite and nitrate concentrations at time 0 were calculated based on their concentrations at the end of the cycle and the dilution in the reactor.

The specific ammonium uptake rate was substantially reduced at salt concentrations exceeding 10 g/L. This is especially the case in the test with non-salt-adapted biomass, in which no ammonium uptake was observed at salt concentration exceeding 20 gNaCl/L (Figure 7.2b). Maximum specific ammonium uptake rates were observed at NaCl concentration of 10 g/L for the tests using biomass acclimated to 11 and 33 gNaCl/L. For

the test where biomass adapted to 22 g/L was used, maximum ammonium uptake rate was obtained at 0 gNaCl/L.

Nitrate and nitrite measurements also were performed for all batch experiments. Nitrate was the main nitrification product at all salt concentrations for the tests using biomass adapted to 0 and 11 g/L of salt, while nitrite concentrations always were lower than 2 mgN/L. For the test using granules acclimated to 22 and 33 g/L, nitrite was the main product of nitrification, reaching concentrations up to 9 mgN/L (for biomass adapted to 22 gNaCl/L) and 20 mgN/L (for biomass adapted to 33 gNaCl/L).

According to the nitrogen balance performed taking into account the nitrogen compounds measured (NH_4^+-N, NO_3^--N, and NO_2^--N), the highest denitrification activity was observed in the experiment using biomass acclimated to 11 gNaCl/L, when nitrogen removal ranged between 70 and 85% for all salt concentrations tested. In the experiment using granules adapted to 22 and 33 gNaCl/L, the maximum N removal obtained was around 45 and 20%, respectively.

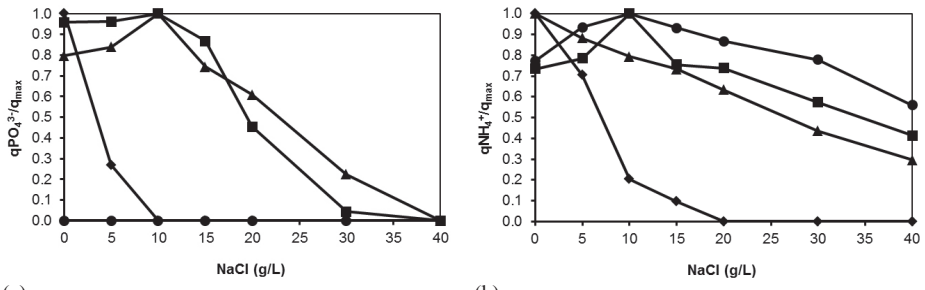

Figure 7.2: Short-term effect of salt on (a) phosphate- and (b) ammonium-specific uptake rates. Biomass adapted to 0 gNaCl/L (♦), 11 gNaCl/L (■), 22 gNaCl/L (▲), and 33 gNaCl/L (●).

7.3.2. Microbial community analysis by DGGE of 16S rRNA gene fragments

The microbial community of the aerobic granular sludge reactor was investigated by DGGE of the 16S rRNA gene fragments. Figure 7.3 shows the DGGE banding patterns representing the general bacterial community of the experimental phases II to IV. DNA extraction of the biomass started to be performed after 275 days of operation, when the reactor had already been run at 11 gNaCl/L for a long time. No biomass was collected during phase I when no NaCl was added to the culture medium fed to the reactor.

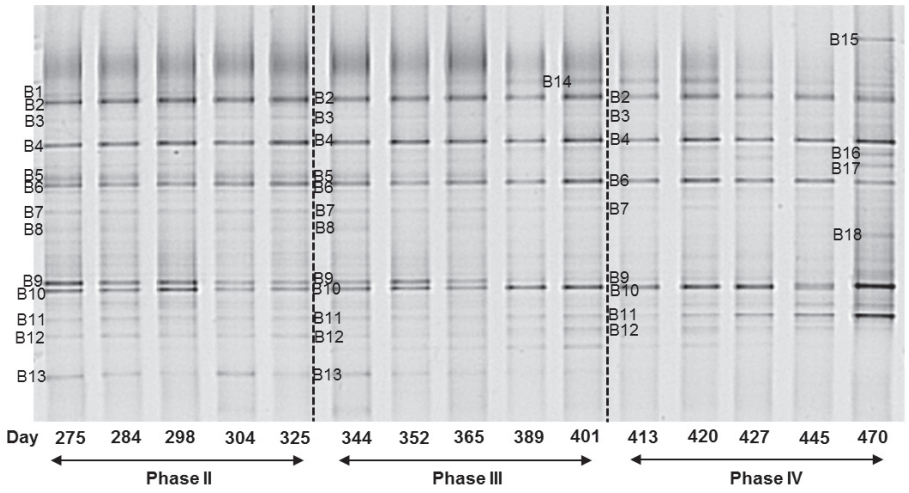

Figure 7.3: Bacterial DGGE results from phases II (11 gNaC/L), III (22 gNaC/L), and IV (33 gNaCl/L). Five representative samples from phases II - IV were taken into account. The operational days can be seen just below gel lanes. Most of the bands indicated by a number were sequenced successfully and used for phylogenetic analysis. Some of them (particularly bands B1, B2, B3, and B16) were not taken into account due to the unsatisfactory sequencing results.

The DGGE profile indicates a greatly diverse bacterial population, with some bands present in all of the experimental phases, albeit at different intensities. Most of the dominant bands appeared over the whole experimental period, while minor variations could be observed. The DNA-derived pattern of the samples taken from the reactor during all the three phases resulted in 18 bands. Some bands were excised from different lanes, and sequencing results showed that their nucleotide sequences were the same. Most of the bands enumerated were sequenced successfully and used for phylogenetic analysis. Conversely, some of them (particularly bands B1, B2, B3, and B16) gave ambiguous sequences and were not included in the analysis. Some bands such as B1, B2 and B3, were even excised from three different gels loaded with the same samples and sequenced, but none of the sequence results showed a pure sequence and therefore had to be neglected.

The banding pattern of phase II resulted in approximately 13 bands (named B1 to B13). These bands remained until the end of this experimental phase, meaning that no variation in the microbial composition occurred within this period. As can be seen from the phylogenetic tree (Figure 7.4), some interesting bacteria were present which are probably directly linked to the main biological conversions taking place in the reactor. Band B9 was closely related to *Candidatus* Accumulibacter, which is commonly considered the main microorganism involved in the enhanced biological phosphorus removal (EBPR) process.

Band B8, which showed high sequence similarity to *Nitrosomonas* sp., and band B13, closely affiliated with *Nitrospira* sp., are representatives of the AOB and NOB populations, respectively. Bands B10 and B11 clustered well with the *Denitromonas* group. Band B5, B6 and B7 were closely related to uncultured bacteria identified in biological phosphorus removal systems belonging to the *Bacteroidetes* phylum. Actually, bands B5 and B6 showed similar mobility in the DGGE gel and gave identical sequences. Therefore, only one was used for the phylogenetic analysis. Band B4 belonged to the Cytophagaceae family, and band B12 was closely related to *Dokdonella soli* (gammaproteobacteria).

In phase III, when the salt concentration was increased to 22 g/L, bands B5, B7 and B9 tended to disappear, showing weaker intensities compared to phase II. A new band (B14), representing an uncultured bacterium belonging to the family Flavobacteriaceae and detected in a saline environment (shoreline), started to appear in the beginning of the phase III, probably due to the affinity for the increased salt concentration.

When the salt concentration was increased from 22 to 33 g/L (transition of phase III to phase IV), bands B8 and B13 completely disappeared. As observed in phase III, the intensity of band B9 continued decreasing, being present just in the beginning of phase IV. The intensity of the band B11 was even increased during the long-term operation at 33 g/L. Curiously, the last sample shown in the gel, collected from the reactor during the end of phase IV (day 470 of operation), showed new bands (B15, B16, B17, and B18). As mentioned before, band 16 did not show satisfactory sequence results. Band B17 is closely related to *Denitromonas* sp., similarly to band B11. Band B18 (*Denitromonas* group) was also closely related to an uncultured bacteria isolated from a denitrifying culture, and band B15 represented an uncultured bacterium belonging to the Cryomorpaceae family, which was isolated from surface seawater.

It should be noted that in two previous DGGE gels where just samples from the phases II and III were loaded, we observed other bands that did not appear in the gel shown in Figure 7.3. All of these extra bands retrieved in preliminary DGGE analysis represented the GAO population and appeared in the down part of the DGGE gel, meaning that these bacteria had a higher GC content than the others. Therefore, it is possible that the bands representing the GAO population have run out of the gel depicted in Figure 7.3. However, even though they were not retrieved in the DGGE gel displayed in Figure 7.3, the bands retrieved in previous DGGE analyses representing the GAO population were taken into account in the phylogenetic analysis.

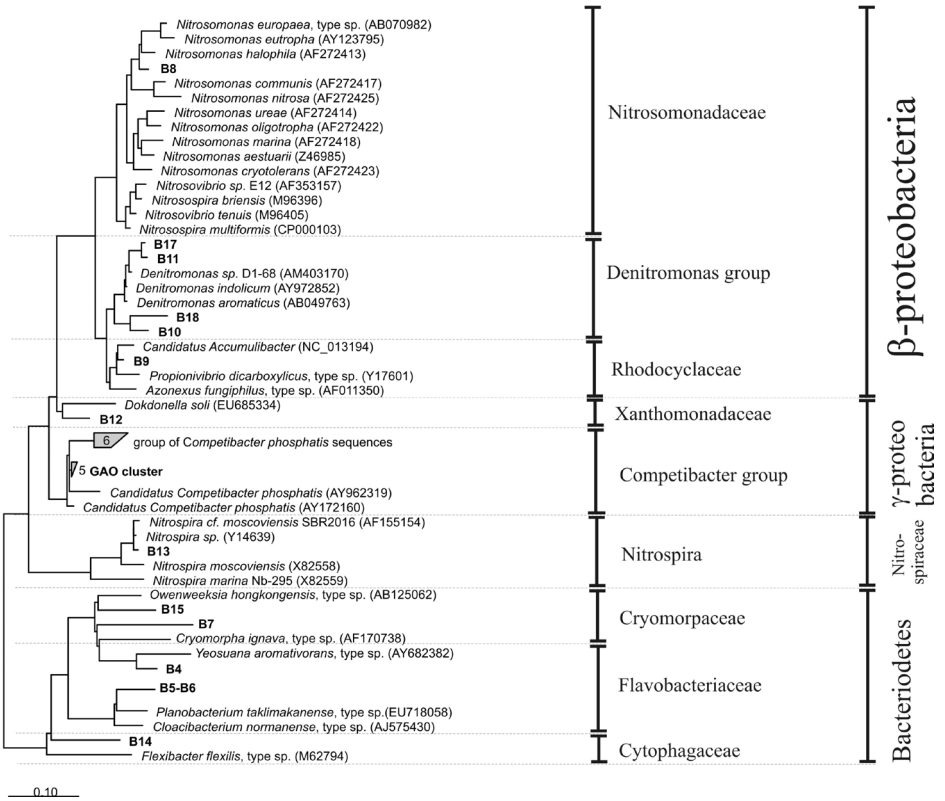

Figure 7.4: Phylogenetic analysis of the bacterial 16S rRNA sequences excised from the DGGE gel. Sequences determined in this study are printed in boldface. The bar indicates 10% sequence difference. The sequence of *Nitrosopumilus maritimus* (Archaea) was used as an outgroup but was pruned from the tree.

