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Modelling and simulation of nitrogen conversion pathways
in aerobic granular sludge

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Le Hong Quan

Gent, 02 June 2014
**List of abbreviations**

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AGS</td>
<td>Aerobic granular sludge</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonia-oxidizing bacteria</td>
</tr>
<tr>
<td>ASM</td>
<td>Activated sludge model</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological oxygen demand</td>
</tr>
<tr>
<td>CANON</td>
<td>Completely Autotrophic Nitrogen Removal Over Nitrite</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>DPAOs</td>
<td>Denitrifying polyphosphate-accumulating organisms</td>
</tr>
<tr>
<td>DGAOs</td>
<td>Denitrifying glycogen-accumulating organisms</td>
</tr>
<tr>
<td>EBPR</td>
<td>Enhanced biological phosphate removal</td>
</tr>
<tr>
<td>GAOs</td>
<td>Glycogen-accumulating organisms</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse gas</td>
</tr>
<tr>
<td>IPCC</td>
<td>Intergovernmental Panel on Climate Change</td>
</tr>
<tr>
<td>NADH2</td>
<td>Nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NOB</td>
<td>Nitrite-oxidizing bacteria</td>
</tr>
<tr>
<td>PAOs</td>
<td>Polyphosphate-accumulating organisms</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly-beta-hydroxyl Butyrate</td>
</tr>
<tr>
<td>poly-P</td>
<td>Polyphosphate</td>
</tr>
<tr>
<td>SBR</td>
<td>Sequencing batch reactor</td>
</tr>
<tr>
<td>SRT</td>
<td>Sludge retention time</td>
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<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
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Summary

Aerobic granular sludge is an innovative technique for simultaneous COD and nutrient removal from wastewater by different groups of bacteria located in different zones inside the granules, which they form. Ammonium is nitrified to nitrite by ammonia-oxidizing bacteria (AOB) and followed by oxidation of nitrite to nitrate by nitrite-oxidizing bacteria. COD, phosphate and nitrate are removed by denitrification activities of phosphate accumulation bacteria. This study deals with the disproportion of nitrite-oxidizing bacteria (NOB) and AOB in aerobic granular sludge reactors as pointed out by Winkler et al. (2012a) An elevated ratio NOB:AOB was observed in an aerobic granular sludge pilot plant treating domestic wastewater and in an acetate-fed aerobic granular sludge lab-scale reactor. This was not the case in the conventional wastewater treatment plant receiving the same wastewater as the pilot plant. Additionally, concerning the distribution of NOB, *Nitrobacter* outcompeted *Nitrospira* and grew till up to 300 μm depth from the granule surface, which was far beyond the penetration depth of oxygen. These findings of Winkler et al. (2012a) led to the conclusion that the growth of the NOB was partly uncoupled from nitrite supplied by AOB, which was explained by the author through two possible hypotheses. The ping-pong hypothesis assumes that *Nitrobacter* uses its capability of dissimilatory nitrate reduction, also denoted as mixotrophic growth of *Nitrobacter*, which enables it to grow in anoxic zones of the granule. The nitrite loop hypothesis suggests that NOB can uncouple their growth from AOB by benefiting from nitrite available from incomplete denitrification by denitrifying polyphosphate-accumulating organisms (DPAOs).

Four different mathematical models were setup in this study to investigate the two hypotheses. In each model, there were four groups of bacteria responsible for the biological nitrogen conversions: the AOB for nitritation, two distinct groups of *Nitrospira* and *Nitrobacter* for nitratation and DPAOs for denitrification. First, a basic model, describing conventional nitrification and denitrification, was setup. Later, to investigate the ping-pong hypothesis, mixotrophic growth of *Nitrobacter* was added to the basic model, resulting in a second model. In the third model (nitrite model), traditional single-step denitrification was replaced by two-step denitrification to describe the nitrite loop hypothesis. Finally, the fourth model combines the ping-pong and nitrite models to study the influence of simultaneous mixotrophic growth of *Nitrobacter* and two-step denitrification.

Simulation results revealed that both hypotheses had an influence on the distribution of nitrifiers in the granule. The ping-pong hypothesis in which mixotrophic growth of *Nitrobacter* was defined, led to the dominance of *Nitrobacter* in the system. The nitrite loop hypothesis facilitated *Nitrobacter*'s growth with extra nitrite from the two-step denitrification processes. The results from the integrated model agreed best with the experimental data showing a high ratio NOB:AOB (of 1.8-3.5) as well as the dominance of *Nitrobacter*. There was no significant effect of the two hypotheses on reactor behaviour in terms of conversion efficiency of ammonium and phosphate, while a 10% increase in the oxygen consumption was observed in case of model with two-
step denitrification. The most significant difference however was the nitrogen removal efficiency. A reduction of nitrogen removal efficiency by 20-25% was obtained by the influence of nitrite loop hypothesis. The reason for this is that not all additional nitrate produced from the loop could be denitrified, as the denitrification of DPAOs was limited by PHB storage pool (a carbon source for all DPAOs' activities). It is known from literature that during the mixotrophic growth of *Nitrobacter*, NO or N₂O are released as the products of dissimilatory nitrate reduction by *Nitrobacter*. These pathways were included in the model and the results suggested that the emissions were up to 4% of incoming nitrogen load under normal operation conditions. The amount of NO or N₂O produced strongly correlated with the ratio of NOB to AOB regardless of different operational conditions. The higher the ratio of NOB to AOB was, the higher NO or N₂O released.

The influence of operating conditions on the distribution of microorganisms, NO (N₂O) emission and reactor behaviour was investigated through additional simulations. An increase in temperature, granule size and a decrease in oxygen concentration led to an increase in the ratio NOB:AOB, an increased NO (N₂O) emission, as well as an increased nitrogen removal efficiency but caused a decrease in phosphate and ammonium removal efficiencies in most cases. These outcomes could be explained by a lower aerobic volume fraction with increasing granule diameter or with a decreased dissolved oxygen concentration whilst at higher temperature, the biological activities largely determined the biomass concentration, which then affected ratio NOB:AOB.
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1 INTRODUCTION

1.1 Aerobic granular sludge for nutrient and COD removal

Wastewater treatment plants (WWTPs) have been developed for carbon, nitrogen, and phosphate removal to prevent eutrophication of water bodies. For this purpose, microorganisms are typically grown in a loose conglomereration of flocs to remove nutrients from the wastewater. In order to offer the bacteria idle conditions and to remove nutrients the activated sludge floc needs to be subjected to different conditions and needs to be recycled over different tanks. After the nutrient removal, the water needs to be separated from the sludge, which typically occurs in clarifiers. Due to their fluffy structure, sludge flocs settle slowly and consequently, a lot of space is required to construct a conventional WWTP. Conversely, space becomes a limiting factor particularly in urban areas. To reduce the footprint of WWTPs, compact treatment systems are required. During the last decades, different compact treatment systems were developed as for instance membrane bioreactors and to date, the application of aerobic granular sludge (AGS), which is regarded as one of the promising biotechnologies in wastewater treatment (de Kreuk et al., 2007a).

Granular sludge has been defined as aggregates of microorganisms, which settle significantly faster than activated sludge flocs (de Kreuk et al., 2007a). Aerobic granules are known to exhibit attributes of the settling velocity associated with granule size and structure from 10 m.h\(^{-1}\) to 80 m.h\(^{-1}\) and can have a sludge volume index 10 (SVI\(_{10}\)) of lower than 50 mL.g\(^{-1}\) (Winkler et al., 2013c). Their densities are much higher than that of conventional activated sludge. Reported values of granular densities vary between 1.005 and 1.080 kg.m\(^{-3}\) (Etterer & Wilderer, 2001; Winkler et al., 2013c). Granules usually have a regular, smooth and nearly round shape with a minimum size was set to 0.2 mm (de Kreuk et al., 2007a) and a hydrophobicity nearly two-fold higher than that of the conventional sludge floc (Liu & Tay, 2004). The high biomass retention without the requirement of large reactors facilitates the selection for slow growing organisms (de Kreuk & van Loosdrecht, 2004; Winkler et al., 2012b). Finally, AGS has the ability to withstand high volumetric and organic loading rates and to tolerate toxicity (Liu & Tay, 2004).

A lot of research has been conducted on aerobic granular sludge and eventually aerobic granules were successfully applied for assuring stable nitrogen, phosphate and carbon removal in one compact reactor. Beun et al. (2001), Yang et al. (2003), and de Kreuk et al. (2005a) successfully applied AGS for simultaneous removal of organics and nitrogen with high efficiency at low DO concentrations (dissolved oxygen). Schwarzenbeck et al. (2005) used aerobic granules for the treatment of dairy waste-water. AGS also has been applied for removal of the heavy metals (Liu et al., 2003), particulate matter (Schwarzenbeck et al., 2004) and for the removal of nuclear waste (Nancharaiah et al., 2006). A pilot plant of 1.5 m\(^3\) with the Nereda system (by DHV Water) treating sewage at a Dutch wastewater treatment plant was successfully operated (de Kreuk et
al., 2007a) and was then followed by a full-scale plant in Epe, the Netherlands treating wastewater for 59,000 population equivalents (van der Roest et al., 2011).

1.2 Thesis outline

In this thesis, mathematic modelling and numerical simulations were used to understand and explain the underlying reasons for the disproportion in the ratio of NOB to AOB in acetate-fed aerobic granular sludge reactors.

Chapter 2 reviews the principle of the aerobic granular sludge technology for the removal of COD and nutrients. This technology is based on a sequencing batch reactor operated with feeding, mixing, and effluent withdrawal phases. The layered biomass structure leads to biological conversions occurring simultaneously in different zones inside the granules. The application of modelling to study aerobic granular sludge systems is discussed. Attention is paid to the possible growth of the nitrite-oxidizing bacteria (Nitrobacter) on organic carbon source and to the two hypotheses explaining the disproportion in the ratio of NOB to AOB. This chapter is concluded by the thesis objectives.

Chapter 3 describes the constructed mathematic models. A well-documented model of AGS for nutrient removal was taken as the starting point and subsequently extended to test the hypotheses. For this purpose, two species of NOB (Nitrospira and Nitrobacter), mixotrophic growth of Nitrobacter and two-step denitrification were introduced, resulting in four model setups (base model, ping-pong model, nitrite loop model and integrated model). In addition, the simulation strategy is presented

Model parameter adjustments are described in Chapter 4. The reactor behaviour and associated biomass distribution of in granular sludge reactor are assessed. The simulation results from different model setups corresponding with the hypotheses are analysed in terms of biomass distribution, especially concerning the ratio of NOB to AOB. Finally, the influence of operating conditions such as granules size, temperature and dissolve oxygen concentration were studied in detail.

Chapter 5 closes this thesis with conclusions and suggestions for future research.
2 LITERATURE REVIEW

2.1 Aerobic granule sludge

Aerobic granular sludge-based wastewater treatment is a promising alternative to activated sludge systems (de Kreuk et al., 2007a; Liu & Tay, 2004). Compared with conventional activated sludge, aerobic granules have a regular and compact physical structure, diverse microbial species, good settle ability, high biomass retention, and ability to withstand shock load or shock of toxic compounds. Aerobic granulation has most often reported in sequencing batch reactors. Many researchers (Beun et al., 2000b; de Kreuk et al., 2005b; Liu & Tay, 2004; Morgenroth et al., 1997; Strous et al., 1998) have extensively investigated the fundamentals of aerobic granular sludge in SBR.

2.2 Reactor characteristic

2.2.1 Cyclic operation

Aerobic granular sludge can be operated in two type of systems. In case of the Nereda system it is operated in a sequencing batch mode but can be also be used in a continuous operation such as it is the case of autotrophic nitrogen removal with constant feeding, withdraw and aeration (Third et al., 2001). The SBR process is characterized by a series of process phases (Figure 2.1) each lasting for a defined period. The cyclic operation of alternate anaerobic and aerobic phases and the application of a short settling time enable the development of stable compact granules and are the two most important operation factors for a successful application.

Figure 2.1 Cycle profile of the aerobic granular sludge sequencing batch reactor consisting of: 1) feeding period either A) mixed feeding or B) feeding from bottom of the reactor in a plug flow regime, 2) aeration period, 3) settling period, and 4) an effluent withdrawal phase.
2.2.2 Feast/famine regime

In the discontinuously fed systems, microorganisms experience a phase of an externally supplied organic electron donor (feast period) and a phase in which autotrophic conditions with ammonium and oxygen being supplied however without any organic carbon source (famine period) (Beun et al., 2001). Van Loosdrecht et al. (1997) stated that microorganisms in general respond to feast-famine regimes by accumulating storage polymers when substrate is present. By applying pulse fed to SBR system, storage processes hence play a dominant role.

A high feast–famine ratio was favourable for the formation of compact and dense aerobic granules. de Kreuk and van Loosdrecht (2004) reported that in acetate-fed SBR, alternating anaerobic feeding (presence of acetate) and aerobic reaction periods with the availability of phosphate in the influent facilitated the selection of slow growing organisms such as polyphosphates-accumulating organisms (PAOs) and additionally improving granular stability. Under these conditions, all acetate can be converted to internal storage polymers (PHB) by PAOs during the anaerobic feeding period. During the aerobic period, growth takes places on the internally stored PHB while phosphate in the liquid is converted into cell as poly-phosphates (poly-P). By the ability of PAOs to anaerobically uptake, all acetate fast growing heterotrophic bacteria are outcompeted. The detailed metabolism of PAOs is discussed in section 2.4.

Substrate concentration also plays a role additional to feast famine regime. A good penetration of acetate through the total granule is important, which can be achieved by a high substrate concentration in a plug-flow feeding regime with up flow of the substrate from the bottom of the reactor through the settled bed. The granules in the lower part of the settled bed faced a high acetate concentration in this way (de Kreuk & van Loosdrecht, 2004).

