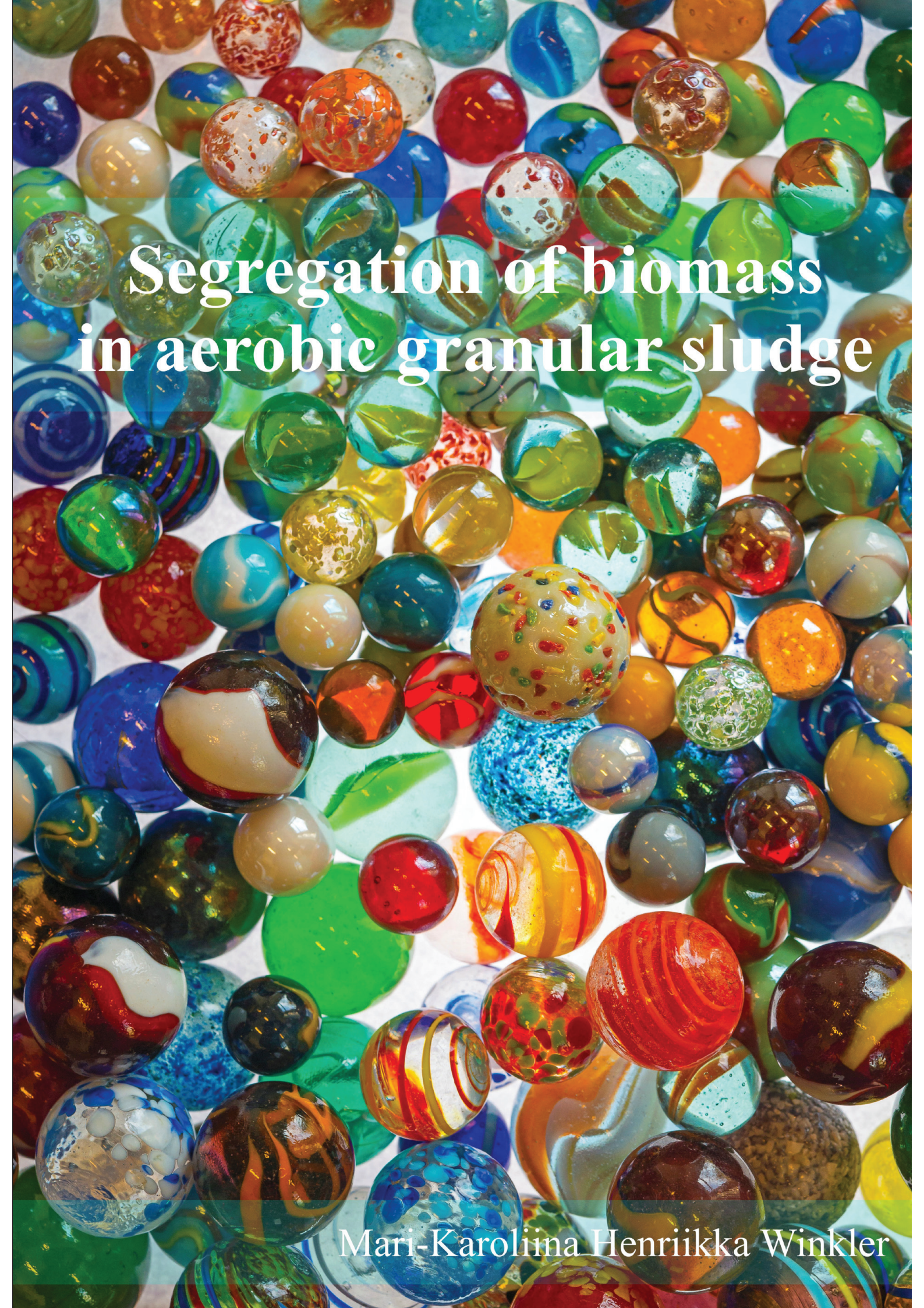
Magic granules

Mari-Karoliina Henriikka Winkler



Segregation of biomass in aerobic granular sludge

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Proefschrift

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Chapter 1

GENERAL INTRODUCTION

1

INTRODUCTION

Granular sludge technology has its origin at Wageningen University where Professor Lettinga developed in the 70-ies of last century the upflow anaerobic sludge bed (UASB) concept (Lettinga et al. 1983; Lettinga and Hulshoff Pol 1986). This concept is based on bacteria grown under anaerobic conditions, which convert organic matter to CO_2, CH_4, volatile fatty acids and H_2 as well as sulphate to H_2S (Beaty et al. 1987; Beaty and McInerney. M.J. 1989). The treatments strategy enriches for anaerobic bacteria like for instance acetogens, methanogenic bacteria or sulfate-reducing bacteria (Tholozan et al. 1990) and the aggregation of these microorganisms into one compact granule supposedly optimizes the processes by reducing the diffusion distance of substrates between different partner organisms (Schink 1997).

While the anaerobic wastewater treatment benefited from the densely packed granules offering a maximal microorganisms-to-space ratio and excellent settling properties, it took two more decades for the aerobic granular sludge technology to get incorporated into the wastewater treatment. The biological removal of nitrogen and phosphorus compounds requires alternate anoxic, anaerobic and aerobic conditions in order to promote the growth of ammonium- and nitrite oxidizing bacteria, Anammox bacteria, denitrifying bacteria as well as polyphosphate accumulating organisms. In conventional wastewater treatment the flocculent biomass needs to be recycled through different tanks to get subjected to aerobic or anaerobic environments and different substrate availability resulting in costly recycle flows and space consuming settling tanks.

It was clear that granular sludge technology would be beneficial for aerobic wastewater treatment as well. However, the conditions and mechanisms to promote bacteria to grow aerobically in granules were unknown. In 1997 laboratory based research between Technical University of Munich and Delft University of Technology developed for the first time aerobically grown granules (Morgenroth et al. 1997; Van Loosdrecht and Heijnen 1993) which yielded the Nereda® technology for the simultaneous treatment of COD, N and P in one single reactor (de Kreuk et al. 2005). But not only heterotrophic granular sludge technology benefited from granules but also the Anammox technology, which is suited for autotrophic nitrogen removal. Anammox bacteria can be either grown in anaerobic granules (two-stage configuration)

in which aerobic nitritation and the Anammox process are separated in two different reactors or in aerobic granules (one stage configuration) in which partial nitritation and anammox take place in the same aerated reactor (Third et al. 2001; Van Dongen et al. 2001). Today aerobic granular sludge technology is applied on full-scale basis to treat real wastewater with technologies as Nereda® and Nitrataion-Anammox reactors (van der Star et al. 2007; Third et al. 2001; Kartal et al. 2010; De Bruin et al. 2004).

NEREDA® –HETEROTROPHIC GRANULES

The Delft University of Technology in cooperation with DHV, the Dutch water boards, STOWA and NWO can look back to a successful history of developing and scaling-up aerobic granular sludge technology from laboratory based reactors to pilot-scale and eventual full-scale systems to treat COD N and P in a more space reducing and cost efficient way. The research started with the PhD student Janneke Beun, who succeeded in growing heterotrophic aerobic granules with integrated COD and nitrogen removal in a discontinuously fed Sequencing Batch Airlift Reactors which also led to a patent (SBAR) (Heijnen. J.J. and M.C.M.; 1998; Beun et al. 2001, 1999, and 2002). This showed that even under the most complex conditions granular sludge could be produced. Merle de Kreuk took over the research and enhanced the process for simultaneous COD- N- and P-removal by integrating an elongated anaerobic feeding period. This was not only needed for efficient nutrient removal but also beneficial for the selection of for slow growing organisms which highly stabilized granular sludge formation (Van Loosdrecht and De Kreuk 2004).

Earlier research has already shown that the biofilm morphology is influenced by the surface substrate loading and applied detachment forces (Kwok et al. 1998; Tijhuis et al. 1995). A moderate surface substrate loading and a high detachment force yielded smooth and strong biofilms, whilst the combination of a high surface substrate loading and low detachment forces led to rough biofilms. This principle is also true for aerobically grown granules and the applied anaerobic feeding period selects for slow growing polyphosphate accumulating organisms (metabolism explained in section ‘Biological removal of Phosphate’) which anaerobically take up all easy degradable organic compounds (Smolders et al. 1994) hence outcompeting aerobic fast growing heterotrophic bacteria for their substrate which hence yields in smoother granules which in turn settle well. Finally, the anaerobic feeding was needed in order to be able to scale-up the process in an

economic fashion with respect to investment in influent pumps and compressors for aeration. Merle worked closely together with the company DHV, which led to a successful pilot-plant research in the Netherlands in Ede and several other wastewater treatment plants. Based on these experiences demonstration plants in South Africa and Portugal where constructed. Currently the world's first full scale plant was started in Epe, The Netherlands treating wastewater for 59,000 population equivalents (van der Roest et al. 2011).

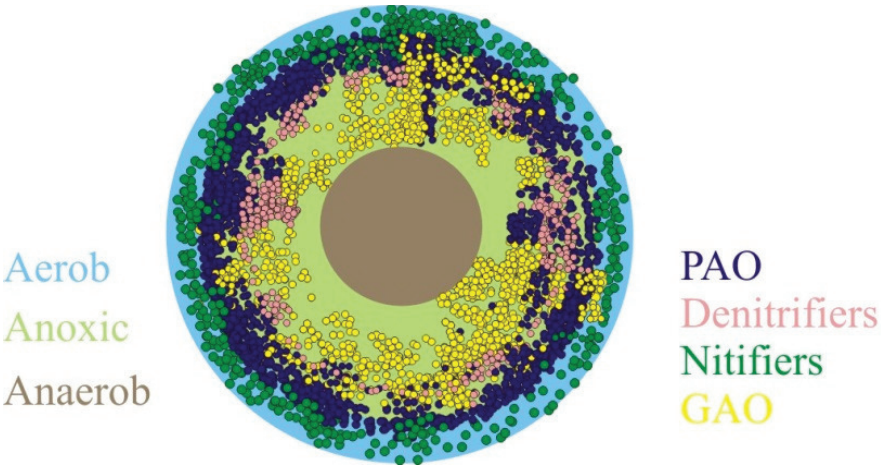

Figure 1 showing the typical structure of an heterotrophic granule with nitrifiers (green) in the aerobic zone followed by PAOs (Blue) GAOs (yellow) and denitrifies (pink) in the up following anoxic zone

NITRATAION-ANAMMOX - AUTOTROPHIC GRANULES

The process of Anammox is a shortcut in the Nitrogen cycle whereby ammonium is oxidized with nitrite to nitrogen gas (Strous et al. 1999). When in 1985 ammonium removal was observed for the first time under anoxic conditions in a pilot scale denitrifying reactor at the company Gist-Brocades, in Delft, The Netherlands, nobody believed that this process could possibly be of microbiological nature (Mulder 1992). The history of enriching this organisms dates back to 1990-ies when Prof. Dr. Gijs Kuenen lead the microbiology department of Delft university of Technology. His PhD students eventually succeeded in the enrichment of this unique bacterium (Strous et al. 1999; Van de Graaf et al. 1996) and in cooperation with the environmental engineering group at TU Delft and the company Paques 10 years later this research led in a successfully running full-scale treatment plant in Rotterdam (van der Star et al. 2007). In this Sharon-Anammox

system the nitrite is produced in a separate aerated (Sharon) reactor and then fed in the second reactor, in which anaerobic granules consisting of Anammox bacteria grow. Since its discovery by Mulder in 1992 many Anammox based treatment systems have successfully been implemented for cost-efficient full-scale nitrogen removal from anaerobic sludge digestion rejection water (Abma et al. 2010; Egli et al. 2001; Sliekers et al. 2003; Third et al. 2001). To establish good autotrophic nitrogen removal in aerobic granules Anammox and AOB need to be enriched in the reactor system (Sliekers et al. 2003; Third et al. 2001). This is possible in oxygen-limited combined process for completely autotrophic nitrogen removal over nitrite (CANON). In this process the AOB grow on the outer oxygen penetrated rim of the granules hence supplying Anammox with nitrite and shielding the strict anoxic Anammox bacteria from oxygen exposure (Figure 2) (Vlaeminck et al. 2010; Winkler et al. 2011).

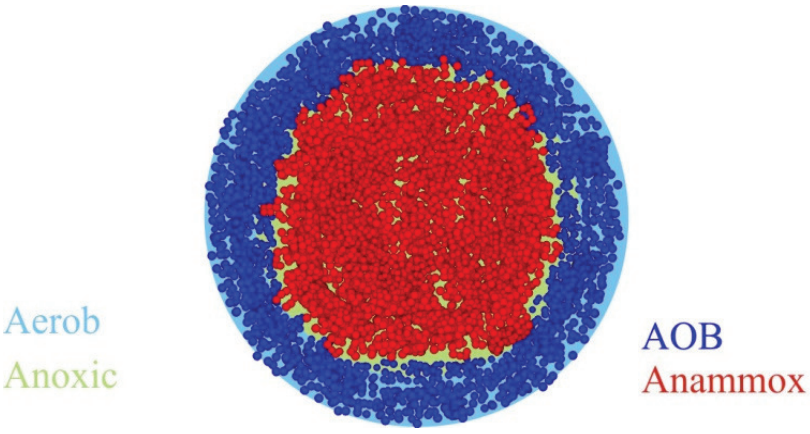

Figure 2 typical structure of a CANON granule with AOB (blue) on the outer oxygen penetrated rim and Anammox (red) in the inner anoxic core

GRANULES VERSUS FLOCS

In granular sludge all conversions are occurring in different layers within the granular biomass located in one reactor compartment with alternating anaerobic and aerobic periods in sequencing batch mode. There will be a stratification of bacteria as a function of depth within the granules. Nitrifiers will be located in the oxygen penetrated outer layers. Denitrifiers and PAOs are located in the inner anoxic layers (Xavier et al. 2007) (Figure 3B). In conventional treatment plants the sludge floc (Figure 3 A) is subjected to aerobic or anaerobic environments and different substrate availability by recycling it over different reactor compartments. For the formation of

granular sludge many parameters play a role but the most important once are substrate composition, loading rates, feeding strategy, reactor design, settling time, seed sludge, and aeration intensity (Beun et al. 1999; de Kreuk et al. 2005; McSwain et al. 2004; Wilén et al. 2008). To distinguish between granules and flocs several parameters are used to identify granules. These parameters include physical properties (settling velocity, density, size) as well as the sludge volume index (SVI). The SVI is the most common tool to define sludge settlability (Martins et al. 2004) and can be used to distinguish between particles with good settlability (granules) and slow settling particles (flocs). Since aerobic granular sludge technology is operated in sequencing batch mode with a short settling period, slow settling biomass (flocs) will be washout and hence removed from the reactor.

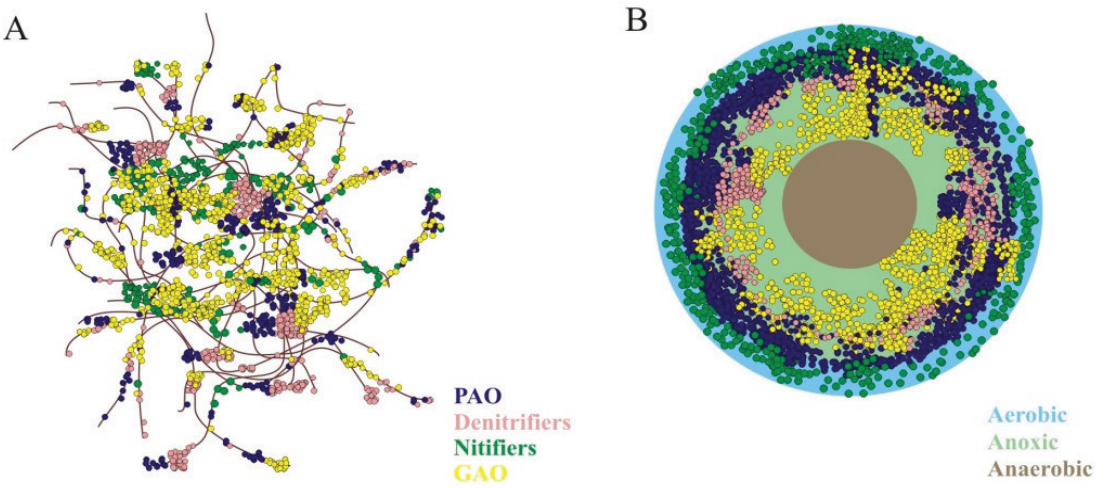

Figure 1 Structural difference differences of A) a sludge floc and B) aerobic granule

BIOLOGICAL REMOVAL OF NUTRIENTS

BIOLOGICAL REMOVAL OF PHOSPHATE

Since wastewater contains phosphate, due to detergents and urine and feces, it is necessary to remove the phosphate before discharging raw water into the receiving water bodies to avoid eutrophication (Höll K. 2002). Phosphorus removal can be achieved either chemically or biologically. Chemically it is removed by the addition of chemical coagulants, like aluminium and iron salts. However, the addition of coagulants results in additional chemical sludge. The resulting disposal of this sludge is costly and harmful to the environment (Grady et al. 1999; Van Loosdrecht et al. 1997). It is also possible to remove phosphate biologically by enhanced biological phosphate

removal (EBPR). The biochemical pathway of the EBPR process is a special adaptation of bacteria in which the phosphate-accumulating organisms (PAOs) are capable of taking up organic matter under anaerobic conditions (i.e., in the absence of an electron acceptor) and convert it to energy-rich intracellular poly-hydroxyalkanoates (PHA) while glycogen is metabolized by glycolysis to produce ATP and NADH (Mino et al. 1998; Seviour et al. 2003; Van Loosdrecht et al. 1997). In the cell, acetate is activated to acetyl-CoA by coupled ATP hydrolysis, yielding ADP. For acetate conversion to poly-hydroxyalkanoates NADH is needed which is generated from the conversion of glycogen. If glycogen is finished, acetate uptake stops and therefore P-release stops. Part of the ATP required for the assimilation of acetate is generated by the cleavage of polyphosphate (poly-P), which leads to the release of orthophosphate from the biomass into the bulk solution (Wentzel et al. 1985) (Figure 3 A).

Under aerobic conditions, the PAOs use oxygen as an electron acceptor. Nitrate and nitrite can be also used as electron acceptor by which denitrification is enabled. The PAOs are then referred in literature to denitrifying PAOs (dPAOs) (Ahn et al. 2001; Kuba et al. 1996; Tsuneda et al. 2006). Orthophosphate is taken up from the mixed liquor to replenish poly-P reserves in the cell. PHA is used as an energy source for the replenishment of glycogen reserves, generation of ATP, and production of new biomass (Figure 3 A). If glycogen reserves are not fully replenished it will lead to problems in the following anaerobic zone because not enough reducing power (NADPH) is supplied by glycolysis of glycogen. However, if energy demands of the cell are in balance the uptake of phosphate during the aerobic phase is always higher than it was released during the anaerobic stage due to new biomass production. By this a net extraction of phosphate from the liquid phase to the biomass is achieved, which in turn can be removed via excess sludge (Comeau et al. 1986; Mino 2000). Glycogen accumulating organisms rely on a very similar metabolism which however does not require the ability to store polyP. Instead of using polyP as energy pool to take up acetate anaerobically they use solely intracellular stored glycogen as both energy and $NADH_2$ source for VFA uptake and sequestration into PHA (Filipe et al. 2001; Mino et al. 1998; Oehmen et al. 2006; Zeng et al. 2003) (Figure 3 B). PAOs and GAOs compete for the same substrate and since GAOs do not contribute to phosphate removal one is trying to select against and GAO dominated system.

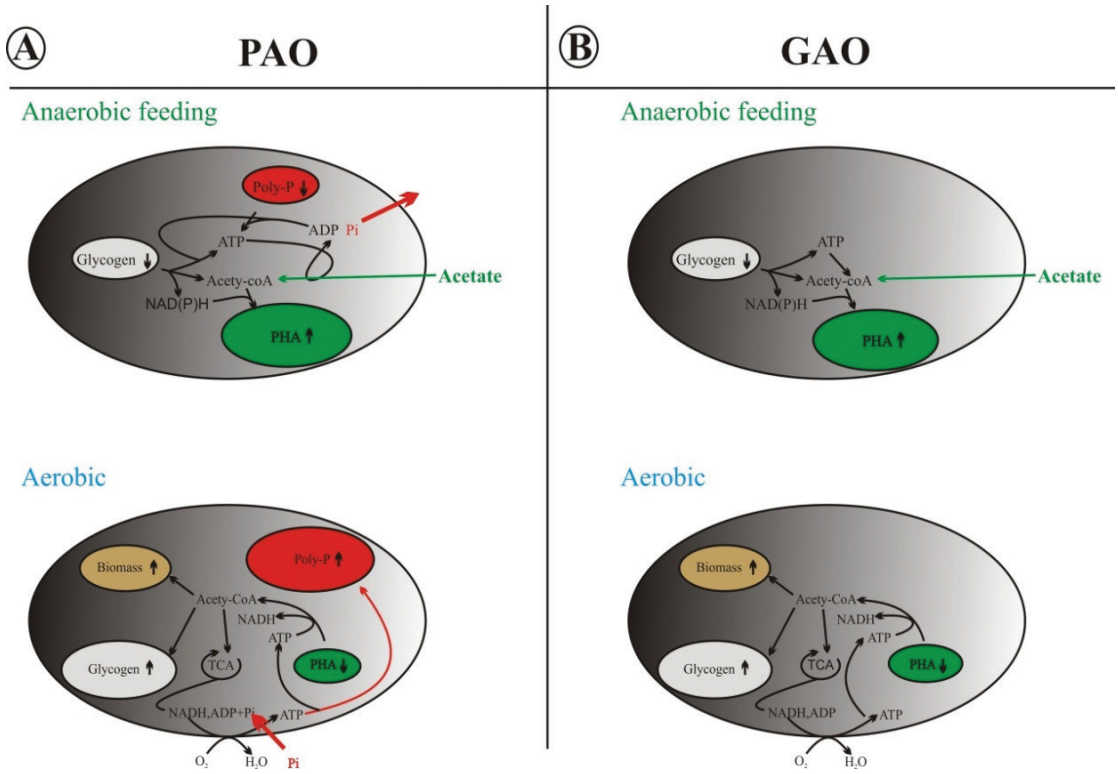

Figure 3 anaerobic conversions of acetate and glycogen to PHA and the aerobic metabolism of polyphosphate and glycogen -accumulating bacteria

BIOLOGICAL REMOVAL OF NITROGEN

The discharge of ammonium from wastewater into water bodies, leads into a disruption of a normal functioning ecosystem. Therefore, ammonium needs to be removed in order to preserve all natural habitats (rivers and lakes). In wastewater treatment ammonium can completely be removed by microorganisms to environmental friendly inert dinitrogen gas. So far there is no bacterium known which completely converts ammonium to dinitrogen gas. Although the anaerobic ammonium oxidizing bacteria are quite close to achieved this nitrite is needed and a fraction of the nitrite is converted to nitrate in the anabolic process leading to an incomplete removal of N (Strous et al. 1999). In general the removal of nitrogen in wastewater treatment is up-to-date either accomplished by nitrification coupled with denitrification or by a combination of aerobic and anaerobic ammonium oxidation.

NITRIFICATION-DENITRIFICATION

Nitrogen in wastewater appears mostly in the form of ammonium (NH_4), which is most commonly removed via autotrophic nitrification followed by heterotrophic denitrification. Ammonium oxidation requires relatively high activation energy and therefore only dedicated organisms with specific enzymes can utilize ammonium as electron donor. These organisms are nitrifying ammonium oxidizing bacteria or Archaea, which aerobically oxidize ammonium to nitrite via the AmoA enzyme (Chain et al. 2003; Könneke et al. 2005). The nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria (NOB) which possess the nitroredoxidase enzyme (nrx) (Starkenburg et al. 2006). Both AOB and NOB are known to grow lithoautotrophically without the need for any organic compounds. However, a discharge of nitrite or nitrate into the water bodies would lead into ecosystem instabilities. Therefore these compounds need to be further reduced by denitrifying bacteria which are using an organic carbon sources as electron donor to form dinitrogen gas. During this denitrification several enzymes are catalysing 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).

ANAMMOX

The anoxic ammonium oxidation (anammox) process is characterized by the reaction of nitrite with ammonium to form dinitrogen gas. In the catabolic reaction of the anammox process ammonium and nitrite are converted into dinitrogen gas. In the anabolic reaction electrons are generated by the oxidation of nitrite to nitrate. These electrons are then further used for the reduction of bicarbonate into biomass (Van de Graaf et al. 1996). Yielding into the experimentally determined stoichiometry of (Strous et al. 1999):

$$1NH_4^+ + 1.3NO_2^- \longrightarrow 1N_2 + 0.3NO_3^-$$

INTEGRATION OF P- N- AND COD-REMOVAL IN GRANULES

Likewise to the anaerobic granular UASB-like reactors aerobic granular sludge technology is characterized by the wastewater stream entering the bottom of the reactor and flowing up along the reactor height. Aerobic granular sludge distinguishes itself from a conventional sludge floc in its compact structure allowing for aerobic, anoxic and anaerobic conditions all at the same time in one compact reactor system (de Kreuk and van Loosdrecht 2004; Kartal et al. 2010). This structure combined with

operational strategies allows for the selection of slow growing organisms. The cycle exists of a feeding period, an aeration period, a settling period and an effluent discharge period (Figure 4). In full-scale installations feeding and discharge are combined. The influent is fed in a plugflow regime from the bottom of the reactor by which the effluent is pushed out of the reactor. Depending on the process one wants to select for feeding can be mixed or in a plugflow regime. For the Nereda® technology an anaerobic plug flow is used to assure high concentrations of organic carbon and to promote the growth of phosphate accumulating organisms.

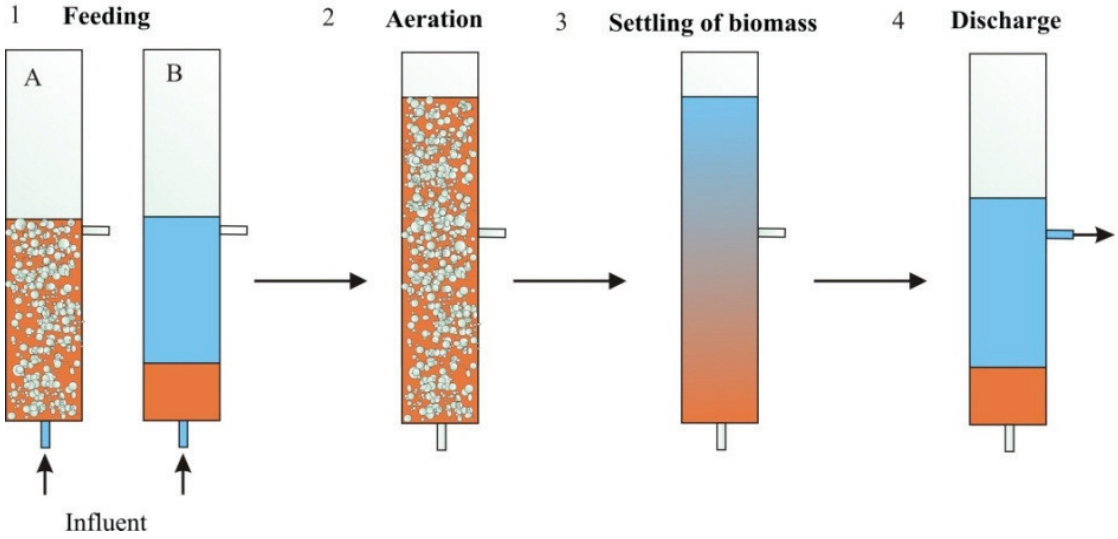

Figure 4 Cycle profile of the aerobic granular sludge sequencing batch reactor of: 1) feeding period either A) mixed or B) from bottom of the reactor in a plug flow regime, 2) aeration period, 3) settling period, and 4) an effluent withdrawal phase

Most ideally all easy degradable organic carbon will be anaerobically incorporated into biomass outcompeting aerobic oxidation of COD by fast growing heterotrophic organisms in the aeration period. During the aeration period PAOs will take up phosphate. Also ammonium is oxidized to nitrate in the oxygen penetrated outer zone of the granules by ammonium and nitrite oxidizing bacteria. The formed nitrate or nitrite can be further reduced to dinitrogen gas in the anoxic core (Figure 1) of the granule by denitrifying PAOs which use internally stored PHB as electron donor to reduce the produced nitrate. For the Anammox integrated in the aerobic granular sludge technology feeding and aeration can be combined since during the aeration

ammonium oxidizing bacteria (AOB) will produce nitrite in the outer oxygen penetrated layer, which diffuses into the inner anoxic core where Anammox can oxidize ammonium at the expense of the nitrite produced. Both Nereda® and Anammox have the fast settling period and effluent withdrawal in common.

MOTIVATION OF THE THESIS

Aerobic granular sludge technology is a recently-developed technology. Phosphate and nitrogen removal are successfully applied on full-scale. Research to better exploit the potential for granular sludge for a enhanced management of microbial communities can further optimize and extent the operation of granular sludge systems. For example is phosphate removal commonly problematic at higher temperatures because the glycogen accumulating organisms win the competition for organic carbon from phosphate accumulation bacteria (Lopez-Vazquez et al. 2009a; Lopez-Vazquez et al. 2009b). A similar problem can occur in the nitritation-Anammox systems, which can be troublesome at lower temperatures at which nitrite oxidizing bacteria are difficult to out-compete, leading to lower nitrogen removal efficiencies (Hellinga et al. 1998).

Clearly, an effective way of controlling microbial community composition would be beneficial for both treatment processes. Therefore, during this study a new method was developed to control the microbial community composition within granular reactors. The solid retention time is an efficient tool to engineer the microbial populations within wastewater treatment systems. The single reactor for high activity ammonium removal over nitrite (Sharon) system is a good example for this (van Dongen et al. 2001). In this system the preferential production of nitrite instead of nitrate from ammonium is accomplished by exploiting the higher growth rate of AOB compared to NOB at higher operational temperatures. This allows for selection of AOB and wash-out of NOB simply by reducing the solid retention time to approximately one day. However at colder temperatures this principle does not apply anymore. Moreover, in a complete autotrophic nitrogen removal over nitrite (CANON) all bacteria grow within one biofilm making a control based on SRT problematic. Therefore, other tools are needed in granular sludge technology to control the presence of bacteria. An option to shorten the SRT of bacteria would be possible if a natural segregation of biomass would occur in granular reactors. The idea of such segregation is very simple and is based on the principle that bigger and

denser granules settle faster while smaller lighter granules settle slower by which a particle-density provoked vertical segregation of biomass will occur. If such a density-size driven segregation would go along with a spatial separation of distinct bacterial groups growing in for instance smaller lighter and hence slower settling granules they would occupy the top layer of the settled sludge bed. This could be used as a powerful tool to remove one (process disturbing) population over another. Therefore, in a first step top and bottom biomass were examined of different reactors with the idea to improve rector performance by selective removal of the unwanted microbial population. After we found that the occurrence of segregation exists in several reactor configurations different questions arose why segregation occurs and how it can be used to control reactor performance:

I) Can segregation be used in Nereda® systems to selectively remove PAOs over GAOs at high temperatures thus yielding higher phosphate removal efficiencies?
II) Can segregation be used in nitritation-Anammox systems to selectively remove NOB over Anammox at low temperatures thus yielding higher nitrogen removal efficiencies?
III) How do precipitates and bacterial densities influence granular settling velocity and in turn the biomass segregation?
IV) How will the physical properties of water affect granular settling velocities of different size and density?

RESEARCH OBJECTIVES

The research had its focus on different methods to influence microbial competition in aerobic granular sludge with the objective to optimize process performance. The main focus of this thesis was hence to investigate parameters influencing the microbial community composition of granules of different size and density of a Nereda® system (**Chapter 2 and 3**) as well as a nitritation-Anammox system (**Chapter 4**). Therefore, for both reactor systems possible factors influencing granular settling velocities were tested. In a first step the physico-bio-chemical composition of a granule (**Chapter 2-4**) as well as the physical characteristics of the water itself (**Chapter 6**) was examined to see how they will influence the settling behaviour of the granules. In addition factors influencing granular densities were investigated to better understand how segregation occurs. Therefore, different bacterial densities as well as the impact of chemical precipitates on granular densities were inspected to evaluate their effect on granular settling velocities (**Chapter 5**).

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Chapter 2

SELECTIVE SLUDGE REMOVAL IN A SEGREGATED AEROBIC GRANULAR BIOMASS SYSTEM AS A STRATEGY TO CONTROL PAO-GAO COMPETITION AT HIGH TEMPERATURES

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ABSTRACT

An aerobic granular sludge (AGS) reactor was run for 280 days to study the competition between Phosphate and Glycogen Accumulating Organisms (PAOs and GAOs) at high temperatures. Numerous researches have proven that in suspended sludge systems PAOs are outcompeted by GAOs at higher temperatures. In the following study a reactor was operated at 30°C in which the P removal efficiency declined from 79% to 32% after 69 days of operation when biomass removal for sludge retention time (SRT) control was established by effluent withdrawal. In a second attempt at 24°C, efficiency of P-removal remained on average at 71±5 % for 76 days. Samples taken from different depths of the sludge bed analysed using *Fluorescent in situ hybridization (*FISH) microscopy techniques revealed a distinctive microbial community structure: bottom granules contained considerably more *Accumulibacter* (PAOs) compared to top granules that were dominated by *Competibacter* (GAOs). In a third phase the SRT was controlled by discharging biomass exclusively from the top of the sludge bed. The application of this method increased the P-removal efficiency up to 100% for 88 days at 30°C. Granules selected near the bottom of the sludge bed increased in volume, density and overall ash content; resulting in significantly higher settling velocities. With the removal of exclusively bottom biomass in phase four, P-removal efficiency decreased to 36% within 3 weeks. This study shows that biomass segregation in aerobic granular sludge systems offers an extra possibility to influence microbial competition in order to obtain a desired population.

INTRODUCTION

Aerobic granular sludge (AGS) reactors are based on the same principle as upflow anaerobic sludge blanket (UASB) reactors in which particles are freely suspended in an upward flow of air and liquid. Contrary to flocculent sludge processes, the biomass in these reactors is not homogeneously mixed. Mixing with gas yields a higher concentration of biomass at the bottom of the reactor than the top. Granules do not have identical physical characteristics and therefore there is a segregation of granules. Granules with larger radius or higher specific density will develop more rapidly settling characteristics, are therefore often at the bottom of the sludge bed. This segregation is viewed as a disadvantage in the particulate biofilm reactors because they lead to instabilities (Ro and Neethling 1994; Safferman and Bishop 1996; Trinet et al. 1991). These instabilities are attributed to a lower shear stress in the top of the sludge bed due to lower density (Gjaltema et al. 1997). The first attempts to numerically-model segregation of biomass in dependency of particle density and diameter caused by outgrowth were made by Di Felice et al in 1997 for fluidized bed reactors. Selection properties can be applied to prevent uncontrolled outgrowth of biomass by using batch wise feeding in granular sludge reactors (Beun et al. 2001). This generates a microbial population with a lower growth rate and hence smoother granules, which makes shear less important for a selection of well settling particles (de Kreuk and van Loosdrecht 2004; Van Loosdrecht and Heijnen 1993). Nicolella and colleagues wrote a review concerning the strength of particle-based biofilm reactors and their potential to develop compact and high rate nutrient removal processes (Nicolella et al. 2000). Nevertheless, for this particular technology segregation of granules is acknowledged to be a difficult process due to, for instance, clogging.

However, researchers have neglected that segregation of differently sized granules may in fact offer different biological niches for bacteria. In UASB reactors as well as in AGS reactors segregation of microbial communities can occur as a function of depth within the different layers of the granules (Macleod et al. 1990; Tsuneda et al. 2003; Xavier et al. 2007). However, it has been neglected that segregation might also occur over the settled sludge bed due to differently sized granules. Circumstances like shear stress or substrate concentrations are different at certain depths. As a result, distinct biological niches can be generated within one reactor, by which one organism can be favoured over others. For instance, it might be possible to influence the SRT of certain organisms independent of other bacteria

depending on the place of excess sludge withdrawal. The importance of controlled biomass removal in biofilm systems has already been experimentally and mathematically discussed earlier (Morgenroth and Wilderer 1999). Previous research has demonstrated that PAOs were prevalent at 10ºC regardless of the specific carbon source or pH (de Kreuk et al. 2005; Lopez-Vazquez et al. 2009b). At temperatures between 20-30°C GAOs are expected to dominate the culture, while at increasing temperatures common heterotrophs dominate the system (Erdal et al. 2003; Lopez-Vazquez et al. 2009a; Panswad et al. 2003; Whang et al. 2007). In our research we hypothesise that if PAOs and GAOs would be differentially distributed over the sludge bed, then selective removal of the GAO dominated section of the settled sludge bed would reduce the SRT for GAOs relative to PAOs. This would in turn make it feasible to obtain good biological phosphate removal at temperatures above 20°C.

MATERIALS AND METHODS

CYCLE OPERATION AND MEASUREMENTS

The granular sludge reactor operation was similar to that described by de Kreuk and van Loosdrecht (de Kreuk and van Loosdrecht 2004). It consisted of a 60 min anaerobic feeding period from bottom of the reactor in a plug flow regime followed by a 111 min period of aeration, 3 min settling, 5 min effluent withdrawal and a 1 min idle period. In the aeration period the dissolved oxygen (DO) concentration and pH were controlled at 20% air saturation and 7±0.2 pH units, respectively. Temperature was held constant at 30°C with a thermocycler and was protected against cooling with a cellular isolation placed around the reactor. The feed medium consisted of 3.1 mM $NaCH_3COO{\cdot}3H_2O$ (400 mg COD/L), 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KCl, 2.1 mM NH_4Cl (60 mg N/L), 0.2 mM K_2HPO_4 and 0.1 mM KH_2PO_4 (20 mg P/L). A ‘Vishniac and Santer’ solution was used to provide trace elements (Vishniac and Santer 1957).

A cycle measurement was conducted and samples were taken during the aerated mixing period to measure system performance of P- and COD-removal during one cycle of operation. Phosphate was analysed spectrophotometrically by the use of standard test kits (Hach-Lange). Calculations for P-removal efficiency are based on influent-effluent basis and 100% efficiency was hence reached when no P was detectable in effluent.

LONG TERM REACTOR OPERATION

The reactor operation can be divided into 4 phases. Firstly, the reactor was run for 69 days at 30°C and was inoculated with granules from an aerobic granular sludge pilot plant in Epe, The Netherlands, treating municipal wastewater and showing excellent N- and P- removal efficiencies. In the second phase, half of the granules were removed and replaced with granular sludge from a lab reactor that was operating at 20°C with an excellent P-removal efficiency. Temperature was decreased to 24°C, to favour PAOs, and the reactor was operated from day 69 until day 150 under the set conditions. During phase one and two the SRT was controlled by the sludge washed out with the effluent withdrawal. In the third phase which lasted from day 150 until day 240, sludge was manually withdrawn from the upper part of the sludge bed and the temperature was raised to 30°C. In the final and fourth phase, sludge was removed from the bottom to provoke the washout of PAOs. In the final two phases, the SRT was controlled at approximately 21 days by removing every third day 15% (phase 3 and 4 a) or every sixth day 30% (phase 4 b) of the settled sludge bed. The SRT was calculated taking the volatile suspended solids (VSS) from the reactor, effluent, and excess sludge into consideration.

A sample was taken and transferred into a small measuring cylinder for determination of the VSS from the reactor and excess sludge. The volume of settled biomass in the small cylinder was used to calculate the ash and dry weight of the biomass. The VSS of settled biomass within the cylinder was then related back to the volume occupied by the settled sludge bed in the reactor as well as to the volume taken out as excess sludge by recording the height of settled sludge in the reactor and the height of excess sludge removed. The VSS was calculated on a mass basis by the following equation $\mathrm{VSS}_{\mathrm{r,ex}} = \mathrm{DW}_{\mathrm{set.\ r,ex}} - \mathrm{ash}_{\mathrm{set.\ r,ex}}\ [\mathrm{gVSS}]$.

The SRT was calculated on the basis of the change in height of the sludge bed occurred due to growth (VSS_r), the amount of excess sludge removed for manual SRT control ($Q_{ex,VSS}$) and by the sludge washed out with effluent withdrawal ($Q_{eff,VSS}$). The calculation was conducted according to the following equation $SRT = \frac{V_r \cdot VSS_r}{Q_{eff,VSS} + Q_{ex,VSS}} [day]$. Please refer to Appendix 1 for the definitions and side calculations.

DENSITY AND SIZE DISTRIBUTION MEASUREMENTS

Bottom and top granules were sampled for measurements of particle size distribution, dry weight, ash content and granule density. Specific biomass density was measured with a pycnometer and size distribution measurements were conducted by the means of an image-analyser. Stokes law for laminar flow was used to calculate settling velocities by applying the equation $SV = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{v_w}$ for $Re_{particle} \leq 1$ and compared to measured settling velocities which were recorded as the time that granules settled in a 30 cm volumetric cylinder. Definitions for Stokes law are given in Appendix 2. In phase four, the removed bottom biomass was sampled on a weekly basis to measure a change in settling behaviour and physical properties caused by selective sludge removal over time.

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

Bottom and top samples were taken over time for FISH analysis in order to assess microbiological properties. FISH was performed on crushed mixed (figure 3.1a 3.1.b), top (3.2a-3.4a) and bottom granules (3.2b-3.4b) in order to determine GAO and PAO microbial populations. Different probes were tested to ensure a good representation of PAO and GAO population and the resulting probes and sequences are listed in Table 1. Crushing was accomplished on 10ml granules by the means of a glass mortar (Glas-Col). From this suspension 500 µl were fixed in 4% paraformaldehyde and incubated for 120 min at room temperature. After fixation, samples were centrifuged for 2 min at 16000 rpm, washed twice in 1x Phosphate buffer saline (PBS), and re-suspended in volume of 1:1 Ethanol/PBS buffer for storage at -20°C.

For hybridisation, the fixed samples were dried on a hybridization slide with 6 wells preventing mixing of probe in adjacent wells and dehydrated by incubating the microscope slides in 50%, 80% and 100% ethanol for 3 min in each solution. After dehydration, the hybridization solution (10 µl) and 25 ng of oligonucleotide probe tagged with a fluorescent label (Fluos, Cy5 or Cy3) was added to each well, and the samples were incubated for 2 hours in a humid chamber at 46°C. The hybridization buffer consisted of a mixture of 360 µl of 5 M NaCl, 40 µl of 1 M Tris (pH 8), 10 µl of a 10% (w/v) sodium dodecylsulfate buffer (SDS), 700 µl of formamide, and 900 µl of MilliQ water (Amann et al. 1990; Crocetti et al. 2002; Crocetti et al. 2000; Daims et al. 1999). After hybridisation, the microscope slides were washed at 48°C for 15 min by immersing them into 50 ml of washing solution consisting of 800 µl of 5 M NaCl, 500 µl of 0.5 M EDTA, 1000 µl of 1M Tris (pH 8), and 50 µl of 10% SDS (w/v). The samples were dried and prepared with 2 µl antifade fluorescent mounting oil and analyzed with an epifluorescence microscope (Axioplan 2, Zeiss). Ratios between PAOs and GAOs were roughly estimated based on visual determination.

Table 1 Oligonucleotide probes, target microorganisms, and references used in this study

Probe	Sequence (from ‘5 to ‘3)	Specificity	Reference
PAO 462	CCGTCATCTACWCAGGGT ATTAAC	Most *Accumulibacter*	(Crocetti et al. 2000)
PAO 651	CCC TCTGCCAAACTCCAG	Most *Accumulibacter*	(Crocetti et al. 2000)
PAO 846	GTTAGCTACGGACTAAAA GG	Most *Accumulibacter*	(Crocetti et al. 2000)
GAO Q989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	(Crocetti et al. 2000)
GAO Q431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	(Crocetti et al. 2002)
EUB 338	GCTGCCTCCCGTAGGAGT	*Most bacteria*	(Amann et al. 1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetales*	(Daims et al. 1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	(Daims et al. 1999)

Probes PAOs were tagged with the fluorescent dye Fluos (green) GAOS with Cy3 (red) and Eub with Cy5 (blue). For analysis probes of one target group were mixed

RESULTS

CYCLE OPERATION

A cycle measurement was conducted in phase three to show a typical reactor performance during one cycle of operation (Figure 1). A classical graph for N and P removal behaviour in aerobic granular sludge based systems is depicted as described by de Kreuk et al. (2005b); nitrification occurs on the outer layers and denitrification and phosphate uptake in the core of the granules. One cycle lasted 3 hours starting with 60 minutes of anaerobic feeding during which all incoming ammonium, phosphate and acetate were fed from the bottom of the reactor in a plug flow regime. All acetate was taken up during the anaerobic feeding period and phosphate was released due to the activity of PAOs. Samples for the cycle measurement were collected only during the aerobic mixing period since sampling is not possible during feeding due to the plug flow regime, which should not be disturbed.

