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AEROBIC GRANULAR SLUDGE

Effect of Substrate on Granule Formation

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 28 April 2016 om 15:00 uur

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geboren te Den Haag, Nederland

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For me

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Summary

Discharging untreated wastewater will contaminate the surface waters and can lead to spread of diseases and long term ecological damage. The most common method for treatment is by the activated sludge process. In this process, nutrients like nitrogen, phosphorus and COD are removed by bacteria grown in flocs. These bacterial flocs are separated from the treated water by settling. Due to the slow settling velocities of these flocs large settling tanks are needed. Settling tanks take up most of the required space for a wastewater treatment plant. Aerobic granular sludge is a compact technology designed to reduce area requirements, save energy while providing excellent effluent quality. Bacteria are grown in granules instead of flocs and have therefore a much higher settling velocity. This reduces the area requirement significantly. So much even so, that external settling tanks are completely omitted. To grow aerobic granules a few selection principles are needed. First, the influent is brought in contact with the biomass in an anaerobic environment. Here COD is converted by the bacteria into storage polymers. These storage polymers are then used for growth in the presence of oxygen hereby removing phosphorus and nitrogen from the bulk liquid. Secondly, a settling pressure is applied by which slow settling biomass is removed from the reactor, thus leading to the formation of granules.

In previous research by the PhD students Janneke Beun (principle of aerobic granulation), Merle de Kreuk (basic process technology for granular sludge nutrient removal) and Mari Winkler and Joao Bassin (Microbiology and process engineering aspects of granular sludge) the basic concepts of the granular sludge technology were worked out. In this thesis the effects of several operational conditions on the conversion processes, formation and stability of aerobic granular sludge was studied. The quick implementation of the technology in practice also meant that several important subjects still needed further investigation. To ensure a well-functioning technology in domestic and industrial applications these subjects were studied in more detail (i.e. adsorption, effect of salinity, higher temperature and other substrates). Besides laboratory work also the start-up and performance of one of the first full-scale aerobic granular sludge reactors treating domestic wastewater is described.

The ammonium adsorption properties of aerobic granular sludge, activated sludge and anammox granules have been investigated in **Chapter 2**. During operation of a pilot-scale aerobic granular sludge reactor, a positive relation between the ammonium influent concentration and the ammonium adsorbed was observed. Aerobic granular sludge exhibited much higher adsorption capacity compared to activated sludge and anammox granules. At an ammonium concentration of 30 mg N L^{-1}, adsorption obtained with activated sludge and anammox granules was around 0.2 mg NH_4^+-N $gVSS^{-1}$, while aerobic granular sludge from lab- and pilot scale exhibited an adsorption of 1.7 and 0.9 mg NH_4^+-N $gVSS^{-1}$, respectively. No difference in the ammonium adsorption was observed in lab-scale reactors operated at different temperatures (20 and 30 °C). In a lab-scale reactor fed with saline wastewater, we observed that the amount of ammonium adsorbed, decreased

considerably when the salt concentration increased. The results indicate that adsorption or better: ion-exchange of ammonium should be incorporated into models for nitrification/denitrification, certainly when aerobic granular sludge is used.

Salinity can adversely affect the performance of most biological processes involved in wastewater treatment (**Chapter 3**). The effect of salt (NaCl) on the main conversion processes in an aerobic granular sludge (AGS) process accomplishing simultaneous organic matter, nitrogen, and phosphate removal was evaluated in this chapter. Hereto an AGS sequencing batch reactor was subjected to different salt concentrations (0.2 to 20 g Cl$^-$ L^{-1}). Granular structure was stable throughout the whole experimental period, although granule size decreased and a significant effluent turbidity was observed at the highest salinity tested. A weaker gel structure at higher salt concentrations was hypothesized to be the cause of such turbidity. Ammonium oxidation was not affected at any of the salt concentrations applied. However, nitrite oxidation was severely affected, especially at 20 g Cl$^-$ L^{-1}, in which a complete inhibition was observed. Consequently, high nitrite accumulation occurred. Phosphate removal was also found to be inhibited at the highest salt concentration tested. Complementary experiments have shown that a cascade inhibition effect took place: first, the deterioration of nitrite oxidation resulted in high nitrite concentrations and this in turn resulted in a detrimental effect to polyphosphate-accumulating organisms (PAOs). By preventing the occurrence of the nitrification process and therefore avoiding the nitrite accumulation, the effect of salt concentrations on the bio-P removal process was shown to be negligible up to 13 g Cl$^-$ L^{-1}. Salt concentrations equal to 20 g Cl$^-$ L^{-1} or higher in absence of nitrite also significantly reduced phosphate removal efficiency in the system.

When aerobic granular sludge is applied for industrial wastewater treatment different soluble substrates can be present. For stable granular sludge formation on volatile fatty acids (e.g. acetate), production of storage polymers under anaerobic feeding conditions has been shown to be important. This prevents direct aerobic growth on readily available COD, which is thought to result in unstable granule formation. In **Chapter 4** we investigate the impact of acetate, methanol, butanol, propanol, propionaldehyde and valeraldehyde on granular sludge formation at 35 °C. Methanogenic archaea, growing on methanol, were present in the aerobic granular sludge system. Methanol was completely converted to methane and carbon dioxide by the methanogenic archaeum *Methanomethylovorans uponensis* during the one-hour anaerobic feeding period, despite the relative high dissolved oxygen concentration (3.5 mg O$_2$ L^{-1}) during the subsequent two-hour aeration period. Propionaldehyde and valeraldehyde were fully disproportionated anaerobically into their corresponding carboxylic acids and alcohols. The organic acids produced were converted to storage polymers, while the alcohols (produced and from influent) were absorbed onto the granular sludge matrix and converted aerobically. Our observations show that easy biodegradable substrates not converted anaerobically into storage polymers could lead to unstable granular sludge formation. However, when the

easy biodegradable COD is absorbed in the granules and/or when the substrate is converted by relatively slow growing bacteria in the aerobic period stable granulation can occur.

The influence of sludge age on granular sludge formation and microbial population dynamics in a methanol- and acetate-fed aerobic granular sludge system operated at 35 °C is investigated in **Chapter 5**. During anaerobic feeding of the reactor, methanol was initially converted to methane by methylotrophic methanogens. These methanogens were able to withstand the relatively long aeration periods. Lowering the anaerobic solid retention time (SRT) from 17 to 8 days enabled selective removal of the methanogens and prevented unwanted methane formation. In absence of methanogens, methanol was converted aerobically, while granule formation remained stable. At high SRT-values (51 days) γ-Proteobacteria were responsible for acetate removal through anaerobic uptake and subsequent aerobic growth on storage polymers formed (so called metabolism of glycogen accumulating organisms). When lowering the SRT (24 days), Defluviicoccus-related organisms (cluster II) belonging to the α-Proteobacteria outcompeted acetate consuming γ-Proteobacteria at 35 ºC. DNA from the Defluviicoccus-related organisms in cluster II was not extracted by the standard DNA extraction method but with liquid nitrogen, which showed to be more effective. Remarkably, the two glycogen accumulating organisms (GAO) types of organisms grew separately in two clearly different types of granules. This work further highlights the potential of aerobic granular sludge systems to effectively influence the microbial communities through sludge age control in order to optimize the wastewater treatment processes.

Recently, aerobic granular sludge technology has been scaled-up and implemented for industrial and municipal wastewater treatment under the trade name Nereda®. With full-scale references for industrial treatment application since 2006 and domestic sewage since 2009 only limited operating data have been presented in scientific literature so far. In this study performance, granulation and design considerations of an aerobic granular sludge plant on domestic wastewater at the WWTP Garmerwolde, the Netherlands were analysed (**Chapter 6**). After a start-up period of approximately 5 months, a robust and stable granule bed (> 8 g L^{-1}) was formed and could be maintained thereafter, with a sludge volume index after 5 minutes settling of 45 mL g^{-1}. The granular sludge consisted for more than 80 % of granules larger than 0.2 mm and more than 60 % larger than 1 mm. Effluent requirements (7 mg N L^{-1} and 1 mg P L^{-1}) were easily met during summer and winter. Maximum volumetric conversion rates for nitrogen and phosphorus were respectively 0.17 and 0.24 kg (m3 d)$^{-1}$. The energy usage was 13.9 kWh (PE150·year)$^{-1}$ that is 58 – 63 % lower than the average conventional activated sludge treatment plant in the Netherlands. Finally, this study demonstrated that aerobic granular sludge technology can effectively be implemented for the treatment of domestic wastewater.

Samenvatting

Het lozen van onbehandeld afvalwater vervuilt het oppervlaktewater en kan leiden tot verspreiding van ziekten en lange termijn ecologische schade. De meest voorkomende methode voor de behandeling van afvalwater is doormiddel van het actief slib proces. In het actief slib proces worden nutriënten zoals stikstof, fosfaat en CZV verwijderd door bacteriën gekweekt in vlokken. Deze bacteriologische vlokken worden gescheiden van het behandelde water doormiddel van bezinking. Door de trage bezinkingssnelheden van deze vlokken zijn grote bezinktanks nodig. Deze tanks nemen het grootste deel van de benodigde ruimte voor een afvalwaterzuiveringsinstallatie in. Aëroob korrelslib is een compacte technologie ontworpen om het vereiste oppervlak te verminderen, energie te besparen met behoud van de effluent kwaliteit. In het aeroob korrelslib proces worden bacteriën gekweekt in korrels in plaats van vlokken. Dit leidt tot een veel hogere bezinksnelheid en hiermee reduceert het vereiste oppervlakte voor een zuivering aanzienlijk. Zozeer zelfs, dat externe bezinking tanks volledig overbodig zijn. Er zijn een aantal selectiecriteria nodig voor de vorming van aeroob korrelslib. Om te beginnen wordt er een anaerobe voeding toegepast waarbij COD wordt omgezet door de bacteriën in opslagpolymeren. Deze opslagpolymeren worden vervolgens gebruikt voor groei in de aanwezigheid van zuurstof en worden fosfaat en stikstof verwijderd uit de bulk. Een tweede selectiecriterium is de bezinkdruk. Hierdoor wordt langzaam bezinkende biomassa verwijderd uit de reactor, wat leidt tot de vorming van snel bezinkende korrels.

In voorgaande onderzoeken van de promovendi Janneke Beun (principe van aerobe korreling), Merle de Kreuk (basis procestechnologie voor korrelslib verwijdering van nutriënten) en Mari Winkler en Joao Bassin (microbiologie en procestechniek van korrelslib) werden de basisbegrippen van de korrelslibtechnologie uitgewerkt. In dit proefschrift worden de effecten van de verschillende operationele omstandigheden op de omzetting, de vorming en de stabiliteit van aeroob korrelslib onderzocht. De snelle invoering van de technologie in de praktijk betekende ook dat een aantal belangrijke onderwerpen onvoldoende zijn onderzocht. Om een goed functionerende korrelslibtechnologie in huishoudelijke en industriële toepassingen te kunnen garanderen zijn deze onderwerpen in meer detail bestudeerd (onder andere adsorptie, het effect van zout, hogere temperatuur en andere substraten). Naast het laboratoriumwerk is ook de opstart van één van de eerste aeroob korrelslib installaties die huishoudelijk afvalwater behandeld beschreven.

Ammonium adsorptie eigenschappen van aeroob korrelslib, actief slib en Anammox-korrels worden beschreven in **hoofdstuk 2**. Tijdens testen in het laboratorium met aeroob korrelslib is een positief verband waargenomen tussen de ammonium influent concentratie en ammonium geadsorbeerd aan het korrelslib. Aeroob korrelslib vertoonde een veel hogere adsorptiecapaciteit vergeleken met actief slib en anammox-korrels. Bij een ammonium concentratie van 30 mg N L^{-1}, adsorptie aan actief slib en anammox-korrels was ongeveer 0,2 mg NH_4^+ -N $gVSS^{-1}$. Aeroob korrelslib uit laboratorium- en pilot-schaal

testen vertoonde een adsorptie van 1,7 en 0,9 mg NH_4^+ -N $gVSS^{-1}$, respectievelijk. Er werd geen verschil waargenomen in de ammonium adsorptie in laboratoriumschaal reactoren bij verschillende temperaturen (20 en 30 °C). In een laboratoriumschaal reactor gevoed met zout afvalwater namen wij waar dat de hoeveelheid geadsorbeerd ammonium aanzienlijk verminderde wanneer de zoutconcentratie werd verhoogd. De resultaten wijzen erop dat adsorptie of beter: ionenwisseling van ammonium in modellen moeten worden opgenomen voor nitrificatie/denitrificatie, zeker als aeroob korrelslib wordt gebruikt.

Zoutgehalte kan een negatieve invloed hebben op de meeste biologische processen die betrokken zijn bij de behandeling van afvalwater (**hoofdstuk 3**). Het effect van zout (NaCl) op de belangrijkste omzettingsprocessen in het aeroob korrelslib proces werd geëvalueerd in dit hoofdstuk. Een aeroob korrelslib reactor werd onderworpen aan verschillende zoutconcentraties (0,2 - 20 g Cl L^{-1}). De korrelstructuur bleef stabiel gedurende de gehele experimentele periode, hoewel korrelgrootte verminderde en een aanzienlijke troebelheid in het effluent werd waargenomen bij de hoogste saliniteit. Een zwakkere gelstructuur bij hogere zoutconcentraties is hier waarschijnlijk de oorzaak van. De ammoniumoxidatie werd niet beïnvloed door de zoutconcentraties die zijn getest. De nitriet oxidatie werd volledig geremd bij zout concentraties hoger dan 20 g Cl L^{-1}. Dit had als gevolg dat er hoge nitriet concentraties werden bereikt in het effluent. De fosfaatverwijdering bleek hierdoor ook te worden geremd. Aanvullende experimenten hebben aangetoond dat een cascade inhibitie effect heeft plaatsgevonden: als eerste, de verslechtering van nitriet oxidatie leidde tot hoge nitriet concentraties in de bulk wat uiteindelijk weer resulteerde in een nadelig effect voor poly-fosfaat accumulerende organismen (PAO's). Zonder het nitrificatieproces bleek de biologische fosfaat verwijdering stabiel tot aan een zoutconcentratie van 13 g Cl L^{-1}. Bij hogere zoutconcentraties zonder nitriet accumulatie verslechterde de fosfaat verwijdering ook significant.

Aeroob korrelslib wordt ook toegepast op industrieel afvalwater. Hier kunnen verschillende oplosbare substraten aanwezig zijn. Voor stabiele korrelvorming bij vluchtige vetzuren (b.v. acetaat), is het belangrijk dat er productie van opslag polymeren onder anaërobe voedingsomstandigheden is. Dit verhindert directe aerobe groei op makkelijk omzetbaar CZV, waarvan men denkt dat dit tot instabiele korrelvorming leidt. In **hoofdstuk 4** wordt het effect van acetaat, methanol, butanol, propanol, propionaldehyde en valeraldehyde op 35 °C onderzocht. Methanogene archaea gebruikten het methanol onder anaerobe omstandigheden voor groei in het aeroob korrelslib systeem. Methanol werd volledig omgezet in methaan en kooldioxide door *Methanomethylovorans uponensis* tijdens de één uur anaërobe voedingsperiode. Dit ondanks de relatief hoge concentratie van opgeloste zuurstof (3,5 mg O_2 L^{-1}) tijdens de daaropvolgende twee uur durende aërobe periode. Propionaldehyde en valeraldehyde werden volledig anaëroob gedisproportioneerd in hun overeenkomstige carbonzuren en alcoholen. De organische zuren die werden geproduceerd werden omgezet in opslagpolymeren, terwijl de alcoholen (geproduceerd en afkomstig van

het influent) werden geabsorbeerd in de korrelslib matrix en werden uiteindelijk aeroob omgezet. Onze waarnemingen tonen aan dat gemakkelijk biologisch afbreekbare substraten die niet anaeroob omgezet worden in opslagpolymeren kunnen leiden tot instabiele korrelslib formatie. Echter wanneer het gemakkelijk biologisch afbreekbaar CZV wordt geabsorbeerd in de korrels en/of wanneer het substraat wordt omgezet door relatief langzaam groeiende bacteriën in de aerobe periode kan stabiele granulatie optreden.

De invloed van slibleeftijd op de korrelvorming en de microbiële populatie dynamica in een met methanol- en acetaat gevoede aeroob korrelslib systeem bedreven bij 35 °C wordt onderzocht in **hoofdstuk 5**. Tijdens de anaërobe voeding van de reactor werd methanol eerst omgezet in methaan door methylotrofe methanogenen. Deze methanogenen konden de relatief lange perioden beluchting overleven. Het verlagen van de anaërobe vaste stoffen retentietijd (SRT) van 17 naar 8 dagen resulteerde in de selectieve verwijdering van de methanogenen en het voorkomen van ongewenste methaanvorming. Bij afwezigheid van methanogenen werd methanol aëroob omgezet, terwijl de korrelvorming stabiel bleef. Bij hoge SRT-waarden (51 dagen) waren γ-Proteobacteria verantwoordelijk voor de verwijdering van acetaat door middel van anaërobe opname en de daaropvolgende aërobe groei op de gevormde opslagpolymeren (het zogenaamde metabolisme van glycogeen accumulerende organismen). Bij het verlagen van de SRT naar 24 dagen, namen de *Defluviicoccus*-verwante organismen (cluster II) behorende tot de α-Proteobacteria de plaats in van actetaat consumerende γ-Proteobacteria bij 35 °C. Het DNA van de *Defluviicoccus*-verwante organismen in cluster II werd niet geëxtraheerd door de standaard DNA-extractie methode, maar wel met vloeibare stikstof. Opmerkelijk is dat de twee GAO soorten afzonderlijk groeiden in twee duidelijk verschillende typen korrels. Dit werk wijst verder op de mogelijkheden van aeroob korrelslib systemen om met de slibleeftijd de microbiële samenstelling te kunnen beïnvloeden. Dit is uiteindelijk van belang om de behandeling van het afvalwater te optimaliseren.

Onlangs is het aeroob korrelslib proces opgeschaald en geïmplementeerd voor industrieel en huishoudelijk afvalwater onder de handelsnaam Nereda®. Sinds de introductie sinds 2006 voor industriële en huishoudelijk afvalwater toepassingen sinds 2009 zijn er slechts beperkte operationele gegevens gepresenteerd in de wetenschappelijke literatuur. In **hoofdstuk 6** zijn de prestaties, granulatie en overwegingen bij het ontwerp van een aeroob korrelslib plant op huishoudelijk afvalwater in Garmerwolde, Nederland, geanalyseerd. Na een opstartperiode van ongeveer 5 maanden, is een robuust en stabiel korrel bed (> 8 gram L^{-1}) gevormd. Het slib volume index na 5 minuten bezinken bedroeg 45 mL g^{-1}. Het korrelslib bestond voor meer dan 80 % uit korrels groter dan 0,2 mm en meer dan 60 % groter dan 1 mm. Gedurende de zomer en winter perioden werd er volledig voldaan aan de effluent eisen (7 mg N L^{-1} en 1 mg P L^{-1}). Maximale volumetrische conversie snelheden voor stikstof en fosfaat waren respectievelijk 0,17 en 0,24 kg $(m^3 d)^{-1}$. Het energieverbruik was 13,9 kWh (PE150 · jaar) $^{-1}$, wat 58 - 63 % lager is dan een gemiddelde conventioneel actief slib zuiveringsinstallatie in Nederland. Tot slot toont deze studie aan dat aeroob korrelslib als technologie effectief kan worden geïmplementeerd voor de behandeling van huishoudelijk afvalwater.

1

General introduction

This chapter starts with an introduction into conventional wastewater treatment and its associated processes, conversions and bottlenecks. Hereafter, a short history of how aerobic granular sludge came into the spotlight. Then, the differences between aerobic granular and activated sludge are discussed as well as the conversions and main advantages one could expect in the aerobic granular sludge process.

Conventional wastewater treatment

Wastewater flushed down our drains (e.g. toilet, shower and industry) into the sewer eventually ends up into our environment. Before it can be safely discharged to the environment, the pollutants it contains have to be removed. Discharging untreated wastewater will contaminate the surface waters, causes oxygen depletion and can lead to spread of diseases and long term ecological damage. Activated sludge was first described by Adern and Locket in (1914). They were the first to use the term 'activated sludge' to describe the treatment of wastewater by microorganisms growing in flocs that are eventually separated from the liquid by settling. Since then, wastewater treatment has become an integral part of our society.

Physical removal of solids

A first important step in the treatment of wastewater is the removal of solids known as the primary treatment. Here, screens, grit removal and a (primary) clarifier are used for the separation of solids and liquid (Fig. 1 and 3). In the latter, suspended solids that were not removed during the screening, are removed by settling. This so-called primary sludge is then tickend and transported to a digester. During primary clarification, fat is also removed to prevent maintenance problems in the remainder of the plant.
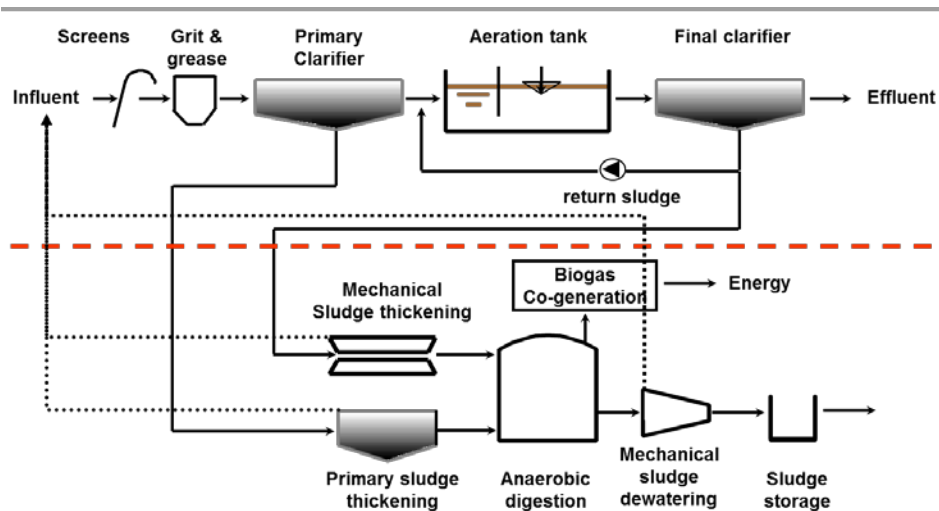
Fig. 1 - schematic representation of a conventional wastewater treatment plant.

Biological removal of contaminants

Although the suspended solids are largely removed during primary treatment, the wastewater is far from suitable to be discharged. The wastewater is still full of dissolved organic matter, nitrogen and phosphorus, all of which have to be removed extensively. Removal of these pollutants is achieved by promoting the growth of specific bacterial species by creating suitable environmental conditions. This step in the treatment of wastewater is called the secondary or biological treatment (aeration tank). Suspended biomass, in the form of sludge flocs, is used to remove the remaining pollutants. Generally, this secondary treatment consists of a biological part that can be separated into an anaerobic, anoxic and an aerobic tank, depending on the selected biological processes to happen. Often, there are still wastewater treatment plants with an anaerobic part. How this influences the process is discussed later on.

The anaerobic part is used to optimize the conversion of easy biodegradable COD into storage polymers by polyphosphate and glycogen accumulating organisms (PAO, GAO) (Fig. 2). Most of this readily available COD are volatile fatty acids (i.e. acetate, propionate etc.) and are fully removed. Besides this, COD is also present in the form of suspended and colloidal solids that were not removed in the primary treatment. These need to be hydrolyzed first before they become available for the bacteria. Since hydrolysis is a relatively slow process not all the solids are converted in the anaerobic part and are thus

passed on to the aerobic part. In the anoxic and aerobic tank, the remainder of these solids will be caught in the flocs and converted by bacteria. Nitrogen and phosphorus that is present in soluble form in the wastewater is removed by bacterial species like nitrifiers and
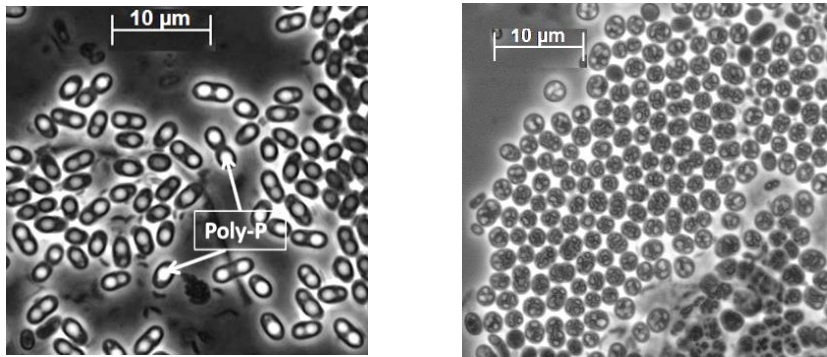
Fig. 2 - (left) phosphate accumulating organisms (Candidatus Accumulibacter phosphatis), (right) glycogen accumulating organisms (Candidatus Competibacter)

PAOs respectively. Under aerobic conditions, PAOs use their internal storage polymers to generate energy for the uptake of phosphate, thus forming poly-phosphate internally. With the conversion of ammonium by ammonium and nitrite oxidizing bacteria (AOB, NOB) nitrate is formed.

In the anoxic tank, the produced nitrate is converted to dinitrogen gas and removed from the system. A recycle flow with high nitrate concentrations is recycled from the aerobic to the anoxic tank via pumps. Storage polymers of the PAOs and GAOs mostly serve here as the electron donor for the denitrification process. In cases where there is a shortage of electron donor an externally dosed substrate (such as glycerol, methanol, bio-ethanol etc.) is used to drive the denitrification process.

Physical separation of effluent

Finally, the treated wastewater has to be separated from the flocculent biomass (Fig. 1 and 3). Large settling tanks called secondary clarifiers are used for this. The flocs settle to the bottom and clear effluent is discharged onto the surface water. Most of the settled sludge is pumped back to the anaerobic tank to maintain a correct biomass concentration in the system. Usually 3 - 4 g L^{-1}, but this can vary greatly between installations. Since the microorganisms use the removed organic matter and nutrients for growth, some sludge has to be removed (surplus sludge) and after thickening it is pumped or transported to anaerobic digesters or directly to incinerations, or landfills and agriculture. Fat (removed

during the primary treatment), primary sludge and surplus sludge are collected and usually further stabilized during digestion. Biogas formed in the digesters is used to generate energy that can be used to (partly) operate the treatment plant itself or provide energy elsewhere.

Fig. 3 - WWTP Harnaschpolder (1.3 million P.E.), Den Hoorn, the Netherlands. 4x Primary clarifiers (1) 8x Secondary treatment (2) and 16x secondary clarifiers (3)

Improved sewage treatment systems

The ever-increasing population in metropolitan areas is leading to more discharge of wastewater in these relatively dense areas. It is here, that the need for compact sewage treatment plants is most obvious. Effluent limits have also become more stringent in the last decades. To accommodate both the increase in wastewater flow and the stricter discharge limits today and in the future, WWTPs need to extend significantly on site or relocate to a new location. Available building areas within the metropolitan areas are limited. Moreover, the construction of large pressure mains through a metropolitan area to a new wastewater treatment plant is very expensive.

Conventional activated sludge plants rely on large volumes with relatively low concentrations of biomass ($3 - 4$ g L^{-1}), although as mentioned before this can vary greatly. For most plants, achieving higher biomass concentrations is not feasible, due to a relatively

high volume per gram settled biomass and a decrease in the oxygen transfer in high concentrated sludge water mixtures (Krampe and Krauth 2003). To separate the slow settling activated sludge flocs from the water, large areas of secondary clarifiers are needed. Designs of the surface area of secondary clarifiers are based on the sludge volume index (SVI) in relation to the expected hydraulic load under rain weather conditions (peak flow) Enforcing higher biomass concentrations in conventional activated sludge plants would therefore demand an increase in the area of the secondary clarifiers. Thus, the usual solution to accommodate a significantly higher wastewater flow in an existing conventianl activated sludge (CAS) plant is to increase the available area. This is in many cases simply not possible. Treating more wastewater with better effluent quality on the same area requires a more efficient and compact plant. Because significant increases in biomass concentration in CAS plant are limited, there are alternative ways to achieve higher biomass concentrations and thus compact plants. Most compact technologies try to reduce or prevent the usage of settling tanks.

Membrane Bioreactors

Membrane bioreactors (MBR) are such a compact system. The sludge is separated from the effluent either with membranes submerged in the sludge tank or via side-stream membrane units. This enables complete separation of biomass and effluent solving the secondary clarifier area 'problems' with its slow settling activated sludge flocs. MBRs are therefore able, like secondary clarifiers in CAS plants, to uncouple the solid from the hydraulic retention time (SRT and HRT) and, in doing so, efficiently allow for very high biomass concentrations (10 - 30 g L^{-1}). In practice, biomass concentrations are maintained between 5 – 10 g L^{-1} due to the aeration efficiency loss at higher biomass concentrations (Melin et al. 2006). Effluent quality is usually very good especially regarding the suspended solids.

However, there are also some drawbacks associated with MBRs. These are primarily all related to bio-fouling of the membranes used to separate the effluent from the biomass, leading to higher energy usage and investments costs (Keerthi and Balasubramanian 2014). This bio-fouling leads to reduced fluxes, higher energy requirements and consumption of cleaning chemicals. The necessity of cleaning the membranes with chemicals is laborious and expensive (Guo et al. 2014). For example with submerged membranes the bio-fouling is partly prevented by turbulent aeration, which requires coarse bubbles. Yet, coarse bubbles are less efficient to transfer oxygen and therefore its use leads to relatively high

energy requirements. Generally $0.5 - 2.5$ kWh m^{-3} of treated wastewater is needed depending on the type of MBR plant (Krzeminski et al. 2012). For CAS this ranges from $0.1 - 0.6$ kWh m^3 (Gnirss and Dittrich 2000, Cornel et al. 2003). In the end, the overall operational costs of MBR's are limiting its application in the treatment of sewage.

Moving Bed Biofilm Reactor

Moving bed biofilm reactors (MBBR) is another example of a compact technology that results in less surface area needed and that makes use of biofilm kinetics. The MBBR was developed in the 1980s (Odegaard et al. 1993, Rusten et al. 1992). MBBR's are normally used to increase the capacity of existing plants. A carrier material is needed in order to form and maintain a biofilm. A variety of carriers (size, density, internal surface area etc.) are commercially available for use. To keep the carriers in the reactor a sieve of some sorts is needed to separate the carriers from the effluent. Aeration is not only used to provide oxygen, but is necessary to keep the carriers moving throughout the reactor. A mechanical stirrer is generally used to obtain anoxic and anaerobic conditions. Although a biofilm will develop on the carriers, still a significant fraction of the biomass is present as suspended biomass. To allow good separation between biomass and effluent an external settler is needed to obtain the required effluent quality. Although this technology makes use of the advantages of biofilms, this necessity of large settlers is a severe drawback.

Aerobic granulation by changing the morphology

Instead of increasing the biomass concentration by means of more energy input (like with MBR plants) or adding carrier materials, another possible option is to change the morphology of the biomass itself so that the settling velocity is increased. This can be achieved with the transition of flocs into granules (Fig. 4). The higher settling velocities (10 - 70 m h^{-1}) of aerobic granules (Winkler et al. 2012a) compared to activated sludge flocs (0.8 – 1.4 m h^{-1}) allow for the integration of the settler in the treatment reactor and thus lead to a compact reactor design.
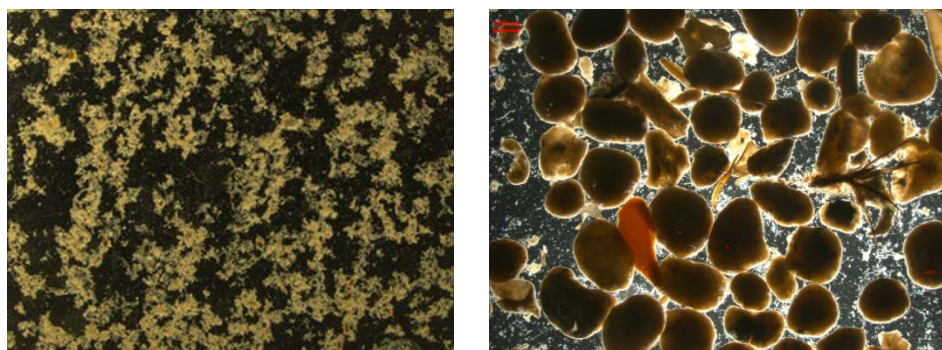
Fig. 4 - (left) activated sludge from WWTP Harnaschpolder, the Netherlands, (right) washed aerobic granules from an aerobic granular sludge plant in Garmerwolde, the Netherlands.

Mid 1990's marked the appearance of aerobic granular sludge in literature (Morgenroth et al. 1997). Until this point in time, granulation was mostly considered to be related to methanogenic processes. It was thought that the complex community structures required to convert substrates anaerobically drove the formation of the granules. Aerobic conversion of such substrates was believed to be much easier and did not need syntrophic interactions between species and would thus not lead to granulation. So granulation studies were conducted primarily on anaerobic reactors.

Reducing costs of wastewater treatment was (and is) particularly of interest for industrial sites. Reducing the area required to treat their wastewater is a strong incentive as this is a direct cost reduction. Mid 1970s the CSM (sugar factory) worked together with Wageningen University to develop a compact anaerobic system to treat wastewater derived from sugar beet processing. This cooperation led to the Upflow Anaerobic Sludge bed Reactor (UASB) (Lettinga et al. 1975). During the 1980s also Gist-Brocades became

interested to develop such a compact system (Heijnen 1984, Heijnen et al. 1990). They used a fluidised bed system with biofilm on a carrier that leads to very short hydraulic retention times. This later led to the development of the Extended Granular Sludge Bed (EGSB) reactor (Lettinga and Hulshoff Pol 1986, Van der Last and Lettinga 1992). Besides the anaerobic treatment, conversion under aerobic conditions in EGSB like systems was also attempted and resulted in the CIRCOX reactor, which is based on the use of a carrier material (basalt) for the formation of biofilms (Frijters et al. 1997a). The CIRCOX reactor was originally developed to treat the effluent of anaerobic reactors by removing COD, ammonium and sulphides aerobically.