7.3.3. Comparative DGGE analysis of DNA and RNA

Since the community structure remained rather stable over time, and taking into account that some bacteria could be present in the granule structure even when they are not active anymore due to the long solid retention time, an analysis to compare the diversity and the activity of the microbial community was performed. The diversity is related to the presence of bacterial populations that are above the detection limit of DGGE and can be inferred by the number of DNA-derived bands in a DGGE gel. Conversely, the activity is assumed to be related to the RNA-derived bands, which reflect the predominant active populations (Dar et al., 2007). Therefore, a RNA analysis was performed to verify which bacterial groups were more metabolically active in the aerobic granules. DGGE profiles obtained with both PCR-amplified bacterial 16S rRNA gene and reverse-transcribed PCR amplified 16 rRNA (cDNA) were compared (Figure 7.5).

DGGE fingerprints of the PCR-amplified and reverse-transcribed PCR amplified 16S rRNA gene fragments were similar. Most of the bands in the DGGE profile from DNA-derived PCR products were also detected in the profiles from RNA-derived PCR products (i.e., cDNA), although their intensities differ in most cases. In this analysis, two representative samples from phases II to IV were taken into account. The respective operational days of each sample are indicated below the gel lanes. Bands were numbered from B1to B13, in the same way as in the previous DGGE gel (Figure 7.3). In accordance with findings for that gel, sequencing results showed that the microorganisms found in each band were the same. The only exception were bands B9 and B10, which switched position according to the sequence determined with the previous DGGE gel. Moreover, the same band excised from different lanes showed identical sequence results.

The intensity of several bands (B1, B2, B3, B4, and B6) decreased from the DNA to the cDNA profile. On the contrary, the intensity of band B8 (closely related to *Nitrosomonas* sp.), increased from the DNA to cDNA banding pattern. This result suggests that *Nitrosomonas sp.* showed high metabolic activity. The intensity of the band referred to *Candidatus* Accumulibacter (retrieved in band B10 but not in band B9, as in the previous DGGE gel) was also higher in the cDNA than in the DNA banding pattern, particularly for phases II and III. During phase IV, band B10 was almost imperceptible. Bands B11 to B13 did not show expression results in the DNA/cDNA DGGE gel.

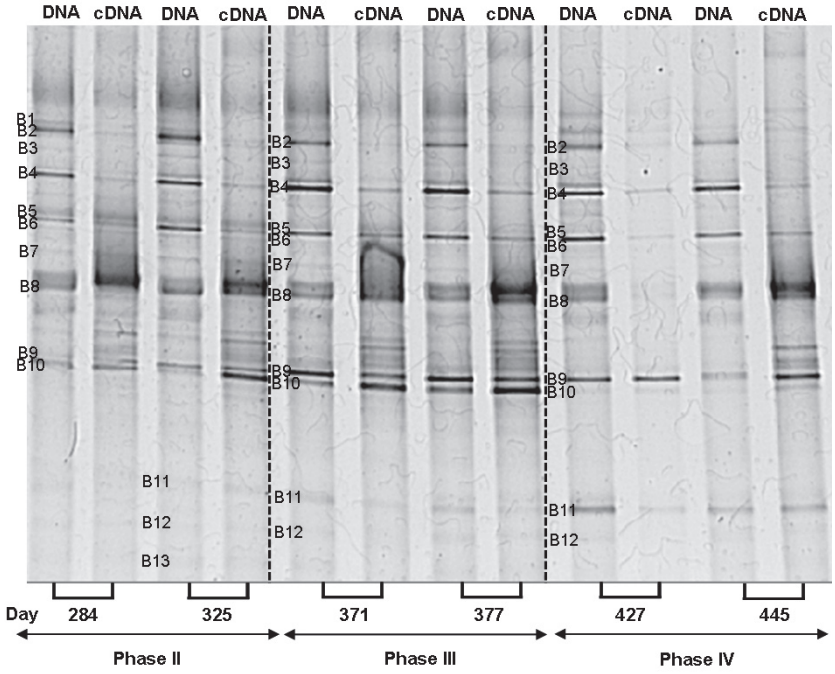

Figure 7.5: DGGE showing the DNA- and RNA(cDNA)-derived bands for each sample. Two representative samples from phases II to IV were taken into account. Bands with the same number retrieved from different lanes gave the identical sequence results.

7.3.4. Ammonia-oxidizing bacteria community structure

To get a further insight into the AOB community in the aerobic granular sludge reactor exposed to increased salt levels, PCR-DGGE analysis using *amoA*-specific primers was performed. The DGGE banding pattern showed only one dominant band in all of the experimental phases (results not shown). The sequence results indicated that this band showed high sequence similarity (more than 99%) with *Nitrosomonas* sp., which is in accordance with the DGGE analysis using 16S rRNA-specific primers. All minor bands excised from the gel gave the same sequence result.

7.3.5. Fluorescent in-situ hybridization (FISH) analysis

Several specific oligonucleotide probes (Table 7.2) were used for FISH analysis in order to complement the PCR-DGGE results and to quantify the different populations among phases II to IV. Results obtained with FISH analysis indicated that almost all bacteria present in the reactor in all operational phases belonged to either the PAO or GAO group (Figure 7.6). From phases II to IV, the fraction of PAO in the whole bacterial community decreased significantly (Figure 7.6a-e). The quantification analysis (Figure 7.7)

of the probe-targeted PAO cells (Cy3, in red) and GAO cells (Fluos, in green) and determination of the fraction of positive signal from each probe relative to the signal visualized with general bacteria probes (Cy5, in blue) revealed that the proportions of PAO and GAO in the whole microbial community in phase II were quite similar. In phase III, the percentage of PAOs decreased while that of GAOs increased. One week after phase IV was started, the fraction of GAOs became even more dominant (Figure 7.6c). Just a few cells of PAOs were present after the second week (Figure 7.6d), and no positive signal was found for these organisms at the end of the third week of phase IV (Figure 7.6e). Nitrifying bacteria cells (AOB + NOB) were detected in substantially lower numbers than the PAOs+GAOs population (Figure 7.6f). FISH analysis also showed that almost all PAOs belonged to clade I (PAOI), which is capable of using nitrate as an electron acceptor for anoxic P uptake.

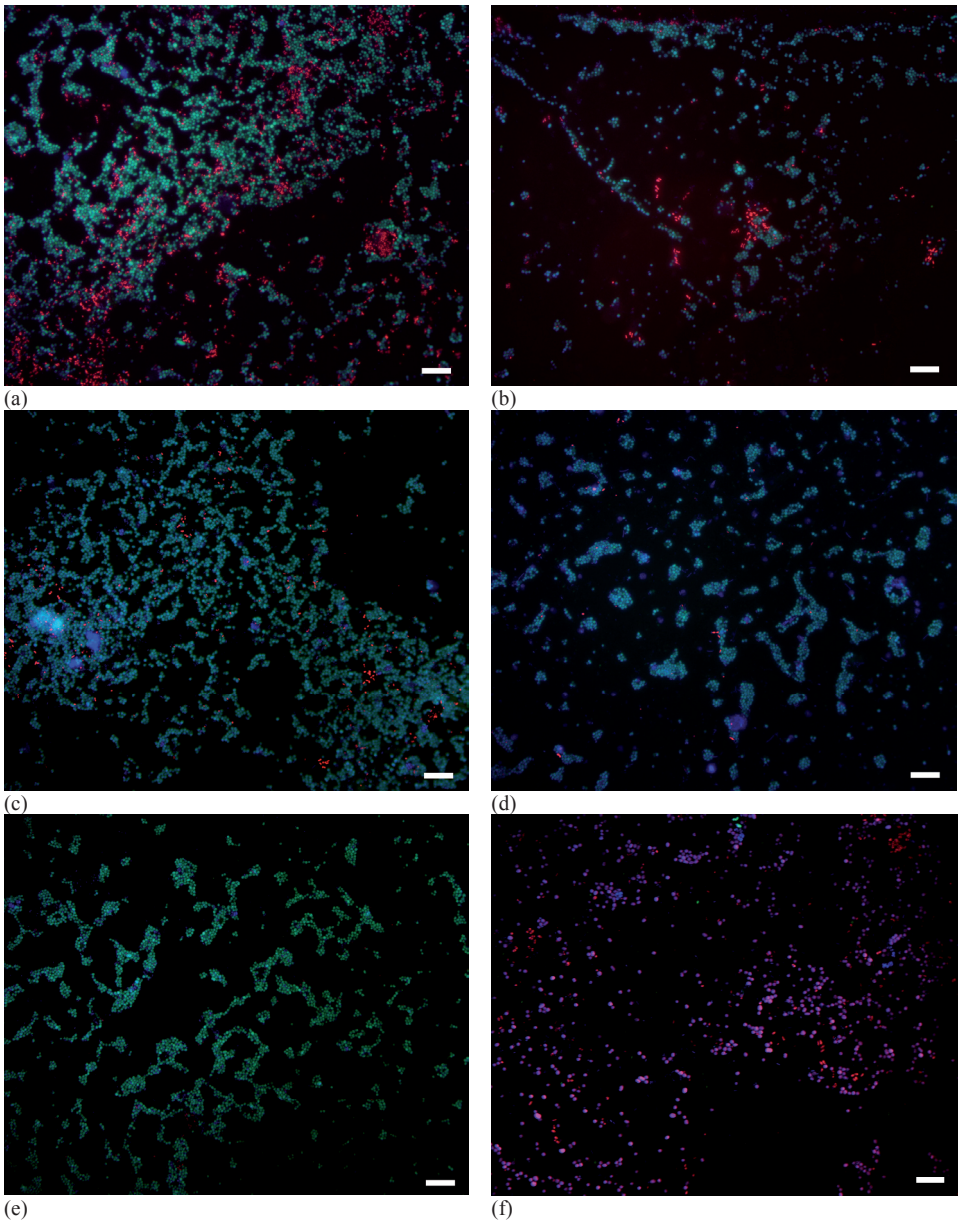

Figure 7.6: Fluorescent in situ analysis of the PAO/GAO populations among all the bacteria present in the reactor at the end of phase II (a), end of phase III (b), first week of phase IV (c), second week of phase IV (d), and third week of phase IV (e) using combinations of specific probes for PAO (PAO462, PAO462, and PAO846, shown in red), GAO (GAOQ431 and GAOQ989, shown in green) and general bacteria EUB338 (EUB338I, EUB338II, and EUB338III; shown in blue). Fraction of PAO+GAO (using PAOmix/GAOmix probes, shown in red) and AOB+NOB (using combination of probes Nso1225/Nso190/Neu653/Ntspa662/Nit1035, shown in green) populations within the whole bacterial community (EUB338 mix) in the granules during phase II (f). Scale bar indicates 20 µm.