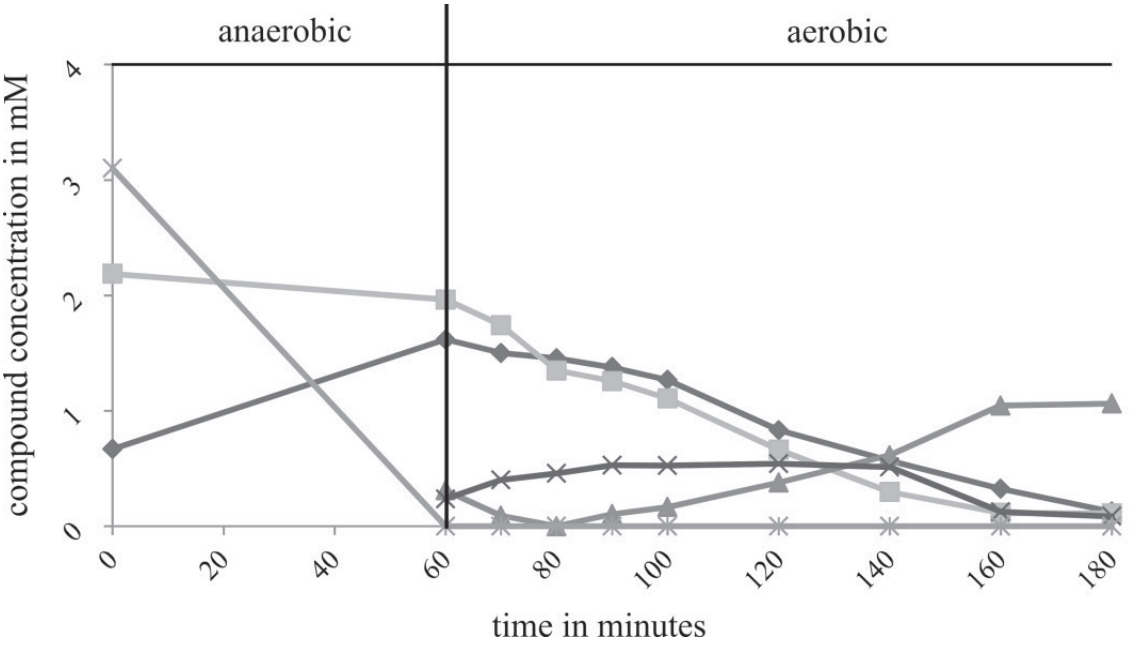

Figure 1 Typical concentration patterns of phosphate (♦), nitrate (▲) nitrite (X) and ammonium (■) during a cycle in an aerobic granular sludge reactor. COD (Ж) is completely consumed in the anaerobic period. Note that measurement during anaerobic phase is not possible due to a strict anaerobic plug flow operation without mixing.

LONG TERM OPERATION

The reactor was operated in four phases over a time period of 280 days. The reactor initially established a P-removal efficiency of 79% when the reactor was run in phase one at 30°C. However, after 69 days of operation P-removal efficiency dropped to 32% (Figure 2a phase 1). In order to enhance P-removal efficiencies in phase two, half of the sludge was discharged and the reactor was inoculated with new granular sludge from a lab-scale AGS reactor run at 20°C showing 100% P removal efficiencies. Following this inoculation, the operational temperature was decreased to 24°C (phase 2) and the P removal efficiency remained on average at 71±5% for 76 days (Figure 2a). During the first two phases, the SRT was defined by washout of sludge during effluent withdrawal.

In the second phase, FISH results illustrated that sludge samples taken from the top of the sludge bed consisted of more GAOs (Figure 3.2a) whereas the bottom sludge contained more PAOs (Figure 3.2b). In order to selectively remove GAOs (*Competibacter*) from the system and hence keep their SRT lower in respect to PAOs (*Accumulibacter*) a third phase was initiated in which biomass was withdrawn from the upper part of sludge bed. The amount of biomass withdrawn was established as such that an average SRT of 25±15 days was achieved according to the SRT calculation as given in the material and methods section. Furthermore, the temperature was raised to 30°C to disfavour PAOs and in order to measure the effect of sludge control on the PAO-GAO competition. Within 3 weeks the P-removal efficiency improved to up to 100% and remained on average at 92±7% for an additional 67 days (Figure 2a phase 3). In the fourth phase sludge was removed from the bottom to determine whether higher P removal efficiencies from phase 3 are indeed due to selective removal of top sludge or solely an effect of lower SRT. Selective removal of the bottom PAO dominated sludge eventually resulted in a decrease in P removal efficiency to 36% within 6 weeks (Figure 2a phase 4). Phase four consisted of two sub phases: in phase 4a sludge was removed in the same manner as it was accomplished for removal of top sludge which was based on removing approximately 15% of the settled sludge bed every three days, achieving on average a SRT of 21±7 days.

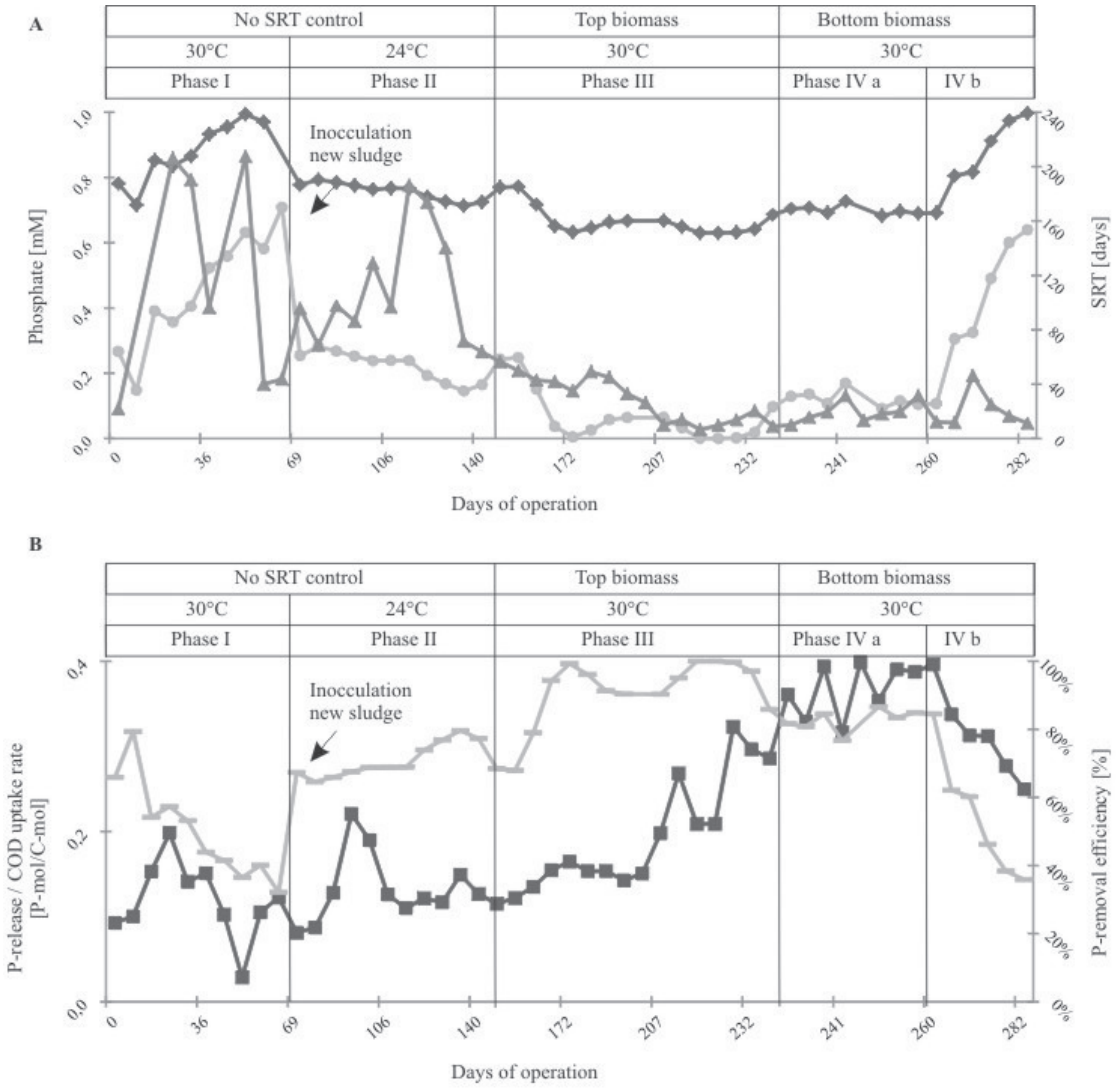

Figure 2 a Phosphate effluent (●) and influent (♦) concentration in mM PO_4-P as well as SRT (▲) in days. Figure 2b shows the P release/COD uptake ratio (■) and the P- removal efficiency (—) over time. Experimental setup was divided in four phases. In phase one the reactor was run at 30°C when removal efficiency dropped to 30%, a second phase started and the system was inoculated with new granular sludge with excellent P-removal capacity, after which P-removal efficiency remained 50%. In phase three, granules were manually withdrawn from the upper part of the sludge bed resulting in 100% P-removal efficiency. In phase four, granules were discarded from the bottom of sludge bed resulting in a collapse in removal efficiency.

During this phase removal efficiency remained on average at 83±8%. In phase 4b the selective removal was changed to see the effect of removing a bigger PAO fraction. Here the SRT was kept constant, however, instead of removing 15% of the settled sludge bed every three days, 30% was removed every six days. During this time the P-removal efficiency dropped from 85% to 36% stressing the importance of proper sludge control. The P release/ COD uptake ratio is depicted in Figure 2b. In a highly enriched PAO culture a P release/COD uptake ratio of about 0.5 P-mol/C-mol can be expected in contrast to a pure GAO culture in which this ratio would decline to zero (Brdjanovic et al. 1997; Smolders et al. 1994). Ratios of about 0.15 P-mol/C-mol were observed in phase 1 and 2 in which removal efficiencies were low and GAOs dominated the system, as indicated by FISH (Figure 3). However, in phase three when SRT was controlled by selectively removing top granules the ratio gradually increased in correlation with removal efficiencies to 0.34 Pmol/Cmol. In phase four the GAOs became prevalent again and the ratio decreased in correlation with the decreasing P-removal efficiencies.

FLUORESCENCE IN SITU HYBRIDIZATION ANALYSIS OF SLUDGE

During the experiment, samples of the sludge were regularly subjected to analysis by FISH. Virtually all cells in the sludge where stained by either *Accumulibacter* (PAO) or *Competibacter* (GAO) probes, indicating that these formed the large majority of the microbial population in the sludge. Figure 3 gives an overview of the most relevant samples. Firstly, the reactor was run at 30°C in which a mixed sludge sample, taken at the end of phase one (no distinction between bottom /top), revealed a higher dominance of GAOs (Figure 3.1.a). At the same time the removal efficiency was also low (32%, day 66). For phase two, half of the reactor sludge was discarded and inoculated with new granular sludge containing mainly PAOs (Figure 3.1b) to ensure an equal starting point for competition of PAOs and GAOs.

P removal efficiency increased instantaneously after inoculation but declined over time. At day 140 bottom and top sludge were checked separately for their microbial community composition. FISH analysis of bottom and top sludge was conducted because stratification of biomass was visually observed in both phases. During the aerobic mixing period biomass density was higher at the bottom. Following the settling period, large, heavy granules remained closer to the bottom whereas smaller granules were concentrated at the top portion of the expanded sludge bed. FISH results

revealed that the top sludge contained considerably more GAOs (Figure 3.2a) whereas the bottom sludge were enriched by PAOs (Figure 3.2b), overall indicating a vertical segregation of microorganisms over the sludge bed. Based on these observations a third phase was initiated in which top sludge was removed to favour PAOs over GAOs. P removal efficiencies increased during this phase to 100% and FISH results of top sludge illustrated an increase in the PAO populations (Figure 3.3a) and a dominance of PAOs in the bottom sludge (Figure 3.3b).

A fourth phase was conducted in order to show that the segregation of community composition over the sludge bed was indeed an effect of sludge control from a specific height of the settled bed. During this phase, sludge was removed from the bottom while keeping the same SRT. P-removal efficiency dropped to 36% and the bottom and top microbial populations were dominated by GAOs (Figure 3.4a; 3.4b).

DENSITY AND SIZE DISTRIBUTION MEASUREMENTS

The physical properties of top and bottom granules during phase three have been evaluated and are given in Table 2. During phase three, the sludge age was manually controlled by sludge removal from the top of the sludge blanket. There was a clear difference in ash percentage between top and bottom sludge. The higher ash content was also reflected in a higher density of the bottom granular sludge. The measured density of 1018±13 g/l for bottom granules versus 1004±4g/l for top granules contributed to the higher settling rates of bottom granules. The diameter derived from the average surface area of bottom granules was also larger. The obtained physical parameters were used to estimate the settling velocity applying Stokes law as given in the material and methods section. There was an estimated 3-4 factor difference in settling velocities between top and bottom granules.

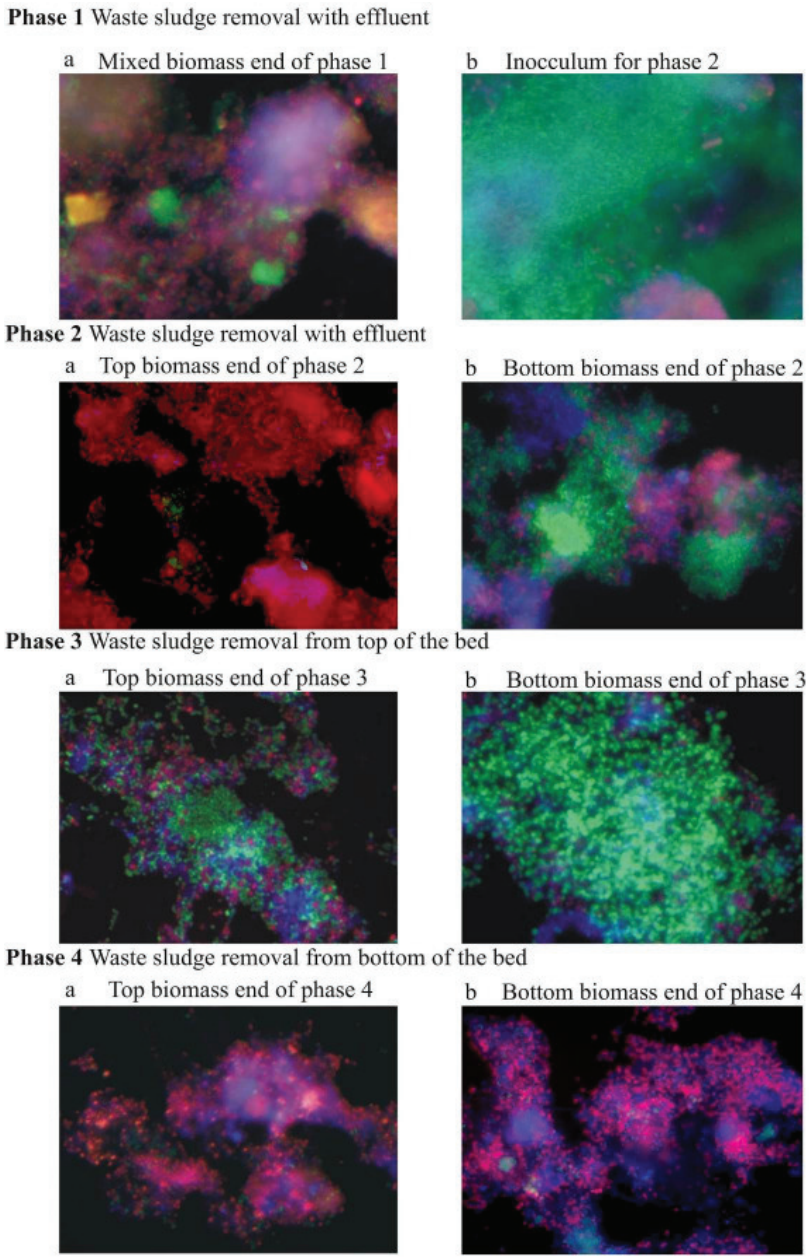

Figure 3 Hybridization with Cy3-red (GAO-competibacter), Cy5-blue (Eub) and Fluos-green (PAO-Accumulibacter)-labelled probes. Epifluorescence photomicrographs are shown for top and bottom biomass at different time points during reactor operation. Picture 3.1a FISH image of mixed biomass at the end of phase 1, Picture 3.1b population used for inocculum to start up phase 2, Picture 3.2 segregation of PAOs and GAOs at a) top and b) bottom of the reactor at the end of phase 2 as well as same relation shown for phase 3 when top sludge was removed and phase 4 when bottom sludge was removed.

Table 2 Physical properties of bottom and top granules during sludge control of top biomass (Phase 2)

Parameter	Top	Bottom
Settling velocity Calculated m/h	20±5	80±9
Settling velocity Measured m/h	n.m	66±9
Ash content %	15±0.1	34±0.1
Density g/l	1004±4	1018±13
Average diameter[1] (mm)	0.8±0.1	1.1±0.2

Average and standard deviations from different days. Measurements were conducted during phase 3 in which SRT was controlled by discarding top biomass. nm: not measured

Furthermore, calculations revealed that differences in settling velocities were influenced equally due to both changes in the radius and the density of the granules. The estimated velocities were similar to the measured velocities. Additionally, the settling properties of removed bottom sludge were measured during phase 4 (see Figure 4). The settling rate of the bottom sludge decreased rapidly and continuously over time. Results revealed that in phase 4a, settling velocities of bottom sludge decreased from 80 m/h to 50 m/h when 15% of the settled sludge bed was removed every third day. However, 30% of the settled sludge bed was removed every six days in phase 4 b. As a consequence, P removal efficiency dropped to 36% while settling velocities of bottom sludge declined to 12 m/h.

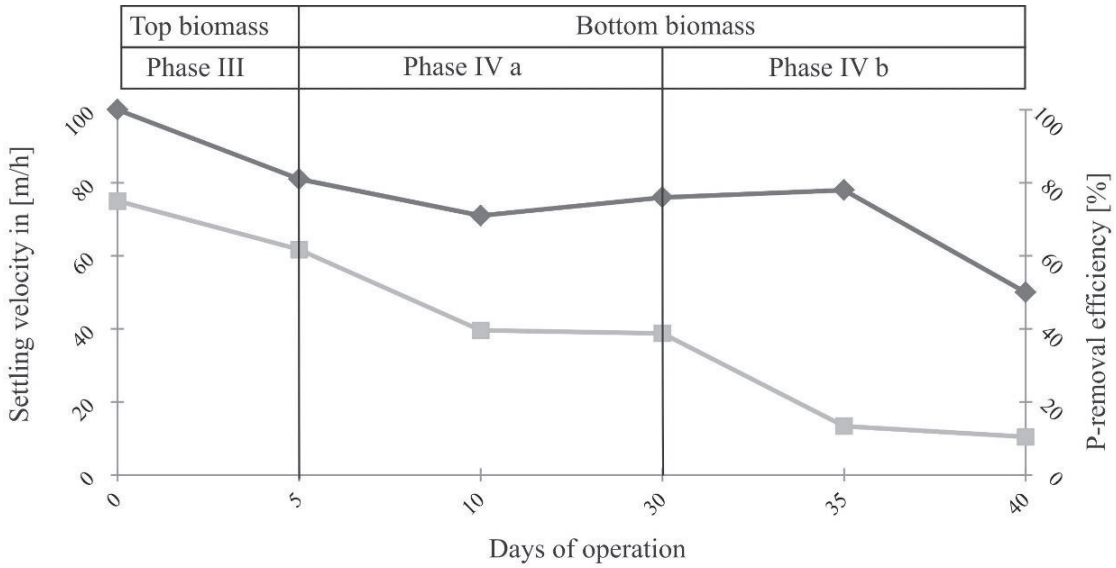

Figure 4 P-removal efficiency (♦) and settling velocity in m/h (■) of bottom granules in phase three (top sludge removal) and in phase four (bottom sludge removal). Settling velocities were calculated by Stokes law based on data obtained from density and size distribution measurements.

DISCUSSION

In granular sludge systems segregation of biomass can easily occur due to slight variations in density and diameter of the particles. The opportunity to select for specific microbial groups at different heights within the column, allows for imposing additional selective pressure in granular sludge systems compared to traditional activated sludge systems. This is delineated by the ability of the granular sludge to maintain PAOs as the dominant group at 30°C by selectively removing top biomass dominated by GAOs and thus keeping their SRT low. Studies which were carried out at higher temperatures have shown that a very short cycle length or a low sludge age (3 days) can also lead to a stable P removal efficiency at 30°C (Freitas et al. 2009; Whang and Park 2006). Without sludge control, flocculent sludge systems have been shown to favour the enrichment of GAOs over PAOs (Lopez-Vazquez et al. 2009a). The results obtained in these experiments revealed that granules dominated by PAOs (*Accumulibacter*) were bigger, more dense and thus have the advantage to remain at the bottom of the reactor due to faster settling velocities. Since the reactor is fed in a plug-flow regime from the bottom, it is evident that bottom granules have more

substrate available, leading to a niche where PAOs were exposed to a higher percentage of the available substrate as compared to GAOs. Since sludge withdrawal is accomplished from the top of the sludge bed the SRT of the GAO population is effectively lower than for PAO dominated granules leading to a washout of GAOs over time. The main heterotrophic microbial population consisted of PAOs (*Accumulibacter*) or GAOs (*Competibacter*) since all acetate was always taken up before the aerobic period started leaving no more organic carbon available for normal heterotrophs. Moreover, the P release/COD uptake ratio positively correlated with removal efficiencies and data derived from FISH analysis. For example, when P-removal efficiencies and PAO content were high within the reactor, the P release/COD uptake ratios were additionally high.

P-removal deteriorated in phase four when the required SRT was established by extracting bottom biomass instead of top biomass as performed in phase 3, demonstrating that the P-removal efficiencies were improved due to selective sludge removal and not only due to the lower SRT values. The difference in P-removal between phase 4a and b demonstrate that selective sludge control is strongly dependent on the amount of biomass extracted. The high values for the P release/COD uptake ratios in phase 4a suggest that although P removal dropped, bottom biomass removal also stimulates new growth of PAOs. In phase 4b, when a larger proportion of bottom granules was removed, P removal declined and at the same time also the settling velocities of bottom granules (due to a decrease in diameter and density), which minimizes segregation and herewith the advantage of bigger PAO dominated granules to always settle first to the bottom. Again, this is of advantage because bottom granules have more substrate available due to the plug flow feeding regime. This highlights the importance in obtaining knowledge concerning mechanisms leading to segregation of biomass, particularly how selective pressure of certain organisms over others can be influenced by removal of sludge from a specific depth within the reactor. In addition it is also significant to gain more knowledge about how selective sludge removal from the top or bottom of the sludge bed is influencing the SRT distribution of different granules in the reactor. For the renewal of bottom biomass and hence the growth of PAOs, sludge removal of bottom biomass could facilitate in avoiding the deterioration in P-removal over time. In order to better understand the effect of sludge removal it would be necessary in future studies to determine the P content of removed sludge and to make a proper P-mass balance.

An explanation of how segregation occurs is that in PAO dominated granules the PO_4 released per unit of acetate removed is higher than in GAO dominated granules, which is attributed to a lack of an active P-uptake/release metabolism in GAOs. Since the settling of biomass occurs after the aeration period PAO dominated granules have accumulated high amounts of poly-P, which will improve their settling properties in comparison to GAO dominated granules. The higher ash content of bottom PAO dominated granules might hence be due to higher poly-P content. Since chemical precipitation is strongly dependent on PO_4 concentrations (Carlsson et al. 1997; Maurer et al. 1999) and only PAOs excrete phosphate, chemical precipitation in a PAO dominated granule might enhance this effect. This way the system has a self-enhancing effect on P removal efficiencies since PAOs can remain in the system despite the high temperatures, which are known to be favourable for GAOs (Lopez-Vazquez et al. 2009b).

Selective sludge removal at different heights in a granular sludge bed might offer a good opportunity to conduct microbial population engineering in AGS and UASB bed technology. Recent research has demonstrated the existence of segregation in other systems. For example, Volcke et al., (2010), illustrated that in a granular sludge nitritation/anammox system nitrite oxidising bacteria accumulate preferentially in smaller granules, again allowing a method for controlling nitrite oxidising bacteria by selective sludge withdrawal. Additionally, selective sludge removal has been shown to enhance granulation processes (Li and Li 2009). It is thus of interest to evaluate the activity of microorganisms in relation to the depth within the sludge bed and to design a sludge extraction protocol based on the specific population one wants to select for.

CONCLUSIONS

In this work it was investigated whether segregation of biomass occurs along the sludge bed. PAOs were prevalent at the bottom, whereas GAOs dominated at the top of the sludge bed. By selective removal of GAO dominated sludge from the top of the sludge bed 100% P removal efficiencies were achieved at 30°C. This study also shows that selective sludge withdrawal in granular sludge reactors can be used as an extra operational parameter to engineer the microbial population in the reactor.

APPENDIX

Equations for calculating VSS of reactor and excess sludge as well as Sludge retention time (SRT) during sludge control

$$SRT = \frac{V_r \times VSS_r}{Q_{eff,VSS} + Q_{ex,VSS}} \quad [day]$$

$$V_{set.\ r,ex} = \pi r^2 \times h_{set.r,ex} \quad [m^3]$$

$$DW_{set.} = \frac{DW}{V_{set.\ cyl}} \times V_{set.\ r,ex} \quad [g\ dry\ weight]$$

$$ash_{set.} = \frac{ash}{V_{set.\ cl}} \times V_{set.\ r,ex} \quad [g\ ash]$$

$$VSS_{r,ex} = DW_{set.\ r,ex} - ash_{set.\ r,ex} \quad [gVSS]$$

V_r = Volume reactor $[m^3]$

$VSS_{r,ex}$ = volatile suspendid solids reactor or excess $[gVSS]$

$Q_{eff,VSS}$ = Outflow VSS effluent $\left[gVSS_{eff} \times \frac{m^3}{day} \right]$

$Q_{ex,VSS}$ = Outflow VSS of bottom or top sludge controle $\left[gVSS_{ex} \times \frac{m^3}{day} \right]$

$h_{set.\ r,ex}$ =bed height settled sludge reactor and removed for excess $[m]$

$V_{set.\ r,ex}$ =Volume settled sludge bed in reactor or excess sludge $[m^3]$

$V_{set.\ cyl}$ =Volume settled sludge bed in measuring cyclinder $[m^3]$

SRT = solid retention time [days]

Equations for calculation of settling velocity by Stokes law

$$SV = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{\nu_w} \text{ for } Re_{particle} \leq 1$$

SV = 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 = denisty 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 $\left[\frac{m^2}{s}\right]$

Re = Re ynolds number of a particle $[-]$

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Chapter 3

IMPROVED PHOSPHATE REMOVAL BY SELECTIVE SLUDGE DISCHARGE IN AEROBIC GRANULAR SLUDGE REACTORS

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ABSTRACT

Two lab-scale aerobic granular sludge sequencing batch reactors were operated at 20°C and 30°C and compared for phosphorus removal efficiency and microbial community composition. P-removal efficiency was higher at 20°C (> 90%) than at 30°C (60%) when the sludge retention time (SRT) was controlled at 30 days by removing excess sludge equally throughout the sludge bed. Samples analysed by fluorescent in situ hybridization (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 P-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% P-removal efficiency was obtained in the reactor operated at 30°C. In the meantime, the biomass in the 30°C reactor changed in colour from brownish-black to white. Big white granules appeared in this system and were completely dominated by PAOs (more than 90% of the microbial population), showing relatively high ash content compared to other granules. In the reactor operated at 20°C, P-removal efficiency remained stable above 90% regardless of the sludge removal procedure for SRT control. The results obtained in this study stress the importance of sludge discharge mainly from the top as well as in minor proportions from the bottom of the sludge bed to control the SRT in order to prevent significant growth of GAOs and remove enough accumulated phosphate from the system, particularly at high temperatures (e.g. 30°C).

INTRODUCTION

Enhanced biological phosphate removal (EBPR) processes are widely applied for phosphorus removal from domestic and industrial wastewaters due to their economic and environmental advantages compared to physical-chemical processes. EBPR systems rely on the activity of organisms capable of storing phosphorus intracellularly as polyphosphate. These organisms, known as polyphosphate accumulating organisms (PAOs), can be enriched by submitting the sludge to alternating anaerobic and aerobic/anoxic conditions (Mino et al. 1998). However, these operating conditions can also favour the growth of other microorganisms which perform similar carbon transformations of that of PAOs (Satoh et al. 1994). These organisms are referred to glycogen accumulating organisms (GAOs), which compete with PAOs for available organic carbon substrates but not contribute for phosphate removal (Oehmen et al. 2006). Therefore, they are undesired organisms generally associated with the deterioration of bio-P removal systems.

Several factors influencing the PAO-GAO competition are reported in literature: pH (Filipe et al. 2001), P/C ratio, SRT (Whang and Park 2006), organic carbon sources(Oehmen et al. 2007) and influent phosphate/volatile fatty acids (P/VFA) ratio (Liu et al. 1997; Schuler and Jassby 2007). Besides all aforementioned factors, other important parameter influencing the PAO-GAO competition is the temperature. In general, temperatures higher than 20°C are reported to cause a deterioration of EBPR processes because GAOs become the dominant microorganisms (Barnard and Steichen 2006; Gu et al. 2005; Panswad et al. 2003). PAOs were found to dominate the microbial community at lower temperatures, such as 10°C (de Kreuk et al. 2005; Lopez-Vazquez et al. 2009).

In order to obtain stable phosphorus removal at high temperatures, such as 30°C, several operational strategies have been reported in literature. The application of a short cycle length in sequencing-batch reactors or low sludge retention time (SRT) are examples (Freitas et al. 2009; Whang and Park 2006). In previous research (Winkler et al. 2011) on PAO-GAO competition in an aerobic granular sludge reactor at 30°C, segregation of biomass over the height of the sludge bed was observed. FISH analysis showed that the granules at the bottom of the sludge bed were dominated by PAOs whereas GAOs were dominant at the top of the sludge blanket.

This different distribution of PAOs and GAOs over the sludge bed was used to control the SRT of the individual populations by selective removing sludge only from the GAO-rich part of the sludge blanket, while no bottom PAO-dominated sludge was removed. Indeed, the specific sludge removal strategy enabled a high P-removal efficiency at 30°C. Several open questions, however, still remained. In the previous study, it was pointed out that eventually it would be necessary to remove part of the bottom sludge to renew that specific biomass in which PAOs are dominant in order to remove P from the system and allow PAOs to grow. Moreover, the selective sludge removal from specific parts of the sludge bed was not studied for lower temperatures. Therefore, a comparative study on the performance, in terms of phosphorus removal, of two aerobic granular sludge reactors operated at different temperatures (20 and 30°C) was performed. The selective sludge removal for SRT control was modified compared to the previous study in order to improve reactor performance and stability.

MATERIALS AND METHODS

EXPERIMENTAL SET-UP

Two lab-scale aerobic granular sludge sequencing batch reactors, designated as SBR_{20} and SBR_{30}, were operated in parallel at 20°C and 30°C, respectively. The volume of both systems was 2.6 L. For the SBR_{30}, the temperature was maintained constant at 30°C by pumping water with the desired temperature through tubes which were placed around the reactor. A thermal insulation surrounded the whole reactor to prevent cooling. Aeration and mixing were supplied through an air diffuser placed in the bottom of the reactors (airflow rate of 4 L/min). The pH was controlled during the aeration phase at 7.0 ± 0.2 by dosing 1 M NaOH or 1 M HCl. Both reactors were operated using a 3 hour-cycle under alternating anaerobic and aerobic conditions. The SBR cycle consisted of an anaerobic feeding phase of 60 min 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 volumetric exchange ratio was 57%, resulting in a hydraulic retention time (HRT) of 5.2 h. A bio controller (Braun DCU4 coupled with mass flow control system and data acquisition software) was used to control and operate the SBRs. The dissolved oxygen (DO) concentration was maintained at 1.8 mgO_2/L by using two mass flow controllers (one for air and other for nitrogen gas).

OPERATING CONDITIONS AND CALCULATION PROCEDURES

SBR_{20} was inoculated with granules from a pilot-scale aerobic granular sludge reactor treating municipal wastewater (Nereda®, EPE, The Netherlands). When a stable operational performance was established in SBR_{20} (full P-removal), half of the sludge was removed from that system to inoculate SBR_{30}. From this point henceforth, the operation of the aerobic granular sludge reactors was divided in different experimental phases, as shown in Table 1.

Table 1 Experimental phases of both aerobic granular SBR reactors.

Experimental Phase[a]	Excess sludge for SRT control	Phosphate influent concentration	Operational time (days)
Phase I	Mixed sample	20	39
Phase II	80% top[b] 20% bottom[b]	20	131
Phase III	100% bottom	2	50

[a] The operational time of SBR_{20} before the inoculation of SBR_{30} was not taken into account here. Instead, it was considered a start-up phase prior to Phase I.
[b] Top and bottom refers to the upper and down part of sludge bed, respectively.

The sludge retention time (SRT) was determined according to Winkler et al. (2011) and was maintained at around 30 days by periodically removing sludge from the reactor (excess sludge) every 2 days throughout the experimental phases. During Phase I, excess sludge for SRT control was removed during aeration (mixed sample). In Phase II, excess sludge was removed during anaerobic feeding, when the granular sludge was settled. In this phase, most of the sludge was removed from the top of sludge bed to favour PAOs over GAOs. In general, 80% of the wasting sludge (in volume basis) was removed from the top and 20% from the bottom of sludge blanket. During Phases I and II, the synthetic feeding medium consisted of two solutions: (A) $NaCH_3COO{\cdot}3H_2O$ 63 mM, $MgSO_4.7H_2O$ 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 media together with 1,200 mL of tap water in order to achieve chemical oxygen demand (COD), ammonium and phosphate influent concentrations of 400 mg/L, 60 mgNH_4-N/L and 20 mgP-PO_4/L, respectively.

The strategy of selective removing sludge mainly from the top of the sludge bed performed in Phase II, was changed in Phase III. In order to observe the link between the key factors influencing segregation within the reactor sludge bed (i.e. chemical precipitation, poly-P and ash content of biomass), sludge was removed mainly from the PAO-rich bottom of the sludge bed and phosphate influent 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 phosphate) immediately after the start of the aeration phase. This procedure was repeated in 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. P-removal efficiency along the operation of the reactors was determined on influent-effluent basis (100% P-removal was achieved when no phosphate was detectable in the effluent). Cycle tests were conducted in the steady state operation of Phases I and II in order to obtain the biomass specific phosphate uptake rates. Samples were collected every 10 to 20 min only during aeration phase when the reactor content was mixed. Sampling during anaerobic feeding was not possible due to the plug-flow operation. Specific phosphate rates were obtained by linear regression of the concentration over time divided by the amount of volatile suspended solids (VSS) in the reactor.

DETERMINATION OF GRANULES PHYSICAL PROPERTIES AND ANALYTICAL MEASUREMENTS

Particle size distribution 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. The procedures used to calculate the reactor biomass concentration and excess sludge are described in Winkler et al. (2011). Phosphate (PO_4-P) was determined spectrophotometrically by Hach Lange cuvette tests.

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 smaller 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 bigger 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.

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

To assess the microbial populations, FISH was conducted on crushed granules. Crushed granules were washed twice with 1x phosphate-buffered saline (PBS) and immediately fixed with 4% (w/v) paraformaldehyde in PBS solution for 3h at 4 ºC. After fixation, cells were centrifuged at 13.000 g for 1 min, washed twice in 1x PBS and re-suspended in an ethanol/PBS solution (1:1) for storage at -20 °C. The hybridization step was performed according to earlier research (Bassin et al. 2012) . Fixed samples were spread on microscope slides and incubated at 46 ºC for drying. The cover slips with the dried cells were dehydrated in three steps 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, 0.02% (w/v) sodium dodecyl sulfate (SDS) and including fluorescently labeled oligonucleotide probes (0.5 pmol for Cy3/Cy5 and 0.83 pmol for fluorescein labeled probes) were added to the different slide wells.

Hybridization was carried out in a humid chamber for 1.5 h at 46 °C. A subsequent washing step to remove unbound oligonucleotides was carried out 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 slide wells 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). An epifluorescence microscope (Axioplan 2, Zeiss) was used for observation of the slides and image acquisition was performed with a Leica D350F camera. The hybridization experiments were performed using different fluorochromes for each probe to make sure that the results were reproducible.

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 2. The PAOmix combination (PAO462, PAO651 and PAO846) was used to target Candidatus *Accumulibacter*. GAO phenotype bacteria was also targeted by combinations of the probes GAOQ431 and GAOQ989 (GAOmix). Sampling for FISH analysis was conducted by selectively removing sludge from either the top or bottom of the sludge bed. Since our columns are made from a transparent material, removal a certain fraction of biomass can be easily controlled. Biomass samples were regularly taken from both SBRs over the whole experimental period.

Table 2 Oligonucleotides probes and their targeted microbial groups.

Probe	Sequence (5'-3')	Target group	Reference
PAO 462	CCGTCATCTACWCAGGGTATTAAC	PAO cluster[a]	(Crocetti et al. 2000)
PAO 651	CCCTCTGCCAAACTCCAG	PAO cluster[a]	(Crocetti et al. 2000)
PAO 846	GTTAGCTACGGCACTAAAAGG	PAO cluster[a]	(Crocetti et al. 2000)
GAO Q431	TCCCCGCCTAAAGGGCTT	Competibacter phosphatis[b]	(Crocetti et al. 2002)
GAO Q989	TTCCCCGGATGTCAAGGC	Competibacter phosphatis[b]	(Crocetti et al. 2002)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	(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 *Candidatus Competibacter phosphatis*

RESULTS

LONG TERM OPERATION

The operation of both reactors was divided in three experimental phases, which lasted in total 220 days. Before the start of Phase I, SBR_{20} was running stable. Phosphate removal was around 90% and P released/COD uptake ratio was 0.37±0.05 P-mol/C-mol (data not shown). In the beginning of Phase I, when half of the biomass was removed from SBR_{20} to inoculate SBR_{30}, phosphate removal and phosphate release were not affected in SBR_{20} (Figure 1). Conversely, the temperature shock severely affected the biomass in SBR_{30}. A significant amount of gelatinous compounds were found in the wall of the reactor, probably as a response of the biomass to the sudden change in the operating temperature from 20°C to 30°C. Even though the SBR_{30} was inoculated with granules from SBR_{20}, which was achieving good P-removal, the start-up period of SBR_{30} (phase I) was characterized by low phosphate removal (around 50%).

Moreover, P released/COD uptake ratio was only around 0.22±0.08 P-mol/C-mol. Samples were collected from the top and bottom of the sludge bed of the reactors for FISH analysis in order to observe the microbial community composition. FISH results indicated that PAOs and GAOs formed the majority of the microbial population. Moreover, a stratification of microbial community structure over the sludge bed was observed during Phase I. In the top of the sludge bed of both SBRs, more GAOs (*Competibacter phosphatis*) than PAOs (*Accumulibacter phosphatis*) were present. On the other hand, in the bottom of the sludge bed, PAOs were dominant. The biomass segregation was more noticeable in the reactor operated at 30°C, where the difference between microbial composition in the bottom and top of the sludge bed was even higher. A similar trend was observed in previous research at 30°C (Winkler et al. 2011).

In order to favour PAOs over GAOs and achieve better and stable phosphate removal, particularly in the SBR operated at 30°C, excess sludge for SRT control started to be removed mainly from the top and in minor proportions from the bottom of the sludge bed in Phase II. In this way, the SRT of top granules (GAO-rich biomass) would be reduced compared to the bottom biomass (PAO-rich biomass).

In order to have the same operational conditions in both reactors, the selective sludge removal mainly of the upper part of the sludge bed was also implemented in SBR_{20}, although phosphate removal in this reactor was around 100% in the end of Phase I. The sludge removal in both reactors was performed every two days and the SRT was kept around 30 days.

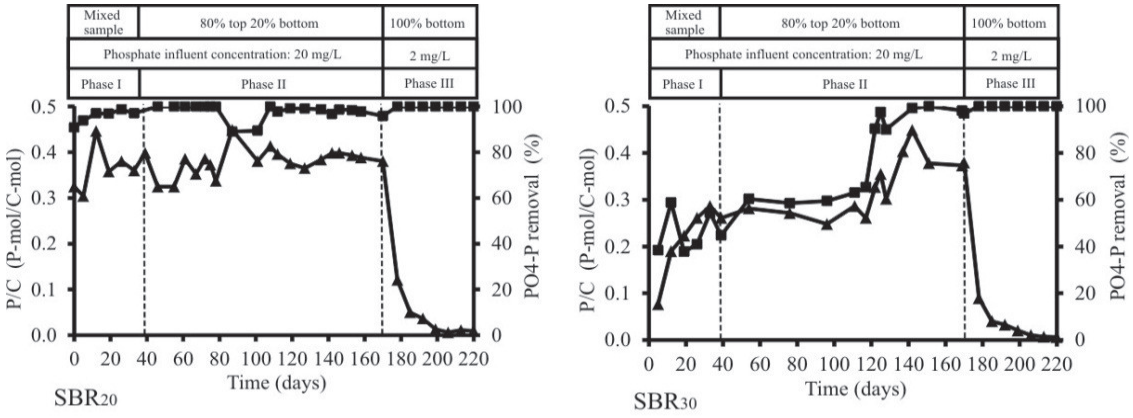
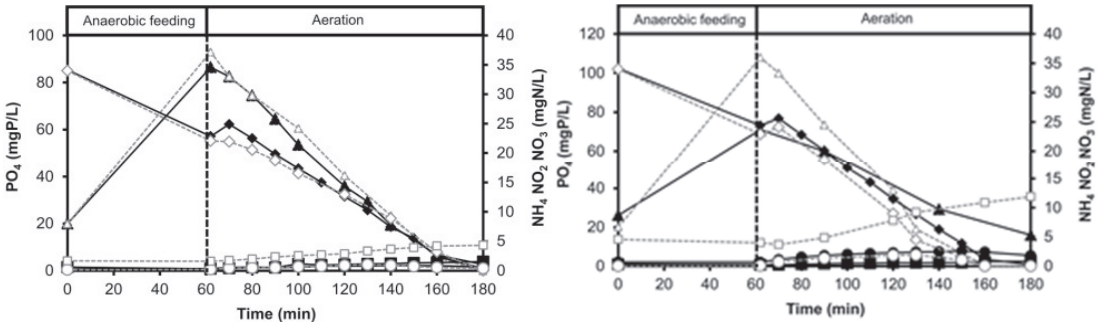


Figure 1 P-release-COD uptake ratio (▲) and Phosphate removal (■) in the long term operation of the SBR_{20} (a) and SBR_{30} (b). Removal of excess sludge for controlling SRT was performed in three different ways: mixed sample during aeration (Phase I), 80% from top and 20% from bottom of the sludge bed (Phase II) and 100% from bottom (Phase III). Phosphate influent concentration was 20 mgP/L in the first two experimental phases and 2 mgP/L in the last phase.

In SBR_{20}, the strategy adopted in Phase II for sludge removal did not change the performance of the reactor, which continued to achieve stable phosphate removal close to 100% (Figure 1). Phosphate release slightly increased indicating a preferential selection of PAO biomass, as shown in the cycle test (Figure 2a). In the SBR_{30}, no immediate improvement in terms of P-removal was observed in the beginning of Phase II, when excess sludge was mainly removed from the top of the sludge bed. P release/COD uptake ratio and phosphate removal remained practically constant (around 0.3 P-mol/C-mol and 60%, respectively). However, more than 90% phosphate removal was achieved after 80 days of operation in Phase II. P release/COD uptake also increased during this phase, amounting around 0.4 P-mol/C-mol at the steady state condition (Figure 1). The increase in the release from Phase I to Phase II can also be observed in the cycle tests of SBR_{30} (Figure 2b).