A translation from the compact anaerobic biofilm systems to a technology for domestic wastewater treatment was still lacking in the early 1990s due to the belief that aerobic conversion of easy biodegradable COD would not lead to stable granule formation. The question was how to change the morphology of a slow settling floc to a dense fast settling granule under aerobic conditions as to make it applicable for the treatment of domestic wastewater. A hypothesis was postulated, that the ratio between biofilm surface loading (and the rate at which new biomass is produced) and shear rate determines the biofilm structure. When shear forces are relatively high, only a patchy biofilm will develop, whereas at low shear rates the biofilm becomes highly heterogeneous with many pores and protuberances. In case of a right balance, smooth and stable biofilms can be obtained (Van Loosdrecht et al. 1995).

First proof of principle for aerobic granulation was eventually shown by Heijnen and van Loosdrecht (1998) with the application of a patent for acquiring aerobic granules. The granules were grown aerobically on molasses, derived from crystallized sugar production from sugar beets (50% saccharose) in a sequencing batch reactor (SBR). By applying a short sedimentation time of 1 minute granulation could be obtained. Fast settling granules were able to remain in the system while the slow settling flocs were washed out. Although granules were formed initially, stable long-term operation was found to be problematic (Morgenroth et al. 1997). The reason for this was not clear at the time. Kwok et al. (1998) showed that by adding basalt a more stable biofilm formation could be achieved. The basalt acted not only as a carrier for the initial biofilm attachment, but also induced enough shear. It was shown that higher basalt concentrations will lead to a higher biofilm density and a higher achievable biomass concentration in the reactor. The conclusion was thus,

that fast growth rates of bacteria grown aerobically on easy biodegradable COD, require a shear rate that balanced outgrowth and detachment. This underlined once more the hypothesis (see above) made previously by Van Loosdrecht et al. (1995).

Beun et al. (1999) showed aerobic granulation (based on the data that was used in the patent) in an SBR system fed with ethanol. Filamentous fungal pellets functioned as an immobilization matrix in which bacteria could grow out to colonies. Once the colonies were large enough the fungal pellets fell apart and the colonies were able to remain and grow in the system. Later (Beun et al. 2002) aerobic granulation was also shown to be stable on acetate in a sequencing batch airlift reactor. In both experiments enough shear was needed to maintain a stable biofilm. High shear forces (in combination with a short settling time) were responsible for the formation of aerobic granular sludge in other aerobically fed reactors, but without a carrier (Beun et al. 2002, Tay et al. 2001, Dangcong et al. 1999).

Slow growth
The aerobic feeding strategy used in combination with a low dissolved oxygen concentration (≤ 40 %) led to filamentous growth and loss of granule stability (Mosquera-Corral et al. 2005a). The oxygen diffusion limitation of this strategy created by applying a low oxygen concentration in the bulk provided excellent conditions for the proliferation of filamentous organisms (Martins et al. 2003, Martins et al. 2011). Filamentous growth will have a detrimental effect on the settling properties and thus on the effluent quality. The stability of aerobic granular sludge under low oxygen concentrations is vital for the success of the technology in practice. A low dissolved oxygen concentration would not only mean a more efficient nitrogen removal, but also improvement of process economy in the full-scale utilization.

The introduction of anaerobic feeding followed by an aerobic reaction period enhanced the stability not only for granulation (without the need for extreme high shear), but also for nitrogen and phosphorus removal (De Kreuk and van Loosdrecht 2004). Converting easy biodegradable COD anaerobically into storage polymers yields an overall lower aerobic growth rate of the heterotrophic bacteria, which results in dense biofilm growth (Van Loosdrecht et al. 1997a, Krishna and Van Loosdrecht 1999). This way fast heterotrophic growth that might lead to filamentous outgrowth and granule instability in aerobically pulse fed SBR systems is avoided. Distribution of easy biodegradable COD throughout the

granule by feeding anaerobically ensures granule stability by allowing growth on the inside of the granules. In the absence of oxygen, nitrate and nitrite (formed during nitrification) can be used as electron donors by PAO and GAO-like organisms. The distribution of substrate combined with slow growth was also found to be the key mechanisms for stable, smooth biofilm formation in several modeling studies (Picioreanu et al. 1998)

Granules versus activated sludge

Besides the obvious physical differences between the biomass in flocs and granules, the conversions present are quite similar and what is good for the one is mostly good for the other. For example; influent dosage to an anaerobic tank or selector first, is crucial in conventional WWTP's to obtain stable nutrient removal, low SVI's and to prevent the proliferation of filamentous organisms (Martins et al. 2003). This is no different in aerobic granular sludge, where the anaerobic feeding also lies at the basis of a stable process as described before. In this respect and many others, aerobic granular sludge is not very different from activated sludge, but there are some profound differences.

Layered structure

One of the great advantages of a biofilm is that factors that inhibit the activity (i.e. temperature, salinity etc.) are less pronounced in a biofilm than for flocs and suspended cells. This was clearly shown by immobilizing nitrifying cells in carrageenan-gel beads (Wijffels et al. 1995, Leenen et al. 1997). The reduced effect of the activity is often mistakenly interpreted as that the biofilm itself is protecting the bacterial community inside. In most cases, the reduced effect of an inhibitant on the conversions present in a biofilm can be explained by the fact that the effect of decreasing activity is less than the effect of substrate limitation. This certainly is true for temperature. A decrease in temperature will decrease activity of all cells present in the biofilm, but due to a lower conversion rate, the substrate can diffuse deeper into the biofilm. Hence, more cells become active than before thereby compensating the overall effect of a temperature decrease on the conversions. This overcapacity is only very limited present in activated sludge flocs, since there diffusion limitation is much less severe, due to their small size (around 40 microns).

For the full-scale application, this means that the conversions in aerobic granular sludge processes are more resistant to decreasing wastewater temperatures than conversions in the activated sludge process. The decrease of temperature (summer – winter) normally requires

higher biomass concentrations in activated sludge plants. This is mainly related to decreasing (de)nitrification rates. In the aerobic granular sludge process, this will be partly compensated by the over-capacity of nitrifiers, due to the high biomass concentrations. If rates are still too low for full nitrification or the load increases dramatically, an increase in oxygen concentration in the bulk liquid will make use of the over-capacity to achieve higher rates. This is only limited possible in activated sludge systems due to the lack of over-capacity (or more accurately diffusion limitation) and thus an increase in biomass concentration is needed.

Simultaneous conversions

The advantage of aerobic granules over activated sludge flocs is not only the easier separation of biomass from the effluent, but also the steeper oxygen diffusion gradients. The dissolved oxygen concentration can be manipulated to perform simultaneous nutrient removal by adjusting the concentration appropriately (Dangcong et al. 1999, Mosquera-Corral et al. 2005a, Beun et al. 2000a, Lochmatter et al. 2013, Kagawa et al. 2014). In most cases, optimization of simultaneous nitrification and denitrification is achieved by lowering the dissolved oxygen concentration by lowering the flow or by pulse aeration. The required dissolved oxygen concentration needed is directly related to the size and activity of the granules. Larger granules have more anoxic volume than smaller granules at the same dissolved oxygen concentration in the bulk (Fig. 5).

Low dissolved oxygen concentrations as mentioned before lead to diffusion limitation inside the granules This means that on the outside of the granules aerobic processes like nitrification can occur, while anoxic conditions occur in deeper layers of the granule (Adav et al. 2009, Vázquez-Padín et al. 2010a, de Kreuk et al. 2005a). In the deeper layers, PAOs and GAOs use the storage polymers formed during the anaerobic feeding as electron donor to convert nitrite and nitrate to di-nitrogen gas (Beun et al. 2000b). PAOs will use oxygen in the aerobic layers and nitrate or nitrite in anoxic layers to take up phosphorus from the bulk and this as store poly-phosphate. This makes the uptake rate of phosphorus by PAOs relatively independent from the oxygen concentration.
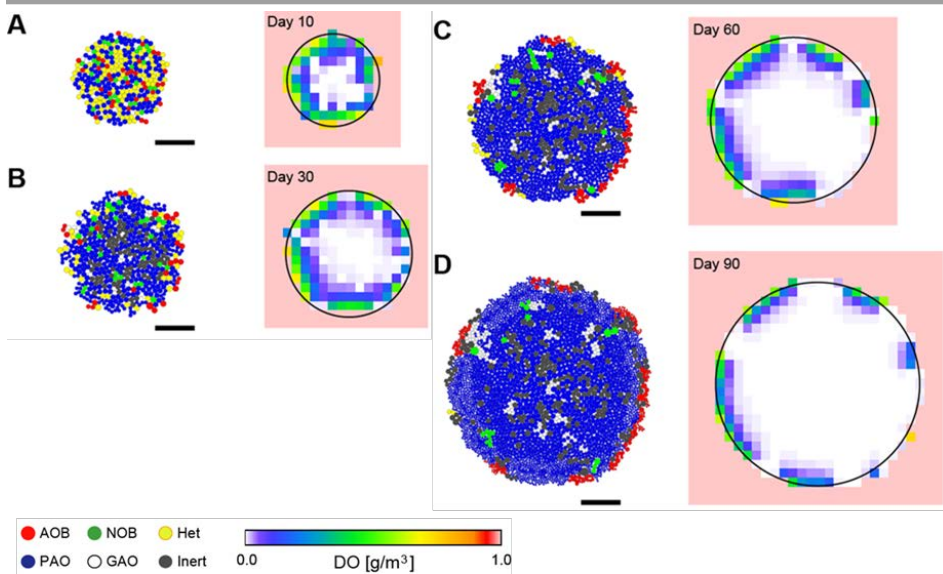
Fig. 5 - Two dimensional simulation results of special distribution of the microbial population in a microbial granule and oxygen concentration at the beginning of the aerobic period. Scale bars, 100 μm. (source: (Kagawa et al. 2015))

Cycle configuration lab versus full-scale reactors

Configuration of the operational cycle in the lab systems differ from those applied in full-scale plants. They are based on the same principle nonetheless. In laboratory systems, the cycle is often started with a (preferentially anaerobic) feeding period. This can last from 20 – 120 minutes, usually 1 hour. This is followed by an aerobic period, where the dissolved oxygen is chosen. This can range anywhere from 10 - 100 % and depends on the purpose of the experiment (i.e. optimization of denitrification requires lower DO values than nitrification or COD removal). Aeration in laboratory systems is also used for mixing purposes in many cases. The aerobic period is normally restricted between 1 – 3 hours and should be based on the COD, nitrogen and phosphorus load, conversion rates and/or another parameter that is investigated. One of the major selection criteria in AGS reactors, besides the anaerobic feeding, is the settling pressure. This is instigated by switching off the aeration to allow for the selection of faster settling biomass versus slower settling biomass. The slower settling biomass is simultaneously drawn from the reactor with the effluent. The effluent decant pipe is usually placed at half of the working volume.

In full-scale plants, the cycle timing can be optimized. This means that during the feeding period effluent is pushed out of the top of the reactor via the weirs. This simultaneous

feeding and effluent discharging saves valuable time that is needed for biological conversions. Feeding times are depended on the wastewater flow. During rain weather events the flow can increase 2 - 5 times that of dry weather flow, thus feeding times are longer and biological (aeration) time is shortened. This ensures that all the wastewater that is received by the plant is treated. To buffer some of the heavier rain weather events an influent buffer tank can be used. Feeding times vary from plant to plant, but are usually somewhere between 0.5 – 1.5 hours. The selection on well settling granules over slow settling flocs is also applied in full-scale plants. However, since the effluent of a full-scale plant is also subjected to guidelines for suspended solids, a 'laboratory' sludge selection via the effluent is not viable. Therefore, effluent discharge and settling are separated. Settling is instigated by switching off the aeration, as is done in laboratory systems, after a certain amount of time the slower settling biomass is wasted from the reactor. Settling times vary greatly from one design to another and depend on the status of granulation. In fully granulated systems, settling times are often in minutes rather than in hours.

Lab scale to practice

The translation from lab-scale to commercial full-scale reactors took approximately 12 years. Fundamental knowledge on formation of biofilms was derived from research topics not directly related to aerobic granular sludge. Biofilm modelling for example gave an early insight in the parameters influencing morphology (Van Loosdrecht et al. 1997a, Picioreanu et al. 1998).

In 2006 - 2008 the design and construction of a demonstration plant in Gansbaai, South Africa was initiated. In 2010 the first Dutch full-scale aerobic granular sludge reactor treating domestic (65 %) and industrial (35 %) (slaughterhouses) wastewater was constructed in Epe, The Netherlands. In 2012 - 2013, a full-scale reactor solely fed with domestic wastewater was built in Garmerwolde, The Netherlands. Several other full-scale installations (Fig. 6) in the Netherlands and other countries are being planned at the moment of writing this thesis.

Fig. 6 - Nereda installations in Garmerwolde, Epe, Dinxperlo, Utrecht, Vroomshoop (The Netherlands) and Gansbaai (South Afrika)

In order to get to the full-scale installations pilot experience and knowledge was imperative. Not only to assess the translation from lab to full-scale, but also to convince the end users that the technology works. It is not uncommon for water authorities to think that their wastewater or situation is unique and thus rigorous testing is usually required repeatedly at each new site. This is not limited to the Netherlands, but is a global mind-set, seen to the many pilot reactors that are now running abroad. Also to that end, the National Nereda Onderzoeks Programma (NNOP) was organised in 2010 for the Dutch water authorities that were involved with AGS. In this program various participants were working together to further develop the Nereda® process. Among the members were Stichting Toegepast Onderzoek Waterbeheer (STOWA), Royal HaskoningDHV, TUDelft and six water association members. The open discussions (once a month) let to fast detection of problems and implementation of solutions between the different water authorities. In addition, the knowledge of the water association was essential to develop the process at full-scale. After the NNOP finished in 2013, a new program was started since there was

still a need to further optimize and exchange experiences from the existing Nereda® plants. The Samenwerking Optimalisatie Opstart Nereda (SOON) consists out of members from the water association, Royal HaskoningDHV and TUDelft.

Outline of the thesis

In previous research by the PhD students Janneke Beun (principle of aerobic granulation), Merle de Kreuk (basic process technology for granular sludge nutrient removal) and Mari Winkler and Joao Bassin (Microbiology and process engineering aspects of granular sludge) the basic concepts of the granular sludge technology were worked out. In this thesis the effects of several operational conditions on the conversion processes, formation and stability of aerobic granular sludge was studied. The quick implementation of the technology in practice also meant that several important subjects still needed further investigation. To ensure a well-functioning technology in domestic and industrial applications these subjects were studied in more detail (i.e. adsorption, effect of salinity, higher temperature and other substrates). Besides laboratory work also the start-up and performance of one of the first full-scale aerobic granular sludge reactors treating domestic wastewater is described.

In **Chapter 2,** the ammonium adsorption phenomenon observed in aerobic granular sludge, anammox granules and activated sludge is investigated.

Chapter 3 focusses on the influence of salt (NaCl) on the conversions and stability of aerobic granular sludge. The main and side effects of the salt on nitrogen and phosphorus removal are investigated.

Chapter 4 deals with the effect of different soluble (industrial relevant) substrates on aerobic granular sludge at 35 °C. The effect and conversion of alcohols and aldehydes with granular sludge is investigated.

In chapter 4, it was found that the presence of methanol leads to the proliferation of methanogens. Methanol was converted in the anaerobic feeding period to methane and carbon dioxide. Methanogenesis is an unwanted conversion in aerobic granular sludge since it can lead to feeding short cuts (because of gas formation), methane emissions and potential explosive situations in practice. **Chapter 5** investigates the effect of sludge age

control on methanogens and glycogen accumulating organisms grown on acetate and methanol at 35 ºC.

In **Chapter 6,** we describe for the first time the performance of one of the first full-scale aerobic granular sludge reactors in the world designed for the treatment of sewage. After start-up and more than one year of operation, this chapter reflects not only on the performance, but also on granulation, COD, nitrogen and phosphorus conversions and especially design considerations.

Finally, in **Chapter 7** some interesting aspects of the aerobic granular sludge technology in practice are given and recommendations for future research are proposed.

2

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

Nomenclature

C_{eq}: ammonium equilibrium concentration (mg N L^{-1})
C_{inf}: ammonium influent concentration (mg N L^{-1})
Γ_{ads}: ammonium adsorbed (mg N gVSS^{-1})
Γ_{ads}^{max} : maximum adsorption constant (mg N gVSS^{-1})

Γ_{ads}^{30} : ammonium adsorbed at a C_{eq} concentration of 30 mg NH$_4^+$-N L^{-1} (mg N gVSS^{-1})
K: half saturation constant (mg N L^{-1})
X: total biomass concentration (gVSS L^{-1})
k_{ads}: adsorption rate constant (L (gVSS h)$^{-1}$)

Introduction

The increasing amounts of nitrogen compounds in water and wastewater contribute to the occurrence of eutrophication of surface waters. Nitrogen removal is generally accomplished by nitrification and denitrification, two important processes involved in wastewater treatment successfully applied for many decades. Nitrification is the microbial oxidation of NH$_4^+$ to NO$_2^-$ and further to NO$_3^-$. The last compound is reduced to nitrogen gas during denitrification in a multi-step reaction (NO$_3^-$→NO$_2^-$→NO→N$_2$O→N$_2$).

Calculation of nitrogen conversion and mass balances in full-scale treatment systems or batch activity tests is a complex task due to the numerous parallel conversions involved in nitrogen removal processes. For instance, regularly the measured production of NO$_2^-$ and NO$_3^-$ by nitrification is higher than the NH$_4^+$ removed from solution. This is generally attributed to simultaneous nitrification of the ammonium generated by ammonification, biomass decay or to analytical problems. There are however indications in literature suggesting that other phenomenon should be taken into account to track the flow of the nitrogen compounds. Among them, adsorption of ammonium to biomass seems to be an important process. The extracellular polymeric substances (EPS) and microbial cell surfaces carry a negative electric charge (Wilkinson 1958). Therefore, the EPS matrix can function as an ion exchanger for cations (e.g. Ca^{2+}, Mg^{2+} and NH$_4^+$) and heavy metals. The binding of heavy metals (Liu et al. 2001, Fukushi et al. 1996, Guibaud et al. 2003, Comte et al. 2006) and some cations such as Ca^{2+} and Mg^{2+} (Dupraz et al. 2004) to EPS has been well studied. However, only a limited number of references point to ammonium adsorption in activated sludge systems or biofilms. Nielsen (1996) studied the extent of adsorption of NH$_4^+$ to activated sludge from full-scale wastewater treatment plants (WWTP) where nitrification and denitrification were occurring. In that study, it was observed that the percentage of ammonium adsorbed to the sludge flocs was between 20 – 25 % at dissolved ammonium concentrations of 1 to 6 mg NH$_4^+$-N L^{-1}. When the bulk concentration was around 15 mg NH$_4^+$-N L^{-1}, the equivalent of 2 mg NH$_4$-N L^{-1} was absorbed. The maximum adsorption capacity reported was in the range of 0.3 – 0.4 mg NH$_4^+$-N gVSS^{-1}.

Wik (1999) estimated an ammonium adsorption of 2.7 mg NH_4^+-N m^{-2} in a trickling filter at an influent ammonium concentration of 15 mg N L^{-1}. During the treatment of municipal wastewater by the BIOFIX-process, Temmink et al. (2001) observed that 9 – 21 % of the ammonium influent was adsorbed by the biofilm when the ammonium influent concentration was 52 ± 20 mg L^{-1} and 37 ± 20 mg L^{-1}, respectively. Schwitalla et al. (2008) found that the adsorption to activated sludge flocs was within a range of 0,07 – 0,20 mg NH_4^+-N $gVSS^{-1}$. Neglecting the ammonium adsorption could therefore in cases lead to underestimations of 10 – 25 % of the ammonium available for nitrification.

In our experiments with lab- and pilot-scale aerobic granular sludge reactors with alternate anaerobic/aerobic phases, it was observed that the ammonium concentration after anaerobic feeding was lower than expected based on the influent concentration. This fact was associated with a possible ammonium adsorption phenomenon to the aerobic granules. Therefore, we decided to perform a study on the ammonium adsorption properties of aerobic granular sludge in comparison with activated sludge and anammox granular sludge. Adsorption kinetics and adsorption isotherms were determined in order to provide a better insight in the ammonium adsorption process and for potential future inclusion in mathematical process models.

Materials and Methods

Lab- and pilot-scale aerobic granular sludge reactors

Two lab-scale aerobic granular sludge sequencing batch reactors with a working volume of 2.6 L were operated at different temperatures (20 and 30 ºC). The cycle time of both reactors was 3 h and comprised 60 min anaerobic feeding from the bottom of the reactor in a plug-flow regime through the settled bed, 112 min aeration provided by an air diffuser, 3 min settling and 5 min effluent withdraw. DO concentration during aeration phase was kept constant at 20 % air saturation by mixing air and nitrogen in the inlet gas by a mass flow controller. The reactors were fed with synthetic wastewater with the following composition: (A) NaAc 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^{-1} trace element solution (Vishniac and Santer, 1957). Each cycle we dosed 150 mL from both media together with 1,300 mL of tap water. The reactor operated at 20 ºC was previously fed with the same wastewater containing different salt concentrations (0 – 30 g NaCl L^{-1}). The pilot-scale aerobic granular sludge reactor had a volume of around 1,5 m^3. The cycle profile was the same of the lab-scale reactors. The influent consisted of domestic sewage, which contained around 600 mg L^{-1} COD and 50 - 100 mg NH_4^+-N L^{-1}.

Adsorption batch tests

Adsorption tests were carried out using granular sludge that was collected from lab- and pilot-scale sequencing batch reactors at the end of their operational cycle. Anammox

granules were collected from Dokhaven WWTP (Rotterdam, The Netherlands). Activated sludge was taken from the outflow of the nitrification tank from two WWTP (Harnaschpolder (biological P-removal) and Kralingseveer (chemical P-removal), The Netherlands). Aerobic granular sludge and activated sludge were aerated for 1 h to minimize residual ammonium that could be present. For the anammox granules, nitrogen gas was supplied instead of compressed air. For the adsorption batch experiments, two types of experiments were conducted: one varying the ammonium concentration and keeping biomass concentration constant and the other varying biomass concentration and keeping the initial ammonium concentration the same. In the experiments with a constant biomass concentration, 250 mL flasks were filled with a fixed amount of biomass (either aerobic granules, activated sludge or anammox granules) and with 0,1 M Tris-HCl buffer (pH 7). In the beginning of the experiment, pulses of ammonium were added to the flasks in order to have different ammonium concentrations. For the second type of experiment (same initial ammonium concentration), the flasks were filled with different amounts of biomass and with 0,1 M Tri-HCl buffer (pH 7). An ammonium pulse was added to have a similar final concentration in each flask. Nitrogen gas was supplied for to all flasks to ensure anaerobic conditions. Samples were taken in different time intervals in order to have an overview of the adsorption kinetics and maximum adsorption capacity of aerobic granules, activated sludge and anammox granules.

Analytical measurements

Ammonium was measured by flow-injection analysis system (QuikChem 8500, Lachat Instruments, Inc.). Biomass concentration was determined according to Standard Methods (APHA, 1998).

Modelling ammonium adsorption

A mathematical tool was developed to predict ammonium adsorption in an aerobic granular sludge reactor as a function of the ammonium concentration in the influent. Ammonium adsorption can be described by an ammonium mass balance (1), and a Langmuir adsorption isotherm (2):

$$C_{\text{inf}} = \Gamma_{ads} \cdot X + C_{eq} \tag{1}$$

$$\Gamma_{ads} = \frac{C_{eq}}{\left(C_{eq} + K\right)} \cdot \Gamma_{ads}^{\max} \tag{2}$$

The equations consist of two known variables (C_{inf} and X), two unknown variables (C_{eq} and Γ_{ads}) and two parameters ($\Gamma_{ads}^{\max}$ and K). Parameter values characterizing the

adsorption capacity of the biomass were estimated by fitting the measured C_{eq}-values to the modelled values that can be obtained from a quadratic solution of equation 1 and 2. For easy comparison of the adsorption capacity of different types of biomass, a characteristic value of the adsorption capacity at an equilibrium concentration of 30 mg N L^{-1} is defined and calculated from equation 1 and 2 (Γ_{ads}^{30}, mg N gVSS^{-1}).

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

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

(3)

In this equation k_{ads} is the biomass specific kinetic constant for ammonium adsorption (L (gVSS/h)$^{-1}$), and $C(t)$ is the time dependent ammonium concentration in the liquid. Integration of this equation allows for description of $C(t)$ as a function of time:

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

(4)

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

Results

Adsorption in pilot-scale aerobic granular sludge reactor

During operation of the pilot-scale aerobic granular sludge reactor, we observed a positive relation between the ammonium influent concentration and the ammonium adsorbed. Based on the ammonium concentration at the end of the anaerobic feeding phase and the ammonium concentration that was expected based on the ammonium influent concentration and the dilution in the reactor, the amount of adsorbed ammonium and the equilibrium ammonium concentration in the bulk were estimated (Fig. 1). Dotted lines in Fig. 1 represent the model fitted to the data and will be discussed in the discussion section. In general, the higher the ammonium influent concentration, the greater the amount ammonium adsorbed. The biomass concentration in the pilot-scale reactor was roughly constant and equal to 8 gVSS L^{-1}. Around 18 – 24 % of the NH$_4$-N influent was adsorbed when the ammonium concentration in the incoming wastewater ranged from 50 to 100 mg NH$_4^+$-N L^{-1}. Since some ammonification may have taken place in the anaerobic period, the data in Fig. 1 do not provide a true adsorption isotherm.
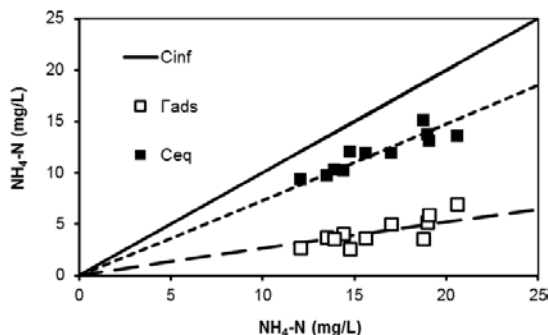

Fig. 1 - Ammonium adsorbed (Γads) and ammonium concentration in the equilibrium (Ceq) at different influent ammonium concentrations (Cinf). Dotted lines represent the model fitted to the experimental data.

Adsorption in lab-scale aerobic granular sludge reactor

In two lab-scale reactors operated at different temperatures (20 and 30 ºC) and fed with the same medium, the adsorption of ammonium was quite similar to the pilot scale system (Γ_{ads}^{30} ~1 mg NH$_4^+$-N gVSS^{-1}). The adsorption varied from 23 – 36 % (for 20 ºC) and from 27 – 37 % (for 30 ºC) of the ammonium influent concentration (34 mg NH$_4$-N) in the aerobic granular sludge reactor. Biomass concentration was kept roughly constant in both reactors (around 12 gVSS L^{-1}). The temperature seems not to influence the adsorption in granules in this range. During operation of the aerobic granular sludge lab-scale reactors, it was also observed that the amount of ammonium adsorbed during anaerobic feeding was reversely proportional to the ammonium concentration remaining in the end of the previous cycle. Therefore, when incomplete nitrification occurred, a smaller amount of

influent ammonium was adsorbed. This observation emphasises the importance of nitrification for the extent of adsorption.
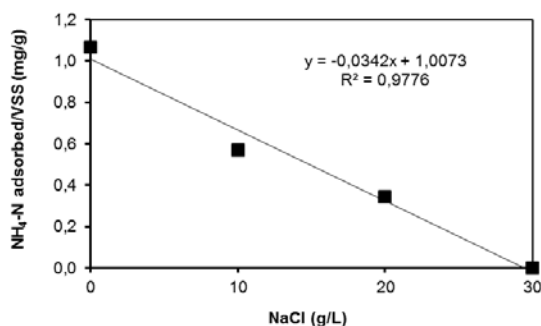

Fig. 2 - Ammonium adsorption in a lab-scale aerobic granular sludge reactor operated at different salt concentrations.

In the reactor fed with synthetic wastewater containing salt (NaCl), it was observed that the amount of ammonium adsorbed considerably decreased when the salt concentration was increased (Fig. 2). At 10 g NaCl L^{-1}, the ammonium adsorption was approximately half of that obtained when no salt was added to the reactor. Moreover, no adsorption was observed at 30 g NaCl L^{-1}.

Adsorption batch tests

Determination of adsorption kinetics
Experiments were conducted in order to determine the kinetics of the adsorption taking place in aerobic granular sludge. No adsorption kinetics study was performed for activated sludge, since the experiments clearly showed that adsorption was very fast (within 5 minutes). Adsorption rates with granular sludge are significantly lower probably due to mass transfer limitations in the biofilm. The data from the adsorption kinetics experiments at variable biomass or initial ammonium concentrations are shown in Fig. 3. In both experiments, we observed a rapid ammonium adsorption in the beginning of the experiment. The adsorption rate gradually decreased until the equilibrium concentration (C_{eq}) was reached. In general, C_{eq} was reached within 60 min of experiment. The anaerobic feeding phase in our aerobic granular sludge reactors have a similar length, suggesting that in this period equilibrium adsorption is reached in the reactors.
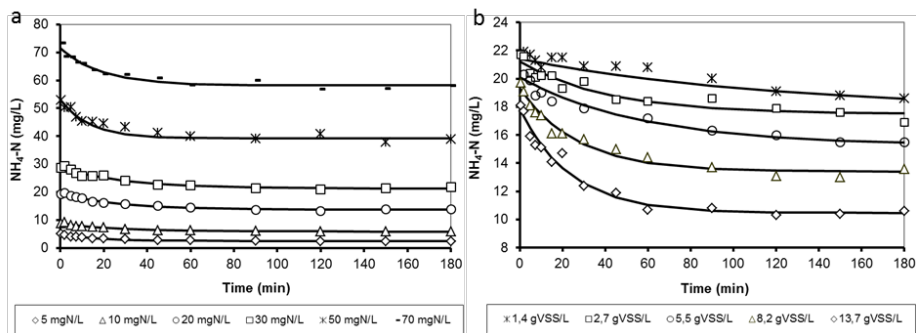
Fig. 3 - Experimental data and model fitted to the adsorption kinetics for the experiment keeping biomass (a) and initial ammonium (b) concentrations constant.

A kinetic model described in the Materials and Methods section was used to characterize the kinetic properties of the adsorption process. The average biomass specific kinetic constant for ammonium adsorption obtained in the experiments with variable biomass and initial ammonium concentrations were comparable and amounted 0.33 ± 0.06 L (gVSS h)$^{-1}$ and 0.31 ± 0.14 L (gVSS h)$^{-1}$, respectively.

Adsorption Isotherms

The ammonium adsorption isotherms obtained in the experiments with activated sludge, in which either ammonium concentration was varied and the amount of biomass was kept constant or the other way around are shown in Fig. 4. A Langmuir's isotherm was fitted to the experimental data. Parameters such as maximum adsorption capacity (Γ_{ads}^{max}) and the half saturation constant (K) are not reported here since measurements were conducted at ammonium concentrations that were too low for identification of the maximum adsorption capacity. At ammonium concentrations lower than 40 mg N L^{-1}, the amount of ammonium adsorbed was directly proportional to the equilibrium concentration, independently of the activated sludge used. Ammonium adsorption obtained at 30 mg NH$_4^+$-N L^{-1} (Γ_{30}) for the activated sludge collected from Harnaschpolder and Kralingseveer WWTP was around 0.18 and 0.16 mg NH$_4$-N gVSS^{-1}, respectively. The sludges of Harnaschpolder and Kralingseveer showed similar ammonium adsorption behaviour. Since typical sludge concentrations are lower than 4 g VSS L^{-1} in activated sludge systems, less than 2 % of the ammonium will be present in an adsorbed form. This amount can, as is usually done, be neglected.
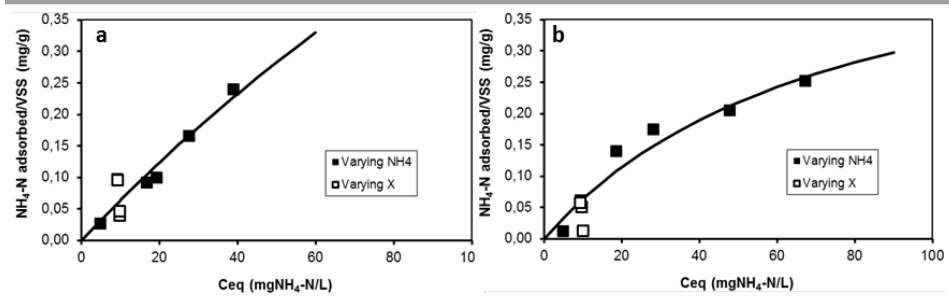
Fig. 4 - Ammonium adsorption isotherms in activated sludge from Harnaschpolder (a) and from Kralingseveer (b) obtained in the batch experiments either keeping biomass concentration constant and varying ammonium concentration (■) or keeping initial ammonium concentration constant and varying biomass concentration (□).

The adsorption of ammonium to aerobic granular sludge from both lab- and pilot- scale reactors is illustrated in Fig. 5. As for activated sludge at lower ammonium concentrations the amount of ammonium adsorbed is linearly proportional to the adsorbed amount. The Γ_{ads}^{30}-values were equal to 1.7 and 0.9 mg NH_4^+-N gVSS^{-1} for the lab-scale and pilot-scale reactor granules. Clearly these values are an order of magnitude higher than for activated sludge. At sludge concentrations of 8 gVSS L^{-1} and higher in aerobic granular sludge reactors, this means that a very significant fraction of ammonium is adsorbed to the granular sludge. A Langmuir-type isotherm was fitted through the experiment data. Values obtained for the maximum adsorption constant (Γ_{ads}^{max}) were 10 and 1.65 mg N VSS^{-1} for lab-scale granules and pilot-scale granules, respectively. Half saturation constants (K) amounted 175 and 28 mg N L^{-1} for lab-scale granules and pilot-scale granules, respectively.
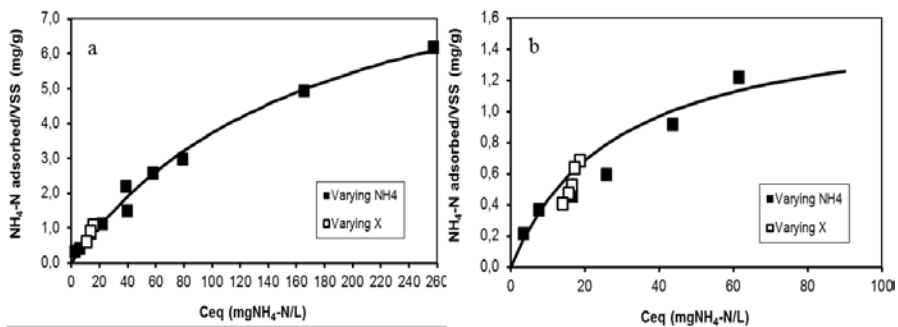
Fig. 5 - Ammonium adsorption isotherms in aerobic granular sludge from lab-scale reactor (a) and pilot-scale reactor (b) obtained in the batch experiments keeping biomass concentration constant and varying ammonium concentration (■) and keeping ammonium concentration constant and varying biomass concentration (□). Γmax: 10 mg N gVSS^{-1}; K: 175 mg N L^{-1} for lab-scale granules; Γmax: 1.65 mg N gVSS^{-1}; K: 28 mg N L^{-1} for pilot-scale granules.