2.2.3 Short settling period

Formation of aerobic granular sludge in laboratory experiments only occurs with a strict and short settling period (less than 5 minutes). The aggregate forming organisms will be maintained in the reactor whilst tiny flocs and hence a certain bacterial community harbouring these flocs are washed out and have no chance to evolve to the environmental changes (de Kreuk et al., 2007a). This screening step lead to the successful cultivation of aerobic granules operated in a SBR (Beun et al., 2000b; de Kreuk et al., 2005b; Liu et al., 2005b).

Fast settling is also the determining factor for the formation of aerobic granules in SBR (Liu et al., 2005b). Conversely, aerobic granulation would fail if the SBR would be operated at longer settling times because the system would be dominated by flocs, which have poorly settling velocity and which hence would be washout out (Liu et al., 2005b). Additionally, Liu et al. (2005b) and Wang et al. (2006) clearly showed the close relation
of exchange ratio and minimum settling velocity. Minimum settling velocity is ratio of the height from the discharging port to the water surface and settling time. One could manipulate exchange ratio and subsequently minimum settling velocity to achieve interested selective pressure.

2.3 Microbial composition of aerobic granular sludge

Functional groups of bacteria in AGS are quite similar to that of conventional sludge (Winkler et al., 2013a). However, because of the sphere shape, substrate gradient, especially oxygen penetration, plays an important role for defining microbial structure of granular sludge (Xavier et al., 2007). Fluorescence in situ hybridization (FISH) and qPCR technique allowed to have insights of the microbial structure, where heterotrophic, nitrifying, denitrifying- phosphorous-accumulating bacteria, and denitrifying glycogen-accumulating bacteria can be identified (Winkler et al., 2013b). Compared to flocs, which have a loose structure, aerobic granules have a layered structure in which different groups of microorganism grow in different redox zones (Figure 2.2).

![Figure 2.2 Structural difference between a conventional sludge floc (left) and aerobic granular sludge (right) (Winkler et al., 2013a)](image)

2.3.1 Autotrophic bacteria

Ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) were found mainly at the depth of 70-100 μm from the granular surface where oxygen was available (de Kreuk et al., 2007b; Xavier et al., 2007). Normally, for conventional sludge system, *Nitrospira* (Nsp) was considered as the most common and important NOB (Daims et al., 2001). However, in AGS, *Nitrobacter* was reported dominate the system (Nogueira & Melo, 2006; Winkler et al., 2012a). Recently, Winkler et al. (2012a) reported that *Nitrobacter* could be found at the depth of 300 μm from the granular surface which then rising several hypothesis explaining this phenomena (further explanation section 2.7).
2.3.2 Heterotrophic bacteria

The introduction of an anaerobic feeding phase followed by a long aerobic phase provided conditions for the proliferation of PAOs (if enough phosphate was provided) or glycogen-accumulating organism (GAOs). According to the similar growth rate of autotrophic organisms (NOB and AOB) and heterotrophic PAOs/GAOs (Brdjanovic et al., 1998) and due to the completion for their electron acceptor oxygen their existence can be expected in the same outer layer. In addition, PAOs and GAOs also can grow in the inner anoxic layer where they can use nitrite and nitrate as electron acceptor instead of oxygen (de Kreuk & van Loosdrecht, 2004). The so-called denitrifying PAOs (DPAOs) and denitrifying GAOs (DGAOs) hence contribute the nitrogen, phosphate (in case of the PAOs) as well as the carbon removal (Kuba et al., 1993).

2.3.3 Inert material

The inner core of granule contains large amounts of precipitate and dead microbial cells (Winkler et al., 2013c). de Kreuk et al. (2005a) reported that calcium-phosphate precipitation was formed in, which lead to an increased ash content of the granules from 6% when pulse feeding was conducted aerobically to 30%-41% with an enriched PAOs culture obtained with an proper feast famine regime. Xavier et al. (2007) studied the microbial structure of AGS which was subjected to anaerobic and aerobic condition. The result biomass distribution profile revealed that mainly the outer layers of the granule will be eroded, which contain less inert material. Therefore, aerobic granular sludge is expected to contain more inert material resulting from biomass decay than activated sludge. A proper sludge wastage is therefore significant not to get infinite sludge retention time (SRT) for large and dense granules.

2.4 Biological conversion processes in an aerobic granular sludge system

2.4.1 Mechanism of COD and nutrient removal

The mechanisms of COD and nutrient removal in AGS are quite similar to the one in activated sludge system. The main difference is that they does not occur in different compartments, but simultaneously in different zones inside the granules (Figure 2.3). Distribution of heterotrophic and autotrophic organisms in granular sludge plays an important role in these processes. The autotrophs are responsible for nitrification, whereas the heterotrophs are responsible for organic carbon oxidation, denitrification, and phosphate removal. During the feast phase, the concentration of external carbon is high. This substrate will diffuse into the granules completely and will anaerobically (PAOs), aerobically or anoxically (other heterotrophs) be stored (30-70%). During the famine phase, on the aerobic layers of granular, the remaining external carbon and stored substrate is consumed by heterotrophs and ammonium will be converted to nitrate by autotrophic organisms. The nitrate can penetrate to the interior of the granule were the stored substrate can serve as carbon source for
denitrification. Phosphate by this way is also removed by presence of PAOs (Beun et al., 2001; de Kreuk et al., 2005a).

2.4.2 Nitrification

Distinct groups of aerobic chemo-lithoautotrophic bacteria such as AOB and NOB are two main groups that are responsible for nitrification. This process consists of two sequential steps: oxidation of ammonia to nitrite by AOB (nitritation) and subsequent oxidation of nitrite to nitrate by NOB (nitratation). Both bacteria obtain their carbon requirement (anabolism) from dissolved CO₂ and their energy requirement (catabolism) for biomass synthesis from oxidizing ammonia to nitrite and nitrite to nitrate, respectively (Ekama, 2008).

2.4.2.1 Nitritation

The overall reaction of ammonia to nitrite is also a two-stage process, but is catalysed by the same organism. Ammonia rather than ammonium is used as the substrate (Suzuki et al., 1974) with hydroxylamine (NH₂OH) as an intermediate. The enzymes involved are ammonia monooxygenase for ammonia oxidation and hydroxylamine oxidoreductase to produce nitrite. The electrons released in the hydroxylamine oxidation are required for ammonia oxidation, the respiratory chain and for the energy expensive CO₂ assimilation (Wood, 1986).

2.4.2.2 Nitratation

Nitrite is further oxidized to nitrate by the enzyme nitrite oxidoreductase and the oxygen atom in the nitrate molecule is derived from water. The two electrons released are used in the electron transport chain and partly also for CO₂ assimilation. Table 2.1 gives the overall formula for the oxidation of nitrite to nitrate (nitratation).
Table 2.1 Overall reactions for the oxidation of ammonia to nitrite (nitritation) and of nitrite to nitrate (nitratation) (Van Hulle et al., 2010)

<table>
<thead>
<tr>
<th>Reaction (AOB)</th>
<th>Equations</th>
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<tr>
<td>Nitritation</td>
<td>(\text{NH}_4^+ + 1.382 \text{O}_2 + 0.091 \text{HCO}_3^- \rightarrow 0.982 \text{NO}_2^- + 1.891 \text{H}^+ + 0.091 \text{CH}_1.4\text{O}_0.4\text{N}_0.2 + 1.036 \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>Nitratation (NOB)</td>
<td>(\text{NO}_2^- + 0.488 \text{O}_2 + 0.003 \text{NH}_4^+ + 0.013 \text{HCO}_3^- \rightarrow \text{NO}_3^- + 0.013 \text{CH}_1.4\text{O}_0.4\text{N}_0.2 + 0.008 \text{H}_2\text{O} )</td>
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The rate of conversion of ammonia to nitrite, by the AOB is generally much slower than that of nitrite to nitrate by the NOB. Therefore, conversion of ammonium to nitrite is the rate-limiting step (Ekama, 2008).

2.4.3 Denitrification and phosphate removal

The denitrifying capability of some PAOs (Kuba et al., 1993) together with the available of anoxic zone in granular sludge, enabled the combined denitrification and phosphate removal. As long as sufficient phosphate is present, PAOs will dominate if the proper conditions (temperature, pH and sort of carbon source) (Mino et al., 1998). If there is a lack of phosphate, the granules will be dominated by GAOs (de Kreuk & van Loosdrecht, 2004; Zeng et al., 2003). However, GAOs are also capable of anaerobic VFA uptake and therefore can also be enriched under essentially the same conditions as PAOs, consuming the generally limited VFA supply, without contributing to phosphate removal (Mino et al., 1998). Both organisms compete from the same substrate (organic carbon) and if GAOs win this competition phosphate removal will deteriorate.

Similar to enhanced biological phosphate removal system (EBPR), in which active sludge is recycled through anaerobic and aerobic compartment, denitrification and phosphate removal in AGS can be demonstrated in the same manner. VFA from the influent are taken up by PAOs/GAOs and stored as PHA. When influent volatile fatty acid (VFA) is mainly acetate, the main storage product is PHB. Anaerobic uptake and storage of acetate requires energy. This is generated by degradation of cell internal glycogen (PAOs/GAOs) and polyphosphate (PAOs only). As a result, under anaerobic conditions they release a large amount of phosphate into the bulk liquid. In a subsequent aerobic (or anoxic) phase, PHA is oxidized. The generated energy is mainly used to restore glycogen and poly-P and the rest is used for growth and maintenance of the cell structure (Mino et al., 1998). Table 2.2 describes those reactions in detail.
Table 2.2 Overall anaerobic and aerobic reactions of PAOs with oxygen as electron acceptor (Murnleitner et al., 1997; Smolders et al., 1994)

<table>
<thead>
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<tbody>
<tr>
<td>Anaerobic acetate up-take</td>
<td>$\text{CH}_2\text{O} + 0.5\text{CH}_3\text{O}<em>6\text{O}</em>{5/6} + 0.44\text{HPO}_3 + 0.023\text{H}_2\text{O} \rightarrow 1.33\text{CH}<em>1.5\text{O}</em>{0.5} + 0.17\text{CO}_2 + 0.44\text{H}_3\text{PO}_4$</td>
</tr>
<tr>
<td>PHB degradation</td>
<td>$\text{CH}<em>1.5\text{O}</em>{0.5} + 0.14\text{NH}_3 + 0.011\text{H}_3\text{PO}_4 + 0.32\text{O}<em>2 + 0.23\text{H}<em>2\text{O} \rightarrow 0.72 \text{ CH}<em>2.09\text{O}</em>{0.54}\text{N}</em>{0.2}\text{P}</em>{0.015} + 0.28\text{CO}_2$</td>
</tr>
<tr>
<td>Poly P formation</td>
<td>$0.19\text{CH}<em>2.09\text{O}</em>{0.54}\text{N}<em>{0.2}\text{P}</em>{0.015} + 0.218\text{O}_2 + 0.997\text{H}_3\text{PO}_4 \rightarrow \text{HPO}_3 + 0.08\text{NH}_3 + 0.19\text{CO}_2 + 1.14\text{H}_2\text{O}$</td>
</tr>
<tr>
<td>Glycogen formation</td>
<td>$0.78\text{CH}<em>2.09\text{O}</em>{0.54}\text{N}<em>{0.2}\text{P}</em>{0.015} + 0.27\text{H}_2\text{O} + 0.22\text{CO}_2 \rightarrow 0.78\text{CH}<em>1.67\text{O}</em>{0.83} + 0.1\text{O}_2 + 0.012\text{H}_3\text{PO}_4 + 0.156\text{NH}_3$</td>
</tr>
</tbody>
</table>

The denitrifying and aerobic metabolisms of PAOs/GAOs are identical except that nitrate or oxygen is the final acceptor in the electron transport phosphorylation process. The energy PAOs/GAOs obtain from aerobic degradation of PHB was reported to be as twice as the one from conventional denitrification (Smolders et al., 1994). The available of genes mediating denitrification are NapAB, NirS, NorBC and NosZ and enable PAOs/GAOs to complete denitrification from nitrate to nitrogen gas. Different groups of PAOs and GAOs have shown varying denitrification capacities depend on their availability of these genes (Oehmen et al., 2010; Zeng et al., 2003) (Table 2.3). It is clear from the studies that the denitrification activity of PAOs and GAOs depends on the abundance of the different subgroups.

Table 2.3 Denitrification capacities of different groups of PAOs and GAOs (Oehmen et al., 2010)

<table>
<thead>
<tr>
<th>Type of bacteria</th>
<th>Denitrification capacities</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPAOs (Type I Accumulibacter)</td>
<td>$\text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$</td>
</tr>
<tr>
<td>PAOs (Type II Accumulibacter)</td>
<td>$\text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$</td>
</tr>
<tr>
<td>DGAOs (Type I Competibacter)</td>
<td>$\text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$</td>
</tr>
<tr>
<td>GAOs (Type II Competibacter)</td>
<td>$\text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$</td>
</tr>
<tr>
<td>GAOs (Defluviicoccus Cluster I)</td>
<td>$\text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$</td>
</tr>
<tr>
<td>GAOs (Defluviicoccus Cluster II)</td>
<td>$\text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$</td>
</tr>
</tbody>
</table>

$\rightarrow$ capable to convert; $\leftrightarrow$ incapable to convert,

Heterotrophic organisms have decreased growth rates when they grow on the slowly biodegradable storage polymer PHB or glycogen compared to the growth on easily biodegradable substrate such as acetate or glucose (Carta et al., 2001).
2.5 Mathematical modelling of AGS

2.5.1 Modelling nutrient removal in AGS

Mathematical modelling has proven to be very useful to study complex processes, such as aerobic granular sludge systems (de Kreuk et al., 2007b). Effect of separate factors cannot be studied experimentally because of the complex interaction of many factors. Aerobic granular sludge systems can be modelled in different ways, using different modelling tools depending on the fields of interest. When the overall reactor behaviours is described (substrate removal or sludge production), traditional biofilm modelling can be used as for instance AQUASIM (Reichert, 1998).