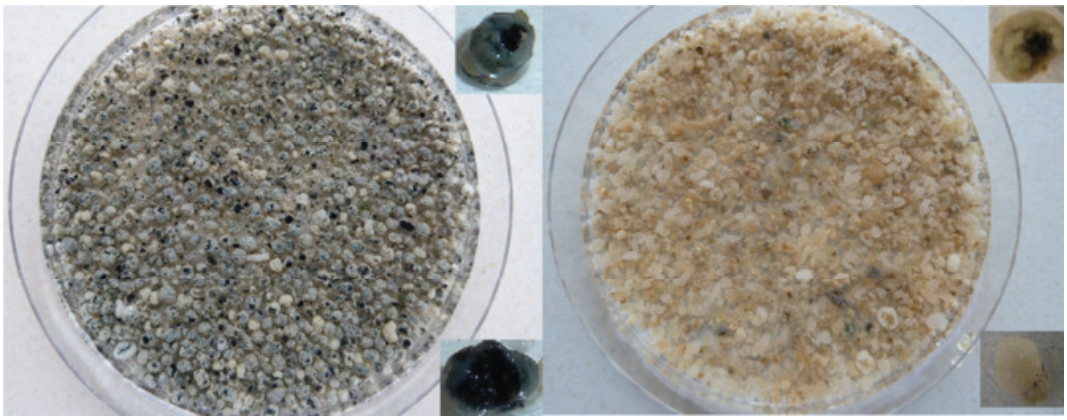
(a) *(b)*

3

Figure 2 Cycle measurements performed in the SBR_{20} (a) and SBR_{30} (b) during different operational phases: Phosphate (▲), Ammonium (♦), nitrite (●) and nitrate (■) in Phase I; Phosphate (Δ), Ammonium (◊), nitrite (○) and nitrate (□) in Phase II. 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-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 specific phosphate uptake rate in both reactors at the end of operation of Phase I and Phase II was obtained from the cycle tests. The results are displayed in Figures 2a (SBR_{20}) and 2b (SBR_{30}). During anaerobic feeding from the bottom of the reactors, all influent acetate (COD of 400 mg/L) was taken up (data not shown) and phosphate was released by PAOs. Phosphate was removed from the bulk liquid by PAO both aerobically (by aerobic PAOs) and anoxically (by denitrifying PAOs) in the aerobic and anoxic zones within the granules, respectively. Ammonium and nitrite were nitrified in the outer aerobic layer by ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively, and nitrite/nitrate was simultaneously denitrified in the anoxic zone of the granules by denitrifying organisms. Since all acetate was consumed in the anaerobic phase (feast phase) by PAOs and GAOs, no external carbon source was available during aeration period for heterotrophic denitrifiers. Therefore, the presence of ordinary denitrifying heterotrophs was minimized and denitrification was performed by denitrifying PAOs (DPAOs) or denitrifying GAOs (DGAOs) in the inside of the granules, which used intracellular PHB as electron donor for this process during the starvation period (famine phase). The activity of DPAOs was confirmed by conducting anoxic cycle tests (continuous supply

of nitrite or nitrate and nitrogen gas instead of air) in the reactor (data not shown). In Phase I, specific phosphate uptake rates observed in SBR_{20} and SBR_{30} were 3.5 and 2.3 $mgPO_4$-P/(gVSS·h), respectively. The lower P-uptake rate obtained in SBR_{30} reflects the results obtained in the long term operation of this system, which showed that the maximum phosphate removal obtained during Phase I was around 60%. The results obtained in SBR_{30} clearly show that the growth and activity of PAOs were severely affected by temperature increase from 20°C to 30°C. With the strategy in which excess sludge for SRT control was mainly removed from the top of sludge bed to favour PAOs over GAOs during Phase II, the specific phosphate uptake rate in SBR_{20} slightly increased from 3.5 to 3.9 $mgPO_4$-P/(gVSS·L). On the other hand, specific P-uptake significantly increased in SBR_{30}, reaching 5.9 $mgPO_4$-P/(gVSS·L). The stratification in the microbial population over the sludge bed observed in Phase I was maintained in both reactors in Phase II. Moreover, during the course of Phase II in SBR_{30}, biomass gradually changed in colour compared to the original biomass collected from SBR_{20} (Figure 3a), and some big white granules appeared (Figure 3b).



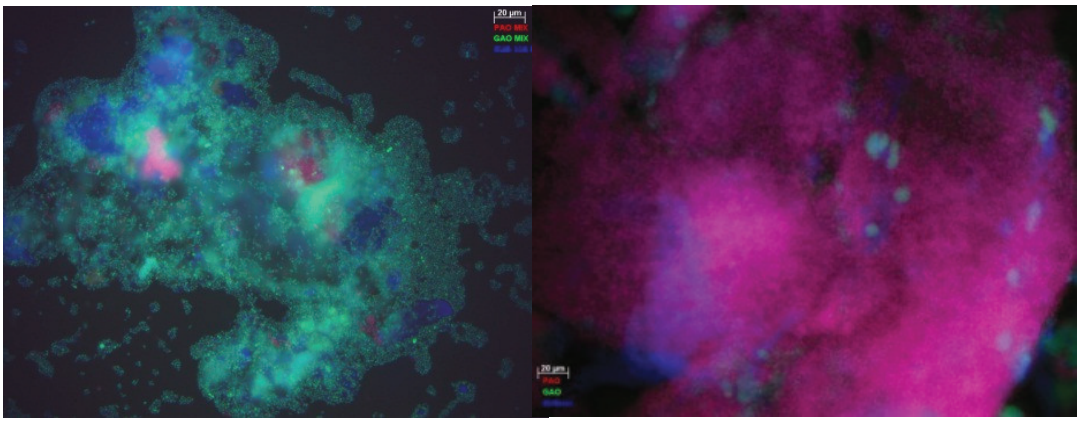
(a) *(b)*

Figure 3 Granules from SBR_{20} (a) and SBR_{30} (b) during operation in Phase II. The core of big granules is shown in detail in the right side of each picture.

FISH analysis showed that these white granules were strongly dominated by PAOs, while GAOs were present in minor proportions (Figure 4b). Some black granules (shown in detail on Figure 3a) were also separately analysed by FISH for comparison. As shown in Figure 4a, these specific granules were composed mainly by GAOs.

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). In general, the ash content of mixed biomass of SBR_{30} considerably increased from around 15% to almost 30% along the operation in Phase II (Figure 5). In SBR_{20}, biomass ash content remained constant in the first two experimental phases.

In Phase III, excess sludge for SRT control started to be removed only from the bottom of the sludge bed and the phosphate influent concentration was decrease from 20 to 2 mgP-PO_4/L. This experimental procedure was adopted to provoke the washout of PAOs and hence have a dominance of GAOs in order to clarify the link between the biomass ash content, poly-P content of the granules and chemical precipitation. The resulted biomass density and settling velocity were also investigated. With the low phosphate influent concentration in Phase III (2 mgP-PO_4/L), P removal efficiency was always 100% in both SBRs. However, the P release/COD uptake ratio decreased gradually and after 20 days of operation in Phase III, almost no phosphate release was observed in both reactors (Figure 1).



(a) black *(b) white*

Figure 4 Fluorescent in situ analysis of the PAO/GAO populations among all the bacteria present in the black (a) and white (b) granules from SBR_{30} (Phase II). 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) were used. Scale bar indicates 20 μm.

In the meantime, FISH pictures showed that the PAO population gradually disappeared from the granular sludge, and after one month of operation in Phase III, only few cells of PAOs were still detected in a GAO-dominated (more than 95% of the total microbial community) culture in both SBRs. In SBR_{30}, the colour of the granules changed back to its original colour (brownish-black) and the ash content of the biomass in both reactors gradually decreased from 30% to 10% (Figure 5).

BIOMASS CONCENTRATION AND PHYSICAL PROPERTIES OF TOP AND BOTTOM GRANULES

The biomass concentration in both aerobic granular sludge SBRs was kept in the range between 10 and 14 gVSS/L. Density, average diameter and settling velocity of both top and bottom granules are displayed in Table 3. In general, both the diameter and the density of top and bottom granules increased from Phase I to Phase II. The increase of these parameters was more noticeable in the bottom biomass. However, in Phase III, the density of both top and bottom granules considerably decreased.

Table 3 Density, diameter and settling velocity of both top and bottom granules

	SBR_{20}					
	Phase I		Phase II		Phase III	
Parameter	Top	Bottom	Top	Bottom	Top	Bottom
Density (g/L)	1003±2	1008±4	1004±3	1010±5	1001±2	1004±5
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
Calculated settling velocity (m/h)	13	26	16	42	12	36

	SBR_{30}					
	Phase I		Phase II		Phase III	
Parameter	Top	Bottom	Top	Bottom	Top	Bottom
Density (g/L)	1004±2	1005±3	1004±1	1008±4	1001±2	1003±2
Average diameter (mm)	0.7±0.1	0.7±0.2	0.6±0.2	0.9±0.3	0.5±0.1	0.8±0.1
Calculated settling velocity (m/h)	20	36	14	43	12	37

Since the granules were composed mainly by GAOs in the last experimental phase, the difference in density of bottom and top granules was marginal. 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. Ash content of the biomass (mixed top and bottom sample) during the whole operational period is shown in Figure 5.

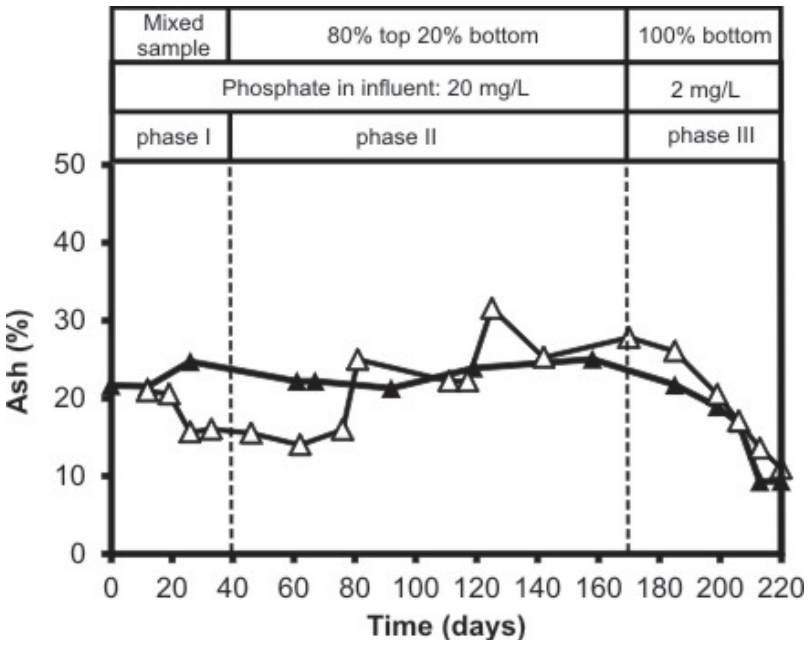


Figure 5 Ash content of biomass (mixed top and bottom samples) from both reactors during operation in Phases I-III.

DISCUSSION

OPERATIONAL FACTORS INFLUENCING PAO/GAO COMPETITION AND P-REMOVAL PERFORMANCE

Two aerobic granular sludge reactors were operated at 20°C and 30°C. Stable P-removal was readily established at 20°C but found to be more troublesome at 30°C. This is in line with results described in literature where higher temperatures (> 20°C) are reported to cause the deterioration of bio-P removal systems (Barnard and Steichen 2006; Gu et al. 2005; Panswad et al. 2003). Indeed, during operation in Phase I, phosphate removal observed in SBR_{30}, operated at 30°C, was considerably lower than that observed in SBR_{20}, operated at 20°C.

The fraction of PAOs and GAOs present in the biomass was not evaluated by quantitative FISH. This technique is more suitable when individual (free) cells are present, whereas clusters of biomass (as in our case) are difficult to be quantified by image analysis software. Instead, we used the ratio between phosphate release and COD (in our case acetate) uptake (P-release/COD uptake), which is a typical parameter used to estimate the amount of organic carbon (COD) taken either by PAOs or GAOs under anaerobic conditions (Schuler and Jassby 2007). 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). During Phase I, we observed that the P-release/COD uptake ratio was indeed higher in SBR_{20} (0.37±0.05) compared to that of SBR_{30} (0.22±0.08). This result suggests that a higher amount of active PAOs was present in SBR_{20} in comparison to that of SBR_{30} in the first experimental phase. However, 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 in Phase I opened a possibility to influence the PAO-GAO competition. This trend was already observed in our previous recent research (Winkler et al. 2011), in which we operated an aerobic granular sludge reactor at 30°C. In order to obtain better phosphate removal, particularly at 30°C (SBR_{30}), we decided to remove excess sludge mainly from the top of the sludge bed (dominated by GAOs) during the feeding period. A similar procedure for excess sludge removal was also conducted in previous work

(Winkler et al. 2011). Indeed, the selective removal of sludge mainly from the GAO-rich upper part of the sludge bed decreased the SRT of GAOs compared to PAOs allowing for complete phosphate removal in SBR_{30} after 80 days under these operating conditions. Moreover, the gradual increase of phosphate release/COD uptake ratio (up to 0.4) suggested that the percentage of active PAOs increased during the course of Phase II. 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 of selective removal of sludge from specific parts of the sludge bed, already suggested in our previous research (Winkler et al. 2011) and adopted again in this study, confirmed to be a good method to favour the development and growth of PAOs at unfavourable high temperatures and therefore improve P-removal capability. Our strategy 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 led 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. 2011). 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 P-removal, allowed obtaining lower amount of ash in the biomass compared to our previous study and avoided the deterioration of P-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 P-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.

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. 2011). 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 complete 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 may have increased the ash content in this specific biomass (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 II 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. 2011). 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).

CONCLUSIONS

We have shown that segregation of biomass in sequencing-batch aerobic granular sludge reactors accomplishing phosphorus removal offers 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 P-removal process. At 20°C, this procedure is not crucial and biomass can be removed equally throughout the sludge bed. The removal of sludge from the PAO-rich part of the sludge bed in minor proportions did not negatively affect P-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. Higher poly-P content and higher temperatures would possibly lead to higher chemical precipitation especially within the granules, which eventually can provoke changes in biomass colour.

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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Chapter 4

SEGREGATION OF BIOMASS IN CYCLIC ANAEROBIC/AEROBIC GRANULAR SLUDGE ALLOWS THE ENRICHMENT OF ANAEROBIC AMMONIUM OXIDIZING BACTERIA AT LOW TEMPERATURES

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ABSTRACT

A cyclic anaerobic/aerobic bubble column reactor was run for 420 days to study the competition for nitrite between nitrite oxidizing bacteria (NOB) and anaerobic ammonium oxidizing bacteria (Anammox) at low temperatures. An anaerobic feeding period with nitrite and ammonium in the influent followed by an aerated period was applied resulting in a biomass specific conversion rate of 0.18±0.02$\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ when the dissolved oxygen concentration was maintained at 1.0 $mgO_2 \cdot l^{-1}$. An increase in white granules was observed in the reactor which were mainly located at the top of the settled sludge bed whereas red granules were located at the bottom. FISH, activity tests and qPCR techniques revealed that red biomass was dominated by Anammox bacteria and white granules by NOB. Granules from the top of the sludge bed were smaller and therefore had a higher aerobic volume fraction, a lower density and consequently a slower settling rate. Sludge was manually removed from the top of the settled sludge bed to selectively remove NOB which resulted in an increased overall biomass specific N-conversion rate of 0.32±0.02 $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$. Biomass segregation in granular sludge reactors gives an extra opportunity to select for specific microbial groups by applying a different SRT for different microbial groups.

INTRODUCTION

The process of Anammox is a shortcut in the Nitrogen cycle whereby ammonium is oxidized with nitrite to nitrogen gas (Strous et al. 1999). For the application of Anammox for ammonium removal from wastewater, it is required to produce nitrite by ammonium oxidizing bacteria (AOB) and combine it with the anaerobic oxidation of ammonium at the expense of the nitrite produced. Since its discovery by Mulder in 1992 (Mulder 1992) many Anammox based treatment systems have successfully been implemented for cost-efficient full-scale nitrogen removal from anaerobic sludge digestion rejection water (Abma et al. 2010; Egli et al. 2001; Sliekers et al. 2003). To establish good nitrogen removal Anammox and AOB need to be enriched in the reactor system while nitrite oxidising bacteria (NOB) need to be outcompeted. Different strategies have been developed to control this competition and a suite of processes is applied at full scale for nitrogen removal from wastewater with relative high ammonium content and higher temperatures (> 20 °C) (van der Star et al. 2007).

In a Single reactor for high activity ammonium removal over nitrite (Sharon) system the preferential production of nitrite instead of nitrate from ammonium is accomplished by exploiting the higher growth rate of AOB compared to NOB at higher operational temperatures. This allows for selection of AOB and wash-out of NOB simply by reducing the solid retention time to approximately one day (Hellinga et al. 1998). In the subsequent Anammox reactor AOB and NOB cannot grow due to absence of oxygen. In processes which combine nitritation and Anammox in one reactor, also the higher growth rate of AOB (as compared to NOB) contributes to preventing nitrate formation by NOB. Current research studying the effect of low temperatures on Anammox activity are showing that Anammox can cope with lower temperatures (Dosta et al. 2008; Isaka et al. 2008; Vazquez-Padin et al. 2009). However, for application of a one-stage Nitritation/Anammox process at lower temperatures, like those encountered in sewage, it is necessary to gain more insight in the microbial competition between AOB, NOB and Anammox. In a one stage system the competition between Anammox and NOB for nitrite and between AOB and NOB for oxygen is the major issue in selecting the desired population. Low oxygen concentrations have been proposed to favour AOB in preference to NOB for their electron acceptor oxygen (Hao et al. 2005).

In oxygen limited one-stage Anammox systems, all 3 groups of bacteria do not grow in suspension but are agglomerated in one compact granule. The minimum sludge retention time (SRT) required is therefore defined by the slow growing Anammox bacteria, making it impossible to select against NOB based on growth rate.

Recently we demonstrated that in aerobic granular sludge vertical segregation of granules occurs based on small differences in settling velocity of the granules (Winkler et al. 2011). Granules with a higher density and diameter accumulate at the bottom of the sludge blanket. Since biomass in the bottom and the top of the sludge bed are exposed to different substrate concentrations during feeding, different microbial communities may develop as a function of height in the sludge bed. Selective sludge withdrawal from either top or bottom of the sludge bed can therefore be used as a possibility to control the microbial community structure in a granular sludge reactor. Interestingly, mathematical models have recently indicated that microbial populations in granules can be influenced by particle size distribution. For a nitritation/anammox granular sludge process it was demonstrated that NOBs are favoured to grow in smaller granules supposedly because of the higher fractional aerobic volume for nitrification (Volcke et al. 2010). Bigger granules have a smaller aerobic volume fraction which is in advantage of Anammox (Nielsen et al. 2005). Anammox can accumulate to larger amounts in these large granules and therefore maintain a low nitrite concentration inside the granule leading to an outcompetition of NOB. If selective particle density/size based sludge control is applicable an extra operational variable would be created in a one-stage complete anaerobic nitrogen removal over nitrite (CANON) system potentially allowing selective removal of NOB from a system at low temperatures. The objective of this work was to investigate the potential to use segregation of granules in a granular sludge bed to obtain a process dominated by nitritation/anammox in the absence of nitrite oxidation.

MATERIALS AND METHODS

CYCLE OPERATION AND MEASUREMENTS

Anammox enriched granular sludge from Rotterdam Dokhaven was used as seed sludge for a laboratory fed batch granular sludge reactor operated with anaerobic aerobic cycles. The operation during one cycle is schematically drawn in figure 1. It consisted of 60 min anaerobic feeding period of 1.5 l media from the bottom of the reactor in a plug flow regime followed by a 112 min of aeration, 3 min settling period and 5 min effluent withdrawal. During the effluent period half of the reactor volume (1.5 l) was discharged and half remained in the system. System performance for N-removal during one cycle was evaluated by sampling every 10-20 minutes to measure ammonium, nitrite and nitrate by means of flow injection analysis (Quick Chem8500, Lachat instruments) (figure 2). Opposed to normal reactor operation substrate was added all at once during a cycle measurement. Mixing was performed with dinitrogen gas during the first 60 minutes to keep anaerobic conditions.

Temperature was not controlled and varied between 17-22 °C. In the aeration period the pH was controlled similar to other studies at 7.0±0.2 using sodium hydroxide and hydrochloric acid (Wett 2007). The dissolved oxygen (DO) concentration was controlled at a specific value between 0.2 and $1.0\,mgO_2 \cdot l^{-1}$. During long-term reactor operation the oxygen set-point was stepwise elevated starting with an initial DO of $0.2\,mgO_2 \cdot l^{-1}$ for the first 90 days (phase I). From day 90 on the oxygen concentration was set to $0.5\,mgO_2 \cdot l^{-1}$ until day 250 (phase II) and from there on $1.0\,mgO_2 \cdot l^{-1}$ until day 400 (phase III and IV). The DO was set by recirculating the off-gas and blending with either dinitrogen gas (anaerobic period) or fresh air (aerobic period). In this way the DO concentration could be regulated while the superficial airflow was kept constant at 2 l/min(Mosquera-Corral et al. 2005). The autotrophic feed medium (1.5 l) consisted of 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KCl, 1.5 mM $NaHCO_3$, 4 mM $NaNO_2$ (41 mgN/l) 0.2 mM K_2HPO_4 and 0.1 mM KH_2PO_4 (9.1 mg P/l). During phase I and II ammonium concentration was 9.1 mM NH_4Cl (129 mg N/l) and was elevated to 13.6 mM NH_4Cl (190 mg N/l) after changing DO from 0.5 to 1 $mgO_2 \cdot l^{-1}$ in phase III and IV. 'Vishniac and Santer' solution was used to provide trace elements (Vishniac and Santer 1957). During phase I, II and III

the SRT was controlled by the sludge which washed-out spontaneously with the effluent withdrawal (1.5 l). In phase IV, sludge was manually withdrawn from the upper part of the sludge bed and SRT was controlled on average at 40 days and hydraulic retention time was 6 hours. Measurements of volatile suspended solids (VSS) as well as calculation of SRT followed the protocol from earlier research (Winkler et al. 2011). Nitrogen removal efficiency and rates are based on the difference in total soluble nitrogen compounds (i.e as measured in the bulk) in the feed and effluent of the reactor. The aerobic NOB activity was evaluated by defining the ratio of NO_3 produced to NH_4 converted during aerobic period, excluding the produced nitrate and consumed ammonium by Anammox from an- and aerobic period $f_{NO_3/NH_4} = NO_{3(aerobic)} / NH^{+}_{4(aerobic)}$ in N-mol/N-mol.

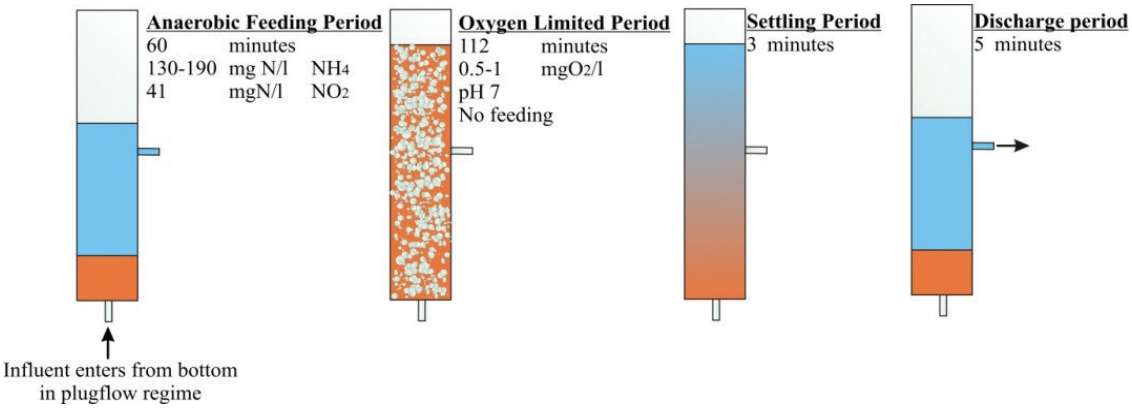

Figure 1 Cycle operation in the laboratory SBR reactor consisting of 1) an anaerobic feeding period of 1.5 l medium from the bottom of the reactor in a plug flow regime 2) an aeration period 3) a settling period and 4) discharge period

GRANULE CHARACTERISATION

Biomass samples were taken from the bottom and the top of the sludge bed over time for microscopic analysis in order to assess morphology and the microbiological community structure. Specific biomass density was measured with a pycnometer (Winkler et al. 2011) and size distribution measurements were conducted by means of an image-analyser. Microscopic images from white and red granules of sliced and regular granules were taken with a light microscope (Axioplan 2, Zeiss). Slicing was accomplished after fixation in 4% paraformaldehyde. Granules where embedded in a tissue freezing medium (Leica Microsystems) hardened by freezing (-20°C) and

cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 25µm thin slices. Dried slices were kept on a microscopic glass slide and Fluorescence in situ hybridization (FISH) was performed on them for determination of Anammox (Cy3) AOB (Cy5) and NOB (Fluos) microbial populations in the same manner as recorded previously (Winkler et al. 2011). Probe sequences are listed in additional material (table 4).

QPCR

qPCR was conducted on white and red granules using primers targeting NOB, AOB as well as Anammox primer sets. Primers were checked in the database Arb as well as with the Ribosomal Database Project (rdp). All samples were measured in triplicates. DNA extraction was conducted with an UltraClean™ Microbial DNA Isolation Kit. First a normal PCR was prepared followed by a purification step with a QIAqiuck PCR Purification Kit. The PCR product was used for a qPCR procedure with a variable primer concentration and 25µM iCycler mix (additional material table 5). All primers were optimized with a gradient qPCR. The resulting conditions, primer concentration as well as the DNA used as a standard for the qPCR are listed in additional material table 5. A picogreen protocol was used to determine the amount of DNA template in order to normalize all C_T values to 5 ng DNA. The ΔC_T was calculated by following equation $\Delta C_T = C_{T(ref)} - C_{T(target)}$ (Zhang et al. 2009). For determination of the ratios of one target organism (e.g. AOB) to the total reference community (here Eub) the following equation was applied: $\text{ratio}_{target/ref} = 2^{\Delta C_T}$.

BIOLOGICAL OXYGEN DEMAND

Maximum aerobic NH_4^+ and NO_2^- oxidizing capacity was analyzed for top and bottom biomass under oxygen saturated conditions. 20 g of granules were transferred into a 50 ml vessel containing water. In order to measure the activity of AOB, NH_4^+ (50 mg/l) was injected and oxygen consumption was monitored over time. For the measurement of the NOB, 80µM Allythiourea (ATU) was used to inhibit AOB (Ginestet et al. 1998). Subsequently NO_2^- was injected reaching an end concentration of 13 mg $NO_2^- \cdot l^{-1}$. Volatile suspended solids (VSS) were determined and the ammonium and nitrite oxidizing capacity was quantified with a linear regression fit of accumulative oxygen uptake rate (OUR).

CALCULATION OF THE OXYGEN PENETRATION DEPTH

The oxygen penetration depth was calculated according to following equation: $\delta_{pf} = \sqrt{\frac{2D \cdot C_{si}}{q_s^{max} \cdot c_{xf}}} [\mu m]$ (Harremoës 1977). With a biomass concentration in the biofilm (c_{xf}) of $3.38 \cdot 10^3 [mol \cdot m^{-3}]$ and $q_{O_2}^{max}$ from a nitrifying culture $9.72 \cdot 10^{-5} [molO_2 / Cmol_x]$, a diffusion coefficient for oxygen of $1.97 \cdot 10^{-9} [m^2/s]$ and an average granular diameter of 0.45mm for small and 1.2 mm for big granules. Based on the oxygen penetration depth the relative volume available for nitrification was calculated using the following equation $V_{pf} = \frac{V_{aerob}}{V_{total}} \left[\frac{\mu m^3}{\mu m^3} \right] \cdot 100[\%] \cdot$

4

MICROELECTRODE MEASUREMENTS

pH profiles were measured with a 25 µm thick micro electrode obtained from Unisense (Arhus, Denmark). Medium concentrations were chosen to be the same as during reactor operation as listed in the section cycle operations. The data acquisition system consisted of a picoamperometer (Unisens, Denmark, model PA2000), an A/D converter (Pico Technology Ltd, UK; model ADC-101) and a computer to retain data and monitor micro sensor motion. Motion control consisted of a motorized two-dimensional stage (Phytroninc., USA, model MT-65) and a motorized micromanipulator (Unisense, Denmark, model MM3M), controlling the position of the micro-electrode in three axis. For the small reactor set-up conditions were chosen to be the similar to the reactor conditions. pH gradients of red granules where tested for Anammox activity. Media contained ammonium and nitrite and was sparged with N_2 for anaerobic conditions. Activity of white granule was tested for pH profiles. Media for the aerobic period contained ammonium only and the dissolved oxygen concentration was 100%.

RESULTS

REACTOR PERFORMANCE DURING ONE CYCLE OF OPERATION

A typical reactor performance during one cycle of operation in phase III is depicted in figure 2. One cycle lasted 3 hours starting with 60 minutes of anaerobic feeding during which all incoming ammonium and nitrite were fed from the bottom of the reactor in a plug flow regime. During the anaerobic feeding period in a plug-flow regime from the bottom all nitrite was metabolized by Anammox to nitrogen gas and a small portion to NO_3^- as its anabolic by-product (Strous et al. 1999).

During the aerobic period ammonium was converted by AOB to nitrite and depending on competition factors further metabolized by either Anammox to nitrogen gas or by NOB to nitrate.

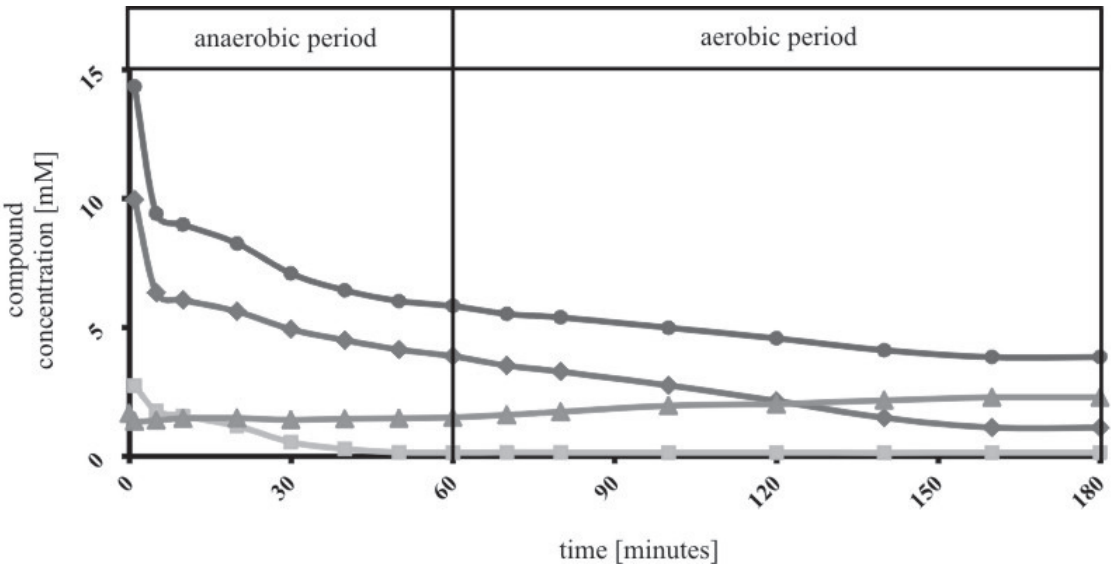

Figure 2 Concentration changes during one cycle in phase III of the aerobic granular sludge reactor. Nitrate (▲) Nitrite (■) Ammonium (♦) and total Nitrogen (●). Aeration starts after 60 min and is controlled at a dissolved oxygen concentration of 1.0 mgO_2/l. Note that first point is calculated and difference to second sampling point is largely due to adsorption of ammonium to the granular sludge.

LONG TERM OPERATION

The reactor was operated during four phases over a time period of 420 days as summarized in table 1. The volumetric and biomass specific nitrogen conversion rates are shown in figure 3. The initial biomass specific nitrogen removal rate was 0.06±0.01 $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ when the reactor was run at a DO of $0.2\,\mathrm{mgO_2} \cdot \mathrm{l}^{-1}$ to slowly adapt the biomass to aerated conditions (figure 3 phase I). The Anammox granules used as inoculum were obtained from a full-scale anaerobic Anammox reactor operated at 35 °C (versus 20 °C in the laboratory reactor). In phase II the oxygen was increased to $0.5\,\mathrm{mgO_2} \cdot \mathrm{l}^{-1}$ to enhance the nitrification in the aerated period. During this period an average biomass specific removal rate of 0.08±0.01 $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ was measured (figure 3 phase II).

In order to further enhance the nitrogen removal capacity in phase III, the oxygen concentration was raised to 1.0 $\mathrm{mgO_2} \cdot \mathrm{l}^{-1}$ resulting in an average biomass specific conversion rate 0.18±0.01 $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ (figure 3 phase III). During this phase there was an increase in white granules observed in the upper part of the settled sludge bed which were dominated by NOB as demonstrated by FISH and qPCR. At the same time the nitrate production in the aerated phase increased gradually (figure 3b). During phase I, two, and three the SRT was not actively controlled and therefore the resultant from the wash-out of sludge during the effluent withdrawal period. In order to selectively remove white NOB-dominated granules from the system in the fourth experimental phase biomass was withdrawn from the upper part of sludge bed. The amount of biomass withdrawn was such that an SRT of 40 days was established. Within 2 weeks the biomass specific nitrogen removal efficiency increased to 0.32±0.02 $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ (figure 3 phase IV) while the volumetric conversion rate remained the same as it was the case in phase III (table 1). The trend of f_{NO_3/NH_4} in the different phases is shown in figure 3b.

The f_{NO_3/NH_4}-value equals 0 if all nitrite produced is consumed by Anammox bacteria and 1 if full oxidation of ammonium to nitrate occurs. The values for f_{NO_3/NH_4} increased with increasing dissolved oxygen concentration reaching the largest value of 0.32 at $1.0\,mgO_2 \cdot l^{-1}$ (phase III). After removal of top biomass in phase IV, while maintaining the same DO concentration, the value for f_{NO_3/NH_4} decreased again to its approximate initial value of 0.1.

Table 1. Dissolved oxygen concentration, application of SRT control, volumetric total nitrogen removal conversion rate and maximum biomass specific conversion rate of total nitrogen removed in the different experimental phases (on influent effluent basis).

Phase	DO mg/l	SRT control	Volumetric Nitrogen conversion rate $\left[gN_2 - N \cdot m^{-3} \cdot day^{-1}\right]$	Max. biomass specific nitrogen removal rate $\left[gN_2 - N \cdot m^{-3} \cdot day^{-1}\right]$
I	0.2	No	905±35	0.06±0.01
II	0.5	No	913±58	0.08±0.01
III	1.0	No	1940±96	0.18±0.02
IV	1.0	Yes	1990±39	0.32±0.02

MICROSCOPIC ANALYSES OF GRANULAR SLUDGE SAMPLES

During the reactor operation the red granules originating from the full scale Anammox reactor system (figure 4a) developed over time into red and a few white granules (4b). Structure and shape differed for red and white granules. Bigger white granules where mostly hollow in the middle (4c) and smaller white granules had a porous structure (image not shown). Red Anammox dominated granules were densely populated regardless of the diameter (4c). In both granules FISH revealed that AOB were the dominant organisms on the outer layers. In the red granules NOB were barely present (4e). However, in white granules a mix of NOB, AOB and Anammox was determined (4f). Overall there was only a limited number of eubacteria present not reacting with the FISH-probes for AOB, NOB and Anammox used.

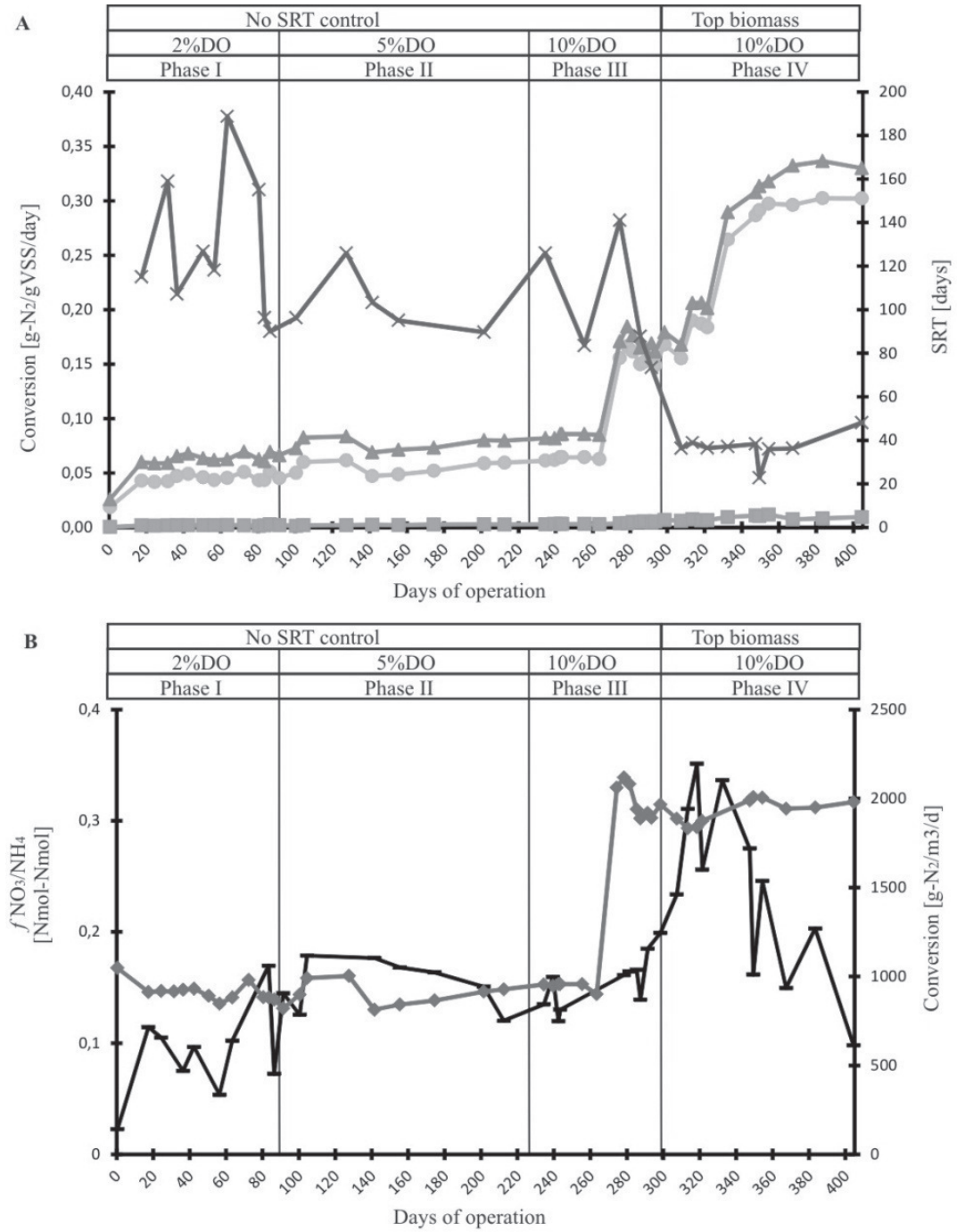

Figure 3 General behaviour of the experimental reactor system. Biomass specific nitrogen conversion rates in $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ *of* NH_4^+ *(●) total nitrogen (▲)* NO_3^- *(■) and SRT (x) over time. The dissolved oxygen during the aerated period was set at different set points for each experimental phase. The* f_{NO_3/NH_4} *–values (-) as well as the volumetric N-conversion rates* $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ *(♦) are shown in 3b.*

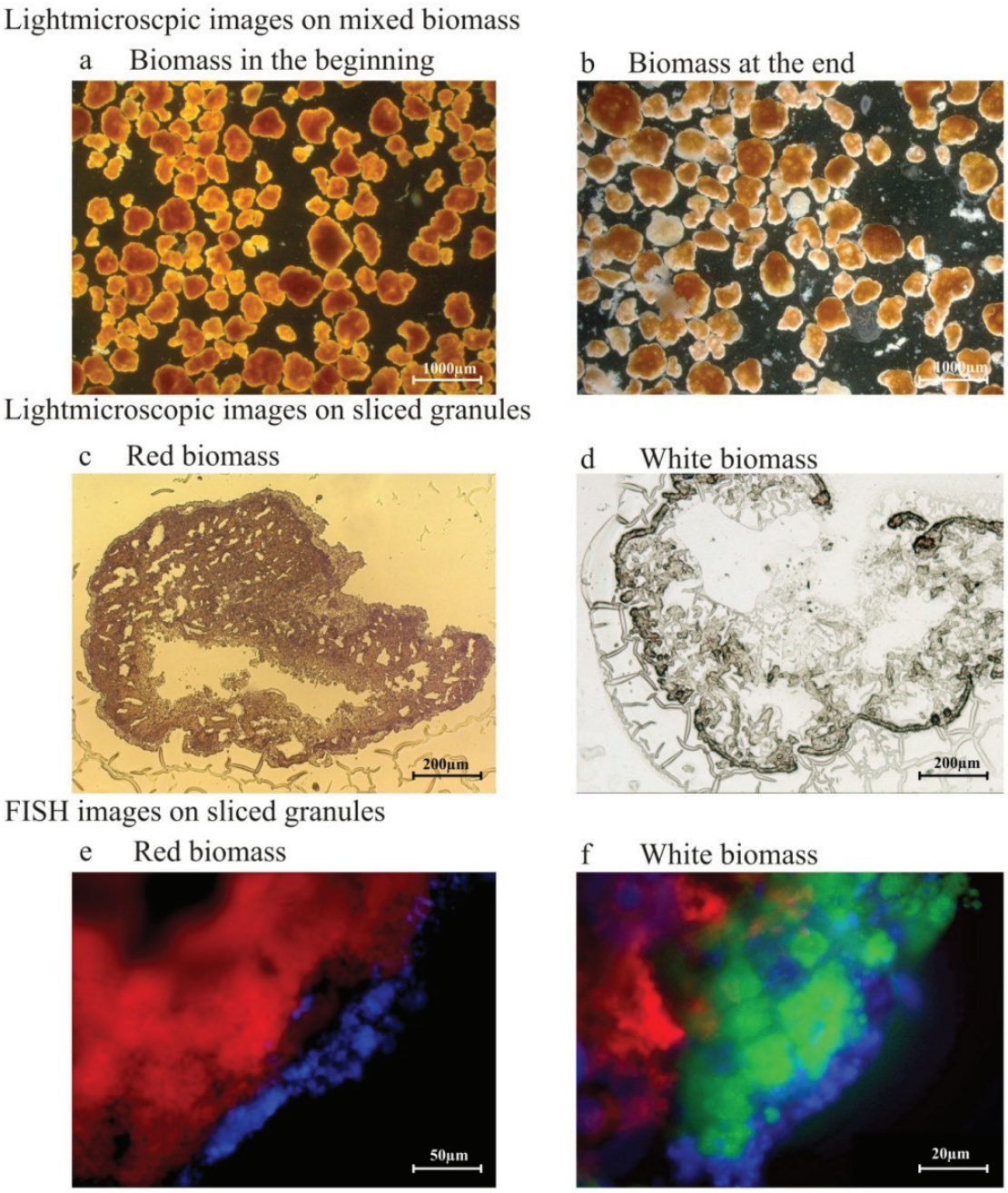

Figure 4 microscopic images of granules a) at the beginning of the experiment and b) at the end. As well as c) Sliced big red granules and d) sliced small white granules e) FISH image of sliced red granule and f) FISH image of sliced white granule. FISH was conducted on sliced granules and hybridization was accomplished with Cy3-red (Anammox), Cy5-blue (AOB) and Fluos-green (NOB)-labelled probes.

MICROBIAL CHARACTERISATION OF GRANULES

Biological oxygen consumption measurements were used to evaluate the activity of AOB and NOB in the granular sludge. These measurements revealed a somewhat higher biomass specific nitrite oxidizing capacity for top granules compared to bottom granules. Aerobic ammonium oxidizing capacities were similar for bottom and top biomass however differed considerably in nitrite oxidizing capacity with a 10 fold higher oxygen consumption for top biomass (table 2). For the experiment white granules were not separated from red granules but 20g of granules where taken from top and bottom biomass, respectively. For qPCR measurement white and red granules were separately analysed. From the derived Ct values ratios were calculated for AOB, NOB and Anammox as a fraction of the general bacterial population (here referred as reference).

The resulting ratios were used to compare e.g. the difference resulting from the ratio between NOB and Anammox from a white and red granule. Ratios were not used to describe community composition within one sample because of the bias that Anammox is not detected by general bacterial primers. In a white granule the ratio for Anammox was 0.6 fold lower and for NOB 33 fold higher than observed in a red granule (table 2). In big and small granules the estimated oxygen penetration depth increased from approximately 13 to 19 µm when dissolved oxygen concentration was increased from 0.5 to $1\,\mathrm{mgO_2 \cdot l^{-1}}$. The volume fraction of a granule available for nitrification $V_{(O_2)}[\%]$ for small granules was circa 3-fold higher as it was for big granules (table 3).