For comparison, adsorption tests with anammox granular sludge were conducted as well. The Γ_{ads}^{30} was around 0.20 mg NH_4^+-N gVSS^{-1}. Anammox granules showed therefore also a low ammonium adsorption capacity. However due to much higher biomass content in these anammox granular sludge reactors (up to 20 - 30 g VSS L^{-1}) the total mass of adsorbed ammonium can be significant in a reactor system.

Discussion

From the literature, it is known that ammonium can be adsorbed to activated sludge flocs (Nielsen 1996, Schwitalla et al. 2008) and to biofilms (Wik 1999, Temmink et al. 2001). In our experiments with lab- and pilot-scale aerobic granular sludge reactors with alternate anaerobic/aerobic phases, we observed that the ammonium concentration after the anaerobic feeding was lower than expected based on the influent concentration, which suggested us the occurrence of a possible ammonium adsorption phenomenon inside the granules. Table 1 summarizes the different investigations on ammonium adsorption obtained from literature and from our research. Some results are difficult to interpret in a proper way due to the lack of information. For instance, the ammonium adsorption found by Temmink et al. (2001) was higher when the ammonium influent concentration was lower, which was possibly caused by different biomass concentrations in the reactor. Unfortunately, the biomass amount or biofilm amount was not mentioned in the publication. The same is true for the work of Wik (1999), who only mentioned the ammonium adsorption per m^2 or m^3 of biofilm, without a reference to the biofilm thickness. In addition, generally the actual equilibrium concentrations at a certain amount of ammonium adsorbed is not presented.
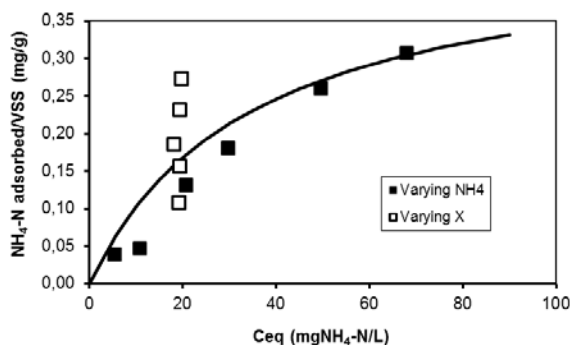
Fig. 6 - Ammonium adsorption isotherms anammox sludge from a pilot-scale reactor obtained in the batch experiments either keeping biomass concentration constant and varying ammonium concentration (■) or keeping ammonium concentration constant and varying biomass concentration (□). Γmax: 0.46 mg N gVSS^{-1}; K: 35 mg N L^{-1}.

During operation of a pilot-scale aerobic granular sludge reactor, we observed a positive relation between the ammonium influent concentration and the ammonium adsorbed. This

is consistent with the results obtained from adsorption batch experiments that showed higher adsorption when C_{eq} was higher. From the operation of two lab-scale reactors, we observed that a temperature variation of 10 °C did not influence the extent of adsorption, which is in line with expectations for adsorption processes. Our results also showed that salt (in our case NaCl) concentrations are also a key factor for the amount of ammonium that will be adsorbed. The fact that the adsorption significantly decreased as the salt concentration was increased can be explained by the competition between Na^+ and NH_4^+ for binding to the negatively charged groups in the EPS or microbial cell walls. The adsorption of the ammonium to the biomass can best be seen as an ion exchange process and the presence of other cations will directly influence the amount of ammonium adsorbed. For experimental determination it is therefore possible to add salt (like Na^+ or K^+) to sludge and measure the amount of ammonium desorbing as e.g. suggested by Nielsen et al. (1996).

The adsorption isotherms obtained in the batch experiments clearly demonstrated that the adsorption in aerobic granular sludge is considerable higher than the one achieved with activated sludge and anammox granules. We estimated Γ_{ads}^{30}-values for activated sludge and anammox granules of 0.16 and 0.20 mg NH_4-N $gVSS^{-1}$, respectively. The adsorption to aerobic granular sludge was characterized by Γ_{ads}^{30}-values for lab- and pilot scale granules of 1.6 and 0.8 mgNH_4-N $gVSS^{-1}$, respectively. From the comparison between anammox granules and aerobic granular sludge, it is clear that granular sludge as such does not lead to a higher ammonium adsorption capacity.

Author (s)	System	Adsorption % [a]	mg N gVSS[-1] at 30 mg N L^{-1}	NH$_4^+$ influent (mg N L^{-1})
Table 1 Ammonium adsorption in different wastewater treatment systems				
Nielsen (1996)	Activated sludge	NA	0.3-0.4 [b]	NA
Wik (1999)	Trickling filter	NA	2.7 [c]	~16
Temmink et al. (2001)	BIOFIX-process	9 – 21 %	NA	30-50
Schwitalla et al. (2008)	Activated sludge	NA	0.07-0.20	NA
Valdivia et al. (2007)	Biofilm SBR	14 – 27 %	NA	22
Our research	Activated sludge	NA	0.16-0.18	20 - 50
Our research	Pilot-scale AGS	18 - 24 %	0.9	50 – 100
Our research	Lab-scale AGS (T=20ºC)	23 – 36 %	1.7	34
Our research	Lab-scale AGS (T=30ºC)	27 – 37 %	NA	34
Our research	Lab-scale AGS (0 to 30 gNaCl L[-1])	0 – 30 %	NA	34

[a] Percentage is relative to influent concentration.
[b] Total absorption capacity at an ammonium concentration of approximately 5 mg N L^{-1}
[c] mg N/m^2
NA: not available

The pilot-scale granular sludge as well as the sludge from Harnaschpolder were grown on wastewater that employed biological P-removal conditions. From the comparison it is therefore clear that the higher adsorption to granular sludge is not directly related to the presence of phosphate accumulating organisms in the sludge. In the adsorption batch tests where biomass was varied and initial ammonium concentration was kept constant, we observed that the ammonium concentration in equilibrium hardly varied, especially in the experiments using activated sludge and anammox granules. This observation can be partly related to the substantially low amount of ammonia adsorbed at low biomass concentrations, which increases the measurement error. Still, the higher adsorption per unit of biomass at lower biomass concentrations is to date not fully understood. Actually, these experiments at low biomass concentrations do not reflect the biomass content in the biological systems from where sludge samples were collected.

As an ion exchange process, the amount of ammonium adsorbed into aerobic granules will be directly related to the compounds functioning as an ion exchange for cations. Among them, extracellular polymeric substances (EPS) likely play a dominant role in the adsorption of ammonium. Sludge contains a mixture of microbial species, which can promote the synthesis of several types of EPS. These polymers can significantly vary in their composition and therefore in their chemical and physical properties. While some are neutral, others are either polyanionic (due to the presence of uronic acids and ketal-linked pyruvate) or polycationic macromolecules (Sutherland 2001). In this respect it is interesting to note that recent studies have described that aerobic granules from different treatment systems can produce exopolysaccharides with a unique composition (Adav and Lee 2008, Lin et al. 2010a, Seviour et al. 2010). Lin et al. (2010a) observed that the dry weight of aerobic granules from the pilot plant reactor treating municipal wastewater (same reactor as in this study however samples at a different time) contained more than 10 % of alginate-like exopolysaccharides. These authors pointed out that this specific exopolysaccharide is only one present in granules, although the amount reported was even higher than the total EPS content reported by Adav and Lee (2008) and Wang et al. (2005). Seviour et al. (2010) also mentioned that granules are characterized by an over-production of a single gel-forming EPS, although the structure of exopolysaccharides was different from that described by Lin et al. (2010). Nevertheless, some similarities in the granules exopolymers are described in these two publications (e.g. presence of uronic acids). Unfortunately there is no information on the ammonium adsorption to the granules.

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

Since the adsorption strongly depends on the type of EPS produced and currently this cannot be predicted from basic principles, adsorption of ammonium has to be considered in each experimental evaluation of nitrogen conversion processes. For example, the adsorption of ammonium to the moving-bed biofilm in the BIOFIX process made the process in practise not feasible due to excessive release of ammonium in the anoxic phase of the process. Also, the ammonium dynamics in the trickling filters described by Wik et al. (1999) was heavily influenced by the ammonium adsorption to the biofilm matrix. Yilmaz et al. (2008) observed a decrease of 30 mg NH_4^+-N L^{-1} during anaerobic feeding of an aerobic granular sludge reactor, but they neglected the occurrence of ammonium

adsorption due to the relatively long time to reach the equilibrium concentration. The assumption of these authors was referred to the publication of Nielsen (1996), who worked with activated sludge. As indicated by our experiments, adsorption in granules takes longer and is not a fast process like in activated sludge. Therefore, ammonium adsorption should not have been neglected.

Modelling ammonium adsorption

Ammonium adsorption was well described using a Langmuir isotherm. Due to the low ammonium concentrations applied, the maximum adsorption capacity (Γ_{ads}^{max}) could only be estimated for the aerobic granular sludges.

The biomass specific adsorption characteristics as determined in the batch experiment were extrapolated to the granular sludge bioreactor conditions to validate the impact of ammonium adsorption on the ammonium concentration after feeding. Fig. 1 demonstrates that using the Γ_{ads}^{max} and K values obtained in the batch experiments and the biomass concentration (X, 8 gVSS L^{-1}) and the influent ammonium concentration (C_{inf} in the pilot could very well be used to predict the ammonium concentration after feeding. The data clearly suggest that biomass characterization in terms of τ_{abs}^{max} and K values is strictly required to describe nitrogen conversion processes in dynamic processes, like SBRs.

Thus, knowing the ammonium influent concentration and the biomass concentration, it is possible to predict the amount of ammonium that will be adsorbed and the amount that will remain in the bulk solution.

The developed approach is valid when no ammonium is present at the end of an SBR cycle (complete nitrification). In the case where ammonium is not completely removed during the cycle, the amount of ammonium adsorbed would be lower and depends on the residual ammonium concentration in the bulk. We are assuming that granules have maximum adsorption capacity when nitrification is complete. However, it is possible that even when ammonium is completely depleted in the liquid phase, a fraction of the adsorbed ammonium remains inside the granules. From the experiments of Nielsen (1996), using activated sludge, it was observed that 0,5 - 0,6 mg NH$_4^+$-N L^{-1} was still adsorbed to the sludge flocs even when the dissolved ammonium was almost completely removed by microbial oxidation. Nielsen (1996) also pointed out that ammonium desorption rate can be quite slow, based on the experiment where the biomass containing 0,5 mg NH$_4^+$-N L^{-1} adsorbed was further oxidized in a vigorously shaken flask for 50 min, and still 0,3 mg NH$_4^+$-N L^{-1} was adsorbed. Therefore, desorption kinetics seems to be important to predict the amount of ammonium that can be exchanged and further oxidized, and should be studied in more details.

Conclusions

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

3

Evaluating the main and side effects of high salinity on aerobic granular sludge

Introduction

Wastewater treatment by conventional activated sludge plants requires a significant amount of space, mainly due to the low settleability of the flocculent biomass resulting in Low biomass concentrations and large settling tanks for these plants. Biofilm-based technologies have much lower footprint and present several advantages compared to suspended biomass processes. One of the emerging and most promising technologies for biological wastewater treatment is the aerobic granular sludge (AGS) process, in which activated sludge flocs are substituted by strong, compact and fast settling granules (de Bruin et al. 2004a). Granules allow separation of the biomass in the treatment reactor and allows higher biomass concentrations, minimising the plant size. Furthermore, the mass transfer limitation which occurs within the granules in conjunction with operation in sequencing batch mode with periods with and without aeration, allows for the presence of different redox conditions (anaerobic, anoxic, aerobic) inside the granular structure. Consequently, several parallel microbial processes (phosphate removal, nitrification and denitrification) can take place in a single reactor compartment. This makes the AGS process a strong candidate for replacing conventional activated sludge processes (De Kreuk and van Loosdrecht 2006a).

Aerobic granular sludge processes have initially been developed for municipal wastewater treatment but currently a wider interest in the technology is emerging. For industrial wastewater, salinity effects are often an important factor, since several industries such as those related to food processing, pickling, pharmaceutical and petrochemical release high quantity of inorganic salts in their effluents (Dahl et al. 1997). The problem of salt is also potentially present in coastal areas, in which a significant seawater intrusion in sewers might occur. The use of seawater for toilet flushing also introduces a significant amount of dissolved salts into the sewer, which will end up in the wastewater treatment plant. (Lu et al. 2011). Salt is known to inhibit several biological wastewater treatment processes such as nitrification, denitrification and phosphate removal (Hunik et al. 1993, 1992, Kargi 2002, Moussa et al. 2006a). Nitrite accumulation, due to the inhibition of nitrite-oxidizing bacteria (NOB), has been associated with elevated salinities in the treatment of wastewater (Hunik et al. 1992, Cui et al. 2006, Ye et al. 2009). In turn, nitrite has an inhibition effect on both anoxic and aerobic phosphate uptake (Meinhold et al. 1999, Saito et al. 2004). Although many researchers have reported on the effect of salt on nitrification processes, few have reported about effects on biological phosphate removal (Uygur and Kargi 2004). Most of the studies focus on single processes, while none have reported on the influence of combined processes within aerobic granular sludge. In a recent study which mainly focused on the microbial diversity analysis of the AGS process under high salt concentration, Bassin et al. (2011a) has shown that at high salt concentrations, polyphosphate-accumulating organisms (PAO) and nitrite oxidizing bacteria (NOB) gradually disappeared from the sludge. However, the cause-effect relationship for their observations was not addressed. In addition, the effect of salt on particular organisms and their response to

increased osmotic pressure as well as the side effects of salt were not evaluated in that particular work. We therefore decided to carry out a complete evaluation on the effect of step-wise increased salinity levels on granule formation and stability, simultaneous nitrification, denitrification, nitrous oxide emissions, phosphate and chemical oxygen demand (COD) removal in aerobic granular sludge grown on synthetic wastewater. Focus has been on the interactions of the various processes involved and the differences compared to activated sludge.

Materials and Methods

Experimental setup and reactor operation

Experiments were conducted in a sequencing batch reactor (SBR) operated at room temperature (20 ± 2°C). The reactor was operated as a bubble column with a working volume of 2.7 litres (diameter 5.6 cm). A gas recirculation system was used to aerate the reactor with a constant airflow of 4 L min^{-1} (superficial gas velocity of 1.51 m min^{-1}). The effluent was extracted at a height of 51 cm, which means that in the end of every cycle 1.2 litres remained in the reactor. This represents a volumetric exchange ratio of 0.56 and a hydraulic retention time (HRT) of 5.4 hrs. The reactor was operated in a 3-hour cycle time with a 60 minutes non-mixed anaerobic feeding period, 112 minutes aeration, 3 minutes settling time, and 5 minutes effluent withdrawal.

A Braun DCU4 controller coupled with Multi Fermentor Control System 3 (MFCS 3) acquisition software (Sartorius Stedim Biotech S.A., Melsungen, Germany) was used to control and operate the SBR. The dissolved oxygen (DO) concentration was controlled by using two mass flow controllers (MFC): one for air and the other for nitrogen gas. The air supply rate applied enabled a DO concentration higher than 90 % oxygen saturation throughout the aeration period. Dinitrogen gas could be added to the sparging gas when a lower dissolved oxygen is required while keeping the superficial gas velocity constant. The pH was measured with a pH electrode and maintained at 7.1 ±0.1 by dosing either 1M NaOH or 1M HCl.

The reactor was inoculated with 1 L of granular sludge from a Nereda® pilot plant treating domestic sewage (Epe WWTP, The Netherlands). The pilot plant was achieving full nitrogen and phosphate removal. The granular sludge was adapted to the laboratory conditions until stable COD, nitrogen and phosphate conversions were obtained (stage 1-I). Stable behaviour was considered when the specific conversion rates (for both the gaseous and soluble concentrations) did not change anymore. After stable operation was obtained, the salinity was increased to 6.6 g Cl$^-$ L^{-1} by addition of NaCl to the influent (stage 1-II). In stage 1-III and 1-IV the salinity was increased to 13 and 20 g CL$^-$ L^{-1}, respectively (Table 1). A second experiment (started at 6.6 g L^{-1}) was performed, in which nitrification was inhibited by dosing allylthiourea (ATU) to the reactor influent.

In order to understand the short and long term effects of increased salinity on biological conversions, cycle tests were conducted one day before and two days after increasing the salt concentration in the reactor. During the cycle tests, COD (acetate), nitrogen and phosphate concentrations were measured offline during the aeration period and the offgas composition (O_2, CO_2, NO and N_2O) was measured online. The effluent concentrations NH_4^--N, NO_2^--N, NO_3^--N and PO_4^{3-}-P were measured weekly to identify long-term changes in conversions. Besides the effluent concentrations of nitrogen and phosphorus, online data such as pH, sodium hydroxide addition rate, off-gas and dissolved oxygen profiles were also used to observe if stable conversions were reached. The sludge retention time (SRT) was controlled at 30 days by manually removing surplus sludge from the reactor once per week. Acetate measurements were used to confirm that all acetate fed to the reactor was fully consumed during the anaerobic feeding phase. Ammonium measurements were corrected for the adsorption of ammonium to the granular sludge in order to calculate the correct conversion rates (Bassin et al. 2011b).

Influent characteristics

The reactor was fed with synthetic medium, which was prepared using demineralized water with the following composition: Medium A (carbon source): 64 mM Sodium acetate; 3.6 mM $MgSO_4 \cdot 7H_2O$; 4.7 mM KCl; and medium B (nitrogen source): 35.4 mM NH_4Cl; 4.2 mM K_2HPO_4; 2.1 mM KH_2PO_4; 10 mL/l trace elements solution according to Vishniac and Santer (1957). From both mediums, approximately 150 mL was dosed to the reactor together with 1.2 l of tap water. After mixing medium A, B and tap water the influent concentrations were 409 mg COD L^{-1}, 60 mg NH_4-N L^{-1}, 20 mg PO_4^{3-}-P L^{-1}. In order to reach the required salinity. Sodium Chloride (NaCl) was added to the medium to obtain a final concentration of 6.6 g Cl^- L^{-1}, 13 g Cl^- L^{-1} and 20 g Cl^- L^{-1} in the influent. In some particular experiments, 5 mg L^{-1} Allylthiourea (ATU) was added to medium B to inhibit nitrification.

Analytical procedures

Ammonium, nitrite, nitrate and phosphate concentrations in the bulk liquid were measured weekly with a spectrophotometer cuvette system (Hach Lange (DR2800)). The acetate concentration was measured by HPLC using an Aminex HPX-87H column from Bio Rad (T=60ºC) coupled to an RI and UV detector. Phosphoric acid (0.01M) was used as the eluent.

Liquid samples from cycle tests were analysed for nitrogen compounds using Flow Injection Analysis (Quikchem 8500, Lachat). All samples were filtered before measurement through a 0.45 μm Millipore filter to ensure sample stability. The staining of acidic polysaccharides was performed as described by McKinney (1953) .

Off-gas measurements

A Rosemount analytical NGA 2000 MLT gas analyser was connected to the gas recycle flow of the reactor to measure the concentrations of CO_2, O_2 and N_2O in the offgas. The pressure sensor built into the analyser corrected the measured off-gas concentration for the changes in atmospheric pressure. The analyser was regularly calibrated with the appropriate gasses. Raw results are given in ppm (parts per million), together with the data of the nitrogen gas and compressed air (nL min^{-1}) supplied, the actual production of N_2O can be calculated.

Biomass measurements

Determining the Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) can be troublesome in aerobic granular reactors operated as a bubble column. The stratification of the aerobic granules prevents homogenous sampling, therefore introducing errors in quantifying the total biomass concentration in the reactor. Serious over or under estimation can occur when taking samples from the bottom or top of granular reactors due to size and density differences. This problematic sampling is often not described and seems to be largely ignored.

In order to deal with this problem, we have adjusted the method for determining the biomass concentration in the reactor. After the biomass has settled, the volume of the settled bed (mL) inside the reactor is recorded. When the reactor is mixed, a sample is taken and put into a volumetric cylinder (mL). The volume of the settled bed in the cylinder is recorded. The biomass in the volumetric cylinder is then filtered over a 0.45μm glass filter, washed with tap water and then dried at 105 °C for at least 24 hours until no weight change occurred anymore. Samples are subsequently placed in a 550°C oven for 1 hour to determine the ash content. The result on the TSS and VSS of the cylinder can be coupled to the reactor biomass concentration by relating it to the ratio between de volume of settled aerobic granules in the volumetric cylinder and that in the reactor. The following formula was used to calculate the biomass concentration (X^R, g L^{-1}) in the reactor:

$$X^R = \frac{M_X^{sample}}{V_X^{sample}} \cdot \frac{V_X^R}{V_L^R} \qquad\qquad [1]$$

Where M_X^{sample} is the amount of dry weight in the sample (g), V_X^{sample} is the volume of the settled bed in the sample (L), V_X^R is the volume of the settled bed in the reactor (L), V_L^R is the total volume of the reactor (L).

Results

General cycle description

A typical operational cycle is shown in Figure. 1. In the first 60 minutes, the influent is fed to the reactor through the settled granule bed from the bottom. Due to the plug flow regime, no homogeneous samples can be taken during this period (see dotted lines for estimated phosphate release or uptake processes). During the feeding phase, acetate is fully consumed by the sludge and no ammonium is oxidized. The decrease in ammonium concentration (indicated in Fig. 1) is due to ammonium adsorption, an important phenomenon taking place in aerobic granular biomass (Bassin et al., 2011b). Once the aeration starts (after 60 minutes in the cycle), a gradual decrease in ammonium concentration and the subsequent production of nitrite and nitrate by nitrification can be observed.
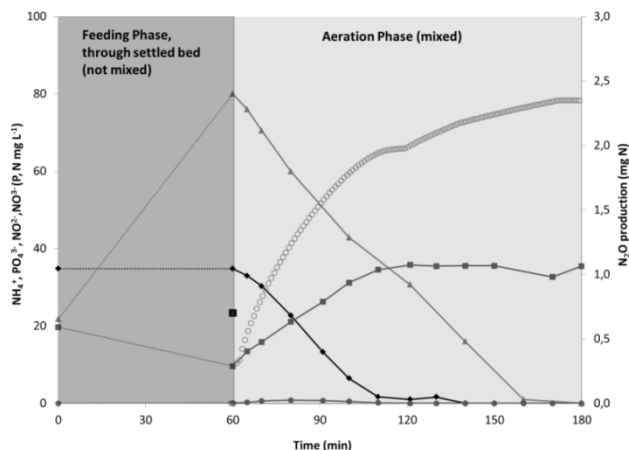

Fig. 1 - Typical change in concentrations during a normal cycle in the aerobic granular sludge reactor. Concentrations at the start of the cycle for nitrite [●] and nitrate [■] are calculated by taking the values at the end of the previous cycle and multiplying them with the volume exchange ratio. For ammonium [◆] and phosphate [▲] the influent concentrations are used to calculate the initial value. The ammonium concentration is corrected for adsorption of ammonium. The actual ammonium measured after feeding without correction is also indicated [▨]. Nitrous oxide produced during the aerobic cycle [O]

About 70 mg P L^{-1} phosphate is released during the anaerobic feeding period, indicating that most of the acetate being consumed by PAOs. This amount is taken up by the PAOs during the aeration period. Denitrification takes place during the feeding phase as well as during the aeration period. Compared to previous research (De Kreuk et al. 2005b) denitrification is relatively low since we operated here under a high dissolved oxygen concentration minimizing simultaneous denitrification. Due to the plug flow pattern of the

influent through the settled bed, there is minimal mixing between influent and treated wastewater remaining in the reactor at the end of the previous cycle.

Characteristics of granular biomass

Stable granules were obtained at each salinity tested and no obvious visual change in granule morphology was observed during the experiments at different salt concentrations (Fig. 2). Image analysis showed that the average granule diameter increased from 0.9 to 1.7 mm from stage 1-I to 1-II.
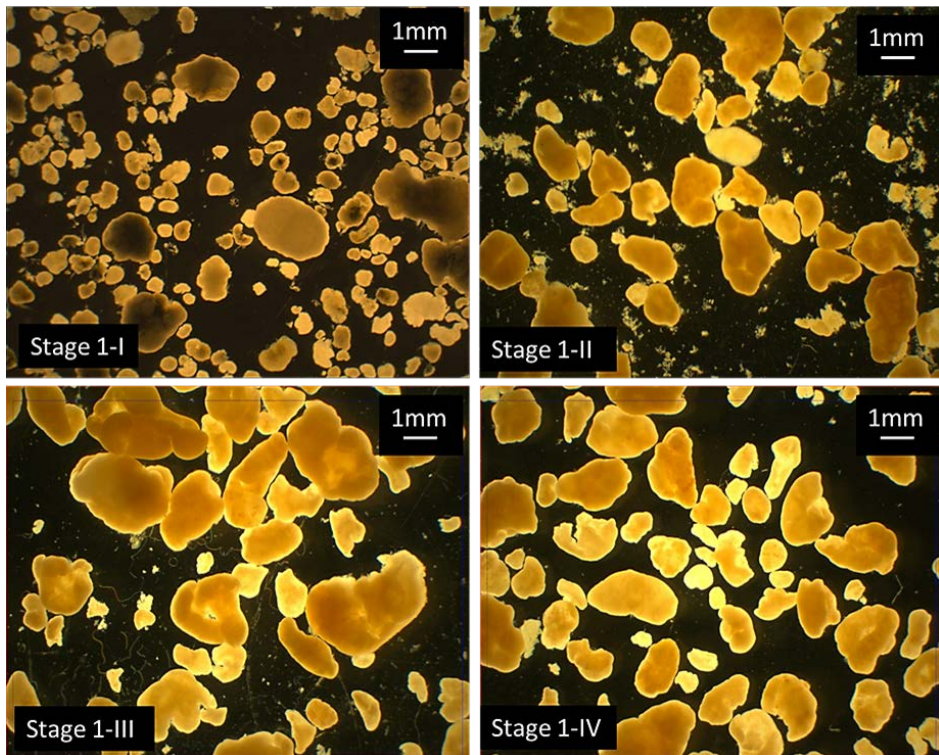

Fig. 2 - Granules grown at different salt concentrations; stage 1-I (0.2g Cl⁻ L⁻¹), stage 1-II (6.6g Cl⁻ L⁻¹), stage 1-III (13g Cl⁻ L⁻¹) and stage 1-IV (20g Cl⁻ L⁻¹).

During Stage 1-II and 1-III, the average granule size (1.7 mm) remained stable. In stage 1-IV, the average granule size decreased to 1.1 mm. No protozoa were observed by microscopy at salinity values of 6.6 g Cl⁻ L⁻¹ or higher, indicating a negative effect of the salt concentration on these higher organisms. Effluent turbidity significantly increased in stage 1-IV (20 g Cl⁻ L⁻¹) and biomass production in the sludge bed was also significantly reduced, although all acetate continued to be totally consumed in the anaerobic feeding period at the highest salinity level. Microscopic analysis revealed the presence of loose cells in the effluent. This was also experienced by the difficulty to filter these effluent samples

over a 0.45 μm filter. Staining of the effluent by Alcian blue showed that acidic polysaccharides were surrounding the individual cells, like a capsule (Fig. 3).
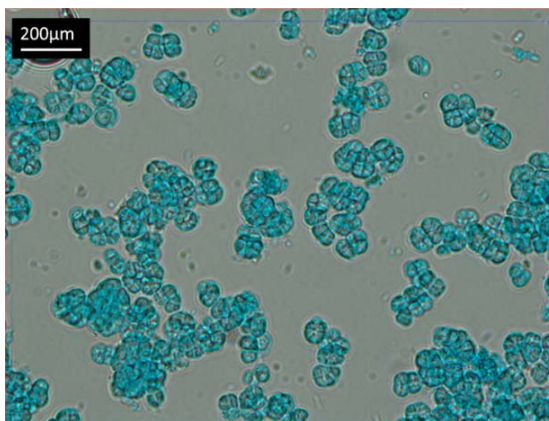

Fig. 3 - Alcian blue stained cells in the effluent at stage 1-IV (20 g Cl⁻ L⁻¹)

Nitrogen conversions

Ammonium was removed within 50 minutes of aeration at all salt concentrations. Nevertheless, although the volumetric removal rate of ammonium remained stable, biomass specific ammonium conversion rates declined in steady state by 55 % when the salinity was increased from 0 to 6.6 g Cl⁻ L⁻¹. The salt increase from 6.6 to 13 g Cl⁻ L⁻¹ had only a slight extra negative effect on the specific ammonium uptake rate (Fig. 4). Ammonium removal rates even increased slightly at 20 g Cl⁻ L⁻¹. After each increase in salinity, the specific ammonium uptake rates initially decreased much stronger, but quickly recovered to values comparable to those shown in Fig. 4.
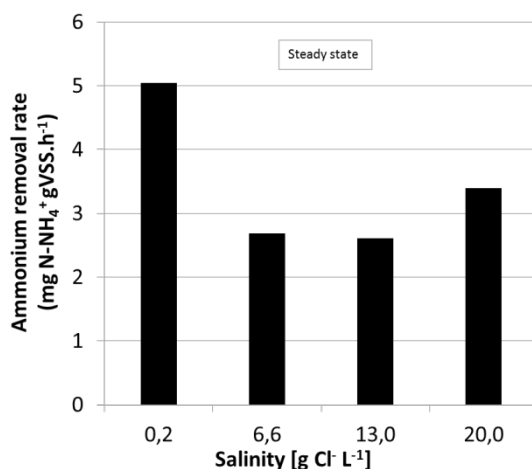

Fig. 4 - Specific ammonium uptake rates during steady state operation at different additional chloride concentrations in the medium.

Maximum nitrite concentrations during steady state conditions for 0.2, 6.6, 13 and 20 g Cl⁻ L⁻¹ were respectively 0.8, 4.90, 13.0 and 47.4 mg NO_2^--N L⁻¹ (Fig. 5a).

The increase in salinity from stage 1-II (6.6 g Cl⁻ L⁻¹) to stage 1-III (13 g Cl⁻ L⁻¹) initially had no effect on the nitrite oxidation capacity as was reflected in the unchanged nitrite accumulation during the cycles (data not shown).
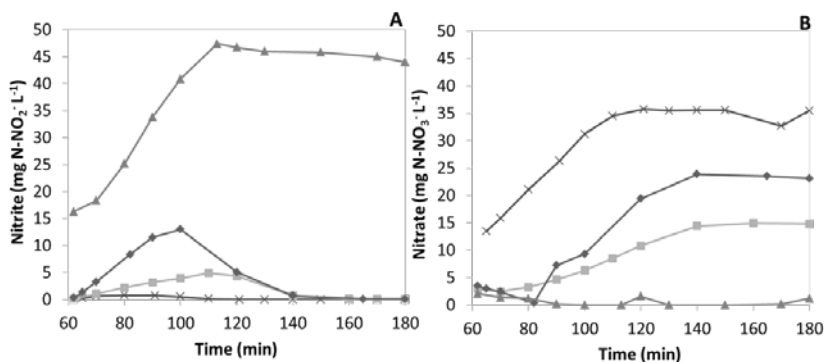
Fig. 5 - Nitrite (A) and Nitrate (B) accumulations during the aerobic part of the cycle at different salinities during steady state; [✕]0.2g Cl⁻ L⁻¹, [▦]6.6g Cl⁻ L⁻¹, [◆]13g Cl⁻ L⁻¹, [▲]20g Cl⁻ L⁻¹

However, during the long-term exposure to elevated salinities during Stage 1-III (10 weeks), nitrite concentration increased along the cycle and amounted up to 13 mg NO_2^--N L⁻¹ (Fig. 5a). An immediate effect of salinity on nitrite oxidizers was observed when the salinity was increased in stage IV to 20 g Cl⁻ L⁻¹, here the nitrite accumulated within 2 days from 13 to 25.5 mg NO_2^--N L⁻¹. The nitrite oxidation capacity completely disappeared over the course of 6 weeks during this period (Fig. 5b). At steady state, nitrite concentrations accumulated to approximately 47 mg NO_2^--N L⁻¹ at the end of the cycle. The nitrite oxidation activity did not return in the following eight weeks, which led to high nitrite concentrations in the effluent.

Nitrate reduction by denitrifiers was observed during the feeding and in the aeration period as long as ammonium was present (Fig. 5b). Denitrification was higher at the intermediate salt concentrations (6.6 and 13 g Cl⁻ L⁻¹) than at the lowest and highest salt concentration during stage 1-I and 1-IV (0.2 and 20 g Cl⁻ L⁻¹), respectively. Nitrous oxide (N₂O) concentrations in the off-gas were also measured (Fig. 6) and showed a dynamic behaviour over the salinities applied. The highest emissions (45 % and 43 %) were observed during stage 1-II and 1-III. The nitrous oxide emissions remained stable and no adaptation was observed.
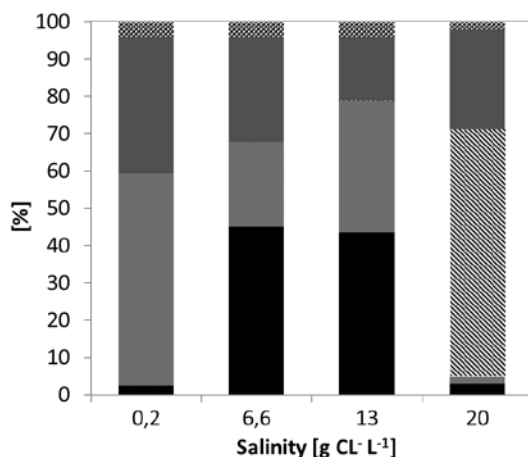
Fig. 6 - Nitrogen balance during steady states at the different Cl- concentrations added to the medium. 100 % is equal to the total ammonium conversion in the reactor (biomass [▦], N_2[▦], NO_2^-[▦], NO_3^-[▯], N_2O[▮]).