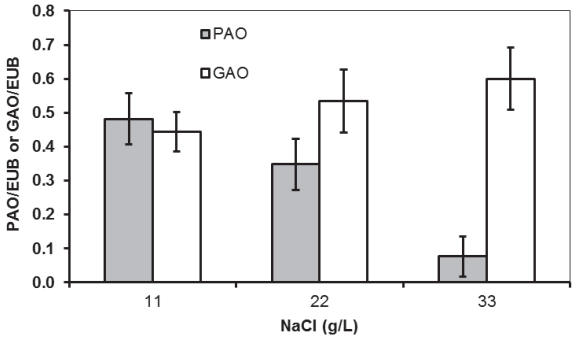

Figure 7.7: Proportion of PAOs and GAOs among the whole microbial community in phases II, III and IV. For the quantification analysis, each data was averaged by at least 10 pictures as described in Materials and Methods.

7.4. Discussion

7.4.1. Effect of increasing NaCl concentrations on PAO-GAO competition

Our results demonstrated that phosphate removal was severely affected by increasing salt concentrations, particularly at 22 and 33 gNaCl/L. Both phosphate release during anaerobic feeding and phosphate uptake in aeration phase were observed to decrease gradually from phases II to IV. P release has been reported to significantly drop when salinity was increased, especially above 5 gNaCl/L (Cui et al., 2009). Phosphorus removal was completely inhibited when chloride concentration was higher than 2.5 gCl^-/L (~4 gNaCl/L) in an experiment performed by Hong et al. (2007) using an anaerobic/anoxic/oxic reactor. The effect of salt on phosphorus removal was also reported by Uygur and Kargi (2004), who described a decrease in P removal from 84 to 22% when the NaCl concentration was increased from 0 to 6% (w/v).

Our results from the reactor cycle measurements suggested that the considerable amount of nitrite accumulated during the operational cycle at salt concentrations equal to or greater than 11 gNaCl/L possibly contributed to the reduction of phosphorous removal capability. As reported in the literature, nitrite can negatively affect P uptake activity of PAOs under both anoxic and aerobic conditions. Concentrations of up to 2 $mgNO_2^-$-N/L are already inhibitory for PAOs according to Saito et al. (2004), who reported the complete inhibition of aerobic P uptake at nitrite concentrations above 6 mgN/L. A severe inhibition of both anoxic and aerobic P uptake at 6 to 8 $mgNO_2^-$-N/L was also reported by Meinhold et al. (1999). In our work, the effect of salt was detrimental to NOB, which was reflected in the accumulation of nitrite. As a cascade effect, phosphate uptake was reduced when nitrite concentrations were above 4 mgN/L.

When nitrite concentrations started to decrease the P uptake rate increased, which suggested a reversible inhibitory effect. Therefore, the gradual deterioration of phosphate removal likely was caused by a combined effect of both salt and nitrite. However, further research is necessary to confirm the detrimental effect of nitrite on the activity of PAOs. Pijuan et al. (2011) reported that free nitrous acid (whose concentration depends on nitrite concentration and pH) had a stronger effect on PAOs than on GAOs, giving a competitive advantage to GAOs in EBPR systems. This observation is also in line with our results, which showed the enrichment of GAOs and the disappearance of PAOs as the salt concentration was increased and nitrite accumulated and started to be left over at the end of the cycle.

From our analysis of the microbial community structure, we conclude that the decrease in phosphate removal observed during phase III (22 gNaCl/L) is directly linked to the gradual disappearance of the microorganism closely related to *Candidatus* Accumulibacter (band B9 of the 16S rRNA DGGE gel). This trend continued in the beginning of phase IV (33 gNaCl/L), when band B9 was hardly detected and P release and uptake still were occurring, although to a minimal extent. A complete absence of those particular microorganisms during the course of phase IV also coincided with the complete deterioration of P release and removal. Batch experiments confirmed that the long exposure of the biomass to 33 gNaCl/L was detrimental for *Candidatus* Accumulibacter and consequently for phosphate removal. In the batch test using biomass adapted to 22 g/L, the P removal still was observed at 30 gNaCl/L. This suggests that *Candidatus* Accumulibacter still is capable of P removal at 30 gNaCl/L, but it is outcompeted by GAOs in the bioreactor. It should be pointed out that even though granulation was stable during the whole experiment, the microbial population structure was changing faster than can be expected if no growth occurs (based on SRT). During phase IV (33 gNaCl/L), PAOs completely disappeared in 3 weeks according to FISH analysis despite an SRT of 30 days.

In parallel to the disappearance of the PAOs, we observed that GAOs became more dominant at increasing salt concentrations, as was demonstrated using FISH analysis. GAOs can perform carbon transformations similar to those of PAOs, but no release/uptake of phosphorus is involved in their metabolism, and they are considered competitors for PAOs (Oehmen et al., 2006). GAOs were shown to dominate laboratory-scale cultures fed with acetate (Crocetti et al., 2002; Zeng et al., 2003; Oehmen et al., 2004) and usually are associated with the deterioration of EBPR systems

(Oehmen et al., 2006). Some operational factors, such as a long SRT (Fukase et al., 1984), excessive aeration (Brdjanovic et al., 1998), high temperatures (Lopez-Vazquez et al., 2007), and a low phosphorus/carbon ratio in the feed (Liu et al., 1997) are described in the literature to favour the growth of GAOs over PAOs. In our study, the dominance of GAOs at the expense of decreasing numbers of PAOs was directly related to the increase in salt concentration. From our experiments, we observed that PAOs could adapt quite well to 11 gNaCl/L and reasonable well to 22 gNaCl/L, but they could not tolerate 33 gNaCl/L, losing the competition with GAOs. So far, no report has shown that high salinity levels favour GAOs over PAOs, and our study is the first to show the complete disappearance of PAOs and dominance of GAOs under such conditions.

7.4.2. Effect of increasing NaCl concentrations on nitrogen conversions

We have shown that the ammonium removal rates were not affected by increases in salt concentration up to 33 gNaCl/L. The only work described in the literature that investigated the effect of salt on nutrient removal using aerobic granules was done by Figueroa et al. (2008), who observed a slight decrease in ammonium removal efficiency when salt content was higher than 10 gCl^-/L (around 16 gNaCl/L). In our study, the relatively long period of operation (more than 300 days) at moderate salt concentration, particularly 11 gNaCl/L, seemed to improve the salt tolerance of the ammonium-oxidizing bacteria, suggesting that this acclimation procedure can be used to successfully achieve good performance of ammonium removal at high salinity levels.

In spite of the fact that ammonium removal was not affected when the salt concentration was increased, we observed that the only AOB encountered by DGGE in the system (belonging to *Nitrosomonas* group) disappeared during the course of phase III. This indicates that either other ammonium-oxidizing bacteria not detected in DGGE analysis were metabolic active in the system (i.e. oxidizing ammonium to nitrite) or the population of *Nitrosomonas* decreased in such way that it was not detected anymore by the DGGE analysis of amplified 16S rRNA fragments. To investigate in more detail the AOB population, PCR-DGGE was done using *amoA* gene-based primers as molecular markers. As opposed to the DGGE profile derived from 16S rRNA genes, the analysis using *amoA*-specific primers showed that *Nitrosomonas* sp. was present during the full experimental period. This observation is in line with the operational performance of the bioreactor. Since the same sequencing result was derived from the bands excised from 16S rRNA and *amoA* DGGE analysis, it can be inferred that the composition of the

AOB population did not change but that the organisms adapted to increased salt concentrations.

As demonstrated by FISH analysis, nitrifying bacteria represented just a small fraction (between 1 and 2%) of the total microbial community dominated by PAOs and GAOs. Given the small fraction of AOB in the system, it is possible that during amplification through PCR using 16S rRNA gene-targeted group-specific primers some AOB could not be amplified. This result emphasizes the importance of using specific primers for functional genes to detect slow-growing microorganisms in a complex mixture of microorganisms. It should be noted that the *amoA* gene analysis does not reflect a quantitative evaluation but a qualitative one. Therefore, it is based simply on the presence or absence of a particular bacterial group, and it does not give information on their quantity. Even if a specific microorganism is present in a substantially small amount, as the nitrifying bacteria in our case, functional genes primers can detect the target bacteria. Moussa et al. (2006) also verified that *Nitrosomonas europaea* was the only detectable ammonium-oxidizing species at high salt concentrations (30 and 40 gNaCl/L), although they observed other species of AOB at lower salt concentrations (up to 16 gNaCl/L).

A perfect match between the analytical data and the microbial diversity also was obtained when considering the nitration step. The change from nitrate to nitrite as the main nitrification product was accompanied by the complete disappearance of the only NOB detected in DGGE analysis, which was closely related to *Nitrospira* sp. This is particularly clear during operation at 33 gNaCl/L (phase IV), when high nitrite concentrations were observed. Batch experiments using biomass adapted to 33 gNaCl/L also showed that practically all the ammonium was oxidized to nitrite at all NaCl concentrations tested. This confirmed that the NOB were strongly inhibited at 33 gNaCl/L. Similarly, Chen et al. (2003) observed that nitrite oxidizers disappeared when salt concentrations were increased from 10 to 18.2 g Cl^-/L (16 to 30 NaCl/L). Moussa et al. (2006) found that *Nitrospira* sp. was the dominant nitrite oxidizer at salt concentrations of up to 10 g/L Cl^- (16.5 gNaCl/L). Several works described in the literature mentioned that *Nitrospira* was the dominant NOB (Schramm et al., 1998; Burrell et al., 1998; Okabe et al., 1999), although most of them are not related to operation at high salinity conditions.

From our experiment, we can conclude that the AOB in the granular sludge system investigated were much less susceptible to osmotic stress than the NOB, which

is in agreement with many former observations (Schenk and Hegemann, 1995; Dincer and Kargi, 1999; Dincer and Kargi, 2001; Cui et al, 2009; Sánchez et al., 2004).