DENSITY AND SIZE DISTRIBUTION MEASUREMENTS OF TOP AND BOTTOM GRANULES

The top layer of the settled sludge bed was dominated by white granules and middle and bottom section of the sludge bed was dominated by red granules (visual observation). The ash content of the red bottom granules was higher then for white top granules. This was also reflected in a higher density of the bottom granular sludge. The measured values were 1008±2.3 $\left[g \cdot l^{-1}\right]$ for bottom granules versus 1003±1.5 $\left[g \cdot l^{-1}\right]$ for top granules. The diameter of bottom granules was also larger (table 2). The physical parameters obtained were used to estimate the settling velocity applying Stokes law as described previously (Winkler et al. 2011). The estimated settling rate of top granules of 5.7 $\left[m \cdot h^{-1}\right]$ was just enough to allow them to settle before effluent was removed from the top half of the reactor.

Table 3. Oxygen penetration depth and aerobic volume fraction available for nitrification at 0.5 and 1 mg $O_2 \cdot l^{-1}$ in big and small granules

	Small granules 0.45 [mm]	Big granules 1.2 [mm]
Dissolved O_2	$0.5 mg O_2 \cdot l^{-1}$	
$\delta_{pf(O_2)}[\mu m]$	13.3	13.1
$V_{pf}[\%]$	16.7	6.4
Dissolved O_2	$1.0 mg O_2 \cdot l^{-1}$	
$\delta_{pf(O_2)}[\mu m]$	18.9	18.8
$V_{pf}[\%]$	23.2	9.1

Table 2. Physical, Kinetic and Microbial properties of bottom and top granules during the period with sludge wasting from the top of the sludge bed (stage IV). The estimated ratio of Anammox, NOB and AOB versus bacterial population as estimated by EUB probe from qPCR data.

Parameter	Top	Bottom
Ash content [%]	7.4±1.5	11.6±2.7
Density $\left[g \cdot l^{-1}\right]$	1003.0±1.5	1008.0±2.3
Average diameter[1] [mm]	0.78±0.30	0.93±0.27
Settling velocity Calculated $\left[m \cdot h^{-1}\right]$	5.7	16.5
$q_{NOB}\left[\frac{mgO_2}{gVSS \cdot h}\right]$	10.5	1.2
$q_{AOB}\left[\frac{mgO_2}{gVSS \cdot h}\right]$	20.1	27.7
	White	Red
$ratio_{AOB/Eub}$	3.5	0.7
$ratio_{AMX/Eub}$	0.6	3.5
$ratio_{NOB/Eub}$	0.1	0.003

PH MICROELECTRODE MEASUREMENTS

pH measurements were conducted on white and red granules. Since it was not possible to fit small white granules in the microelectrode set-up a bigger white granule was picked to see difference in pH profiles. In white granules the pH dropped under aerated conditions from 7 to 5.5 as expected for nitrifying granules. When red granules were anaerobically incubated with nitrie and ammonium a pH increase of 0.2 pH units was measured (figure 5).

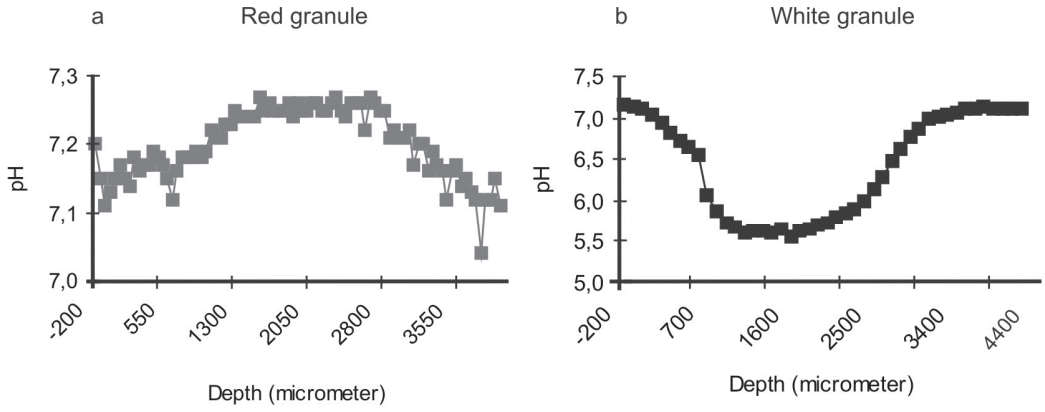

Figure 5 pH profile over a granule a) a red granule and b) a white granule. Media conditions where chosen to be the same as during reactor operation.

DISCUSSION

Earlier research has shown that particle size- and density-induced segregation of granules occurs as a function of height over a settled sludge bed in the aerobic granular sludge process (Winkler et al. 2011). Larger and denser granules accumulate due to their higher settling rate at the bottom of the sludge bed. In a plug flow reactor fed from the bottom larger granules get more substrate and grow faster as compared to smaller granules at the top of the sludge bed. Granules that are exposed to higher substrate concentrations grow more which may enhance the segregation of biomass. In this study we succeeded to favour the development of bigger Anammox-dominated granules by an anoxic feeding period with nitrite and ammonium in the feed, and there with a segregation of biomass. An extra factor contributing to the segregation of the granular sludge bed is the observations that Anammox dominated granules tend to have an elevated internal pH, whereas nitrification dominated particles have a decreased pH (figure 5). In the anabolic pathway of anaerobic ammonium oxidation carbonate is reduced and assimilated into biomass. This is a proton consuming process which causes an increase of the pH in the bulk (Van de Graaf 1996). At higher pH chemical precipitation is provoked (Carlsson et al. 1997; Maurer et al. 1999) which might explain the higher observed ash percentage in Anammox dominated granules, leading to a larger specific density and thereby a higher settling velocity of these granules.

At limiting oxygen concentrations AOB already get a preferential advantage over NOB. However, in the competition between AOB, NOB and Anammox bacteria not only the oxygen concentration in the bulk liquid but also the particle size seems to be a determining factor (Volcke submitted). It has been proposed based on mathematical modelling that Anammox will grow in bigger granules, while NOB grow in smaller (Volcke et al. 2010; Volcke submitted). This was also experimentally observed for a CANON reactor (Nielsen et al. 2005; Vlaeminck et al. 2010). Since small and large granules are subjected to the same bulk oxygen concentrations, the oxygen penetration depths are similar. The aerobic volume fraction for larger granules is therefore smaller (table 3), and Anammox can likely more efficiently outcompete the NOB for nitrite.

FISH images demonstrated that larger granules were dominated by AOB and Anammox whereas smaller granules contained also NOB (figure 4). qPCR revealed the same trend with more Anammox in the large granules and more AOB and NOB in the small granules. Activity measurements showed similar ammonium oxidizing capacity for top and bottom biomass but a higher nitrite oxidizing capacity for top biomass, which had a large fraction of smaller white granules (table 2). The N-removal capacity was measured over time and the removal rate increased from 905±35 to 1940±96 $\left[gN_2 - N \cdot m^{-3} \cdot day^{-1} \right]$ when dissolved oxygen concentration was elevated from $0.2\,\mathrm{mgO_2 \cdot l^{-1}}$ to eventually $1.0\,\mathrm{mgO_2 \cdot l^{-1}}$ (table 1).

Without selective sludge wasting from the top of the bed white-NOB dominated granules accumulated at the top of the sludge bed. When the solid retention was manually maintained by removing granular sludge from the top of the bed a considerable increase in biomass specific removal rate from 0.18±0.02 to 0.32±0.02 $\left[gN_2 - N \cdot gVSS^{-1} \cdot day^{-1} \right]$ (figure 3 phase III-IV, table 1) was observed. The observed volumetric conversion was in the order of magnitude with reported values of other Anammox based treatment systems ran at higher temperatures(Arrojo et al. 2008; Fernandez et al. 2009). Moreover, the ratio for aerobic $\mathrm{NO_3^-}$ produced / $\mathrm{NH_4^+}$ consumed positively correlated with the overall results. The ratio was low when the dissolved oxygen concentration was low ($0.3\text{-}0.5\,\mathrm{mgO_2 \cdot l^{-1}}$) and increased with increasing oxygen concentration ($1\,\mathrm{mgO_2 \cdot l^{-1}}$) indicating an ingrowth of

NOB. When top biomass was extracted to selectively remove NOB the ratio dropped to its approximate initial ratio of 0.1 for pure anammox sludge, in spite of a higher oxygen concentration ($1\,mgO_2 \cdot l^{-1}$). This further indicates the selective removal of NOB.

Like in our previous work related to competition between phosphate and glycogen accumulating bacteria, biomass segregation in aerobic granular sludge reactors was shown to enable removal of an unwanted microbial population by a combination of minimising SRT for that population (here the NOB) and feeding preferentially the desired population (here Anammox) (Winkler et al. 2011). This study has shown that it was possible with selective sludge control to counter select against NOB in preference to AOB and Anammox bacteria in a granular sludge reactor at ambient temperatures. It was reported that at high temperature in a continuously operated granular sludge (CANON) systems, selective washed-out of samller particles lead to higher nitrogen convension rates (De Clippeleir et al. 2009; Vlaeminck et al. 2010), suggesting that smaller aggregates were enriched in NOB. The exact mechanism why NOB are outcompeted in larger granules needs further investigation, but it is likely that even at lower temperatures NOB would be preferentially accumulating in smaller granules due to their affinity to oxygen. Feeding of nitrite and ammonium could be used to promote the growth of bigger Anammox dominated granules and hence enhance segregation during a CANON stratup process at mesophilic temperatures. Modelling work showed that thicker biofilms (i.e. larger granules) are needed to sustain Anammox conversion at lower temperature(Hao et al. 2002). A selective wash-out of smaller NOB dominated granules gives the opportunity to introduce Anammox conversions in main stream wastewater treatment plants. This will greatly increase the sustainability of the process with respect to energy (Kartal et al. 2010; van Loosdrecht et al. 2004).

APPENDIX

$$\delta_{pf} = \sqrt{\frac{2D \cdot C_{si}}{q_{O_2}^{max} \cdot c_{xf}}} [\mu m]$$

δ_{pf} = oxygen penetration depth $[m]$

D = oxygen diffusion coefficient $\left[\frac{m^2}{s}\right]$

C_{si} = oxygen concentration in the biofilm interphase $\left[\frac{mol}{m^3}\right]$

$q_{O_2}^{max}$ = maximal oxygen specific uptake rate $\left[\frac{1}{s}\right]$

c_{xf} = biomass in the biofilm $\left[\frac{mol}{m^3}\right]$

$$V_{pf} = \frac{V_{aerob}}{V_{total}} \left[\frac{\mu m^3}{\mu m^3}\right] \cdot 100 [\%]$$

$$r_{anaerob} = \delta_{pf} - d_{granule}$$

$$V_{tot} = \frac{4}{3} \pi r_{granule}^3$$

$$V_{anaerob} = \frac{4}{3} \pi r_{anaerob}^3$$

$$V_{aerob} = V_{tot} - V_{anaerob}$$

V=Volume $[m^3]$

d=diameter $[m]$

r=radius $[m]$

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Chapter 5

FACTORS INFLUENCING THE DENSITY OF AEROBIC GRANULAR SLUDGE

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M-K H. Winkler, R. Kleerebezem, M. Strous, K. Chandran, M.C.M. van Loosdrecht, Factors influencing the density of aerobic granular sludge.

ABSTRACT

In the present study the factors influencing density of granular sludge particles were evaluated. Granules consist of microbes, precipitates, and of extracellular polymeric substance. The volume fractions of the bacterial layers were experimentally estimated by FISH staining. The volume fraction occupied by precipitates was determined by CT scanning. PhreeqC was used to estimate potential formation of precipitates to determine a density of the inorganic fraction. Densities of bacteria were investigated by Percoll density centrifugation. The volume fractions were then coupled with the corresponding densities and the total density of a granule was calculated. The sensitivity of the density of the entire granule on the corresponding settling velocity was evaluated by changing the volume fractions of precipitates or bacteria in a settling model. Results revealed that PAOs had a higher density than GAOs leading to significantly higher settling velocities for PAO dominated granules explaining earlier observations of the segregation of the granular sludge bed inside reactors. The model showed that a small increase in the volume fraction of precipitates (1-5%) strongly increased the granular density and thereby the settling velocity. For nitritation-anammox granular sludge mainly granular diameter and not density differences are causing a segregation of the biomass in the bed.

INTRODUCTION

Aerobic granular sludge offers an interesting alternative for conventional activated sludge systems. This technology relies on compact and self-immobilized granulated biomass. By applying short settling times in a sequencing batch process, only big and rapidly-settling biomass aggregates are selected, while flocculent sludge is washed out (Beun et al. 1999; de Kreuk et al. 2005; Etterer and Wilderer 2001; Morgenroth et al. 1997). The parameters determining the settling velocity of particles and in turn biomass wash-out are of crucial importance to granular sludge technology. The settling velocity is influenced by particle size and shape, and the difference between the density of the water and the particles (Winkler et al. 2012).

Recently we demonstrated that in aerobic granular sludge vertical segregation of granules occurs, resulting from small differences in granular settling velocity. Fast or slow settling particles showed different microbial composition and activities. Selective sludge withdrawal from either the top or bottom of the sludge bed was used to control the microbial community structure and enhance desired removal processes (Bassin et al. 2012; Volcke et al. 2012; Winkler et al. 2011a; Winkler et al. 2011b). In a heterotrophic system it was shown that glycogen accumulating organisms (GAO) dominated the slower settling granules and the preferred phosphate accumulating bacteria dominated the fast settling granules which were hence favored for substrate since the reactor was fed in a plugflow regime from the bottom at the reactor. In an autotrophic reactor system the fast settling granules consisted of aerobic (AOB) and anaerobic ammonium oxidizing (Anammox) bacteria, whereas slow settling particles also harboured the undesired nitrite oxidizing bacteria (NOB) (Vlaeminck et al. 2010; Volcke et al. 2012; Winkler et al. 2011b). In both cases sludge withdrawal from the top of the sludge bed ensured a stable and well working process. Segregation was also observed in fluidized bed biofilm reactors where bigger granules were reported at the bottom of the reactor setup (DiFelice et al. 1997; Ro and Neethling 1994; Sliekers et al. 2003).

The major factors for fast settling granules are size and density (Nicolella et al. 2000; Nor Anuar et al. 2007; Winkler et al. 2011b; Winkler et al. 2012). Factors influencing granular density are chemical precipitates and the microbial community structure itself. Calcium phosphate precipitations are formed easily in the enhanced biological phosphorous removal (EBPR) process (Angela et al. 2011; Arvin 1983; Carlsson et al. 1997; Clark et al.

1997; Maurer et al. 1999) and are also reported in granular systems (Angela et al. 2011; Bassin et al. 2012; de Kreuk et al. 2005; Yamaguchi et al. 2001). Precipitates in the inner core of the granule are known for anaerobic and aerobic granules and are in both systems discussed to be induced due to pH shifts within the granules caused by microbiological activities (Angela et al. 2011; Yamaguchi et al. 2001). Reported values of granular densities vary between 1005-1070 kg/m^3 (Bassin et al. 2012; Batstone and Keller 2001; Etterer and Wilderer 2001). However, not only precipitates influence the overall granular density. Bacteria can occupy large volume factions of the granules and although less dense than precipitates they will contribute to granular densities and hence settling characteristics. Storage polymers such as polyphosphate, glycogen and PHA will contribute to the overall density (Oshiki et al. 2010). Given the importance of particle density for segregation of biomass in granular sludge beds it is interesting to investigate the factors influencing the density of sludge particles (Bin et al. 2011; Volcke et al. 2012; Winkler et al. 2011b). The goal of this study was therefore to determine the influence of precipitates and the microbial community structure on the density of granular sludge and in turn evaluate their effect on settling velocity.

MATERIALS AND METHODS

FLUORESCENT IN-SITU HYBRIDIZATION (FISH)

Granules from CANON and EBPR reactors as previously described (Winkler et al. 2011a; Winkler et al. 2011b) were inspected by microscopic analysis to assess morphology and microbiological composition. Slicing was performed after fixation in 4% paraformaldehyde. Granules were embedded in a tissue freezing medium (Leica Microsystems) hardened by freezing (-20°C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 25µm thin slices. Dried slices were kept on a microscopic glass slide and FISH was performed for determination of Anammox (Cy3) AOB (Cy5) and NOB (Fluos) (for CANON reactor) as well as PAO (Cy5), GAO (Fluo) and nitrifyers (Cy3) (for the EBPR reactor) following the same procedure as previously described (Winkler et al. 2011b). Sequences are listed in Table 1.

Table 1 Oligonucleotide probes, target organisms, and references

Probe	Specificity	Reference
PAO 462	Most *Accumulibacter*	(Crocetti et al. 2000)
PAO 651	Most *Accumulibacter*	(Crocetti et al. 2000)
PAO 846	Most *Accumulibacter*	(Crocetti et al. 2000)
GAO Q989	Some *Competibacter*	(Crocetti et al. 2002)
GAO Q431	Some *Competibacter*	(Crocetti et al. 2002)
Amx 368	All Anammox bacteria	(Schmid et al. 2003)
Ntspa662	*Nitrospira like organisms*	(Daims et al. 2001)
NIT1035	*Nitrobacter*	(Wagner et al. 1996)
NSO190	All AOB	(Mobarry et al. 1996)
NSO1225	All AOB	(Mobarry et al. 1996)

ORIGIN OF BACTERIA AND PERCOLL DENSITY DISTRIBUTION

Different bacteria were either grown in our laboratories or provided by other researchers. All strains provided by others were fixed in 4% paraformaldehyde and incubated for 120 min at room temperature. After fixation, samples were centrifuged for 2 min at 16,000 rpm, washed twice in 1x phosphate buffered saline (PBS), and re-suspended in a volume of 1:1 Ethanol/PBS buffer for storage at -20°C. *Nitrobacter* and *Nitrosomonas* were harvested from chemostat reactors and their purity and cell concentration was controlled via direct cell counts using a Brightline hemocytometer (Hausser Scientific, Horsham PA). For the bacteria from other laboratories purity was given by the researchers, who determined their purity by FISH as well as qPCR and results are given in table 2. Percoll density centrifugation is a useful tool to measure bacterial densities and has been mainly used for cell separation (Beaty et al. 1987; Scherer 1983; Strous et al. 1999) and by some researcher also to directly measure the density of cells (Putzer et al. 1991; Woldringh et al. 1981) or the density of extracted storage polymers (Oshiki et al. 2010).

An isotonic stock solution was made by mixing 45 ml of Percoll (GE, Healthcare) with 5ml of 1.5M NaCl solution. Next, this isotonic Percoll stock solution was used to create an 80% (v/v) working solution by using NaCl (1.5 mM). The tubes were centrifuged in a fixed angle rotor (Beckmann Optima TL Ultracentrifuge) at 27.000x g for 45 min at room

temperature to allow a self-forming gradient to develop by isopycnic centrifugation. All cell pellets and aggregates were crushed by the means of a glass mortar (Glas-Col) and 25 µl of the resulting cell suspension was loaded on the gradient tubes. Five density marker beads (Cosmopheric) in the range of 1.028-1.13 [g/ml] were used as internal standards and their height was recorded to fit the measured bacterial densities by interpolation using a polynomial regression fit. All measurements were repeated 5-10 times to determine a standard deviation.

Table 2 bacterium, purity, source and reference for media and growth conditions

Bacterium	Purity [%]	Source	Reference
Nitrobacter winogradskyi	100	Our laboratory	ATCC: medium 203
Nitrosomonas europea	100	Our laboratory	(Chandran and Love 2008)
PAO aerobic phase	80	Unesco IHE	(Smolders et al. 1994)
PAO anaerobic phase	80	Unesco IHE	(Smolders et al. 1994)
GAO aerobic phase	90	Unesco IHE	(Lopez-Vazquez et al. 2009a)
GAO anaerobic phase	90	Unesco IHE	(Lopez-Vazquez et al. 2009a)
Anammox fulgida	80	TUDelft	(Van Der Star et al. 2008)

CHEMICAL PRECIPITATION

CT SCAN

Granules of bottom and top sludge were analysed by a nanofocus computed tomography (CT) Micro Scanner (phoenix nanotom s, GE). Granules were placed into a straw and fixed on the rotating plate and were exposed to a 180 kV / 15 W ultra-high performance nanofocus X-ray tube enabling a non-destructive visualization of the granules. Components of higher densities appeared white on the images (Figure 2).

MICROELECTRODE MEASUREMENTS

pH profiles were measured with a 25 µm micro electrode (Unisense , Aarhus, Denmark). Medium concentrations were as described previously (Winkler et al. 2011a; Winkler et al. 2011b). The data acquisition system consisted of a picoamperometer (Unisense, Denmark; model PA2000), an A/D converter (Pico Technology Ltd, UK; model ADC-101) and a computer to log the sensor data and the microsensor motion. Motion control consists of a motorized two-dimensional stage (Phytron Inc., USA, model MT-65) and a motorized micromanipulator (Unisense, Denmark, model MM3M),

controlling the position of the micro-electrode in three axis. Sensors were equipped with silver/silver chloride anode and a gold plated platinum cathode. Bulk dissolved oxygen concentration was controlled using a mass flow controller for air and dinitrogen gas and medium was then fed into to chamber used for microelectrode measurements.

PHREEQC

PHREEQC for Windows (version 2) was used to calculate the saturation index for potential precipitates. The program uses a Newton-Raphson numerical method to solve a system of non-linear equations. The sensitivity to pH and phosphate concentration was evaluated. The saturation index was calculated based on the concentration as present in the media of our lab reactors (Winkler et al. 2011a) and concentration are listed in online material. The sensitivity analysis for acidity was performed in the range from 5.5 until 10, in step sizes of 0.5 pH units. The phosphate concentrations were varied as well to see the effect of biological phosphate release on precipitation equilibria. Saturation indices (SI) larger than 1 indicate supersaturation and hence the increased ability of precipitation of components.

SETTLING MODEL

In our observations granules can be divided in two to three layers consisting of either bacteria, EPS and or chemical precipitates (Figure 1). According to these observations we evaluated our settling model. Heterotrophic granules from our reactors generally showed a densely populated outer rim of nitrification and heterotrophic bacteria (De Kreuk et al. 2007; Xavier et al. 2007). Underneath this outer layer a low density layer was found consisting mainly of EPS. The inner core of the granules generally contained large amounts of precipitates (Figure 1 A).

Autotrophic, nitrogen removing granules generally consisted of a dense outer rim with ammonium oxidising bacteria (AOB) and a dense inner core with Anammox bacteria. When nitrite oxidisers were present, they usually resided between the AOB and Anammox bacteria (Ni et al. 2009; Volcke et al. 2010b; Volcke et al. 2010a; Winkler et al. 2011b) (Figure 1B). For all simulations performed, the settling velocity was calculated based on granular diameter and granular densities. The thickness of each layer was estimated by Ct scan and FISH pictures (Figure 2) to calculate the volume fractions occupied by each compound. The structure as schematically drawn in Figure

1 was used to predict the settling velocity of granules within an EBPR system and CANON system. The average density of the whole granule (ρ_p) was calculated by multiplying the different volume fraction by the density of the components consisting of either bacteria, EPS or precipitates $\rho_p = (V_A' \cdot \rho_A) + (V_B' \cdot \rho_B) + (V_C' \cdot \rho_C)$. A density of EPS could not be found in literature but since EPS largely consists of water it was assumed to be equal to the density of water (Flemming et al. 2007; Flemming and Wingender 2010). The density of Calciumphosphate (2.2 [g/ml]) was used for the precipitates (International Union of Pure and Applied Chemistry . Nomenclature of Organic Chemistry 1979). The overall density (ρ_p) of the granule was then used to calculate the theoretical settling behaviour of a granule according to the procedure by Winkler et al. 2012.

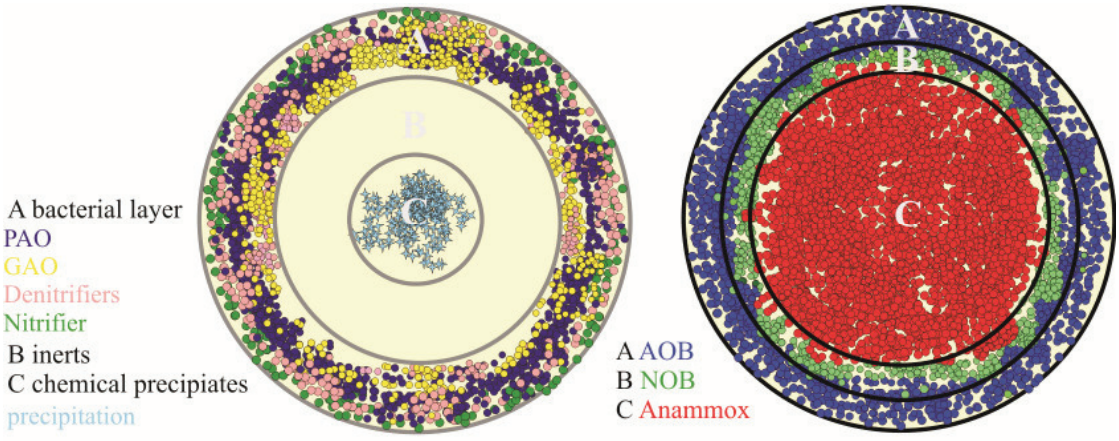

Figure 1 schematic view of a granule originating from 1) an enhanced biological phosphorous removal (EBPR) system consisting of three layers of bacteria (A), EPS (B) and chemical precipitates (C) as well as a granule originating from 2) an complete autotrophic nitrogen removal of nitrite (CANON) process consisting of three layers of bacteria: AOB (A), NOB (B) and Anammox (C)

RESULTS

CHARACTERISATION OF THE SLUDGE GRANULES

The results from the Ct scan, pH microelectrode measurements as well FISH for heterotrophic and autotrophic sludge granules are presented in Figure 2. The Ct scan originating from the heterotrophic EBPR system (left) and autotrophic CANON system (right) indicated that the granular makeup was very different for these two cases. In the EBPR system higher densities were measured in the middle of the granule whereas this was not observed for the Nitritation-Anammox granules originated form a CANON reactor. FISH on sliced granules confirmed these differences. The autotrophic granules showed AOB in the outer layers and Anammox bacteria growing equally distributed in the inner core, whereas the EBPR granules contained few bacteria in the core of the granule. The EPBR granules showed the typical spatial distribution of nitrifyers in the oxygen penetrated layers (Cy3) as well as PAOs (Cy5) and GAOs (Fluos) in the outer layers (Figure 4C-D). The pH profiles from granules obtained from a measurement mimicking anaerobic conditions within a EBPR system showed a more significant pH increase towards the core of the granule, as compared to an Anammox granule (0.6 instead of 0.1) (Figure 4 E-F).

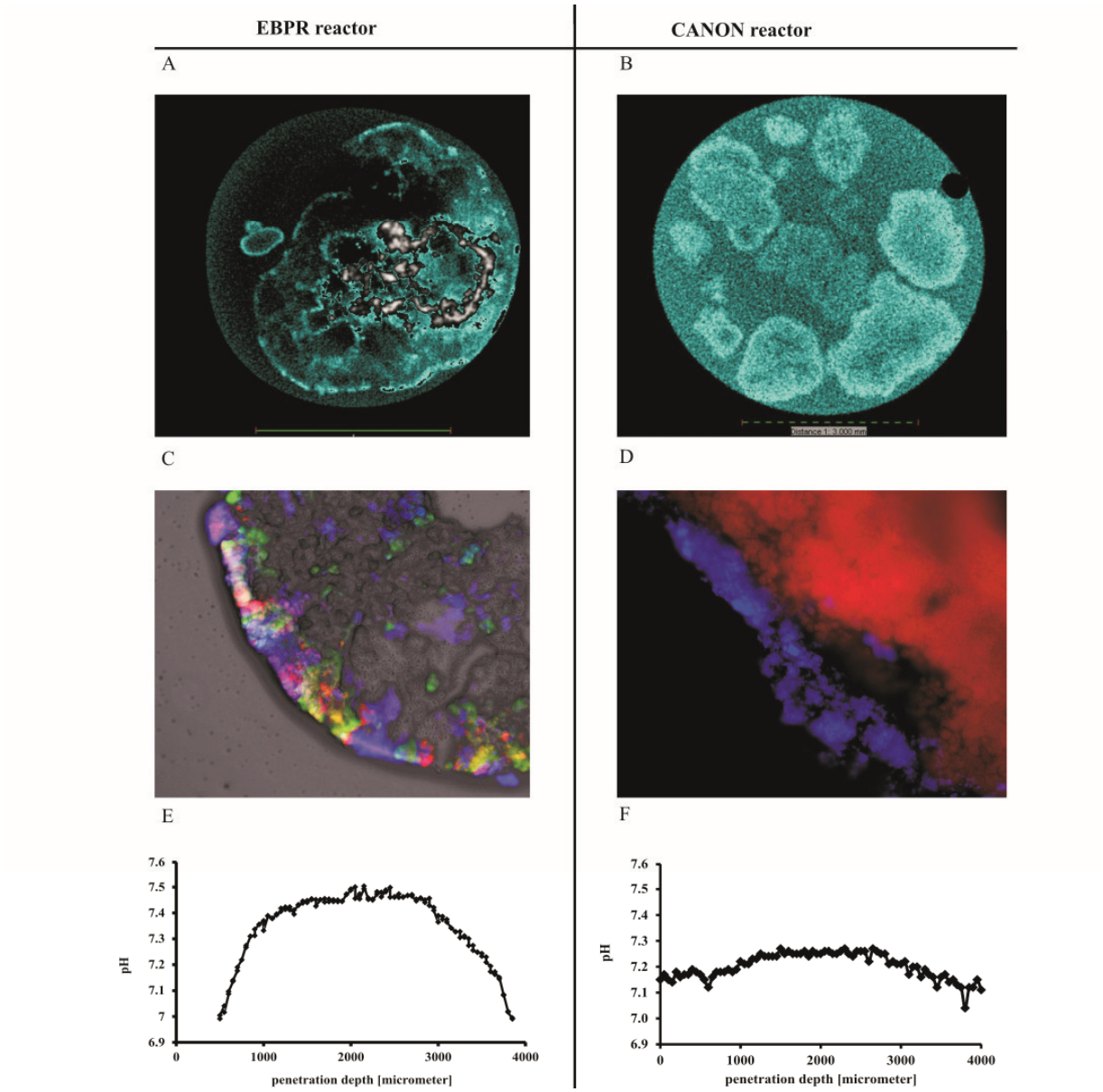

Figure 2 showing images of a CT scan (A-B) sliced FISH images (C-D) and pH electrode images (E-F) of sludge taken from an EBPR system (left) and an CANON reactor (right). The white colour indicates high density in the CT scans.

DENSITY OF BACTERIA

Percoll particles have a very dense (2.2 g/ml) inner core of silica with an average particle size of 29–30 nm (in 0.15 M NaCl). These particles partially settle during centrifugation, forming an uneven distribution of particles by which a density gradient is formed (Laurent T.C et al. 1979). The bacteria loaded on this gradient will sediment to an equilibrium position, at which the gradient density is equal to the density of the bacteria. During this process, the bacteria are separated solely on the basis of differences in their densities, irrespective of their size (Applications). The densities of different bacteria are summarized in table 3. The effect of fixation with 4% Paraformaldehyde was tested on 4 different strains and the density difference [g/ml] was negligible small (less than 0.1%) .

Table 3 name of bacterium and corresponding density

Bacterium	Density fixed [g/ml]
Nitrobacter winogradskyi	1.108±0.007
Nitrosomonas europea	1.081±0.003
PAO aerobic phase	1.077±0.003
PAO anaerobic phase	1.065±0.003
GAO aerobic phase	1.031±0.003
GAO anaerobic phase	1.032±0.003
Candidatus *Brocardia fulgida*	1.048±0.002

CHEMICAL PRECIPITATION

We calculated the saturation index values with PhreeqC based on the media composition as given in the online material. PhreeqC calculated circa 40 minerals but we only showed those which had the highest (positive) SI values. If the SI is equal to one, equilibrium between the mineral and the solution is reached. In the case that a SI of a mineral increases above zero it is thermodynamically possible that the mineral can precipitate. A higher pH and phosphate concentration increase the SI for all minerals and led to supersaturation especially for Fluoroapatite, Hydroxydicalciumphosphate and Hydroxyapatite (Figure 3).

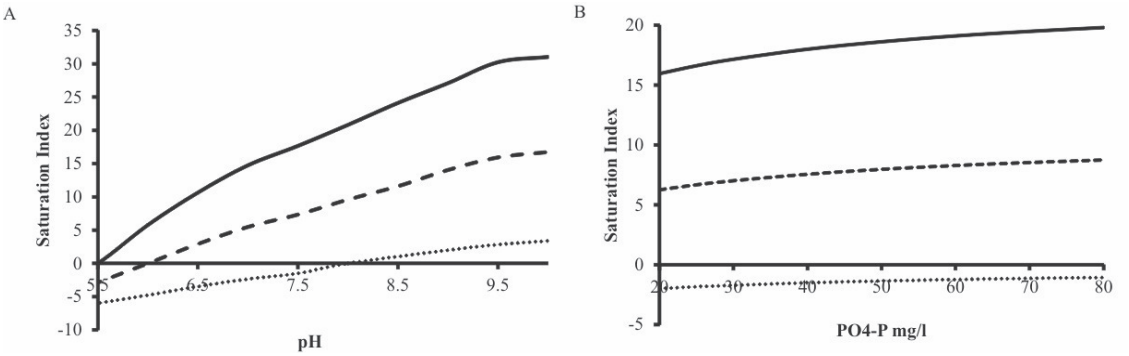

Figure 3 Influence of A) pH and B) phosphate concentration (pH 7) on precipitation equilibria of Fluoroapatite (straight line), Hydroxydicalciumphosphate (dashed line) and Hydroxylapatite (dotted line) at 20°C.

EVALUATIONS OF THE SETTLING MODEL

We evaluated three different cases with our settling model (Figure 4 A-C). All cases are based on the observed volume fractions from Ct scan and FISH pictures (Figure 2) as well as based on evaluated densities (Table 2; Figure 3). Case A and B are based on the results gained from the EBPR system (Figure 1 picture 1). In case A we assumed that there is no precipitation and the outer layer (A) is either occupied by PAOs or by GAOs. The thickness was chosen to be not deeper than 200 µm (corresponds to a volume fraction of 70% at a fixed diameter of 1.2 mm; Figure 4A) to be consistent with the observed bacterial layer from sliced granules stained by FISH (Figure 2C).

We choose the densities of the organisms in the end of the aerobic phase (Table 2) because in granular reactors the settling occurs immediately after the aeration stops. During this phase PAO are expected to be enriched with poly-phosphate granules. Our results show that the settling velocity of PAO granule was significantly increased when compared to GAO dominated granules by up to a factor 2. In case B the thickness of the bacterial layer (aerobic PAO and GAO) was fixed at 50µm (corresponding to 23% volume fraction) and only the volume fraction occupied by precipitates was varied showing that small changes in the inorganic volume fraction (5%) are severely affecting settling velocities by a factor of up to 6.

Case C refers to the CANON reactor (Figure 1 picture 2). Since nitrifiers only occupy the oxygen penetrated outer layer, which is typically not deeper than 80 µm we assumed for AOBs and NOBs a maximal thickness of 80µm (corresponding to a volume fraction of 35%). Here we once assumed a granule consisting of an outer layer of AOB (80 µm) and an inner anoxic layer consisting of Anammox and once a granule consisting of an outer layer of AOB (50 µm) followed by NOB (30 µm) and an inner layer consisting of EPS. We then tested the effect on settling velocity by changing the diameter of the granule. Results showed that at smaller granular diameters the settling velocities and granular densities were very similar for both types of granules and increased for the Anammox dominated granules when granular diameter was increased (Figure 4 C).

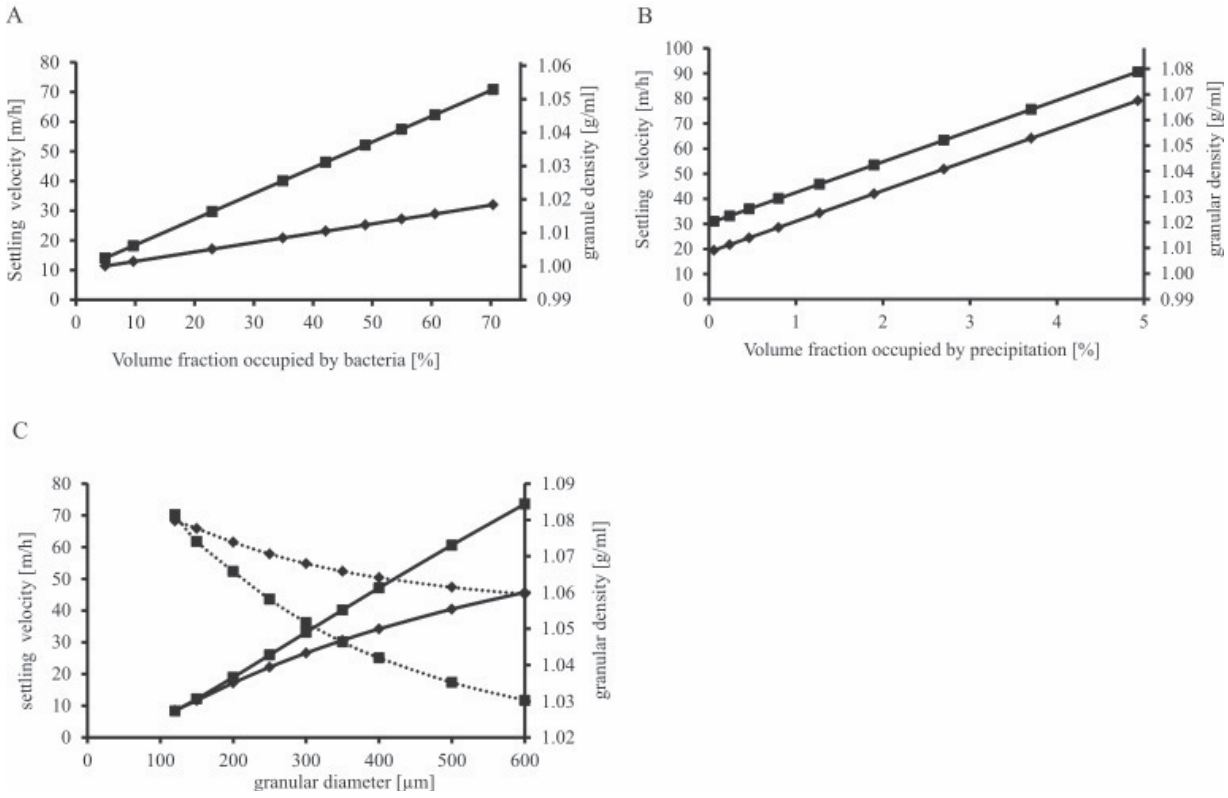

Figure 4 Calculated settling velocities and granular densities with respect to varying volume fractions of: A) bacteria and B) precipitates for a PAO (■) and GAO (♦) dominated granules as well as C) calculated settling velocities (straight line) and corresponding granular densities (dashed line) for a CANON granule with respect to varying diameter of a granules dominated by either AOB and Anammox (●) or AOB and NOB (▲).

DISCUSSION

The results presented here show that differences in density of different types of bacteria and differences in the inorganic volume fraction can significantly alter settling velocity of granules and hence influence the earlier described segregation effect within granular sludge systems (Bassin et al. 2012; Volcke et al. 2012; Winkler et al. 2011a; Winkler et al. 2011b). The higher density of PAOs in combination with intracellular stored poly-P resulted in higher settling velocities of PAO-dominated granules when compared to a GAO dominated granule (Figure 4A). Another factor influencing the settling velocity is the inorganic fraction. The contribution of precipitates significantly increased granular settling velocity as shown by our settling model (Figure 4B). A change in volume fraction from 1 to 5% accounts for a change in settling velocity from 20 to 90 m/h. The small volume fraction occupied by inorganic components will account for 10-30% of the total weight as indicated by general ash contents measured in other granular studies (Bassin et al. 2012; Batstone and Keller 2001; Etterer and Wilderer 2001). The precipitation of components is provoked at higher pH and phosphate concentration (Figure 3). The observation that precipitation was detected mainly in inner core is in line with earlier research (Angela et al. 2011; Maurer et al. 1999; Yamaguchi et al. 2001) and can be explained by the higher pH in the middle of the granules and higher phosphate concentrations caused by phosphate release of PAOs during the anaerobic feeding period (Figure 2 E-F).

The strong increase of the pH inside an EBPR granule can be explained by the uptake (H^+ removal from bulk) of the acidic component acetic acid by PAOs and GAOs. However magnesium- and potassiumphosphates (Mg^{2+}, K^+ are counter ions from the poly-P) released by PAOs will have a buffering effect, reducing the pH. Although this buffering effect disfavours precipitation, the higher phosphate release by PAOs will favour precipitation of phosphate minerals (Figure 3) (Lopez-Vazquez et al. 2009a; Lopez-Vazquez et al. 2009b; Lopez-Vazquez et al. 2008; Smolders et al. 1994). The complexity of precipitation equilibria within a PAO or GAO dominated granules would need to be modelled mathematically to further investigate in which granule one can expect more precipitates. Experimental data showed a considerably higher PAO fraction and higher ash contents at the bottom of the settled sludge bed (Bassin et al. 2012; Winkler et al. 2011a). Segregation in an EBPR system is clearly influenced by higher PAO densities and internally stored polyP which will be accumulated in the biomass after the

aerobic period (Figure 4A). Internal storage compounds can influence the density of bacteria. This was also found previously by others who measured the effect of sulphur inclusions on the density of bacteria *Chromatium warmingii* (this resulted in a density range of 1.71-1.108 [g/ml] (Guerrero et al. 1984). Our results show that in granular sludge systems segregation of biomass can easily occur due to slight variations in density of bacteria and precipitates.

For the CANON system our results from FISH and Ct scan show that precipitation is playing a less important role than in an EBPR system and also the detected pH shift was lower than in EBPR granules indicating that the internal pH plays an important role in the formation of precipitates in granular systems (Figure 2). The settling model shows that in the competition for settling speed between a granule occupied by either Anammox and AOB or by NOB and AOB granular diameter is playing an important role (Figure 4 C). Experimental and mathematical models have shown that in bigger granules NOB bacteria are outcompeted by Anammox bacteria. NOB are expected to grow in smaller granules due to a higher aerobic volume fraction (Vlaeminck et al. 2010; Volcke et al. 2012; Volcke submitted; Winkler et al. 2011b). Since the settling velocity of a granule is influenced by size and density and NOB are expected to grow in smaller granules this fact will lead to slower settling velocities. While Anmmox bacteria can grow in deeper layers of the granules, AOB and NOB are restricted to grow in oxygen penetrated layers of the granules. This will hence lead to a decrease in density with increasing diameter (Figure 4C). Oxygen penetration depth can be calculated by standard formulas and can be expected to not be higher than circa 80 µm in CANON systems since it is run under lower oxygen concentrations to keep the accumulation of NOB bacteria low (Hao et al. 2005; Harremoës 1977; Winkler et al. 2011b).

Therefore, the contribution of the density from nitrifying bacteria will be limited in systems consisting of mainly bigger granules. However in systems consisting of mostly granules with a small diameter a segregation will be difficult to achieve because the density of NOB is much higher than the density of AOB or Anammox (Table 3). For this reason it is important to select for bigger granules in CANON system (Figure 4 C). We observed significant density differences among the autotrophic bacteria. *Nitrobacter winogradskyi* (NOB) had the highest density and Candidatus *Brocardia fulgida* (Anammox) had the lowest density. It is likely that these differences

can be explained by the cellular makeup of these organisms, but it is presently impossible to say which factor may determine this differences and whether the observed densities are intrinsic properties of these organisms or caused by the cultivation conditions. For E. coli different studies have reported densities between 1.05 and 1.11 g/ml which is most likely due to different cultivation methods, strains and different Percoll procedures used (Koch and Blumberg 1976; Martinez-Salas et al. 1981; Woldringh et al. 1981). Selective sludge removal has successfully been implemented to control microbial populations in aerobic granular sludge systems. Since anaerobic granules also have a multistructural layer of microorganisms a segregation based on different physico-chemical differences of granules can be equally expected in these systems underlining the importance of this research also for other reactor systems (Macleod et al. 1990). Our results show that microorganisms which adhere to the granule strongly influence subsequent physical (densities) and chemical (precipitates) properties of the granule and hence their settling velocity.

CONCLUSIONS

Our results revealed that bacterial densities as well as precipitates have an impact on granular settling velocity. PAOs had a higher density than GAOs leading to significantly higher settling velocities for PAO dominated granules explaining earlier observations of segregation in granular sludge reactors. Moreover, it was shown that a small increase in the volume fraction of precipitates (1-5%) strongly increased the granular density and thereby the settling velocity. For nitritation-anammox granular sludge mainly granular diameter and not density differences were shown to impact a segregation of the biomass in the sludge bed.