Interestingly, during the first and last stage, the nitrous oxide emissions were only 2.5 % and 3.2 % of the total nitrogen fed. Nitrous oxide was mainly produced when ammonium was present in the bulk.
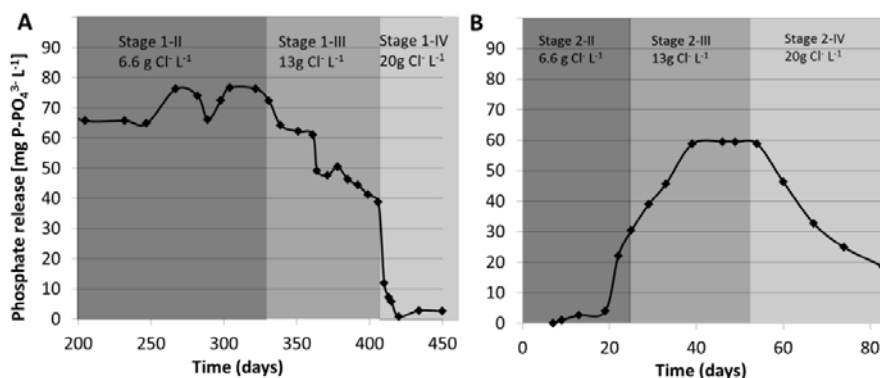
Fig. 7 - (a) Phosphate release after anaerobic feeding period in time in a system with nitrification (b) phosphate release in the anaerobic feeding period in time in a system without nitrification (ATU inhibited).

Phosphate removal

Phosphate release in the anaerobic feeding period (Fig. 7a) increased from approximately 50 mg PO_4^{3-} -P L^{-1} in stage 1-I to 70 mg PO_4^{3-} -P L^{-1} at stage 1-II (6.6 g Cl- L^{-1}). At 13 g Cl- L^{-1} (stage 1-III), phosphate release was initially not affected. In the long term under these conditions, however, phosphate release declined steadily from approximately 70 to 42 mg PO_4^{3-} -P L^{-1}. In stage 1-IV (20 g Cl- L^{-1}), a dramatic and instantaneous decrease in

phosphate release was observed. After about 10 days, the phosphate uptake and release stopped completely.

The increase in salinity from 0.2 g Cl$^-$ L^{-1} to 6.6 g Cl$^-$ L^{-1} at day 17 had a strong effect on the biomass specific phosphate uptake rate, which decreased from 4.8 to 2.3 mg P gVSS h^{-1} (Fig. 8). Measurements on the soluble phosphate concentrations and online measured pH and offgas O_2 and CO_2 time dependent variations indicated that, immediately after the salinity increase, the P-uptake rates decreased even further. However, the uptake recovered again within a few days. In stage 1-III (13 g Cl$^-$ L^{-1}), phosphate uptake rates were initially not affected, but started to decline over time from 2.3 to 1.3 mg P gVSS h^{-1}. The increase of salinity to 20 g Cl$^-$ L^{-1} had an immediate and permanent detrimental effect on the phosphate uptake rate.
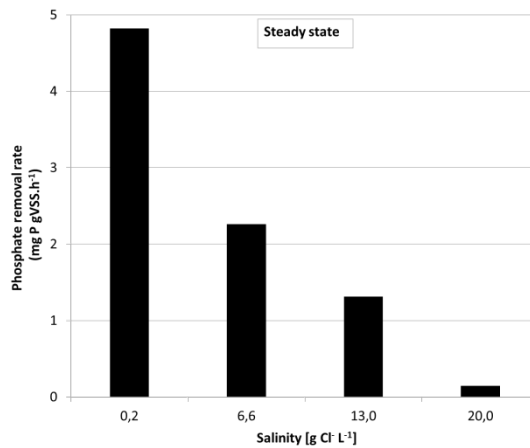
Fig. 8 - Specific phosphate uptake rates during steady state (stage 1-I through 1-IV).

Nitrite inhibition
Phosphate uptake rates were found to decrease when nitrite was accumulating during the cycle at increased salinity. The phosphate uptake (Fig. 9) declined to approximately 40 % of the maximum uptake rate when nitrite accumulated up to 13 mg N-NO$_2$ L^{-1}. Phosphate uptake was gradually recovering when nitrite was consumed. The maximum phosphate uptake rate is observed directly after the 1-hour anaerobic feeding period, when nitrite concentrations are less than 4 mgNO$_2$-N L^{-1}. To evaluate separately the impact of salinity from that of nitrite, a second independent reactor was operated at elevated salinities with the addition of allylthiourea (ATU) to inhibit nitrification and thus prevent nitrite build-up. Without nitrite present (Fig 7b), PAO activity increased at 13 g Cl$^-$ L^{-1} (stage 2-III) and a phosphate release of 60 mg PO$_4^{3-}$ -P L^{-1} was reached. The ratio between PAO and GAO populations revealed a PAO fraction of approximately 50 %. Switching the salinity to 20 g

Cl⁻ L⁻¹ (stage 2-IV) resulted in a gradual, but steady decrease in phosphate release. After 27 days, the PAO fraction reduced to only 16 %.
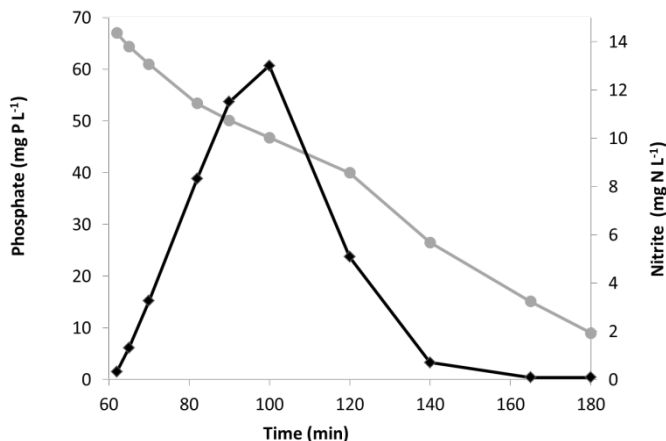
Fig. 9 - Phosphate [●] and nitrite [♦] concentrations during aeration at stage 1-III (13 g Cl⁻ L⁻¹).

Discussion

Impact of salt on granule formation and on its physical properties

Granule formation was achieved at all salinities applied. The smaller granule size during the start-up period is likely a result of the short duration of this period (17 days). The conversions at the end of this initial stage were however already highly similar to those observed in a similar reactor running on steady-state for many months by De Kreuk et al. (2005c). It is a common observation that the time needed to obtain stable conversions is significantly shorter than the time needed for establishment of a stable granule size distribution, which may take many weeks. During stage 1-IV (20 g Cl⁻ L⁻¹), the granule size decreased, the biomass yield dropped and a high effluent turbidity was observed. There is no clear explanation yet for this observation. Potentially the growth yield is decreased due to higher energy requirements. Besides a lower biomass production, a compacting effect of the biofilm due to saline conditions (Vendramel et al. 2011, Li and Wang 2008) has been proposed to lead to a smaller granule size. Microscopy of the effluent liquid samples revealed the presence of single bacterial cells encapsulated in extracellular polymeric substances (EPS). This points to general instability of the granule (i.e., higher erosion rates) leading to smaller granules at higher salt concentrations. An increase in acidic polysaccharides in the effluent was also observed by Bassin et al. (2011c) when treating high salinity (8 g Cl⁻ L⁻¹) wastewater. Figueroa et al. (2008) reported that granule formation was hampered when treating high saline (9 g Cl⁻ L⁻¹) wastewater from a fish canning

industry. They partly related the slowdown in granule formation to the lack of EPS production of the biomass. However, we observed no detrimental effect on granule formation or effluent quality at the chloride concentrations applied by Figueroa et al. (2008). The observation of acidic EPS in the system coincides with recent reports by Lin et al (2010b) that alginate- like polysaccharides might form the major EPS constituents in aerobic granular sludge. Acidic polysaccharides form a gel due to bridging between the carboxylic acid groups and divalent ions such as Calcium. The observed weakening of the EPS at higher NaCl concentrations might be induced by the replacement of Ca^{2+} by Na^+ in the EPS matrix, especially in highly saline conditions (Ismail et al. 2010). Currently this seems the best explanation for the observed decrease in granule size. The decreased strength of the gel shifts the balance between growth and detachment leading to a decreased granule size. This explains the detachment of small pieces of the granules and single cells incorporated in a gel capsule from the granule and washout of those cells with effluent.

Impact of salt on nitrogen conversions

From the operation of the granular sludge bioreactor we observed that the ammonium removal efficiency of the granular sludge was largely unaffected at all salinities applied (0.2 – 20 g Cl^- L^{-1}). This confirms results from previous studies on the effect of salinity on suspended and immobilised nitrifying cultures (Hunik et al. 1992, Moussa et al. 2006a, Figueroa et al. 2008, Campos et al. 2002). Biomass specific ammonium uptake rates remained relatively stable between stages 1-II and 1-III (salinity of 6.6 – 13 g Cl^- L^{-1}) as shown in Figure 4. However, during stage 1-I and 1-IV (0.2 and 20 g Cl^- L^{-1}) rates were higher, in phase 1-I the absence of salinity is most likely responsible together with a smaller granule size. Smaller granules have a higher specific surface area and thereby a higher specific oxidation capacity, this can also explain the slight increase in rates during phase 1-IV. In suspended cell cultures, increasing salt concentrations generally have been shown to strongly inhibit the nitrifying activity (Hunik et al. 1993, 1992, Moussa et al. 2006a, Moussa et al. 2006b). Moussa (2006a) reported only approximately 15 % of the maximum activity at a salt concentration of 20 g Cl^- L^{-1}, while in this experiment 60 % of the maximum ammonium oxidation rates were obtained at the highest salinity. This difference in inhibition sensitivity between suspended cultures and biofilm systems has been mainly explained by an increased aerobic zone in the biofilms/granules when the cell specific activity decreases (Wijffels et al. 1995, Leenen et al. 1997, De Kreuk et al. 2005c). Although these results were obtained from temperature experiments, salinity would have a similar effect on the concentrations inside the granules. The reduction of specific activity by salinity leads to less oxygen consumption by the biomass, much like lower temperature would. In aerobic granular sludge this means that the oxygen penetration depth inside the granule will increase. This gives an increase of aerobic biomass volume, which compensates for the decreased cell specific ammonium oxidation rates. Suspended biomass does not have this advantage. Moreover, the relative high biomass capacity in granular sludge

compared to suspended systems leads to a decreased sensitivity towards adverse conditions. This is equally true for the impact of stress factors and toxicities on biofilms compared to suspended systems (Frijters et al. 1997b, Artiga et al. 2003, Liu et al. 2009, Buitrón and Moreno-Andrade 2011, Ogugbue et al. 2011).

Compared to ammonium-oxidizing bacteria, nitrite-oxidizing bacteria were severely inhibited at increasing salt concentrations and showed complete inhibition at 20 g Cl^- L^{-1}. It is widely reported that nitrite-oxidizing bacteria are more sensitive to salt stress (Moussa et al. 2006a, Figueroa et al. 2008, Vredenbregt et al. 1997). The generally reported disappearance of nitrite oxidizers at higher salinities is in agreement with our observations. This fact means that at higher salt concentrations it might be easier to develop granular sludge processes with nitrite as the main intermediate of nitrification-denitrification processes. These nitrite-based processes should certainly be considered for more saline type of industrial wastewater.

Impact of salt on nitrous oxide emissions
High nitrous oxide emissions were measured during stage 1-II and 1-III (6.6 and 13 g Cl^- L^{-1}). In both cases, around 45 % of the influent ammonium was converted into nitrous oxide, while only 2 – 3 % was emitted as nitrous oxide at low and high salinities. Various process parameters can cause nitrous oxide emissions. Among some of the generally accepted parameters are salinity and high and low dissolved oxygen concentrations. Denitrification as well as nitrification processes have been reported as the cause of nitrous oxide formation (Kampschreur et al. 2009).

Zeng et al. (2003) reported denitrifying glycogen-accumulating organisms (DGAOs) were most likely responsible for the N_2O emissions measured. Nitrite accumulation (> 1mg N L^{-1}) in their nitrifying GAO reactor had a major influence on the N_2O emissions. Accumulation of nitrite inside the flocs was given as a potential cause for N_2O emission by DGAOs when nitrite bulk liquid concentrations were almost zero. Also Lemaire et al. (2006) concluded that DGAOs were more likely to be responsible for the nitrous oxide emissions in simultaneous nitrification, denitrification and phosphorus removal (SNDPR) systems.

In our experiments, almost no nitrite accumulated in stage 1-I (0.2 g Cl^- L^{-1}) with the corresponding Low emissions of nitrous oxide. Remarkably, at 20 g Cl^- L^{-1} (higher salt concentration tested), the nitrite accumulated to roughly 45 mg N-NO_2^- L^{-1}, but the N_2O emissions remained low. Nevertheless, in this stage, GAO were the dominant organisms and were practically fully responsible for the acetate uptake under anaerobic conditions while AOB were still fully active (Bassin et al. 2011a). This suggests that nitrifiers are potentially responsible for the low nitrous oxide emissions measured at the lowest and highest salinity. Denitrification and the enrichment of DGAOs are expected to be the main cause for the nitrous oxide emissions during the intermediate salinities in our system.

Recently, Ye et al (2010) showed that at nitrite concentrations above 30 mg N-NO$_2$ L^{-1} (at pH 7) completely inhibited the growth of GAOs, while glycogen production and PHA (polyhydroxyalkanoates) consumption severely decreased. This could possibly explain our observation at the highest salinity where not only the nitrous oxide reductase but, denitrification by GAO as a whole, was inhibited. However, further research and more detailed experiments are needed to determine the responsible parameters and organisms which would trigger nitrous oxide emissions in aerobic granular biofilms in general, but also in more challenging conditions (i.e., high salinity). This should especially focus on the interaction between the different species present in aerobic granular sludge.

Impact of salt on biological phosphate removal

Biological phosphate removal was found to be largely unaffected as long as the salinity did not exceed 6.6 g Cl$^-$ L^{-1}. Concentrations of 13 g Cl$^-$ L^{-1} resulted in gradual loss of biological phosphate removal capacity. Instantaneous deactivation of phosphate accumulating organisms was observed when the salinity was increased to 19.9 g Cl$^-$ L^{-1}, when few PAO cells were still observed by fluorescent in situ hybridization (FISH). From our understanding, dual inhibition of salinity and nitrite on PAOs are the two main reasons for the loss of phosphate removal activity in the first experiment. This became apparent when, in the second experiment (Fig. 7b) with high salinity (13 g Cl$^-$ L^{-1}) and suppressed nitrification by ATU the phosphate release after feeding increased from 2.7 to 60 mg P-PO$_4$$^{3-}$ L^{-1}. Nitrite concentrations obviously did not accumulate in this specific test showing that PAOs are able to compete with GAOs at this salinity when no nitrite was present. Even at 20 g Cl$^-$ L^{-1} the PAOs remained active in the second experiment. Also from a parallel study mainly focused on microbiological aspects, microbial diversity analysis by FISH and denaturing gradient gel electrophoresis (DGGE) confirmed the decline of the PAOs and the subsequent dominance of GAOs at the highest salinity (Bassin et al. 2011a). These results point to inhibition of PAOs at elevated salinity, but the major inhibition effect below 20 g Cl$^-$ L^{-1} is attributed to nitrite accumulation.

Nitrite inhibition of aerobic bacteria and anoxic/aerobic phosphate uptake in PAOs has been widely reported in Literature (Saito et al. 2004, Yarbrough et al. 1980, Rowe et al. 1979). Saito et al. (2004) showed that nitrite concentrations of 2 mg NO$_2$$^-$-N L^{-1} resulted in a severe decrease of activity and a complete inhibition on the aerobic phosphate uptake above 6 mg NO$_2$$^-$-N L^{-1}. Meinhold et al.(1999) reported that nitrite concentrations up to 4-5 mg NO$_2$$^-$ -N L^{-1} had no detectable effect on both anoxic and aerobic uptake of phosphate. These authors indicated that higher concentrations of nitrite ($\sim$ 8 mg NO$_2$$^-$-N L^{-1}) can inhibit anoxic phosphate uptake completely, while aerobic uptake is only severely affected, which is in Line with our results. In contrast with the report by Saito et al.(2004), Meinhold et al.(1999) and Pijuan et al.(2010) reported a reversible inhibition effect of nitrite, even though recovery times of several hours were needed before original rates were restored.

Our experimental results indicated that P-uptake rates were recovered directly when nitrite concentrations decreased due to nitrite oxidation and/or nitrite reduction by denitrification. Also Zeng et al.(2003) found a short temporarily inhibition for nitrite, that immediately disappeared when nitrite concentrations declined. It might be that different PAO clades (Flowers et al. 2009), present in the experiments, are the main cause of the conflicting reports. Although PAO seemed to be able to recover from the inhibition, they have a competitive disadvantage compared to GAO when nitrite accumulates, assuming that GAOs are not or less inhibited at these nitrite concentrations (Saito et al. 2004). If nitrite concentrations remain high for long periods during the aerobic period, PAO will have a disadvantage since they will store less polyphosphate, which will eventually lead to deterioration of phosphate removal efficiency. These results show that in simultaneous phosphate and nitrogen removal processes cascade effects could play an important role that should not be overlooked

4

Effect and behaviour of various substrates in relation to the formation of aerobic granular sludge

Introduction

Aerobic granular sludge (AGS) is an innovative technology to simultaneously remove nitrogen, phosphorus and carbon from wastewater with bacterial granules. This is achieved in one reactor compartment, therefore the requirement of space and energy are highly reduced (De Kreuk and van Loosdrecht 2006b). The process is operated as a sequencing batch system allowing for optimal process control and flexibility. Full-scale aerobic granular reactors treating domestic wastewater are currently operational in The Netherlands, Portugal and South Africa (Giesen et al. 2013). Aerobic granular sludge is formed by applying selective environmental pressures on bacteria generally found in sewage treatment sludge (Beun et al. 2000a, McSwain et al. 2004). Selection of fast settling over slower settling biomass is commonly reported for the selection of AGS (Lochmatter and Holliger 2014a, Jungles et al. 2011, Liu et al. 2005a). However, the selection of relative slow growing bacteria is even more or equally important to fully utilize the potential of AGS (De Kreuk et al. 2005d). Easy biodegradable COD when supplied under anaerobic conditions can be converted into storage polymers, by organisms such as polyphosphate accumulating organisms (PAO). The easily degradable COD is in this way stored in a larger fraction of the granule volume. When all soluble COD is removed, the bacteria are supplied with oxygen (and nitrate) and they slowly convert the substrate into CO_2 and new biomass. This eliminates the presence of fast growing aerobic heterotrophs on dissolved COD and results in smooth dense granules due to growth of heterotrophs throughout the granule (De Kreuk and van Loosdrecht 2004).

As AGS is quickly adopted as technology for the treatment of domestic and some industrial wastewaters, so does the need for further knowledge on the effect of different carbon compounds on the granulation process. As for now mainly acetate, propionate, ethanol, sugars, molasses, and sewage have been used in Lab scale reactors to investigate granulation (Morgenroth et al. 1997, Beun et al. 1999, Beun et al. 2002, Tay et al. 2002, de Kreuk and van Loosdrecht 2006c, Weissbrodt et al. 2013a).

The ability of bacteria to store substrates anaerobically plays an important role in the effective formation and stability of AGS. Some substrates might be very difficult or even impossible for bacteria to utilize anaerobically without special requirements and their impact on the granulation potential and stability is still largely unknown.

In this work we studied the conversion and evaluated the granulation potential of a set of carbon compounds frequently encountered in industrial wastewaters i.e. acetate, methanol, butanol, propanol, propionaldehyde and valeraldehyde. Based on the results obtained in this work and those already present in Literature the general effect on AGS of various types of carbon compounds and operational conditions is discussed.

Material and methods

Experimental setup

A double walled glass sequencing batch reactor (SBR) with an internal diameter of 6.25 cm, 1.5 m in height and 2.7 litres working volume was operated as a bubble column. The temperature of the reactor was controlled at 35 $\pm$0.5 °C by means of a cryostat similar to the reactor used by de Kreuk et al. (2005e) and Winkler et al. (2011). The influent was preheated to ensure that the reactor remains at the correct temperature during feeding. The off gas was recirculated with a constant flow of 5 L min^{-1} to keep the dissolved oxygen at its desired set point of 3.5 mg O_2 L^{-1}. The dissolved oxygen concentration in the reactor medium was controlled by supply of a nitrogen gas and air mixture via mass flow controllers. A bio controller (Braun DCU4 coupled with Multi Fermentor Control System acquisition software; Sartorius Stedim Biotech S.A., Melsungen, Germany) was used to control and operate the sequencing batch reactor. The volume exchange ratio was 0.56. The reactor was operated at a cycle length of 3 hours, following an anaerobic - aerobic regime as shown in Table 1. The dosage of 1 M NaOH and HCl controlled the pH at 7.1 $\pm$0.05 during the aeration period. The sludge used for inoculation of the reactor was obtained from an activated sludge treatment plant treating domestic wastewater with phosphorus and nitrogen removal. The total suspended solids (TSS) and volatile suspended solids (VSS) were calculated as described in Pronk et al. (2014).

Table 1: Operation of the cycles in the aerobic granular sludge reactor		
Phases	Time	Volume
	[min]	[L]
Anaerobic feeding	60	1.5
Aeration	100 - 112	
Settling	3 - 15	
Effluent withdrawal	5	1.5
Total cycle length	180	

Medium

The synthetic medium consisted of 150 mL medium A and 150 mL medium B dosed together with 1200 mL heated tap water, achieving an influent temperature of 35 ºC. The composition of medium A consisted of 1.13 g L^{-1} NH$_4$Cl, 0.13 g L^{-1} K$_2$HPO$_4$, 0.05 g L^{-1} KH$_2$PO$_4$, 0.88 g L^{-1} MgSO$_4$·7H$_2$O, 0.35 g L^{-1} KCl, and 90 mL L^{-1} trace element solution with the following composition: 63.7g L^{-1} C$_{10}$H$_{14}$N$_2$Na$_2$O$_8$•2H$_2$O (EDTA TITRIPLEX® III), 4.99 g L^{-1} FeSO$_4$.7H$_2$O, 2.2 g L^{-1} ZnSO$_4$.7H$_2$O, 7.34 g L^{-1} CaCl$_2$2H$_2$O, 5.06 g L^{-1} MnCL$_2$.4H$_2$O, 1.51 g L^{-1} Na$_2$MoO$_4$.2H$_2$O, 1.57 g L^{-1} CuSO$_4$.5H$_2$O, 3.22 g L^{-1} CoCl.6H$_2$O.

The composition of medium B was changed after 64 days of operation. First medium B consisted of: 4.87 g L^{-1} HAc, 0.5 g L^{-1} MeOH, 0.53 g L^{-1}PrOH, 0.29 g L^{-1}BuOH. After 63 days, the HAc contribution to the chemical oxygen demand (COD) was halved and substituted by propionaldehyde and valeraldehyde. This changed the concentration of HAc to 2.4 g L^{-1}, propionaldehyde to 0.27 g L^{-1} and valeraldehyde to 0.22 g L^{-1} in medium B. The total COD concentration of the influent fed to the reactor was 509 mg COD L^{-1} and the total nitrogen was 29.5 mg N L^{-1}.

Analytical procedures

The chemical oxygen demand (COD), ammonia, nitrite, nitrate and phosphate concentrations in the bulk liquid were measured weekly with a spectrophotometer cuvette system from Hach Lange (DR2800). Acetate, methanol, propanol, pentanol, propionaldehyde, butanol, propionic acid, valeric acid and valeraldehyde were quantified by gas chromatography (GC) (Oudshoorn et al. 2009).

Methane concentrations from the anaerobic batch tests were determined with a Varian 3800 custom solution Gas Chromatograph. Gas samples were injected with a 100-µL gastight Hamilton syringe in a Varian Ultimetal 1079 split/splitless, which was operated at 200 °C at a split ratio of 100. A CP-Sil-5CB (50m x 0.32 mm) capillary column was used isothermally at 100 °C at a constant gas flow rate of 10 mL min^{-1}. The used carrier gas was Helium. Methane peaks were detected with a Varian Flame Ionisation Detector, which was operated at 300 °C. The Helium make-up flow was 25 mL min^{-1}, hydrogen flow was 30 mL min^{-1} and the airflow was 300 mL min^{-1}. Carbon dioxide samples were separated on a Hayesep Q 80/100 mesh 0.25 x 1/16" x 1 mm Ultimetal micro packed column via a Varian 1041 on-column direct injector. Helium was applied as carrier gas at a pressure of 12.5 PSI. Temperatures of the TCD detector, column and injector used were 300, 50 and 120 °C, respectively.

Pottered and sliced granules were examined with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 06 (bp 436/10 FT 460). To visualize methanogens in these samples the fluorescence of coenzyme F$_{420}$, present in most methanogens, was used as described by Reuter et al. (1986).

Analysis of polyhydroxyalkanoates

Biomass samples were collected in 15 mL plastic falcon tubes and freeze-dried. Approximately 40 mg of homogenized freeze dried sample, 2 mg of mixed standard (88 % PHB, 12 % PHV) and 2 mg of 2-hydroxyhexanoic acid 98 %, 2 mL of chloroform and 2 mL of acidified methanol were put in borosilicate glass tubes and mixed vigorously. The closed tubes were heated for 1 day at 95 – 100 °C in a heating block. After which the tubes were cooled down to 4 °C for 30 minutes. One millilitre of aqueous ammonia solution (14 %) was added and vigorously mixed before centrifugation with 2500 rpm for 5 minutes. The samples were stored at 4 °C for 1 hour to prevent methanol evaporation.

About 1.5 mL of the chloroform phase of each tube was collected and inserted into closed GC ampules. One microliter of the chloroform phase was injected in an Agilent 6890N gas chromatograph. The chromatograph was operated with a HP-innowax Column (60 m x 0.25 mm X 0.15 µm), Helium as a carrier gas (1.7 mL min^{-1}). The flame ionization detector (FID) unit was operated at 300 °C with an injection port temperature of 250 °C. The oven temperature was set to 80 °C for 1 min, increased at 10 °C min^{-1} to 120 °C, and then to 270 °C at 45 °C min^{-1} and held for 3 min. The column used was a ZB-Wax (20m x 0,18mm x 0,18µm) with helium as a carrier gas (230 kPa). Injector temperature was set at 240 °C; transfer line temperature was 250 °C, the split ratio 1:8. Electron impact ionization was set to 70 eV with a source temperature of 250 °C.

Off-gas measurements

Gas analysis of the recycle flow was measured online with a Rosemount analytical NGA 2000 MLT gas analyser for carbon dioxide and oxygen. Methane was measured with a Servomex 4900 infrared gas analyser. Both analysers were calibrated regularly with the corresponding gasses. The built in pressure sensor corrected automatically for changes in atmospheric pressure.

Batch experiments

Anaerobic batch experiments for the different substrates were performed with OxiTop® Control AN6/AN12. The measuring heads were equipped with a pressure sensor (-360 hPa, +360 hPa). Every individual bottle used was carefully calibrated for its volume; the increase of the pressure in the headspace (hPa) could then be related to the conversion of the added substrate via the ideal gas law. About 0.6 gVSS of granular sludge was added per bottle and filled with influent from the reactor (without the carbon sources) together with a 25 mM HEPES buffer solution set to pH 7.2 (purged with nitrogen gas for 5 minutes) to a total volume of 200 mL. The bottles were then incubated on a shaker at 190 rpm and at 35 °C. After reaching the required temperature, the elevated pressure (due to the temperature increase) was released with a water lock to maximize the available pressure range. Recording was started and 50 mg MeOH, PrOH, BuOH, PeOH, propionaldehyde, valeraldehyde and 120 mg acetate was injected. Soda lime pellets present in the headspace were used to absorb carbon dioxide in some experiments. An Oxitop® OC110 remote controller was used to monitor and gather the data without the need to disturb the measurement. Batch tests performed with granules from the reactor are summarized in Table 2.

Sample collection

Granules were taken from the reactor and pottered to create a cell suspension. The cell suspension was washed two times with PBS buffer. The supernatant was discarded and the pellet was stored at -80˚C.

Substrate	Product				
	CH_4	CO_2	PHA	PrOH	PeOH
Methanol	+	+	-	-	-
Acetate	-	+	+	-	-
Propanol	-	-	-	-	-
Butanol	-	-	-	-	-
Pentanol	-	-	-	-	-
H_2/CO_2	-	-	-	-	-
Propionaldehyde	-	+	+	+	-
Valeraldehyde	-	+	+	-	+

Table 2: Anaerobic batch tests with aerobic granules

DNA extraction

The pelleted cell suspension was extracted after a pre-treatment of grinding under liquid nitrogen, which was repeated three times followed by the usage of the Ultraclean Microbial DNA extraction kit (Mobio, USA) according to the supplied protocol. After extraction 5 µl of a total of 50 µl of gDNA solution was subjected to gel electrophoresis to check for quality and quantity.

PCR and DGGE analysis

For the universal detection of the 16s-rRNA gene from the archaeal domain we used the following PCR primer set, Parch519fm (Øvreås et al. 1997) in combination with a modified primer Arc934r (5'- GTGCTCCCCCGCCAA- 3') originating from the probe Arc915r developed by Stahl and Amann (1991) which is more specific in the detection of only archaea. For DGGE analysis a GC-clamp (Muyzer et al. 1993) was added to the 5'-end of the Arc934r primer. For amplification the following temperature program was used, an initial denaturation of 5 minutes at 95 °C followed by 28 cycles of 30 seconds at 95 °C, 40 seconds at 62.5 °C, 30 seconds at 72 °C and a final elongation of 30 minutes at 72 °C. The product (250 ng) was subsequently analysed on DGGE according to Bassin et al. (2011d) with the exception that we used a modified Urea-Formamide gradient, 30 – 60 % and a running protocol of 5h at 200V. As an alternative the near full 16s-rRNA genes from archaea were amplified using the primers S-D-Arch-0025-a-S17 and S-*-Univ-1517-a-A-21 as describes by Vetriani et al. (1999) and subjected to DGGE analysis. We used a different PCR annealing temperature of 57 °C instead of 48 °C and an elongation time of 90 seconds at 72 °C. This analysis was performed to confirm the results of the partial 16s-rRNA gene DGGE.

RESULTS

Description of start up

Activated sludge from a conventional wastewater treatment plant in the Netherlands (WWTP Harnaschpolder, Den Hoorn, Netherlands) that had good nitrogen and phosphate removal capability was used to inoculate the reactor. The carbon medium used during the start-up contained; acetic acid, propanol, butanol and methanol (see materials and methods). A settling time of 20 minutes was selected at first to accommodate the slower settling velocity of the activated sludge and allow the biomass to adapt to the synthetic substrate. This was gradually decreased by 3 - 5 minutes over the course of three weeks to a final three-minute settling time corresponding to a minimum settling velocity of 12 m h^{-1}.

The SRT was not actively controlled and was therefore determined by the solids in the effluent. First granulation occurred after 15 days although flocculent biomass was still predominantly present. Analysis of the bulk liquid after the anaerobic feeding period indicated that roughly 40 % of the COD was not removed by the sludge. The incomplete removal was mainly because methanol, butanol and propanol were initially not taken up during the feeding period. This led to floc formation in the mixed aeration period as can be seen in Figure 1a. After 64 days, propionaldehyde and valeraldehyde were introduced in the feed replacing a part of the acetate. Total COD was kept constant. Full ammonium removal was already present early in the experimental run, but let to nitrite accumulation (4 - 6 mg N L^{-1}) in the effluent until approximately day 80 (Fig. 2c). The decrease in dissolved oxygen (from 7 to 3.5 mg O^2 L^{-1}) at day 83 resulted in a decrease of nitrite in the effluent due to a higher simultaneous denitrification. During the aeration period, 50 % of the nitrogen was removed via denitrification.

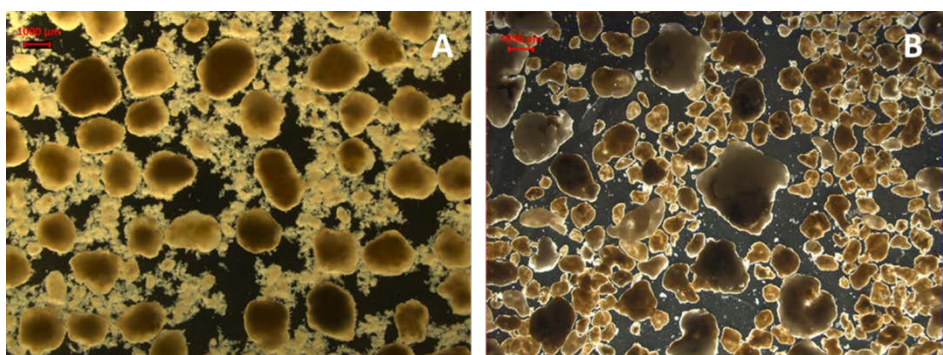**Fig. 1 - Stereoscopic view of aerobic granules (a) day 35 grown on acetate, methanol, propanol and butanol, (b) day 225 grown on acetate, methanol, propanol, butanol, propionaldehyde and valeraldehyde, size bar is equivalent to 1000 μm.**

Overall nitrogen removal remained roughly 75 % throughout the experiment. Nitrogen removal was not optimized during this experiment. Full removal of the biodegradable

COD during the anaerobic feeding period was achieved after approximately 160 days. At this time, the biomass in the reactor had reached approximately 8 - 9 g VSS L^{-1} (Fig. 2a) and a solid retention time (SRT) of 30 days (Fig. 2b). From this time onward also the flocculent biomass and the many protozoa previously observed by microscopy disappeared completely from the reactor and the sludge volume index stabilized at a low value (Fig. 2b). At day 256 approximately 100 mL of granules were removed from the reactor for an unrelated experiment; hence the decline in biomass concentration. Alcohol concentration after the anaerobic feeding; (d) decrease of propanol and butanol (closed diamonds) found after the anaerobic feeding period versus the volatile suspended solids over time, expected (calculated) concentration of propanol (open diamond) in the bulk liquid after the anaerobic feeding period without conversion in a completely mixed reactor.
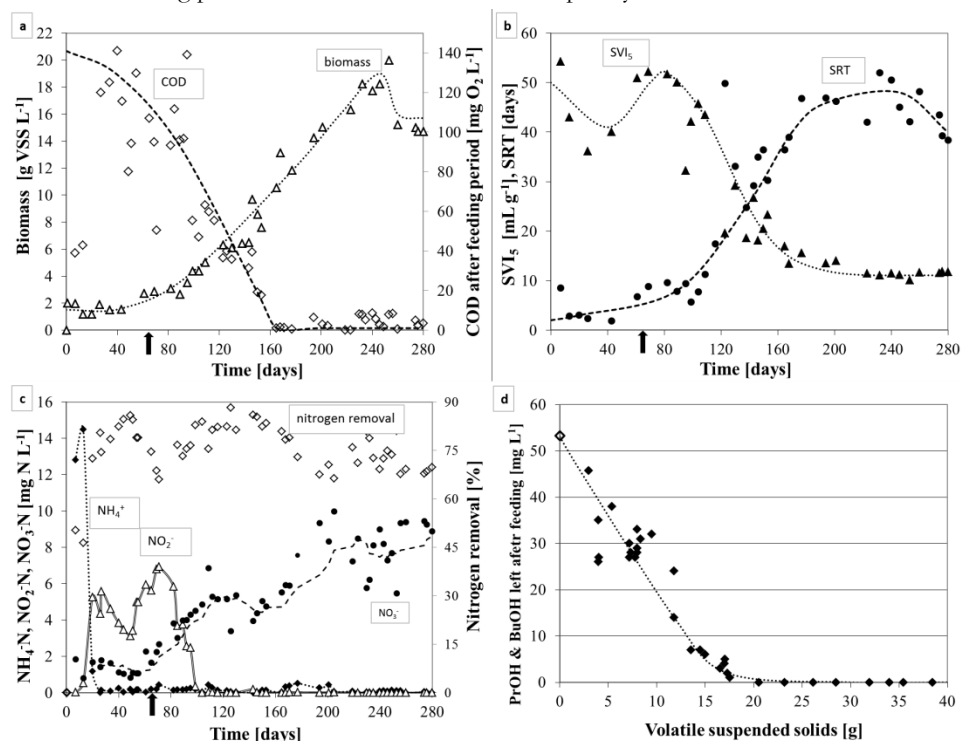
Fig. 2 - Evolution of biomass growth; (a) volatile suspended solids concentration in the reactor (open triangles) and chemical oxygen demand in the reactor after the feeding period (open diamonds). Evolution of granular sludge; (b) sludge volume index (closed triangles) and solid retention time (filled circles). Nitrogen conversions; (c) total nitrogen removal efficiency (open diamonds), ammonium (filled diamonds), nitrite (open triangles) and nitrate (closed circles). Black arrow indicates the time at which halve of the acetate (COD) was replaced by COD-equivalent amounts of propionaldehyde and valeraldehyde in the medium. Lines are shown to indicate trends.