7.4.3. Additional microbial community structure changes at increasing salt concentrations

Apparently, microorganisms belonging to the *Denitromonas* group have higher affinity for high salt concentrations, since the intensity of some bands, such as B10 and B11, increased as salinity levels were increased. Moreover, other bands, like B17 and B18, representing *Denitromonas* species, also appeared during the long-term operation at the highest salinity level tested, when a highly turbid effluent was observed. In our previous work regarding the effect of salt on the microbial diversity of nitrifying sludge (Bassin et al., 2012), we also observed the appearance of *Denitromonas sp*. at high salt concentrations (20 gNaCl/L). *Denitromonas* is a genus of *Betaproteobacteria,* which are found in considerable numbers in denitrifying reactors (Etchebehere et al., 2003). These organisms are scarcely mentioned in the literature, and the only research which reported the presence of *Denitromonas* in very high salinity conditions (6% NaCl) was performed by Xiao et al. (2010).

Giver that actively growing cells contain increased levels of rRNA, as has been described in previous research (DeLong et al., 1989; Poulsen et al., 1993; Pichard and Paul, 1993; Aoi et al., 2004), while dormant cells showing low metabolic activity are associated with low rRNA content (Witzig et al., 2002), the band intensity of the cDNA profile in DGGE analysis can be related to the ribosome content and provides information about the activity of the microbial population. From the comparative analysis of the diversity (DNA-derived pattern) and the activity (cDNA-derived pattern) of the microbial population, we observed that the high metabolic active microorganisms were those related to ammonium (*Nitrosomonas* sp.) and phosphate removal (*Candidatus* accumulibacter), a result supported by the increased intensity of their corresponding bands from the DNA to cDNA banding patterns. For the *Candidatus* Accumulibacter in particular, the high metabolic activity was detected only up to 22 gNaCl/L, again confirming the detrimental effect of their exposition at 33 gNaCl/L. All other bands corresponded to microorganisms with low metabolic activity, most of them belonging to the Bacteroidetes group, which represents heterotrophic bacteria probably growing at the expense of other active bacteria (i.e., PAOs, GAOs, and nitrifiers), and they were not crucial for the biological processes occurring within the granules. The

relevance of this finding lies in the fact that although the microbial population within the granules is considerably diverse, just the microorganisms directly related to the main biological conversions, such as PAOs and AOB, have shown to have high metabolic activity.

7.5. Conclusions

Our results demonstrate that *Nitrosomonas sp.* and *Nitrospira* were the only AOB and NOB, respectively, found in the system. AOB could tolerate NaCl concentrations up to 33 g/L (stable and complete ammonium removal), while NOB were severely affected (high nitrite accumulation). The increase in salt concentration had a strong effect on phosphorus removal. This observation could be directly linked to the gradual disappearance of *Candidatus* Accumulibacter phosphatis. P release and uptake stopped completely at steady-state operation at 33 gNaCl/L. At high salinity, PAOs were outcompeted by GAOs in the bioreactor. Our findings related to process performance and molecular diversity analysis could help further optimization of the simultaneous biological nitrogen and phosphorus removal processes in high salinity environments.

References

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Clean water drop
Photo credit: Edward Aish

Outlook

Chapter 8

Content

8.1. Aim of the research and evaluation

The main objective of this thesis was to investigate important aspects related to nutrient removal in currently emerging biofilm reactor technologies. A wide range of topics involving the nitrogen and phosphorus removal processes were evaluated. Moving bed biofilm reactors and aerobic granular sludge reactors are currently among the most studied emerging technologies. These systems were studied on bench-scale models combined with biomass characterisation in batch experiments and molecular ecology tools. The combination between molecular ecology techniques to study microbial diversity of engineered bioreactors and process conversions significantly enhanced the understanding of the reactor systems studied in this research.

Besides increasing knowledge of several important theoretical aspects regarding biological nutrient removal processes, the findings of this research will be useful for diverse practical applications in the wastewater treatment segment. The main conclusions obtained from the different topics investigated in this thesis are summarized below:

- The application of a heterotrophic start-up phase as a strategy to decrease the time necessary for developing nitrifying biofilms in moving bed biofilm reactors was proposed. The experimental findings of this research can potentially be used in industrial settings, especially when nitrification should be accomplished in wastewaters with limited or no organic carbon (**Chapter 2**).
- In a comprehensive characterization of the main process conversions taking place in two aerobic granular sludge reactors performing simultaneous nitrogen and phosphate removal and operated at different temperatures (20 and 30°C), low dissolved oxygen concentration was observed to favour the development of denitrifying polyphosphate-accumulating organisms (DPAOs). Hence, denitrification coupled to anoxic phosphate uptake (denitrifying dephosphatation) was sustained in the granular systems. Based on experimental evidence, denitrification was proposed to proceed mainly via the nitrate pathway. Denitrifying glycogen-accumulating organisms (DGAOs) were the dominant organisms reducing nitrate to nitrite in both reactors, which was further reduced to nitrogen gas concomitant with anoxic phosphate uptake by PAO clade II (PAOII). An improved operational strategy developed to control PAO-GAO competition by selective sludge discharge mainly from the GAO-

rich top of the segregated reactor sludge bed proved to enhance phosphate removal efficiency, particularly at tropical temperatures (30°C) (**Chapter 3**).

- From the analysis of the nitrifying microbial population of an aerobic granular reactor operated at 30°C, an unexpected high nitrite-oxidizing bacteria/ammonium-oxidizing bacteria (NOB/AOB) ratio was observed by means of quantitative polymerase chain reaction (qPCR). Interestingly, nitrite oxidation capacity was found to be around three times higher than the ammonium oxidation capacity in separate activity batch tests. These unexpected results suggested that the growth of NOB (*Nitrobacter*) was partly uncoupled from the lithotrophic nitrite supply by AOB. Based on the experimental evidence and literature review, two hypotheses were considered to explain the NOB/AOB ratio obtained: either *Nitrobacter* grew mixotrophically by acetate-dependent dissimilatory nitrate reduction ('ping-pong' effect) or a nitrite oxidation/nitrate reduction loop (*nitrite loop*) occurred in which denitrifiers reduced nitrate to nitrite supplying additional nitrite for the NOB apart from the AOB (**Chapter 4**).
- Aerobic granular sludge from lab- and pilot-scale reactors was observed to adsorb a considerable amount of ammonium under anaerobic conditions. The amount of ammonium adsorbed to aerobic granules was much higher than that occurring in activated sludge and anammox biomass. The high ammonium adsorption to the granular biomass was attributed to the presence of K-struvite (potassium magnesium phosphate), which acted as potassium source for ion-exchange with ammonium. The impact of this research is that ammonium adsorption cannot be neglected, particularly in granular sludge bioreactors that are characterized by strongly variable ammonium concentrations as a function of place (plug flow systems) or time (batch systems) (**Chapter 5**).
- Different salt (NaCl) adaptations strategies applied to two sequencing batch reactors containing suspended nitrifying sludge caused different changes in microbial community structure. However, no change in the nitrification performance was observed, despite of the different microbial populations in each reactor. Specific ammonium oxidation rates were more affected when salt increase was performed more rapidly. A gradual increase in NaCl concentration had a positive effect on the settling properties, as evidenced by a decrease in the sludge volume index (SVI). However, higher washout of light and poor settling

flocs was observed as water density was increased with salt increase (**Chapter 6**).

- In an aerobic granular sludge subjected to salt increase from 0 to 33 gNaCl/L, ammonium removal efficiency was not affected even at the highest salt concentration tested. However, a high nitrite accumulation was observed at salt concentrations exceeding 22 gNaCl/L, which coincided with the disappearance of *Nitrospira* sp. The salt increase severely affected the phosphate removal process, which completely deteriorated at 33 gNaCl/L. The polyphosphate-accumulating organisms (PAOs), *Candidatus* Accumulibacter phosphatis, were not detected anymore at this salinity level and were outcompeted by glycogen-accumulating organisms (GAOs) in the bioreactor (**Chapter 7**).

8.2. Outlook

8.2.1. Compact biofilm systems versus conventional activated sludge systems

The use of biofilm-based systems within the environmental biotechnology field offer several advantages compared to conventional activated sludge processes. One of the most important characteristics of biofilm systems is the biomass retention, which allows increasing the volumetric conversion capacity when the conversion is limited by the amount of biomass present in the reactor. In bioreactors with no biomass retention, like for instance in chemostats, the biomass concentration primarily depends on the influent substrate concentration. As a result, large hydraulic retention times are necessary to treat diluted influent wastewaters (Nicolella et al., 2000).

Depending on the settling properties of the microbial aggregates, biomass can be easily separated by sedimentation from the bulk liquid and returned to the reactor. However, settling problems are common in activated sludge-based systems, leading to washout of biomass, loss of volumetric treatment capacity and poor effluent quality. Presenting better settling properties than activated sludge flocs, particle-supported biofilms and self-immobilized granular biomass can be more easily separated from the bulk liquid, allowing higher biomass concentration in the reactor and higher reactor specific biofilm surface area. In these biofilm systems, settlers for biomass retention and recirculation are usually not needed, decreasing the plant footprint and complexity to a large extent.

Biofilm systems are spatially heterogeneous. Different redox conditions result from mass transfer limitations in the biofilm and an operation in sequencing batch mode allows for introduction of periods with and without aeration. Thus, simultaneous biological processes, such as nitrification, denitrification, and phosphate removal can occur in the aerobic and anaerobic/anoxic microenvironments. Aerobic granular sludge, which is a promising technology in environmental biotechnology, was used in this thesis for simultaneous organic matter, nitrogen and phosphorus removal.

The use of biofilm-based systems is also especially relevant when slow-growing organisms, such as nitrifying bacteria, must be retained in the reactor. Moving bed biofilm reactors were applied in this thesis to favour the attachment of nitrifiers to the plastic carrier materials, protecting these bacteria from washout. In the case of reactors with suspended biomass, process disturbances caused by external inhibitors or inadequate operation can easily lead to biomass washout. In this thesis, sequencing batch reactors containing suspended nitrifying sludge were subjected to high salt concentrations. An increased concentration of solids in the effluent was observed as salinity was increased. Elevated salt concentration increases the density of water, which can potentially cause the washout of poor settling sludge flocs (light flocs). In turn, the volumetric treatment capacity drastically decreases. The increase of effluent solids content due to biomass washout can be prevented by using particle-supported biomass or even biomass with high settling velocities, such as aerobic granular sludge.