2.5.1.1 Nitrogen removal in a granular sludge sequencing batch airlift reactor (Beun et al., 2001)

Beun et al. (2001) have developed a simulation model to evaluate the effects of several operating factors on nitrogen removal in a granule-based SBR. Based on balance equations connecting conversion processes and transport processes the model could predict the N-conversion processes in the reactor under different conditions by using stoichiometry and kinetics reported by Beun et al. (2000a). Biological conversion processes (acetate uptake, biomass growth, PHB production, and maintenance) in feast and famine phase have been described by using following linear equations. In this model, decay of biomass resulted in formation of inert particulate compounds. The SBR performance was described using a model implemented in AQUASIM. It has been shown that nitrification, denitrification, and removal of COD can occur simultaneously.

2.5.1.2 Kinetic model of simultaneously COD, nitrogen and phosphate removal (de Kreuk et al., 2007b)

This model has been proposed for an aerobic granular sludge reactor that simultaneously removed COD, nitrogen and phosphate operated at SBR mode. The biological conversion processes are described using stoichiometric and kinetic parameters from the model published by Hao et al. (2002a), Hao et al. (2002b) and Meijer et al. (2001). The model described the experimental data well. The effect of process parameters such as oxygen concentration, temperature, granule diameter, sludge load rate, SBR cycle configuration on the nutrient removal rates of aerobic granules could be reliably evaluated using this model. Oxygen penetration depth in combination with the position of the autotrophic biomass played a crucial role in the conversion rates of the different components and thus on overall nutrient removal efficiency. The ratio between the aerobic and anoxic volumes in the granule strongly determined the nitrogen removal efficiency, model simulations with varying oxygen concentration, temperature, and granule size showed it. The optimum granule diameter for maximum N- and P-removal at DO of 2 g.m⁻³ and 20°C was found between 1.2 and 1.4 mm, and the optimum COD loading rate is 1.9 kg COD.m⁻³.day⁻¹. When all ammonia is oxidized oxygen can diffuse into the core of the granule by which inhibiting the denitrification capacity significantly. In order to optimize the process, anoxic
phases could be implemented in the SBR-cycle configuration, leading to a more efficient overall nitrogen removal. Phosphate removal efficiency depends (beside from a strict anaerobic aerobic/anoxic phase) on the sludge age and hence on a proper sludge wasting. At an SRT longer than 30 days, no sufficient sludge renewal is occurring leading in a gradual increase of effluent phosphate concentrations (de Kreuk et al., 2007b).

2.5.1.3 Multi-scale modelling of AGS (Xavier et al., 2007)

Xavier et al. (2007) proposed a multi-scale model of aerobic granular sludge SBR reactor by considering a two-dimensional spatial arrangement of four bacterial groups: heterotrophic bacteria (HE), AOB, NOB and PAOs. The simulations provided insight into the bioconversion processes with short-term dynamics and long reactor operation and integrated dynamics of microbial metabolisms, and the diffusion reaction with 2-D spatial organization. Extended ASM1 kinetic model was used to include PAOs metabolism with 3 internal storage (PHB, glycogen and poly-P) and separate description of AOB and NOB. Five simulation cases with different aeration schemes have been analysed for long-term and short-term operation. The results showed that the microbial population and activity depends on the operating conditions. The simulations on short-term dynamics of solute bulk concentrations are comparable with experimental results from a lab scale reactor. Multi-scale models suggested that nitrogen removal in AGS occurs mostly via alternating nitrification/denitrification rather than simultaneous nitrification/denitrification, supporting an alternative strategy to improve nitrogen removal in this promising wastewater treatment process (Xavier et al., 2007).

2.5.2 Modelling other features of AGS

Table 2.4 provides an overview of other modelling approaches for aerobic granular sludge.

<table>
<thead>
<tr>
<th>Model type</th>
<th>Description</th>
<th>Developer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic granulation process</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empirical models for aerobic granulation</td>
<td>Linear phenomenological equation describing growth</td>
<td>Yang et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>of mean diameter of aerobic granules.</td>
<td></td>
</tr>
<tr>
<td>Selection pressure for aerobic granulation</td>
<td>Modified logistic model describing growth of mean</td>
<td>Su and Yu (2005)</td>
</tr>
<tr>
<td></td>
<td>diameter of aerobic granules.</td>
<td></td>
</tr>
<tr>
<td>Selection pressure for aerobic granulation</td>
<td>Combines the three major selection pressures: depth</td>
<td>Liu et al. (2005c)</td>
</tr>
<tr>
<td></td>
<td>of discharged outlet designed settling time and preset discharge time.</td>
<td></td>
</tr>
<tr>
<td>Biomass dynamics in the granulation process</td>
<td>Biomass dynamics in aerobic granulation in an SBR</td>
<td>Li and Li (2009)</td>
</tr>
<tr>
<td></td>
<td>using the sectional approach</td>
<td></td>
</tr>
<tr>
<td>Settling velocity in aerobic granulation</td>
<td>Settling velocity of aerobic granules as a function of SVI,</td>
<td>Liu et al. (2005a)</td>
</tr>
<tr>
<td></td>
<td>mean size of granules and biomass concentration.</td>
<td></td>
</tr>
</tbody>
</table>
Quantitative simulation of aerobic granulation
Incorporating microbial growth, oxygen transfer, substrate diffusion, increased granule size, and biomass detachment.

**Modelling the physicochemical processes in aerobic granule**

**Mass transfer**
One-dimensional conservation laws.  
The two-dimension spatial solute distributions can be calculated from a steady-state diffusion-reaction equation for each of the solutes.  
Classified the aerobic granules into various size fractions.

**Detachment**
A detachment rate was introduced when the growth velocity of granules was positive.

**Porosity of granules**
Linear function to describe the variation of porosity along the inner (anoxic) zone of granules. Porosity was considered constant through the outer (aerobic) shell.

**Species growth and storage modelling the bioconversion processes in aerobic granules**

**Microbial species growth**
Autotrophs and heterotrophs growth. The biological conversion processes are modeled using a modified ASM3 with a consideration of carbon removal, nitrification and denitrification

**Microbial storage**
Simultaneous growth and storage processes in aerobic granules cultivated with soybean-processing wastewater by using a modified ASM3.

**Formation and utilization of microbial products**
Formation of EPS, soluble microbial products and internal storage products to describe the fate of these microbial products in aerobic granules

2.6 **Growth of Nitrobacter in presence of organic carbon source**

2.6.1 **Under anoxic conditions - dissimilatory nitrate reduction of Nitrobacter**

Schramm et al. (1999) postulated that *Nitrospira*-like bacteria are K-strategists, which have a low growth rate but which can exploit low amounts of nitrite and oxygen more efficiently than *Nitrobacter*. The latter is termed r-strategist, which has faster growth rate but required higher substrate concentration. This K/r hypothesis was used for explaining the predominance and wide distribution of *Nitrospira* in conventional activated sludge system where sludge retention times are sufficiently low to sustain them in the reactor and where concentration of nitrite was low (Kim & Kim, 2006; Nogueira & Melo, 2006). In contrast, in granular sludge, *Nitrobacter* was the main NOB present (Winkler et al., 2012a). Their predominance could be explained by their dissimilatory
nitrate reduction capability, which is the capacity to use organic compounds as carbon and energy source (Spieck & Bock, 2005; Starkenburg et al., 2006; Starkenburg et al., 2008).

Freitag et al. (1987) provided evidence that *Nitrobacter* was a facultative anaerobic organism, which grows aerobically by nitrite oxidation and anoxically by denitrification of NO$_3^-$. Seven out of eight strains of the genus *Nitrobacter* were cultivated in gas-tight, totally filled bottles, which were equipped with a loop of gas-permeable silicone tubing of 20 cm length to provide oxygen which could penetrate into the medium. In these flasks, two morphologically and physiologically distinguishable cell types developed from a pure culture. One cell type grew as a biofilm on the surface of the tubing and oxidized nitrite to nitrate. The other cell type grew in the anoxic medium at low nitrite concentration by dissimilatory nitrate reduction. The "ping-pong mechanism" was introduced to explain their occurrence. Nitrate produced from the nitrite oxidation was metabolized by nitrate-reducing cells in the medium. Conversely, nitrite, the product of the nitrate reduction, was consumed within the biofilm. Pyruvate served as the energy source and electron donator during the nitrate reduction. The cell yield exceeded up to 3 times the yield that was obtained by aerobic cultures. Nitrite, ammonia, and especially N$_2$O were produced. Nitrogen balance calculations revealed a loss of about 40% in form of N$_2$O. It was also reported that cells accumulated PHB in large amounts under anoxic conditions.

Similar to the study of Freitag et al. (1987), Bock et al. (1988) reported that *Nitrobacter* could grow under aerobic/lithotrophic (with oxygen), aerobic/mixotrophic (with oxygen and organic carbon source), and anoxic/heterotrophic (with nitrite and organic carbon source) conditions. *Nitrobacter* are not only able to oxidize nitrite but also to reduce nitrate to nitrite, to ammonia and/or to nitrogen gases, in particular to N$_2$O. Beside, cells growing under anoxic conditions contained up to 10 mg of PHB per mg of cell protein. Bock et al. (1990) also reported the capability of heterotrophic growth and PHB storage of *Nitrobacter vulgaris*. In the presence of nitrite and organic substances, the organisms often showed diphasic growth. First nitrite and then the organic material were oxidized. In the absence of oxygen, growth was possible by dissimilatory nitrate reduction. Nitrite, nitric and nitrous oxide as well as ammonia were formed.

More recently, research of Gieseke et al. (2003) on structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm also reported that a ‘nitrifier denitrification’ is likely to play a role during extended periods of reactor operation. Nitrogen loss was report in the absence of highly active denitrifying zone while only part of the oxygen taken up from the biofilm was released in the form of nitrite or nitrate especially under oxygen-limited condition.

These observations have been further confirmed by the genome sequence of *Nitrobacter hamburgensis* X14, *Nitrobacter winogradskyi*, *Nitrobacter-255* and comparative genomic analysis of species within the genus *Nitrobacter* (Starkenburg et al., 2006; Starkenburg et al., 2008). The observations were that nitrite oxidation
mediated by $NXR$ is reversible, and that this enzyme presumably provides the nitrate reductase activity initiating denitrification. Enzyme kinetic studies led to the conclusion that the reduction of nitrate proceeds at a higher velocity than oxidation of nitrite (Bock et al., 1988). However, the pathway by which $N_2O$ would be formed is uncertain because the cultures lacked the nitric oxide reductase. Nevertheless, genome sequence of other *Nitrobacter* strains have not been analysed so far, while at least 5 strains were reported to give rise to a loss of nitrogen in form of $N_2O$ (Freitag et al., 1987).

### 2.6.2 Under aerobic conditions

Smith and Hoare (1968) reported that in culture of *Nitrobacter agilis* fed with nitrite and acetate under aerobic conditions, carbon from acetate contributed for 33 to 39% of newly synthesized cell carbon of *Nitrobacter agilis* (beside carbon from autotrophic CO$_2$-fixation mechanism), including most of the amino acids of cell protein and PHB in cell. There was accumulation of PHB in the “post-exponential phase”, when the supply of nitrite was exhausted. The rate of acetate assimilation was stimulated by nitrite (two times higher). This research also indicated that *Nitrobacter agilis* could also use acetate both as a source of cell carbon for the synthesis of a variety of cell constituents and as a source of energy.

Vangool et al. (1971), reported that *Nitrobacter Winogradskyi*, when subjected to nitrite and acetate, could use energy from autotrophic nitrite oxidation to take up PHB. As soon as the nitrite supply was depleted, the stored PHB content rapidly decreased but the cell activities remained the same. This indicated that the storage polymers supported the maintenance of the microorganism either as carbon or as energy sources to grow under aerated conditions.

### 2.7 The hypotheses

During nitrification, AOB obtain about four times more energy from the oxidation of ammonium ions (58 - 84 kcal/mole of ammonium) than NOB can obtain from the oxidation of nitrite ions (15.4-20.9 kcal/mole of nitrite) (EPA., 1993). However, due to ammonium oxidation by ammonia monooxygenase, only half of these energy obtained by AOB is available for CO$_2$ assimilation (Wood, 1986). Therefore, a ratio NOB:AOB of 0.15-0.4 can be expected. In simultaneous nitrification-denitrification (SND) systems, if denitrification over nitrite is the main pathway, NOB would have to compete for nitrite source leading to the ratio NOB:AOB even lower than 0.15.

In case of AGS, when PAOs Type II (Table 2.3) are present, the one that only uses nitrite for generating nitrogen gas, a similar ratio NOB:AOB to that of SND system is expected.

However, recently, Winkler et al. (2012a) observed an elevated ratio of NOB to AOB in an aerobic granular sludge pilot plant treating domestic wastewater and in an acetate-fed aerobic granular sludge lab reactor. This ratio was not observed in the conventional treatment plant, which received the same wastewater as the pilot
plant. This elevated ratio led to the conclusion that the growth of the NOB was partly uncoupled from nitrite supplied of AOB. qPCR showed a high ratio NOB:AOB in the pilot plant as well as lab reactor (3 – 4), a typical ratio in the conventional treatment plant (0.2 ± 0.1) and a low ratio in a CANON reactor, which was used as negative control (0.004 ± 0.002). In addition, *Nitrobacter* was the main NOB in the granular sludge while in conventional treatment plant; both *Nitrospira* and *Nitrobacter* coexisted in equal amounts. The disproportion in the ratio of NOB to AOB in aerobic granular sludge was also reported by Carvalho *et al.* (2006). Their FISH quantification of the two groups after separation of granules from flocs in nitrifying/denitrifying SBR revealed that NOB were more than five times as abundant as AOB in the granules, whereas in the floccular part of the biomass, AOB were almost twice as abundant as NOB. Winkler *et al.* (2012a) show that *Nitrobacter* grew in a zone up to 300 μm depth from granular surface which was far beyond the penetration depth of oxygen (< 100 μm) (Picioreanu *et al.*, 2004). This led to the two hypotheses to explain the unexpected high amount of NOB, which are summarized in Figure 2.4.