SYMBOL LIST

Thickness of different layers

r_{total} = radius granule$[\mu m]$; r_A = thickness layer A$[\mu m]$; r_C = thickness layer C$[\mu m]$

r_{C+B} = thickness layer C+B $\Rightarrow$ $r_{C+B} = (r_{total} - r_A)[\mu m]$

r_B = thickness layer B $\Rightarrow$ $r_B = r_A - r_C\,[\mu m]$

Volume fractions of different layers

$$V_{total} = \frac{4}{3}\pi r_{total}^3\left[\mu m^3\right];\ V_{B+C} = \frac{4}{3}\pi r_{C+B}^3\left[\mu m^3\right];\ V_B = V_{B+C} - \frac{4}{3}\pi r_C^3\left[\mu m^3\right];$$

$$V_C = V_B - V_{B+C}\left[\mu m^3\right];\ V_A = V_{total} - V_{B+C}\left[\mu m^3\right]$$

Volume percentrages of different layers

$$V_A^{'} = \frac{V_A}{V_{total}}\left[\frac{\mu m^3}{\mu m^3}\right]\cdot 100[\%]; V_B^{'} = \frac{V_B}{V_{total}}\left[\frac{\mu m^3}{\mu m^3}\right]\cdot 100[\%]; V_C^{'} = \frac{V_C}{V_{total}}\left[\frac{\mu m^3}{\mu m^3}\right]\cdot 100[\%]$$

Volume fractions coupeled with desnities

$\rho_A; \rho_B; \rho_C$ = density of layer A;B;C [kg/m^3]

$\rho_p = (V_A^{'}\cdot\rho_A) + (V_B^{'}\cdot\rho_B) + (V_C^{'}\cdot\rho_C)$[kg/m^3]

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Chapter 6

TEMPERATURE AND SALT EFFECTS ON SETTLING VELOCITY IN GRANULAR SLUDGE TECHNOLOGY

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ABSTRACT

Settling velocity is a crucial parameter in granular sludge technology. In this study the effects of temperature and salt concentrations on settling velocities of granular sludge particles were evaluated. A two-fold slower settling velocity for the same granules was observed when the temperature of water decreases from 40ºC to 5ºC. Settling velocities also decreased with increasing salt concentrations. Experiments showed that when granules were not pre-incubated in a solution with increased salt concentration, they initially floated. The time dependent increase in mass and hence in settling speed of a granule due to salt diffusion into the granule was dependent on the granule diameter. The time needed for full salt equilibrium with the bulk liquid took 1 minute for small particles from the top of the sludge bed and up to 30 minutes for big granules from the bottom of the sludge bed. These results suggest that temperature and salt concentration are important parameters to consider in the design, start-up and operation of granular sludge reactors and monitoring of these parameters will aid in a better control of the sludge management in anaerobic and aerobic granular sludge technology. The observations also give an explanation for previous reports which were suggesting that a start-up of granular sludge reactors is more difficult at low temperatures.

INTRODUCTION

In activated sludge wastewater treatment processes the sludge-liquid separation in secondary clarifiers is an important step to assure the effluent quality. Activated biosolids however frequently show poor settling properties, which in turn can potentially harm the process efficiency (De Los Reyes Ill and Raskin 2002). Recently, interesting alternatives for the conventional activated sludge systems were developed relying on compact and self-immobilized granulated biomass. Examples are for instance Aerobic Granular Sludge (AGS), Upflow Anaerobic Sludge Bed (UASB), as well as Expanded Granular Sludge Bed technology (EGSB) (Beun et al. 1999; Lettinga and Hulshoff Pol 1991; Morgenroth et al. 1997; Rinzema et al. 1993). One of the most important parameters to select for granular sludge is the settling velocity. By applying short settling times in reactors operated as sequencing batch process, only big and fast settling biomass aggregates are selected, while flocculent sludge is washed out (Beun et al. 2000). As a result, granular sludge technology has a small area requirement due to the absence of large clarifiers and increased sludge content in the bioreactors (De Bruin et al. 2004).

The parameters determining the settling velocity of particles and in turn biomass wash-out are of crucial importance to granular sludge technology. The balances of forces for the sedimentation of a spherical particle are depending on the buoyancy, gravity and drag force (Giancoli 1995). From this relation, the settling velocity is influenced by the viscosity of water, particle size and shape, and the difference between the density of the water and the particles. The density and viscosity of the medium depend on the temperature and solutes present in water. With increasing temperature, the viscosity and density of the water decrease even though the density is not as much influenced as the viscosity. At high temperature, water molecules are more mobile than at low temperature decreasing its viscosity from e.g. 10 to 40 ºC by a factor two (Podolsky 1994). Salts are dissolved as ions, which enhance the water structure and increase the density of the fluid. The density of ocean water at the sea surface is 1028 kg/m^3, which is much higher than the density of fresh water (998 kg/m^3 at 20ºC). Since the density of granules is only slightly different from water (reported values vary between 1005 and 1070 kg/m^3) (Bassin et al. 2012; Batstone and Keller 2001; Etterer and Wilderer 2001) changes in the density of the water will have a significant impact on the settling behaviour of granules. Many researchers measured,

calculated, and compared the settling properties of granular sludge and activated sludge under different process conditions (de Kreuk et al. 2005; Grant and Lin 1995; Lew et al. 2003; Nor Anuar et al. 2007; Liu et al. 2008). However, no study focussed on the influence of the viscosity and density changes of water due to changes in temperature and salt concentrations on the granular sludge settling. This is of special importance during operation of full-scale operation plants, which are susceptible to changes in temperature and in salt content. We measured in this study the settling velocity of laboratory grown aerobic granules under different temperatures and NaCl concentrations. The relevance of considering the physical properties of water for the design and operation of aerobic granular sludge reactors is discussed.

MATERIALS AND METHODS

DENSITY AND SIZE DISTRIBUTION MEASUREMENTS

Biomass was collected from a lab-scale aerobic granular sludge reactor operating with simultaneous nitrogen and phosphorus removal. This granular system was operated at 20°C and was inoculated with granules from a pilot-scale aerobic granular sludge reactor treating municipal wastewater (WWTP Epe, The Netherlands). The composition of the synthetic media fed to the reactor can be found elsewhere (Bassin et al. 2012). Granules were sampled for measurement of particle size distribution, dry mass, ash content and granule density similar to earlier research (Kwok et al. 1998; Mosquera-Corral et al. 2005; Winkler et al. 2011a). Size distribution measurements were conducted by the means of an image-analyser using the averaged projected surface area of the granules. Specific biomass density was measured with the pycnometer method. In this method, a known amount of biomass is placed inside a pycnometer, with a known volume and weight (m_o). The weight of the pycnometer together with the inserted biomass is determined ($m_o + m_s$). Subsequently, the pycnometer is filled with water (m_T) and the weight m'_{H2O} (m_T minus $m_o + m_s$) is determined. The volume of added water (V'_{H2O}) is obtained according to $V'_{H_2O} = \frac{m'_{H_2O}}{\rho_{H_2O}}$. The volume of measured solid V_S is obtained from the difference between the volume of water that fills the empty pycnometer (V) and the previously determined volume of water (V'_{H2O}). $V_S = V - V'_{H_2O} = \frac{m_{H_2O} - m'_{H_2O}}{\rho_{H_2O}}$. Finally, the density of the granules (ρ_S) can be calculated as: $\rho_S = \frac{m_S}{V_s}$.

EXPERIMENTAL DETERMINATION OF SETTLING VELOCITIES AT DIFFERENT TEMPERATURES AND SALT CONCENTRATIONS

The settling velocity of granules at different temperatures (5 - 40°C) and salt concentrations (0 - 40 g/L NaCl) was determined in a 3 meter long column with a diameter of 5.6 cm. The tap water composition was well within the European drinking water quality standards with negligible amount of salts present. There was no chlorination applied to the distributed tap water. The experimental column comprised a water jacket for temperature control. Temperature was controlled online within the reactor and was kept constant through the water jacket in which the water was constantly circulated. No temperature gradient within the water column was measured. The experiment was not started before a stable temperature value was reached. Granules from a lab-scale aerobic granular sludge reactor were collected from a settled sludge bed. During the settling phase of the sequencing-batch AGS reactor, bigger and denser granules settle faster and occupy the bottom of the sludge bed, whereas smaller and lighter granules form the top layer of the sludge bed (Winkler et al. 2011a).

Granules from top and bottom of the reactor sludge bed were collected and their diameter and density were measured as described in section 2.1. Henceforth the granules from the top were called small and granules derived from the bottom were called big granules. For visual representation images from top and bottom fraction were taken with a light microscope (Figure 1). For the measurement of settling velocities at different temperatures, the column was filled with tap water and temperature was adjusted between 5ºC and 40ºC. For the settling experiments at different salt concentrations (0 - 40 g/L NaCl), the temperature of the water was kept constant at 20°C. Moreover, settling experiments for different salt concentrations were conducted a) without pre-incubation b) after 15 minutes pre-incubation and c) after a day of incubation of the granules in the same salt concentration as used in the experimental setup.

For salt and temperature experiments, 5 g of wet granules were placed on a spoon and were released for every experiment into the reactor column. The bottom of the spoon was placed on the top rim of the column and the granules were released at time zero all at once into the water column. The water column height was always kept constant for all experiments. The time necessary to reach half of the column height (a mark was set at 1.5 meters)

was recorded with a chronometer. The settling velocity of granules was determined by building the average time between the first and last granule passing the marked column. Each experiment was carried out 5 times and graphs are based on average values. The amounts of granules used were kept constant (5 g) and the same granules were used throughout the experimental setup. At the end of one measurement (1 measurement consisted of 5 repetitions) all granules were removed from the water column and used for the next measurement. This was easily possible because the lower part of the water column was removable enabling a smooth removal of granules from the column without any rupture.

CALCULATION SETTLING VELOCITY

The measured average density and diameter of granules obtained in the settling experiments were used to calculate the theoretical settling behaviour. For particle Reynolds numbers smaller or equal to 1, Stokes' law was used to calculate the settling velocity of a particle $\upsilon_s = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{\nu_w}$. For particle Reynolds numbers in the range $1<Re_p<10^3$, the theoretical settling behaviour was calculated by iteratively solving the particle Reynolds (Re_p) number and using the equation $C_d \, Re_p^2 = \frac{3}{4}\left(\frac{d_p^3 \cdot p_w \cdot (p_p - p_w) \cdot g}{\nu_w^2} \right)$ to solve the stationary sedimentation velocity of a single spherical particle: $\upsilon_s = \frac{Re \cdot \nu_w}{d_p}$ (Hallermeier 1981). Definitions for equations are given in symbol list. Changes in density and viscosity of water were adjusted according to the temperature and salt conditions as applied in the experimental setup (Viswanath et al. 2007; Weast and Lide 1990). It was further assumed that inside the granules the same salinity occurred as in the liquid. The granular density was corrected for the increase in density of water at different temperature or salt concentrations.

CALCULATION SALT PENETRATION

The penetration time of a known salt concentration (C_L = 41 g/L NaCl, density 1030 kg/m^3) into a granule and the resulting specific mass increase and settling velocities over time were calculated. In these calculations, spherical granules were assumed to have equal diameter and composition. Furthermore, granules were assumed to not influence each other while

settling. The chosen diffusion coefficient of salt in solution (D_L) was 9×10^{-10} m^2/s and $0.8D_L$ for the diffusion coefficient of salt into the granules (D_G) (Pajonk et al. 2003; Stewart 2003). For calculations, an initial particle density of 1010 kg/m^3 and a diameter (d) of 1 mm or 3 mm were assumed. Absorption of salt into the particle can be simulated using the diffusion equation, but here the simple standard solution was used. First absorption follows penetration theory, but after a critical time the mass transfer coefficient becomes constant, given by $Sh = \frac{2}{3}\pi^2$. This period we call permeation. The salt penetration depth $\delta = \sqrt{\pi D_L \tau}$ at the critical time τ between penetration and permeation was calculated using $\tau = Fo\ d^2/D_G$ assuming a critical Fourier (Fo) of 0.02. The overall mass transfer coefficient $K_L = \left(\frac{1}{k_o} + \frac{1}{m k_i} \right)^{-1}$ was derived using an external mass transfer coefficient $k_o = Sh \frac{D_L}{d}$ (with Sh = 2) and an internal (average) mass transfer coefficient $k_i = 2\frac{D_G}{\delta}$.

The relative concentration of salt in the granule compared to the salt solution (m) was arbitrarily assumed to be 0.8. This assumes that cells and EPS molecules in the granule take up volume leading to a lower volumetric salt concentration in the granules. The mass entering the granule during this period follows from $\Delta M = K_L A C_L \tau$. After the penetration period (Fo > 0.02), during permeation, the internal mass transfer coefficient $k_i = Sh \frac{D_G}{d}$ was used with a Sherwood of $Sh = \frac{2}{3}\pi^2$. Now the change in mass follows from a mass balance: $\frac{dM}{dt} = \frac{dVC_G}{dt} = K_L A \left(C_L - \frac{C_G}{m} \right)$. Combining, the density of the granule follows from $\rho_G = \rho_{Gi} + mC_L - \left(mC_L - 6\frac{K_{Lp}}{d} C_L \tau \right) \exp\left(-\frac{K_L}{d\,m} (t - \tau) \right)$, with subscript p referring to the penetration period.

RESULTS

SETTLING VELOCITY AT DIFFERENT TEMPERATURES

Top (small) and bottom (big) granules with known density, diameter and ash content (table 1) were used to determine the settling velocity at different temperatures and salt concentrations. The results presented in this work are in alignment with general physical mass and heat transfer theorems.

Table 1 Physical properties of small and big granules from settling velocities test

Parameter	Small	Large
Ash content %	15±6	34±7
Density g/L	1020±5	1037±8
Average diameter (mm)	1.5±0.3	2.3±0.5

Heat transfer was quicker than transfer of salts and did not require additional pre-incubation. Results of settling velocities at different temperatures showed a good fit with the calculated settling velocities which were based on the average measured diameter and density of the granules used in the experiment. Smaller and lighter granules settled slower than bigger and denser granules. Results revealed a two-fold difference in settling velocity for the same granule at 5ºC and 40ºC with values increasing from 84 to 145 m/h for big granules and from 35 to 63 m/h for smaller granules (Figure 2).

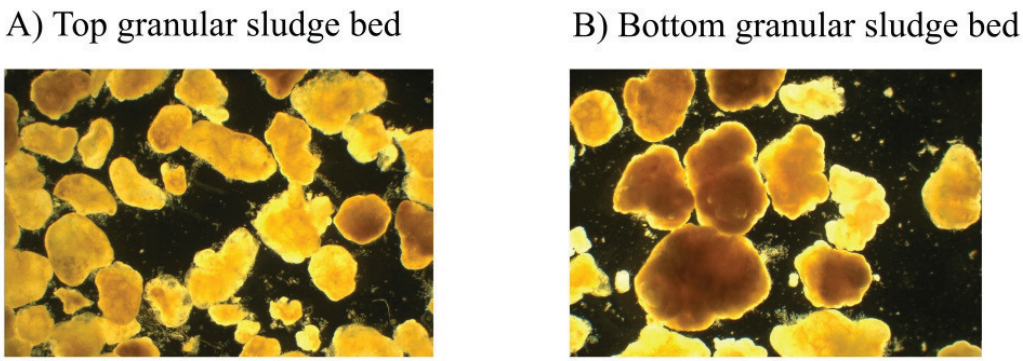

Figure 1 Light microscopic image (7.5 x magnifications) of granules taken from the A) top and B) bottom layer from the settled sludge bed

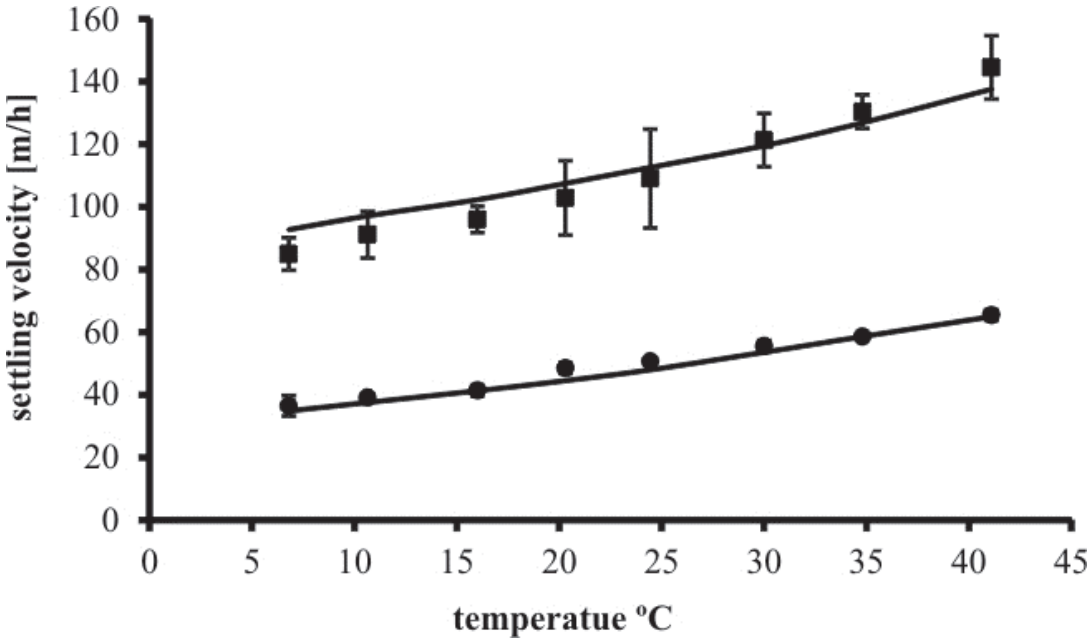

Figure 2 Measured settling velocities and standard deviations at different temperatures for small (●) and big (■) granular sludge particles and the corresponding theoretical settling velocities (lines) based on the measured values in table 1

SETTLING VELOCITY AT DIFFERENT SALT CONCENTRATIONS

Opposed to heat transfer, mass transfer of e.g. salt was much slower which can be seen from experiments showing that for temperature measurements no pre-incubation was needed. For the salt measurements settling tests without a pre-incubation of the granules in the salt concentration in which the experiment was conducted in, most of the granules floated making a measurement of the settling velocity impossible. In order to experimentally show the effect of salt penetration on the density and hence the settling velocity of granules a pre-incubation of 15 minutes and of one day was chosen. Results showed that the longer the incubation was chosen the faster the granules settled. To calculate the theoretical settling behaviour the density and size from measured granules (table 1) were used in the model. Theoretical settling behaviour of granules at different salt concentrations gave a good fit (Figure 3).

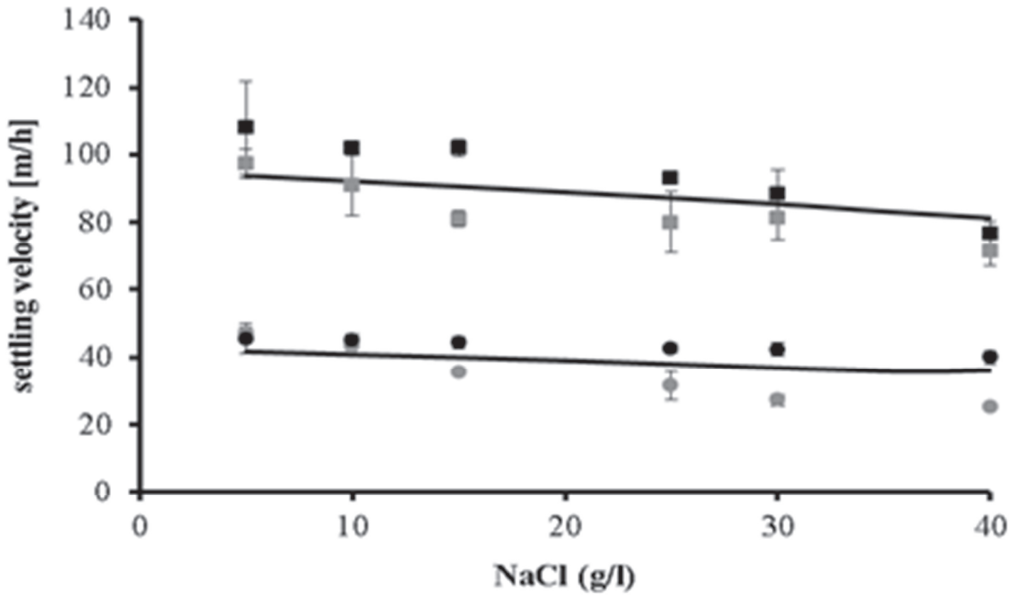

Figure 3 Measured settling velocities and standard deviations at different salt concentrations for small (●) and big (■) granular sludge particles after incubation in corresponding salt solution for 15 minutes (grey) and 24 hours day (black)

TIME DEPENDANT SALT PENETRATION

The time dependent increase in density and settling velocity of small and big granules was calculated during incubation in a 40g/L NaCl solution. Settling velocity of granules increased due to the increase of density caused by the penetration of salt into the granule. Settling velocity of smaller granules was accelerated whereas for bigger granules no settling occurred until 11 minutes. Only after this time the settling increased linearly due to full salt penetration (Figure 4A). Figure 4B shows the time needed for a particle to get as dense as the solution. For large particles the time was in the order of 5-30 minutes.

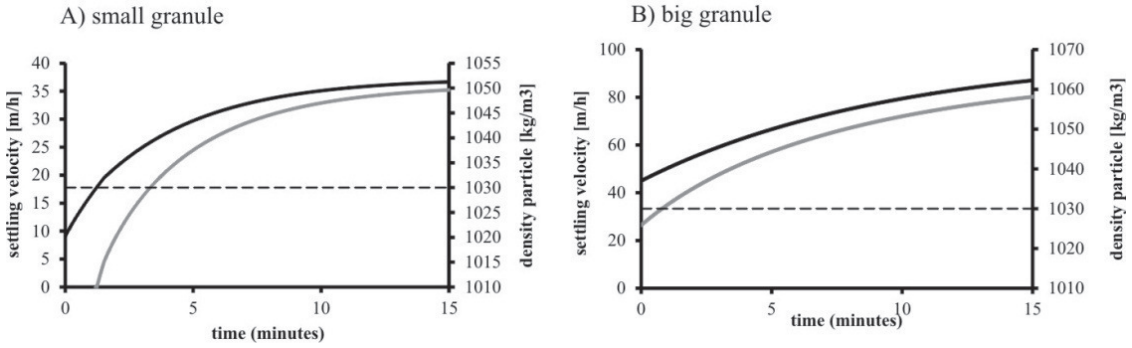

Figure 4 Calculated time dependent increase in density (black) and settling velocity (grey) of a A) small and B) big granule during incubation in a 40g/L NaCl solution. Diameter and density of granules are based on experimentally determined values (table 1). Reference line (dashed) corresponds to density at which granules start to settle.

DISCUSSION

In this study the effects of temperature and salt concentrations on settling velocities of granules were evaluated. Results revealed a two-fold faster settling velocity for the same granules when temperature of water was increased from 5ºC to 40ºC. Measured and calculated settling velocities within this study gave a good fit for fresh water (Figure 2). Other researchers who measured and calculated settling velocities of aerobic granular aggregates in fresh water also found a good fit of measured and theoretical values (Xiao et al. 2008). In addition, settling velocities were measured at different salt concentration and results revealed as expected in a decrease in settling velocity with increasing salt concentrations. This decrease was however lower than expected from a calculation based on the originally measured density (table 1). The density of a granule is very sensitive in the calculation of the settling velocity.

Therefore we corrected the density of the granule for the salt water inside the granule which also leads to an increased density of the particles. It is simply assumed that the density of the liquid inside and outside of the granule is the same. Our model (Figure 4) shows that after 15 minutes the small granules should be saturated and hence the terminal settling velocity should be reacted. However we could still measure a difference in settling velocity between 15 minutes and 24 hours of incubation (Figure 3). Earlier studies

reported a shrinking of alginate gels at increasing salt concentration (Moe et al. 1993; Tierney et al. 2010). Since density is defined as mass per volume granular density will be increased by shrinkage which in turn affects the settling velocity. Since granules are reported to consist of alginate this effect is likely to have played a role in our experiments as well (Lin et al. 2010). The corresponding shrinking of the particle was too small to be measurable with an image analyser. In case of the temperature, the main factor influencing the settling velocity is the change in viscosity of the water, whereas for salt water the major factor influencing settling velocity is the water density. Temperature and salt have a significant effect on granular sludge settling rate. This is due to the fact that at higher temperature the viscosity of water is decreased hence decreasing resistance, while at higher salt concentrations resistance is increased due to the water density increase (Judd 1970; Thomas and Stevenson 1973).

Certainly when temperature and salt content rapidly vary, like in industrial wastewaters or e.g. due to melt water or storm water in municipal wastewater treatment plants, the effect of these parameters should be considered and appropriate measures need to be taken to prevent process instabilities due to biomass washout. For municipal wastewater during cold winter periods also salt peaks from di-icing road salts might influence the settling rate. The concentrations of salts in runoff water can be up to 4 g chloride per litere (Bubeck et al. 1971) and can lead in extreme cases to floatation and washout of the sludge since the diffusion of salt into the granule can take up to 30 minutes (Figure 4A,B). Salt events will therefore decrease system functioning not only by toxification of microorganisms (increase in osmotic pressure) (Uygur and Kargi 2004) but also by wash-out events due to biomass floating as we encountered in our salt experiments when granules were not pre-incubation in the working salt solution. For a continuous operation of granular sludge reactors at high salt concentrations of up to 30 g NaCl/L floating has not be reported to be problematic (Figueroa et al. 2008) which is in line with our experiments showing well settling biomass after a pre-incubation (Figure 3).

However, when granular sludge reactors are run in sequencing batch mode, with e.g. 3-4 parallel reactors fed sequentially, the wastewater characteristic can significantly vary in between two influent additions. A strong change in temperature or salt might disturb an even upflow of the influent through the settled sludge bed.

The formation of granular sludge at lower temperatures is hindered by many factors. Earlier research on aerobic granular sludge reactor has experimentally shown that a start-up process at cold temperatures is troublesome (de Kreuk et al. 2005). All microbial processes run slower at lower temperatures (Brdjanovic et al. 1997; Kettunen and Rintala 1997; Lettinga et al. 2001) which could limit the granulation at a lower temperature. This research shows that not only microbial factors are hindered at lower temperatures but also that settling velocity is much reduced at lower temperatures (Figure 2). During a start-up process of a granular sludge system, granular biomass has to be separated from flocculent sludge in order to have selective washout of the latter. Initially granular sludge particles are only small (0.2 mm), which implies that the settling velocity differences between flocculent and granular biomass are small. At lower temperature, this difference is even decreased. The settling velocity of a granule with a diameter of 200 µm and a density of 1010 kg/m^3 will not be higher than 1 m/h regardless of the temperature. At these low temperatures the separation of initial granules and flocs becomes therefore troublesome explaining the reported problematic start-up under cold conditions.

Recently we demonstrated that in aerobic granular sludge vertical segregation of granules occurs based on small differences in settling velocity of the granules and that different types of bacteria grow either in bigger or smaller granules due to physico-bio-chemical reasons (Winkler et al. 2011a; Winkler et al. 2011b). Granules with a higher density accumulate at the bottom of the sludge blanket. Since the feeding is from the bottom of the reactor, these granules get a higher substrate load and therefore a higher radius, which enhances the segregation effect. Segregation of biomass has been reported earlier and is hence a common occurrence in wastewater treatment based on granular sludge or biofilms (DiFelice et al. 1997; Ro and Neethling 1994). Selective sludge withdrawal from either top or bottom of the sludge bed can therefore be used as a possibility to control the microbial community structure granular sludge technology. Since we have shown that salt events mainly cause bigger granules to float (Figure 4 A,B) and our previous studies have shown that polyphosphate accumulation organisms are mainly located in bigger granules (Bassin et al. 2012; Winkler et al. 2011a), salt events might lead to a significant washout of this functional group and hence to a loss in P removal efficiency. The settling of granule depends on its diameter and density (and the physical properties of the fluid immersed

in). Both parameters can differ between anaerobic and aerobic granules which will have an influence on their settling velocity (Bassin et al. 2012; Batstone and Keller 2001; Etterer and Wilderer 2001). In our tests we showed a good fit between settling model and measurements from small and big granules (Figure 2, 3). The authors believe that the principles found from this study are hence transferable to any kind of granules of known density and diameter regardless of their origin. We can conclude that in order to optimise biomass retention within granular sludge reactors a monitoring of a variation in physical water properties like temperature and conductivity (to measure salts) is of importance to understand potential disturbances of the reactor system.

CONCLUSIONS

In this study we showed that the temperature and salt concentration dependent density and viscosity changes of water have great impact on settling velocity of granular sludge. The corresponding slow settling of small granules at decreased water viscosities and increased water densities as caused by a lower temperature can be an important reason for the reported troublesome start-up of granular sludge reactors. Conductivity and temperature measurements can therefore be used as an additional operational factor to stabilise and improve biomass retention in granular sludge technology.

SYMBOL LIST

δ Salt penetration depth

$$\delta = \sqrt{\pi D_L \tau}[mm]$$

d diameter particle [mm]

D_L diffusion coefficient (Liquid or Granule)[m^2/s]

Fo Fourier group (dimensionless time)[-]

k_i Internal mass transfer coefficient in penetration period

$$k_i = 2\frac{D_G}{\delta}[m/s]$$

k_i' Internal mass transfer coefficient after penetration period

$$k_i' = Sh\frac{D_G}{d}[m/s]$$

k_o External mass transfer coefficient

$$k_o = Sh\frac{D_L}{d}[m/s]$$

K_L Overall mass transfer coefficient

$$K_L = \left(\frac{1}{k_o} + \frac{1}{mk_i}\right)^{-1}[-]$$

m Distribution coefficient (solubility salt in granule versus salt solution)[-]

Sh Sherwood group (dimensionless mass transfer coefficient)[-]

τ Penetration time (during which penetration theory holds)[sec]

Calculation of settling velocity

$$C_d \, \mathrm{Re}_p^2 = \frac{3}{4}\left(\frac{\mathrm{d}_p^3 \cdot \mathrm{p}_w \cdot (\mathrm{p}_p - \mathrm{p}_w) \cdot g}{\nu_w^2} \right)$$

$$K = \frac{3}{4}\left(\frac{\mathrm{d}_p^3 \cdot \mathrm{p}_w \cdot (\mathrm{p}_p - \mathrm{p}_w) \cdot g}{\nu_w^2} \right)$$

$$\mathrm{C}_d = 24 \cdot \mathrm{Re}_p \cdot (1 + 0.15\mathrm{Re}^{0.687}) - K$$

$$\upsilon_\mathrm{s} = \frac{\mathrm{Re}_\mathrm{p} \cdot \nu_\mathrm{w}}{\mathrm{d}_\mathrm{p}} \text{ for } \mathrm{Re}_\mathrm{p} \geq 1$$

$$\upsilon_s = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{\nu_w} \text{ for } Re_\mathrm{p} \leq 1$$

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

d_p = particle diameter $[\mathrm{m}]$

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

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

g = gravitational constant 9,81 $\left[\frac{\mathrm{m}}{\mathrm{s}^2}\right]$

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

C_d = Continuum, intermediate $[-]$

K = particle – liquid constant $[-]$

Re_p = Particle Reynoldsnumber $[-]$

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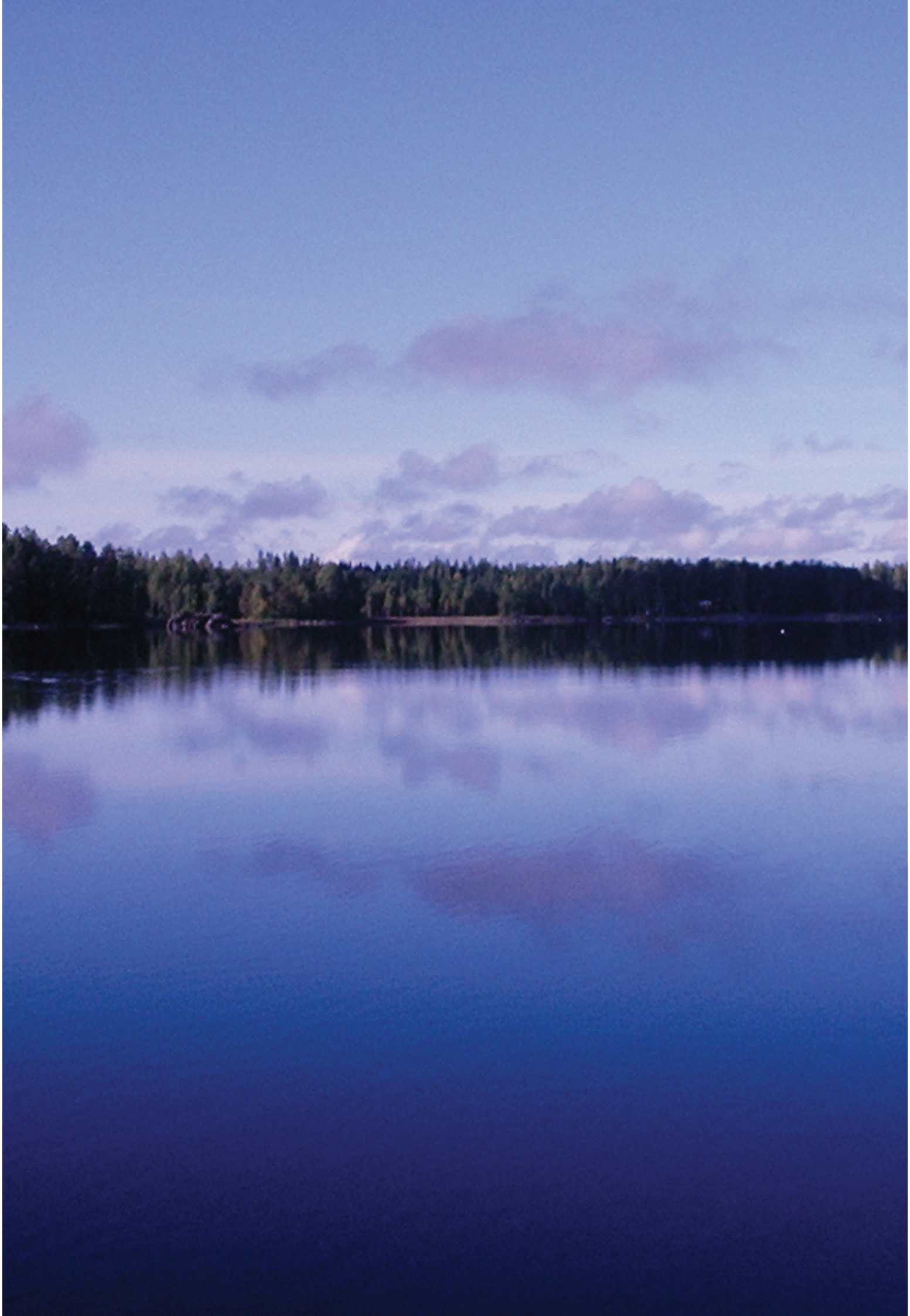

Chapter 7

CONCLUDING REMARKS

EVALUATION AND OUTLOOK

SEGREGATION IN NEREDA® REACTORS

The segregation of microbial populations over the height of the sludge bed has been shown to offer new opportunities in controlling microbial communities in aerobic granular wastewater treatment processes. This might well also give the opportunity of microbial population control for other granular sludge reactors such as upflow-anaerobic-sludge bed (UASB) systems. Not only the research conducted in this thesis but also other research demonstrated the existence of segregation (Li and Li 2009; Volcke et al. 2010). Our results showed that in aerobic granular sludge systems segregation of biomass can easily occur due to slight variations in density of bacteria and precipitates. Segregation in an EBPR system was clearly influenced by higher PAO densities and internally stored polyP (**Chapter 5**).

The contribution of precipitates significantly increased granular settling velocity. To provoke precipitation in a granule dominated by a certain type of bacteria known to be beneficial for process performance would be a nice way to create a physico-bio-chemical induced segregation and might be of interests for future research objectives. It might also be attractive for future research to recover the phosphate from aerobic granules, since it is a limiting resource in agriculture. This would also increase the value of wasted sludge and hence decrease overall costs of the granular technology. Moreover, an evaluation of the activity of micro-organisms in relation to the depth within the sludge bed might be an interesting aspect for a design of a sludge extraction protocol based on the specific population one wants to select for. For instance selective sludge removal within an EBPR system will be of specific interest for warm weather countries or warm industrial wastewaters. At high temperatures usually GAOs become the dominant bacterial group (Lopez-Vazquez et al. 2009a; Lopez-Vazquez et al. 2009b; Lopez-Vazquez et al. 2008). In this thesis, at ambient temperatures selective sludge removal was not crucial for a good bioP-removal and biomass can be removed equally throughout the sludge bed such as it is applied in most wastewater treatment plants (**Chapter 3**).

However, at high temperature (30°C) phosphate removal was troublesome when no specific sludge control was applied but could be improved by a selective removal of top-GAO dominated biomass (**Chapter 2**). The biomass segregation observed was favoured due to a plug-flow feeding regime from the bottom of the reactor, where bottom PAO-dominated biomass had more substrate available.

SEGREGATION IN NITRITATION-ANAMMOX REACTORS

In another reactor based on nitritation/Anammox segregation of biomass could also be used to influence reactor performance by selective sludge removal. Bigger granules were dominated by Anammox bacteria due to a larger anoxic fraction available for Anammox bacteria and NOB were effectively out-competed as also suggested by previous model based evaluation (Volcke et al. 2010). In addition the feeding of nitrite and ammonium from the bottom also promoted the growth of bigger Anammox dominated granules. Smaller granules were dominated by the process disturbing NOB bacteria and were located at the top of the settle sludge bed. A selective removal of top biomass led to better nitrogen removal efficiencies at unfavorable low temperatures opening a wider application of Anammox, which was so far mostly implemented in warm side-stream processes (**Chapter 4**).

The importance of the selection of larger Anammox dominated granules was confirmed by density measurements of bacteria since NOBs were having a higher density as Anammox bacteria (**Chapter 5**). Oxygen penetration depth can expected to be restricted to a several micrometres. Therefore, in a CANON system the contribution of the density from NOBs, which effectively occupy this outer layer will be limited if a granular systems is consisting of mainly bigger granules. A selective wash-out of smaller NOB dominated granules can hence give the opportunity to introduce Anammox conversions in main stream wastewater treatment plants. This will greatly increase the sustainability of the process with respect to energy (Kartal et al. 2010; van Loosdrecht et al. 2004).

GENERAL FACTORS INFLUENCING SEGREGATION

Selective sludge removal has successfully been implemented to control microbial populations in aerobic granular sludge systems (**Chapter 2-4**). Since anaerobic granules also have a multistructural layer of microorganisms a segregation based on different physico-chemical differences of granules can be equally expected in these systems underlining the importance of this research also for other reactor systems (Macleod et al. 1990). Further it is of interest to monitor temperature and salt changes of the wastewater to prevent biomass washout. Salt events can cause bigger granules to float due to slow diffusion of salt into the granules (**Chapter 6**). Since it was shown that polyphosphate accumulation organism are mainly located in bigger granules (**Chapter 1,2**), salt events might lead to a significant washout of this functional group. We can conclude that in order to optimize biomass retention within granular sludge reactors monitoring of the variation in physical water properties like temperature and conductivity (to measure salts) are of importance to understand potential disturbances of the reactor system. The corresponding slow settling of small granules at decreased water viscosities and increased water densities as caused by a lower temperature can be an important reason for the reported troublesome start-up of granular sludge reactors (de Kreuk et al. 2005). At colder temperatures the difference in settling velocity between flocs and smaller granules is critical. It is therefore suggested to conduct a start-up of a granular reactor during warmer summer seasons.

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SUMMARY

Chapter 1 gives a short overview of the history of aerobic granular sludge technology and finishes with an outline of the thesis. **Chapter 2** deals with segregation of biomass as a function of height of the sludge bed. Phosphate accumulating organisms were found to dominate at the bottom of the sludge bed, whereas Glycogen accumulating organisms dominated at the top of the sludge bed. By selective removal of glycogen accumulating organisms dominated sludge from the top of the sludge bed, more than 95% P removal efficiencies were achieved at 30°C. Based on current knowledge this is the first process in which a stable biological P-removal could be maintained at 30°C. In **chapter 3** the selective sludge removal was studied within this enhance biological phosphorous removal (EBPR) system in more detail. At 30°C the removal of bottom sludge from the PAO-rich part of the sludge bed in minor proportions did not negatively affect P-removal and allowed to obtain biomass with a lower ash content. The research further shows that at 20°C, selective removal of PAOs was not crucial for a stable bioP removal and biomass can be removed equally throughout the sludge bed. Our results indicate that high ash content and density of bottom granules positively correlated with the presence of PAO-dominated granules.

In **chapter 4** we studied the competition for nitrite between nitrite oxidizing bacteria (NOB) and anaerobic ammonium oxidizing bacteria (Anammox) at low temperatures. White granules were dominated by NOB bacteria and were mainly located at the top of the settled sludge bed whereas red granules were dominated by Anammox bacteria and were located at the bottom. Granules from the top of the sludge bed were smaller and therefore had a larger aerobic volume fraction. These smaller granules also furthermore had a lower density then larger granules and consequently a slower settling rate. Selective sludge removal from the top of the settled sludge bed selectively removed NOB resulting in an increased overall biomass specific N-conversion. This forms an option for obtaining a stable Anammox process at lower temperatures in municipal wastewater treatment systems. **Chapter 5** investigates the effect of granular density on the settling velocity of individual granules. The granule was divided in different layers each occupying a certain volume fraction consisting out of either bacteria, extra polymeric substances or precipitates. The density of each fraction was estimated experimentally. Each volume fractions was coupled with the corresponding densities to calculate a total density of a

granule. This was used to calculate settling velocities. Results revealed that Phosphate accumulating organisms (PAO) had a higher density than glycogen accumulating organisms leading to significantly higher settling velocities for PAO dominated granules explaining earlier observations of the segregation of the granular sludge bed inside reactors. The model showed that a small increase in the volume fraction of precipitates (1-5%) strongly increased the granular density and thereby the settling velocity. For nitritation-anammox granular sludge the settling model shows that density differences are not very important and segregation of the biomass in the bed is mainly caused by variations in granule radius.

In **chapter 6** we showed that the temperature and ionic strength dependent density and viscosity changes of water have great impact on settling velocity of granular sludge. The corresponding slow settling of small granules at decreased water viscosities and increased water densities as caused by a lower temperature can be an important reason for the reported troublesome start-up of granular sludge reactors at low temperatures. Settling velocities also decreased with increasing salt concentrations. Changes in salt concentration will cause a strong time dependent effect of settling of granules due to the slow diffusion of salts into the granules. Conductivity and temperature measurements can therefore be used as an additional operational factor to stabilize and improve biomass retention in granular sludge technology.

SAMENVATTING

Hoofdstuk 1 geeft een kort overzicht van de geschiedenis van aërobe korrelslib technologie. **Hoofdstuk 2** beschrijft de segregatie van biomassa binnen een slibbed. PAOs (P accumulerende organismen) waren de dominante bacteriesoort in het onderliggende deel van het slibbed, en GAOs (glyciogeen accumulerende organismen) domineerden het bovenliggende deel. Het selectief verwijderen van slib van de door GAO gedomineerde topfractie leidde tot een systeem met 95% fosfaatverwijderingsefficiëntie bij 30 ^{o}C. Voor zover bij ons bekend is hiermee voor het eerst een stabiele fosfaatverwijdering aangetoond bij een temperatuur van 30 ^{o}C. In **hoofdstuk 3** is het proces uit hoofdstuk 1 in detail bekeken. Omdat biologische fosfaatverwijdering gebaseerd is op het vastleggen van fosfaat in PAOs die vervolgens uit het systeem verwijderd dienen te worden, wordt in dit hoofdstuk onderzocht in welke mate de selectiedruk voor het verkrijgen van PAO bij 30 ^{o}C door middel van segregatie werkt als er ook uit het onderste compartiment slib wordt verwijderd om de fosfaatverwijdering uit het systeem te faciliteren. De uitkomst was dat fosfaatverwijdering mogelijk is, zelfs als een gedeelte van het slib specifiek uit het onderste compartiment wordt verwijderd. Verder hebben wij aangetoond dat bij 20 ^{o}C segregatie minder sterk optreedt dan bij 30 ^{o}C; maar aangezien bij 20 ^{o}C de competitie tussen PAO en GAO in het voordeel van PAO's uitvalt is het selectief slibverwijderen op deze temperatuur niet noodzakelijk.