8.2.2. Aerobic granular sludge (AGS) versus moving bed biofilm reactor (MBBR)

Different studies were carried out in this thesis making use of aerobic granular sludge and moving bed biofilm systems. A general comparison can be made between these two technologies applied in wastewater treatment. An important difference is that the biomass concentration that can be retained in AGS systems is much higher than in MBBRs. Biomass concentrations (expressed as total suspended solids (TSS)) as high as 6 – 12 g/L have been obtained in AGS SBRs (Tay et al., 2002; Liu and Tay, 2004; de Kreuk et al., 2005). In this thesis, experiments with AGS allowed to reach biomass concentrations up to 20 g/L. As a result of high solids retention capacity, higher organic and nitrogen loading rates can be applied to the granular biomass reactors.

On the other hand, Johnson et al. (2000) reported that the total suspended solids in different full-scale MBBR systems was lower than 2 g/L. The maximum biomass concentration fixed to the carriers materials in a pilot-scale MBBR was around 2.9

gTSS/L (Broch-Due et al., 1994). Salvetti et al. (2006) obtained a maximum solids concentration of approximately 3.0 gTSS/L, which is similar to that observed in our experiments. The volumetric conversion rates that can be obtained in MBBR systems are therefore much lower than in AGS reactors. However, MBBR technology can easily be applied to upgrade and enhance the capacity of existing conventional biological wastewater treatment plants with minimal plant downtime, no need for extra tank (small footprint) and reduced process complexity and operator attention. In space-limited sites, the system improvement can be carried out without additional use of land. By retrofitting the conventional plant with retention screens, plastic carrier materials (biomedias) and sufficient aeration, the performance of the treatment system can be enhanced significantly to meet more stringent regulatory effluent discharge requirements at reduced costs. Moreover, the treatment capacity can be simply extended by adding more biofilm carrier elements to the existing plant, increasing the active surface area the system with minimal process adjustments. The combination of MBBR technology (biofilm process) and conventional activated sludge process (suspended biomass) is often regarded as Integrated Fixed Film Activated Sludge (IFAS®). The MBBR/IFAS® system allows nitrification to occur even at low sludge retention time (no need to increase the hydraulic retention time), since nitrifiers can grow attached to the biomass moving carriers. Furthermore, if process disturbances occur and lead to considerable biomass washout, the biomass attached to the carriers remains in the aeration tank, allowing quicker recovery of the biological system.

The capacity and performance of existing conventional activated sludge-based plants can also be enhanced by using aerobic granular sludge. Classified as a type of self-immobilized microbial consortium, aerobic granular sludge does not require carrier materials as MBBR does. This represents an important feature of the granular technology. MBBR carriers are often patented by commercial companies, increasing the implementation costs of this technology. However, for practical application of AGS technology, either a certain amount of granules should be available to speed up the granulation process or the operational conditions of conventional activated sludge plants should be changed in order to favour only the development of fast settling particles, while washing out light activated sludge flocs. To accomplish this, important selection pressures (such as short settling time) should be applied to successfully obtain aerobic granulation in reactors operated in sequencing batch mode.

8.2.3. Ammonium adsorption to aerobic granular sludge and struvite formation: potential for nutrient removal and recovery

A considerable amount of ammonium adsorbed to aerobic granular sludge was attributed to the presence of potassium magnesium phosphate (K-struvite) in the biomass, acting as a potassium source for ion-exchange with ammonium. Struvite (ammonium magnesium phosphate) or K-struvite (potassium magnesium phosphate) was found to accumulate in aerobic granular sludge due to induction of precipitation by higher pH levels inside the granular sludge. The alginate-like exopolysaccharides likely function as a crystallisation nucleus. Whether K-struvite or ammonium struvite is the type of struvite present depends on the availability of potassium and ammonium ions in the bulk. If complete nitrification is obtained, as in our experiments with aerobic granular sludge, K-struvite is the predominant stuvite form. Ammonium struvite will dominate if residual ammonium (as a consequence of incomplete nitrification) remains in the granular sludge reactor.

Stuvite is formed when the chemical constituents reach levels of supersaturation. Struvite formation requires nucleation sites and is more likely to occur in systems in which CO_2 is released to the air, causing a pH increase (Baker et al., 2006). Besides the operating pH, the adjustment of the molar ratios of magnesium, ammonium and phosphate are the most important process parameters in ammonium magnesium phosphate crystallization (Ohlinger et al., 1998). Furthermore, the presence of calcium has been shown to be determinative for both crystal size and purity (Le Corre et al., 2005; Pastor et al., 2007).

Traditionally, phosphate removal technologies in wastewater treatment plants have consisted of biological processes, chemical precipitations and combinations of biological process with tertiary treatment (Momberg and Oellermann, 1992). Due to the limited natural resources of phosphorus, a suitable recovery of phosphorus from wastewater is an important feature of future wastewater treatment processes. There is an increasing interest in struvite precipitation as an interesting alternative and feasible approach to achieve nutrient (phosphorus and nitrogen) removal (von Munch and Barr, 2001; Gonzalez and De Sa, 2007; Battistoni et al., 1997). Phosphorus recovery as struvite from several wastes, like for instance agricultural manure and municipal sewage, makes the recycle of this nutrient into agriculture as fertilizer possible. In this context, integration of struvite formation as a side-stream treatment of phosphate-rich wastewaters in crystallisation reactors is becoming a common practise (Caffaz et al.,

2008) and a significant amount of research is ongoing into chemical and reactor parameters, which will facilitate optimization of process operation.

The considerable amount of research on aerobic granular sludge highlights the importance of investigating further the potential nutrient recovery through struvite precipitation, which can contribute to enhance phosphate removal efficiency. To effectively include biologically induced struvite precipitation in aerobic granular sludge, some process conditions should be assured. Since local precipitation as stuvite depends on the levels of supersaturation of its chemical constituents, the simultaneous presence of considerable amount of ammonium, magnesium and phosphate is necessary. K-struvite is the predominant form if complete nitrification is accomplished, whereas ammonium struvite will dominate if residual ammonium is present.

Up to now, few works have assumed (but not directly demonstrated) the occurrence of phosphate precipitation in aerobic granular sludge (de Kreuk et al., 2005; Yilmaz et al., 2007; de Kreuk and van Loosdrecht, 2006). Angela et al. (2011) first proved that the biologically induced precipitation of hydroxyapatite can be relevant for the overall phosphate removal. In our study, K-struvite was the predominant precipitate found in the granular biomass, since no residual ammonium was observed (complete nitrification achieved). Major factors influencing biologically induced precipitation (e.g., pH gradients, CO_2 stripping caused by aeration, anaerobic phosphate release by polyphosphate-accumulating organisms, nitrification and denitrification) require further investigation since this phenomenon consists of an interesting way to immobilize phosphorus in a stable and reliable product.

8.2.4. Effect of salt on suspended and granular biomass reactors

The performance of biological treatment processes is known to decrease at high salt concentrations due to adverse effect of salt on microbial flora. In this thesis, two different investigations on the effect of salt were conducted. Firstly, the effect of different salt adaptation strategies on microbial diversity and activity of suspended sludge accomplishing nitrification was addressed. Secondly, the effect of elevated salt concentrations on the aerobic granular sludge process developing simultaneous organic matter, nitrogen, and phosphate removal was investigated.

Even though the objective and research questions were quite different between these two works, some comparisons can be made. As salt was increased, the microbial population structure assessed by 16S rRNA targeted DGGE remained much more stable

in the aerobic granular sludge system compared to the suspended nitrifying sludge reactor. Moreover, the ammonium oxidation capacity in the aerobic granular sludge reactor was stable regardless the salt concentration applied (from 11 to 33 gNaCl/L). Conversely, ammonium uptake rates considerably decreased in the suspended nitrifying sludge as salinity was increased until 20 gNaCl/L. The strong and compact structure of aerobic granules likely provided a protection to the microorganisms against the adverse effect of salt. The microorganisms present in the outer layer of the granules (for instance ammonium-oxidizing bacteria (AOB)) could indeed be negatively affected by the salt, leading to a decrease on the activity of this particular bacterial population. As a consequence of biomass decay in the outer layer of the granule, oxygen penetration may have reached deeper regions of the granular biomass (in which diffusional resistance to salt may occurred), where other AOB may have had the chance to grow. This could have in turn compensated the loss in ammonium oxidation activity due to salt increase in the granular system.

Another important aspect to be discussed is the higher biomass washout due to settling problems at high salt concentrations and consequent loss of volumetric capacity in the study with suspended nitrifying sludge. Lighter flocs may not settle fast enough to be maintained in the sequencing batch reactor when the density of the bulk liquid is increased as a result of salt increase. Conversely, in the aerobic granular sludge systems, where biomass is mainly composed by fast-settling particles, problems related to poorly settling biomass is not an important issue.

It would be interesting to carry out studies on the effect of salt on aerobic granular sludge and conventional activated sludge processes, both developing similar biological processes (e.g., nitrification, denitrification, and phosphate removal). These investigations should not only be limited to the main biological conversions but should point to the differences between the microbial community structures of the two processes running on similar operational conditions (e.g., influent composition, and dissolved oxygen concentration). The results obtained from chemical analysis and molecular approaches should be combined for a complete overview of the susceptibility of each type of biomass to inhibition caused by high salt concentrations.

8.2.5. The relevance of molecular techniques applied to environmental engineering processes

Microbial ecology provides the scientific foundation for the processes applied to accomplish the practical goals of environmental biotechnology, whereas the processes in environmental biotechnology consist of interesting ecosystems for microbial ecologists to advance their methods and concepts (Rittmann, 2006). Besides highlighting the importance of microbial ecology, the revolutionary advances in molecular methods which have occurred in the past two decades have been improving the understanding of biological processes taking place in wastewater treatment systems, helping to increase their stability and efficiency (Daims et al., 2006; Rittmann, 2006). Thanks to cultivation-independent molecular techniques (some of them described in item 1.1.7), the study of complex microbial communities of engineered treatment plants turned out to be possible. As a consequence, new insights into uncultured bacteria were provided and key microorganisms involved in treatment processes were identified.

Some research topics studied in this thesis are examples of how microbial ecology of wastewater treatment systems, in combination with modern molecular approaches, offers outstanding possibility to investigate the role of some microorganisms in specific conversions, observe the selection and enrichment of specific microbial populations according to the experimental conditions applied, improve treatment efficiency and better understand biochemical conversion pathways. In Chapter 2, for instance, fluorescent in situ hybridization (FISH) technique was applied to observe the enrichment of biofilm for nitrifiers in MBBR systems. This technique was also applied in the study described in Chapter 3, allowing the observation of biomass segregation along the sludge bed of aerobic granular sludge sequencing batch reactors. This, in turn, offered a good opportunity to conduct microbial population engineering to select for the desired microorganisms and hence increase phosphate removal efficiency. Moreover, the identification of different lineages of organisms responsible for the bio-P removal process by using specific oligonucleotide FISH probes allowed for the clarification of the denitrification pathway in the aerobic granular systems.