Methanol

During steady operation of the reactor, gas bubbles could be observed escaping from the settled bed during the anaerobic feeding period. Online off gas measurements showed high concentrations of methane shortly after the aerobic period started. Online quantification of the methane after feeding was found to be troublesome due to strong concentration dynamics in the off gas. It was therefore difficult to quantify the methane production during the feeding period. In order to quantify methane production during feeding, anaerobic batch experiments were performed. Batch tests with methanol as the only substrate showed that methane was indeed formed. To verify that the methane produced during the anaerobic feeding period was only derived from methanol, also the other carbon compounds present in the feed were evaluated in batch tests. Acetate, propanol, butanol, propionaldehyde and valeraldehyde were not contributing to the production of methane (Table 2). Furthermore, various combinations of the above-described substrates did not yield any methane production, except when methanol was present.

A batch test with a gas mixture of hydrogen and carbon dioxide in the headspace did also not yield any methane. The obtained anaerobic methanol conversion rate in batch tests was determined to be 0.4 - 0.6 mM MeOH g VSS^{-1} h^{-1} at day 200. With these conversion rates, only 1.4 – 2.2 g VSS L^{-1} is required to completely convert methanol during the one-hour feeding period. During the start-up period methanol was not completely converted during the anaerobic feeding period (Fig. S1). To detect the responsible methanogen species Denaturing Gradient Gel Electrophoresis (DGGE), separating amplified archaeal 16s rRNA gene fragments, was performed (Fig. S2). A single methanogenic archaea with 99.7 % – 99.9 % similarity to *Methanomethylovorans uponensis*, a species out of the *Methanosarcinaceae* family, was found to dominate the aerobic granules. Sequences were deposited into GenBank under accession number KP064473 - KP064477. No other methanogenic species were detected. Methanogens in the granules were also detected under ultra violet light (excitation at 420 nm, emission 470 nm) using an epifluorescence microscope (Fig. 3). Mainly coenzyme F$_{420}$ present in most methanogens will fluoresce under these conditions, making the presence of methanogens that have this coenzyme easily visible (Reuter et al. 1986). The observed methanogens were growing in large clusters of two to four cells (Fig. 3a, b). They mainly seemed to grow a bit more in the depth of the granule in dense clusters just beneath the surface (Fig. 3c, d).
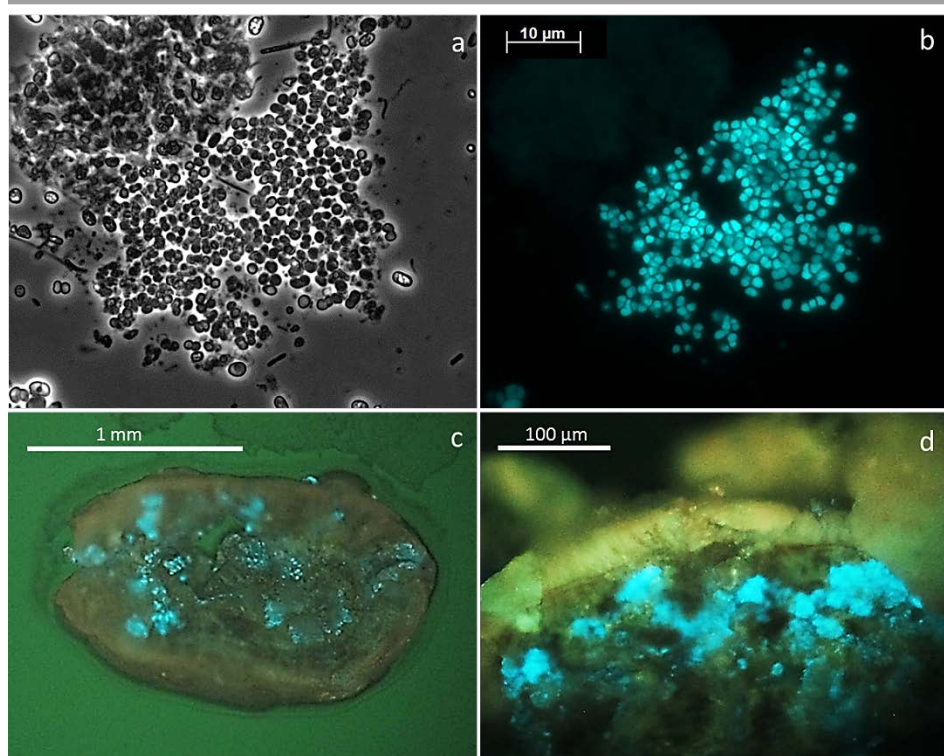
Fig. 3 - fluorescence of *Methanomethylovorans uponensis* by excitation of coenzyme F_{420} (excitation 420, emission 470nm) in pottered granules; phase contrast (a) and fluorescence (b) and in a sliced granule (c and d).

Propanol and butanol

Figure 2d indicates that an increase in biomass corresponded with a higher removal of both propanol and butanol during the anaerobic feeding period. Once a significant granular bed ($\pm$ 8 - 9 g L^{-1}) had developed, both alcohols could not be detected anymore after the anaerobic period and no more flocculent growth was observed (Fig. 1b). This is also reflected by the decrease in COD found after the feeding period (Fig. 2a). At day 64 propionaldehyde and valeraldehyde were introduced in the feed, which led to the release of extra propanol and pentanol from propionaldehyde and valeraldehyde disproportionation respectively (see below). The extra effective alcohol load was not fully removed in the anaerobic feeding period, and the remaining alcohols in the liquid phase were converted aerobically. This temporarily induced more floc formation and worsened the SVI_5 of the biomass (Fig. 2b). To investigate the fate of propanol and butanol during the anaerobic feeding period, a variety of anaerobic batch tests were performed (Table 2). These tests showed no conversion of the alcohols in the storage polymer PHA. Neither were carbon dioxide or methane produced from these substrates, indicating that no anaerobic bioconversion occurred.

Aldehydes

Propionaldehyde and valeraldehyde were not detected after the anaerobic feeding period. To investigate the behaviour during the anaerobic feeding, anaerobic barometric batch tests were performed. The measured pressure build-up during the Oxitop batch tests originated from carbon dioxide production (confirmed with GC). Interestingly propanol and pentanol in the propionaldehyde and valeraldehyde tests respectively were detected as being produced. Supplementary batch tests with propionaldehyde and valeraldehyde showed the anaerobic disproportionation reaction of the aldehydes into their corresponding alcohols and carboxylic acids (Fig. 4a, b). Propionic acid produced was completely removed from the liquid by the granules, while valeric acid was only partly removed during the batch tests. Conversions of the aldehydes also lead to PHA accumulation. A carbon balance over the test showed that indeed the conversion of both propionaldehyde and valeraldehyde was almost completely balanced by production of the alcohol, carbonic acid compounds and PHA. The carbon balance closed for 92 % and 90 % for propionaldehyde and valeraldehyde respectively. The theoretical glycogen conversion into PHA has been subtracted, since it was not separately measured (Lopez-Vazquez et al. 2009a). Conversion rates derived from batch tests were 1.17 and 0.98 mmol (g VSS.h)$^{-1}$ for respectively propionaldehyde and valeraldehyde at 35 ºC.
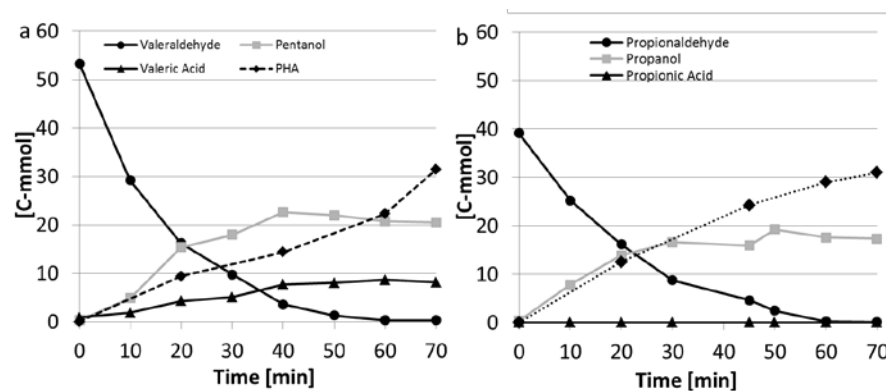
Fig. 4 - Anaerobic conversion of valeraldehyde (a) and propionaldehyde (b) at 35 ºC, pH 7.2 in batch with granular sludge with 16 and 15 g VSS L-1 respectively.

Discussion

Methane production by aerobic granular sludge

Methanol was completely converted to methane and carbon dioxide during the one-hour anaerobic feeding despite the relative high dissolved oxygen concentration (3.5 mg O_2 l^{-1}) during the two-hour aeration period. The high average SRT of approximately 50 days obtained, most likely allowed the growth of *M. uponensis*, which was identified as the only methanogen present in this system. The results from the various carbon sources in the

anaerobic batch tests showed that only methanol was converted to methane. This merits the results also found by Ach et al. (2013) with *M. uponensis*. The reported catabolic substrates are trimethylamine, dimethylamine, monomethylamine, methanol, dimethyl sulphide, and methanethiol, while H_2 - CO_2, 2-propanol and acetate are not. Both the reported optimal temperature and pH range of 37 ºC and 6.5 – 7.0 respectively for *M. uponensis* meet the operational characteristics of the reactor described in this paper.

Utilization of acetic acid by methanogens is likely prevented in AGS systems by the fast anaerobic acetic acid uptake of phosphate accumulating organisms (PAOs) or glycogen accumulating organisms (GAOs) that are normally present in these systems. In prolonged tests without methanogens present, methanol did not lead to PHA production in the anaerobic period (data not shown). Production of storage polymers from methanol in mixed cultures is often only achieved by nutrient limitation (Dobroth et al. 2011) in aerobic conditions, a situation which will not be met in standard AGS reactors. This absence of bacteria utilizing the methanol for the anaerobic storage of PHA is what allowed the methanogens to thrive in this system providing that the SRT is sufficient. Typically, intensely aerated systems are not associated with methanogenic activity as they are strict anaerobes. However, some methanogen species are aero-tolerant (Morozova and Wagner 2007). Perhaps *M. uponensis* shares this trade. This could explain why methanogens are active in this system where they are potentially exposed to oxygen from time to time. The dense structure of the granule possible also further facilitates the correct environment for methanogens due to the inherent oxygen gradients that are associated with biofilms (Harremoes 1982, Gonenc and Harremoes 1985). Aerobic species on the outer zones of the granules will consume the oxygen, creating oxygen-limiting conditions in the deeper layers. At the end of the cycle when substrates are fully converted, oxygen is expected to be present throughout the granules. Currently no oxygen inhibition data is available for *M. uponensis*, but due to the presence of a two hour aerobic period a certain tolerance or reversible inhibition to oxygen is to be expected.

Our results show that methylotrophic methanogenic archaea can survive in aerobic granular sludge if specific substrates, like methanol, are present in the wastewater. From a wastewater treatment point of view, methane production in AGS is unwanted as it is a potent greenhouse gas. It could potentially lead to explosive situations as methane and oxygen can both be present concurrently in this system. Likely, a lower SRT can be used to prevent methanogens from flourishing when methanol is present in the wastewater. Further experiments are required to investigate possible methods for restricting the proliferation of methanogens in AGS systems, especially if methanol or other similar one-carbon compounds are present.

Removal of propanol and butanol by aerobic granular sludge
Propanol and butanol did not lead to any significant storage polymer formation during the anaerobic feeding period. In addition, anaerobic batch tests showed no production of CO_2

or CH_4 from the alcohols (Table 2). Storage polymer formation from alcohols has been observed, but only in selected strains under nutrient limitation in aerobic conditions and anaerobically with ethanol, albeit very limited (Alderete et al. 1993, Puig et al. 2008). The absence of polymer formation and formation of catabolic products such as CO_2 or CH_4 strongly indicate there is no conversion of the alcohols under anaerobic conditions. Even after considerable adaptation time (280 days).

What was observed was that with increasing biomass, less propanol and butanol were found after the anaerobic feeding period (Fig. 2d). Since there is no sign of bioconversion during the anaerobic feeding period, and the compounds can only be detected in the liquid at high alcohol to biomass ratios, absorption to the granular sludge matrix seems the most logical process occurring. Future experiments are needed to investigate the absorption mechanism for aliphatic alcohols.

Butanol and propanol, but actually most aliphatic alcohols, are well-known to be able to dissolve in lipids due to their hydrophobic properties, which are determined by their partition coefficient with octanol and water (log P(ow)) (Rowe et al. 1987, McKarns et al. 1997, Ly and Longo 2004). Lipids are a major constituent in the bilayer-water interface of bacterial cell membranes, so a certain absorption capacity can be expected (Ly and Longo 2004, Weber and De Bont 1996). In fact, Thérién et al. (1984) concluded that the longer the carbon chain of the alcohol, the greater the solubility in Lipids, when they investigated the influences of aliphatic alcohols on activated sludge. Possibly further facilitation of the possible absorption in AGS is the presence of a considerable amount of (approximately 15 - 25 % w/w) exopolysaccharides (EPS). This EPS has been found to have hydrophobic properties mainly due to the presence of lipids (Adav and Lee 2008, Lin et al. 2010b, Zheng et al. 2005, Artiga et al. 2008). Indeed, other hydrophobic compounds such as fluor-quinolones, nitrobenzene and malachite green have been observed to absorb more in AGS than activated sludge (Amorim et al. 2014, Adav et al. 2008, Zhao et al. 2011, Sun et al. 2008).

A beneficial effect of absorption is that both propanol and butanol will be distributed throughout the granule before aeration starts. In the aerobic period, these easy degradable compounds are then converted inside the granules, which prevent deterioration of the granule stability and structure. If easy degradable material diffuses from the liquid to the granules in aerobic conditions it will be mainly converted in the outer fraction of the granules. This will lead to fluffy outgrowth of the granule surface and less stable granule formation (Van Loosdrecht et al. 1997b). This was actually observed in the early stages of the experiment (Fig. 1a) and after an increase in alcohol load due to the addition of the aldehydes (Fig. 2b). In both instances alcohols remained in the liquid after the anaerobic period and more instable granule formation was observed. By this absorption mechanism, easily biodegradable substrates that are not converted into storage polymers during the anaerobic period will not lead to granule instability or fluffy outgrowth (Fig. 1b).

Disproportionation of aldehydes

Propionaldehyde and valeraldehyde were disproportionated into their corresponding carboxylic acids and alcohols during the anaerobic feeding period. In turn, the alcohols and acids produced were absorbed and converted into storage polymers respectively (Fig. 4a, b). Disproportionation of aldehydes by various dehydrogenases has been reported in yeast and bacteria alike (Thielen and Ciriacy 1991, Mee et al. 2005). The biological treatment of aldehydes is possible in both anaerobic and aerobic systems (Qaderi et al. 2011, Pereira and Zaiat 2009, Eiroa et al. 2005). In AGS systems, the disproportionation of the aldehydes is not only removing the toxicity anaerobically, but also prevents acidification through conversion of the produced acids to storage polymers. Consequently, the transfer of easy biodegradable substrates to the aeration period is limited and fast heterotrophic growth is reduced. Stable granule formation is therefore expected.

Influence of substrates and feeding strategy on granular morphology

Wastewater is in general composed of a mixture of substrates. Feeding regimes of AGS bioreactors may vary widely. Both will influence the morphology of the aerobic granules and its stability. The basic principle of stable aerobic granular sludge is the selection of slower growing bacteria and distributing the substrates throughout the granule. This will increase the formation of compact biofilms or granular sludge (Van Loosdrecht et al. 1995). Based on the results of this study and existing knowledge from other substrates present in Literature, we have summarized the different feeding conditions and their impact on granular sludge morphology in Figure 5.

(A) Easily biodegradable soluble substrates (i.e. acetate) when fed anaerobically are taken up by PAO or GAO type of bacteria and converted in storage polymers. In a subsequent aerobic period these storage polymers are used for growth at a relatively slow rate (De Kreuk and van Loosdrecht 2004). By ensuring a relatively high substrate concentration (a few milligrams per litre), due to the anaerobic plug flow feeding, distribution throughout the granules is ensured. This leads to biomass production throughout the granule ensuring granule stability. While no oxygen will be present in deeper regions of the granules, the storage polymers can be oxidized by other electron acceptors such as nitrite and nitrate. These will be produced by nitrification on the aerobic outer layer of the granules. Without nitrification, oxygen will penetrate deeper inside the granule.

Absorption of substrates (such as the alcohols in this study) that are not sequestered by PAO or GAO like organisms will not lead to granule instability or fluffy outgrowth. Due to absorption, the substrate is not present in the bulk liquid anymore and growth will occur throughout the granule. In this respect, alcohols do not induce granule formation, but rather; they do not affect granule formation negatively. This anaerobic feeding strategy not only selects for stable granulation, but also ensures optimal phosphate and nitrogen removal which is important for the treatment of domestic wastewaters (Mosquera-Corral et al. 2005a).
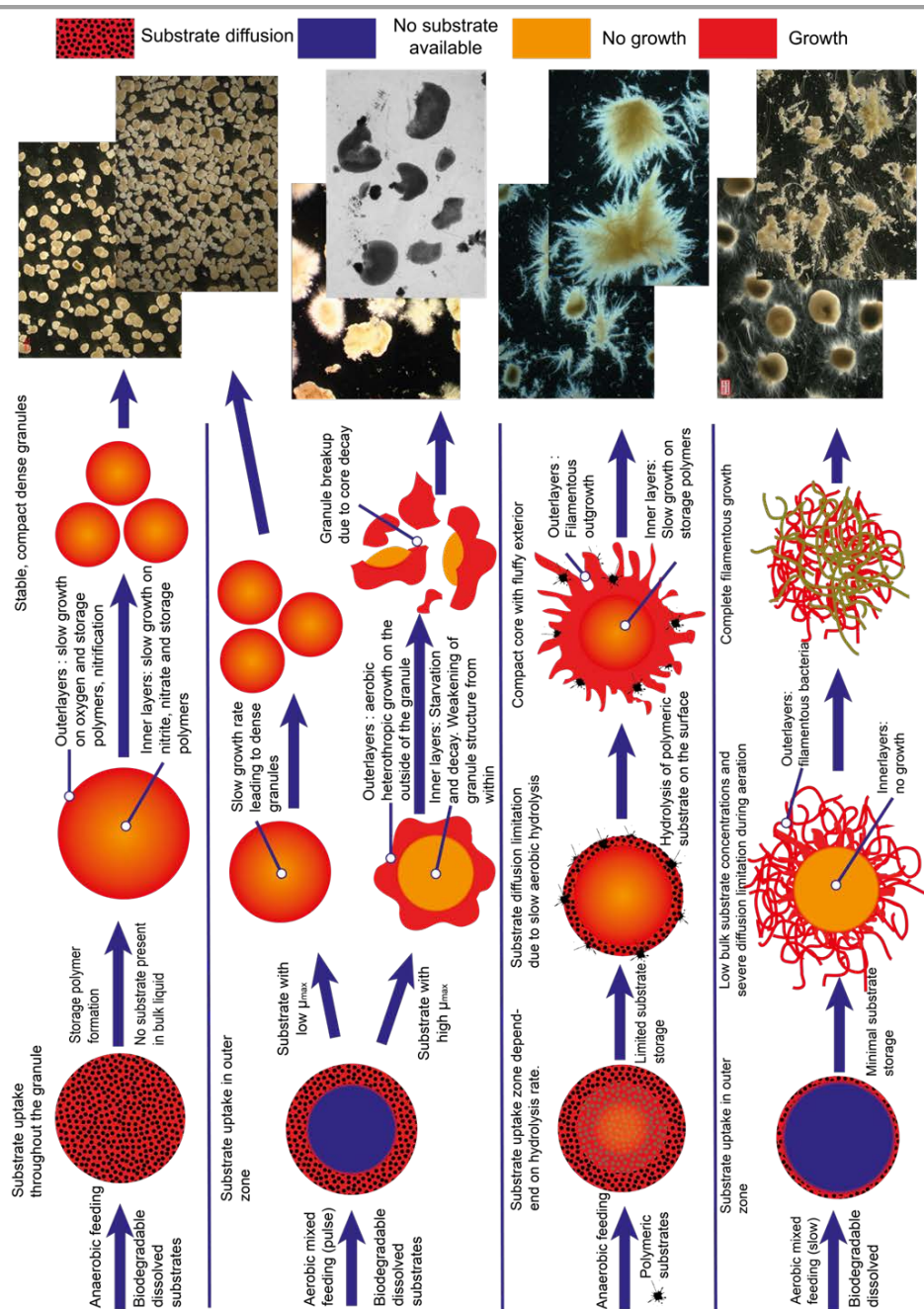
Fig. 5 - Effect on aerobic granule formation of different carbon sources and feeding regimes in sequencing batch reactors selecting for aerobic granular sludge; (a) biodegradable dissolved substrates fed anaerobically, (b) aerobic feeding of biodegradable dissolved substrates into a mixed reactor, (c) anaerobic feeding of polymeric substrates through a settled granular bed, (d) slow aerobic feeding of biodegradable dissolved substrates in a mixed reactor.

(B) Easy biodegradable soluble substrates dosed fast in an aerobic feeding strategy will lead to substrate or oxygen diffusion limitation. The substrate is used for simultaneous growth and formation of storage polymers mainly limited to outside areas of the granule, while the inner regions are deprived of oxygen (Beun et al. 2002). The fast consumption of easy biodegradable substrates in the presence of oxygen on the outside fraction of the granule will lead to formation of filamentous outgrowth, increased shear is needed to ensure smooth and stable granulation (Beun et al. 1999). Filamentous growth will increase when the dissolved oxygen concentration in the bulk is decreased below saturation levels (Mosquera-Corral et al. 2005b). Granules formed under this regime are more prone to breaking under shear stress since the inner regions are inactive and will eventually decay and weaken the granule (Beun et al. 2002). This will result in unstable granulation and poor settling characteristics (higher SVI) combined with higher suspended solids (flocs and loose cells) in the liquid after fast settling. Besides this, also the nitrification and phosphorus removal potential is decreased. The slow growing nitrifying bacteria will be overgrown and pushed down to oxygen limited layers by the faster growing heterotrophs (Gonenc and Harremoes 1990, Elenter et al. 2007).

Some substrates also lead to good granulation even if they are converted aerobically. Ammonium and methanol are such substrates. Both these substrates are converted with oxygen by relatively slow growing bacteria, which leads to a denser biofilm formation (Villaseñor et al. 2000, Mosquera-Corral et al. 2003). In AGS systems, substrates that induce slow growth aerobically are therefore generally expected to lead to stable granulation.

(C) Particulate substrates (i.e. starch, proteins) present another challenge, because of the need for hydrolytic conversions. Particulate substrates are mainly hydrolyzed at the surface of the granules during steady state (de Kreuk et al. 2010a). The hydrolysis products will thereafter be converted into storage polymers. Under anaerobic conditions, PAO and GAO like organisms will be selected and good granulation will occur. Depending on the anaerobic hydrolysis rate, also aerobic hydrolysis will occur. Under aerobic conditions the hydrolysis product will be directly used for growth by the organisms at the surface of the granules with steep substrate diffusion limitation gradients (Mosquera-Corral et al. 2003). This will induce filamentous outgrowth, less stable granule formation and higher suspended solids in the liquid phase.

(D) Easy biodegradable substrates fed slowly in a mixed aerobic environment will lead to severe substrate diffusion limitation gradients. This provides very good conditions for the proliferation of filamentous organisms (Martins et al. 2003, Martins et al. 2011). Aerobic granular sludge fed under these conditions will therefore quickly deteriorate. Breakage of the granules will occur as the inside will not receive any substrate and die. Filamentous growth will have detrimental effect on the settling properties of the sludge and thus on the effluent quality. Granulation formation is thus unlikely.

To summarize, easy biodegradable substrates can have different behaviours during the anaerobic period of the aerobic granular sludge process. Volatile fatty acids are converted by PAO and GAO like organisms into storage polymers, methanol can be converted by methylotrophic methanogens to methane, high carbon alcohols (i.e. propanol and butanol) adsorb in the granule, while the aldehydes are disproportionated in an alcohol and a volatile fatty acid. Easy biodegradable substrates not converted into storage polymers would lead to unstable granular sludge formation unless the substrate is absorbed in the granules and/or select for relatively slow growing bacteria in the aerobic period.

5

Effect of sludge age on methanogenic and glycogen accumulating organisms in an aerobic granular sludge process fed with methanol and acetate

Introduction

Aerobic granular sludge (AGS) is a new emerging wastewater treatment technology that provides several advantages over conventional activated sludge based systems, such as: reduction of surface area, lower energy demand, lower reactor volume, easier process control and excellent effluent quality (Morgenroth et al. 1997, de Kreuk and van Loosdrecht 2006c, Wilderer and McSwain 2004). A short settling time imposes a selective pressure for compact dense granules at the expense of flocculent sludge. Aerobic granules allow for simultaneous nitrogen, chemical oxygen demand (COD) and phosphate removal in one reactor compartment, excluding the need for recirculation loops resulting in a significant reduction of energy consumption (de Kreuk et al. 2007a, Lochmatter and Holliger 2014b, Isanta et al. 2012a). AGS systems depend on the anaerobic uptake of carbon sources and the subsequent storage of polyhydroxyalkanoates (PHA) to prevent growth of fast growing strict aerobic heterotrophic bacteria (De Kreuk and van Loosdrecht 2004, Weissbrodt et al. 2013b). This is a key strategy to achieve long-term stable granulation and stable nitrogen and phosphorus removal.

Pronk et al. (2015) showed that the operational conditions in AGS systems fed with methanol might provide an opportunity for methanogens to proliferate. This is mostly because the formation of storage polymers from methanol is challenging under the conditions that are common in AGS systems (Dobroth et al. 2011) Methanol is present in many industrial wastewaters, since it is a key solvent in many processes. Treatment of such wastewaters could consequently lead to unwanted formation of methane, a greenhouse gas and potentially explosive when mixed with air. Controlling the anaerobic solid retention time (SRT) was suggested as a potential effective method to prevent proliferation of methanogens feeding on methanol or other single carbon compounds that do not lead to storage polymer formation and are excellent precursors for biogas production (Pronk et al. 2015).

Acetate has been the main carbon source in aerobic granular sludge reactors operated in laboratories (Weissbrodt et al. 2013a, De Kreuk et al. 2005e, Carucci et al. 2008). Its influence on the microbial population has been well described. In most experiments, it leads to the proliferation of polyphosphate-accumulating organisms (PAO) or glycogen-accumulating organisms (GAO). This is mostly due to anaerobic-aerobic regimes, temperature, solid retention time (SRT), phosphate-limiting conditions etc. (Winkler et al. 2011, Lopez-Vazquez et al. 2009a, Barr et al. 2010, Rocktäschel et al. 2013). However, temperature experiments in AGS have generally focused on the competition between PAO and GAO as this is of vital importance for phosphate removal during sewage treatment (De Kreuk et al. 2005e, Bassin et al. 2012). Very few reports focus on the competition between different types of GAOs in AGS reactors especially at temperatures above 30 °C, as is common in industrial wastewaters. Furthermore, previous studies did not

unambiguously clarify the effect of elevated temperatures (30 °C and 35 °C) on the stability of AGS. Ebrahimi et al. (2010) report poor granulation and incomplete removal of acetate during the anaerobic feeding while Pronk et al. (2015) reports excellent granulation and full acetate consumption during the anaerobic feeding period at 35 °C. Cui et al. (2014) also report stable granulation, albeit with an aerobic feeding regime. Until now, the fate of acetate fed anaerobically in the AGS process at mesophilic temperatures has not been clarified yet.

In this study, we investigated the conversion of methanol and acetate by AGS at 35 °C, with an emphasis on preventing methanogenic conversion. We hypothesized that methanogens are effectively washed out from the system by lowering the anaerobic SRT. Hereto, the average SRT in an AGS bioreactor was reduced from originally 51 days to 24 days, resulting in an anaerobic SRT of approximately 8 days. Additionally, the effect of the decreasing SRT on the acetate-consuming (GAO) population was intensively monitored.

Experimental Procedures

The experimental setup and analytical procedures are identical as described in Pronk et al (2014). In short, the sequencing batch reactor (SBR; 2.7 Litre) cycle consisted of a 60 min feeding, 112 minutes aeration, 3 minutes settling and a 5-minute effluent discharge period. The pH was controlled at 7 by addition of 1M HCl and 1M NaOH. The dissolved oxygen concentration was controlled at 50 % saturation. The temperature of the reactor was controlled at 35-$\pm$1 °C. An average SRT of 24 days was maintained by manually removing biomass from the reactor on a weekly basis. The SRT was calculated by the total biomass present in the system, removed via the effluent and the biomass that was manually removed. The reactor was inoculated with granular sludge from a previous experiment on a complex carbon medium, which contained acetate, methanol, propanol, butanol, propionaldehyde and valeraldehyde (Pronk et al. 2015). Granule size distribution was performed by measuring at least 500 granules in a mixed reactor sample using a Leica Microsystems Ltd stereo zoom microscope (M205 FA) in combination with Leica Microsystems Qwin (V3.5.1) image analysis software.

Detection of methanogenic activity in batch

Granules from the reactor were separated manually and transferred to reactor medium without a carbon source in a 20 mL glass bottle. The pH was set to 7.2. The bottles were then flushed with nitrogen gas to make them anaerobic. Methanol (20 mM) was injected and the bottles were incubated in a shaker set at 190 rpm at 35 °C. Samples were taken from the headspace at regular time intervals and the methane concentration in the headspace was determined on a Varian 3800 gas chromatograph. Gas samples were injected with a 100 µL gastight Hamilton syringe in a Varian Ultimetal 1079 split/splitless, which was operated at 200 °C at a split ratio of 100. A CPSil5CB 5 (50m x 0.32 mm) capillary column was used isothermally at 100 °C at a constant gas flow rate of 10 mL min^{-1}. The used carrier gas was helium. Methane peaks were detected with a Varian Flame

Ionisation Detector which was operated at 300 °C. The helium make-up flow was 25 mL min^{-1}, hydrogen flow was 30 mL min^{-1} and air flow was 300 mL min^{-1}.

Medium

The synthetic medium consisted of 150 mL medium A and 150 mL medium B dosed (Table 1) together with 1200 mL heated tap water, achieving an influent temperature of 35 ºC. Influent was fed to the bottom of the reactor column. No additional mixing was applied during this period. The carbon composition of medium A consisted of a mixture of methanol and acetate (Table 1). Trace elements solution had the following composition: 63.7g L^{-1} C$_{10}$H$_{14}$N$_2$Na$_2$O$_8$•2H$_2$O (EDTA TITRIPLEX® III) (171.1 mM), 4.99 g L^{-1} FeSO$_4$.7H$_2$O (17.95 mM), 2.2 g L^{-1} ZnSO$_4$.7H$_2$O (7.6 mM), 7.34 g L^{-1} CaCl$_2$.2H$_2$O (49.9 mM), 5.06 g L^{-1} MnCL$_2$.4H$_2$O (51.1 mM), 1.51 g L^{-1} Na$_2$MoO$_4$.2H$_2$O (6.2 mM), 1.57 g L^{-1} CuSO$_4$.5H$_2$O (6.3 mM), 3.22 g L^{-1} CoCl.6H$_2$O (15.9 mM).

Medium composition			
Carbon Medium (A)	**[mM]**	**Nitrogen Medium (B)**	**[mM]**
Acetate	67.6	NH$_4$Cl	21.1
Methanol	15.6	K$_2$HPO$_4$	0.7
		KH$_2$PO$_4$	0.4
		MgSO$_4$.7H$_2$O	3.6
		KCl	4.7
		Trace element solution	9 [mL L^{-1}]

Fluorescent *in situ* hybridization

The FISH probes used in this study to show all present bacteria is a mixture of EUB338, EUB338II and EUB338III, referred to in the text as EUBmix. DF1mix (*Defluviicoccus*-related organisms in cluster I) consisted out of TFO_DF218 plus TFO_DF618 (Wong et al. 2004) and DF2mix (*Defluviicoccus*-related organisms in cluster II) consisted out of DF988, DF1020, helper probes H966 and H1038 (Meyer et al. 2006) to target tetrad-forming *a*-Proteobacteria. Glycogen accumulating organisms belonging to *γ-protebacteria* were stained using GAO-Q431 and GAOQ-989 (in text as GAOmix) probes developed by (Crocetti R et al. 2002a). GAM42A and BET42A developed by Manz et al. (1992) were used to detect *γ*-and β-Proteobacteria in the samples. All probes were prepared with a concentration of 35 % formamide. Due to difficulty in the adhesion of the biomass to the glass slides, the drying time was done overnight at 45 °C. The samples were examined with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575 - 625/ FT645/ bp 660 - 710), 20 (bp 546/12/ FT560/ bp 575 - 640), 17 (bp 485/20/ FT 510/ bp 5515 - 565) for Cy5, Cy3 and fluos respectively.