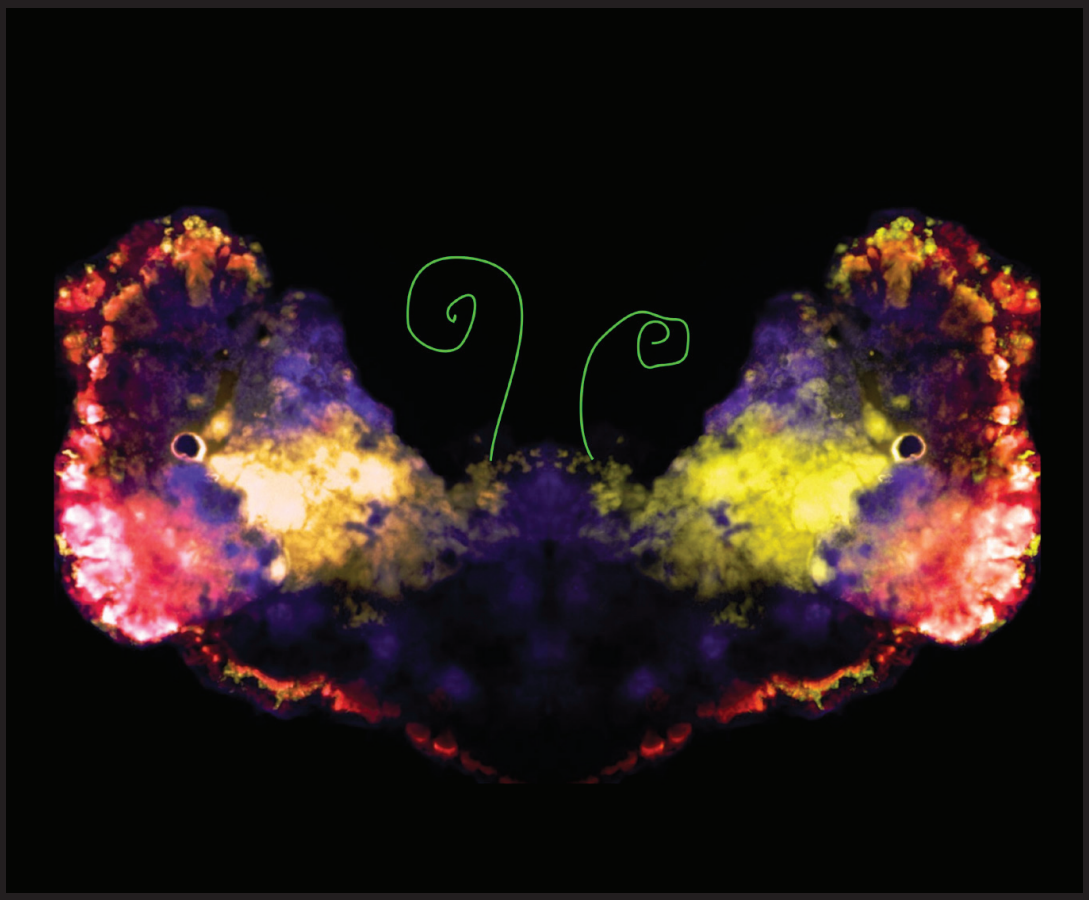
In **hoofdstuk 4** hebben wij de competitie factoren tussen nitriet oxideerde bacteriën (NOB) and ammonium oxiderende bacteriën (Anammox) bij relatief lage temperaturen (rond 18 ^{o}C) onderzocht. Witte korrels waren gedomineerd door NOB en kwamen meer op het boven liggende bed terecht. Rode korrels waren echter gelokaliseerd in het onderste deel van het slibbed en gedomineerd door Anammox bacteriën. Korrels van de toplaag waren kleiner in diameter en daarom was in een relatief groter gedeelte van de korrel zuurstof aanwezig, wat gunstig bleek voor de groei van NOBs. Bovendien hadden de korrels in de toplaag een geringere dichtheid hetgeen bijdroeg aan een langzamere bezinksnelheid dan voor grotere korrels. Het was mogelijk om surplusslib selectief uit de bovenliggende laag te verwijderen om anammox competitief voordeel te geven over NOBs. Hierdoor kan in autotrofe systemen verbeterde stikstofverwijdering worden gerealiseerd en ook bij lagere temperaturen een stabiel anammox gebaseerd proces worden bedreven. **Hoofdstuk 5**

behandelt het effect van de dichtheid van een korrel op hun bezinksnelheid. De korrels zijn hierbij onderverdeeld in verschilde lagen op basis van hun samenstelling, waartoe de volgende categorieën zijn gebruikt: bacteriesoorten, EPS of anorganische precipitaten. Iedere laag is geïdentificeerd door middel van FISH/CT scan. Om een beeld te krijgen van de bezinksnelheid van een korrel was het nodig de gemiddelde dichtheid van de korrel vast te kunnen stellen. Hiertoe is van elke type bacteriesoort, EPS of precipitaat eerst de afzonderlijke dichtheid bepaald om deze vervolgens te kunnen uitmiddelen op basis van de FISH/CT gebaseerde topografie van de korrel. Met onze resultaten hebben wij laten zien dat PAOs een hogere dichtheid hebben dan GAOs wat tot een hogere bezinksnelheid leidde voor een PAO gedomineerd korrel, wat eveneens eerdere observaties van segregatie van biomassa in aeroob korrel slib verklaard. Verder heeft het model laten zien dat een kleine verhoging van slechts 1-5% van anorganische volumefractie de bezinksnelheid van een korrel 9 keer verhoogd. Voor een Nitritatie-Anammox korrel heeft het model laten zien dat voor de selectie van snell bezinkende Anammox gedomineerde korrels de korrel diameter de doorslaggevend punt is (hoe groter hoe beter).

In **hoofdstuk 6** hebben wij het effect van verschillen in dichtheid en viscositeit van water, die worden veroorzaakt door verschillen in temperatuur en ionsterkte, op de bezinksnelheid van korrelslib onderzocht. De corresponderende langzame bezinksnelheid van een korrel in water met verhoogde viscositeit en dichtheden zoals veroorzaakt door lagere temperaturen zouden een belangrijke reden kunnen zijn voor de gerapporteerde start-up problemen van aëroob korrelslib bij lagere temperaturen. Bezinksnelheden worden ook lager door verhoogde zoutconcentraties, die een rol kunnen spelen in winterperiodes waar zoutconcentraties in het afvalwater tijdelijk verhoogd kunnen zijn door strooizout. De tijdafhankelijke verhoging in massa en dus bezinksnelheid veroorzaakt door zoutdiffusie in de korrel was afhankelijk van de korreldiameter. Conductiviteits- en temperatuurmetingen zouden dus gebruikt kunnen worden om operationele factoren te stabiliseren en om de retentietijd van biomassa te verhogen. **Hoofdstuk 7** geeft een evaluatie over toekomstige toepassingen van slibsegregatie in afvalwater.

LIST OF PICTURES

Microbial diversity in aerobic granular sludge



Mari-Karoliina Henriikka Winkler

Microbial diversity in aerobic granular sludge

Mari-Karoliina Henriikka Winkler

Magic granules

Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. ir. K.C.A.M. Luyben,
voorzitter van het College voor Promoties
in het openbaar te verdedigen
op woensdag 5 september 2012 om 10:00 uur

door

Mari-Karoliina Henriikka WINKLER

Master in Chemistry, Universität Duisburg-Essen, Duitsland
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Prof. dr. D. Brdjanovic	UNESCO-IHE, *reservelid*

Magic granules

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Chapter 1

GENERAL INTRODUCTION

1

INTRODUCTION

STRUCTURAL DIFFERENCES OF FLOCS AND GRANULES

Aerobic granular sludge is a new wastewater treatment strategy in which the same functional groups of bacteria as existing in a conventional sludge floc are present. From an ecological point of view these two systems are interesting to compare because one can ask the question how structural differences of an ecosystem can influence community assemblage. Flocs and granules are clearly different in structure and shape (Figure 1) but in both systems the microbial communities have comparable functionalities. During nitrification ammonium is oxidized via nitrite to nitrate under aerobic conditions. Nitrate is in turn reduced to nitrogen gas with an organic electron donor. Phosphate removal is carried out by bacteria which are subjected to altering anaerobic-aerobic conditions (Smolders et al. 1994). In a conventional treatment plant the sludge floc is subjected to aerobic or anaerobic environments and different substrate availability by recycling it over different reactor compartments. In comparison to the distribution of bacteria in granules the bacteria in a floc are homogenously distributed and are equally subjected to shear stress and substrate concentrations. In granular sludge, all conversions are occurring in different layers within the granular biomass located in one reactor compartment with alternating anaerobic and aerobic periods in a sequencing batch mode.

A granule – as opposed to a flock – has a compact structure in which nitrifiers will be located in the oxygen penetrated outer layers. Denitrifiers and PAOs are located in the inner anoxic layers (De Kreuk et al., 2005) (Figure 1). Granules are also used to enrich for anaerobic ammonium oxidizing bacteria (Anammox). To establish good autotrophic nitrogen removal in aerobic granules, Anammox and AOB need to be enriched in the reactor system (Third et al., 2001; Sliekers et al., 2003). This is possible in oxygen-limited combined process for completely autotrophic nitrogen removal over nitrite (CANON). In this process the AOB grow on the outer oxygen penetrated rim of the granules hence supplying Anammox with nitrite and shielding the strict anoxic Anammox bacteria from oxygen exposure (Hao et al., 2005, Vlaeminck et al., 2010; Volcke et al., 2010; Winkler et al., 2011a).

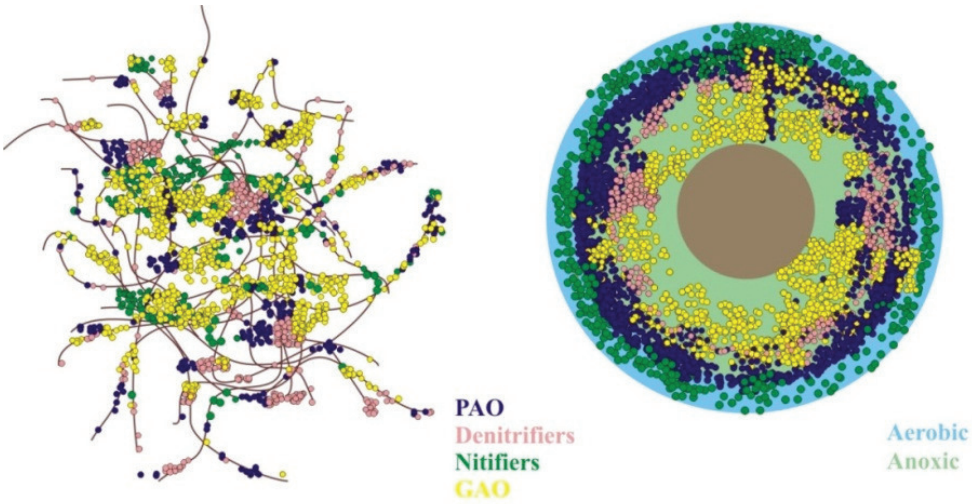

Figure 1 structural difference of a sludge flock (left) and a granule (right)

MICROBIAL COMMUNITIES

Communities can be defined as multi-species assemblages, in which individuals interact and influence each other. In granules and flocs microbial communities are located, which are in both cases responsible for the removal of nitrogen, phosphate and organic substances from wastewater. To analyze a community it is of interest to investigate how they are structured, and how their functional interactions influence environmental conditions and vice versa (Clements, 1916; Baas-Becking, 1934). In environmental biotechnology engineers are aiming to design a system (environment) that selects for a certain type of bacterium carrying out the desired engineering purpose or function (Temudo et al., 2008; Jiang et al., 2011). By this concept one assumes that species with a specific metabolic capacity get enriched within a reactor system because they out-compete others without this capacity. This behavior is also known as exclusion principle, which states that two species competing for the same resources cannot coexist if ecological factors are constant. In such a case the species with the slightest advantage will dominate in the long term (Gause, 1932; Hardin, 1960).

FUNCTIONALITY DRIVEN ENRICHMENT STRATEGIES

In order to make use of a bacterial function (e.g. nitrogen removal) within wastewater treatment bacteria need to be enriched in large quantities. Especially granular sludge systems are supreme for this, since they offer a maximal microorganisms to space ratio yielding in multiple times higher biomass concentration and hence in a higher volumetric removal capacity as compared to flocculent systems. Enrichment cultures were already used to

study bacteria by Sergei Winogradsky and Martinus Beijerinck (Winogradsky, 1890; Beijerinck, 1901). Beijerinck, who was a Dutch chemical engineer and microbiologist concluded that *everything is everywhere*, by which he build the first part of Baas Beckings theory, who completed this theorem to *everything is everywhere* but *the environment selects* (Baas-Becking, 1934; De Wit and Bouvier, 2006). Bioreactors can be considered as engineered ecosystems in which conditions are created selecting for a specialist rather than generalists and hence for the bacterium being the fittest / most adapted under the given circumstances. The theory of the survival of the fittest, which Darwin and Spencer developed in the early 19th century, are hence also driving the processes of innovation and adaptation in modern biotechnology (Darwin, 1859; Spencer, 1864). The principle that selection forces a population in an environment for adaption is also valid within bioreactor systems. In the following two examples of how reactor systems can be used to enrich bacteria are described. Since in aerobic granular sludge technology the inclusion of biological phosphate removal as well as the autotrophic nitrogen removal are of great importance, Anammox and *Accumulibacter* were used as example organisms to describe how these bacteria were enriched by imposing a selection pressure in bioreactor system.

ACCUMULIBACTER –BIOLOGICAL PHOSPHATE REMOVAL

Since Phosphate is often a limiting element in nature the storage of phosphate in the form of polyphosphate is a common phenomenon in Archaea, Eubacteria and also Eukaryotes, since it is needed for biomass production (Langen, 1958; Rao and Kornberg, 1999; Cardona et al., 2002). However, the energy generation in form of ATP from polyphosphate appears to be unique to only a few bacteria. This mechanism is today thought to be the basis of biological phosphorus removal and it has been reported for the first time in 1959 when Srinath and coworkers observed that aerated sludge of waste water treatment plants contained higher phosphate concentrations than un-aerated sludge of the same plant (Srinath et al., 1959). Since biological phosphate removal is of engineering and microbiological interest researchers tried to enriched and study the PAOs in order to identify them and to biologically remove phosphate in wastewater treatment processes (Smolders et al., 1994; Seviour et al., 2003). Microbiologist tried to isolate this bacterium, by growing it on agar plates, and identified it as belonging to the genus *Acinetobacter* (Fuhs and Chen, 1975). Then engineers started to mathematically model the metabolism of this phosphate accumulating

organism (PAO) and the models were fed with data from simple measurements and concluded that for an active Phosphate uptake/release mechanism the capability of an anaerobic acetate uptake as well as PHB synthesis is required (Mulder and Rensink, 1987; Wentzel et al., 1989; Smolders et al., 1994; Henze et al., 1999). However, it was shown that all of the *Acinetobacter* isolates could neither assimilate acetate nor synthesize PHA anaerobically (Tandoi et al., 1998).

Only after molecular tools could eventually show that *Acinetobacter* only composed a very minor proportion within a phosphate removing population the discussions, which lasted over almost 3 decades, to show that *Acinetobacter* was of importance for phosphate removal, stopped (Wagner et al., 1994). Today it is know that PAOs cannot be enriched on agar plates and are instead grown in enrichment cultures by applying alternating anaerobic feeding and aerobic mixing to suit the needs of the PAOs. The so enriched and in the meantime identified *Accumulibacter spp*. indeed fulfills all criteria of the engineering models and is up-to-date used to validate and optimize these models (Lopez-Vazquez et al., 2009). Accumulibacter spp. was finally found in natural habitats (freshwater sediments) but it was present in much lower densities as usually found in specialized, discrete habitats such as bioreactors or wastewater treatment plants (Peterson et al., 2008). The success in finally enriching PAOs, was that conditions were created, which specifically selected for the bacteria capable of anaerobic substrate uptake in form of energy-rich PHB granules, by which they could outcompete other bacteria for their substrate.

ANAEROBIC AMMONIUM OXIDYXING BACTERIA (ANAMMOX)

A similar example is the anaerobic ammonium oxidizing (Anammox) bacterium, which was discovered by a gap in the Nitrogen balance in an anoxically operated wastewater treatment plant (Mulder, 1992). Because of microbiological as well as engineering interests in the function to autotrophically (no organic electron donor) remove ammonium under anoxic conditions it was enriched in well-defined bioreactor studies (Van de Graaf, 1996; Strous et al., 1999) and is up-to-date used to treat ammonium rich wastewater in a more cost efficient manner (Kartal et al., 2010). Anammox was discovered to be performed by novel organisms related to Planctomycetales and it revolutionized the view on the worldwide Nitrogen cycle (Kuenen, 2008, Francis et al., 2007). In the anaerobic ammonium oxidation (anammox) reaction, ammonia is oxidized with nitrite as electron

acceptor in strictly anoxic environments. Today Anammox is detected in many marine and freshwater ecosystems and is estimated to contribute up to 50% of oceanic nitrogen loss (Kuypers et al., 2003). Engineers and microbiologist hence select for bacterial functions, and therefore they create conditions which are most ideal for the bacterium carrying out this desired function and hence out-competing their competitors and by this enriching it in large quantities.

ENGINEERS IN MICROBIOLOGY

Engineers typically narrow down microbes to their functionality and simplify their behavior by describing their specific rates and yields and express their thermodynamic and kinetic properties (Kleerebezem and Van Loosdrecht, 2010). This enables to build processes around these bacteria offering supreme conditions for them to achieve the highest removal or production rates. Most ideally environmental engineers created conditions, which enrich for one specific bacterium or a specific bacterial group carrying the desired function. However, a simplification of microbial communities is only valid if the major community acts according to the made assumptions. This is especially difficult when the environment is very complex, which is typically the case, if well-defined lab reactor systems are scaled-up from pilot to eventually full-scale reactor systems. Temporal fluctuations of the influent (nutrient availability, temperature, pH) as encountered in e.g. full-scale wastewater treatment plants can alter population growth rates, potentially resulting in shifts in population dynamics driven by for instance species competition or stress adaptation (Müller, 2000; Carrero-Colon et al., 2006). In order to understand this complexity microbial ecologists and engineers try to define microbial populations in mathematical terms and couple them with engineering functions to understand explain and eventually improve the predictability of a system behavior, examples are given in the following.

CORRELATING COMMUNITY ASSEMBLY WITH SYSTEM FUNCTIONING

Microbial ecologists are trying to manipulate reactor conditions with the intention of influencing community assembly (species level), or shifts within bacteria on a proteomic or genomic level. Although it is currently not possible to confidently predict community assembly as it appears to be a chaotic event (Curtis et al., 2003), intuition gathered from years of operating and studying mixed communities gives us a sense of what factors are

important. For example, we know that process loading rates (F/M), solids retention time (SRT) and shifts in redox conditions can influence the development of a functionally stable microbial community (Lee et al., 2003; Saikaly et al., 2005; Li et al., 2008). Community shifts can be recorded by the usage of for instance denaturing gradient gel electrophoresis (DGGE) or Terminal restriction fragment polymorphism (TFRLP). These techniques will give a fingerprint from the microbial population. With these techniques it is possible to explore community-level responses to environmental changes. The emergence of molecular methods like pyrosequencing and quantitative polymerase chain reaction (qPCR) gives us an unprecedented ability to characterize species level shifts in stable and unstable communities (Werner et al., 2011). This data has allowed us to examine how community resistance and resilience is impacted by microbial diversity (Briones and Raskin, 2003). Studies have already attempted to quantify community shifts over time and partly correlated these changes to system performance (Ayala-Del-Río et al., 2004; Pholchan et al., 2010; Cai-Yun et al., 2011; van Nostrand et al., 2011), and there are also studies showing a temporal pace of microbial and operational changes within full-scale systems (Wells et al., 2011; Winkler et al., 2012a).

Despite the direction of correlating system functioning to microbial community shifts there are only few attempts which enable a translation of these findings towards defining guidelines for reactor design and operation. The attempt to use molecular tools to effectively calculate key engineering parameters potentially yield in a more stable reactor performance due to better control strategies. In the research of Winkler et al 2012a such a combination was achieved. In the research, a dimensionless wash-out ratio from reactor and effluent sludge was determined for one specific bacterium by the means of qPCR. This ratio was then coupled with the total SRT derived from the suspended solids [days] to calculate the specific retention time of one functional group [days] (Winkler et al., 2012b). Recent opinions argue that the full potential of the on-going revolution of molecular techniques to improve our understanding of community assembly, will not be realized if research is not directed and driven by theory (Prosser et al., 2007). Therefore, interdisciplinary research is needed to optimize and understand microbial communities better.

HOW DIVERSITY IS AFFECTING FUNCTIONALITY

To get better insights into species-species interaction it is of importance to understand the structure of the community as well as the relationship between diversity and system functioning. This ecological aspect is of engineering interest because the functional stability (e.g. N- P- COD-removal efficiency) in biological system such as in wastewater treatment plants has been proposed to be correlated to species richness and evenness (Naeem and Li, 1997; Stirling and Wilsey, 2001; Wittebolle et al., 2009). Species diversity is reported to have functional consequences because the number and kinds of species determine ecosystem processes (Tilman et al., 1997; Bell et al., 2005). Diversity is generally assumed to be positively correlated to functional stability because independent species can degrade similar compounds or create a competition network of many species (McCann, 2000; Cardinale et al., 2002; Rowan et al., 2003).

There are highly stable systems known to consists of only a few species (Temudo et al., 2008; Jiang et al., 2011) hence speaking against the necessity of species diversity for functional stability. However, diversity might be positive in unstable systems experiencing a disturbance (e.g. pH shock), after which less abundant functional groups within a diverse ecosystem can act as a buffer, herewith replacing a negatively affected functional group thus conserving functional stability (Yachi and Loreau, 1999). In the bacterial kingdom millions of bacterial species are contributing to diversity and, we are only beginning to investigate patterns in their diversity (Beijerinck, 1901; Ward et al., 1998; Tunlid, 1999; Curtis and Sloan, 2005). The underestimation of diversity is partially due to DNA complexity and a detection bias of molecular tools and cultivation methods (Torsvik et al., 1990; Hong et al., 2009). Laboratory reactor systems can allow simplification of processes appearing in nature in order to understand them better and - in the longer run - predict their behaviour appearance and define their ecological importance within nature. An appreciation for the tight correlation between species and their environment is hence crucial for the delineation of microbial communities (O'Donnell et al., 2007).

DIVERSITY STUDIES IN GRANULAR SLUDGE SYSTEMS

It is an interesting question whether aerobic granular sludge or activated sludge has a higher diversity and if this offers a more stable system performance. Ecological niches are important for the maintenance of species diversity (Levine and HilleRisLambers, 2009). Granular sludge provides

many ecological niches due to substrate gradients, whereas a sludge floc is exposed to different reactor units of different environmental conditions (Nicolella et al., 2000; Picioreanu, 2000; Xavier et al., 2007). In granular sludge there is also segregation of biomass over the sludge bed column, in which the microbial population in the bottom of the reactor will be different from those present in the top (Winkler et al., 2011b; Winkler et al., 2011c). Granules can harbor slow growing organisms due to higher biomass retention time and an applied feast famine regime (de Kreuk and van Loosdrecht, 2004). There has been research studying the diversity within different aerobic granular sludge reactors (Li et al., 2008; Ebrahimi et al., 2010) and also studies correlating community shifts to environmental disturbance, because it is believed that the community within granules are more resistance to shock events and that granules offer the potential to select for bacteria capable of degrading toxic (e.g. phenol) compounds or none toxic compounds under difficult conditions (e.g higher salt concentrations) (Jiang et al., 2004; Bassin et al., 2011).

Moreover, a study on a flocculent system and granular system receiving the same wastewater has shown that the community composition of both sludge types was very dissimilar. Despite this difference, general bacterial population of both systems had on average a comparable species richness, entropy, and evenness, suggesting that different bacteria were sharing the same functionality, resulting in a stable system performance in both systems (Winkler et al., 2012a). In another study comparing the community structure between different granular systems it got clear that the structural differences of sludge composition (floc versus granules) as well as operational differences (reactor configuration) lead to differences in microbial community composition. It was hypothesized that the growth of *Nitrobacter* within aerobic granular sludge, in particular, was partly uncoupled from the lithotrophic nitrite supply from AOB. Fluorescent in situ hybridization (FISH) and quantitative-PCR (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. This was supported by activity measurements which showed an approximately 3 fold higher nitrite oxidizing capacity compared to ammonium oxidizing capacity (Winkler et al., 2012c). These studies show that the different environmental conditions (flocs and granules) select for different microbial populations. Therefore, granular systems are of ecological and engineering interest.

MOTIVATION OF THE THESIS

New technologies such as aerobic granular sludge need first to demonstrate that they can perform equally well or even better than the old well approved technology (activated sludge technologies). This is not only valid for the achievement of engineering purposes such as good removal rates but also for economical purposes of being less space and energy consuming and by this the economically more profitable technology as it is the case for granular sludge (van der Roest et al., 2011). However, there is also an ongoing discussion about the microbial community composition and if there are differences in the population harbored in granules and flocculent sludge and if this differences has functional consequences for the system performance of both reactors. It is of interest to see if the structural differences of granules and flocs have consequences for the sludge retention time for the different functional groups (e.g. NOB, AOB, PAOs, GAOs) present within both systems. Furthermore it is of interest to investigate if bacteria or the relationship between bacteria is influenced differently in flocs and granules. Therefore, several questions arose to study the microbial community between these two systems in more detail:

I) Is the microbial community structure of aerobic granules and flocs different and what does it mean for functional stability?
II) Is the sludge retention time of specific bacterial groups different for granules and flocs?
III) Will the nitrifying bacteria in aerobic granular sludge be different from those present in flocculent systems?

RESEARCH OBJECTIVES

In this part of the thesis the structural differences of flocs and granules were investigated with classical molecular tools. The goal was to describe their differences in microbial population and to see if changes in their structure can be correlated with operational changes (**Chapter 2**). Further the nitrifying population was takes as exemplary microbial group to see if differences in community structure can be discovered between granular sludge and flocculent sludge systems (**Chapter 3**). Furthermore, it was of interest to investigate if the sludge retention time of specific bacterial groups differs between both systems (granules and flocs) and therefore a new methodology needed to be defined to combine molecular tools with engineering parameters with the goal to understand and in turn predict system behavior better. The goal was to find a way to calculate the specific sludge retention time for bacterial groups and then use this technique to compare granules and flocs with each other (**Chapter 4**).

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Chapter 2

CORRELATING MICROBIAL DIVERSITY WITH PROCESS PERFORMANCE IN AEROBIC GRANULAR SLUDGE AND ACTIVATED SLUDGE

Submitted as:

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ABSTRACT

In this study we investigated during 400 days the microbial community variations as observed from 16S-rDNA gen DGGE banding patterns from aerobic granular sludge from a pilot plant in Epe (NL). As comparison we used the activated sludge community variations from the full scale plant at Epe. Both plants obtained the same wastewater and had the same relative hydraulic variations and run stable over time. For the total bacterial population a similarity analysis was conducted showing that the community composition of both sludge types was very dissimilar. Despite this difference, general bacterial population of both systems had on average comparable species richness, entropy, and evenness, suggesting that different bacteria were sharing the same functionality. Moreover, multi-dimensional scaling analysis revealed that the microbial populations of the flocculent sludge system moved closely around the initial population whereas the bacterial population in the aerobic granular sludge moved away from its initial population representing a permanent change. In addition the ammonium oxidizing community of both sludge systems were studied in detail showing more unevenness than the general bacterial community. A correlation analysis of process data and microbial data from aerobic granular sludge showed that the microbial diversity shift in ammonium oxidizing bacteria clearly correlated with fluctuations in temperature.

INTRODUCTION

The functional stability (e.g. nutrient removal efficiency) in biological wastewater treatment systems has been proposed to be correlated to species richness (number of bacteria) and evenness (distribution of bacteria in a population) (Stirling & Wilsey 2001, Naeem & Li 1997, Wittebolle *et al.* 2009). Species diversity is reported to have functional consequences because the number and kinds of species determine ecosystem processes (Bell *et al.* 2005, Tilman *et al.* 1997). Diversity is generally assumed to be positively correlated to functional stability because independent species can degrade similar compounds or create a competition network of many species (Cardinale *et al.* 2002, McCann 2000, Rowan *et al.* 2003) whereas mono- and highly enriched cultures are reported to negatively influence ecosystem functioning (Chapin *et al.* 2010, Wilsey & Potvin 2000). Even though most of the studies confirm the positive diversity-stability relationship, the opposite has been described as well (Jiang *et al.* 2011, Manefield *et al.* 2002, Temudo *et al.* 2008). Diversity is thought to be positive for system functioning because after a disturbance, less abundant functional groups within a diverse ecosystem can act as a buffer, herewith replacing a negatively affected group thus conserving functional stability (Yachi & Loreau 1999). Aerobic granular sludge is a new wastewater treatment strategy in which the same functional groups of bacteria as present in a conventional sludge floc (nitrifiers, denitrifiers, Phosphate Accumulating Organisms (PAOs)) are present. Flocs and granules are clearly different in structure, shape and substrate availability (Figure 1).

It can therefore be questioned whether aerobic granular sludge or activated sludge has a higher diversity and if a different diversity offers a more stable system performance. Ecological niches are important for the maintenance of species diversity (Levine & HilleRisLambers 2009). Granular sludge provides many ecological niches due to substrate gradients, shear stress phenomena (Nicolella *et al.* 2000, Xavier *et al.* 2007) as well as protozoa grazing on the outer layers of the granules (Huws *et al.* 2005, Winkler *et al.* 2012). In addition there is also segregation of biomass over the sludge bed column. The microbial population in the bottom of the reactor will be different from those present in the top (Winkler *et al.* 2011b, Winkler *et al.* 2011a). Also granules can harbor slow growing organisms due to higher biomass retention time for the biomass in the deeper parts of the granule (de Kreuk & van Loosdrecht 2004). One might argue that granular sludge selection leads to a lower biodiversity since only organisms capable of

forming granular sludge might be enriched, which might decrease diversity based evenness. On the other hand the substrate gradients in granular sludge might lead to more ecological niches and in turn a higher biodiversity. Clearly there are pros and cons why aerobic granular sludge should hold a more or less diverse microbial population and hence offer more/less freedom for functional stability than it is the case for an activated sludge floc. Studies have shown that the community composition within a reactor changes despite a functional stability of the reactor (Carrero-Colon *et al.* 2006, Fernandez *et al.* 1999, Lee *et al.* 2002).

The diversity within one and among different aerobic granular sludge reactors has been described earlier (Ebrahimi *et al.* 2010, Li *et al.* 2008), but a comparison of the bacterial population of an aerobic granular and a suspended sludge system has not been described. From an engineering point of view it is appealing to correlate microbial shifts to system performance. Studies have already attempted to quantify community shifts over time and partly correlated these changes to system performance (Ayala-Del-Río *et al.* 2004, Cai-Yun *et al.* 2011, Pholchan *et al.* 2010, van Nostrand *et al.* 2011), however studies showing a temporal pace of microbial and operational changes within full-scale systems are sparse (Wells *et al.* 2011). In this study, two industrial reactor system (granules and flocs), were investigated for their microbial community composition. The reactor operation of both systems was clearly different but both systems were run on the same municipal wastewater and experienced the same seasonal changes. We then made a direct comparison of population dynamics within both treatment systems and correlated these changes to changes in operational conditions (N- P- COD-removal as well as temperature) to investigate potentially interesting conclusions from an ecological and engineering point of view.

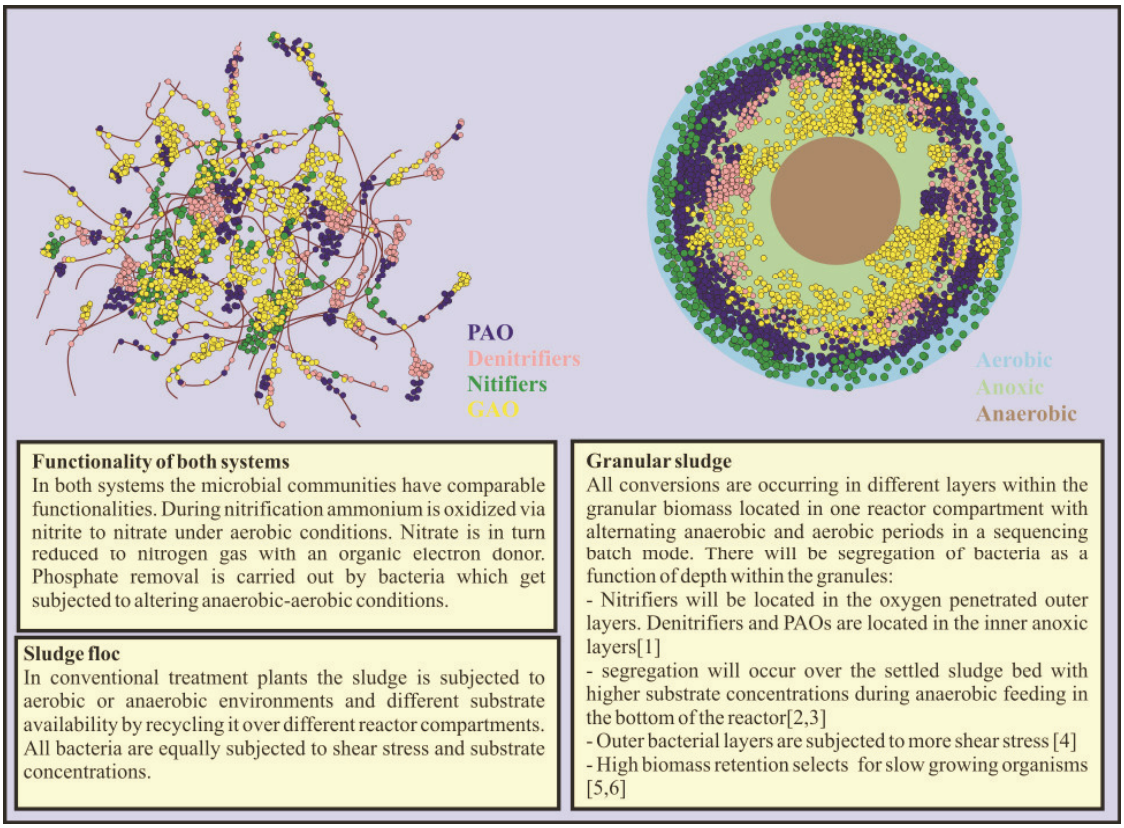

Figure 1 Structural difference and resulting functional difference in aerobic granular sludge and a conventional sludge floc [1]*(Xavier et al. 2007)* , [2,3]*(Winkler et al. 2011b, Winkler et al. 2011a),* [4]*(Picioreanu 2000),* [5]*(de Kreuk & van Loosdrecht 2004),* [6]*(de Bruin 2004).*

MATERIALS AND METHODS

SAMPLE COLLECTION OPERATIONAL DATA

Samples from an aerobic granular sludge pilot plant in Epe and from a full scale activated sludge plant were collected over a period of 400 days (November 2008 to January 2010). The granular reactor ran as a sequencing batch reactor similar to our lab reactors (Winkler et al. 2011a), whereas the flocculent system ran in a continuous mode. Despite the difference in operational conditions both installations were fed with the same wastewater, experienced the same temperature fluctuations and were operated on the same relative hydraulic flow variations. The aerobic granular sludge pilot plant was operated by the company DHV, situated in the Netherlands. At the moment sampling was started the granular system already ran stable for a period of 2 years. The flocculent sludge treatment plant was operated by the municipal wastewater treatment authorities of Epe, The Netherlands. Throughout the sampling period influent parameters of COD, phosphate,

ammonium and temperature from both reactors and effluent values for the granular sludge properties were collected on a daily basis.

DNA EXTRACTION

Total genomic DNA from the reactor sludge of both systems was extracted using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA USA). The manufacturer's protocol was applied, based on the principle of combined mechanical/chemical cell lysis (chemical enhanced bead beating). The DNA extraction was tested by electrophoresis on a 1.5 % Agarose Gel, at 90 V for 32 minutes with Syngene Gel Doc G:BOX (Syngene, Cambridge CB4 1TF, UK) and the software Gene Snap (Syngene). The DNA extract was checked on quantity and quality by gel-electrophoresis.

PCR PROCEDURE - GENERAL PRIMERS

A set of universal primers, Bac341f and Bac907rM (Muyzer & Smalla 1998), were used targeting the 16S rRNA gene fragment including the hypervariable regions V3 to V5 (Neefs et al. 1990). The PCR mixture contained 0.25 µl (50 µM) of each of the primers, 12.5 µl Qiagen PCR Taq master mix (1.25 units Taq Polymerase, dNTPs, buffer), 11.5 µl PCR water (Qiagen) and 0.5 µl DNA-template, with a content of 1.5 mM $MgCl_2$. The whole reaction mix and one positive control (*E.coli*), as well as a negative control, were loaded in a Biometra T-Gradient Thermocycler (Biometra, Goettingen, Germany). The following program was used for PCR: 5 min denaturing at 95 °C followed by 32 equal cycles of 30 sec, denaturing at 95 °C, 40 sec at 57 °C for annealing and 40 sec at 72 °C for extension. The quality and quantity of amplicons of all the dilutions were tested on a 2% agarose gel.

FUNCTIONAL PRIMERS

For the functional population analysis ammonia monooxygenase specific primer set were optimized with amoA-1F_deg6 (5'-GGGGHTTYTACTGGTGGT-3')+GC (Rotthauwe *et al.* 1997) and amoAr-i (5'-CCCCTCIGIAAAICCTTCTTC-3') (Hornek *et al.* 2006, Stephen *et al.* 1996). The PCR mixture contained 0.25 µl (50 µM) of forward and reverse primer, 12.5 µl of Qiagen PCR Taq master mix (Taq Polymerase, dNTPs, buffer), 0.25 µl (5 units/µl) extra Taq polymerase, 0.5 µl $MgCl_2$ (50 mM), 10.75 µl PCR-H_2O (Qiagen) and 0.5 µl extracted DNA template (undiluted). The resulting reaction mix of 25 µl contained 2.5 units Taq polymerase, 0.5

µM of each primer and 2.5 mM $MgCl_2$. The following, program was used for the PCR: 5 min denaturing at 95 °C followed by 41 equal cycles of 30 sec denaturing at 95 °C, 40 sec at 50 °C for annealing and 40 sec at 72 °C for extension. An extension step of 30 min at 72 °C followed and the procedure concluded with a final resident temperature of 12 °C. The reaction mix and one positive control (a mixture of *Nitrosomonas europaea* 43 µg/ml, *Nitrosomonas oligotropha* 41.9 µg/ml, *Nitrosospira multiformis* 42 µg/ml, 1000 or 20000 times diluted), as well as a negative control, were loaded in a Biometra T-Gradient Thermocycler (Biometra, Goettingen, Germany).

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

The gel was prepared in order to obtain 6% acrylamide-bis-acrylamide mix content (W/V) in 1x TAE buffer and a gradient of 20-70 % of denaturant. The electrophoresis was run for 16 h in a 1x TAE buffer, 100 V and 60 °C. The running conditions were set to 5 h and 100 V. The best fitting dilutions of PCR-products were chosen by the densitometry estimation in order to load 200-300 ng DNA per slot. 2.4 µl of loading dye (10x concentrated "Blue juice", Invitrogen) was added as well as PCR-water to fill up to a volume of 24µl/lane. The lanes on the edges of the gel were filled with 25 µl anti-smiling liquid (anti-smiling solution, Ingeny, Netherlands). After electrophoresis the gel was stained with SYBR-gold solution in 1x TAE and visualized in the Syngene Gene Doc by a digital camera. The image processing was accomplished to convert the data stored as a digital camera picture of the DGGE-Gel into numerical values in order to use them for statistical analysis. In this research the main steps of image processing for the alignment of bands were done using the software GelCompare II ® (Version 5.0) from the company "Applied Maths NV". Heatmaps of DGGE gels were created with Visual basics and are based on band intensities derived from GelCompare II ®.

SEQUENCING OF AMOA BANDS

Bands were cut from the ammonia monooxygenase (amoA)-denaturing Acrylamide gels using flame sterilized blade. The cut material was soaked in 40 µl 1xTris buffer (pH 8) for two days at 4°C. For re-amplification the amoA-1F_deg6 primer was used without GC clamp. The PCR procedure was kept as previously described, however reducing the cycle number to 25 and using 1.25 units Polymerase per vial and 0.5 µl of eluted DNA. The the resulting PCR products were purified and sequenced (Macrogen Inc. South Korea). The obtained amoA genes sequences were imported into the ARB

software (http://www.arb-home.de) version 5.1 and aligned by using the integrated aligner. The alignment was further verified and corrected manually. A phylogenetic tree was generated using a maximum likelihood algorithm (RAxML).

STATISTICAL ANALYSIS

ENTROPY AND PARETO-LORENZ

For statistical analysis two evenness measurements were used. Firstly the evenness according to Pielou was calculated which is based on the entropy measurements of the Shannon-Weaver index $H_1 = -\sum_{i=1}^{q} p_i \log_2(p_i)$, where p_i is the probability of having species i present (Pielou 1981). The measurement of order one (H_1) is the logarithm of intensities (abundance). A closely related evenness measure is the Partic index $H_0 = \log_2 q$ (Legendre & Legendre 1979), where q is the number of bands. The base of the logarithms was chosen to be 2 for both entropy based measures. The measurement of zero order (H_0) is the logarithm of the number of bands (richness) in each DGGE lane. The Pielou evenness is defined as $J=H_1/H_0$. The second measure of evenness is derived from the Pareto-Lorenz curve (Naeem 2009, Wittebolle et al. 2009). The reason to choose these two classes of measures is that they have been often used in ecological literature (Buzas & Hayek 2005).

TIME CONSTRAINED ANALYSIS

In our study we have chosen to use asymmetrical indices only; i.e. only the bands in the DGGE gel are compared. Symmetrical indices were not considered, because in these indices the absence of a band (double zero) in two different patterns is also considered as a factor, increasing the similarity between these two patterns. Resemblance analysis for 16S primer sets were checked with chord and Ruzicka dissimilarity index (Legendre & Legendre 1979). Only the Ruzicka index was used for data representation. All banding patters were converted into a binary (presence / absence) and quantitative (band intensities) matrix to compare if species abundance or presence absence changes the community more.

TREE CLUSTERING AND MDS OF BANDING PATTERNS

For tree clustering the average dissimilarity (Ruzicka) and chord distance were calculated (Legendre & Legendre 1979). Multi-dimensional scaling was performed based on the chord distance. All statistical analyses were performed using the Vegan package in the software program R! (http://www.r-project.org/).

CORRELATION OF FUNCTIONAL AND PROCESS DATA

A correlation coefficient R was calculated to determine the relationship between functional data y (COD, total N, and temperature) and microbial data x (similarity matrix from DGGE gels of amoA and bacterial primers) for the aerobic granular sludge system over a time period of 400 days. All similarity indices (chord, Ruzicka) were tested for quantitative and binary data sets. The correlation was calculated by $r(x,y) = \frac{\sum (x-\overline{x})(y-\overline{y})}{\sqrt{\sum (x-\overline{x})^2 (y-\overline{y})^2}}$.

Operational data were averaged according to the time elapsed between samples taken for DGGE analysis. To test for statistical significance for each data sets the P-value was calculated (Table 1). At a P-value of ≤ 0.05 a statistically significant correlation exists whereas a value of ≤ 0.1 is reviewed to be of marginal significance and P-values of ≥ 0.1 no statistical significant is given (Lipson *et al.* 2006).

RESULTS

HEATMAPS AND SEQUENCING

DGGE gels of bacterial 16S-rDNA gene from aerobic granular sludge (AGS) and Activated sludge where aligned for cluster analysis and are presented in one heatmap; each band on one height is assumed to represent the same species (Figure 2). For the amoA gel a heatmaps was constructed for the aerobic granular sludge system and conventional sludge (Figure 3). Sequences from selected bands are represented in the amoA heatmap (Figure 3). Sequenced bands clustered in tree with *Nitrosomonas* sp. and *Nitrosospira* sp. related bacteria (Figure 3A, Figure 3B).