Quantitative PCR (qPCR) was another molecular tool applied in a study of this thesis in which the nitrifying microbial community within aerobic granular sludge systems was investigated (Chapter 4). The results brought up from this technique were quite interesting, showing that nitrite oxidizers were in higher numbers than ammonium

oxidizers. This unexpected result was in line with activity measurements, showing that nitrite oxidation capacity was higher than the ammonium oxidation capacity. These surprising findings provoked us to find out the possible reasons for such unusual proportions of ammonium and nitrite oxidizers in aerobic granular sludge reactors.

Denaturing gradient gel electrophoresis (DGGE) was employed to monitor shifts in microbial population of bioreactors exposed to increasing salt concentrations. In Chapter 6, the application of this molecular method enabled us to observe how different salt adaptation strategies influence the bacterial community in sequencing batch reactors with suspended nitrifying sludge. DGGE was also very useful to detect variation over time in the microbial community structure as salinity was increased in an aerobic granular sludge reactor (Chapter 7). The comparison of DNA and cDNA DGGE fingerprint profiles enable us to observe high metabolically active bacteria. In this particular work, FISH was used to quantify dominant bacterial populations and validate PCR-based results.

The studies developed in this thesis are only few of the many promising opportunities arising from the use of molecular tools to better understand and manage biological wastewater treatment systems, enhancing their performance. The benefit obtained from the molecular studies can be much greater. For instance, environmental genomics provides a deeper insight into ecophysiology and evolution of microorganisms present in wastewater treatment reactors. Comparative genomics can provide information about the genomic variability among functionally similar organisms, allowing us to understand in a better way the relationship between structure and function of microbial communities (Daims et al., 2006). As a consequence, the stability of key processes in wastewater treatment plants can be better understood. On the other hand, the applicability of the information gained from the molecular ecology studies depends on the ability of environmental engineers, who often consider biological treatment systems as a "black box", to transform all the accumulated knowledge into new technical implementations to obtain the desired success. To achieve that, one of the best ways is to merge the practical and fundamental experience of these engineers with the fundamental research in microbial ecology developed by microbiologists. Better integration between these two inherently tied disciplines is absolutely needed to make the best use of the great potentials of environmental biotechnology.

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Summary

Summary

Biological Nutrient Removal in Compact Biofilm Systems

The removal of nutrients such as nitrogen and phosphorus from both domestic and industrial wastewaters is imperative since they potentially harm the environment. One of the main consequences of excessive availability of nitrogen and phosphorus in aquatic ecosystems (freshwater, marine and estuarine) is the overgrowth of algae and other aquatic plants, a phenomenon designated as eutrophication. Algae and aquatic plants induce depletion of oxygen in water basins, resulting in massive death of eukaryotic organisms in the ecosystem and a decrease in biodiversity. Human activities have increased the input of nutrients to aquatic resources beyond its natural capacity to assimilate them, resulting in acceleration of the eutrophication process.

Nutrient removal from wastewaters by biological processes is cost effective and offers many advantages compared to physical-chemical processes. Essentially, biological nitrogen (nitrification and denitrification) and phosphorus removal involves a series of biochemical processes carried out in an appropriate sequence of aerobic, anoxic and anaerobic environments. In conventional activated sludge-based processes, these conditions are provided in separate tanks (multistage treatment processes), demanding large land areas for the wastewater treatment plant installation. The low biomass concentrations in the traditional activated sludge systems and especially the need for clarifiers for biosolid-liquid separation contribute to the large footprint of activated sludge-based sewage treatment plants.

Generally, wastewater treatment systems are located in densely populated urban areas, where space is scarce. In this context, new compact technologies with a reduced footprint are required. Moreover, the increasing pollution in water resources as a consequence of enormous discharge of untreated sewage into receiving waters imposes the necessity for upgrading the wastewater treatment plants to meet stringent effluent regulations posed by environmental agencies. Most of the new technological developments in compact sewage treatment processes rely on biofilm- and granular-based systems with a high biomass retention capacity, resulting in higher volumetric treatment capacities and minimized space requirement.

The research described in this thesis aimed at the investigation of several important aspects regarding biological nutrient removal in compact systems such as moving-bed biofilm reactors (MBBR), aerobic granular sludge reactors (AGS) and

sequencing batch reactors with suspended biomass. Different issues related to organic matter, nitrogen and phosphorus removal were addressed in this work.

The first aim of the research described in this thesis was to investigate how different operational conditions (e.g., influent composition and feeding regime) influence the development of nitrifying biofilms in moving bed biofilm reactors (MBBR). In this particular study, the nitrification process was also carefully evaluated. To fulfil the objectives, analytical methods and molecular techniques (such as fluorescent in situ hybridization) were combined to provide further insight into the enrichment of the biofilm for nitrifiers. In order to obtain better control of the different moving bed systems operated in parallel and to understand the effect of particular variables on biofilm development and nitrification process, a synthetic medium was used to feed all the MBBRs. Strategies to speed up the formation of enriched nitrifying biofilms and the feasibility of applying a sequentially operated moving bed reactor for the treatment of high-strength ammonium wastewater are described in this work, presented in **Chapter 2**. It was shown that the application of a heterotrophic start-up phase decreased the time required for the development of nitrifying biofilms in MBBRs. The findings of this research can potentially be used in industrial applications, most notably when nitrification should be accomplished in wastewaters with limited or no organic carbon. Inoculation of a MBBR operated on a pulse-feeding sequencing batch regime with biomass detached from other MBBR systems was also found to reduce the time necessary to develop an enriched nitrifying biofilm.

Aerobic granular sludge (AGS) is a promising technology for wastewater treatment. Several studies have investigated the simultaneous nitrogen and phosphate removal in AGS systems. However, none of them specified the impact of specific subpopulations of polyphosphate-accumulating organisms (PAOs) on phosphate and nitrogen conversions. Recent research efforts exploring the characteristics of PAOs have shown new insights about these microorganisms, classifying them according to their capability of using nitrite and/or nitrate as electron acceptor for denitrification. Taking into account the new discoveries regarding these organisms, a complete characterization of the main process conversions occurring in aerobic granular sludge reactors applied for simultaneous organic matter, nitrogen and phosphorus removal and operated at different temperatures (20 and 30°C), was conducted. In this research project, described in **Chapter 3**, full nitrification/denitrification was achieved at low dissolved oxygen concentrations (less than 2 mgO_2/L). A stratification of the microbial

community structure over the settled sludge bed was noticed by means of fluorescent in situ hybridization (FISH). In the top of the sludge bed in both SBRs, glycogen-accumulating organisms (GAOs) clearly dominated over PAOs. Conversely, in the bottom of the sludge bed, PAOs were the dominant organisms. The segregation offered the possibility to control PAO-GAO competition to enhance the phosphate removal efficiency. A selective sludge discharge mainly from the GAO-rich top of the segregated reactor sludge bed as an operational strategy to favour PAOs over GAOs proved to enhance phosphate removal efficiency, particularly at tropical temperatures (30°C). The development of denitrifying polyphosphate-accumulating organisms (DPAOs) was found to be favoured at low dissolved oxygen concentrations. Hence, denitrification coupled to anoxic phosphate uptake was sustained in the aerobic granular sludge systems, which is advantageous since the same carbon source (usually intracellular polymers such as polyhydroxybutyrate) is used for both denitrification and anoxic phosphate removal.

According to experimental results from this research, denitrification was proposed to run mainly via the nitrate route. Denitrifying glycogen-accumulating organisms (DGAOs) were the principal organisms responsible for reduction of nitrate to nitrite in both reactors. Nitrite was further reduced to nitrogen gas concomitant with anoxic phosphate uptake by PAO clade II (PAOII). A method to stimulate this conversion relative to the nitrate based nitrification-denitrification certainly would be interesting for the optimisation of aerobic granular sludge processes.

During the investigation of the nitrifying bacterial diversity in a lab-scale aerobic granular sludge (AGS) reactor operated at 30°C **(Chapter 4)**, a nitrite-oxidizing bacteria/ammonium-oxidizing bacteria (NOB/AOB) ratio higher than 1 was observed by means of quantitative PCR (qPCR). This was not observed in the samples from a conventional activated sludge system. The NOB/AOB ratio higher than 1 was an unexpected result, since the theoretical NOB/AOB ratio based on the biomass yield of these organisms in the nitrification process is approximately 0.5. This ratio is expected to be even lower in aerobic granular sludge systems where simultaneous nitrification/denitrification takes place, since NOB have to compete for nitrite with heterotrophic denitrifying bacteria. In general, the amount of AOB would be always higher than that of NOB unless the metabolism of NOB is changed in such a way that their biomass yield increases. This is possible if the growth of these organisms would be partly uncoupled from the lithotrophic nitrite supply by AOB and would be capable of

using other substrates (e.g., organic compounds) as well. In this particular case, NOB would grow mixotrophically. Surprisingly, the nitrite oxidation capacity was found to be around three times higher than the ammonium oxidation capacity in separate activity batch tests. Taking into account the experimental evidence and literature information, two hypotheses were proposed to explain why the NOB/AOB ratio was higher than 1 in the granular system. In the first assumption, designated as *ping-pong effect*, *Nitrobacter* (the NOB found in the aerobic granular system) could have grown mixotrophically by acetate-dependent dissimilatory nitrate reduction. In the second hypothesis, a nitrite oxidation/nitrate reduction loop (designated as *nitrite loop*) may have occurred within the granular sludge. In the *nitrite loop*, denitrifiers reduced nitrate to nitrite supplying additional nitrite for the NOB. This would support the growth of NOB apart of the nitrite supply from AOB. Further investigation is needed to identify the mechanistic rationale for the disproportion of the amount of AOB and NOB in aerobic granular sludge.

During operation of lab- and pilot-scale aerobic granular sludge reactors with alternate anaerobic/aerobic phases, ammonium concentrations after anaerobic feeding were found to be lower than expected based on the influent concentration and dilution in the reactor. This fact was attributed to ammonium adsorption to the granular biomass. A detailed study on adsorption of ammonium in aerobic granular sludge and the main causes of this phenomenon is described in **Chapter 5**. By comparing the extent of ammonium adsorption in several types of biomass, the amount of ammonium adsorbed to aerobic granules was found to be much higher than that occurring in activated sludge and anammox granules. Kinetic experiments with granules showed that ammonium adsorption in granules is much slower than in activated sludge. The high ammonium adsorption to the granular biomass can be attributed to the presence of K-struvite (potassium magnesium phosphate), which functions as potassium source for ion-exchange with ammonium. Overall, this study has shown that the phenomenon of ammonium adsorption to aerobic granules cannot be neglected particularly in granular sludge bioreactors that are characterized by strongly variable ammonium concentrations as a function of place (plug flow systems) or time (batch systems).