![Figure 2.4](image)

**Figure 2.4** Schematic view of two hypotheses (a) the ping-pong and (b) nitrite loop.

Both theories have the conventional steps (1) oxidation of ammonium to nitrite by AOB and (2) oxidation of nitrite NOB in common. In case of the ping-pong theory, the third step assumes that *Nitrobacter* uses its capability of dissimilatory nitrate reduction by using nitrate as electron accepter and organic carbon as carbon source for its growth in anoxic zone of granule. In case of nitrite loop theory, NOB not relied only on nitrite provided autotrophically by AOB but also on nitrite from other sources such as from incomplete denitrification (till nitrite) by DPAOs and DGAOs.
2.8 Thesis objectives

The objectives of this thesis are following:

- To setup a mathematical model that describes the cyclic operation of an aerobic granular sludge reactor for COD and nutrient removal.

- To extend the model in view of investigating the two hypotheses that were proposed by Winkler *et al.* (2012a) to explain how NOB could grow uncoupled from the nitrite supply of AOB in aerobic granular sludge.

- To investigate influence of the two hypotheses and operation conditions on performance of granular sludge reactor in term of oxygen consumption, nitrogen removal efficiency, and potential of greenhouse gas emission.
3 MODEL DESCRIPTION

3.1 Overview

A basic model was setup first, which served as a base case for all model extensions to describe the reactor performance and to test the two hypotheses. This model considered four groups of bacteria: the denitrifying polyphosphate-accumulating organisms (DPAOs), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria, which then includes two species Nitrospira (NOB1), and Nitrobacter (NOB2). Subsequently, the basic model was extended by including mixotrophic growth of Nitrobacter (ping-pong model) and two-step denitrification (nitrite loop model) as well as the combined processes (integrated model).

The biological processes and involved bacteria groups in the different model setup are summarized in Table 3.1. The model stoichiometry and kinetics are detailed in Appendix 1 and Appendix 2, respectively. The model parameters are listed in Appendix 3. The same parameters’ values were applied for all models.

<table>
<thead>
<tr>
<th>Process/Model</th>
<th>Basic</th>
<th>Ping-pong</th>
<th>Nitrite loop</th>
<th>Integrated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heterotrophic processes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic acetate uptake</td>
<td>DPAOs</td>
<td>DPAOs, NOB2</td>
<td>DPAOs</td>
<td>DPAOs, NOB2</td>
</tr>
<tr>
<td>Anaerobic maintenance</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
</tr>
<tr>
<td>Aerobic PHB degradation</td>
<td>DPAOs</td>
<td>DPAOs, NOB2</td>
<td>DPAOs</td>
<td>DPAOs, NOB2</td>
</tr>
<tr>
<td>Aerobic glycogen production</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
</tr>
<tr>
<td>Aerobic poly-P formation</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
</tr>
<tr>
<td>Aerobic maintenance</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
</tr>
<tr>
<td>Anoxic PHB degradation (on NO₃⁻)</td>
<td>DPAOs</td>
<td>DPAOs, NOB2</td>
<td>DPAOs</td>
<td>DPAOs, NOB2</td>
</tr>
<tr>
<td>Anoxic glycogen production (on NO₃⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
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<tr>
<td>Anoxic poly-P formation (on NO₃⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
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<tr>
<td>Anoxic maintenance (on NO₃⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
</tr>
<tr>
<td>Decay</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td>DPAOs</td>
</tr>
</tbody>
</table>
### Process/Model

<table>
<thead>
<tr>
<th>Single-step denitrification</th>
<th>Basic</th>
<th>Ping-pong</th>
<th>Nitrite loop</th>
<th>Integrated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Autotrophic processes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic growth</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
</tr>
<tr>
<td>Aerobic endogenous respiration</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
</tr>
<tr>
<td>Anoxic endogenous respiration (NO₃⁻)</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
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<tr>
<td><strong>Heterotrophic processes</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Anoxic PHB degradation (on NO₂⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anoxic glycogen production (on NO₂⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anoxic poly-P formation (on NO₂⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anoxic maintenance (on NO₂⁻)</td>
<td>DPAOs</td>
<td>DPAOs</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Additional processes in two-step denitrification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anoxic endogenous respiration (NO₂⁻)</td>
<td>AOB, NOB1, NOB2</td>
<td>AOB, NOB1, NOB2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3.2 Basic model

In the reactor, acetate was consumed by DPAOs and converted to a storage polymer (PHB) during the anaerobic period. Converting glycogen and degrading poly-P generates the energy needed for the active uptake of acetate. During the aerobic period, the stored PHB is used for growth, maintenance and restoring the glycogen and poly-P pools. The formation of glycogen and the formation of poly-P are always coupled to the consumption of PHB. The model describing the DPAOs in SBR reactors followed the kinetic structure proposed by Oehmen et al. (2010) with the difference that the denitrification of DPAOs was defined in the base model as single-step denitrification.

Nitrification takes place in the aerated period, in which ammonia is converted via nitrite into nitrate. The biological conversion processes of nitrifiers were modeled using the model published by Volcke et al. (2010), which was then extended with two species of NOB. In the basic model, denitrification occurs by DPAOs under anoxic conditions in the inner oxygen free zone during aeration and is defined as single-step denitrification which takes place on nitrate as electron acceptors and convert nitrate to dinitrogen gas. When neither PHB, glycogen nor acetate is available, DPAOs are defined to decay (de Kreuk et al., 2007b). For simplicity and maintaining of the granule structure, the decayed biomass was considered to be turned into inert material...
(Beun et al., 2001). No other heterotrophic organisms were included in the model by the fact that a very small amount of the incoming acetate is left at the start of the aerobic period, which could be consumed by aerobic heterotrophs and by DPAOs. In addition, endogenous respiration for autotrophic organisms is included in the basic model.

To describe above processes, the model accounts for biomass distribution among eight particulate components which are four active, one inactive biomass type and three internal storage compounds: denitrifying polyphosphate-accumulating organisms ($X_{DPAO}$), ammonia-oxidizing bacteria ($X_{AOB}$), two nitrite-oxidizing bacteria, *Nitrospira* ($X_{NOB1}$) and *Nitrobacter* ($X_{NOB2}$), inert biomass ($X_i$), polyphosphate ($X_{DPAO_PP}$), poly-hydroxybutyrate ($X_{DPAO_PHB}$), and glycogen ($X_{DPAO_Gly}$). Six soluble components were taken into account: dissolve oxygen ($S_{O2}$), acetate ($S_A$), ammonium ($S_{NH4}$), phosphate ($S_{PO4}$), nitrate ($S_{NO3}$), and dinitrogen-N ($S_N2$). Eleven processes were necessary to describe the DPAOs, which were named as anaerobic acetate uptake, anaerobic maintenance, aerobic PHB degradation, aerobic glycogen formation, aerobic poly-P formation, aerobic maintenance, anoxic PHB degradation (on NO$_3$), anoxic glycogen formation (on NO$_3$), anoxic poly-P formation (on NO$_3$), anoxic maintenance (on NO$_3$) and decay of DPAOs. Each of the nitrifiers had three similar processes for autotrophic growth: aerobic growth, aerobic endogenous respiration, and anoxic endogenous respiration (on NO$_3$).

### 3.3 Ping-pong model

To explain and model the ping-pong hypothesis, a ping-pong model was setup. Additional processes and parameters were included in the basic model to describe the mixotrophic growth of *Nitrobacter* in presence of organic carbon source. As discussed in the literature review (Chapter 2, section 2.6), it is assumed that *Nitrobacter* is capable of acetate uptake, PHB storage and PHB oxidation for growth (Freitag et al., 1987; Smith & Hoare, 1968; Vangool et al., 1971). These features were described in this model. Three additional processes of *Nitrobacter* (NOB2) were defined similar to DPAOs to describe anaerobic acetate uptake, aerobic PHB degradation and anoxic PHB degradation (on NO$_3$) (Appendix 2, Table A.2.1, process 28, 29 and 30). Additional parameters and coefficient of *Nitrobacter* such as internal storage biomass PHB ($X_{PHB}^{NOB2}$), maximum anaerobic acetate uptake rate ($q_{AC,AN}^{NOB2}$), maximum aerobic PHB degradation rate ($q_{PHB,OX}^{NOB2}$), maximum storage of PHB, affinity constant of *Nitrobacter* to nitrate, PHB, and acetate ($K_{NO3}^{NOB2}$, $K_{PHB,DX}^{NOB2}$, $K_{AC}^{NOB2}$) were also assumed. Rate of PHB degradation of NOB2 under anoxic condition was assumed half of that in aerobic condition (Appendix 3, Table A.3.2).

In the feeding phase, when NO$_3^-$ and NO$_2^-$ can be still present from previous cycle, *Nitrobacter* can use them as electron acceptor to uptake acetate and store it as PHB. In the aeration phase, PHB is oxidized with either NO$_3^-$ or O$_2$ as electron acceptor. The possible products of both processes were reported to be N$_2$O and NH$_3$. 
(Freitag et al. (1987). However, the pathway by which N₂O would be formed is uncertain because recent genome studies showed that *Nitrobacter* lacks nitric oxide reductase but still has enzyme to reduce NO₃⁻ until NO (Starkenburg et al., 2006). Therefore, in this study, the final product of dissimilatory nitrate reduction by *Nitrobacter* was NO. In this thesis, was the growth of *Nitrobacter* was defined according to following pathways:

**Feeding phase:** \[\text{Acetate} + \text{NO}_3^- \rightarrow \text{NH}_4^+ + \text{PHB} + \text{Biomass} \]  

(1)

**Aeration phase:** 
\[\text{PHB} + \text{O}_2 \rightarrow \text{Biomass} \]  

(2)

\[\text{PHB} + \text{NO}_2^- \rightarrow \text{NO} + \text{Biomass} \]  

(3)

The energy necessary for the assimilation of acetate in reaction (1) will be supplied from endogenous reserves or from the oxidation of acetate itself (Smith & Hoare, 1968). For this reason, stoichiometric yields of above reactions can be calculated from oxidative phosphorylation δ (ratio of ATP produced to NADH2 oxidized mol.mol⁻¹). This method was also used for mixed (but highly enriched) cultures in the model proposed by Johnson et al. (2009), for the evaluation of polyhydroxybutyrate production under aerobic conditions (Table 3.2). In model of Johnson et al. (2009), which describes the PHB uptake and consumption of bio plastic (PHB) accumulating bacteria subjected to a feast famine regime, part of energy derived from oxidation of acetate was used for growth and the other part for the assimilation of acetate and PHB storage.

<table>
<thead>
<tr>
<th>Process</th>
<th>Formula¹</th>
<th>Nitrobacter δ = 2 &amp; 1.2²</th>
<th>Nitrobacter δ = 1.1 &amp; 0.7³</th>
<th>DPAOs</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anoxic PHB stored on acetate consumed</td>
<td>(\frac{2\delta - 1}{2.25\delta})</td>
<td>0.58</td>
<td>0.29</td>
<td>1.35</td>
<td>g COD.g⁻¹</td>
</tr>
<tr>
<td>Anoxic ammonium release per acetate consumed</td>
<td>(\frac{0.25\delta + 1}{2\delta - 1})</td>
<td>0.41</td>
<td>1.29</td>
<td></td>
<td>g N.g⁻¹</td>
</tr>
<tr>
<td>Aerobic yield for PHB degradation (PHB per biomass)</td>
<td>(\frac{2.25\delta - 0.25}{2.16 + 2.16})</td>
<td>1.77</td>
<td>2.38</td>
<td>1.3</td>
<td>g COD.g⁻¹</td>
</tr>
<tr>
<td>Anoxic yield for PHB degradation on nitrate (PHB per biomass)</td>
<td>(\frac{2.25\delta - 0.25}{2.16 + 2.16})</td>
<td>2.26</td>
<td>3.24</td>
<td>1.7</td>
<td>g COD.g⁻¹</td>
</tr>
</tbody>
</table>

The ratio δ was reported to be 2 and 1.2 for mixed microbial cultures under aerobic and anoxic condition, respectively (Beun et al., 2002). For *Nitrobacter agilis* these values were reported to be 1.1 and 0.7 (Sewell & Aleem, 1979). The calculated yields of *Nitrobacter* from these δ values were used to evaluate the model with experimental data (see Chapter 4, section 4.1). Uptake rate of acetate and PHB degradation rate of *Nitrobacter*
were assumed similar to the ones of DPAOs (Appendix 3, Table A.3.2). To make it the consistence with DPAOs models, no growth was considered during feeding for *Nitrobacter*. Result of calculations is summarized in Table 3.2. The yield calculated from δ = 2 & 1.2 were quite similar to the mixotrophic yield reported by Hahne (2014) being 0.4-0.6 g COD per g VSS, depending on the substrate provided.

### 3.4 Nitrite loop model

The nitrite loop model was also based on the basic model with the modification that single-step denitrification of DPAOs was replaced by two-step denitrification. The model evaluates the impact on the NOB and AOB ratio by incomplete denitrification and hence the effect of additional nitrite supply for NOB beside the nitrite being supplied by AOB. Instead of denitrification by DPAOs from nitrate to dinitrogen gas (nitrate → dinitrogen gas), nitrite was considered as an intermediate of denitrification (nitrate → nitrite → dinitrogen gas). To implement this, four additional processes of DPAOs using nitrite as electron acceptor were considered: anoxic PHB degradation (on NO$_2^-$), anoxic glycogen formation (on NO$_2^-$), anoxic poly-P formation (on NO$_2^-$), and anoxic maintenance (on NO$_2^-$). Three additional endogenous respiration processes on nitrite for AOB, NOB1, and NOB2 were considered. Another important parameter, which was replaced, is the reduction factor and anoxic maintenance of DPAOs under anoxic condition. Under anoxic conditions, these parameters were expected to be different for single-step and two-step denitrification due to the ratio of electrons being involved (and therefore fraction of biomass produced). In the two-steps denitrification, 2e- are involved for nitrate and 3e- for nitrite reduction compared to the 5e- being involved during completed denitrification of nitrate to dinitrogen (Kaelin *et al.*, 2009). Chosen values were tested and evaluated to ensure that growth of DPAOs in single-step and two-step denitrification were identical. Table 3.3 summarizes the values of DPAOs considered in this study.