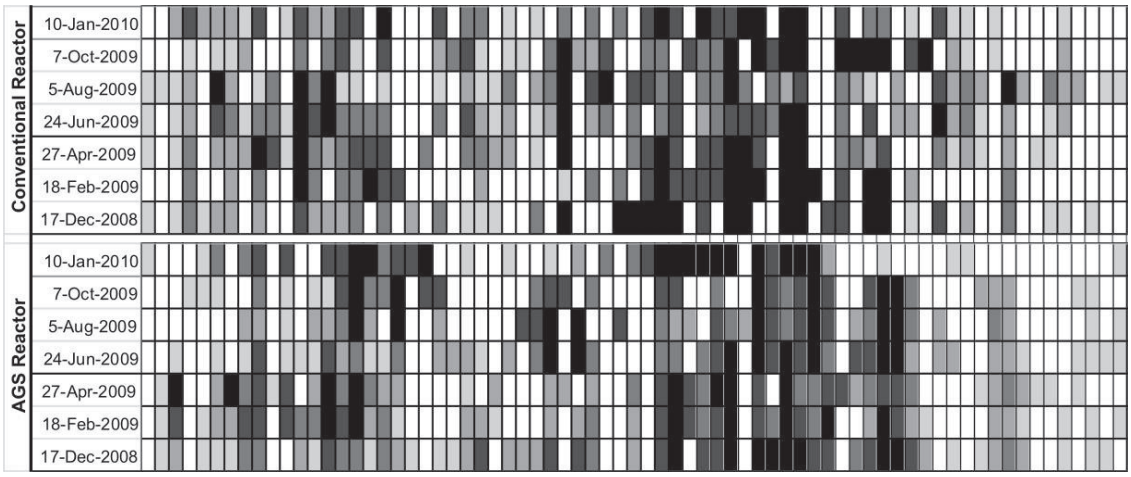

Figure 2 Heatmaps derived from constructed densiometric curves (GelCompare) of the bacterial 16S DNA DGGE gel of the aerobic granular sludge system and flocculent sludge treatment system from 7 samples taken over a period of 13 month. The grey-scale corresponds to bins of intervals of relative band intensity

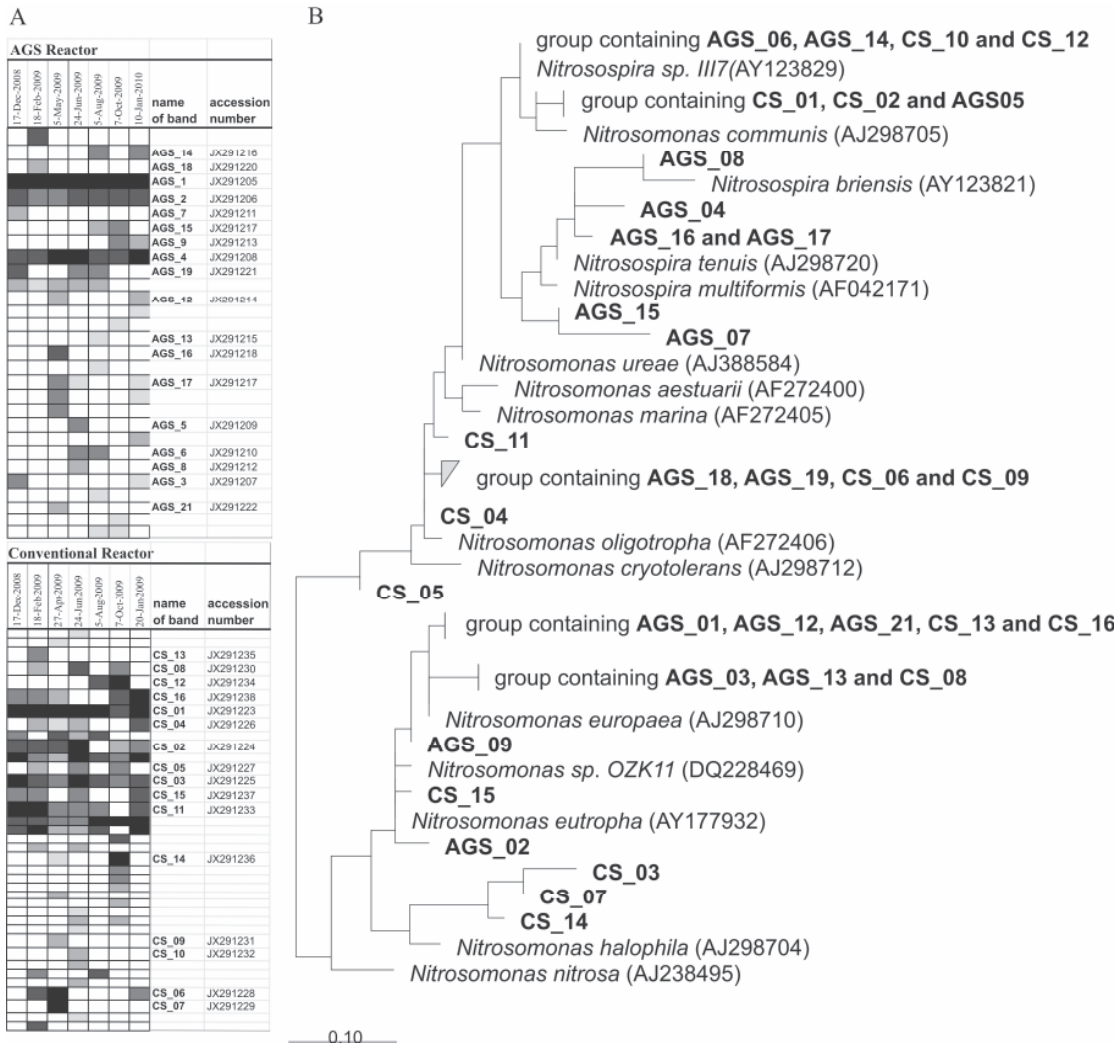

Figure 3A Heatmaps derived from constructed densiometric curves (GelCompare) of the amoA DNA DGGE gel of the aerobic granular and flocculent sludge system as well as sequencing accession numbers for selected bands from 7 samples taken over a period of 400 days. The grey-scale corresponds to bins of intervals of relative band intensity B) The tree was constructed using the full amoA gene length, the cultured ammonium oxidizing bacteria were used as references with the addition of unknown sequences (AGS_# for granules and CS_# for flocks) derived from DGGE gel (3A). For the calculation of the tree the maximum likely-hood algorithm (RAxML) was used. A total of 94 amino acid positions were used for analysis. The sequence of Nitrosococcus oceani ATCC 19707 (AF047705) was used as an out-group, but was pruned from the tree.

EVENNESS ESTIMATION

The evenness according to Pielou and the Pareto-Lorenz curve were calculated based on banding patterns of DGGE gels from both treatment system (Figure 4 and 5). Both methods measure evenness and indicate how heterogeneous the value of a measured variable in a sample series is. The evenness according to Pielou is a concentration measurement which will takes its maximum value (1) when all species are equally represented (Pielou 1981). The Pielou index of the microbial community is expressed in one value at a certain time point resulting from zero (or maximum) and first order entropy measurements (Figure 4).

Both systems show similar evenness for the bacterial primer sets. The average number of bands was 42±6 for the conventional system and 43±6 for aerobic granular sludge with the biggest difference of 15 bands (31 for conventional; 46 for aerobic granular sludge). For both systems the evenness according to Pareto Lorenz was also evaluated for amoA and bacterial primer sets. The average number of bands for the AOB community was 14±5 for flocculent sludge system and 9±2 for aerobic granular sludge. The Pareto-Lorenz curve belongs to the category of concentration statistics which shows the relation between the cumulative proportions of band intensities (abundance) and cumulative proportions of number of bands (richness) (Figure 5) (Naeem 2009, Wittebolle et al. 2009). The reference line in the graph corresponds to a perfect evenly distributed community composition. In this case all bands would have the same abundance / band intensity. Results reflect the same trend as the Pielou index (Figure 4) and show that the general bacterial community of both systems was more even than the AOB community, in particular in the aerobic granular sludge (Figure 5).

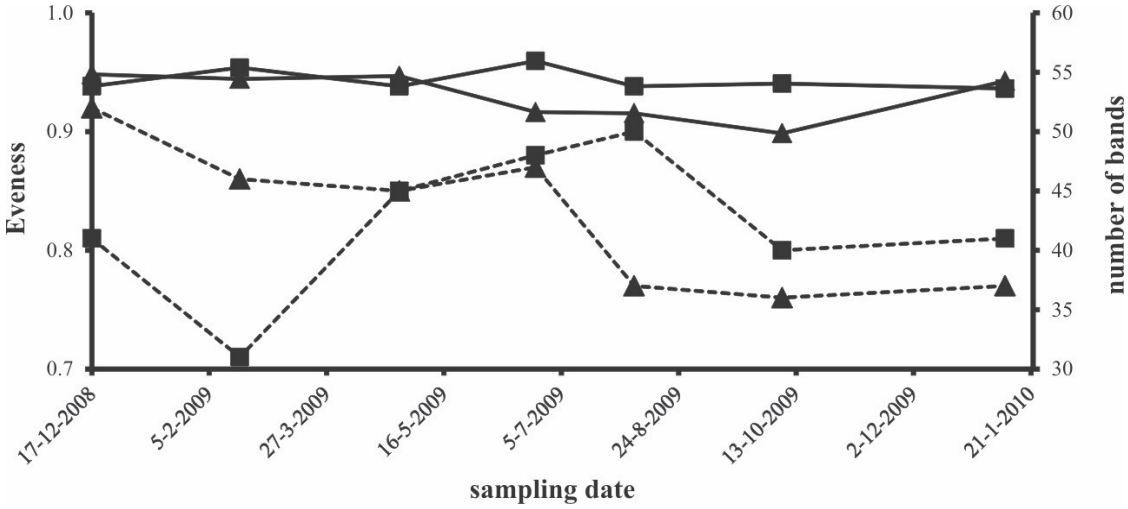

Figure 4 Profiles of evenness according to Pielou of general bacterial 16SDNA-DGGE gels (solid line), the number of bands on the gels (dashed line) both for flocculent sludge (■) and aerobic granular sludge (▲). Each sampling point represents banding patterns (species) of one lane (sampling day) on the DGGE gel.

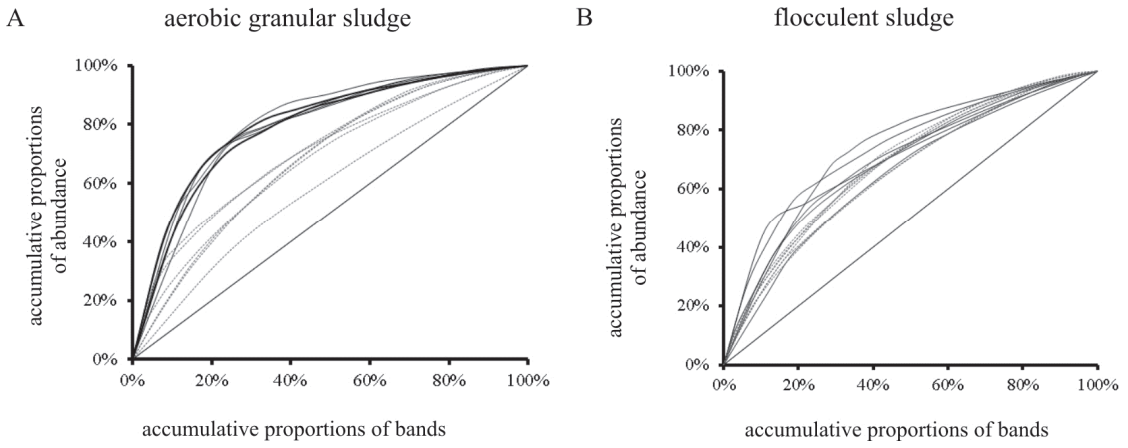

Figure 5 Relation between the cumulative proportions of band intensities (abundance) and cumulative proportions of number of bands (richness) of A) aerobic granular sludge and B) conventional sludge floc from general bacterial 16SDNA-DGGE gels (gray dashed lines) and amoA DGGE gels (black solid lines) each line is for one of the samples in figure 2, 3 and 4. The reference line in the graph corresponds to a theoretical perfect evenly distributed community composition.

TIME CONSTRAINED ANALYSIS

The Pielou index (Figure 4) and Pareto-Lorenz curve (Figure 5) give an indication on how uniform a population distribution is in a certain sample. However, the linkage between the sampling dates is lacking. For instance if from one month to the next, the whole population is replaced by other species while their abundance and richness remains comparable, the evenness measurement will not shift. In similarity measurements, which are based on a comparison of community data, the population of one sampling day is compared with the population on the following sampling dates and is hence showing a relation among them.

A population of 2 sampling dates, having everything in common, is identical and has a similarity of 1 whereas, a population which does not have anything in common has similarity of zero. Here we addressed this with the Ruzicka similarity index the question what is changing the community more either species richness (presence and absence of a band) or species abundance (band intensity). There was not a strong trend observable but we can report that in the flocculent sludge system the similarity based on species richness (present [1] absence [0]) changed in time more significantly than it was the case for the data taking species abundance into consideration whereas the reverse was true for the granular sludge system (Figure 6).

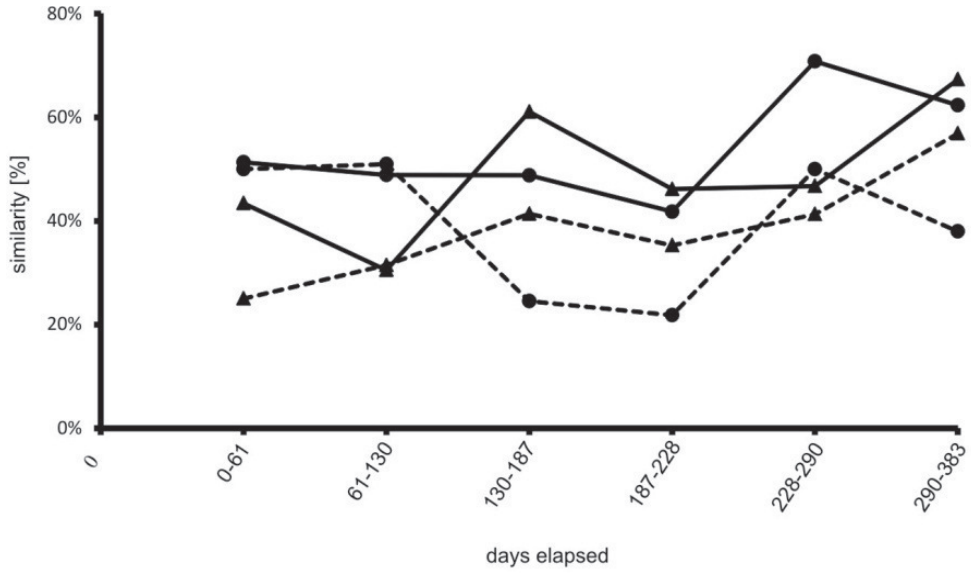

Figure 6 The times constraint similarity measurement based on the Ruzicka index for binary data (dashed line) and quantitative data (solid line) from flocculent sludge (●) and aerobic granular sludge (▲) 16S DNA DGGE band patterns.

CLUSTER ANALYSIS

Clustering analysis for the DGGE band patterns was conducted because it has the advantage of not only showing the relation within a community between one sampling point to the next but also compares the similarity among all samples and clusters them into groups (Figure 7). Here we present the results of one dissimilarity measurements (1-similarity) (Ruzicka) and one distance measurement (chord) on both systems. Measurements on populations which have no species in common can have distances that depend on the number and abundance of species. Therefore, distance measurements are not bounded to one or zero and in chord distance the highest possible distance will be √2 (=1.414). Results revealed that the flocculent and granular systems are very different. They share only a similarity of approximately 30% (1-0.7) and a distance of 1 (Figure 7).

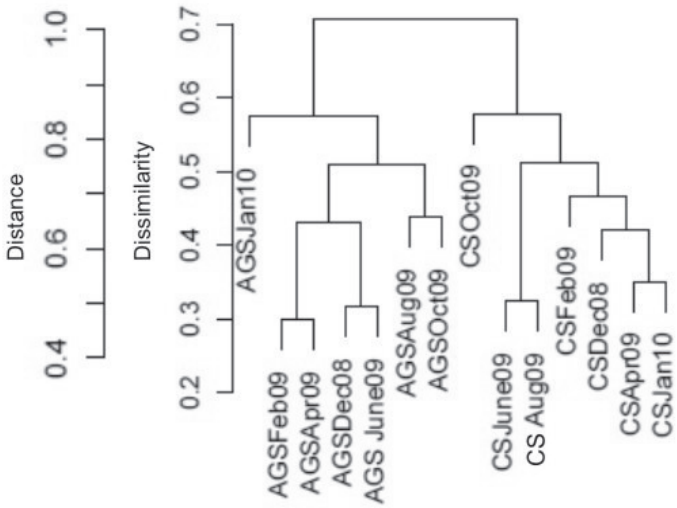

Figure 7 Tree cluster diagram showing the average Ruzicka dissimilarity (1-similarity) and chord distance between samples of flocculent sludge treatment plant (CS) and aerobic granular sludge treatment plant (ASG) on quantitative data set (16S DNA DGGE gel) at different time points.

MULTI-DIMENSIONAL SCALING ANALYSIS

In a tree diagram the population (bands) from different sampling dates are compared according to their similarity and are then clustered together in groups hence showing the relation among different sampling dates (Figure 7). However when clustering is applied some information is lost after certain groups (sampling points) are clusters to each other. This can be overcome with a multi-dimensional scaling analysis. In a multi-dimensional scaling

analysis all objects (species in a population) are ordered in a multivariate graph, with as many axes as there are descriptors (sampling dates). The data representation is only possible in a reduced dimension (2D, 2 axes) and shows the distance between all objects as well as all descriptors (Legendre & Legendre 1979). It therefore shows the direction in which a population shift and also shows the distance of the population shifts. An eigenvalue analysis was conducted and more than 50% of the information from the microbial data set was condensed in dimension 1 and 2. The same analysis with random numbers spread 50% of the information on the first 4 dimensions. The major variability is represented in these first two dimensions. The multi-dimensional scaling analysis based on Euclidean distance revealed that the microbial populations of the flocculent sludge system movement around the initial population whereas in the aerobic granular sludge the bacterial population moved away from its initial populations (Figure 8).

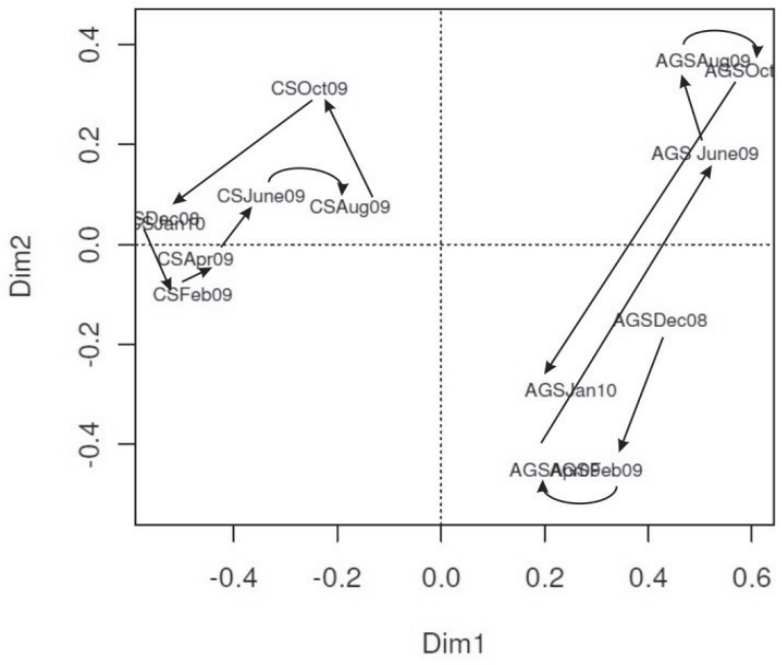

Figure 8 Multi-dimensional scaling analysis of the distance matrix based on the Euclidean distance matrix of aerobic granular sludge system (left) and flocculent sludge treatment system (right). The points (A, C) represent the centroid of the clusters determined by time-constraint cluster analysis.

CORRELATION ANALYSIS

Correlation analysis was conducted to express the degree of relationship between the microbial community composition (x) of a DGGE-similarity

matrix and operational data (y). For the microbial community composition all indices (chord and Ruzicka) were tested and the best fit to operational data gave the binary data from the Ruzicka similarity indices, which are presented in Table 1. The analysis shows to what extent two variables show similar or equivalent information about subjects of these two variables x and y. The P-value shows the statistical significance of a correlation. A correlation analysis between functional data and microbial data was conducted for both systems of the entire community as well as the ammonium oxidizing bacterial population.

Results showed that there was no statistically significant correlation between total phosphate and ammonium influent concentrations and microbial community changes (data not shown). None of the fluctuations measured in influent concentrations resulted in a statistically significant correlation to microbial data sets (P>0.1) except for COD, which correlated statistical marginal significantly with shifts in bacterial population of flocculent sludge (r=0.64, P=0.08). The ammonium oxidizing bacteria showed in both systems a statistically significant positive correlation to temperature shifts (granules: r=0.78, p=0.03 and flocs r=0.79, P=0.03) and a negative correlation with fluctuations in ammonium removal efficiencies (granules: r=-0.59, P=0.1 and flocs n.a). The bacterial population negatively correlated with fluctuations in total nitrogen removal efficiency (granules: r=-0.67, P=0.11 and flocs r=-0.82, P=0.02) (Table 1).

Table 1 Correlation analysis and corresponding P-values between changes in operational data (COD, P, N, effluent and influent concentrations as well as temperature) and changes in microbial community differences derived from DGGE gels of amoA and bacterial primer sets from aerobic granular sludge samples only over a time period of 400 days.

Treatment system:	Aerobic granular sludge				Conventional sludge			
Primer set:	bacterial		amoA		bacterial		amoA	
Parameter:	r	P-value	r	P-value	r	P-value	r	P-value
Influent								
COD [mg/l]	0.02	0.49	-0.18	0.37	0.64	0.08	0.46	0.18
Temperature [°C]	0.07	0.45	0.78	0.03	-0.07	0.45	0.79	0.03
Effluent								
NH_4 removal [%]	-0.12	0.43	-0.59	0.10	n.a	n.a	n.a	n.a
N- removal [%]	-0.67	0.11	-0.08	0.44	-0.82	0.02	0.08	0.44

DISCUSSION

Here we compared the microbial community changes derived from DGGE banding patterns from an aerobic granular sludge pilot plant in Epe (NL) with the community changes occurring in a full scale activated sludge plant over a period of 400 days. Despite the fact that both systems are structurally different, it must be emphasised that both systems received the same wastewater (composition and variation in flow rate) and were subjected to the same seasonable variations. The strongest conclusions within this study could be drawn from the similarity tree, the evenness measurements as well as the multi-dimensional scaling analysis.

The similarity cluster analysis showed that the community composition within the reactors was very dissimilar and distant (Figure 7). This can be explained by the very different operational conditions and structural differences of aerobic granular and flocculent sludge technology (Figure 1). Despite the difference derived from our cluster analysis, general bacteria community assessment showed that both systems had on average highly similar species richness, entropy, and evenness (Figure 2,4,5), suggesting that in both systems different bacteria had the same functionality. The multi-dimensional scaling clearly showed that the populations of conventional and flocculent sludge were separated in two district groups. The microbial populations of the flocculent sludge system moved closely around the initial population whereas the bacterial population in the aerobic granular sludge moved away from its initial population representing a permanent change (Figure 8). Beside these clear results we also conducted more analysis, which yielded in less obvious conclusions as it was the case for the similarity tree, the evenness measurements as well as the multi-dimensional scaling analysis.

We addressed the question if species abundance or presence and absence is causing more shifts within the population. Our analysis on binary (presence-absence) and quantitative (abundance) data suggested that community changes in aerobic granular sludge were rather affected by a change in species abundance than due to appearance and disappearance of bacteria whereas the opposite was true for the flocculent sludge system (Figure 6). This is also in line with the multi-dimensional scaling analysis and hence shows a different development in time of the microbial communities in both systems which is likely due to the different process characteristics as

presented in Figure 1. In order to test if a subpopulation is distributed in a similar way a DGGE was run for AOB of both systems. Sequencing results yielded in only AOB strains showing the specificity of used primer sets (Figure 3AB). Similar to other research conducted on granular sludge systems the sequencing results indicated the presence of sequences highly similar with *Nitrosospira* sp. and *Nitrosomonas* sp. (Bassin *et al.* 2011). In the flocculent sludge system *Nitrosomonas* sp. and not *Nitrosospira* sp. was the dominant AOB, whereas in the granular system sequences equally clustered within both AOB types (Figure 3 B). *Nitrosospira* has been shown to be negatively impacted at higher dissolved oxygen concentrations and potentially suppressed by *Nitrosomonas* (Wells *et al.* 2009), which is in line with other research showing mainly the appearance of *Nitrosospira* in environments low in DO (Park *et al.* 2002). In the granular sludge diffusion limitation is more severe and finding organisms more adapted to low DO environments is therefore not surprising. The AOB population of both systems showed less evenness than the bacterial population (Figure 4). This can be explained by the fact that nitrifiers in a granule can only grow in the outer oxygen penetrated layers, which are subjected to more shear stress than bacteria in the inner core (Figure 1). However, in principle the fluctuations in community composition were behaving very similar to what has been reported in other nitrifying bioreactors (Egli *et al.* 2003, Falk *et al.* 2009, Wittebolle *et al.* 2008) suggesting that a lower evenness of this microbial group might be related to a narrow substrate spectrum (only ammonium) as opposed to an heterotrophic organism (different electron donors and acceptors) (Curtis & Sloan 2006, McGuinness *et al.* 2006).

Several studies have attempted to quantify community shifts over time and they partly correlated these changes to system performance (Cai-Yun et al. 2011, Pholchan et al. 2010). Therefore, one of our attempts was to correlate microbial data with process data. Both processes in this study were reported to be run without process disturbances and were removing COD, Nitrogen and Phosphate to effluent levels as required by the European standards (European-Water-Framework-Directive 2000). Temporal fluctuations of the influent (nutrient availability, temperature) can alter population growth rates, potentially resulting in shifts in population dynamics driven by for instance species competition or stress adaptation (Müller 2000, Carrero-Colon et al. 2006). The combination of chemical and microbial community data showed a coherent correlation between system performance and community changes. This was indicated by our correlation analysis, which showed a statistically

significant (P<0.05) positive correlation between temperature shifts and AOB population (Table 1). Since the growth rate of ammonium oxidizing bacteria is significantly lowered at lower temperatures a positive correlation to species richness is hence in line with general expectations (Gujer 1977). The correlation between the ammonium removal efficiency and the microbial shifts in the AOB population was with a P-value of 0.1 less evident. A weak correlation was measured between in the similarity shifts derived from bacterial primer sets and total nitrogen removal efficiency (r=-0.59, P=0.1). Since AOBs account for ammonium removal and most of general bacteria are capable of denitrification, in both cases a negative correlation is expected and is hence reported here despite of a P-value higher than 0.05. Here we demonstrated that statistical and ecological modelling of molecular microbial community data can be used to identify differences between processes with the same functionality. Especially the similarity tree, evenness measurements as well as the multi-dimensional scaling analysis offered clear results showing that statistical analysis are worth to conduct. Our study gives evidence that statistical and ecological model approaches can couple shifts in microbial communities with process data (Table 1). We have explored many different similarity measures and methodologies, and the choices made here, are those we evaluated as the optimal ones.

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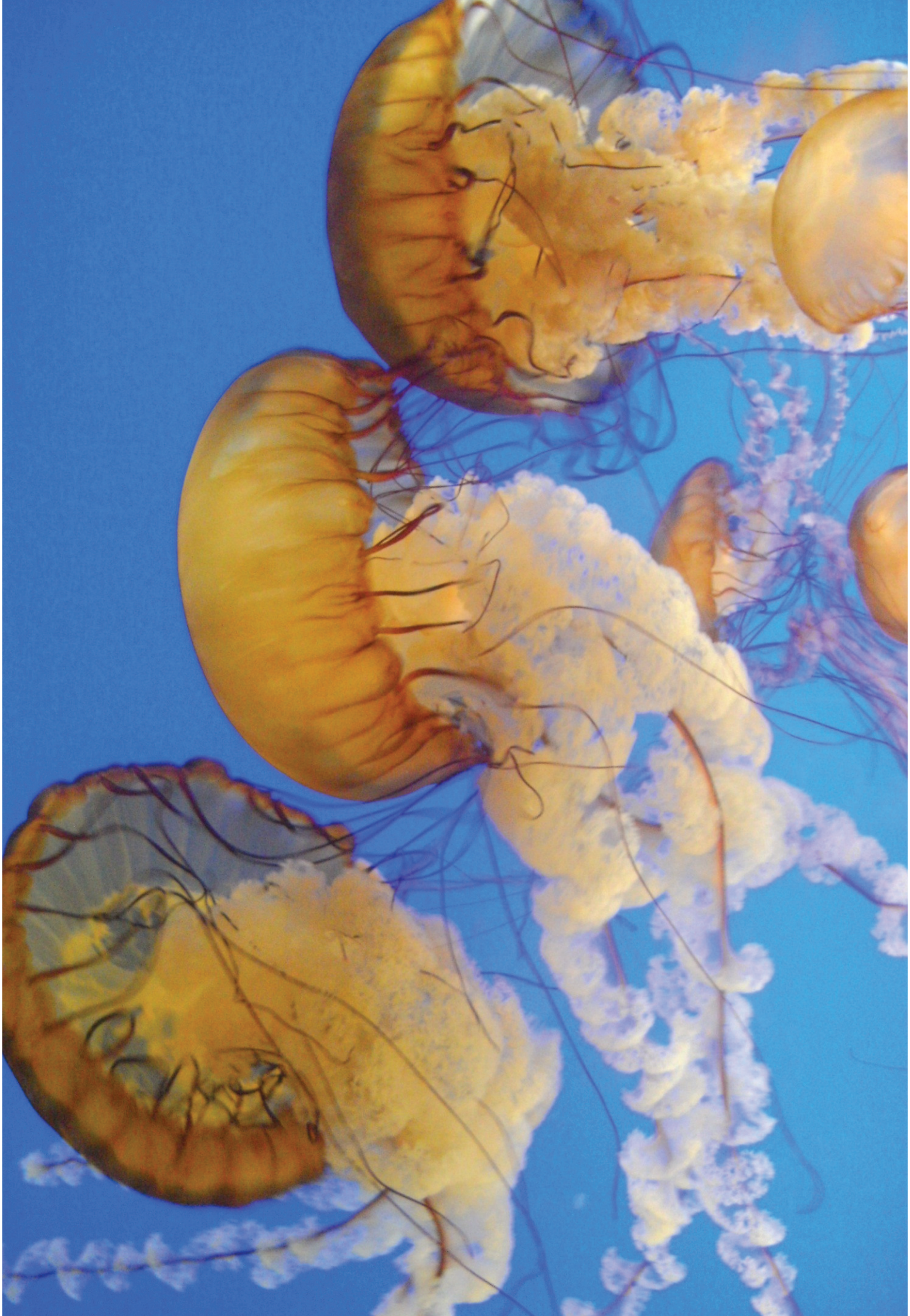

Chapter 3

UNRAVELLING THE REASONS FOR DISPROPORTION IN THE RATIO OF AOB AND NOB IN AEROBIC GRANULAR SLUDGE

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ABSTRACT

In this study, we analysed the nitrifying microbial community (ammonium-oxidizing bacteria - AOB and nitrite-oxidizing bacteria - NOB) 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 polymerae 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 oxidizing 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.

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. 2001a, 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.

MATERIALS AND METHODS

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 2.0±3 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, 60 mgNH_4-N/L and 20 mgP-PO_4/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.

ANAMMOX/NITRITION - CANON REACTOR

An autotrophic partial-nitritation/Anammox granular based on complete autotrophic nitrogen removal over nitrite (CANON) reactor 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).

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.

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.

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 media 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.

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.

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-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).

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.

QUANTITATIVE PCR (QPCR)

Primers and PCR conditions are listed in Table 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 QIAqiuck PCR Purification Kit (250) (Germany, QIAGEN). The PCR product was used for a qPCR procedure with a variable primer concentration and 25 µM iQ SYBR® Green Supermix (BIO-RAD Laboratories, USA) (Table 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 1.

Table 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		95°/6' [95°/0.30' 55°/0.4' 52°/0.4'80°/0.25'] x40	Sludge from wwtp[2]	(Muyzer et al. 1993)
Bac905r	0.355	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

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.

FLUORESCENT IN SITU HYBRIDIZATION

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 25µm 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 2. Samples were analysed with an epifluorescence microscope (Axioplan 2, Zeiss) equipped with filters suited for Cy3, Cy5 as well as Fluos.

Table 2 Oligonucleotide probes, primers target microorganisms and references

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

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 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).

RESULTS

REACTOR CYCLE MEASUREMENT

Figure 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. 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.

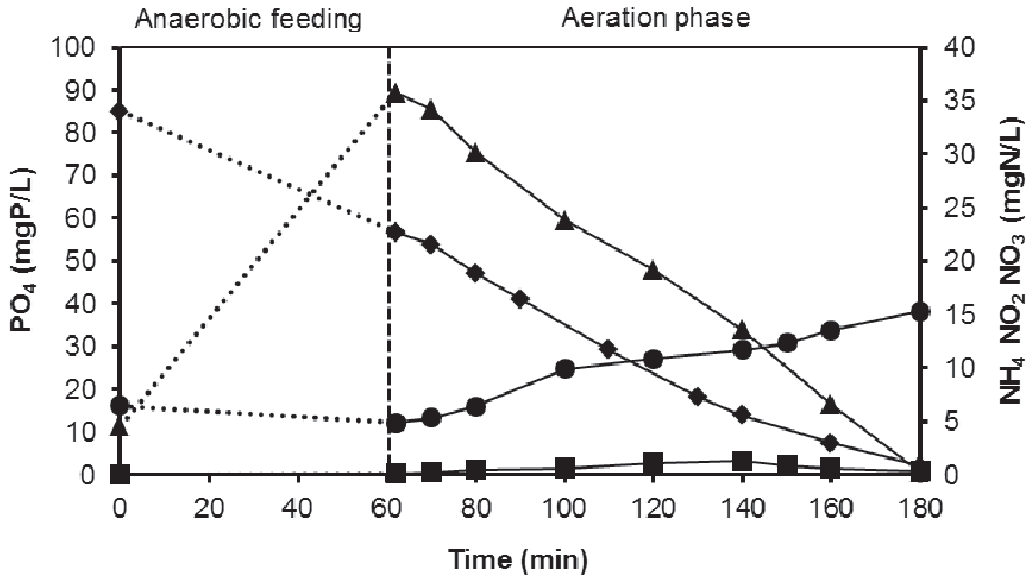

Figure 1 Typical cycle test showing the profiles of (♦) ammonium, (●) nitrate and (■) nitrite (right y-axis) and (▲) phosphate (left y-axis). The starting ammonium and phosphate concentrations depicted at time 0 were calculated based on the influent concentration (60 mgNH_4-N/L and 20 mgPO_4-P/L) and 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.

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 2a and Figure 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 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 3).

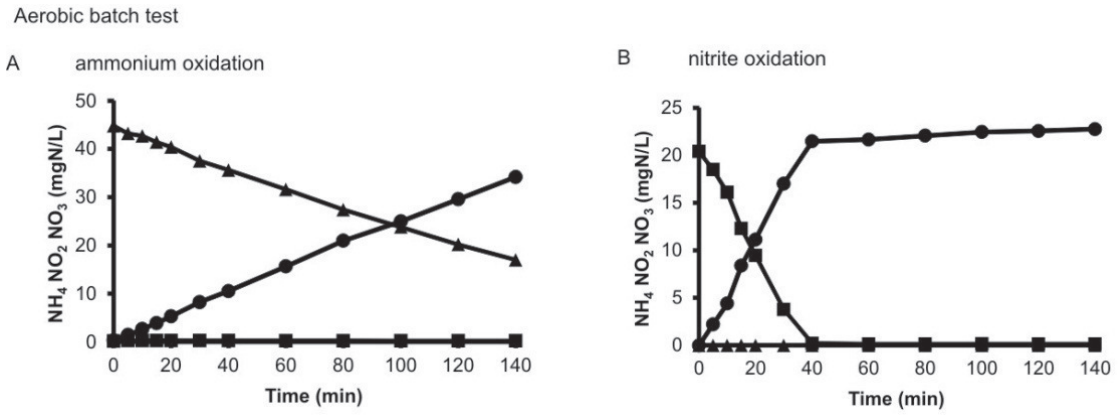

Figure 2 Aerobic batch tests dosing either ammonium (A) or nitrite (B). Legend description: ammonium (▲), nitrite (■) and nitrate (●).

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 3a) and a batch experiment in which nitrate was added as a pulse in the beginning of the test (Figure 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 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.

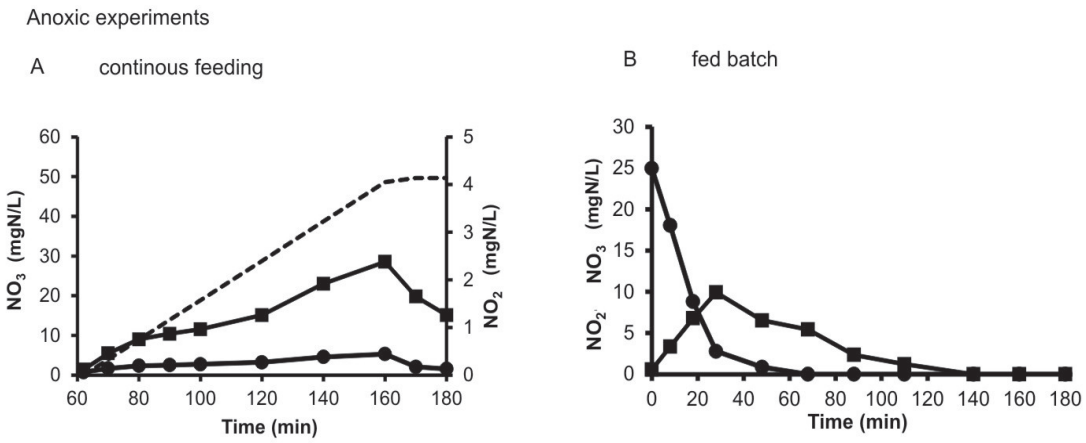

Figure 3 Anoxic experiments dosing nitrate (A) in a continuous feeding regime and (B) pulse feeding regime. Legend description: nitrite (■), nitrate (●). The dashed line shows the calculated amount of nitrate added.

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). 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.

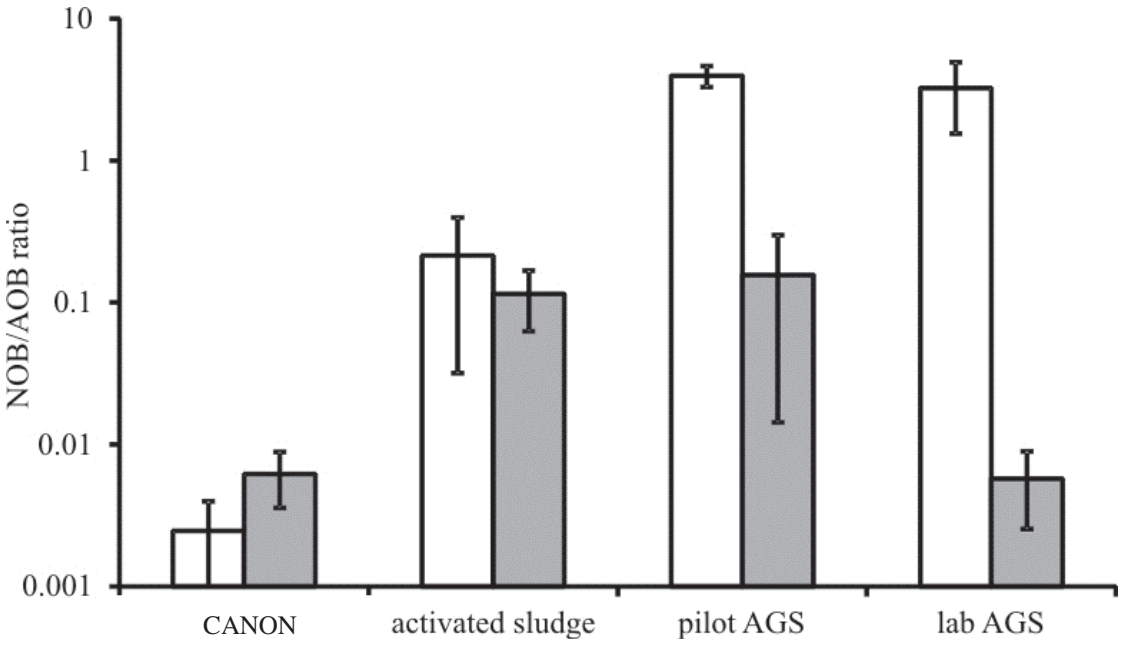

Figure 4 Average of NOB/AOB ratios build on the basis 16S rRNA gene analysis of Nitrobacter/AOB ☐ and Nitrospira/AOB ▭ of (left to right) autotrophic partial nitritition/Anammox (CANON) reactor, activated sludge, aerobic granular sludge pilot plant, and aerobic granular sludge lab reactor. Error bars indicate maximum and minimum observed ratios.

FLUORESCENT IN SITU HYBRIDIZATION

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 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 5A).

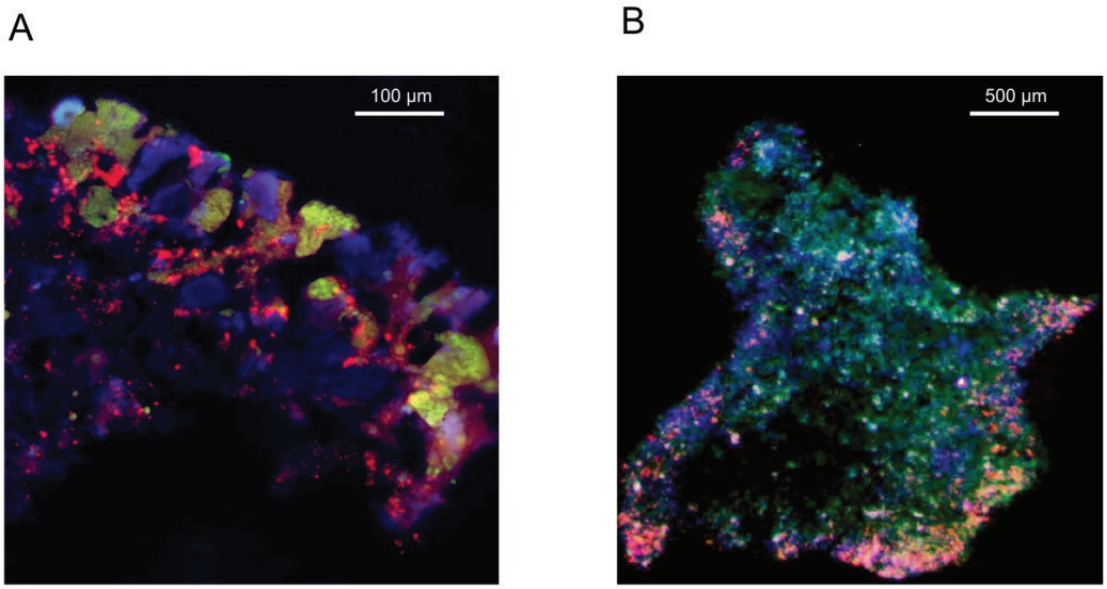

Figure 5 Microscopic FISH image on sliced granule showing A) AOB (green), NOB (red) and PAO (blue) as well as B) nitrifiers (mix of AOB and NOB) (red), PAO (blue) and GAO (green)

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 amount of 37 mg of COD/L is needed to elevate the expected autotrophic NOB/AOB ratio of 0.5 by a factor 6 (Table 3). This amount is less than 10% of the total COD (as acetate) fed into the system (400 mg/L).

Table 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 (37mgCOD/L ; 60mgNH_4-N/L) Calculations were based on the assumption of a) autotrophic growth of nitrifiersn and b) mixotrophic growth of NOB on 37 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**	

DISCUSSION

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. 2001a, 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 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 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.

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). 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.