Sampling, Nucleic Acids Extraction, and PCR amplification

Granules taken from the reactor were separated (black and white) and pottered to create a cell suspension. The cell suspension was washed two times with PBS buffer (pH 7). The supernatant was discarded and the pellet was stored at -80 °C. Default (routine) DNA extractions were performed using the Ultraclean Microbial DNA extraction kit from Mobio (USA) according to manufactures recommendations.

Modified DNA extraction method using liquid nitrogen

A more stringent extraction was also performed by modifying the standard extraction protocol. The pellet was incubated with 3 mg mL^{-1} Lysozyme for 30 minutes at 37 °C at 800 rpm (Sigma Aldrich L7651) in a bead buffer followed by Proteinase-K (500 mg mL^{-1}) digestion and 0.5 % SDS incubation of 15 minutes at 56 °C at 800 rpm (#EO0491, Thermo scientific). The subsequent, lysate was transferred into resistant metal bead tubes with a special silicon cap (Biospec, Cat. No. 2007 / 2008) and frozen into liquid nitrogen with small beads supplied with the kit, (~50 mg large volcanic beads from the Ultraclean soil DNA extraction kit and 2 x 3.2 mm chrome-steel beads (Cat. No. 11079132c)). Before bead beating the resulting 50-µl solution, MD1 was added. The total sample was homogenized on a Mini-Beadbeater-16 (Biospec, USA) for 5 minutes and refrozen with liquid nitrogen and the procedure was repeated another time. After the second homogenizing step, the sample was refrozen and homogenized for a final 3 minutes. The resulting lysate was extracted using the Ultraclean Microbial DNA extraction kit (Mobio, USA) as mentioned above by following the supplied protocol from step 6 on.

After extraction 5-µL of a total of 50-µL was subjected to gel electrophoresis to check for quality and quantity. PCR amplification of the 16s rDNA gene from the whole bacteria kingdom was performed as described in Bassin et al. (2011d). Sequences were deposited into Genbank under accession number KP064478 - KP064500.

RESULTS

The influence of SRT on methylotrophic methanogens

The reactor was inoculated with granular sludge from a previous experiment in which the influent COD consisted of a mixture of organic substrates including 15 % of methanol. During these previous experiments, methanol was fully utilized during the anaerobic feeding period by methanogens, resulting in high methane emissions in the off gas due to stripping at the start of the aeration period. The responsible methanogenic organism was identified by DGGE as *Methanomethylovorans uponensis* (Pronk et al. 2015). To evaluate this methanol conversion in more detail and to investigate the conditions inhibiting methanogenesis in AGS systems, the medium was simplified to a mixture of acetate (85 COD %) and methanol (15 COD %). By increasing the acetate concentration, the volumetric COD loading was kept constant (760 mg COD L^{-1} d^{-1}) (Table 1). Acetate was

fully consumed during the anaerobic feeding period over the course of the experiment. No degradable soluble COD was detected once the aeration period started. Also in these conditions methanol was entirely converted to methane by methanogens during the anaerobic feeding period. Online gas analysis revealed methane concentrations of approximately 2600 ppm being released right after aeration was started (Fig. 1a). The COD-equivalent amount of methane produced was similar to the amount of methanol converted. After 17 days of operation weekly removal of biomass from the reactor at the end of the aeration period was introduced in order to obtain an average total SRT of 24 days.
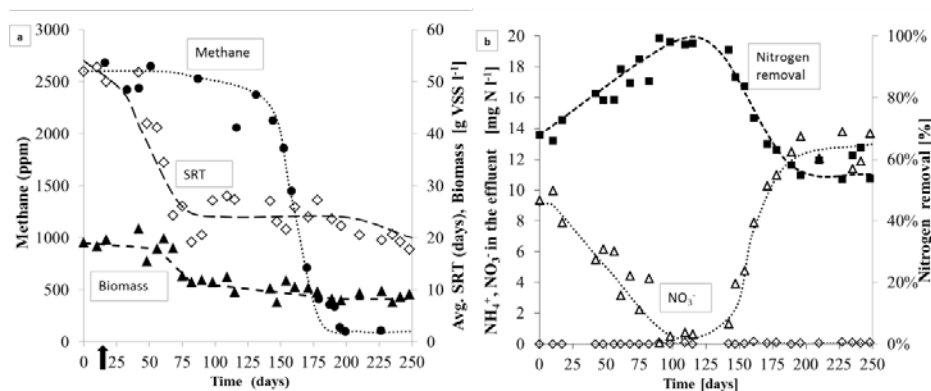

Fig. 1 - (a) Evolution in time of; methane concentration measured just after the anaerobic feeding period when the aeration started (filled circle), average anaerobic solid retention time (days) (open diamond) and biomass concentration (gVSS L⁻¹) (filled triangle) in an aerobic granular sludge reactor fed with acetate and methanol at 35 ºC. (b) Nitrogen species in the effluent; ammonium in the effluent (open diamonds), nitrate in the effluent (open triangles), nitrogen removal efficiency (closed squares). Arrow indicates when the solid retention time of 24 days was introduced. Lines are shown to indicate trends.

This decreased the anaerobic SRT from approximately 17 to 8 days (Fig. 1a). Initially, methane emissions remained high due to the overcapacity for methanogenesis in the system and slow removal of methanogens from the reactor through SRT control. Only after 150 days of operation at reduced SRT, a decline in methane production was observed. The methanol concentration in the bulk liquid after the feeding increased simultaneously. This was accompanied by a higher oxygen consumption of the system. This indicates that the methanol was not used anaerobically anymore, but was instead converted aerobically. Aerobic conversion of methanol, however, did not lead to any significant visual granule deterioration. The average granule size did decrease after introduction of the shorter SRT regime from 1.7 mm (day 17) to 0.6 mm (day 226), but this had no adverse effect on the COD effluent quality. The system was fully nitrifying to nitrate and partial simultaneous denitrification (not optimized) occurred (Fig. 1b). Nitrogen removal efficiency increased from 65 % at the start of the experiment to about 99 % on day 90. This was most probably

induced by a lower biomass concentration in the reactor and thus an increase in storage polymer formation per biomass, which then became available for denitrification. Methanol that was not converted anaerobically could also contribute to the denitrification either as a carbon source or by creating a larger anoxic zone in the biofilm due to the consumption of oxygen at the granule surface (see supplementary materials Fig. S1 and S2 in online version). However, since methanol was quickly consumed during the first 5 – 10 minutes of the aerobic period it is unlikely to have contributed significantly to the denitrification. The nitrogen removal efficiency gradually decreased from 98 % on day 135 to 54 % on day 190. A smaller granule size coupled with an increase of oxygen penetration depth in the granules leading to less denitrification potential was the most likely reason for this decline.

Segregation of two types of granules

A remarkable phenomenon was observed after lowering the total SRT from 51 to 24 days (17 and 8 days anaerobic respectively): white smaller granules started to develop separately from the already present large black granules. In the beginning of the experiment, separate white granules were rarely observed, and they were mostly attached as small white colonies to the outside of the large black granules.
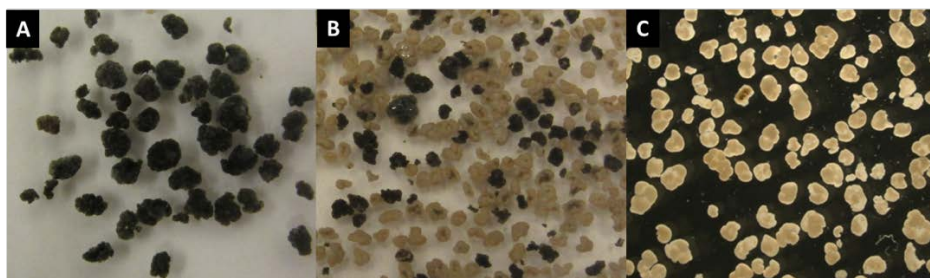

Fig. 2 - (a) black granules at the start of the experiment, (b) black and white granules after 120 days of operation at reduced SRT, (c) white granules after 250 days with a solid retention time of 24 days fed with acetate (85 % COD) and methanol (15 % COD). Average granule size distribution for a, b and c were 1.7, 0.9 and 0.6 mm respectively.

Over the course of this experiment, the white granules slowly started to dominate the reactor (Fig. 2). Besides the obvious difference in color, also the morphology of the white granules differed strongly from the morphology of the black ones. White granules had a more open structure than the black granules, even though the settling rate was similar, as indicated by the black and white mixed granule bed after settling. In order to elucidate the difference between the two types of granules, anaerobic batch tests were performed with manually separated black and white granules. Under anaerobic conditions, 20 mM methanol was dosed to investigate methanogenic activity in both types of granules. Although the separation of black and white granules was based on color only, a distinctive difference in conversion was observed.

The black granules were responsible for the anaerobic conversion of methanol to methane, while the white granules had no methanogenic activity at all (Fig. 3). The maximum methane production rate in the black granules was 19.2-mmol CH_4 $(gVSS\ d)^{-1}$.
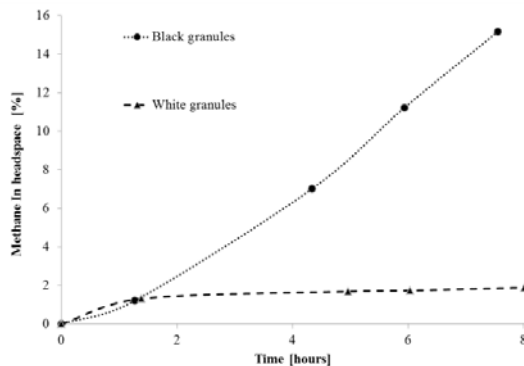
Fig. 3 - Methane measured in the headspace during anaerobic batch tests with methanol on selected white and black granules taken at day 140.

Microbial community analysis

In the black granules, *Methanomethylovorans uponensis* was previously detected as the dominant methanogen responsible for the anaerobic conversion of methanol to methane (Pronk et al. 2015). From the white granules, no archaeal PCR products were retrieved. Moreover, fluorescence of co-enzyme F_{420} present in *M. uponensis* was only observed during microscopy in the black granules. Adjacent to the apparent difference in methanogenic activity and color, also the microbial community structure was investigated separately in black and white granules. PCR-DGGE analysis was performed based on the 16S rRNA gene of bacteria to determine the microbial community structure. Fig. 4 shows the banding pattern obtained for manually separated black and white granules taken at day 150. The profile shows a limited number of bands at the same height in both granules suggesting a significant difference in community structure. The sequenced bands are numbered B1 to B25, in both the banding pattern (Fig. 4) and the phylogenetic tree (Fig. 5).
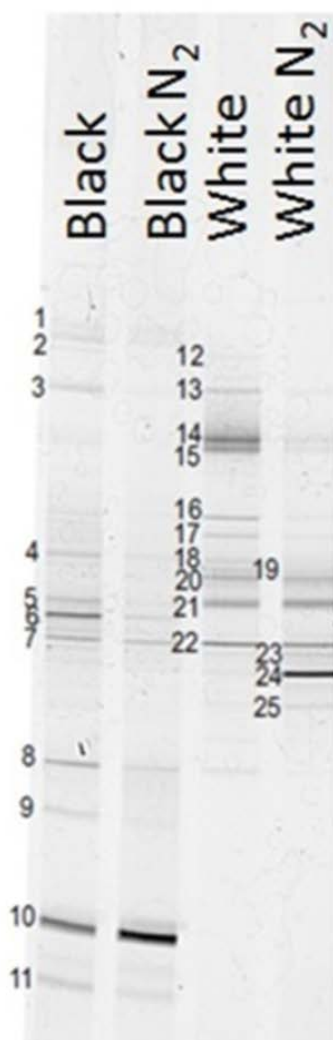
Fig. 4 - DGGE on bacterial 16s rDNA gene isolated from black and white granules (day 120) from an aerobic granular sludge reactor extracted with the protocol described in the standard extraction kit from (Mobio, USA) and the adjusted liquid nitrogen (N₂) extraction method (as described in materials and methods).
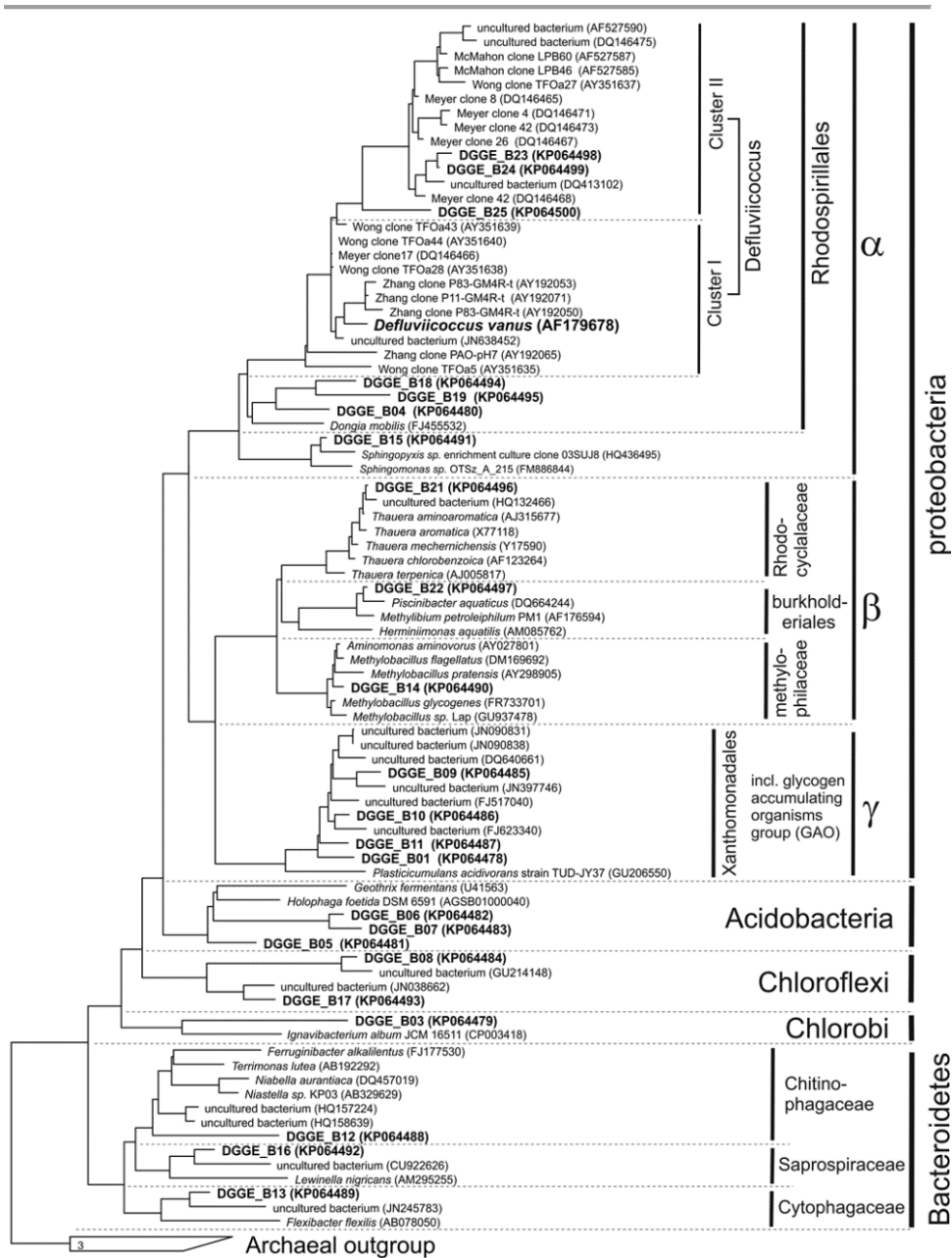
Fig. 5 - The phylogenetic tree was calculated using maximum-likelihood algorithm implemented in ARB (RAxmL module). Full sequences from the SSU115-NR99 database were used for calculation together with an SSUref: bacteria filter (resulting in 1125 base pairs). DGGE bands and clones with a variable length (311-962 base pairs) were added later using parsimony algorithm. Bootstrap (250rxn) was performed but not shown. Sequences shown were deposited into Genbank under accession number KP064478 - KP064500.

α- and γ -Proteobacteria

Black granules were populated by *γ*-Proteobacteria (B1, B9, B10 and B11). Remarkably, the white granules seemed devoid of *γ*-Proteobacteria. Instead, *β*-Proteobacteria of the genus *Thauera* colonized the white granules (B21). However, FISH staining with a beta-probe failed to verify a significant presence of *Thauera* sp. related species in the white granules, suggesting that part of acetate consuming microorganisms were not identified. Furthermore, microscopic examination of the white granules unmistakably revealed tetrad-forming bacteria (Fig. 6). It was then assumed that a bias had occurred during extraction of the DNA. Therefore, the standard DNA extraction protocol was changed by including (among others) a liquid nitrogen step (see methods for details). With this modified extraction protocol, a new strong band (B24) appeared in the DGGE gel. Once sequenced, it was identified as a member of the *Defluviicoccus* cluster II in the *a*-Proteobacteria and is a known tetrad-forming organism (TFO). The very weak band B23 is closely related (98.6 %) to band B24. Bands B24 and B25 only have 94.5 % similarity, nevertheless bands B23, 24 and 25 all belong to the genus *Defluviicoccus sp.* in cluster II. The closest cultivated species to B24 is *Defluviicoccus vanus*, but it has only 92 % 16S-rRNA gene similarity and belongs to in cluster I. *Defluviicoccus* was not found in the black granules where *γ*-Proteobacteria were dominating (Fig. 4). Bands B14, B15 and B22 were also exclusively present in the white granules. Band B14 is most closely affiliated with *Aminomonas aminovorus* (99.1 %) in the *Methylophilaceae* family, which are known for their methylothrophic capabilities. Methylotrophic potential is also present within the family *Burkholderiales* (B22). Band B15 belongs to the genus *Sphingopyxis*.
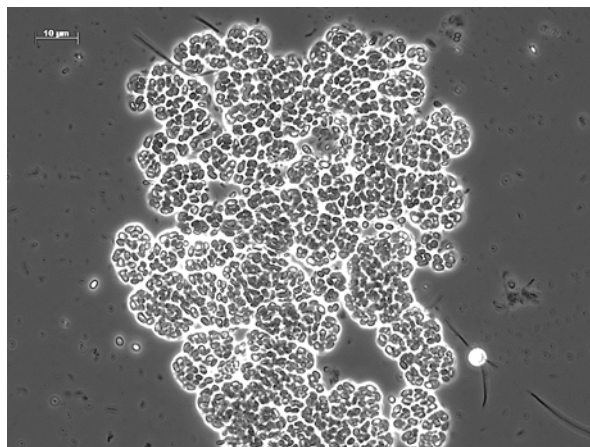
Fig. 6 - Microscopic examination of crushed white granules showing the tetrad-forming *Defluviicoccus*-related organisms in phase-contrast.

Fluorescent *in situ* hybridization

To gain more insight into the bacterial abundance and diversity in both types of granules, FISH staining was performed. In the black granules, in agreement with the DGGE results, the *γ*-Proteobacteria were visibly dominant (Fig. 7a). In the white granules, however, hardly

any (< 5 %) γ-Proteobacteria could be detected. Probes DF1 and DF2 targeting tetrad-forming a-Proteobacteria of *Defluviicoccus* clusters I, and II respectively, were used.
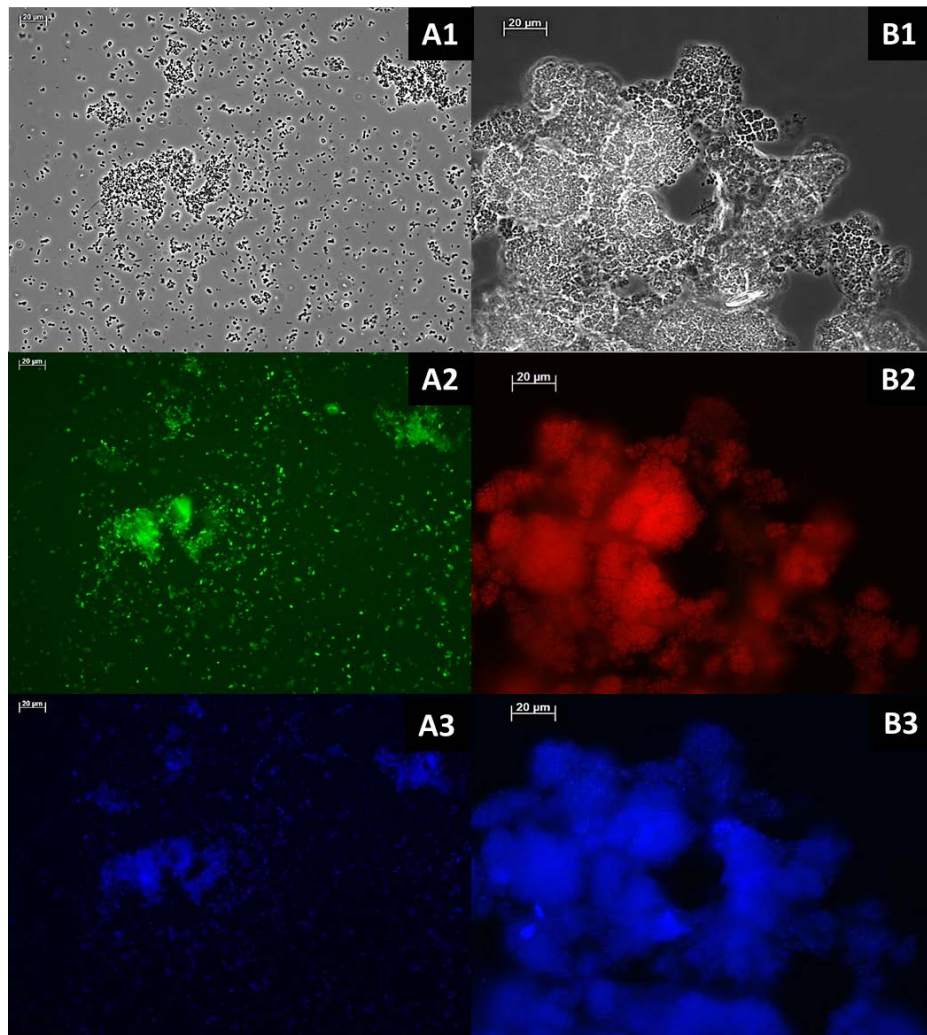
Fig. 7 - FISH performed on a black granule with (a1) phase-contrast, (a2) GAOmix targeting γ-Proteobacteria (fluos), (a3) EUBmix targeting all bacteria (Cy5); white granule (b1) phase-contrast, (b2) *DF* 2 targeting α-Proteobacteria (Cy3), (b3) EUBmix targeting all bacteria (Cy5).

Only probe DF2 successfully stained the bacteria in the different samples, confirming the DGGE results. In the white granules, the tetrad-forming *Defluviicoccus*-related bacteria were a dominant form (Fig. 7b).

DISCUSSION

Preventing growth of methanogens

Methanogenesis from e.g. methanol during anaerobic feeding is an unwanted process during AGS treatment of industrial wastewater. When aeration starts, a large fraction of methane, a potent greenhouse gas, will be stripped to the air. Moreover, methane accumulation might also lead to safety risks and poor plug flow conditions during the anaerobic feeding. Control of the methanogenic archaea is therefore essential for effective implementation of the AGS process.

Most methanogens are very sensitive towards high red-ox, but this sensitivity varies widely depending on the species (Morozova and Wagner 2007, Kiener and Leisinger 1983). Methanogens are relatively slow-growing archaea (0.03 - 0.06 h^{-1} for *M. uponensis* on methanol) and as suggested previously by Pronk et al. (2015), lowering the anaerobic SRT below 10 days indeed resulted in the successful washout of *M. uponensis* from the aerobic granular sludge system. The relative long washout time needed to remove the methylotrophic methanogens, was mainly due to a large population initially present combined with an SRT of 24 days. The reason might be that the SRT calculated is an average SRT. Biomass in the effluent contributes significantly to the biomass wasted from the system. Effluent biomass concentrations might seem insignificant (0.04 – 0.1 g l-1), but with 12 liters of effluent per day this accounts for nearly 20 – 30 % of the waste sludge. This has a large impact on the SRT, lowering it significantly. Therefore, the SRT calculated here (24 days) is derived from the biomass that is removed deliberately as well as by effluent extraction. The SRT of bacteria present in granules might be higher than the SRT calculated for the entire system since the effluent mainly contains suspended or flocculent material. Because of this, washout times can be prolonged significantly compared to suspended systems.

The growth rate of *Methanomethylovorans* members on methanol was reported by to be in the range of 0.03 - 0.06 h^{-1} (Cha et al. 2013, Lomans et al. 1999). This should be more than sufficient to remain in the reactor, even when running at the lowest anaerobic SRT (8 days). However, this growth rate applies in optimal conditions only. In this experiment, the conditions are not optimal for methanogenic growth. Long aeration times would most definitely lead to oxygen inhibition and result in decay of the methanogenic archaea. The washout of the methanogens was most probably also further facilitated by the decreased granule size, since smaller granules allow for a relative deeper oxygen penetration depth (Vázquez-Padín et al. 2010b, Kishida et al. 2006). Because of this strategy, methanol was not consumed during the anaerobic feeding period, but it was oxidized in the aerobic period. Usually, aerobic growth on readily available COD will result in fast heterotrophic growth and instable granulation (De Kreuk and van Loosdrecht 2004, Kishida et al. 2006). However, aerobic growth on methanol is relatively slow and has also led to compact

biofilms in other research (Villaseñor et al. 2000). This is in line with the general hypothesis on biofilm structure formation that suggests that low growth rates result in dense and compact biofilms (Van Loosdrecht et al. 1995, Van Loosdrecht et al. 1997c).

In order to prevent methylotrophic methanogens in aerobic granular systems, the anaerobic SRT should be kept low enough, preferable below 10 days with wastewaters of 30 to 40 °C. Likely, under full-scale conditions this is the standard operational condition. Oxygen concentrations during the aerobic period can also be increased to minimize growth of methanogens. Special care has to be taken to control the SRT in regard to segregation as a result of granule size differences if present (Winkler et al. 2011). As bigger granules provide more protection against oxygen for the methanogens, their proliferation might be more effectively prevented by removing bigger granules from the bottom of the reactor first.

Competition between α- and γ-Proteobacterial GAOs for acetate
Acetate was fully converted to storage polymers during the anaerobic feeding period. Aerobic and anoxic growth occurred subsequently during the aerated mixing period. This anaerobic formation of PHA from volatile fatty acids (VFA) and the subsequent growth once oxygen is available suggests that microorganisms performing the so-called GAO metabolism were present in the reactor. DGGE and FISH analysis identified *Defluviicoccus* cluster II members of the *a*-Proteobacteria as the major bacterial population. Most of the *Defluviicoccus*-related organisms found until now are reported to perform the GAO metabolism on acetate, propionate and lactate (Wong and Liu 2007). They have been suggested as effective competitors for PAOs in the activated sludge process (Burow et al. 2007, Lanham et al. 2008, Mielczarek et al. 2013).

Tetrad-forming bacteria in the α-GAOs of genus *Defluviicoccus,* a novel cluster within its subgroup II, outcompeted the γ-GAOs for anaerobic acetate uptake when the SRT was lowered. Although the γ-GAOs and *Defluviicoccus*-related organisms have a highly similar metabolism, it has been reported that *Defluviicoccus*-related organisms are mainly proliferating when propionate is the main carbon source (Meyer et al. 2006, Oehmen et al. 2005). Acetate uptake rates of *Defluviicoccus*-related organisms have been reported as being much lower than for the γ-GAOs (Oehmen et al. 2005, Lopez-Vazquez et al. 2009b). On the other hand, *Defluviicoccus*-related organisms have also been reported as dominant in bioreactors with acetate as the sole carbon source (Dai et al. 2007, Wang et al. 2008). As yet, there is no clear consensus on the competition between γ-GAOs and *Defluviicoccus*-related organisms for acetate. Perhaps, the outcome may depend on other environmental factors, like temperature, high COD/P ratios or different dissolved oxygen concentrations during the famine period as Oehmen et al. (2007) already suggested.

One problem in the analysis of the phylogeny of *Defluviicoccus*-related organisms is the recognition of separate subgroups. It is therefore unclear, whether the same subgroup was

present in previous studies and if there are major functional differences between the subgroups. Moreover, in granular sludge significant simultaneous denitrification occurs, suggesting that the microbial community inside the granules mainly experiences anaerobic/anoxic conditions, which have hardly been studied under controlled conditions.

An extra complication in the research on *Defluviicoccus*-related organisms is the difficulty to obtain its DNA using standard extraction methods (McIlroy et al. 2008). In this study, the DNA extraction of *Defluviicoccus*-related organisms (B24) from the granules with the standard extraction procedure also led to unsatisfactory results. Meyer et al. (2006) also reported DNA extraction problems, even from sludge that comprised more than fifty percent of *Defluviicoccus*-related organisms. To successfully identify *Defluviicoccus*-related organisms in cluster II, rRNA-based stable isotope probing (SIP) followed by full-cycle rRNA analysis was needed (Meyer et al. 2006). In the present study, the problem was solved by using liquid nitrogen in the extraction procedure, omitting arduous methods that include SIP, chloroform, phenol or sodiumtrichloroacetate (McIlroy et al. 2008, McMahon et al. 2002). This seems to suggest that this particular cluster (II) within the *Defluviicoccus*-related organisms group is difficult to extract using normal extraction kits. This raises the question whether or not the cluster has been overlooked in community analysis studies in the past. Moreover, this shows again that genomic results should always be verified by complementary methods like FISH to ensure adequate interpretation of experimental results.

What is the cause of the segregation?

Segregation of biomass in different types of granules in a laboratory operated AGS system have recently also been reported by Barr et al. (2010) and Winkler et al. (2011). The segregation as reported by Winkler et al. (2011) was due to different microbial populations in fast and slow settling particles, largely due to differences in particle size and to lesser extent density variations. In this case, the slower settling microbial population was assumed to be exposed to different substrate concentrations during feeding, due to its localization in the top of the sludge bed. Furthermore, the fraction of cells that is exposed to oxygen during the aerobic period depends a lot on the particle size as well. Moreover, due to sludge removal during settling at a specific height in the reactor, both populations get a different SRT. In the case described in this study, as well as in the study by Barr et al. (2010), both types of granules were fully mixed and no difference in feeding regime or SRT between the different granules occurs. Additionally, Barr et al. (2010) noticed that white granules comprised more than 97 % *Candidatus Accumulibacter phosphatis* (PAO) and about 0.9 % of *Competibacter phosphatis* (γ-GAO), as measured by quantitative FISH, while in the yellow granules these species were distributed vice versa, 12 % and 58 % respectively. Barr et al. (2010) suggested that the separation was partly induced by excessive production of exopolysaccharides (EPS) by PAOs and the segregation could not be attributed to specific operational reasons. Winkler et al. (2011) also indicated a segregation for the same

microbial populations in top and bottom granules, induced by a difference in density of the granules and thereby a difference in settling velocity. In addition, PAOs seemed to proliferate in smooth white granules.

In our case, there is no big difference in settling velocity as inferred from the mixed presence of both granule types in the settling bed. We postulate therefore that the difference in EPS produced by the different species is the reason for this separation, as Barr et al. (2010) already suggested. Initially, white micro colonies grew on the surface of the black granules, but as the conditions changed, these micro-colonies gradually detached and became independent granules. Substantial amounts of EPS are visible around the *Defluviicoccus*-related clusters as illustrated in Figure 6.

It could be hypothesized that *Defluviicoccus*-related organisms in cluster II are excreting exopolysaccharides with different characteristics compared to that of for example the γ-GAOs, which were found mainly in the black granules. This could also lead to segregation if the two polysaccharides produced repulse each other. This idea is also supported by Seviour et al. (2011, 2012), who postulated that there might be several exopolysaccharides produced by different bacteria that have variable properties. *Defluviicoccus*-related organisms themselves are known for their clustering and heavy encapsulation with EPS (McIlroy et al. 2008). Initial extraction of EPS as reported by Lin et al. (2010) before showed a large fraction of alginate like polymers (> 15 %) in the black granules while the white granules had no or very limited alginate like polymer content (< 4 %) (results not shown). Clearly, besides alginate-like exopolysaccharides other polymers could be used by bacteria to construct granular sludge. Further experiments on the exopolysaccharides and species present in the two different granule types should elucidate the segregation behaviour in the future.

Conclusions

Stable aerobic granular sludge formation was shown for mesophilic conditions. Controlling the anaerobic solid retention time in an aerobic granular sludge reactor below 10 days (at 35 °C) enabled prevention of growth of methylotrophic methanogens, thereby preventing methane emissions. No detectable anaerobic methanol conversion occurred in the absence of methanogens. Aerobic oxidation of methanol did not deteriorate the stability of the sludge granules. Defluviicoccus-cluster II-related organisms belonging to the α-Proteobacteria replaced the γ-Proteobacteria (so called γ -GAOs) when the SRT was decreased. Remarkably, the two GAO types of organisms grew segregated in two clearly distinct types of granules. In addition, we demonstrated that a liquid nitrogen extraction step proved an easy and reliable method to obtain DNA from Defluviicoccus-related organisms in cluster II, since the standard extraction procedure led to unsatisfactory results. This work further highlights the potential of aerobic granular sludge systems to influence the microbial communities through sludge age control in order to optimize the wastewater treatment processes.

6

Full-scale performance of the aerobic granular sludge process for sewage treatment

Introduction

Aerobic granular sludge (AGS) technology is an upcoming technology for the treatment of domestic and industrial wastewater (Morgenroth et al. 1997, Heijnen and Van Loosdrecht 1998, de Bruin et al. 2004b, de Kreuk et al. 2007b, Coma et al. 2012, Show et al. 2012, Morales et al. 2013). AGS technology for combined carbon, nitrogen and phosphorous removal is based on a repeated fed batch process and relies on microorganisms selected to grow in granules rather than flocs. As a result of the high settling rate of the sludge granules, separate settling tanks are not needed and an 80 % reduction in area use is possible (de Bruin et al. 2004b).