**Table 3.3 Reduction factors and maintenance under anoxic condition**

<table>
<thead>
<tr>
<th>Process parameter</th>
<th>Two-step de Kreuk <em>et al.</em> (2007b)</th>
<th>Single-step (Kaelin <em>et al.</em>, 2009)</th>
<th>Considered values (This study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction factor</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3-0.7</td>
</tr>
<tr>
<td>Anoxic maintenance</td>
<td>0.11</td>
<td>0.22</td>
<td>0.13-0.22</td>
</tr>
</tbody>
</table>

### 3.5 Integrated model

In addition to the previous three models setup, an integrated model was also established. In this model the mixotrophic growth of *Nitrobacter* (ping-pong model) and two-step denitrification of DPAOs (nitrite loop) were combined to evaluate the influence of both hypotheses (nitrite loop and ping-pong hypotheses) that lead to decoupled growth of NOB apart from the chemolithotrophic nitrite supply from the AOB.
3.6 Reactor setup and operation

The reactor model was based on the experimental setup described by Winkler et al. (2012a). A lab-scale aerobic granular sludge sequencing batch reactor (SBR) of 2.6 L was operated at constant temperature (20°C). The operational cycle lasted 3h and was divided into the following phases: 60-min anaerobic feeding from bottom of the reactor in a plug flow regimen; 112-min aeration period; 3-min settling and 5-min effluent withdrawal. Dissolved oxygen (DO) concentration was fixed at 2.0 g O₂.m⁻³. pH was kept at 7.0. The volume exchange ratio and the hydraulic retention time (HRT) were 57 % and 5.2h, respectively. Per cycle, influent concentrations of 400 g COD.m⁻³, 50 g NH₄–N.m⁻³ and 20 g Pₒ₄-P.m⁻³ were introduced with total volume of 1.5 L being added during 1 hour of feeding phase.

The reactor model was implemented in AQUASIM software (Reichert et al., 1996). Simulating the fill and discharge processes of SBR reactor in this study was adopted from the methods proposed by Beun et al. (2001). In the AQUASIM, the volumes of the biofilm compartments have to be constant over time (Reichert, 1998). Therefore, two compartments were defined. First, the biofilm compartment was defined to have a fixed volume and was then connected to a completely mixed compartment by an advective link. Second, a high recirculation flow rate (Q recirculation) was incorporated from the mixed compartment to the biofilm compartment to ensure the same concentrations in both compartments. A schematic drawing of the aerobic-granule-based SBR configuration is illustrated in Figure 3.1. The volume of biofilm compartment was set at 1 L; including 0.9 L of sludge and 0.1 L of liquid. The completely mixed compartment contained the remaining liquid volume of the reactor, with a maximum volume of 1.6 L.

![Figure 3.1 Schematic diagram of the granule-based SBR as implemented in the AQUASIM proposed by Beun et al. (2001). Each compartment contains all soluble and particulate components.](image-url)
3.7 Granule characteristics

The diameter of granules in steady state was chosen to be 1.2 mm, which is a typical diameter for aerobic granules (de Kreuk et al., 2007b). The number of granules was calculated from the volume of sludge in the reactor (0.9 L), resulting in a comparable amount of biomass as present in the laboratory scale reactor. A radius-dependent biofilm area was defined in AQUASIM to correctly represent the spherical symmetry of the granules. Biomass granules, are typically dense, and have very small pores in which no relevant motion of suspended solids is taking place. The granule structure is assumed rigid, meaning that particulate components are displaced only by the expansion or shrinkage of the biofilm solid matrix (Volcke et al., 2010).

The porosity of granules was assumed constant ($\varepsilon = 0.8$). This value is determined by the initial biomass volume fractions of DPAOs, AOB, NOB1, NOB2 and inert material, which were 0.1487, 0.0243, 0.0135, 0.0135 and 0 corresponding to initial concentration of these biomass of 55000, 9000, 5000, 5000 and 0 g COD.m$^{-3}$ respectively. These initial biomass values were calculated based on the total VSS in reactor of 52,000 g VSS or MLVSS of 20,000 g VSS.m$^{-3}$ as measured in earlier research of Winkler et al. (2012a). The concentration of internally stored storage polymers of DPAOs and NOB2 was set to 90% of their maximum storage capacity (de Kreuk et al., 2007b).

3.8 Simulation setup

The standard simulation conditions were set to a temperature of 20°C, dissolved oxygen concentration of 2 g O$_2$.m$^{-3}$ and to a diameter of granules in steady state of 1.2 mm. Each type of model was simulated for at least 500 days of operation, which corresponded to 24-36 hours of computing time on a core i5 processor. Steady state was considered sufficiently reached if after 200 days, effluent concentration and active biomass concentration of 5 days interval did not show to change more than 1% (de Kreuk et al., 2007b). The operation of SBR under different conditions from standard condition (i.e. different of temperature, DO and/or granular size) were simulated with the model that described the reactor the best and started from initial condition, which is listed in Appendix 3, Table A.3.3.

In order to assess the influence of the granular size on nitrogen removal efficiency, the ratio of NOB to AOB as well as on the NO emission, simulations were done for different granules radius size ranging from 0.4 mm to 0.8 mm whilst DO and temperature were kept at 2 mg O$_2$.m$^{-3}$ and 20°C. The total volume of sludge in the reactor was set to be constant at 0.9 L, which means that the number of granules varied from 4.2x10$^5$ to 3.36x10$^6$ according to the size of the granules.

To evaluate temperature effects, temperature dependency for maximum acetate uptake, PHB degradation and maintenance kinetic coefficient were defined by introducing the term $e^{0.069(T-20)}$ (Henze et al., 2000). The
temperature dependency of nitrifiers’ growth and decay rates were incorporated according to Arrhenius type equation (Henze et al., 2000) considering the activation energies of each bacterial group (Hao et al., 2002a). Nevertheless, in this study, temperature dependency was not setup for the affinity constants, the interphase transport rates (i.e. oxygen transfer rate), the Henry coefficients, and the diffusion coefficients. Details of temperature dependent parameters can be found in Appendix 3, Table A.3.2. Each simulation conducted with the integrated model ran for at least 500 days of operation with temperature ranging from 10°C to 30°C and at DO concentrations of 1, 2, 4, 6 and 10 g O₂.m⁻³ to assess long-term influence of both parameters.

3.9 Model calibration

Model calibration was done to better describe the experimental data of Winkler et al. (2012a). Parameters adjustment and visual examination of the modelled curves were used for the parameter evaluation rather than the use of statistical indicators such as correlation coefficient, average error, root mean square error, or modelling efficiency. Selection of sensitive parameters were based on previous studies on modelling AGS such as Ni et al. (2008b), Kaelin et al. (2009) and Zhou et al. (2013). Table 3.1 summarized considered parameter for adjustment (other unchanged parameters can be found in Appendix 3, Table A.3.1 and A.3.2).

Two criteria were used for the calibration of the model. Firstly the substrate profile during one cycle of operation at steady state and secondly the biomass distribution of nitrifiers especially the ratio of NOB to AOB (based on of total biomass of NOB and AOB). For the evaluation of the substrate profile during one typical cycle of operation, kinetic parameters of DPAOs and AOB were adjusted. Subsequently, conversion rates measured in the experiments of Winkler et al. (2012a) were compared to the ammonium and phosphate consumption and to nitrate production rates obtained from the models simulation at steady state (defined in Chapter 3, section 3.8). Maximum conversion rates observed in the experiments were calculated by taking the largest slope of at least three data points in the cycle (Appendix 4). These were compared to the average conversion rates over the same time interval in the simulation (de Kreuk et al., 2007b). For biomass distribution of nitrifiers, results from the models were compared to the qPCR results of Winkler et al. (2012a) which showed that *Nitrobacter* and not *Nitrospira* was the dominant NOB in granules with *Nitrobacter*:AOB ranging from 1.5 – 5 and *Nitrospira*:AOB < 0.1. Finally, biological conversion rate and growth rate of DPAOs need to be identical in both single-step and two-step denitrification models (basic and nitrite loop model) by adjusting reduction factors and maintenance under anoxic condition.
<table>
<thead>
<tr>
<th>Process parameter</th>
<th>Considered values</th>
<th>Reference value from</th>
<th>Adjusted value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum aerobic poly-P formation rate (DPAOs)</td>
<td>0.10-0.45</td>
<td>Murnleitner et al. (1997)</td>
<td>0.10</td>
</tr>
<tr>
<td>Anaerobic yield phosphate release</td>
<td>0.20-0.50</td>
<td>Murnleitner et al. (1997)</td>
<td>0.30</td>
</tr>
<tr>
<td>Reduction factor of DPAOs under anoxic condition for two-step denitrification</td>
<td>0.20-0.60</td>
<td>de Kreuk et al. (2007b)</td>
<td>0.50</td>
</tr>
<tr>
<td>Anoxic maintenance for two-step denitrification</td>
<td>0.13-0.22</td>
<td>de Kreuk et al. (2007b)</td>
<td>0.20</td>
</tr>
<tr>
<td>Maximum specific growth rate (AOB)</td>
<td>0.20-0.40</td>
<td>Blackburne et al. (2007a)</td>
<td>0.25</td>
</tr>
<tr>
<td>Anaerobic PHB stored on acetate taken up (Nitrobacter)</td>
<td>0.29-1.35</td>
<td>de Kreuk et al. (2007b)</td>
<td>1.35</td>
</tr>
<tr>
<td>Anoxic ammonium release per acetate consumed (Nitrobacter)</td>
<td>0.20-1.29</td>
<td>Sewell and Aleem (1979)</td>
<td>0.20</td>
</tr>
<tr>
<td>Anoxic yield for PHB degradation on nitrate (Nitrobacter)</td>
<td>1.30-2.38</td>
<td>de Kreuk et al. (2007b)</td>
<td>1.39</td>
</tr>
<tr>
<td>Aerobic yield for PHB degradation (Nitrobacter)</td>
<td>1.70-3.24</td>
<td>Johnson et al. (2009)</td>
<td>1.70</td>
</tr>
</tbody>
</table>
4 RESULTS AND DISCUSSION

4.1 Model evaluation with experimental data

4.1.1 Model structure selection for calibration

Prior to the adjustment of the parameters’ values, it is important to select the model structure that describes the experimental data the best (Chapter 3, section 3.9). The first simulation results showed that all models had a similar substrate profile during one cycle operation. However, the results from the basic and nitrite model resulted the in the coexistence of Nitrospira and Nitrobacter, while in the ping-pong model and the integrated model, Nitrobacter out competed Nitrospira. This trend was always the same irrespectively of the value of the model parameter’s values. In the integrated model, the ratio of Nitrobacter to AOB was higher than that in the ping-pong model (1.5 vs 0.5, respectively). Therefore, the integrated model was concluded to described the experimental results the best. Another reason for choosing the integrated model was that it was based on two-step denitrification, which is commonly used and well documented (de Kreuk et al., 2007b; Hellinga et al., 1999; Kaelin et al., 2009; Moussa et al., 2005; Sin & Vanrolleghem, 2006; Volcke et al., 2007; Xavier et al., 2007; Zhou et al., 2013). Thus, the integrated model was used for the model calibration by parameter adjustment and visual inspection of the simulated curves.

Table 4.1 Comparison of biological conversion rates between experimental and model results

<table>
<thead>
<tr>
<th></th>
<th>$\text{r}_{\text{O}_2}$ (mg COD.h$^{-1}$)</th>
<th>$\text{r}_{\text{NH}_4}$ (mg N.h$^{-1}$)</th>
<th>$\text{r}_{\text{PO}_4}$ (mg P.h$^{-1}$)</th>
<th>$\text{r}_{\text{NO}_3}$ (mg N.h$^{-1}$)</th>
<th>NOB1: AOB</th>
<th>NOB2: AOB</th>
<th>$\text{r}_{\text{DPAOs}}$ (mg. h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured</td>
<td>64.74</td>
<td>167.70</td>
<td>33.15</td>
<td>&lt;0.1</td>
<td>1.5-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrated model</td>
<td>372.75</td>
<td>60.87</td>
<td>181.76</td>
<td>30.86</td>
<td>0.01</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>Base model</td>
<td>331.34</td>
<td>58.27</td>
<td>194.69</td>
<td>9.91</td>
<td>0.49</td>
<td>0.17</td>
<td>106.28</td>
</tr>
<tr>
<td>Nitrite loop model</td>
<td>363.07</td>
<td>59.60</td>
<td>184.99</td>
<td>27.74</td>
<td>1.01</td>
<td>0.32</td>
<td>102.02</td>
</tr>
<tr>
<td>Ping-pong model</td>
<td>333.76</td>
<td>58.52</td>
<td>195.92</td>
<td>9.94</td>
<td>0.02</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

The adjusted parameter values after calibration are presented in the last column of Table 3.1. These values were then used for all model simulation. By using these adjusted parameters, simulations were performed again for all four models and the obtained biological conversion rates and ratio NOB2:AOB are presented in Table 4.1. The results clearly showed that the conversion rates obtained from the integrated model and from experimental data as well as the conversions of single-step (basic model) and two-step denitrification (nitrite loop model) (Table 4.1) did not differ more than 10%. This is acceptable considering the uncertainty associated with other parameters (e.g., exact granule size distribution and granule surface area). Similar evaluation was done in de Kreuk et al. (2007b) with difference of 30%.
4.1.2 Steady state substrate profile during one typical cycle

Since the integrated model described the reactor performance the best, it was used to discuss the cyclic operation of the reactor. The simulated substrate profiles fitted experimental data very well (Figure 4.1a). Steady state was reached after 400 days of simulation (Figure 4.1b).

![Figure 4.1](image)

**Figure 4.1** a) Steady state substrate profile of typical operation cycle (experimental data: O phosphate; □ ammonium; V nitrate) and b) effluent concentration and granule radius as function of time during 1000 days of simulation. Simulation results were obtained from the integrated model with granule radius = 0.6 mm, DO = 2 g O₂.m⁻³, t = 20°C.