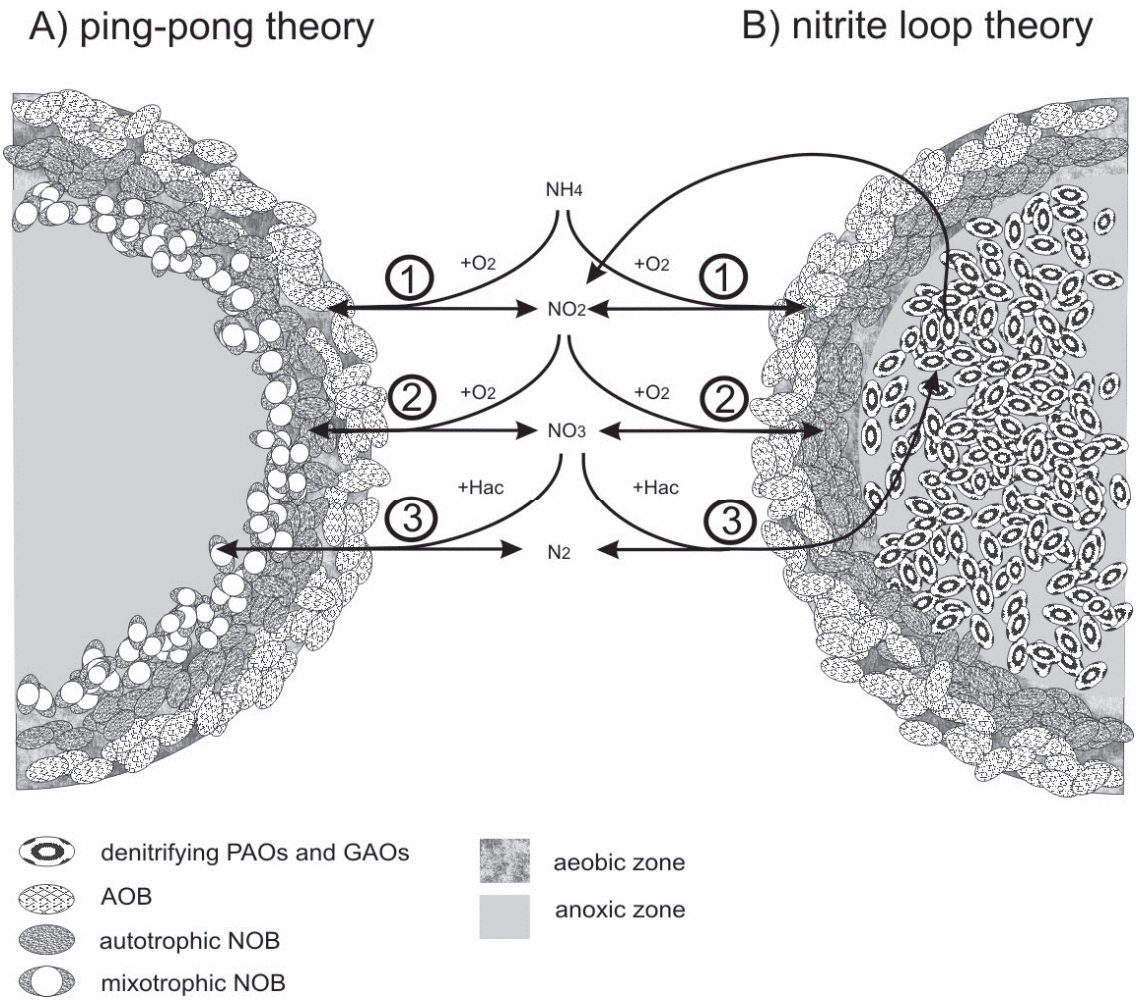

Figure 6 Schematic view of a) the ping pong theory and b) nitrite loop theory. Both theories have step 1) oxidation of ammonium to nitrite via ammonium-oxidizing bacteria (AOB) and step 2) oxidation of nitrite by autotrophic nitrite-oxidizing bacteria (NOB) in common. In case of the ping-pong effect 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 hence increasing its cell yield. In 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.

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 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 (Jiang et al. 2011, Johnson et al. 2010).

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.

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 an accumulation was not measurable during normal reactor operation (Figure 1). However, in a batch test in which we dosed twice the amount of nitrate denitrified under normal operation (Figure 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).

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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Chapter 4

EVALUATING THE SOLID RETENTION TIME OF BACTERIA IN FLOCCULENT AND GRANULAR SLUDGE

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ABSTRACT

The specific solid retention time for different bacteria within flocculent and granular sludge was determined. Samples were collected from reactor and effluent sludge and the number of a specific bacterial group was evaluated in respect to the total bacterial community with quantitative polymerase chain reaction (qPCR). The ratio of the relative presence of a specific bacterial group in the reactor sludge and wasted sludge was established to observe if preferential wash-out occurred. From the data also the solid retention time for different microbial groups can be estimated. Using this tool, we were able to show that the SRT of populations found on the exterior of granules is slightly lower than the SRT for population in the interior. Archaea were not found in the flocculent system but were present in small amounts within the granular system. It was further observed that protozoa were grazing on the bacterial community within the system indicating that they have the potential to shorten the specific SRT of bacteria.

INTRODUCTION

Microbial population engineering for the purpose of developing a bacterial community that performs a specific function requires the presence of niches that select for the retention of the desired populations (Grady and Filipe 2000). This can be accomplished by manipulating reactor conditions with the intention of influencing community assembly. Although we currently cannot confidently predict community assembly at the species level as it is discussed to be a chaotic event (Curtis et al. 2003), intuition gathered from years of operating and studying mixed communities gives us a sense of what factors are important. For example, we know that process loading rates (F/M), solids retention time (SRT) and shifts in redox conditions can influence the development of a functionally stable microbial community (Lee et al. 2003; Li et al. 2008; Saikaly et al. 2005).

Of the multitude of operational parameters that can be used to constrain niche development, SRT (also known as sludge age and mean cell retention time) is one of the most powerful. This parameter is traditionally used in the design and operation of biological nutrient removal (BNR) systems (Ekama 2010) to control for the presence or absence of desired microbial populations (e.g. nitrifiers). Practically, this is achieved by removing biomass at specific intervals and assuming a homogenous distribution of bacterial populations within the sludge. This assumption is not always valid as has been demonstrated in biofilm systems and granular sludge (Bassin et al. 2012; Winkler et al. 2011a; Winkler et al. 2011b) where stratification of populations (over the depth of the biofilm as well as over the height of reactor/sludge bed) results in non-homogenous biomass distribution. Moreover, in granules bacteria are not equally distributed since, the outer oxygen penetrated layer selects for the growth of aerobic bacteria (nitrifiers), whereas the microaerobic/anoxic conditions of the interior allows for the growth of denitrifying phosphate accumulating (PAO) and glycogen accumulating (GAO) organisms (De Kreuk et al. 2005). Since it is clear that an uneven distribution of bacteria within the sludge is more the norm than exception, there is a need to properly assess the total bacterial SRT as well as the SRT for individual functional groups. From a practical perspective, knowledge of these parameters can help engineers operate systems to preferentially enrich or select against bacteria with a desired function.

The emergence of molecular methods like pyrosequencing and quantitative polymerase chain reaction (qPCR) gives us an unprecedented ability to characterize species level shifts in stable and unstable communities (Werner et al. 2011). This data has allowed to examine how community resistance and resilience is impacted by microbial diversity (Briones and Raskin 2003); however, translation of these findings towards defining guidelines for reactor design and operation are lacking. From this perspective, we studied flocculent and granular sludge with the intent to understand how molecular tools can be used to derive essential engineering parameters. Specifically, we use qPCR to derive the SRT for different microbial populations in the different sludges. We then used this approach to test the hypothesis that the SRTs of microbial groups in flocculent sludge and aerobic granules performing the same function are different due to biomass stratification.

MATERIALS AND METHODS

REACTOR OPERATION AND SAMPLE COLLECTION

Samples were taken from an aerobic granular pilot plant and activated sludge plant from Epe the Netherlands over a period of 1 year. Both treatment systems were operated at the same site treating the same wastewater. The conventional plant removed phosphate chemically whereas the granular system was based on biological phosphate removal. Further description of these reactors and the wastewater composition are provided elsewhere (van der Roest et al. 2011).

The reactors were operated under steady state conditions and both reactors removed COD, N and P according to the effluent guidelines of the European water frame work directive From both reactors, biomass samples were collected (discharged reactor effluent and mixed liquor from the aerated period) for molecular analyses as described below. In this study, 6 sampling events were conducted. For the granular sludge reactor samples were also taken during the anaerobic feeding period from the top and bottom of the settled sludge bed.

QPCR PROCEDURE

DNA extraction was conducted according to the manufacturer's recommendation using an UltraClean™ Microbial DNA Isolation Kit. qPCR was then performed to monitor the community composition. Primer specificity was checked *in silico* using the SILVA database (Pruesse et al. 2007) and the Ribosomal Database Project ProbeMatch tool (Cole et al. 2009). All primers were optimized using temperature gradient qPCR. The resulting conditions, primer concentrations as well as DNA used as a standard for the qPCR calibration are listed in table 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. All qPCR assays were performed in triplicate.

COMBINATION OF MOLECULAR DATA WITH OPERATIONAL DATA

All functional primers were adapted for their copy number. ΔC_T was calculated by following equation $\Delta C_T = C_{T(Eub)} - C_{T(A)}$ (Zhang et al. 2009). For determination of the proportion of one species (denoted as A) to the total Eubacterial community (denoted as Eub) the following equation was applied: $p_A = 2^{\Delta C_T}$. This approach is based on the $2^{-\Delta\Delta CT}$ method for quantifying gene expression (Livak and Schmittgen 2001). Species proportion ratios (SPR) were calculated for samples obtained from the effluent and mixed liquor of both reactors. SPRs were also calculated for samples obtained at the top (p_{Atop}) and the bottom (p_{Abot}) of the granular reactor. Ratios between top and bottom samples were calculated ($ratio_{A^*} = \frac{p_{A\_bot}}{p_{A\_top}}$). A comparison of SPRs for the same species in the effluent (p_{Aout}) versus mixed liquor (p_{Ain}) was also performed ($ratio_A = \frac{p_{Ain}}{p_{Aout}}$). Using the overall SRT Θ_{total} ($\Theta_{total} = \frac{V_R \times x_R}{Q \times x_{runoff} + x_{ex} \times Q_{ex}}[day]$) of the system derived from mixed liquor suspended solid concentrations, the SRT of specific microbial groups/species (Θ_A) was calculated: $\Theta_A = \Theta_{total} \cdot ratio_A [day]$. Explanations of calculations are given in the appendix.

Table 1. Primers, qPCR conditions and primer concentrations used in this study

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)
AmoA1R/ AmoA 2F	0.2	94/4m, (94/60s, 57/45s, 72/60s, 80/25s)*40, 72/10min, 12/∞	*Nitrosomonas europaea*	(Rotthauwe 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	(Lim et al. 2008)
NxrB F706/ NxrB R1431	0.2	95/5m, (95/40s, 56/30s, 72/30s)*35, 72/10min, 12/∞	Nitrobacter	(Degrange and Bardin 1995)
FGPS872/ FGPS 1269	0.2	95/3m, (95/60s, 50/60s, 72/60s)*35, 72/3min, 12/∞	Nitrobacter	(Degrange and Bardin 1995)
Bac341F	0.35	95°/6' [95°/0.30' 55°/0.4' 52°/0.4'80°/0.25'] x40	Sludge from wwtp[2]	(Muyzer et al. 1993)
Bac905r	0.35	52°/5'10°/∞	Sludge from wwtp[2]	(Weisburg et al. 1991)
ARC519fc & ARC519fa	0.2	95°/5' [95°/0.30' 62.5°/0.4'	Nitrosopumilus maritimus	(Øvreås et al. 1997)
ARC934r	0.2	72°/0.4'/80°/0.25'] x40	Nitrosopumilus maritimus	(DeLong et al. 1989)
ARC915r	0.2	72°/7' 10°/∞		(Raskin et al. 1994)
cren_amoAf	0.2	95°/5' [95°/0.30' 62.5°/0.4' 72°/0.4'/80°/0.25'] x40	Nitrosopumilus maritimus	(Hallam et al. 2006)
amoAr	0.2	72°/7' 10°/∞		(Francis et al. 2005)
SRB-Dsr4R	0.2	95°/5' [95°/0.40' 55°/0.4' 72°/0.6'/72°/0.6' 80°/0.25']	Sludge from wwtp[2]	(Wagner et al. 1996a)
SRB-Dsr2060F	0.2	x35 72°/10' 10°/∞	Sludge from wwtp[2]	(Geets et al. 2006)
Univ518f	0.2		Sludge from wwtp[2]	(He et al. 2007)
PAO-846r	0.2	94°/5' [94°/0.15' 57°/0.20' 68°/0.15'68°/0.15' 80°/0.25']	EBPR sludge[1]	(He et al. 2007)
PAO-651f	0.2	x38 68°/10' 10°/∞	EBPR sludge[1]	(Fukushima et al. 2007)
APAO-184f	0.2	95°/2' [95°/0.1' 64°/0.1' 72°/0.2' 80°/0.25']	EBPR sludge[1]	(Okunuki et al. 2007)
A445r	0.2	X50 72°/10' 10°/∞	EBPR sludge[1]	(Okunuki et al. 2007)
GAO-Gbf	0.2	94°/2' [94°/0.15' 57°/0.15' 68°/0.25' 80°/0.25']	EBPR sludge[1]	(Fukushima et al. 2007)
GAO-Gbr	0.2	x38 68°/10' 10°/∞	EBPR sludge[1]	(Fukushima et al. 2007)

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] Sludge from the aerobic granular sludge pilot plant in Epe, The Netherlands

[2] Sludge from the conventional wastewater treatment plant in Epe, The Netherlands

CLONE LIBRARY

Since only the primer Arc934R but not Arc915R resulted in a positive outcome in the qPCR protocol, a clone library was constructed using Univ518f and Arc934R to confirm the presence of Archaea (Gábor et al. 2006). The reaction conditions were similar to those described in Table 1 except 36 cycles were used. 16 clones were picked and sequenced.

FLUORESCENT IN SITU HYBRIDIZATION

Samples from the granular treatment plant in Epe were fixed in 4% paraformaldehyde. Granules were embedded in a tissue freezing medium (Leica Microsystems), hardened by freezing (-20°C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 25µm thin slices. Dried slices were kept on a microscopic glass slide and FISH was performed to detect the presence of nitrifiers (Cy3), PAO (Cy5), and GAO (Fluos) microbial populations using protocols previously described (Winkler et al. 2011b) FISH probes used in this study are described in Table 2.

Table 2 Oligonucleotide probes, target organisms, and references

Probe	Sequence (from ‘5 to ‘3)	Specificity	Reference
PAO 462	CCGTCATCTACWCAGGGTATTAAC	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)
GAO Q989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	(Crocetti et al. 2002)
GAO Q431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	(Crocetti et al. 2002)
Ntspa662	GGAATTCCGCGCTCCTCT	*Nitrospira like organisms*	(Daims et al. 2001)
NIT1035	CCTGTGCTCCATGCTCCG	*Nitrobacter*	(Wagner et al. 1996b)
NSO190	CGATCCCCTGCTTTTCTCC	All AOB	(Mobarry et al. 1996)
NSO1225	CGCCATTGTATTACGTGTGA	All AOB	(Mobarry et al. 1996)

RESULTS

STRUCTURE AND COMPOSITION OF GRANULES AND FLOCS

FISH analyses on sliced granules showed the typical spatial distribution of nitrifiers in the oxygen penetrated outer layer (Cy3) as well as PAOs (Cy5) and GAOs (Fluos) in the outer as well as interior layers (Figure 1C). Sulphate reducing bacteria and ammonium oxidizing archaea were not detected in these granules. Archaea were only present in aerobic granular sludge as confirmed by qPCR and a clone library. One out of sixteen picked clones resulted in a positive result for an uncultured crenarchaeote clone. Only the primer Arc934R but not Arc915R resulted in a positive outcome (qPCR signal). Ciliated protozoa (*Vorticella*-like) were also identified on the outer layer of the granules through light microscopy (Figure 1A-B). FISH images indicated the simultaneous presence of GAO (red), PAO (blue) and nitrifiers (green) within these cells, indicating active grazing (Figure 1D).

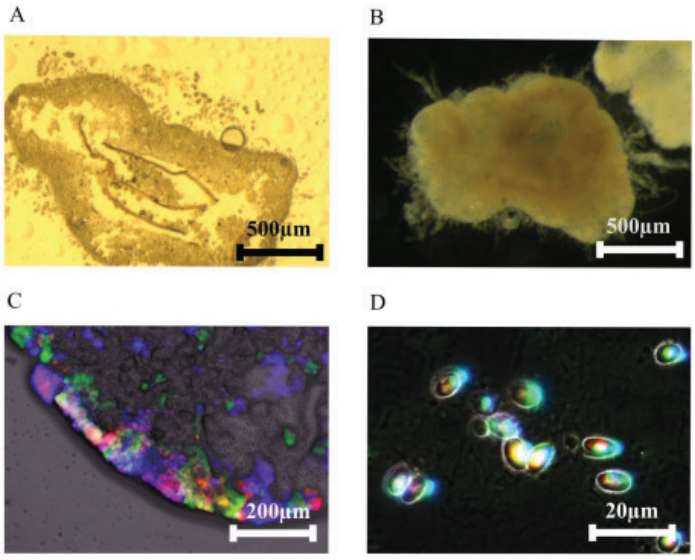

Figure 1 FISH and light microscopy images. A-B) light microscopic image of a granule with Protozoa on outer layers. C-D) FISH image of C) PAO (blue) GAO (green) and nitrifiers (red) on a sliced granule as well as D) the presence of GAO (red) nitrifiers (green) PAO (blue) within protozoa.

MICROBIAL POPULATION DISTRIBUTION AND CORRESPONDING SRT VALUES

Total bacterial SRT for the flocculent sludge and aerobic granular sludge systems were 11 and 20 days, respectively. To determine the effective SRT of the individual microbial populations, species proportion ratios (SPR) were calculated for both granular and conventional systems (Figure 2). A SPR smaller than one indicates a relative short SRT for the group of organisms, whereas a SPR higher than 1 a longer SRT for the concerned bacterium. Results from granular sludge samples indicated that the SRT of nitrifying bacteria (exterior layer of granules; 11±3 days) was slightly lower than the SRT for bacteria present within the interior of the granules (PAO, GAO, actinoPAO, archaea) (13±4 days; Table 3). For the conventional systems, the SRT of nitrifying bacteria ranged from 7-15 days, with the average SRT of AOB being 15±4 days versus 8±2 days for NOB (average from *Nitrobacter* and *Nitrospira*). For the granular systems, the SRT of nitrifying bacteria ranged from 6-14 days, with the average SRT of AOB being 12± 4 days versus 9±3 days for NOB.

SPRs for PAOs and GAOs present in the bottom versus top sludge of the granular sludge reactor suggested that PAOs were circa 1.5-2 times more abundant in the bottom sludge. In contrast, GAOs were twice as abundant in the top sludge. These results were consistent within lab reactor and pilot scale reactors in Epe/Netherlands (Figure 2C). The reactor configuration of the lab scale system is described in detail in earlier research (Winkler et al., 2011a).

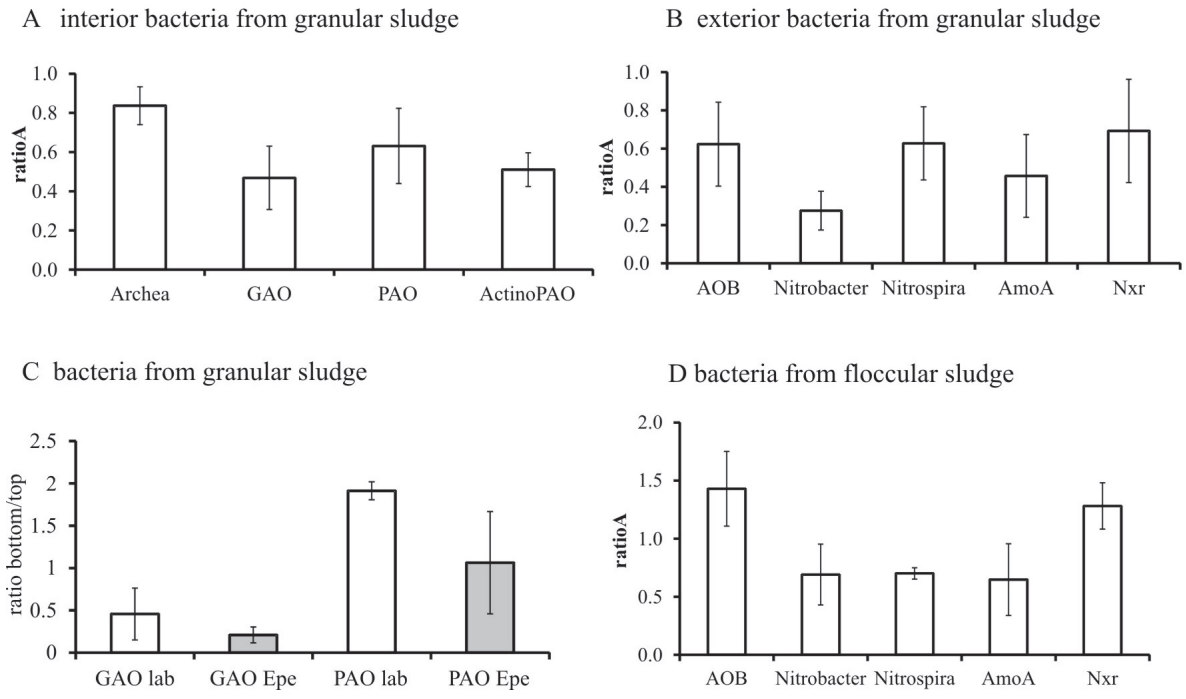

Figure 2 Calculated Species proportion ratios (SPR) for different microbial populations in granules (A-B) and flocculent sludge (D) as well as bottom/top ratios of GAOs and PAOs from aerobic granular sludge samples (C) collected from the laboratory ☐ *and the pilot scale plant in Epe/The Netherlands* ▭

Table 3 SRT of bacteria based on calculated wash-out ratios as given in Figure 2 and the SRT of the conventional system and the granular sludge system

bacterium	**SRT [days]**	**bacterium**	**SRT [days]**	**SRT [days]**
	Interior layer		Exterior layer	flocs
Archea	17±2	**AOB**	12±4	15±4
GAO	9±3	***Nitrobacter***	6±2	8±3
PAO	13±4	***Nitrospira***	12±4	8±1
ActinoPAO	10±2	**AmoA**	11±5	7±3
		Nxr	14±5	14±2
Average	13±4		11±3	11±4

DISCUSSION

In this study, we examined granular and flocculent sludge from a full-scale reactor as case studies for demonstrating the use of molecular tools for calculating key engineering parameters. We succeeded in linking operational data (SRT from suspended solids) with a wash-out ratio derived from qPCR and enabled herewith a direct combination of traditional engineering with classical molecular tools. Studies connecting microbial and operational data within full-scale systems are sparse (Wells et al., 2011) but are of importance to better understand reactor performance. Here we present a new powerful approach to combine these two fields enabling a direct determination of the specific SRT of bacteria and hence define the wash-out rate of distinct bacterial groups. The techniques presented here are relatively easy to use and interpret and could allow for the utilization of this approach at utilities to more carefully manage specific solids inventory. This is particularly important at facilities that encounter frequent weather events that compromise aerobic SRT control which leads to the loss of nitrification (Giokas et al., 2002). This tool could also be used to define design criteria (e.g. Aerobic SRT and Anoxic SRT) that are needed for operation and management of facilities intentionally performing simultaneous nitrification and denitrification through the use of dynamic aeration control strategies (Thauré et al., 2008).

We initially hypothesized that bacterial populations (nitrifiers) that colonize the exterior of aerobic granules are more rapidly eroded and subsequently washed out in higher numbers than biomass on the interior of the granule (PAO, GAO, Archaea) (Figure 3A). Thus, the SRT of these exterior populations would be lower in respect to the interior populations. For flocculent sludge we expect that the SRT of the individual populations is similar to the SRT of the total solids since there will be hardly stratification. When granule breakage (Figure, 3B) dominates over erosion there will also be equal SRT for total granular sludge as well as individual populations.

From an engineering point of view it is appealing to correlate microbial shifts to system performance by the usage of molecular tools. Studies have already attempted to quantify community shifts over time and partly correlated these changes to system performance (Ayala-Del-Río et al., 2004; Cai-Yun et al., 2011; Pholchan et al., 2010; van Nostrand et al., 2011). Instead of correlating operational data with molecular data in this study we directly linked molecular techniques with operational system performance to investigate the SRT of district bacterial groups. Specifically, we calculated

wash-out ratios for microbial populations in granular and conventional system using qPCR data. These ratios allowed us to describe how an individual microbial population SRT is related to the overall system SRT. Specifically, we calculated wash-out ratios for microbial populations in granular and conventional system using qPCR data. These ratios allowed us to describe how an individual microbial population SRT is related to the overall system SRT. The results obtained here show that in aerobic granular sludge, the SRT of the nitrifiers growing on the oxygen penetrated outer layer was 4 days shorter (Figure 1B) than the SRT of bacteria growing in the interior of the granule (11±3 days versus 14±4 days) (Table 3). Although the differences are not very strong, this difference suggests that granular disassembly occurs from outer layer erosion (Figure 3A) versus intra-granule rupture (Figure 3B). Due to the fast settling properties of granular sludge, small fragments and flocculent material are removed preferential over large granules, which might additionally influence the results obtained in this study. Further work is needed to confirm this finding.

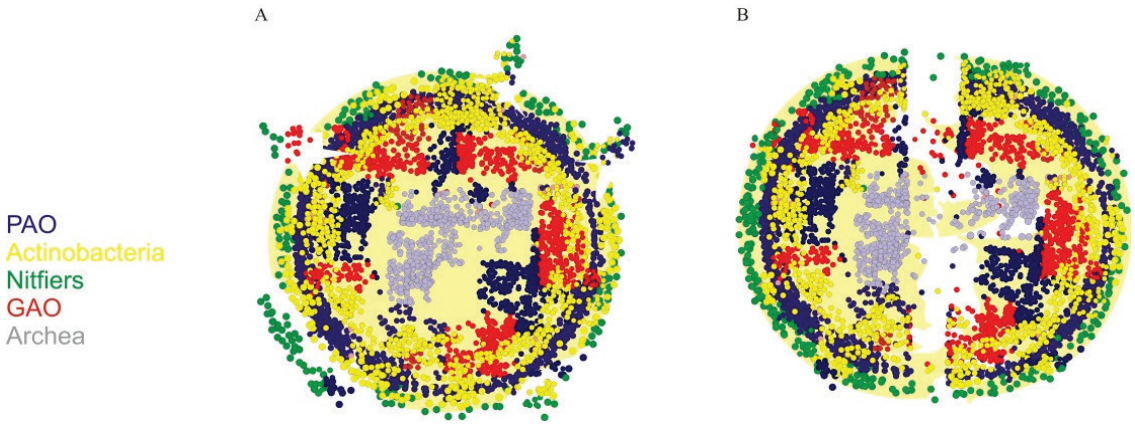

Figure 3 schematic view of a granules the with typical multi structural layer with nitrifies in the outer oxygen penetrated layer followed by PAOs (blue) ActinoPAOs (yellow) GAOs (red) and with Archea (gray) in the interior anaerobic core. The graphical model shows A) breakage of mainly the outer layers hence decreasing the SRT of nitrifiers and B) granule breakage in the middle leading to an equal wash-out of bacteria and hence to the same SRT for the whole bacterial population

It should be mentioned that the technical replicates (3 per sampling day) had a very small standard deviations for the ΔC_T-values of all primer sets (0.9±0.2% for granules, 2±1.3% for flocs). The relatively big standard deviation as shown in the results (Figure 1) were due to different wash-out ratios ($ratio_A$) between the samples from different sampling days (6 samples per 1 calculated SRT of one bacterium). Therefore, we suggest that future investigations perform extensive sampling across multiple days to further characterize inherent biological variations that exist. It should also be noted that even if a certain fraction of the population is underestimated (e.g. due to primer unspecificity etc.), the proposed method will not be affected since the calculation is based on a ratio between sludge samples (which are all exposed to the same bias). Thus, this proposed method is robust for calculating population SRTs (Θ_A) if enough sampling days are used.

In this study, we also investigated the segregation of biomass in a granular sludge bed. Our results confirmed earlier reports (Winkler et al. 2011a) that glycogen accumulating organisms dominated the slower settling granules and the preferred phosphate accumulating bacteria dominated the fast settling granules. Thus, PAOs would preferentially benefit from substrate supply since the reactor was fed in a plug-flow regime from the bottom. These results are consistent with our prior findings, whereby we used FISH to study biomass stratification (Bassin et al. 2012; Winkler et al. 2011a).

The effect of protozoa grazing on microbial population SRT has not been extensively studied to date (Huws et al. 2005; Moussa et al. 2005; Van Loosdrecht and Henze 1999). In this study, we were able to identify that *Vorticella*-like ciliated protozoa were actively grazing on bacteria in the aerobic granules (Figure 1 D). While it may be tempting to hypothesize that the exterior populations (e.g. nitrifiers) are more prone to grazing events, our current results give no clear indication that this is the case. Instead, FISH analyses suggested that protozoa incorporated all bacteria and not only nitrifiers. Additional work is needed to understand whether protozoa and metazoa can selectively impact population dynamics of functional groups.We also identified that archaea were present within granular sludge but not within flocculent sludge.

Archaea comprised ca 5% of the total population detected by qPCR, as well as with a clone library. It is possible that granules offer better growing conditions for archaea (e.g. continuous anaerobic conditions in the interior) as opposed to a sludge floc. This work represents a successful example in which molecular techniques are used to improve our understanding of how to engineer mixed cultures with multiple functional identities.

CONCLUSIONS

In this study, we showed that molecular tools can be used to effectively calculate key engineering parameters e.g. specific retention time of bacteria. A dimensionless wash-out ratio from reactor and effluent sludge was determined for one specific bacterium by the means of qPCR. This ratio was then coupled with the total SRT derived from the suspended solids [days] to calculate the specific retention time of one functional group [days]. Using this tool, we are able to confirm that the SRT of populations found on the exterior of granules is lower than the SRT for population in the interior. Further we found evidence that protozoa can influence microbial populations numbers, however, it is unclear whether individual populations are preferentially consumed.

APPENDIX

To normalize all samples to 5 ng DNA and to adapted calculations to the PCR efficiency (E) following equations were applied to all samples:

$$ratio_5 = \frac{DNA_{measured}}{5}\left[\frac{ng}{ng}\right] \quad (1)$$

$$C_{Tcor} = {}^{ratio_5}\log(1+\frac{E}{100}) \quad (2)$$

If measured DNA >5 ng then ΔC_T shifted towards higher ct values
If measured DNA <5 ng then ΔC_T t shifted towards lower ct values
If measured DNA = 5 ng then ΔC_T remains the same

C_T values of functional primers were corrected for their copy number:

$$C_{Tcopy} = {}^{copynr}\log(1+\frac{E}{100}) \quad (3)$$

If copy number >1 then ΔC_T shifted towards higher ct values
If copy number =1 then ΔC_T remains the same

The originalΔC_T was corrected for copy number and amount in DNA:

$$\Delta C_{TspeciesA} = C_{Torg} + C_{Tcor} + C_{Tcopy} \quad (4)$$

To adapt 1 species to the whole population a species proportion ratios (p_A) was build:

$$p_A = 2^{(\Delta CT_{Eub} - \Delta CT_{species_A})} \quad (5)$$

If pure culture than 2^0=1

The formula below was used for construction of a ratio between samples from the effluent (out) and the reactor (in):

$$ratio_A = \frac{p_{Ain}}{p_{Aout}} \quad (6)$$

In order to calculate the SRT of the total suspended solids following calculation was used:

$$\Theta_{total} = \frac{V_R \times x_R}{Q \times x_{runoff} + x_{ex} \times Q_{ex}} \quad [day]$$

V_R	=	Volume reactor	$[m^3]$
x_R	=	Biomass reactor	$[kg/m^3]$
Q	=	Flow rate	$[m^3/day]$
Q_{ex}	=	Flow rate	$[m^3/day]$
x_{runoff}	=	Sludge in runoff	$[kg/m^3]$
x_{ex}	=	Sludge in effluent	$[kg/m^3]$
Θ_{total}	=	SRTof all bacteria	[day]

(7)

In order to calculate the SRT of one specific organism within the whole community the following calculations was used:

$$\Theta_{speciesA} = \Theta_{total} \cdot ratio_A [day] \quad (8)$$

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Chapter 5

CONCLUDING REMARKS

EVALUATION AND OUTLOOK

In the research presented in this thesis it was shown that the total bacterial community structure of flocculent sludge and granular sludge were very dissimilar (similarity tree) whereas their species richness entropy, and evenness were very similar. These results suggest that different types of bacteria were establishing a functional stability in both systems. Our study gave evidence that statistical and ecological model approaches can couple shifts in microbial communities with process data (**Chapter 2**). Future research and development should exploit this link and its use for process optimisation and control. Sample collection as well as the laboratory and computer based analyses are still very time consuming and cause a major time shift between the operational data and the results obtained from numerical ecological data sets.

The current rapid development of genomic and bioinformatics technology might overcome this barrier in the coming years. Proteomics is one tool, which might lead into a better option to combine molecular data with operational data. Proteomics and transcriptomics are widely used in medicine to identify potential new drugs by determining a specific protein being or not being expressed as a result of specific diseases. While in medicine these tools are a commonly used it has widely been ignored in environmental biotechnology. The genome contains the complete set of genes required to build all functionality within bioreactors (e.g nutrient removal). Since there is direct linkage between the amount of mRNA and proteins expressed to a specific functionality within a bioreactor, transcriptomics and proteomics can be useful tools to test for specific circumstances improving reactor performance and hence increase a product or removal efficiency.

Microarrays can be used to analyze the expression of tens of thousands of genes simultaneously and help to identify the mechanisms, which are essential to provide system functioning. Therefore, proteomics and transcriptomics can be very useful tools to answer fundamental questions about the molecular state of a bacterium. In reactor technology they can be used to understand how bacteria are reacting to changes in reactor operation. For instance proteomics can tell what kind of proteins are being made under which circumstances and can give information about the amounts of proteins being produced. Although these techniques are very appealing it must be emphasised that the full potential of the on-going revolution of molecular

techniques to improve our understanding of community assembly, will not be realized if research is not directed and driven by theory (Prosser et al. 2007). Also relatively simple classical microbiological experiments with pure and mixed culture experiments will be still necessary in future to gain more knowledge about kinetic parameters of microorganisms using for instance mixed substrates as it is the case for wastewater treatment systems (Egli et al. 2010). Therefore, interdisciplinary research is needed to optimize and better understand microbial communities in complex systems as it is the case for aerobic granules. Biofilm systems (such as granules) do not only offer attractive options for engineered systems for better removal efficiencies but are also interesting from a microbiological point of view.

Since granules consist of a great variety of coagulated microorganisms in one small ecosystem the processes occurring between the bacteria can be of interest to study fundamental questions in microbiology. In this thesis for instance an elevated ratio between NOB and AOB indicates that the NOB (in specific *Nitrobacter*) grew independently from the nitrite supply of AOB suggesting that either denitrifying bacteria created additional nitrite by forming a nitrite loop in which nitrate gets several times reduced to nitrite or that *Nitrobacter* used its capacity to conduct dissmiliatory nitrate reduction (**Chapter 3**). This hence shows that biofilm systems offer an interesting environment to test for other microbial phenomena. In this research we have shown that a combination of molecular tools (qPCR) and operational data (SRT from suspended solids) gives new insight about how process performance shapes the microbial community structure (**Chapter 4**). Since the bacteria are responsible for reactor performance this methodology is a new approach to understand and eventually control reactor performance better potentially yielding in higher removal efficiencies. The techniques presented in this thesis are relatively easy to use and interpret and could allow for the utilization of this approach at utilities to more carefully manage specific solids inventory. In specific this technique can be of importance to aerobic granular sludge technology however also for flocculent sludge this technique can find its application. For instance at facilities that encounter frequent weather events that compromise aerobic SRT control, which leads to the loss of nitrification (Giokas et al., 2002). This tool could also be used to define design criteria (e.g. Aerobic SRT and Anoxic SRT) that are needed for operation and management of facilities intentionally performing simultaneous nitrification and denitrification through the use of dynamic aeration control strategies (Thauré et al., 2008).

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SUMMARY

Chapter 1 gives a short introduction in microbial ecology. **Chapter 2** of the second book reports the differences in the microbial community composition of flocculent sludge and granular sludge. Their total bacterial community composition were very dissimilar whereas the community assessment showed that both systems had on average a similar species richness entropy, and evenness, suggesting that although the bacterial groups where very dissimilar a same stability in microbial community and function was obtained. The AOB population showed more unevenness than it was the case for the total bacterial populations. A correlation between the ammonium oxidizing bacterial population and changes in ammonium removal efficiency as well as temperature was found for both systems, whereas the bacterial population correlated with total nitrogen removal efficiencies.

Chapter 3 aims to unravel the reasons for the disproportion in the ratio of AOB and NOB in aerobic granular sludge. In this study, we analysed the nitrifying microbial community (ammonium-oxidizing bacteria - AOB and nitrite-oxidizing bacteria - NOB) within three different aerobic granular sludge treatment systems as well as within one flocculent sludge system. Fluorescent in situ hybridization (FISH) and quantitative-PCR (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. This suggested that the growth of *Nitrobacter* within aerobic granular sludge was partly uncoupled from the lithotrophic nitrite supply from AOB. This was supported by activity measurements which showed a 3 fold higher nitrite oxidizing capacity than ammonium oxidizing 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*) occured in which denitrifiers reduced nitrate to nitrite supplying 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.

In **chapter 4** the specific solid retention time for different bacteria within flocculent and granular sludge was determined. Samples were collected from reactor and effluent sludge and the number of a specific bacterial group was evaluated in respect to the total bacterial community by the means of quantitative polymerase chain reaction (qPCR). Operational data were combined with molecular techniques and the SRT of each individual microorganism could be calculated. It was further observed that protozoa were grazing on the bacterial community within the system indicating that they have the potential to shorten the specific SRT of bacteria. Archea were not found in the flocculent system but were present in small amounts within the granular system. **Chapter 5** gives outlook about possible applications molecular techniques in wastewater treatment.

SAMENVATTING

Hoofdstuk 1 geeft een kort overzicht over microbiële populatie dynamica in afvalwater. **Hoofdstuk 2** van het tweede boek beschrijft de verschillen in de microbiële samenstelling van slibvlokken en korrelslib. De bacteriële samenstelling was zeer verschillend maar desondanks hadden de beide systemen een zeer vergelijkbare soortenrijkdom en verdeling tussen de verschillende soorten qua aantallen. Bovendien waren beide systemen functioneel stabiel in termen van CZV en N verwijdering ondanks deze verschillen in microbiële samenstelling. De AOB populatie heeft meer ongelijkheid laten zien als het bacteriële systeem. Een correlatie tussen de AOB populatie en de ammonium verwijderingcapaciteit en temperatuur kon gevonden worden voor allebei systemen. Het was verder mogelijk de bacteriële populatie te correleren met de totale stikstof verwijdering capaciteit.

Hoofdstuk 3 is uitgevoerd om de reden achter de in het systeem gevonden hoge ratio tussen AOB/NOB beter te kunnen begrijpen. De nitrificerende gemeenschap in drie verschillende korrel systemen en in een system gebaseerd op vlokken zijn daarom geïnspecteerd. Fluorescent in situ hybridization (FISH) and quantitative-PCR (qPCR) hebben laten zien dat *Nitrobacter* de dominante NOB in anorganisch gevoed aeroob korrelslib was. In de conventionele system waren *Nitrobacter* en *Nitrospira* aanwezig in gelijke hoeveelheden. Dit geeft aan dat de groei van *Nitrobacter* in aeroob korrelslib gedeeltelijk onafhankelijk van de nitriet aanvoer van de AOB was. Dit werd ondersteund door activiteitsmetingen die een 3 keer hogere nitriet oxidatie capaciteit aantoonden. Gebaseerd op deze uitkomsten zijn er twee hypothesen gemaakt: of *Nitrobacter* groeit mixotrofisch op acetaat afhankelijke dissimilative nitraat reductie (*Ping-pong effect*) of een nitriet oxidatie/nitraat reductie loop (*nitriet loop*) heeft plaats gevonden waarbij denitrificerende bacteriën nitraat naar nitriet gereduceerd hebben en dus extra nitriet voor de groei van NOB beschikbaar gemaakt hebben. De disproportionele fracties AOB en NOB zouden beter onderzocht moeten worden om de hypotheses gemaakt in dit werk te kunnen bevestigen.

Hoofdstuk 4 is gericht op de vraagstelling of de structuur van een korrel en een vlok het slib retentie tijd van individuele bacteriële groepen verschillend beïnvloed. Effluent and reactor slib zijn van een korrel en vlokken reactor bemonsterd worden en de aantallen van een specifieke bacteriële group was geëvalueerd in betrekking tot de totale bacteriële populatie aan hand van quantitative polymerase chain reaction (qPCR). Operationele data zijn gecombineerd worden met moleculaire technieken en de SRT van een individuele bacterium was berekend. Verder is uitgevonden dat protozoa bacteriën als voedsel bron gebruiken. Dit geeft aan dat protozoa de potentiaal hebben om het slib retentie tijd van de bacterie te verlagen. Verder zijn archea niet gevonden in vlokken maar waren wel aanwezig in kleine hoeveelheden in een korrel. **Hoofdstuk 5** geeft een overzicht over toekomstige combinatie mogelijkheden van moleculaire en operationeel data in biotechnologie.

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CURRICULUM VITAE

Mari-Karoliina Henriikka Winkler was born on July 9th 1980 in Hannover, Germany. Since her mother is from Finland she has a German-Finnish background. In 2001 she started to study Water Science at the University of Duisburg Essen, Germany. Her bachelor thesis she did in the field of microbiology at the University of New South Wales in Sydney, Australia in 2004. Her Master thesis she accomplished in the field of biotechnology at the University of British Columbia, in Vancouver Canada and received her Master degree in 2006. During her study time she worked part-time in several research institutes to gain some practical knowledge in different research fields. After the university she worked for circa 2 years in the industry at Macherey-Nagel as Sales manager in Germany and Austria. In 2008 she joined the Environmental Biotechnology group of Mark van Loosdrecht at Delft University of Technology, The Netherlands to start her PhD. Part of her research she accomplished at the Columbia University, in New York, USA.

ACCEPTED OR SUBMITTED PUBLICATIONS

Winkler, M-K.H., J. P. Bassin, R. Kleerebezem, M.C.M. van Loosdrecht, and T. P. H. van den Brand, 2011, Selective sludge removal in a segregated aerobic granular biomass system as a strategy to control PAO-GAO competition at high temperatures: Accepted in: *Water Research*

Winkler, M-K.H., R. Kleerebezem, J.G. Kuenen, J. Yang, and M.C.M. van Loosdrecht, 2011, Segregation of biomass in cyclic anaerobic/aerobic granular sludge allows the enrichment of Anaerobic Ammonium Oxidizing Bacteria at low temperatures: Accepted in: *Environmental Science and Technology*

Winkler, M-K.H., Kleerebezem R., van Loosdrecht. M.C.M., 2012 Integration of Anammox into the aerobic granular sludge process for mainstream wastewater treatment at ambient temperatures, Accepted in: *Water Research*

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Winkler M-K.H., Bassin Joao. P., Kleerebezem R, Sorokin D, van Loosdrecht M.C.M, Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge, Accepted in: *Applied environmental biotechnology*

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Winkler M-K H., Bassin J.P, Kleerebezem R., van der Lans R. G. J. M, van Loosdrecht M.C.M. Temperature and salt effects on settling velocity in granular sludge technology, Accepted in: *Water Research*

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PUBLICATIONS IN PREPARATION

Kim Y, Winkler, M-K.H., Kleerebezem R., van Loosdrecht M.C.M., Chandran K, Monitoring the effect of inorganic carbon limitation on a pure *Nitrobacter winogradskyi* culture

PRESENTATIONS AND CONFERENCE PROCEEDINGS

WEF/IWA Biofilm Reactor Technology Conference 2010, Portland, Oregon, (USA) Winkler M-K H., Kleerebezem R., Bassin J. P.,. de Kreuk M.K , de Bruin L.M.M, van Loosdrecht M.C. M. Segregation of biomass in aerobic granular sludge favours phosphate accumulating organisms over glycogen accumulating organisms at high temperatures (oral presentation)

IWA Leading Edge Technology Conference 2011, Amsterdam, (THE NETHERLANDS) Segregation of biomass in cyclic anaerobic/aerobic granular sludge allows the enrichment of Anaerobic Ammonium Oxidizing Bacteria at low temperatures (poster presentation)

IWA Biofilm Conference 2011: Processes in Biofilms, Shanghai, (CHINA), Winkler M-K H., Kleerebezem R., de Bruin L.M.M, Verheijnen P, van Loosdrecht M.C.M. Microbial diversity differences in aerobic granular sludge in comparison to conventional treatment plant (oral presentation by a co-author)

Microbial resource management in biotechnology, Ghent, (BELGIUM) Winkler, M. K. H., R. Kleerebezem, J. G. Kuenen, J. Yang, and M. C. M. van Loosdrecht, 2011, Segregation of biomass in cyclic anaerobic/aerobic granular sludge allows the enrichment of Anaerobic Ammonium Oxidizing Bacteria at low temperatures (poster presentation)

Invited colloquium at the Institute of Water Quality Control and Waste Management Technical University of Munich, (GERMANY) topic "aerobic granular sludge technology"

Colloquium "Segregation in aerobic granular sludge" at Cornell University, Ithaca, NY, (USA) (oral presentation)

Colloquium "Segregation of biomass: Selective sludge control as a method to engineer reactor performance" at University of Cape Town, (AFRICA) (oral presentation)

IWA BeNeLux Young Water Professionals 2nd Regional Conference presentation on "Nereda and Anammox as case studies" (THE NETHERLANDS) (oral presentation)

Presentation "Anammox in the mainstream" at IFAT 2012, Munich, (GERMANY) (oral presentation)

AWARDS AND SCHOLARSHIPS

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HUBER TECHNOLOGY PRIZE 2012: international contents, 1st prize for best article on energy from wastewater, Bavarian Minister of Environment, (GERMANY)

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