The effluent of chemical, pharmaceutical and petroleum industries can contain high salt concentrations. High osmotic pressure is a consequence of elevated salinity, affecting the metabolism of most fresh water-based microbial ecosystems. As a consequence, high salt concentrations negatively influence the main biological

processes (e.g., organic matter, nitrogen and phosphorus removal) occurring in wastewater treatment plants. The adverse effect of salt on these processes was reported to be minimized with gradual adaptation of microorganisms to high salt concentrations. In **Chapter 6**, a study investigating the role of changes in the microbial community structure of suspended nitrifying sludge during adaptation to salt (NaCl) was conducted. Two sequencing batch reactors (SBR_1 and SBR_2) treating synthetic wastewater were subjected to increasing salt concentrations. In SBR_1, four salt concentrations (5, 10, 15, and 20 gNaCl/L) were tested, while in SBR_2, only two salt concentrations (10 and 20 gNaCl/L) were applied in a more shock-wise manner. The different salt adaptation strategies provoked different shifts in the microbial community structure, although they did not influence the nitrification performance. This finding suggests that independently of the different nitrifying bacterial populations present in the reactor, the nitrification process can be maintained stable within the salt range and operating conditions tested. On the other hand, the more rapid salt increase imposed to SBR_2 caused a higher decrease in the specific ammonium oxidation rates. The gradual increase in NaCl concentration positively affected the settling properties, as demonstrated by the reduction of sludge volume index. However, higher washout of light and poor settling flocs was observed due to increasing water density, which is a consequence of gradual salt increase. Higher organisms (e.g., protozoa, nematodes, and rotifers) as well as filamentous bacteria could not withstand salt concentrations over 10 gNaCl/L.

The effect of salt on aerobic granular sludge (AGS) is scarcely reported in literature. In **Chapter 7**, a detailed study regarding the long- and short-term effect of salt on organic matter, nitrogen and phosphate removal in an AGS system was conducted. The dynamics of the microbial community structure within the granules at increasing salt concentrations (0 – 33 gNaCl/L) was also addressed in this research. Ammonium removal efficiency was not affected when salt (NaCl) was increased until 33 gNaCl/L. Ammonium uptake rates remained stable, differently from the study carried out with suspended nitrifying sludge (**Chapter 6**). However, nitrite accumulation was observed to occur at salt concentrations higher than 22 gNaCl/L, which coincided with the disappearance of *Nitrospira sp*. The increase of salt severely affected the phosphate removal process, which completely deteriorated at 33 gNaCl/L. Polyphosphate-accumulating organisms (*Candidatus* Accumulibacter phosphatis) were no longer detected at this salt concentration. Batch experiments confirmed that phosphate removal could still occur at 30 gNaCl/L, but the long exposure of the

biomass to this salt concentration was detrimental to phosphate-accumulating organisms (PAOs), which were outcompeted by glycogen-accumulating organisms (GAOs) in the bioreactor. GAOs became the dominant microorganisms at increasing salt concentrations, especially at 33 gNaCl/L.

Herewith, we hope that the studies described in this thesis contribute to improved understanding of key aspects of biological nutrient removal processes in compact biofilm based bioprocesses. This will enable the development of improved wastewater treatment processes and the protection of natural environments from human activities.

João Paulo Bassin

Samenvatting

Samenvatting

Biologische Verwijdering van Nutriënten in Compacte Biofilm Systemen

De verwijdering van nutriënten zoals stikstof en fosfaat van zowel huishoudelijk als industrieel afvalwater is noodzakelijk omdat ze in grote hoeveelheden schadelijk zijn voor het milieu. Een van de belangrijkste gevolgen van overmatige beschikbaarheid van stikstof en fosfaat in aquatische ecosystemen (zoet water, mariene en estuariene) is de overmatige groei van algen en andere waterplanten, een fenomeen aangeduid als eutrofiëring. Algen en waterplanten kunnen leiden tot depletie van zuurstof in het water, waardoor massale sterfte van hogere organismen, zoals vissen, aanwezig in het ecosysteem kan plaatsvinden. De proliferatie van bacteriën en algen die afgestorven algen en planten afbreken draagt ook bij aan depletie van zuurstof. Menselijke activiteit verhoogt de toevoer van nutriënten en is dus direct verantwoordelijk voor de versnelling van het eutrofiëring proces en de daaruit voorkomende gevolgen.

De verwijdering van nutriënten uit afvalwater door biologische processen is kosteneffectief en biedt tal van voordelen ten opzichte van fysisch-chemische processen. Biologische stikstof- (nitrificatie en denitrificatie) en fosfaatverwijdering omvatten een reeks biochemische processen die plaats vinden in opeenvolgdende milieus (van aërobe, anoxische tot anaerobe milieus). In conventionele actief slib-gebaseerde processen, wordt aan deze voorwaarden voldaan door incubatie in aparte tanks (meertraps behandeling van processen). De lage biomassaconcentraties in de traditionele actiefslibsystemen, en in het bijzonder de moeilijke scheiding van het gevlokte slib van het gezuiverde water dragen bij aan een zeer grote ruimtelijke voetafdruk van traditionele afvalwaterzuiveringsinstallaties.

In zijn algemeenheid zijn afvalwaterzuiveringssystemen in dichtbevolkte stedelijke gebieden gesitueerd, waar ruimte schaars is. In dit kader zijn er nieuwe technologieën benodigd met een gereduceerde ruimtelijke voetafdruk. en een hoog zuiveringsrendement, die door de jaren heen aan de strenge effluent regelgeving voor afvalwaterlozingen kunnen voldoen. Het merendeel van de nieuwe technologische ontwikkelingen zijn gericht op compacte biofilm- en korrelslib gebaseerde systemen. Deze systemen hebben een hoog biomassaretentie vermogen, en daardoor een hogere volumetrische verwerkingscapaciteit.

Het in dit proefschrift beschreven onderzoek had tot doel om verschillende belangrijke aspecten te onderzoeken van nutriëntenverwijdering in compacte

zuiveringssystemen zoals bewegend bed biofilm reactoren (*Moving bed biofilm* reactor (MBBR), aeroob korrelslib (AGS), en repeterende batch reactoren met gesuspendeerde biomassa. Verschillende aspecten van de verwijdering van organisch koolstof, stikstof en fosfaat zijn onderzocht.

Het eerste doel van het onderzoek beschreven in dit proefschrift was om te onderzoeken hoe de verschillende operationele omstandigheden (bijv. influentsamenstelling en voedingsregime) de ontwikkeling van nitrificerende biofilms in een MBBR beïnvloeden. In dit onderzoek werd het nitrificatie proces ook in detail geëvalueerd. Om aan onze doelstellingen te voldoen, zijn analytische methoden en moleculaire technieken (zoals fluorescente in situ hybridisatie (FISH)) gecombineerd om meer inzicht te geven in de verrijking van nitrificeerders in de biofilm. Strategieën om de vorming van verrijkte nitrificerende biofilms te versnellen en de toepassingsmogelijkheden van een MBBR voor de behandeling van ammonium verrijkt afvalwater worden beschreven in **hoofdstuk 2**. In dit hoofdstuk wordt beschreven dat het toepassen van een heterotrofe startfase de tijd die nodig is voor de ontwikkeling van nitrificerende biofilms in MBBRs, kan reduceren. De resultaten van dit onderzoek kunnen eventueel worden gebruikt voor industriële toepassingen, met name als nitrificatie wordt toegepast in afvalwater met geen of een beperkte hoeveelheid organische koolstof. Het enten van een MBBR bedreven als een opeenvolgende batch reactor met biomassa van andere MBBR systemen versnelde de verrijking van een nitrificerende biofilm. Aëroob korrelslib is een nieuwere veelbelovende technologie voor de behandeling van afvalwater. Verschillende studies hebben de gelijktijdige stikstof en fosfaatverwijdering in aëroob korrelslib systemen onderzocht. Echter, geen van de studies toonden het belang van specifieke subpopulaties van polyfosfaat accumulerende organismen (PAOs) aan op fosfaat en stikstofconversies. Recent wetenschappelijk onderzoek naar de kenmerken van PAOs laten nieuwe inzichten zien, onder andere een nieuwe classificatie van denitrificerende PAOs. Rekening houdend met de nieuwe ontdekkingen, met betrekking tot deze organismen, werd een volledige karakterisering van de belangrijkste processconversies gedaan, die plaatsvinden in aërobe korrelslib reactoren. Dit betreft gelijktijdige chemisch zuurstofverbruik (CZV), stikstof en fosfaat verwijdering bij verschillende temperaturen (20^{o}C en 30^{o}C) Bij het onderzoek beschreven in **hoofdstuk 3** werd volledige nitrificatie/denitrificatie bereikt bij lage concentraties opgeloste zuurstof (minder dan 2 mgO_2/L). Ook werd verticale stratificatie van de microbiële samenstelling van bezonken slib bed aangetoond middels

fluorescente in situ hybridisatie (FISH). Biomassa in de bovenste lagen van het bezonken slibbed werd gedomineerd door glycogeen accumulerende micro-organismen (GAOs). Echter, in de onderste laag van het slib bed waren PAOs de dominante organismen. Dit verschil biedt de mogelijkheid om de PAO-GAO competitie te beïnvloeden en de efficiëntie van fosfaatverwijdering te verbeteren. Een selectieve verwijdering van voornamelijk de GAO-rijke bovenste laag van van het slib-bed resulteerde in een verrijking van de PAO populatie en daarmee een verhoging van de fosfaatverwijderingsefficiëntie, vooral bij tropische temperaturen (30°C). De verrijking van denitrificerende PAOs (DPAOs) kon worden verkregen door toepassing van lage concentraties opgeloste zuurstof. Denitrificatie gekoppeld aan anoxische fosfaatopname in de aërobe korrelslib systemen is voordelig omdat dezelfde koolstofbron (meestal intracellulaire polymeren in de vorm van polyhydroxybutyraat) wordt gebruikt voor zowel denitrificatie als anoxische fosfaatopname.