During the anaerobic feeding phase, the acetate concentration was almost zero due to fast consumption and storage as PHB by DPAOs (Figure 4.1a). Phosphate was released by DPAOs during this phase from degradation of internal poly-P gaining ATP for acetate uptake (Smolders et al., 1994). The phosphate concentration in the reactor increased to 90 g P.m⁻³ by the end of the feeding phase. The measured concentration of ammonium at the end of feeding phase was lower than the simulated one (24.39 g NH₄-N.m⁻³ vs 28.84 g NH₄-N.m⁻³). Since there was no process that consumed ammonium in this phase, this difference could be explained by the chemical adsorption of ammonium into the granule sludge, which was reported earlier in literature but not considered in the model. Bassin et al. (2011) reported that aerobic granular sludge exhibits an adsorption of 1.7 mg NH₄-N.g⁻¹ VSS. In Winkler et al. (2012a)’s study, there was total 5.2 g VSS in reactor, hence, there could be 8.8 mg ammonium adsorbed in granules, which corresponds to 3.4 g NH₄-N.m⁻³. This fits the gap between model and experiment very well.

The aeration phase started right after the anaerobic phase. The concentration of oxygen was kept at a constant 2 g O₂.m⁻³. Nitrification took place, with ammonia converted via nitrite into nitrate. No nitrite accumulation was simulated during aerobic period in contrast what was experimentally observed (< 5 mg NO₂-N.L⁻¹, not shown on figure). Simulation show that, ammonia was not only consumed by AOB also DPAOs and NOBs used ammonium for growth hence consuming at least 10% of the incoming ammonia during the aeration phase.
Growth, formation of glycogen and poly-P by DPAOs with oxygen, nitrite, or nitrate as electron accepter are always coupled to the consumption of PHB and this internal storage compound was one of most important limiting factors for the growth of DPAOs. Consumption of phosphate, led to a sharp drop of phosphate concentration, mainly due to poly-P formation besides a very small portion of phosphate being used by DPAOs to grow (Figure 4.1a). Since the rate of acetate consumption in anaerobic period was high (up to 8 g COD acetate per g COD DPAOs per day), no acetate remained in aeration period leading to a lack of substrate availability for other heterotrophic organisms. Actually, other heterotrophs, which can growth on decay material, could be included in this model; however, their contribution to total biomass would be negligible or even not present as reported by Xavier et al. (2007).

During the last 8 minutes of settling and effluent withdrawal, nitrate slightly decreased whereas nitrite increased. Maintenance processes of DPAOs, which perform denitrification and hence consume nitrate and release nitrite (Appendix 2, Table A.2.1, processes 10 and 14), can explain this observation. The increased nitrite and decreased nitrate during the last 8 minutes of each cycle (consisting of settling and effluent withdrawal) was not observed in the experiment since during this settling period no representative sample could be taken because there was no mixing in the reactor.

4.1.3 Biomass distribution in granule

The simulated oxygen penetration profiles in aerobic granules are shown in Figure 4.2a. For half of aeration time (from minute 60th to minute 120th of the cycle), ammonium was still available in the bulk (Figure 4.1a), which led to high microbial activities in outer layers of the granule. Oxygen was continuous consumed in outer layers and prevented from penetrating deeper than 150 \( \mu \text{m} \). As soon as ammonium was depleted, oxygen could penetrate throughout the granule (Figure 4.2a, minute 144th to minute 168th).

The simulated biomass profile was in accordance with the FISH analysis (Figure 4.2b and Figure 4.3), in which the spatial distribution of the autotrophs (ammonia- and nitrite-oxidation bacteria) and heterotrophs (DPAOs) were observed. The nitrifiers (AOB and NOB) were mainly located on the outer layer of granules, while the DPAOs could be found throughout the granule (Figure 4.2b). More *Nitrobacter* could be found deeper inside the granule than AOB, presumably, because they used their mixotrophic capacities allowing them to grow in anoxic layers.
Figure 4.2  a) oxygen profile in granule and b) Absolute quantity o biomass in granules
Result of simulation of integrated model. Granule radius = 0.6 mm, DO = 2 g O₂.m⁻³, temperature = 20°C

Figure 4.3 Microscopic FISH image on sliced granule. a) AOB (green), NOB (red) and DPAOs (blue)
b) nitrifiers (mix of AOB and NOB) (red), DPAOs (blue) and GAO (green) (Winkler et al., 2012a)

In AGS, acetate can penetrate the entire granules because of high substrate concentrations in the liquid, but
oxygen will only be present on the outer layers of granules due to diffusion limitation and fast consumption.
DO has a substantial effect on the microbial population distribution in aerobic granules (de Kreuk et al., 2007).
Low oxygen penetration will create an anoxic zone and the volume fraction of this zone determines the
abundance of denitrifiers and hence the denitrification capacity in the reactor. This fraction itself is highly
depended on the diameter of the granule and concentration of oxygen in the bulk. Consequently, competition
between the autotrophs and heterotrophs for DO and space is expected and additionally that the autotrophs
are mainly located on the outer oxygen penetrated layers. In contrast to the nitrifiers, the location of the
heterotrophs in aerobic granules is less restricted. They can occupy deeper layers in the granule, where they
grow with organic substrates from the feast phase, using NOx as the electron acceptor. The autotrophs can
only successfully compete for DO and space with the heterotrophs on the outer layers of granules, where
oxygen and ammonium concentrations are high, because of their lower growth rate (Ni & Yu, 2008b).

It is important to note that, in the experiments at laboratory scale, experiments usually run 4-18 month to
achieve stable operation (de Kreuk et al., 2007b). However, the definition of steady state (see section 3.8)
concerning the bulk liquid concentration and is thus related to operational stability and not microbial stability
(Johnson, 2010). A true long-term steady state where no changes would be observable could be found if simulation kept running for a much longer time. Therefore, despite the fact that steady state could be reach within 200-400 days, the models were simulated for at least 500 days of operation to ensure the stabilization of biomass and to accurately present the ratio of nitrifiers.

Overall, removal efficiency of COD, phosphate, and nitrogen removal of 100%, 100% and 62.8%, respectively, were achieved in the simulation. However, simulation showed that a small change in a conversion rate could easily lead to an increased concentration of ammonium and phosphate in the effluent. The trends of the conversion rates are comparable to practice, so the model can be used to obtain insights in the process rates and in the granule structure (de Kreuk et al., 2007b). The main reason for the model to be less predictive is the complexity of the granular sludge itself. The granules in the model are of a certain diameter and are assumed perfect spheres. In reality, a wide range of granule sizes and shapes in a reactor are expected (de Kreuk & van Loosdrecht, 2004). Less regularly shaped granules will lead to an increased surface to volume ratio, which would influence the oxygen transport into the granules.

4.1.4 Conclusion

Simulation results of four models showed a similar substrate profile during one cycle operation at steady state except the profiles of nitrate, which were much lower in basic, and ping-pong model compare to those of nitrite loop and integrated model. However, in term of nitrifiers’ distribution, the integrated model described the experimental data well. The simulated substrate and biomass profile at steady state of calibrated model were in accordance to experimental data showing high ratio NOB:AOB and the dominance of Nitrobacter in nitrifiers population. The ratio of NOB2 to AOB was one of the most important factors to select model structure for calibration and the mass transfers of oxygen and granule diameter seem to be the decisive factors for distribution of autotrophs and heterotroph in granules.
4.2 Unravelling the two hypotheses

4.2.1 Compare ratio of NOB to AOB in four model setup to experiment data

Simulation results of the basic and nitrite loop model revealed that both *Nitrospira* and *Nitrobacter* could coexist in granules but that *Nitrospira* was presented in higher amounts. The situation was different in the ping-pong as well as in the integrated model, in which *Nitrobacter* outcompeted *Nitrospira* (Figure 4.4a). It has to be noted that the total quantity of AOB in four models was essentially the same (around 2.3 g per 2.6 L of reactor). Obtained results indicated that both nitrite loop and ping-pong theory had certain effects on distribution of nitrifiers in granules.

![Figure 4.4 Ratio of NOB1 to AOB and ratio of NOB2 to AOB from a) models simulation and b) experimental data of Winkler et al. (2012a).](image)

Experimental data from Winkler et al. (2012a) (Figure 4.4b) reported with qPCR measurements on samples from a conventional activated sludge treatment plant a very low ratio of *Nitrobacter* to AOB, biomass ratio of *Nitrobacter* to AOB (<0.3). The negative control samples collected from a CANON reactor revealed low *Nitrobacter* and AOB ratios (i.e. ca. 100-fold lower than AOB) since NOB were suppressed in this reactor. *Nitrobacter* and not *Nitrospira* was the dominant NOB in aerobic granular sludge pilot plant and lab-scale reactor (ratio of *Nitrobacter* to AOB of 1.5-5). Next sections from 4.2.2 to 4.2.5 will discuss individual model results.

4.2.2 Basic model

Results of simulations showed that both type of NOB coexisted in one granule and that *Nitrospira* was the predominate species. The values of *Nitrospira*:AOB and *Nitrobacter*:AOB were 0.61 and 0.25, respectively (Figure 4.4a). In terms of biomass distribution of NOB and AOB, the basic model could describe conventional activated sludge well but failed to explain observations from the granular pilot and lab-scale reactor (Figure 4.4b). Nitrite affinity and growth yield rather than oxygen affinity seemed to be the factor determining the
distribution of NOB populations in the base model. The half saturation constant of nitrite (K_s values) for *Nitrospira* is significantly lower than the one of *Nitrobacter* while the growth rate of *Nitrobacter* is higher (Table 4.2). Simulation data indicated that there was no or very low nitrite accumulation in the bulk and in side granule (<0.5 mg N.L\(^{-1}\)) during the aeration phase, which means that the conditions would favour *Nitrospira* in the granules. Nevertheless, the K_o values of both nitrifiers are not significantly different (Table 4.2).

**Table 4.2** Affinity constant and kinetic parameter of *Nitrospira* and *Nitrobacter* used in the model (Blackburne *et al.*, 2007b)

<table>
<thead>
<tr>
<th></th>
<th>Nitrospira</th>
<th>Nitrobacter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half saturation constant for nitrate K_s (g.m(^{-3}))</td>
<td>0.27</td>
<td>0.39</td>
</tr>
<tr>
<td>Half saturation constant for oxygen K_o (g.m(^{-3}))</td>
<td>0.57</td>
<td>0.43</td>
</tr>
<tr>
<td>Maximum growth rate (d(^{-1}))</td>
<td>0.372</td>
<td>0.495</td>
</tr>
<tr>
<td>Growth yield (g COD.g(^{-1})N)</td>
<td>0.1</td>
<td>0.072</td>
</tr>
</tbody>
</table>

*Nitrospira* bacteria are widely distributed in natural and engineered ecosystems (Daims *et al.*, 2001). Many research attempted to explain the predominance of *Nitrospira* over *Nitrobacter* in most WWTP by their different survival strategies, which are based on their affinity constants and growth rates (K/r strategy). Nogueira and Melo (2006) postulated that *Nitrospira* bacteria are K-strategists and can exploit low amounts of nitrite much more efficiently than *Nitrobacter*. In contrast, *Nitrobacter* are r-strategists that can grow faster than *Nitrospira*, but depend on significantly higher nitrite concentrations. This K/r-hypothesis can explain the predominance of Nsp-like bacteria in activated sludge, where nitrite concentrations are usually low. Other authors also indicated that also *Nitrospira* and not *Nitrobacter* could be the predominant species of nitrite oxidizers in granular nitrifying systems (Carvalho *et al.*, 2006; Wang *et al.*, 2007).

### 4.2.3 Influence of ping-pong hypothesis

The model was setup with single-step denitrification and the capacity of *Nitrobacter* to denitrify nitrate until NO. The purpose of using single-step denitrification in the model was to exclude the effect of extra nitrite from denitrification and to hence to show the effect of the mixotrophic capacity of *Nitrobacter* only. Figure 4.4a shows quite different ratio of ping-pong model compare to the one of the basic model. The values of *Nitrospira*:AOB and *Nitrobacter*:AOB were 0.08 and 0.64, respectively. *Nitrobacter* outcompeted *Nitrospira* and became the only NOB in the reactor despite the similar total ratio of NOB to AOB in comparison to the basic model. This result clearly showed that the ping-pong hypothesis or the growth of *Nitrobacter* on organic carbon did affect biomass distribution of *Nitrobacter* and *Nitrospira* in the granule but still did not explain the elevated ratio NOB2:AOB of the experimental data in Figure 4.4b.
In the presented case, *Nitrobacter* can grow on nitrite produced by AOB as well as on acetate. *Nitrobacter* has lower autotrophic yield (0.072 g COD.g⁻¹ N) compared to the autotrophic yield of *Nitrospira* (0.1 g COD.g⁻¹ N) (Blackburne et al., 2007b) (Table 4.2). This means that *Nitrospira* produces 0.028 g more biomass per unit of nitrogen (nitrite being oxidized) than *Nitrobacter*. Theoretically spoken, hence the ratio NOB:AOB will be lower in a system dominated by autotrophic *Nitrobacter* if compared to a reactor receiving the same amount of nitrite but which is dominated by *Nitrospira*. When *Nitrobacter* is using its mixotrophic capacities it can elevate the NOB:AOB ratio. However, because (autotrophic) *Nitrobacter* has lower yield than that of Nitrospira, the mixotrophic growth (on nitrate and acetate) does elevate the ratio of *Nitrobacter* to AOB. However, the ratio of NOB to AOB of both model were very similar (Figure 4.4). Nitrate generated during aeration phase from previous cycle triggered acetate uptake up take by mixotrophic *Nitrobacter*. In this case, mixotrophic growth contributed to 30% of the total biomass of *Nitrobacter* and could hence explain why *Nitrobacter* completely dominated over *Nitrospira*. Activities of *Nitrobacter* in early feeding phase were in high competition with DPAOs, which also consumed nitrate and acetate in early period of feeding phase for denitrification. Another key point was that the calibrated model resulted in a yield of *Nitrobacter* was similar to that of DPAOs. This is in agreement with the study of Hahne (2014) the mixotrophic yield in a pure Nitrobacter culture was 0.4-0.6 g COD per g VSS, depending on substrate provided (different electron acceptors and donors were used).