Aerobic granules are characterised by a compact structure, without the need for carrier material, resulting in high settling velocities and a low sludge volume index (SVI). A good indication of granulation is the limited difference between SVI after 5 and 30 minutes (Etterer and Wilderer 2001). Aerobic granules are also characterised by their layered structure. The presence of an aerobic outer layer and an anaerobic or anoxic core, facilitates co-existence of nitrifying organisms in the outer layers of the granules and denitrifying phosphate accumulating organisms (dPAO), as well as (facultative) anaerobic organisms towards the centre of the granules (Pronk et al. 2015, Gieseke et al. 2001, Winkler et al. 2012b). Due to this structure, aerobic granular sludge can simultaneously remove phosphorus, nitrogen and COD (chemical oxygen demand) from the liquid (de Kreuk et al. 2005a, Gonzalez-Gil and Holliger 2011).

Aerobic granular sludge technology was developed during the last decade at laboratory scale (Morgenroth et al. 1997, Beun et al. 1999, Zeng et al. 2003, Tay et al. 2002), as well as pilot scale (Morales et al. 2013, Liu et al. 2010, Isanta et al. 2012b, Wei et al. 2012, Li et al. 2014). Aerobic granular sludge is applied by RoyalHaskoningDHV in the Nereda® technology which was first adapted for industrial applications and then further scaled-up for domestic sewage treatment. Valuable scale-up experience gained from full-scale demonstration plants in Gansbaai, South Africa and Frielas, Portugal were used for optimization of the process design and construction in 2010 of the first full-scale AGS wastewater treatment plant in Epe, The Netherlands (Giesen et al. 2013, van der Roest et al. 2011). The AGS technology used relies on a sequencing fed-batch process with a constant working volume. This is possible due to simultaneous feeding and effluent discharge, that relies on a plug-flow pattern for displacement of effluent from the reactor (de Kreuk et al. 2005a). In addition, because of an oxygen gradient within the granular sludge particle during aeration, extensive biological phosphate removal and simultaneous nitrogen removal can be achieved during one aeration step. The absence of conventional recycle pumps, sludge return pumps and mixers provides a significant reduction in electricity consumption compared to standard nutrient removal plants.

In July 2013, a full-scale installation based on the aerobic granular sludge process was taken into operation in Garmerwolde, the Netherlands.

Few papers have been published so far describing full-scale operation of the AGS process on domestic wastewater. Li et al. (2014) showed the performance of an full-scale AGS plant fed with 30 % domestic and 70 % industrial wastewater (BOD / COD = 0.23). The full-scale installation in Epe, the Netherlands briefly described by Giesen et al. (2013) also treats wastewater that is derived for a large part (35 %) from industry (mainly slaughterhouses). Operational data provided in these studies are very general. More detailed descriptions of the process, conversions, energy usage and design considerations when treating domestic wastewater are lacking.

After start-up and more than one year of operation, this paper reflects not only on the performance, but also on granulation, COD, nitrogen and phosphorus conversions and especially design considerations. The measured energy requirement of the AGS process is compared to conventional activated sludge systems. Furthermore, differences between the full-scale granular sludge process, conventional activated sludge and laboratory reactors are discussed in detail.

Materials and Methods

Description of plant

In 2012 water board Noorderzijlvest decided that the treatment plant in Garmerwolde would be upgraded with the aerobic granular sludge process. The extension of the existing activated sludge based sewage treatment process (STP) was necessary to meet the effluent requirements. The STP in Garmerwolde treats approximately 27 million m³ of wastewater per year and 0.8 MW of electricity is generated by the use of the biogas formed during sludge digestion. The existing WWTP consists of a two-stage activated sludge plant (so-called AB-process), with chemical phosphorus removal and either glycerol or methanol for denitrification (Böhnke 1978) and a SHARON reactor to treat side-streams from the plant's digester and sludge thickeners (Hellinga et al. 1998).

The AGS plant was designed by Royal HaskoningDHV and is operated in parallel with the existing AB-plant. The AGS plant treats 41 % (28600 m³ d⁻¹) of the total influent received at Garmerwolde WWTP during dry weather flow with a maximum of 4,200 m³ h⁻¹. The average flow received by AGS and the AB-plant totals approximately 70,000 m³ d⁻¹ with a peak flow of 11,600 m³ h⁻¹.
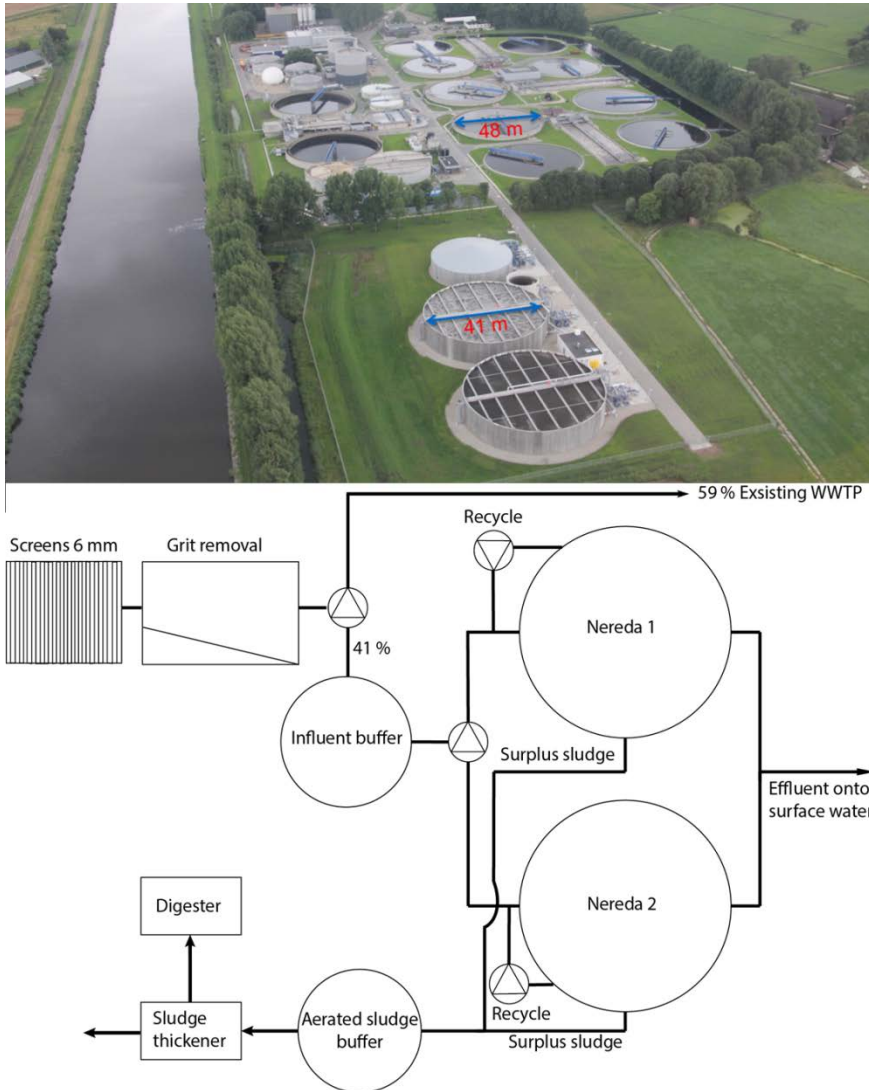
Fig. 1 - Garmerwolde WWTP in the Netherlands. The photograph displays the existing AB-treatment system in the background with the aerobic granular sludge plant extension in front. The figure below shows the process scheme of the aerobic granular sludge plant.

Wastewater characteristics are given in Table 1. The designed sludge loading rate was 0.10 kg COD (kg TSS d)$^{-1}$ at an expected sludge concentration of 8 kg m^{-3}. The sludge-loading rate is calculated by dividing the treated kg COD per day by the total biomass present in the reactor (Table 2). The volumetric loading rate of the AGS reactors is 1.5 m^3 (m^3 d)$^{-1}$. Wastewater enters the plant by a pressure main. After screening by 6 mm screens, the wastewater goes to a grit removal plant and an influent buffer (4000 m^3) (Fig. 1). From the influent buffer, the wastewater is fed to two AGS reactors (height 7.5 m, volume 9600 m^3

each) that are equipped with an internal recirculation system (top to bottom of reactor) with a capacity of 2500 m³ h⁻¹ for each reactor. Treated effluent is directly discharged from the reactors to the surface water via static fixed overflow weirs. Biological phosphorus removal in the AGS process can be supplemented by metal salt addition directly in the bulk if necessary. Surplus sludge is stored in a sludge buffer tank (400 m³). To prevent anaerobic phosphorus release and to ensure continuous discharge towards the mechanical belt thickeners, the retention time in the surplus sludge buffer is kept to a minimum.

Dry weather cycle

Time [min]	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300	315	330	345	360	375	390
REACTOR 1																										
Fill/ draw	■	■	■	■																						
Reaction/Aeration					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■						
Settling																									■	■
REACTOR 2																										
Fill/ draw															■	■	■	■								
Reaction/Aeration	■	■	■	■	■	■	■	■	■										■	■	■	■	■	■	■	■
Settling												■	■													

Rain weather cycle

Time [min]	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300	315	330	345	360	375	390
REACTOR 1																										
Fill/ draw	■	■	■	■	■	■								■	■	■	■	■							■	■
Reaction/Aeration							■	■	■	■										■	■	■	■			
Settling											■	■												■	■	
REACTOR 2																										
Fill/ draw							■	■	■	■	■	■						■	■	■	■	■				
Reaction/Aeration	■	■	■	■									■	■	■	■							■	■		
Settling					■	■													■	■						

Fig. 2 - Typical cycle timing during dry and rain weather operation of the aerobic granular sludge plant.

The AGS plant is operated as a sequencing fed batch process, consisting of a simultaneous feeding and effluent withdrawal period, a reaction period, and a settling/sludge withdrawal/idle period. Nitrogen removal is predominantly established by simultaneous nitrification and denitrification, but for maximisation of nitrogen removal (non-mixed) anoxic periods with a recycle from top to bottom can be provided. The cycle can be adjusted to the influent characteristics (rain or dry weather conditions), the actual sludge conversion rates, the desired effluent conditions and the granular sludge selection pressure. During aeration periods, the dissolved oxygen concentration was maintained between 1.8 - 2.5 mg L⁻¹. The total operational cycle time of the reactors is 6.5 hours at dry weather conditions. During rainy weather, the cycle time is shortened to 3 hours by decreasing the aeration and increasing the feeding time in order to treat the increased influent flow (Fig. 2). The reactor was seeded with surplus sludge from an existing full-scale AGS plant in Epe, the Netherlands, treating wastewater that consist out of a large industrial part (slaughterhouses) to a concentration of 1 g L⁻¹. In the surplus sludge, no granules were present (SVI_{30} 140 mL g⁻¹).

Online measurements

Each reactor is equipped with measurements for dissolved oxygen concentration, redox potential, temperature, water level, dry matter and turbidity. Ammonium and phosphate are semi continuously measured (5 – 10 minute interval) during the cycle by an automatic sampling and analysis device (Hach Lange; Filtrax, AMTAX and PHOSPHAX). Sampling points for ammonium and phosphate are located on 0.5 meters under the water surface in the reactors. This means that during feeding, when the reactor is not mixed the concentrations measured are the effluent concentrations. During the feeding, the liquid an S::CAN spectro::lyser[tm] probe from Interline was used to continuously monitor nitrate concentrations at 0.5 meter under the water surface.

Table 1: Influent and effluent data of the Nereda® granular sludge plant in Garmerwolde, Netherlands						
	Influent				Effluent	
	Min.	Max.	Average	Load	Effluent req.	March – Dec. 2014 avg.
Parameter	[mg L^{-1}]	[mg L^{-1}]	[mg L^{-1}]	[kg d^{-1}]	[mg L^{-1}]	[mg L^{-1}]
Suspended Solids	101	465	236	8000	30	**20**
COD	146	715	506	14636	125	**64**
BOD$_5$	60	420	224	5495	20	9.7
Total Phosphorus	1.9	9.7	6.7	212	**1**	0.9
PO$_4^3$-P dissolved	1.5	6.8	4.4	-	-	0.4
Total Nitrogen	14	81	49.4	1387	**7**	6.9
NH$_4^+$-N dissolved	13.4	56.5	39.0	-	-	1.10

Grab samples

Periodic sampling for the determination of the concentration of the biomass present in the reactor is performed by a grab sampler. Samples can be acquired pressureless at various depths to account for possible segregation over the depth of the reactors due to

granulation. Normally, samples are only taken when the reactor is well mixed during the reaction period.

Physical characteristics of granules

1500 mL of reactor volume was washed over a 212 and 600 µm sieve. The sieved granules and 1500 mL of un-sieved sample were dried at 105 °C until no weight change was measured. The dried fractions were then used to calculate the percentage of granules and the total biomass concentration in the reactor. Sludge volume index was measured by pouring 1000 mL of sample in a graduated measuring cylinder. Volume of the biomass was consequently recorded after 5 and 30 minutes. Only undiluted samples were used in determining the SVI.

Fluorescent in situ hybridisation

Sample handling, fixing and staining was performed as described in Bassin et al. (Bassin et al. 2011a). Probes used to stain all bacteria consisted out of a mixture of EUB338, EUB338II and EUB338III (Amann et al. 1990, Daims et al. 1999). To stain the PAOs present, a mixture of PAO462, PAO651 and PAO846 described by Crocetti et al. (2000) was used. Probes GAOQ431 and GAOQ989 were used to stain the glycogen accumulating organisms (GAO) population (Crocetti R et al. 2002b). The samples were examined with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575 - 625/ FT645/ bp 660 - 710), 20 (bp 546/12/ FT560/ bp 575 - 640), 17 (bp 485/20/ FT 510/ bp 5515 - 565) for Cy5, Cy3 and fluos respectively.

Results

Description of start up

The first year of operation of the treatment plant can be divided into two periods, based on the extent of granulation and conversion efficiencies established. The first period was characterised by a gradual increase of the volumetric loading rate to the design-loading rate and the development of granules from the flocculent sludge (September 2013– February 2014). Reaching design-loading rate took about 3 months with fluctuating conversion efficiencies. In period two, reactor operation and process efficiencies were optimized (March – December 2014).

Preceding period one, technical testing was performed in July and August 2013. Both AGS plant and the existing conventional activated sludge treatment plant were expected to meet nitrogen and phosphorus effluent standards of 15 and 1 mg L^{-1} respectively. To safeguard these effluent quality requirements, the loading rate towards the AGS reactors was increased only as far as the effluent quality allowed. This meant that on some occasions during the start-up period, the loading rate had to be temporarily reduced. Within 3 months the design flow rate was reached and 41 % of the wastewater received at the WWTP was treated by the aerobic granular sludge plant (Fig. 3a). Nitrogen, phosphorus and COD removal were already stable during a part of period 1, but were not optimized yet (Fig. 5). Sludge volume indexes, decreased significantly during start-up (Fig. 3b). Initially, the SVI_5 and SVI_{30} were 145 and 90 mL g^{-1}, but they decreased during the start-up period to approximately 70 and 50 mL g^{-1} respectively. The difference in SVI_5 between the samples from the reactor and those from the surplus sludge continued in the second period. SVI values kept improving until stable values were reached of 45 and 35 mL g^{-1} for SVI_5 and SVI_{30} respectively. Biomass concentration increased from 3 to 6.5 kg m^{-3} in the first period (Fig. 4). At the end of the second period it reached a concentration of > 8 kg m^{-3}. The percentage of sludge present as granules increased from 30 to > 80 % over this period.
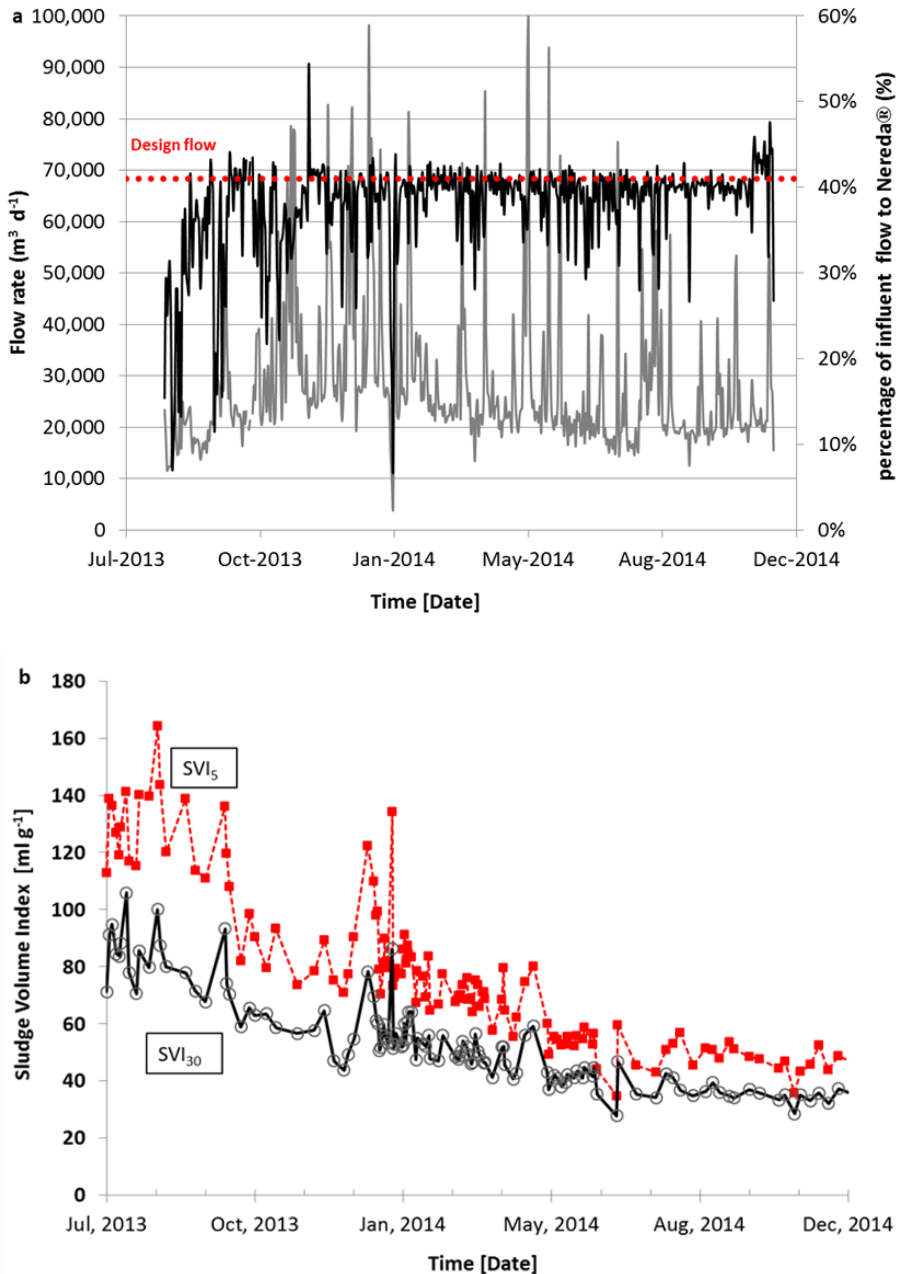
Fig. 3 - **Flow and biomass characteristics during start-up of the aerobic granular sludge reactor: (a) percentage of the influent flow towards the aerobic granular sludge plant (black line; five day moving average), total influent flow received at the Garmerwolde plant (light grey line) and the design influent percentage towards the aerobic granular sludge plant (dotted line); (b) sludge volume index after 5 (filled cubes) and 30 (open circles) minutes of settling.**

Performance results during period 2

February 2014 was considered as the end of period 1. Nutrient removal as well as a significant granule bed was present from that time onward. Performance results and operational parameters obtained between March and December 2014 are summarized in Table 1 and 2. Monthly average nutrient concentrations are given in Fig. 5. Effluent guidelines were met for all parameters, with an average N_{tot} and P_{tot} of 6.9 and 0.9 mg L^{-1}, respectively, in the period March - December 2014. Elevated nitrogen and phosphorus concentrations in the effluent always coincided with heavy rain weather events. Due to presence of a large sewer system, heavy rain events provoke a flow rate that can be 2 – 5 times as high (Fig. 3a) with nutrient concentrations as occurring in dry weather flow (so-called first flush). The combination of increased flow and loading rates leads to shorter cycle times and resulted in elevated effluent concentrations. At these times, in accordance with the design set-up targeting an optimized installed reactor volume, metal salts are used for the removal of phosphorus at the end of the cycle. The Fe(III)/P molar ratio during the operational period was 0.18 with a total P-removal efficiency of 90 %. During normal rain and dry weather flow the removal of phosphorus is completely biological.
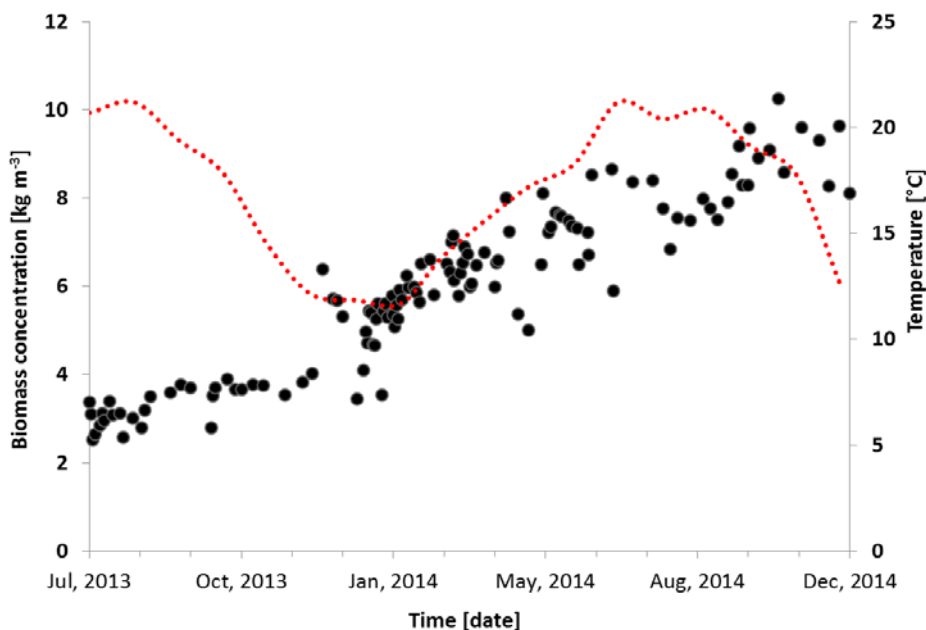

Fig. 4 - Evolution of biomass concentration with time (filled circles) and temperature of the bulk liquid (dotted line) in the aerobic granular sludge installation.

Granulation

As the dry weight increased, so did the percentage of granules in the total biomass. In period 2 more than 80 % of the TSS consisted of granules larger than 0.2 mm with more than 60 % above 1 mm. Since selection pressure on settling behaviour is needed for the development of well settling granular sludge during start-up (Beun et al. 1999, Qin et al. 2004), the smaller and less settling sludge should be removed with the surplus sludge, while larger granules should be maintained in the reactor.

Barely any granules larger than 0.2 mm were detected in the surplus sludge and consisted mostly out of relatively slow settling flocs, which indicates good separation between slow and fast settling biomass (Fig. 7a, b, c). Protozoa, mainly ciliated, are present on the outside of the granules. Sliced granules examined under a stereo zoom microscope revealed a layered structure with micro colonies present inside (Fig. 7d).
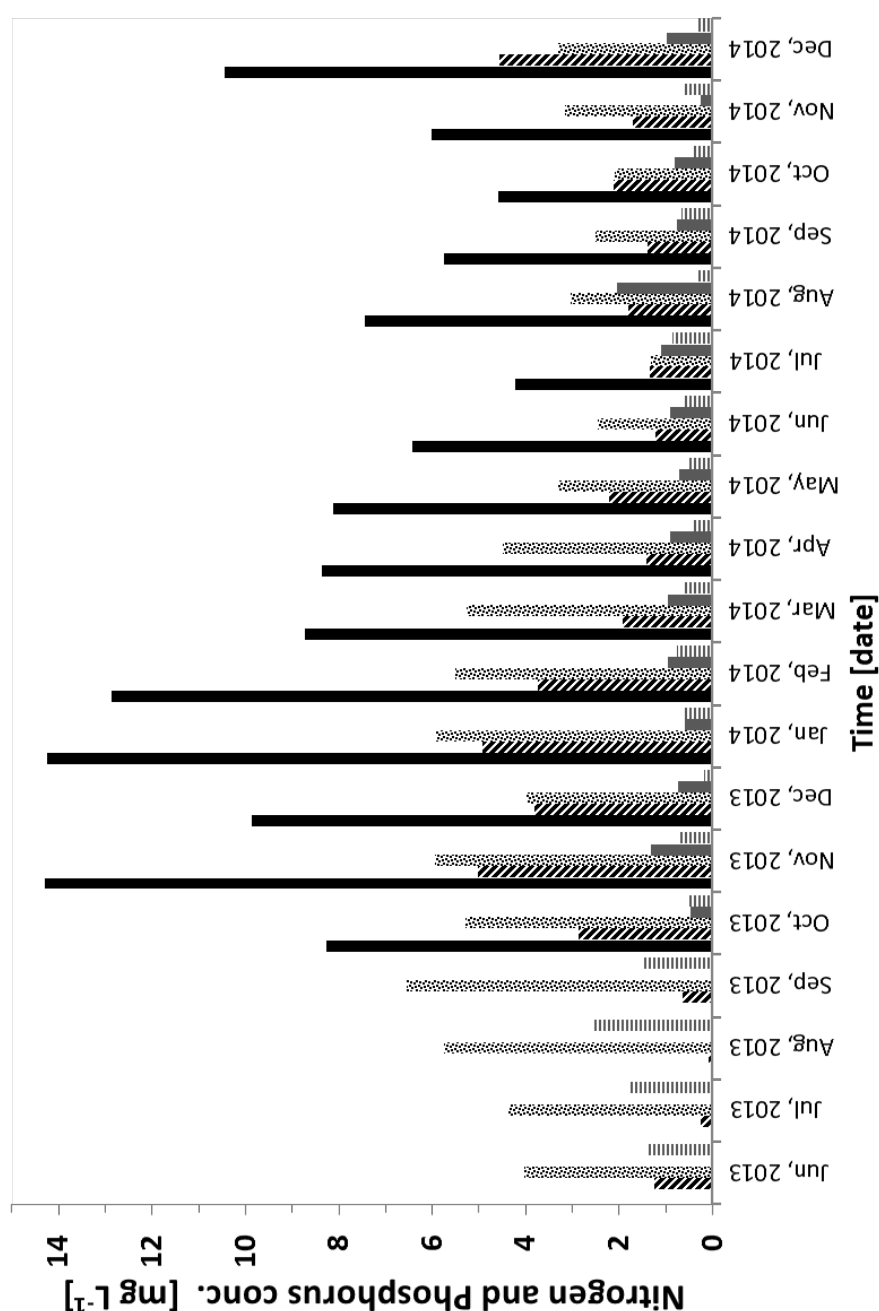
Figure 5: Monthly nutrient averages in the effluent of the aerobic granular sludge system with (■) total nitrogen, (▨) ammonium, (▧) nitrate, (▤) P-total, (▤) ortho-phosphate. Total nitrogen and phosphorus measurements for the aerobic granular sludge plant separately were only available from October 2013.

| Table 2: Operational parameters of the Nereda® granular sludge plant in Garmerwolde, Netherlands					
Data obtained between March and December 2014					
Parameter	**Unit**	**Value**	**Parameter**	**Unit**	**Value**
Solid retention time	d^{-1}	20 - 38	Max Recycle ratio	-	0.3
Dry weight	$kg\ m^{-3}$	6.5 – 8.5	Fe(III)/P ratio [4]	-	0.18
Ash content	%	25	Volumetric phosphate uptake rate [2]	$kg\ P\ (m^3\ d)^{-1}$	0.011
Sludge loading total [1]	$kg\ COD\ (kg\ TSS\ d)^{-1}$	0.10	Max. volumetric phosphate uptake rate [3]	$kg\ P\ (m^3\ d)^{-1}$	0.24
Sludge loading biological [2]	$kg\ COD\ (kg\ TSS\ d)^{-1}$	0.12	Volumetric ammonium uptake rate [2]	$kg\ N\ (m^3\ d)^{-1}$	0.058
Sludge production	$kg\ d^{-1}$	3900	Max. volumetric ammonium uptake rate [3]	$kg\ N\ (m^3\ d)^{-1}$	0.17
Hydraulic retention time	h^{-1}	17	Energy	$kwh\ m^{3-1}$	0.17
Volumetric load	$m^3\ (m^3\ d)^{-1}$	1.45	Energy	$kwh\ (kg\ N)^{-1}$	3.6
Nitrogen Load	$kg\ N_{tot}\ (kg\ TSS\ d)^{-1}$	0.011	Energy [5]	$Kwh\ (PE_{150,\ rem.}\ year)^{-1}$	13.9

[1] based on kg COD received per day over the total biomass present in the reactor, [2] based on the aerated time, [3] based on actual rates measured inside the cycle at 20 °C, [4] Only during heavy rain weather events. Molar ratio is given. [5] P.E.: Population equivalent or 1 i.e. equals 150 mg TOD per day.

FISH

Fluorescent in situ hybridisation performed on pottered granules showed the presence of a significant PAO population (Fig. 7e, f). Most of the bacteria stained with the EUB probe (blue), also stained with the PAO probe (red) resulting in the pink colour. In fact, hardly any GAOs could be detected (< 5 %). Other heterotrophs that are typically stained with the EUB probe were present, but were not further investigated.

Conversions within a typical Nereda® cycle

The cycle begins with simultaneous influent feeding and effluent discharge (Fig. 2 and Fig. 6). Influent is pumped into the reactor from the bottom with an upward velocity of 3 – 3.3 m h^{-1} without further mixing or aeration. Simultaneously, effluent is pushed over the overflow weirs at the top of the reactor. In these anaerobic feeding conditions, easy biodegradable organic carbon will be converted into storage polymers by glycogen (GAO) and phosphorus (PAO) accumulating organisms in the granule bed. The uptake of easy biodegradable substrates by PAOs (Fig. 7h) is accompanied by the release of ortho-phosphate into the bulk liquid as can be seen from the relative high phosphate peak at the beginning of the aeration period.
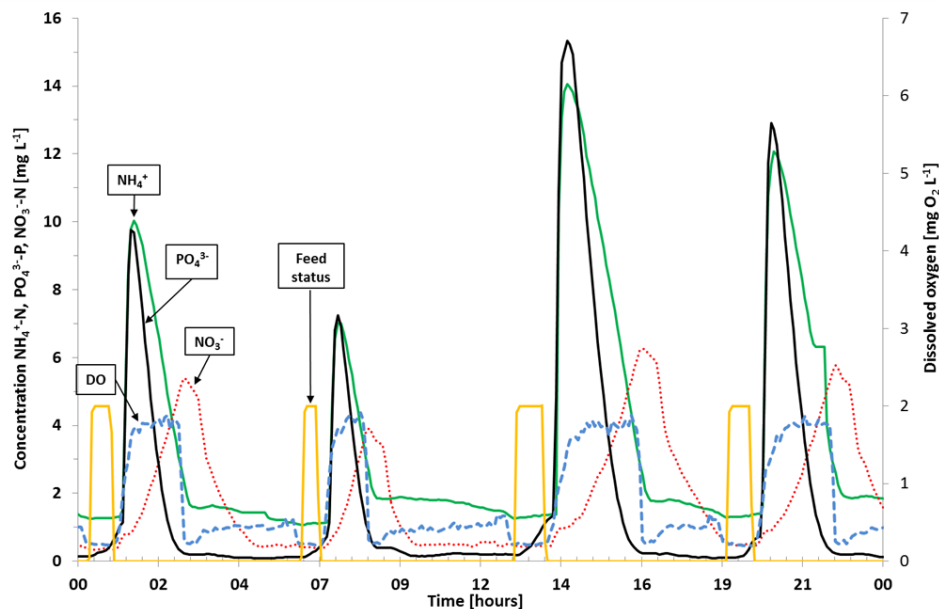
Fig. 6 - Typical operational cycle in the aerobic granular sludge plant with concentration of ammonium (green solid line), phosphate (black solid line), nitrate (red dotted line), oxygen (blue dashed line) and feed (yellow solid line).

The volume exchange ratio in each cycle is limited by dispersion of the influent and thus the imperfection in the plug flow pattern during feeding. Since the sampling points for nitrogen and phosphorus are located 0.5 m under the liquid surface (just below the effluent weirs), the influent feeding can and needs to be stopped as soon as the phosphate concentration increases at this sampling point, since this indicates that a small fraction of the influent is reaching the effluent weir. This occurs when the effective volume exchange ratio is 65 %, while during dry weather flow in accordance with the design this effective volume exchange ratio is typically between 30 – 40 %. Ammonium present in wastewater is partially adsorbed by the biomass during the feeding period (Bassin et al. 2011b). Adsorbed ammonium will however, slowly desorb from the biomass during the aerobic period and consequently be converted (see Chapter 2). After the feeding period, the reactor is aerated and thus mixed, while the dissolved oxygen concentration is controlled at 1.9 mg L^{-1}. At the start of the aeration period ammonium and phosphate concentration peak due to the mixing of the clean water in the top (where the measurement system is) and the influent in the lower part of the reactor. The concentrations after mixing, combined with the amount of wastewater fed, can be used as an indication of the loading rate of the cycle, but need to be corrected for phosphorus release and ammonium adsorption.