Volgens de experimentele resultaten van dit onderzoek, lijkt de denitrificatie vooral te lopen via de conventionele nitraat route. Hierin waren de denitrificerende glycogeen accumuleren organismen (DGAOs) de belangrijkste organismen, verantwoordelijk voor de reductie van nitraat naar nitriet in beide reactoren. Nitriet werd verder gereduceerd tot stikstofgas gecombineerd met anoxische fosfaatopname door PAO clade II (PAOII). Een methode om deze conversie te stimuleren teneinde denitrificatie en fosfaatverwijdering via de nitrietroute te verwezenlijken is een interessante optie voor optimalisatie van aërobe korrelslib processen.

Tijdens het onderzoek van de nitrificerende bacteriële diversiteit in een aëroob korrelslib reactor (**hoofdstuk 4**), werd een nitrietoxiderende bacterie/ammonium-oxiderende bacterie (NOB/AOB) ratio van meer dan 1 waargenomen met behulp van kwantitatieve PCR. Dit was een onverwacht resultaat, omdat de theoretische NOB/AOB-verhouding op basis van de biomassa opbrengst van deze organismen in het nitrificatie proces minder dan 0,5 zou moeten zijn. Deze verhouding zal naar verwachting nog lager zijn in aërobe korrelslib systemen waar gelijktijdig nitrificatie/denitrificatie plaatsvindt, omdat NOB in competitie zijn met heterotrofe bacteriën om het nitriet. In stikstof verwijderende systemen zou de NOB/AOB ratio altijd lager moeten zijn dan 0.5 tenzij het metabolisme van de NOB anders is als algemeen aangenomen. Ook de in batchexperimenten bepaalde nitrietoxidatiecapaciteit was driemaal hoger dan de ammonium oxidatiecapaciteit. Gebaseerd op de

experimentele bewijs en literatuurinformatie, hebben we twee hypothesen geformuleerd die een verklaring geven voor de verhoogde NOB/AOB-ratio:

De eerste hypothese, aangeduid als ping-pong effect, is gebaseerd op de capaciteit van Nitrobacter (de NOB in het aerobe granulaire systeem) om mixotroof te kunnen groeien door acetaat-afhankelijke dissimilatieve nitraatreductie. De tweede hypothese is dat door anoxische reductie van nitraat naar nitriet, een nitraat-nitriet lus kan ontstaan binnen de slibkorrel. Hierbij wordt nitraat tot nitriet gereduceerd door denitrificeerders, gevolgd door heroxidatie van het nitriet door de NOB. Dit resulteert in extra groei van NOB t.o.v. AOB. Nader onderzoek is nodig om deze hypotheses te testen.

Tijdens het gebruik van lab- en pilotschaal aërobe korrelslibreactoren met alternerende anaërobe / aërobe fases bleek de ammoniumconcentratie na anaërobe voeding lager te zijn dan verwacht op basis van de influentconcentratie en verdunning met de vloeistof in de reactor. Deze waarneming kan worden toegeschreven aan ammonium adsorptie aan de korrelvormige biomassa. Een gedetailleerde studie over de adsorptie van ammonium in aëroob korrelslib, en de belangrijkste oorzaken van dit fenomeen, wordt beschreven in **hoofdstuk 5**. De adsorptiecapaciteit van ammonium aeroob korrelslib bleek veel hoger dan die in actief slib en anammox korrelslib. Ook bleek dat ammoniumadsorptie aan korrelslib veel langzamer is dan in actief slib. De hoge ammonium adsorptie aan aeroob korrelslib kon worden toegeschreven aan de aanwezigheid van K-struviet (kalium magnesiumfosfaat) dat fungeerde als kalium bron voor ionenuitwisseling met ammonium. De algemene conclusie van dit onderzoek is dat ammonium adsorptie aan aeroob korrelslib niet te verwaarlozen valt met name in processen die gekenmerkt worden door sterk varierende ammonium concentraties als een functie van de plaats (propstroom systemen) of tijd (batch systemen).

Het afvalwater van de chemische, farmaceutische en petrochemische industrieën kan hoge zoutconcentraties bevatten. De hoge osmotische druk als gevolg van hoge zoutgehaltes beïnvloedt het metabolisme van de meeste in zoetwater milieus levende micro-organismen. Dit heeft tot gevolg dat hoge zoutconcentraties nadelige gevolgen kunnen hebben voor de belangrijkste biologische processen in rioolwaterzuiveringsinstallaties. De nadelige gevolgen van verhoogde zoutconcentraties op deze processen als gerapporteerd in vakliteratuur kunnen geminimaliseerd worden door geleidelijke adaptatie van het slib aan de hoge zoutconcentraties. In hoofdstuk 6, werd de rol van de veranderingen in de microbiële gemeenschap van gesuspendeerd

nitrificerend slib tijdens aanpassing aan zout (NaCl) onderzocht. Twee Sequencing Batch Reactors (SBR_1 en SBR_2) gevoed met synthetisch afvalwater werden onderworpen aan toenemende zoutconcentraties. In SBR1 werd de zoutconcentratie in drie stappen verhoogd (5, 10, 15 en 20 gNaCl/L) en in SBR2 in een stap (10 en 20 gNaCl/L). De verschillende bedrijfsvoeringen veroorzaakten verschuivingen in de samenstelling van microbiële gemeenschap, maar hadden geen effect op de nitrificatiecapaciteit van het systeem. Dit suggereert dat onafhankelijk van de verschillende nitrificerende bacteriële gemeenschappen, de nitrificatie stabiel kan worden gehandhaafd bij de toegepaste zoutconcentraties en bedrijfsvoeringen. Wel bleek dat een snellere toename van zout concentratie als in SBR_2 een sterkere afname van de specifieke ammonium oxidatiesnelheid te veroorzaken. De geleidelijke stijging van de NaCl concentratie heeft een positief effect op de bezinkingbaarheid van het slib. Echter, de uitspoeling van slecht bezinkende vlokken werd ook waargenomen, mogelijk veroorzaakt door de toenemende water dichtheid bij verhoogde zoutconcentraties. Hogere organismen (bijv. protozoa, nematoden en rotiferen) en filamenteuze bacteriën konden zoutconcentraties tot 10 gNaCl/L. niet weerstaan.

Het effect van zout op aeroob korrelslib is nauwelijks beschreven in de literatuur. Hoofdstuk 7 beschrijft een gedetailleerde studie naar de lange-en korte-termijn effecten van zout op organisch materiaal, stikstof-, en fosfaatverwijdering in aërobe korrelslib-systeem plaats. De dynamiek van de microbiële gemeenschap in de korrels als gevolg van het verhogen van de zoutconcentraties (0 tot 33 gNaCl/L) werd ook behandeld in dit onderzoek. Het ammonium verwijderingsrendement werd niet beïnvloed wanneer zout (NaCl) werd verhoogd tot 33 gNaCl/L. De ammoniumopname bleef stabiel, dit in tegenstelling tot de studie uitgevoerd met gevlokt nitrificerend slib (**hoofdstuk 6**). Er werd echter nitrietaccumulatie waargenomen bij zoutconcentraties hoger dan 22 gNaCl/L, wat samenviel met het verdwijnen van *Nitrospira sp* uit het slib. De toename van de zoutconcentratie had een negatief effect op de fosfaatverwijdering. Bij een concentratie van 33 gNaCl/L vond slechts marginale fosfaatverwijdering plaats. Polyfosfaat accumulerende organismen werden niet gevonden bij deze zoutconcentratie. Batch experimenten bevestigden dat fosfaatverwijdering niet volledig is geremd bij 30 gNaCl/L, maar op de langere termijn leidt de verhoogde zoutconcentratie tot verminderde groei van fosfaat accumulerende organismen met uitspoeling tot gevolg. Bij deze hoge zoutconcentraties worden PAOs weggeconcureerd door glycogeen-accumulerende organismen (GAOs) in de bioreactor.

We hopen dat de verschillende studies beschreven in dit proefschrift bijdragen aan een beter begrip van fundamentele aspecten van biologische nutriënten-verwijdering in compacte biofilm processen. Deze inzichten faciliteren de ontwikkeling van verbeterde wasterzuiveringssystemen en leveren daarmee een bijdrage aan de bescherming van natuurlijke milieus tegen menselijke activiteiten.

João Paulo Bassin

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Curriculum Vitae

João Paulo Bassin was born on April 16th 1982, in Erechim, Rio Grande do Sul State, Brazil. In 1999, he concluded middle school in the same city he was born in. In 2001, he moved to Florianópolis, Santa Catarina State, to study Chemical Engineering at Federal University of Santa Catarina. He obtained the bachelor's degree in 2006. In the same year, he started his Master's studies in Chemical Engineering at the Federal University of Rio de Janeiro, Rio de Janeiro. In 2008, he obtained the Master's degree and in the same year, he became a PhD student at the same university. In October 2009, he moved to the Netherlands to carry out part of his studies in the Environmental Biotechnology group of the Delft University of Technology, The Netherlands. He later became a PhD student from this university as well. The PhD thesis he wrote is a combination of his studies performed both in Brazil and in the Netherlands.

List of Publications

Journal articles (thesis chapters)

Bassin, J.P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M., van Loosdrecht, M.C.M. (2011) Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. Appl. Environ. Microbiol. 77(22), 7942-7953.

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* These authors contributed equally to this work

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The supervisors of this thesis

Mark van Loosdrecht
(Promotor of this thesis)

Professor, Group leader of Environmental Biotechnology, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, The Netherlands

M.C.M.vanLoosdrecht@tudelft.nl

Marcia W.C. Dezotti
(Promotor of this thesis)

Associate Professor, Chemical Engineering Program – COPPE, Federal University of Rio de Janeiro, Brazil

mdezotti@peq.coppe.ufrj.br

Robert Kleerebezem
(Co-promotor of this thesis)

Assistant Professor, Environmental Biotechnology, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, The Netherlands

R.Kleerebezem@tudelft.nl

Gerard Muyzer
(Supervisor of this thesis)

Professor of Microbial Systems Ecology, Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

g.muijzer@uva.nl

Alexandre Soares Rosado
(Supervisor of this thesis)

Associate Professor, Director of Microbiology Institute, Federal University of Rio de Janeiro, Brazil

asrosado@micro.ufrj.br

The two Universities I worked during the PhD research years

Delft University of Technology
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Department of Biotechnology, Faculty of Applied Sciences
(View of the Kluyver laboratory from the Botanical Garden).

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(View of the laboratories)

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The Markt square, Delft, The Netherlands (Photo credit: Christiaan Brugge)

Copacabana Beach, Rio de Janeiro, Brazil.

Closing this book, the author of this thesis (João Paulo Bassin) in:

- **One of the Conferences during the PhD research study**

IWA Biofilm Conference 2011: Processes in Biofilms.
October 27-30, 2011, Tongji University, Shanghai, China.

- **One of his first snow days**

Snowman carrying me in a supermarket cart.
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