An oxygen limitation term was included in acetate take-up process of *Nitrobacter* meaning that acetate uptake was only possible during the feeding phase. This term prevented the growth of *Nitrobacter* from growing on available organic carbon from decay and lysis in aeration phase (this was also true for DPAOs). The growth of *Nitrobacter* on acetate under aerated condition was proven possible. It means that *Nitrobacter* can simultaneously oxidize nitrite, store PHB from available acetate, and then use this preserved polymer for growth when nitrite is depleted under aerobic condition (discussed in Chapter 2, section 2.6). When this restriction term was removed, hence enabling growth of both DPAOs and *Nitrobacter* on lysis products the biomass concentration of *Nitrobacter* was not changed. It indicated that the growth of *Nitrobacter* on acetate available from decay and lysis in the aeration phase was negligible due to all organic carbon which might available in aeration phase from decay processes was assimilated by DPAOs due to much higher biomass concentration of DPAOs in granules.

### 4.2.4 Influence of nitrite loop hypothesis

This model is based on two-step denitrification and normal autotrophic nitrification to evaluate the impact of incomplete denitrification (with nitrite as intermediate) and hence the impact of additional nitrite supply for the uncoupled growth from NOB beside from the one generated by AOB. The results were that ratio of *Nitrobacter* to AOB, and ratio of *Nitrospira* to AOB were 1.11 and 0.61, respectively (Figure 4.4a). *Nitrospira* and *Nitrobacter* increase their total biomass by almost 100% if compared to the results of base model while the total biomass of AOB remained the same as basic case (2.3 g biomass of AOB in reactor).
An increased total biomass of both *Nitrospira* and *Nitrobacter* in this model setup indicated that extra nitrite from incomplete denitrification (nitrite loop) did play an important role in decoupled growth of NOB from nitrite originated from AOB. In the model, there was no nitrite accumulation in the reactor during the aeration phase. This can be explained by the biomass distribution in the granules. Nitrite from DPAOss, which are located in anoxic zones of the granule, could not diffuse out of granule since it was quickly oxidized to nitrate by NOB in outer zone of granules. This nitrate from NOB was then denitrified again to generated more nitrite (due to incomplete denitrification) hence looping it back to NOB (Figure 2.4). In conventional wastewaters treatment systems such a nitrite loop is unlikely to occur, since nitrification and denitrification processes are usually carried out in different compartments.

![Figure 4.5](image)

**Figure 4.5** Schematic representation of the nitrification/denitrification route in the aerobic granular sludge, structure for two separate reactors run at 30°C (SBR$_{30}$) and reactor at 20°C (SBR$_{30}$).

The main pathway of denitrification used in this model was over nitrate with nitrite as intermediate, which was also observed experimentally. In a very similar experimental setup and operation, Bassin *et al.* (2012) reported that DGAOss were the main organisms responsible for reducing nitrate to nitrite, which then was used by DPAOss of clade II for full denitrification. DPAOss type II are capable of nitrite reduction only (and hence not nitrate). DPAOss type II were represent in the reactor at 30°C and dominated the reactor at 20°C (Figure 4.5). The involvement of different denitrifying heterotrophs made extra nitrite available in granules for NOB to decouple their growth from the AOB. Including several types of PAOss and GAOss in the here presented model is unnecessary for this study in terms of nitrogen removal evaluation since the biological conversion of DGAOss, and DPAOss are very similar and they both coexist in the AGS system. The significant difference in between those organisms is that GAOss cannot uptake phosphate (Filipe *et al*., 2001).

### 4.2.5 Integrated model

The combined effect of the nitrite loop and ping-pong was assessed by setting up an integrated model in which two-step denitrification of DPAOss and mixotrophic growth of *Nitrobacter* were included. The results obtained from ping-pong model have already shown that as soon as *Nitrobacter* mixotrophic capacities were introduced in the model, *Nitrospira* was outcompeted, which hence was also expected to occur in the integrated model.
Indeed, Figure 4.4a illustrates that the ratio Nitrospira:AOB and Nitrobacter:AOB were 0.00 and 1.97, respectively and this clearly, underlines that both ping-pong and nitrite loop had combination influence on biomass distribution of nitrifiers in the granular sludge reactor. The integrated model hence describes the observation of the experiment by Winkler et al. (2012a) in pilot and lab-scale reactor very well.

The data of one cycle operation simulated in this model can clearly illustrate that due to the mixotrophic activity of Nitrobacter, about 8.2 mg COD acetate was up taken but resulted in 8.84 mg biomass in addition to 11.34 mg biomass from autotrophic growth of Nitrobacter (Table 4.3). This is hence a significant advantage of mixotrophic capacity, which contributed to 44% total biomass of NOB. Table 4.3 and Figure 4.4 clearly show that the mixotrophic advantage of Nitrobacter lead to their complete dominance over Nitrospira. The extra nitrite being supplied from incomplete denitrification facilitated their autotrophic growth hence increasing the ratio Nitrobacter:AOB even more. Without mixotrophic capacity of Nitrobacter, both species of NOB would coexist as in the case of nitrite loop model.

| Table 4.3 Growth of Nitrobacter during a typical cycle in two models |
|----------------------|----------------------|----------------------|----------------------|
|                      | COD assimilated (mg) | Autotrophic growth (mg) | Heterotrophic growth (mg) | Total Nitrobacter biomass in reactor (mg) |
| Ping-pong model      | 1.15                 | 2.03                  | 0.79                  | 1234                  |
| Integrated model     | 8.2                  | 11.76                 | 4.91                  | 3652                  |

4.2.6 Conclusion

Simulation results underlined that the combined effect of both ping-pong and nitrite loop leads to a higher ratio of NOB to AOB due to the uncoupled growth of NOB from nitrite originated from AOB. With the different models, different nitrifiers distributions were obtained. A clear distinction was found between single-step and two-step denitrification and between models with and without mixotrophic capacity of Nitrobacter. Both Nitrospira and Nitrobacter benefited from in reactor and nitrite available from two-step denitrification, which facilitated their growth and doubled their total biomass. When Nitrobacter mixotrophic ability was included in the model, Nitrobacter out competed Nitrospira and became the only nitrite-oxidizing bacteria in AGS reactor. In case both mixotrophic capacity of Nitrobacter and two-step denitrification were included in one model, a much higher biomass and hence higher ratio of Nitrobacter to AOB were found due to significant advantage of mixotrophic capacity and the availability of additional nitrite originated from denitrification.
4.3 Influence of the two hypotheses on reactor performance

4.3.1 Oxygen consumption and nutrient removal efficiency

The influence of the ping-pong and nitrite loop on biological conversion rates of ammonium, phosphate, and especially oxygen was investigated for all models. Relatively same rate of ammonium and phosphate consumption of all models clearly showed that there was no significant effect of the two hypotheses on these substrates conversion (Figure 4.6a). However, there were differences in oxygen consumption in between the model based on two-step denitrification (nitrite loop and integrated model) and single-step denitrification (basic and ping-pong model). Results from the two-step denitrification suggested a 10% higher oxygen consumption compared to the single-step denitrification. This can be explained by the fact that the process of nitrite oxidation by NOB and the proceeding nitrate reduction by DPAOs (until nitrite) is repeated several times hence increasing the nitrification capacity and therefore the oxygen consumption. This henceforth implies that the nitrite loop will lead to a higher aeration demand. Since approximately 60% of all costs of a WWTP are due to aeration, the nitrite loop will severely affect operational costs. In addition, Table 4.3 showed that growth of *Nitrobacter* in integrated model was much higher than that in ping-pong model, which suggest that more oxygen was consumed to gain that growth. The autotrophic growth of *Nitrobacter* was 5 times higher if compared to the base model. However, NOB are not the only bacteria that consume oxygen but also DPAOs which have the biggest contribution to oxygen consumption in the system. The higher oxygen consumption can hence not entirely be contributed to the NOB. In addition, it must be stated that a 10% difference was also accepted in the evaluation of the reduction factors (see section 4.1) and therefore the 10% increase in oxygen consumption should be seen as a trend rather than a fixed number.

A significant difference was shown in the rate of nitrate production and corresponding nitrogen removal efficiency among different models (Figure 4.6b). The basic and ping-pong model (single-step denitrification) resulted in similar nitrogen removal efficiency whereas the nitrite loop and the integrated model (two-step denitrification) had comparable removal efficiencies (70.7% and 62.8%, respectively) which were much lower compared to the single-step denitrification (over 90%). These simulation results indicated that the more nitrate are available from denitrification, the lower nitrogen removal efficiency are. Indeed, the integrated model had a lowest nitrogen removal efficiency due to the combined effect of the ping-pong and nitrite loop. In this case, total biomass of NOB was higher than that of other models, therefore more nitrate could be produced from nitrite loop. While the denitrification capacity of DPAOs was limited by PHB storage pool under given operation condition (see section 4.1.2), as soon as this storage of PHB was emptied, extra nitrate would not be convert to dinitrogen gas and subsequently lowered nitrogen removal efficiency.
Analysing conversion rate implied that measuring ammonium or phosphate consumption rate could yield very good estimation for biomass of *Nitrobacter* in the reactor since there were high correlation in between those parameters (R\(^2\) > 0.8). In addition, ammonium conversion rate could be used to explain nitrogen removal efficiency (R\(^2\) > 0.7). Low ammonium conversion rate lead to better nitrogen removal efficiency since whenever ammonium is available in the bulk, high activity of organisms in outer layer create anoxic zone for denitrification. The longer this zone last the better performance of denitrification can be.

### 4.3.2 NO and N\(_2\)O emission from mixotrophic growth of *Nitrobacter*

In the integrated model, which could describe the reactor performance the best, nitric oxide was considered as main product for mixotrophic growth of *Nitrobacter* in the aeration phase (Chapter 3, section 3.3). Amount of released NO obtained from standard case was 0.45 mg NO-N.L\(^{-1}\) per cycle, which was corresponding to 1.41% of incoming nitrogen load. Strictly taken NO was not emitted through the gas phase in this model but remained in the bulk liquid since there was no interphase transport and gas compartment setup in this study. NO is not typically measured in the off-gas from wastewater treatment system, although it can cause ozone depletion.

<table>
<thead>
<tr>
<th></th>
<th>Concentration (g N.m(^{-3}))</th>
<th>% nitrogen incoming load</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>0.45</td>
<td>1.41</td>
</tr>
<tr>
<td>N(_2)O</td>
<td>0.58</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Beside NO, *Nitrobacter* can also produce N\(_2\)O (Freitag *et al.*, 1987) but the pathway in which they releases nitrous oxide is unknown (see Chapter 2, section 2.6). N\(_2\)O was also considered in an additional model setup.
(result not shown), the amount of N₂O released was higher than that of NO, which was 0.58 g N₂O-N.m⁻³ or 2.2% of incoming nitrogen load (Table 4.4) because oxygen equivalent of N₂O (2.28 g COD.g⁻¹ N) and NO (1.43 g COD.g⁻¹ N) are quite different. This N₂O emission is high compared to the factor of 0.5% of the nitrogen load to assess N₂O emission from wastewater treatment plants used by the Intergovernmental Panel on Climate Change (IPCC). From this study, it seems that only small amounts of nitrate and acetate could lead to significant amount of N₂O emission. To date, there have been very limited studies carried out on N₂O emissions from aerobic granular sludge bioreactors (to removal nutrient and COD). The complexity of granules itself as well as the complexity of interactions between organism in granules (de Kreuk et al., 2007a) will cause difficulties to draw an ambiguous conclusions about which organism or metabolic pathway contributes to nitrous oxide emission. Nevertheless, nitrous oxide emitted from *Nitrobacter* could be one of the possibility pathways.

![Figure 4.7 Relation between NO emission and a) ratio of NOB2 to AOB, R² = 0.77 and b) phosphate consumption rate, R² = 0.87.](image)

Simulation results with various operational condition showed a strong correlation in between *Nitrobacter*/AOB and NO emission (R² = 0.77, Figure 4.7a) regardless of the operational conditions. The higher the *Nitrobacter*/AOB is the more NO emission was obtained. High ratios of NOB2 and AOB were found in conditions in which high anoxic volume fractions were found (big granules size or low oxygen concentration in the bulk). Also in these cases, measuring phosphate consumption rate was a good indicator for NO emission since there was good agreement in between those parameters (R²=0.87, Figure 4.7b).

### 4.3.3 Conclusion

The four models had similar substrate profiles during one cycle of operation but the integrated model described the ratio NOB:AOB the best. Biomass distribution reflected the availability of oxygen thought the granule during the aeration phase. There was no effect of the ping-pong on the reactor behaviour in terms of substrate consumption rate, but nitrite loop did increase oxygen consumption by 10%. On the other hand, nitrogen removal efficiency was significantly influenced by two-step denitrification (nitrite loop). A reduction up to 20%
in nitrogen removal efficiency was obtained in the nitrite loop and the integrated model, in which, relatively more nitrate was produced by *Nitrobacter* while the denitrification capacity of DPAOs to convert nitrate to dinitrogen gas was limited by their PHB storage. The possible emission of NO or N₂O need to be considered during mixotrophic growth of *Nitrobacter*. The amount of NO (N₂O), which was released under typical reactor conditions, was 1.41% (2.2%) of incoming nitrogen loading. A strong correlation (R²=0.76) was found between the ratio of NOB2 to AOB and the amount of NO (N₂O) emitted. The higher the ratio is the higher the emission will be.
4.4 Influence of operation conditions on reactor performance

4.4.1 Influence of granule size

Figure 4.8 summarizes the effect of changing the granule sizes on the ratio NOB2:AOB, nitrogen removal efficiency, rate of consumption of phosphate/ammonium, and NO emission. As fraction DPAOs decreased with bigger granule due to lower conversion rate, ammonium competition between DPAOs and nitrifiers was ease. More available ammonium for AOB and NOB led to more biomass produced (Figure 4.8d). The ratio NOB2:AOB varied in a narrow range from 1.65-1.85 (Figure 4.8a). Figure 4.8b clearly shows that nitrogen removal increased with increased granule size, which was confirmed by experimental data (de Kreuk et al., 2007b), meaning that nitrate concentration at the end of cycle decreased with bigger granules. In a smaller granule, the surface to volume ratio will be higher and therefore oxygen can diffuse deeper inside granules. With more oxygen available, ammonium can be quickly oxidized by AOB or consumed by DPAOs for aerobic growth. As soon as ammonium was depleted, oxygen started to penetrate the whole granule and denitrification was inhibited. Therefore, with smaller granule radius, lower nitrogen removal was obtained in simulations (Figure 4.8b).