In the aerated period ammonium and phosphorus uptake occurred, while nitrate was produced. Once ammonium reached its desired set-point the oxygen concentration in the

bulk was lowered to allow for maximum denitrification potential. Denitrification is taking place inside the granule. Since the diffusion of oxygen into the granule is slower than the conversion rate, an anoxic volume is created inside the granules (de Kreuk et al. 2005a, Osman et al. 2001). Storage polymers of the GAOs and PAOs are delivering the reducing power for the conversion of nitrate to dinitrogen gas. Once both nitrogen and phosphorus have reached the required effluent quality, the reactor is ready to receive new influent.
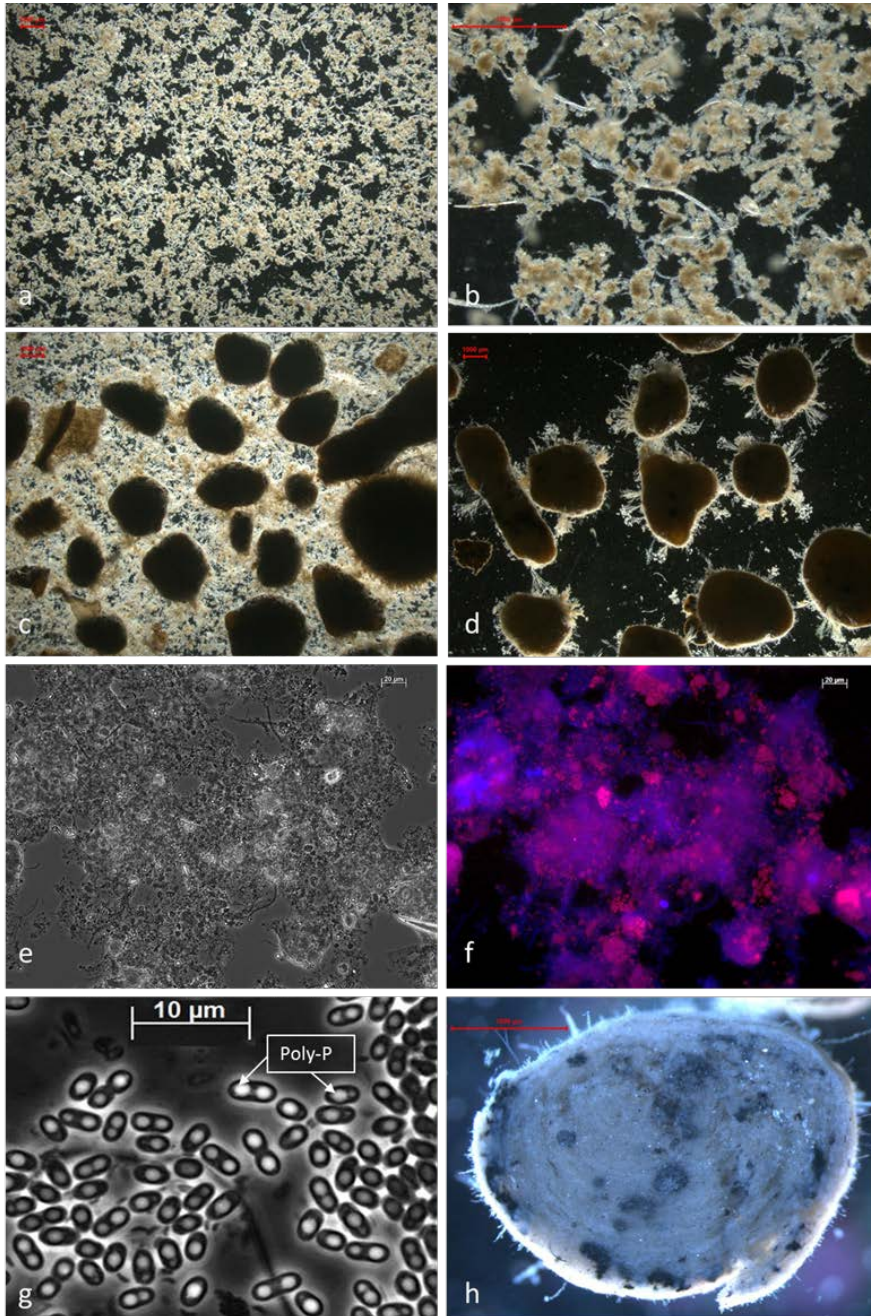
Fig. 7 - Aerobic granular sludge: (a, b) biomass on the top of the granular sludge bed, (c) mixed sample of the bulk liquid, (d) granules sieved and washed with tap water, (e) phase contrast and (f) fluorescent in situ hybridisation with blue EUB (Cy5) and red PAOmix (Cy3). Overlay of blue and red gives the pink colour, (g) 1000x magnification of phosphate accumulating organisms found in pottered granules and (h) sliced granule.

Discussion

Start-up

Generally, technological start-up times for nutrient removing conventional activated sludge (CAS) plants range anywhere between 30 and 40 days, assuming the biomass inoculated is adapted to the wastewater and adequate amounts are available. Otherwise, longer start-up times are needed to grow and adapt the biomass. Start-up times with respect to effluent quality required for the AGS plant were similar. In this case, the start-up period was a bit longer due to the requirement to achieve full effluent quality standards at all times, even during start-up conditions. This resulted in a 3 months stepwise increase in redirected influent flow from the AB-plant towards the aerobic granular sludge plant.

This study demonstrated that a good effluent quality can be achieved long before granulation is complete. The required flow and treatment capacity was reached in only 3 months, but the intended biomass concentration of 8 g L^{-1} was only achieved after 9 months. The studied reactors were started with surplus sludge of the full-scale Nereda in Epe, The Netherlands. Although this surplus sludge was well settling (SVI$_{30}$ =140 mL g^{-1}), it hardly contained any granular sludge. Development of granular sludge is expected to be shortened drastically by starting with granular sludge from other plants (Pijuan et al. 2011). Also, when the effluent criterium is less critical during start-up, the time to reach the design sludge mass can be significantly reduced by increasing the COD load. It should be noted that a treatment plant is designed for the highest load at the coldest temperature. For this reason, the required treatment capacity can be achieved during most of the year, even if full granulation is not yet reached.

Granulation

Successful formation of AGS in a full-scale plant treating domestic wastewater was demonstrated in this study. A high percentage (> 80 %) of the sludge consisted of aerobic granules larger than 0.2 mm. Aerobic granulation on domestic wastewater was shown before in lab-and pilot scale studies, but the full granulation of biomass on full-scale was not (Giesen et al. 2013, de Kreuk and van Loosdrecht 2006c, Coma et al. 2012, Wagner and Da Costa 2013).

As proposed previously in lab-scale and pilot-scale studies, granule formation could also be monitored satisfactorily with the SVI$_5$/SVI$_{30}$ ratio for this full-scale reactor start-up (de Kreuk et al. 2007b, Etterer and Wilderer 2001). More complicated tests as granular size distribution measurements give more insight, but are not required for full-scale operations. In full-scale plants there will always be a fraction of flocculent sludge resulting in an SVI$_5$/SVI$_{30}$ ratio higher than 1 (Fig. 7 a, b). This flocculent material is resulting from influent particulates, detached pieces of granules and growth on polymeric substrates (De Kreuk et al. 2010b).

Many factors have been mentioned as critical for the formation of granular sludge, such as high shear, very short settling times and high DO concentrations (Beun et al. 2000a, Liu et al. 2005b, Lee et al. 2010). Most of these are related to experiments with aerobic or completely mixed feeding. In the case of anaerobic plug flow feeding through the granule bed, most of these factors (like high settling pressure, shear, high DO etc.) become less relevant, although not irrelevant, during start-up (de Kreuk et al. 2005a). Anaerobic feeding selects for relatively slow growing bacteria such as PAOs, that convert the easy degradable COD under anaerobic conditions into storage polymers, resulting in smooth and compact biofilms (Van Loosdrecht et al. 1997a). The plug flow feeding furthermore ensures high substrate concentrations near the granule surface allowing substrate to diffuse through the entire granule, further contributing to the formation of stable, compact and dense granular biomass.

Suspended solids in the effluent

Suspended solids in the effluent can originate from the influent, as well as from detached biomass. In the present case the influent contained on average 236 mg SS L^{-1} while in the effluent on average only 20 mg SS L^{-1} was present. The exact removal mechanism of suspended solids in aerobic granular sludge systems is not fully understood yet, however a few relevant observations were made. Suspended material flocculates and can be retained in the AGS plant despite the up flow velocity (3 - 3.3 m h^{-1}), after which it is removed with the excess sludge. Cellulose fibers, which are present in domestic wastewater (Ruiken et al. 2013), are predominately removed with the excess sludge (Fig. 7b), and do not end up in the effluent. Particles can also be entrapped in the granules themselves (Fig. 7h), where they are degraded or finally end up in the excess sludge as well. Finer material and colloidal particles that are not entrapped can be further removed by protozoa (Lemaire et al. 2008, Priya et al. 2007, Li et al. 2013). In granular sludge, stalked protozoa such as *Vorticella* and *Carchesium*-like organisms attach to the granules (Fig. 7c). Protozoa have also been related to particulate starch removal in laboratory experiments (De Kreuk et al. 2010b, Lemaire et al. 2008, Schwarzenbeck et al. 2004) and in biofilm plants fed with suspended bacteria (Lee 1996), however, their exact contribution to the removal of suspended solids from municipal wastewater has not been quantified yet. Although these protozoa slightly hinder settling, they do not disturb the plant and should be seen as a sign of a healthy microbial ecosystem in the treatment of domestic wastewater.

Nutrient removal

In sewage treatment, the nitrogen removal efficiency depends on the incorporation of nitrogen in the growing biomass, and the nitrification and denitrification capacity of the microbial community. The effluent nitrogen concentration of conventional activated sludge plants with pre-denitrification is furthermore limited by the recycle flow from the aerobic to the anoxic tank. Through post denitrification very low nitrogen concentrations can be established, but this requires the supply of external carbon. External carbon dosage is

costly and can lead to elevated BOD concentrations in the effluent when overdosed (Jobbágy et al. 2008). One of the main advantages of the aerobic granular sludge plants is that the biological nitrogen removal processes are not separated in different anoxic and aerobic tank volumes, but can be optimised by a flexible aeration and denitrification period. During aeration, ammonium will be converted to nitrate and due to the inherent oxygen gradients present in the granules, a part of the nitrogen will be simultaneously denitrified. The ratio of nitrification to denitrification can therefore be regulated by changing the DO set point. When the nitrate concentration at the end of the nitrification process is still too high for effluent discharge, a recycle from the bulk liquid at the top of the reactor towards the settled granules bed can be introduced for enhanced nitrate removal. As an alternative, an intermittent aeration strategy can be applied. The effective recycle flow rate can be much smaller than is usually used in activated sludge plants and in the present study, the average was only 0.3 of the influent flow rate to the reactors during the operational period. Compared to the AGS plant (0.3), a conventional pre-denitrification system would require a recycle ratio of roughly 10 - 15 times higher to achieve the same total nitrogen removal of over 90 % (excluding nitrogen in SS effluent). In the aerobic granules, internally stored polymers of the PAO and the GAO serve as electron donor in the denitrification process. Simultaneously, the PAOs store phosphate as poly-P, hereby fulfilling a double function (denitrification and phosphate removal). The phosphate uptake is therefore less sensitive to aeration than nitrification, making the process control more flexible. As long as anoxic conditions prevail, the PAOs do not release phosphate in the bulk liquid, which results in very low nitrogen and phosphorus concentrations in the effluent.

Due to the high amount of phosphorus stored inside the sludge as poly-P, a large amount of phosphorus is released during the anaerobic periods. This is especially true during heavy rain events, when the increased flow rate is combined with concentrations that initially remain as high as during dry weather flow (so-called first flush). The resulting increased COD loading rate triggers extra phosphate release. The high hydraulic loading rate, however, enforces a short hydraulic residence time with a shorter cycle time as a consequence (Fig. 2). This reduces the time for aerobic P-uptake significantly. In the design of the Garmerwolde WWTP, this decreased biological phosphorous removal capacity under extreme rain events is tackled by iron dosing, rather by tuning the reactor size. This enables further reduction of the installed reactor volume. During normal rain – and dry weather - events metal dosing was not needed as phosphate was always removed biologically.

Volumetric conversion rates
As a result of the higher biomass concentrations (> 8 g L[1]), the batch wise operation and the parallel occurrence of conversions that can be reached with granular sludge plants, volumetric conversion rates are higher than in conventional activated sludge plants and thus volumetric loading rates can therefore be increased accordingly. The simultaneous

nitrification and denitrification in granular sludge plants, can even lead to a more than proportional increase of volumetric loading rate. The volumetric nitrogen conversion rate (based on the aerated time) of this plant was 0.058 kg N $(m^3$ d)$^{-1}$. The maximum volumetric ammonium conversion rate was 0.17 kg N $(m^3$ d)$^{-1}$ at an average temperature of 20 °C (Table 2). The obtained conversion rates underestimate the actual rate, since they are based on on-line ammonium measurements, while a fraction of the influent ammonium is initially adsorbed or incorporated in struvite (Bassin et al. 2011b, Lin et al. 2012). The granular sludge allows for a compact treatment process, which is reflected in the volumes of the existing old conventional plant, treating 59 % of the sewage in 28400 m^3 of activated sludge tanks plus 24800 m^3 of clarifiers, while the AGS reactors treats 41 % of the sewage in 19200 m^3 tanks plus 4000 m^3 of rainwater buffer. This amounts to 0.8 m^3 d^{-1} of wastewater treated per m^3 volume needed for the AGS plant compared to 1.2 m^3 d^{-1} for the existing treatment plant. Leading to a volume reduction of 33 % for the AGS plant compared to the existing plant.

Effluent nutrient concentrations were also higher for the conventional AB-plant (9.9 mg N L^{-1} and 0.9 mg P L^{-1}) in the same period. It should be noted that there is no biological phosphorus removal in the AB-plant; hence most of the phosphorus is removed chemically (metal dosing).

Generally, the ammonium and phosphorus removal rates are such that both compounds can be easily removed well before the end of the cycle, thus creating room for polishing the effluent quality if desired (Fig. 6). However, in light of saving energy, effluent concentrations are steered towards the demands instead of the lowest concentration obtainable.

The oxygen gradients inside the granules, due to diffusion limitation, and the batch wise operation of the plant allow for enhanced process control via DO (Lochmatter et al. 2013, Kagawa et al. 2015, Isanta et al. 2013). An increase of the DO will lead to a larger aerobic volume fraction of the biofilm and thus an increase in volumetric nitrification rate (Beun et al. 2000b, Aravinthan et al. 1998, Wijffels and Tramper 1995). Likewise, lowering the DO creates more anoxic volume leading to higher denitrification rates. This opens up process control opportunities that are difficult to achieve with activated sludge plants. A proper DO control strategy will therefore enable accurate steering of effluent parameters by manipulating the oxygen gradients inside the granules.

Finally, transport characteristics are less temperature dependent than bacterial growth rates (Wijffels et al. 1995). This implies that biofilm systems are less sensitive to temperature changes than suspended and flocculated systems (Leenen et al. 1997). So the effect of decreasing temperature (summer - winter) in AGS systems on the conversion rates is expected to be less than that of activated sludge systems.

Energy

We have estimated the electricity consumption of the AGS plant in the total energy balance of the Garmerwolde WWTP. The calculation includes the fraction of energy used in the influent pumping station for the AGS plant influent, but dewatering of the biomass and the energy generation by sludge digestion is not taken into account. The reason for this is that the digester (onsite) receives sludge from the AGS as well as the AB-plant plus external sludge from neighbouring plants. Determining the exact contribution of the waste aerobic granular sludge to the total biogas production is therefore impossible. The energy consumption of the full-scale AGS plant is compared to the average energy use of all Dutch WWTP's (Table 3). These are partitioned in plants with a good effluent quality (N ≈ 10 mg N L^{-1}; P ≈ 1 mg L^{-1}) and plants with an excellent effluent quality due to post treatment (N = 3 - 10 mg N L^{-1} and P = 0.1 - 0.5 mg L^{-1}) (STOWA 2013). These data for Dutch WWTP's include energy generation by sludge digestion, which is present at most of the Dutch WWTPs. The AGS process should be compared to the higher effluent quality WWTP's. The AGS process shows very low energy consumption. Due to the lack of mixers, conventional recycle pumps, settlers and sludge return pumps, energy usage of sequencing batch systems will be lower than that of conventional activated sludge plants. Moreover, energy usage for aeration is affected by the water depth of the tanks (Fernández-Álvarez et al. 2014). The AGS tank studied had a height of 7.5 meters, while activated sludge tanks are often limited to 6 metres. This potentially adds 5 – 10 % extra to the aeration energy saving. Overall, the studied AGS plant consumed 58 - 63 % less energy compared to conventional activated sludge plants.

Table 3: Comparison between the average energy demand between various WWTP's in the Netherlands	
Type	**Specific energy demand [kWh (PE$_{150, removed}$ year)$^{-1}$]**
WWTP with polishing (Total N effluent < 10 mg N L^{-1})	37.5
WWTP without polishing (Total N effluent ≥ 10 mg N L^{-1})	33.4
WWTP Nereda® Garmerwolde	13.9

When comparing the energy usage of the existing conventional AB-plant (0.33 kWh m^{-3}) with the AGS plant (0.17 kWh m^{-3}) at WWTP Garmerwolde over the same period per m^3 of treated influent, the AB-plant consumed 51 % more energy. To make a proper comparison between the two plants the energy required for the sludge treatment and the SHARON plant were omitted in both energy calculations.

Due to the low energy use of the AGS plant, the concept contributes to the development of energy neutral or energy producing WWTP's. In addition, preliminary results showed that the total bio-methane potential of aerobic granular sludge during digestion is similar to

the digestion of activated sludge (data not shown), but more detailed studies on the digestion and biogas production of aerobic granules grown on sewage are needed to be able to give a detailed energy balance over the plant (Val Del Río et al. 2014).

Conclusion

The present study described the start-up and operation of one of the currently largest full-scale aerobic granular sludge plants treating domestic sewage. The results illustrate that the technology has grown in a decade from a promising innovation to a mature technology capable of competing with established wastewater treatment technologies. The operational data show that in Dutch climate conditions the effluent requirements (7 mg N L^{-1} and 1 mg P L^{-1}) were easily reached, while maintaining a high granular biomass concentration (> 8 g L^{-1}) with SVI_5 values of 45 mL g^{-1} and process stability for both summer and winter periods. Because of the high biomass concentration, the volumetric loading rate could be increased accordingly. Energy usage of the aerobic granular sludge system was 13.9 kWh $(PE_{150} \cdot year)^{-1}$. This is 58 – 63 % lower than the average conventional activated sludge plant in the Netherlands, with an effluent quality which is comparable or better. The specific volume that was needed for the aerobic granular sludge based system was 33 % lower than the volume needed for the existing conventional activated sludge plant. Finally, the results show that, aerobic granular sludge technology can be effectively implemented for the treatment domestic wastewater. Further enhancement, mainly based on improved process control, is still possible. The full operational window with respect to influent variations has still to be established

.

7

Outlook

In this thesis, the effects of several operational conditions on the conversion processes in aerobic granular sludge, and the formation and stability of granular sludge was studied. Most experiments were conducted in laboratory reactors with synthetic wastewater, but Chapter 6 describes the evaluation of a full-scale aerobic granular sludge reactor. The results obtained led to better understanding of the aerobic granular sludge process and were used to design and adjust operational procedures in both domestic and industrial applications of the technology.

For example, the results obtained for the ammonium adsorption (**Chapter 2**) have led to better understanding of the ammonium removal process in aerobic granular sludge plants. Ammonium adsorption explains why reactor ammonium concentrations after feeding, are generally lower than expected based on influent measurements. This improves the interpretation of on-line ammonium measurement and process control and is especially important for designing an aerobic granular sludge plant. The ammonium adsorption should be introduced into biofilm models in the future to shed light on the influence the adsorption might have on the selection of species, conversions and precipitates. Optimization of adsorption combined with precipitation may lead the way to a new recoverable resource from aerobic granular sludge in practice. Another example is the conversion of substrates in the aerobic granular sludge process that were previously not recognized (**Chapter 4**). Methanogenic conversions in a system that is mainly aerobic has several practical consequences. The observation that aldehydes dismutate under anaerobic conditions into a carboxylic acid (consequently stored as PHA) and an alcohol (partly adsorbed onto the biomass) resulted in a better understanding of the conversions and granulation potential that one can expect when working with such substrates as frequently encountered in industrial wastewaters.

Future of aerobic granular sludge

First experiences with a full-scale municipal aerobic granular sludge sewage treatment plant revealed that it can be successfully implemented for the treatment of domestic wastewater (**Chapter 6**). Based on these results it may seem that no further investigation into the process and microbiology is needed. However, due to the emphasis on rapid development and scale-up of the process many aspects of the aerobic granular sludge technology remain unknown and deserve in-depth research. Differences observed between laboratory and full-scale results are the main driving force for these new research questions. Other aspects of aerobic granular sludge have not (yet) been studied or are very difficult to simulate in laboratory conditions and therefore require access to full-scale reactors. Since full-scale aerobic granular sludge reactors are relatively new, very few studies have thus been performed in such plants. In the future, the implementation of the process in more extreme conditions like alternating salinities, low (< 5 °C) and high temperatures (> 30 °C), high COD and Nkj concentrations should be investigated. A few aspects of the aerobic

granular sludge technology that might lead to a better understanding and distribution of the process in practice and research in laboratory studies are given below:

Process control

Aerobic granular sludge needs a different process control strategy compared to conventional activate sludge plants, because of the batch wise operation of the process and the fact that the system is a biofilm-based process. Ideally, the enhanced process control should make full use of the strengths a batch process using biofilms has to offer. Batch wise operation allows for extensive variability of the cycle layout in order to obtain the effluent quality required while minimising running costs.

Different from laboratory studies, the cycle length in practice is not always fixed. This is mainly due to daily flow variations especially under dry and rain weather conditions. The feeding time can be increased or decreased at the expense of available aeration time leading to changes in exchange rates. All these parameters (and more) can be controlled per cycle providing great flexibility without the adjustment of the system. This is, in many aspects, very different from conventional systems. Other controllable parameters that could be used to control the system are; sludge loading rates (also by adjusting the amount of granular sludge), dissolved oxygen concentrations (creating higher nitrification or more available denitrification capacity), selection pressures, sludge discharge etc. All this is of course related to the initial design, the required effluent quality and local conditions of the plant. Here, the translation from laboratory or modelling studies to practice and vice versa is not always that easy. Many more factors influencing the process are present in practice that cannot be all simulated in the lab or in a model. Process controls developed in literature have been mainly using pure SBR systems with synthetic influent and fixed batches controlling the dissolved oxygen concentration in the bulk to optimise the nitrogen removal (Lochmatter et al. 2013, Isanta et al. 2013). For now, the process control in practice is sufficient, but not (yet) smart enough. The batch wise operation in itself provides much more information (see **Chapter 6**) about the process than in continues systems like a flocculated continuous activated sludge based process. This is because the concentrations in such systems are much less time variable and the conversions have to be derived from solving complex mass balances. Measuring process variables is also easier in a one-tank layout such as the AGS plants than in activated sludge plants where processes are separated in place instead of time. The data gathered could be used to obtain the best process control strategy using for example a fuzzy logic approach.

Mass balances

In current full-scale plants, measurement of the off-gas composition is missing. This is also true for most of the laboratory studies on aerobic granular sludge found in literature. Off-gas data can be used to calculate mass-balances and directly provides alpha factors and k_La values of the reactor in different conditions (e.g. dry weight concentrations, batch sizes, flow rates, temperature etc.). This would help in designing new plants as well as to optimise

the previously discussed process control for existing and future plants. One of the more important questions regarding off-gas measurements is if the measurement can be used as a control parameter. In aerobic granular sludge reactors, there is just one reactor compartment and it is nearly perfectly mixed during aeration. This makes off-gas measurements relatively easily implementable compared to the often poorly mixed conventional activated sludge systems. It might also lead to new insights, as off-gas measurements are not that often applied in practice as a control parameter.

Automation

A complete autonomic running wastewater treatment plant could be very efficient. Human mistakes or misinterpretations of data are hereby limited. The question is however, how much autonomic the process can become in such variable systems. A certain human presence is still needed to oversee the main performance of the plant. In practice, it already shows that the transfer from an activated sludge process to a batch wise operation of a biofilm process requires significant training and adaptation of the end users. What is true for an activated sludge plant is not always directly translatable to a biofilm reactor, but this is something that can be solved by automation. Sampling, calibration of sensors and maintenance all need to be accommodated to the AGS cycle. Requirements of the sensors (DO, ammonium, phosphate etc.) are also different than in a conventional activated sludge plant, since large variations occur in the measured concentrations. The problems arise when the autonomic control fails or other less predictable issues occur that require human input. Education and training programs should therefore be present when switching from conventional to plants with aerobic granular sludge. Both the marketing company and the university should be involved in such programs. Like this, the cooperation between water authority, university and commercial would also get stronger and might lead to better performance, more insights and efficient designs.

Conversion of suspended solids

Usually, in domestic wastewater a large part of the total COD is in the form of suspended solids. It consists of a large range of organic and inorganic particulates that are normally not removed in the pre-treatment. Effluent standards for suspended solids vary greatly from plant to plant and even more from country to country. Nevertheless, it is an important parameter that should not be overlooked. In conventional activated sludge plants, the not convertible inorganic and organic particulates are removed by incorporation into the activated sludge flocs. Eventually, the particulates leave the process via the waste sludge route. In aerobic granular sludge plants, the most likely removal route of the particulates that are not converted is also via the sludge selection (**Chapter 6**). A detailed study on the fate of particulates in full-scale reactors is still lacking. The conversion of COD into storage polymers is important for the formation and stability of aerobic granular sludge as well as the proper functioning of bio-P removal. The conversion of particulates requires a hydrolysis step before bacteria can utilize it as a substrate. Hydrolysis of the

suspended solids is therefore the rate-limiting step (Morgenroth et al. 2002). To which extent the particulates are hydrolysed and converted in aerobic granular sludge plants is still unknown. Laboratory studies with particulate starch and protein (unpublished) showed that the aerobic conversion of these particulates have a significant impact on granule formation and morphology (De Kreuk et al. 2010b). Longer feeding times will lead to more anaerobic hydrolysis and better granule formation (Wagner et al. 2015). This will take away valuable aeration time in practice. When and how these particulates are removed depends on factors like; type, origin, hydraulic circumstances, temperature, SRT etc. A recent study showed that the percentage of granulation was directly related to the effluent suspended solids (Rocktäschel et al. 2015). This seems to be in contradiction to what is observed in practice and thus requires further investigation. In literature the conversions of particulate substrates and the impact on granule morphology and stability are virtually absent as is the impact on important conversions like (de)nitrification and phosphorus removal.

Are bigger granules better?
In practice, the consensus is that the bigger the granule the better it is, but is there an ideal granule size considering all the requirements? Or should there be a wide distribution of sizes for optimal performance? Bigger granules have a larger anoxic potential than smaller ones, which is beneficial for denitrification, but might limit certain aerobic conversions like nitrification. Smaller granules in turn have a lower settling velocity and might be more prone to washout in adverse conditions. Small granules furthermore require very low oxygen concentrations to establish simultaneous nitrification and denitrification. In earlier models the optimal granule size for nitrogen removal growing on acetic acid was proposed to be on average 1.5 mm (de Kreuk et al. 2007a). However, the conditions found for aerobic granular sludge in practice were not taken into account and this should thus be re-evaluated in accordance with data obtained from full-scale plants.

Modelling
So far, modelling of organic carbon conversions in aerobic granular sludge has been limited to easy biodegradable COD like acetate and propionate in standard SBR modes (Kagawa et al. 2015, Isanta et al. 2013). Models on the formation of biofilms and the microbial community structure and distribution are as of yet neglecting the variation in influent composition, hydraulic behaviour and reactor operation as is performed in practice. Data generated by these models are thus of limited value for practical applications of the aerobic granular sludge process. To be able to model the aerobic granular sludge process successfully in practise, the data collected should be used for validation. One important question regarding modelling of aerobic granular sludge reactors is on which level the process is to be modelled. Describing mixing, settling and diffusion requires two times 3D, the question is how to simplify without losing too much accuracy? Adapting existing activated sludge models to describe the aerobic granular sludge process might be a quick way to determine what is truly needed to describe the process. Such a top down method

where one is starting simple, getting more complex as it is needed prevents overly complex models. This does not exempt the need for more detailed models describing certain aspects of granular sludge in practice that are still unknown. Knowledge obtained from such detailed models could then provide input for the more simplistic models describing the overall process. Like this, the 'problem' of modelling aerobic granular sludge is targeted from top and bottom. It is good to realize that models can be based on data or knowledge. Empirical models using data (e.g. from practice) can be used to enhance performance relatively quickly, while the more fundamental models are based on knowledge, can provide valuable insights and lead to better understanding of the AGS process in different conditions.

Potential subjects of interest are;

- Microbial population model with a focus on biological phosphorus removal and glycogen accumulating organisms.
- Where and when does denitrification take place and by whom and what is the best cycle configuration
- Solid retention time differences between small and larger granules and how the SRT influences the species and conversions.
- Determining the optimal granule size. Taking into account hydraulic behaviour and particulate conversion process.

Microbiology and molecular analysis

Research on the microbial community structure of aerobic granular sludge from practice treating domestic wastewater is still absent in literature. Recent studies targeting the bio-P removal in activated sludge systems showed that the actinobacterial Tetrasphaera might play a larger role than previously thought (Hanada et al. 2002, Kong et al. 2005, Nguyen et al. 2011). An experiment with Tetrasphaera *elongata* has shown its ability to take up glycine, a small amino acid and glucose (Nielsen et al. 2012, Nguyen et al. 2015). Most laboratory studies use volatile fatty acids (e.g. acetate, propionate) in their studies, which generally lead to the proliferation of Candidatus Accumulibacter Phosphatis species in an alternating anaerobic, aerobic cycle (Weissbrodt et al. 2013a, Lopez-Vazquez et al. 2009a). The presence of Tetrasphaera species should thus be investigated as to clarify their role in aerobic granular sludge plants in practice. This might lead to new insights and potentially better selection criteria for bio-P removal in aerobic granular sludge systems.

Batch systems have, as discussed before, a variety of cycle configurations that can be applied. Together with site-specific conditions, this most likely leads to differences in microbial community structure in the biomass. Eventually this will also influence the required conversions of COD, N and P. Due to minor differences in competitive advantages between different microorganisms models could have problems predicting the

actual population distribution and this gives an erroneous prediction of the actual conversions (Lopez-Vazquez et al. 2009a). It might be more accurate to obtain the influence of cycle configuration directly by determining the microbial population. As the aerobic granular sludge technology is spreading around the world, a more varied set of environmental conditions come into play (e.g. temperature, salinity and industrial influences). The impact of these influences on the microbial population should also be investigated.

Resource recovery potential of aerobic granular sludge

Most of the sludge produced in wastewater treatment is still considered waste. A larger part of the operational cost of a treatment plant is due to sludge handling (Kroiss 2004). Recovery of valuable biomaterials from sludge reduces waste generated and relieves consumption of virgin sources. As aerobic granular sludge is an emerging technology the full biomaterials recovery potential is not well known. A few aspects of resource recovery are:

Methane
One of the most widely recovered resources out of wastewater is energy in the form of methane. Methane is obtained by anaerobic digestion of (waste) sludge (Soto et al. 1993). The methane is subsequently used to generate electrical energy and heat. Little is known on the anaerobic biodegradability of aerobic granular sludge grown on domestic wastewater. Recent laboratory studies have shown that aerobic granular sludge grown on pig slurry and synthetic wastewater has similar digestibility compared to activated sludge (Val Del Río et al. 2014, Val del Río et al. 2011). It makes sense that the granular structure would influence the digestibility. The compact structure of the granules would make it harder for the enzymes to work properly and so the reaction time required would increase. The high SRT obtained in the aerobic granular sludge process might be of influence on the digestibility. Higher SRT's often lead to lower sludge production and higher inorganic fractions in the sludge that will decrease methane production potential. Studies into the digestibility of aerobic granular sludge and the exopolysaccharides present have not been published at all. A comparison study of the digestibility of aerobic granular sludge and activated sludge should be conducted.

Phosphorus

Another popular resource for recovery is phosphorus. Phosphorus can be considered as one of the most valuable compounds in waste sludge today, not only from a sustainability point of view, but also in light of economic value (Kroiss 2004). Phosphorus is a finite resource, which is expected to be depleted in the next 30 to 50 years (Cordell et al. 2009, Neset and Cordell 2012). Various technologies exist today that harvest phosphorus back from wastewater. Phosphorus is primarily recovered after anaerobic digestion by struvite formation (Doyle and Parsons 2002). Aerobic granular sludge with its batch wise operation could potentially offer alternatives. Fixing phosphorus before it enters a digester might prevent maintenance and expensive replacements as normally struvite precipitates in the pipelines. Ways to fix phosphorus with use of aerobic granular sludge should thus be investigated. Another possibility is to use the granular sludge directly as a nutrient resource in agricultural lands. The presence of other compounds besides phosphorus like calcium, magnesium and potassium make it a good soil fertilizer. Strict laws in many countries however, prevent the direct use waste sludge on agricultural lands due to hygienic concerns.

Biopolymers

The formation of aerobic granular sludge is made possible by bacteria that produce exopolysaccharides. These polymers or bacterial glue as it is sometimes called, form a matrix in which bacteria are immobilized. The characteristics are in some ways very similar to commercial alginate, but differ in other aspects (e.g. they were dissimilar with commercial alginate in the reactions with acid ferric sulfate, phenol-sulfuric acid and Coomassie brilliant blue G_{250} (Lin et al. 2010a). It is therefore commonly referred to as alginate like exopolysaccharides (ALE). Recent studies have shown that more than 10 % (w/w) of ALE can be extracted from aerobic granular sludge. Some applications of ALE were patented (Van Loosdrecht et al. 2015) underlining the potential of aerobic granular sludge as a sustainable biopolymer resource. At present, the exact composition and characteristics of ALE is still unknown. The impact of microorganism species, environmental conditions and reactor operational conditions on ALE production and property is waiting for exploration. In addition, the combination of ALE recovery and the anaerobic digestion is another interesting topic. The ALE characteristics produced by different bacterial species might vary significantly. Some bacteria may produce more or longer polymers than others. Substrate and environmental conditions might also affect the polymer composition. Eventually this could lead to aerobic granular sludge reactors treating specific waste streams to generate ALE with known properties and yields for use in agricultural or civil applications.

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Mario Pronk was born on the 6th of May 1977 in Den Haag the Netherlands. In September 1996 he started Life Sciences at the Hogere Laboratorium Onderwijs (HLO) in Delft (Biological and Medical laboratory research), but switched to Environmental Technology, with a specialisation in wastewater treatment at the HAS in Delft. He obtained his bachelor in 2002. After continuing a few months with his one-man company in interior care (1999 - 2003), he started to work (2002) as a research technician in the Environmental Biotechnology group in Delft until 2007. He then changed to the Fermentation Technical Service group in the same department where he stayed until 2010. At the end of 2010 he started his PhD in aerobic granular sludge under the supervision of Prof.dr.ir. M.C.M. (Mark) van Loosdrecht and Dr. ir. R. (Robbert) Kleerebezem.

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