![Diagram](image)

**Figure 4.8** Influence of granules size on a) Ratio of *Nitrobacter* to AOB b) Nitrogen removal efficiency and NO emission c) Phosphate and ammonium consumption rates d) Fraction of biomass in granules. Simulations results of the integrated model at temperature = 20°C, DO = 2 g O₂·m⁻³.

The phosphate and ammonium consumption rate followed the opposite trend to granule size (Figure 4.8c). As granules became smaller, those conversion rates became higher. More aerobic volume fraction with smaller
granules was the main reason for this obtained trend. Smaller granule facilitated the aerobic poly-P formation of the DPAOs, which can proceed faster with oxygen as electron acceptor (and slower with NOx) (Appendix 2, Table A.2.1, process 5, 9 and 13) and facilitated a faster ammonium oxidation due to favoured growth of AOB (Appendix 2, Table A.2.1, process 16). In contrast, with larger granules, the total surface area of the granules became smaller. In accordance, surface to volume ratio (which is the total surface of granules divide by the volume of reactor) became smaller; and hence, became the surface area limited for oxygen transport and consequently, for conversion processes. If more simulations were done for even bigger granule sizes (bigger than 0.8 mm), lower conversion rates were expected and at certain granule size, these conversion rates became so low that phosphate and ammonia accumulated in the effluent. Since denitrification depends on nitrification, denitrification will automatically be limited to the ammonium being nitrified hence leading to lower nitrogen removal efficiency.

NO emission fluctuated in a narrow range (from 1.2 to 1.6% of nitrogen loading). More NO was produced for bigger granules due to a higher anoxic volume fraction available for mixotrophic growth within bigger granules (Figure 4.8b). Since NO was defined as production of anoxic PHB degradation of *Nitrobacter* on nitrate (Chapter 3, section 3.3), more available anoxic volume fraction led to more NO released. However, from a certain granule size, in this case, from granule size of 0.7 mm, NO emission was lowered. This was because anoxic activities of *Nitrobacter* depended on stored PHB. As N-removal increased and then corresponded nitrate from previous cycle was lowered with increasing granule size, PHB storage of *Nitrobacter* (which much depend on nitrate) decreased and subsequently, NO production (which in turn depends on PHB) was lowered. This trend clearly indicated that there is an optimum in operational condition for granules size and NO emission. The highest NO concentration was found at granules’ radius of 0.7 mm. Any deviation from this size could result in less NO emission.

### 4.4.2 Influence of temperature

The ratio of NOB2 to AOB increased from 1 to 3.6 with increasing temperature (Figure 4.9a). This can be explained by the fact that endogenous respiration rate of AOB is higher than that of NOB2 (Blackburne *et al.*, 2007a). An increase in the temperature from 10 to 30°C implied a decrease of active biomass of organisms (Figure 4.9d) which can be explained due to higher endogenous respiration and maintenance rate.
Simulations indicated that, at 30°C, ammonium was completed consumed within 60 min of the aeration phase. Henceforth, no ammonium was available for heterotrophic growth and due to their higher maintenance and endogenous respiration; a lower biomass production was expected. Nitrifiers shifted into deeper layers inside of the granule at lower temperature. This shift was mainly due to higher penetration depth of oxygen in early periods of the aeration phase, caused by lower activities of DPAOs in outer layers. The DPAOs population decreased at higher temperatures, leading to a lower phosphate consumption rate. These simulation results coincided with the trend of phosphate removal observed in experimental result (unpublished data from Winkler et al. (2012a)), from which, 40% of added phosphate accumulated in the effluent when the reactor operated at 30°C.

An increased NO emission was obtained in this model at higher temperature (Figure 4.9b). NO released up to 0.85 g N.m⁻³ at 30°C (1.2 g N.m⁻³ for N₂O), which corresponded to 2.8% (or 4% for N₂O) of incoming nitrogen load. As shown in Figure 4.9b, nitrogen removal efficiency did not differ largely with different temperatures meaning that the nitrate being available for Nitrobacter from previous cycle were more or less the same. The higher NO emissions can be explained by the degradation rate of PHB by Nitrobacter, which will be higher at higher temperature due to increased bacterial activity. Another key point was that, at the early aeration phase, when ammonium and phosphate were still present, the bacteria could be more active, which lead to a lower
oxygen penetration depth and then created a bigger anoxic zone for anoxic PHB degradation of *Nitrobacter* (the pathway of NO emission).

### 4.4.3 Influence of oxygen concentration

The ratio of *Nitrobacter* to AOB increased with decreasing of DO concentrations (Figure 4.10a) due to lower fractions of DPAOs at lower DO. This then led to lower competition between DPAOs and *Nitrobacter* during the feeding phase for nitrate and acetate. *Nitrobacter* could assimilate more acetate and then use it to growth more in aeration phase. Ratio of NOB2 to AOB was correlated with NO emission. At lower DO, high NO emissions were obtained due to higher fraction of *Nitrobacter* and high anoxic fraction that facilitated anoxic PHB degradation of *Nitrobacter* (Figure 4.10b). At higher DO concentrations, less anoxic volume fraction was available for denitrification leading to a decrease in total nitrogen removal efficiency (Figure 4.10b).

![Figure 4.10](image)

**Figure 4.10** Influence of oxygen concentration on a) Ratio of *Nitrobacter* to AOB b) Nitrogen removal efficiency and NO emission c) Phosphate and ammonium consumption rate d) Fraction of biomass in granules Simulations results of the integrated model with granules radius = 0.6 mm and t = 20°C.

The phosphate removal at lower oxygen concentrations was slower than at higher oxygen concentrations. This is due to the significantly faster storage of poly-P under aerobic conditions than it is the case under anoxic conditions. At lower oxygen concentrations and thus at a limited oxygen penetration depth, DPAOs will not be
able to store their phosphate aerobically, resulting in a decreased P-removal rate. At all simulated oxygen concentrations, the storage was high enough to have complete phosphate removal. Ammonium was consumed slower and nitrate production rates decreased, due to higher denitrification rates in the presence of more anoxic zones (Figure 4.10c).

In a very similar experimental setup, de Kreuk et al. (2005a) reported that at 10 gO₂.m⁻³, only 10% nitrogen was removed. In this study, simulations showed that at 2 gO₂.m⁻³ the simulated total nitrogen removal efficiency was 68% while at 10 g O₂.m⁻³ it still was 57%, which is not expected. It seemed that this model overestimated anoxic activities of DPAOs. Any influence on anoxic activities of DPAOs such as increased DO concentration or decrease granules size did not show to have a high influence on nitrogen removal efficiency. More model calibrations could be done to evaluate this especially with respect to the reduction factor and the maintenance for anoxic conditions of DPAOs.

### 4.4.4 Conclusion

The effect of granule size, temperature, and DO on the ratio *Nitrobacter* to AOB, rate of ammonium consumption, rate of phosphate and ammonium consumption, the nitrogen removal efficiency, as well as the NO emission are summarized in Table 4.5. Limited oxygen penetration/diffusion became the decisive factor in the rate of biological conversion process and the available anoxic zone for denitrification whilst temperature played the key role in determining biomass concentration. Further investigation is necessary to find optimal condition for nitrogen removal and NO (N₂O) emission.

**Table 4.5** Summarized influence of operation conditions.

<table>
<thead>
<tr>
<th></th>
<th>Granule size</th>
<th>Temperature</th>
<th>DO</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOB2:AOB</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Nitrogen removal efficiency</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>NO (N₂O) emission</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Rate of NH₄⁺</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Rate of PO₄⁻</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑ Increase, ↓ decrease, ↔ variations are negligible
5  GENERAL CONCLUSIONS AND PERSPECTIVES

In this thesis, a mathematical model was setup to describe the performance of an aerobic granular sludge reactor (AGS). The model was then extended to investigate the two hypotheses that were previously proposed by Winkler et al. (2012a). These two hypotheses (ping-pong and nitrite loop), were proposed to explain how nitrite-oxidizing bacteria (NOB) could grow uncoupled from the nitrite supply of ammonia-oxidizing bacteria (AOB), which led to elevated ratios of NOB to AOB and to the dominance of *Nitrobacter* in this system. Besides the influence of the two hypotheses, also the influence of operating conditions on the distribution of microorganisms, NO (N₂O) emission and reactor performance were investigated, through additional simulations.

5.1  Modelling simultaneous COD and nutrient removal in an AGS

Four models were successfully set up and calibrated to describe the performance of a lab-scale AGS, capable of simultaneously removing COD, nitrogen and phosphate in one reactor. These models comprised a basic model describing conventional nitrification and denitrification, a ‘ping-pong model’ with mixotrophic of *Nitrobacter*, a nitrite loop model including two-step denitrification, and an integrated model combining the ping-pong model and the nitrite model. The available lab-scale experimental data of Winkler et al. (2012a) were best described by the integrated model, showing a good match for the biological conversion rate as well as concerning the ratio of NOB to AOB. The simulation results underlined the importance of the oxygen penetration depth into the granules on the biomass distribution.

5.2  Two hypotheses

Both hypothesis proposed by Winkler et al. (2012a) gave possible reasons on why there was an elevated ratio of NOB to AOB in AGS, which could be 4 times higher than the one in conventional activated sludge system (NOB:AOB up to 2 compared to about 0.5) and why *Nitrobacter* but not *Nitrospira* was the dominant NOB species. The first hypothesis (ping-pong) assumes the experimentally proven capacity of *Nitrobacter* to grow on acetate by dissimilatory nitrate reduction. In this hypothesis, *Nitrobacter* derives extra energy from the nitrate being generated during the autotrophic pathway and additional acetate being present in the granular reactor. The second hypothesis (nitrite loop) assumes that a nitrite oxidation/nitrate reduction loop occurs within the granules in which denitrifying bacteria reduced nitrate to nitrite providing additional nitrite for the NOB apart from the one originated form AOB.

The simulation results showed that only the combination of the ping-pong and the nitrite loop led to an elevated ratio NOB:AOB close to the experimentally observed one and to the dominance of *Nitrobacter*. The ratio
NOB:AOB was elevated because the additional nitrite produced as an intermediate during denitrification was reused by NOB. In the combined model, the ratio of *Nitrobacter* to AOB was about 2 and increased with increasing temperature (up to 3.5 at 30°C), which fitted the experimental observation of Winkler et al. (2012a) very well. The dominance of *Nitrobacter* over *Nitrospira* was explained by the fact that *Nitrobacter* utilized the nitrate, which was produced during nitrification and incomplete denitrification and reduced it with available acetate, which constituted a competitive advantage for this species.

### 5.3 Reactor performance

From the nitrite loop hypothesis, it was expected that more oxygen needs to be supplied to the system because more nitrite is generated, which needs to be oxidized. The expected higher oxygen consumption was confirmed in this study. While the mixotrophic growth of *Nitrobacter* (ping-ping) only affected the NOB distribution, incomplete denitrification (nitrite loop) did affect reactor performance by an increase in the oxygen consumption of about 10% and a lower nitrogen removal efficiency (by 20%). These results were explained by the fact that more nitrite from incomplete denitrification became available. This increased the oxygen consumption due to more nitrite oxidized to nitrate by NOB. In addition, because of the limited denitrification capacity of DPAOs (limited by PHB storage pool, only carbon source of DPAOs to grow) not all additional nitrate from the loop could be denitrified, resulting in lower nitrogen removal efficiency.

Mixotrophic growth of *Nitrobacter* could lead to the potential greenhouse gas (GHS) emissions during operation of aerobic granular sludge reactor. It was experimentally determined in earlier research that a product of dissimilatory nitrate reduction by *Nitrobacter* can be either NO or N$_2$O. The amount of NO (N$_2$O) released under typical reactor conditions, ranged between 1% and 4% of incoming nitrogen loading. A strong correlation ($R^2=0.77$) was found between the ratio of *Nitrobacter* to AOB and amount of NO (N$_2$O) emitted showing that higher this ratio will lead to higher GHG emissions.

### 5.4 Influence of operation conditions on reactor performance

In most cases, an increased temperature, granule size and decreased oxygen concentration lead to an increase in the nitrogen removal efficiency, the ratio NOB:AOB, and the NO (N$_2$O) emission, but in a decrease of biological conversion rates of phosphate and ammonium. Limited oxygen penetration/diffusion occurring in bigger granules or at low oxygen concentrations will lower the rate of biological conversion process and will influence the anoxic volume fraction needed for denitrification. The temperature played a key role in biomass concentration due to increased decay rates at increased temperature.
5.5 Perspectives

- The temperature dependency of diffusion coefficients could be taken into account to refine the model for assessing the influence of temperature on reactor behaviour.

- Other pathways of mixotrophic *Nitrobacter* could be considered in future works including the release of NO (N₂O) during the feeding phase, the use of nitrite as electron acceptor for acetate uptake or the growth on decay material in the aeration phase. Experimental investigations of NO and N₂O production in mixotrophically growth of pure *Nitrobacter* cultures are necessary for a better understanding of the mechanisms behind the associated greenhouse gas emissions.

- Computing time is one of the constraint of the presented models. It took about 24-36 hours to complete one simulation. Optimizing the model for faster simulation is surely necessary for future simulations.
REFERENCES


